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PROGNOSTIC AND PREDICTIVE MARKERS IN BREAST CANCER – PI3K SIGNALING PATHWAY

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PROGNOSTIC AND PREDICTIVE MARKERS IN BREAST CANCER – PI3K SIGNALING PATHWAY

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I hereby declare that I have written the present thesis myself under the expert guidance of my two supervisors: MUDr. Kateřina Bouchalová, PhD and PharmD. Ivan Bieche, PhD from the Laboratory of Experimental Medicine (Institute of Molecular and Translational Medicine, Czech Republic) and the Oncogenetic Laboratory (Institut Curie, Hospital René Huguenin, France). A complete review of the literature used and cited in the thesis is listed in Chapter 7.

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Contents

Abbreviations

3,4-PIP2/PtdIns(3,4)P2	Phosphatidylinositol-3,4-bisphosphate
4,5-PIP2/PtdIns(4,5)P2	Phosphatidylinositol-4,5-bisphosphate
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
AKT 1-3/PKB	v-akt murine thymoma viral oncogene homolog 1-3/protein kinase B
AMPK	Protein kinase, AMP-activated, alpha 1 catalytic subunit
ANPEP	Alanyl (membrane) aminopeptidase
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
BAD	BCL2-associated agonist of cell death
BID	BH3 interacting domain death agonist
Bp	Base pairs
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BRCA1	Breast cancer 1, early onset
cDNA	Complementary DNA
CI	Confidence interval
CIT	Carte d'Identité des Tumeurs
Cmax	Maximal concentration
Ct	Cycle threshold
CYP3A4/5	Cytochrome P450, family 3, subfamily A, polypeptide 4/5
CYP4B1	Cytochrome P450, family 4, subfamily B, polypeptide 1
CYP4X1	Cytochrome P450, family 4, subfamily X, polypeptide 1
CYP4Z1	Cytochrome P450, family 4, subfamily Z, polypeptide 1
CYP4Z2P	Cytochrome P450, family 4, subfamily Z, polypeptide 2 pseudogene
DAVID	Database for annotation, visualization and integrated discovery
Deptor	DEP domaincontaining mTOR-interacting protein
DFS	Disease free survival
dNTP	Deoxyribonucleotide triphosphates
EGF	Epidermal growth factor
EGFR/ERBB1	Epidermal growth factor receptor
EGFRvIII	Mutant form of EGFR, generated by in-frame deletion of exons 2 – 7
eIF4E	Eukaryotic translation initiation factor 4E
ER	Estrogen receptor
ERK1/2	Extracellular regulated MAP kinase 1/2
Erα-	Estrogen receptor alpha negative

ERα+	Estrogen receptor alpha positive
FASL	Fas ligand
FC	Fold change
FDA	Food and drug administration
FFPE	Formalin-fixed paraffin-embedded tumor tissue samples
FGFR 1-5	Fibroblast growth factor receptor 1-5
FISH	Fluorescence in situ hybridization
FKBP1A/FKBP12	FK506 binding protein 1A
FOXO	Forkhead transcriptor factor
GAP	GTPase-activating protein
GEO	Gene expression omnibus
GO	Gene ontology
GOLPH3	Golgi phosphoprotein 3 (coat-protein)
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HB-EGF	Heparin-binding EGF
HER	Epidermal growth factor receptor family
HER2/ERBB2	Epidermal growth factor receptor 2
HER3/4	Epidermal growth factor receptor 3/4
HIF-1	Hypoxia inducible factor 1
HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)
HR	Hazard ratio
HR	Hormonal receptors
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
Hsp70	Heat shock 70kDa protein
ID4	Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein
IDC	Invasive ductal cancer
IGF-1R	Insulin-like growth factor 1 receptor
IHC	Immunohistochemistry
IKK	IkappaB kinase
ILC	Invasive lobular cancer
INPP4B	Inositol polyphosphate-4-phosphatase, type II, 105kDa
IR	Insulin receptor
IRS-1-4	Insulin receptor substrate 1-4
IS	Intensity score
KEGG	Kyoto encyclopedia of genes and genomes
Ki-67/MKI67	Antigen identified by monoclonal antibody Ki-67
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LIMCH1	LIM and calponin homology domains 1
LKB1/STK11	Serine/threonine kinase 11
LTF	Lactotransferrin

mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MAPT	Microtubule-associated protein tau
MCC	Matthews' correlation coefficient
MDM2	Mdm2 p53 binding protein homolog
MEK	Mitogen activated protein kinase kinase
MFS	Metastasis-free survival
MIAME	Minimum information about a microarray experiment
miRNA	Micro RNA
mLST8	Mammalian lethal with SEC13 protein 8
MNK1/2	MAPK-interactring protein kinases 1/2
mSIN1	Mammalian stress activated protein kinase interacting protein 1
MSX2	Msh homeobox 2
mTOR	Mammalian target of rapamycin
mTORC1/2	mTOR complex 1/2
MYC	v-myc myelocytomatosis viral oncogene homolog
NCOA3/AIB1	Nuclear receptor coactivator 3
NKAIN1	Na+/K+ transporting ATPase interacting 1
NR2F2	Nuclear receptor subfamily 2, group F, member 2
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
NRG1-6	Neuregulins 1-6
NRIP3	Nuclear receptor interacting protein 3
NTN4	Netrin 4
p110	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha protein
p27	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)
p53/TP53	Tumor protein p53
p85	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha) protein
PAM	Prediction Analysis for Microarrays
pCR	Pathological complete response
PCR	Polymerase chain reaction
PDGFRα/β	Platelet-derived growth factor receptors α/β
PDK1/PDPK1	Phosphoinositide-dependent kinase 1
PH	Pleckstrin homology domain
PHLPP 1/2	PH domain and leucine rich repeat protein phosphatase 1/2
PI3K	Phosphatidylinositol 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PIK3CB	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit beta

PIK3CD	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)
PIK3R2	Phosphoinositide-3-kinase, regulatory subunit 2 (beta)
PIK3R3	Phosphoinositide-3-kinase, regulatory subunit 3 (gamma)
PIP3/PtdIns(3,4,5)P3	Phosphatidylinositol-3,4,5-triphosphate
PR	Progesterone receptor
PRAS40	Proline rich Akt substrate 40
Protor1/PRR5	Proline rich 5 (renal)
PS	Proportion score
PTEN	Phosphatase and tensin homologue deleted on chromosome ten
Q	Quantitative
Raptor	Regulatory associated protein of mTOR
RAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
RBL2	Retinoblastoma-like 2
REDD1	DNA-damage-inducible transcript 4
REEP1	Receptor accessory protein 1
Rheb	Ras homolog enriched in brain
Rictor	Rapamycin insensitive companion of mTOR
RPS6	40S ribosomal protein S6
RSK1	Ribosomal protein S6 kinase, 90kDa, polypeptide 1
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase polymerase chain reaction
S6K1/2/p70S6K	S6 kinase 1/2
SEC14L2	SEC14-like 2 (S. cerevisiae)
Ser	Serine, amino acide
SH2	Src-homology 2
SHC	Src homology 2 domain containing
SLC40A1	Solute carrier family 40 (iron-regulated transporter), member 1
SLC4A4	Solute carrier family 4, sodium bicarbonate cotransporter, member 4
TBP	TATA box binding protein
TCF-4	Transcription factor 4
TCF7L2	Transcription factor 7-like 2 (T-cell specific, HMG-box)
T-DM1	Trastuzumab emtansine
TFAP2B	Transcription factor AP-2 beta (activating enhancer binding protein 2 beta)
TGF- α	Transforming growth factor-α
Thr	Threonine, amino acide
TIS	Total immunostaining score
TMC5	Transmembrane channel-like 5

TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b
TOS	TOR signaling
TPD52	Tumor protein D52
TSC1/2	Tuberous sclerosis complex 1/2 (hamartin and tuberin, respectively)
TUSC3	Tumor suppressor candidate 3
VANGL2	Vang-like 2 (van gogh, Drosophila)
VEGF	Vascular Endothelial Growth Factor
VEGFR1-3	Vascular Endothelial Growth Factor Receptor 1-3
WEE1	WEE1 homolog (S. pombe)
Wnt	Wingless signaling pathway
WNT5A	Wingless-type MMTV integration site family, member 5A
-	Negative
+	Positive

1 Introduction and Background

1.1 Introduction

The present work focuses on the prognostic and predictive markers in breast cancer that are connected with the PI3K signaling pathway. The main interest was phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (*PIK3CA*) gene mutations and their role in breast cancer prognosis and treatment response prediction. Other markers related to the PI3K signaling were also assessed. For this reason, the background on the following pages covers current knowledge of PI3K pathway signaling in normal conditions and in tumor cells where it can be activated by multiple hits affecting the genes and their associated protein products implicated in the pathway. The research results are then described in the form of original articles introduced by a brief results summary and discussion of recent papers on related topics.

1.2 Background

1.2.1 Breast cancer

Breast cancer is the most common malignancy in women. Data from 2008 showed an incidence of more than 1,300,000 and mortality of 450,000 women worldwide. In the European Union, the incidence and mortality were more than 330,000 and 80,000 women, respectively (http://globocan.iarc.fr/). These statistics make breast cancer an important research subject in order to gain more information about its nature for use in assessing disease prognostic and treatment outcome prediction. Breast cancer in reality is a heterogeneous group of tumors. Thus, current knowledge distinguishes several breast cancer subgroups, which differ in multiple characteristics at a genetic, histological and clinical level (Malhotra *et al*, 2010; Russnes *et al*, 2011).

Histologically, breast cancer is divided into two main subgroups with the highest incidence and then minor subgroups which are found with much lower frequency. The two most common histological subgroups of breast cancer are ductal cancers comprising up to 75% cases and lobular cancers comprising about 10% of cases. Among the less

common histological breast cancer subtypes are medullary (up to 7% cases), invasive cribriform (up to 3.5% cases), tubular (less than 2% cases), metaplastic carcinomas (less than 1% cases) and other less frequent subtypes (Tavassoli *et* Devilee, 2003). However, histological subtype definition has a minor impact on clinical treatment choice. Other tumor characteristics and histological features such as tumor grade (tumor differentiation, nuclear pleomorphism and number of mitoses), tumor extension and lymph node status provide additional information in clinical practice.

Further, there is classification based on molecular markers of breast cancer tumor cells such as hormonal estrogen and progesterone receptors (ER and PR), epidermal growth factor receptor 2 (ERBB2/HER2), proliferation (Ki-67) and other markers. This classification of molecular subtypes describes: luminal A (ER and/or PR-positive, HER2-negative, low proliferation signs, about 40%), luminal B (ER and/or PR-positive, HER2-positive, signs of increased proliferation, about 20%), HER2-related (ER- and PR-negative, HER2-positive, 10-15%), basal-like (ER, PR and HER2-negative, cytokeratin 5/6-positive and/or epidermal growth factor receptor-positive, about 15-20%), normal breast-like (adipose tissue gene signature) and claudin-low tumors (claudin 3/4/7-low, vimentin-positive, E-cadherin-low, 12-14%). Identification of these subtypes is based on microarray gene expression analysis and hierarchical clustering. As a substitute for this classification, immunohistochemical detection of tumor receptors has become well established in everyday clinical practice for outcome prediction and therapy selection. This classification describes breast cancer tumors according to expression of hormonal receptors (ER and PR) and HER2 to create the 4 subgroups: hormonal receptor (HR)-positive/HER2-negative, HR-positive/HER2-positive, HR-negative/HER2-positive, HR and HER2 negative (triple negative subgroup) (Sørlie *et al*, 2006; Reis-Filho *et* Tutt, 2008; Malhotra *et al*, 2010; Gruver *et al*, 2011; Russnes *et al*, 2011; Saxena *et* Dwivedi, 2012). In reality, despite the different assessment approaches, there are associations and overlaps between subgroups derived from a histological, immunohistochemical and molecular basis as shown in Figure 1 (Russnes *et al*, 2011). It remains true however that breast cancer is a heterogenous disease and even the above described subgroups provide only approximative classification. Further research might detect smaller and more numerous, but better characterized subgroups of tumors. Recently, Curtis and coworkers (Curtis *et al*, 2012) described 10 breast cancer clusters based on acquired somatic gene copy number aberrations that influence gene expression.

Distinct breast cancer subgroups also differ at the level of disease prognosis and prediction of treatment response. Generally, favorable prognosis is connected with invasive lobular, HR-positive and luminal tumors. As shown in Figure 1, these subgroups describe overlapping populations of breast cancer tumors using differing assessment approaches (Russnes *et al*, 2011). These tumors also respond to hormonal treatment. On the other hand, invasive ductal and HER2-related tumors have worse prognosis. In the case of HER2 positivity, the outcome in patients is improved if HER2-targeted therapy (trastuzumab, lapatinib) is applied. The worst prognosis is attributed to some minor histological subgroups such as medullary or metaplastic cancers which are commonly triple negative using immunohistochemistry and/or basal-like using molecular markers. For these tumors, there is no targeted therapy available in clinical practice (Reis-Filho *et al*, 2006; de Ruijter *et al*, 2011). All these breast cancer subgroups display additional deregulations in cellular signaling pathways (Shah *et al*, 2012; Stephens *et al*, 2012; Martins *et al*, 2012). Better knowledge of such changes will assist in understanding differing nature of breast cancer subtypes and offer options for new treatment approaches.

1.2.2 PI3K signaling pathway

This signaling pathway is one of the crucial and central signaling pathways in normal cells as well as in tumor cells, and in particular in breast cancer. The pathway is activated by receptor tyrosine kinases such as HER family (epidermal growth factor receptor family; EGFR, HER2, HER3, HER4), insulin receptor tyrosine kinase or insulin-like growth factor 1 receptor which are anchored in the cellular membrane and pass signals from the outside environment into the cell. The scheme of the pathway signaling cascade is shown in Figure 2. The PI3K pathway integrates multiple signals and regulates important functions in the cell such as glucose homeostasis and metabolism (particularly in muscle and fat), protein synthesis, cellular proliferation and survival, motility and cellular polarity (Wickenden *et* Watson, 2010). Importantly, this pathway appears to be the most frequently deregulated pathway in breast cancer and its components have been reported to be mutated, amplified and/or altered at the expression level in more than 70% breast cancers (Wickenden *et* Watson, 2010; Miller *et al*, 2011).

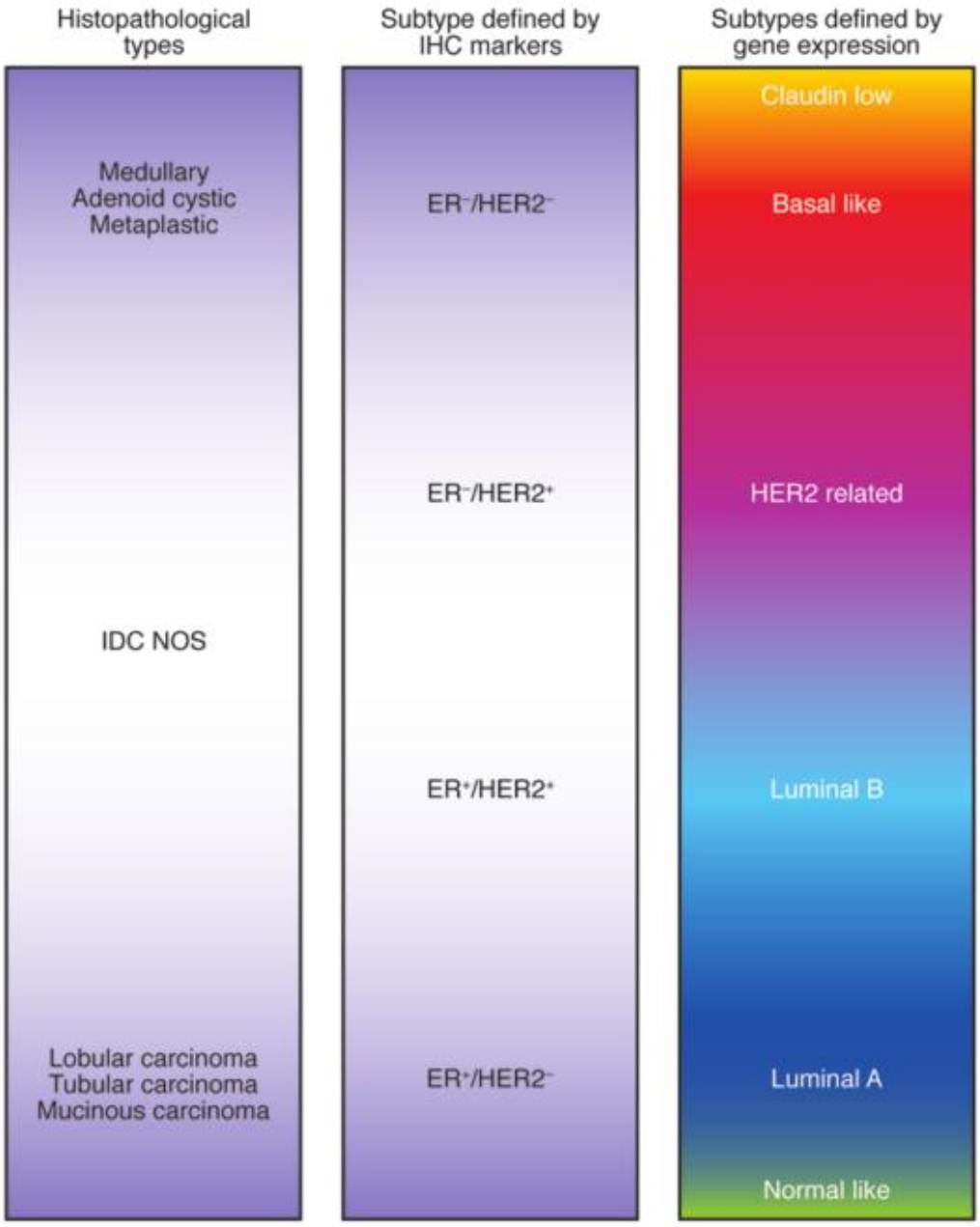

Figure 1. Associations between breast cancer subtypes based on different assessment approaches (adapted from Russnes *et al*, 2011).

1.2.2.1 Receptor tyrosine kinases

The PI3K signaling pathway is activated by multiple receptor tyrosine kinases. There are several which are substantially implicated in breast cancer development and progression.

Well-described is the epidermal growth factor receptor family containing four members: EGFR (ERBB1), HER2 (ERBB2), HER3 (ERBB3) and HER4 (ERBB4). These receptors

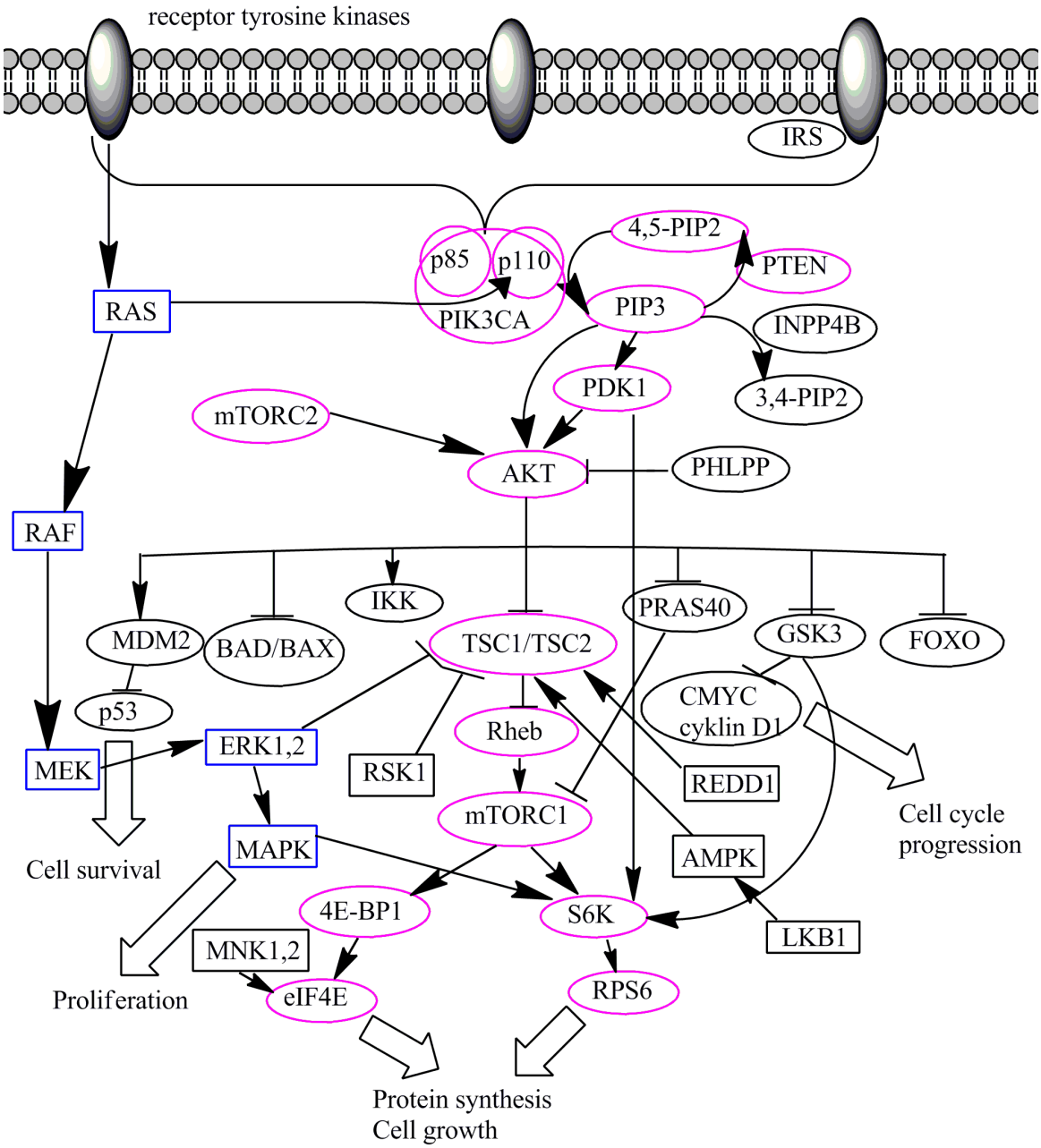

Figure 2. The PI3K signaling pathway (adapted from Castaneda *et al*, 2010; Wickenden *et* Watson 2010; Baselga 2011; Saxena *et* Dwivedi, 2012).

consist of an extracellular ligand-binding domain, a single membrane spanning region and a cytoplasmic domain with tyrosine kinase activity. The domains however, show functional differences in particular receptors such as HER2 with no known ligand and HER3 lacking tyrosine kinase activity. HER family receptors are activated by ligands that can be divided into subgroups following their specificity for particular receptors. EGFR binds epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin, betacellulin, heparin-binding EGF (HB-EGF) and epiregulin. HER3 binds ligands from neuregulin family (NRG1, NRG2, NRG6). HER4 binds betacellulin, HB-EGF and epiregulin that also binds EGFR and neuregulins NRG1-5. Since HER2 has no known ligand, its activation occurs in heterodimer formation with other family members. Dimerized activated HER family receptors transmit signals on both the PI3K and the mitogen-activated protein kinase (MAPK) pathways (Hynes *et* Lane, 2005; Bouchalova *et al*, 2009; Koutras *et al*, 2010; Saxena *et* Dwivedi, 2012; Seshacharyulu *et al*, 2013; Iwakura *et* Nawa, 2013).

The insulin receptor (IR) and the insulin-like growth factor 1 receptor (IGF−1R) are both dimeric receptors composed of two extracellular α-subunits and two β-subunits with three parts: extracellular, intramembranous and intracellular tyrosine kinase-containing. Despite high level similarities of amino acid sequences, these two receptors have different functions and trigger different cellular processes. However, both IGF-1R as well as IR transmit signals through the PI3K among other pathways. Activation of these downstream pathways is mediated by phosphorylation of adaptor proteins, the insulin receptor substrate 1-4 (IRS-1, IRS-2, IRS-3, IRS-4) and SHC (Src homology 2 domain containing) transforming protein (Larsson *et al*, 2005; Baselga, 2011). Platelet-derived growth factor receptors α and β (PDGFR α and β), fibroblast growth factor receptor family of 5 members (FGFR 1-5), and vascular endothelial growth factor receptors 1, 2 and 3 (VEGFR 1, 2 and 3) are closely related receptors composed of extracellular, transmembrane and intracellular tyrosine kinase including domains. These receptors act in a similar manner and require dimerization in order to activate downstream pathways such as PI3K and MAPK, except for FGFR5 which lacks the tyrosine kinase domain (Andrae *et al*, 2008; Jiang *et* Liu, 2009; Turner *et* Grose, 2010).

1.2.2.2 PI3K pathway components

1.2.2.2.1 PI3K

The phosphatidylinositol 3-kinase (PI3K) superfamily contains kinases grouped in 3 classes – class I, II and III. Various isoforms form each of these classes. Class I consists of subclass IA activated by receptor tyrosine kinases and subclass IB activated by G-protein-coupled receptors. However, only subclass IA activates AKT and subsequently also downstream levels of the pathway (Jiang *et* Liu, 2009; Wickenden *et* Watson, 2010). Subclass IA PI3K is a heterodimer protein composed of two subunits: regulatory (p85) and catalytic (p110). Both proteins exist in isoforms encoded by distinct genes. The isoforms are namely p85α (p85α, p55α and p50α), p85β, and p55γ (encoded by *PIK3R1-3*) and p110α, p110β and p110δ (encoded by *PIK3CA*, *PIK3CB* and *PIK3CD*). In terms of breast cancer development and progression, p85α (*PIK3R1*) and p110α (*PIK3CA*) have been studied the most as two genes undergoing tumoral deregulations (Jiang *et* Liu, 2009; Castaneda *et al*, 2010; Courtney *et al*, 2010; Hernandez-Aya *et* Gonzalez-Angulo, 2011). Under non-activated conditions, p85α stabilizes the p110α subunit. Besides the PI3K dimer, p85 (*PIK3R1*) has also been found to positively regulate the lipid phosphatase activity of PTEN. For this reason, p85 (*PIK3R1*) has also been proposed as a player in tumor suppression in opposition to p110α (*PIK3CA*) that plays an oncogenic role in cells (Luo *et* Cantley, 2005; Chagpar *et al*, 2010).

Receptor tyrosine kinases interact with the p85 regulatory subunit releasing the p110 catalytic subunit from p85 suppression (Jiang *et* Liu, 2009; Adams *et al*, 2011). p85 contains two Src-homology 2 (SH2) domains which serve to bring PI3K to the membrane and recognize upstream activating molecules such as receptor tyrosine kinases or IRS (Luo *et* Cantley, 2005). Upon activation by receptor tyrosine kinases, PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (4,5-PIP2) at its 3'-hydroxyl group and generates the the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 further binds to the pleckstrin-homology domains of downstream targets including v-akt murine thymoma viral oncogene homolog (AKT) and 3-phosphoinositide-dependent kinase 1 (PDK1). The signal transmission leads to PDK1 autophosphorylation at position Ser241 followed by transphosphorylation of AKT (Wickenden *et* Watson, 2010). In addition to AKT, PDK1 also activates other kinases (Blanco-Aparicio *et al*, 2007). Besides the described interactions, p110 has also been found to interact with RAS protein at its RAS-

binding domain that leads to subclass IA PI3Ks activation (Jiang *et* Liu, 2009; Hernandez-Aya *et* Gonzalez-Angulo, 2011; Adams *et al*, 2011).

1.2.2.2.2 PTEN

Phosphatase and tensin homolog (PTEN) is a phosphatase acting as a negative regulator of the pathway by dephosphorylating PIP3 and thus reversing the activity of PI3K (Wickenden *et* Watson, 2010). Since PTEN has an important role in 4,5-PIP2/PIP3 homeostasis maintenance, it plays a tumor suppressor role in the PI3K pathway signaling. As an important regulator, expression and activity of PTEN have been found to be controlled at multiple levels including gene transcription, mRNA stability, translation and protein modification such as phosphorylation or acetylation (Jiang *et* Liu, 2009). Besides PIP3, PTEN can dephosphorylate other lipids and proteins as well. Additional to its cytoplasmic localization, PTEN has also been found in the nucleus, acting as a cell cycle inhibitor. This effect has been described as PTEN phosphatase activity directed at mitogen-activated protein kinase pathway and cyclin D1. Further, PTEN has been found to regulate p53 activity in the nucleus as well (Blanco-Aparicio *et al*, 2007; Wickenden *et* Watson, 2010; Adams *et al*, 2011).

1.2.2.2.3 AKT

AKT is a serine-threonine protein kinase, the central player of the pathway integrating upstream signals and regulating multiple downstream effectors. AKT exists in three isoforms: AKT1, AKT2, AKT3, which vary by expression in different tissues. Using mice models, the AKT1 isoform has been linked to breast gland tissue development and differentiation whereas AKT2 has been suggested to act in rather the opposite way. AKT3 appears to have a minor role in normal breast tissue (Wickenden *et* Watson, 2010).

For AKT activation, principal is its localization to cellular membrane where AKT is recruited by PIP3 which is followed by conformational changes in AKT (Blanco-Aparicio *et al*, 2007; Maurer *et al*, 2009). Interaction with activated PDK1 leads to AKT partial activation by phosphorylation at position Thr308 (catalytic loop) and complete activation occurs when there is also phosphorylation at position Ser473 (C-terminus regulatory domain) (Luo *et* Cantley, 2005; Hernandez-Aya *et* Gonzalez-Angulo, 2011). AKT activation is negatively regulated by the PH domain and leucine rich repeat protein phosphatase (PHLPP), a phosphatase containing a lipid-binding pleckstrin homology domain. PHLPP dephosphorylates AKT specifically at position Ser473. Studies on the two

isoforms of PHLPP suggest that PHLPP1 interacts with AKT2 and AKT 3 (but not AKT1) and PHLPP2 with AKT1 and AKT3 (but not AKT2) (Shaw *et* Cantley, 2006; Brognard *et* Newton, 2008). Activated AKT regulates multiple proteins and as a result it also regulates multiple cellular functions leading to increased metabolism, proliferation and survival (Jiang *et* Liu, 2009).

AKT phosphorylates and so inactivates tuberous sclerosis (TSC) 2 protein which is associated with TSC1 in the TSC1-TSC2 complex (also known as hamartin and tuberin). In active status, TSC2 acts as a GTPase-activating protein (GAP). Upon phosphorylation, this complex loses its ability to activate hydrolysis of GTP bound to Ras homologue enriched in the brain (Rheb). Thus in consequence of TSC1-TSC2 complex inactivation, Rheb remains linked with GTP and can activate the mechanistic target of rapamycin (mTOR) kinase domaine at position Ser2448. The TSC1-TSC2 complex is also phosphorylated by extracellular signal-regulated kinase 1/2 (ERK1/2) and by p90 ribosomal S6 kinase 1 (RSK1) (Laplante *et* Sabatini, 2009; Pópulo *et al*, 2012). The TSC1-TSC2 complex inactivation can be reversed by DNA damage response 1 (REDD1) which is activated by hypoxia and transcriptionally dependent on hypoxia-inducible transcription factor (HIF-1). There is also evidence showing that TSC1-TSC2 complex may additionally regulate mTORC2 (Laplante *et* Sabatini, 2009; Pópulo *et al*, 2012).

AKT also phosphorylates AKT1 proline-rich substrate 1 (PRAS40) at position Thr246, which is another negative regulator of mTOR, leading to PRAS40 sequestration by 14-3-3 proteins (Oshiro *et al*, 2007; Adams *et al*, 2011; Hernandez-Aya *et* Gonzalez-Angulo, 2011). FOXO transcription factors are other AKT downstream targets that are inhibited by AKT activity leading to decreased expression of cell cycle regulating proteins. FOXO targets comprise the retinoblastoma-like 2 (RBL2), the cell cycle inhibitor p27 and FASL, among other genes (Wickenden *et* Watson, 2010). Among other AKT targets, there are glycogen synthase kinase 3 (GSK3), Bcl2 antagonist of cell death (BAD), IkappaB kinase (IKK), v-myc myelocytomatosis viral oncogene homolog (MYC) and Mdm2 p53 binding protein homolog (MDM2) (Jiang *et* Liu, 2009).

1.2.2.2.4 mTOR

mTOR exists in cells in the form of two distinct complexes with other proteins: mTORC1 and mTORC2 (Figure 3). The functions of these two complexes differ from each other, but signaling and activation is less understood in the case of mTORC2. Both mTOR complexes

contain mLST8 protein which is necessary for complete activity of mTOR (Wullschleger *et al*, 2006). The two complexes differ in response to rapamycin which blocks mTORC1 whereas mTORC2 is generally insensitive (Shaw *et* Cantley, 2006).

The mTORC1 complex consists of mTOR, a regulatory-associated protein of mTOR (Raptor), a mammalian LST8 (mLST8/GβL), a DEP-domain-containing mTOR-interacting protein (Deptor), and PRAS40 and is activated by GTP-bound Rheb. PRAS40 has also been proposed to act as an mTORC1 substrate that is phosphorylated by mTORC1 after binding to Raptor at position Ser183. In the mTORC1 complex, the mTOR acts as the catalytic subunit and Raptor

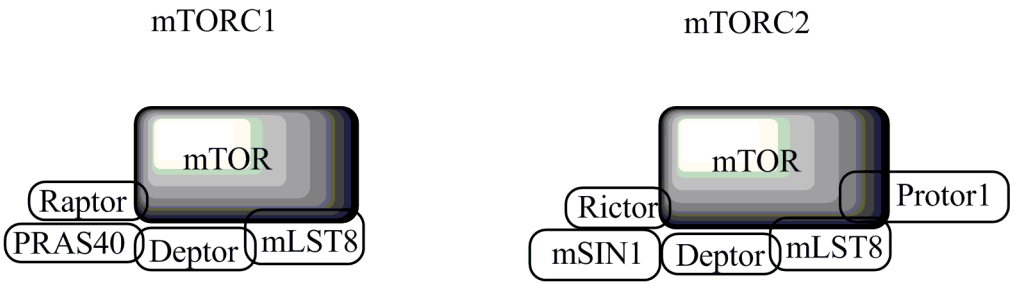

Figure 3. Schema of the two mTOR functional complexes (adapted from Pópulo *et al*, 2012).

is suggested to regulate the complex and recruit mTOR substrates (Oshiro *et al*, 2007; Laplante *et* Sabatini, 2009; Wickenden *et* Watson, 2010; Hernandez-Aya *et* Gonzalez-Angulo, 2011). However, the precise functions of the other proteins in the complex are less well known. mLST8 was proposed to regulate positively mTOR activity and might play a role in shuttling mTOR between the two complexes. PRAS40 and Deptor have also been described as mTORC1 regulators. PRAS40 was proposed to inhibit mTOR activity by preventing substrate binding. PRAS40 and Deptor were found directly phosphorylated upon mTORC1 phosphorylation which effects their interaction with mTORC1 and enables further signaling. As described above, PRAS40 can be directly phosphorylated by AKT which also leads to mTORC1 activaton in a TSC1-TSC2 independent manner (Laplante *et* Sabatini, 2009; Adams *et al,* 2011; Pópulo *et al*, 2012).

In the case of low cellular energy represented by increased AMP levels, AMP-activated protein kinase (AMPK) phosphorylates TSC2 leading to mTORC1 inhibition. AMPK in these conditions is phosphorylated and activated by serine/threonine kinase 11 (STK11, also known as LKB1). Thus, LKB1 plays a tumor suppressor role in PI3K pathway signaling. Moreover, AMPK can negatively regulate mTORC1 activity by direct phosphorylation of Raptor. AMPK is also activated by p53 in response to DNA damage (Shaw *et* Cantley, 2006; Huang *et al*, 2008; Zhong *et al*, 2008; Laplante *et* Sabatini, 2009). The Rag family of GTPases responding to amino acid levels regulates mTORC1 in a TSC-independent manner. Rag proteins assist with mTORC1 activation by binding Raptor and enabling complex shifting in the proximity of its activator Rheb. This process is disrupted in conditions of amino acid deprivation (Laplante *et* Sabatini, 2009; Adams *et al*, 2011). Low nutrient availability in cells increases autophagy and is connected with mTORC1 inhibition. On the other hand, mTORC1 activation inhibits cellular autophagy. Besides the described mechanisms of mTORC1 activation, other forms of regulation have been described through TSC1-TSC2 inactivation by pro-inflamatory cytokines or TSC1-TSC2 inactivation by Wnt signaling pathway-mediated GSK3 inhibition (Laplante *et* Sabatini, 2009).

mTORC1 activates downstream protein synthesis through regulation of proteins controlling translation: eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4EBP1) and p70 ribosomal protein S6 kinase (S6K) (Wickenden *et* Watson, 2010; Hernandez-Aya *et* Gonzalez-Angulo, 2011). Activated mTORC1 is also implicated in the regulation of other proteins leading to increase in protein translation, glucose uptake, glycolysis and other metabolic processes also in response to cellular energy status. mTORC1 activity is involved in angiogenesis by expression induction of hypoxia inducible factor 1 (HIF-1) and vascular endothelial growth factor (VEGF) (Laplante *et* Sabatini, 2009; Castaneda *et al*, 2010; Pópulo *et al*, 2012).

The mTORC2 complex is composed of mTOR, a mitogen-activated protein kinase associated protein 1 (mSIN1, also known as MAPKAP1), mLST8, proline rich 5 (Protor1, or PRR5), Deptor, and a rapamycin-insensitive companion of mTOR (Rictor). Rictor and mSIN1 have been found to stabilize each other, but functional interaction among other components of the complex are less known. mSIN1 is further important for mTORC2 activity toward AKT phosphorylation. Deptor inhibites both mTORC2 as well as mTORC1 (Laplante *et* Sabatini, 2009; Wickenden *et* Watson, 2010; Adams *et al*, 2011;

Hernandez-Aya *et* Gonzalez-Angulo, 2011). Heat shock protein 70 (Hsp70) was found to be needed for mTORC2 formation and its kinase activity (Pópulo *et al*, 2012). Recently published evidence also suggests that both mTOR complexes might be positively regulated by golgi phosphoprotein 3 (GOLPH3) activity (Scott *et al*, 2009; Scott *et* Chin, 2010).

The mTORC2 complex regulates activity of proteins implicated in cell survival and migration. The complex phosphorylates AKT at position Ser473 which is required for its full activation (Wickenden *et* Watson, 2010; Hernandez-Aya *et* Gonzalez-Angulo, 2011; Pópulo *et al*, 2012). Besides mTORC2, there is also phosphatidylinositol 3,4-bisphosphate (3,4-PIP2) that contributes to AKT phosphorylation at position Ser473. The concentration of 3,4-PIP2 is regulated in cellular cytoplasm by inositol polyphosphate 4-phosphatase type II (INPP4B) which hydrolyzes the 4-position phosphate and acts as a negative regulator and tumor suppressor of the pathway at the level of AKT phosphorylation (Agoulnik *et al*, 2011). Another role of mTORC2 is cytosceletal organization. mTORC2 has been found to regulate actin polymerization and perturbation of this mechanism leads to deviations in cellular morphology (Wullschleger *et al*, 2006; Laplante *et* Sabatini, 2009; Pópulo *et al*, 2012).

1.2.2.2.5 p70S6K

The 70 kDa ribosomal protein S6 kinase (p70S6K/S6K) is a serine/threonine kinase. S6K exists in two isoforms in humans – S6K1 and S6K2. However, it is mostly S6K1 which is found deregulated and linked to diseases such as insulin resistance and cancer (Shin *et al*, 2011). A recent study has suggested that both S6K isoforms have distinct functions in cellular signaling. The authors proposed that knockdown of S6K2 might cause AKT inhibition resulting in apoptotic cell death through the mitochondrial pathway involving BH3 interacting domain death agonist (BID) (Sridharan *et* Basu, 2011). While S6K2 plays an activating role in the PI3K pathway, S6K1 activation is involved in the pathway negative regulatory feedback by promoting IRS1 phosphorylation which leads to its destabilization and degradation (Laplante *et* Sabatini, 2009). Additionally, S6K1 has been described to regulate negatively PDGFR and the ERK/MAPK pathway (Efeyan *et* Sabatini, 2010). On the other hand, S6K1 deficiency has been proposed to protect against death receptor–mediated apoptosis in hepatocytes. Moreover, signaling through mTOR/S6K1 was shown to activate p53-dependent cell death in response to DNA damage (Sridharan *et* Basu, 2011).

Both mTORC1 and PDK1 phosphorylate S6K1 at positions Thr389 (C-terminal hydrophobic motif) and Thr229 (the kinase domain T loop), respectively, when phosphorylation by mTORC1 allows following phosphorylation by PDK1. Recently, it has been shown in cell lines including breast cancer that phosphorylation of Ser371 is essential for Thr389 phosphorylation and is positively coregulated by GSK3. S6K1 can also be coactivated by other pathways as MAPK or stress-activated protein kinase (SAPK), but phosphorylation at position Thr389 remains essential for its activity (Wullschleger *et al*, 2006; Shin *et al*, 2011; Pópulo *et al*, 2012). Activated S6K1 subsequently controls synthesis of cellular proteins by phosphorylation and activation of 40S ribosomal protein S6 (RPS6) at several sites, including Ser235 and Ser236 (Boulay *et al*, 2004; Iwenofu *et al,* 2008; Heinonen *et al*, 2008, Shin *et al*, 2011). S6K and RPS6 are crucial downstream effectors of the PI3K pathway affecting ribosome biogenesis, synthesis of cellular proteins, metabolism and cell cycle progression. S6K has been also shown to phosphorylate mTOR at positions Thr2446/Ser2448 (Wullschleger *et al*, 2006).

1.2.2.2.6 4E-BP1

Eukaryotic translation initiation factor 4E (eIF-4E) binding protein 1 (4E-BP1) is a protein that acts as a repressor of translation by negative regulation of eukaryotic initiation factor 4E (eIF-4E). This effect is mediated by control of phosphorylation status of the involved proteins. 4E-BP1 is constitutively phosphorylated at positions Ser37 and Ser46 and activated by mTORC1-mediated phosphorylation. Activation process involves phosphorylation of 4E-BP1 at positions Thr70 and Ser65, where Ser65 is the final phosphorylation site. Phosphorylated 4E-BP1 releases eIF-4E to form the initiation factor complex. eIF-4E can be additionally phosphorylated by active MAPK-interactring protein kinases 1 and 2 (MNK1 and 2; Pópulo *et al*, 2012). eIF-4E binds 5',7-methylguanosine cap of mRNAs and subsequently delivers mRNAs to the eIF4F translation initiation complex. This leads to translation of proteins involved in transition from G1 to S phase (Boulay *et al*, 2004; Rojo *et al*, 2007; Graff *et al*, 2008; Iwenofu *et al,* 2008; Pópulo *et al*, 2012). Both S6K and 4E-BP1 interact with Raptor by a TOR signaling (TOS) motif in order to become activated by the mTORC1 complex (Wullschleger *et al*, 2006).

1.2.2.3 PI3K pathway crossactivation

As mentioned earlier, crosstalk exists between RAS/MAPK and PI3K signaling. This occurs on different levels of the signaling cascades. Firstly, there is interaction of RAS and 110α proteins enabled by RAS-binding domain of p110α activating PI3K. Additionally, there is crossactivation of lower levels of PI3K pathway by RAS/MAPK components. This activation is mediated by ERK which negatively regulates TSC2 and disables its interaction with TSC1. At downstream level, there is also phosphorylation of eIF-4E mediated by the ERK activated MNK1 and MNK2 (Shaw *et* Cantley, 2006; Jiang *et* Liu, 2009; Laplante *et* Sabatini 2009; Wee *et al*, 2009; Pópulo *et al*, 2012).

Several studies have focused on cross activation between Wnt signaling and EGFR-induced pathways. Connection of these signaling pathways has been found showing the PI3K mediated activation of β-catenin and conversely Wnt-generated EGFR/PI3K/AKT activation in APC-deficient model. There is further evidence of Wnt pathway involved in expressional regulation of AKT1. In glioma, β-catenin/TCF-4 has been shown to regulate directly AKT1 expression. As mentioned earlier, TSC1-TSC2 inactivation can be mediated by GSK3 inhibition through Wnt signaling pathway. On the other hand, there are also reports disproving crosstalk potencial of Wnt and PI3K pathways caused by cellular compartmentalization of the proteins involved in both signaling pathways (Laplante *et* Sabatini 2009; Ng *et al*, 2009; Hu *et* Li, 2010; Chen *et al*, 2011).

There is also evidence of signaling cooperation between the PI3K pathway and hormonal receptors. As mediator of this was suggested to be nuclear receptor coactivator 3 (NCOA3, also called amplified in breast cancer 1, AIB1) which is a coactivator of ERα and PR activity. AIB1 is phosphorylated by GSK3 what leads to AIB1 proteosomal degradation. As described earlier, GSK3 is one of AKT downstream targets that are inhibited by AKT activity (Lahusen *et al*, 2009). Also more recent studies support the finding that the PI3K pathway plays a role in activation of steroid receptors (Riggio *et al*, 2012). Furthermore, an extra-nuclear pool of ER has been found in cytoplasm or bound to cellular membrane having an ability of direct interaction with signaling proteins as EGFR, HER2, p85 or IGF-1R (Osborne *et al*, 2005).

1.2.3 Changes in the PI3K signaling in tumors

The PI3K pathway is frequently deregulated in many cancer types including breast cancer. Tumoral changes affecting distinct levels of the signaling pathway comprise gene mutations, amplifications or epigenetic silencing as well as changes at mRNA and protein expression. Activation of the pathway has been described in up to 70% of breast cancer cases. The overview of tumoral changes affecting the PI3K pathway is given in Table 1. Interestingly, the rate of particular alterations in the pathway components differs often in breast cancer subtypes. Multiple studies have suggested association between the PI3K pathway activation, aggressive tumor features and poor patient outcome (Castaneda *et al*, 2010). Moreover, alterations of the PI3K pathway components are important also in the point of view of low incidence of changes occuring in other signaling pathways. This is the case of rare mutations in RAS (HRAS, KRAS, NRAS) proteins occuring in about 5% cases, but that are common in different cancer types as colorectal cancer and also neurofibromin 1, a negative regulator of RAS signaling pathway, that is mutated in about 3% breast tumors (Sánchez-Muñoz *et al*, 2010; Stephens *et al*, 2012; The Cancer Genome Atlas Network, 2012).

1.2.3.1 Receptor tyrosine kinases

HER family receptors have been extensively studied in breast cancer. Currently the main focus is on HER2 and EGFR, but it is only HER2 that is used as a clinical prognostic and predictive marker. HER2-positive tumors form a distinct subgroup of 20-25% breast cancers, as described above, characterized by HER2 gene amplification and/or HER2 protein overexpression. HER2 gene copy number is caused commonly by gene amplification, but chromosome 17 polysomy is also found in a considerable number of cases (20-40%). Tumors displaying gene amplification commonly also present HER2-protein overexpression. However, effect of chromosome 17 polysomy on HER2 overexpression is less clear and chromosome 17 polysomic samples have been described showing all degrees of HER2 expression intensity by immunohistochemistry. Natural history of HER2-positive breast cancer drives tumors to unfavorable prognosis of increased metastatic potential and shortened survival which can be counteracted by

Table 1. Approximate frequences of tumor changes in the receptor tyrosine kinases and the PI3K pathway components in unselected breast cancer series (Laplante *et* Sabatini, 2009; Castaneda *et al*, 2010; Wickenden *et* Watson, 2010; Baselga, 2011; Hernandez-Aya *et* Gonzalez-Angulo, 2011; The Cancer Genome Atlas Network, 2012; Pópulo *et al*, 2012; http://www.sanger.ac.uk/genetics/CGP/cosmic/ - further on referred to as Cosmic database).

Gene	Genomic localisation	Gene-affecting changes (frequency)	Expression-affecting changes (frequency)
HER2	17q21.1	amplification (20-25%) mutation (1%)	protein overexpression (20-25%)
EGFR	7p12	amplification (15%) deletion (about 30%) mutation (point mutations rare, truncating large deletions 4-78%)	protein expression (40%)
HER3	12q13	amplification (10-30%) deletion (2%) mutation (about 1%)	protein overexpression (20-30%)
HER4	2q33.3-q34	amplification (15%) deletion (7%) mutation (about 1%)	protein expression (up to 50%)
IGF-1R	15q26.3	15q26 chromosome area copy gain (10%)	protein overexpression
PDGFRa	4q12	mutation (1%)	expression (about 40%)
FGFR1	8p12	amplification (10%) mutation (1%)	protein overexpression
FGFR2		amplification (rare) mutation (1%)	protein overexpression
VEGFR1	13q12	no changes well characterized	strong protein expression (6%)
VEGFR2	4q11-q12	mutations none or rare	strong protein expression (15%)
VEGFR3	5q35.3	no changes well characterized	strong protein expression (13%)
PIK3CA	3q26.3	mutation (20-40%) amplification (8%)	protein overexpression
PIK3R1	5q13.1	mutation (5%)	mRNA expression loss (18%)
PTEN	10q23.3	mutation (5%)	decreased protein expression (50%)
PDK1	16p13.3	amplification (20%)	activated protein expression (80%)

		mutation (rare)	
AKT1	14q32.32	mutation (2-8%) amplification and deletion (rare)	activated protein expression (50%)
AKT2	19q13.1-q13.2	amplification (3%)	protein expression (up to 50%)
AKT3	1q44	amplification (rare)	protein expression
TSC1	9q34	mutation (1%)	low protein expression
TSC2	16p13.3	mutation (<1%)	low protein expression
LKB1	19p13.3	mutation (1%)	decreased protein expression (30%)
mTOR	1p36.2	mutation (1%)	activated protein overexpression (40%)
INPP4B	4q31.21	loss of heterosigosity (5-60%)	mRNA and protein expression loss (20%)
S6K1	17q23.1	amplification (30%)	cytoplasmic protein expression (15%)
S6K2	11q13.2	11q13 chromosomal area copy gain	cytoplasmic protein expression (25%)
RPS6	9p21	no changes well characterized	activated protein overexpression (72%)
4E-BP1	8p12	8p12 chromosomal area copy gain	activated protein overexpression (up to 60%)
eIF-4E	4q21-q25	amplification	increased protein expression (50%)

HER2-targeted treatment (Shah *et al*, 2009; Mukohara, 2011; Zhu *et al*, 2011; Banerji *et al*, 2012). Moreover, some rare *HER2* mutations found preferentially in HER2-negative breast cancer were identified. These amino acide substitutions or short deletions/insertions occur the most often in the region of kinase domain and in majority act as activating mutations. Such potentionally driver events might be overcome by HER2-targeted treatment (Bose *et al*, 2012).

EGFR gene status has been found changed in breast cancer in a way of gene amplification, mutation or increased gene copy number due to chromosome 7 polysomy. EGFR gene copy gain occurs in up to 15% unselected breast cancer samples, but mostly is caused by only a low gene amplification. In metaplastic breast tumors, related to triple negative and basal-like subtypes, the number of samples with EGFR gene copy gain increases up to 30%. EGFR gene copy loss was also described in 31% unselected breast cancers.

Nevertheless, chromosome 7 aneusomy has been found quite frequently reaching up to 75% depending on breast cancer series and presenting mostly as chromosome 7 polysomy. Thus, EGFR gene copy number gain has been suggested caused in considerable number by chromosome 7 polysomy (Kapranos *et al*, 2005; Gilbert *et al*, 2008; Sassen *et al*, 2008; Zaczek *et al*, 2008; Bouchalova *et al*, 2009; Kadota *et al,* 2009; Hu *et* Li, 2010). EGFR gene copy increase has been observed to present in approximately one third of EGFR expressing samples suggesting other ways of its expression regulation (Reis-Filho *et al*, 2006). *EGFR* was found underexpressed on mRNA level in about 80% unselected breast cancers in comparison with normal breast tissue (Bièche *et al*, 2003). Expression of EGFR protein is observed in up to 40% all breast cancer cases and increases in up to 80% triple negative, basal-like or metaplastic tumors (Rojo *et al*, 2007; Bouchalova *et al*, 2009; Foley *et al*, 2010). Besides whole gene changes, *EGFR* was found also mutated with varying frequency in breast cancer. Exon 19 and 21 in-frame deletions and point mutations of *EGFR*, which are well described in lung cancer, are rare in breast cancer. On the other hand, EGFRvIII mutant form has been reported in 4–78 % depending on methods and set of breast cancer tissue samples used. This mutation is generated by in-frame deletion of exons 2–7 (Moscatello *et al*, 1995; Kuan *et al*, 2001; Lynch *et al*, 2004; Nieto *et al*, 2007; Sequist *et al*, 2008; Yu *et al*, 2008). The negative prognostic effect of EGFR expression on patient survival have been found in many studies, but there are also studies that failed to find any link between EGFR expression and patient outcome. This might be caused by differing methods and cutoff levels defining EGFR positivity in the current literature which leads to results that are not easily comparable (Tsutsui *et al*, 2002; Reis-Filho *et al*, 2005; Nieto *et al*, 2007; Foley *et al*, 2010).

In breast cancer, changes on the gene level were also reported for *HER3* and *HER4* showing them amplified in 10-30% and in about 15%, respectively. Deletions of these two genes have been observed in low frequency below 10% of breast cancer cases (Zaczek *et al*, 2008). The authors of the latter study described at least one abnormal HER family gene copy number in 65% and two or more abnormal gene copy counts in 31% breast cancers. Both *HER3* and *HER4* were also found mutated in about 1% breast cancers (The Cancer Genome Atlas Network, 2012). On the mRNA level in comparison with normal breast tissue, *HER3* was found overexpressed in less than 50% breast cancers and *HER4* was found both overexpressed and underexpressed in about 30% and 25%, respectively (Bièche *et al*, 2003). HER3 protein has been observed overexpressed in 20-30% and its

overexpression associated with HR-positive status. This might play an important role in signaling of the affected cells, since HER3 receptor has been proposed to signal potently through the PI3K pathway after dimerization with other members of the HER family. However, the prognostic impact of HER3 overexpression is not clear, because poor as well as improved patient outcomes have been reported (Koutras *et al*, 2010). On the other hand, HER4 has been found to be expressed in less than 50% breast cancers. The expression of this protein may be a positive marker due to the contribution to activation of differentiation, and antiproliferative and pro-apoptotic activities. In addition, favorable patient outcome has been reported in association with *HER4* expression, but there are also contradictory reports on its negative influence on survival (Koutras *et al*, 2010).

Other tyrosine kinase receptors also undergo changes leading to signaling deregulation in breast cancer. The chromosome 15q26 harboring IGF-1R was found amplified in some breast cancer tumors. Moreover, IGF-1R overexpression was described in tumors. Similarly, the genes encoding adaptor proteins acting at intracellular domains of receptor tyrosine kinases have been found amplified or mutated with low frequency in breast cancer (Almeida *et al*, 1994; Adams *et al*, 2011; Curtis *et al*, 2012; The Cancer Genome Atlas Network, 2012). PDGFRα have been found expressed in considerable numbers of breast cancer cases and its expression associated with lymph node metastasis and HER2 and Bcl2 expression. An additional small number of tumors presented with *PDGFRa* mutation (Carvalho *et al,* 2005; Cosmic database). *FGFR1* amplification was found in about 10% of breast cancers and correlates strongly with *FGFR1* overexpression at the mRNA level, both presenting preferentially in luminal B breast cancer subtype. *FGFR1* amplification/overexpression associated with markers of poor prognosis and resistance to hormonal therapy. However, the latter effect may be mediated by MAPK pathway activation. Likewise, FGFR3 and FGFR4 expression has been linked to tamoxifen therapy resistance (Kadota *et al*, 2009; Turner *et* Grose, 2010; Karlsson *et al*, 2011; Tomlinson *et al*, 2012; Cosmic database). Gene copy gain causing overexpression and missense mutations were found in the case of FGFR2 leading to signaling activation and tumor cell proliferation and survival (Katoh *et* Katoh, 2009). Expression of VEGFR family receptors have been studied in breast cancer tissue samples showing strong protein expressions in up to 15% of breast cancers. At the DNA level, alterations in VEGFR gene family seem to be rare (Rydén *et al*, 2005; Longatto Filho *et al*, 2005; Denduluri *et al,* 2008; Schmidt *et al,* 2008).

1.2.3.2 PIK3CA

The *PIK3CA* oncogene encodes p110α, the PI3K catalytic subunit, which is particularly often found mutated in distinct cancers such as endometrial and breast cancer, accounting for up to 40% cases in both these cancer types. In breast cancer, the *PIK3CA* mutations occur in 10 to 40% of unselected cases, but frequency variations appear between breast cancer subtypes (Barbareschi *et al*, 2007; Stemke-Hale *et al*, 2008; Castaneda *et al*, 2010; Baselga, 2011; Hernandez-Aya *et* Gonzalez-Angulo, 2011). The spectrum of the mutations differ with cancer types, but most of the mutations (80% in breast cancer) are found in "hot-spots" in exons 9 (E542K and E545K) and 20 (H1047R) coding the protein's helical and kinase domain (Figure 4). E542K and E545K mutations cause release of p110α from the inhibitory interaction with p85, and H1047R mutation facilitates access of p110α to the membrane and promotes constitutive activation. Thus all three major mutations generate an increase in signaling activity (Bouchalova *et al*, 2010; Castaneda *et al*, 2010; Cheung *et al*, 2011; Banerji *et al*, 2012; Boyault *et al*, 2012).

Apropos the three hot-spot mutations, H1047R mutation of exon 20 has been reported more frequent than exon 9 mutations. *PIK3CA* mutations are the most common in HR-positive breast cancer (in around 30 to 40%) and less common in triple negative tumors (in up to 10%), while *PIK3CA* mutations are found with medium frequency in HER2-positive cases (in around 20%) (Saal *et al*, 2005; Barbareschi *et al*, 2007; Stemke-Hale *et al*, 2008). In triple negative/basal-like breast cancer, mutations in *PIK3CA* remain, despite only about 10% occurrence, second most common after *TP53* mutations (Shah *et al*,

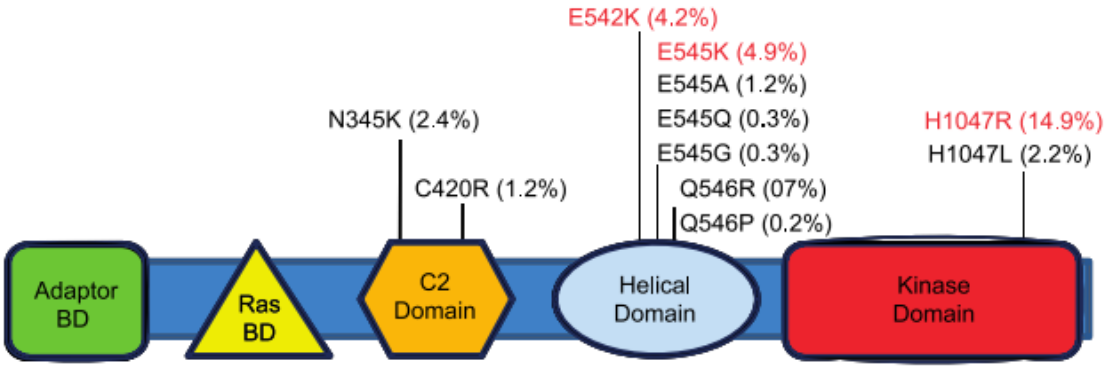

Figure 4. Frequent (red) and some rare (black) *PIK3CA* mutations found in breast cancer (adapted from Kalinsky *et al*, 2009).

2012; The Cancer Genome Atlas Network, 2012). This encourages further research on *PIK3CA* mutations in triple negative/basal-like breast cancer. Interestingly, particular mutations seem to be associated with distinct breast cancer subtypes (e.g. E545K was found predominantly in luminal A tumors). Frequencies of *PIK3CA* mutations in exons apart from 9 and 20 are reported less frequently. *PIK3CA* mutations were found with low incidence in exons 1, 4, 6, 7, 13 and 18 in breast cancer. In contrast, in endometrial cancer, the mutations are similarly the most common in helical and kinase domain, but *PIK3CA* mutations are found in about 20% also in exons coding for the adaptor-binding and C2 domains encoded by exons 1 and 4-7, respectively (Bachman *et al*, 2004; Campbell *et al*, 2004; Saal *et al*, 2005; Wu *et al*, 2005; Liedtke *et al*, 2008; Stemke-Hale *et al*, 2008; Dunlap *et al*, 2010; Cheung *et al*, 2011; The Cancer Genome Atlas Network, 2012). Double mutations present in the same tumor in two exons of *PIK3CA* gene, are uncommon in breast cancer. In the case of double mutation, one of these is usually present in exon 20 (Saal *et al*, 2005; Stemke-Hale *et al*, 2008).

It has been shown that the most common hot-spot *PIK3CA* mutations have strong oncogenic ability to activate the PI3K pathway independently of growth factor activation in mammary epithelial cells and enhance cellular growth in *in vitro* colonies as well as implanted *in vivo*. Other less common and rare *PIK3CA* mutations have oncogenic capabilities in the PI3K pathway activation varying from marked to low (Zhao *et al*, 2005; Bader *et al*, 2006; Gymnopoulos *et al*, 2007; Zhang *et al*, 2008). Similarly, mutations in particular *PIK3CA* domains present with modified abilities to interact with signaling molecules as is demonstrated in the case of helical domain mutants responding to RAS but not to p85. The opposite ability to respond to these two proteins was observed for the H1047R kinase domain mutant. Kinase domain mutations were described as changing the tertiary structure of the protein and facilitate accessibility of substrates (Adams *et al*, 2011; Dumont *et al*, 2012). However, there are also studies that found no other signs of the PI3K pathway activation in *PIK3CA* mutated breast cancer cell lines or human tumors. This suggests that *PIK3CA* mutations alone might not have the *in vivo* ability to activate downstream levels of the PI3K pathway, unlike PTEN loss that seems to be a potent activator (Stemke-Hale *et al*, 2008; Loi *et al*, 2010).

However, the results from studies based on breast cancer patient samples and clinical data show rather contradictory results. Although *PIK3CA* mutations have been shown to be associated with metastatic lymph node involvement (Saal *et al*, 2005), the prognostic

impact of *PIK3CA* mutations remains controversial. There are increasing numbers of studies suggesting that these mutations are associated with good clinicopathological characteristics and favorable clinical outcome (Baselga, 2011). However, other studies have also described no or a negative prognostic impact of these mutations or different survival impact of exon 9 and 20 mutations (Barbareschi *et al*, 2007; Stemke-Hale *et al*, 2008; Castaneda *et al*, 2010). Kalinsky et al. suggested that it is mostly exon 20 hot-spot mutations that contribute to favorable survival outcome in breast cancer patients (Kalinsky *et al*, 2009). Recently however, Mangone et al. (Mangone *et al*, 2012) reported that on the contrary exon 20 mutations might be associated with poorer prognosis. Dunlap et al. compared *in situ* and invasive ductal carcinoma samples for *PIK3CA* mutations and found concordance, suggesting that *PIK3CA* mutations are early events in breast tumor development (Saal *et al*, 2005; Dunlap *et al*, 2010; Nik-Zainal *et al*, 2012, [A]). Overall, the precise role of *PIK3CA* mutations in breast cancer remains unsolved.

PIK3CA is also amplified in many cancer types including ovarian, head and neck or urinary cancer. Increased *PIK3CA* copy number is associated with increase in its expression and PI3K enzymatic activity. Amplification of the gene occurs with considerably lower frequency than mutation, in about 8% of unselected breast cancer cases, but in up to 50% in basal-like breast tumors. *PIK3CA*-amplified cases were reported with *PIK3CA* mutation in up to 50% of cases (Campbell *et al*, 2004; Wu *et al*, 2005; Kadota *et al*, 2009; Adams *et al*, 2011; The Cancer Genome Atlas Network, 2012). Interestingly, p110 protein expression appears to play a partly opposite role to *PIK3CA* mutations. Increased p110 expression was found in hormonal receptor-negative and HER2-positive breast cancer subtypes and associated with higher tumor grade, larger tumor size and distant metastisis. Further, p110 protein expression also associated with shorter breast cancer survival and distant metastasis-free survival (Aleskandarny *et al*, 2010).

1.2.3.3 PIK3R1

The protein p85α encoded by the *PIK3R1* gene has been described as stabilizing the p110α subunit of PI3K (Yu *et al*, 1998; Shekar *et al*, 2005; Taniguchi *et al*, 2010). The *PIK3R1* gene appears to play a tumor suppressor role since loss of p85α suppressing effect on p110α leads to PI3K pathway activation at downstream levels. Data collection of mRNA array studies show *PIK3R1* expression loss in multiple solid tumors including prostate, lung,

ovarian and breast cancer, where *PIK3R1* decreased expression presented in 18% of cases. In addition, simultaneous decrease in p110α and PTEN expression has been observed with p85 loss. *PIK3R1* has also been found mutated in breast cancer, but at a considerably lower levels than in the case of *PIK3CA* mutations. Other members of the same family of proteins have been found mutated with even lower frequency than *PIK3R1* (Jaiswal *et al*, 2009; Taniguchi *et al*, 2010; Adams *et al*, 2011). *PIK3R1* C-terminal truncating and small internal deletion mutations as well as p85α expression loss lead to the pathway activation in tumor models. Further, there are also some mutations described in the N-terminal domain. These mutations were observed to increase p110α kinase activity but not affect interaction between PI3K subunits (Shekar *et al*, 2005; Luo *et* Cantley, 2005; Jaiswal *et al*, 2009; Courtney *et al*, 2010; Taniguchi *et al*, 2010). In contrast to breast cancer where *PIK3R1* mutations present in less than 5% of cases, these mutations occur in up to 24% of endometrial cancer (where the frequency increases to 43% in endometrioid endometrial cancers) and in 8% of gliomas (Figure 5). *PIK3R1* mutations are described in the majority as point mutations or short in-frame deletions that cluster in the regions of nSH2 and iSH2 domain mediating interaction with p110α. In endometrioid endometrial and breast cancers, *PIK3R1* mutations associate with *PIK3CA* wild-type status (Jaiswal *et al*, 2009; Parsons *et al*, 2008; Cheung *et al*, 2011; Urick *et al*, 2011; The Cancer Genome Atlas Network, 2012). *PIK3R1*/p85α expression could be additionally regulated by miRNA expression since few miRNAs such as miR-126 and miR-155 have been identified as potentially targeting *PIK3R1* transcripts (Bueno *et al*, 2008; Huang *et al*, 2012).

PIK3R1 loss caused the development of aggressive hepatocellular cancer in murine model with liver-specific *PIK3R1* loss (Taniguchi *et al*, 2010). At the level of cell lines, loss of *PIK3R1* mRNA expression associated with more migratory and invasive phenotype of MCF-7-14 cells compared to the parental MCF-7 cell line (Uchino *et al*, 2010). The impact of *PIK3R1* mutations and expression loss on breast cancer patient survival is not well understood. Lu et al. described a *PIK3R1*-including gene expression signature that distinguished between low and high-risk stage I lung adenocarcinoma. The authors found *PIK3R1* overexpressed in low-risk compared to high-risk lung adenocarcinoma (Lu *et al*, 2006). On the other hand, reports on glioblastomas suggested that these tumors might be negatively influenced by *PIK3R1* expression in cell lines as well as in patients (Serão *et al*, 2011, Weber *et al*, 2011).

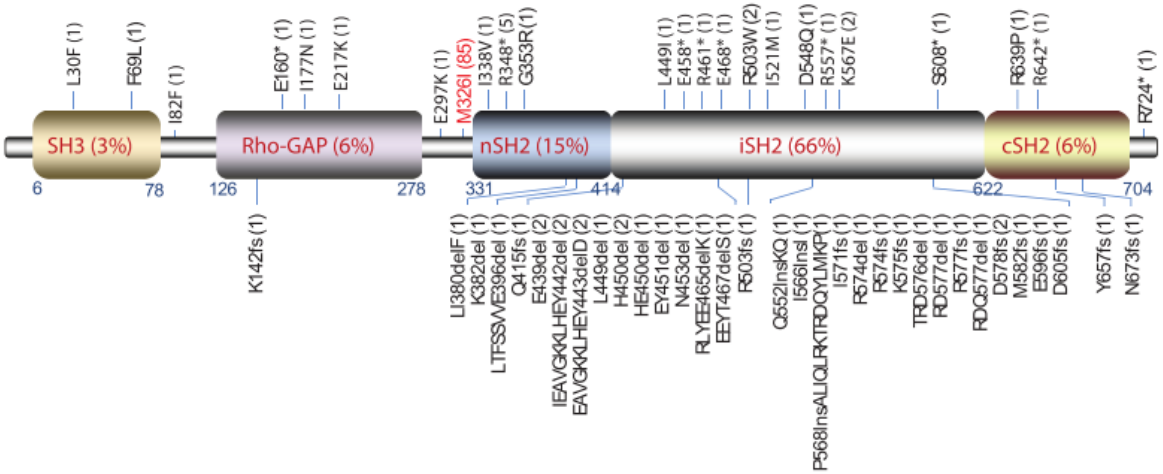

Figure 5. *PIK3R1* mutations described by Cheung *et al*, 2011 in endometroid tumors.

1.2.3.4 PTEN

PTEN gene encodes the tumor suppressor PTEN which negatively regulates PI3K signaling and its loss leads to pathway activation. PTEN expression loss has been found in many cancer types including breast cancer where loss or decreased expression presents in up to 50% of cases. In breast cancer subgroups, PTEN loss is associated with hormonal receptor negativity and is frequent in basal-like cancers. PTEN loss at the protein level has been observed accompanied by *PTEN* mRNA loss (Depowski *et al*, 2001; Bose *et al*, 2006; Marty *et al*, 2008; Bouchalova *et al*, 2010; Wickenden *et* Watson, 2010; Adams *et al*, 2011; Hernandez-Aya *et* Gonzalez-Angulo, 2011). Somatic predominantly truncating mutations cause PTEN loss in about 5% of breast cancer cases. The expression loss can be generated by promoter hypermethylation and deletion (loss of heterozygozity). Comparison with normal tissue samples confirms that DNA methylation around the transcription start site of PTEN is absent in normal and present in cancer samples. There is also growing evidence of miRNA targeting PTEN causing expression loss. PTEN loss can present as loss of heterozygozity in about 25% of patients with hereditary inactivation of one allele. However, in sporadic breast cancer, the most common cause of PTEN expression loss appears to be driven by posttranslational modifications and not genetic or epigenetic alterations which have been reported as rather rare events in unselected breast cancer cohorts (Depowski *et al*, 2001; Bueno *et al*, 2008; Castaneda *et al*, 2010; Muggerud *et al*, 2010; Wickenden *et* Watson, 2010; Adams *et al*, 2011; Boyault *et al*, 2012; Fata *et al*, 2012;

The Cancer Genome Atlas Network, 2012). On the other hand, reduced *PTEN* DNA copy number was observed in 46% basal-like breast cancers and correlated significantly with PTEN protein expression loss (Marty *et al*, 2008). Moreover, PTEN expression loss was described as one of the earliest changes and tumor evolution drivers especially in triple negative/BRCA1 (breast cancer 1, early onset gene) mutation-associated tumors (Martins *et al*, 2012). Considering other frequent changes in the PI3K pathway, it is important to point out that PTEN loss is almost mutually exclusive with *PIK3CA* and *AKT1* mutations (Saal *et al*, 2005; Stemke-Hale *et al*, 2008).

Heterozygous germline mutations of *PTEN* are found in familial cancer predisposition syndromes presenting with hamartomas such as Cowden, Bannayan-Riley-Ruvalcaba (also called Bannayan–Zonana syndrome) or Proteus-like syndromes (Shaw *et* Cantley, 2006; Blanco-Apparitio *et al*, 2007; Adams *et al*, 2011). Cowden syndrome, a disease with a frequency estimated as 1 in 200 000 individuals, is characterized by hamartomas found possibly in every organ, but the most common sites are the skin and gastrointestinal tract. Other usual features are macrocephaly, trichilemmomas and papillomatous papules and development of benign thyroid, breast and uterine lesions. Importantly, these patients are at increased risk of breast (life time risk of 50% and average age of the disease diagnoses at 36–46 years), thyroid and endometrial cancer. On the genetic level, about 80% of Cowden syndrome patients present with mutation of the *PTEN* gene, another roughly 10% have mutated *PTEN* promoter region, and in the rest, PTEN is probably inactivated by other mechanisms. Bannayan-Riley-Ruvalcaba syndrome presents with benign hamartomas, lipomas, hemangiomas, macrocephaly, developmental delay and pigmented macules of the glans penis. About 60% of patients displaying these syndrome features have *PTEN* germline mutations. Proteus syndrome is a rare and variable disease presenting with post-natal mosaic growth dysregulation consisting of progressive, asymmetric, and disproportionate overgrowth. There are reports of heterozygous germline *PTEN* mutations in Proteus syndrome patients, but there are also studies which failed to find *PTEN* mutations in syndrome patients. On the other hand, a mosaic of *AKT1* mutations has recently been described in Proteus syndrome patients (Blumenthal *et al*, 2008; Adams *et al*, 2011; Lindhurst *et al*, 2011).

The negative prognostic role of PTEN loss in patient survival has been demonstrated in multiple studies, but there are also studies which found no prognostic impact of PTEN expression loss. However, loss commonly correlates with markers of worse disease

outcome such as high tumor grade and markers of downstream PI3K pathway activation (Bose *et al*, 2006; Depowski *et al*, 2001; Castaneda *et al*, 2010; Hernandez-Aya *et* Gonzalez-Angulo, 2011). Multiple results suggest that PTEN loss might be an early event in breast cancer development since e.g. *PTEN* promoter hypermethylation has already been found in ductal carcinoma *in situ* and germline *PTEN* mutations cause cancer-predisposition syndromes (Muggerud *et al*, 2010; Hernandez-Aya *et* Gonzalez-Angulo, 2011; Adams *et al*, 2011).

1.2.3.5 PDK1

The PDK1 kinase is encoded by the *PDPK1* gene which is amplified in about 20% breast cancer samples (Maurer *et al*, 2009). Increased expression of its protein product was found in up to 72% and the phosphorylated form of the protein in about 80% breast cancer cases. Maurer et al. described slightly higher PDK1 expression in samples with *PDPK1* amplification, but these results suggested that PDK1 overexpression arises not only from the background of the gene amplification. The authors also searched for *PDPK1* gene somatic mutations and found only one (P340A) in 124 breast cancer samples, a frequency of mutations similar to that of colorectal cancer (Lin *et al*, 2005; Maurer *et al*, 2009). *PDPK1* increased copy number in the latter study associated with other upstream activating hits in the pathway signaling and increased PDK1 enhanced AKT activation (Maurer *et al*, 2009).

1.2.3.6 AKT

The *AKT1* gene rarely bears oncogenic changes. Mutation (E17K) in exon 2 is found in small number of breast cancer cases (in up to 8%). E17K mutation causes one amino acid substitution in the protein pleckstrin homology (PH) domain leading to alteration in enzymatic activity and constitutive membrane localization since the PH domain interacts with membrane bound PIP3. This mutation appears in many cancer types besides breast including thyroid and urinary tract cancer in about 4% and recently in meningiomas without mutation of neurofibromin 2 as well. E17K in breast cancer cases associates with HR-positive and luminal subtypes. Importantly, mutations in *AKT1*, *PIK3CA* and *PTEN* present mostly with mutual exclusivity (Maurer *et al*, 2009; Castaneda *et al*, 2010; Dunlap

et al, 2010; Kirkegaard *et al*, 2010; Wickenden *et* Watson, 2010; Adams *et al*, 2011; Hernandez-Aya *et* Gonzalez-Angulo, 2011; Banerji *et al*, 2012; Boyault *et al*, 2012; Clark et al, 2013; Cosmic database). Increased expression of phosphorylated AKT was observed in up to 50% of invasive breast cancers and was found associated with activation of downstream signaling proteins as S6K1 or 4E-BP1 (Zhou *et al*, 2004; Lin *et al*, 2005; Bose *et al*, 2006; Rojo *et al*, 2007; Gershtein *et al*, 2007).

As mentioned earlier, *AKT1* mutations have recently been found associated with Proteus syndrome. The syndrome is charecterized by segmental, disproportionate overgrowth and hyperplasia that appears to be caused by a somatic mutation in *AKT1* gene presenting as a mosaic disorder. The causative mutation was identified as c.49G→A. Proteus syndrome patients are also susceptible to tumor development, besides the somatic presentation of the disease (Lindhurst *et al*, 2011).

There are opposing reports on the role of AKT status in tumor cell survival and disease outcome prediction. At the level of cell migration, invasion and epithelial-mezenchymal transition, AKT activity was observed to enhance these processes. On the other hand, activated AKT1 has also been reported to block tumor cell migration and invasion. Such studies in cell cultures suggest that AKT isoforms might have distinct non-abundant activities in cells. Reports from breast cancer patient series show that phosphorylated AKT1 associated with generally unfavorable tumor features such as larger tumors with increased tumor grade and also those with ER-positive status. However, in colorectal cancer phophorylated AKT associates with low-stage tumors and favorable patient outcome (Zhou *et al*, 2004; Toker *et* Yoeli-Lerner, 2006; Gershtein *et al*, 2007; Baba *et al*, 2011).

Considering other AKT family genes, *AKT2* was described as amplified in a small number of breast cancers (about 3%). In ER-positive samples, *AKT2* gene deletion was described in 21% and AKT2 high expression in 50% which interestingly associated with better prognosis. A missense mutation corresponding to E17K in *AKT1* was also found infrequently in *AKT2* in breast cancer (Bellacosa *et al*, 1995; Kirkegaard *et al*, 2005; Kirkegaard *et al*, 2010; Courtney *et al*, 2010; Wickenden *et* Watson, 2010; Stephens *et al*, 2012). In contrast, AKT3 bears changes at the gene or protein level in breast cancer rather rarely. Amplification was observed in 10% ER-positive breast cancer samples. However, increased expression of *AKT3* was observed in triple negative/basal-like breast tumors. A

translocation of MAGI3 (membrane associated guanylate kinase, WW and PDZ domain containing 3)-AKT3 was described in a small number of breast cancer samples. Slightly increased frequency of this translocation of about 7% was observed in triple negative breast cancer. Increased expression was reported predominantly in ER-negative breast cancer cell lines. For all three *AKT* genes, their mRNA expression was observed in normal as well as tumor breast tissue samples pointing to changes at protein level as the most important events affecting the pathway signalization. However, AKT1 activation is the most important player among AKT family members in breast cancer (Nakatani *et al,* 1999; Zinda *et al*, 2001; Wu *et al*, 2008; Courtney *et al*, 2010; Kirkegaard *et al*, 2010; Banerji *et al*, 2012; The Cancer Genome Atlas Network, 2012).

1.2.3.7 PHLPP

PHLPP is a phosphatase that specifically dephosphorylates Ser473 of the AKT1 protein and it plays a role in negative regulation of AKT1 activity. Chromosomal regions coding for two related PHLPP genes were reported to carry mutations and present with loss of heterozygosity in colon, ovarian and breast cancer. Further, metastatic breast cancer cells were found to have decreased levels of PHLPP (Shaw *et* Cantley, 2006; Brognard *et* Newton, 2008).

1.2.3.8 TSC1-TSC2

Hamartin (TSC1) and tuberin (TSC2) are two proteins related by function in a complex which plays a tumor suppressor role. Since hamartin stabilizes tuberin by preventing its ubiquitination, both proteins are needed in the TSC1-TSC2 complex. The strongest interactions between the two proteins were observed to include amino acids 335-430 in hamartin and 1-418 in tuberin. Small in frame deletions and missense mutations disrupting interaction of the proteins have been described. Such germline mutations in either TSC1 or TSC2 lead to tuberous sclerosis, a syndrome associated with malignancy predisposition (Hodges *et al*, 2001; Jiang *et al*, 2005; Cosmic database).

Tuberous sclerosis caused by inherited mutations in *TSC1* and *TSC2* is a disease characterized by multiple hamartomatous tumors in various organs including preferentially kidneys, brain and skin. Intragenic mutations in the second allele or loss of

heterozygosity at the mutant locus have been found in hamartomas and cancers associated with tuberous sclerosis (Jiang *et al*, 2005; Cully *et al*, 2006). Familial cancer syndromes associated with inactivation of TSC1, TSC2 as well as of LKB1 or PTEN share clinical features of phakomatoses (neurocutaneous syndromes) (Shaw *et* Cantley, 2006). Decrease of hamartin and tuberin expression in breast cancer is associated with poor prognosis and positive lymph nodes (Jiang *et al*, 2005).

Studies on sporadic breast cancer tissue showed rare mutations, and decreased mRNA and protein expression of both these genes. *TSC1* mutations were observed in other cancer types such as in endometrium, bladder and urinary tract cancer showing about 33% and 18% mutated cases, respectively (Iyer *et al*, 2012; Cosmic database). One of the causal changes in tumor cells might be promoter methylation as was observed in cell lines. However, the methylation level was found to be lower in breast cancer samples (Hodges *et al*, 2001; Jiang *et al*, 2005).

1.2.3.9 LKB1

Expression of the LKB1 tumor suppressor is lost in about 30% of breast cancers (Shen *et al*, 2002). The *LKB1* gene (also known as *STK11*) appears to suffer from loss of heterozygosity events, epigenetic deactivation and mutations which are however rare in sporadic breast cancer (Bignell *et al*, 1998; Forster *et al*, 2000; Zhuang *et al*, 2006; Cosmic database). Germinal mutations are found in the majority of cases of autosomal dominant Peutz-Jeghers syndrome. The inherited mutations of the *LKB1* gene mostly affect its kinase domain and present as point and truncation mutations. In syndrome-associated tumors, the other wild-type gene allele is commonly targeted by somatic mutations.

The typical characteristics of Peutz-Jeghers syndrome are gastrointestinal polyposis and mucocutaneous melanin pigmentation. This disease also increases susceptibility to other cancers apart from colon cancer. This includes pancreatic, lung, gynecological, and breast tumors (Bignell *et al*, 1998; Forster *et al*, 2000; Shen *et al*, 2002; Zhuang *et al*, 2006; Zhong *et al*, 2008). A notable frequency of *LKB1* mutations (>10%) was found in cervical, lung, skin or gastroinestinal tract cancers (Cosmic database). In a breast cancer cell line model, high LKB1 expression has been found to be associated with G1 cell cycle arrest, decrease in number of lung metastases and microvessel density as well as down-regulation in expression of VEGF and matrix metalloproteinases 2 and 9 (Shen *et al*, 2002; Zhuang *et al*,

2006). On the other hand, LKB1 has also been reported as having possibly an oncogenic role, since its activity seems needed for deactivation of some proapoptotic proteins by AKT1 (Zhong *et al*, 2008). These observations point to the complexity of cellular signaling mediated by LKB1. In a breast cancer patient series, low LKB1 associated significantly with markers of tumor aggressivity such as higher histological grade, tumor size, and presence of lymph node metastasis. Survival analysis showed shorter recurrence free survival and overall survival in case of LKB1-low tumors (Shen *et al*, 2002).

1.2.3.10 mTOR

mTOR is a central member of the PI3K signaling and a component of the two distinct protein complexes. Deregulation of mTOR in tumoral cells may be caused by overactivation of positive regulators or functional loss of negative regulators in upstream signaling levels, as was described above. Overexpression of activated mTOR has been observed in more than 40% breast cancer tumor cells (Zhou *et al*, 2004; Lin *et al*, 2005; Bose *et al*, 2006; Shin *et al*, 2011). mTOR truncating and mostly point mutations have been found in human solid tumors conferring pathway activation (Sato *et al*, 2010; Hardt *et al*, 2011; Robbins *et al*, 2011; Cosmic database). Increase in phosphorylated mTOR expression in invasive breast cancer was found to be associated with markers of worse prognosis. Its expression is repeatedly also associated with shorter survival (Zhou *et al*, 2004; Bose *et al*, 2006).

1.2.3.11 INPP4B

As a regulator of AKT activity, INPP4B has the potential to play a tumor suppressor role in cancer development. Its expression is associated with hormonal receptor status and INPP4B expression loss is presented in hormonal receptor negative and basal-like breast cancer. Its expression loss accounts for about 20% cases in unselected breast cancer series and in up to 90% in the basal-like subtype. Moreover, INPP4B loss is associated with PTEN loss which may contribute to downstream activation of the pathway (Fedele *et al*, 2010; Adams *et al*, 2011; Agoulnik *et al*, 2011). The *INPP4B* gene is located on chromosome 4q31.21 and the region 4q31.1-31.21 has been described as deleted in about 40% of primary breast cancers. Gewinner and coworkers found loss of heterozygosity in the

region 4q31.21 in 60%, 55.6%, and 5% of BRCA1-mutant, sporadic basal-like, and high grade non-basal-like tumors, respectively. Mutations of *INPP4B* were also observed in prostate and with very low frequency also in breast cancer (Gewinner *et al*, 2009; Agoulnik *et al*, 2011; The Cancer Genome Atlas Network, 2012).

In vitro studies showed increase proliferation, cellular motility and AKT activation in the case of *INPP4B* knockdown (Gewinner *et al*, 2009). INPP4B loss is associated with markers of aggressive tumors such as increased tumor grade, size and proliferation besides hormonal receptor negativity and PTEN expression loss in breast cancer samples (Fedele *et al*, 2010; Agoulnik *et al*, 2011). Moreover, loss of INPP4B expression correlates with decreased patient overall survival (Gewinner *et al*, 2009).

1.2.3.12 S6K

Ribosomal protein S6 kinase in one of the final downstream mediators of the PI3K pathway since it transmits the pathway signaling on ribosomal protein S6, but more is known about S6K1 than S6K2. Despite the localization of the coding gene in a frequently amplified area of 17q23, *S6K1* was found amplified in up to 30% of breast cancers. *S6K2* mRNA level elevation was observed along with amplification of chromosomal area 11q13 (Bärlund *et al,* 2000; Andersen *et al*, 2002; Hennessy *et al*, 2005; Karlsson *et al*, 2011; Sridharan *et* Basu, 2011). However, increase in S6K cytoplasmic expression was found in about 15% of breast tumors in the case of S6K1 and 25% in the case of S6K2. In one study (Bärlund *et al,* 2000), the gene and protein S6K1 data were compared and marked expression was found in 41.2% of *S6K1*-amplified cases and showed statistical association between *S6K1* amplification and high expression. Interestingly, both S6K1 and S6K2 display additional nuclear expression. Specifically, S6K2 nuclear expression was found in more than 50% cases and S6K1 in 8%, but with partial overlap of cytoplasmic and nuclear positivity and a limited number of cases displaying expression uniquely in cytoplasm. The authors suggested that nuclear translocation might be important for the kinase activation and signal transduction. Studies on the phosphorylated form of S6K1 found marked expression in nuclei of less than 40% of assessed breast cancer samples (Bärlund *et al,* 2000; Filonenko *et al*, 2004; Rojo *et al*, 2007; Noh *et al*, 2008; Song *et al*, 2010).

In one study (Andersen *et al,* 2002), the 17q23 chromosomic region was amplified in 14% of primary breast tumors and in 36% metastases. Additionally, statistically significant

association was found between increased copy number of the region and disease progression. Tumors with *S6K1* amplification as well as high expression were found to have worse survival than tumors without these changes in whole patient series and subpopulations. Poor prognosis was also found in patients with tumors expressing phosphorylated S6K1 (Bärlund *et al,* 2000; Andersen *et al*, 2002; Rojo *et al*, 2007; Noh *et al*, 2008).

1.2.3.13 RPS6

Ribosomal protein S6 is encoded by the *RPS6* gene located at chromosome 9. This protein is one of the important downstream effectors of the PI3K pathway. Activation of RPS6 points to increased cellular metabolism and protein synthesis. Overexpression of phosphorylated RPS6 protein was observed in up to 72% invasive breast cancers (Lin *et al*, 2005; Bose *et al*, 2006; Rojo *et al*, 2007; Song *et al*, 2010).

Overexpression of phosphorylated RPS6 is associated with poor differentiation of breast tumors and other markers of poor prognosis (Bose *et al*, 2006; Song *et al*, 2010). The negative effect of RPS6 activation on patient survival is supported by similar observations from other cancer types even if RPS6 phosphorylation seems dispensable for cancer development (Pantuck *et al*, 2007; Villanueva *et al*, 2008; Hsieh *et al*, 2010; Golfinopoulos *et al*, 2012).

1.2.3.14 4E-BP1

Like S6K, 4E-BP1 is one of the crucial downstream mediators of the PI3K pathway. Its phosphorylation leads to activation of protein translation. In unselected breast tumor samples, overexpression of phosphorlylated 4E-BP1 was observed in about 59% of samples (Zhou *et al*, 2004; Rojo *et al*, 2007). Increased mRNA expresion of *4E-BP1* was described along with 8p12 chromosomal area copy gain. However, the association of 4E-BP1 phosphorylation status with activation status of other PI3K pathway proteins is unclear. Besides results showing association of activated AKT, mTOR, S6K and 4E-BP1, there are also results showing lack of associations between phosphorylation of 4E-BP1 and upstream signaling proteins in tumor samples. These *in vitro* observations may signify that 4E-BP1 is one of integrating points between multiple cellular pathways such

as PI3K/AKT and ERK signaling (Zhou *et al*, 2004; Rojo *et al*, 2007; She *et al*, 2010; Karlsson *et al*, 2011).

High expression of *4E-BP1* mRNA was found to predict poor outcome in breast cancer patients as well as other solid tumor patients (Karlsson *et al*, 2011). Similarly, increased expression of phosphorylated 4E-BP1 correlates with higher tumor grade, size, lymph node positivity and poor prognosis (Rojo *et al*, 2007). In a study (Coleman *et al*, 2009) comparing expression of 4E-BP1 protein and its phosphorylated form, the former was associated with lower tumor grade whereas the latter with increased tumor grade, supporting results of other studies and pointing again to the important role of protein phosphorylation (Rojo *et al*, 2007; Graff *et al*, 2008; Coleman *et al*, 2009; She *et al*, 2010; Karlsson *et al*, 2011).

1.2.3.15 eIF-4E

This factor is one of key regulators of protein translation in normal as well as tumor cells. Additionally, this protein also regulates expression of some genes. eIF-4E has been found overexpressed in many tumor types in comparison with adjacent healthy tissue. Moderate to strong expression has been found in about 50% of breast cancers. The *eIF-4E* gene has been also found amplified in solid tumors including breast cancer (Sorrells *et al*, 1998; Haydon *et al*, 2000; Zhou *et al*, 2006; Holm *et al*, 2008; Coleman *et al*, 2009; Flowers *et al*, 2009). It has been found that eIF-4E overexpression triggers tumor formation in various tissue types. Moreover, its overexpression is associated with increased tumor grade and worst prognosis in breast cancer patients (Ruggero *et al*, 2004; Zhou *et al*, 2006; Holm *et al*, 2008; Graff *et al*, 2008; Coleman *et al*, 2009; Flowers *et al*, 2009).

1.2.4 PI3K pathway-targeted treatment

Since the PI3K pathway is found deregulated in many cancer types and subtypes, targeting this signaling provides promising possibilities for cancer treatment. Several molecules have been designed and tested in various solid cancers. These include monoclonal antibodies designed to block extracellular domains of tyrosine kinase receptors and small molecules inhibiting protein kinases of the signaling pathway (Table

2, Figure 6). Some of these drugs have already entered clinical practice whereas other are currently being tested in different clinical trials.

Deregulation of the pathway can explain some mechanisms of primary or aquired resistance to targeted drugs (Garrett *et* Artega, 2011; Saxena *et* Dwivedi, 2012). Newer inhibitors targeting downstream signaling, targeting more signaling molecules at the same time or effective combinations of the inhibitors might help overcome therapy resistances. This shows the importance of studying the PI3K signaling pathway and its deregulation.

Table 2. Targeted drugs in breast cancer treatment and research (Courtney *et al*, 2010; Garrett *et* Artega, 2011; Pópulo *et al*, 2012; Saxena *et* Dwivedi, 2012; Zardavas *et al*, 2013; www.fda.gov).

Drug name	Classification	Target	Clinical status of breast cancer treatment
Trastuzumab	monoclonal antibody	HER2	in use since 1998
Pertuzumab	monoclonal antibody	HER2	in use since 2012
Lapatinib	kinase inhibitor	HER2, EGFR	in use since 2007
Neratinib	kinase inhibitor	HER2, EGFR	preclinical tests and clinical trials
AMG-888, MM‑121	monoclonal antibodies	HER3	preclinical tests and clinical trials
Dalotuzumab (MK-0646), Ganitumab (AMG 479), Figitumumab (CP-751,871), Cixutumumab (IMC-A12)	monoclonal antibodies	IGF-1R	preclinical tests and clinical trials
AG 1024, NVP-AEW541	kinase inhibitors	IGF-1R	preclinical tests and clinical trials
Ramucirumab (IMC-1121B)	monoclonal antibody	VEGFR1	preclinical tests and clinical trials
BYL719, GDC-0032, INK-	kinase inhibitors	p110α	preclinical tests and clinical trials

1117, GDC-0941

GSK2636771	kinase inhibitor	p110β	preclinical tests and clinical trials
CAL-101	kinase inhibitor	p110δ	preclinical tests and clinical trials
BKM120, PX-866, GDC-0941, CH5132799, XL-147	kinase inhibitors	pan-class IA PI3K	preclinical tests and clinical trials
AZD5363, GDC-0068, GSK690693, VQD002, AT-13148, A-443654	kinase inhibitors	AKT	preclinical tests and clinical trials
MK-2206	allosteric inhibitor	AKT	preclinical tests and clinical trials
Everolimus	allosteric inhibitor	mTORC1	in use since 2012
Temsirolimus (CI-779), Ridaforolimus (MK-8669)	allosteric inhibitors	mTORC1	preclinical tests and clinical trials
Torin, PP242, PP30, Ku-0063794, OSI-027, AZD8055, WAY-600, INK-128, WYE-687, WYE-354	kinase inhibitors	mTOR	preclinical tests and clinical trials
XL-765, PI-103, NVP-BEZ235, PKI-587, BEZ235, BGT226, PF-4691502, GDC-0980, SF1126, GSK1059615	kinase inhibitors	PI3K/mTOR	preclinical tests and clinical trials

1.2.4.1 Monoclonal antibodies

Trastuzumab (Herceptin) is a humanized monoclonal antibody which has been used for breast cancer treatment in all settings in clinical practice since 1998. This treatment is indicated for patients with HER2-overexpressing and/or –amplified breast cancer. Binding of trastuzumab to HER2 receptor inhibits the pathway signaling by several mechanisms such as preventing HER2 dimerization or antibody-dependent cell-mediated cytotoxicity. Typical side effects on trastuzumab treatment are cardiomyopathy (increased incidence when administered in combination with anthracyclines), diarrhea, skin rash and infusion reactions (Arteaga *et al*, 2011; Saxena *et* Dwivedi, 2012; www.fda.gov). Pertuzumab (Perjeta) is a recombinant humanized monoclonal antibody newly approved

by the American Food and Drug Administration (FDA) for clinical use. Its target is also HER2, but pertuzumab binds to HER2 at different position than trastuzumab and blocks heterodimer formation, for which it is called as a HER dimerization inhibitor. Pertuzumab is a promising drug and has been approved in combination with trastuzumab and docetaxel for advanced HER2-positive breast cancer. In this combination, pertuzumab administration was not associated with increased incidence of cardiomyopathy (Arteaga *et al*, 2011; Saxena *et* Dwivedi, 2012; www.fda.gov).

Several mechanisms leading to HER2-targeting monoclonal antibodies and especially trastuzumab resistance have been suggested, including increased signaling through other HER family members or tyrosine kinase receptors outside HER family, expression of truncated HER2 and the PI3K pathway activation by PTEN loss or *PIK3CA* mutation (Garrett *et* Artega, 2011; Mukohara, 2011; Saxena *et* Dwivedi, 2012; Dave *et al*, 2011; Jensen *et al*, 2012). There are several drugs in clinical trials that target various levels of the signaling pathway. Inhibition of the pathway at downstream levels might help overcome such resistance and introduce new effective treatment options for clinical practice. Furthermore, an approach using monoclonal antibody linked with a cytotoxic agent is being tested to increase therapeutic potency. This is the case of trastuzumab emtansine (T-DM1), a conjugate of trastuzumab with the microtubule polymerization inhibitor DM1 (a derivate of maytansine), that delivers the cytotoxic compound more specifically to cancer cells and lowers the systemic side effects (Arteaga *et al*, 2011; Garrett *et* Artega, 2011).

Like HER family receptors, other cell membrane receptors can serve as targets for monoclonal antibodies and these drugs are being tested in clinical trials. For example figitumumab (CP-751,871), dalotuzumab (MK-0646), ganitumab (AMG 479) or cixutumumab (IMC-A12) are monoclonal antibodies against IGF-1R that have been tested in the treatment of a wide range of solid tumors. These antibodies might benefit breast cancer patients especially when given in combination with HER2 inhibitors (Haluska *et al*, 2011; Reichert, 2011; Natha, 2012; Tinoco *et al*, 2013). Another monoclonal antibody, ramucirumab (IMC-1121B), a VEGFR2 inhibitor, has also been tested alone or in combination with other active compounds in cancer treatment (Reichert, 2011). Both drugs might become useful treatment options for breast cancer patients, especially if these drugs are combined with targeted therapies that inhibit lower signaling levels.

1.2.4.2 Small molecule inhibitors

This group of drugs contains numerous small molecule tyrosine kinase and allosteric inhibitors which bind to components of the PI3K signaling pathway and block signal transduction. The compounds targeting signaling levels downstream of tyrosine kinase receptors are assumed to have antiproliferative and proapoptotic activity as well as the potential to restore sensitivity to receptor inhibitors in the case of resistance. Currently there are a large number of small molecules being tested that target different levels of the pathway.

1.2.4.2.1 HER family targeting tyrosine kinase inhibitors

Lapatinib is a tyrosine kinase inhibitor which is currently used in clinical practice for breast cancer patient treatment. It has dual specificity, inhibiting reversibly HER2 and EGFR at their intracellular tyrosine kinase sites. Lapatinib is approved for the treatment of HER2-positive advanced-stage breast cancer patients in combination with capecitabine or letrozole. Besides cardiotoxicity, hepatotoxicity, diarrhea and skin lesions are also reported on lapatinib treatment (Bouchalova *et al*, 2010; Arteaga *et al*, 2011; Saxena *et* Dwivedi, 2012; www.fda.gov). Alone as well as in combination with trastuzumab, lapatinib showed increased clinical activity over trastuzumab alone including some trastuzumab-resistant tumors. However, lapatinib resistance has been found to be partly caused by similar events to trastuzumab. Despite results showing that PTEN loss and *PIK3CA* mutations might play a smaller role in resistance to lapatinib, downstream inhibitors of the pathway might improve treatment outcomes (Arteaga *et al*, 2011; Garrett *et* Artega, 2011; Dave *et al*, 2011).

Neratinib (HKI-272) is another dual tyrosine kinase inhibitor that targets HER2 and EGFR, and is currently being tested in clinical trials. This inhibitor binds to ATP-binding site of the two receptors in a covalent, irreversible manner. Thus, neratinib has the advantage of inhibiting cells with acquired mutations in HER2-positive tumors where reversible inhibitors loose their activity. Moreover, rare *HER2* somatic mutations presenting commonly in HER2-negative cancers are sensitive to neratinib and this extends the range of neratinib efficacy beyond HER2-positive tumors (Bose *et al*, 2012). There are multiple clinical trials assessing neratinib treatment alone or in combination in advance-stage breast cancer patients. So far, diarrhea has been reported as the predominant adverse event in terms of frequency (Arteaga *et al*, 2011; Garrett *et* Artega, 2011; Saxena *et*

Dwivedi, 2012). Similarly, canertinib (CI-1033) is an irreversible pan-HER inhibitor that is highly specific to EGFR (Saxena *et* Dwivedi, 2012).

Other membrane receptors can also be blocked by tyrosine kinase inhibitors. Some of these are being tested in clinical and preclinical studies. IGF-1R kinase inhibitors are e.g. AG 1024 or NVP-AEW541 (Natha, 2012). A combination of such drugs with established breast cancer treatments such as chemotherapy and HER2-blocade could improve patient survival.

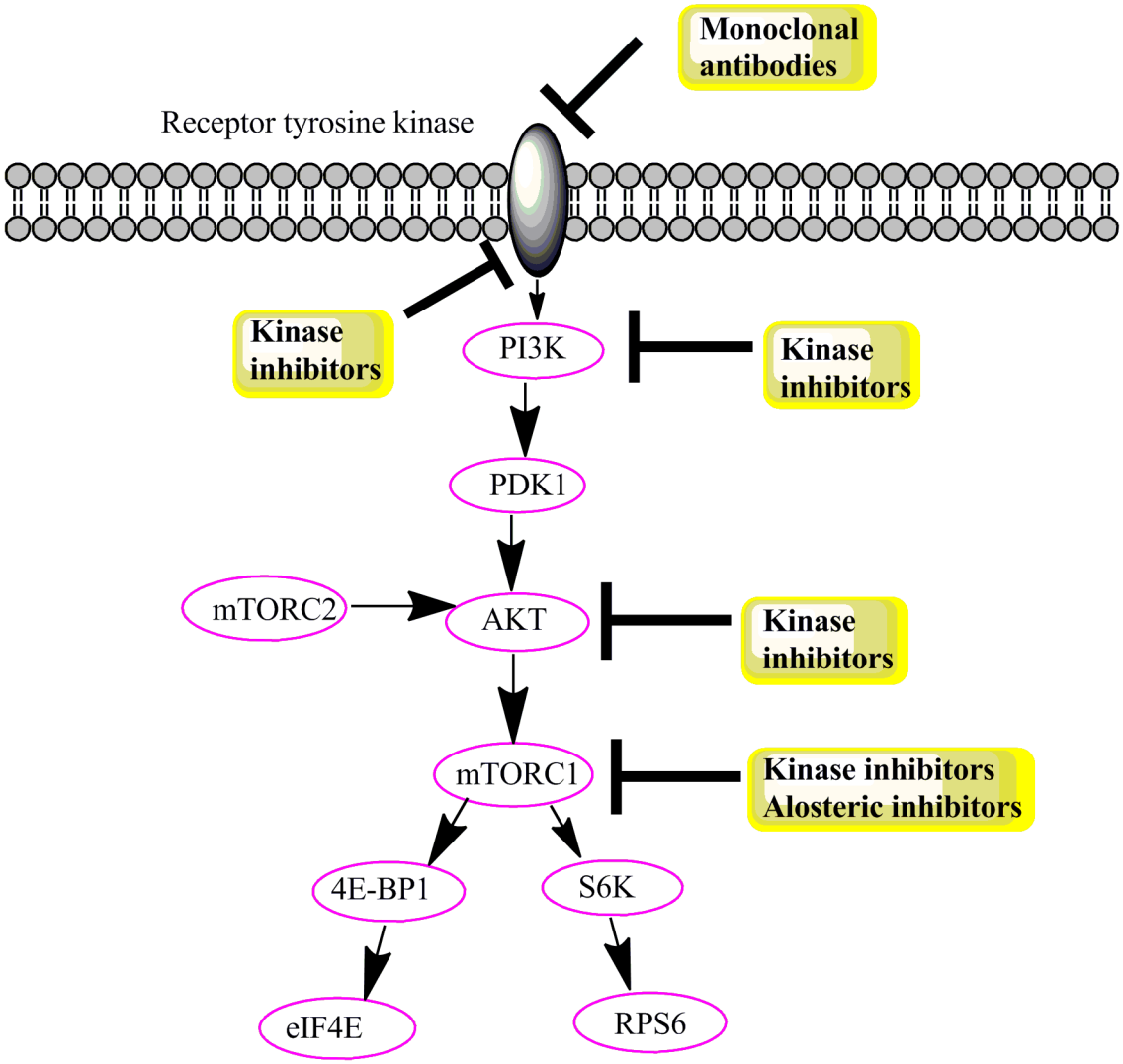

Figure 6. Scheme of therapeutic agents targeting PI3K pathway signaling (adapted from Miller *et al*, 2011; Saxena *et* Dwivedi, 2012; Artega *et al*, 2012).

1.2.4.2.2 PI3K inhibitors

PI3K inhibitors are basically divided into two subgroups: isoform-specific and pan-class IA inhibitors. BYL719, GDC-0032 and INK-1117 are specific inhibitors of p110α, GSK2636771 is a selective inhibitor of p110β and CAL-101 is a selective inhibitor of p110δ. Pan-class IA inhibitors include wortmannin derivates and prodrugs. Currently, there are several pan-class IA PI3K inhibitors being tested in low-phase clinical trials. These drugs are e.g. BKM120, CH5132799 and XL-147, which might be efficacious in trastuzumab-resistant cells (Hernandez-Aya *et* Gonzalez-Angulo, 2011; Miller *et al*, 2011; Dumont *et al*, 2012; Zardavas *et al*, 2013). Both p110α and pan-PI3K inhibitors appear to be active against *PIK3CA* mutants. However, PI3K inhibitors cause myocardial toxicity and insulin resistance causing type II diabetes. From this point of view, the subunit-specific drugs might be more active in mutated cancers and also present with a more favorable profile of side effects than pan-PI3K inhibitors avoiding or at least minimizing the effect on e.g. metabolism of glucose (Liu et al, 2009; Courtney *et al*, 2010; Wickenden *et* Watson, 2010; Arteaga *et al*, 2011; Zardavas *et al*, 2013).

1.2.4.2.3 AKT inhibitors

Several types of drugs targeting AKT have been developed, including catalytic ATP-competitive inhibitors and allosteric inhibitors. ATP-competitive inhibitors of AKT isoforms as well as pan-AKT inhibitors have been tested at the level of cell cultures and some also in low-phase clinical trials. The results of preclinical studies show that combined inhibition of AKT1 and AKT2 might be more effective and useful in breast cancer treatment. Allosteric inhibitors of AKT promote inactive conformation, and prevent AKT phosphorylation and localization to the plasma membrane. This inhibition seems more specific than kinase inhibition and some of these inhibitors such as MK-2206 have entered low-phase clinical trials. However, AKT-targeting inhibitors might increase activation of PI3K-dependent non-AKT effectors by blocking negative feedback (Courtney *et al*, 2010; Hernandez-Aya *et* Gonzalez-Angulo, 2011; Miller *et al*, 2011).

1.2.4.2.4 mTOR inhibitors

Inhibitors of mTOR can also be divided into two distinct groups: the allosteric mTOR inhibitors such as rapamycin and its analogues and the small molecule tyrosine kinase inhibitors targeting mTOR. So far, mTOR inhibitors have been reported to cause mostly

disease stabilization rather than tumor regression and cell growth arrest rather than apoptosis (Efeyan *et* Sabatini, 2010; Pópulo *et al*, 2012).

Rapamycin was isolated from the bacterium *Streptomyces hygroscopicus* and found to have anti-tumor and immunosuppressive effects. Derivatives of rapamycin with better pharmacological properties were synthesized and tested in the treatment of various solid tumor types. mTORC1 complex is considered sensitive to rapamycin whereas mTORC2 appears generally resistant, but some signs of sensitivity were found in mTORC2 in the case of long-lasting treatment. The therapeutic effect is mediated by rapamycin binding to FK506 binding protein 1A (FKBP1A, also known as FKBP12). This complex further binds to mTOR and directly inhibits mTORC1 activity but not mTORC2 where inhibition was found after prolonged treatment (Wullschleger *et al*, 2008; Efeyan *et* Sabatini, 2010; Miller *et al*, 2011; Pópulo *et al*, 2012). Rapamycin analogues that have been tested in cancer treatment include temsirolimus (CI-779), everolimus (RAD001) and ridaforolimus (MK-8669). These drugs have been used in clinical trials alone, in combination with other pathway inhibitors or with chemotherapy. Everolimus in combination with hormonal drug (aromatese inhibitor) exemestrane has been newly approved for the treatment of postmenopausal women with HR-positive, HER2-negative breast cancer after treatment failure with letrozole or anastrozole (aromatese inhibitors). Moreover, temsirolimus and everolimus have also been approved by the FDA for the treatment of renal cell carcinoma and everolimus additionally for progressive endocrine tumors of pancreatic origin and subependymal giant cell astrocytoma. Tumors appear to be particularly sensitive to everolimus in case of *TSC1* mutation. Search for other markers predictive of everolimus treatment response is ongoing including assessment of *PIK3CA*-related gene signatures (Pópulo *et al*, 2012; Baselga *et al*, 2012 [A]; Iyer *et al*, 2012; Loi *et al*, 2013; Tinoco *et al*, 2013; www.fda.gov).

However, rapamycin analogues were not found to block all mTOR functions but to stimulate AKT as well as MAPK through IRS1-mediated feedback. The anti-tumor activity of rapamycin analogues seems increased when given in combination with MAPT pathway inhibitors or other inhibitors of the PI3K pathway. Thus, such combination treatment could provide optimal results in breast cancer patient treatment and overcome resistance to trastuzumab and lapatinib (Shaw *et* Cantley, 2006; Efeyan *et* Sabatini, 2010; Castaneda *et al*, 2010; Garrett *et* Artega, 2011; Hernandez-Aya *et* Gonzalez-Angulo, 2011; Sridharan *et* B**asu**, 2011; Pópulo *et al*, 2012).

Catalytic mTOR inhibitors effectively block both mTORC1 as well as mTORC2. Thus, mTOR kinase inhibitors are more efficient than rapamycin and its analogues in blocking downstream effectors. The group of mTOR catalytic inhibitors already has multiple representatives such as Torin, PP242, WYE-687 and WYE-354. Tests conducted on animal tumor models suggest that inhibition of mTORC2 might be well tolerated and useful in cancer treatment (Efeyan *et* Sabatini, 2010; She *et al*, 2010; Garrett *et* Artega, 2011; Hernandez-Aya *et* Gonzalez-Angulo, 2011; Miller *et al*, 2011; Pópulo *et al*, 2012).

1.2.4.2.5 Dual PI3K–mTOR inhibitors

The first inhibitor of this group tested was LY-294002, but this compound is too toxic to be used in clinical patient treatment. Nevertheless, LY-294002 served well in preclinical studies to obtain information on the expected activity of PI3K-mTOR dual inhibition in tumor cells. LY-294002 and related drugs were found to inhibit the PI3K signaling pathway more effectively than rapamycin and its analogues. However, activation of MAPK signaling pathway might interfere with treatment effects suggesting therapeutic combination of inhibitors targeting these two pathways. Data from cell lines and model organisms suggest that PI3K-mTOR inhibitors could be active in tumors with HER2 overexpression, PTEN loss and *PIK3CA* mutation. Nonetheless, it remains unclear whether these inhibitors cause only tumor stasis or also regression. Currently, there are several compounds being tested in low-phase clinical trials such as XL-765, PI-103 and NVP-BEZ235. As for PI3K inhibitors, insulin resistance is the surveyed side effect even for the dual inhibitors (Courtney *et al*, 2010; Efeyan *et* Sabatini, 2010; Hernandez-Aya *et* Gonzalez-Angulo, 2011; Pópulo *et al*, 2012).

Results for the efficacy of the PI3K inhibitors downstream of membrane receptors so far show only modest activity in tumor suppression and alone cause mostly growth inhibition. Nevertheless, these inhibitors may be beneficial in clinical practice under certain conditions including *HER2*-amplification, *PIK3CA* mutations, and PTEN-deficiency as has been suggested from *in vitro* studies. As mentioned above, therapeutic targeting of the PI3K pathway is complicated by feedback activation. This occurs when mTORC1 is inhibited but there are also reports on feedback activity in the case of targeting other signaling levels of the pathway. Activation of other related pathways such as MAPK is also possible contributing to treatment failure. Combined therapy of receptor

tyrosine kinase inhibitors and lower-level pathway inhibitors could show increased potency in tumor treatment (Courtney *et al*, 2010; Arteaga *et al*, 2011; Miller *et al*, 2011). Furthermore, the PI3K pathway and its targeting have been studied in relation to hormonal and chemotherapy treatment outcome in breast cancer patients (Castaneda *et al*, 2010). Taken together, the complexity of the pathway signaling and tumor deregulations in breast cancer warrant for further understanding of the PI3K pathway changes. The work described on the following pages provides new evidence and missing knowledge about this pathway deregulation in breast cancer. The results should help as a basis for further research leading to useful treatment options.

2 Aims

The overall aim of this thesis was to obtain new information on the PI3K signaling pathway role in breast cancer development and treatment. Potential cancer markers related to the PI3K pathway were assessed in particular sub-studies to evaluate the applicability of the markers for further research and future clinical practice. Special focus was aimed at PI3K and particularly at *PIK3CA*, which codes for one of the two subunits of PI3K. *PIK3CA* mutations have been extensively studied in the recent years in cell cultures as well as in tumor samples, but discordances remain in the results describing the effects of these mutations on the pathway signaling and its prognostic and predictive role in breast cancer patients.

The work described in the following pages is divided into four main sections according to the general focus of individual 6 studies. The four sections contain research projects on *PIK3CA* mutations and PI3K pathway deregulation in breast cancer, their prognostic and predictive roles, and clinical practice from the viewpoint of trastuzumab and lapatinib treatment and EGFR assessment.

PIK3CA mutations in association with gene expression deregulation (Chapter 4.1)

Study 1. The project was focused on search for *PIK3CA* mutations-associated gene expression signature in ERα-positive breast cancer.

Prognostic role of PI3K pathway deregulation (Chapter 4.2)

Study 2. The first project of this section was focused on the prognostic role of *PIK3CA* mutations in unselected breast cancer series and its subtypes.

Study 3. The next project included assessment of *PIK3CA* and *PIK3R1* mutations and expression levels in unselected breast cancer and its subtypes. mRNA expression deregulations in other PI3K signaling components and patient survival were also studied.

HER2-targeting treatment response in HER2-positive breast cancer patients (Chapter 4.3)

Study 4. The first project in this section focused on prediction of treatment response to neoadjuvant chemotherapy with concomitant or delayed trastuzumab in association with *PIK3CA* mutations in HER2-positive breast cancer patients.

Study 5. The other project focused on lapatinib plasma levels and their impact on the treatment response in lapatinib plus capecitabine-treated HER2-positive breast cancer patients.

EGFR status assessment in archival breast cancer samples (Chapter 4.4)

Study 6. A pilot study comparing EGFR status assessment approaches.

3 Materials and Methods

The following paragraphs cover information on materials and methods that are described only briefly in the individual articles incorporated in the results section.

3.1 Materials and patient cohorts

Table 3 showes a list of patient cohorts studied and samples used in the individual projects. The patients were treated for breast cancer in France or in the Czech Republic, as is indicated in Table 3. Total RNA was extracted from liquid nitrogen-stored tumor samples. Formalin fixed-paraffin embedded (FFPE) tumor tissues were cut in 4 - 6 µm sections and used for fluorescence *in situ* hybridization marking and immunohistochemistry staining. Blood samples from lapatinib-treated patients were used for plasma separation.

3.2 Methods

3.2.1 Primer design and testing

The list of PCR primers used is shown in Table 4. Primers were chosen with the assistance of the computer program Oligo 6.0 (National Biosciences, Plymouth, MN). We performed BLASTN (Altschul *et al*, 1990) searches against dbEST and nr (the nonredundant set of GenBank, EMBL, and DDBJ database sequences) to confirm the total gene specificity of the nucleotide sequences chosen as primers. In the case of mRNA amplifying primers to avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons or in a different exon. Primer sets were, furthermore, checked on PCR reaction for a single band on agarose gel, and their products were purified and sequenced to confirm the specificity.

Table 3. Overview of samples used in particular research projects with detailed information about origin and methods used.

Number of samples	Breast cancer subtypes	Sample type *Material used*	Years of treatment	Origin *Collaboration*	Study key words
452	All subtypes	Liquid nitrogen-stored tissues *RNA extracted*	1978 to 2008	France	*PIK3CA* mutations and patient survival
458	All subtypes	Liquid nitrogen-stored tissues *RNA extracted* Formalin fixed-paraffin embedded tissues *4 µ m slides*	1978 to 2008	France *Department of pathology, St. Cloud*	*PIK3CA* and *PIK3R1* mutations, PI3K pathway components mRNA expression and patient survival
292	ERα-positive	Liquid nitrogen-stored tissues *RNA extracted*	1978 to 2008	France Ligue contre le Cancer (CIT2)	*PIK3CA* mutation-associated mRNA expression signature
28	26 HER2-positive, 2 HER2-negative	Formalin fixed-paraffin embedded tissues *4 µ m slides*	2007 to 2009	Czech Republic *Laboratory of molecular pathology, Olomouc*	EGFR assessment
55	21 HER2-positive	Blood samples *Plasma extracted*	2007 to 2009	Czech Republic *Laboratory for Inherited Metabolic Disorders,*	Lapatinib plasma levels
80	HER2-positive	Liquid nitrogen-stored tissues *RNA extracted*	2004 to 2007	France Institut Gustave Roussy, Hospital Saint-Louis, Institut Curie Paris	Neoadjuvant trastuzumab and chemotherapy, *PIK3CA* mutations, treatment response prediction

Table 4. Primers used.

Gene	Oligonucleotide	Sequence	PCR Product Size (bp)	Method
AKT1	Forward primer	5' – CCCAGGTCACGTCGGAGACT – 3'	99	q real-time RT-PCR
	Reverse primer	5' – ACTCCATGCTGTCATCTTGGTCA – 3'		
AKT1 mutation E17K	Forward primer	5' – GGAGCCTCGGGCACCATGA – 3'	199	q real-time RT-PCR, HRM
	Reverse primer	5' – GCTGGCACTGCGCCACAGA – 3'		
AKT1	Forward primer	5' – CCCAGGTCACGTCGGAGACT – 3'	99	q real-time RT-PCR, HRM
	Reverse primer	5' – ACTCCATGCTGTCATCTTGGTCA – 3'		
AKT2	Forward primer	5' – ACGGCTCCTTCATTGGGTACA – 3'	98	q real-time RT-PCR
	Reverse primer	5' – CTTCATCAGCTGGCATTCTGCTA – 3'		
AKT3	Forward primer	5' – AACAGAACGACCAAAGCCAAACACAT – 3'	114	q real-time RT-PCR
	Reverse primer	5' – GCTTCTGTCCATTCTTCCCTTTCCTC – 3'		
ALB	Forward primer	5' – GCTGTCATCTCTTGTGGGCTGT – 3'	139	q real-time RT-PCR
	Reverse primer	5' – ACTCATGGGAGCTGCTGGTTC – 3'		
ANPEP	Forward primer	5' – AACATGCTTCCCAAAGGTCCCA – 3'	76	q real-time RT-PCR
	Reverse primer	5' – GCGTGGTGTGGAACTCAGTGACA – 3'		
CYP4B1	Forward primer	5' – AGGACTTCTTCCAGTGGGATGAT – 3'	126	q real-time RT-PCR
	Reverse primer	5' – CAAAGGTGACAGGCTTGCTGA – 3'		
CYP4X1	Forward primer	5' – TCAGGACACAAGCGTGGAGGTCTA – 3'	121	q real-time RT-PCR
	Reverse primer	5' – TGCATAAGGATCATGGGTGCTGTT – 3'		
CYP4Z1	Forward primer	5' – GATGATCAGAGCCCTGCACCT – 3'	103	q real-time RT-PCR
	Reverse primer	5' – CAGCTTATGATACACCTCAAACTCCT – 3'		
CYP4Z2P	Forward primer	5' – GGTTCTATGGCCACAAGGAGTCTTA – 3'	76	q real-time RT-PCR
	Reverse primer	5' – CATGGGTATTTTTCCATCAGCTCA – 3'		
EGFR	Forward primer	5' – GGAGAACTGCCAGAAACTGACC – 3'	106	q real-time RT-PCR
	Reverse primer	5' – GCCTGCAGCACACTGGTTG – 3'		
ERα	Forward primer	5' – CCACCAACCAGTGCACCATT – 3'	108	q real-time RT-PCR
	Reverse primer	5' – GGTCTTTTCGTATCCCACCTTTC – 3'		
ERBB2	Forward primer	5' – AGCCGCGAGCACCCAAGT – 3'	147	q real-time RT-PCR
	Reverse primer	5' – TTGGTGGGCAGGTAGGTGAGTT – 3'		
GOLPH3	Forward primer	5' – CCTCCAGAAACGGTCCAGAACT – 3'	61	q real-time RT-PCR
	Reverse primer	5' – TTAATGGATTCCATGTCTCACCACTA – 3'		

HMGCS2	Forward primer Reverse primer	5' – TCCAGTTCCTGGGATGGTCGTT – 3' 5' – TACCACTGGGATAGACGGCAATGT – 3'	67	q real-time RT-PCR
ID4	Forward primer Reverse primer	5' – CCCGCTCACTGCGCTCAACA – 3' 5' – CACAGAATGCTGTCGCCCTGCTT – 3'	66	q real-time RT-PCR
LIMCH1	Forward primer Reverse primer	5' – TGGATTCCTTTGGCTCTCGCTCT – 3' 5' – GCTTCCTCTCCCATCGCTGCTT – 3'	80	q real-time RT-PCR
LTF	Forward primer Reverse primer	5' – CCTTCGCAGGACCGCTGGAT – 3' 5' – CCTGGCCACAGCTGCCTCAA – 3'	100	q real-time RT-PCR
MAPT	Forward primer Reverse primer	5' – ACACCACCCAGCTCTGGTGAA – 3' 5' – CTGCTGTAGCCGCTGCGAT – 3'	110	q real-time RT-PCR
MKI67	Forward primer Reverse primer	5' – ATTGAACCTGCGGAAGAGCTGA – 3' 5' – GGAGCGCAGGGATATTCCCTTA – 3'	105	q real-time RT-PCR
MSX2	Forward primer Reverse primer	5' – TCGCCGCCGCCAAGACATA – 3' 5' – GGCGAGGAGCTGGGATGTGGTA – 3'	102	q real-time RT-PCR
NKAIN1	Forward primer Reverse primer	5' – CTGCAGATCTTCCTGGCACTGTT – 3' 5' – AAAGCCGCCGATGAAGTCAA – 3'	99	q real-time RT-PCR
NR2F2	Forward primer Reverse primer	5' – GCCATAGTCCTGTTCACCTCAGAT – 3' 5' – GCTCCTAACGTATTCTTCCAAAGCA – 3'	105	q real-time RT-PCR
NRIP3	Forward primer Reverse primer	5' – CCAGTGTGCTGGAAAGGATGTGAA – 3' 5' – TGACATGCTCCTTGAGTCCCAATCT – 3'	104	q real-time RT-PCR
NTN4	Forward primer Reverse primer	5' – CCATGCACTGGAGGAGAGGTTA – 3' 5' – GGTTGGTGATCTTCAGCTGCTC – 3'	103	q real-time RT-PCR
P70S6K	Forward primer Reverse primer	5' – AGGACGCGGGCTCTGAGGAT – 3' 5'– ATTTCTCACAATGTTCCATGCCAAGT – 3'	108	q real-time RT-PCR
PIK3CA	Forward primer Reverse primer	5' – CCTGATCTTCCTCGTGCTGCTC – 3' 5' – ATGCCAATGGACAGTGTTCCTCTT – 3'	91	q real-time RT-PCR
PIK3CA exon 9	Forward primer Reverse primer	5' – TGGCCAGTACCTCATGGATTAGAA – 3' 5' – GAGGCCAATCTTTTACCAAGCAA – 3'	439	PCR, direct sequencing
PIK3CA exon 20	Forward primer Reverse primer	5' – ATGCACAAAGACAAGAGAATTTGA – 3' 5' – AGTGTGGAATCCAGAGTGAGCTT – 3'	341	PCR, direct sequencing
PIK3R1	Forward primer Reverse primer	5' – GATTCTCAGCAGCCAGCTCTGAT – 3' 5' – GCAGGCTGTCGTTCATTCCAT – 3'	91	q real-time RT-PCR
PIK3CA exon 1	Forward primer Reverse primer	5' – GTTACTCAAGAAGCAGAAAGGGAAGA – 3' 5' – GTTGAAAAAGCCGAAGGTCACA – 3'	73	q real-time RT-PCR, HRM

PIK3CA exon 2	Forward primer Reverse primer	5' – GTTACTCAAGAAGCAGAAAGGGAAGA – 3' 5' – CCATATCAAATTCACACACTGGCA – 3'	175	q real-time RT-PCR, HRM
PIK3R1 exons 11-13	Forward primer Reverse primer	5' – TCCAAATACCAACAGGATCAAGT – 3' 5' – ACACCTTTTTGAGTCAACCACAT – 3'	482	q real-time RT-PCR, HRM
PIK3R1 exons 14-15	Forward primer Reverse primer	5' – GCTGAGAAAGACGAGAGACCAA – 3' 5' – CCGTCCACCACTACAGAGCA – 3'	279	q real-time RT-PCR, HRM
PDK1	Forward primer Reverse primer	5' – TCCAGATAATCTTCTCAGGACACCAT – 3' 5' – CATAAATAGCTTTAGCATCCTCAGCA – 3'	119	q real-time RT-PCR
PTEN	Forward primer Reverse primer	5' – GTGGCGGAACTTGCAATCCT – 3' 5' – ATGAACTTGTCTTCCCGTCGTGT – 3'	97	q real-time RT-PCR
REEP1	Forward primer Reverse primer	5' – AGACCGAAGTTACGATGCCCTTGT – 3' 5' – AAGGCACCCTGTCCCTTGGAA – 3'	99	q real-time RT-PCR
SEC14L2	Forward primer Reverse primer	5' – CTTCCTGAGTGAGGACACTCGTAAGA – 3' 5' – TGGTCAGGGCTGATATGTTTCAGTA – 3'	87	q real-time RT-PCR
SLC40A1	Forward primer Reverse primer	5' – TCTGGTCCTGGGAGCCATCAT – 3' 5' – AGCGAGGTCTGGGCCACTTTA – 3'	72	q real-time RT-PCR
SLC4A4	Forward primer Reverse primer	5' – CCAGCCATGACCCATAGGAATC – 3' 5' – CATGAACTTATTCTTCAGCTGGTCCTT – 3'	90	q real-time RT-PCR
TBP	Forward primer Reverse primer	5' – CACGAACCACGGCACTGATT – 3' 5' – TTTTCTTGCTGCCAGTCTGGAC – 3'	89	q real-time RT-PCR
TCF7L2	Forward primer Reverse primer	5' – TCACCGGCACACATTGTCTCTAA – 3' 5' – GGCGTGAAGTGTTCATTGCTGTA – 3'	104	q real-time RT-PCR
TFAP2B	Forward primer Reverse primer	5' – CCTAGAGACCAGGCTGCCATCAT – 3' 5' – ACCATCGTGCCGGTCCTCAT – 3'	84	q real-time RT-PCR
TMC5	Forward primer Reverse primer	5' – CAACATGCAGCTGGCCTACATCTT – 3' 5' – GGAAATACTTGGCCATGCTGAACA – 3'	89	q real-time RT-PCR
TNFRSF11B	Forward primer Reverse primer	5' – AAAGGAAATGCAACACACGACAACA – 3' 5' – TCCTCACACAGGGTAACATGTATTCCA – 3'	86	q real-time RT-PCR
TPD52	Forward primer Reverse primer	5' – ATCAAGCGGAAACTTGGAATCAAT – 3' 5' – CCACCCTTTGGCAATGTTCTGT – 3'	63	q real-time RT-PCR
TUSC3	Forward primer Reverse primer	5' – ATTATTCTGGTACTGAATGCCGCTA – 3' 5' – CGTCTTTTTCCAACATCGCCT – 3'	89	q real-time RT-PCR
VANGL2	Forward primer	5' – GGGAGCCCCTGCTGGACAA – 3'	85	q real-time

	Reverse primer	5' – GTGCCCGTTACTACTGTCGTCGTT – 3'		RT-PCR
VTCN1	Forward primer Reverse primer	5' – GGGCAGATCCTCTTCTGGAGCATAA – 3' 5' – CCCTGAAATACCAAAGCCAATGATG – 3'	87	q real-time RT-PCR
WEE1	Forward primer Reverse primer	5' – TACTCCGGATTCTTTGTTGCTTCAT – 3' 5' – GTCTTCACCACAGGAATCATTCCA – 3'	85	q real-time RT-PCR
WNT5A	Forward primer Reverse primer	5' – AGCCAATTCTTGGTGGTCGCTA – 3' 5' – TGCAGAGAGGCTGTGCTCCTATAA – 3'	83	q real-time RT-PCR

3.2.2 Polymerase chain reaction (PCR) conditions

3.2.2.1 cDNA synthesis

RNA was reverse transcribed in a final volume of 20 ml containing 13 RT buffer [500 mM each dNTP, 3 mM MgCl2, 75 mM KCl, and 50 mM Tris-HCl (pH 8.3)], 10 units of RNasinTM RNase inhibitor (Promega, Madison, WI), 10 mM DTT, 50 units of Superscript II RNase H-reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD), 1.5 mM random hexamers (Pharmacia, Uppsala, Sweden), and 1 mg of total RNA. The samples were incubated at 20°C for 10 min and 42°C for 30 min, and reverse transcriptase (RT) was inactivated by heating at 99°C for 5 min and cooling at 5°C for 5 min.

3.2.2.2 Quantitative real-time polymerase chain reaction (q PCR)

The PCR reactions intended to quantify cDNA gene expression were performed using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Courtabæuf, France). PCR was performed using the SYBR Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems, Courtabæuf, France). The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 50 cycles at 95°C for 15 s and 65°C (or 60°C depending on specific primer design) for 1 min. Specific PCR amplification products were detected by the fluorescent double-stranded DNA-binding dye, SYBR Green (Schmittgen *et al*, 2000). Experiments were performed with duplicates for each data point. All of the samples with a coefficient of variation for *Ct* value higher than 1% were retested.

3.2.2.3 High-melting resolution curve assessment

The qPCR was performed on a LightCycler 480 (Roche Diagnostics, Penzberg, Germany) using LCGreen Plus+ Melting Dye fluorescence (Biotech, Idaho Technology Inc., Salt Lake City, UT) and included the following steps: 40°C for 10 min, 95°C for 10 min, 50 cycles of 95°C for 15 s and 65°C (or 60°C depending on specific primer design) for 45 s (Rouleau *et al*, 2009). The high-melting resolution curve was obtained for the range from 60°C to 95°C, rising 1°C per second with 25 acquisitions per degree.

3.2.2.4 Polymerase chain reaction for sequencing

The polymerase chain reaction used to amplify gene exons for following direct sequencing was performed on a GeneAmp 9700 PCR Thermo Cycler (Perkin Elmer, Waltham, MA) using TaqGold polymerase (Applied Biosystems, Courtabæuf, France) and including the following steps: 95°C for 10 min, 38 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 45 s, followed by 72°C for 10 min.

3.2.3 Sequencing conditions

3.2.3.1 Direct Sanger's sequencing

The sequencing reaction was performed on a GeneAmp 9700 PCR Thermo Cycler (Perkin Elmer, Waltham, MA) using BigDye Terminator sequencing kit (Applied Biosystems, Courtabæuf, France) and including the following steps: 96°C for 1 min, 25 cycles of 96°C for 10 s, 58°C for 4 s and 60°C for 1 min. The sequences were determined on an ABI Prism 3130 automatic DNA sequencer (Applied Biosystems, Courtabæuf, France).

3.2.4 Immunohistochemistry (IHC)

Immunohistochemistry staining was performed on FFPE using EGFR (clone 111.6) mouse monoclonal antibody (NeoMarkers, Fremont, CA) diluted 1:25 using a standardized protocol (Hlobilkova *et al*, 2007). The tissue sections were treated with proteinase K (37°C, 5 minutes). The incubation with primary antibody (30 minutes) was followed by standard

indirect immunohistochemical method with Envision plus kit labelled polymer HRP (Dako, Glostrup, Denmark). Diaminobenzidine (Fluka, Buchs, Switzerland) was used as chromogenic substrate and tissues were counterstained with hematoxylin (Merk, Darmstadt, Germany). The membrane expression intensity was evaluated as 0, no staining; 1, weak; 2, moderate; and 3, strong and the proportion as pecentage of expressing cells (%).

3.2.5 Fluorescence *in situ* hybridization (FISH)

Tumor sections from FFPE tumor tissue samples immobilized on "Plus Slides" (Superfrost Plus, BDH, Germany) were baked overnight (56°C) and deparaffinized before hybridization using hydrochloric acid and sodium thiocyanate pre-treatment (Hedley *et al*, 1983; Hopman *et al*, 1991). Protease treatment was then performed using pepsin solution (2 mg/ml in saline pH 2; catalytic activity 2500–3000 U/mg, Sigma, St.Louis, MO). The slides were fixed in 10% buffered formaldehyde solution, washed twice in sodium saline citrate solution (SSC) and dried at 45–50°C.

The genetic status of EGFR and chromosome 7 were analyzed using the two-color FISH on FFPE tissue sections after deparaffinization, applying directly labelled locus specific EGFR (Orange, IntellMed Ltd., Olomouc, Czech Republic) and centromeric 7 (Green, IntellMed Ltd., Olomouc, Czech Republic) DNA probes. Tissue sections were hybridized with the probes overnight in a hybridizer (HYBriteTM, Vysis, Downers Grove, IL) at 80°C. Slides were washed in 2xSSC/0.3% NP-40 and counterstained with DAPI III (Vysis, Downers Grove, IL) (Pinkel *et al*, 1986; Mark, 1994; Mark *et al*, 1999). FISH signals were evaluated using fluorescence microscopy Olympus BX60, and computer imaging system ISIS (MetaSystems, Altlussheim, Germany). Hybridization signals of EGFR and chromosome 7 were counted in at least 60 non-overlapping nuclei per section.

3.2.6 Lapatinib plasma levels assessment

Plasma samples were obtained from 3mL of whole blood treated with an anticoagulant (ethylenediaminetetraacetic acid). After centrifugation at 2,200 rotations per minute for 5 minutes, the plasma was carefully separated from sedimented blood cells. Plasma samples were stored at -20 °C until analysis.

For the analysis, 20µL of plasma samples were deproteinized using 180µL of methanol with the addition of deuterium-labeled imatinib (Novartis, Zurich, Switzerland) as an internal standard diluted in LC-MS methanol (Sigma - Aldrich, Steinheim, Germany) to obtaind final concentration of 50ng/mL. Samples were then sonified, vortexed and shaken for 5 minutes in a thermostat agitator, followed by freezing at -20°C for 30 minutes and centrifugation at 14,000 rotations per minute for 5 minutes. Supernatant was used for the assessment of lapatinib plasma levels.

The method was validated for imatinib assessment and then optimized for lapatinib. Dionex UltiMate 3000 Rapid Separation LC (Thermo Scientific, Thermo Scientific, Waltham, MA) and API 4000™ LC/MS/MS System (AB SCIEX, Framingham, MA) were used for the analysis, separation conditions were adopted from a previously published approach (Titier *et al*, 2005). Lapatinib standard (LC Laboratories, Woburn, MA) was diluted in LC-MS methanol to obtain a final concentration of 0.5mg/mL. A C18 column filled with 1.7µm BEH particles (Waters, Milford, MA, USA), which provides high resolution of more than 100,000 theoretical plates per meter and fast separation with a retention time of 1.98 min under a back-pressure of 400 bar, was used. Tandem mass spectrometer with positive electrospray ionization operated in MRM (multiple reaction monitoring) mode was used for lapatinib determination. The ion source temperature was set to 500°C, capillary voltage of 5500 V, nebulizer gas 50 psi, auxiliary gas 50 psi, curtain gas 20 psi and highpurity nitrogen as the collision gas 6 psi. Declustering potential (11/97 V), cillision energy (47/37 V) and collision exit potential (24/26 V) were determined for lapatinib/D8-imatinib using standard solutions, respectively. Transitions *m/z* 582→366 was used for detection of lapatinib and *m/z* 502→394 for monitoring of deuterated imatinib (ISTD). This method offers linear correlation in the range of 0.1-15.0 µg/mL($y = 0.000301x - 0.0212$; $R = 0.9946$), a limit of quantification of 18.2 ng/mL (signal-to-noise ratio of 10), recovery 102.5% and 107.9% (addition of 1 and 5 µg/mL, n = 6) and within-day and between-day precisions better than 4.5% and 8.6% (n = 6).

4 Results

The research outputs are described in the following pages. In the majority of cases, the published articles are inserted. All 6 articles (published as well as only submitted) are always accompanied by a brief summary of results and a discussion taking into account recently published reports on related topics. The results section is divides as described previously into four main sub-chapters:

4.1 PIK3CA mutations in association with gene expression deregulation

4.2 Prognostic role of PI3K pathway deregulation

4.3 HER2-targeting treatment response in HER2-positive breast cancer patients

4.4 EGFR status assessment in archival breast cancer samples

4.1 PIK3CA mutations in association with gene expression deregulation

4.1.1 Gene expression profiling reveals new aspects of *PIK3CA* mutation in ERα-positive breast cancer: major implication of the Wnt signaling pathway

The study focused on *PIK3CA* mutation-related changes in gene expression at mRNA level in ERα-positive breast cancer. Mutations of *PIK3CA* exons 9 and 20 were assessed in 292 patient tumor samples on the mRNA level by direct sequencing. The first part of this study was conducted on a pangenomic oligonucleotide microarray level to obtain initial information on gene expression changes in 14 *PIK3CA* mutated and 29 *PIK3CA* wild-type ERα-positive breast tumors. The microarray analysis contained 54675 probe sets and revealed 2538 probes as up-regulated and 3586 as down-regulated. Of these, 216 up-regulated probes (153 unique genes) and 28 down-regulated probes (18 unique genes) showed at least a 2-fold change.

We further focused on gene ontology analysis of the identified genes. The DAVID database annotated the 6124 probes and categorized them by function involving mostly the regulation of transcription, cell cycling, proliferation, death, adhesion and cytoskeleton organization, and also ion binding and transport, and ATP and RNA binding activity. Two-class prediction analysis with the Prediction Analysis for Microarrays (PAM) applying a threshold of 2.81 identified 56 differentially expressed probes corresponding to 39 unique genes that best characterized *PIK3CA*-mutated and wild-type tumors. Detailed analysis of the cellular processes and pathways using Gene Ontology and Kyoto Encyclopedia of Genes and Genomes tools together with the PAM algorithm results identified 29 promising genes differentially expressed between *PIK3CA*-mutated and wild-type tumors.

The expression levels of the 29 genes selected by microarray analysis were then verified by quantitative RT-PCR in a large independent cohort of 249 ERα-positive breast tumors, of which 157 were *PIK3CA* wild-type (63%) and 92 were *PIK3CA*-mutated (37%). Almost all the tumors had a single mutation, 44 (47.8%) in exon 9 (helical domain) and 46 (50%) in exon 20 (kinase domain). Two tumors (2.2%) carried two mutations, located in exons 9 and 20 in one case, and in exon 20 in the other. Among the 26 up-regulated genes, 18 were

also up-regulated in the validation set. Among the three down-regulated genes of interest in the screening set, only one was significantly down-regulated in the validation set. The 19-gene set including one down-regulated and 18 up-regulated genes showed the best ability to classify the 249 breast tumors according to *PIK3CA* mutation status in supervised hierarchical clustering analysis. The 19-gene set included several genes involved in Wnt signaling (*WNT5A*, *TCF7L2*, *MSX2* and *TNFRSF11B*), regulation of gene transcription (*SEC14L2*, *MSX2*, *TFAP2B* and *NRIP3*) and metal ion binding (*CYP4Z1*, *CYP4Z2P*, *SLC40A1*, *LTF* and *LIMCH1*). Several of these genes have been linked to breast cancer (*MAPT*, *HMGCS2*, *NR2F2*, *TFAP2B*, *NTN4*, *SEC14L2* and *LTF*).

Current reports on the role of *PIK3CA* mutations in breast cancer patient survival showed that both exon 9 and 20 mutations associate frequently with superior survival. Dumont et al have recently suggested that the beneficial effect of the mutations might be associated mainly with ER-positive tumors since these are the most frequent and comprise the majority of evaluated cases (Dumont *et al*, 2012). However, the underlining mechanisms of this positive survival effect remain unknown. The present study revealed the 19-gene set differentially expressed between ERα-positive *PIK3CA*-mutated and wild-type breast cancer that provides new information on *PIK3CA* mutation-associated gene expression changes and also opens new possibilities for cancer treatment. This is particularly the case of the Wnt signaling pathway where four of the identified genes are implicated. This observation is supported by findings at a functional level showing adaptive transcriptional response to glucose deprivation mediated by Wnt signaling in conditions of E545K *PIK3CA* mutant (Cardone *et al*, 2012). Other identified genes also point to cellular processes that have the ability to affect tumor development and growth. Alteration in gene transcription plays an important role in cancer associated processes. MSX2 was identified as one of proteins implicated in RAS/MAPK pathway signaling that is the pathway that interacts with the PI3K pathway at several levels and is activated by upstream receptor tyrosin kinases (Pópulo *et al*, 2012; Satoh *et al*, 2012). Iron metabolism apart from other metals is growing in importance as a cause of diseases including cancer. LTF is an iron-binding glycoprotein with many effects including the ability to induce apoptosis and inhibit proliferation in cancer cells (Gibbons *et al*, 2011; Jomova *et* Valko, 2011) and thus its deregulation in *PIK3CA*-mutated ER-positive tumors might participate on the disease outcome.

The Wnt pathway is important for the regulation of proliferation, differentiation, growth and survival from the embryo stage (Reya *et* Clevers, 2005; Nteliopoulos *et al*, 2009). Furthermore, the Wnt pathway was also suggested to play a role in cancer development and progression as it takes part e.g. in epithelial-mesenchymal transition. Many studies propose that Wnt signaling crosstalk with EGFR and HER2 downstream signaling pathways such as MAPK and PI3K/AKT. The connection between Wnt and PI3K pathways has been described at different levels of the signaling cascades (Laplante *et* Sabtini, 2009; Hu *et* Li, 2010; Steelman *et al*, 2011; Khalil *et al*, 2012). On the other hand, cellular compartmentalization of GSK3, one of proteins mediating the connection between the PI3K and Wnt pathways, may prohibit interaction of the two pathways (Ng *et al*, 2009). Genetic and expression alterations in the Wnt pathway components were observed with high frequency in all breast cancer subtypes pointing to the importance of the pathway signaling changes. Interestingly, the pattern of deregulation differs in HR-positive and negative tumors suggesting various mechanisms of pathogenesis (Mukherjee *et al*, 2012). Mutations and other alterations in Wnt signaling components have also been described in many other cancer types besides breast cancer including melanomas, liver or kidney cancer (Tarapore *et al*, 2012).

Moreover, targeting Wnt signaling might improve anti-cancer treatment. Since the Wnt pathway is mostly implicated in developmental signaling of normal cells, targeting deregulations in this pathway could present with low level of side effects affecting healthy cells (Barker *et* Clevers, 2006). There are multiple approaches to Wnt pathway inhibition considered and already tested. Receptor ligands present one possible treatment target since these can be neutralized by specific antibodies or ligand production can be blocked. Wnt pathway antagonist proteins and their regulators could be also used in cancer treatment in the case of an effective way to re-establish their expression (Herr *et al*, 2012; Izrailit *et* Reedijk, 2012; Veeck *et* Dahl, 2012). Similarly, other signaling components of the Wnt pathway can be potentially targeted in cancer treatment. Interestingly, there are multiple natural compounds that present with anti-Wnt signaling activity such as flavonoids, retinoids and curcumin. Furthermore, some small molecule inhibitors targeting Wnt pathway are also being tested (Izrailit *et* Reedijk, 2012; Tarapore *et al*, 2012). Thus, studies of functional connections between the PI3K and Wnt pathways might be useful for the development of new therapeutic strategies.

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Gene Expression Profiling Reveals New Aspects of *PIK3CA* Mutation in ERalpha-Positive Breast Cancer: Major Implication of the Wnt Signaling Pathway

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Abstract

Background: The PI3K/AKT pathway plays a pivotal role in breast cancer development and maintenance. *PIK3CA*, encoding the PI3K catalytic subunit, is the oncogene exhibiting a high frequency of gain-of-function mutations leading to PI3K/AKT pathway activation in breast cancer. *PIK3CA* mutations have been observed in 30% to 40% of ERα-positive breast tumors. However the physiopathological role of *PIK3CA* mutations in breast tumorigenesis remains largely unclear.

Methodology/Principal Findings: To identify relevant downstream target genes and signaling activated by aberrant PI3K/AKT pathway in breast tumors, we first analyzed gene expression with a pangenomic oligonucleotide microarray in a series of 43 ERα-positive tumors with and without *PIK3CA* mutations. Genes of interest were then investigated in 249 ERα-positive breast tumors by real-time quantitative RT-PCR. A robust collection of 19 genes was found to be differently expressed in *PIK3CA*-mutated tumors. *PIK3CA* mutations were associated with over-expression of several genes involved in the Wnt signaling pathway (*WNT5A*, *TCF7L2*, *MSX2*, *TNFRSF11B*), regulation of gene transcription (*SEC14L2*, *MSX2*, *TFAP2B*, *NRIP3*) and metal ion binding (*CYP4Z1*, *CYP4Z2P*, *SLC40A1*, *LTF*, *LIMCH1*).

Conclusion/Significance: This new gene set should help to understand the behavior of *PIK3CA*-mutated cancers and detailed knowledge of Wnt signaling activation could lead to novel therapeutic strategies.

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Introduction

Deregulation of the phosphatidylinositol 3-kinase (PI3K) signaling pathway is frequent in human cancers. Activation of PI3K, which catalyzes inositol lipid phosphorylation to produce phosphatidylinositol-3,4,5-trisphosphate, is one of the most important downstream molecular events following tyrosine kinase receptor activation. Phosphatidylinositol-3,4,5-trisphosphate activates the serine/threonine kinase AKT, which in turn regulates several signaling pathways controlling cell survival, apoptosis, proliferation, motility, and adhesion [1]. PI3K is a heterodimeric enzyme composed of a p110α catalytic subunit encoded by the *PIK3CA* gene, and a p85 regulatory subunit encoded by the *PIK3R1* gene [2].

Gain-of-function mutations in *PIK3CA* have recently been found in several malignancies, including breast cancer [1,3,4]. *PIK3CA* is frequently mutated at "hotspots" in exons 9 and 20, corresponding to the helical (E542K and E545K) and kinase (H1047R) domains, respectively. P110α carrying a hotspot mutation has oncogenic activity, transforming primary fibroblasts in culture, inducing anchorage-independent cell growth, and causing tumors in animals [5,6].

After the *TP53* suppressor gene, the *PIK3CA* oncogene is the most frequently mutated gene in human breast cancers (up to 40% of breast tumors) [7,8]. Activating somatic mutations of other oncogenes (*EGFR*, *KRAS*, *HRAS*, *NRAF*, *BRAF* and *AKT1*) involved in downstream molecular events following tyrosine kinase receptor activation are frequent in several malignancies but rare in breast cancer. Several studies suggest that *PIK3CA* mutations are more frequent in estrogen receptor alpha (ERα)-positive breast tumors (30–40%) than in ERα-negative breast tumors (10–20%) [7].

The pathological role of these gain-of-function *PIK3CA* mutations in breast tumors, and particularly in ERα-positive breast tumors, is largely unknown. Better knowledge of *PIK3CA* mutation impact requires the identification of downstream target genes and signaling pathways activated by aberrant PI3K/AKT signaling. Here, we compared gene expression in *PIK3CA*-mutated and *PIK3CA* wild-type ERα-positive breast tumors, using a

genome-wide microarray and subsequently real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

Materials and Methods

Patients and Samples

We analyzed samples of 292 primary unilateral non metastatic ERα-positive postmenopausal breast tumors excised from women at René Huguenin Hospital (Saint-Cloud, France) from 1978 to 2008. Other characteristics of the patients are listed in Table S1. Each patient gave written informed consent and this study was approved by the Local Ethical Committee (Breast Group of René Huguenin Hospital). Immediately after surgery the tumor samples were stored in liquid nitrogen until RNA extraction. The samples analyzed contained more than 70% of tumor cells. ERα status was determined at the protein level by using biochemical methods (Dextran-coated charcoal method until 1988 and enzyme immunoassay thereafter) and was confirmed at mRNA level by real-time RT-PCR. Forty-three samples were used as a microarray and RT-PCR screening set to identify differentially expressed genes. These genes were then validated in the remaining 249 ERα-positive tumors by means of RT-PCR. Control samples consisted of eight specimens of normal breast tissue collected from women undergoing cosmetic breast surgery or adjacent normal breast tissue from breast cancer patients.

RNA extraction

Total RNA was extracted from breast tissue by using the acid-phenol guanidium method, and its quality was determined by agarose gel electrophoresis and ethidium bromide staining. The 18S and 28S RNA bands were visualized under ultraviolet light.

PIK3CA mutation screening

PIK3CA mutation screening was performed on cDNA fragments obtained by RT-PCR amplification of exons 9 and 20 and their flanking exons. Details of the primers and PCR conditions are available on request. The amplified products were sequenced with the BigDye Terminator kit on an ABI Prism 3130 automatic DNA sequencer (Applied Biosystems, Courtabœuf, France). Sequences thus obtained were compared with the corresponding cDNA reference sequence (NM_006218).

Microarray analysis

Microarray experiments used Human Genome U133 Plus 2.0 arrays from Affymetrix, containing 54675 probe sets. Gene chips were hybridized and scanned using standard Affymetrix protocols. Expression data were obtained as CEL files. BRB ArrayTools (version 3.6.0 available on http://linus.nci.nih.gov/BRB-ArrayTools.html) were used to import CEL files with Robust Method Average (RMA) normalization, and to analyze gene expression. A class comparison based on a univariate *t* test applied to log-normalized data was used to identify genes differentially expressed in breast tumors with and without *PIK3CA* mutations. Supervised class prediction analysis was implemented with the Prediction Analysis for Microarrays (PAM) algorithm to identify genes required for optimal prediction [9].

The Database for Annotation, Visualization and Integrated Discovery (DAVID, available on http://david.abcc.ncifcrf.gov/) was used to interpret the lists of differentially expressed probes and to identify statistically overrepresented biological function categories of Gene Ontology (GO) and biological pathways, as defined in the Kyoto Encyclopedia of Genes and Genomes (KEGG).

In compliance with the Minimun Information About a Microarray Experiment (MIAME) recommendations, raw data were deposited in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under series accession number GSE22035.

Real-time quantitative RT-PCR

RT-PCR was applied to the selected genes, as well as *ERα* (NM_000125), *MKI67* (NM_002417), and *TBP* (NM_003194; endogenous mRNA control). Primers and PCR conditions are available on request, and the RT-PCR protocol using the SYBR Green Master Mix kit on the ABI Prism 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) is described in detail elsewhere [10]. The relative mRNA expression level of each gene, expressed as the N-fold difference in target gene expression relative to the *TBP* gene, and termed "N*target*", was calculated as $N_{target} = 2^{\Delta Ct}{}_{sample}$. The value of the cycle threshold (ΔCt) of a given sample was determined by subtracting the average Ct value of the target gene from the average Ct value of the *TBP* gene. The N*target* values of the samples were subsequently normalized such that the median N*target* value of the normal breast samples was 1. The relative expression of each gene was characterized by the median and range, and the differences in gene expression between tumors with and without *PIK3CA* mutations were analysed for significance with the non parametric Mann-Whitney *U* test.

Clustering analysis

Hierarchical clustering analyses of gene expression and samples were performed using BRB ArrayTools. Classification performance was calculated as overall accuracy, defined as the proportion of correctly classified tumors in each cluster, using Matthews' correlation coefficient (MCC) [11]. This parameter was used to discriminate identical accuracies. The chi-square test was used to determine the statistical significance of the clustering.

Results

Analysis of differentially expressed genes in 43 ERα-positive tumors

Overview of transcriptome changes in PIK3CA-mutated tumors. To identify *PIK3CA* mutation-related genes, microarray analysis (Affymetrix U133 Plus 2.0 arrays) was first applied to 43 ERα-positive breast tumors, of which 14 were *PIK3CA*-mutated and 29 were wild-type (Table S1). We found that 6124 probes were differentially expressed between breast tumors with and without *PIK3CA* mutations, with *P* values <0.05. Of these, 2538 probes (1630 unique genes) were up-regulated (Table S2) and 3586 (2672 unique genes) were down-regulated (Table S3). Only 216 up-regulated probes (153 unique genes) and 28 down-regulated probes (18 unique genes) showed at least a 2-fold change (FC).

Gene ontology analysis of differentially expressed genes. To identify families of genes that might have significant roles related to specific biological or molecular processes, we used the DAVID database to annotate the 6124 probes and categorize them by function. As shown in Table 1, these genes were mainly involved in the regulation of transcription, cell cycling, proliferation, death, adhesion and cytoskeleton organization, and also ion binding and transport, and ATP and RNA binding activity.

The 2672 down-regulated genes were mainly associated with ATP binding, acetylation and ion transport (Table 1). Among the down-regulated genes with FC≥2, no significantly overrepresented GO categories appeared.

Table 1. Selected categories significantly over-represented in *PIK3CA*-mutated breast tumors.

	Up- and down-regulated genes		Up-regulated genes		Down-regulated genes	
Gene Category	**Number of genes**	***P* value**	**Number of genes**	***P* value**	**Number of genes**	***P* value**
GENE ONTOLOGY						
• Biological Process						
Regulation of transcription	581 (14%)	0.0100	282 (17%)	<0.0001	–	–
Regulation of cell cycle and proliferation	203 (4.8%)	0.0002	94 (5.8%)	0.0004	115 (4.3%)	ns
Regulation of cell death	198 (4.7%)	0.0052	84 (5.2%)	0.0360	120 (4.5%)	0.0430
Cell adhesion	171 (4.1%)	0.0073	81 (5.0%)	0.0027	94 (3.5%)	ns
Ion transport	169 (4.0%)	ns	–	–	130 (4.9%)	0.0003
Cytoskeleton organization	116 (2.8%)	0.0014	58 (3.6%)	0.0004	63 (2.4%)	ns
• Molecular Function						
Ion binding	936 (22%)	0.0040	417 (26%)	0.0007	543 (20%)	ns
Metal ion binding	920 (15%)	0.0019	411 (25%)	0.0003	533 (20%)	ns
Zinc ion binding	518 (22%)	0.0140	268 (16%)	<0.0001	269 (10%)	ns
ATP binding	339 (8.1%)	0.0130	130 (8.0%)	ns	218 (8.2%)	0.0048
RNA binding	182 (4.4%)	0.0009	87 (5.3%)	0.0008	108 (4.0%)	0.0260
Acetylation	–	–	–	–	378 (14%)	0.0004
KEGG PATHWAY						
Pathways in cancer	100 (2.4%)	<0.0001	55 (3.4%)	<0.0001	47 (1.1%)	ns
MAPK signaling pathway	76 (1.8%)	0.0011	32 (2.0%)	0.0200	47 (1.1%)	0.0190
Calcium signaling pathway	50 (1.2%)	0.0093	10 (0.6%)	ns	44 (1.0%)	<0.0001
Jak-STAT signaling pathway	43 (1.0%)	0.0210	17 (1.0%)	ns	28 (0.7%)	0.0470
Wnt signaling pathway	41 (1.0%)	0.0370	24 (1.5%)	0.0015	17 (0.4%)	ns
Apoptosis	27 (0.6%)	0.0130	12 (0.7%)	ns	15 (0.4%)	ns

ns: not significant.

The biological processes, molecular functions and physiological pathways of genes were obtained from the DAVID database using GOTERM_BP_FAT, GOTERM_MF_FAT and KEGG PATHWAY, respectively. The two first tools (Gene Ontology) annotated 4202 genes (1630 up- and 2672 down-regulated genes) while KEGG annotated 960 genes (385 up- and 601 down-regulated genes). The gene enrichment of a given class was measured by determining the number of genes belonging to the class in the list of significantly altered genes, weighed against the total human genome, and was tested using Fisher exact probability test. Not all significant categories are included here in order to reduce redundancy. A given gene can belong to several processes.

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Most of the 1630 up-regulated genes were involved in transcriptional regulation (17.3%) (biological process) and ion binding (25.6%) (molecular function) (Table 1). The latter included the metal ion-binding and zinc ion-binding categories (Table 1). As shown in Figure 1A, the 216 probes most strongly up-regulated in *PIK3CA*-mutated tumors (153 unique genes) belonged mainly to the ion-binding category (35.5%) but also to categories of structural molecule activity (including structural cytoskeleton constituents) (9.3%), transcription regulatory activity (9.3%) and nucleotide binding (including ATP and GTP binding) (7.5%).

In the ion-binding category, the genes corresponded to genes encoding metal ion-binding proteins in 95% of cases: 28% encoding iron ion-binding and 23% with zinc ion-binding proteins (Figure 1B), pointing to a role of ion-binding proteins, and especially iron ion-binding proteins, in breast cancer with *PIK3CA* mutations. Interestingly, the genes belonging to the metal ion-binding category (Table 2) included two families of genes that were among the most strongly up-regulated in *PIK3CA*-mutated tumors. They comprised four genes of cytochrome P450 family 4 (*CYP4Z1*, *CYP4X1*, *CYP4B1* and the pseudogene (*CYP4Z2P*) and two solute carrier genes (*SLC4A4* and *SLC40A1*). All these genes, with exception of *SLC4A4*, are associated with iron ion binding. In addition to these genes, we found on the top of the list *lactoferrin* (*LTF*), also known to be involved in iron metabolism. Among the genes encoding zinc ion-binding proteins, three (*ANPEP*, *LIMCH1* and *NR2F2*) are known to be cancer-related.

Besides *NR2F2*, five other transcription factors, all known to be involved in tumorigenesis, were identified (Table 2): (a) *TFAP2B*, a tumor suppressor gene in breast cancer [12], (b) *SEC14L2*, a gene possibly involved in the antiproliferative effect of vitamin E in cancer [13], (c) *ID4*, which has been proposed to be involved in breast cancer, inhibiting mammary epithelial cell differentiation and stimulating mammary epithelial cell growth [14], (d) *TCF7L2*, also named *TCF4*, a cancer-promoting gene involved in the Wnt signaling pathway [15], and (e) *MSX2*, a gene implicated in mammary gland and breast cancer development [16], and which is also activated by Wnt signaling [17].

These five transcriptional factors (*TFAP2B*, *SEC14L2*, *ID4*, *TCF7L2* and *MSX2*), as well as ten genes involved in metal ion binding (*CYP4Z1*, *CYP4X1*, *CYP4B1*, *CYP4Z2P*, *SLC4A4*, *SLC40A1*, *LTF*, *ANPEP*, *LIMCH1* and *NR2F2*), were selected for validation by RT-PCR.

Pathway analysis of differentially expressed genes. By applying KEGG pathway analysis to the 6124 probes differentially expressed in *PIK3CA*-mutated tumors, we identified physiological pathways directly or indirectly associated with *PIK3CA* mutations. The most significantly overrepresented pathways are shown in Table 1. In addition to signaling pathways in cancer cells, the

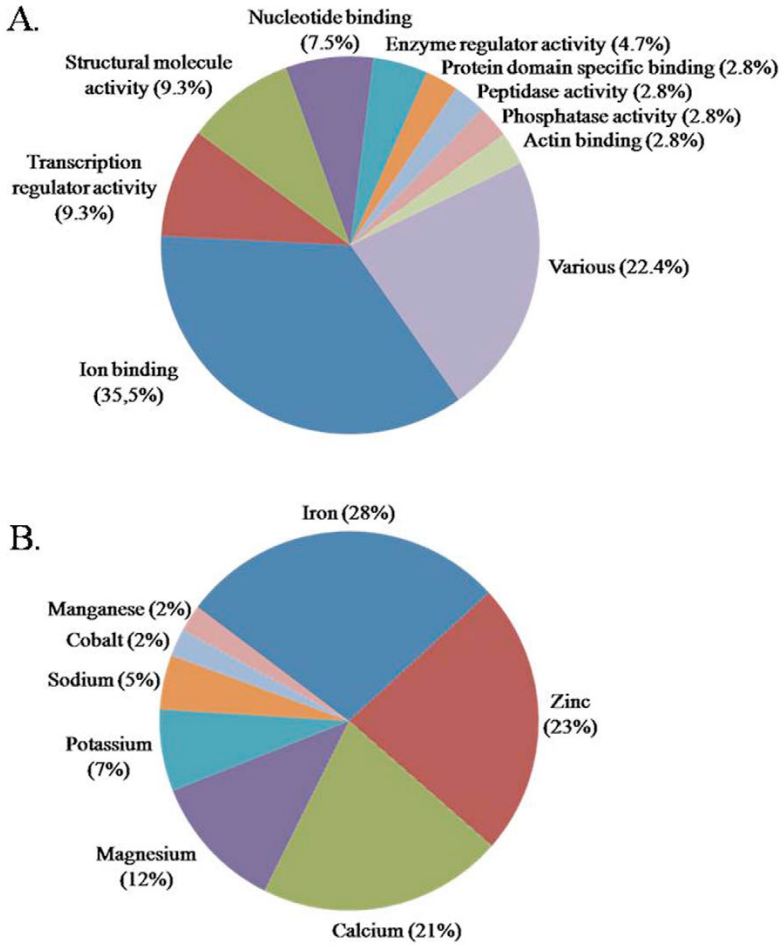

Figure 1. Molecular function classifications of genes up-regulated with a FC≥2 in the *PIK3CA*-mutated tumors. Molecular functions were attributed to 107 of the 153 genes using GOTERM_MF_FAT from the DAVID database. Categories with at least three genes are represented in A. Subclassification of the 36 genes belonging to the metal ion-binding category is shown in B. All categories were represented and several genes were common to more than one category. Genes belonging to the metal ion-binding and transcription activity categories are listed in Table 2.
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following five signaling networks were thus identified: MAPK, Calcium, Jak-STAT, Wnt and apoptosis. The Calcium signaling pathway was specifically altered by the down-regulated genes, whereas the Wnt signaling pathway was specifically altered by the up-regulated genes. The same method applied to the 216 probes (153 unique genes) that were up-regulated with FC≥2 also revealed the Wnt signaling pathway ($P = 0.015$) (data not shown), highlighting the importance of this pathway in *PIK3CA*-mutated tumors. Five major genes of the Wnt signaling were thus recognized among the 216 probes (Table S2): *MSX2* and *TCF7L2* (already cited), and *WNT5A*, *VANGL2* and *TNFRSF11B/osteoprotegerin*. These genes were also selected for RT-PCR validation.

Finally, among the genes up-regulated with FC≥2 (Table S2), we identified *PIK3R1*, the gene encoding the PI3K regulatory subunit, and two other genes of interest: *HMGCS2*, a nuclear gene encoding a mitochondrial matrix enzyme involved in ketogenesis and cholesterol synthesis, processes possibly implicated in the etiology or progression of breast cancer [18] and *MAPT*, a protein enhancing microtubule assembly and stability, that might be involved in taxane resistance [19]. These three genes were added to the RT-PCR validation set.

Two-class prediction analysis of differentially expressed genes. Two-class prediction analysis with the PAM algorithm was used to identify the group of genes that best characterized *PIK3CA*-mutated and wild-type tumors and that classified the tumors with the smallest number of predictive features. A threshold of 2.81, that minimized the error, identified 56 differentially expressed probes corresponding to 39 unique genes (Table S4). Thirty-eight of these 39 unique genes were over-expressed in ERα-positive breast tumors with *PIK3CA* mutations, 16 being up-regulated at least 3-fold, while only one gene (*NKAIN1*, encoding Na+/K+ ATPase interacting protein) was down-regulated, with a FC of 3.52. Interestingly, two major genes involved in the Wnt signaling pathway were also identified by PAM, namely *WNT5A* (the most predictive gene) and *TCF7L2*, further confirming the importance of this pathway in *PIK3CA*-mutated tumors. The previously selected up-regulated genes were almost all included in the list of the most predictive genes. PAM analysis identified five interesting new genes that were

Table 2. List of genes belonging to the metal ion-binding and transcription regulation categories.

Probe set	FC	*P* value	Gene symbol	Probe set	FC	*P* value	Gene symbol
METAL ION BINDING				221584_s_at	2.11	0.0023	*KCNMA1*
Iron ion binding				1564241_at	2.07	0.0257	*ATP1A4*
202018_s_at*	10.52	0.0005	*LTF*	230364_at	2.00	0.0217	*CHPT1*
237395_at*	7.76	0.0035	*CYP4Z1*	Sodium ion binding			
227702_at*	5.57	0.0032	*CYP4X1*	203908_at*	4.81	0.0005	*SLC4A4*
239723_at*	4.42	0.0005	*SLC40A1*	201242_s_at	2.76	0.0001	*ATP1B1*
210096_at*	4.12	0.0011	*CYP4B1*	201243_s_at	2.71	0.0002	*ATP1B1*
1553434_at*	3.80	0.0009	*CYP4Z2P*	210738_s_at*	2.13	0.0023	*SLC4A4*
225871_at	2.34	0.0188	*STEAP2*	211494_s_at*	2.13	0.0025	*SLC4A4*
1555497_a_at*	2.34	0.0061	*CYP4B1*	Potassium ion binding			
233123_at*	2.29	0.0139	*SLC40A1*	244623_at	2.30	0.0152	*KCNQ5*
223044_at*	2.26	0.0066	*SLC40A1*	221584_s_at	2.11	0.0023	*KCNMA1*
205542_at	2.17	0.0266	*STEAP1*	1564241_at	2.07	0.0257	*ATP1A4*
219232_s_at	2.15	0.0006	*EGLN3*	Cobalt ion binding			
222453_at	2.14	0.0119	*CYBRD1*	205513_at	2.87	0.0009	*TCN1*
204446_s_at	2.19	0.0003	*ALOX5*	Manganese ion binding			
224996_at	2.10	0.0135	*ASPH*	230364_at	2.00	0.0217	*CHPT1*
Zinc ion binding							
202888_s_at*	3.52	0.0008	*ANPEP*	TRANSCRIPTION REGULATION			
212774_at	2.97	0.0320	*ZNF238*	214451_at*	6.68	0.0020	*TFAP2B*
212325_at*	2.96	0.0002	*LIMCH1*	1553394_a_at*	4.34	0.0035	*TFAP2B*
225728-at	2.72	0.0141	*SORBS2*	223864_at	4.25	0.0399	*ANKRD30A*
207981_s_at	2.69	0.0213	*ESRRG*	230316_at*	3.05	0.0006	*SEC14L2*
212328_at*	2.69	0.0001	*LIMCH1*	204541_at*	3.03	0.0004	*SEC14L2*
204288_s_at	2.69	0.0073	*SORBS2*	209292_at*	3.03	0.0002	*ID4*
212327_at*	2.49	0.0008	*LIMCH1*	212774_at	2.97	0.0320	*ZNF238*
241459_at*	2.35	0.0003	*LIMCH1*	209291_at*	2.96	0.0001	*ID4*
227811_at	2.20	0.0051	*FGD3*	207981_s_at	2.69	0.0213	*ESRRG*
211965_at	2.18	0.0002	*ZFP36L1*	226847_at	2.61	0.0020	*FST*
215073_s_at*	2.08	0.0063	*NR2F2*	243030_at	2.49	0.0006	*MAP3K1*
231929_at	2.07	0.0039	*IKZF2*	226992_at	2.23	0.0064	*NOSTRIN*
214761_at	2.05	0.0016	*ZNF423*	212762_s_at*	2.18	0.0000	*TCF7L2*
Calcium ion binding				210319_x_at*	2.17	0.0011	*MSX2*
219197_s_at	3.08	0.0173	*SCUBE2*	216511_s_at*	2.16	0.0000	*TCF7L2*
204455_at	2.70	0.0065	*DST*	224975_at	2.13	0.0003	*NFIA*
229030_at	2.42	0.0370	*CAPN8*	240024_at*	2.12	0.0016	*SEC14L2*
209369_at	2.42	0.0174	*ANXA3*	209706_at	2.12	0.0292	*NKX361*
203887_s_at	2.20	0.0006	*THBD*	221666_s_at	2.09	0.0050	*PYCARD*
204446_s_at	2.19	0.0003	*ALOX5*	215073_s_at*	2.08	0.0063	*NR2F2*
224996_at	2.10	0.0135	*ASPH*	216035_x_at*	2.08	0.0000	*TCF7L2*
221584_s_at	2.11	0.0023	*KCNMA1*	231929_at	2.07	0.0039	*IKZF2*
1564241_at	2.07	0.0257	*ATP1A4*	214761_at	2.05	0.0016	*ZNF423*
Magnesium ion binding				220625_s_at	2.02	0.0286	*ELF5*
227556_at	2.99	0.0007	*NME7*	226806_s_at	2.02	0.0006	*NFIA*
243030_at	2.49	0.0006	*MAP3K1*				

These genes are ranked according to the fold change (FC) in tumors with *PIK3CA* mutations relative to non mutated tumors. Several genes were common to more than one category. The genes marked with an asterisk were selected for RT-PCR validation.

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up-regulated with FC≥3, namely *VTCN1*, *TMC5*, *NTN4*, *REEP1* and *NRIP3*, which were added to the RT-PCR validation set.

Among the down-regulated genes, *NKAIN1* was selected for RT-PCR validation, along with two other genes known to be involved in cancer biology: *TUSC3* and *TPD52*, that were among the 28 most strongly down-regulated probes (FC≥2) (Table S3) and that were also among the most predictive genes in PAM analysis with a lower FC threshold of 2.5 (data not shown).

Combined analysis of the GO, KEGG and PAM approaches identified 29 most promising genes (26 up-regulated and 3 down-regulated) for RT-PCR validation. The expression status of these genes was first confirmed in the same series of 43 breast tumors (Table 3). Strong positive correlations were observed between the microarray and RT-PCR expression levels of each gene (Spearman's correlation coefficients ranged from 0.69 to 0.97 and were all significant, at $P<0.0001$; data not shown).

mRNA expression of the 29 genes of interest in 249 ERα-positive breast tumors

Overall expression of the 29 differentially expressed genes. The expression levels of the 29 genes selected by microarray analysis were then verified by RT-PCR in a large independent cohort of 249 ERα-positive breast tumors, of which 157 were *PIK3CA* wild-type and 92 were *PIK3CA*-mutated (Table S1). This *PIK3CA* mutation frequency of 37% was in keeping with the results of previous studies showing a mutation rate of up to 40% in ERα-positive breast tumors [7,8]. Almost all the tumors had a single mutation, 44 (47.8%) in exon 9 (helical domain) and 46 (50%) in exon 20 (kinase domain) [7]. Two tumors (2.2%) carried two mutations, located in exons 9 and 20 in one case, and in exon 20 in the second case.

Nineteen (66%) of the 29 selected genes showed significantly different expression between mutated and wild-type tumors in the

Table 3. Microarray and RT-PCR analyses of the 29 genes in 43 ERα-positive breast tumors.

		Microarray analysis		RT-PCR analysis			
Symbol Gene	**GenBank**	**FC**	***P* value**	***PIK3CA* non mutated (n = 29)**	***PIK3CA* mutated (n = 14)**	**FC**	***P* value**
UP-REGULATED GENES							
*ANPEP**	NM_001150	3.52	0.0008	0.17 (0.03–1.52)	0.37 (0.10–23.7)	2.16	0.0033
*CYP4B1**	NM_000779	4.12	0.0011	3.13 (0.11–71.5)	10.6 (2.14–431)	3.40	0.0033
CYP4X1	NM_178033	5.57	0.0032	1.04 (0.05–73.3)	5.85 (0.63–97.7)	5.62	0.0124
CYP4Z1	NM_171834	7.76	0.0035	0.36 (0.01–220)	9.17 (0.10–311)	25.15	0.0085
*CYP4Z2P**	NR_002788	3.80	0.0009	34.8 (0.12–1457)	160 (22.9–2103)	4.59	0.0007
*HMGCS2**	NM_005518	5.31	0.0003	0.10 (0.00–11.1)	3.40 (0.07–16.3)	32.56	0.0011
*ID4**	NM_001546	3.03	0.0002	0.07 (0.02–0.61)	0.16 (0.05–1.03)	2.13	0.0133
*LIMCH1**	NM_014988	2.96	0.0002	0.54 (0.10–3.83)	1.66 (0.48–2.87)	3.06	0.0014
*LTF**	NM_002343	10.52	0.0005	0.03 (0.00–11.3)	0.86 (0.00–37.4)	31.54	0.0012
*MAPT**	NM_016835	2.82	0.0004	1.09 (0.02–12.1)	4.40 (0.04–10.2)	4.02	0.0010
MSX2	NM_002449	2.17	0.0011	1.74 (0.09–4.56)	3.32 (1.56–8.57)	1.91	0.0025
NR2F2	NM_021005	2.08	0.0063	0.51 (0.14–2.02)	1.06 (0.58–2.20)	2.09	0.0009
*NRIP3**	NM_020645	3.28	0.0002	0.94 (0.05–18.9)	3.64 (0.64–33.9)	3.87	0.0025
*NTN4**	NM_021229	4.21	0.0008	0.48 (0.05–3.07)	1.87 (0.68–3.19)	3.91	0.0004
*PIK3R1**	NM_181523	2.45	<0.0001	0.28 (0.07–0.89)	0.49 (0.18–1.61)	1.74	0.0053
*REEP1**	NM_022912	3.30	0.0005	1.15 (0.16–14.4)	3.49 (1.36–8.99)	3.04	0.0446
*SEC14L2**	NM_012429	3.03	0.0006	0.98 (0.13–16.1)	5.54 (0.37–24.4)	5.68	0.0049
*SLC4A4**	NM_003759	4.81	0.0005	0.28 (0.10–8.45)	3.45 (0.00–116)	12.15	0.0190
*SLC40A1**	NM_014585	4.42	0.0005	0.37 (0.11–7.79)	1.14 (0.26–6.62)	3.12	0.0068
TCF7L2	NM_030756	2.08	<0.0001	0.24 (0.00–0.64)	0.35 (0.23–0.91)	1.45	0.0010
*TFAP2B**	NM_003221	6.68	0.0020	0.09 (0.00–26.0)	1.32 (0.00–34.7)	15.23	0.0164
*TMC5**	NM_024780	4.27	0.0022	2.53 (0.05–36.4)	9.45 (1.26–37.8)	3.74	0.0177
TNFRSF11B	NM_002546	2.12	0.0023	0.67 (0.13–10.6)	2.64 (0.44–31.3)	3.91	0.0004
VANGL2	NM_020335	2.49	0.0009	0.64 (0.03–3.44)	1.90 (0.13–5.37)	2.99	0.0018
*VTCN1**	NM_024626	5.47	0.0007	0.19 (0.00–4.89)	1.12 (0.22–23.3)	5.97	0.0025
*WNT5A**	NM_003392	3.43	<0.0001	0.56 (0.05–6.03)	2.10 (0.37–6.17)	3.75	0.0013
DOWN-REGULATED GENES							
*NKAIN1**	NM_024522	−3.52	0.0006	137.8 (0.94–560)	12.13 (1.39–389)	−11.36	0.0124
TPD52	NM_005079	−2.17	0.0014	6.29 (3.13–81.8)	3.88 (1.20–11.68)	−1.62	0.0020
TUSC3	NM_006765	−2.48	0.0026	0.58 (0.09–9.35)	0.32 (0.12–0.63)	−1.83	0.0092

For each gene, we report the fold change (FC) between tumors with and without *PIK3CA* mutations. RT-PCR results are expressed as the median (range) mRNA level for each gene relative to normal breast tissues. Genes identified by PAM analysis are marked with an asterisk.

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validation cohort, with a distribution similar to that observed in the screening cohort (Table 4). Among the three down-regulated genes of interest in the screening set, only *NKAIN1* was significantly down-regulated in the validation set. Among the 26 up-regulated genes, 18 were also up-regulated in the validation set. With exception of *VANGL2*, up-regulation of the genes involved in Wnt signaling pathway, namely *WNT5A*, *MSX2*, *TCF7L2* and *TNFRSF11B*, was confirmed in the validation set, further emphasizing the important role of the Wnt signaling pathway in *PIK3CA*-mutated breast cancer. Up-regulation was also confirmed for genes related to breast cancer (*MAPT*, *HMGCS2*, *NR2F2* and *TFAP2B*), genes involved in metal ion binding (*CYP4Z1*, *CYP4Z2P*, *SLC40A1*, *LTF* and *LIMCH1*) and also *NRIP3*, *NTN4*, *REEP1*, *SEC14L2* and *TMC5*. Deregulation of these genes was not related to ERα status or proliferation since similar expression levels of *ERα* and *MKI67* were observed in *PIK3CA*-mutated and -non mutated tumors (Table 4). Only 2 of the 29 selected genes showed significantly different expression between *PIK3CA* exon 9- and exon 20-mutated tumors, namely *TFAP2B* and *NRIP3* (Table S5). Interestingly, *TFAP2B* was over-expressed in exon 20-mutated tumors and *NRIP3* in exon 9-mutated tumors.

Identification of the most discriminatory genes. PAM prediction analysis was then used to test the ability of each gene to classify the 249 ERα-positive breast tumors according to *PIK3CA* mutation status. *NKAIN1* was the most predictive gene (PAM rank) (Table 4). *NKAIN1* was also an essential classifier in supervised

Table 4. Relative mRNA expression levels of the 29 genes in 249 ERα-positive breast tumors.

Symbol Gene	GenBank	*PIK3CA* non mutated (n = 157)	*PIK3CA* mutated (n = 92)	FC	*P* value	Rank in PAM
UP-REGULATED GENES						
ANPEP	NM_001150	0.46 (0.00–154)	0.39 (0.06–18.3)	−0.84	ns	15
CYP4B1	NM_000779	6.59 (0.00–222)	5.72 (0.00–178)	−1.12	ns	20
CYP4X1	NM_178033	2.34 (0.02–59)	3.78 (0.05–101)	1.62	ns	11
CYP4Z1	NM_171834	1.15 (0.01–140)	2.97 (0.01–254)	2.58	0.0134	4
CYP4Z2P	NR_002788	38.3 (0.00–1815)	66.4 (0.00–1069)	1.74	0.0060	8
HMGCS2	NM_005518	0.29 (0.00–24.8)	0.60 (0.00–25.7)	2.09	0.0487	10
ID4	NM_001546	0.13 (0.00–9.10)	0.17 (0.02–9.57)	1.30	ns	28
LIMCH1	NM_014988	0.73 (0.05–6.59)	1.09 (0.08–8.58)	1.49	0.0007	19
LTF	NM_002343	0.08 (0.00–14.7)	0.14 (0.00–41.8)	1.74	0.0036	7
MAPT	NM_016835	3.03 (0.07–71.1)	4.52 (0.15–26.2)	1.49	0.0039	14
MSX2	NM_002449	2.26 (0.00–13.9)	3.69 (0.11–39.3)	1.63	0.0003	5
NR2F2	NM_021005	0.79 (0.06–10.8)	1.00 (0.11–7.27)	1.25	0.0415	24
NRIP3	NM_020645	1.55 (0.00–168)	2.69 (0.10–105)	1.73	0.0250	16
NTN4	NM_021229	0.75 (0.03–5.47)	1.17 (0.04–10.2)	1.57	0.0002	12
PIK3R1	NM_181523	0.32 (0.06–1.38)	0.37 (0.08–1.30)	1.16	ns	27
REEP1	NM_022912	1.85 (0.00–12.1)	2.59 (0.19–21.8)	1.40	0.0053	6
SEC14L2	NM_012429	2.49 (0.00–24.0)	4.51 (0.16–39.1)	1.81	<0.0001	2
SLC4A4	NM_003759	0.29 (0.00–178)	0.42 (0.00–128)	1.43	ns	17
SLC40A1	NM_014585	0.88 (0.03–7.81)	1.22 (0.00–17.9)	1.38	0.0311	13
TCF7L2	NM_030756	0.26 (0.03–1.05)	0.32 (0.06–1.26)	1.21	0.0373	26
TFAP2B	NM_003221	1.28 (0.00–35.7)	5.53 (0.00–179)	4.31	0.0055	3
TMC5	NM_024780	4.79 (0.01–69.0)	5.77 (0.11–46.2)	1.20	0.0331	9
TNFRSF11B	NM_002546	1.25 (0.00–50.3)	1.90 (0.15–21.8)	1.52	0.0068	21
VANGL2	NM_020335	0.73 (0.03–4.64)	0.82 (0.07–9.09)	1.12	ns	23
VTCN1	NM_024626	0.61 (0.00–10.3)	0.64 (0.01–15.4)	1.05	ns	22
WNT5A	NM_003392	0.74 (0.03–12.4)	1.17 (0.18–7.27)	1.59	<0.0001	18
DOWN-REGULATED GENES						
NKAIN1	NM_024522	81.1 (0.54–1648)	57.7 (0.71–560)	−1.41	0.0471	1
TPD52	NM_005079	6.01 (1.30–115)	5.34 (1.75–80.9)	−1.12	ns	25
TUSC3	NM_006765	0.68 (0.08–3.72)	0.63 (0.08–6.31)	−1.09	ns	29
CONTROL GENES						
ERα	NM_000125	8.77 (1.27–68.9)	8.86 (1.59–39.8)	1.01	ns	–
MKI67	NM_002417	12.1 (0.86–57.2)	11.0 (1.79–313)	0.91	ns	–

ns: not significant.
Results are expressed as the median (range) mRNA level for each gene relative to normal breast tissues. For each gene, we report the fold change (FC) between tumors with and without *PIK3CA* mutations and the PAM rank.
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hierarchical clustering analysis. Indeed, the 19-gene set including *NKAIN1* classified the 249 breast tumors significantly more accurately than the set of 18 up-regulated genes without *NKAIN1* (accuracy 59% and 57%, X^2 test P values of 0.0006 and 0.0141, respectively) (Table S6). Three different minimal sets of 4, 5 and 6 genes, all including both *NKAIN1* and *CYP4Z2P*, showed the same overall clustering accuracy of 59.4% (Table S6). However, the 5-gene group (*NKAIN1-CYP4Z2P-NRIP3-SEC14L2-TFAP2B*) had the most significant discriminatory value (MCC = 0.2334, $P = 0.0002$) correctly clustering 66 of the 92 mutated tumors and 82 of the 157 non mutated tumors. Notably, this 5-gene set contained the two genes that were differently expressed between exon 9- and exon 20-mutated tumors, and thus had the best capacity to distinguish between these two tumor categories (data not shown). The other two gene sets, both comprising genes involved in Wnt signaling (*NKAIN1-CYP4Z2P-WNT5A-TMC5* and *NKAIN1-CYP4Z2P-WNT5A-MAPT-MSX2-TFAP2B*), classified 65 mutated and 83 non mutated tumors correctly (MCC = 0.2286, $P = 0.0003$).

Discussion

We used a two-step strategy to identify downstream target genes and signaling pathways affected by *PIK3CA* mutations in breast tumors. We first applied a pangenomic oligonucleotide microarray approach to a series of 43 ERα-positive tumors with and without *PIK3CA* mutations, and then validated genes of interest by RT-PCR in an independent series of 249 ERα-positive tumors. A robust set of 19 genes differentially expressed in *PIK3CA*-mutated and wild-type tumors was thus identified.

Over-expression of several genes involved in Wnt signaling (*WNT5A*, *TCF7L2*, *MSX2* and *TNFRSF11B*), regulation of gene transcription (*SEC14L2*, *MSX2*, *TFAP2B* and *NRIP3*) and metal ion binding (*CYP4Z1*, *CYP4Z2P*, *SLC40A1*, *LTF* and *LIMCH1*) was observed in *PIK3CA*-mutated tumors. Several of these genes have been linked to breast cancer (*MAPT*, *HMGCS2*, *NR2F2*, *TFAP2B*, *NTN4*, *SEC14L2* and *LTF*).

The human Wnt signaling network is important for regulation of proliferation, differentiation, growth and survival from the embryo stage [20,21]. Crosstalk of complex pathways belonging to Wnt signaling has been observed leading to, when altered, disparate effects in different tumor types [22-24]. We observed over-expression of four major genes involved in the Wnt pathway, namely *WNT5A*, *TCF7L2*, *MSX2* and *TNFRSF11B*. *WNT5A* encodes a major Wnt ligand affecting tumor cell motility and metastasis, but its role in breast cancer is controversial [23]. The emerging view is that, in breast cancer, *WNT5A* has a suppressive effect, inhibiting migration and invasion of breast cancer cell lines [24]. Moreover, *WNT5A* over-expression observed in invasive breast tumors has been associated with a favorable outcome [24]. *PIK3CA* mutations have also been associated with favorable outcome of breast cancer patients [1,3,4,25]. We can thus suggest a link between gain-of-function mutation in *PIK3CA*, up-regulation of *WNT5A* and favorable outcome in breast cancer. We also observed over-expression of *TCF7L2*, which encodes one of the four major transcription factors involved in the Wnt signaling pathway [20,26], as well as two other genes (*MSX2* and *TNFRSF11B*) known to be downstream targets of the Wnt signaling pathway [27–29]. Wnt signaling has a major role in cancer stem cell self-renewal and tumor maintenance [20,30] and contributes to tumor invasion, metastasis and angiogenesis [31]. Recent studies have identified a role of Wnt pathway in epidermal-mesenchymal transition during breast cancer development [32,33]. Thus, Wnt pathway activation appears to be an important consequence of *PIK3CA* mutations in breast tumors, in keeping with recently observed crosstalk between the PI3K/Akt and Wnt pathways in both physiological (myeloid progenitor cells) [21] and pathological conditions (medulloblastoma) [34].

Better understanding of the biological functions of the Wnt and PI3K/Akt pathways and their interplay could have therapeutic implications for breast cancer. Drugs targeting the PI3K/Akt pathway have given promising preliminary results in human malignancies [35,36]. However, as the PI3K pathway is crucial for metabolic processes, PI3K inhibitors might also have side effects, especially affecting insulin signaling and cardiac functions [36,37]. In contrast, targeting of downstream Wnt signaling events might have fewer adverse effects, considering their crucial importance in embryonic development [23,38].

Genes encoding metal ion-binding proteins were also over-expressed in *PIK3CA*-mutated tumors. Such metal ion-binding proteins have regulatory roles in central cellular processes such as gene expression, proliferation, differentiation and survival. Increased expression of these proteins in ERα-positive breast tumors has also been reported by Abba et al. [39]. We observed over-expression of *LIMCH1*, a gene encoding zinc-binding protein, and also four genes encoding iron-binding proteins (*LTF*, *SLC40A1*, *CYP4Z1* and *CYP4Z2P*) previously linked to breast cancer. *LTF* encodes lactoferrin, a protein involved in non specific immunity and that may inhibit carcinogenesis and tumor growth [40]. *CYP4Z1* and its pseudogene *CYP4Z2P* are two members of cytochrome P450 family 4 which have been found to be over-expressed in about 50% of breast cancers relative to normal breast tissue from the same patients [41]. Here, we confirm that the pseudogene *CYP4Z2P* is expressed in both *PIK3CA*-mutated and -non mutated ERα-positive breast tumors, by using specific primers unambiguously distinguishing *CYP4Z2P* from *CYP4Z1*. Thus, *CYP4Z2P* is transcriptionally active, but its translation remains to be studied. *CYP4Z2P* is located in a head-to-head orientation close to *CYP4Z1* in chromosome region 1p33 [41], raising the possibility that expression of these two genes is co-regulated in *PIK3CA*-mutated breast tumors.

We identified several genes previously implicated in breast cancer development or outcome. The proteins encoded by *TFAP2B*, *NTN4* and *SEC14L2* have been linked to tumors with less aggressive features and better outcome [12,13,42]. *MAPT* has been proposed as a predictive marker of taxane responsiveness in breast cancer [43]. *NR2F2* has been also detected up-regulated in breast cancer, but its involvement in tumor development remains elusive because of its ability to affect both pro-oncogenic and anti-oncogenic proteins [44,45]. *HMGCS2* was recently shown to be regulated in response to hormonal stimulation [18].

NRIP3, *TMC5*, *REEP1* and *NKAIN1*, whose expression had not previously been described in breast cancer, were also deregulated in the *PIK3CA*-mutated breast tumors. *NRIP3*, *TMC5* and *REEP1* are differentially expressed in various other tumor types [46–48]. Interestingly, *NKAIN1* was the only gene under-expressed in *PIK3CA*-mutated tumors and was also the most discriminatory gene for these tumors. The role of these genes in breast cancer development remains to be evaluated in following studies.

Recently, Loi et al. identified a 278 gene-expression signature associated specifically with *PIK3CA* exon 20-mutated ER-positive/ERBB2-negative tumors [25]. These authors observed an unexpected significant down-expression of some Akt-regulated genes such as *RPS6KB1* in their *PIK3CA*-mutated tumor series, but a normal level of *AKT1* and *mTOR* transcripts. They also showed that phosphor-Akt expression was not significantly up-regulated at the protein level. In the present study, we did not identify *RPS6KB1*, *AKT1* and *mTOR* in our final 19-gene set nor in the list

of 6124 genes differentially expressed in *PIK3CA*-mutated tumors (Table S2 and S3). Interestingly, among the 168 significantly up-regulated genes detected by Loi et al., *WNT5A* and *MSX2*, as well as *HMGCS2* and *LTF*, were identified in agreement with our results. The data of Loi et al. [25] confirm thus the positive association between *PIK3CA* mutation and Wnt signaling pathway activation reported in the present manuscript.

In conclusion, this gene expression profiling study suggests that over-expression of genes belonging to the Wnt signaling pathway may play a pivotal role in *PIK3CA*-mutated breast tumors, in particular *WNT5A*. Further studies of biological mechanisms affected by *PIK3CA* mutations may have therapeutic implications.

Supporting Information

Table S1 Molecular, pathological and clinical characteristics of patients in relation to metastasis free survival (MFS) in the 43 ERα-positive and 249 ERα-positive patient series.
(PDF)

Table S2 2538 probes up-regulated in tumors with *PIK3CA* mutations (Mutated) compared to tumors without *PIK3CA* mutation (Normal) with a *P* value <0.05 identified by a parametric *t* test using BRB ArrayTools. These genes were ranked according to fold change (FC) calculated between expression intensities of tumors with *PIK3CA* mutations and those of tumors without *PIK3CA* mutation. The 216 probes with a FC≥2 are put in bold. In this list, the probes belonging to Wnt signaling pathway are shaded in light grey, and *PIK3R1*, *HMGCS2* and *MAPT* are shaded in dark grey.
(PDF)

Table S3 3586 probes down-regulated in tumors with *PIK3CA* mutations (Mutated) compared to tumors without *PIK3CA* mutation (Normal) with a *P* value <0.05 identified by a parametric *t* test using BRB ArrayTools. These genes were ranked according to fold change (FC) calculated between expression intensities of tumors with *PIK3CA* mutations and those of tumors without *PIK3CA* mutation. The 28 probes with a FC≥2 are put in bold.
(PDF)

Table S4 List of 56 probes (39 unique genes) deregulated in REα-positive breast tumors with *PIK3CA* mutations compared to those without *PIK3CA* mutation identified by PAM. These genes are presented according to the rank in PAM output. For each gene, we report the fold change (FC) calculated between expression intensities of tumors with and without *PIK3CA* mutations using BRB Arrays Tools. The genes with a FC≥3 are put in bold.
(PDF)

Table S5 Relative mRNA expression levels of the 29 genes in the 44 ERα-positive breast tumors with exon 9 *PIK3CA* mutations compared to the 47 tumors with exon 20 *PIK3CA* mutations. The tumor with *PIK3CA* mutations in both exon 9 and exon 20 was excluded from the analysis. For each gene, we report the median (range) of the mRNA levels of each gene relative to normal breast tissue samples, the fold change (FC) between tumors with exon 9-mutated and exon 20-mutated *PIK3CA* and the *P* value associated to Mann-Whitney *U* test.
(PDF)

Table S6 Supervised hierarchical clustering analysis of the 249 ERα-positive breast tumors. Classification performance of discriminating gene sets identified from the 19 significantly deregulated genes in tumors with *PIK3CA* mutations (18 up-regulated genes + *NKAIN1*). Each gene set separates the 157 tumors without *PIK3CA* mutation (N) and the 92 tumors with *PIK3CA* mutations (M) in two main clusters (cluster 1 and 2).
(PDF)

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Author Contributions

Conceived and designed the experiments: IB RL. Performed the experiments: SV AS CA. Analyzed the data: GCC. Contributed reagents/materials/analysis tools: GCC IB RL. Wrote the paper: MC GCC IB MS GCC.

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4.2 *Prognostic role of PI3K pathway deregulation*

4.2.1 *PIK3CA* mutation impact on survival in breast cancer patients and in ERα, PR and HER2 (ERBB2)-based subgroups

This study focused on *PIK3CA* mutations in unselected breast cancer series and the prognostic role of these mutations on patient survival. Mutations of *PIK3CA* exons 9 and 20 were assessed in 452 patient tumor samples at the mRNA level by direct sequencing. *PIK3CA* mutations were identified in 151 (33.4%) of the tumors of which 64 tumors (42.4%) bore exon 9 mutations and 86 (57.0%) exon 20 mutations. Three tumors presented with double mutation: two tumors with mutations in exon 20 and only one tumor (0.6%) with one mutation in exon 9 and the other in exon 20. The frequency of the *PIK3CA* mutations differed markedly across four major tumor subgroups: HR-positive/HER2-positive (28.3%, 15 out of 53), HR-positive/HER2-negative (41.1%, 118 out of 287), HR-negative/HER2-positive (20.8%, 10 out of 48), and HR-negative/HER2-negative (12.5%, 8 out of 64) (P = 0.00009).

PIK3CA mutations were significantly associated with low histopathological grade, small macroscopic tumor size, and ERα-positive, PR-positive, and HER2-negative tumors. In the overall population of 452 patients, *PIK3CA* mutation was associated with more favorable metastasis-free survival (MFS; P = 0.0056). More interestingly, *PIK3CA* mutations were associated with markedly better MFS in patients with PR-positive (P = 0.0064) than in those with PR-negative (P = 0.71) tumors and also in patients with HER2-positive (P = 0.014) than in those with HER2-negative (P = 0.12) tumors. In contrast, *PIK3CA* mutation was associated with only a trend toward better MFS in patients with ERα-positive (P = 0.082) and ERα-negative (P = 0.098) tumors. In the multivariate analysis, the prognostic significance of *PIK3CA* mutation status persisted in the HER2-positive tumor subgroup (P = 0.023) but not in the total tumor population or in the PR-positive tumor subgroup.

The composition of the patient series describes a natural collection of breast cancer patients diagnosed between the years 1978 and 2008 with the commonly found proportion of the particular breast cancer subtypes based on HR and HER2 states. Importantly, the patient survival demonstrates response to non-targeted treatment modalities only including surgery, chemotherapy and hormonal therapy. The distribution of *PIK3CA* mutations reflects well the frequencies of these mutations in breast cancer and

its subgroups described by others showing the greatest number of mutated cases in the HR-positive and the lowest in the HR-negative/HER2-negative tumors. Similarly, the representation of hot-spot and rare mutations in both exons too, is in good accord with previously published observations (Barbareschi *et al*, 2007, Stemke-Hale *et al*, 2008; Castaneda *et al*, 2010; Hernandez-Aya *et* Gonzalez-Angulo, 2011; The Cancer Genome Atlas Network, 2012). A recent comprehensive study described exome sequencing of 510 breast cancer samples finding *PIK3CA* mutations in 36%. Moreover, the authors showed distribution of *PIK3CA* mutations along the whole *PIK3CA* coding sequence with a focus on breast cancer subtypes. Thus, they demonstrated specific associations of particular mutations with breast cancer subtypes including mutations in rarely mutated exons. Among the most frequently found mutations, E542K occured with varying rate in all subtypes, but e.g. E545K was almost exclusively associated with luminal A tumors (The Cancer Genome Atlas Network, 2012).

There are increasing numbers of studies reporting favorable survival in *PIK3CA*-mutated breast cancer patients. Despite some reports showing none or negative impact on patient outcome, or alternatively showing different impact of exon 9 and 20 mutations, the majority of the published studies agree on better survival associated with *PIK3CA* mutations (Li *et al*, 2006; Barbarechi *et al*, 2007; Maruyama *et al*, 2007; Pérez-Tenorio *et al*, 2007; Kalinsky *et al*, 2009; Loi *et al*, 2010; Mangone *et al,* 2012). Tumor samples assessed in these studies come from retrospective patient series with breast cancer diagnosis dating from 1970's to late 1990's and only rarely include samples from the 2000's. Thus, the patients described in these studies like ours were treated with surgery, radiotherapy, chemotherapy or hormonal therapy. This makes the reported data generally comparable but specific treatment sequence and chemotherapy choices varied between studies. Recently, a study by Mangone et al. (Mangone *et al*, 2012) suggested in contrast, that kinase domain mutations might be associated with poorer prognosis. Dumont et al. reviewed recently multiple retrospective studies focusing on *PIK3CA* mutation impact on breast cancer patient survival (Dumont *et al*, 2012). Interestingly, the authors suggested that the beneficial effect of the mutations might be associated mainly with kinase domain mutations and ER-positive tumors since these are the most frequent. Furthermore, these authors also proposed hypotheses explaining the favorable survival in *PIK3CA*-mutated cases: *PIK3CA* mutations may induce cellular senescence and suppress tumor metastasis and so lead to better outcome; *PIK3CA* mutations may cause tumors prone to earlier

detection in more favorable disease stages; finally these mutations might enhance treatment response on hormonal therapy (Dumont *et al*, 2012). In the past years, several other hypotheses have been proposed to explain the favorable prognostic impact of *PIK3CA* mutations suggesting that: *PIK3CA* mutations themselves as the only hit to the PI3K signaling pathway may have a limited oncogenic potential; *PIK3CA* mutation-bearing cells might also be more sensitive to chemotherapy and/or other treatment modalities; or *PIK3CA* mutation-induced signaling could trigger a negative feedback loop inhibiting lower levels of the pathway (Stemke-Hale *et al*, 2008; Di Cosimo *et* Baselga, 2009; Loi *et al*, 2010). However, currently none of these options prevails. Nevertheless, our data support the observation showing that *PIK3CA* mutations associate with better prognosis in breast cancer patients without targeted therapy.

PIK3CA mutations were also proposed as predictive markers for targeted inhibitors of PI3K downstream signaling components of the pathway. A recent report showing *in vitro* response to mTOR inhibition suggested that *PIK3CA* mutations and PTEN loss predict good treatment response to rapamycin (Meric-Bernstam *et al*, 2012). Thus, the subgroup of patients bearing *PIK3CA* mutations could benefit from treatment targeting the PI3K pathway signaling (PI3K or its downstream major effectors) (Kataoka *et al*, 2010; O'Brien *et al*, 2010; Tanaka *et al*, 2011). Fleming et al. described phase II clinical trial testing temsirolimus monotherapy in 31 heavily pretreated breast cancer patients, but they found no association between treatment response and *PIK3CA* status (Fleming *et al*, 2012). However, these results might be caused by small patient cohort (5 *PIK3CA*-mutated out of 23 assessed patient samples), treatment under-dosing or other factors and the authors concluded that mTOR inhibitors might be better used in combination with other treatments. Despite these initial negative results, PI3K pathway inhibitors should be further tested in breast cancer treatment and the treatment response to these inhibitors should be evaluated in connection with PI3K pathway deregulations as *PIK3CA* mutations.

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PIK3CA mutation impact on survival in breast cancer patients and in ERα, PR and ERBB2-based subgroups

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Abstract

Introduction: *PIK3CA* is the oncogene showing the highest frequency of gain-of-function mutations in breast cancer, but the prognostic value of *PIK3CA* mutation status is controversial.

Methods: We investigated the prognostic significance of *PIK3CA* mutation status in a series of 452 patients with unilateral invasive primary breast cancer and known long-term outcome (median follow-up 10 years).

Results: *PIK3CA* mutations were identified in 151 tumors (33.4%). The frequency of *PIK3CA* mutations differed markedly according to hormone receptor (estrogen receptor alpha [ERα] and progesterone receptor [PR]) and ERBB2 status, ranging from 12.5% in the triple-negative subgroup (ER-/PR-/ERBB2-) to 41.1% in the HR+/ERBB2- subgroup. *PIK3CA* mutation was associated with significantly longer metastasis-free survival in the overall population (P = 0.0056), and especially in the PR-positive and ERBB2-positive subgroups. In Cox multivariate regression analysis, the prognostic significance of *PIK3CA* mutation status persisted only in the ERBB2-positive subgroup.

Conclusions: This study confirms the high prevalence of *PIK3CA* mutations in breast cancer. *PIK3CA* mutation is an emerging tumor marker which might become used in treatment-choosing process. The independent prognostic value of *PIK3CA* mutation status in ERBB2-positive breast cancer patients should be now confirmed in larger series of patients included in randomized prospective ERBB2-based clinical trials.

Introduction

Dysregulation of tyrosine kinase receptor (TKR)-phosphatidylinositol 3-kinase (PI3K) signaling pathways is frequent in human cancers. Among the most important molecular events downstream of TKR activation is PI3K activation, which catalyzes the phosphorylation of inositol lipids to phosphatidylinositol-3,4,5-trisphosphate. Phosphatidylinositol-3,4,5-trisphosphate activates the serine/threonine kinase AKT, which in turn regulates several signaling pathways controlling cell survival, apoptosis, proliferation, motility, and adhesion [1]. PI3K is a heterodimeric enzyme composed of a p110α catalytic subunit encoded by the *PIK3CA* gene and a p85 regulatory subunit encoded by the *PIK3R1* gene [2].

Recently, gain-of-function mutations in *PIK3CA* have been found in several cancers, including breast cancer [1,3,4]. *PIK3CA* is frequently mutated at 'hotspots' in exons 9 and 20, corresponding to the helical (E542K and E545K) and kinase (H1047R) domains, respectively. P110α carrying a hotspot mutation shows oncogenic activity: it can transform primary fibroblasts in culture, induce anchorage-independent growth, and cause tumors in animals [5,6].

After the *TP53* suppressor gene, the *PIK3CA* oncogene is the most frequently mutated gene in human breast cancers; mutations are observed in 20% to 40% of cases [7,8]. Mutation is an early event in breast cancer and is more likely to play a role in tumor initiation than in invasive progression [9]. It is noteworthy that

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activating somatic mutations of other oncogenes (*EGFR, KRAS, HRAS, NRAF, BRAF, AKT1*, and so on) involved in molecular events downstream of TKR activation and frequently observed in other cancers are rare in breast cancer. Several studies of breast cancer suggest that *PIK3CA* mutations are more frequent in estrogen receptor-alpha- positive ($ER\alpha^+$) breast tumors (30% to 40%) than in receptor-alpha-negative ($ER\alpha^-$) breast tumors (10% to 20%) [3,7,10,11].

The prognostic value of *PIK3CA* mutation status in breast cancer is controversial. Li and colleagues [12] suggested that mutations in any part of the gene may be related to poor clinical outcome. On the contrary, Maruyama and colleagues [13], Pérez-Tenorio and colleagues [14], and Kalinsky and colleagues [11] suggested that *PIK3CA* mutations were significantly and independently associated with better recurrence-free survival. In particular, Kalinsky and colleagues [11] studied a series of 590 patients with breast cancer with a median follow-up of 12.8 years and found 32.5% of *PIK3CA* mutations. *PIK3CA*-mutated status was associated with markers of good prognosis and with significant improvement in overall (P = 0.03) and breast cancer-specific (P = 0.004) survival [11]. A study focused specifically on recurrent and metastatic breast cancer found a significant association of *PIK3CA* mutations and longer relapse-free survival [15]. Barbareschi and colleagues [16] reported that only *PIK3CA* exon 9 mutations were independently associated with early recurrence and death but that exon 20 mutations were associated with favorable outcome. Several teams have found no significant effect of *PIK3CA* mutations on patient outcome [7,8,17,18]. It is, however, noteworthy that Loi and colleagues [18] identified an expression signature derived from exon 20 *PIK3CA*-mutated tumors. This signature predicted better outcome in ER^+ breast cancer. In particular, the clinical consequences of *PIK3CA* mutations might vary according to the status of well-known molecular markers in breast cancer, namely *ERα*, progesterone receptor (*PR*), and *ERBB2*. Here, we examined the prognostic value of *PIK3CA* mutation status in a series of 452 patients with unilateral invasive primary breast cancer and known long-term outcome, taking *ERα, PR*, and *ERBB2* status into account.

Materials and methods

Patients and samples

We analyzed samples of 452 primary unilateral invasive primary breast tumors excised from women at the Institut Curie/Hôpital René Huguenin (Saint-Cloud, France) from 1978 to 2008. All patients who entered our institution before 2007 were informed that their tumor samples might be used for scientific purposes and had the opportunity to decline. Since 2007, patients entering our institution have given their approval also by signed informed consent. This study was approved by the local ethics committee (Breast Group of René Huguenin Hospital). The samples were examined histologically and were considered suitable for this study if the proportion of tumor cells exceeded 70% with sufficient cellularity as was proven by evaluation of tumor samples stained by hematoxylin and eosin. Immediately after surgery, the tumor samples were placed in liquid nitrogen until RNA extraction.

The patients (mean age of 61.6 years and range of 31 to 91) met the following criteria: primary unilateral non-metastatic breast carcinoma, with full clinical, histological and biological data; no radiotherapy or chemotherapy before surgery; and full follow-up at Institut Curie/Hôpital René Huguenin.

One hundred sixty patients (35.4%) had breast-conserving surgery plus locoregional radiotherapy, and 292 patients (64.6%) had modified radical mastectomy. Clinical examinations were performed every 3 or 6 months during the first 5 years, according to the prognostic risk of the patients, and then yearly. Mammograms were done annually. Three hundred sixty-six patients received adjuvant therapy, consisting of chemotherapy alone in 94 cases, hormone therapy alone in 177 cases, and both treatments in 95 cases. None of the $ERBB2^+$ patients was treated with anti-ERBB2 therapy. The histological type and number of positive axillary nodes were established at the time of surgery. The malignancy of infiltrating carcinomas was scored with the Scarff-Bloom-Richardson histoprognostic system'.

ER and PR status was determined at the protein level by using biochemical methods (dextran-coated charcoal method or enzymatic immunoassay) until 1999 and later by using immunohistochemistry. Cutoff for ER and PR positivity was set at 15 fm/mg (dextran-coated charcoal or enzyme immunoassay) and at 10% immunostained cells (immunohistochemistry). A tumor was considered $ERBB2^+$ by immunohistochemistry if it scored 3 or more with uniform intense membrane staining of greater than 30% of invasive tumor cells. Tumors scoring 2 or more were considered to be equivocal for ERBB2 protein expression and were tested by fluorescence *in situ* hybridization for ERBB2 gene amplification. In all cases, the *ERα, PR*, and *ERBB2* status was confirmed by real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) with cutoff levels based on previous studies comparing results of the mentioned methods [19-22]. On the basis of hormone receptor (HR) (*ERα* and *PR*) and *ERBB2* status, we subdivided the 452 patients into four subgroups: HR^+ (ER^+ or PR^+ or both)/$ERBB2^+$ (n = 53), HR^+ (ER^+ or PR^+ or both)/$ERBB2^-$ (n = 287), HR^- (ER^- and PR^-)/$ERBB2^+$ (n = 48), and HR^- (ER^- and PR^-)/$ERBB2^-$ (n =

64). Standard prognostic factors are reported in Table S1 of Additional file 1. The median follow-up was 10.0 years (range of 13 months to 28.9 years). One hundred seventy patients developed metastases.

RNA extraction

Total RNA was extracted from breast tumor samples by using the acid-phenol guanidium method. RNA quantity was assessed by using a NanoDrop Spectrophotometer ND-1000 with its corresponding software (Thermo Fisher Scientific Inc., Wilmington, DE, USA). RNA quality was determined by electrophoresis through agarose gel and staining with ethidium bromide. The 18S and 28S RNA bands were visualized under ultraviolet light. DNA contamination was quantified by using a couple of primers located in an intron of gene coding for albumin (ALB) (Gene ID: 213). Samples were further used only when the cycle threshold (Ct) obtained by using these ALB intron primers was greater than 40.

PIK3CA mutation screening

PIK3CA mutations were detected by screening cDNA fragments obtained by RT-PCR amplification of exons 9 and 20 and their flanking exons. Details of the primers and PCR conditions are available on request. The amplified products were sequenced with a BigDye Terminator kit on an ABI Prism 3130 automatic DNA sequencer (Applied Biosystems, Courtabæuf, France) with detection sensitivity of 5% mutated cells, and the sequences were compared with the corresponding cDNA reference sequence (NM_006218). All of the detected *PIK3CA* mutations were confirmed in the second independent run of sample testing.

Statistical analysis

Relationships between *PIK3CA* mutation status and clinical, histological, and biological parameters were estimated with the chi-squared test. Differences between the mutated and non-mutated populations were judged significant at confidence levels of greater than 95% ($P < 0.05$). Metastasis-free survival (MFS) was determined as the interval between diagnosis and detection of the first metastasis. Survival distributions were estimated with the Kaplan-Meier method [23], and the significance of differences between survival rates was ascertained with the log-rank test [24]. The Cox proportional hazards regression model [25] was used to assess prognostic significance.

Results and Discussion

PIK3CA mutations were identified in 151 (33.4%) of 452 primary breast tumors, in keeping with the results of the largest previous studies, showing mutation rates of 25% to 40% [7,8,11,14,16,18,26-30]. Sixty-four tumors bore *PIK3CA* mutations located in exon 9, 86 tumors bore mutations in exon 20, and one tumor bore mutations in both exons 9 and 20 (Table 1). Exon 20 was thus the most frequently mutated *PIK3CA*

Table 1 *PIK3CA* mutation profiles

Exon	Nucleotide	Codon	Number of mutations
9	c.1634A > C	Glu545Ala	2
9	c.1636C > A	Gln546Lys	2
9	c.1624 G > A	Glu542Lys	20
9	c.1634A > G	Glu545Gly	1
9	c.1633 G > A	Glu545Lys	32
9	c.1633 G > C	Glu545Gln	1
9	c.1490A > G	Asn497Ser	1
9	c.1636C > A	Gln546Lys	2
9	c.1637A > C	Gln546Pro	1
9	c.1637A > G	Gln546Arg	2
20	c.3203dupA	Asn1068Lys	2
20	c.3140A > T	His1047Leu	8
20	c.3140A > G	His1047Arg	70
20	c.3132T > A	Asn1044Lys	1
20	c.3145 G > C	Gly1049Arg	2
20	c.3155C > A	Thr1052Lys	1
20	c.[3085 > C(+)3140A > T]	p.[Asp1029His(+)His1047Leu]	1
20	c.[3140A > T(+)3197C > T]	p.[His1047Leu(+)Ala1066Val]	1
9+20	c.[1624 G < A(+)3127A > G]	p.[Glu542Lys(+)Met1043Val]	1
			Total = 151

PIK3CA, phosphatidylinositol 3-kinase, catalytic, alpha polypeptide gene.

exon, in keeping with most other studies [7,8,11,14,26,28-30]. Among the 151 tumors with *PIK3CA* mutations, three bore double mutations: two in exon 20 (D1029H and H1047R, H1047R and A1066V) and one in exons 9 and 20 (E542K and M1043V). Rare double *PIK3CA* mutations have been reported elsewhere [7,8,30]. We also observed two c.3203dupA frameshift mutations that would change the last C-terminal amino acid (N1068K) of the PIK3CA protein and add another three amino acids. N1068K represents 50% of all *PIK3CA* mutations in hepatocellular carcinoma [28] but its possible role in tumor initiation or progression is unknown.

Table 2 shows links between *PIK3CA* mutation status and standard clinical, pathological, and biological characteristics of breast cancer. *PIK3CA* mutations were significantly associated (chi-squared test) with low histopathological grade, small macroscopic tumor size, and ERα$^+$, PR$^+$, and ERBB2$^-$ tumors. For example, *PIK3CA* mutations were observed in 52.7% (29 out of 55) of histopathological grade I tumors, 36.8% (84 out of 228) of grade II tumors, and 23.3% (37 out of 159) of grade III tumors. These relationships have also been found in most previous studies [3,7,10,11]. For example, Kalinsky and colleagues [11], like us, found that *PIK3CA* mutations were associated with low histopathological grade and ERα$^+$, PR$^+$, and ERBB2$^-$ tumors. However, it is noteworthy that, in several studies, no significant association between *PIK3CA* mutations and important clinical or pathological features was found [30]. A high frequency of *PIK3CA* mutations has also been found in lobular carcinoma [16,31]. In agreement with other authors [27,30], we observed a similar frequency of *PIK3CA* mutations in lobular carcinomas (34.5%, 10 out of 29) and ductal carcinomas (33.2%, 129 out of 388) of the breast (Table 2).

Table 2 Relationship between *PIK3CA* mutation status and standard clinical, pathological, and biological features of breast cancer

		Number of patients (percentage)		
	Total population number (percentage)	*PIK3CA* wild-type	*PIK3CA*-mutated	*P* value[a]
Total	452 (100.0)	301 (66.6)	151 (33.4)	
Age, years				
≤ 50	96 (21.2)	66 (21.9)	30 (19.9)	NS
> 50	356 (78.8)	235 (78.1)	121 (81.1)	
SBR histological grade[b, c]				
I	55 (12.4)	26 (8.9)	29 (19.3)	0.00021
II	228 (51.6)	144 (49.3)	84 (56.0)	
III	159 (36.0)	122 (41.8)	37 (24.7)	
Lymph node status[d]				
0	115 (25.5)	78 (26.0)	37 (24.5)	
1-3	237 (52.5)	157 (52.3)	80 (53.0)	
> 3	99 (22.0)	65 (21.7)	34 (22.5)	NS
Macroscopic tumor size[e]				
≤ 25 mm	217 (48.8)	135 (45.2)	82 (56.2)	0.029
> 25 mm	228 (51.2)	164 (54.8)	64 (43.8)	
ERα status				
Negative	117 (25.9)	97 (32.2)	20 (13.2)	0.000014
Positive	335 (74.1)	204 (67.8)	131 (86.8)	
PR status				
Negative	194 (42.9)	150 (49.8)	44 (29.1)	0.000028
Positive	258 (57.1)	151 (50.2)	107 (70.9)	
ERBB2 status				
Negative	351 (77.7)	225 (74.8)	126 (83.4)	0.036
Positive	101 (22.3)	76 (25.2)	25 (16.6)	
Histology				
Ductal	388 (85.8)	259 (86.0)	129 (85.5)	NS
Lobular	29 (6.4)	19 (6.3)	10 (6.6)	
Others	35 (7.8)	23 (7.7)	12 (7.9)	

[a]Chi-squared test. [b]Scarff-Bloom-Richardson (SBR) classification. [c]Information available for 442 patients. [d]Information available for 451 patients. [e]Information available for 445 patients. ERα, estrogen receptor-alpha; NS, not significant; *PIK3CA*, phosphatidylinositol 3-kinase, catalytic, alpha polypeptide gene; PR, progesterone receptor.

Functional genomic studies have recently shown that breast cancer is a highly heterogeneous disease. Several tumor subtypes, such as basal-like, *ERBB2*$^+$, and HR$^+$ (luminal A and luminal B), can be distinguished on the basis of their gene expression profiles, pointing to the involvement of different oncogenetic pathways. In keeping with this possibility, we observed a marked difference in the *PIK3CA* mutation frequency across four major tumor subgroups: HR$^+$/ERBB2$^+$ (28.3%, 15 out of 53), HR$^+$/ERBB2$^-$ (41.1%, 118 out of 287), HR$^-$/ERBB2$^+$ (20.8%, 10 out of 48), and HR$^-$/ERBB2$^-$ (12.5%, 8 out of 64) (P = 0.00009). Being found in 41.1% of cases, *PIK3CA* mutations might thus be characteristic of the luminal subtype (HR$^+$/ERBB2$^-$). We also observed a low frequency (12.5%) of *PIK3CA* mutations in triple-negative tumors (ER$^-$/PR$^-$/ERBB2$^-$), a subgroup reported to overlap with the basal-like subtype of breast cancer. Stemke-Hale and colleagues [8] also observed a marked difference in *PIK3CA* mutation frequency across breast tumor subtypes, and *PIK3CA* mutations were more common in HR$^+$ tumors (39%) and ERBB2$^+$ tumors (25%) than in basal-like tumors (13%).

In the overall population of 452 patients, *PIK3CA* mutation was associated with more favorable MFS (P = 0.0056) (Table 3 and Figure 1a). The outcome of the 151 patients with *PIK3CA* mutations was thus significantly better than that of the 301 wild-type patients, as was demonstrated by 5-year and 15-year survival rates in these two groups (5-year MFS of 81.0% versus 69.6% and 15-year MFS of 65.8% versus 53.4%). Differences in treatment are unlikely to account for this difference, as *PIK3CA* mutations were as frequent in patients who received postoperative adjuvant chemotherapy or hormone therapy or both (126 out of 366, 34.4%) as in those who received neither treatment (25 out of 86, 29.1%).

These data confirm the results of smaller series of breast tumors, in which *PIK3CA* mutations were significantly associated with more favorable MFS [13,14]. However, unlike Barbareschi and colleagues [16], who found that mutations in the helical (exon 9) and kinase (exon 20) domains of the *PIK3CA* gene had different prognostic values, we found that MFS was similar in patients with mutations in one exon or the other when we compared these two subgroups together and with the wild-type subgroup (Figure 1b).

More interestingly, *PIK3CA* mutation was associated with markedly better MFS in the patients with PR$^+$ tumors (P = 0.0064) than in those with PR$^-$ tumors (P = 0.71) (Table 3 and Figure 2a) and also in patients with ERBB2$^+$ tumors (P = 0.014) than in those with ERBB2$^-$ tumors (P = 0.12) (Table 3 and Figure 2b). In contrast, *PIK3CA* mutation was associated only with a trend toward better MFS in patients with ERα$^+$ (P = 0.082) and ERα$^-$ (P = 0.098) tumors (Table 3). Accordingly, Loi and colleagues [18] did not find statistically significant difference in survival between *PIK3CA* wild-type and *PIK3CA*-mutated tumors in the ER$^+$ population. However, it is noteworthy that these authors described a *PIK3CA* mutation-associated gene expression signature predicting favorable survival in ER$^+$ breast cancer [18].

Table 3 *PIK3CA* mutation status according to hormone receptor and ERBB2 status and relation to metastasis-free survival

	Number of patients	5-year MFS	HR (95% CI)	*P* value[a]
Total population	452			
Wild-type	301	69.6%	1	0.0056
Mutated	151	81.0%	0.62 (0.44-0.87)	
ERα$^+$	335			
Wild-type	204	75.6%	1	NS
Mutated	131	81.9%	0.71 (0.49-1.04)	
ERα$^-$	117			
Wild-type	97	56.9%	1	NS
Mutated	20	75.0%	0.46 (0.18-1.15)	
PR$^+$	258			
Wild-type	151	77.8%	1	0.0064
Mutated	107	86.6%	0.52 (0.33-0.83)	
PR$^-$	194			
Wild-type	150	61.3%	1	NS
Mutated	44	67.6%	0.91 (0.55-1.50)	
ERBB2$^+$	101			
Wild-type	76	59.9%	1	0.014
Mutated	25	88.0%	0.31 (0.12-0.79)	
ERBB2$^-$	351			
Wild-type	225	72.9%	1	NS
Mutated	126	79.7%	0.75 (0.51-1.08)	

[a]Univariate Cox analysis. CI, confidence interval; ERα, estrogen receptor-alpha; HR, hazard ratio; MFS, metastasis-free survival; NS, not significant; PR, progesterone receptor.

Using a Cox proportional hazards model, we also assessed the MFS predictive value of the parameters that were significant in univariate analysis (that is, Scarff-Bloom-Richardson histological grade, lymph node status, macroscopic tumor size, and ERα, PR, and ERBB2 status (Table S1 of Additional file 1) and *PIK3CA* mutation status). The prognostic significance of *PIK3CA* mutation status persisted in the ERBB2$^+$ tumor subgroup (P = 0.023) (Table 4) but not in the total tumor population or in the PR$^+$ tumor subgroup. Since the patients were not treated with ERBB2-targeted

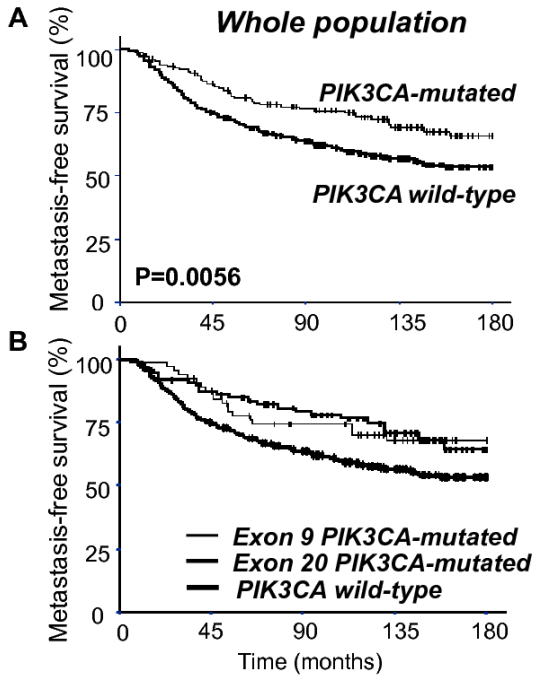

Figure 1 Whole population survival curves. **(a)** Metastasis-free survival curves of patients with *PIK3CA* wild-type and -mutated tumors. **(b)** Metastasis-free survival curves of patients with exon 9 *PIK3CA*-mutated tumors, exon 20 *PIK3CA*-mutated tumors, and *PIK3CA* wild-type tumors. Comparison of these curves did not show any statistically significant difference. *PIK3CA*, phosphatidylinositol 3-kinase, catalytic, alpha polypeptide gene.

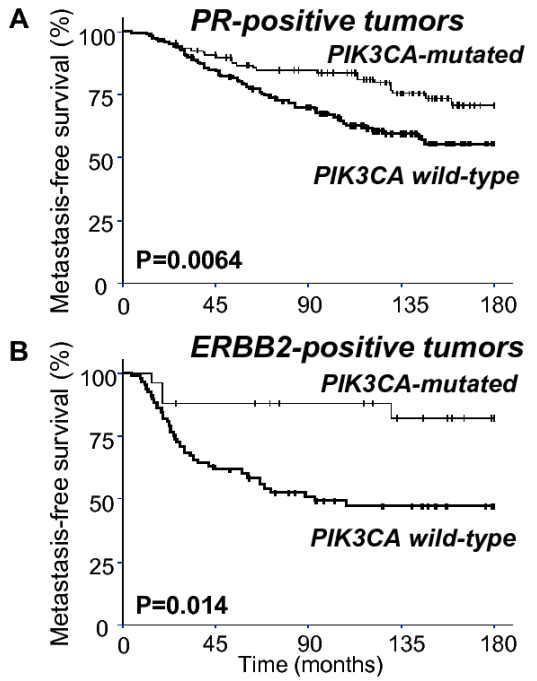

Figure 2 Subgroup analysis survival curves. **(a)** Metastasis-free survival curves of progesterone receptor-positive (PR^+) patients with *PIK3CA* wild-type and -mutated tumors. **(b)** Metastasis-free survival curves of $ERBB2^+$ patients with *PIK3CA* wild-type and -mutated tumors. *PIK3CA*, phosphatidylinositol 3-kinase, catalytic, alpha polypeptide gene.

Table 4 Multivariate Cox analysis of metastasis-free survival in the total population and in subgroups of patients with breast cancer

Variables	Total population		ER^+ patients		ER^- patients		PR^+ patients		PR^- patients		$ERBB2^+$ patients		$ERBB2^-$ patients	
	HR (95% CI)	*P* value[a]	HR (95% CI)	*P* value[a]	HR (95% CI)	*P* value[a]	HR (95% CI)	*P* value[a]	HR (95% CI)	*P* value[a]	HR (95% CI)	*P* value[a]	HR (95% CI)	*P* value[a]
SBR		0.038		0.019		NS		0.00077		NS		NS		0.0043
I	1		1		1		1		1		1		1	
II	1.34 (1.02-1.76)		1.45 (1.06-1.98)		1.02 (0.57-1.80)		1.88 (1.30-2.71)		0.93 (0.63-1.38)		0.89 (0.51-1.55)		1.60 (1.16-2.21)	
III	1.79 (1.03-3.11)		2.11 (1.13-3.92)		1.03 (0.33-3.26)		3.52 (1.69-7.32)		0.87 (0.40-1.91)		0.79 (0.26-2.39)		2.56 (1.34-4.90)	
pN		0.00014		0.00093		NS		0.0068		0.01		0.000049		NS
0	1		1		1		1		1		1		1	
1-3	1.58 (1.25-1.99)		1.60 (1.21-2.11)		1.45 (0.94-2.25)		1.61 (1.14-2.27)		1.51 (1.10-2.07)		2.61 (1.63-4.18)		1.26 (0.97-1.66)	
> 3	2.48 (1.56-3.96)		2.56 (1.47-4.47)		2.11 (0.88-5.05)		2.59 (1.30-5.18)		2.28 (1.21-4.29)		6.83 (2.67-17.44)		1.60 (0.93-2.74)	
pT		0.01		0.00041		NS		0.0023		NS		NS		0.0054
≤ 25 mm	1		1		1		1		1		1		1	
> 25 mm	1.53 (1.11-2.13)		2.05 (1.38-3.05)		0.80 (0.44-1.47)		2.09 (1.30-3.36)		1.17 (0.74-1.84)		1.06 (0.54-2.08)		1.72 (1.17-2.52)	
ER		NS		-		-		NS		NS		NS		NS
Negative	1		-		-		1		1		1		1	
Positive	1.04 (0.68-1.60)		-		-		2.09 (0.28-15.63)		0.82 (0.51-1.32)		0.78 (0.36-1.70)		1.23 (0.71-2.11)	
PR		NS		NS		NS		-				NS		NS
Negative	1		1		1		-		-		1		1	
Positive	0.77 (0.52-1.14)		0.81 (0.54-1.23)		0.31 (0.04-2.33)		-		-		0.56 (0.23-1.36)		0.79 (0.51-1.23)	
ERBB2		NS		NS		NS		NS		NS		-		-
Negative	1		1		1		1		1		-		-	
Positive	1.12 (0.77-1.62)		1.01 (0.60-1.70)		1.33 (0.74-2.38)		0.98 (0.50-1.93)		1.17 (0.74-1.85)		-		-	
PIK3CA		NS		NS		NS		NS		NS		0.023		NS
Wild-type	1		1		1		1		1		1		1	
Mutated	0.75 (0.53-1.07)		0.84 (0.57-1.23)		0.49 (0.19-1.26)		0.62 (0.38-1.01)		0.92 (0.55-1.54)		0.31 (0.12-0.79)		0.96 (0.65-1.44)	

[a]Multivariate Cox analysis. CI, confidence interval; ER, estrogen receptor; HR, hazard ratio; NS, not significant; *PIK3CA*, phosphatidylinositol 3-kinase, catalytic, alpha polypeptide gene; pN, number of positive axillary lymph nodes assessed by a pathologist at the time of surgery; PR, progesterone receptor; pT, size of the primery tumor assessed by a patologist at the time of surgery}; SBR, Scarff-Bloom-Richardson classification.

treatment, these results address the outcome of $ERBB2^+$ tumors affected by surgery and chemotherapy but not targeted therapy like trastuzumab or lapatinib. The independent prognostic value of *PIK3CA* mutation status in patients with $ERBB2^+$ breast cancer should now be tested in a larger series of patients included in randomized prospective ERBB2-based clinical trials.

PIK3CA mutation is also an emerging tumor marker that, in the future, might be used in the process of choosing a treatment. Indeed, ERBB2 inhibitors

(trastuzumab and lapatinib) are clinically active in women with ERBB2$^+$ breast cancer, but recent studies suggest that *PIK3CA*-mutated tumors could be resistant to these drugs [32,33]. There is also evidence showing that tumors with PI3K/AKT pathway activation including PTEN loss or *PIK3CA* mutation or both are less sensitive to trastuzumab treatment [17]. Interestingly, this resistance appears to be reversed by mammalian target of rapamycin (mTOR) or PI3K inhibitors [33]. A final validation of *PIK3CA* mutation as an independent predictor of the response to trastuzumab treatment in ERBB2$^+$ breast cancer needs a prospective randomized study. Our results also support the emerging role of *PIK3CA* mutation status in the management of future gene-based therapies (ERBB2, mTOR, or PI3K inhibitors used alone or in combination) for breast cancer, particularly in patients with tumors with activated PI3K/AKT pathway [34,35]. *ERBB2* amplification and *PIK3CA* mutation were recently validated as biomarkers of sensitivity to single-agent PI3K inhibitor (GDC-0941) therapy in breast cancer models [35].

Conclusions

This study of 452 breast tumors confirms the high prevalence (33.4%) of *PIK3CA* mutations. The frequency of *PIK3CA* mutations differed markedly according to *ERα, PR*, and *ERBB2* status, from 12.5% in triple-negative tumors to 41.1% in the HR$^+$/ERBB2$^-$ subgroup. Subgroup analysis of patient survival identified *PIK3CA* mutation status as an independent prognostic value in patients with ERBB2$^+$ breast cancer. These findings should be confirmed in larger series of patients included in a randomized prospective ERBB2-based clinical trial. Then *PIK3CA* mutation status could serve as a new independent prognostic tool when selecting targeted therapies for patients with ERBB2$^+$ breast cancer.

Additional material

Additional file 1: Table S1. Characteristics of the 452 primary breast tumors, and relation to metastasis-free survival. A table showing metastasis free survival of the patients in relation to pathological data.

Abbreviations

ALB: albumin; ERα: estrogen receptor-alpha; HR: hormone receptor; MFS: metastasis-free survival; mTOR: mammalian target of rapamycin; PCR: polymerase chain reaction; PI3K: phosphatidylinositol 3-kinase; *PIK3CA*: phosphatidylinositol 3-kinase: catalytic: alpha polypeptide gene; PR: progesterone receptor; RT-PCR: reverse transcriptase-polymerase chain reaction; TKR: tyrosine kinase receptor.

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Authors' contributions

AS and SV helped to conceive the approach to mutational analysis, design the primers, and carry out the mutational analysis. CA helped to conceive the approach to mutational analysis, design the primers, and perform the DNA extraction. MC helped to carry out the mutational analysis and draft the manuscript. GC-C and EF performed the statistical analysis. IB and RL helped to draft the manuscript and conceive the study and participated in its design and coordination. KD helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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4.2.2 *PIK3R1* underexpression is an independent prognostic marker in breast cancer

After analysis of *PIK3CA* mutations, we focused on a selection of crucial players (in addition to *PIK3CA* gene) in the PI3K pathway. *PIK3CA*, *PIK3R1* and *AKT1* were screened for mutations known to occur in these genes. *PIK3CA* status was previously assessed in exons 9 and 20 and for the present publication also in exons 1 and 2. In all 4 screened exons, *PIK3CA* mutations were identified in 151 (33.0%). *PIK3R1* mutations were found in 11 (2.4%) cases in exons 11, 12, 13 and 15. *AKT1* mutation (E17K) was found in 15 (3.4%) cases. Taken together, we observed 175 (38.5%) breast cancer tumors mutated in *PIK3CA* and/or *PIK3R1* and/or *AKT1*. We also assessed mRNA expression of these 3 genes and other important genes implicated in the pathway (*EGFR*, *PDK1*, *PTEN*, *AKT2*, *AKT3*, *GOLPH3*, *WEE1*, *P70S6K*). Interestingly, *PIK3R1* underexpression was found in 283 (61.8%) of cases, predominantly in HR-negative tumors. Decreased expression of *PIK3R1* was previously described in breast cancer with a frequency of 18% (Taniguchi *et al*, 2010). On the other hand, expression of *PIK3CA* was found deregulated only in a minority of the tumor samples: overexpressed in 18 (3.9%) and underexpressed in 40 (8.7%) cases. Increased expression of *AKT1* was found in 116 out of 458 (25.3%) available samples and presented mostly in HER2-positive tumors. Increase in expression was found in known key players of the PI3K pathway, namely in *PDK1*, *AKT2*, *GOLPH3* and *P70S6K* in 81 (17.7%), 116 (25.3%), 89 (19.4%) and 83 (18.1%) tumor samples, respectively. On the other hand, decrease in expression was observed in the case of *EGFR*, *PTEN*, *AKT3*, *WEE1* and interestingly also in some cases in *PDK1* in 389 (84.9%), 78 (17%), 307 (67.1%), 84 (18.3%) and 61 (13.3%) samples, respectively. *PTEN* underexpression was significantly mutually exclusive with *PIK3CA*, *PIK3R1* and *AKT1* mutations (P = 0.00016) being found only in one tumor, mutated also in *AKT1* and in 14 tumors mutated in *PIK3CA*.

Since there is growing evidence suggesting that *PIK3CA* mutations associate with favorable prognosis and prolonged survival in breast cancer (Maruyama *et al*, 2007; Pérez-Tenorio *et al*, 2007; Cizkova *et al*, 2012), we focused on patient survival in *PIK3CA* mutated versus wild-type tumors. Furthermore, we also evaluated the survival impact of *PIK3R1* expression deregulation in our patients. Opposite effects of *PIK3CA* mutation and *PIK3R1* underexpression on patient survival were found. *PIK3CA* mutation associated with better and *PIK3R1* loss with worse MFS (P = 0.016 and P = 0.00028, respectively). Multivariate analysis showed a strong trend to better outcome of *PIK3CA* mutations in HER2-positive

tumors (P = 0.051). Furthermore, the prognostic significance of *PIK3R1* underexpression persisted in the entire series (P = 0.0013) and in breast cancer subgroups characterized by ERα+ (P = 0.0076), PR-positive (P = 0.043), HER2-positive (P = 0.018) and also HER2-negative (P = 0.024). Important is that similar to the previous study, the composition of the patient series was a natural collection of breast cancer patients diagnosed between years 1978 and 2008 with the commonly found proportion of the particular breast cancer subtypes based on HR and HER2 states. Noteworthy, the patient survival demonstrates response to non-targeted treatment modalities only including surgery, chemotherapy and hormonal therapy.

p85 protein, encoded by *PIK3R1* gene, is essential for the stability of the p110 protein and its membrane recruitment and activation. Furthermore, under resting state, monomeric p85 might act as a negative regulator of PI3K signaling, but this effect of p85 is uncertain because of equimolar levels of p85 and p110 observed in mammalian cells (Luo *et* Cantley, 2005; Geering *et al*, 2007). The results of the present study show that the favorable survival associated with *PIK3CA* mutations remains in the case of normal expression of *PIK3R1* where its functions are preserved and worsens in the case of *PIK3R1* decreased expression. Similarly in the case of wild-type *PIK3CA*, patient survival is better when *PIK3R1* is normally expressed and worse when *PIK3R1* expression is decreased. This new observation suggests that normal *PIK3R1* function is needed for mutated as well as normal *PIK3CA*. Moreover, the favorable survival observed in *PIK3R1* expressing cases could be connected with the stabilizing effect of p85 protein on PTEN protein, one of the crucial negative regulators of the PI3K pathway (Chagpar *et al*, 2010; Cheung *et al*, 2011). Our results are based on *PIK3R1* mRNA levels, but expression concordance was confirmed on a small subset of samples by immunohistochemistry (IHC). Taniguchi et al. (Taniguchi *et al*, 2010) described the development of aggressive tumors in a background of organ-specific *PIK3R1* loss. On the other hand, studies on other cancer types and p85 at the protein level reported p85 expression associated with advanced stage disease, markers of poor prognosis and inferior survival (Elfiky *et al*, 2011; Zito *et al*, 2012). The effect of *PIK3CA* and *PIK3R1* on patient survival described in this study raises an interesting question about the tumor suppressor role of p85 in breast cancer that might be useful in clinical practice.

PIK3R1 underexpression is an independent prognostic marker in breast cancer

(Submitted in Clinical Cancer Research)

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Abstract:

Background: PI3K is a heterodimer kinase which consists of a p110α catalytic subunit encoded by the *PIK3CA* gene and a p85α regulatory subunit encoded by the *PIK3R1* gene. The present study focused on the prognostic roles of these two genes and additional PI3K pathway-associated genes in breast cancer.

Materials and methods: The mutational status of *PIK3CA, PIK3R1* and *AKT1*, and the mRNA expression status of these three genes and other genes involved in the PI3K pathway (*EGFR*, *PDK1*, *PTEN, AKT2, AKT3, GOLPH3, WEE1, P70S6K)* were assessed in a series of 458 breast cancer samples. Protein expression of PTEN and PI3K subunit p85 was confirmed by immunohistochemistry in a subset of samples.

Results: *PIK3CA* mutations were identified in 151 samples (33.0%) in exons 1, 2, 9 and 20. *PIK3R1* mutations were found in 11 samples (2.4%) and underexpression in 283 samples (61.8%). *AKT1* mutations were found in 15 samples (3.3%) and overexpression in 116 samples (25.3%). *PIK3R1* underexpression was mutually exclusive with *PIK3CA*

mutations (p=0.00097), predominantly observed in triple-negative and hormone receptor-positive tumors, respectively. Tumors characterized by *PIK3R1* underexpression were associated with *PDK1* overexpression (p=0.000004) and *EGFR* and *PTEN* underexpression (p=0.0096 and p<0.0000001, respectively). Opposite effects of *PIK3CA* mutation and *PIK3R1* underexpression on patient survival were observed. *PIK3CA* mutations were associated with better metastasis-free survival and *PIK3R1* underexpression was associated with poorer metastasis-free survival (p=0.014 and p=0.00028, respectively). By combining *PIK3CA* mutation and *PIK3R1* expression status, four prognostic groups were identified with significantly different metastasis-free survival (p=0.00046). On Cox multivariate regression analysis, the prognostic significance of *PIK3R1* underexpression was confirmed in the total population (p=0.0013) and in breast cancer subgroups.

Conclusion: The results of the present study show that alterations in *PIK3CA* and *PIK3R1* have a complementary impact on PI3K/AKT pathway activation, demonstrated by *PIK3CA* mutations in hormone receptor-positive tumors and *PIK3R1* underexpression in triple-negative tumors. As prognostic factors in breast cancer patient survival, these alterations in *PIK3CA* and *PIK3R1* show opposite effects on patient outcome. Combinations of *PIK3CA* mutation and *PIK3R1* expression could be useful prognostic factors and predictive factors of targeted therapy response in breast cancer.

Introduction

The phosphatidylinositol 3-kinase (PI3K) pathway has been identified as an important player in cancer development and progression. Following receptor tyrosine kinase activation, PI3K kinase phosphorylates inositol lipids to phosphatidylinositol-3,4,5-trisphosphate. The level of phosphatidylinositol-3,4,5-trisphosphate is regulated by phosphatase activity of PTEN. Signal transmission subsequently leads to PDK1 followed by activation of AKT. AKT then regulates activation of the pathway downstream effectors, including mTOR and subsequently P70S6K as well as other targets such as GSK3, WEE1 or BAD. mTOR has been found to be positively regulated by GOLPH3. The PI3K pathway controls important cellular processes such as protein synthesis, cell growth and proliferation, angiogenesis, cell cycle and survival (Katayama *et al*, 2005; Scott *et al*, 2009; Baselga, 2011).

PI3K pathway deregulation is frequent in tumor cells and can be caused by multiple changes affecting different levels of the signaling cascade. These changes include gene amplifications, mutations and expression alterations. However, various patterns of PI3K pathway changes have been identified in different cancer types. In breast cancer, such events commonly affect receptor tyrosine kinases, *PTEN*, *PIK3CA* and, to a lesser degree, *AKT1*. *PIK3CA* as well as *AKT1* mutations have been described as early events in the breast cancer development process (Stemke-Hale *et al*, 2008; Dunlap *et al*, 2009; Castaneda *et al*, 2010; Baselga, 2011).

PI3K is a heterodimer and consists of a p110α catalytic subunit encoded by the *PIK3CA* gene and a p85 regulatory subunit alpha encoded by the *PIK3R1* gene (Shekar *et al*, 2005; Barbareschi *et al*, 2007; Maruyama *et al*, 2007; Pérez-Tenorio *et al*, 2007; Kalinsky *et al*, 2009). The *PIK3CA* oncogene is a well known site of activating hot spot mutations located in exons 9 and 20, corresponding to the helical (E542K and E545K) and kinase (H1047R) domains, respectively. *PIK3CA* mutations are among the most common mutations, as they are observed in 10 to 40% of breast cancer cases, depending on the breast cancer subtype (Barbareschi *et al*, 2007; Stemke-Hale *et al*, 2008; Baselga, 2011; Cizkova *et al*, 2012). *PIK3CA* carrying a hotspot mutation exerts an oncogenic activity: it can transform primary fibroblasts in culture, induce anchorage-independent growth, and cause tumors in animals (Zhao *et al*, 2005; Bader *et al*, 2006). Apart from exons 9 and 20, *PIK3CA* has been recently shown to be also mutated frequently in other exons, as demonstrated by Cheung *et al.* in the case of endometrial cancer (Cheung *et al*, 2011). On the contrary, the *PIK3R1* gene appears to play a tumor suppressor role because PI3K subunit p85α (p85α) regulates and stabilizes p110α (Shekar *et al*, 2005; Taniguchi *et al*, 2011). *PIK3R1* has also been recently found to be mutated in breast cancer, but with a considerably lower frequency (about 3%) than *PIK3CA* (The Cancer Genome Atlas Network, 2012). The impact of its suppressor activity needs to be further described in breast cancer. Loss of PTEN expression, observed in about 20-30% of cases, is known to be one of the most common tumor changes leading to PI3K pathway activation in breast cancer (Stemke-Hale *et al*, 2008).

Discordant reports have been published concerning the prognostic role of *PIK3CA* mutations (Saal et al, 2005; Li *et al*, 2006; Stemke-Hale *et al*, 2008). These mutations appear to be preferentially associated with more favorable clinicopathologic characteristics and more favorable outcome in breast cancer patients (Baselga, 2011). *PIK3R1* underexpression

might possibly lead to PI3K pathway activation and confer tumor development and progression in humans in a similar way to that observed in a mouse model of hepatocellular cancer (Taniguchi *et al*, 2011).

In the present study, we explored the two genes encoding PI3K subunits and their role in PI3K pathway deregulation and patient survival. *PIK3CA*, *PIK3R1* and *AKT1* mRNA expression levels and mutations were studied. We also assessed mRNA expression levels of other genes involved in the PI3K pathway, namely *EGFR*, *PDK1*, *PTEN*, *AKT1*, *AKT2, AKT3, GOLPH3*, *P70S6K*, and *WEE1* to elucidate the pathway deregulations associated with changed *PIK3CA* and *PIK3R1* states. PTEN and p85 protein expression were also assessed by immunohistochemistry.

Materials and methods

Patients and Samples

We analyzed 458 samples of unilateral invasive primary breast tumors excised from women at the Institut Curie / Hôpital René Huguenin (Saint-Cloud, France) from 1978 to 2008 (Table 1'). All patients admitted to our institution before 2007 were informed that their tumor samples might be used for scientific purposes and they were given the opportunity to refuse the use of their samples. Since 2007, patients admitted to our institution also give their approval by signing an informed consent form. This study was approved by the local ethics committee (René Huguenin Hospital Breast Group). Patients (mean age: 61.7 years, range: 31-91) met the following criteria: primary unilateral non-metastatic breast carcinoma, with full clinical, histological and biological data; no radiotherapy or chemotherapy before surgery; and full follow-up at Institut Curie / Hôpital René Huguenin. Median follow-up was 8.6 years (range: 4.3 months to 28.9 years). One hundred and seventy patients developed metastases.

Samples were examined histologically and were considered suitable for this study when the proportion of tumor cells exceeded 70% with sufficient cellularity, as demonstrated by evaluation of tumor samples stained by hematoxylin and eosin. Immediately following surgery, tumor samples were placed in liquid nitrogen until RNA extraction and also stored as formalin-fixed paraffin-embedded tumor tissue sample blocks for immunohistochemistry analysis.

Table 1′. Characteristics of the 458 primary breast tumors.

	Number of patients (%)	Number of distant relapses (%)	*P-value*[a]
Total	*458 (100.0)*	*170 (37.1)*	
Age			
≤50	*99 (21.6)*	*38 (38.4)*	*NS*
>50	*359 (78.4)*	*132 (36.8)*	
SBR histological grade[b,c]			
I	*58 (12.6)*	*8 (13.8)*	
II	*230 (50.2)*	*84 (36.5)*	***0.000079***
III	*161 (35.2)*	*74 (46.0)*	
Lymph node status[d]			
0	*120 (26.2)*	*35 (29.2)*	
1-3	*237 (51.7)*	*77 (32.5)*	***0.00000064***
>3	*100 (21.8)*	*58 (58.0)*	
Macroscopic tumor size[e]			
≤25mm	*223 (48.7)*	*63 (28.3)*	***0.00002***
>25mm	*227 (49.6)*	*106 (46.7)*	
ERα status			
Negative	*119 (26.0)*	*51 (42.9)*	***0.0086***
Positive	*339 (74.0)*	*119 (35.1)*	
PR status			
Negative	*195 (42.6)*	*86 (44.1)*	***0.0011***
Positive	*263 (57.4)*	*84 (31.9)*	
ERBB2 status			
Negative	*359 (78.4)*	*128 (35.7)*	*NS*
Positive	*99 (21.6)*	*42 (42.4)*	
Molecular subtypes			
HR- ERBB2-	*69 (15.1)*	*27 (39.1)*	
HR- ERBB2+	*45 (9.8)*	*23 (51.1)*	***0.0087***
HR+ ERBB2-	*290 (63.3)*	*101 (34.8)*	
HR+ ERBB2+	*54 (11.8)*	*19 (35.2)*	

[a]Log-rank test. NS: not significant.

[b]Scarff-Bloom-Richardson classification.

[c]Information available for 449 patients.

[d]Information available for 457 patients.

[e]Information available for 450 patients.

Treatment consisted of modified radical mastectomy in 283 cases (63.9%) and breast-conserving surgery plus locoregional radiotherapy in 160 cases (36.1%). None of the ERBB2-positive patients was treated by anti-ERBB2 therapy. Clinical examinations were performed every 3 or 6 months for the first 5 years according to the prognostic risk of the patients, then yearly. Mammograms were done annually. Adjuvant therapy was administered to 358 patients, consisting of chemotherapy alone in 90 cases, hormone therapy alone in 175 cases and both treatments in 93 cases. The histological type and number of positive axillary nodes were established at the time of surgery. The malignancy of infiltrating carcinomas was scored with Bloom and Richardson's histoprognostic system.

Estrogen receptor (ER) and progesterone receptor (PR) status was determined at the protein level by using biochemical methods (dextran-coated charcoal method or enzyme immunoassay) until 1999 and then by immunohistochemistry. The cutoff for estrogen and progesterone receptor positivity was set at 15 fm/mg (dextran-coated charcoal or enzyme immunoassay) and 10% immunostained cells (immunohistochemistry). A tumor was considered ERBB2-positive by IHC when it scored 3+ with uniform intense membrane staining > 30% of invasive tumor cells. Tumors scoring 2+ were considered to be equivocal for ERBB2 protein expression and were tested by FISH for ERBB2 gene amplification. In all cases, the ERα, PR and ERBB2 status was also confirmed by real-time quantitative RT-PCR with cutoff levels based on previous studies comparing results of the these methods (Bièche *et al*, 1999; Bièche *et al*, 2001; Ondy *et al*, 2001; Bossard *et al*, 2005). Based on HR (ERα and PR) and ERBB2 status, the 458 patients were subdivided into 4 subgroups as follows: HR- (ER- and PR-) / ERBB2- (n=69), HR- (ER- and PR-) / ERBB2+ (n=45), HR+ (ER+ or/and PR+) / ERBB2- (n=290) and HR+ (ER+ or/and PR+) / ERBB2+ (n=54).

RNA extraction

Total RNA was extracted from breast tumor samples by using the acid-phenol guanidium method. The quantity of RNA was assessed by using an ND-1000 NanoDrop Spectrophotometer with its corresponding software (Thermo Fisher Scientific Inc., Wilmington, DE). RNA quality was determined by electrophoresis through agarose gel and staining with ethidium bromide. The 18S and 28S RNA bands were visualized under ultraviolet light. DNA contamination was quantified by using a primer pair located in an

intron of the gene encoding albumin (gene *ALB*). Only samples with a cycle threshold (Ct) using these *ALB* intron primers greater than 35 were used for subsequent analysis.

Mutation screening

PIK3CA mutations (exons 1, 2, 9, 20), *PIK3R1* (exons 11-15) and AKT1 (exon 4) were detected by sequencing of cDNA fragments obtained by RT-PCR amplification. Screening by high-resolution melting curve analysis was performed on *PIK3CA* exons 1 and 2, *AKT1* exon 4 and *PIK3R1* exons 10 to 14 on a LightCycler 480 (Roche Diagnostics, Penzberg, Germany) using LCGreen Plus+ Melting Dye fluorescence (Biotech, Idaho Technology Inc., Salt Lake City, UT). Details of the primers and PCR conditions are available on request. The amplified products were sequenced with the BigDye Terminator kit on an ABI Prism 3130 automatic DNA sequencer (Applied Biosystems, Courtaboeuf, France) with detection sensitivity of 5% mutated cells, and the sequences were compared with the corresponding cDNA reference sequences (*PIK3CA* NM_006218, *PIK3R1* NM_181523, *AKT1* NM_005163). All detected mutations were confirmed in the second independent run of sample testing.

Real-time quantitative RT-PCR

RT-PCR was applied to the selected genes and to *TBP* (NM_003194) as endogenous mRNA control. PCR conditions are available on request. The RT-PCR protocol using the SYBR Green Master Mix kit on the ABI Prism 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA) is described in detail elsewhere (Bieche et al, 1999). The relative mRNA expression level of each gene, expressed as the N-fold difference in target gene expression relative to the *TBP* gene, and termed "N*target*", was calculated as $N_{target}=2^{\Delta Ct_{sample}}$. The value of the cycle threshold (ΔCt) of a given sample was determined by subtracting the average Ct value of the target gene from the average Ct value of the *TBP* gene. The N*target* values of the samples were subsequently normalized so that the median N*target* value of normal breast samples was 1. Cut-offs for normalized values ≤ 0.5 and ≥ 2.0 were used to determine gene underexpression and overexpression, respectively.

Immunohistochemistry

PTEN and p85 protein expression levels were assessed by immunohistochemistry staining on tumor sections from formalin-fixed paraffin-embedded blocks. Indirect

immunoperoxidase staining was performed using mouse monoclonal antibody directed against human PTEN protein (Dako, Glostrup, Denmark) and rabbit polyclonal antibody directed against human p85 protein (Signalway Antibody, Baltimore, Maryland). The localization and intensity of staining were assessed by two independent pathologists blinded to real-time RT–PCR results.

Both antibodies were used at a 1/50 dilution. The immunohistochemical procedure was performed as described below, using a water bath antigen-retrieval technique in each case. Sections were mounted on precoated slides (Dako, Glostrup, Denmark) and allowed to dry at 50^0C overnight. Sections were then dewaxed in xylene and hydrated by graded dilutions of ethanol. Endogenous activity was blocked with 1% hydrogen peroxide for 15 min. Sections were then immersed in a heat-resistant plastic box containing 10 ml of pH 9.0 citrate buffer and processed in the water bath for 40 min. Sections were then allowed to cool to room temperature for 20 min before rinsing in H_2O. The blocking reagent was poured off and the primary antibodies were left for 25 min. A standard avidin–biotin–peroxidase complex (LSAB) method was used to reveal the antibody–antigen reaction (Dako, Glostrup, Denmark). Autostainer link 48 was used for the staining process (Dako, Glostrup, Denmark).

Statistical analysis

Relationships between tumor changes (expressed as mutational or expression status) and clinical, histological and biological parameters were estimated with the Chi^2 test. Differences between the population subgroups were considered significant at confidence levels greater than 95% ($p<0.05$). Metastasis-free survival (MFS) was determined as the interval between diagnosis and detection of the first metastasis. Survival distributions were estimated by the Kaplan-Meier method (Kaplan et al, 1958), and the significance of differences between survival rates was ascertained with the log-rank test (Peto *et al*, 1977). Cox's proportional hazards regression model (Cox *et al*, 1972) was used to assess prognostic significance in multivariate analysis.

Results

PIK3CA, PIK3R1 and *AKT1* mutational analysis

The present study extends our previously published data describing the positive effect of *PIK3CA* exon 9 and 20 mutations on breast cancer patient survival (Cizkova *et al*, 2012). In the present study, *PIK3CA* mutations were additionally assessed in exons 1 and 2. *PIK3CA* mutations were identified in 151 (33.0%) of the 458 samples, in line with previous studies in which *PIK3CA* mutations were found in 10 to 40% of breast cancer cases (Barbareschi *et al*, 2007; Stemke-Hale *et al*, 2008; Baselga, 2011). Sixty-three tumors showed *PIK3CA* mutations located in exon 9, 85 tumors showed mutations in exon 20, and one tumor showed mutations in both exon 9 and exon 20. Five mutations were found in exon 1, including two cases with 3 nucleotide deletions (c.305_307del and c.328_330del). Three other mutated tumors showed point mutations (R115L in one case and R108H in two cases). Two tumors showed mutations in exon 2 (both G118D). Point mutations in exons 1 and 2 were always found in cases mutated in either exon 9 or exon 20, but the two tumors with deletions did not present any additional *PIK3CA* mutations in other exons. Breast cancer subgroup analysis demonstrated *PIK3CA* mutations with the lowest frequency (10/69; 14.5%) in HR-/ERBB2- tumors and the highest frequency (118/290; 40.7%) in HR+/ERBB2- tumors, while an intermediate frequency of *PIK3CA* mutations was observed in HR-/ERBB2+ and HR+/ERBB2+ tumors (9/45; 20.0% and 14/54; 25.9%, respectively).

PIK3R1 mutations were screened in exons 11 - 15 and were present in 11 (2.4%) of the 454 available samples (Table 2'). Seven cases of deletions of 3-nucleotide multiples were observed in exons 11 and 13 (in the area between nucleotides 1345-1368 and 1701-1743, respectively), 2 cases of duplications of 3-nucleotide multiples were observed in exon 13 (in the area between nucleotides 1650-1723) and 2 cases of point mutations were observed in exons 13 and 15 (c.1590G>A, c.1925G>T). It is noteworthy that the AAG-->AAA (Lys) nucleotide substitution located at codon 1590 is probably a polymorphism with no amino acid change. *PIK3R1* mutations were found in only 1 of the 151 *PIK3CA*-mutated cases and in 10 of the 297 *PIK3CA* wild-type cases and were therefore mutually exclusive with *AKT1* mutations. The low frequency of *PIK3R1* mutations did not allow any further statistical analysis concerning a possible association between *PIK3R1* mutations and clinical, histological and biological parameters.

Table 2'. List of *PIK3R1* mutations found in the present study.

Sample	*PIK3R1* mutation			*PIK3CA* mutation
	Nucleotide	Codon	Exon	
1	c.1345_1347del	p.Leu449del	11	no
2	c.1351_1368del	p.Glu451_Phe456del	11	no
3	c.1590G>A	p.=(Lys530Lys)	13	no
4	c.1650_1688dup	p.Lys551_Met563dup	13	no
5	c.1701_1727del	p.Pro568_Thr576del	13	no
6	c.1718_1723dup	p.Arg574_Lys575insMetArg	13	p.His1047Arg
7	c.1723_1731del	p.Lys575_Arg577del	13	no
8	c.1727_1729del	p.Thr576del	13	no
9	c.1738_1743del	p.Tyr580_Leu581del	13	no
10	c.1738_1743del	p.Tyr580_Leu581del	13	no
11	c.1925G>T	p.Arg642Leu	15	no

AKT1 mutation (E17K) was found in 15 (3.3%) of the 457 available samples. *AKT1* mutations were found in only 1 of the 161 *PIK3CA/PIK3R1*-mutated cases and 14 of the 297 *PIK3CA/PIK3R1* wild-type cases and were therefore mutually exclusive with *PI3K* mutations (p=0.019).

Altogether, we observed *PIK3CA* and/or *PIK3R1* and/or *AKT1* mutations in 175/454 (38.5%) breast cancer tumors. Breast cancer subgroup analysis demonstrated mutation of at least one of the three genes with the highest frequency in HR+/ERBB2- tumors (134/289; 46.4%). The other 3 breast cancer subtypes showed a lower frequency of these mutations: HR+/ERBB2+ in 15/54 (27.8%), HR-/ERBB2+ in 10/43 (23.3%) and HR-/ERBB2- in 16/68 (23.5%).

mRNA expression

The *PIK3CA*, *PIK3R1* and *AKT1* mRNA expression levels were assessed in the whole series of 458 samples. *PIK3R1* underexpression was found in 283 (61.8%) cases, indicating a relevant tumor alteration occurring in the majority of tumor samples (Table 3'). Moreover, when assessing breast cancer subgroups, *PIK3R1* was predominantly underexpressed in HR-/ERBB2- and HR-/ERBB2+ tumors (p<0.0000001) (Table 4'), while *PIK3CA* was deregulated in only a minority of tumor samples: overexpressed in 18 (3.9%)

Table 3'. Gene mRNA levels in 458 breast tumors.

Genes	Median Ct of normal breast tissue (n=10)	Normal breast tissue (n=10)	Breast tumors n=458	Percentage of underexpressed tumors (Ntarget ≤0.5)	Percentage of normal expressed tumors	Percentage of overexpressed tumors (Ntarget ≥2)
EGFR	30.2 (29.3-31.5)[a]	1.0 (0.7 -1.3)[b]	0.2 (0.0-112.9)[b]	**84.9%**[c]	**13.3%**[c]	1.8%[c]
PIK3CA	29.7 (28.4-31.0)	1.0 (0.7-1.3)	0.9 (0.2-33.4)	8.7%	**87.4%**	3.9%
PIK3R1	26.8 (25.8-28.1)	1.0 (0.7-1.5)	0.4 (0.0-5.2)	**61.8%**	**36.0%**	2.2%
PDK1	31.8 (29.7-33.5)	1.0 (0.5-1.9)	1.0 (0.0-14.7)	**13.3%**	**69.0%**	**17.7%**
PTEN	26.4 (25.3-31.3)	1.1 (0.7-2.0)	0.8 (0.1-9.0)	**17.0%**	**81.0%**	2.0%
AKT1	28.7 (27.5-30.1)	1.0 (0.7-1.5)	1.5 (0.0-11.1)	1.3%	**73.4%**	**25.3%**
AKT2	26.7 (25.4-29.7)	1.0 (0.7-2.0)	1.7 (0.5-12.2)[d]	0.0%	**64.0%**	**36.0%**
AKT3	26.0 (23.8-28.4)	1.0 (0.6-1.9)	0.4 (0.0-7.5)[d]	**67.1%**	**31.1%**	1.8%
GOLPH3	27.9 (26.4-29.0)	1.0 (0.8-1.6)	1.4 (0.3-6.7)	0.7%	**79.9%**	**19.4%**
P70S6K	31.2 (29.9-32.7)	1.0 (0.7-1.8)	1.2 (0.0-19.6)	2.2%	**79.7%**	**18.1%**
WEE1	28.4 (26.1-29.8)	1.0 (0.5-1.6)	0.8 (0.2-6.9)	**18.3%**	**77.3%**	4.4%

[a]Median (range) of gene Ct values.

[b]Median (range) of gene mRNA levels; the mRNA values of the samples were normalized so that the median of the 10 normal breast tissue mRNA values was 1.

[c]Percentages of underexpressing, normal and overexpressing tumors using cut-offs of N*target* ≤0.5 and N*target* ≥2.

[d]Data available in 456 samples.

and underexpressed in 40 (8.7%) cases (Table 3'). *PIK3CA* expression did not vary significantly between the four breast cancer subgroups based on hormone and ERBB2 receptor status (Table 4'). Expression levels of *PIK3CA*, the oncogene bearing the highest number of mutations in breast cancer, were therefore mostly stable in breast cancer subgroups indicating that mutations constituted the main tumor change affecting *PIK3CA*. These results show that changes of expression of *PIK3R1* but not *PIK3CA* play a role in breast cancer, specifically in hormone receptor-negative cases. *AKT1* overexpression was present in 116 (25.3%) of the 458 available samples, mostly in HR-/ERBB2+ and HR+/ERBB2+ tumors (p=0.00019) (Table 4'). Seven of the 15 *AKT1* mutated tumors also showed increased *AKT1* expression. However, *AKT1* mutation and expression status as well as expression changes in other genes of the PI3K/AKT pathway did not show any statistically significant association (data not shown) possibly because of the small number of *AKT1* mutated cases.

mRNA expression levels of other genes involved in the PI3K/AKT pathway were also evaluated., i.e. *EGFR*, *PDK1*, *PTEN*, *AKT2* and *3*, *GOLPH3*, *P70S6K*, and *WEE1* (Table 3'). Marked overexpression with a frequency > 10% was observed for the known key players of the PI3K/AKT pathway, namely *PDK1, AKT2, GOLPH3* and *P70S6K* in 81 (17.7%) 116 (25.3%), 89 (19.4%) and 83 (18.1%) tumor samples, respectively. On the other hand, decreased expression was observed for *EGFR*, *PTEN, AKT3, WEE1* and, interestingly, in some cases, also for *PDK1* in 389 (84.9%), 78 (17%), 307 (67.1%), 84 (18.3%) and 61 (13.3%) samples, respectively. *PTEN* underexpression was significantly mutually exclusive with *PIK3CA*, *PIK3R1* and *AKT1* mutations (p=0.00016), as it was observed in only one *AKT1* mutated tumor and 14 *PIK3CA* mutated tumors.

Expression levels were also compared in the four breast cancer subgroups as shown in Table 4'. Interestingly, gene expressions were deregulated in different ways in the 4 subgroups. *EGFR* underexpression was demonstrated in all subgroups, as previously published (Meseure *et al*, 2011). In contrast, *PDK1* was mostly overexpressed in HR- and underexpressed in HR+ tumors (p<0.0000001). *PTEN* underexpression and *WEE1* underexpression were predominantly observed in HR-/ERBB2- tumors (p=0.0000066 and 0.0014, respectively). *P70S6K* and *AKT1* was predominantly overexpressed in ERBB2+ tumors (p<0.0000001 and 0.00019, respectively). This increased expression of these two genes might be linked to the PI3K/AKT pathway activated by ERBB2 overexpression. On the other hand, expression changes in HR-/ERBB2- tumors might indicate downstream activation of the pathway occurring despite the negativity of ERBB2. Alterations of the remaining 3 genes i.e. *AKT2*, *AKT3* and *GOLPH3* showed few or no associations with the 4 subgroups. The 4 molecular subgroups of breast cancer therefore appeared to undergo distinct changes at the levels of expression of the genes involved in the PI3K/AKT pathway.

The next step of analysis focused on PI3K constituents, specifically *PIK3R1* expression and *PIK3CA* mutations in relation to expression levels of the other genes evaluated. Tumors characterized by *PIK3R1* underexpression were associated with deregulation of other genes involved in the PI3K/AKT pathway (Table 5'). *PIK3R1* underexpression was associated with *PDK1* overexpression (p=0.000004) and *EGFR* and *PTEN* underexpression (p=0.0096 and p<0.0000001, respectively). *PIK3R1* underexpression was also associated with *AKT3* and *WEE1* underexpression (0.00000013 and 0.000063, respectively). *PIK3R1*

underexpression was negatively associated with *PIK3CA* mutations (p=0.00097) and these two parameters were therefore predominantly mutually exclusive.

Table 4'. Genes mRNA levels in the 4 breast tumor subtypes.

	Normal breast tissues	***All tumors***	***Tumor subtypes***				
	n = 10	*n = 458*	***HR-ERBB2-*** *n = 69*	***HR-ERBB2+*** *n = 45*	***HR+ERBB2-*** *n = 290*	***HR+ERBB2+*** *n = 54*	*P-value*[a]
PIK3CA values: median [range]	*1.0 (0.7-1.3)*	*0.9 (0.2-33.4)*	*0.9 (0.3-33.4)*	*0.7 (0.3-1.7)*	*0.9 (0.2-5.9)*	*1.0 (0.4-5.6)*	
Underexpressed tumors (%)		**40 (8.7)**	*6 (8.7)*	*5 (11.1)*	*25 (8.6)*	*4 (7.4)*	*NS*
Non-underexpressed tumors (%)		418 (91.3)	*63 (91.3)*	*40 (88.9)*	*265 (91.4)*	*50 (92.6)*	
PIK3R1 values: median [range]	*1.0 (0.7-1.5)*	*0.4 (0.0-5.2)*	*0.2 (0.0-2.2)*	*0.3 (0.1-1.5)*	*0.5 (0.1-4.4)*	*0.4 (0.1-5.2)*	
Underexpressed tumors (%)		**283 (61.8)**	*61 (88.4)*	*40 (88.9)*	*150 (51.7)*	*32 (59.3)*	***<0.0000001***
Non-underexpressed tumors (%)		175 (38.2)	*8 (11.6)*	*5 (11.1)*	*140 (48.3)*	*22 (40.7)*	
PDK1 values: median [range]	*1.0 (0.5-1.9)*	*1.0 (0.0-14.7)*	*2.4 (0.5-14.7)*	*1.7 (0.4-6.2)*	*0.9 (0.0-3.3)*	*0.9 (0.1-2.4)*	
Underexpressed tumors (%)		**61 (13.3)**	*0*	*1 (2.2)*	*51 (7.9)*	*9 (16.6)*	***<0.0000001***
Normally expressed tumors (%)		316 (69.0)	*25 (36.2)*	*27 (60.0)*	*221 (90.0)*	*43 (79.6)*	
Overexpressed tumors (%)		**81 (17.7)**	*44 (63.8)*	*17 (37.8)*	*18 (2.1)*	*2 (3.7)*	
PTEN values: median [range]	*1.1 (0.7-8.9)*	*0.8 (0.1-9.0)*	*0.6 (0.1-1.5)*	*0.8 (0.3-1.9)*	*0.8 (0.1-9.0)*	*0.9 (0.4-3.3)*	
Underexpressed tumors (%)		**78 (17.0)**	*27 (39.1)*	*6 (13.3)*	*39 (13.4)*	*6 (11.1)*	***0.0000066***
Non-underexpressed tumors (%)		380 (83.0)	*42 (60.9)*	*39 (86.7)*	*251 (86.6)*	*48 (88.9)*	
AKT1 values: median [range]	*1.0 (0.7-1.5)*	*1.5 (0.0-11.1)*	*1.1 (0.0-11.1)*	*2.0 (0.6-10.0)*	*1.4 (0.4-6.1)*	*1.8 (0.6-9.9)*	
Non-overexpressed tumors (%)		342 (74.7)	*55 (79.7)*	*24 (53.3)*	*230 (79.3)*	*33 (61.1)*	***0.00019***
Overexpressed tumors (%)		**116 (25.3)**	*14 (20.3)*	*21 (46.7)*	*60 (20.7)*	*21 (38.9)*	
AKT2 values: median [range][b]	*1.0 (0.7-2.0)*	*1.7 (0.5-12.2)*	*1.7 (0.7-12.2)*	*1.4 (0.8-8.7)*	*1.8 (0.5-10.6)*	*1.8 (0.5-7.0)*	
Non-overexpressed tumors (%)		293 (64.3)	*46 (67.6)*	*38 (84.4)*	*180 (62.3)*	*29 (53.7)*	***0.0097***
Overexpressed tumors (%)		**163 (35.7)**	*22 (32.4)*	*7 (15.6)*	*109 (37.7)*	*25 (46.3)*	
AKT3 values: median [range][b]	*1.0 (0.6-1.9)*	*0.4 (0.0-7.5)*	*0.5 (0.0-2.2)*	*0.3 (0.1-0.9)*	*0.4 (0.0-7.5)*	*0.4 (0.1-2.3)*	
Underexpressed tumors (%)		**306 (67.1)**	*38 (55.9)*	*32 (71.1)*	*198 (68.5)*	*38 (70.4)*	*NS*
Non-underexpressed tumors (%)		150 (32.9)	*30 (44.1)*	*13 (28.9)*	*91 (31.5)*	*16 (29.6)*	
GOLPH3 values: median [range]	*1.0 (0.8-1.6)*	*1.4 (0.3-6.7)*	*1.2 (0.6-3.3)*	*1.4 (0.7-5.0)*	*1.3 (0.3-6.7)*	*1.7 (0.8-5.4)*	
Non-overexpressed tumors (%)		369 (80.6)	*54 (78.3)*	*36 (80.0)*	*241 (83.1)*	*38 (70.4)*	*NS*
Overexpressed tumors (%)		**89 (19.4)**	*15 (21.7)*	*9 (20.0)*	*49 (16.9)*	*16 (29.6)*	
P70S6K values: median [range]	*1.0 (0.7-1.8)*	*1.2 (0.0-19.6)*	*1.0 (0.0-5.4)*	*1.9 (0.6-9.9)*	*1.2 (0.3-8.0)*	*1.4 (0.4-19.6)*	
Non-overexpressed tumors (%)		375 (81.9)	*64 (92.8)*	*24 (53.3)*	*250 (86.2)*	*37 (68.5)*	***<0.0000001***
Overexpressed tumors (%)		**83 (18.1)**	*5 (7.2)*	*21 (46.7)*	*40 (13.8)*	*17 (31.5)*	
WEE1 values: median [range]	*1.0 (0.5-1.6)*	*0.8 (0.2-6.9)*	*0.7 (0.2-6.9)*	*0.9 (0.3-2.8)*	*0.8 (0.2-3.9)*	*0.8 (0.3-4.1)*	
Underexpressed tumors (%)		**84 (18.3)**	*24 (34.8)*	*6 (13.3)*	*43 (14.8)*	*11 (20.4)*	***0.0014***
Non-underexpressed tumors (%)		374 (81.7)	*45 (65.2)*	*39 (86.7)*	*247 (85.2)*	*43 (79.6)*	

[a]Chi^2 test. NS: not significant.

[b]Data available in 456 samples.

In contrast to *PIK3R1*, deregulation of the expression of genes involved in the PI3K/AKT pathway was almost exclusively associated with *PIK3CA* wild-type tumors. *PTEN* and

PIK3R1 underexpression and *P70S6K* overexpression (p=0.0019, p=0.0017 and p=0.0000022, respectively) were negatively associated with *PIK3CA* mutation (Table 6'). Interestingly, *PDK1* was predominantly underexpressed in *PIK3CA* mutated tumors and overexpressed in *PIK3CA* wild-type tumors (p=0.0011).

Immunohistochemistry

Alteration of p85 (encoded by *PIK3R1*) and PTEN expression was also verified at the protein level by immunohistochemistry in randomly selected samples with low and high mRNA expression. In both cases, samples showing decreased mRNA expression (5 *PIK3R1* underexpressed- and 5 *PTEN* underexpressed-tumors) also presented low immunohistochemical staining intensity. Similarly, samples showing normal mRNA expression (7 *PIK3R1* expressing and 8 *PTEN* expressing tumors) presented strong immunohistochemical staining intensity. The only exceptions were two samples stained for PTEN (one showing low mRNA expression and more intense immunohistochemistry staining, the other showing opposite features). A good match (23/25 samples tested) was therefore obtained between mRNA and protein expression status for both *PIK3R1* and *PTEN* (Figure 1'). These results suggest that the regulation of p85 (and PTEN) expression is mainly transcriptional.

Survival analysis

Survival curves were compared to assess the possible impact of these expression changes and mutations on patient outcome. Table 7' summarizes survival analysis performed on the overall patient series. Patients presenting any of the mutations assessed in this study (*PIK3CA*, *PIK3R1* or *AKT1*) had a significantly poorer MFS (p=0.024). Among the 11 genes studied, only *PIK3CA* mutations and *PIK3R1* underexpression, as separate markers, were associated with MFS and had opposite effects on patient survival: *PIK3CA* mutation was associated with better MFS and *PIK3R1* underexpression was associated with poorer MFS (p=0.016 and p=0.00028, respectively). *PIK3R1* underexpression was associated with histological grade 3 status and an increased rate of positive axillary lymph nodes (p<0.0000001 and p=0.013, respectively). HR- and ERBB2+ tumors were also more likely to present *PIK3R1* underexpression (p<0.0000001 and p=0.011, respectively). These results show that *PIK3R1* underexpression predominantly occurred in tumors with poorer prognostic markers (Table 8'). The combination of these two molecular markers (*PIK3CA*

Table 5′. Comparison of *PIK3R1* expression status and alterations of other genes of interest.

	Number of patients (%)			
	Total population (%)	*PIK3R1* underexpression	*PIK3R1* non-underexpression	*P-value*[a]
Total	*458 (100.0)*	*283 (61.8)*	*175 (38.2)*	
EGFR values: median [range]	*0.2 (0.0-112.9)*	*0.1 (0.0-7.3)*	*0.2 (0.0-112.9)*	
Underexpressed tumors (%)	**389 (84.9)**	250 (88.3)	139 (79.4)	***0.0096***
Non-underexpressed tumors (%)	69 (15.1)	33 (11.7)	36 (20.6)	
PDK1 values: median [range]	*1.0 (0.0-14.7)*	*1.2 (0.1-14.7)*	*0.9 (0.0-6.2)*	
Underexpressed tumors (%)	**61 (13.3)**	26 (9.2)	35 (20.0)	
Normally expressed tumors (%)	316 (69.0)	189 (66.8)	127 (72.6)	***0.000004***
Overexpressed tumors (%)	**81 (17.7)**	68 (24.0)	13 (7.4)	
AKT1 values: median [range]	*1.5 (0.0-11.1)*	*1.4 (0.4-10.0)*	*1.6 (0.0-11.1)*	
Non-overexpressed tumors (%)	342 (74.7)	216 (76.3)	126 (72.0)	*NS*
Overexpressed tumors (%)	**116 (25.3)**	67 (23.7)	49 (28.0)	
AKT2 values: median [range][b]	*1.0 (0.7-2.3)*	*1.6 (0.5-10.6)*	*1.8 (0.5-12.2)*	
Non-overexpressed tumors (%)	293 (64.3)	189 (67.0)	104 (59.8)	*NS*
Overexpressed tumors (%)	**163 (35.7)**	93 (33.0)	70 (40.2)	
AKT3 values: median [range][b]	*1.0 (0.4-1.9)*	*0.3 (0.0-2.4)*	*0.5 (0.1-7.5)*	
Underexpressed tumors (%)	**306 (67.1)**	215 (76.2)	91 (52.3)	***0.00000013***
Non-underexpressed tumors (%)	150 (32.9)	67 (23.8)	83 (47.7)	
GOLPH3 values: median [range]	*1.4 (0.3-6.7)*	*1.3 (0.3-5.2)*	*1.7 (0.7-6.7)*	
Non-overexpressed tumors (%)	369 (80.6)	242 (85.5)	127 (72.6)	***0.00067***
Overexpressed tumors (%)	**89 (19.4)**	41 (14.5)	48 (27.4)	
P70S6K values: median [range]	*1.2 (0.0-19.6)*	*1.2 (0.4-19.6)*	*1.2 (0.0-8.9)*	
Non-overexpressed tumors (%)	375 (81.9)	226 (79.9)	149 (85.1)	*NS*
Overexpressed tumors (%)	**83 (18.1)**	57 (20.1)	26 (14.9)	
WEE1 values: median [range]	*0.8 (0.2-6.9)*	*0.7 (0.2-4.1)*	*0.9 (0.2-6.9)*	
Underexpressed tumors (%)	**84 (18.3)**	68 (24.0)	16 (9.1)	***0.000063***
Non-underexpressed tumors (%)	374 (81.7)	215 (76.0)	159 (90.9)	
PTEN values: median [range]	*0.8 (0.1-9.0)*	*0.7 (0.1-9.0)*	*1.0 (0.4-5.8)*	
Underexpressed tumors (%)	**78 (17.0)**	71 (25.1)	7 (4.0)	***< 0.0000001***
Non-underexpressed tumors (%)	380 (83.0)	212 (74.9)	168 (96.0)	
PIK3CA				
Wild-type (%)	307 (67.0)	*205 (72.4)*	*102 (58.3)*	***0.0017***
Mutation (%)	**151 (33.0)**	*78 (27.6)*	*73 (41.7)*	
PIK3R1[c]				
Wild-type (%)	433 (95.4)	*276 (98.6)*	*167 (96.0)*	*NS*
Mutation (%)	**11 (2.4)**	*4 (1.4)*	*7 (4.0)*	
AKT1[d]				
Wild-type (%)	442 (96.7)	272 (96.5)	170 (97.1)	*NS*
Mutation (%)	**15 (3.3)**	10 (3.5)	5 (2.9)	

[a]Chi^2 test. NS: not significant.

[b]Data available in 456 samples.

[c]Data available in 454 samples.

[d]Data available in 457 samples.

Table 6′. Comparison of *PIK3CA* mutational status and alterations in other genes of interest.

	Number of patients (%)			
	Total population (%)	*PIK3CA* wild-type	*PIK3CA*-mutated	*P-value*[a]
Total	*458 (100.0)*	*307 (67.0)*	*151 (33.0)*	
EGFR values: median [range]	*0.2 (0.0-112.9)*	*0.2 (0.0-112.9)*	*0.2 (0.0-7.3)*	
Underexpressed tumors (%)	**389 (84.9)**	256 (83.4)	133 (88.1)	*NS*
Non-underexpressed tumors (%)	69 (15.1)	51 (16.6)	18 (11.9)	
PIK3R1 values: median [range]	*0.4 (0.0-5.2)*	*0.3 (0.0-4.4)*	*0.5 (0.1-5.2)*	
Underexpressed tumors (%)	**283 (61.8)**	205 (66.8)	78 (51.7)	***0.0017***
Non-underexpressed tumors (%)	175 (38.2)	102 (33.2)	73 (48.3)	
PDK1 values: median [range]	*1.0 (0.0-14.7)*	*1.1 (0.0-14.7)*	*0.8 (0.1-4.5)*	
Underexpressed tumors (%)	**61 (13.3)**	35 (11.4)	26 (17.2)	
Normally expressed tumors (%)	316 (69.0)	204 (66.5)	112 (74.2)	***0.0011***
Overexpressed tumors (%)	**81 (17.7)**	68 (22.1)	13 (8.6)	
PTEN values: median [range]	*0.8 (0.1-9.0)*	*0.8 (0.1-5.8)*	*0.9 (0.1-9.0)*	
Underexpressed tumors (%)	**78 (17.0)**	64 (20.8)	14 (9.3)	***0.0019***
Non-underexpressed tumors (%)	380 (83.0)	243 (79.2)	137 (90.7)	
AKT1 values: median [range]	*1.5 (0.0-11.1)*	*1.5 (0.0-11.1)*	*1.5 (0.4-9.9)*	
Non-overexpressed tumors (%)	342 (74.7)	230 (74.9)	112 (74.2)	*NS*
Overexpressed tumors (%)	**116 (25.3)**	77 (25.1)	39 (25.8)	
AKT2 values: median [range][b]	*1.0 (0.7-2.3)*	*1.7 (0.5-12.2)*	*1.6 (0.5-10.6)*	
Non-overexpressed tumors (%)	293 (64.3)	190 (62.3)	103 (68.2)	*NS*
Overexpressed tumors (%)	**163 (35.7)**	115 (37.7)	48 (31.8)	
AKT3 values: median [range][b]	*1.0 (0.4-1.9)*	0.4 (0.0-3.6)	*0.4 (0.1-7.5)*	
Underexpressed tumors (%)	**306 (67.1)**	206 (67.5)	100 (66.2)	*NS*
Non-underexpressed tumors (%)	150 (32.9)	99 (32.5)	51 (33.8)	
GOLPH3 values: median [range]	*1.4 (0.3-6.7)*	*1.4 (0.5-6.7)*	*1.3 (0.3-5.4)*	
Non-overexpressed tumors (%)	369 (80.6)	242 (78.8)	127 (84.1)	*NS*
Overexpressed tumors (%)	**89 (19.4)**	65 (21.2)	24 (15.9)	
P70S6K values: median [range]	*1.2 (0.0-19.6)*	*1.2 (0.0-19.6)*	*1.1 (0.4-8.0)*	
Non-overexpressed tumors (%)	375 (81.9)	233 (75.9)	142 (94.0)	***0.0000022***
Overexpressed tumors (%)	**83 (18.1)**	74 (24.1)	9 (6.0)	
WEE1 values: median [range]	*0.8 (0.2-6.9)*	*0.8 (0.2-6.9)*	*0.7 (0.3-3.4)*	
Underexpressed tumors (%)	**84 (18.3)**	61 (19.9)	23 (15.2)	*NS*
Non-underexpressed tumors (%)	374 (81.7)	246 (80.1)	128 (84.8)	
PIK3R1[c]				
Wild-type (%)	433 (95.4)	293 (96.7)	150 (99.3)	*NS*
Mutation (%)	**11 (2.4)**	10 (3.3)	1 (0.7)	
AKT1[c]				
Wild-type (%)	442 (96.7)	292 (95.4)	150 (99.3)	*NS*
Mutation (%)	**15 (3.3)**	14 (4.6)	1 (0.7)	

[a]Chi[2] test. NS: not significant.

[b]Data available in 456 samples.

[c]Data available in 454 samples.

[d]Data available in 457 samples.

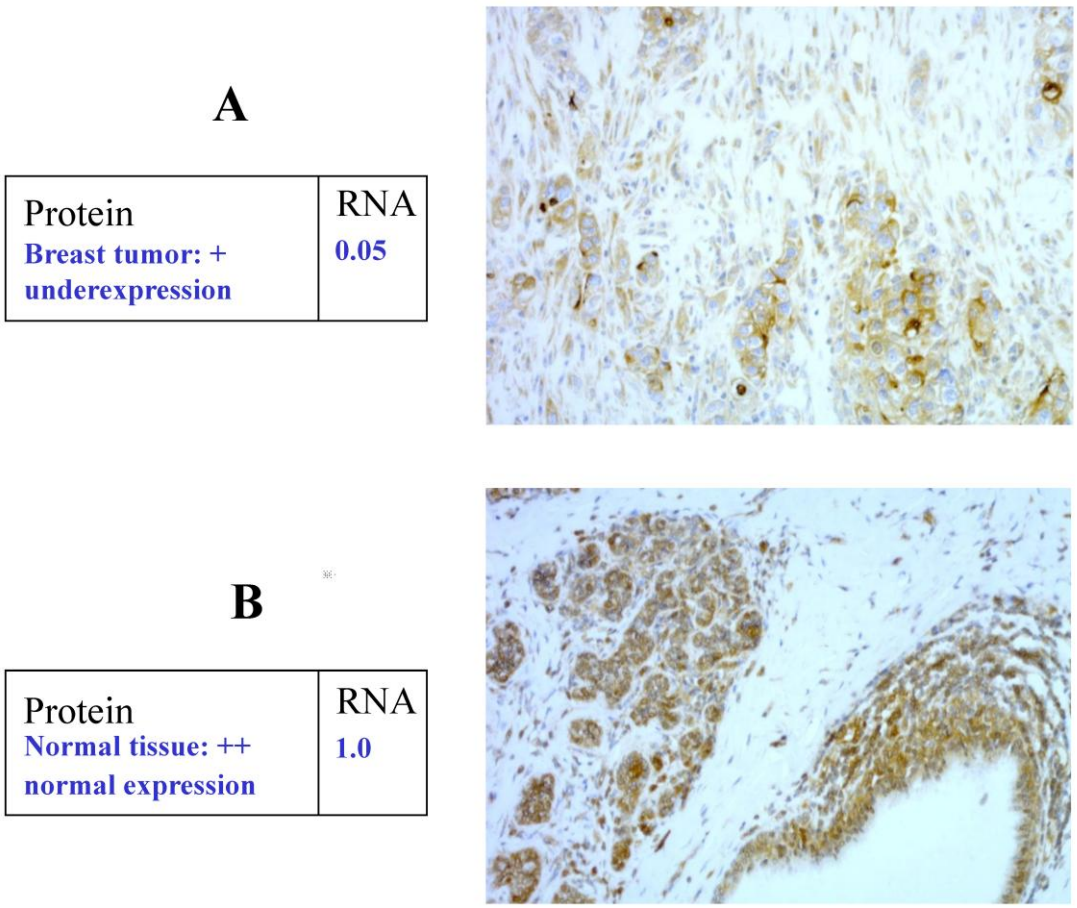

Figure 1′. Comparison of *PIK3R1*/p85 immunohistochemistry and mRNA expression results. A. Tumor sample with protein underexpression (expression intensity +) and decreased mRNA expression (normalized mRNA expression value 0.05). B. Healthy tissue sample with normal protein expression (expression intensity +++) and normal mRNA expression (normalized mRNA expression value 1.0).

mutations and *PIK3R1* underexpression) can be considered to provide more accurate prediction of patient survival than when they are considered separately. Combined analysis of *PIK3CA* mutations and *PIK3R1* expression status defined four separate prognostic groups with significantly different survivals (p=0.00046, Figure 2′). The least favorable survival was observed in the subgroup characterized by *PIK3CA* wild-type and *PIK3R1* underexpression and the most favorable survival was observed in the subgroup characterized by *PIK3CA* mutation without *PIK3R1* underexpression.

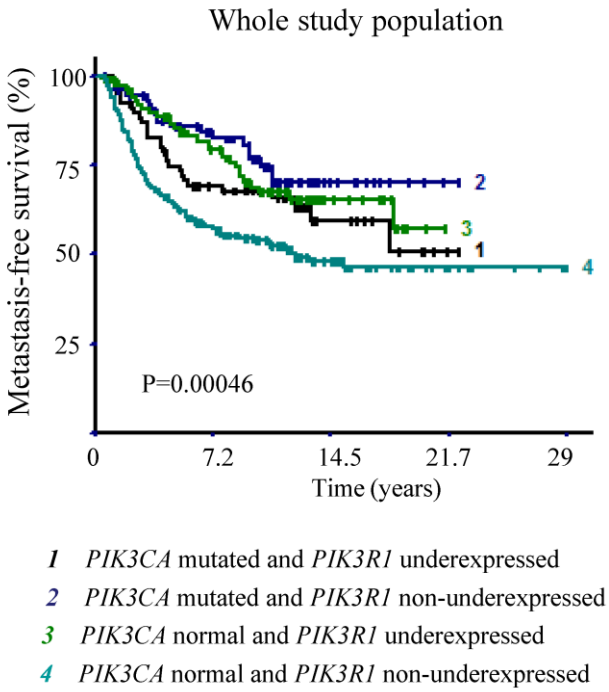

Figure 2'. Survival curves based on *PIK3R1* expression status and *PIK3CA* mutations.

Multivariate analysis using a Cox proportional hazards model (Table 9') assessed the predictive value for MFS of the parameters found to be significant on univariate analysis (i.e. Scarff-Bloom-Richardson histological grade, lymph node status, macroscopic tumor size, and ERα, PR, and ERBB2 status, as well as *PIK3CA* mutation and *PIK3R1* expression status). This analysis confirmed a trend towards an independent prognostic significance of *PIK3CA* mutations only in ERBB2+ tumors (p=0.051). Furthermore, the prognostic significance of *PIK3R1* underexpression persisted in the overall series (p=0.0013) and in breast cancer subgroups characterized by ERα+ (p=0.0076), PR+ (p=0.043), ERBB2+ (p=0.018) and also ERBB2- (p=0.024).

Discussion

This study extends the previously obtained data concerning the positive prognostic role of exon 9 and 20 *PIK3CA* mutations in breast cancer (Cizkova *et al*, 2012). This study focused on PI3K signaling pathway, particularly the two subunits of PI3K encoded by *PIK3CA* and *PIK3R1* genes. In addition to our previous study, *PIK3CA* mutations were also assessed in exons 1 and 2 that have been recently shown to be frequently mutated in

endometrial cancer (Cheung *et al*, 2011). *PIK3CA* mutations were detected in 33.0% of cases (exons 1, 2, 9, 20) and *PIK3R1* mutations were detected in 2.4% of cases (exons 11, 12, 13, 15). The low frequency of about 3% *PIK3R1* mutations is in agreement with published studies (Jaiswal *et al*, 2009; The Cancer Genome Atlas Network, 2012). *AKT1* mutations (exon 4) were also assessed and detected in 3.3% of tumors. This finding is also in agreement with previous studies describing a moderate frequency of *AKT1* mutations in breast cancer and their association with positive hormone receptor status (Castaneda *et al*, 2010). *PIK3CA*, *PIK3R1* and *AKT1* mutations were mutually exclusive and were observed in a total of 175 breast cancer tumors. Interestingly, *PIK3R1* underexpression was observed in 61.8% of breast cancer tumors. *PIK3CA* mutations were associated with better MFS and *PIK3R1* underexpression was associated with poorer MFS (p=0.014 and p=0.00028, respectively). By combining *PIK3CA* mutation and *PIK3R1* expression states, we identified four prognostic groups with significantly different MFS (p=0.00046). These new results suggest that *PIK3CA* mutations and *PIK3R1* underexpression are associated with opposite prognostic impacts on breast cancer patient survival. Multivariate analysis showed that *PIK3R1* expression status was an independent predictor of MFS in the total population (p=0.0013), whereas *PIK3CA* mutation status only showed a trend in the ERBB2+ population (p=0.051).

The frequency and associations of genomic and protein expression alterations in the PI3K pathway differ in the various breast cancer subgroups. Additionally, some alterations may co-exist, while others are mutually exclusive. Mutually exclusive mutations have been previously reported for *PIK3CA* and *AKT1* mutations (Stemke-Hale *et al*, 2008). We and other teams have found *PIK3CA* mutations in 10 to 40% of breast cancer cases and *AKT1* mutations in less than 10% of cases (Barbareschi *et al*, 2007; Stemke-Hale *et al*, 2008; Dunlap *et al*, 2009; Castaneda *et al*, 2010; Baselga, 2011; The Cancer Genome Atlas Network, 2012). Our data are in agreement with the mutational frequencies described by other authors. Our findings also support the data recently published by Ellis et al., who described a low frequency of exon 1 and 2 mutations in breast cancer. They also observed missense mutations in these two exons occurring in cases bearing additional *PIK3CA* mutations, whereas one deletion in exon 1 was not accompanied by another *PIK3CA* mutation (Ellis *et al*, 2012). The most frequent mutations were E542K and E545K in exon 9 and H1047R in exon 20 in keeping with most other studies (Saal *et al*, 2005; Barbareschi *et al*, 2007; Stemke-Hale *et al*, 2008; The Cancer Genome Atlas Network, 2012). We also found

Table 7′. Relationship between gene status and MFS.

	Total population (%)	Number of relapses (%)	*P-value*[a]
Total	*458 (100.0)*	*170 (37.1)*	
EGFR			
Underexpression	389 (84.9)	*153 (39.3)*	*NS*
Non-underexpression	69 (15.1)	*17 (24.6)*	
PIK3R1			
Underexpression	283 (61.8)	*122 (43.1)*	***0.00028***
Non-underexpression	175 (38.2)	*48 (27.4)*	
PDK1			
Underexpression	61 (13.3)	*22 (36.1)*	
Normal expression	316 (69.0)	*116 (36.7)*	*NS*
Overexpression	81 (17.7)	*32 (39.5)*	
PTEN			
Underexpression	78 (17.0)	*31 (39.7)*	*NS*
Non-underexpression	380 (83.0)	*139 (36.6)*	
AKT1			
Non-overexpression	342 (74.7)	*123 (36.0)*	*NS*
Overexpression	116 (25.3)	*47 (40.5)*	
AKT2[b]			
Non-overexpression	293 (64.3)	*119 (40.6)*	*NS*
Overexpression	163 (35.7)	*50 (30.7)*	
AKT3[b]			
Underexpression	306 (67.1)	*120 (39.2)*	*NS*
Non-underexpression	150 (32.9)	*49 (32.7)*	
GOLPH3			
Non-overexpression	369 (80.6)	*131 (35.5)*	*NS*
Overexpression	89 (19.4)	*39 (43.8)*	
P70S6K			
Non-overexpression	375 (81.9)	*134 (35.7)*	*NS*
Overexpression	83 (18.1)	*36 (43.4)*	
WEE1			
Underexpression	84 (18.3)	*34 (40.5)*	*NS*
Non-underexpression	374 (81.7)	*136 (36.4)*	
PIK3CA			
Non-mutated (%)	307 (67.0)	*124 (40.4)*	***0.016***
Mutated (%)	151 (33.0)	*46 (30.5)*	
PIK3R1[c]			
Non-mutated (%)	443 (97.6)	*162 (36.6)*	*NS*
Mutated (%)	11 (2.4)	*5 (45.5)*	
AKT1[d]			
Non-mutated (%)	442 (96.7)	*164 (37.1)*	*NS*
Mutated (%)	15 (3.3)	*5 (33.3)*	
All mutations[d]			
Non-mutated (%)	279 (61.5)	*112 (40.1)*	***0.024***
Mutated (%)	175 (38.5)	*55 (31.4)*	

[a]Log-rank Test. NS: not significant.

[b]Data available in 456 samples.

[c]Data available in 454 samples.

[d]Data available in 457 samples.

Table 8′. Characteristics of the 458 primary breast tumors correlated with *PIK3R1* expression status.

	Total population (%)	Number of patients (%) *PIK3R1* underexpression	Number of patients (%) *PIK3R1* non-underexpression	*P-value*[a]
Total	*458 (100.0)*	283 (61.8)	175 (38.2)	
Age				
≤50	*99 (21.6)*	61 (61.6)	38 (38.4)	*NS*
>50	*359 (78.4)*	222 (61.8)	137 (38.2)	
SBR histological grade[b,c]				
I	*58 (12.6)*	23 (39.7)	35 (60.3)	
II	*230 (50.2)*	125 (54.3)	105 (45.7)	***<0.0000001***
III	*161 (35.2)*	127 (78.9)	34 (21.1)	
Lymph node status [d]				
0	*120 (26.2)*	82 (68.3)	38 (31.7)	
1-3	*237 (51.7)*	131 (55.3)	106 (44.7)	***0.013***
>3	*100 (21.8)*	69 (69.0)	31 (31.0)	
Macroscopic tumor size [e]				
≤25mm	*223 (48.7)*	139 (62.3)	84 (37.7)	*NS*
>25mm	*227 (49.6)*	140 (61.7)	87 (38.3)	
ERα status				
Negative	*119 (26.0)*	103 (86.6)	16 (13.4)	***<0.0000001***
Positive	*339 (74.0)*	180 (53.1)	159 (46.9)	
PR status				
Negative	*195 (42.6)*	156 (80.0)	39 (20.0)	***<0.0000001***
Positive	*263 (57.4)*	127 (48.3)	136 (51.7)	
ERBB2 status				
Negative	*359 (78.4)*	211 (58.8)	148 (41.2)	***0.011***
Positive	*99 (21.6)*	72 (72.7)	27 (27.3)	
Molecular subtypes				
HR- ERBB2-	*69 (15.1)*	61 (88.4)	8 (11.6)	
HR- ERBB2+	*45 (9.8)*	40 (88.9)	5 (11.1)	***<0.0000001***
HR+ ERBB2-	*290 (63.3)*	150 (51.7)	140 (48.3)	
HR+ ERBB2+	*54 (11.8)*	32 (59.3)	22 (40.7)	

[a]Chi[2] test. NS: not significant.

[b]Scarff-Bloom-Richardson classification.

[c]Information available for 449 patients.

[d]Information available for 457 patients.

[e]Information available for 450 patients.

that *PIK3R1* mutations were mutually exclusive with *PIK3CA* and *AKT1* mutations. PTEN loss occurring in up to 30% of unselected breast tumor cohorts is also predominantly mutually exclusive with *PIK3CA* and *AKT1* mutations (Saal *et al*, 2005; Stemke-Hale *et al*, 2008). *PIK3R1* mutations as well as combined mutations of the three genes studied were also found to be mutually exclusive with *PTEN* underexpression (p=0.00016). As *PIK3CA* and *AKT1* are oncogenes activated by mutations and as *PIK3R1* and *PTEN* are tumor suppressors mainly inactivated by underexpression, respectively, all these alterations result in PI3K pathway activation. The frequencies of *PIK3CA*, *PIK3R1* and *AKT1* alteration differ according to breast cancer subtypes. *PIK3CA* mutations have been previously described to occur most frequently in HR+ breast tumors (Stemke-Hale *et al*, 2008; Cizkova *et al*, 2012). The highest mutational frequency for all of the genes assessed in this study (*PIK3CA* and/or *PIK3R1* and/or *AKT1*) was observed in HR+/ERBB2- tumors (134/289; 46.4%), while mutations were observed in up to 28% of cases in other breast cancer subtypes. In terms of expression, *PIK3R1* was underexpressed in about 90% of HR- tumors, but only in about 55% of HR+ breast cancers. Similarly, *PTEN* underexpression was observed in 40% of triple-negative tumors versus 13% in other breast cancer subtypes, suggesting different mechanisms underlining PI3K pathway deregulation in specific breast tumor subtypes.

The protein p85α encoded by the *PIK3R1* gene has been described to play an important role in PI3K pathway signaling by stabilizing the other PI3K subunit – p110α – encoded by *PIK3CA* gene (Yu *et al,* 1998; Shekar *et al*, 2005; Taniguchi *et al*, 2010). Loss of the p85α tumor suppressor effect leads to downstream PI3K pathway activation. The impact of *PIK3R1* deregulation on pathway signaling could be caused by the impaired ability of interaction of the two subunits and loss of the inhibitory effect of p85α on p110α and PI3K activity (Shekar *et al*, 2005; Jaiswal *et al*, 2009). *PIK3R1* has been reported to play a tumor suppressor role in hepatocellular cancer and this tumor suppressor effect is lost in the case of gene underexpression (Kalinsky *et al*, 2009; Taniguchi *et al*, 2010). Mostly point mutations and deletions have been reported for *PIK3R1*, but much less frequently in breast cancer (<5% of cases) than in other cancer types, such as endometrial cancer (about 20% of cases) (Jaiswal *et al*, 2009; Cheung *et al*, 2011). *PIK3R1* mutations were observed in 2.4% of cases in the present study. *PIK3R1* mutations and p85 loss have also been associated with PI3K pathway activation and increased oncogenic potential. However, the fact that *PIK3R1* mutations are rare in breast cancer indicates that *PIK3R1* mRNA/p85α

Table 9'.Results of Cox multivatiate analvsis.

Variables	Total population		ER-positive patients		ER-negative patients		PR-positive patients		PR-negative patients		ERBB2-positive patients		ERBB2-negative patients	
	HR (95% CI)[a]	*P* -value[b]	HR (95% CI)[a]	*P* -value[b]	HR (95% CI)[a]	*P* -value[b]	HR (95% CI)[a]	*P* -value[b]	HR (95% CI)[a]	*P* -value[b]	HR (95% CI)[a]	*P* -value[b]	HR (95% CI)[a]	*P* -value[b]
SBR														
I	1		1				1						1	
II	1.27 (1.01-1.61)	**0.041**	1.42 (1.06-1.90)	**0.018**		NS	1.87 (1.30-2.68)	**0.00068**		NS		NS	1.49 (1.14-1.95)	**0.0034**
III	1.62 (1.02-2.59)		2.02 (1.13-3.61)				3.49 (1.70-7.17)						2.23 (1.31-3.82)	
pN														
0	1		1		1		1		1		1			
1-3	1.54 (1.22-1.93)	**0.00021**	1.53 (1.16-2.01)	**0.0024**	1.61 (1.08-2.40)	**0.02**	1.57 (1.12-2.22)	**0.0097**	1.53 (1.13-2.07)	**0.0055**	2.91 (1.82-4.64)	**0.0000076**		NS
>3	2.36 (1.50-3.72)		2.34 (1.35-4.04)		2.58 (1.16-5.74)		2.47 (1.24-4.91)		2.34 (1.28-4.28)		8.47 (3.32-21.57)			
pT														
≤25mm	1	**0.004**	1	**0.00014**		NS	1	**0.00096**		NS		NS	1	**0.00034**
>25mm	1.62 (1.17-2.24)		2.16 (1.45-3.20)				2.22 (1.38-3.55)						1.97 (1.36-2.86)	
ER														
Negative		NS		-		-		NS		NS		NS		NS
Positive														
PR														
Negative		NS		NS		NS		-		-		NS		NS
Positive														
ERBB2														
Negative		NS		NS		NS		NS		NS		-		-
Positive														
PIK3R1														
Underexpression	1		1				1				1		1	
Normal	0.59 (0.43-0.81)	**0.0013**	0.62 (0.44-0.88)	**0.0076**		NS	0.65 (0.43-0.99)	**0.043**		NS	0.40 (0.19-0.85)	**0.018**	0.66 (0.46-0.95)	**0.024**
Overexpression	0.35 (0.18-0.66)		0.38 (0.19-0.77)				0.42 (0.18-0.97)				0.16 (0.03-0.73)		0.44 (0.21-0.89)	
PI3KCA														
Normal		NS		NS		NS		NS		NS	1	**0.051**		NS
Mutated											0.39 (0.15-1.00)			

[a] Hazard ratio (95% Confidential Interval). NS: not significant.

[b] Multivariate Cox analysis. NS: not significant.

expression loss is the main deregulation occurring in breast tumors, particularly in HR- breast tumors. Another player affecting the PI3K pathway activation is PTEN, a tumor suppressor phosphatase which negatively regulates the PI3K pathway. Loss of PTEN expression is frequently observed in various cancer types and in up to 30% of breast cancers, leading to PI3K pathway activation (Stemke-Hale *et al*, 2008). Interestingly, p85 has also been suggested to have a positive regulatory effect on PTEN function via stabilization of this protein (Taniguchi *et al*, 2010; Cheung *et al*, 2011). *PTEN* underexpression was found in 17% cases in our series (39% in triple-negative tumors) and was associated with *PIK3CA* wild-type status and *PIK3R1* underexpression, in line with previous findings.

There is growing evidence in the literature concerning the favorable outcome of *PIK3CA*-mutated breast cancer, as supported by the results of this study (Maruyama *et al*, 2007; Pérez-Tenorio *et al*, 2007; Kalinsky *et al*, 2009; Cizkova *et al*, 2012). These mutations are known to play an activating role in cell lines and animal models (Zhao *et al*, 2005; Bader *et al*, 2006). Several hypotheses are currently proposed to explain the favorable prognostic impact of *PIK3CA* mutations: 1, *PIK3CA* mutations, when they are the only hit to the PI3K signaling pathway, have a limited oncogenic potential; 2, *PIK3CA* mutations result in oncogene-induced senescence; 3, *PIK3CA* mutation-bearing cells are more sensitive to chemotherapy and/or other treatment modalities; 4, *PIK3CA* mutation-induced signaling triggers a negative feedback loop inhibiting lower levels of the pathway (Barbareschi *et al*, 2007; Baselga *et* Di Cosimo, 2009). *PIK3CA* mutations might affect the PI3K/AKT pathway in different ways in patient tumors and cell lines. The difference between *PIK3CA* mutation-related activation of the pathway in cell lines or animal models and patient outcome could be related to the treatment received by patients, as suggested above. In contrast with the *PIK3CA* mutation-associated survival advantage in anti-ERBB2 untreated patients, *PIK3CA* mutations appear to predict resistance to treatment including ERBB2 inhibitors such as trastuzumab (Dave *et al*, 2011; Jensen *et al*, 2012).

The present study demonstrates that *PIK3R1* underexpression is associated with decreased patient survival. Immunohistochemical analysis showed that *PIK3R1* transcripts are translated into p85 protein in epithelial tumor cells (Figure 1'). A strong correlation was also demonstrated between *PIK3R1* mRNA underexpression and decreased p85 protein levels. Immunohistochemistry could be the method of choice to routinely determine p85 expression status. *PIK3R1* underexpressing tumors were also

prone to cumulate other changes of the PI3K/AKT pathway, i.e. *PDK1* overexpression and *EGFR, AKT3, PTEN* and *WEE1* underexpressions. *PIK3R1* underexpression is therefore associated with additional pathway deregulation and increased signaling activation. In a murine model with liver-specific *PIK3R1* loss, this condition led to development of aggressive hepatocellular cancer (Taniguchi *et al*, 2010). Loss of *PIK3R1* mRNA expression in cell lines was associated with a more migratory and more invasive phenotype of MCF-7-14 cells compared to the parental MCF-7 cell line (Uchino *et al*, 2010). Lu *et al.* described a gene expression signature including *PIK3R1* distinguishing between low- and high-risk stage I lung cancer. The authors found low *PIK3R1* expression in high-risk compared to low-risk lung cancers (Lu *et al*, 2006). Studies concerning glioblastomas have also suggested that these tumors might be negatively influenced by *PIK3R1* expression at the level of cell lines and in terms of patient survival (Serão et al, 2011; Weber *et al*, 2011). The recently observed role of *PIK3R1* expression deregulation in breast cancer survival needs to be further assessed, preferably in a prospective clinical study.

Our results suggest that *PIK3R1* could potentially become a clinically useful independent prognostic marker in breast cancer. *PIK3R1* underexpression (as well *PIK3CA* mutation) might also predict a favorable response to treatment with PI3K inhibitors or inhibitors of lower levels of the signaling pathway, such as mTOR inhibitors (Bader *et al*, 2006; Jaiswal *et al*, 2009; Kataoka *et al*, 2010; Tanaka *et al*, 2011). Finally, *PIK3R1* underexpression (and *PIK3CA* mutation) could be used as predictors of resistance to treatment with ERBB2 inhibitors (Cizkova *et al*, 2012).

In conclusion, *PIK3CA* and *PIK3R1* are genes encoding two subunits of the PI3K enzyme, p110α and p85α, respectively. The present study showed that alterations in these two genes have a complementary impact on PI3K/AKT pathway activation and breast cancer patient survival. There is growing evidence supporting *PIK3CA* mutations as good prognostic markers in breast cancer, but the negative impact of *PIK3R1* underexpression on patient survival has been less extensively studied. These two potential tumor markers warrant further assessment, preferably in prospective clinical studies.

4.3 *HER2-targeting treatment response in HER2-positive breast cancer patients*

4.3.1 Outcome impact of *PIK3CA* mutations in HER2-positive breast cancer patients treated with trastuzumab

The present study focused on *PIK3CA* mutations in neoadjuvant chemotherapy and neoadjuvant/adjuvant or adjuvant only treated series of 80 HER2-positive breast cancer patients. All the patients received preoperatively 4 cycles of anthracycline-based chemotherapy followed by 4 cycles of docetaxel and one year of trastuzumab, starting either before surgery combined with docetaxel (n = 43), or only after surgery (n = 37). The *PIK3CA* mutations were assessed by direct sequencing on pre-treatment samples at cDNA level. *PIK3CA* mutations were found in 17 tumors (21.3%) of which 4 were in exon 9 and 13 in exon 20. We found only *PIK3CA*-wild type tumors responding well to trastuzumab added to chemotherapy in terms of disease free survival. Despite no association between *PIK3CA*-mutated or wild-type tumors in terms of pathological complete response to the treatment, superior disease free survival was found in patients with *PIK3CA* wild-type tumors compared with mutated tumors (P = 0.0063). Furthermore, disease free survival (DFS) varied significantly in subgroups based on treatment arms and *PIK3CA* mutation status (P = 0.0013). Improved DFS was found in neoadjuvant trastuzumab and chemotherapy-treated *PIK3CA* wild-type patients.

PI3K pathway activation, mostly represented by *PIK3CA* mutation and/or PTEN loss, was observed to cause resistance to trastuzumab in *in vitro* studies (Köninki *et al*, 2010; Dave *et al*, 2011; Jensen *et al*, 2012). Similarly, reports from clinical studies agree on the negative predictive role of *PIK3CA* mutations and PI3K pathway activation on trastuzumab treatment response (Dave *et al*, 2011; Razis *et al*, 2011; Wang *et al*, 2011; Jensen *et al*, 2012). The present study describes treatment response on neoadjuvant chemotherapy and trastuzumab started also in a neoadjuvant setting or delayed as adjuvant therapy in 80 HER2-positive breast cancer patients. The results show that treatment response was generally inferior in *PIK3CA*-mutated patients despite some insignificant improvement in case of neoadjuvant trastuzumab. Thus, this suggests that *PIK3CA* mutations confer partial resistance to trastuzumab in HER2-positive breast cancer and only *PIK3CA* wild-type patients benefit well from trastuzumab treatment. Our study supports previously described observations based on smaller patient cohorts than ours. In

contrast to trastuzumab, the situation with lapatinib, HER2 tyrosine kinase inhibitor, is less clear. There are reports describing lapatinib resistance associated with *PIK3CA* mutations as well as results describing favorable response to lapatinib in conditions of PI3K pathway activation (Eichhorn *et al*, 2008; Köninki *et al*, 2010; Dave *et al*, 2011; Wang *et al*, 2011).

Inconsistencies between *PIK3CA* mutations in primary breast tumors and their metastases were found and this might influence the results of studies based on retrospective sample collection and advance treatment lines. This could explain some discordances described in treatment response results mostly in studies assessing advanced line treatment based on primary tumors samples evaluated for *PIK3CA* mutations (Dupont Jensen *et al*, 2011). From this point of view, it is important that our study shows *PIK3CA* status assessed on pre-treatment tumor samples. Since the patients in the present study were treated with neoadjuvant chemotherapy and concomitant or delayed trastuzumab, the given *PIK3CA* status distinguishes well between *PIK3CA*-mutated and wild-type tumors.

Patients bearing tumors with *PIK3CA* mutations causing anti-HER2 resistance might benefit from the PI3K pathway downstream inhibition. Janku et al. (Janku *et al*, 2012) described the results of phase I clinical trials of 140 cancer patients including advanced breast, ovarian, endometrial and cervical tumors treated with mTOR and PI3K inhibitors in monotherapy or in combination with other agents. The authors found that patients with a *PIK3CA* mutation experienced a response rate of 39% (9/23), higher than patients with wild-type tumors [response rate of 10% (7/70)]. Interestingly, a large number of patients with H1047R *PIK3CA* mutation treated with the combinational therapy were found among the good responders. The future role of *PIK3CA* mutations in treatment efficacy prediction of PI3K pathway inhibitors is supported by a recent study that tested mTOR-inhibitors on a panel of cell lines and found association of *PIK3CA* and *PTEN* mutations with rapamycin sensitivity (Meric-Bernstam *et al*, 2012). On the other hand, a phase II clinical trial testing mTOR-inhibitor temsirolimus in monotherapy in 31 breast cancer patients did not find any association between *PIK3CA* mutations and treatment response, but this could be due to a variety of reasons including small patient sample (Fleming *et al*, 2012). More studies in the coming years will provide additional evidence and describe better the predictive role of *PIK3CA* mutations on anti-PI3K pathway inhibitors. Taken together, our results and the reports of other research groups, this

evidence supports the importance of *PIK3CA* mutation assessment in treatment outcome prediction of anti-HER2 and downstream anti-PI3K pathway inhibitors.

Outcome impact of *PIK3CA* mutations in HER2-positive breast cancer patients treated with trastuzumab

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Abstract:

Background: PI3K pathway activation has been suggested to negatively influence response to anti-HER2 therapy in breast cancer patients. The present study focused on mutations of the *PIK3CA* gene, encoding one of the two PI3K subunits.

Methods: *PIK3CA* mutations were assessed by direct sequencing in 80 HER2-positive patients treated with one year of trastuzumab**.** All patients preoperatively received 4 cycles of anthracycline-based chemotherapy followed by 4 cycles of docetaxel and one year of trastuzumab, starting either before surgery with the first cycle of docetaxel and continuing after surgery (neoadjuvant trastuzumab arm, n=43), or only after surgery (adjuvant trastuzumab arm, n=37).

Results: *PIK3CA* mutations were found in 17 tumors (21.3%). Better disease-free survival was observed in patients with *PIK3CA* wild-type compared to mutated tumors (P=0.0063). By combining *PIK3CA* status and treatment arms, four separate prognostic groups with significantly different disease-free survival (P=0.0013) were identified.

Conclusion: These results confirm that the outcome of HER2-positive patients treated with trastuzumab is significantly worse in patients with *PIK3CA*-mutated compared to wild-type tumors.

Introduction

The phosphatidylinositol 3-kinase (PI3K) pathway has been identified as an important player in cancer development and progression. Upon receptor tyrosine kinase activation, the PI3K kinase phosphorylates inositol lipids to phosphatidylinositol-3,4,5-trisphosphate. PI3K is a heterodimeric enzyme composed of a p110α catalytic subunit encoded by the *PIK3CA* gene and a p85 regulatory subunit encoded by the *PIK3R1* gene. Phosphatidylinositol-3,4,5-trisphosphate activates the serine/threonine kinase AKT, which in turn regulates several signaling pathways controlling cell survival, apoptosis, proliferation, motility, and adhesion (Zhao *et* Vogt, 2008; Baselga, 2011).

Recent reports suggest that the PI3K pathway activation could negatively influence response to trastuzumab therapy. This observation was described on both retrospective and prospective patient series (Dave *et al*, 2011; Wang *et al*, 2011; Jensen *et al*, 2012). Jensen *et al*. (2012) described a statistically significant poorer survival in 240 HER2-positive breast cancer patients with *PIK3CA* mutations treated with trastuzumab and chemotherapy in the adjuvant setting.

PIK3CA, encoding one of the two PI3K subunits, is an oncogene exhibiting gain-of-function mutations in several cancers, including breast, colorectal or endometrial cancer. These mutations are present in 20% to 40% cases of breast cancer. *PIK3CA* is frequently mutated at hot-spots in exons 9 and 20, corresponding to the helical and kinase domains, respectively (Saal *et al*, 2005; Stemke-Hale *et al*, 2008; Zhao *et* Vogt, 2008; Baselga, 2011). In this study, we assessed the influence of *PIK3CA* mutations on patient survival in a series of HER2-positive breast cancer patients treated with neoadjuvant chemotherapy and one

year of trastuzumab starting either before surgery with the first cycle of docetaxel and continuing after surgery, or only after surgery.

Materials and Methods

Tumor samples from 80 HER2-positive breast cancer patients were tested. All patients were participating in the phase II randomized neoadjuvant Remagus 02 trial (Pierga *et al*, 2010). The study was approved by the French Ethics Committee (03-55, RO2) and patients gave their written informed consent. All patients preoperatively received 4 cycles of anthracycline-based chemotherapy followed by 4 cycles of docetaxel and one year of trastuzumab, starting either before surgery with the first cycle of docetaxel and continuing after surgery (neoadjuvant-trastuzumab arm, n=43), or only after surgery (adjuvant-trastuzumab arm, n=37). Complete follow-up data were available for the entire patient series with a median follow-up of 51 months (range: 7-76 months).

Frozen pretreatment tumor biopsies from the patients were used for total RNA extraction. *PIK3CA* mutations were detected by screening cDNA fragments obtained by RT-PCR amplification of exons 9 and 20 and their flanking exons. Details of the primers and PCR conditions are available on request. The amplified products were sequenced with the BigDye Terminator kit on an ABI Prism 3130 automatic DNA sequencer (Applied Biosystems, Courtaboeuf, France), and the sequences were compared with the corresponding cDNA reference sequence (NM_006218).

Response to neoadjuvant therapy was determined as pathological complete response (pCR). Follow-up data for disease-free survival (DFS) and overall survival (OS) were analyzed using the Kaplan-Meier method and comparisons between groups were performed with a log-rank test.

Results

PIK3CA mutations were found in 17 tumors (21.3%), of which 4 were in exon 9 and 13 were in exon 20. No significant associations were found between *PIK3CA* mutations and classical clinicopathological characteristics (Table 1''). No significant difference in pCR was observed between *PIK3CA*-mutated and wild-type tumors.

Table 1''. Description of the study patients - overview of clinicopathologic characteristics in wild-type and *PIK3CA* mutated tumors.

	Total (%)	*PIK3CA* wild-type (%)	*PIK3CA*-mutated (%)	*P-value*[a]
Total	80 (100.0)	63 (78.8)	17 (21.3)	
Treatment group				
Adjuvant trastuzumab arm	37 (46.2)	29 (46.0)	8 (47.1)	NS
Neoadjuvant (plus adjuvant) trastuzumab arm	43 (53.8)	34 (54.0)	9 (52.9)	
Histological grade				
II	31 (38.7)	25 (39.7)	6 (35.3)	NS
III	46 (57.5)	35 (55.5)	11 (67.0)	
unknown	3 (3.8)	3 (4.8)	0 (0.0)	
Clinical tumor size				
T2	39 (48.8)	29(46.0)	10 (58.8)	NS
T3	29 (36.2)	24 (38.1)	5 (29.4)	
T4	12 (15.0)	10 (15.9)	2 (11.8)	
Clinical nodal involvement				
N0	27 (33.8)	19 (30.1)	8 (47.1)	NS
N1	52 (65.0)	43 (68.3)	9 (52.9)	
N2	1 (1.2)	1 (1.6)	0 (0.0)	
ER status				
Negative	35 (43.8)	28 (44.4)	7 (41.2)	NS
Positive	45 (56.3)	35 (55.6)	10 (58.8)	
PR status				
Negative	49 (61.4)	40 (63.5)	9 (52.9)	NS
Positive	30 (37.5)	22 (34.9)	8 (47.1)	
unknown	1 (1.3)	1 (1.6)	0 (0.0)	

[a]Chi^2 test. NS: non significant.

Survival analysis found significantly lower DFS in *PIK3CA*-mutated cases in the overall population (P=0.0063; Figure 1''). More detailed analysis of the 4 patient subgroups based on treatment arm and *PIK3CA* status demonstrated statistically significant differences in patient outcome (P=0.0013; Figure 2''). The most favorable survival was observed in the subgroup of patients without *PIK3CA* mutations treated in the neoadjuvant trastuzumab arm and the poorest prognosis was observed in the subgroup of patients with *PIK3CA* mutations treated in the adjuvant trastuzumab arm. OS curves also differed significantly in the overall population (P=0.035) and in the treatment-based subgroups (P=0.028) in favor of *PIK3CA* wild-type tumors (data not showed).

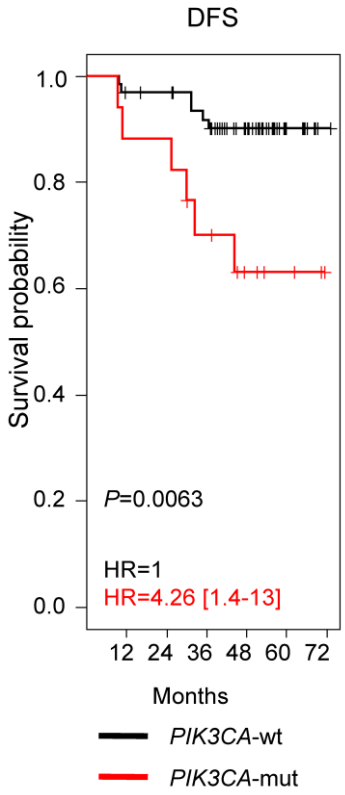

Figure 1''. Disease-free survival curves according to *PIK3CA* status in the overall population. (DFS=disease-free survival; wt=wild type).

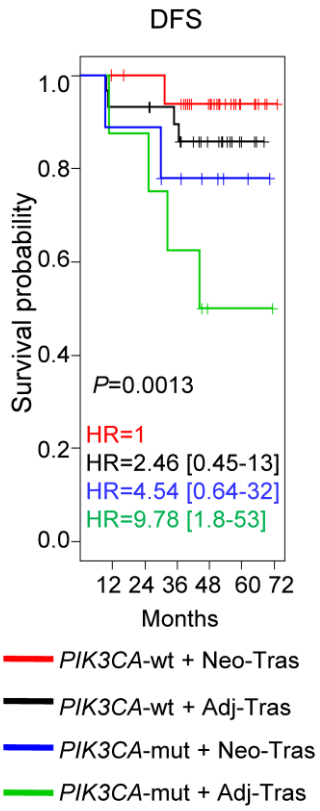

Figure 2''. Disease-free survival curves according to *PIK3CA* status and treatment arm. All patients preoperatively received 4 cycles of anthracycline-based chemotherapy followed by 4 cycles of docetaxel and one year of trastuzumab, starting either before surgery with the first cycle of docetaxel and continuing after surgery (neoadjuvant trastuzumab arm, n=43), or only after surgery (adjuvant trastuzumab arm, n=37). (DFS=disease-free survival; wt=wild type; mut=mutation; Neo-Tras=trastuzumab starting before and continuing after surgery; Adj-Tras=trastuzumab starting after surgery).

Discussion

PIK3CA is the most frequently mutated oncogene in human breast cancers and shows activating mutations ranging from 10% in the triple-negative subgroup to 40% in the hormonal receptor-positive/ERBB2-negative subgroups. Moreover, *PIK3CA*-mutated status confers a more favorable outcome in breast cancer patients without trastuzumab treatment (Baselga, 2011). We confirm previously published data showing *PIK3CA* mutations in exon 9 and 20 hot-spots in about 20% of HER2-positive breast cancers and occurring more frequently in exon 20 (Baselga, 2011; Dave *et al*, 2011; Jensen *et al*, 2012). In

the present study focusing on one year of trastuzumab treatment, patients with *PIK3CA*-mutated tumors had a poorer outcome than *PIK3CA* wild-type cases (Figure 1''). A favorable survival benefit was observed when neoadjuvant trastuzumab was added early to neoadjuvant chemotherapy, particularly in patients with *PIK3CA* wild-type tumors (Figure 2'').

These data therefore support the negative influence of PI3K pathway activation on response to trastuzumab therapy described by Jensen *et al*. (2012). Moreover, based on a larger series, we confirm the data reported by Dave *et al*. (2011), who studied the effects of *PIK3CA* mutations on response to neoadjuvant trastuzumab therapy in a small series of 32 HER2-positive breast cancer patients. It is noteworthy that these authors similarly did not find any difference in pCR associated with *PIK3CA* mutations. Importantly, the results described here are derived from a prospective clinical trial of neoadjuvant patients with pre-treatment tumor samples available for assessment and with well documented follow-up. Thus, the mutational status assigned to each patient showed the therapy-naive tumor condition before initiation of study treatment. This is an important point especially in the light of a report by Dupont Jensen *et al.* (2011) showing discordances between *PIK3CA* mutations in primary breast tumors and their metastases that might influence the results of studies based on retrospective sample collection and advanced treatment lines.

Furthermore, the negative effect of *PIK3CA* mutations on response to trastuzumab therapy is also supported by similar observations in breast cancer cell lines (Berns *et al*, 2007; Dave *et al*, 2011; Jensen *et al*, 2012). This extends and underlines the knowledge of the effect of *PIK3CA* mutations and PI3K pathway activation on HER2-inhibitor treatment response observed on patient breast tumor samples. In the light of published data, PI3K pathway activation also appears to predict treatment response to the HER2-targeting tyrosine kinase inhibitor lapatinib (Eichhorn *et al*, 2008).

Altogether, these data suggest that only *PIK3CA* wild-type cancers clearly benefit from neoadjuvant trastuzumab therapy added to chemotherapy. On the other hand, the subgroup of patients bearing *PIK3CA* mutations could further benefit from treatment targeting PI3K pathway signaling (PI3K or its downstream major effectors) (Kataoka *et al*, 2010; Tanaka *et al*, 2011; Jensen *et al*, 2012). Such treatment may be able to overcome the activation effect of *PIK3CA* mutations and block the PI3K pathway signaling. Our results support the importance of *PIK3CA* mutational status assessment in the management of

future gene-based therapies (HER2, mTOR or PI3K inhibitors used alone or in combination) for HER2-positive breast cancer.

In conclusion, these results confirm that *PIK3CA* mutations are a pejorative factor in HER2-positive breast cancer patients receiving trastuzumab. *PIK3CA* mutations should be assessed in clinical trials testing anti-HER2 therapies and, in the future, in clinical practice.

4.3.2 High lapatinib plasma levels in breast cancer patients: risk or benefit?

Lapatinib is a HER2 and EGFR-targeting tyrosine kinase inhibitor that is administered to advanced breast cancer patients once daily in a dose of 1250mg. The present pilot study focused on lapatinib plasma levels in HER2-positive advanced breast cancer patients treated with a combination of lapatinib plus capecitabine in recommended dosing (lapatinib 1250mg daily, capecitabine 2000mg/m^2 taken in 2 doses 12 hours apart on days 1-14 in a 21 day cycle). Fifty five plasma samples from 21 patients were used for lapatinib level assessment by liquid chromatography tandem mass spectrometry. The median lapatinib plasma level was 5.09µg/mL, with large interindividual differences. This concentration exceeded twice the recommended clinically effective steady-state geometric mean Cmax.

The lapatinib plasma levels of one patient were markedly higher than those of the others, reaching a median of 11.25µg/mL and repeatedly exceeding 7.80µg/mL. The treatment was terminated in her case after 8 months of lapatinib plus capecitabine administration when grade II hyperbilirubinemia developed. It is important to note that the patient was 150cm tall and weighed 48kg at the time of treatment initiation and 42kg at the time of its withdrawal. She had no liver function impairment at the time of treatment initiation and the bilirubin level was restored after treatment withdrawal within 3 weeks and 4 days.

Lapatinib plasma levels are influenced by multiple factors associated with a particular patient's status, treatment and life style. Reports on increase in lapatinib concentrations caused by co-administration with high fat food were published earlier (Bouchalova *et al*, 2010). Moreover, the patient-related factors may also play a role in lapatinib metabolism and excretion. In our patient's case we observed increased lapatinib plasma levels in conditions of low height and weight. Since the same doses of lapatinib are given to all patients despite body weight or surface, the doses could be too high for patients of small body size. Similar observations were reported in the case of imatinib treatment of chronic myeloid leukemia patients, but the final conclusion in this matter needs more evidence (Takahashi *et* Miura, 2011).

Treatment toxicity can be the limiting factor for treatment duration and subsequently also for patient outcome. Patients presenting with severe side effects as is the case of hepatotoxicity can thus terminate otherwise effective anticancer treatment prematurely. In

the present study, we described a patient with lapatinib plasma levels exceeding recommended effective plasma level more than three times. Importantly, the patient developed hyperbilirubinemia that caused treatment termination. In the described case, we cannot be sure about the causal connection between increased lapatinib plasma levels and hepatotoxicity. However, reports on hepatotoxicity occurrence in lapatinib-treated patients show up with increasing frequency (Gomez *et al*, 2008; Capri *et al*, 2010; Baselga *et al*, 2012[B]). Similarly, there have been hepatotoxicity reports for other tyrosine kinase inhibitors such as imatinib (Castellino *et al*, 2012).

Excretion of lapatinib and mostly its metabolites is mediated partly by urine but mostly via the feces (more than 90%). Lapatinib is metabolized in the liver, mainly with the participation of CYP3A4/5. However, both CYP3A4 and CYP3A5 were suggested to be inactivated by lapatinib and its metabolites. Specifically, lapatinib more potently inactivates CYP3A4 than CYP3A5. Further, some metabolites contain structures that can be further metabolized to reactive compounds capable of interaction with cellular proteins initiating processes leading to toxic effects (Teng *et al*, 2010; Castellino *et al*, 2012; Chan *et al*, 2012). Spraggs et al observed a significant association between the histocompatibility complex HLA-DQA1*02:01 and hepatotoxicity in lapatinib-treated metastatic breast cancer patients (Spraggs *et al*, 2011). Additionally, a combination of direct mitochondrial cytotoxicity and inhibition of bile salt efflux was proposed for explaining the clinical hepatoxicity observed by a structurally similar tyrosine kinase inhibitor CP-724,714 (Castellino *et al*, 2012). Idiosyncratic lapatinib-caused hepatotoxicity could be attributed to multiple combination factor interactions including polymorphisms of CYP3A5, other genetic characteristics or host immune status (Chan *et al*, 2012). In the light of these recent observations, the increase of lapatinib plasma levels points to increased supply of the compound to be metabolized in the liver and also increased production of toxic metabolites. The cause of lapatinib treatment hepatotoxicity is complex and based on our results we cannot claim that increased plasma levels are the main danger for liver damage. However, the possible link between lapatinib blood concentration and hepatotoxicity should be further investigated on larger patient cohorts.

In the clinical practice, hepatotoxicity on lapatinib treatment was observed in 0.4% of metastatic breast cancer patients from the Lapatinib Expanded Access Program, which evaluated 4283 patients, and in 11.7% of 154 patients treated in lapatinib arm from the neoadjuvant NeoALTTO clinical trial (Capri *et al*, 2010; Baselga *et al*, 2012[B]). Hepatic

toxicity was reported to be lapatinib-related by Gomez et al. in one patient after more than 7 months of treatment (Gomez *et al*, 2008). Peroukides et al. described a case of jaundice on lapatinib in less than one month of the treatment. Liver biopsy was performed and showed acute drug-induced hepatitis with necrosis of contiguous hepatocytes in portal-to-portal and portal-to-central fashion (bridging necrosis) and also foci of severe hemorrhage and hepatocellular dropout around the centrilobular areas (Peroukides *et al*, 2011). As with our patient, the hepatotoxicity subsided within 3 months after lapatinib discontinuation. Besides hepatotoxicity occurring on the treatment combination of lapatinib and capecitabine, there are also reports of this type of toxicity on treatment combinations of lapatinib with other chemotherapies (Baselga *et al*, 2010; Park *et al*, 2012). All these reports show lapatinib treatment-associated hepatotoxicity as an increasingly important matter to study. Lapatinib in breast cancer treatment is further discussed in the review article on page 135.

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High lapatinib plasma levels in breast cancer patients: risk or benefit?

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ABSTRACT

Aims and background. Lapatinib is a tyrosine kinase inhibitor targeting epidermal growth factor receptors 1 (EGFR/HER1) and 2 (HER2) used in the treatment of patients with HER2-positive breast cancer. The aim of the present study was to determine lapatinib plasma levels in breast cancer patients treated with lapatinib plus capecitabine.

Patients and methods. We assessed lapatinib plasma levels in blood samples from 21 breast cancer patients treated with lapatinib plus capecitabine using the standard regimen in an expanded access program. Liquid chromatography tandem mass spectrometry was used for measuring lapatinib plasma concentrations. The validated method was applied for measurement of 55 plasma samples.

Results. The median lapatinib plasma level was 5.09 μg/mL, with large interindividual differences. Patients of lower weight tended to have higher lapatinib plasma levels (Spearman correlation coefficient R = -0.435, $P = 0.055$). One patient's lapatinib plasma levels were markedly higher than those of the others, with a median level of 11.25 μg/mL and repeatedly exceeding 7.80 μg/mL. The treatment was terminated after 8 months when hyperbilirubinemia occurred.

Conclusions. The lapatinib plasma levels reported here are twice as high as the clinically effective steady-state geometric mean maximum concentration. We conclude that increased lapatinib body levels occur when patients are in a nonfasting state at the time of drug intake and when lapatinib doses are not adjusted to low body weight or weight loss during treatment. In Europe, dose adjustments are not recommended in the case of hepatic function impairment. Thus, attention should be paid to changes in liver function test results in clinical practice, especially in patients of small stature and weight, given the risk of high plasma concentrations. Prospective lapatinib plasma level assessment in treated patients might be useful to confirm or refute the possible correlation of high lapatinib plasma levels with hepatic and/or other toxicities.

Key words: lapatinib plasma level, tyrosine kinase inhibitor, epidermal growth factor receptor 1 (EGFR/HER1), epidermal growth factor receptor 2 (HER2), hepatic toxicity.

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Introduction

Lapatinib is a tyrosine kinase inhibitor targeting epidermal growth factor receptors 1 (EGFR/HER1) and 2 (HER2). Currently, it is being used in the treatment of patients with HER2-positive breast cancer in combination with capecitabine, or letrozole, in the case of hormone receptor positivity, at daily doses of 1,250 mg and 1,500 mg, respectively. Dose adjustments are generally recommended if cardiac toxicity occurs. The Food and Drug Administration also recommends lapatinib dose modifications in case of impaired liver function or concomitant treatment with a strong CYP3A4 inhibitor/inducer[1,2].

In earlier pharmacokinetic evaluations, at a daily dose of 1,250 mg, steady-state geometric mean values were reported to reach 2.43 μg/mL (95% confidence interval 1.57 to 3.77 μg/mL) and 36.2 μg.h/mL (95% confidence interval 23.4 to 56 μg.h/mL) for maximum concentration (Cmax) and area under the curve[1,2]. However, in one study, when the medication was taken with food, specifically with a high-fat breakfast (50% fat; 1,000 calories), the lapatinib plasma Cmax and area under the curve were found to be up to 3-fold and 4-fold higher, respectively[3]. Currently, patients are advised to take lapatinib in 1 morning dose at least 1 hour before or 1 hour after a meal[1,2].

Patients and methods

The aim of the present study was to determine lapatinib plasma levels in breast cancer patients treated with lapatinib plus capecitabine and to correlate these levels with the treatment outcome. The project was approved by the hospital ethics committee.

We assessed lapatinib plasma levels in a series of prospective and retrospective blood samples from 21 breast cancer patients treated with lapatinib plus capecitabine using the standard regimen in an expanded access program. The clinical and histopathological data of the patients are summarized in Table 1. Two patients had both prospective and retrospective plasma samples analyzed. Blood sampling was done 16-30 hours after lapatinib administration 29 days or more after treatment initiation when steady-state concentrations had been reached. Plasma samples obtained by centrifugation and separation were stored at -20 °C until analysis. Patient samples were collected between April 2007 and November 2008. Multiple blood samples of each patient were taken on different days during the treatment period. Both retrospective and prospective plasma samples came from regular clinical blood testing. The availability of retrospective samples depended on the number of tests performed previously. A modification of liquid chromatography tandem mass spectrometry, which was originally developed for determination of imatinib in plasma from patients with chronic myeloid leukemia, was used for measuring lapatinib plasma concentrations[4]. A C18 column filled with 1.7-μm BEH particles (Waters, Milford, MA, USA), which provides high resolution of more than 100,000 theoretical plates per meter and fast separation with a retention time of 1.98 min under a back-pressure of 400 bar, was used. This method offers linear correlation in the range of 0.1-15.0 μg/mL($y = 0.000301x - 0.0212$; $R = 0.9946$), a limit of quantification of 18.2 ng/mL (signal-to-noise ratio of 10), recovery 102.5% and 107.9% (addition of 1 and 5 μg/mL, n = 6) and within-day and between-day precisions better than 4.5% and 8.6% (n = 6). The validated method was applied for measurement of 55 plasma samples from 21 patients. The number of plasma samples available for evaluation varied between 1 and 6 per patient. The results were evaluated using standard statistical methods (nonparametric Spearman correlation coefficient).

Table 1 - Overview of patients' histopathological and clinical data

Characteristic	Patients percentage (ratio)	Characteristic	Patients percentage (ratio)
Characteristics at primary diagnosis		Metastatic sites in progressive disease	
Stage		Visceral	62% (13/21)
I	5% (1/21)	Nonvisceral	67% (14/21)
II	48% (10/21)	Both	29% (6/21)
III	38% (8/21)		
IV	9% (2/21)	Overview of treatment before lapatinib plus capecitabine	
Grade			
I	9% (2/21)	Chemotherapy	
II	24% (5/21)	Neoadjuvant only	9% (2/21)
III	62% (13/21)	Adjuvant only	67% (14/21)
Unknown	5% (1/21)	Neoadjuvant and adjuvant	24% (5/21)
Histological subtype			
IDC	86% (18/21)	Advanced therapy	
ILC	5% (1/21)	1 or 2 further lines	67% (14/21)
Other	9% (2/21)	3 or more further lines	33% (7/21)
Hormone receptors		Other treatment	
HR negative	57% (12/21)	Hormonal	38% (8/21)
HR positive	43% (9/21)	Trastuzumab	100% (21/21)

IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; HR, hormone receptors.

Results

The median lapatinib plasma level was 5.09 μg/mL, with large interindividual differences, in agreement with already reported variations (76% coefficient of variation for the maximum concentration[2]). However, the clinically effective steady-state geometric mean Cmax has been described as 2.43 μg/mL[1,2]. In our sample, patients with a lower weight tended to reach higher lapatinib plasma levels (Spearman correlation coefficient R = -0.435, $P = 0.055$). The lapatinib plasma levels of 1 patient were markedly higher than those of the others, reaching a median of 11.25 μg/mL and repeatedly exceeding 7.80 μg/mL. Blood samples evaluated in this woman were prospective as well as retrospective. With foreknowledge of the risk of lapatinib-caused hepatic toxicity[5], the treatment was terminated after 8 months when hyperbilirubinemia occurred (grade II according to Common Terminology Criteria for Adverse Events v3.0[6], total bilirubin >twice the upper limit of normal at the time of treat-

ment withdrawal). It is important to note that the patient was 150 cm tall. She weighed 48 kg at the time of treatment initiation and 42 kg at the time of its withdrawal. She had no liver function impairment at the time of treatment initiation and the bilirubin level was restored after treatment withdrawal within 3 weeks and 4 days. Apart from lapatinib plus capecitabine, the patient was not regularly taking any additional medication. She was only advised to use metoclopramide 10 mg tablets (maximum dose 30 mg daily) in case of nausea.

Discussion

Compared with published data[1,2], the lapatinib plasma levels reported here are twice as high as the clinically effective steady-state geometric mean Cmax. These high lapatinib plasma levels could be caused by several concomitant factors including non-compliance with fasting recommendations, taking lapatinib before blood sampling, hepatic dysfunction, other medication/herbal supplements, or low weight. No case of severe toxicity occurred in patients with high lapatinib plasma levels but the treatment in the case of the patient described above was terminated because of the increase in bilirubin levels. We can only speculate whether liver impairment would have progressed if the treatment had continued. Hepatic toxicity was reported to be lapatinib-related by Gomez *et al.*[7] in 1 patient after more than 7 months of treatment and possibly linked to lapatinib treatment in 0.4% of treated patients from the Lapatinib Expanded Access Program, which evaluated 4283 patients[5]. Abnormalities of liver function were detected in a significant proportion of lapatinib-treated patients in a phase II study[8,9]. Moreover, hepatic toxicity (grade ≥3) was reported in 13% (20/154) of patients in the lapatinib arm based on first data from the NeoALTTO trial (BIG 01-06/EGF 106903): a phase III, randomized, open label, neoadjuvant study of lapatinib, trastuzumab, and their combination with paclitaxel in women suffering from HER2-positive primary breast cancer[10].

Currently, lapatinib-related hepatotoxicity is considered idiosyncratic and might be caused by reactive–metabolite-related inactivation of CYP3A4[11]. HLA-DQA1*02:01 was found to be a risk factor for lapatinib-induced hepatotoxicity[12]. On the other hand, capecitabine cannot be excluded as the cause or an additional cause of hyperbilirubinemia. However, the time of capecitabine-caused hyperbilirubinemia onset was reported previously as occurring at a median of 64 days after the start of treatment[1].

Conclusion

We conclude that increased lapatinib body levels occur when patients are in a nonfasting state at the time of drug intake and when lapatinib doses are not adjusted to low body weight or weight loss during treatment. In Europe, dose adjustments are not recommended in the case of hepatic function impairment. Thus, attention should be paid to changes in liver function test results in clinical practice, especially in patients of small stature and weight, given the risk of high plasma concentrations. Prospective lapatinib plasma level assessment in treated patients (followed for other medication and/or herbal supplements) might be useful to confirm or refute the possible correlation of high lapatinib plasma levels with hepatic and/or other toxicities.

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LAPATINIB IN BREAST CANCER – THE PREDICTIVE SIGNIFICANCE OF HER1 (EGFR), HER2, PTEN AND PIK3CA GENES AND LAPATINIB PLASMA LEVEL ASSESSMENT

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Background. Breast cancer treatment trends are currently based on tailored therapies using tumor and patient biomarkers. Lapatinib is the first dual inhibitor of HER1 (EGFR, ErbB1) and HER2 (ErbB2, Neu) tyrosine kinases to be used in clinical practice. However, only HER2 is currently used for therapy indications and new predictors for the treatment with lapatinib are sought.

Methods and results. This minireview focuses on lapatinib and its role in breast cancer treatment. Preclinical and clinical studies as well as pharmacological characteristics are briefly reviewed while the focus is on efficacy assessment including predictive factors for therapy outcome.

Conclusion. Lapatinib (Tykerb/Tyverb) was Food and Drug Administration (FDA) approved in 2007 for use in combination with capecitabine for the treatment of HER2-positive advanced or metastatic breast cancer in patients who had received previous treatment (including anthracycline, taxane and trastuzumab containing regimens) and in 2010 for use in combination with letrozole for postmenopausal women with hormonal receptor positive and HER2-positive metastatic breast cancer. In contrast to trastuzumab (Herceptin), lapatinib is orally administered and it targets both HER2 and HER1 receptors. As a synthetic and oral tyrosine kinase inhibitor (TKI), it is convenient, cheaper and easier to produce than monoclonal antibodies. The recommended dosage is not dependent on body weight either. Lapatinib plasma level measurement could be an approach to tailored therapy for further optimizing the dose and prolonging this efficient therapy. New lapatinib response predictors are being evaluated. At this time, only HER2 amplification/overexpression is used to choose lapatinib therapy candidates. Further studies on concurrent HER1 fluorescent *in situ* hybridization (FISH)/immunohistochemistry (IHC) assessment and/or microarray analyses may produce new data on the predictive role of the HER1 (EGFR) gene/protein. PTEN loss and PIK3CA gene mutations are other markers that may predict lapatinib poor response.

INTRODUCTION

Breast cancer (BC) is the most common malignancy in females affecting around 1.3 million women worldwide each year and causing about 460,000 deaths annually[1,2]. Data from the Czech National Oncology Registry[3] indicate that the incidence of BC has doubled since 1977 and in 2007 BC affected 123.2/100,000 women with a mortality of 31.9/100,000 (ref.[3,4]). Metastatic breast cancer (MBC) is found at initial diagnosis in up to 10% of patients[3,5,6]. Tailored therapy based on biological markers of tumor and patient is the trend in clinical practice these days. Lapatinib (Tykerb/Tyverb, GlaxoSmithKline, Research Triangle Park, NC) was introduced into routine clinical settings and follows success of hormonal therapy (used in hormonal receptor positive BC) and the monoclonal antibody trastuzumab (Herceptin, Genentech, South San Francisco, CA) indicated in HER2 overexpressed and/or amplified breast cancers[7-10]. This minireview focuses on lapatinib in BC treatment. Preclinical and clinical studies as well as pharmacological characteristics are briefly reviewed while the focus is on efficacy assessment including predictive factors for therapy outcome.

THE HER FAMILY AND ITS BLOCKADE

The family of cell receptors called human epidermal growth factor receptors (HER) plays an important role in tumor development via influence on cell proliferation, migration, angiogenesis and protection against apoptosis in many cancer types. The HER family consists of four

members – HER1 (also known as epidermal growth factor receptor, EGFR), HER2, HER3 and HER4. These receptors are composed of an N-terminus extracellular ligand-binding domain, a single membrane spanning region and a C-terminus cytoplasmic domain which exhibits tyrosine kinase activity. However, HER2 has no known ligand and HER3 lacks tyrosine kinase activity[6,11,12]. After ligand binding on extracellular domains, receptors homo- or heterodimerize and become active through autophosphorylation. This activation allows further signal transduction. The intracellular downstream signal is split into two important pathways: the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K)-Akt pathways[6,12].

Two main approaches are used in cancer therapy to block membrane receptors and thus their signaling – via monoclonal antibodies or blocking kinase activity using small molecules - TKIs. Monoclonal antibodies are intravenously administered. They bind the extracellular domain of the receptor, inhibit signaling and attract immune response. On the other hand, TKIs are orally administered small molecules targeting the intracellular part of the receptors. In the case of BC, both approaches are used in clinical practice[8,13-16]. Both trastuzumab, and lapatinib, have been approved by the FDA and the European Medicines Agency (EMEA) for BC treatment. Lapatinib (Tykerb/Tyverb) was FDA approved in 2007 for use in combination with capecitabine for the treatment of HER2-positive (HER2+) advanced or MBC in patients who had received previous treatment (including anthracycline, taxane and trastuzumab containing regimens) and in 2010 it was approved in combination with letrozole for postmenopausal women with hormonal receptor positive and HER2+ MBC[8,10,17-20].

TKIs are cheaper and easier to produce than monoclonal antibodies[21]. As they are taken orally this is a great advantage to cancer patients, 83-92% of whom prefer this form of administration according to various studies[22-26].

Lapatinib – mechanism of action

Lapatinib (GW572016) was derived from the quinazoline core found to be active in other HER TKIs. Its chemical name is N-[3-chloro-4-[(3-fluorobenzyl)oxy]-phenyl]-6-[5-([[2-(methylsulfonyl)ethyl] amino]methyl)-2-furyl]-4-quinazolinamine[27]. It has been shown to inhibit the intracellular domain phosphorylation of both HER2 and HER1 in a reversible manner with a long dissociation time of receptor-drug complex estimated as ≥ 300 min. The described effect is due to the lapatinib structure and its ability to bind at an ATP binding site in inactive form[6,28]. In humans, lapatinib is administered as the monohydrate ditosylate salt[6]. The specificity of lapatinib has been tested on a wide range of protein kinases. An affinity was found only for HER4 and c-Src apart, that is from HER2 and HER1[18]. Lapatinib blocks, by inhibition of HER2 and HER1, activation of subsequent intracellular pathways leading through extracellular signal-related kinase (ERK)-1/2 and PI3K/Akt[29-31]. Lapatinib can inhibit both wild-type and truncated forms of HER2 receptors (p95HER2) both *in vitro* and *in vivo*[32,33].

In vitro and xenograft studies

Rusnak et al.[29] showed growth inhibition of tumor cells overexpressing both receptors – HER1 (head and neck cancer, vulvar cancer cell lines) and HER2 (breast, gastric, lung cancer cell lines). The ability of lapatinib to inhibit the proliferation of tumor cells overexpressing HER1 was compared with erlotinib and a similar impact on growth was found. Inhibition of HER1 and HER2 receptor autophosphorylation and phosphorylation of the downstream modulator, Akt, was verified by Western blot in the BT474 and HN5 cell lines. HER1 and HER2 receptor autophosphorylations were similarly inhibited by lapatinib. However the level of Akt phosphorylation was, post-treatment, lower in HER2+ samples than in HER1 positive samples. In proliferation and cell cycle assays, lapatinib proved to be more effective against HER2-overexpressing cell lines than against HER1-overexpressing cell lines. However these results might be due to the specific cell lines used. The results suggest that HER1 inhibition leads preferentially to cell growth arrest and HER2 inhibition causes both growth arrest and cell death after 72 h *in vitro*. The authors also confirmed that lapatinib was capable of inhibiting the growth of human tumor cells *in vivo*, using HN5 and BT474 xenograft models. Taken together, these results indicate that lapatinib achieves excellent potency on tumor cells with selectivity for tumor versus normal cells and they suggest that lapatinib would benefit patients with tumors overexpressing either HER1 or HER2. Another study showed potent inhibition of both HER1 and HER2 tyrosine kinases leading to growth arrest and/or apoptosis in HER1 and HER2-dependent tumor cell lines as a response to lapatinib treatment. Lapatinib markedly reduced tyrosine phosphorylation of both HER1 and HER2, and inhibited activation of Erk1/2 and Akt. However, the inhibition of phosphorylated (p)-Akt in HN5 cells overexpressing HER1 was smaller than in HER2-overexpressing tumor cells. Lapatinib inhibited activation of HER1, HER2, Erk1/2 and Akt in human tumor xenografts as well[34]. Lapatinib efficacy both *in vitro* and *in vivo* was also confirmed by Konecny et al[31].

Clinical studies

Phase I clinical studies have proven the safety of lapatinib administration either alone[35,36] or in combination with another oral agent, capecitabine[37,38]. In the phase I study of Burris et al.[36], out of 67 patients with advanced solid tumors displaying HER1 expression by immunohistochemistry (IHC) and/or HER2 overexpression by IHC or amplification by fluorescence *in situ* hybridization (FISH) 30 (44.7%) were treated for BC. Patients who experienced complete remission, partial response (PR) or stable disease in the phase I studies were mainly those suffering from BC[36,38]. The phase II study in HER2+ MBC patients after trastuzumab treatment failure, produced more results supporting the safety of lapatinib and it evaluated its benefits[39,40]. Lapatinib demonstrated modest activity as a single agent[40]. A combination of capecitabine and lapatinib significantly prolonged treatment efficacy with acceptable toxicity[19,41]. The phase III clinical evalua-

tion showed better response to lapatinib with capecitabine than capecitabine alone in a group of HER2+ BC patients suffering from locally advanced or metastatic disease[41]. Lapatinib also inhibited truncated forms of HER2 receptor (p95HER2) in BC patients, partially explaining its activity in trastuzumab resistant disease[32,33]. The development of CNS metastases is a serious clinical problem occurring in approximately one third of women with MBC who receive trastuzumab[42,43]. The phase II studies using lapatinib in BC with brain metastases showed volumetric changes in the metastases[44]. Lapatinib plus capecitabine resulted in 20% CNS objective response and in 40% a ≥ 20% volumetric reduction in their CNS lesions was observed[45]. More recently, other studies on lapatinib in combinations have been published: with trastuzumab[46], paclitaxel[47] and hormonal treatment[48,49], some also in neoadjuvant settings[47]. One study exploring the combination of anthracycline-based chemotherapy plus trastuzumab, lapatinib, or both in a neoadjuvant setting is ongoing[50]. Important and relevant outcomes are expected from an international ALTTO phase III adjuvant trial that will evaluate 8,000 early HER2+ BC patients and will produce data on lapatinib and trastuzumab in combination, both alone and in sequence. The first patient was enrolled in 2007. BC patients will receive study treatment for one year, and will be followed for a total of 10 years[51]. A companion trial will evaluate lapatinib in the neoadjuvant treatment of BC (Neo-ALTTO)[51].

Pharmacokinetics

Absorption: Lapatinib is a small orally administered molecule, whose absorption depends on concurrent conditions. Detectible levels of lapatinib are found in the blood after 0.25 hours ranging from 0 to 1.5 hours with the maximum concentration reached approximately after 3 to 4 hours. A daily dose of 1,250 mg causes steady state levels of Cmax 2.43 mcg/ml (1.57 to 3.77 mcg/ml) and the area under the curve (AUC) 36.2 mcg.hr/ml (23.4 to 56 mcg.hr/ml). With multiple daily dosing, a steady state was achieved within 6 to 7 days[6,17,18,52-54]. When taken with food, lapatinib absorption is increased[52,53]. In the case of high-fat food (characterized as 50% of fat and 1,000 calories) AUC values were approximately 4-fold higher (Cmax approximately 3-fold higher)[17,18,52-54]. Lapatinib plasma levels were also higher when lapatinib was combined with capecitabine compared to lapatinib alone but the difference did not reach statistical significance[38]. *Distribution:* Lapatinib is bound (>99%) to albumin and alpha-1 acid glycoprotein in the blood stream but it does not undergo erythrocyte binding which creates a blood to plasma ratio <1. *In vitro* studies have shown that lapatinib is a substrate for and inhibitor of P-glycoprotein (Pgp)[6]. *Metabolism and elimination*: The metabolism depends primarily on CYP3A4 and CYP3A5, with minor contributions from CYP2C19 and CYP2C8 followed by biliary elimination and stool excretion. Renal excretion accounts for less than 2% of the given dose[6,17,18]. The elimination $t_{1/2}$ was 14.2 hours after a single dose administration, and 24 hours with repeated dosing (result of drug accumulation)[6,17,18,52-54]. In patients with severe hepatic dysfunction (Child-Pugh class C), the AUC of lapatinib was increased by >60% and the $t_{1/2}$ was 3 times that of individuals without hepatic disorder. Thus, dose reductions to 750 mg/d are recommended in patients with liver disease. Ketoconazole, a CYP3A4 inhibitor, increases the AUC of lapatinib and $t_{1/2}$. The package insert recommends avoidance of strong CYP3A4 inhibitors. If co-administration is necessary, reduction of the lapatinib dose to 500 mg/d is advised. On the other hand, carbamazepine, a CYP3A4 inducer, decreases the AUC of lapatinib. Avoidance of strong CYP3A4 inducers is recommended, and if it is necessary to receive a strong CYP3A4 inducer in combination with lapatinib, the dose of lapatinib should be titrated gradually from 1,250 mg/day up to 4,500 mg/day (HER2 positive MBC indication) or from 1,500 mg/day up to 5,500 mg/day (hormone receptor positive, HER2+ BC indication) based on tolerability as recommended by the FDA[6,17,55]. However, there are no published clinical data with this dose adjustment in patients receiving strong CYP3A4 inducers.

Resistance to lapatinib

As a small tyrosine kinase molecule, lapatinib affects receptors and signal transduction at a different level than trastuzumab. Moreover, different modes of cellular drug resistance have been suggested for trastuzumab and lapatinib and this underlines the rationale of lapatinib administration after trastuzumab failure[56]. However, some BCs do not respond or develop resistance to lapatinib too (e.g. tumors with HER2 tyrosine kinase domain mutations; HER1 tyrosine kinase domain mutations; PIK3CA mutation, PTEN loss, AXL overexpression, RelA activation)[57-62]. Enhanced estrogen signaling described *in vitro* may also be a route for increased tumor cell survival on lapatinib treatment. Combining lapatinib treatment with fulvestrant reduced the rate of lapatinib resistance[63]. One *in vitro* study has shown AXL overexpression as a novel mechanism of acquired resistance to HER2-targeted agents, which can be overcome by a new multikinase (AXL, MET, and VEGFR) inhibitor foretinib. Further, AXL expression *in vitro* was also decreased using small interfering RNA to AXL, estrogen deprivation or estrogen receptor antagonist fulvestrant and, sensitivity to lapatinib was restored[60]. These findings also suggest that epigenetic changes may play a role in lapatinib resistance[60]. Taken together, the results support the use of different targeted therapeutics in combination.

Treatment tailoring and efficacy

Response to lapatinib administration may be influenced both by tumor and patient characteristics. The tumor phenotype, predictor status, drug dose as well as other factors may play a role here. Lapatinib has been proven to be more absorbed by a high-fat diet[52,54]. The following steps of lapatinib body passage depend on liver metabolism and cytochrome inducers/inhibitors. The level of proteins in blood available to bind lapatinib plays another role by changing the free drug fraction[17,18]. Lapatinib insensitivity can also be caused by intratumoral signal pathway changes leading to an overwhelming of its inhibitory effect[57-62].

THE HER FAMILY

The HER1 gene is located on 7q12 and its protein product plays an important role in cell proliferation, migration and protection against apoptosis[12]. In contrast, to HER2, overexpression of HER1 appears to be a later event in tumorigenesis[14]. Increased HER1 protein expression is described in about 40% of BC, (ranging from 14% to 91% of all primary BC). High expression is described in triple negative breast cancer and metaplastic cancer (mostly basal-like)[7,14,64-72]. HER1 overexpression was found in 30% of inflammatory breast cancer (IBC). Patients with HER1-positive tumors have worse 5-year overall survival than patients with HER1-negative tumors, and HER1 expression is associated with an increased risk of recurrence in patients with IBC[73]. The HER1 gene was amplified in a nonselected series in 0-14%, in metaplastic cancer up to 28% (ref.[7,14,64-69]). In a study by Reis-Filho et al.[69], which assessed 47 metaplastic cancers, HER1 amplification showed a significant association with HER1 overexpression and was restricted to cancers with homologous metaplasia. Coexpression of HER1 and HER2 has been observed in 10–36% primary BC, and it is associated with a poorer prognosis than in cases with expression of a single receptor[74-77]. In one study, where HER1 expression was found in only 15% of 807 invasive BC, the majority of HER1-positive tumors (87%) coexpressed HER2. Moreover, almost all the tumors that expressed the HER2 phosphorylated form (pHER2), coexpressed HER1, and expression of pHER2 or coexpression of HER2 and HER1 was associated with the shortest patient survival[78]. The HER2 gene is localized in the 17q12-q21 amplicon and its amplification occurs in approximately 20-35% of invasive BC[79-84].

HER2 overexpression/amplification is widely accepted as a lapatinib therapy response predictor based on the results of several clinical studies[15,85,86]. Coexpression of pHER2 and pHER3 in IBC seems to predict a favorable response to lapatinib even more accurately[87]. On the other hand, HER1 did not predict lapatinib response in various studies[15,40,85,88]. However, HER1 protein was assessed using IHC but no HER1 gene examination by FISH was performed[15,30,36,40,85,88]. In another study, additional correlative tumor tissue analyses including HER1 were conducted, but the small number of responders precluded any useful interpretation of these results[89]. Further, the HER1 status was not published in some studies[38,44,45]. In a phase I study of 67 patients with metastatic solid malignancies and using 6 different lapatinib doses ranging from 500 to 1,600 mg/d, 59 patients were assessed for treatment results. Breast cancer presented in 15 out of 33 patients with assessed biomarkers. Four PR were observed: these were all in BC and all of them overexpressed HER2 at 3+ level by IHC and three also displayed HER1 expression. HER1 IHC results were described as positive or negative[30,36]. Detailed receptor status evaluation showed PR in patients with high HER2 activated level. Lapatinib treatment lowered both HER2 and HER1 phosphorylation but did not change overall receptor expression[30]. In a phase II study, HER2 overexpression but not HER1 expression alone, predicted sensitivity to lapatinib in IBC; high HER2, pHER2 and insulin-like growth factor receptor-1 (IGF-IR) coexpression predicted clinical response to lapatinib monotherapy in patients with relapsed/refractory IBC[85]. In a phase III study, investigators revealed the low frequency of HER1 IHC 2+ or 3+ overexpression (44/320, 14% in both arms, 25/163, 15% in patients treated with lapatinib+capecitabine and in 19/157, 12% treated with capecitabine) in the available tumor specimens. The results suggested that HER1 overexpression did not play a significant role in the biology of the HER2+ BC of women included in this trial. The authors were unable to identify a subgroup of patients who fail to benefit from the addition of lapatinib to capecitabine based on the biomarker studies. There was no association identified between level of HER1 expression and progression-free survival (PFS)[15,41]. Burstein et al.[40] found no correlation between HER1 expression level assessed via IHC and response to lapatinib (six patients had an objective response to lapatinib by investigators review, two by independent review). Combined biomarker analysis was performed by Blackwell et al.[90] in two large phase II studies with refractory MBC and they published initial data suggesting that expression levels of estrogen, progesterone and HER1 receptors may be related to lapatinib response in trastuzumab pretreated patients. Tumor tissues were obtained from each patient from the time of most recent biopsy. Phase II study EGF20009 assessed lapatinib as first line monotherapy in advanced or metastatic BC and for the initial 65 patient samples analyzed, an elevation of HER2 expression was significantly associated with response to treatment with lapatinib ($p=0.02$) and a longer time to progression following treatment with lapatinib ($p<0.0025$). Further, of the 17/65 responders in this preliminary study, SpotFire™Decision Tree Analysis demonstrated that 16/17 (94%) who responded to lapatinib had a gene expression signature combining HER1, 2, and 3 (ref.[86]). Further large studies focusing on concurrent HER1 FISH/IHC assessment or microarray analyses on samples from metastatic sites if available could produce new data on the predictive role of this marker. Comparison of HER1 gene/protein status in primary and metastatic sites may also be a tool to better assess the role of HER1 receptor in BC patients.

PIK3CA

Activation of the phosphoinositide 3-kinase (PI3K) pathway plays an important role in the pathogenesis of a variety of cancers. The gene encoding the p110alpha catalytic subunit of PI3K (PIK3CA) can be mutated in up to 40% of BC. The majority of PIK3CA mutations lie in two hotspot regions, including the central helical domain encoded by exon 9 and the COOH-terminal kinase domain encoded by exon 20(ref[91]). The PIK3CA activating mutations (E545K and H1047R) cause resistance to lapatinib[58]. Patients with tumors harboring an H1047R PIK3CA mutation or low expression of PTEN, derived clinical benefit from lapatinib in one phase II study[92].

On the other hand, PIK3CA mutations have recently been found to sensitize cancer cells (with *KRAS/BRAF* normal status) to the mammalian target of the rapamycin (mTOR) inhibitor everolimus[61]. Clinical trials testing mTOR inhibitors are currently ongoing, and this includes BC patients[8,61,93]. Taken together, PIK3CA mutations may serve as both positive (mTOR inhibition) and negative (lapatinib) therapy predictors in BC.

PTEN

The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a tumor suppressor identified in 1997 in the 10q23 region. PTEN phosphatase negatively regulates the PI3K pathway and is inactivated in many human malignancies, including BC[91,94,95]. However, there are no uniform study results. Knockdown of PTEN did not alter response to lapatinib *in vitro* and PTEN loss was not associated with reduced response to lapatinib in a phase II monotherapy trial in IBC, as approximately 70% of responders showed PTEN deficiency[9,87,96]. Patients with tumors harboring an H1047R PIK3CA mutation or low expression of PTEN derived clinical benefit from lapatinib in one phase II study[92]. On the other hand, a combined *in vitro* and *in vivo* study using a genome wide loss-of-function short hairpin RNA (shRNA) screen identified loss of PTEN expression as one of causes of lapatinib resistance[58]. Recently, a distinct resistance mechanism has been proposed. PTEN inactivation specifically raised HER1 activity by impairing the ligand-induced ubiquitinylation and degradation of the activated receptor through destabilization of newly formed ubiquitin ligase Cbl complexes. This resistance can be overcome by more complete HER1 kinase inhibition[97]. PTEN deficient cells are also extremely sensitive to poly(ADP-ribose) polymerase (PARP) inhibitors and thus PARP inhibition is another hope for patients with PTEN deficiency[98,99].

LAPATINIB PLASMA LEVELS

Effective plasma concentrations of lapatinib might be assessed using the approaches similar to common therapeutic drug monitoring. Such testing has already been suggested in the case of another TKI, imatinib which has been studied in relation to plasma concentration impact on treatment response in patients treated with the compound for chronic myeloid leukemia (CML) and gastrointestinal stromal tumors[100-102]. In the case of imatinib, higher plasma levels correlated with complete cytogenetic responses in CML patients. Plasma levels lower than those assessed as effective were significantly associated with worse treatment response[102-105].

Imatinib plasma level evaluation supports the idea that a similar approach could be useful in lapatinib-treated patients. Lapatinib can be evaluated in patient blood samples. The recommended dose is not dependent on body weight. Lapatinib plasma level assessment could be a tool to identify patients in danger of treatment failure because of too low or too high lapatinib levels. Methods based on liquid chromatography and mass spectrometry have already been tested to determine lapatinib level in human plasma[106,107]. Haouala et al.[107] described a method useful for a wide range of currently used TKIs including lapatinib and they suggest that free plasma levels should be assessed to obtain accurate estimates of drug quantity available to affect tumor cells. Nevertheless, lapatinib plasma levels have not been published in association with therapy outcome[38,106,107]. We applied a previously developed method for determination of imatinib in plasma for lapatinib, which is separated in 1.9 min under the same chromatographic conditions[108-110] and we are currently evaluating the role of lapatinib plasma level assessment in therapy tailoring.

CONCLUSION

Lapatinib is a new therapeutic option for HER2+ BC patients. Interactions with other drugs metabolized by cytochromes P450 can influence lapatinib effectiveness, mainly with regard to CYP3A4 inhibitors and inducers. Resistance to lapatinib can be caused by genetic/epigenetic changes in tumor cells as well as by other factors leading to low and ineffective lapatinib concentrations in a tumor, e.g. reasons described in association with pharmacokinetics. At this time, only HER2 amplification/overexpression is used to select the best lapatinib therapy candidates in routine clinical settings. Further studies focusing on HER1 (EGFR) gene/protein status assessment promise to provide new data on its predictive role. PTEN loss and PIK3CA gene mutations are markers that could also predict treatment response. Secondary resistance appearing during lapatinib treatment caused by tumor cell changes in DNA or protein expression is difficult to assess in patients on lapatinib treatment. Repeated tumor samples are needed for such an examination and these are not usually available in clinical practice. Thus markers quickly and easily available for assessment may play an important role in therapy tailoring. Pharmacokinetic influence on lapatinib efficacy might be easily assessed by lapatinib plasma levels representing changes in drug metabolism. Lapatinib plasma level assessment may also be a tool for identifying patients at risk of treatment failure or toxicity because of too low or too high lapatinib levels. A prospective clinical study evaluating such an approach would provide evidence of lapatinib plasma level assessment and its application in routine clinical practice with the aim of optimizing and prolonging this efficient therapy.

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4.4 EGFR status assessment in archival breast cancer samples

4.4.1 EGFR (HER1) gene and protein assessment by fluorescence *in situ* hybridization and immunohistochemistry in breast cancer: the search for optimal method and interpretation

EGFR is one of the two HER family members that are targeted by lapatinib. However, EGFR status is not considered as predictive for lapatinib treatment response and EGFR assessment is not routinely evaluated in clinical practice. One explanation for this situation is that EGFR assessment lacks standardization and studies on a predictive role in breast cancer treatment use different approaches to EGFR status evaluations. The following pilot study describes multiple approaches to interpretation of EGFR status assessment by fluorescence *in situ* hybridization (FISH) and IHC. FISH assessment included counting signals representing *EGFR* gene and chromosome 7. The FISH results were interpreted using different methods: determining gene/chromosome ratio (Sauer *et al*, 2005), counting the proportion of cellular clones bearing a defined number of gene copies (Reis-Filho *et al*, 2005) and counting the mean chromosome copy number per nucleus to assess chromosome polysomy (Kaplan *et al*, 2010). IHC assessment included counting the proportion of cells with membrane expression and the expression intensity. The IHC results were also interpreted using three different methods: Allred score which sums the proportion of expressing cells and staining intensity (Harvey *et al*, 1999), histoscore obtained by summing multiples of the proportion of expressing cells and staining intensity (Yang *et al*, 2008) and total immunostaining score (TIS) obtained by multiplying proportion score described as estimated fraction of positively stained cells (PS) and intensity score (IS) of expressing cells (Spizzo *et al*, 2011).

FISH counts varied depending on the interpretation method: only one sample 5% (1/20) displayed *EGFR* amplification according to gene/chromosome 7 ratio, 20% (4/20) cases presented with *EGFR* amplification in cellular clones, and chromosome 7 polysomy was observed in 30% (6/20) cases. IHC counts showed: 22% (5/23) EGFR expressing cases assessed by Allred scoring, the same 22% (5/23) expressing cases assessed by histoscore, and only one EGFR expressing case 4% (1/23) evaluated by TIS following the given thresholds. The subsequent statistical analysis found that increased IHC staining

positivity of EGFR assessed by Allred score and histoscore associated with *EGFR* gene amplification in cellular clones (both P = 0.0485) and TIS tended to the same association (P = 0.0624). The present data point to highly differing results using particular interpretation methods especially in the case of FISH and suggest that the used methods should be further evaluated. Standardization of EGFR status assessment might enable coherent results from independent studies and clarify the prognostic and predictive role of EGFR status in breast cancer.

In contrast to EGFR, HER2 assessment has already been standardized and entered everyday clinical practice as a prognostic and predictive marker in breast cancer. The recommendations of the American Society of Clinical Oncology/College of American Pathologists to HER2 assessment now consist of IHC staining accompanied by FISH to identify equivocal IHC results as positive or negative cases. The standardized approach to sample assessment is as important as standardized handling of a tissue starting with tumor sampling (Wolff *et al,* 2007; Deyarmin *et al*, 2013). Even if such standardization might not have the power to decrease the number of inconclusive cases or increase concordance between the two methods (Vergara-Lluri *et al*, 2012), the inter-institutional standardization is important to obtain comparable results. Similarly, standardization in IHC staining assessment has been also sought in the case of HR and American Society of Clinical Oncology/College of American Pathologists recommended an optimal approach to sample handling and HR status assessment by IHC (Hammond *et al*, 2011). The guidelines like the ones described help substantially with the whole procedure of receptor status assessment. EGFR assessment in breast cancer lacks such recommendations.

As shown in the present study, EGFR FISH and IHC count interpretation depends significantly on method and thresholds used. We used three different approaches to interpretation of both FISH and IHC counts. There are also other interpretation methods used in different cancer types. In lung adenocarcinoma, *EGFR* amplification is defined using Colorado scoring criteria that take into account gene/chromosome ratio as well as gene copy number increase in a defined proportion of cellular clones (Varella-Garcia *et al*, 2009). Counting the average number of gene signals in tumor cells is also a possible approach to determine *EGFR* amplification (Bhargava *et al,* 2005). In the case of colorectal cancer, EGFR expression is defined as ≥1% positive cells (http://www.ema.europa.eu/). There are also publications on staining intensity with positive breast tumors defined as intensities 3+, 2+ (or also 1+) (Cameron *et al*, 2008; Press *et al*, 2008). The lack of

standardization in EGFR assessment might influence the unclear results of studies looking for association of patient survival and lapatinib treatment. Assessment standardization might resolve the question whether there is any predictive effect of EGFR status on lapatinib treatment response in breast cancer. The role of EGFR in breast cancer is further discussed in the review article on page 156.

EGFR (HER1) gene and protein assessment by fluorescence *in situ* hybridization and immunohistochemistry in breast cancer: the search for optimal method and interpretation

(Draft of manuscript)

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Abstract:

EGFR status assessment is not currently used in the clinical management of breast cancer patients. Approaches to EGFR assessment are also not standardized and its prognostic and predictive role in breast cancer remains unclear. We evaluated the *EGFR* gene status using fluorescence *in situ* hybridization (FISH) and the corresponding EGFR protein by immunohistochemistry (IHC) in a cohort of 28 breast cancer patients to find an optimal interpretation approach to these methods. Archival formalin-fixed paraffin-embedded tumor tissue samples were used. Both methods were interpreted in 3 different ways: FISH by gene/chromosome ratio, cellular clones, and chromosome 7 polysomy, IHC by Allred scoring, histoscore, and total immunostaining score (TIS). Only one sample (5%) displayed *EGFR* amplification according to gene/chromosome 7 ratio, four samples (20%) according to cellular clones, and six samples (30%) displayed chromosome 7 polysomy. IHC counts showed EGFR expression in: five samples (22%) by Allred scoring and by histoscore, and only one case (4%) by TIS. Increased IHC staining positivity of EGFR

assessed by Allred and histoscore significantly associated with the *EGFR* gene amplification in cellular clones (both p=0.0485). This shows that IHC and FISH EGFR status assessment depends in both methods on the interpretation approach used. EGFR assessment should be standardized to obtain reproducible data on the EGFR prognostic and predictive role in breast cancer.

Introduction

Assessment of human epidermal growth factor receptor 2 (HER2/ERBB2) expression and the gene amplification are important in breast cancer as they allow identification of a relatively small subgroup of patients who will benefit from targeted treatment including trastuzumab, lapatinib, and more recently, pertuzumab and trastuzumab emtansine (Melichar *et* Plebani, 2012). Since HER2 has no natural ligand, the receptor activation and signal transduction are dependent on the formation of dimers with other HER family receptors including epidermal growth factor receptor (EGFR/HER1). EGFR is a membrane receptor encoded by a gene located on chromosome 7p12. Upon activation, EGFR stimulates intracellular signaling pathways leading to tumor growth, proliferation and survival by the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K)-Akt pathways (Hynes *et* Lane, 2005). The prognostic and predictive role of EGFR in breast cancer has been extensively studied in recent years (Bouchalova *et al*, 2009; Bouchalova *et al*, 2010). EGFR protein expression was reported in about 40% and *EGFR* gene amplification was found in 0-14% of breast cancer cases (Bouchalova *et al*, 2010). The EGFR expression could, in a subset of breast cancer cases be underlined by *EGFR* gene copy gain similar to the case of HER2 (Reis-Filho *et al*, 2005; Gumuskaya *et al*, 2010). Association between EGFR gene and expression status as well as the role of EGFR in breast cancer prognosis and treatment prediction remain unclear. A possible explanation for these conflicting results regarding the significance of EGFR in breast cancer could be the lack of standardization of EGFR status assessment.

The most commonly used methods for EGFR assessment are immunohistochemistry (IHC) and *in situ* hybridization (ISH) techniques, including fluorescence *in situ* hybridization (FISH) and chromogenic *in situ* hybridization (CISH) (Sauer *et al*, 2005; Reis-Filho *et al*, 2005; Kaplan *et al*, 2010). In the case of ISH methods, there are several ways of signal assessment, such as: determining gene/chromosome ratio (Sauer *et al*, 2005), counting the proportion of cellular clones bearing a defined number of gene copies (Reis-

Filho *et al*, 2005) or counting the mean chromosome copy number per nucleus to assess chromosome polysomy (Kaplan *et al*, 2010). While a number of techniques have also been used for IHC, the 3 most common interpretation methods currently in use are Allred score summing proportion of expressing cells and staining intensity (Harvey *et al*, 1999), histoscore obtained by summing multiples of proportion of expressing cells and staining intensity (Yang *et al*, 2008) and total immunostaining score (TIS) obtained by multiplying proportion score described as estimated fraction of positively stained cells and intensity score of expressing cells (Spizzo *et al*, 2011).

In the present pilot study, we compared EGFR gene and protein assessment by FISH and IHC using different interpretation methods in a cohort of breast cancer patients. The aim was to compare the results of FISH and IHC interpreted by different approaches.

Materials and methods

Patients and clinical data

Twenty-eight female patients treated with lapatinib plus capecitabine at the Department of Oncology, University Hospital Olomouc, Czech Republic between February 2007 and January 2009 were included in the study. The majority of the patients were previously included in another study focusing in particular on patient clinical outcome (Cizkova *et al*, 2012). They were originally diagnosed with stage I to IV disease. All patients were treated with lapatinib plus capecitabine in a palliative setting after tumor metastasis. Samples from primary tumors were available for further assessment in the majority of patients. Tumors in 26 patients were confirmed as HER2 positive by FISH and/or IHC. This patient cohort was chosen for the present pilot study because it represented the target population that might benefit from standardization of EGFR assessment.

Tumor tissue assessment

Archival formalin-fixed paraffin-embedded tumor tissue samples (FFPE) were cut into 4 - 6 µm sections and immobilized on "Plus Slides" (Superfrost Plus, BDH, Germany).

Fluorescence in situ *hybridization*

FFPE were prepared for the two-color FISH as previously described (Bouchalova *et al*, 2006). The cytogenetic states of the *EGFR* gene and chromosome 7 were analyzed. Directly

labeled locus specific EGFR (Orange, IntellMed Ltd., Olomouc, Czech Republic) and chromosome 7 centromere (Green, IntellMed Ltd., Olomouc, Czech Republic) DNA probes were used. The gene and chromosome 7 signals were counted in 100 tumor nuclei when possible or in at least 60 tumor nuclei in the case of small tissue samples. The gene and chromosome 7 signal counts were interpreted using different approaches. First, gene/chromosome 7 ratio was counted and the ratios <0.8 and >1.2 were considered as deletion and amplification, respectively (Sauer *et al*, 2005). Then only gene signals were taken into account and percentage representation of tumor cells displaying a defined numbers of gene signals were counted (cellular clones). In this case, samples were considered amplified if >50% of the neoplastic cells were represented by cellular clones exhibiting >5 signals per nucleus or large gene signal clusters (Reis-Filho *et al*, 2005). Finally, a mean chromosome copy number per nucleus was counted and the cases displaying ≥2.5 copies of chromosome 7 per nucleus were considered as polysomic (Kaplan *et al*, 2010).

Immunohistochemistry

Immunohistochemistry staining was performed on FFPE using EGFR (clone 111.6) mouse monoclonal antibody (mAb) (NeoMarkers, Fremont, CA) following standardized protocols (Hlobilkova *et al*, 2007). The membrane expression intensity was evaluated as 0, no staining; 1, weak; 2, moderate; and 3, strong and proportion as percentage of expressing cells (%). The counts of expression were interpreted using the three well-described IHC assessment methods. Allred score was counted as described previously (Harvey *et al*, 1999) using proportion of positively stained cells evaluated as 0, none; 1, <1⁄100; 2, 1⁄100 to 1⁄10; 3, 1⁄10 to 1⁄3; 4, 1⁄3 to 2⁄3; and 5, >2⁄3 and the intensity of the staining. A final value ranging from 0 to 8 was then obtained by summing proportion and intensity scores. The cutoff value for positivity was set as >2. Histoscore was defined using percentage of positively stained tumor cells and the staining intensity (Yang *et al*, 2008). The final histoscore was counted as the sum of (1x% weakly positive tumor cells) + (2x% moderately positive tumor cells) + (3x% intense positive tumor cells). The cutoff value for positivity was set as ≥5. TIS was obtained by multiplying proportion score (PS) and intensity score (IS) (Spizzo *et al*, 2011). PS described a fraction of positively stained tumor cells as 0, none; 1, <10%; 2, 10-50%; 3, 51-80%; 4, >80% and IS corresponded to the staining intensity. TIS ranged from 0 to 12 with only nine possible values (0, 1, 2, 3, 4, 6, 8, 9 and 12). The cutoff value for positivity was >4.

Statistical analysis

Standard statistical tests were used to process the data. The EGFR gene and protein states were assessed using the Spearman test, Mann-Whitney U test and Chi^2 test. For all analyses, a p-value of <0.05 was considered to be statistically significant. Statistical analyses were performed using Statistica 8.0 software.

Results

FISH results

Twenty of the available tumor sections were successfully deparaffinized, hybridized applying *EGFR* and centromere 7 probes and evaluated (71%). The median *EGFR* gene copy number per nucleus was 2.35, the median chromosome 7 copy number per nucleus was 2.17 and the median *EGFR*/chromosome 7 ratio was 1.06. All the 3 interpretation approaches were applied showing varying results. Counting gene/chromosome 7 ratio, only one sample 5% (1/20) displayed *EGFR* amplification. When cellular clones were counted, 20% (4/20) of cases showed *EGFR* amplification. Chromosome 7 polysomy was observed in 30% (6/20) of cases. The results are summarized in Table 1'''. These data indicate that results of FISH were strongly dependent on the interpretation method used.

IHC results

Twenty three tumor sections available for IHC were successfully evaluated for EGFR expression (82%). EGFR membrane expression was found to range from 0 to 2. In three samples, a range of intensity was given (e.g. 1-2) and higher value of the range was further used to interpret the results in these cases. The counts of intensity and proportion of expressing cells were interpreted by the three methods. The Allred score was found ranging 0-7. Following the cutoff value, 22% (5/23) cases were positive. The histoscore was found ranging 0-160. Positivity was found in the same 22% (5/23). TIS was found ranging 0-6 and according to the given cutoff, only one case was positive among TIS score results 4% (1/23). IHC results are summarized in Table 1''.

Statistical assessment

The results obtained by IHC and FISH were compared, to investigate correlations between *EGFR* amplification and chromosome 7 polysomy and EGFR protein expression. The assessment of continuous variables obtained by applying different FISH and IHC interpretation methods showed no statistically significant correlations between genomic and protein states (Spearman test, Table 2‘’’). EGFR results of FISH and IHC marked as positive and negative according to described cutoffs (Table 1‘’’) were also analyzed. Both Allred score and histoscore results showed a trend to association with gene amplification assessed as cellular clones (Chi^2 test, p=0.0644 in both cases) but not with chromosome 7 polysomy (Chi^2 test, p=0.9295 in both cases). Results of TIS interpretation of IHC and gene/chromosome 7 ratio of FISH were not analyzed because only one positive case was detected by each method. Further, we also tested these data as continuous variables for IHC results and categorical for FISH results. The EGFR gene amplification assessed as cellular clones was associated with expression positivity determined by Allred scoring system (Mann-Whitney U test, p=0.0485) and histoscore (Mann-Whitney U test, p=0.0485). The EGFR expression evaluated by TIS also showed a trend to association with *EGFR* amplification based on cellular clones (Mann-Whitney U test, p=0.0624). These findings demonstrate that increased IHC staining positivity of EGFR associates with increased *EGFR* gene copy number in cellular clones. No associations were found between EGFR protein expression and chromosome 7 polysomy or gene/chromosome ratio (Mann-Whitney U test, data not shown). This shows that statistical correlations between IHC and FISH results depended significantly on the assessment method used.

Discussion

The present study compared different methods of assessment and interpretation of EGFR status in breast cancer patients. Based on the described data, it is evident that the results can differ markedly depending on the interpretation approach. The method used affected final results including associations between EGFR status assessed by IHC and FISH. These results show clearly that different interpretation approaches to both IHC and FISH counts

Table 1‴. Overview of IHC and FISH results.

Sample	IHC results						FISH results					
	1. Allred score		2. Histoscore		3. TIS		1. Ratio		2. Clones		3. Polysomy	
	Values	Results	Values	Results	Values	Results	Values	Results	Values[a]	Results	Values[b]	Results
1	*x*	*x*	*x*	*x*	*x*	*x*	1.0	*normal*	2.0	*normal*	2.0	*normal*
2	3	***positive***	5	***positive***	1	*normal*	1.1	*normal*	2.2	*normal*	2.0	*normal*
3	7	***positive***	160	***positive***	6	***positive***	1.0	*normal*	3.7	***amplified***	3.6	***polysomic***
4	0	*normal*	0	*normal*	0	*normal*	1.1	*normal*	1.9	*normal*	1.8	*normal*
5	0	*normal*	0	*normal*	0	*normal*	1.0	*normal*	2.3	*normal*	2.2	*normal*
6	5	***positive***	40	***positive***	4	*normal*	1.5	***amplified***	3.2	***amplified***	2.1	*normal*
7	3	***positive***	5	***positive***	1	*normal*	1.1	*normal*	4.4	***amplified***	3.9	***polysomie***
8	0	*normal*	0	*normal*	0	*normal*	1.1	*normal*	2.4	*normal*	2.2	*normal*
9	0	*normal*	0	*normal*	0	*normal*	*x*	*x*	*x*	*x*	*x*	*x*
10	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*
11	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*
12	0	*normal*	0	*normal*	0	*normal*	1.1	*normal*	6.0	***amplified***	5.5	***polysomie***
13	2	*normal*	1	*normal*	1	*normal*	1.0	*normal*	2.0	*normal*	1.9	*normal*
14	0	*normal*	0	*normal*	0	*normal*	1.1	*normal*	2.9	*normal*	2.7	***polysomie***
15	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*
16	0	*normal*	0	*normal*	0	*normal*	1.0	*normal*	2.2	*normal*	2.1	*normal*
17	0	*normal*	0	*normal*	0	*normal*	1.0	*normal*	2.1	*normal*	2.0	*normal*
18	0	*normal*	0	*normal*	0	*normal*	1.0	*normal*	2.3	*normal*	2.2	*normal*
19	0	*normal*	0	*normal*	0	*normal*	1.1	*normal*	2.1	*normal*	1.9	*normal*
20	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*
21	0	*normal*	0	*normal*	0	*normal*	1.1	*normal*	2.7	*normal*	2.5	***polysomie***
22	0	*normal*	0	*normal*	0	*normal*	*x*	*x*	*x*	*x*	*x*	*x*
23	0	*normal*	0	*normal*	0	*normal*	1.1	*normal*	2.6	*normal*	2.5	***polysomie***
24	0	*normal*	0	*normal*	0	*normal*	1.2	*normal*	2.6	*normal*	2.3	*normal*
25	0	*normal*	0	*normal*	0	*normal*	*x*	*x*	*x*	*x*	*x*	*x*
26	4	***positive***	20	***positive***	2	*normal*	1.0	*normal*	2.5	*normal*	2.5	*normal*
27	0	*normal*	0	*normal*	0	*normal*	1.0	*normal*	1.9	*normal*	2.0	*normal*
28	0	*normal*	0	*normal*	0	*normal*	*x*	*x*	*x*	*x*	*x*	*x*

X=not available or not assassable sample.

[a]Average number of gene signals per nucleus.

[b]Avarage number of chromosome signals per nucleus.

Table 2'''. Correlations between EGFR gene and protein continuous proper values using Spearman test.

		FISH results		
	p-value Spearman R	1. Ratio	2. Average gene copy number per nucleus	3. Chromosome 7 polysomy
IHC results	1. Allred score	*p=0.67* -0.11	*p=0.2* 0.31	*p=0.5* 0.16
	2. Histoscore	*p=0.67* -0.11	*p=0.2* 0.31	*p=0.5* 0.16
	3. TIS	*p=.61* -0.13	*p=0.23* 0.29	*p=0.55* 0.15

display varying ability to identify EGFR positive cases. Association of IHC and FISH data was found when the *EGFR* gene amplification was evaluated based on the gene copy number in cellular clones and EGFR protein expression was interpreted by Allred score or histoscore (p=0.0485). In these conditions, *EGFR* was amplified in 20% of cases (gene signals in cellular clones) and expressed in 22% cases (both Allred score and histoscore). The results of protein expression support the previously described observations, but the frequency of the gene amplification was higher (Bouchalova *et al*, 2010).

Studies on the prognostic and predictive significance of the EGFR expression have provided conflicting results (Viale *et al*, 2009; Kallel *et al*, 2012; Malorni *et al*, 2012; Olsen *et al*, 2012; Liu *et al*, 2012; Tang *et al*, 2012). However, it is difficult to compare the results of such studies due to the use of various methods, interpretation approaches and cutoff levels for the EGFR status assessment. Nevertheless, a number of studies have reported associations of EGFR positivity with some negative prognostic markers or poor breast cancer patient outcome (Al-Kuraya *et al*, 2004; Siziopikou *et al*, 2006; Dihge *et al*, 2007; Nieto *et al*, 2007; Viale *et al*, 2009; Kallel *et al*, 2012; Liu *et al*, 2012). This suggests there is value in EGFR assessment in breast cancer. Current studies evaluating EGFR expression

focus on the staining intensity only or on both staining intensity and proportion of expressing cells. Cutoff levels used to distinguish positive and negative cases vary as well. Considering EGFR gene status assessed by ISH methods, two main assessment approaches are used in breast cancer studies: counting only the signals representing gene alleles or counting the ratio including the signals representing the chromosome copy number (Bouchalova *et al*, 2009; Sauer *et al*, 2005; Reis-Filho *et al*, 2005; Kaplan *et al*, 2010). Positive association between EGFR IHC and ISH status have been reported in some studies (Bhargava *et al*, 2005; Reis-Filho *et al*, 2005; Gumuskaya *et al*, 2010), but there are also studies that found no link between EGFR expression and gene status (Sauer *et al*, 2005; Umemura *et al*, 2005; Park *et al*, 2007; Gilbert *et al*, 2008). As our results showed, association between EGFR status assessed by IHC and FISH is strongly dependent on a method used to interpret the counts and also on the threshold used. There are greater differences between interpretation methods in the case of FISH than in IHC. However, the present pilot study comprises only a limited number of cases and should be confirmed on a larger sample.

These data have important implications for the development of EGFR-targeted treatment in breast cancer. Currently, several agents targeting EGFR are available, including monoclonal antibodies cetuximab and panitumumab, and small-molecular-weight inhibitors erlotinib and gefitinib. EGFR-targeted therapy is a standard component of therapy in advanced colorectal, head and neck and pancreatic cancer as well as in lung adenocarcinoma (Wheeler *et al*, 2010). In colorectal cancer, none of the tests determining EGFR expression has so far been found useful in response prediction, while in lung adenocarcinoma the presence of specific *EGFR* mutations has been shown to predict treatment response. It has been suggested that *EGFR* gene copy gain could be a better predictor of EGFR-inhibitor treatment response than EGFR expression in some tumor types but e.g. in head and neck cancer, *EGFR* gene status does not appear to be a good predictor (Varella-Garcia *et al*, 2009; Wheeler *et al*, 2010; Ålgars *et al*, 2011; Licitra *et al*, 2011). Currently, there is no evidence available showing that EGFR status predicts response to lapatinib in HER2-positive breast cancer. In preclinical studies, lapatinib was proven to inhibit EGFR and subsequently signal transduction leading to cell growth arrest (Xia *et al*, 2002; Wood *et al*, 2004), but following clinical studies failed to provide any evidence that EGFR expression plays any role in lapatinib treatment efficacy (Spector *et al*, 2005; Johnston *et al*, 2008; Press *et al*, 2008). Johnston et al. focused on the phosphorylated

form of EGFR protein while Press et al. assessed the non-activated form of the protein. Both studies considered EGFR positive in the case of 1+, 2+ or 3+ expression, but EGFR status was not found to predict lapatinib treatment response in any of these studies (Johnston *et al*, 2008; Press *et al*, 2008). Standardization of EGFR status assessment could help to clarify the predictive role of EGFR in lapatinib treatment response. Prospective clinical studies should focus on EGFR assessment by IHC and FISH using differing interpretation approaches as in our study. Identified methods with sufficient potential for predicting patient survival and lapatinib treatment response should be further tested in independent prospective clinical studies. This approach might have the statistical power needed to resolve persisting questions about the role EGFR in breast cancer. Moreover, standardized assessment would help avoid other interlaboratory differences such as chemicals used, staining procedures and differences in the visual assessment itself.

In conclusion, the present data demonstrate that association between EGFR gene and protein status assessed by IHC or FISH varies depending on the method and interpretation used. EGFR assessment should be standardized, following the example of HER2. A series of prospective clinical studies would best answer these open questions. These studies could include exploratory studies using several interpretation approaches and then independent confirmatory studies on a sufficient patient sample.

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TRIPLE NEGATIVE BREAST CANCER – CURRENT STATUS AND PROSPECTIVE TARGETED TREATMENT BASED ON HER1 (EGFR), TOP2A AND C-MYC GENE ASSESSMENT

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Background: Every year about one million women worldwide are diagnosed with breast cancer which is the most common malignancy in female. Of these, triple negative breast carcinoma represents 10–17 %. Triple negative breast carcinomas, characterized by estrogen, progesterone and HER2 receptor negativity are very aggressive tumours with poor prognosis. Individualized treatment (tailored therapy) based on molecular biology markers of tumor and patient is the trend in clinical practice these days. However, molecular targets and predictors for the treatment of triple negative breast carcinoma do not currently exist.

Methods and results: This minireview focuses on biomarkers (HER1/EGFR, TOP2A and C-MYC genes) that may predict the response of triple negative breast carcinoma patients to chemotherapy and/or targeted biological treatment with a summary of current knowledge about them.

Conclusion: HER1 belonging to the HER family of receptors plays an important role in cell proliferation, migration and protection against apoptosis. HER1 protein could be targeted by monoclonal antibodies and/or tyrosine kinase inhibitors (TKIs). Given signal pathway complexity and HER family member cooperation, it may be better to simultaneously target a number of these receptors (e.g. HER1/HER2 by lapatinib). Thus, HER1 assessment could reveal a particular breast cancer patient group with probably good response to HER1 targeted therapy. TOP2A gene, encoding topoisomerase II alpha (target for anthracyclines) is predictive of response to anthracycline therapy. TOP2A aberrations (amplification, deletion) are found in up to approximately 30-90 % of HER2 amplified breast cancer and amplifications are more common than deletions. Recent publications describe TOP2A amplification also in 2.7–8.8 % HER2 nonamplified breast cancers. Patients with a pathologic complete response to anthracycline based neoadjuvant chemotherapy had a good overall prognosis regardless of molecular subtype of breast cancer. These results suggest that particularly tumors with a complete pathological response to anthracyclines could have TOP2A amplification. C-MYC encodes nuclear DNA binding proteins that regulate proliferation and apoptosis; amplification is associated with poor prognosis and hormonally negative breast carcinoma.

INTRODUCTION

About one million women worldwide are diagnosed with breast cancer (BC) every year. BC is the most common cancer in women. Data from the Czech National Oncology Registry (www.svod.cz) indicate the incidence has doubled since 1977 and in 2005 it reached 105.5/100 000 women with a mortality of 36.5/100 000. Due to mamographic screening, stage I of the disease is diagnosed in nearly 35 % of patients and stage II in more than 30 %[1-2]. Triple negative breast carcinoma (TNBC) represents 10-17 % of all BC[3]. This minireview focuses on potential molecular targets and biomarkers that could be aimed or analyzed for prediction of response of TNBC patients to chemotherapy and/or biological targeted therapy.

Pathology and Cytogenetics

Ductal carcinoma is the most common histological category of malignant breast tumors, lobular carcinoma is the second major type while medullary carcinoma is relatively rare entity[4]. On diagnosis, the various presentations are classified on the basis of morphological and molecular examination. Prognosis is defined according to a number of parameters: tumor size and grade, the presence/absence of estrogen (ER) and/or progesterone (PR) receptors, HER2/neu (HER2, c-erbB2) protein, lymph node metastases and vascular or perineural tumor invasion. Other parameters, such as the proliferating index, ploidy, the presence of P53, cytokeratins (Ck), HER1 (EGFR), or TOP2A alterations, may also be useful for prognostic evaluation or as predicting therapeutic re-

sponse. Classification according to immunohistochemistry (IHC) (based on expression profiles) distinguishes HER2+ (HER2+, ER+/-, Ck5/6 and EGFR +/-), luminal (HER2 -, ER+, Ck5/6 and EGFR +/-) and basal-like (ER -, HER2 -, Ck5/6 and/or EGFR +) carcinomas [5-7].

New insights into the molecular pathogenesis of BC, with prognostic and predictive impact, have been gained using cytogenetics. BC tumor genomes have undergone major rearrangements. Hot spots for gains are routinely observed at 1q31-q32, 8q12 and 8q24 (MYC), 17q12 (HER2), 17q23-24 and 20q13, recurrent losses are present at 1p, 6q, 8p, 11q23qter, 13q, 16q, 17p and 22q. The number of changes accumulates in advanced tumors[8-9].

Triple negative breast carcinomas

Triple negative breast carcinomas (TNBC), characterized by absence of ER, PR and HER2 expression, are very aggressive tumors with poor prognosis. They more frequently affect younger patients (<50 years), are more prevalent in African-American women, often present as interval cancers, initially are highly chemosensitive, but are significantly more aggressive than tumors pertaining to other molecular subgroups. This aggressiveness is best illustrated by the fact that the peak risk of recurrence is between the first and third years and the majority of deaths occur in the first 5 years following therapy. The majority of TNBC are high-grade invasive ductal carcinomas of no special type, metaplastic and medullary carcinomas[3, 7].

Individualized treatment (tailored therapy)

Personal, custom made, therapy based on molecular biology markers of tumor and patient is the trend in clinical practice these days. The first clinically used predictive markers in BC were ER/PR tailoring response to antihormonal therapy[10-11]. The first cytogenetic predictor for BC treatment is the HER2 (HER2/neu, c-erbB2) gene amplification and protein overexpression. Monoclonal antibody trastuzumab (Herceptin) is used in the treatment of BC in patients who display HER2 positivity in invasive carcinoma component[12-16]. Nevertheless, predictors for the therapy of TNBC do not exist yet.

HER1 gene and targeted therapy

HER1 (also known as epidermal growth factor receptor, EGFR) belongs to the HER family of transmembrane receptors. HER1 gene is located on 7q12. Its protein product - 170-kD glycoprotein - plays an important role in cell proliferation, migration and protection against apoptosis mediated by subsequent activation of intracellular pathways. HER1 receptor can dimerize with all members of HER family and it has to create homo - or heterodimers to be functionally active[17]. Worse prognosis of breast tumors overexpressing HER1 is connected with the above-mentioned effects on proliferation, migration and apoptosis. A study by Filardo et al. focuses on a receptor called G protein-coupled receptor 30 (GPR30), a member of the seven transmembrane receptor superfamily which is associated with specific estrogen binding and HER1 activation[18]. This crosstalk between receptors together with the described influence on cell biology makes HER1 status assessment valuable even in the context of tumor hormonal dependence.

HER1 protein could be targeted by monoclonal antibodies and/or synthetic tyrosine kinase inhibitors (TKIs). Monoclonal antibodies (cetuximab, panitumumab) are now clinically used in the treatment of colorectal cancer and head and neck carcinoma. TKIs are also important in the therapy of pancreatic and non-small cell lung cancer (NSCLC). HER1 targeted treatment with cetuximab in breast cancer have not produced satisfactory results probably because of the activation of downstream signal pathways[7] or inadequate patient selection. TKIs are an option for targeted therapy in BC that is focused on HER1 in particular. Agrawal et al. evaluated the results of studies testing TKIs (erlotinib, gefitinib) in BC and pointed out that HER1 protein must be present in targeted tumor tissue to obtain valuable treatment results. They also concluded that because of signal pathway complexity and HER family member cooperation it might be better to target more of these receptors at the same time[19]. Thus, HER1 assessment could reveal a particular BC patient group with probably good response to HER1 targeted therapy. Dual HER1/HER2 inhibitor lapatinib is now approved for BC patients with HER2 amplification/overexpression when trastuzumab therapy has failed. However, HER1 gene status is not used in clinical practice to guide therapy in BC, although increased HER1 expression is detected in about 40 % of BC. Particularly, HER1 expression is higher (up to 80 %) in TNBC and metaplastic carcinoma (mostly basal-like), where it possibly substitutes ineffective, but otherwise major proliferation/survival pathways of BC induced by expression and activation of HER2, ER and PR proteins. HER1 gene is amplified in nonselected series in 0-14 %, in metaplastic carcinoma up to 28 %[20-26]. Interestingly, HER1 and C-MYC coamplification can be also present[24]. More insights into significance of HER1/HER2 status in outcome of patients treated with TKIs should provide undergoing phase II clinical trial which examines the effect of lapatinib monotherapy in metastatic breast cancer patients with HER2 positive vs. HER1 positive circulating cells in peripheral blood[27].

TOP2A

The TOP2A gene, located on 17q21-22, encoding topoisomerase II alpha (molecular target for anthracyclines) is predictive of response to anthracycline therapy. TOP2A aberrations (amplification, deletion) are found in up to approximately 30-90 % of HER2 amplified BC and amplifications are more common than deletions. Good response to anthracyclines is associated with TOP2A amplification while deletion may be accompanied by resistance. On the other hand, clinical study results are not uniform. Knoop et al. reported in a nonselected series, an association between TOP2A amplification and good response to anthracycline based regimens. Surprisingly, better response to anthracyclines than CMF [cyclophosphamid, methotrexat, 5-fluorouracil (5-FU)] was found in subgroup of patients with TOP2A deletion compared to normal TOP2A status[12, 28-36], probably demonstrating high-

er overall efficacy of antracycline based therapies. One recent study showed that TOP2A deletion was associated with poor prognosis in HER2 amplified BC. Clarification of the mechanism of this association will require additional studies[37]. Burgess et al. identified in a nonselected series, TOP2A expression levels as major determinants of response to the topoisomerase II inhibitor doxorubicin and showed that suppression of TOP2A levels produces resistance to doxorubicin in vitro and in vivo[38]. However in the case of TOP2A there is no correlation between amplification and overexpression[28, 39]. Moreover, our study in locally advanced BC showed HER2 and TOP2A gene status changes after antracycline based chemotherapy[40], and thus number of published data can be biased by treatments preceding tumor biopsy.

Recent publications describe TOP2A amplification in 2.7–8.8 % of HER2 non-amplified BC[35, 41-43]. Tan et al. found TNBC associated with TOP2A protein expression and poor response to adjuvant anthracyclines; in this study including 31 cases of TNBC TOP2A amplification was not detected using chromogenic in situ hybridization[25]. However, as pointed out above, in contrast to HER-2 status, there is no correlation between TOP2A gene amplification and overexpression[28, 39]. Patients with basal-like BC (overlapped with TNBC)[3] treated with neoadjuvant anthracyclines also have poor prognosis (distant disease free survival, DDFS and overall survival, OS)[44]. It may be hypothesized that the lack of HER2 and TOP2A co-amplification could be the cause. However, patients with a pathologic complete response to anthracycline based neoadjuvant chemotherapy had a good prognosis regardless of subtype (basal-like, luminal-like, HER2+/ER-) (TN paradox)[44]. These results suggest that at least individuals with a complete pathological response to anthracyclines could have TOP2A amplification or overexpression. Unfortunately, in the above discussed study by Carey et al. TOP2A status was not assessed. Weigelt et al. studied metaplastic BC (a subgroup of TNBC) by microarray expression analysis and found significant downregulation in PTEN and TOP2A which might partly explain observed differences in response to chemotherapy in TNBC[45].

C-MYC

The 8q chromosome arm that harbors the C-MYC gene is frequently altered in BC. C-MYC encodes nuclear DNA binding proteins that regulate proliferation and apoptosis. The MYC protein is directly involved in regulating more than 1500 genes[46-52]. C-MYC amplification is one of the most frequent aberrations in BC that has been detected in 1-94 % of patients in different studies. Amplification is clearly associated with poor prognosis: patients suffer from early relapses and have poor OS. C-MYC amplification is associated with ER – and PR – breast carcinoma. C-MYC deregulation occurs preferentially in young patients[53]. C-MYC protein may affect the response to chemotherapy probably through DNA damage response regulation[46, 54-60]. Interestingly, C-MYC amplification in colon carcinoma predicts better response to 5-FU adjuvant chemotherapy [disease free survival (DFS) and OS have been improved by 30 %], but only in p53 wild tumors[61-62]. This type of study in BC has not been published. Nonetheless, Rakha et al. described improvement of the poor prognosis of TNBC by treatment with the CMF regimen[63]. TNBC often have amplified HER1 gene[21] and according to the described C-MYC coamplification in BC[24], so we may hypothesize the possibility that the tumors responding favorably to the 5-FU containing regiment CMF were those with C-MYC amplification. Suppression of C-MYC transcription in BC cells after 5-FU treatment supports the direct effect of 5-FU on the oncogene activity, probably mediated by upstream signaling inhibition[64].

Adjuvant/neoadjuvant chemotherapy

Adjuvant chemotherapy is recommended according to the international guidelines for patients in clinical stage IB to IIIB of breast carcinoma, when the tumor is larger than 1cm and/or lymphonodes are positive, respectively. However in TNBC even smaller tumors are recommended to consider adjuvant chemotherapy with respect to diseases recurrence risk and high aggressivity of this tumor type. For treating TNBC, anthracycline regimens are mostly used[65]. However, there are no data on the real patient benefits. It is assumed that chemotherapy is more successful in ER – than ER+ patients and appears to be more appropriate for young premenopausal women[10-11, 66]. Since the chemotherapy has serious side-effects, finding an accurate predictor of response could determinate patients who would profit from adjuvant chemotherapy. However, current knowledge indicates the possibility of CMF renascence in treatment of TNBC associated with poor prognosis and limited therapeutic options, particularly in adjuvant settings. It is hoped that poor prognosis of TNBC could be improved using CMF treatment[25, 63].

Neoadjuvant chemotherapy is a newer possibility recommended for patients with expected good response to chemotherapy administration (ER-, PR-, non-lobular, fast proliferating, luminal B and high grade tumors), mainly in patients with locally advanced disease potentially indicated for breast saving surgery. Currently applied regimens are mostly based on a combination of anthracyclines and taxanes which suggests again the importance of further comparative studies evaluating the efficacy of CMF, anthracycline based or other therapies in TNBC[65].

CONCLUSION

Triple negative breast carcinoma (TNBC) represents 10-17 % of all BC[3] with poor prognosis. Specific predictors for its targeted treatment are still lacking. However, TOP2A status could predict sensitivity to anhracycline therapy in a small proportion of TNBC patients. Chemotherapy optimization (CMF vs. other regimens) needs to be evaluated in large clinical studies. The complexity of intracellular signal transducing pathways also demands further investigations. This raises the importance of dual inhibitors like lapatinib or molecules preventing dimerisation of receptors like pertuzumab at the

level of HER2 and other members of the HER family[19]. Together with the treatment approaches described there is also the possibility of combination with drugs acting at lower levels of signal transmission.

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5 Discussion and prospective

Breast cancer is a highly heterogeneous group of the disease subtypes differing genetically, biologically, histologically and clinically, due to their extreme molecular complexity. The vast number of tumor alterations affects important signaling pathways leading to cell proliferation and survival. The PI3K signaling pathway belongs to the most commonly deregulated pathways in breast cancer together with other signaling pathways such as MAPK, p53 and Wnt. Deeper understanding of alterations affecting these pathways at the genetic as well as functional level in tumor cells is needed for more accurate patient survival prediction and treatment choice. Recently, there are an increasing number of publications that search for tumor changes underlining cancer development using pangenomic approaches. Nik-Zainal and coworkers have published a comprehensive study focusing on development of specific mutational process taking place in the breast cancer tumor cells of 21 patients (Nik-Zainal *et al*, 2012 [B]). The authors focused on the phenomenon of localized hypermutation and identified distinct mutational signatures characteristic for breast cancer development. Curtis et al. (Curtis *et al*, 2012) focused on the genomic and transcriptomic architecture of 2,000 breast tumors in the search for a novel molecular stratification. A recent study focused on 510 breast cancer exomes and provided a comprehensive description of alterations in breast tumors. This study described alterations specific for the main breast cancer subtypes as well as heterogeneities in these alterations found within the subtypes (The Cancer Genome Atlas Network, 2012). Based on various deregulations taking place in breast cancer cells, new subgroups might be defined in future presenting more homogenous types suitable for uniform tailored therapies. Such new breast cancer subgroups might further widen our knowledge currently associated with breast cancer sorting based on cellular receptors. Thus we might be able to predict more precisely patient prognosis and expected treatment response (Banerji *et al*, 2012; Nik-Zainal *et al*, 2012 [B]).

Prognostic and predictive markers of the PI3K pathway activation have great potential to serve in clinical practice, but more is needed to understand well the underlying mechanisms of action of the pathway. Multiple specific hits have been described that alter signaling in the PI3K pathway leading to its activation. Among the most frequent alterations, are receptor tyrosine kinase deregulations, *PIK3CA* and *AKT1* mutations and

PTEN expression loss (Bièche *et* Lidereau, 2011; The Cancer Genome Atlas Network, 2012). Other signaling pathways such as MAPK are also altered in breast cancer. New methods that allow assessment of whole genome in considerable numbers provide valuable information on the range of alterations occurring in breast tumors. In a small subset of breast tumors (less than 10%), likely driver oncogenic mutations were described in *KRAS*, neurofibromin 1, *MAP2K4, MAP3K1,* and *MAP3K13.* Inactivating mutations in *MAP3K1*, *MAP2K4* and *MAP3K13* are predicted to abrogate signaling pathways that activate JUN kinases and specifically *MAP3K1* and *MAP2K4* mutations are the most frequently found in ER-positive breast tumors (Curtis et al, 2012; Stephens *et al*, 2012; The Cancer Genome Atlas Network, 2012). However, the PI3K signaling pathway plays a crucial role in breast cancer development and progression because it is probably the most frequently activated signaling pathways in breast tumors. Moreover, the mutations found in the PI3K pathway are often present in oncogenes (as *PIK3CA*, *AKT1*) and occur in a few hot-spots which makes assessment of these mutations easier than in the case of tumor supressors where mutations are often spread through the whole gene requiring extensive sequencing.

We have searched for new pieces of information to enrich current understanding of the PI3K pathway activity in breast cancer. The particular projects cover subjects connected with the pathway at levels of the HER family receptors activating the PI3K pathway as well as PI3K itself and its downstream effectors. All these subjects are connected by the PI3K pathway, the need to deepen current knowledge and bring new useful information applicable in future in clinical practice.

In the presented studies, the first focus was aimed at *PIK3CA* mutations that are common in breast cancer occurring in 10-40% tumors. Among thousands to tens of thousands of somatic mutations found in whole-cancer genome by sequencing, the *PIK3CA* has a prominent position being mutated more frequently than other oncogenes in breast cancer. A recent study describing analysis of 510 exomes confirmed *PIK3CA* as the most commonly mutated oncogene in breast cancer (36%) followed by mutations in *AKT1* oncogene occurring in about 3% (The Cancer Genome Atlas Network, 2012). Another important fact that turns attention to *PIK3CA* among the great number of various mutations described in breast cancer cell is that majority of mutations found in cancer cells have probably no biological relevance what brings value to mutations occurring repeatedly in know oncogenes and tumor suppressors. Moreover, *PIK3CA* mutations

were found to transform primary fibroblasts in culture, induce anchorage-independent growth, and cause tumors in animals (Bader *et al*, 2006, Zhao *et al*, 2005, Nik-Zainal *et al*, 2012 [A]; Stephens *et al*, 2012).

Above all, the results described in the included articles show varying effects of *PIK3CA* mutations on survival in anti-HER2-treated and anti-HER2-naïve breast cancer patients. Whereas *PIK3CA* mutations act as good prognostic markers in conventional therapy-treated patients undergoing surgery, radiotherapy, chemotherapy and hormonal treatment (Study 2, Chapter 4.2.1), these mutations are on the contrary negative predictors of monoclonal antibody trastuzumab treatment response (Study 4, Chapter 4.3.1). Based on other published studies, the same negative effect of *PIK3CA* mutations on treatment prediction might be connected with HER2 tyrosine kinase inhibition (Eichhorn *et al*, 2008; Mukohara, 2011). However, there are opposite reports on this matter and good treatment response to tyrosine kinase inhibitor lapatinib was also observed (Dave *et al*, 2011). There are multiple theories providing explanation of the positive prognostic role of activating mutations in *PIK3CA* oncogene in patients without HER2-targeted therapy (Di Cosimo *et* Baselga, 2009; Dumont *et al*, 2012). The opposite role of these mutations in anti-HER2-treated patients might be associated with downstream activation of the pathway. We could also speculate about a feedback mechanism that is blocked by HER2 inhibitors or about another pathway inactivation that otherwise crosstalks with PI3K and renders tumor cells more sensitive to conventional antitumor treatment and that is blocked by anti-HER2 treatment. Despite the lack of knowledge of the precise mechanisms underlying contradictory prognostic and predictive effects of these mutations, assessment of the *PIK3CA* mutational status appears beneficial in breast cancer patients. Regular assessment of *PIK3CA* mutations in prospective clinical studies might also help to clarify such unanswered questions.

Other genes implicated in the PI3K signaling pathway are altered much less frequently than *PIK3CA*. These include tumor suppressors *PTEN*, *PIK3R1* or *INPP4B*, and oncogenes as *AKT1* or receptor tyrosine kinases activating the PI3K pathway (Bièche *et* Lidereau, 2011; The Cancer Genome Atlas Network, 2012; Study 3, Chapter 4.2.2). These alterations affect the genomic level as well as gene expression. Mutations appear in those genes in unselected breast cancer series in only up to 10% cases. PTEN expression loss occurring in around 20-30% is well established as one of the common tumor changes leading to the pathway activation in breast cancer (Stemke-Hale *et al*, 2008; Martins *et al*, 2012).

Regarding breast cancer subtypes, alterations of PTEN or HER4 affect mostly triple negative tumors whereas *PIK3CA* and *AKT1* alterations are associated with luminal tumors (Marty *et al*, 2008; Stephens *et al,* 2012; The Cancer Genome Atlas Network, 2012). Our data obtained at the mRNA level also show the relevance of *PTEN* expression loss in breast cancer, since we found underexpression of *PTEN* mRNA transcripts in 17% primary breast cancer samples and in particular in 39% triple negative breast cancer samples (Study 3, Chapter 4.2.2). Nonetheless, specific effects of the PI3K pathway gene mutations on cancer outcome and patient treatment are less well described in triple negative/basal-like tumors because of low representation of these tumor subtypes in unselected breast cancer patient series.

As described above, *PIK3R1* mutations are rare in breast cancer accounting for about 3% as was also shown by our results (Study 3). However, *PIK3R1* and its encoded protein p85 have a potential to affect PI3K signaling in tumor cells by affecting p110α and PTEN activity (Luo *et* Cantley, 2005; Geering *et al*, 2007; The Cancer Genome Atlas Network, 2012). As suggested in our Study 3, decreased expression of *PIK3R1*/p85 could deregulate the pathway signaling and participate in reduced survival of some breast cancer patients. Especially the subgroup of triple negative breast cancer patients showed significant association with *PIK3R1* underexpression in our series. These observations are interesting and demand further investigation and confirmation at protein levels in larger patient cohorts. Further assessment is attractive especially because there are opposing reports concerning some other tumor types (Elfiky *et al*, 2011; Zito *et al*, 2012). Detailed assessment of deregulations of the PI3K pathway signaling downstream of PI3K proteins might also provide additional new information on the roles of altered pathway signaling in breast cancer. In our tumor samples, we observed expression deregulations in PI3K pathway associated genes cumulated in *PIK3R1* underexpressing cases, such as in *EGFR*, *PTEN* or *AKT3*.

The study searching for *PIK3CA* mutation-associated gene expression signature provides information on the genes and pathways that are transcriptionally deregulated specifically in ERα-positive *PIK3CA*-mutated breast cancers. These deregulated pathways and cellular processes might be implicated in the features of ERα-positive *PIK3CA*-mutated tumors and *vice versa* also affected by the pathway activation caused by the mutations. Identification of the Wnt pathway deregulation in particular might play an important role in the nature of ERα-positive *PIK3CA*-mutated breast cancers since Wnt signaling was

found to crosstalk with PI3K as well as with MAPK pathways (Study 1, Chapter 4.1.1). Connection between Wnt and the PI3K pathways was described at different levels of the signaling cascades (Laplante *et* Sabtini, 2009; Hu *et* Li, 2010; Steelman *et al*, 2011; Khalil *et al*, 2012). Moreover, other deregulated genes and pathways found in ERα-positive *PIK3CA*-mutated tumors might also participate in the nature of these tumors and patient outcome. Metal binding processes and especially iron metabolism including LTF was associated with breast cancer (Study 1). Importantly, the effects of LTF include the ability to induce apoptosis and inhibit proliferation in cancer cells (Gibbons *et al*, 2011; Jomova *et* Valko, 2011). As in our study, Loi et al. (Loi *et al*, 2010) studied *PIK3CA* exon 20 mutation-associated gene expression signature on ER-positive/HER2-negative breast cancers at the mRNA expression level. A study focusing on the protein level might provide additional information on expression deregulations and further support the findings at the mRNA level.

Last but not least, improving assessment methods and gaining new information on potential factors affecting treatment outcome will be useful for future clinical practice. In Study 6 (Chapter 4.4.1), we focused on EGFR status assessment by FISH and IHC in breast cancer samples. EGFR, as a member of HER family of receptors and one of the lapatinib targets, might become a prognostic as well as predictive marker for breast cancer patients, but current studies on this subject are often contradictory (Press *et al*, 2008; Kallel *et al*, 2012; Liu *et al*, 2012; Malorni *et al*, 2012; Olsen *et al*, 2012; Tang *et al*, 2012). The incoherencies in EGFR status role in breast cancer could be due to lack of standardization of assessment. Furthermore, lapatinib treatment response might be influenced not only by the two targeted HER family receptors but also by other factors. As we showed in our Study 5 (Chapter 4.3.2), lapatinib plasma levels can be increased above recommended effective levels which could subsequently cause treatment toxicity. A prospective clinical trial regarding EGFR status using a standardized assessment method and therapeutic drug monitoring of lapatinib could answer such questions. Similar problems with reaching effective plasma levels were also described in other tyrosine kinase inhibitors as is the case of imatinib in chronic myeloid leukemia patients (Takahashi *et* Miura, 2011). Both improved standardized assessment of cancer markers and deeper knowledge of causes leading to treatment side effects should help in clinical practice. Thus, we could establish disease prognosis and treatment prediction more precisely.

This thesis was focused principally at the genomic and mRNA level. Unfortunately, the time frame did not allow confirmation of the results at the protein level. A project assessing the PI3K pathway protein expression in breast cancer samples is ongoing in collaboration with a laboratory using reverse phase protein arrays (RPPA) technique. The study might provide additional information extending further already described data since the analysis is based on assessment of 20 proteins associated with the PI3K pathway in 185 protein extracts from the samples previously included in the patient cohort described in Chapters 2.2.1 and 2.2.2. This project forms basis for the future postdoctoral studies.

For the future research work, there is also a project involving assessment of *PIK3CA* and *PIK3R1* mutations at DNA level in a large serie of triple negative breast cancer cases from the Czech Republic. Considering the extent of the series, this research work might provide insides into the role of PI3K subunit mutations in triple negative breast cancer. Since the majority of the previous studies have been focused on unselected patient cohorts with only small patient subpopulations with triple negative tumors (Li *et al*, 2006; Barbareschi *et al*, 2007; Kalinsky *et al*, 2009; Martin *et al,* 2012), the planned project has the potential to provide new information on the PI3K pathway activation caused by the PI3K subunit mutations in triple negative tumors. Clinical follow-up data are available for the entire patient cohort which will provide reliable survival information.

The crucial application of the knowledge about alterations in breast cancer is the utilization in establishment of disease prognosis, therapy choice and treatment response prediction. Further research describing changes occurring in tumor cells on all levels from genomic alterations to functional signaling will bring the necessary information needed for improvement of treatment approaches. Currently, there are only a limited number of markers used in clinical practice. Besides tumor stage and grade, breast cancer prognosis is established and treatment chosen based on expression of hormonal and HER2 receptors (Baselga, 2011). However, the PI3K pathway reveals promising new markers that should become useful for everyday clinical use. Moreover, therapeutic targeting of multiple signaling levels of the PI3K pathway is being tested in breast cancer patients in clinical trials (Arteaga *et al*, 2011; Hernandez-Aya *et* Gonzalez-Angulo, 2011; Miller *et al*, 2011). Novel markers from the PI3K pathway, especially *PIK3CA* mutations, should help to choose the best treatment combination for a particular patient.

The PI3K pathway targeting in cancer treatment begins with inhibition of receptor tyrosine kinases. Currently, there are HER family inhibitors used in clinical practice to treat breast cancer patients (trastuzumab, lapatinib, pertuzumab). However, many more molecules targeting PI3K signaling on downstream levels are tested. These compounds target PI3K pathway components as p110, AKT or mTOR and promise improved treatment outcome alone or in combination with chemotherapy (Arteaga *et al*, 2011; Hernandez-Aya *et* Gonzalez-Angulo, 2011; Miller *et al,* 2011; Zito *et al*, 2012). The *PIK3CA* gene is particularly important as a potential marker for PI3K pathway targeting. Despite its rather negative predictive effect (i.e. biomarker of drug resistance) on treatment with HER2 inhibitors, *PIK3CA* mutations seem to be a powerful predictive marker for treatment response (i.e. biomarker of drug sensitivity) on downstream PI3K pathway inhibitors such as everolimus (Dave *et al*, 2011, Janku *et al*, 2012; Jensen *et al*, 2012). Other useful prognostic and predictive markers could emerge from PI3K pathway components thanks to new technologies providing detailed insides into alterations in breast cancer. Additionally, improving assessment methods is as important as gaining new facts on tumor deregulations.

6 Summary and Key Words

6.1 English

Results of the presented research projects bring information about several aspects of the PI3K signaling pathway roles in breast cancer development and treatment response. The particular projects covered the subjects connected with the signaling pathway, ranging from the HER family receptors activating the pathway, and PI3K to the downstream levels of signalisation. The prognostic and predictive effect of PI3K deregulation was the central subject of the described research. The decreased expression of *PIK3R1* associated with reduced survival of our patients. A special focus was put on the *PIK3CA* mutations which are common in breast cancer. Whereas the *PIK3CA* mutations act as a good prognostic marker in patients non-treated with the HER2 inhibitors, these mutations predict a negative response to trastuzumab treatment. The described results, furthermore, draw attention to the role of several altered molecular signaling pathways in breast cancer development, especially to the Wnt signaling pathway. The lapatinib plasma levels showing the relevant increase in comparison with the already described efficient steady-state levels were also described in one of the projects. Moreover, various modifications to EGFR status assessment were compared and showed that EGFR FISH and IHC count interpretation depended significantly on method and thresholds used. All these subjects are connected by the PI3K pathway, the need to deepen current knowledge and bring new useful information applicable in future clinical practice.

Key words: breast cancer, PI3K pathway, *PIK3CA*, *PIK3R1*, survival, trastuzumab, lapatinib

6.2 Czech

Prezentované výsledky přinášejí nové informace popisující různé aspekty vlivu signální dráhy PI3K na vývoj charakteristických znaků karcinomu prsu a jeho odpověď na léčbu. Jednotlivé projekty se zabývaly tématy spojenými s touto signální dráhou počínaje receptory rodiny HER, které dráhu aktivují, přes PI3K až k nižším úrovním signalizace. Prognostický a prediktivní efekt deregulace PI3K byl hlavním tématem popisovaných projektů. Snížená exprese *PIK3R1* asociovala s kratším přežitím našich pacientek. Zvláštní pozornost byla věnována mutacím *PIK3CA*, které jsou u karcinomu prsu velice časté.

Zatímco se mutace *PIK3CA* projevují jako dobrý prognostický marker u pacientek neléčených inhibitory HER2, tyto mutace naopak predikují špatnou odpověď na trastuzumab. Popisované výsledky též upozorňují na roli dalších signálních drah v rozvoji karcinomu prsu, především signální dráhy Wnt. V jednom z projektů byly testovány plazmatické hladiny lapatinibu s nálezem jejich významného zvýšení oproti popisovaným účinným hladinám. Bylo provedeno zhodnocení EGFR pomocí IHC a FISH ve vzorcích karcinomu prsu. Použití různých přístupů k interpretaci odečtů ukázalo výrazné rozdíly ve finálních výsledcích. Všechna studovaná témata jsou propojena signální dráhou PI3K a potřebou prohloubit aktuální znalosti o nové užitečné informace využitelné v budoucí klinické praxi.

Klíčová slova: karcinom prsu, dráha PI3K, *PIK3CA*, *PIK3R1*, přežití, trastuzumab, lapatinib

6.3 French

Les résultats des projets actuels apportent une information, sur différents aspects des rôles de la voie PI3K, dans le développement du cancer du sein, et la réponse au traitement. Les projets particuliers couvrent des sujets liés à la voie aux niveaux concernant les récepteurs de la famille HER, activant la voie PI3K, ainsi que PI3K et les effecteurs en découlant. Les effets pronostic et prédictif de la dérégulation de PI3K sont les sujets centraux de la recherche décrite ici. Une baisse d'expression de *PI3KR1* est associée à une survie réduite dans notre cohorte de patients. Une attention particulière a été portée aux mutations de *PIK3CA* communes dans le cancer du sein. Tandis que les mutations de *PIK3CA* agissent comme des marqueurs de bon pronostic chez les patients anti-HER2-naïfs, ces mutations agissent au contraire comme prédicteurs négatifs de la réponse au traitement par trastuzumab. Les résultats décrits mènent un peu plus vers l'implication de plusieurs voies moléculaires altérées, en particulier la voie de signalisation Wnt, dans la tumorigénèse des cancers du sein *PIK3CA* mutés. De plus, nous avons testé les taux de lapatinib plasmatique montrant une augmentation pertinente dans les périodes d'état d'équilibre du traitement. Par ailleurs, nous avons démontré des incohérences dans l'évaluation de l'EGFR et proposé des approches pour l'interprétation des comptages d'immunohistochimie et de FISH. Tous ces sujets sont connectés par la

voie PI3K, et le besoin d'approfondir les connaissances actuelles, et d'apporter de nouvelles informations utiles applicables dans le futur dans les pratiques cliniques.

Mots-clés: cancer du sein, voie PI3K, *PIK3CA*, *PIK3R1*, survie, trastuzumab, lapatinib

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8 Overview of the published manuscripts and abstracts

8.1 Publications associated with the thesis

8.1.1 List of original articles in journals with IF

1. **Cizkova M**, Cizeron-Clairac G, Vacher S, Susini A, Andrieu C, Lidereau R, Bieche I. Gene expression profiling revers new aspects of PIK3CA station in ERalpha-positive breast cancer: major implication of the Wnt signaling pathway. PloS ONE 2010; 5(12): e15647. IF (2010) 4.411. Published.

2. **Cizkova M**, Bouchalova K, Friedecky D, Polynkova A, Janostakova A, Radova L, Cwiertka K, Trojanec R, Zezulova M, Zlevorova M, Hajduch M, Melichar M. High lapatinib plasma levels in breast cancer patients: risk or benefit? Tumori 2012; 98(1): 162-165. IF (2011) 0.606. Published.

3. **Cizkova M**, Susini A, Vacher S, Cizeron-Clairac G, Andrieu C, Driouch K, Fourme E, Lidereau R, Bièche I. PIK3CA mutation impact on survival in breast cancer patients and in ERa, PR and ERBB2-based subgroups. Breast Cancer Res 2012; 14(1): R28. IF (2011) 5.245. Published.

4. **Cizkova M**, Dujaric ME, Lehmann-Che J, Scott V, Tembo O, Asselain B, Pierga JY, Marty M, de Cremoux P, Spyratos F, Bieche I. Outcome impact of *PIK3CA* mutations in HER2-positive breast cancer patients treated with trastuzumab. Br J Cancer, IF (2011) 5.042. Accepted.

5. **Cizkova M**, Vacher S, Meseure D, Trassard M, Susini A, Mlcuchova D, Callens C, Rouleau E, Spyratos F, Lidereau R, Bièche I. *PIK3R1* underexpression is an independent prognostic marker in breast cancer. Submitted in Clin Cancer Res, IF (2011) 7.742.

8.1.2 A review published in a journal with IF

1. Bouchalova K, **Cizkova M**, Cwiertka K, Trojanec R, Friedecky D, Hajduch M. Lapatinib in breast cancer – the predictive significance of HER1 (EGFR), HER2, PTEN and PIK3CA genes and lapatinib plasma level assessment. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2010; 154(4): 281–288. IF (2010) 0.716. Review. Published.

8.1.3 A review published in a journal without IF

1. Bouchalova K, **Cizkova M**, Cwiertka K, Trojanec R, Hajduch M. Triple negative breast cancer – current status and prospective targeted treatment based on HER1 (EGFR), TOP2A and C-MYC gene assessment. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2009; 153(1): 13–18. Review. Published.

8.1.4 List of published abstracts

1. **Čížková M**, Bouchalová K, Cwiertka K, Kolář Z, Trojanec R, Mlčochova S, Furstová J, Radová L, Hajduch M. Triple negative breast cancer – cytogenetic changes assessment of EGFR (HER1) and TOP2A genes with clinical and histopathological data analysis. XXXIII. Brno Oncology Days, April 16th-18th, 2009, Brno, Czech Republic. Edukační sborník (Abstract book); page 88-89.

2. Palková V, **Čížková M**, Trojanec R, Radová L, Mlčochová S, Melichar B, Kolář Z, Hajdúch M. Cytogenetické změny u karcinomu prsu s polyzomií chromozomu 17 a jejich význam pro diagnosticko-léčebnou rozvahu. XXXIII. Brno Oncology Days, April 16th-18th, 2009, Brno, Czech Republic. Edukační sborník (Abstract book); page 43.

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8.1.5 List of oral and poster presentations

1. **Čížková M**, Bouchalová K, Cwiertka K, Kolář Z, Trojanec R, Mlčochova S, Furstová J, Radová L, Hajduch M. Triple negative breast cancer – cytogenetic changes assessment of EGFR (HER1) and TOP2A genes with clinical and histopathological data analysis. XXXIII. Brno Oncology Days, April 16th-18th, 2009, Brno, Czech Republic. Edukační sborník (Abstract book); page 88-89.

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8.2 Other publications

8.2.1 An original article published in a journal with IF

1. Kharaishvili G, **Cizkova M**, Bouchalova K, Mgebrishvili G, Kolar Z, Bouchal J. Collagen triple helix repeat containing 1 protein, periostin and versican in primary and metastatic

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8.2.2 A review published in a journal without IF

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