NEW STRATEGIES FOR GENOTYPING OF *KRAS* AND *BRAF* ONCOMARKERS IN HETEROGENEOUS CLINICAL TUMOUR SAMPLES

PhD thesis

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Olomouc 2015

PALACKÝ UNIVERSITY IN OLOMOUC

FACULTY OF MEDICINE AND DENTISTRY

NEW STRATEGIES FOR GENOTYPING OF *KRAS* AND *BRAF* ONCOMARKERS

IN HETEROGENEOUS CLINICAL TUMOUR SAMPLES

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Olomouc 2015

Declaration: *I declare that the work performed for this project was done by me and the studies which resulted in this thesis were carried out in the Institute of Molecular and Translational Medicine, Department of Pediatrics, Faculty of Medicien and Dentistry, Palacký University and Faculty Hospital in Olomouc, under the supervision of Doc. Mgr. Jiří Drábek, Ph.D.*

ACKNOWLEDGEMENTS

In Olomouc, January 12, 2015   Mgr. Sylwia Jančík

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CONTENT

Theoretical Part

1.Introduction

Mortality rate among cancer patients remains one of the important public health problems in Europe and World. The number of cancer cases increases every year due to overall longer lifespan of general population, exposure to pollution and smoking, bad eating habits, exposure to specific viruses which were shown to be linked to cancerogenesis, insufficient preventive screening and ineffective intervention. The database summarizing data regarding 25 different types of cancer in 40 European Union countries in year 2008 pinpoints that there are about 3.2 million reported cancer cases and 1.7 cancer deaths per year `(Ferlay, 2010)`.

The Czech Republic has been ranked among countries with highest incidence of cancer in Europe. According to the Czech National Cancer Registry (CNCR) which contains information about all cancer diagnoses collected during the last 30 years, there was estimated that more than 27,000 persons in the Czech Republic die of cancer each year (about 263 deaths per population of 100,000). The mortality rates are stabilized; however, the incidence of primary tumours continues to increase. The most frequently diagnosed types of cancer were: colorectal cancer, lung cancer, breast cancer, and prostate cancer, see www.svod.cz. Although some types of cancers get diagnosed at earlier stage than before, early diagnosis in general is still insufficient in the Czech Republic `(Dusek, 2010)`. Improved methods of early diagnosis, novel biomarkers and novel screening methods are sought to improve cancer treatment success.

1.1 Cancer biomarkers

According to the National Cancer Institute (NCI), a biomarker has been defined as *"a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease. A biomarker may be used to see how well the body responds to a treatment for a disease or condition"* (**NCI Dictionary of Cancer Terms. National Cancer Institute http://www.cancer.gov/dictionary?cdrid=45618**).

Cancer biomarkers, also called molecular markers and signature molecules, are helpful during the process of assessment of cancer staging and diagnosis of a cancer patient. They are also helpful in making adequate prognosis regarding disease progression, cancer patient survival, and prediction of response to anticancer drugs. Biomarkers help physicians to select patients who are more likely to benefit from personalized medical therapy `(Madu, 2010; Mattos-Arruda, 2011)`.

During the cancer progression, there are interactions among cancer cells, stromal cells, extracellular matrix, and immune system. This process leads to modulation of specific genes

expression, which subsequently can be used as biomarkers. Overall, the biomarkers can be divided into few distinct categories: *genetic* biomarkers which include: a) structural alternations in the gene sequence, such as mutations, insertions, deletions, chromosomal rearrangements, and increase of gene expression specific for cancer epithelium; b) *epigenetic* biomarkers which might results from DNA methylation changes throughout the genome as well as alterations in the post-translational chromatin proteins modifications (Chan, 2012); c) *metabolic* biomarkers include oxidative stress changes in plasma metabolites and the activity of endogenous antioxidant enzymes, and d) *immunological* biomarkers include dynamic changes in inflammatory or lymphocytic cells and their cytokines (Ascierto, 2013).

Prognostic biomarkers help to assess the risk of developing a particular cancer, to determine prognosis and help doctors to decide how invasive the anticancer treatment should be (e.g. low level of tissue inhibitor of metalloprotease-1 -TIMP1- gives a better prognosis to the myeloma patients). Predictive biomarkers enable to forecast the response of patients to cancer drug/s or treatment/s (e.g. metastatic colorectal patients with aberrant *RAS* are not recommended for therapy with panitumumab monoclonal antibodies or only breast cancer patients with human epidermal growth factor 2 -HER2- overexpression are recommended to monoclonal antibodies therapy with Herceptin (Thibault, 2013; van Krieken, 2008).

Diagnostic biomarkers help to diagnose a particular type of cancer when pathologists are unable to identify the specific type of cancer by immunohistochemistry, and recurrence biomarkers help to predict if cancer is likely to regress after treatment. For example an increase in the expression of breast cancer anti-estrogen resistance 1 gene - BCAR1 - suggests possibility of recurrence of prostate cancer following radical prostatectomy; Oncotype DX® breast cancer assay screens several genes for mutations within breast tumor specimens and quantitatively indicates the probability of recurrence of that cancer (Fromont, 2012; Varga, 2013).

Pharmacodynamics and pharmacokinetics biomarkers help determine the most effective dosage of drug or therapy needed for specific patient. For example, testing patients' thiopurine methyl-transferase (TPMT) gene should be mandatory for chemotherapeutic drugs which are known to utilize mercaptopurine pathway, since the patients with the mutation in the TPMT gene should bebe treated with lower dose of a chemotherapeutic drugs to prevent a fatal decrease in while blood cells (Dorababu, 2012).

1.2 EGFR signal induced pathways

Epidermal Growth Factor Receptor (EGFR) is the cell surface receptor with signal activated by binding of specific EGF ligands outside cell. It elicits downstream signaling activation by several other proteins inside cell, transferring signal into cell nucleus. EGFR signal is transduced by RAS/RAF/MEK/ERK pathway and by PI3K/AKT pathway. These two signaling pathways play a very important role in the regulation of fundamental cellular processes including: proliferation, differentiation, and cell survival/cell death. Aberrant regulation of the genes engaged in these

two cascades may result in the abnormal increase of the cell proliferation and survival, and consequently might become pivotal during carcinogenesis, cancer progression, and metastasis. (McCubrey, 2006; Roberts, 2007).

Figure 1. EGFR pathways
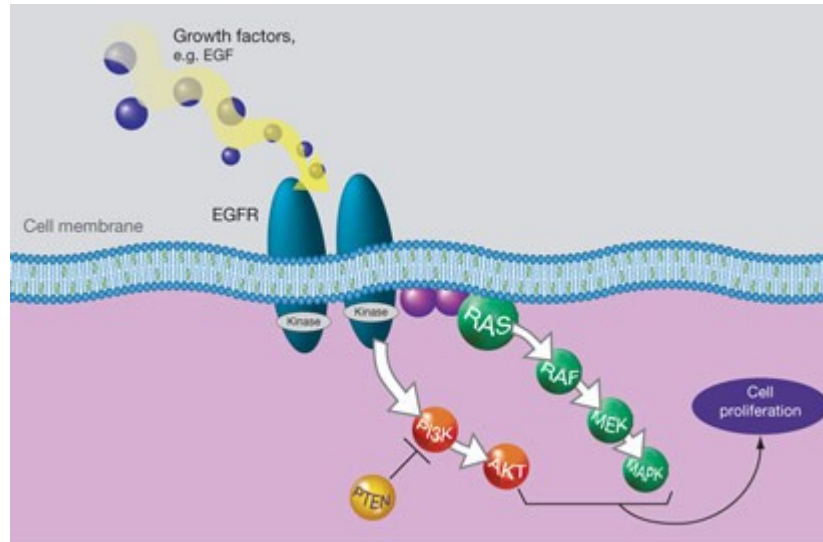
Figure adapted from:

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1.2.1 EGFR/RAS/RAF/MEK/ERK signaling pathway

EGFR/RAS/RAF/MEK/ERK pathway (also known as mitogen activated protein kinase pathway (MAPK) or extracellular signal-regulated kinase cascade (ERK)) transduces signal from the cell membrane to the nucleus, thus affecting activity of transcription machinery.

Her1/erbB1/EGFR (Epidermal Growth Factor Receptor), first protein of the kinase cascade, is a 170 kDa transmembrane glycoprotein. The gene encoding this protein is located on chromosome 7p. EGFR is member of structurally related receptor family which also includes: Her2/erbB2/Her2neu, Her3/erbB3, and Her4/erbB4. All these receptors consist of main extracellular ligand-binding domain, hydrophobic transmembrane domain, and intracellular catalytic domain containing ATP binding site and enzymatic-tyrosine kinase activity involved in the signal transduction. Only Her3 does not have intrinsic tyrosine kinase activity, whereas Her2 receptor is constitutively activated and no ligand is required for its activation. Many of cellular ligands can activate EGFR. The EGFR ligands include EGF, TGF-α, epiregulin, amphiregulin, betacellulin, and others (Figure 2). Ligand binding to EGFR receptor induces conformational

change of the EGFR receptor and promotes its dimerization with itself to form homodimer or with other member of HER family (HER2, HER3, or HER4) to form heterodimer, subsequently leading to receptor autophosphorylation at its tyrosine residues.

Figure 2. Ligands binding to the EGFR family of receptors
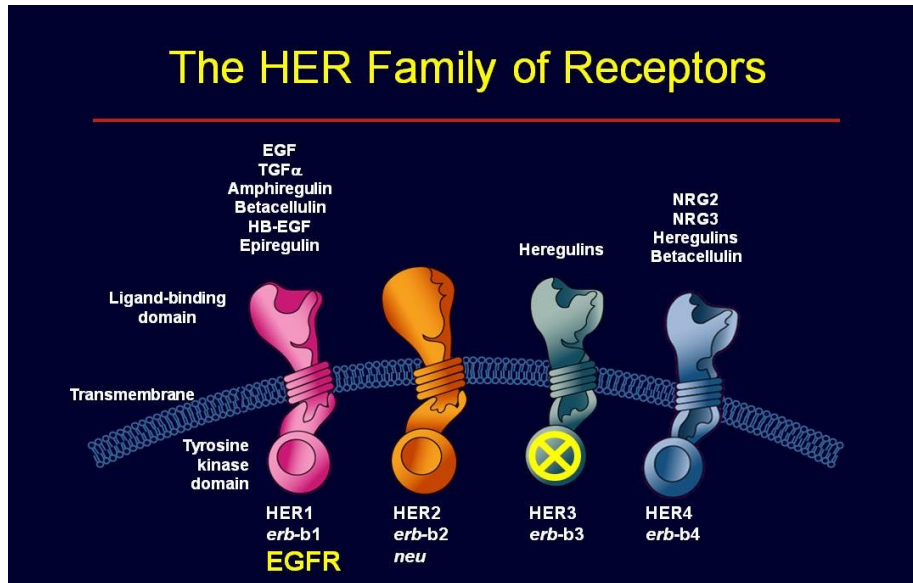
Figure adapted from:

Rowinsky. Annu Rev Med; 2004; 55:433-57.`(Rowinsky, 2004)`

Another protein of the EGFR/ERK pathway, GRB2 (Growth Factor Receptor Bound Protein-2, GRB2 adaptor protein) using SH2 domain binds to the phosphorylated tyrosine residues of the EGFR and using SH3 domain creates complex with another cytoplasmatic protein, guanine nucleotide exchange factor SOS (Son of Sevenless). The GRB2-SOS complex can bind directly to the active residue of EGFR receptor, or indirectly through Src protein (each dimeric receptor complex initiates distinct signaling by recruiting different Src homology SH2 domain). Activated SOS in this complex promotes activation of transduction of small G protein RAS by exchanging its activity from inactive state with GDP to activated state with GTP (**Fig**.**2**). GTPase-activating proteins (GAPs) that accelerate intrinsic KRAS GTPase activity help KRAS turn off and form again inactive GTP state.

RAS family includes KRAS (KRAS - Ki-ras2 Kristen Rat Sarcoma Viral Oncogene Homologue), HRAS (Harvey-Ras), and NRAS (Neuroblastoma-Ras). *KRAS* gene is located on short arm of the 12 chromosome and codes for a 21 kDa cytoplasmatic protein localised on the inner surface of the plasma membrane downstream from the EGFR.

Activated RAS activates serine/threonine kinase cascade, including RAF (MAPKKK), which promotes phosphorylation and activation of another serine/threonine kinase MEK1/2 (MAPKK) and then ERK1/2 kinase (MAPK). That protein kinase downstream pathway can activate several transcription factors like c-myc, c-fos, AP-1, and Elk-1 that promote gene expression responsible for cell cycle, proliferation, and cytoskeleton organisation (e.g. microtubule association protein, MAP).

Figure 3. KRAS molecule transducing signal
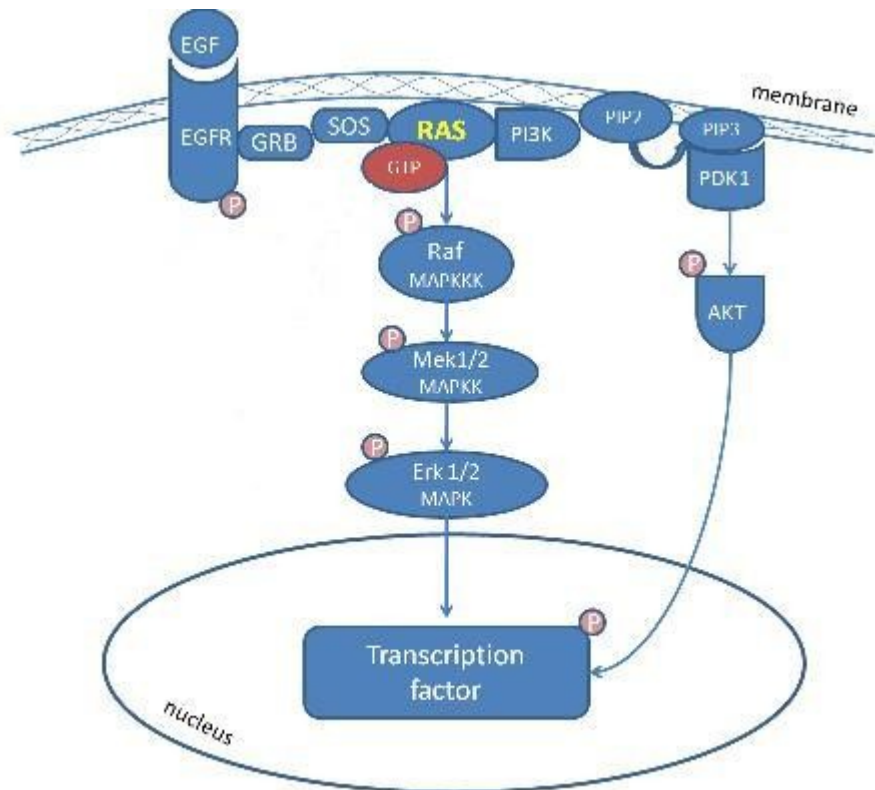
Figure adapted from:

,,Clinical Relevance of KRAS in Human Cancers''
Jančík S, Drábek J, Radzioch D, Hajdúch M - J. Biomed. Biotechnol. (2010) `(Jancik, 2010)`

Of course the MAPK-EGFR/ERK signaling downstream cascade of kinase activation is more complicated and does not represent only a simple linear process. It is also affected by activation and interactions with others signaling pathways like: PI3K/AKT pathway, JAK/STAT3 pathway, and/or SAPK/JNK pathway (Kumar, 2010; Recchia, 2009; Steelman, 2008).

1.3 Biomarkers of EGFR/ERK/MEK pathway

Currently, commonly mutated genes which regulate or constitute EGFR/MEK/ERK pathway are undergoing extensive research to dissect specific regulatory mechanisms in the cancer cells. These investigations may also result in generation of useful information for predicting of response efficiency to both standard chemotherapies and novel selectively targeting therapies, as well as prognostic biomarkers suggesting potential clinical outcomes such us: OS (overall survival), PFS (progression free survival), and RR (response rate).

1.3.1 EGFR

Figure 5. EGFR molecule
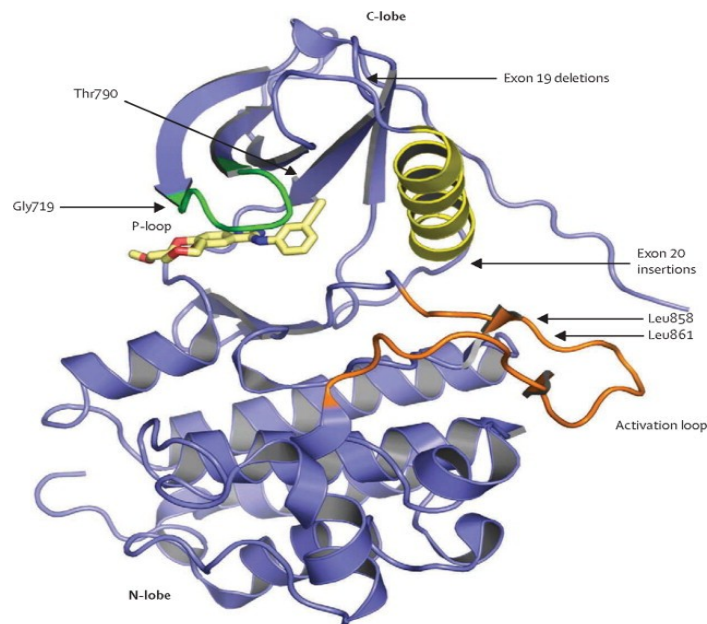
Figure adapted from:

"EGFR exon 20 insertion mutations in non-small-cell lung cancer: preclinical data and clinical implications." Yasuda H, Kobayashi S, Costa DB.-Lancet Oncol (2012)(Yasuda, 2012).

EGFR-Epidermal Growth Factor Receptor also known as HER1 or c-erbB1 is generally expressed in normal tissue at low level and its expression is important in the regulation of the cell proliferation and survival. An increase of the *EGFR* gene expression may result from the gene copies amplification, overproduction of EGFR ligand(s), or gene activation by mutation, and these processes were shown to induce malignant transformation (Liang, 2010).

EGFR is frequently overexpressed in many human cancers including hormone sensitive tumors such as breast cancer (15-90 %), prostate cancer (40-80 %), ovarian cancer (35-70 %), and also in tumors that are not hormone-dependent such as head and neck cancer (SCCHN) (80-100 %), colorectal cancer (CRC) (25-70%), glioma (40-60%), pancreatic cancer (30-90%), esophagial cancer (40-80%), and bladder cancer (30-40%)(Harari, 2004). Appearance of a mutant or amplified form of *EGFR* has been also linked with genesis of lung tumors, especially NSCLC (40-80%). An increase in EGFR activation is found in about 40 % of adenocarcinoma, 30 % of mixed adenosquamous carcinoma and about 5 % of large-cell or squamous carcinoma(Sanders, 2010).

Mutations in tyrosine kinase domain coding by exons 18-21 lead to constitutive increase in the activation of the EGFR (Sahoo, 2011). These mutations within catalytic domain are primarily responsible for ATP binding inside ATP-binding pocket and include point mutations, deletions, and insertions. The most frequently found mutations (about 90 % of all described alternations), are the substitution of leucine by arginine at the codon 858 (L858R) in the exon 21 (about 40 %) and deletion in the exon 19 (about 45 % of cases, deleting codons 746-750 Glu-Leu-Arg-Glu–Ala, so called ELREA-deletion). Other less common mutations include eg. G719X in the exon 18 and L861Q in the exon 21, and some insertions in the exon 20 (nearby codons 770-771) (Sugio, 2006; Yatabe, 2007). Tumors containing these types of somatic mutations within the tyrosine kinase domain or *EGFR* amplification are known to be associated with a poor prognosis but also with an increase in sensitivity to treatment which in some cases might result in significant benefit from tyrosine kinase inhibitors therapy and prolonged disease stabilization in patients undergoing this particular treatment protocol (Uramoto, 2006).

In NSCLC, about 10-20 % patients have objective response from tyrosine kinase therapy and molecular analyses identified mutation in the kinase domain affecting residues of the ATP binding pocket in approximately 80 % of responders. *EGFR* mutations in tyrosine kinase domain were found mainly in adenocarcinoma histological subtypes, more frequently in well differentiated tumors; other risk factors included women gender, never smoker status, and Asiatic origin (Sasaki, 2006; Shigematsu, 2005; Sugio, 2006).

Mutation T790M appears in the exon 20 encoding tyrosine kinase domain. This mutant aberration is often referred to as a secondary mutation, because it is mostly elicited during the course of tyrosine kinase targeted therapy. T790M mutation has been shown to increase the affinity to ATP in the ATP-binding pocket, thus conferring resistance to EGFR-TKIs (such as

gefitinib and erlotinib) by blocking their binding to this domain (Janne, 2008; Pao, 2005; Pao, 2010).

Furthermore, the expression of rearranged EGFRvIII form was also documented in many human tumors. Rearrangement occurs because of a deletion of exons 2-7 resulting in the generation of novel glycine residue within extracellular domain. This aberration results in a disruption of extracellular domain of the receptor and constitutive activation of the protein even in the absence of a ligand binding. EGFRvIII form has been rarely observed in normal tissue. In tumor tissue it has been associated with increased cell survival, and resistance not only to biological therapies (i.e. cetuximab) but also to standard chemotherapy (i.e. cisplatin) (Dreier, 2012; Gupta, 2010; Kuan, 2000).

1.3.2 KRAS

Figure 6. KRAS molecule
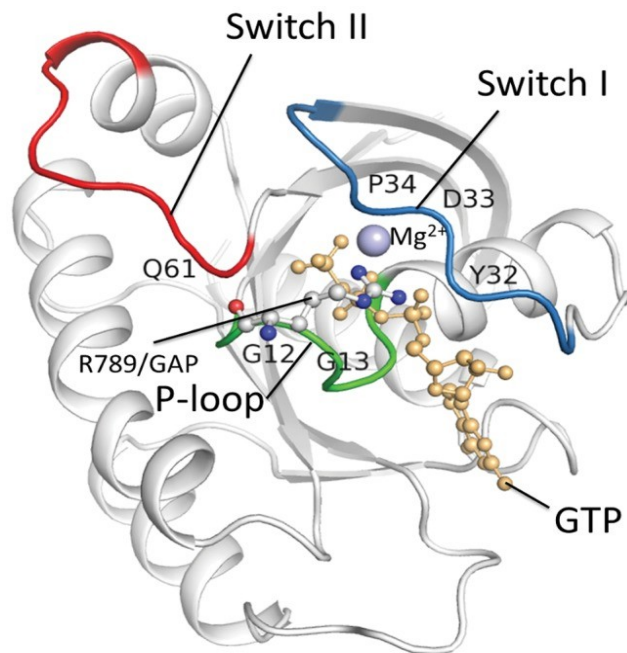
Figure adapted from:

"Computational analysis of KRAS mutations: implication for different effects on the KRAS p.G12D and p.G13D mutations."

Chen CC, Er TK, Liu YY, Hwang YK, Barrio MJ, Rodrigo M, Garcia-Toro E, Herreros Villanueva M.- PloS One. (2013)(Chen, 2013).

KRAS-Ki-ras2 Kristen Rat Sarcoma Viral Oncogene Homolog in normal cells, small GTPases protein KRAS acts as an intracellular signal transductor that transports signals from the cell surface to the nucleus through the cytoplasm. Activation of KRAS triggers mainly the activation of Mitogen Activation Protein Kinase (MAPK) pathway, including RAF, MEK, and ERK serine/threonine kinases but also play a role in the regulation of cell cycle through the activation of numerous downstream pathways (PI3K/AKT/mTOR, RAL, and PKC pathways) essential for the proliferation and survival of normal cells.(Kolch, 2000; Mansi, 2011; McCubrey, 2007).

Mutated oncogenic form of KRAS affecting canonical mitogenic cascades has been found in many cancers. It is mainly common in pancreatic carcinoma (~ 90 %) (Yachida, 2012), colon carcinomas (~ 40 %)(Vaughn, 2011), and lung carcinomas, especially NSCLC (15-30 %). Activating mutation by single nucleotide change leading to aminoacid change in *KRAS* happens mainly in the codon 12 and 13 of exon 2 (95% of all mutations). Less frequent mutations occur in codons 59, 61, 117, and 146 (Hinoda, 2011; Loupakis, 2009).

Mutation impairs the ability of the KRAS protein to switch between active GTP state and inactive GDP state by reducing GTPase activity and affinity for GTPase activating proteins (Andreyev, 2001).

Figure 7. KRAS GTPase activity
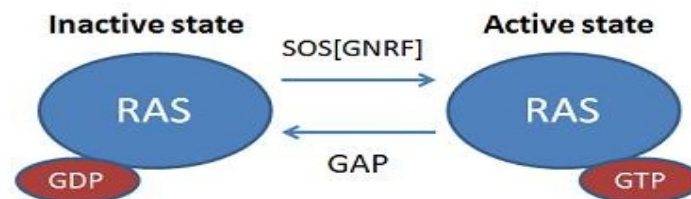
Figure adapted from:

"Clinical Relevance of KRAS in Human Cancers"
Jancík S, Drábek J, Radzioch D, Hajdúch M - J. Biomed. Biotechnol (2010) (Jancik, 2010).

Many reports have shown that *KRAS* mutations seem to be mutually exclusive with *EGFR* and *BRAF* mutations (Agarwal, 2008; Kosaka, 2004; Shigematsu, 2005).

In lung cancer, the mutated form of *KRAS* is more predominantly associated with the NSCLC adenocarcinoma (about 25 %) than other histological types (about 5 %) and it is mainly associated with history of smoking. However, the absence of history of tobacco use does not eliminate definitively the possibility of *KRAS* mutation in lung cancer patient (Riely, 2008; Sugio, 2006).

Based on meta-analysis of 22 studies (up to 2009 year) including 1470 NSCLC patients, *KRAS* mutations were identified in 231 cases (16%). These studies showed that current and former smokers had frequency of *KRAS* mutation of 25% and never smokers had the frequency of only 6%. Mutation was also more common in adenocarcinoma type than in other histological types (26% vs 16%). There were no significant differences in frequency of *KRAS* mutation between men (22%) and women (20%); however, there was a difference in frequency of *KRAS* mutation between ethnic groups: higher frequency of *KRAS* mutations is seen in African Americans and lower frequency in Asians (Hunt, 2002; Mao, 2010).

In a large RASCAL II study (The Kristen ras in-colorectal-cancer collaborative group) which included enrolment of 4268 colorectal cancer patients from 21 countries, there had been shown that different mutations can have different association with biological behaviour of cancer. *KRAS* mutation glycine to valine (G12V), which is found under 10% of colorectal patients, was associated with an increased risk of relapse and death (Andreyev, 2001).

Other study suggests that mutations in codon 13 (G13D) have similar behavior as *KRAS* wild-type. Patients with metastatic colorectal cancer carrying G13D type of mutation appear to benefit more from anti-EGFR therapy with monoclonal antibodies (cetuximab) than patients who carry mutations in codon 12. If confirmed, such surprising behaviour may be explained by finding that GTP-binding pocket in the *KRAS* wildtype and G13D mutation is more closed than in different type of mutation with more aggressive behavior. However, the role of this mutations still need to be more investigated (Chen, 2013; Mao, 2013; Tejpar, 2012).

1.3.2.1 Clinical significance of KRAS in Colorectal Cancer

In recent years, *KRAS* mutation status has been recognized as a predictive marker of resistance to EGFR treatment with tyrosine kinase inhibitors (TKs) and monoclonal antibodies (mAb) (Falchook, 2013). Mutation presence seemed to be associated with diminished response to

small molecular weight EGFR inhibitors, such as gefitinib (Iressa) and erlotinib (Tarceva) in NSCLC and to diminished response to therapies with humanized monoclonal antibody cetuximab (Erbitux) and panitumumab (Vectibix) in patients with advance or metastatic colorectal carcinoma (mCRC) who had progressed after standard chemotherapy. U.S. recommendation and guidelines for testing *KRAS* mutations have been issued by the American Society of Clinical Oncology (ASCO) and by the National Comprehensive Cancer Network (NCCN) after the results of large study phase III CRYSTAL and phase II OPUS were presented in May, 2008 in Chicago, USA.

These studies have demonstrated that mCRC patients in first line treatment with cetuximab added to FOLFIRI (5-fluorouracil, leucovorin, and irinotecan) or FOLFOX (5-fluorouracil, leucovorin, and oxaliplatin) had better overall response (OR) and progression free survival (PFS) if they had wildtype genotype at the *KRAS* locus than those with a mutation in *KRAS*.

In phase II OPUS study, in group of patients with *KRAS* wildtype, median PFS was 8.3 months for patients receiving FOLFOX plus cetuximab and 7.2 months for patients receiving only FOLFOX, median OS was 22 and 18.5 months, and median OR was 57% vs. 34% accordingly(Cartwright, 2012).

CRYSTAL study provided retrospective KRAS data in archived tumor tissues obtained from 1198 metastatic colorectal cancer patients with EGFR positive tumour; KRAS gene status was determined in 1063 patients. Mutated *KRAS* was detected in 36% of tumour patients, and 64% patients were wildtype. In *KRAS* wildtype group PFS was 9.9 months for patients who receive FOLFIRI and cetuximab, and 8.4 months for patients who get only FOLFIRI, OS was 23.5 vs. 20.0 months, and RR was 57.3% vs. 39.7%. In *KRAS* mutated group there was no statistical difference in efficacy parameters between treated arms of the study (Cartwright, 2012; Van, 2011).

Since 2009, *KRAS* gene has been approved for screening as an oncomarker. The testing for wildtype status of KRAS has become a gold *standard of care* in routine clinical practice regarding the use of monoclonal antibodies (cetuximab or panitumumab) therapy in metastatic colorectal cancer (mCRC) (Morton, 2009).

Dahabreh *at al*. (2011) has summarized the results of many published studies assessing whether *KRAS* mutation status have modified effect on treatments with anti-EGFR monoclonal antibodies. Based on the results of these studies it has been concluded that OS in *KRAS* mutated patients was shorter compared to patients with wildtype status of KRAS, ranging from 4.4 to 17.5 months for patients with mutation, and from 6.6 to 24.9 months for patients with wildtype status of KRAS; median PFS ranging from 1.3 to 7.6 months and from 1.4 to 12.3 months, respectively(Dahabreh, 2011b).

In colorectal cancer, response and clinical benefits from monoclonal antibodies are observed in only part of patients with *KRAS* wildtype (tested in codons 12 and 13). These results indicate that absence of *KRAS* mutation in exon 2 not always guarantees an improved response to EGFR target therapies and additional mechanisms of EGFR-pathway' activation exists, potentially

BRAF, *NRAS*, and *PI3KCA* mutations or loss of *PTEN* gene expression (Bando, 2012; Mao, 2012; Sood, 2012).

Indeed, in August 2013 Direct Healthcare Communication written by Amgen highlighted the importance of monitoring mutations not only in the *KRAS* but also in the *NRAS* gene. The reports points out that the sequence of exon 2 including codons 12 and 13, exon 3 including codons 59 and 61, and exon 4 including codons 117 and 146, should be assessed before treatment with panitumumab (Vectibix). Study called PRIME (ClinicalTrials.gov number NCT00364013) showed that patients with wildtype *KRAS* and *NRAS* in these particular codons, have advantage both in PFS and OS(Douillard, 2013).

1.3.2.2 Clinical significance of KRAS in NSCLC

In NSCLC patients, predicting interaction between mutated *KRAS* and erlotinib and gefitinib therapy resistance is not so clear. Results of large multicentric phase III trials, including INTEREST, SATURN, and BR21 did not show any predictive value of *KRAS* status assessment, and did not confirm hypothesis that mutated *KRAS* invalidates the target therapy with small molecules gefitinib or erlotinib(Brugger, 2011; Douillard, 2010; Roberts, 2010; Zhu, 2008).

However, the results from different studies showed that the status of *KRAS* has a predictive value. In two big meta-analyses by Linardou *et al*. 2008 and Mao *et al*. 2010 based on 17 studies (including 1008 patients) and 22 studies (including 1470 patients) in NSCLC, *KRAS* mutation positive status was found in 16.3% (165 patients) and 17.6% (235 patients), respectively. Occurrence of mutations was generally associated with lack of response to TKIs therapy, only 3% percent *KRAS* mutation positive patients were responders vs. 26% wildtype (Linardou, 2008; Mao, 2010).

Overall, prognostic significance of mutations in *KRAS* gene on clinical outcome NSCLC such as disease free survival or/and overall survival has been reported in a number of publications with conflicting results. Some but not all studies demonstrated that the active *KRAS* mutation was associated with tumor progression and poor survival.

For example, the results generated from phase III TRIBUTE trials with the group of advanced NSCLC, *KRAS* mutated patients after treatment with erlotinib (Tarceva) plus chemotherapy (carboplatin and paclitaxel) indicated that *KRAS* mutated patients showed poorer clinical outcome including decreased time to progression (3.4 month vs. 5.3 month) and survival (4.4 month vs. 12.1 month) compared to non-mutated patients (Eberhard, 2005). The results of large study SATURN with erlotinib in unresectable NSCLC have not confirmed *KRAS* predictive value; however, this study concluded that *KRAS* mutated status was a significant negative prognostic factor for PFS (Brugger, 2011).

On the contrary, some studies showed that mutated *KRAS* was not associated with poor prognosis and pathological stage of disease: the results of the large trial INTEREST III which investigated efficacy of gefitinib (Iressa) versus docetaxel after prior-chemotherapy did not show differences between overall survival and time to progression in group with *KRAS* mutation indicating that *KRAS* is not prognostic biomarker for this group of NSCLC patients (Douillard, 2010). No differences in survival outcome between mutated and wildtype *KRAS* group was also observed in a group restricted I to III stage NSCLC patients (Ragusa, 2013).

Results of study published by Wang and colleagues on *KRAS* mutation analysis using serum and/or plasma samples from advanced NSCLC patients showed the predictive value (5% RR for *KRAS* mutated patients vs. 29% for *KRAS* wildtype) and prognostic value (median PFS for *KRAS* mutated status 2.5 months vs. 8.8 months for wildtype *KRAS* status) to EGFR-TKIs treatment results (Wang, 2010). On the contrary, Kim and colleagues were unable to confirm any importance of *KRAS* testing using serum plasma from cancer patients. However, lower frequencies of *KRAS* mutation (in comparison to colorectal cancer) and poorer availability of tumor tissue in pairs with circulating tumor DNA in advanced NSCLC have made the statistical assessments of these associations for patients with lung cancer more difficult (Kim, 2013). Promising treatment results of small molecule drug selumetinib (inhibitor of MEK1/2) in combination with standard chemotherapy with docetaxel compared to docetaxel alone, currently gave a new perspective to advanced NSCLC patients who have mutation in *KRAS* gene; however, more clinical investigations are needed to validate these findings (Paolo, 2013).

1.3.3 BRAF

Figure 8. BRAF molecule
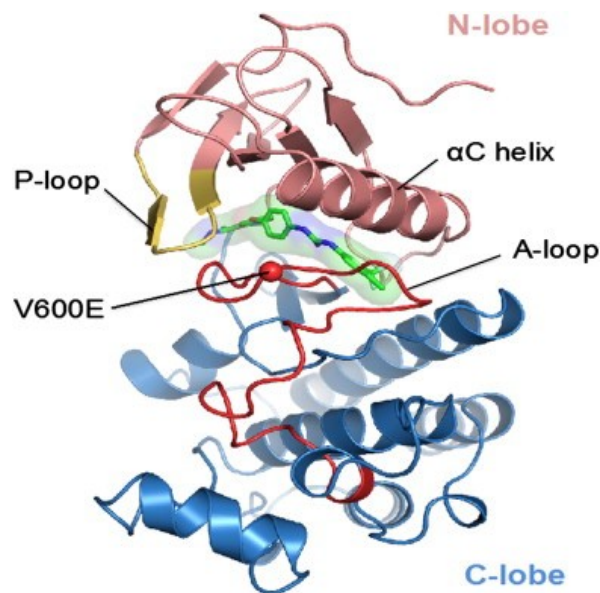
Figure adapted from:

,,Characteristics and prevalence of KRAS, BRAF, and PIK3CA mutations in colorectal cancer by high-resolution melting analysis in Taiwanese population''

Li-Ling Hsieh, Tze-Kiong Er, Chih-Chieh Chen, Jan-Sing Hsieh, Jan-Growth Chang, Ta-Chih Liu- Clinica Chimica Acta (2012) (Hsieh, 2012).

BRAF-Murine Sarcoma Viral Oncogene Homolog is one of three members of serine/threonine RAF - kinase family that acts in the MAPK pathway and gets upregulated by binding RAS. *BRAF* mutations were found in almost all hairy cell leukemia cases (Arcaini, 2012), 50-80% of melanomas (Huang, 2013), ~ 45% of papillary thyroid carcinomas (Kopczynska, 2006), ~ 40% hepatocellural carcinoma (Huang, 2013), 30-60% ovarian carcinoma (Pakneshan, 2013; Singer, 2003), 8-13 % colorectal carcinoma (Borras, 2011; Mao, 2011), and 7-16% gliomas (Myung, 2012;

Schindler, 2011). The vast majority of somatic mutations were detected in the exon 15 coding kinase domain and more than 80 % of the mutations were a single substitution of adenine to thymine at nucleotide position 1799, converting valine to a glutamic acid at amino acid position 600 (V600E). The activating mutation disrupts phosphorylation of serine/threonine residue of this kinase within the activation loop of BRAF, resulting in appearance of the protein with higher kinase activity and subsequently increasing activity of the entire ERK-pathway (Mercer, 2003; Pratilas, 2007). As stated above, mutated forms of *EGFR*, *BRAF*, and *KRAS* are rarely identified together (Singer, 2003). BRAF inhibitors (eg. vemurafenib, dabrafenib, and/or sorafenib) have been developed to treat various human cancers including melanoma, kidney carcinoma, liver carcinoma, and currently have been also investigated for thyroid carcinoma and brain carcinoma (Al-Marrawi, 2013; Arcaini, 2012) (Klinac, 2013; Tang, 2010).

1.3.3.1 Clinical significance of BRAF in colorectal cancer

The reports from randomized clinical trials in advanced CRC cancer patients treated with oxaliplatin or irinotecan showed that *BRAF* mutation status is associated with poorer survival compared to patients with wildtype *BRAF* status, but was not considered to be a predictive factor regarding the efficacy of these therapies (Richman, 2009; Yuan, 2013). In mCRC patients with *KRAS* wildtype treated with cetuximab in combination with chemotherapy, or with chemotherapy alone, mutation in *BRAF* also appears to be negative prognostic biomarker. A retrospective study analyzed, if additional biomarkers can be used to further focus selection of patients who are wildtype *KRAS*. In the CRYSTAL trial on mCRC patients, *BRAF* mutations were identified in 59 of 625 (9.4%) *KRAS* wildtype patients. Patients with *BRAF* mutations had poorer survival ratio after treatment with cetuximab compared to FOLFIRI alone. Additionally, in the cohort *BRAF*/*KRAS* wildtype, improvement of OS (25.1 vs. 21.6 moths) and PFS (10.9 vs. 8.8 moths) was observed (Cartwright, 2012).

CRYSTAL and OPUS studies analyzed whether there is difference between cetuximab in combination with FOLFIRI and FOLFIRI alone as a first line treatment of mCRC. They demonstrated that patients with *KRAS* wildtype tumours harbouring *BRAF* mutations had poorer prognosis across all outcomes compared with those with *KRAS* and *BRAF* wildtype samples in both treatment protocols tested. When cetuximab in combination plus FOLFIRI was compared to FOLFIRI alone, clinical outcomes were better in cetuximab plus FOLFIRI arm (Bokemeyer, 2012).

1.4 Targeted therapies

Novel molecular strategies designed to target specific molecules affecting regulatory mechanisms involved in the control of cancer cell proliferation enable to improve cancer therapy efficiency compared to conventional chemotherapy and/or radiotherapy protocols. Cellular processes governing proteins like EGFR are often deregulated by gene amplification or mutation, leading to cancer. However, during the last few years this perturbation in genes is being specifically targeted in attempt to develop new personalized cancer treatments. The aim of targeted therapies is inhibition and down-regulation or inactivation of overactive proteins responsible for triggering of aberrant cellular pathways.

1.4.1 Anti-EGFR targeted therapies

EGFR –MEK1/2-ERK1/2 kinase pathway became a major focus of investigations attempting to identify specific tumor biomarkers which could be useful in increasing effectiveness of new cancer therapeutics. Mutations and amplifications in the *EGFR* gene represent important hallmarks of lung cancer, colorectal cancer, pancreatic cancer, and head and neck cancer, so inhibition of EGFR pathway is one of the targets for effective anticancer therapies (Elferink, 2011; Markman, 2010; Marks, 2008; Viel, 2011). Currently two distinct therapeutic EGFR inhibitors have been developed: 1) monoclonal antibodies (mAbs) that target the extracellular domain of the EGFR receptor 2) small molecule tyrosine kinase inhibitors (TKIs) targeting intracellular catalytic domain of the receptor (Harari, 2004). Both classes of agents show antitumor activity, both *in vivo*, in human cancer cells as well as *in vitro* using animal models and cell lines (Prewett, 2011). Thereby, various medical protocols are under investigation to test the most efficient strategy, including a targeted monotherapy, targeted therapy with combination with cytotoxic agent, and dual therapy to overcome resistance in secondary EGFR-mutation positive patients (i.e. with MET amplifications or T790M mutation) (Lin, 2011; Murphy, 2011; Nguyen, 2009; Okabe, 2009).

1.4.1.1 Monoclonal antibodies-mAbs

First and the best known monoclonal antibodies targeting EGFR are called cetuximab and panitumumab. This class of molecular inhibitors serves as an efficient therapy in a group of patients who display activating *EGFR* gene mutation or a gene amplification which increases EGFR protein levels.

Figure 1. Schema of Monoclonal antibodies targeted therapy
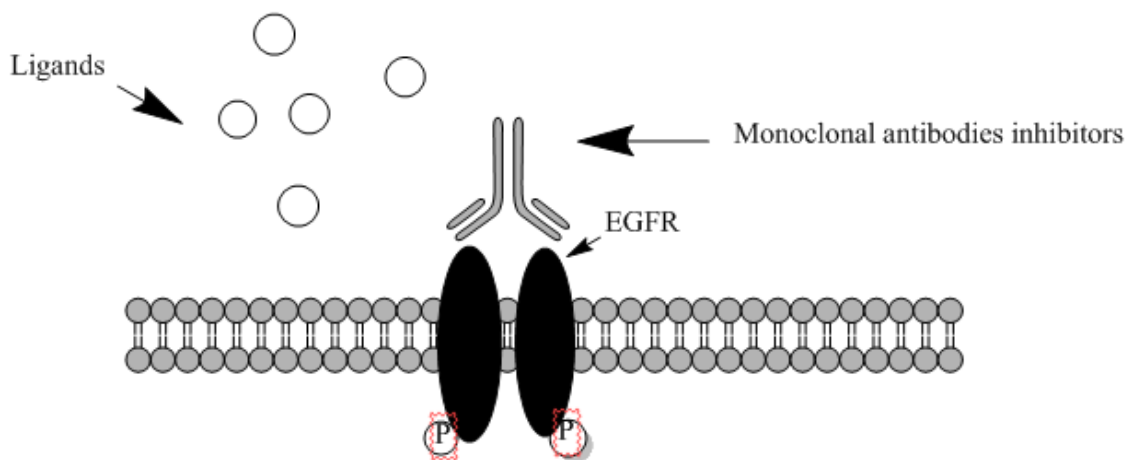
Figure created by author.

Cetuximab (IMC-C225/ Erbitux) made by ImClone Systems, Inc. is the recombinant humanized monoclonal IgG1 antibody able to bind to EGFR, thus inhibiting the binding of the EGF and other ligands to the EGFR (Vincenzi, 2008). Cetuximab humanized chimeric antibody was developed by combining variable region of the mouse antibodies with human immunoglobulin G1 constant region to reduce the possibility of adverse immunological reactions (Ciardiello, 2005). Cetuximab targeting demonstrates enhanced receptor binding to the extracellular domain of EGFR with higher affinity than its endogenous ligand and ability to specifically block EGFR receptor activity by causing receptor internalization and by blocking receptor phosphorylation (Hildebrandt, 2007). Direct inhibition of EGFR tyrosine kinase activity influences a wide range of biological processes including inhibition of tumor cell cycle process, angiogenesis, invasion and metastasis, activation and increase of apoptosis, and synergic cytotoxicity effect with chemotherapy or radiation therapy (Vincenzi, 2008).

In Europe, in February 2004, cetuximab has been approved by European Medicines Agency (EMEA) as a first specific monoclonal antibody targeting EGFR in the cancer patients with

metastatic colorectal cancer (mCRC). Treatment protocol regarding potential application of cetuximab has been accepted by the regulatory agencies in combination with a standard irinotecan-based chemotherapy (FOLFIRI) as well as a monotherapy for patients who were intolerant to irinotecan. Subsequently, this protocol was accepted by the regulatory agencies also for mCRC patients who failed chemotherapy based on oxaliplatin (FOLFOX). Since 2006 Cetuximab have been approved for use in combination with radiation therapy (RT) for treatment of locally or regionally advanced squamous cell carcinoma of the head and neck (SCCHN) or as a single agent for treatment of patients who had recurrent or metastatic carcinoma of SCCHN and had no benefit from platinum-based therapy (Hildebrandt, 2007; Vincenzi, 2010). National Cancer Institute of Canada Clinical Trials Group demonstrated the monoclonal inhibitor EGFR-cetuximab improves OS and PFS in patients with chemotherapy-refractory advanced colorectal cancer patients, particularly in patients with wildtype *KRAS* status (Au, 2009). The recent study published by Di and colleagues also indicated that patients who lack mutations in *KRAS* and also in *NRAS, BRAF, PI3KC,* and *TP53* are likely to benefit the most from the cetuximab therapy(Di, 2013).

Panitumumab (ABX-EGF/Vectibix) Amgen Inc.,Thousand Oaks, CA, is a fully humanized monoclonal IgG2 antibody specifically targeting the EGFR ligand binding domain. Panitumumab was approved in September 2006 and is used to treat metastatic colorectal cancer (mCRC) patients showing disease progression or whose status worsened following prior chemotherapy regimens such us oxaliplatin, fluoropyrimidine, and irinotecan based regimens. Approval of of panitumumab administration was based on multinational, randomized clinical trial (Open-labeled III trial for panitumumab) that enrolled EGFR-expressing mCRC patients. Approval was based on demonstrating that panitumumab treatment made progression free survival (PFS) approximately 50% longer (PFS mean 13.7 weeks for panitumumab vs. 8.5 weeks for best supportive care). However, there was no statistically significant difference in overall survival (OS). Objective response rate for panitumumab was 8% and the median duration of response was 17 weeks. Like in case of cetuximab, the most often observed adverse events consisted of skin rash and dermatologic toxicity (Giusti, 2007; Giusti, 2008). Increase in PFS from panitumumab monotherapy was demonstrated in non-mutated *KRAS* patients group while in *KRAS*-mutated patients no benefit from panitumumab treatment was observed (Weber, 2008). In mCRC patients with wildtype *KRAS* status, panitumumab has been recommended in combination with prior chemotherapy as a first and a second-line of treatment, or as a monotherapy for the treatment of chemotherapy resistant patients(Keating, 2010).

1.4.1.2 Tyrosine kinase inhibitors-TKIs

Another class of EGFR inhibitors are small molecules TKIs gefitinib and erlotinib. Both agents bind to the adenosine triphosphate-binding site of the receptor, inhibiting intracellular tyrosine kinase domain of the receptor. Cancer patients with point mutations (mainly L858R), deletions

(in the 19th exon), insertions in exon 20 and EGFR amplification benefit from gefitinib and erlotinib. In contrast, a mutation in exon 20 (T790M) was shown to lead to resistance to these drugs. Gefitinib and erlotinib are compounds primarily indicated for non-small cell lung cancer (NSCLC).

Figure 2. Schema of the Tyrosine kinase targeted therapy
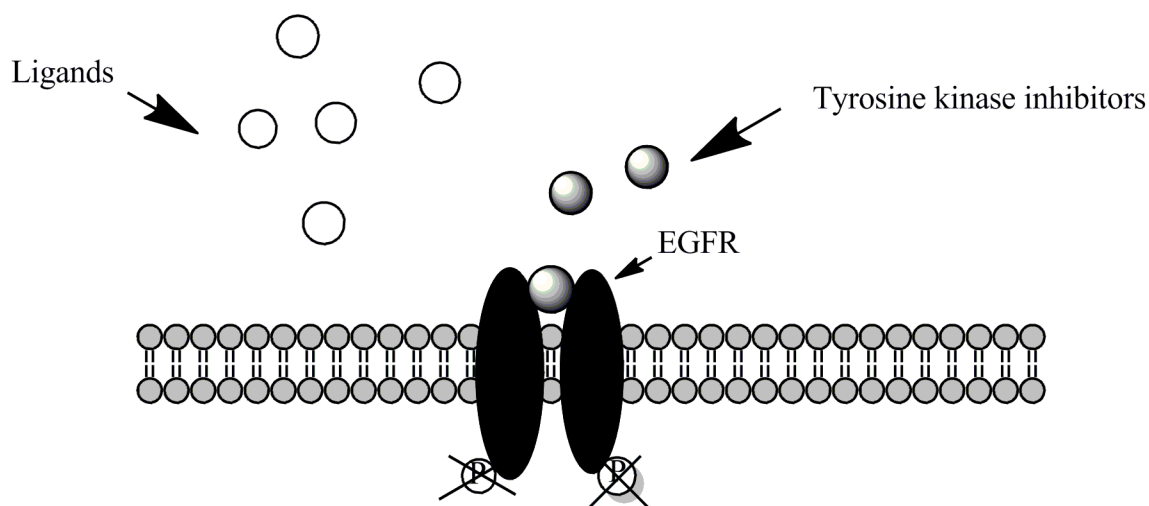
Figure created by author.

Gefitinib (ZD1839/Iressa, marketed by AstraZeneca and Teva) represents the first small molecule shown to act as a selective inhibitor of EGFR targeting the tyrosine kinase domain of the receptor. Gefitinib is orally active, low molecular weight anilinoquinazoline. In 2003 gefitinib was approved by United States Food and Drug Administration as monotherapy for patients with locally advanced or metastatic NSCLC after failure of the basic chemotherapy with platinum and docetaxel (Cohen, 2004).

Phase III trial IPASS IRESSA study which included 1217 patients (enrolled in 87 centers in East Asia) who had never smoked or were former light smokers and who get gefitinib versus carboplatinium and paclitaxel as a first line treatment, demonstrated that gefitinib was more effective and induced significant improvement in progression free survival (PFS) in the clinical trial arm containing patients with sensitizing *EGFR* mutations (Armour, 2010; Brown, 2010). Also the results of the large meta-analysis studies in which advanced or recurrent NSCLC patients treated gefitinib and erlotinib were enrolled showed that increase in *EGFR* gene copy number correlated with improved overall survival (OS) and improvement in the time to

progression (TTP). Gefitinib treated patients had also prolonged OS and improved their quality of live (QOL) (Allegra, 2009; Dahabreh, 2011a). Response to gefitinib is mainly correlated with mutated *EGFR* gene status and clinical features like adenocarcinoma histology, no smoking history, female sex, and Asian ethnicity (Oxnard, 2010; Sanford, 2009).

In July 2009, European Medicines Agency granted marketing authorization for the use of gefitinib, across all lines of treatment for locally advanced or metastatic NSCLC patients with sensitive *EGFR* mutations. Currently gefitinib treatment represents one of the best first-line treatment options for this selected group of patients with molecularly documented mutations in *EGFR* (Gridelli, 2011). Potential benefit of gefitinib therapy has also been investigated in wide range of solid tumor types including lung, head, neck, colon, and breast cancer (Von Pawel J., 2004).

Another anti-EGFR drug **Erlotinib hydrochloride (Tarceva, marketed by Roche)** is a small molecule drug which specifically targets the EGFR tyrosine kinase domain in the same way as gefitinib, by competition with ATP for binding to the adenosine triphosphate (ATP)–binding side of the receptor. In May 2003, Erlotinib was approved as monotherapy by U.S. FDA for the treatment of patients locally advanced or metastatic NSCLC after failure of at least one standard chemotherapy regiment. The most common adverse events were similar as observed in patients treated with gefitinib: skin rash and diarrhea. Currently, skin rash is considered to be a positive indicator of good response on erlotinib treatment(Gridelli, 2010).

In placebo controlled study of advanced NSCLC after the failure of first-line or second-line chemotherapy, erlotinib prolonged survival (6.7 months-erlotinib vs. 4.7 months-placebo group), and the median duration of the response (7.9 months vs. 3.7 months, respectively) (Shepherd, 2005). However, it was proven in many clinical studies that the presence of EGFR activating mutations facilitated the selection of patients and increased benefit from erlotinib/ gefitinib therapy of advanced NSCLC (Pallis, 2013). In OPTIMAL study, in group of EGFR mutation positive patients, erlotinib vs. chemotherapy (gemcitabine/carboplatin) was compared in first line treatment. Erlotinib conferred a significantly better progression free survival (13.1 month for erlotinib arm vs. 4.6 months chemotherapy arm) (Zhou, 2011). Importance of proper patients' selection for treatment was demonstrated on the results of two large studies, TRIBUTE and TALENT. In these studies, NSCLC patients were not selected for treatment based on the aberration in their EGFR expression, and erlotinib treatment was not beneficial when it was combined with standard platinum based chemotherapy (ciplatin and gemcitabine: TALENT, carboplatin and paclitaxel: TRIBUTE) (Gatzemeier, 2005; Herbst, 2005).

In November 2005, erlotinib in combination with gemcitabine was also approved by the U.S. Food and Drug Administration (FDA) as a first line treatment for locally advanced or metastatic pancreatic cancer patients (Burris, III, 2008; Senderowicz, 2007).

1.4.1.3 Other anti-EGFR pathway strategies

Several different anti-EGFR (HER1) targeted strategies have been investigated in various clinical trials because vast majority of patients treated with EGFR inhibitors, such as cetuximab, panitumumab, erlotinib and gefitinib, have eventually developed resistance to these drugs. Therefore, the search for novel drug with superior activity has continued. Lapatinib (GW-572016, Tykerb) is one of the first inhibitors displaying dual activity binding to EGFR (HER1) and HER2, and inhibiting their kinase activity. This drug was approved by Food and Drug Administration in 2007 for the treatment advanced or metastatic breast cancer (Medina, 2008). Another second generation drug called afatinib (BIBW2992, anilino-quinazoline), was shown to be capable of displaying dual activity of irreversibly binding to EGFR (HER1) and HER2, and inhibiting their kinase activity. Afatinib is designed to suppress both the wildtype and the activated forms of EGFR including erlotinib-resistant form of EGFR with T790M mutation and HER2 mutants form. Afatinib has been used to treat advanced solid tumors, particularly NSCLC (Li, 2008; Subramaniam, 2011). Dual activity inhibitors for EGFR/ HER2 are also neratinib (HKI-272) and blockers of EGFR family: pelitinib (EKB-56) and cenertinib (CI-1033) that covalently binds to EGFR 1, 2, and 4 (Majem, 2013).

The other examples of directed tyrosine kinase inhibitors under investigations include matuzumab (EMD 72000), humanized IgG1 monoclonal antibodies. The treatment with matuzumab was shown to prolong survival among the treated patients. Another type of monoclonal antibodies developed by the Ludwig Institute called necitumumab (IMC-11 F8) which had displayed less frequent hypersensitivity reaction in comparison to cetuximab (Hartmann, 2013; Pirker, 2013).

Humanized monoclonal IgG1 antibody trastuzumab (Herceptin) was developed and approved to specifically target HER2 (also HER2neu or EGFR2), by binding to extracellular domain. Results of clinical studies have shown that trastuzumab alone or in combination with paclitaxel or carboplatine represents a good treatment option for women with metastatic breast cancer overexpressing HER2 (Goldenberg, 1999; Shak, 1999). Recently trastuzumab in combination with pertuzumab and docetaxel was approved by US FDA as a first dual anti-HER2 regiment for HER2 positive metastatic breast cancer patients (Blumenthal, 2013).

EGFR inhibitor panitumumab and cetuximab, as well as gefitinib and erlotinib have been tested for its efficacy in combination with other monoclonal antibodies, eg. sorafenib, bevacizumab, or small molecules dasatinib, bortezomib, or sirolimus in patients who develop resistance to first line chemotherapy (Falchook, 2013; Wheler, 2013).

Sorafenib (BAY 43-9006, Nexavar) represents example of a different strategy of targeting cancer cells. It has been designed to inhibit several kinases, especially BRAF by targeting

RAF/MEK/ERK pathway in the tumor cells, vascular endothelial growth factor receptors VEGFR and platelet-derived growth factor receptor PDGFR in tumor vasculature. Following successful completion of Phase III clinical trial, sorafenib started to be marketed for advanced renal cell carcinoma and hepatocellular carcinoma (Adnane, 2006; Mellor, 2011). It was also the first agent developed to target BRAF mutant melanoma and was approved for therapy of kidney, and subsequently liver cancer. Currently sorafenib is in clinical trials for thyroid, lung and brain cancer and in combination with panitumumab showing improved clinical outcome in metastatic colorectal cancer (Al-Marrawi, 2013).

Bevacizumab (Avastin) is recombinant humanized monoclonal antibody that selectively binds and blocks vascular endothelial growth factor receptor (VEGFR) and currently is approved for use in patients with metastatic cancers, including colorectal cancer, breast cancer, renal cancer, grade IV glioma, and non-small cell lung cancer non-squamous subtype (Mellor, 2011). So far, there are no approved biomarkers for bevacizumab which could be considered to be predictive..
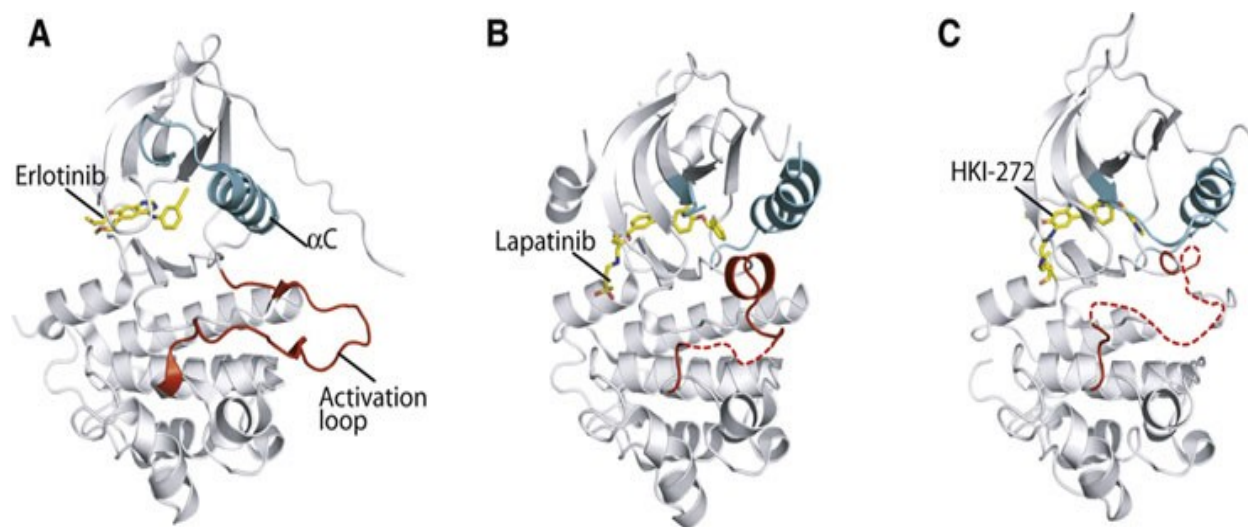
Figure 3. Structures of EGFR kinase domain bound to small molecule kinase inhibitors

Figure adapted from:

http://hematology.wustl.edu/faculty/bose/boseBio.html **Ron Bose, M.D., Ph.D.** Department of Medicine Oncology Division Breast Oncology Section Department of Cell Biology & Physiology Washington University in St. Luis

1.5 Genotyping methods for detection of KRAS gene mutations

KRAS screening has been practiced in colorectal cancer, lung cancer (NSCLC), and cancer of head and neck prior to initiation of treatment with EGFR inhibitors.

National Comprehensive Cancer Network (NCCN) and American Society of Clinical Oncology (ASCO) issued guidelines for treatment of metastatic colorectal cancer (mCRC) which recommended that anti-EGFR therapy decision should take into consideration *KRAS* gene status in addition to other disease associated factors (Allegra, 2009).

Diagnostics of *KRAS* gene mutations in clinical setting might have been limited by two factors: first, at the time of testing, KRAS mutated tumor cells frequently are in minority, outbalanced by wildtype tumor cells and wildtype non-tumor cells present in the collected sample. Secondly, analytically preferable snap-frozen tumor samples are rarely available for *KRAS* mutation testing. Instead, formaline fixed paraffin-embedded (FFPE) tissue is usually collected. Integrity of DNA extracted from FFPE gets severely compromised by non-optimal procedure of formaline fixation (i.e. non.-buffered formaldehyde and prolonged incubation), by disease itself, and by previous anticancer treatment.

Many different methods of detection of *KRAS* mutations were described. These methods can be divided into sequencing-based (Sanger sequencing, pyrosequencing, and Next generation sequencing)(Tuononen, 2013; Wang, 2013) methods, which are based on specific cutting by restriction enzymes (RFLP-restriction fragment length polymorphism) (Zhang, 2013), methods based on detection using denaturing high performance liquid chromatography (DHPLC) (Karim, 2010), methods based on hybridization of primer to template (such as ARMS-amplification refractory mutation system)(Hamfjord, 2011), methods based on hybridization of specific probes (TaqMan or ASO-allele specific oligonucleotide such as StripAssay)(Buxhofer-Ausch, 2013), and methods based on changes in the shape of the amplicon melting curve profiles (HRMA-high resolution melting analysis)(Guedes, 2013).

Such methods need to be evaluated and procedures need to be standardized regarding their sensitivity, specificity, and cost per analysis before they might become considered universally as standard reliable practice to be employed for diagnostics purposes. There are two main challenges which need to be standardized to achieve more reproducible and consistent results: heterogeneity of the testing materials, and differences in the detection limits among methods.

The Table 1 included below summarizes different types of genotyping methods: those which are not available commercially (research methods validated for clinical application) including few methods which are certified for the use in clinical practice diagnostics, such as CE-IVD (Communauté Européenne in vitro diagnostics) marked TheraScreen® kit which is based on the combination of ARMS primer and Scorpion probes, KRAS LightMix®, utilizing clamped hybridization based probes, KRAS Cobas® Mutation Test (real time PCR based specific probe

31

kit with closed system process including standardization of DNA isolation, AmoyDx[TM] KRAS (real time PCR with sequence mutation specific primers), RealQuality KRAS MuST (real time PCR based on sequence specific primers with intercalating dye), Devyser AB KRAS-BRAF which are based on multiplex PCR and capillary electrophoresis, KRAS-StripAssay® (multiplex PCR and reverse dot blot hybridization), TheraScreen®KRASPyro® (quantitative detection assay using pyrosequenicng system), Surveyor®Scan KRAS Kit (PCR and celery nuclease cleavage in combination with high performance liquid chromatography technology - DHPLC, confirmed by Sanger sequencing).

Table 1. Summary of methods described in literature and propose by company allowing detection of *KRAS or KRAS and BRAF* (Domagala, 2012; van Krieken, 2008).

Electrophoresis assays	Detection limit if published	
Temporal temperature gradient electrophoresis		[1]NC-LBM
Denaturing gradient gel electrophoresis		[1]NC-LBM
Constant denaturant capillary electrophoresis		[1]NC-LBM
Single strand conformation polymorphism assay (SSCP)		[1]NC-LBM
Sequencing		
Dideoxy capillary sequencing	25%	[1]NC-LBM, [2]RUO kit
Pyrosequencing	5-10%	[1]NC-LBM
Next generation sequencing	adjustable	[1]NC-LBM
Pyrosequencing- PyroMark™ KRAS kit-Biotage	5-10%	[2]RUO kit
TheraScreen ® KRASPyro® Kit-Qiagen	5%	[3] CE-marked kit
Devyser KRAS-BRAF kit- Devyser	~ 3%	[3] CE-marked kit
Allele-specific PCR assays		
Allele discrimination based on primer design		
Allele refractory mutation system (ARMS)-PCR		[1]NC-LBM
K-ras TheraScreen® kit –Qiagen	1%	[3] CE-marked kit
AmoyDx[TM] KRAS, BRAF-Amoy Diagnostics	1%	[3] CE-marked kit
KRAS LightMix® kit –TIB Molbiol	1%	[3] CE-marked kit
KRAS Cobas® Mutation Test kit-Roche	<5%	[3] CE-marked kit
RealQuality KRAS MuS-AB Analitica	1%	[3] CE-marked kit
Restriction endonuclease mediated selective (REMS)-PCR		[1]NC-LBM
Fluorescent amplicon generation (FLAG) assay		[1]NC-LBM
Restriction fragment length polymorphism -(RFLP)-PCR		[1]NC-LBM
Allele discrimination based allele-specific ligation detection reaction		
Multiplex polymerase chain reaction ligase detection reaction(LDR)-PCR		[1]NC-LBM
PCR-LDR spFRET assay		[1]NC-LBM
Allele discrimination based on discriminating amplification efficiencies at low melting temperatures COLD-PCR		[1]NC-LBM
PNAClamp[TM] K-ras Mutation Detection kit-Panagene	<1%	[3]CE-Marked kit
KRAS Muatation Detection Kit-DiaCarta	0.1%	[3]CE-Marked kit
K-ras/B-rafMutation Analysis kit –EntroGen	I >1%	[3]CE-Marked kit
K-ras StripAssay ® kit –ViennaLab	0.5%	[3]CE-Marked kit
KRAS/BRAF StripAssay ® kit –ViennaLab	1%	[3]CE-Marked kit

Other methods		
Surface ligation reaction and biometallization		NC-LBM
Multi-target DNA assay panel		NC-LBM
Surveyor®Scan KRAS Kit,-Transgenomic	2-5%	[3]CE-Marked kit

[1]NC-LBM: Laboratory-based method, not commercially available,

[2]RUO: research use only, not validated for clinical applications

[3]CE-IVD kit for clinical use (Approach Directives. 'CE' stands for 'Conformité Européenne')

http://www.cesolutions.eu/

*For use in clinical application, NC-LBM or RUO methods should be validated and accredited according to standards proposed by the *European Quality Assurance Program for *KRAS* mutation testing.

From the variety of available detection methods for screening important key region of the KRAS or BRAF oncomarker, the study presented in this thesis includes selected methods which were investigated in our laboratory based on the available equipment: Real-Time PCR Light Cycler 480 (Roche), Capillary Electrophoresis 8800 (Beckman), Next Generation Sequencing GS Junior-454 (Roche), and MiSeq (Illumina), Pyrosequencing (Biotage) (outsourced use of Pyrosequencing instrument (Boston,USA) although the sample preparation and data analysis of the data was done in our laboratory). We have used commercial kits KRAS TheraScreen DxS (Qiagen), KRAS Strip Assay (ViennaLab) BRAF PNA Clamp (Panagene) and BRAF (IntellMed), and the methods were designed in our laboratory: Direct sequencing, COLD-PCR, High Resolution Melting Analysis, Next Generation Sequencing. Characteristics of the methods are described in detail below.

1.5.1 Direct sequencing

This method is based on principle of Dideoxy Sanger termination (BigDye-termination) which uses modified deoxyribonucleotide triphosphate called termination dideoxynucleotide (ddNTPs), fluorescently labelled by four different dyes, mixed with standard non labelled and non terminating chain deoxyribonucleotide triphosphates (dNTPs). A ladder of DNA fragments produced by elongation by polymerase and randomly terminated is generated as a result a sequencing reaction. Denatured fragments are separated in capillaries-based gel (polymer) electrophoresis and whole process is monitored by measuring the change in fluorescence signal. By analyzing the four colour signals, the base sequence of the template can be determined. Although an advantage of this method is its potential for detection of all mutations in a fragment being sequenced, the sensitivity of this method is relatively low (in our experience requires presence of at least 25 % of mutated DNA (Jancik, 2010).
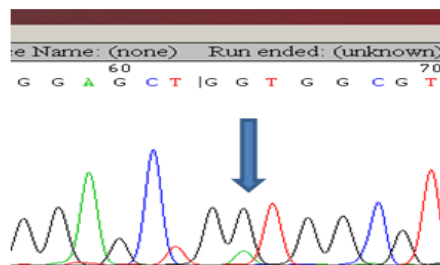
Figure: *KRAS* somatic mutation G**A**T 25%, in the contexts of 75% GGT wildtype background.

1.5.2 Pyrosequencing

Pyrosequencing method of DNA sequence analysis represents quantitative method of DNA sequencing. For *KRAS* mutation detection in current time the PyroMark KRAS Kit is marked for *in vitro* diagnostic use. The method is based on the principle of sequencing by synthesis of small fragments of DNA. The reagents and machine for sequencing of short fragments DNA are offered by Swedish Pyrosequencing AB company–renamed Biotage and currently distributed by Qiagen company. This kit enables to detect and quantify mutations in *KRAS* gene codons 12, 13, and 61 including rare or unknown mutations in these codons. Procedure of pyrosequencing is based on chemical light-producing enzymatic reaction. Single strand of DNA is used as a template for synthesizing its complementary strand which grows by incorporating one nucleotide at the time. Sequencing primer is hybridized to a ssDNA template and incubated with four enzymes: DNA-polymerase, ATP-sulfurylase, luciferase, and apyrase, and with the substrate adenosine 5' phosphosulphate. During incorporation of a nucleotide using polymerase enzyme, diphosphate group-(PPi) is releasing. Later ATP sulphurylase converts PPi to ATP which acts as source of energy (*fuel*) to the luciferase. Enzyme luciferase with D-luciferin and oxygen mediated conversion of luciferin to oxyluciferin that generates visible light proportional to the amounts of ATP. At the end of the entire process, the light signal is detected by a charge coupled device camera (CCD). The amount of light signal is proportional to the number of fluorescently-labelled nucleotides incorporated in the synthesized strand. Unincorporated nucleotides and ATP are then degraded by another enzyme - apyrase, making place for restarting next cycle with another nucleotide. Each PyroMark Research test contains quality controlled and tested reagents validated by Biotage/Qiagen (Ahmadian, 2006). Limitation of the method is the length of DNA fragment which can be assessed in a single sequencing run, which does not exceed 25-70 bp. The sensitivity of the pyrosequencing from our experience has been estimated to be between 5-10%.
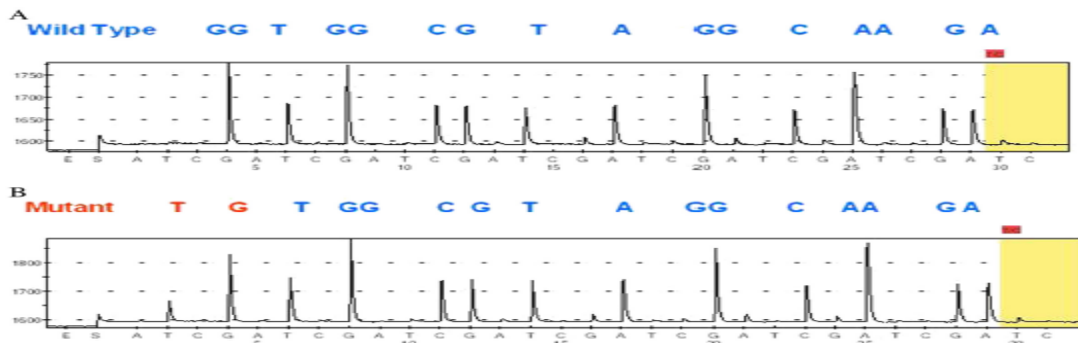
Figure: *KRAS* somatic mutation A) negative-GGT codon B) positive-TGT codon.

1.5.3 Scorpion primers and ARMS (TheraScreen KRAS Mutation Kit)

TheraScreen KRAS Mutation Kit manufactured at DxS (Diagnostic Innovation) Manchester, UK and now distributed by Roche. DxS TheraScreen which specifically detects *KRAS* mutations is CE-marked product for clinical use under the IVD Directive 98/79/EC and registered in many European countries, including the Czech Republic. TheraScreen is able to detect six mutations at the codon 12 (mutated codon 12; Ala, Asp, Arg, Cys, Ser, and Val), and one at the codon 13 (mutated codon 13: Asp) of the *KRAS* oncogene. This assay utilizes a Real-time PCR-Scorpions® methodology and allele specific PCR-ARMS® methodology. ARMS® method is employed for specific PCR to selectively amplify of mutated DNA in abundant background of non-mutated DNA by specific nucleotide at the 3′ end of the primer. This test kit includes separate primers for 7 specific assays and one single control reaction which reflects the amount of *KRAS* template in the sample. Whole reaction is monitored in a real time by the Scorpions® PCR fluorescence detection system.

Scorpions® system consists of a primer linked with a probe that contains a fluorophore and quencher. When probe hybridizes to the specific complementary DNA target sequence, fluorophore separates from quencher and fluorescence is emitted. The test kit includes also an internal control in all reaction mixes, controlling the presence of inhibitors which may lead to false negative results. The delta-CT values between the control reaction and the allele specific reaction reflect the amount of *KRAS* mutation within the sample and should not be higher than 8 to 9 cycles compared to a positive reaction. Detection limit for TheraScreen should be approximately 1% of mutated DNA in the background of wildtype DNA (Cross, 2008; Thelwell, 2000) (http://www.qiagen.com).
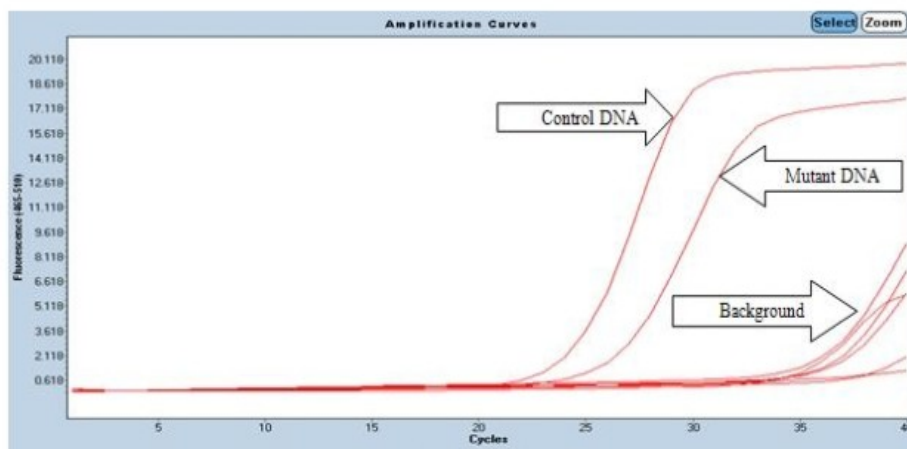
Figure: Positive *KRAS* mutation result. Delta-Ct values between the control reaction and allele specific reaction lower than 8 cycles.

1.5.4 Reverse dot-blot hybridization (K-ras StripAssay)

K-ras StripAssay is manufactured by ViennaLab and distributed by PentaGen (Czech Republic). This test of *KRAS* gene mutation detection is designed to detect 10 most frequently occurring mutations in the *KRAS* gene based on multiplex mutant-enriched PCR and reverse-hybridization of the amplification products to nitrocellulose test strips. Oligonucleotides used for hybridization are synthesized as probes which are specifically targeting eight mutations in the codon 12 (Ala, Arg, Asp, Cys, Ile, Leu, Ser, and Val) and two mutations in the codon 13 (Asp and Cys) of the *KRAS* gene (http://www.viennalab.com).
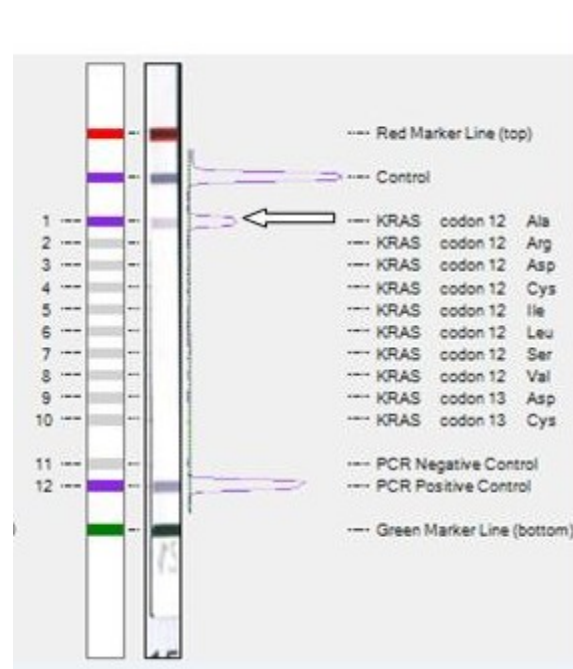
Figure: Positive *KRAS* somatic (KRAS, p.Gly12Ala) mutation on nitrocellulose test strip.

1.5.5 High Resolution Melting Analysis (HRMA)

High-resolution melting (HRM) analysis assay represents a real time PCR based mutations detection platform for identification of differences in DNA sequence by changes in the shape of the melting curve profiles compared to a profile obtained for a standard wildtype DNA-PCR product. HRM was developed using a new family of DNA-binding dyes which include SYTO 9, LC Green or LC GreenPLUS+. These dyes uniformly adhere to DNA double strand at low temperatures and reproducibly jump off from a dsDNA at higher and specific melting

temperature during melting process. This unique feature of the dye enables detection of heteroduplexes formed in the sample (Reed, 2007).

For detection of *KRAS* mutations, primers were created to achieve short PCR-product (up to 100bp). These short fragments are more efficiently detected when differences in changes in the shape of melting curves are monitored. This method is also effective only when good quality of DNA is used, and is important to use the same PCR (and preferably also DNA extraction) conditions.
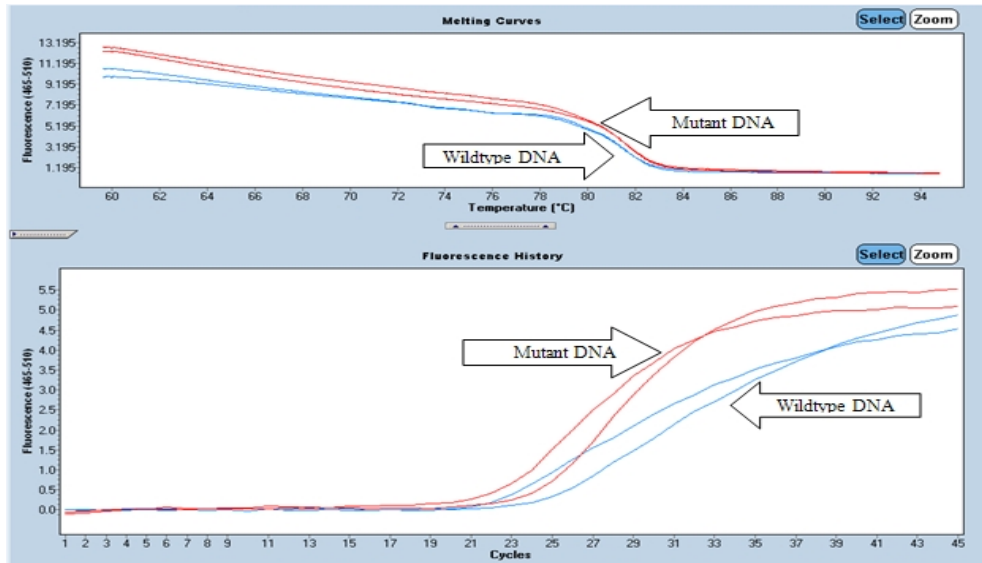
Figure: HRM result, difference between standard wildtype melting curves profiles and mutant melting curves profile.

1.5.6 Coamplification at lower denaturation temperature (COLD-PCR)

COLD-PCR–Coamplification at lower denaturation temperature-PCR is a modification of PCR that increases sensitivity of mutation detection. Principle of this method is based on critical denaturation temperature for each DNA sequence, which is lower than its melting temperature. Temperature is "critical" regarding aspect that reduction in denaturation temperature allows only heteroduplexes to become denatured and amplified, whereas homoduplexes are not amplified efficiently. This modification can be applied to many methods of detection, including HRM, Sequencing, and Pyrosequencing (Pinzani, 2011; Song, 2011; Wang, 2012).

1.5.7 Next generation sequencing (NGS)

NGS is a general term describing novel multiparallel sequencing technologies with high-throughput capacity, also called deep sequencing, massively parallel sequencing, or sequencing by synthesis. These methods can be used for revealing the nucleotide sequence of whole genome, whole exome, transcriptome, targeted region of interest or amplicon etc. Recent advances in NGS technology have enabled improved sensitivity of detection of single nucleotide polymorphisms even in the clinical specimens with high heterogeneity. NGS generates hundreds of megabases to gigabases of nucleotide sequences in a single instrument run, depending of the capacity platform used. Currently, MiSeq from Illumina is the most popular NGS bench top diagnostic sequencer, replacing GS 454 Junior from Roche. The platforms differ in sequencing chemistries and configurations, but share principle based on clonally amplified DNA that are spatially separated in a flow cell and sequenced with high repetition (coverage) (Desai, 2012; Voelkerding, 2009).
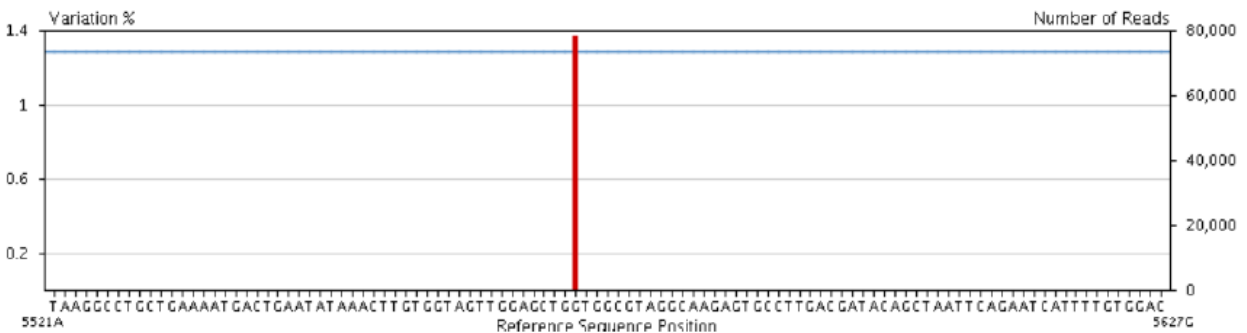
Figure: NGS GS 454 Junior result. Detection 1.4% mutation (GGT/G**A**T) in second position 12 codon *KRAS* gene.

Roche 454 Junior from Life Science technology combines two technologies: single-molecule emulsion-PCR and Pyrosequencing. For amplicon sequencing, library of template DNA is prepared by amplification of the region of interest using specific primers. Library of the template is then diluted to single molecule concentration and denatured to generate single strands of DNA. Single stranded fragments of DNA are then hybridized to the individual beads which are compartmentalized into water-in-oil emulsion and template of DNA is enriched by clonal amplification. After clonal amplification emulsion is completed, the sequencing primers anneal. Beads are separated and deposited onto picotiter plate wells (flow cell). Each well in the picotiter plate is able to hold only one single DNA enriched bead and surrounding mixture of smaller beads with enzymes necessary for pyrosequencing step to generate light. Intensity of the light is proportional to the pyrophosphate release during successive nucleotide incorporation. The nucleotides are added in fixed predefined order. Generated luminescence is transmitted through

fiber optic plate-well and read using specially charge-coupled device and camera. Sequencing coverage depth capacity of Roche GS 454 Junior for amplicon sequencing is ~ 70 000 reads per one run.

Principle of Illumina MiSeq analysis is based on bridge-PCR clonally amplified DNA and fluorescence emission using reversible dye terminators in four colours. Illumina technology includes flow cell consisting of an optically transparent slide with individual lines on the surface to which oligonucleotide anchors are bound. Adaptors with a modified single-stranded DNA are immobilized by hybridization to anchors, and DNA templates are clonally amplified in the flow cell by so called bridge amplification. Multiple amplifications convert single-molecule DNA template into several million clusters (one cluster contains approximately 1000 clonal molecules); clusters are generated in each lane of the flow cell. During sequencing which is performed in cycles with addition of primer and polymerase, clusters are denatured and cleaved. Four differently coloured reversible dye terminators are incorporated into each strand in each clonal cluster giving post incorporation fluorescence which is subsequently recorded by charge-couple device type of camera. Sequencing coverage depth capacity of Illumina-MiSeq for amplicon sequencing is ~ 15 000 000 reads per one run (http://www.illumina.com).

Experimental part

2. Aims of the research

Novel therapeutic agents targeting the epidermal growth factor receptor signaling have improved outcomes for patients with colorectal patients, lung cancer, and cancer of head and neck; however, these therapies are effective only in subset of cancer patients selected based on specific biomarkers (e.g. mutation in *KRAS* biomarker is negative predictive factor for EGFR targeted therapy in colorectal cancer, and *BRAF* is biomarker of poor prognosis in colorectal cancer or thyroid cancer, or some *EGFR* mutations or amplifications are positive predicting factor anti-EGFR therapy in lung cancer). In order to select subpopulation of patients which are most likely to benefit from EGFR targeted therapies, several recommendations were proposed by the European Quality Assurance Program regarding screening procedures (van Krieken, 2008). Selection of the most reliable method of detection of mutations is very important to provide oncologists biomarker of high quality for choosing the appropriate therapy for each patient, influencing progression free survival, overall survival, and overall quality of life. Ideally, cancer biomarkers should be measured easily, reliably, and cost-effectively. An assay used should have high analytical sensitivity and specificity to be clinically valid even if limited samples are analyzed. In the laboratory, other parameters such as workflow performance including handling and time consumption of the methods should also be considered.

The major aims and hypotheses of the study were:

1) To introduce and compare new strategies for genotyping of *KRAS* oncomarker in heterogeneous clinical tumor samples and to evaluate their analytical parameters (specificity, sensitivity, detection limit, cost, and working time). To compare five different commercial methods. We expected that new methods do not have identical analytical parameters.
2) To apply COLD-PCR amplification as the first step in Sanger capillary direct sequencing for genotyping of *KRAS* oncomarker. It was expected that COLD-PCR amplification can improve detection limit of Sanger capillary direct sequencing technology in comparison to standard PCR amplification.
3) To design and optimize NGS for genotyping of *KRAS* and *BRAF* oncomarkers. To evaluate if the NGS method have potential to achieve high quality analytical parameters regarding sensitivity and specificity when compared to CE-IVD PCR Real-Time methods. To analyze other parameters important for daily laboratory routine (i.e. cost, handling time, and robustness) are appropriate for routine clinical diagnostics in the field of cancer biomarkers.
4) To apply B-Raf method from IntellMed to testing of *BRAF* oncogene mutation in papillary thyroid carcinoma (PTC) patients. It was expected that $BRAF^{V600E}$ mutation detection status can have prognostic value regarding association with tumour metastasis

and other clinicopathological parameters, and can have different prevalence among individual histological PTC variants.

2.1 Comparison of KRAS genotyping methods in NSCLC

A comparison of Direct sequencing, Pyrosequencing, High resolution melting analysis, TheraScreen DxS, and the K-ras StripAssay for detecting *KRAS* mutations in non-small cell lung cancer.

Journal of Experimental &
Clinical Cancer Research

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A comparison of Direct sequencing, Pyrosequencing, High resolution melting analysis, TheraScreen DxS, and the K-ras StripAssay for detecting *KRAS* mutations in non small cell lung carcinomas

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Abstract

Background: It is mandatory to confirm the absence of mutations in the *KRAS* gene before treating metastatic colorectal cancers with epidermal growth factor receptor inhibitors, and similar regulations are being considered for non-small cell lung carcinomas (NSCLC) and other tumor types. Routine diagnosis of *KRAS* mutations in NSCLC is challenging because of compromised quantity and quality of biological material. Although there are several methods available for detecting mutations in *KRAS*, there is little comparative data regarding their analytical performance, economic merits, and workflow parameters.

Methods: We compared the specificity, sensitivity, cost, and working time of five methods using 131 frozen NSCLC tissue samples. We extracted genomic DNA from the samples and compared the performance of Sanger cycle sequencing, Pyrosequencing, High-resolution melting analysis (HRM), and the Conformité Européenne (CE)-marked TheraScreen DxS and K-ras StripAssay kits.

Results and conclusions: Our results demonstrate that TheraScreen DxS and the StripAssay, in that order, were most effective at diagnosing mutations in *KRAS*. However, there were still unsatisfactory disagreements between them for 6.1% of all samples tested. Despite this, our findings are likely to assist molecular biologists in making rational decisions when selecting a reliable, efficient, and cost-effective method for detecting *KRAS* mutations in heterogeneous clinical tumor samples.

Keywords: SNP - single nucleotide polymorphism, KRAS - Kiras2 kristen rat sarcoma viral oncogene homolog, NSCLC - Non-small cell lung cancer, Genotyping

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Background

At present, identifying targeted anticancer treatment suitable for a given patient requires the availability of accurate diagnostics. Diagnostic techniques therefore have a significant impact on patients' survival and quality of life [1]. In recent years, it has become apparent that certain types of tumors undergo mutations that either originate from the aberrant physiology of the tumor or are induced/selected by mutagenic cancer therapies [2-4]. Failure to detect mutations in important regulatory genes in tumor specimens may have serious consequences for the patients, because these alterations can significantly reduce the effectiveness of certain biological and cytotoxic therapies. Mutations in the KRAS oncogene are often found in human cancers. They are most common in pancreatic cancer, which can exhibit mutation rates of 80 - 90%. KRAS mutations are also observed in 40 – 50% of colorectal cancers and 10 - 30% of Non-Small Cell Lung Cancers (NSCLCs).

Recent studies have shown that some anticancer drugs are only effective against tumors in which the KRAS signaling pathway has not undergone oncogenic activation. These include the small-molecule epidermal growth factor receptor inhibitors erlotinib (Tarceva®) and gefitinib (Iressa®), which are used to treat NSCLC patients, and monoclonal antibody therapies such as cetuximab (Erbitux®) and panitumumab (Vectibix®), which are primarily used in the treatment of metastatic colorectal cancers (mCRC) [5-7]. According to the U.S. National Comprehensive Cancer Network (NCCN) guidelines from November 2008 (http://www.nccn.org/about/news/newsinfo.asp?NewsID=194) and recommendations of the American Society of Clinical Oncology (ASCO)[8], screening of the status of the KRAS gene is mandatory when deciding whether or not a patient with colorectal cancer should receive anti-EGFR drugs. Similar rules are being considered for NSCLC where KRAS mutations have prognostic value for progressive disease in adenocarcinoma [9,10].

There are multiple methods for detecting KRAS mutations in patient tissues, with varying analytical parameters. Individual methods need to be evaluated in terms of their sensitivity, specificity, and cost per analysis before they can be considered to meet acceptable gold standards in clinical practice. A standardized European quality assurance program for tests to detect mutations in KRAS was proposed at the Third International Congress of Pathology, held by the European Society of Pathology (ESP) in Barcelona in May 2008. This program is focused on achieving optimal accuracy and proficiency across the European Union [11]. However, there are many methods in current use, some of which are only employed by individual laboratories and are not commercially available. These typically include sequencing assays [12] and gel-based DNA conformation assays [13,14]. Some of the commercial assays for detecting mutations in the KRAS gene have not yet been validated for clinical use (i.e.: Allele-specific oligonucleotide hybridization - Invigene®, KRAS mutation test kit - EntroGen®). At the time of writing, only the TheraScreen® kit sold by QiaGen, the KRAS LightMix® kit sold by TIB MolBiol, and the K-ras StripAssay® sold by ViennaLab had received the Conformité Européenne (CE) mark certifying them as being suitable for diagnostic use in the clinic under the terms of the European IVD Directive 98/79/EC.

In order to assess the specificity, sensitivity, cost, and working time of five frequently used methods for detecting mutations in KRAS, we performed parallel tests using DNA extracted from 131 frozen NSCLC tissue samples. The methods examined were Sanger cycle sequencing, Pyrosequencing, High-resolution melting analysis (HRM), and the CE-marked TheraScreen DxS and K-ras StripAssay kits. Our data demonstrate that there are important differences between these methods, which should be considered in routine clinical testing for KRAS mutations.

Methods
Pathological assessment

The experimental research presented in this manuscript was performed in compliance with the Helsinki Declaration according to the study ethics proposal approved by Ethical Board of Palacky University in Olomouc. Written informed consent was obtained from all patients for the use of the collected samples in the research projects which includes studies for publication of this report or any accompanied images.

Diagnosis of NSCLC was initially performed at the time of surgery and later confirmed from leftover by histological subtyping performed by experienced pathologist. All samples were found to contain more than 70% of tumour cells from at least 200 cells.

DNA extraction from cell lines and primary tumor samples

Genomic DNA was extracted from 131 frozen Non Small Cell Lung Cancer (NSCLC) tissue specimens removed from patients undergoing surgery for lung cancer. Tissue was snap frozen in liquid nitrogen immediately after surgery and stored at –80°C until analyzed. Cell lines with specific KRAS mutations were obtained from the American Tissue Culture Collection (ATCC, Rockville, MA) and cultured according to ATCC instructions. DNA extraction and purification was performed using the QIAquick (QIAGEN, Hilden, Germany) isolation kit according to manufacturer's instructions; in each case examined, the five methods were tested against the same DNA isolate, so potential differences in percentage of

tumor cells does not confound the method comparison. Concentrations of DNA samples were measured spectrophotometrically using a NanoDrop ND 1000 spectrophotometer (NanoDropTechnologies, Wilmington, USA).

Genotyping methods

Analyses were performed according to a blinded design, in which the experimentalist was not aware of the KRAS mutation status of any given sample. 131 NSCLC samples were analyzed using four methods: Direct sequencing, Pyrosequencing, and the TheraScreen DxS and K-ras StripAssay kits. Due to limited amount of tissue, only 116 samples from this group were also subjected to HRM analysis and 114 yielded usable data. Significance of the concordance of mutation detection with different methods for two categories (wildtype and mutant) was assessed by κ statistics (http://faculty.vassar.edu/lowry/kappa.html).

Direct sequencing method

Two primers were used to prepare amplicons for use in Sanger dideoxy termination sequencing [15]: a forward (FW) primer, 5'AAA AGG TAC TGG TGG AGT ATT TGA, and a 3' reverse (REV) primer, 5' TCA TGA AAA TGG TCA GAG AAA CC 3' (Generi-Biotech, Hradec Králové, Czech Republic). PCR was performed with a reaction volume of 50 μl in an MJ Research PTC-200 Peltier Thermal Cycler (Watertown, USA). The composition of the PCR reaction mixture was as follows: $MgCl_2$ (3 mM, ThermoScientific, Waltham, USA), dNTPs (0.2 mM, ThermoScientific), ThermoStart DNA polymerase (2U, ThermoScientific), FW-primer (0.3 μM), REV-primer (0.3 μM), 1xPCR buffer, and between 10 ng and 100 ng of genomic DNA per reaction. The following amplification program was used: 95°C/15 min to activate the Taq polymerase; 35x (95°C/30 s, 58°C/30 s 72°C/30 s) for denaturation, annealing, and extension; and finally 75°C/5 min to finalize the extension, followed by cooling to 15°C. The PCR product was separated using a 2% agarose gel and purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany). For each sample specimen, separate sequencing reactions were performed using the forward (FW) and reverse (REV) primers. The sequencing primers were internal to the amplicons from the previous PCR cycles: FW - 5' TTA ACC TTA TGT GTG ACA TGT TCT AA 3', REV - 5' AGA ATG GTC CTG CAC CAG TAAT 3'. Sequencing reactions were performed according to the manufacturer's protocol in a 20 μl reaction volume containing 4 μl DTCS Quick Start kit (Beckman Coulter, Brea, USA), 1 μl (10 μM) of the FW or REV primer, 10 μl nuclease-free water, and 5 μl of 25x diluted template PCR product. After cleaning, precipitated DNA was diluted in SLS-formamide (Beckman Coulter, Brea, USA) and dideoxylabelled fragments were size-separated using an automated CEQ

8800 Genetic Analysis System (Beckman Coulter, Brea, USA) (Figure 1). Sequence identification was performed using version 1.42 of the Chromas software package (Conor McCarthy, Southport, Australia). For all analyses, data obtained with the forward and reverse primers were combined and aligned to the consensus sequence obtained from the BLAST GenBank database http://www.ncbi.nlm.nih.gov/nuccore/166706780?report=genbank.

Pyrosequencing

In the pyrosequencing method for DNA sequence analysis [16,17], inorganic phosphate released in the course of nucleotide incorporation serves as the initial substrate in a sequence of four successive enzymatic reactions. This result in the emission of light, which functions as a signal that is proportional to the number of nucleotides incorporated.

In this project, the PyroMark K-ras assay test (Biotage, Uppsala, Sweden) was used for primary amplification and pyrosequencing of both the 12th and the 13th codons of the KRAS oncogene (Figure 2). The following amplification program was used: the mixture was heated at 95°C for 5 min, then subjected to 45 cycles of 95°C for 15 s, 57°C for 30 s, and 72°C for 15 s. It was then held at 72°C for 5 min, and finally cooled to and held at 4°C. The final concentrations of the PCR components were: 1x PCR buffer, 2 mM $MgCl_2$, 0.125 mM dNTPs, 0.2 μM FW primer and 0.2 μM REV biotinylated primer, 1U of AmpliTaq polymerase (Perkin Elmer, Waltham, USA) and 2 ng/μl DNA template. Fifteen μl of the PCR product was run on a 1,5% agarose gel (Sigma-Aldrich, St. Louis, USA) to confirm successful amplification, and 100 ng of PCR products were sent to the EpigenDX company (Worcester, USA) to be analyzed using the PyroMark MD System and the Pyromark ID analysis Software with previously validated cut-off of 5%.

K-RAS TheraScreen DxS

The TheraScreen DxS KRAS Mutation Kits KR-21 and KR-22 (QiaGen, Hilden, Germany) are designed to detect six mutations in codon 12 (Gly>Ala, Asp, Arg, Cys, Ser, and Val) and one in codon 13 (Gly>Asp) of the KRAS oncogene. The primers used in the assay have two characteristic features: sequence-specific 3′ ends (which comprise the PCR-Amplification Refractory Mutation System, PCR-ARMS®) to identify specific mutations, and Real-time PCR-Scorpion® primer tags, which fluoresce when incorporated into double-stranded DNA (Figure 3).

The commercial test kit includes an internal reaction control and a synthetic control template. The degree of mutation of KRAS is calculated on the basis of the difference between the control reaction and the allele-specific reaction in terms of the number of cycles required for the

Figure 1 Sequencing of the KRAS gene in DNA isolated from NSCLC tissues. (A) Wild type-(12Gly-GGT, 13Gly-GGC), **(B)** Mutant- (12Asp-GAT).
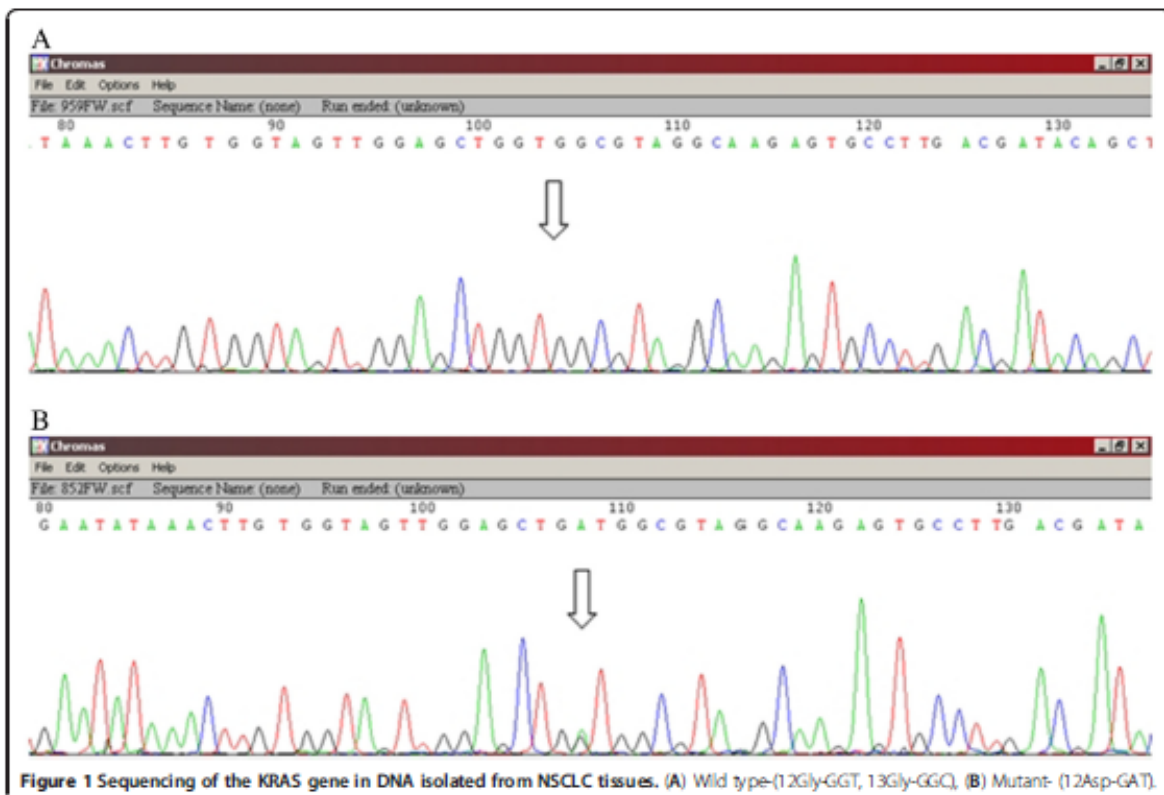
fluorescence of the reaction mixture to exceed the background level (Δ-C_T) [18].

PCR reactions were performed according to the protocol recommended by the manufacturer (TheraScreen K-RAS Mutation Kit version DU001PE) using a LightCycler[®]480 II (Roche Applied Science, Penzberg, Germany), with a final reaction volume of 25 µl. An initial denaturation step at 95°C for 4 min was followed by 45 cycles of 95°C for 30 sec and 60°C for 1 min. Analysis was performed using a predefined absolute quantification algorithm implemented in the LightCycler Analysis Software 1.5.0 SP3 program (Roche Applied Science, Penzberg, Germany) and by visual inspection conducted by two different researchers.

K-ras StripAssay

The K-ras StripAssay REF 5–590 (ViennaLab Diagnostics GmbH, Vienna, Austria) detects the 10 most common mutations in the *KRAS* gene by using multiplex mutant-enriched PCR and reverse-hybridization of the amplification products to nitrocellulose test strips (oligonucleotides used in the subsequent hybridization reactions are synthesized as probes targeting 8 mutations in codon 12 of the *KRAS* gene (Gly > Ala, Arg, Asp, Cys, Ile, Leu, Ser, and

Val) and two mutations in codon 13 (Gly > Asp and Gly > Cys). Specifically hybridized biotinylated oligonucleotides are visualized using streptavidin-alkaline phosphatase and colored substrates (Figure 4).

The KRAS StripAssay was performed according to the manufacturer's protocol (K-ras StripAssay™, ViennaLab Diagnostic GmbH, Vienna, Austria). Samples were diluted using deionized water to a concentration of 10 ng/µl. Five µl of diluted DNA was added to the multiplex PCR reaction with biotinylated primers, and PCR was conducted according to the manufacturer's instructions. All of the incubation steps were performed using a PST-60 HL Plus thermoshaker (Biosan, Riga, Latvia) platform with the temperature set to 45°C. Scanning was performed using the EPSON Perfection V30 scanner (Epson America, Inc., Long Beach, USA) and bands were analyzed by StripAssayEvaluator software (ViennaLab, Vienna, Austria) and by visual inspection.

High resolution melting analysis

The high-resolution melting (HRM) assay is a platform for real time detection of mutations that can be used to identify small differences in DNA sequences, even in heterozygous samples, by assessing changes in the shape

47

Figure 2 Pyrosequencing of the KRAS gene in DNA isolated from NSCLC tissues. (A) Wild type-(12Gly-GGT, 13Gly-GGC), (B) Mutant-*KRAS* (12Cys-TGT).

of their melting curve profiles compared to profiles generated using standard (wild-type) DNA [19] (Figure 5). The HRM assay was developed using a new family of DNA-intercalating dyes including SYTO 9, LC Green and LC Green^{PLUS+} that display strong intercalation and even association with DNA.

HRM-PCR reaction components were used according to the instructions provided with the LightCycler® 480 II (Roche) instrument. The LC Green^{PLUS} (Idaho Technology, Salt Lake City, USA) intercalating dye was used, together with the primers FW-5'-AAA CTT GTG GTA GTT GGA GCT-3' (forward) and REV-5'-ATT AGC TGT ATC GTC AAG GCA-3' (reverse). The final concentrations of the components of the reaction mixture were: 1x PCR buffer, 2 mM MgCl$_2$, 0.2 μM dNTP, 0.5x LC Green^{PLUS}, 0.2 U ThermoTaq polymerase (Thermo Scientific), and 0.3 μM FW and REV primer. The cycling and melting conditions were as follows: one cycle at 95°C for 15 min., followed by 45 cycles of 95°C for 10 s, 63°C for 10 s, and 72°C for 10 s. The sample was then melted by raising the temperature from 60°C to 95°C at a rate of 0.02 C/s. Mutations were analyzed using LightCycler Analysis Software 1.5.0 SP3 program (Roche Applied Science, Penzberg, Germany).

Results

Five genotyping methods for determining the status of mutation of *KRAS* were assessed using frozen tissue from primary NSCLC tumor specimens. 131 DNA samples were analyzed with 4 of the methods (Direct sequencing, K-ras StripAssay, TheraScreen DxS, and Pyrosequencing), and 116 of these were also analyzed using the High resolution melting (HRM) technique. In the absence of a gold standard, we adopted a consensus method for assigning each sample's mutation status. The results obtained and methodology used are shown in Table 1.

As expected, the percentage of the DNA samples in which mutations were detected varied (from 20% to 5%) depending on the method of detection used. The Kras-StripAssay had the highest likelihood of referring a mutation in the *KRAS* locus, followed by TheraScreen DxS, HRM, Pyrosequencing, and Direct sequencing (Table 2).

However, on the basis of our evaluation criteria (Table 1), the most sensitive tool was the TheraScreen DxS kit (95%), followed by the K-ras StripAssay (90%), HRM (70%), Pyrosequencing (48%), and Sequencing (29%). The most specific tools were the TheraScreen DxS kit, Sequencing, and Pyrosequencing (100%), followed by HRM (98%) and the K-ras StripAssay (95%) (Table 3).

48

Figure 3 TheraScreen analysis of the *KRAS* gene in DNA isolated from NSCLC tissue. (A) Wild type. The figure shows a positive PCR control in which the quantity and quality of the DNA were assessed (red curve) without any other amplification of ARMS mutation positive primers (green curve) **(B)** Mutant. The figure shows a positive PCR control and a mutation signal (12Asp) generated by one tube of the ARMS-primers. The upper limit on ΔCt, which corresponds to a mutant DNA content of 1%, is for the mutant PCR to be 8 cycles behind the control PCR (here $\Delta Ct = 26.44 - 24.03 = 2.41$).

The number of false positives and false negatives obtained with each method would change if one were to change the interpretation criteria. For example, if the K-ras StripAssay is taken to be the gold standard, then the false positives detected by this method would become false negatives detected by the other methods. To eliminate this potential ambiguity, we performed more tests to assess and compare the sensitivity thresholds of

the tested methods. We used three ATCC cell lines whose *KRAS* mutation statuses are known and recorded in the COSMIC database: A549 (p.Gly12Ser), NCI-H620 (p.Gly12Val), and NCI-H2009 (p.Gly12Ala). We extracted sample DNA from the cell lines, measured its concentration by spectrophotometry, and then made dilution series of the DNA from the *KRAS* mutant cell lines in DNA from the NCI-H1975 *KRAS* wild-type cell

Figure 4 StripAssay analysis of the *KRAS* gene in DNA isolated from NSCLC tissue. (A) Wild type-(12Gly, 13Gly) (B) Mutant-(12Ala, 13Gly).
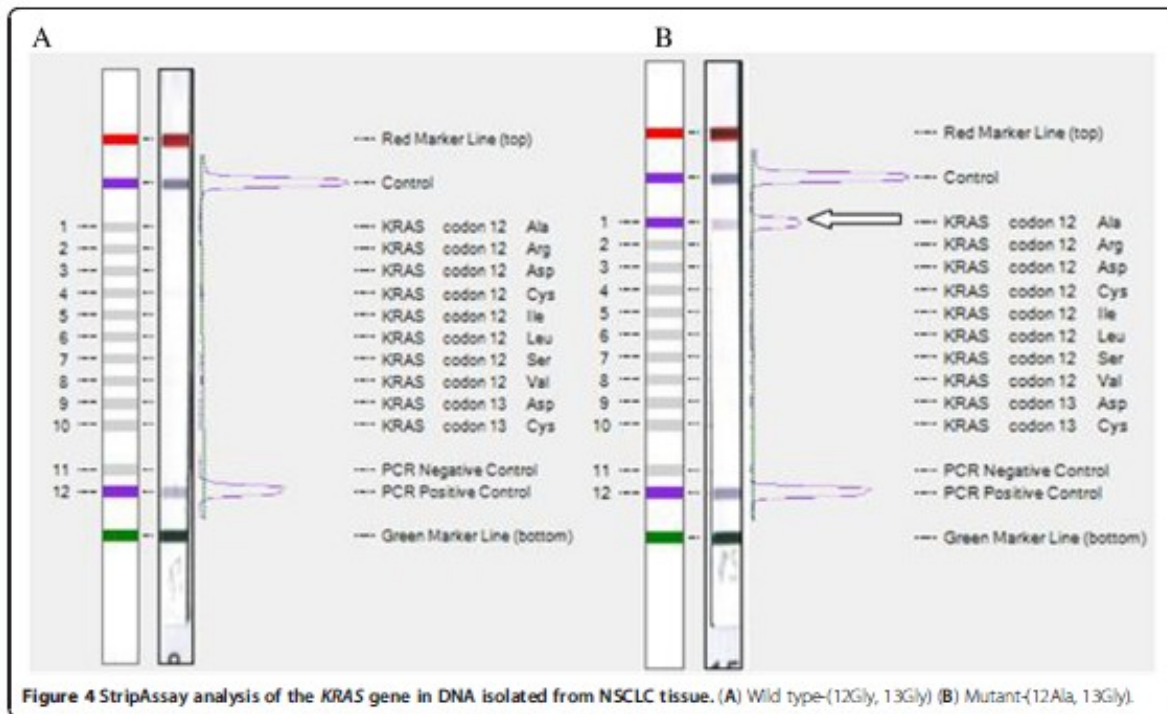
line such that the mutant DNA comprised 25%, 20%, 15%, 10%, 5%, 1%, 0.5%, 0.25%, or 0.125% of the total *KRAS* DNA (Figure 6).

At a mutant minority of 1%, only TheraScreen and StripAssay were capable of detecting mutations in *KRAS*, while other methods have detection limit at 10% (Pyrosequencing), and 25% (HRM and Sanger sequencing). Interestingly, in one technical replicate the mutation detected by the TheraScreen DxS kit in cell line A549 (p.Gly12Cys) was inconsistent with what was actually

Figure 5 HRM melting profile result of the *KRAS* gene isolated from NSCLC tissue. The figure shows the results of Gene Scanning analyses (using the LC Green^PLUS dye with the sensitivity set to 0.3) of wild type samples (yellow line) and mutant cell line samples (curves with other than yellow colours).

Table 1 Summary of the genotyping results obtained with the five tested methods in 131 NSCLC samples

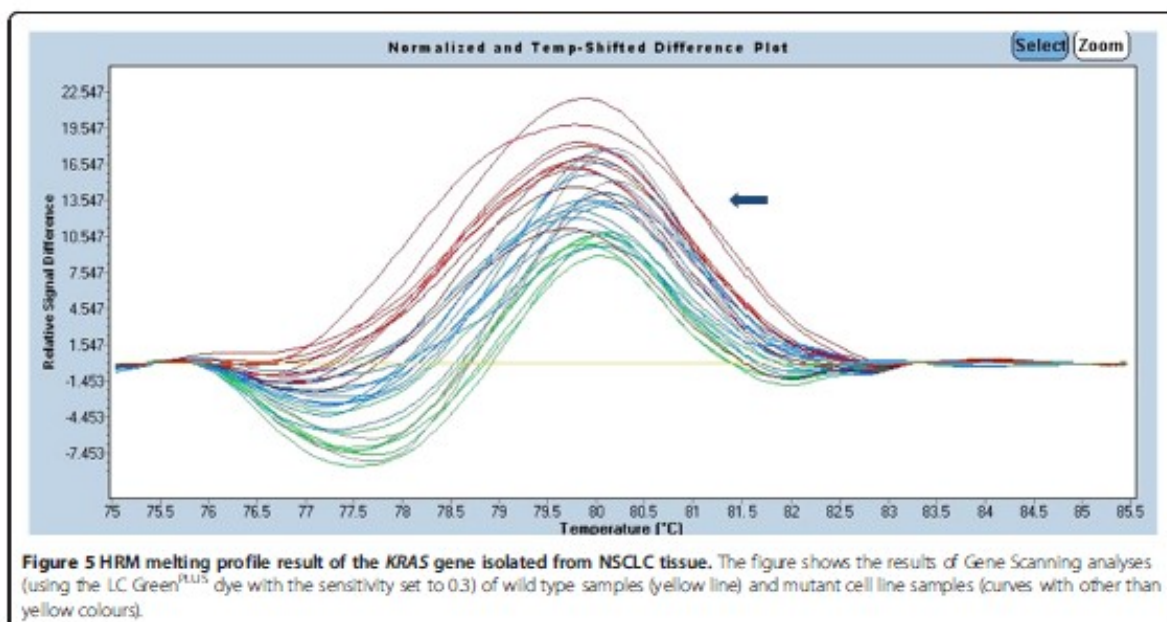| DNA sample number	Direct sequencing	Pyrosequencing	TheraScreen DxS	K-ras StripAssay	HRM	Consensus
1	12Cys	12Cys	12Cys	12Cys	Mutation	12Cys
2	13Cys	13Cys	Wt	13Cys	Mutation	13Cys
3	12Cys	12Cys	12Val	Wt	Mutation	12Cys
4	12Asp	12Asp	12Asp	12Asp	Mutation	12Asp
5	12Cys	12Cys	12Cys	12Cys	Mutation	12Cys
6	12Cys	12Cys	12Cys	12Cys	Mutation	12Cys
7	Wt	12Cys	12Cys	12Cys	Mutation	12Cys
8	Wt	12Val	12Val	12Val	Mutation	12Val
9	Wt	12Cys	12Cys	12Cys	Mutation	12Cys
10	Wt	12Cys	12Cys	12Cys	Wt	12Cys
11	Wt	Wt	12Cys	12Cys	Wt	12Cys
12	Wt	Wt	12Cys	12Cys	Wt	12Cys
13	Wt	Wt	12Val	12Val	Wt	12Val
14	Wt	Wt	12Cys	12Cys	Wt	12Cys
15	Wt	Wt	12Cys	12Cys	Wt	12Cys
16	Wt	Wt	12Arg	13Cys	Inconclusive	Mutation
17	Wt	Wt	12Cys	12Cys	Mutation	12Cys
18	Wt	Wt	12Asp	13Asp	Not tested	Mutation
19	Wt	Wt	12Asp	Wt	Mutation	12Asp
20	Wt	Wt	12Cys	Wt	Not tested	Inconclusive
21	Wt	Wt	13Asp	Wt	Not tested	Inconclusive
22	Wt	Wt	Wt	12Cys	Mutation	12Cys
23	Wt	Wt	Wt	12Cys	Mutation	12Cys
24	Wt	Wt	Wt	12Arg	Wt	Wt
25	Wt	Wt	Wt	12Val	Wt	Wt
26	Wt	Wt	Wt	12Cys	Wt	Wt
27	Wt	Wt	Wt	13Cys	Wt	Wt
28	Wt	Wt	Wt	12Ala,13Cys	Wt	Wt
29	Wt	Wt	Wt	12Ser	Not tested	Inconclusive
30	Wt	Wt	Wt	12Ala	Wt	Wt
31	Wt	Wt	Wt	Wt	Mutation	Wt
32	Wt	Wt	Wt	Wt	Mutation	Wt
33 - 131	Wt (99 samples)	Wt (99 samples)	Wt (99 samples)	Wt (99 samples)	Wt (87 samples), 11-no DNA template, 1 –Inconclusive sample.	Wt

The consensus result for a given sample was taken to be that obtained when the two CE-marked methods (K-ras StripAssay and TheraScreen DxS) were concordant with one-another (results that do not match this consensus are highlighted with a dark background). The detection of different types of mutation by different methods (e.g. in sample 3, p.Gly12Cys vs p.Gly12Val; in sample 16, p.Gly12Arg vs p.Gly13Cys; and in sample 18, p.Gly12Asp vs p.Gly13Asp) was not considered indicative of discrepancy because the precise identity of the mutation present is clinically irrelevant in this case (instances of type-of-mutation discordance are highlighted with a light background). In cases where the K-ras StripAssay and TheraScreen DxS kit generated inconsistent results, the sample was considered to be mutated only if one of the other three methods indicated the presence of a mutation. Thus, three samples (samples 20, 21, and 29) generated inconclusive results. Inconclusive results were excluded from further analysis.

present. At a mutant minority of 0.5%, the TheraScreen DxS kit only detected mutation in the NCI-H620 cell line (p.Gly12Val); the K-ras StripAssay failed to yield any positive results when analyzed using the StripAssay Evaluator software, but was judged to have correctly detected a mutation in the NCI-H620 line on the basis of visual inspection. At a mutant minority of 0.25%, only the K-ras StripAssay yielded a positive result. Remarkably,

Table 2 Number and percentage of mutations detected by methods

Methods	Mutations/ samples	%	Mutations/ samples	%
Direct sequencing	6/131	4.5	6/116	5.2
Pyrosequencing	10/131	7.6	10/116	8.7
HRM	-	-	15/116	13.1
TheraScreen DxS	20/131	15.2	17/116	14.6
K-ras StripAssay	26/131	19.8	24/116	20.7

To allow comparison with HRM, results are provided not only for 131 but also for 116 samples.

the K-ras StripAssay was able to detect the mutation in the NCI-H2009 line (p.Gly12Ala) even at a mutant minority of 0.125%.

Discussion

We have examined the ability of five different methods to detect mutations in the *KRAS* gene in 131 DNA samples. *KRAS* mutations were detected in 21 samples (16.0%), 107 samples were found to contain wild-type DNA (81.7%), and three yielded inconclusive results (2.2%) (Table 1). Of the 21 samples in which mutation was detected by one or more methods, there were only four for which all five yielded a positive result (19.0%). Of the 95 wild-type samples analyzed by all five methods, concordance was observed in 87 (91.6%); overall, the five methods were in agreement with one-another for 78% of the samples examined. Excluding HRM, the four remaining analytical methods all generated positive results for 4 out of 21 samples found to be positive by one or more method (4.4%) and all generated negative results for 101 of 107 samples found to be negative by one or more method (94.4%), giving an overall agreement of 82%.

Our findings concerning the ability of these methods to detect mutations in *KRAS* are similar to those of Whitehall et al. (2009), who compared Dideoxy sequencing, HRM, the TIB Molbiol kit (Berlin, Germany), and the TheraScreen DxS (Manchester, UK) kit using DNA isolated from frozen colorectal cancer tissues. In their study, all five methods were found to be in concordance with regard to the *KRAS* mutation status of 66 of the 80 samples tested (83% agreement) [20].

Both our results and those obtained by Whitehall [20] show that a significant number of samples from colorectal tumor and NSCLC contain mixtures of *KRAS* wild-type and *KRAS* mutant cells, and that in many cases the percentage of mutant cells is below the threshold that can be detected by direct sequencing. This inherent heterogeneity of bioptic tumor tissues is an universal problem, albeit one that can be partially addressed by concentrating the tumor cells (e.g. by laser capture microdissection) before extracting their DNA. However, the fact that even a pure sample of tumor cells may contain large quantities of wild-type *KRAS* further complicates the selective identification of mutations in this gene. Consequently, it is desirable that methods for detecting *KRAS* mutations should be highly sensitive, and this point should be borne in mind when selecting a proper diagnostic method. Our study identified the TheraScreen DxS kit as having the best ability to detect *KRAS* mutations in clinical samples, followed by the K-ras StripAssay (Table 4).

Our results also indicate that direct sequencing is only of limited utility when trying to detect mutations in the *KRAS* gene in cancer tissues, since this method only detected *KRAS* mutations in 6 of the 131 DNA samples tested, even though 21 were found to contain mutations by other methods. Though direct sequencing is still being advocated as *KRAS* genotyping method of choice [21], it missed 72% of all mutations in our cohort. Obviously, the sensitivity of the sequencing methods could be further improved by using laser microdissection [22], preferential preamplification [23], inclusion of both primary and metastatic tissues in the analysis, or by using clamping to suppress PCR amplification of the wild-type gene [24]. However, we agree with Pinto et al. that Sanger sequencing (without the first steps of COLD-PCR) [25] is currently outperformed by more sensitive techniques [26].

Pyrosequencing is easily capable of detecting PCR fragments that are 25–50 bp in length while longer fragments may pose a problem. However, this is not the case of detecting mutations in *KRAS*, because the most frequent mutations in this gene are adjacent, occurring in codons 12 and 13. It may even be advantageous to use short fragments when diagnosing mutations because DNA may be fragmented during the processing of clinical tissue samples. In accordance with results of others [27,28], Pyrosequencing outperformed conventional sequencing for

Table 3 False positive and false negative rates of the different methods

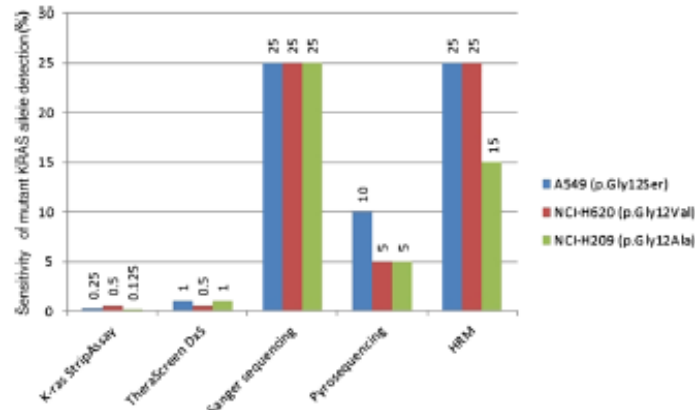|	Sequencing (n=131)	Pyrosequencing (n=131)	TheraScreen DxS (n=131)	K-ras StripAssay (n=131)	HRM (n=116)
False positives (1 - specificity)	0/110 (0 %)	0/110 (0 %)	0/110 (0 %)	6/110 (5 %)	2/96 (2 %)
False negatives (1 - sensitivity)	15/21 (71 %)	11/21 (52 %)	1/21 (5 %)	2/21 (10 %)	6/20 (30 %)

Figure 6 Comparative sensitivity analysis of KRAS typing kits in dilution series, where DNA from three mutated cell lines was diluted in wild-type DNA. Results of dilution series consisted of 25%, 20%, 15%, 10%, 5%, 1%, 0.5%, 0.25%, and 0.125% of mutated DNA in wild-type DNA. For threshold found in the first dilution experiment and one adjacent concentration from each side, typing was performed three times. Resulting consensus thresholds (found two or three times out of three repeats) for cell lines A549 (p.Gly12Ser), NCI-H620 (p.Gly12Val), and NCI-H209 (p.Gly12Ala) are shown in the graph.

detecting *KRAS* mutations in samples with levels of mutant cells ranging from 5 to 25% (Table 4) while quantification of mutated portion of DNA was not possible. This is probably due to preferential amplification of the mutated samples by the primers designed for the particular Biotage kit used. This shortcoming could be obviated by a better primer design or other modification of the kit and/or improvements in the interpretation algorithm [29,30]. Promisingly, a massively parallel pyrosequencing system using nanoliter reaction volumes has yielded satisfying results in an interlaboratory comparison [28]. While this probably represents the future of testing in predictive oncology, such systems are prohibitively costly for most laboratories at the present.

HRM proved to be the least expensive and the most rapid method, as it requires only standard real-time PCR reagents and a slightly prolonged PCR protocol. Despite the optimistic references from other laboratories [31], the analysis of the melting profiles in our hands remains less reliable than other methods, and even repeated testing of our reference DNA did not always yield consistent results. Because of this, the typing of two samples by this method was inconclusive. We may speculate with Do [32] that treatment of DNA with uracil glycosylase or special step of DNA cleaning would help standardize the method and better its analytical parameters. Interestingly, HRM analysis identified mutations in the *KRAS* locus of two DNA samples (samples 31 and 32) for which none of the other methods detected any mutation (Table 1). In keeping with the findings of

Table 4 Pairwise concordance between methods for *KRAS* mutation detection

		Direct sequencing		TheraScreen DxS		K-ras StripAssay		Pyrosequencing		HRM	
		+	-	+	-	+	-	+	-	+	-
Direct sequencing	+				0.338		0.257		0.735		0.537
	-										
TheraScreen DxS	+	5	15				0.790		0.555		0.739
	-	1	110								
K-ras StripAssay	+	5	21	19	7				0.438		0.500
	-	1	104	1	104						
Pyrosequencing	+	6	4	9	1	9	1				0.687
	-	0	121	11	110	17	104				
HRM	+	6	9	12	3	11	4	9	6		
	-	0	99	4	95	12	87	1	98		

Every intersection of method row and method column corresponds to a 2x2 contingency table for two methods. The upper right part of the table is filled with κ concordance metrics.

other authors [33], we interpret these results as reflecting a tendency of HRM to generate false positives. However, it is possible that they reflect rare mutations outside codon 12 and 13 that destabilize heteroduplex DNA even in the presence of an excess of wild-type DNA. Although cost and time efficiency are important factors in clinical diagnosis, the reproducibility of the HRM method will need to be improved before it can be considered viable. This could potentially be done by changing primers [34], adding melting standards [35], spiking with oligonucleotides [36], or combination with SNaPshot [37].

The StripAssay was the most analytically sensitive test (Table 2) of those we examined. On the basis of the results obtained with this method in the series of tests conducted with dilution series of mutant *KRAS* DNA (Figure 6), one could even argue that samples 24 to 30 should be reassigned as mutants (Table 2), thereby changing the false positive rate for the K-ras StripAssay to 0/128 and the false negative rate for TheraScreen DxS to 7/128. However, the interpretation of StripAssay results can be quite problematic for samples whose mutant DNA content is below 1% (see the result obtained with a mutant minority of 0.5% NCI-H620 in Figure 6). Insofar, it was not tested in clinical studies what is a significance of fraction of mutated cells below 1%, regardless of the typing method used.

During time of submitting this article, company´s software was upgraded to follow more precisely the requirements of ISO15189 norm (scanner calibration standard was added and manual baseline correction feature was removed). It remains to be seen if such changes bring any improvement to diagnostic accuracy.

Of the methods examined in this study, the TheraScreen DxS kit was the fastest method and exhibited the highest sensitivity and specificity. However, it was also the most expensive method that is not free of false reactions. Specifically, the kit failed to detect the p.Gly13Cys mutation in sample 2 because it is not designed to detect this mutation. Although the frequency of the mutations that are not covered by the TheraScreen DxS test is very low and clinically not highly relevant, this nevertheless constitutes an inherent limitation of the kit. In addition, the precise allelic mutation detected by this kit in samples 3, 16, and 18 differed from the consensus result. While this could potentially be due to stochastic variation in the early events of PCR priming, there is no firm evidence to support this hypothesis. Although discrepancies in the precise identity of the mutation are not yet clinically relevant, and these results were not scored as errors in this study, this finding warrants caution when using the ARMS Scorpions assay in different diagnostic setups, where the type of mutation is important (e.g. when looking at the T790M and S768I activating mutations in *EGFR* genotyping). As discussed above, samples 24 to 30 gave positive results in the StripAssay but were negative when analyzed with the TheraScreen DxS kit, and they seem to have a mutant population in the clinically "grey area," having less than 1% of the cells in the sample containing *KRAS* mutation. Ideally, their status should have been resolved by PCR amplicon cloning, followed by sequencing of the clones, digital PCR,

Table 5 Summary of the properties of the different methods

	Sanger sequencing	Pyrosequencing	TheraScreen DxS	StripAssay	HRM	
CE mark	no	no	yes	yes	no	**CE mark**
Limit of detection*	25-30 %*	5-10 %*	1 %	below 1 %	5-10 %*	**Limit of detection***
Turnaround time	2-3 days	2 days	1/2 day	1 day	1/2 day	**Turnaround time**
Ease of interpretation	easy	easy	easy	medium	difficult	**Ease of interpretation**
Technician time	6 hrs	4 hrs	2 hrs	5 hrs	2 hrs	**Technician time**
Amount of input DNA	1 reaction	1 reaction	8 reactions	1 reaction	1 reaction	**Amount of input DNA**
Detection of rare mutations	Yes – can detect any mutation located between the primers.	Yes – can detect any mutation within the short sequencing fragments.	No – can only detect 7 specific mutations.	No – can only detect 10 specific mutations.	Yes – can detect some mutations located between the primers.	**Detection of rare mutations**
Reagent cost	20 €	40 €	120 €	60 €	4 €	**Reagent cost**
Special equipment required	Sequencer 70 000 €	Pyrosequencer 150 000 €	Real time PCR cycler 30 000 €	PCR cycler 7 500 €	HRM Real time PCR cycler 75 000 €	**Special equipment required**

* from reference of Tsiatis[26] and Ogino[27].

or ultradeep sequencing. However, this approach is not practical for routine work and we did not have sufficient DNA to perform this experiment. Moreover, the low frequency of *KRAS* mutations in patient tumors have unknown clinical relevance, since all drug registration trials were performed using 1% of mutant *KRAS* cells as a low detection limit of the method.

The properties of the different methods examined in this work are summarized in Table 5.

We agree with Tsiatis et al. [27] that for research purposes more than one genotyping platform is necessary to reveal double mutations and to provide complementary data. In clinical settings, the most readily accessible NSCLC sample type is needle or brush biopsy, which is examined cytologically while resected, or biopsied tumors processed by formaldehyde fixation and paraffin embedding (FFPE). Proportion of FFPE samples from all samples usually reflects the best local practice and experience. Unfortunately, the FFPE process alters significantly the quality of DNA, and in many cases the DNA isolation from cytology smears yields higher quality albeit lower quantity of DNA.Very low quantity of available DNA isolated from cytological preparations was a major limiting factor in our comparative study, which we tried to overcome using frozen tissue from biobank, since it provides both high quality and quantity of DNA. Moreover, due to recent biobanking initiatives [38], we are more frequently facing situations, where the tumor molecular diagnostics is performed from frozen tissues. Of the 11 FFPE samples genotyped using both the StripAssay and TheraScreen, 5 samples could not be typed by at least one method, 2 samples were wildtype by both methods, 3 samples were mutant by both methods, and one sample was p.Gly12Asp by TheraScreen and wildtype by StripAssay. From one point of view, it could be argued that our genotyping results obtained using frozen samples are transferable to genotyping of FFPE samples because the mechanisms by which the methods work are not dependent on the nature of the input sample. On the other hand, it should be noted that improperly-performed paraffin embedding damages DNA and can favor methods that are more robust to variation in the amount and quality of the starting material (this would arguably disfavor TheraScreen because it requires eight PCR reactions whereas the other methods require only one equivalent reaction). It has been suggested that the issue of limited material for testing can be largely circumvented by using whole genome amplification techniques [39,40], although the potentially biasing impact of the genome amplification techniques on low frequency somatic mutation genotyping is still not fully addressed. However, we suppose that our tests of kit performance on frozen tissue samples provide useful insights into their general utility and will be valuable for orchestrating genotyping efforts across molecular pathology laboratories.

Conclusions

The performance of five methods (Direct sequencing, Pyrosequencing, High resolution melting analysis, the TheraScreen DxS kit, and the K-ras StripAssay) for detecting mutations in the *KRAS* gene was compared using DNA extracted from 131 frozen NSCLC samples. The TheraScreen DxS kit was found to be the most effective, followed by the StripAssay kit. However, because of the heterogeneity of typical cancer tissue samples and the differences in the two methods' mechanisms of action, there are still unsatisfactory numbers of discrepancies between these two 'best' methods, which failed to agree on 8 of the 131 specimens examined in this work. Nevertheless, our findings should facilitate the rational selection of methods for detecting mutations at the *KRAS* locus using heterogeneous clinical samples obtained from biopsies of cancer patients.

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2.2 COLD-PCR for improvements of mutation detection threshold

2.2.1 Introduction and aim of the experiment

COLD-PCR is a modification of a conventional PCR method, based on replacing usual denaturation temperature with "critical" denaturation temperature (Tc) for each amplified DNA sequence. Tc is lower (colder) than melting temperature (Tm) of the same amplicon and is chosen to allow denaturation of mainly heteroduplexes which then become amplified, whereas homoduplexes are not quantitatively denatured and amplified efficiently. This method is able to selectively amplify minority mutant alleles in a wild-type DNA background, what is situation often found in heterogeneous cancer tissue. Thus, it has capacity to increase the mutation detection sensitivity (mutant to wild-type ratio) which is extremely important when using clinical specimens.

The aim of this study was to test whether this method can improve the detection sensitivity of Direct capillary sequencing for detecting *KRAS* gene mutations. Direct sequencing has very unfavourable detection limit (25 %) for mutated gene in the context of the surplus of wild-type DNA.

Scheme of the method

Figure 1. Determination of critical temperature for *KRAS* DNA PCR amplified–fragment and sensitivity of COLD-PCR was assessed and compared to standard PCR.
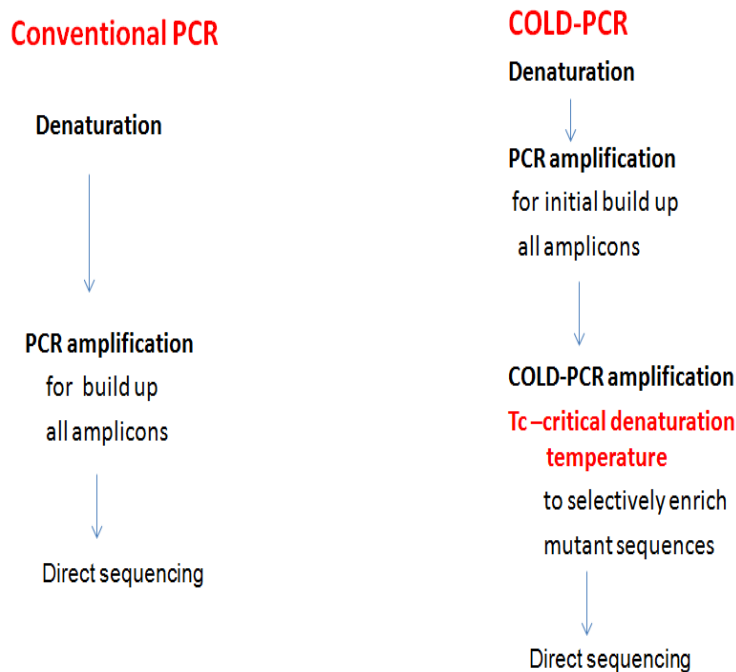
2.2.2 Materials and methods

Samples:

For this experiment sample DNA from homozygote cell line A549 (including mutation in *KRAS* G12S -AGT, exon 2) and homozygote cell line ASPC-1 (including mutation in *KRAS* G12D-GAT, exon 2, according to COSMIC/Catalogue of somatic mutation in cancer Database, (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) and wild-type *KRAS* DNA isolated from patients blood was mixed in different dilution ratio of mutated DNA (50%, 25%, 12.5%, 6.25%, 3.12%, 1.5%).

Cell line cultivation, DNA isolation and concentration estimation:

Cell lines were ordered from ATCC (American Type Culture Collection) and cultivated according to ATCC description for individual cell lines, see http://www.lgcstandards-atcc.org/?geo_country=cz. Extraction of DNA from blood and cell lines was performed using the DNAeasy® Blood and Tissue purification kit, Qiagen®, performed according to manufacturer's instructions in DNeasy Blood & Tissue Hadbook (Edition 07/2006). Concentrations of DNA samples were measured spectrophotometrically using a NanoDrop ND 1000 Spectrophotometer V3.7 (NanoDropTechnologies, Thermo Scientific Wilmington, USA) according to User's Manual.

PCR primer design:

KRAS specific primers were designed using Primer3 program (http://simgene.com/Primer3). For design of primers input parameters used were: product size 100-300bp, primer length 18-30 bp, primer annealing temperature 56-65°C, GC content 30-70%. Primer positions on chromosome and length of the primers were confirmed using UCSC database In Silico PCR (http://genome.ucsc.edu/cgi-bin/hgPcr). Primers were ordered from Generi Biotech (the Czech Republic).

Table 1. *KRAS* specific fragment primers

Primers	nucleotide sequence	Product (bp)
Forward	5'- AAAAggTACTggTggAgTATTTgA-3'	293
Reverse	5'- TCATgAAAATggTCAgAgAAACC--3'	

USSC-In Silico PCR result (KRAS fragment product, yellow marked codons 12 and 13)

```
>chr12:25398126-25398418 fragment length (293bp)
293bp AAAAGGTACTGGTGGAGTATTTGA
TCATGAAAATGGTCAGAGAAACCAAAAGGTACTGGTGGAGTATTTGAtagtgtattaaccttatgtgtgacatgttc
taatatagtcacattttcattatttttattataaggcctgctgaaaatgactgaatataaacttgtggtagttggag
ctggtggcgtaggcaagagtgcccttgacgatacagctaattcagaatcattttgtggacgaatatgatccaacaata
gaggtaaatcttgttttaatatgcatattactggtgcaggaccattctttgatacagataaaGGTTTCTCTGACCAT
TTTCATGA
```

Validation of the critical temperature COLD-PCR:

Critical temperature of COLD-PCR was assessed using gradient of temperature ranging from 79°C to 84°C (79.0°C, 79.2°C, 79.5°C, 80°C, 80.6°C, 81.2°C, 81.8°C, 82.4°C, 83°C, 83.5°C, 83.8°C, 84.0°C). Testing and optimization was performed using PCR Gradient Cycler Biometra and PCR-chemicals (ThermoScientific). DNA including 75% of wildtype and 25% of *KRAS* mutant G12D (AGT) allele was tested. This experiment was repeated for 12.5% mutated DNA.

Table 2. PCR-reagents concentration used for assessing the critical temperature:

Reagents concentration	Final conc.	µl/1 reaction
H_2O		13.04
10xPCR buffer	1x	2
dNTPs 25mM	0.2mM	0.16
$MgCl_2$ 25mM	2mM	1.6
Primer Forward 10µM	0.5µM	1
Primer Reverse 10µM	0.5µM	1
ThermoTaq polymerase 5U/µl	1U	0.2
DNA 20 ng/µl	1µl	1
Total volume		v=20µl

Table 3. Temperature condition used for PCR reaction:

Gradient-Cycler Biometra			
PCR-program:			
Polymerase initialization	95°C	15min	
Denaturation	95°C	15s	
Annealing	65°C	30s	10 cycles
Elongation	72°C	20s	
Gradient	79-84°C	15s	
Melting	56°C	50s	35cycles
Elongation	72°C	20s	
Cooling	25°C	hold	

Melting profile of PCR gradient temperature was checked on LightCycler480

Figure 2. Melting temperature plot for samples amplified at Ct range 79.0-84.0°C
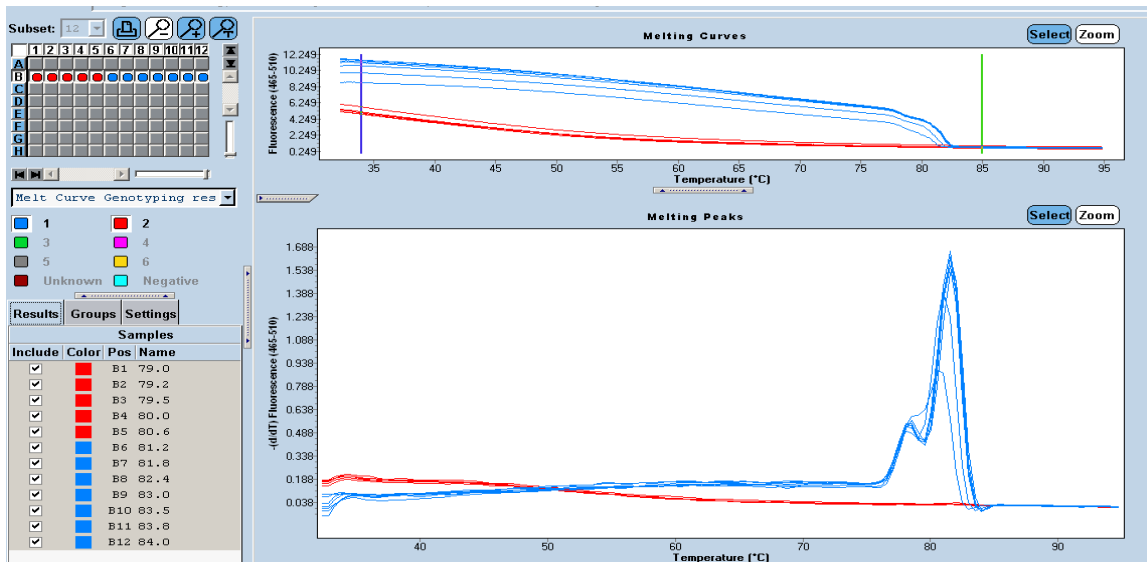
red curve- melting temperature gradient for PCR with Tc from 79.0°C to 80.6°C (DNA product not amplified)
blue curve- melting temperature gradient for PCR with Tc from 81.2°C to 84.0 °C (DNA product amplified)

According to melting temperature plot results of amplified target DNA, temperature range 81-84°C was chosen for later analysis on capillary electrophoresis system Beckman 8800. Sequencing was done according to standard Beckman Coulter sequencing user's guide CEQ^{TM} 8800 (April 2004) using Beckman Coulter GenomeLab DTCS Terminator Quick Start kit (December 2009).

Table 4. Primer used for sequencing reaction:

Sequencing primer	nucleotide sequence
	5'-TTAACCTTATgTgTgACATgTTCTAA-3'

Figure 3. Sequencing result for COLD-PCR using Sanger sequencing on Beckman 8800

25% mutated DNA_grad-**81°C**

effect of COLD-PCR
(mutated DNA is preferentially amplified)

25% mutated DNA _ grad-**82°C**
effect of COLD-PCR

(mutated DNA is preferentially amplified)

25% mutated DNA_grad- **83°C**
no effect of COLD-PCR
mutated DNA is in standard dilution rate
(25% mutation allele vs.75% wild type allele)
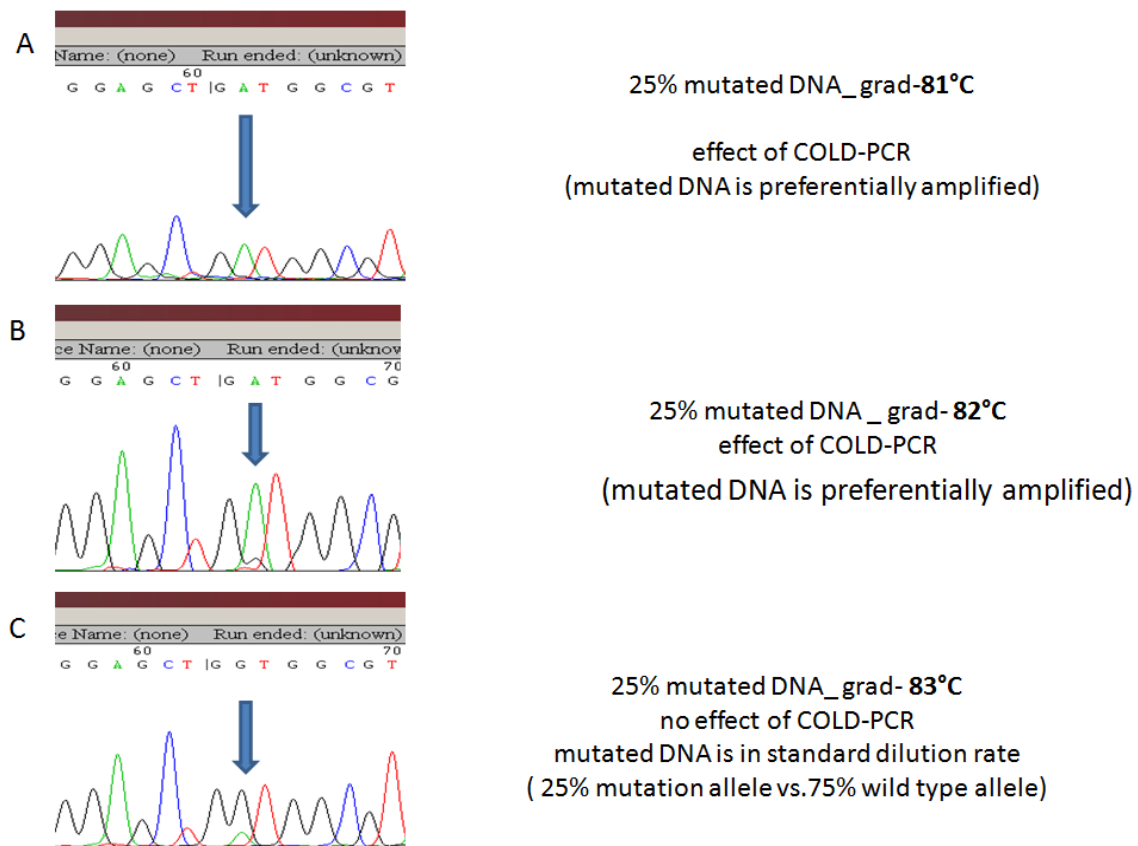
 Picture A and B showing preferentially amplified mutated DNA (wild type GGT codon replaced to GAT mutated codon with A depicted as green peak) Critical melting temperature for COLD-PCR was found to be in the range 81-82°C.
 Picture C showing no effect of 83°C denaturation temperature to amplification of (25% mutated DNA in 75% wildtype DNA).
DNA was mixed to reach 25% mutated allele vs. 75% wildtype allele. Mutated DNA green peak is small, sequencing result is showed by automatic software analyzer to be a wild-type GGT result.

Determination of detection limit of COLD-PCR in comparison to standard PCR (in *KRAS* mutation detection):

According to temperature optimization results, COLD-PCR was run at 82°C critical temperature (Tc). Six different percentages of mutated DNA were evaluated. Dilution series was prepared using DNA from cell line A549 homozygous for *KRAS* G12S (AGT). Mutation and wildtype DNA from patient's blood in ratio was: 50%, 25%, 12.5%, 6.25%, 3.12%, 1.5%. Experiment was run twice.

2.2.3 Result

wildtype codon- GGT (black peak) / **A**GT- mutated codon (A is green peak)

COLD-PCR

6.25% mutated DNA

3.12% mutated DNA
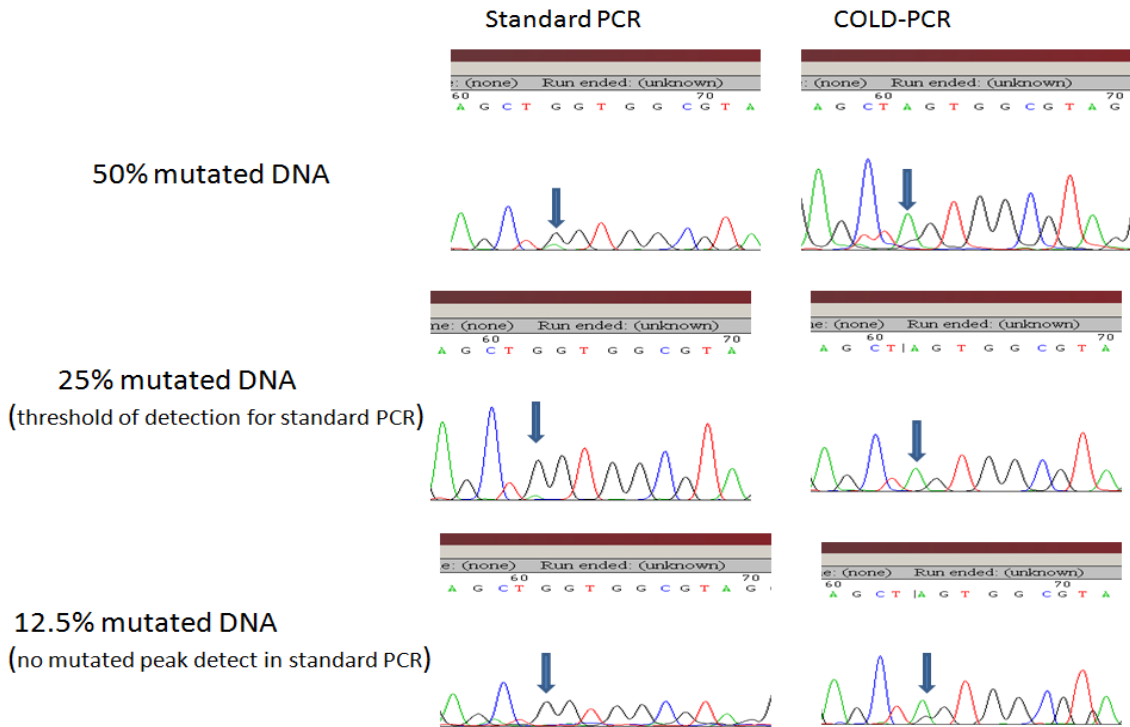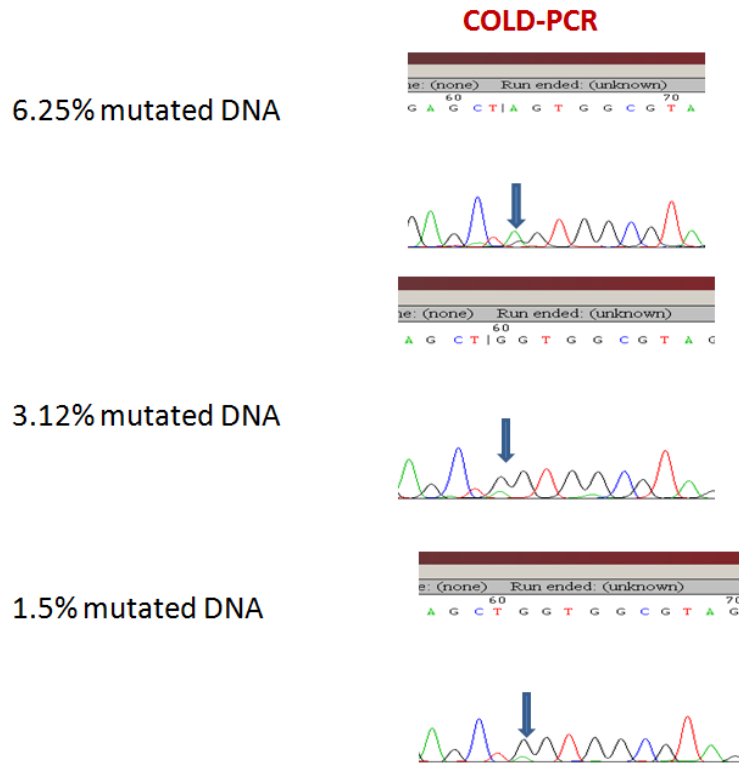
1.5% mutated DNA

2.2.4 Summary of the COLD-PCR result

Using standard PCR and Sanger capillary sequencing method, the mutated (green) peak can be detected only when minimally 25% of mutated DNA was present in the context of wild-type background (detection limit for capillary sequencing when standard PCR is used is only 25%). This detection limit was also confirmed and described by other authors (Arcila, 2011; Obradovic, 2012)

For COLD-PCR method, critical temperature (Tc) of *KRAS* fragment was found to be 82°C. At this critical temperature, mutation of *KRAS* gene fragment in exon 2 has been consistently detectable by capillary Sanger sequencing when minimally 6.25% of mutated DNA was present in the context of non mutated DNA. Frequently, mutation signal could be still detected when only 1.5% mutated DNA was mixed with wildtype DNA. These results demonstrated that COLD-PCR can preferentially amplify minority of mutated DNA in high background of non-mutated DNA, and can be appropriate method for improving detection limit of capillary sequencing on Beckman 8800 Sequence Analyzer. It changed detection limit from 25% (when using standard PCR) to 6.25% (1.5%) (when using COLD-PCR).

2.3 Application of the Next Generation Sequencing (NGS) for testing of KRAS and BRAF oncomarkers, and comparison of NGS with CE-methods.

2.3.1 Introduction and aim of the experiment

As stated previously in this thesis, NGS is a general term describing novel multiparallel sequencing technologies with high-throughput capacity. From a clinical perspective, advantage of NGS lays in the possibility of designing variable panels with flexible throughputs and adjusted minority variant detection limit by defined coverage.

Sequencing coverage refers to the number of times a single base within genome is sequenced, which provides truly superior quantitative ability of mutation detection threshold. This approach offers advantage for detection of mutations in highly heterogeneic tumor samples.   The observed heterogeneity is due to variable content of normal cells, inflammatory cells, and existence of sub-clones of cancer cells in the cancer biopsy which may carry mutated oncogenes *KRAS/BRAF*.

KRAS mutation appears mainly in the codons 12 and 13, and is usually found in about 40% colorectal cancer patients (mainly adenocarcinoma type), and in about 25% lung cancer patients (mainly of NSCLC type). In *BRAF* gene, the most often found mutation is V600E. This mutation is present in about 15% colorectal cancer patients tested, and in about 45% thyroid cancer patients (mainly papillary carcinoma type).

We expect that NGS protocols already reached the level of the quality similar to CE-IVD methods based on Real-Time PCR, regarding analytical parameters as sensitivity, specificity, and limit of detection. Also, we expect that other parameters important for daily laboratory routine i.e. cost, handling time, and robustness are appropriate for routine clinical diagnostics in the field of cancer biomarkers.

2.3.2 Materials and methods

Samples:

A series of 36 DNA samples from formalin fixed paraffin embedded tissue (FFPE) biopsies were investigated including colorectal cancer (20 samples), lung cancer (4 samples), thyroid cancer (12 samples), BRAF V600E FFPE reference control standard (Horizon diagnostics) with pre-defined mutant alleles ~1.5%, and KRAS G12V control standard ~1.5% constructed by mixing SW620 homozygous cell line (ATCC-American Type Culture Collection) with wild-type DNA (MG7003) isolated from blood.

DNA isolation and concentration measurement:

Extraction of DNA from formalin fixed paraffin embedded tissue (FFPE) was performed by Cobas® DNA Sample Preparation Kit, Cobas® (Roche Molecular System, Inc.) as per manufacturer's instructions (Handbook Edition 05/2011 Rev 1.0). This kit contains glass fibers filter columns which bind nucleic acids allowing efficient purification of DNA. Deparafinization was done using xylene on (5µm) slide sections of an FFPE slice. Tissues were lysed using lysis/binding buffer that releases nucleic acid while protecting it from DNases. Subsequently, released DNA was precipitated by mixing with isopropanol and mixture was spun down. During centrifugation of lysate mixture, the genomic DNA bound to the surface of the glass fibers filters. Unbound component of the mixture such as proteins, salt, and other cellular impurities, were removed during centrifugation with ethanol-based washing buffers. Subsequently, DNA was released from filter surface by elution to the 50µl elution buffer (10 mM Tris HCl, pH=8.5).

For DNA isolation from cell lines and blood, and to prepare KRAS Reference Standard with pre-defined mutant alleles ~1.5%, DNAeasy® Blood and Tissue purification kit, Qiagen® extraction was performed according manufacturer's instructions (DNeasy Blood & Tissue Hadbook, Edition 07/2006). For adsorption of DNA and removal of cellular contamination and enzyme inhibitors, this kit contains silica-based membrane spin column with DNA-binding properties. DNA was released from filter surface by elution to the 100 µl elution buffer (10 mM Tris HCl pH=8.5).

Concentration and purity of isolated DNA was subsequently assessed using NanoDrop 2100 spectrophotometer (NanoDrop Technologies, Wilmington, USA) and using Agilent DNA 1000 Kit, according to instructions (Agilent DNA 1000 Kit Quick Start Guide, Edition 04/2007).

Samples investigated by methods:

In this study, two NGS platforms were used: GS Junior 454 (Roche) and MiSeq (Illumina). Both techniques were optimized and applied for screening of oncomarkers *KRAS* exon 2, codon 12 and 13, and *BRAF* exon 15, codon 600. Sensitivity and specificity of the NGS methods were compared to Real Time PCR methods, according to manuals supplied with CE-IVD kits. Real Time TheraScreen® K-RAS (Qiagen) Mutation Detection Kit was used for *KRAS* gene testing, and PNAClamp™ (Panagene) BRAF Mutation Detection Kit, and *BRAF* Mutation Detection Kit (IntellMed) for detection of mutation in the *BRAF* gene.

From 36 samples, 23 samples (including 22 patients samples with unknown mutation status and one control standard sample ~1.5% mutation) were tested on *KRAS* by TheraScreen® K-RAS (Qiagen) Mutation Detection Kit, and 13 (including 12 patients samples with unknown mutation status and one control standard sample ~1.5% mutation) were tested on *BRAF* by PNAClamp™ (Panagene), and BRAF Mutation Detection Kit (IntellMed). All of 36 tested samples were later investigated by NGS Roche and Illumina methods.

2.3.2.1 CE-certified Real-Time PCR Mutation Detection diagnostic methods used for detection of *KRAS* and *BRAF* genes

TheraScreen® K-RAS (Qiagen) Mutation Detection Kit is *in vitro* diagnostic test intended for detection of 7 somatic mutations (codon 12: Ala, Asp, Arg, Cys, Ser, and Val, codon 13: Asp) in the human *KRAS* oncogene on exon 2. This method provides qualitative assessment of mutation status and can detect 1% of mutant in the background of wildtype genomic DNA. TheraScreen Real-time assay is based on combination of ARMS® (Amplification Refractory Mutation System) and Scorpions® (primer linked with the probe). ARMS system enables allele-mutation specific amplification, and the use of Scorpions probe technology enables detection of amplified DNA by fluorescence signal in real time cycler. The test kit includes also an internal synthetic template for quality control of every individual primer, and control reaction for amplification of a region in the exon 4 of the *KRAS* gene to assess the total DNA in the sample. The delta-C_T values between the control reaction and the allele specific reaction reflect the amount of *KRAS* mutation within the sample and should not be higher than 8 to 9 cycles compared to a positive reaction. This kit was used according to Roche user manual (LightCycler®480 Real Time PCR System, Version 05/2009). Ct values were analyzed manually according to instructions in the manual without use of any specific analyzer software.

PNAClamp^{TM} BRAF Mutation Detection Kit (Panagene) is a diagnostic test intended for detection of *BRAF* V600E mutation in the codon 600, exon 15. Technology of PNAClapm® is based on Real-Time PCR with peptide nucleic acid clamp probe which is artificially created DNA analog in which phosphodiester backbone is replaced by a peptide-like one formed by 2-aminoethyl-glycine units. Wildtype-specific PNA clamp probe is not recognized by DNA polymerase as primer and it has strong binding affinity to the wildtype DNA, inhibiting its amplification. Only mutated *BRAF* DNA sequences are selectively amplified from the mixture of wildtype DNA and mutated DNA. Sensitivity of the test is <1%. The kit was used according to user manual instructions (Version 1.5 Rev.12/2012), and PCR was run on Roche LightCycler®480 Real Time PCR System. Analysis was done according to instructions in the manual without use of any specific analyzer software (only Excel).

BRAF Mutation Detection Kit (IntellMed) is diagnostic test for identification of *BRAF* V600E mutation in the codon 600, exon 15. This test is based on Real-Time PCR with fluorescency measurement of amplification using two fluorescent channels HEX and JOE. Test also includes internal quality control with pair of primers for globin gene, present in every human sample. Internal control must yield signal when amplifiable DNA is present in the sample. Sensitivity of the test is at least 1% mutated DNA in background of wildtype DNA. The kit was used according to user manual instructions (Version 06/2013), and PCR was run on Roche LightCycler®480 Real Time PCR System. Analysis was done according to instructions in the manual without use of any specific analyzer software (only MS Excel).

2.3.2.2 Experimental design for Next Generation Sequencing on GS Junior 454 (Roche) technology

Schema of the method

1 PCR amplification
M13 sequence (UnivA/B)+template specific primers (KRAS/BRAF)

1000x dilution of
amplified product

2 PCR amplification
Universal primers A/B –MID- M13 sequence (Univ A'/B')

Purification of the product,
dilution of the product and
pooling

Clonal amplification on beads
Emulsion PCR amplification (LibA)

Sequencing
GS Junior 454-Roche

Data analysis
AVA software
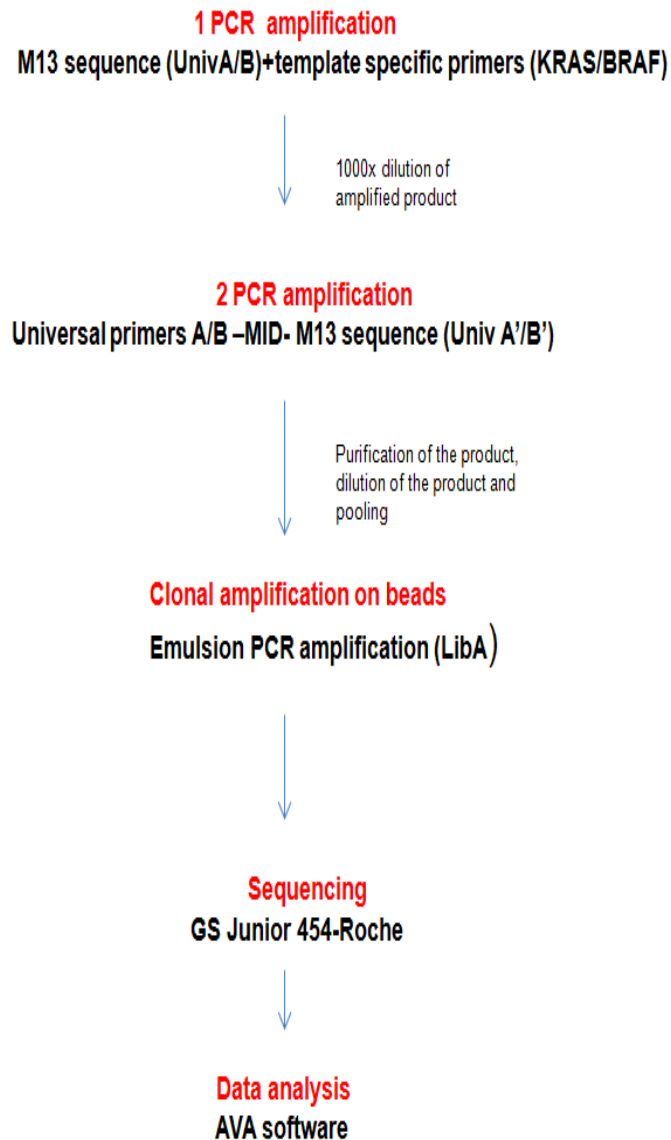
Library preparation:

In this study the *Universal Tailed Amplicon design* was performed. This particular design includes two step PCR amplification process and use of designed fusion primers which are created according to GS Junior System Guidelines for Amplicon Sequencing Design, (Edition 04/2010).
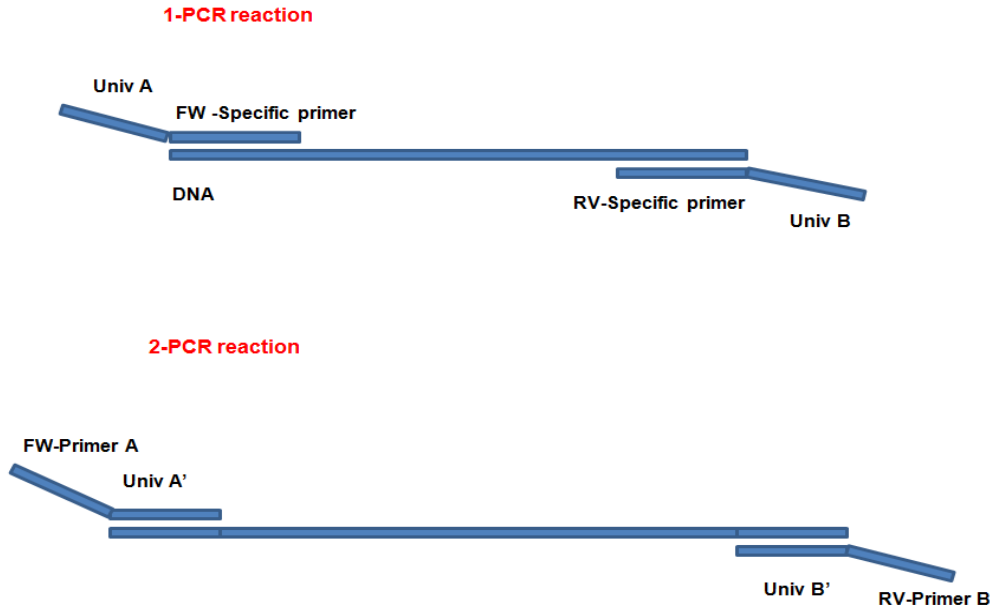
Schema of *Universal Tailed Amplicon design* including two step amplification process.

In the first PCR reaction, to generate basic library, the fusion primers were composed from two parts fused together: universal part (universal sequencing primer A and B) and specific part (specific primers for *KRAS* gene fragment or *BRAF* gene fragment, respectively).

In the second PCR, universal tail A or universal tail B of the 5' end fusion primers from the first PCR was used to bind universal forward and reverse sequencing primers that possess 17-mer M13 sequence. This enabled us to target the fusion primers in the second PCR reaction (Table1).

Table 1. Sequence of M13 primers (these sequences create 5' part of fusion primers)

M13 universal sequencing primers	nucleotide sequence
Forward primer (A)	5'-gTTTTCCCAgTCACgAC-3'
Reverse primer (B)	5'-CAggAAACAgCTATgAC-3'

Specific, 3′ part of the fusion primers, were designed so it can amplify the desired sequence of interest: *KRAS* and *BRAF* fragment products. UCSC (http://genome.ucsc.edu) was used as source information about reference sequence of the genes, position of the genes in the genome. Primers were designed and selected from DNA sequences using free online Primer3 program (http://simgene.com/Primer3). For design of primers standard condition was used: product size 200-300bp, primer length 18-30bp, primer annealing temperature 57-60°C, and 30-70% of GC content.Primer positions on chromosome and length of the primers were confirmed using In Silico PCR (http://genome.ucsc.edu/cgi-bin/hgPcr). All primers were ordered from Generi Biotech (the Czech Republic).

Table 2. Specific sequence of primers for *KRAS* and *BRAF* genes (this sequence creates 3' part of fusion primers)

Specific primer	Exon (including codons)	Nucleotide sequence	Product (bp)
KRAS Forward	exon 2 (codon 12, 13)	TggTggAgTATTTgATAgTgTATTAACCT	282
KRAS Reverse	exon 2 (codon 12, 13)	ATgAAAATggTCAgAgAAACCTTTATC	282
BRAF Forward	exon 15 (codon 600)	TgCTTgCTCTgATAggAAAATg	222
BRAF Reverse	exon 15 (codon 600)	TCAgggCCAAAAATTTAATCA	222

USSC-In Silico PCR result (*KRAS* fragment product, codons 12 and 13 are highlighted with yellow)

```
>chr12:25398128-25398409 282bp (fragment length)
TGGTGGAGTATTTGATAGTGTATTAACCTtatgtgtgacatgttctaata
tagtcacattttcattattttttattataaggcctgctgaaaatgactgaa
tataaacttgtggtagttggagctggtggcgtaggcaagagtgccttgac
gatacagctaattcagaatcattttgtggacgaatatgatccaacaatag
aggtaaatcttgtttttaatatgcatattactggtgcaggaccattctttg
atacaGATAAAGGTTTCTCTGACCATTTTCAT
```

UCSC-In Silico PCR result (*BRAF* fragment product, codon 600 is highlighted with yellow)

```
>chr7:140453029-140453250 222bp (fragment length)
TGCTTGCTCTGATAGGAAAATGagatctactgttttcctttacttactac
acctcagatatatttcttcatgaagacctcacagtaaaaataggtgattt
tggtctagctacagtgaaatctcgatggagtgggtcccatcagtttgaac
agttgtctggatccatttttgtggatggtaagaattgaggctatttttcca
cTGATTAAATTTTTGGCCCTGA
```

Table 3. Fusion primers created for 1-PCR reaction

(including M13 sequence and specific primer sequence):

Fusion primers	nucleotide sequence
Forward primer	5'-universal tail A (Univ-A) M13- template-specific sequence-3'
Reverse primer	5'-universal tail B (Univ-B) M13 - template-specific sequence-3'
Forward primer KRAS (exon2, includes codon 12 and 13)	5'-gTTTTCCCAgTCACgACTggTggAgTATTTgATAgTgTATTAACCT -3'
Reverse primer KRAS (exon2, includes codon 12 and 13)	5'-CAggAAACAgCTATgACATgAAAATggTCAgAgAAACCTTTATC-3
Forward primer BRAF (exon 15, includes codon 600)	5'gTTTTCCCAgTCACgACTgCTTgCTCTgATAggAAAATg-3
Reverse primer BRAF (exon15, includes codon 600)	5'-CAggAAACAgCTATgACTCAgggCCAAAAATTTAATCA-3

Fusion primers for first 1-PCR reaction, gDNA 10-200 ng/µl templates, and FastStart High Fidelity PCR system dNTPack (Roche, Manual version 07, 2011), were amplified on Roche 480 LihgtCycler. High-fidelity polymerase was used to avoid amplification-derived variations in the sequence that we encountered when low-fidelity polymerase was used (data not shown). Primer binding was optimized by annealing temperature to achieve one specific product for *KRAS* and *BRAF* in one PCR amplification step, respectively. In this study, presumably fragmented FFPE DNA was used as a template which is likely to include very low amount of not degraded DNA copy template resulting in very late cycle amplification. Therefore, PCR was run for 50 cycles. Melting program was run to check for primer dimers, non specific products, and/or not amplified products.

Table 4. Final reaction concentration used for 1-PCR reaction

1-PCR		
Reagents concentration	**Final conc.**	**µl/1 reaction**
H_2O		13.64
10xPCR buffer with 18mM $MgCl_2$	1x, 1.8 mM	2
dNTPs 25 mM	0.2 mM	0.16
Eva Green 20x	1x	1
Primers mix 10 µM fusion primers (Fw and Rv mix) KRAS or BRAF	1 µM each	2
High fidelity polymerase 5U	1 U	0.2
DNA	1 µl	1
Total volume		**V=20 µl**

Fusion primers used–(including M13 nucleotide sequence part and *KRAS/BRAF* specific sequence part).

Table 5. Final temperature condition used for 1-PCR reaction:

LightCycler 480 Roche			
PCR-program:			
Polymerase initialization	95°C	5 min	
denaturation	95°C	30 s	
annealing	59°C	30 s	50 cycles
elongation	72°C	40 s	
melting	95°C	10 s	
	60°C	1 min	
	95°C	Continuous fluorescence signal gathering	
cooling	37°C		

For the second PCR reaction, the samples from first PCR were diluted 1:1000.

Fusion primers Forward A and Reverse B used in the second step PCR (2-PCR) were created according to GS Junior System Guidelines for Amplicon Experimental Design (Edition 04/2010).

Fusion primers were composed of three parts which are linked together: 25-mer sequence including key sequence, mid sequence, and universal tail A' or B'.

The first part, 25-mer sequence (Forward and Reverse) are dictated by requirements of sequencing system to enable binding to the DNA Capture Beads (Lib-A), and to enable annealing the em-PCR Amplification Primers and the Sequencing Primers. These primers part always end with the sequencing key TCAG for library identification. Second part, named Multiplex Identifiers (MIDs) labels every individual sample to distinguish between library during sequencing process and analysis. The third part, named universal tail A' (Univ-A') and universal tail B' (Univ B'), is complementary to the universal tails A and B from first PCR reaction. Fusion primers were created according to GS Junior System Guidelines for Amplicon Experimental Design (Edition 04/2010).

Table 6. The fusion primers A and B created for the 2-PCR reaction:

Forward-Primer A	5'-Forward primer A-KEY-(MID)-universal tail A'(Univ-A')-3'	
Forward-Primer A	5-' CgTATCgCCTCCCTCgCgCCATCAg -(MID)- gTTTTCCCAgTCACgAC-3'	
Forward-Primer A	5'-CgTATCgCCTCCCTCgCgCCATCAg-(ACgAgTgCgT)- gTTTTCCCAgTCACgAC-3'	MID1
Forward-Primer A	5'-CgTATCgCCTCCCTCgCgCCATCAg(ACgCTCgACA)-gTTTTCCCAgTCACgAC-3'	MID2
Forward-Primer A	5' -CgTATCgCCTCCCTCgCgCCATCAg-(AgACgCACTC)-gTTTTCCCAgTCACgAC-3'	MID3
Forward-Primer A	5'-CgTATCgCCTCCCTCgCgCCATCAg - (AgCACTgTAg)-gTTTTCCCAgTCACgAC-3'	MID4
Forward-Primer A	5'-CgTATCgCCTCCCTCgCgCCATCAg-(ATCAgACACg)-gTTTTCCCAgTCACgAC-3'	MID5
Forward-Primer A	5'-CgTATCgCCTCCCTCgCgCCATCAg-(ATATCgCgAg)-gTTTTCCCAgTCACgAC-3'	MID6
Forward-Primer A	5'-CgTATCgCCTCCCTCgCgCCATCAg-(CgTgTCTCTA)-gTTTTCCCAgTCACgAC-3'	MID7
Forward-Primer A	5'-CgTATCgCCTCCCTCgCgCCATCAg- (CTCgCgTgTC)- gTTTTCCCAgTCACgAC-3'	MID8
Forward-Primer A	5'-CgTATCgCCTCCCTCgCgCCATCAg(TAgTATCAgC)-gTTTTCCCAgTCACgAC-3'	MID9
Forward-Primer A	5'-CgTATCgCCTCCCTCgCgCCATCAg-(TCTCTATgCg)- gTTTTCCCAgTCACgAC-3'	MID10
Forward-Primer A	5'-CgTATCgCCTCCCTCgCgCCATCAg -(TgATACgTCT)- gTTTTCCCAgTCACgAC-3'	MID11
Forward-Primer A	5'- CgTATCgCCTCCCTCgCgCCATCAg-(TACTgAgCTA)-gTTTTCCCAgTCACgAC-3'	MID12

Reverse-Primer B	5'-Reverse primer B-KEY-(MID)-universal tail B'(Univ-B')-3'	
Reverse-Primer B	5-'CTATgCgCCTTgCCAgCCCgCTCAg-(MID)-CAggAAACAgCTATgAC-3'	
Reverse-Primer B	5'-CTATgCgCCTTgCCAgCCCgCTCAg-(ACgAgTgCgT)-CAggAAACAgCTATgAC-3'	MID1
Reverse-Primer B	5'- CTATgCgCCTTgCCAgCCCgCTCAg-(ACgCTCgACA)-CAggAAACAgCTATgAC-3'	MID2
Reverse-Primer B	5'- CTATgCgCCTTgCCAgCCCgCTCAg -(AgACgCACTC)- CAggAAACAgCTATgAC-3'	MID3
Reverse-Primer B	5'- CTATgCgCCTTgCCAgCCCgCTCAg-(AgCACTgTAg)-CAggAAACAgCTATgAC-3'	MID4
Reverse-Primer B	5'- CTATgCgCCTTgCCAgCCCgCTCAg-(ATCAgACACg)-CAggAAACAgCTATgAC-3'	MID5
Reverse-Primer B	5'- CTATgCgCCTTgCCAgCCCgCTCAg- (ATATCgCgAg)-CAggAAACAgCTATgAC-3'	MID6
Reverse-Primer B	5'- CTATgCgCCTTgCCAgCCCgCTCAg-(CgTgTCTCTA)-CAggAAACAgCTATgAC-3'	MID7
Reverse-Primer B	5'- CTATgCgCCTTgCCAgCCCgCTCAg-(CTCgCgTgTC)-CAggAAACAgCTATgAC -3'	MID8
Reverse-Primer B	5'- CTATgCgCCTTgCCAgCCCgCTCAg-(TAgTATCAgC)-CAggAAACAgCTATgAC-3'	MID9
Reverse-Primer B	5'- CTATgCgCCTTgCCAgCCCgCTCAg-(TCTCTATgCg)- CAggAAACAgCTATgAC-3'	MID10
Reverse-Primer B	5'- CTATgCgCCTTgCCAgCCCgCTCAg-(TgATACgTCT)-CAggAAACAgCTATgAC-3'	MID11
Reverse-Primer B	5'- CTATgCgCCTTgCCAgCCCgCTCAg-(TACTgAgCTA)-CAggAAACAgCTATgAC-3'	MID12

Table 7. Final reaction concentration used for 2-PCR reaction with fusion primers A and B

2-PCR		
Reagents concentration	Final conc.	µl/1 reaction
H20		13.64
10xPCR buffer with 18 mM MgCL$_2$	1x, 1.8 mM	2
dNTPs 25 mM	0.2 mM	0.16
Eva Green 20x	1x	1
Primers mix 10 µM fusion primers (Fw and Rv mix)	1 µM each	2
High fidelity polymerase 5U	1 U	0.2
DNA (diluted 1:000 from 1-PCR)	1 µl	1
Total volume		V=20µl

Table 8. Final temperature condition used for 2-PCR reaction:

LightCycler 480 Roche			
PCR-program:			
Polymerase initialization	95°C	5 min	
denaturation	95°C	30 s	
annealing	64°C	30 s	20 cycles
elongation	72°C	40 s	
melting	95°C	10 s	
	60°C	1min	
	95°C	Continuous fluorescence signal athering	
cooling	37°C		

After second PCR reaction, PCR products were purified using QIAquick PCR Purification Kit (Qiagen) according to manufacturer's protocol (Edition 10/2010). The final product was eluted using 25 µl of molecular grade water.

Subsequently, concentration of DNA in the eluted samples was tested on Agilent Bioanalyzer 2100, using Agilent DNA 1000 Assay Protocol (Edition 04/2010). The final product was diluted to obtain the standardized concentration of 20ng/µl for all prepared samples. All samples library were then pooled together to generate one pooled library.

Clonal amplification–emulsion PCR (emPCR):

Pooled library was then diluted to obtain a working solution concentration of 1×10^6 molecules/µl according calculation *molecules/µl=[sample concentration (ng/µl) x 6.022 x 10^{23}]/[656.6 x 10^9x library length (pb)]* and volume of DNA library calculation *µl of DNA library per tube =[desired molecules per bead (2 molecules per bead)x 5milion beads]/[library concentration (in molecules/µl)]* that is translated into required molar equivalent of 2-DNA molecules of library DNA per one capture bead. This step allows obtaining appropriate level of bead enrichment required for satisfactory sequencing results.

DNA library molecules were clonally amplified on beads which are compartmentalized into water-in-oil emulsion. During this process, one DNA molecule per bead is amplified to ~ 10 million copies. Clonal amplification using emPCR was performed according to manufacturer instruction regarding GS Junior Titatum series, emPCR Amplification Method Manual-Lib-A (Edition 05/2010 Rev. 04/2011)

Sequencing:

DNA enriched beads were separated and deposited into picotiter plate along with small enzyme beads. This process was provided using GS Junior Titanium Sequencing kit in combination with GS Junior Titanium PicoTiterPlate kit, according to Sequencing Method Manual, GS Junior Titatnium Series (Edition 05/2011, Rev.06/2010). This kit provides reagents and components for single sequencing run. Sequencing run was performed using GS 454-Junior (Roche) sequencer.

Data analysis:

To analyze generated data, we used GS Roche 454 Amplicon Variant Analyzer (AVA) 2.6 version software (Roche). This software provides easy and robust analysis enabling identification of variation and quantitation of sequence variants. The Human Genome 19 (HG19) was used as the reference sequence for alignment (the analysis was performed by the Ph.D candidate - Mgr.Sylwia Jancik and confirmed by Mgr. Petr Vojta).

2.3.2.3 Experimental design for Next Generation Sequencing MiSeq (Illumina) technology

Schema of the method

PCR amplification
template specific primers (KRAS/BRAF)

Purification of the product

End repair PCR products

Adaptor ligation

Purification of the product

PCR amplification
unique barcodes-Index

Purification of the product,
dilution and pooling

Sequencing
MiSeq-Illumina

Data analysis
MiSeq reporter
IGV software
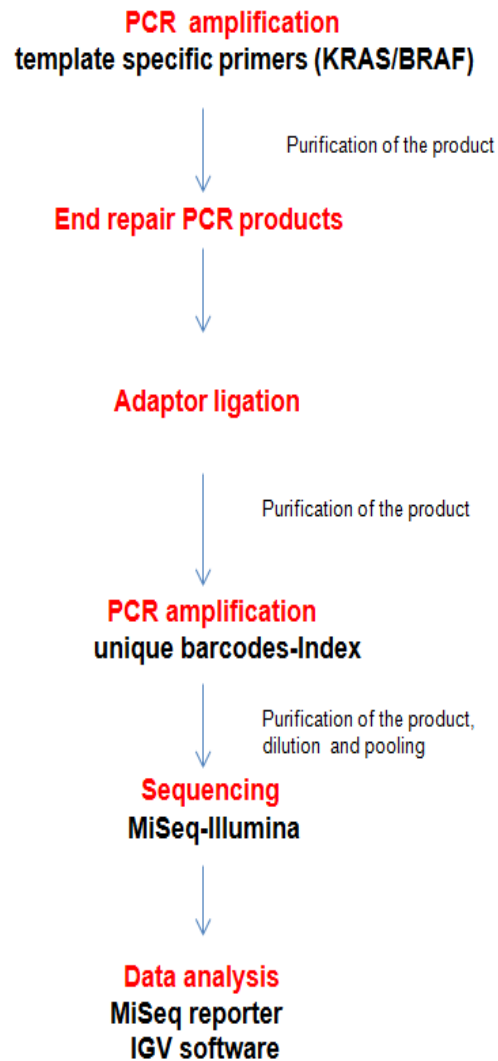
Library preparation:

For PCR amplification, the same specific primers for *KRAS* exon 2, codons 12, 13 and *BRAF* exon 15 codon 600 were used as for GS Junior 454 (Roche) technology.

Table 1. PCR-specific primers for *KRAS* and *BRAF* genes fragment.

	5- template-specific sequence-3' (Forward)	xxx bp product size
Forward primer		
Reverse primer	5'- template-specific sequence-3' (Revers)	xxx bp product size
Forward primer KRAS (exon2, includes codon12 and 13)	5'-TggTggAgTATTTgATAgTgTATTAACCT-3'	282 bp product size
Reverse primer KRAS (exon2, includes codon 12 and 13)	5'-ATgAAAATggTCAgAgAAACCTTTATC-3'	282 bp product size
Forward primer BRAF (exon 15, includes codon 600)	5'TgCTTgCTCTgATAggAAAATg-3'	222 bp product size
Reverse primer BRAF (exon15, includes codon 600)	5'-TCAgggCCAAAAAATTTAATCA-3'	222 bp product size

PCR amplification conditions and *in silico* results are described in Roche experiment design section (see above).

Table 2. Final reaction concentration used for specific PCR amplification (*KRAS* or *BRAF*)

Specific PCR amplification KRAS/BRAF		
Reagents concentration	**Final conc.**	**µl/1 reaction**
H$_2$O		13.64
10xPCR buffer with 18 mM MgCL$_2$	1x, 1.8 mM	2
dNTPs 25mM	0.2 mM	0.16
Eva Green 20x	1x	1
Primers (Fw and Rv mix) 10 µM KRAS or BRAF	1 µM each	2
High fidelity polymerase 5U	1 U	0.2
DNA	1 µl	1
Total volume		**V=20 µl**

Table 3. Final PCR program

LightCycler 480 Roche			
PCR-program:			
Polymerase initialization	95°C	5min	
denaturation	95°C	30 s	
annealing	59°C	30 s	50 cycles
elongation	72°C	40 s	
melting	95°C	10 s	
	60°C	1 min	
	95°C	Continuous fluorescence signal gathering	
cooling	37°C		

In the following step, amplified products were end-repaired. This step is needed to allow for hybridization of adaptor-modified DNA to oligonucleotide anchor in the flow cell. Ligation of the adaptors is only possible when the PCR fragments are end-repaired. During end-repairing process, the generated fragments are phosphorylated at the 5' ends, and 3' ends are adenylated by single A-nucleotide added to the fragments. This allows for ligation of the fragment to an adaptor that has a single base –T overhange (AT cloning).

Amplified PCR products were purified using QIAquick PCR Purification Kit (Qiagen) according to manufacturer's QIAquick Spin Handbook (Edition 05/2012), elution of product to the 25 µl molecular grade water. Alternatively, when unspecific products occurred, purification was provided using SPRI Based Size Selection (Beckman Coulter) according to manual instructions (Revision version 10/2012 eluting DNA to 20 µl molecular grade water.

To generate end-repair fragments, GS FLX Titanum Rapid Library Preparation Kit (Roche, Content version 12/2010, Version 4.0) manual was followed. Procedure was modified to be compatible with the use of Illumina by using adaptors compatible with Illumina system and DNA amplicons products (originally this kit is aimed to Roche NGS system).

Modified manual process for Illumina is described below:

16 µl DNA of PCR amplicon with minimal concentration 5 ng/µl was added to the 9 µl of Fragment End- Repair Mix, vortexed for 5 seconds, spun shortly and run using an End Repair program:

Table 4. Reagents and enzymes used for End-repair process:

Fragment End-Repair Mix	
Reagents	µl/1-rce.
RL 10x Buffer	2.5
RL ATP	2.5
RL dNTP	1
RL T4 Polymerase	1
RL PNK	1
RL Taq Polymerase	1
Total	9

Table 6. Thermal conditions used for End-repair:

PTC-200 Peltier Thermal Cycler		
Program:	End-Repair	
25°C	20 min	
72°C	20 min	1x cycle
4°C	hold	

1µl of 20µM adaptor (Illumina) and 1µl Ligase was added to the reaction tube which included end repaired PCR fragments sequence, and the mixture was incubated according to Adaptor-Ligation program:

Table 7. Thermal condition for Adaptor-ligation:

PTC-200 Peltier Thermal Cycler		
Program:	**Adaptor- Ligation**	
25°C	20 min	
16°C	20 min	1 x cycle
4°C	hold	

Table 8. Primer sequences and temperature conditions for creating adaptors:

Before process with adaptor ligation, adaptors were created using Forward and Reverse primers. They were then ligated to each other using the following program: 95°C 10 min, 70°C 30 min.

Adaptor_Illumina	Adaptor sequence – compatible with Nextera Indexes	modification
Forward-adaptor-i5	5'-TCgTCggCAgCgTCAgATgTgTATAAgAgACAgT-3'	5' phosphorylated
Revers-adaptor-i5	5'-CTgTCTCTTATACACATCTgACgCTgCCgACgA-3'	5' phosphorylated
Forward-adaptor-i7	5'-gTCTCgTgggCTCggAgATgTgTATAAgAgACAgT-3'	5' phosphorylated
Revers-adaptor-i7	5'-CTgTCTCTTATACACATCTCCgAgCCCACgAgAC-3'	5' phosphorylated

Oligonucleotide sequences © 2007-20012 Illumina, Inc. All right reserved. Derivative work created by Illumina customers are authorizied for use with Illumina instruments and products only. Other use is strictly prohibited.

After adaptor ligation process was completed, the products were purified using QIAquick PCR Purification Kit (Qiagen) according to manufacturer's QIAquick Spin Handbook (Edition 05/2012), and they were eluted using 25µl molecular grade water. During this step, unligated adaptors and all reagents used in previous steps were removed.

Subsequently, purified products were barcoded with multiplex indexes and amplified. Nextera short (7-8bp) Index fragments (i5- index and i7-index) are unique codes used to label every individual sample to identify them during sequencing and analysis process.

Table 8. Index primers sequences

Nextera Index -primers	Index primers sequences
Index i5	**AATgATACggCgACCACCgAgATCTACAC [i5-8 bases codes fragment]TCgTCggCAgCgTC**
Index i7	**CAAgCAgAAgACggCATACgAgAT [i7-bases codes fragment]gTCTCgTgggCTCgg**

Indexing of primer sequences with unique barcode was performed using MID Kit for Illumina MiSeq.

Table 9. Reagents used for PCR Index annealing process:

PCR-Index annealing		
Reagents concentration	Final conc.	µl/1-rce.
H2O		13.4
10xPCR buffer/MgCL2 18 mM	1x/1.8 mM	2
10mM dNTPs	0.2 mM	0.4
Eva Green 20x	1x	1
High fidelity polymerase 5U	1U	0.2
		Mix=17µl
mid i5 (Fw and Rv) 10 µM	0.5 µM	1
mid i7 (Fw and Rv) 10 µM	0.5 µM	1
DNA (after adaptor ligation)		1
Total		V=20 µl

Table 9. Thermal condition used for PCR Index annealing process:

Lihght Cycler 480 Roche			
Program: Index annealing			
polymerase activation	95°C	5 min	
denaturation	95°C	30 s	
annealing	55°C	30 s	20 cycles
elongation	72°C	1 min	
extension	72°C	5 min	
cooling	37°C	for ever	

The products resulting from the annealing reaction including specific sequence with ligated adaptors and index, and concentration of individual samples were analyzed using Agilent 2100 Bioanalyzer, using DNA Chips Technologies according to DNA 1000 Kit Quick Start Guide (Edition 10/2010). All concentrations of the samples were calculated and adjusted to reach the same concentration and then they were pooled together.

Sequencing:

Pooled library was diluted to a final concentration of 12 pM and denatured using NaOH. DNA libraries were spiked-in 5% of 12 pM PhiX control (spiking using PhiX control is necessary for amplicon sequencing, especially when few genes are being sequenced. This allows obtaining higher quality data, with high diversity. Low diversity can occur when pools consist of one or few amplicons and significant number of reads contains the same sequence. This can shift the base composition because the reads are no longer random). Subsequently, 600 µl of sample from the library was loaded onto sequencing cartridge and 15 M flow cell was used in each sequencing run. Library preparation and sequencing process preparation was performed according to manual protocol Preparing DNA Libraries for Sequencing on the MiSeq® (Verison 03/2013, Rev.B).

Data analysis:

Analysis was performed using MiSeq reporter 2.2.29. Output .vcf and .bam files were processed using Microsoft Office Excel and IGV 2.3 software. The HG19 reference was used for alignment (analysis of data were done with a bioinformatics help of Ing. Rastislav Slavkovsky, PhD.).

2.3.4 Results

To attest whether NGS is technically competent and able to produce precise and accurate data meeting the standard for further validation under quality management for medical laboratory (ISO15198), the samples were analyzed in relation to presence and type of *KRAS/BRAF* mutation. All NGS results were compared with results of CE-marked Real-Time PCR methods. Percentage correlation and mutational status was also compared between two NGS technologies, and test repeatability was obtained for MiSeq (Illumina) sequencing technology.

Result of adaptor ligation process for MiSeq (Illumina) system using Agilent 2100 Bioanalyzer, DNA Chips Technologies:

BRAF , KRAS fragments before adaptor ligation

BRAF, KRAS fragments after adaptor ligation
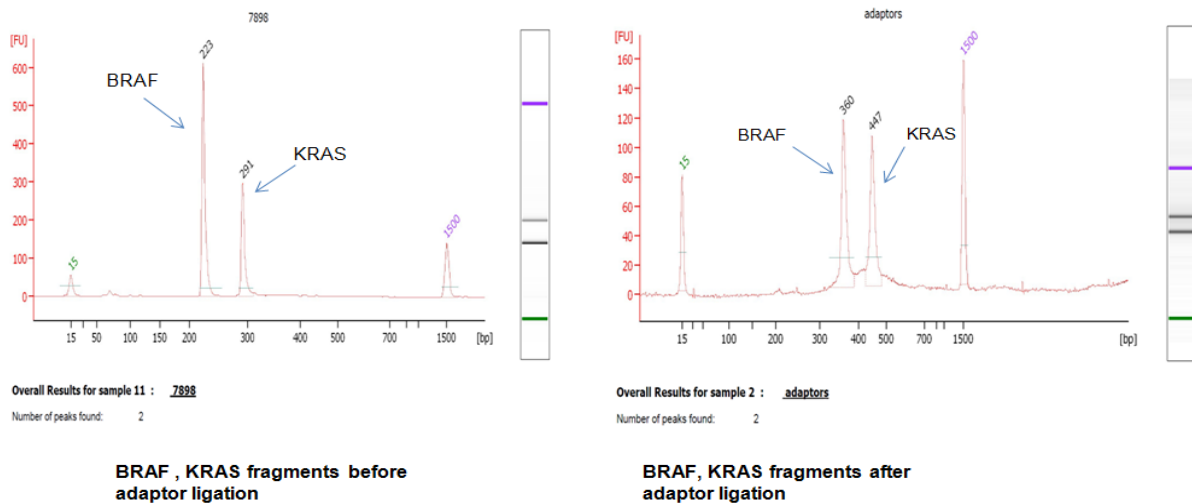
NGS system optimization:

NGS Illumina and NGS Roche technology systems were tested using 1% mutation detection threshold during analysis. Achieved coverage during sequencing process for GS 454-Junior (Roche) system was: minimal coverage ~3500 reads/1sample (35 reads per 1% threshold), maximal coverage ~10 000 (100 reads per 1%). Achieved coverage during sequencing process for NGS MiSeq (Illumina) was: minimal coverage ~2500 reads/1sample (25 reads per 1%),

.maximal coverage ~50 000 (500 reads per 1%). 36 samples analyzed previously for *KRAS* or *BRAF* mutation by Real-Time PCR methods were used for system optimization (see below).

NGS versus TheraScreen Mutation kit (Qiagen) for *KRAS* mutation detection:

Detection of *KRAS* gene mutations, exon 2 (codon 12 and 13) was done using 23 samples including one control sample with pre-defined percentage of mutated DNA (~1.5% G12V), and 22 patients samples with unknown mutation status. *KRAS* gene was tested by K-ras TheraScreen Mutation Detection kit (Qiagen) with detection limit 1%. Between 23 samples tested samples, 15 samples were mutation positive and 8 samples were mutation negative (Table 1).

Table 1. Results of *KRAS* mutation detection by NGS GS 454-Junior (Roche) and NGS MiSeq (Illumina) in comparison to TheraScreen Real-Time method.

Nr.	Sample	Real-time PCR KRAS_TheraScreen	NGS Roche 454 Junior	NGS Illumina MiSeq
1	KRAS 1.5%	G12V	G12V	G12V
2	7889	G12V	Wild-type	G12V
3	7964	Wild-type	12Ser	Wild-type
4	8233	Wild-type	Wild-type	Wild-type
5	7857	Wild-type	Wild-type	Wild-type
6	7886	Wild-type	Wild-type	Wild-type
7	7935	Wild-type	Wild-type	Wild-type
8	8235	Wild-type	Wild-type	Wild-type
9	8236	Wild-type	Wild-type	Wild-type
10	7934	Wild-type	Wild-type	Wild-type
11	7847	G13D	G13D	G13D
12	6981	G12D	G12D	G12D
13	7849	G12V	G12V	G12V
14	6977	G12C	G12C	G12C
15	8210	G13D	G13D	G13D
16	8424	G12V	G12V	G12V
17	8425	G12A	G12A	G12A
18	8426	G12S	G12S	G12S
19	8281	G13D	G13D	G13D
20	8394	G12V	G12V	G12V
21	8254	G12V	G12V	G12V
22	7848	G12R	G12R	G12R
23	7825	G12A	G12A	G12A

Sequencing by NGS GS Junior-454 (Roche) 23 tested samples yielded one false positive sample (MG7964) and one false negative (MG7889) sample in comparison to TheraScreen Mutation Detection kit and NGS MiSeq (Illumina) (Table 1). This missing sample (MG7889) was found subsequently to contain 0.8% mutated DNA. In AVA software during analysis, detection threshold was set to 1%, so taking this criteria, this one sample was consider to be wildtype; however, it was close to detection threshold. False positive sample (MG7964) was also found near to detection limit with 1.4% mutation detection positivity (Table 3).

From our experience, false negativity or false positivity in samples with mutation percentage near the detection threshold is problem during data analysis using any genotyping methods (data not showed). Thus, for results close to detection limit of NGS, we recommend to perform sequencing twice or using different detection method to confirm the result.

 Sequencing by NGS MiSeq (Illumina) results were in all 23 cases tested samples concordant with TheraScreen Mutation Detection kit. Samples MG7889 and MG7964, discordant with NGS Junior result, were considered in the context of Real-Time (CE) methods results to be true negative and true positive for NGS MiSeq sequencing system (Table 1).

According to these results, GS Junior-454 (Roche) sensitivity (true positive rate) was estimated to be 95.7%, and specificity (false positive rate) was estimated to be 95.7% as well.

NGS MiSeq sensitivity and specificity was estimated to be 100% for both parameters.

NGS versus PNAClamp Mutation Detection kit (Panagene) and *BRAF* mutation Detection kit (IntellMed) for *BRAF* mutation detection:

Detection of *BRAF* gene mutation, exon 15 (codon 600) was done on 13 samples including one control standard sample with pre-defined percentage of mutated DNA (~1.5% V600E) and 12 patients samples with unknown mutation status. All samples were tested by PNAClamp Mutation Detection kit (Panagene); results were also confirmed by BRAF Mutation Detection kit (IntellMed). Detection limit of the kits is about 1%. Between 13 samples tested on *BRAF*, 10 samples were mutation positive and 3 samples were mutation negative (Table 2).

NGS Junior and Illumina sequencing results were completely concordant with Real-Time methods PNAClamp Mutation Detection kit and IntellMed Mutation Detection kit in all 13 samples cases (Table 2).

According to these results, sensitivity and specificity were 100% for BRAF mutation detection status of both NGS methods.

Table 2. Results of *BRAF* mutation detection by GS 454 Junior (Roche) and NGS MiSeq (Illumina) in comparison to PNAClamp and IntellMed Real-time methods

Nr.	Sample	Real-time PCR PNA Clamp and IntellMed	NGS Roche 454 Junior	NGS Illumina MiSeq
1	BRAF 1.5%	V600E	V600E	V600E
2	7888	Wild-type	Wild-type	Wild-type
3	7898	Wild-type	Wild-type	Wild-type
4	7911	Wild-type	Wild-type	Wild-type
5	7011	V600E	V600E	V600E
6	7116	V600E	V600E	V600E
7	7030	V600E	V600E	V600E
8	7092	V600E	V600E	V600E
9	7094	V600E	V600E	V600E
10	7088	V600E	V600E	V600E
11	7100	V600E	V600E	V600E
12	7106	V600E	V600E	V600E
13	7114	V600E	V600E	V600E

Very similar result was also described in publication by Katja Tuononen *et. al*. The *KRAS* and *BRAF* mutation detection was carried out using TheraScreen KRAS PCR test (Qiagen) and BRAF AmoyDx^{TM} BRAF V600E PCR test (Amoy Diagnostics). *KRAS* and *BRAF* mutation status was determined on 78 patient samples isolated from formalin fixed paraffin embedded tumour tissue. Percentage of concordance detected mutations between NGS GS 454-Junior (Roche) sequencing and Real-Time PCR analysing in these study was 98.7% for *KRAS* gene and 100% for *BRAF* gene (Tuononen, 2013).

Quantitative comparison between two NGS methods: Roche 454-Junior versus Illumina MiSeq technology:

Control sample with known percentage ~1.5% *KRAS* (G12V) mutated DNA was identified as 1.7% by Roche Junior-454 system, and as 1.8% by Illumina MiSeq system. Control sample with ~1.5% BRAF (V600E) mutated DNA was identified as 1.7% and 1.3 % respectively (Table 3., Table 5). 1.5% KRAS control samples measured as 1.7% by Junior and 1.8% by MiSeq, giving 0.2% and 0.3% percentages discrepancy between true value and measured value, this giving 0.88 and 0.83 ratio, respectively. This error of calculated ratio of NGS, giving satisfied result near to ideal ratio=1. From results of control sample analyzed for detection of *KRAS* gene, we can conclude, that detection error (defined as percentage ratio between true value and measured value of the NGS methods) is acceptable for detection threshold set to 1%.

Table 3. *KRAS* mutation results (percentage correlation between NGS GS 454-Junior (Roche) and NGS MiSeq (Illumina) technology

| Nr.	Sample	Real-time PCR KRAS_TheraScreen	NGS Roche 454 Junior	% mutation	NGS Illumina MiSeq	% mutation
1	KRAS 1.5%	G12V	G12V	1.7%	G12V	1.8%
2	7889	G12V	Wild-type/G12V	0.8%	G12V	**4.3%**
3	7964	Wild-type	12Ser	1.4%	Wild-type	**0.0%**
4	8233	Wild-type	Wild-type	0.0%	Wild-type	0.0%
5	7857	Wild-type	Wild-type	0.0%	Wild-type	0.0%
6	7886	Wild-type	Wild-type	0.0%	Wild-type	0.0%
7	7935	Wild-type	Wild-type	0.0%	Wild-type	0.0%
8	8235	Wild-type	Wild-type	0.0%	Wild-type	0.0%
9	8236	Wild-type	Wild-type	0.0%	Wild-type	0.0%
10	7934	Wild-type	Wild-type	0.0%	Wild-type	0.0%
11	7847	G13D	G13D	37.1%	G13D	41.3%
12	6981	G12D	G12D	5.4%	G12D	7.3%
13	7849	G12V	G12V	43.2%	G12V	37.6%
14	6977	G12C	G12C	1.2%	G12C	1.6%
15	8210	G13D	G13D	41.7%	G13D	45.7%
16	8424	G12V	G12V	17.9%	G12V	19.4%
17	8425	G12A	G12A	6.8%	G12A	5.7%
18	8426	G12S	G12S	12.1%	G12S	12.7%
19	8281	G13D	G13D	99.3%	G13D	99.7%
20	8394	G12V	G12V	13.4%	G12V	20.8%
21	8254	G12V	G12V	7.0%	G12V	16.9%
22	7848	G12R	G12R	33.1%	G12R	9.6%
23	7825	G12A	G12A	13.3%	G12A	4.4%

 BRAF control sample with pre-defined 1.5% mutated DNA were measured as 1.7% by Junior and 1.3% by MiSeq, giving 0.2% and -0.3% percentages discrepancy between true value and measured value (0.88 and 1.15 ratio, respectively). This error is acceptable.

Between 34 patients samples with unknown mutation percentage (including 12 samples tested on *BRAF* and 22 samples tested on *KRAS*), quantitative results identified mutations with some high percentage correlation or/ discrepancy in some samples cases. Result is showed like a ratio percentage mutation between NGS methods. (Table 4, Graph 1, Table 6, Graph 2) (all samples were measured ones).

Table 4. Percentage correlation between NGS GS 454-Junior (Roche) and NGS MiSeq (Illumina) technology for *KRAS* mutation detection. Ratio of percentage mutation detection between NGS technologies

Nr.	Sample	NGS Roche 454- Junior	% mutation	NGS Illumina MiSeq	% mutation	Ratio of % mutation Junior/MiSeq	Ratio of % mutation MiSeq/Junior
1	KRAS 1.5%	G12V	1.7%	G12V	1.8%	0.94	1.0
2	7889	Wild-type/G12V	0.8%	G12V	4.3%	-	x
3	7964	12Ser	1.4%	Wild-type	0.0%	-	x
4	8233	Wild-type	0.0%	Wild-type	0.0%	x	x
5	7857	Wild-type	0.0%	Wild-type	0.0%	x	x
6	7886	Wild-type	0.0%	Wild-type	0.0%	x	x
7	7935	Wild-type	0.0%	Wild-type	0.0%	x	x
8	8235	Wild-type	0.0%	Wild-type	0.0%	x	x
9	8236	Wild-type	0.0%	Wild-type	0.0%	x	x
10	7934	Wild-type	0.0%	Wild-type	0.0%	x	x
11	7847	G13D	37.1%	G13D	41.3%		1.11
12	6981	G12D	5.4%	G12D	7.3%	0.73	1.35
13	7849	G12V	43.2%	G12V	37.6%	1.14	0.87
14	6977	G12C	1.2%	G12C	1.6%	0.75	1.33
15	8210	G13D	41.7%	G13D	45.7%	0.91	1.09
16	8424	G12V	17.9%	G12V	19.4%	0.92	1.08
17	8425	G12A	6.8%	G12A	5.7%	1.19	0.83
18	8426	G12S	12.1%	G12S	12.7%	0.95	1.04
19	8281	G13D	99.3%	G13D	99.7%	0.99	1.00
20	8394	G12V	13.4%	G12V	20.8%	0.64	1.55
21	8254	G12V	7.0%	G12V	16.9%	0.41	2.41
22	7848	G12R	33.1%	G12R	9.6%	3.44	0.29
23	7825	G12A	13.3%	G12A	4.4%	3.02	0.33

Graph 1.
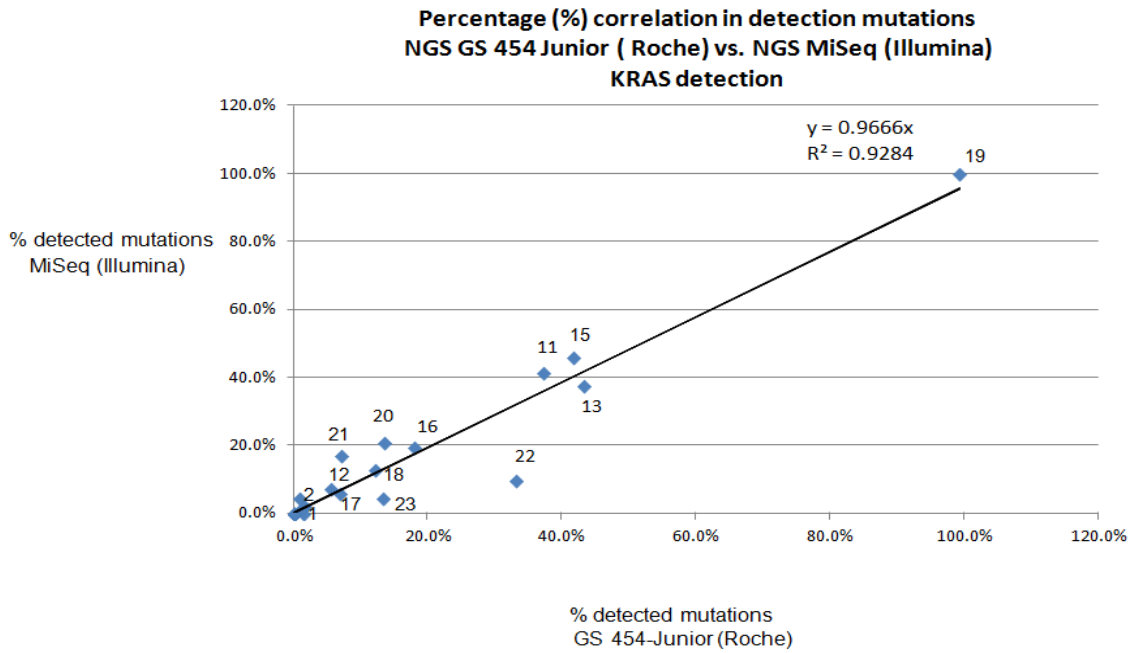
Scatter plot presented percentage correlation KRAS mutation detection between two NGS methods (numbers) number-Nr. of sample in the table, (blue points) indicate correlation between the percentages of detected mutation using GS 454 Junior Roche and percentages of detected mutations using MiSeq Illumina.

Table 5. *BRAF* **mutation results (percentage correlation between NGS GS 454-Junior (Roche) and NGS MiSeq (Illumina) technology)**

Nr.	Sample	Real-Time PCR PNA Clamp and IntellMed	NGS Roche 454 Junior	% mutation	NGS Illumina MiSeq	% mutation
1	BRAF 1.5%	V600E	V600E	1.7%	V600E	1.3%
2	7888	Wild-type	Wild-type	0.0%	Wild-type	0.0%
3	7898	Wild-type	Wild-type	0.0%	Wild-type	0.0%
4	7911	Wild-type	Wild-type	0.0%	Wild-type	0.0%
5	7011	V600E	V600E	3.1%	V600E	2.7%
6	7116	V600E	V600E	18.2%	V600E	18.2%
7	7030	V600E	V600E	8.0%	V600E	8.6%
8	7092	V600E	V600E	21.3%	V600E	24.0%
9	7094	V600E	V600E	22.4%	V600E	25.1%
10	7088	V600E	V600E	44.8%	V600E	23.4%
11	7100	V600E	V600E	43.3%	V600E	23.2%
12	7106	V600E	V600E	42.2%	V600E	36.4%
13	7114	V600E	V600E	25.6%	V600E	31.5%

Table 6. Percentage correlation between NGS GS 454-Junior (Roche) and NGS MiSeq (Illumina) technology for BRAF mutation detection.

Nr.	Sample	NGS Roche 454 Junior	% mutation	NGS Illumina MiSeq	% mutation	Ratio of % mutation Junior/MiSeq	Ratio of % mutation MiSeq/Junior
1	BRAF 1.5%	V600E	1.7%	V600E	1.3%	1.30	0.76
2	7888	Wild-type	0.0%	Wild-type	0.0%	x	x
3	7898	Wild-type	0.0%	Wild-type	0.0%	x	x
4	7911	Wild-type	0.0%	Wild-type	0.0%	x	x
5	7011	V600E	3.1%	V600E	2.7%	1.14	0.87
6	7116	V600E	18.2%	V600E	18.2%	1.00	1.00
7	7030	V600E	8.0%	V600E	8.6%	1.00	0.93
8	7092	V600E	21.3%	V600E	24.0%	0.88	1.12
9	7094	V600E	22.4%	V600E	25.1%	0.89	1.1
10	7088	V600E	44.8%	V600E	23.4%	1.91	0.52
11	7100	V600E	43.3%	V600E	23.2%	1.86	0.53
12	7106	V600E	42.2%	V600E	36.4%	1.15	0.86
13	7114	V600E	25.6%	V600E	31.5%	0.81	1.23

Statistic analysis of percentages correlation or/and discrepancy Junior (Roche) vs. MiSeq (Illumina) was done using Exact Wilcoxon signed rank test 23 sample/KRAS p-value=0.4637 (alternative hypothesis: true mu is not equal to 0) and for 13 sample/BRAF p-value=0.7109 (alternative hypothesis: true mu is not equal to 0). In both cases, we cannot reject that median difference between percentage targeted by two methods is zero (cannot reject that median differences two method give the same results).

Discrepancy was also observed when repeating measurements by only one NGS method (MiSeq). Results of three measurements of the 6 samples by NGS MiSeq (Illumina) sequencing method is presented in Table 7.

Table 7. Test repeatability by NGS MiSeq (Illumina) sequencing technology

Nr.	Type	measure 1	measure 2	measure 3	average value of three measurements	mutation detected
	MiSeq (Illumina)					
Sample 1	FFPE	40.7%	20.7%	40.3%	33.9%	G13D
Sample 2	FFPE	15.7%	12.3%	6.7%	11.5%	G12R
Sample 3	FFPE	40.0%	44.0%	40.30%	41.4%	G12V
Sample 4	FFPE	7.3%	6.9%	7.20%	7.1%	G12D
Sample 5	Cell line A549	20.2%	19%	20.6%	19.9%	G12S
sample 6	Cell line SW620	29.4%	34%	42.4%	35.2%	G12V

Three measurements of the same samples by NGS MiSeq (Illumina). Sample 1-4 DNA isolated from fixed tissue embedded in paraffin, samples 5-6 DNA isolated from cell line mixed with wild type DNA isolated from blood.

We suppose that this slight percentage discrepancy in some sample measurements can be result of very high heterogeneity of cancer material and in some cases very low quality of DNA (some copy of mutated DNA can be significantly degraded and therefore not amplified efficiently). These factors can slightly change ratio of mutated and non mutated alleles during repeated process of amplification and can result in discrepancy in quantitative result.

Graph 2.
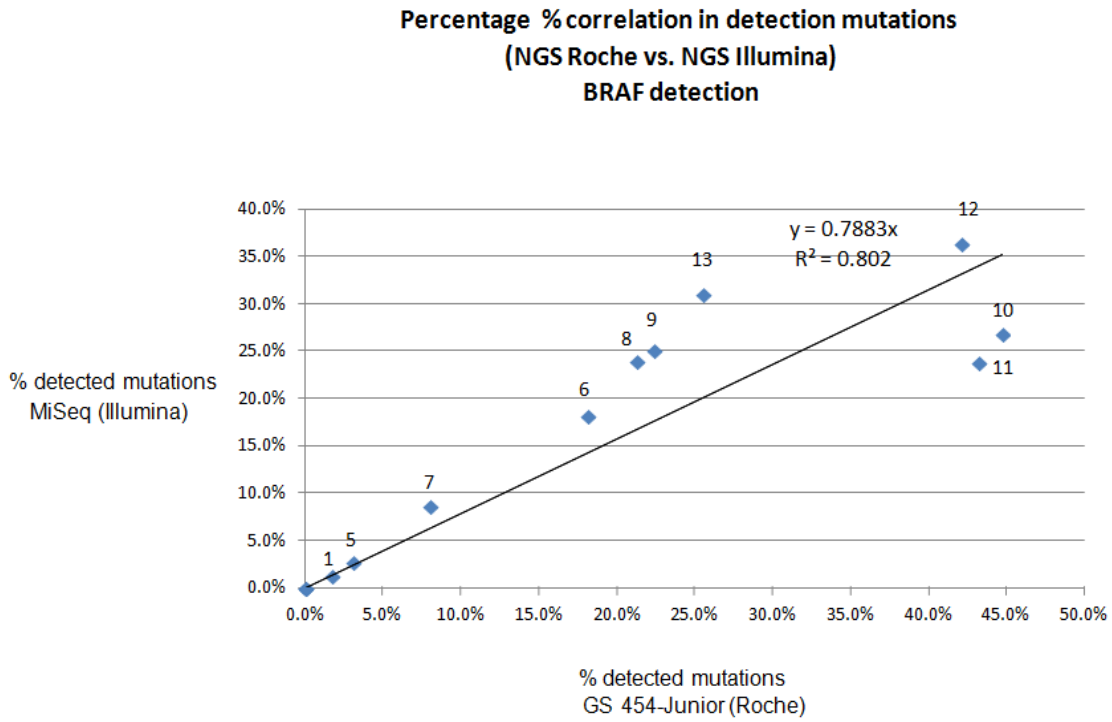
Scatter plot presented percentage correlation BRAF mutation detection between two NGS methods (numbers) number-Nr.of sample in the table, (blue points) indicate correlation between percentages of detected mutations using GS 454-Junior Roche and percentages detected mutations using MiSeq Illumina.

Comparison of analytical parameters of the NGS systems and Real Time PCR test:

NGS methods are sufficiently robust with regards to specificity and sensitivity of detected mutation when compared to Real-Time CE-IVD certified methods.

Advantage of NGS method is easy readjustment of variable testing panel according to type of gene and mutation (different genes and exons, eg. *KRAS* and *BRAF* can be tested in one sequencing run using variable number of samples). This factor is especially promising in context of the new PRIME study which showed that also different type of mutation in *KRAS* gene than in codon 12 and 13 and also *NRAS* mutations are clinical relevant. Real-Time PCR methods are usually developed to detect one specific mutation in one tube, while NGS can detect even unknown mutations. This is big advantage for NGS system, especially when more than one gene and codons needs to be tested.

NGS methods allowed direct quantitation of mutation, and adjustable detection threshold during analysis and sorting of result according to percentage value. In our study, detection threshold was deliberately chosen to be 1%, with regards of commercially available Real-Time PCR methods used in diagnostic laboratory. This detection threshold was successfully tested with good results using DNA samples with pre-defined percentage mutation (~1.5%) for *KRAS* and *BRAF* gene.

Disadvantage of NGS is that it is more time consuming and less simple regarding handling protocol process and data analysis compared to Real-Time methods (~3/5 days vs. ~ 1/2day, respectively). Also, it is more problematic method according to time and price when only few samples are run in one sequencing run. To achieve price approximately equal to TheraScreen Kit (Qiagen) which is ~ 4000CZK, at least 12 samples should be analyzed in one sequencing run (Table 8). On the other hand, with growing number of samples including more genes and/or exons which are supposed to be tested in one sequencing run, the price becomes competitive since the cost per sample diminishes (Table 9). This represents significant advantage especially when using MiSeq Illumina system since the coverage per sequencing run can be even as high as 15 000 000 reads in one sequencing run and is dependent on capacity of flow cell chosen (1M, 4M, and 15M with MiSeq reagent Kit v2). Maximal amount of tested samples is shown in the table below (Table 10) , which for MiSeq was calculated based on 15M flow cell (15 000 000

reads/run; calculation criteria were based on recommended coverage for amplicon sequencing 5000-reads per one exon to achieve "safely" the coverage of 50 reads-per 1% mutation. Using GS 454-Junior (Roche) machine, total coverage for amplicon sequencing is 70 000 reads. To achieve good quality results, theoretically, maximal cohort of tested samples in one sequencing run should be no more than 14.

Table 8. Example of price calculation for genotyping 12 samples using MiSeq Illumina system

MiSeq Illumina (cost per 1 sample when 12 samples is sequenced in one run)				
Flow cell capacity	Flow cell cost	Flow cell cost/1samples	Chemicals cost/1 sample	Total price/1sample (Flow cell+ chemicals cost)
1M	15 000CZK	1250CZK	2750CZK	4000CZK
4M	24 000CZK	2000CZK	2750CZK	4750CZK
15M	30 000CZK	2500CZK	2750CZK	5250CZK

Approximate calculation cost for different flow cell capacity is provided in the table.

1M flow cell is sufficient for 12 samples. Less than 12 samples makes price higher than calculated in the table (4000CZK). However, when 12 samples are sequenced using 1M flow cell, more than one gene and more exons can be sequenced in one run, elucidating the advantage of NGS to Real-Time PCR (TheraScreen) which is designed for analyzing only one gene in one detection test for price of 4000CZK.

Table 9. Example of price calculation for genotyping 96 samples using MiSeq Illumina system

MiSeq Illumina (cost per 1 sample when 96 samples is sequence in one run)				
Flow cell capacity	Flow cell cost	Flow cell cost/1samples	Chemicals cost/1 sample	Total price/1sample (Flow cell+ chemicals cost)
1M	15 000CZK	157CZK	2750CZK	2907CZK
4M	24 000CZK	250CZK	2750CZK	3000CZK
15M	30 000CZK	313CZK	2750CZK	3063CZK

1M flow cell capacity (1 000 000 reads) is sufficient for 96 samples when one gene/exon is tested in one sequencing run (when more genes or/and exons are tested, we would recommend higher capacity of flow cell to reach 1% threshold for mutation detection).

Table `10. Comparison analytical parameters between detection methods.

	TheraScreen	PNAClamp	IntellMed	Roche GS Junior	Illumina MiSeq
Sensitivity/detection limit	1%	<1%	1%	adjustable	adjustable
Procedure and analysis time	1/2 day	1/2 day	1/2 day	3/5 days	3/5 days
Simplicity of wet procedure Low *, high ****	****	****	****	**	***
Simplicity of data processing and analysis	****	****	****	***	**
Maximum tested genes in one run	KRAS only	BRAF only	BRAF only	more	more
Maximum tested samples in one run	10 (1 exon)	50 (1 exon)	94 (1 exon)	14 (1 exon) to achieve sensitivity 1%	96 (25 exons) to achieve sensitivity 1% 15M (flow cell)
Cost per sample	4000 CZK	1500 CZK	800 CZK	4000 CZK when 12 samples are run	4000 CZK when 12 samples are run 1M (flow cell)
CE-certified	yes	yes	yes	no	no

All calculation for NGS was done for detection limit of 1% and must be taken with caution because it assumes that all quality control steps were passed and technician reached a needed level of personal skill using this method.

2.3.5 Summary of the NGS results

In summary, NGS stands as a technically competent method able to produce precise and accurate results with high sensitivity and specificity compared to CE Real-Time PCR test. Price of NGS is comparable with commercially used method in diagnostic practices. Time of method processing and data analysis is longer compared to Real-Time methods but within accepted range of two weeks which is currently acceptable by clinicians.

According to our analysis, NGS method can be standardized for clinical practice and is ready for the full validation to suit requirements for ISO15198 accredited medical laboratory.

2.4 BRAF (IntellMed) p.Val600Glu testing in thyroid carcinoma patients.

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Correlation among the BRAF Gene Mutation Status, Clinicopathological Features of Primary Tumour, and Lymph Node Metastasizing of Papillary Thyroid Carcinoma

Thieme

Correlation among the *BRAF* Gene Mutation Status, Clinicopathological Features of Primary Tumour, and Lymph Node Metastasizing of Papillary Thyroid Carcinoma

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Abstract
▼

Background: Papillary thyroid carcinoma (PTC) is the most common malignant thyroid tumour. A common mutation of papillary thyroid carcinoma (PTC) is the somatic mutation of the *BRAF*[V600E] gene.

Aim: The aim was to 1) determine the association of lymph node metastases of PTC with the *BRAF* gene mutation of primary tumour; 2) evaluate association of the *BRAF* mutation in the primary tumour with clinicopathological parameters; 3) examine the extent of genetic heterogeneity by monitoring the *BRAF* mutation in multicentric tumours.

Subjects and methods: Retrospective analysis of the *BRAF*[V600E] mutation in PTC and PTC neck lymph node metastases in 156 patients operated from 2003 to 2012 in Prague and Zlín, the Czech Republic, using a qPCR assay. The results were correlated with clinicopathological factors.

Results: DNA was successfully extracted from 137 samples. The *BRAF*[V600E] mutation was detected in 78 cases (56.9%). The patients with *BRAF* p.Val600Glu mutation of primary tumour had only non-significantly higher risk of cervical lymph node metastases [OR = 2.39 (95%) CI 1.00–5.75, p = 0.052]. In the classic papillary variant, the *BRAF*[V600E] mutation was found significantly more often than in other PTC subtypes (p = 0.022). We did not confirm any significant association between the *BRAF*[V600E] mutation and other clinicopathological findings.

Conclusion: Except for the higher prevalence in papillary variant of PTC, *BRAF* p.Val600Glu mutation was not associated with other prognostic clinicopathological factors of PTC. *BRAF* mutation cannot be regarded as a reliable marker of node metastases in patients with PTC.

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Introduction
▼

Well-differentiated thyroid carcinomas (WDTC) originate from follicular cells and they produce colloid and thyroid hormones. WDTCs can be subdivided into papillary carcinomas (PTC) and follicular carcinomas (FTC). However, there is a growing incidence of the so-called papillary microcarcinomas (PTMC), i.e., tumours ≤ 10 mm (T1aN0M0) [1–3]. Even though the PTC has a good prognosis and a 10-year survival rate is reported in more than 90% of patients [1], the presence of node metastases at the time of diagnosis is confirmed in 40–90% of cases [4,5]. A common genetic change in PTC is somatic mutation within the *BRAF* gene, which activates the MAPK (mitogen-activated protein kinase) signal-

ling pathway that plays a constitutive role in cell proliferation, differentiation and apoptosis [6,7]. The most common *BRAF* gene mutation is the T (thymine)→A (adenine) substitution at nucleotide position 1799, resulting in a valine to glutamic acid substitution at residue 600 (p.Val600Glu) within exon 15 of the kinase domain [8,9]and constitutive kinase activity. Varied prevalence of the *BRAF*[V600E] mutation is described in the literature [10,11]. Gene Set Enrichment Analysis revealed that the *BRAF*[V600E] mutation increased the expression of some collagens and laminins in extracellular matrix and thus promoted the cancer migration and invasion [12]. Some sources claim that it correlates with predictors of poor prognosis (extracapsular tumour spread, higher TNM stage, formation of node metastases, and recurrence) [8,10,13] while others did not confirm the association between the *BRAF*[V600E] mutation and these clinical-pathological parameters [14–16].

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*Both authors contributed equally to this work.

The aims of this study were: 1) to determine the association of lymph node metastases of PTC with the *BRAF* gene mutation of primary tumour; 2) to evaluate association of the *BRAF*[V600E] mutation in the primary tumour with other monitored clinico-pathological parameters; 3) to examine the extent of genetic heterogeneity of multicentric tumours by monitoring the *BRAF* gene mutational status, focus by focus.

Subjects and Methods
▼

Subjects
Retrospective dicentric analysis of 156 consecutive patients with PTC operated in the period from 2003 to 2012 at the Otolaryngology-Head and Neck Surgery Department of the Na Homolce Hospital in Prague and the Department of Surgery, Atlas Hospital a.s. in Zlín. The monitoring time of patients from surgery to last check-up ranged from 1 to 95 months, median 14 months, interquartile range 41 months. During the monitoring time, 3 patients died. The extent of neck metastases and stage were evaluated in compliance with the TNM classification of malignant thyroid s (7th edition/2009, Czech version 2011). The retrospective study was approved by the Ethics Committee of the Na Homolce Hospital in Prague and Atlas Hospital a.s in Zlín. Besides basic demographic data, i.e., sex and age, we also evaluated the following clinical and pathological parameters: tumour size and histological characteristics, mono/multifocality, extracapsular growth, angioinvasion, metastasis into neck nodes, presence of distant metastases, and disease stage.

Methods
Detection of the *BRAF* mutation from paraffin block carcinoma tissue and node metastases fixed in neutral formalin was carried out at the Institute of Molecular and Translation Medicine of the Faculty of Medicine in Olomouc. Microtome sections (5 μm) were cut from paraffin block carcinoma tissue and neck node metastases were cut out. After deparaffinization of sections in xylene and immersion in absolute alcohol, DNA was isolated using the Nucleospin kit (Macherey-Nagel, Germany). The presence of the *BRAF* Val600Glu mutation was determined by the 2-colour genotyping system with internal amplification control on the qPCR basis with hydrolysis probes (IntellMed, the Czech Republic) on LightCycler 480 II instrument (Roche, the Czech Republic), following the manufacturer's protocol. In multifocal tumours with a confirmed *BRAF* mutation, the *BRAF* genotyping for individual foci was carried out using laser-capture microdissection to assess the genetic heterogeneity. The MAPK signalling pathway activity was tested immunohistochemically using the antibodies Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mA (Cell Signalling Technology, USA).

Statistical analysis
Data are presented as absolute and relative frequencies (percents). Comparisons between groups were made using Fisher's exact test, odds ratio and logistic regression. Multiple logistic regression model was used to adjust for relevant covariates and factors and to compare observed and predicted outcomes. All tests were 2-tailed, at the 5% significance level. The data were analysed by the statistical software Stata 9.2 (Stata CorpLP, College Station, USA).
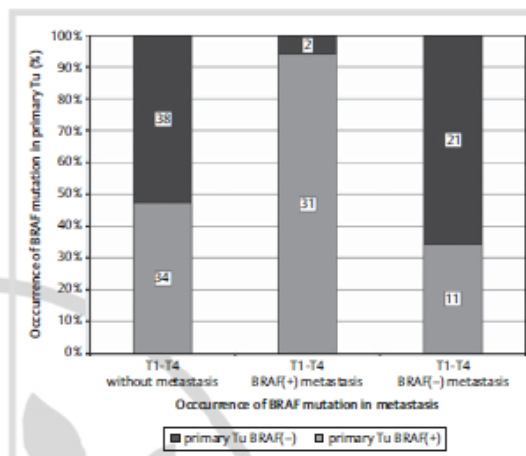

Fig. 1 Absolute and relative frequencies of patients according to the *BRAF*[V600E] mutation occurrence in primary PTC and in metastases.

Results
▼

In total, 380 paraffin blocks of papillary thyroid carcinomas from 156 patients with PTC were assessed. DNA was extracted in 137 (87.8%) patients (326 paraffin blocks), 34 men and 103 women (75.2%). Reasons for omitting 19 patients from analysis were: cut away paraffin (8 patients), degraded DNA in primary tumour (7 patients), and degraded DNA in metastasis (4 patients). In multicentric tumours, the mutation status of the *BRAF* gene was studied in all foci to assess the genetic heterogeneity. Among 25 multifocal tumor patients, 13 patients had difference in mutation status among foci. In 137 patients, there was detected the *BRAF*[V600E] mutation in 78 cases (56.9%), 31 both in primary tumour and metastasis, 45 in primary tumour only and 2 in metastasis only. When comparing patients with node metastases, we observed the phenomena, that in the case of positive *BRAF* mutation in the metastasis, there is a high probability of the presence of the *BRAF* positive mutation in the primary tumour. In node metastases with negative finding of the *BRAF* mutation, there is also a negative finding of the *BRAF* mutation in the primary tumour in almost 2/3 of cases (◦ Fig. 1).

Within bar numbers represent absolute numbers of cases, y-axis scale characterizes proportion of cases with and without *BRAF* mutation in primary tumour within particular category (bar).

A significant difference was found for overall *BRAF*[V600E] mutation frequency between the 2 participating institutions (p = 0.038). There was a statistically significant difference (p = 0.027) in the presence of individual PTC histological subtypes between the 2 institutions as well (◦ Table 1).

The average age of operated patients was 47.4 years (median 47; range 7–86 years). 2 age categories of patients (under 45 and over 45 years of age) varied in disease stages present (p = 0.012). In this study, 59.2% patients over 45 years had *BRAF* mutation. Nearly three quarters of patients < 45 years of age were in stage I. No statistically significant difference in percentage of *BRAF*[V600E] mutations in primary tumour (p = 0.604) was found between the 2 age groups. An average tumour size was 17 mm (range 2–60 mm), median 15 mm. No significant correlation was found between the *BRAF*[V600E] mutation and tumour size (p = 0.605);

Table 1 Individual histological variants of PTC (papillary thyroid cancer) at the participating institutions.

Histological variant PTC	Institution n (%) Prague	Zlín	Total n (%)
cystic variant	0	1 (3.23)	1 (0.73)
follicular v.	31 (29.2)	3 (9.7)	34 (24.8)
classic papillary v.	63 (59.4)	25 (80.6)	88 (64.2)
papillo-follicular v.	10 (9.4)	1 (3.2)	11 (8.0)
oncocytic v.	1 (0.94)	1 (3.2)	2 (1.5)
undifferentiated v.	1 (0.94)	0	1 (0.73)
total	106	31	137

Table 2 Comparison of clinical and pathological parameters with the detection of the $BRAF^{V600E}$ mutation in the primary tumour of patients with PTC (papillary thyroid cancer).

	Total	BRAF+n (%)	BRAF−n (%)	p-value
sex				0.432
men	34	21 (61.8)	13 (38.2)	
women	103	55 (53.4)	48 (46.6)	
age				0.604
<45 years	59	31 (52.5)	28 (47.5)	
≥45 years	78	45 (57.7)	33 (42.3)	
tumor size				0.605
≤10 mm	51	27 (35.5)	24 (38.3)	
10–40 mm	83	48 (63.2)	35 (57.4)	
>40 mm	3	1 (1.3)	2 (3.3)	
histological variant of PTC				0.022
classic papillary	88	55 (62.5)	33 (37.5)	
follicular	34	12 (35.3)	22 (64.7)	
papillo-follicular	11	6 (54.5)	5 (45.4)	
oncocytic	2	2	0	
cystic	1	1	0	
non-differentiated	1	0	1	
tumor angioinvasion				0.280
yes	48	30 (62.5)	18 (37.5)	
no	89	46 (51.7)	43 (48.3)	
node metastases				0.058
yes	65	42 (64.6)	23 (35.4)	
no	72	34 (47.2)	38 (52.8)	
distant metastases				0.585
yes	3	1	2	
no	134	75 (56.0)	59 (44.0)	
extracapsular growth				0.205
yes	47	30 (63.8)	17 (36.2)	
no	90	46 (51.1)	44 (48.9)	
mono/multifocal localisation				1.000
monofocal	80	44 (55.0)	36 (45.0)	
multifocal	57	32 (56.1)	25 (43.9)	
disease stage				0.145
I.–II.	91	46 (50.5)	45 (49.5)	
III.–IV.	46	30 (65.2)	16 (34.8)	
centres				0.038
Prague	106	55 (51.9)	51 (48.1)	
Zlín	31	23 (74.2)	8 (25.8)	

neither in mono- nor in multifocal locations (p = 1.000) as shown in ○ Table 2.

We found that the $BRAF^{V600E}$ mutation occurred in the classic papillary variant significantly more often (p = 0.008) than in follicular histological variant, while low numbers for papillofollicular, oncocytic, cystic, and undifferentiated variants do not allow for statistical analysis (○ Table 3).

Table 3 Incidence of the $BRAF^{V600E}$ mutation in individual PTC (papillary thyroid cancer) subtypes in analysed samples.

Histological variant	BRAF (+) n (%)	BRAF (−) n (%)	Total
classic papillary	112 (51.1)	107 (48.9)	219
follicular	15 (23.4)	49 (76.6)	64
papillo-follicular	17 (50.0)	17 (50.0)	34
oncocystic	5	0	5
cystic	1	1	2
undifferentiated	0	2	2
total	150	176	326

Table 4 Risk factors for cervical lymph node metastases occurrence (multiple logistic regression model).

Variable	Odds ratio	95% confidence Interval for odds ratio	p-value
BRAF mutation in primary tumour (yes vs. no)	2.39	1.00–5.75	0.052
sex (male vs. female)	3.72	1.34–10.36	0.012
extracapsular growth (yes vs. no)	4.29	1.70–10.78	0.002
primary tumour size (mm)	1.09	1.04–1.14	<0.001

Detection of the $BRAF^{V600E}$ mutation in the primary tumour tended to associate with the presence of neck node metastases but without statistical significance (p = 0.052, ○ Table 2). Other independent predictors of cervical lymph node metastases identified in the multiple logistic regression model included: male sex (p = 0.012), extracapsular growth (p = 0.002), and especially the tumour size (p < 0.001) (○ Table 4). In our sample, patients with tumours > 10 mm had neck node metastases in 66.3 % of cases.

The odds ratio of node metastases in patients with the $BRAF^{V600E}$ mutation, compared with patients without the mutation, was 2.39 (95 % CI 1.00–5.75) after adjustment for the 3 above mentioned independent predictors. Logistic regression model correctly classified 78.8 % of cases. The analytical parameters for $BRAF^{V600E}$ mutation test for the prediction of node metastases were: sensitivity 78.5 %, specificity 79.2 %, positive predictive value 77.3 % and negative predictive value 80.3 %. When using BRAF mutation status as the primary information without patient stratification due to 3 independent predictors, sensitivity is lowered to 64.6 %, specificity to 52.8 %, positive PPV to 55.3 %, and NPV to 62.3 %.

Discussion

▼

The incidence of PTC has been growing almost worldwide in the last 30 years. Some publications claim that thyroid gland tumours peak between 45–59 years [1,2]. Firstly, this is attributed to the improved diagnostic of thyreopathies and secondly to the increased ionizing radiation exposure [1,8,10]. The following clinical-pathological features are accepted reliable predictors of unfavourable prognosis for thyroid carcinoma: patient age ≥45 years, children age, tumour size, extracapsular growth, presence of node metastases at the time of diagnosis, and the disease stage [8,10,15,17]. In the literature, the average frequency of the $BRAF^{V600E}$ mutation in PTC is around 45 % [8,11,18,19], ranging from 22.9 % [20] to 83 % [21], with several studies surpassing 50 % [19,22,23]. The large spread of mutation frequencies may reflect the histological heterogeneity of PTC subtypes, heterogeneity of mutational status across tumour

[24], geography, epidemiologic factors, and, especially, patient age [8, 13].

In our study, the different prevalence of BRAF^{V600E} mutation among individual histological PTC variants was statistically significant. In accordance with other authors, we demonstrated that the BRAF^{V600E} mutation occurred more often in the classic papillary variant than in the follicular or papillo-follicular variant (p = 0.001) [10, 18].

Certain limitations of our study were: a) its retrospective nature; b) the small sample size; c) the absence of an a priori power analysis; d) the fact that thyroidectomies were performed by more than one surgeon and the specimens were histologically subtyped and interpreted by more than one pathologist.

The striking difference in BRAF mutation frequency (33.5% vs. 56.9%) between 2 Czech studies (Sykorova et al. and our own) may be caused by above mentioned limitations of our study, stochastic effect, patient selection bias, and/or difference in the method of BRAF genotyping [25]. We speculate that methodological difference in BRAF status assessment may have caused the difference: while low mutation frequency was found using Single Strand Conformational Polymorphism (SSCP) confirmed by Sanger sequencing (detecting 25% of mutated DNA in surplus of 75% of wildtype DNA [26]), high mutation frequency was found using real time Amplification Refractory Mutation System (ARMS) with internal control of amplification efficiency and hydrolysis probe (detecting 1% of mutated DNA in 99% of wildtype DNA [27]). Such a varied prevalence of the BRAF mutation in the populations from the same region obtained using different genotyping techniques (i. e., PCR-RFLP vs. sequencing) were already reported for Nagasaki [28,29].

We found a statistically significant difference in the frequency of the BRAF^{V600E} mutation between the participating institutions (p = 0.038). These inter-regional differences are probably influenced by the limitations of our study and the fact that Zlín is a region with one of the highest incidences of malignant thyroid tumours in the Czech Republic (10.4 cases/100 000 inhabitants/year) [30]. We can speculate about slight differences in the biogeographical ancestry of cosmopolitan Prague and the Wallachian Zlín populations and/or mutagenic differences between the metropolis and rural area, environmental factors e. g. iodine and selenium deficiency and regional ionizing radiation.

In our analysis, we found in 2 cases BRAF negative primary tumor site and BRAF positive metastasis in PTC. This finding may be caused by a heterogeneity of the primary tumour, an artefact of laser capture microdissection, and/or accumulation of mutations in metastasis [31,32].

Research into the BRAF mutation has been a subject of growing attention. One of the reasons is that inhibition of the mutated BRAF^{V600E} reveals a high response rate, fast regression and significant prolongation of patient survival [33]. Xing M. confirmed the prognostic value of the BRAF^{V600E} mutation and its correlation with clinical and pathological parameters, especially with the extracapsular growth of the presence of neck nodal metastases and advanced stages III–IV [34]. Kim TH et al. found in their meta-analysis that patients with PTC and BRAF ^{V600E} mutation had 2.14 times increased risk of extrathyroidal invasion, 1.54 times increased risk of a lymph node metastasis, 2.0 times increased risk of an advanced TNM stage, and 2.14 times increased risk of recurrent and persistent disease [18].

Lee JH et al. reported a 2-fold increase in extracapsular growth and higher disease stage in patients with the BRAF^{V600E} mutation

[13]. Even though the presence of nodal metastases and extracapsular growth was almost 30% lower than in the group of patients with BRAF^{WT}, they did not confirm any association between nodal metastases and extracapsular growth and the incidence of the BRAF^{V600E} mutation (<50% and ≥50%, respectively) [16]. Sykorova V. et al. and other authors found that the BRAF^{V600E} mutation in PTC significantly correlates with the following clinicopathological characteristics: the presence of nodal metastases (p = 0.029), more advanced TNM stage (p = 0.014), recurrence of disease (p = 0.008), and greater size (p = 0.041) [10,13,34]. In accord with some authors [14–16,35,36], we found no significant association between the BRAF^{V600E} mutation and other measured clinical parameters, sex (p = 0.432), extracapsular growth (p = 0.205), angioinvasion (p = 0.365), and presence of distant metastases (p = 0.585), suggesting that BRAF mutation is a clonal event only rarely in PTC. Furthermore, we found only non-significantly higher prevalence of the BRAF mutation in patient with metastases than in patients without metastases (p = 0.052). Similarly, the patients with BRAF p.Val600Glu mutation of the primary tumour had only non-significantly higher risk of cervical lymph node metastases. In our study, male sex (p = 0.001), extracapsular growth (p = 0.007), and especially the size (p < 0.001) were independent predictors of node metastases. According to our study, analytical parameters of positive predictive value and negative predictive value (55.3% and 62.3%, respectively; after adjustment for male sex, extracapsular growth, and especially the tumour size 77.3% and 80.3%), BRAF mutation seems to be less significant predictor of cervical lymph node metastases than other clinicopathological factors and thus, of low clinical value.

Conclusion
▼

Despite the worldwide efforts to improve prognosis, prediction, and therapy for PTC by targeting BRAF, the correlation between BRAF mutation and patient survival is still a matter of controversy. With except of higher prevalence in papillary variant of PTC, BRAF p.Val600Glu mutation was not associated with other prognostic clinicopathological factors of PTC in our study. Patients with BRAF p.Val600Glu mutation or primary tumour had only non-significantly higher risk of cervical lymph node metastases. BRAF p.Val600Glu mutation testing cannot be regarded as a reliable marker of node metastases in patients with PTC. Thus, search for further PTC prognostic biomarkers needs to be broadened, i. e., to microRNAs or methylation status [37].

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2.5 Whole genome amplification (WGA) before *KRAS* genotyping

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Short Communication

Whole genome amplification induced bias in the detection of *KRAS*-mutated cell populations during colorectal carcinoma tissue testing

Whole genome amplification replicates the entire DNA content of a sample and can thus help to circumvent material limitations when insufficient DNA is available for planned genetic analyses. However, there are conflicting data in the literature whether whole genome amplification introduces bias or reflects precisely the spectrum of starting DNA. We analyzed the origins of discrepancies in *KRAS* (Kirsten rat sarcoma viral oncogene homolog gene) mutation detection in six of ten samples amplified using the GenomePlex® Tissue Whole Genome Amplification kit 5 (WGA5; Sigma-Aldrich, St. Louis, MO, USA) and KRAS StripAssay® (KRAS SA; ViennaLab Diagnostics, Vienna, Austria). We undertook reextraction, reamplification, retyping, authentication, reanalysis, and reinterpretation to determine whether the discrepancies originated during the preanalytical, analytical, and/or interpretative phase of genotyping. We conclude that a combination of glass slide/sample heterogeneity and biased amplification due to stochastic effects in the early phases of whole genome amplification (WGA) may have adversely affected the results obtained. Our findings are relevant for both forensic genetics testing and massively parallel sequencing using preamplification.

Keywords:

Bias / Colon cancer / FFPE sample / *KRAS* mutation genotyping / Whole genome amplification

DOI 10.1002/elps.201400136

The small amount of sample material can severely limit the number of genetic tests from tumor [1, 2], crime scene [3, 4], preimplantation [5, 6], and metagenomic samples [7]. This problem could potentially be overcome using WGA techniques such as multiple displacement amplification using the bacteriophage φ29 DNA polymerase [8], primer extension preamplification [9], degenerate oligonucleotide-primed PCR [10], the OmniPlex technology (Rubicon Genomics, Inc.) [11], or primase-based WGA methods [12].

However, any preamplification strategy may introduce a degree of bias due to an uneven start or continuation of amplification. Various levels of bias have been reported for WGA [13–16], although in many studies no significant bias

was observed and the WGA approach was recommended for future use [17–19].

We recently participated in an external quality assessment (EQA) organized by European Society of Pathology focusing on the detection of the *KRAS* mutation in colon carcinoma tissue. We were using the WGA5 kit (Sigma-Aldrich) together with the KRAS SA (ViennaLab Diagnostics) (see Fig. 1). This combination had previously been internally validated on 301 samples in our laboratory for cases where only small quantities of input DNA were available. For validation purposes, two methods, NucleoSpin® Tissue XS (MACHEREY-NAGEL, Düren, Germany) extraction followed by genotyping using kit QGENEKRAS (Institute of Applied Biotechnologies, Prague, the Czech Republic) and WGA5 kit (Sigma-Aldrich) running on PTC-200 Peltier Thermal Cycler (MJ Research Inc., St. Bruno, Canada) followed by genotyping using KRAS SA (ViennaLab Diagnostics) were compared to gold standard of NucleoSpin Tissue extraction followed by TheraScreen DxS®: KRAS Mutation kit (KRAS TS; Roche Diagnostics, Mannheim, Germany). None of the two methods tested had false-negative samples. However, QGENEKRAS (Institute of Applied Biotechnologies) had four false-positive samples while WGA-KRAS SA had 14 false-positive samples.

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Abbreviations: EQA, external quality assessment; **FFPE tissue**, formalin-fixed paraffin-embedded tissue; *KRAS*, Kirsten rat sarcoma viral oncogene homolog gene; **KRAS SA**, KRAS StripAssay; **NGS**, next-generation sequencing; **WGA**, whole genome amplification

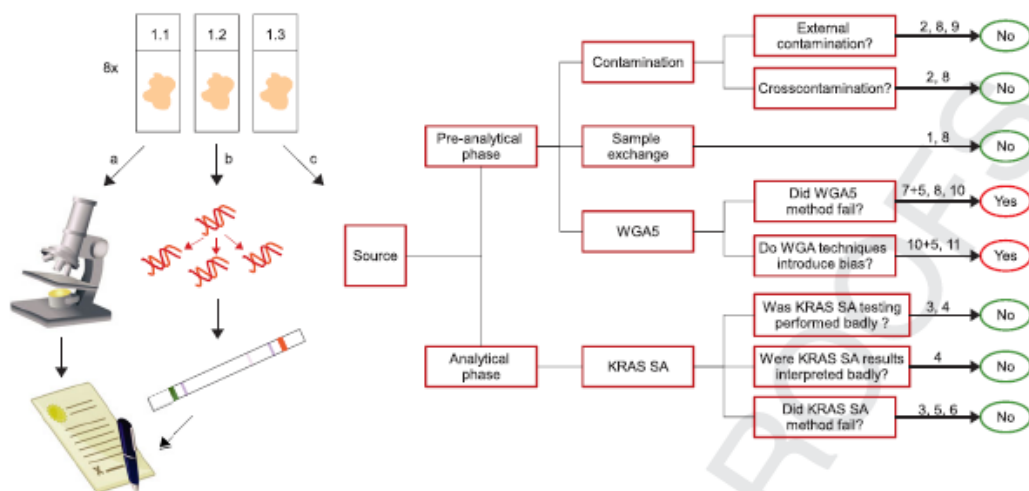**Colour Online**: See the article online to view Fig. 1 in colour.

Figure 1. Workflow for the analysis of KRAS-EQA FFPE samples in our laboratory and in the experiments performed to identify the origin of the observed bias. We received three glass slides of 6 μm sections of eight different colorectal carcinomas in paraffin-embedded material and two external quality control samples in sample tubes. (A) The first FFPE section was hematoxylin-eosin stained and its tumor content was evaluated by a pathologist. (B) The second section and external control samples were used for WGA5 (Sigma-Aldrich) amplification and KRAS mutation analysis with the KRAS SA (ViennaLab Diagnostics). All remaining sections were used for corrective actions. (C) Corrective actions were performed to identify sources of bias. 1, sample registration was performed well; 2, WGA5 NTC control did not show any detectable contamination; 3, KRAS SA (ViennaLab Diagnostics)—amplification products were present as checked by electrophoresis, while NTC and positive controls yielded expected results; 4, blind reinterpretation of the KRAS SA (ViennaLab Diagnostics) results yielded the same results; 5, internal retesting with KRAS TheraScreen (KRAS TS, Roche Diagnostics) yielded the same result (see Table 1); 6, external retesting with the cobas® KRAS Mutation Test yielded the same result (see Table 1); 7, DNA was extracted from the remaining FFPE slice using the cobas® DNA Sample Preparation kit; 8, both WGA5 (Sigma-Aldrich) and cobas-extracted samples were identity checked by STR typing (results not shown); 9, an open-tube experiment did not reveal any detectable contamination; 10, cobas-extracted DNA was used as a target for the WGA2 kit and one incorrect mutation was detected (see Table 1); 11, subsequent WGA experiments (see Table 2).

Table 1. *KRAS* genotyping results obtained during EQA examination and corrective actions

Sample no.	Correct EQA result	WGA5			cobas Reextraction	
		StripAssay	TheraScreen	cobas Mutation Test	TheraScreen	Reamplification and TheraScreen
1	No mutations	p.Gly13Asp	p.Gly13Asp	Mutation present	No mutations	No mutations
2	p.Gly12Asp	No mutations	Impossible	Impossible	p.Gly12Asp	Consumed
3	p.Gly12Val	No mutations	Impossible	No mutations	Consumed	Consumed
4	No mutations	p.Gly13Asp	p.Gly13Asp	Mutation present	No mutations	Impossible
5	No mutations	p.Gly12Cys	p.Gly12Cys	Mutation present	No mutations	p.Gly12Ser
6	No mutations	p.Gly13Asp	p.Gly13Asp	Mutation present	No mutations	No mutations

This result was interpreted within acceptable limits for both tested methods, reflecting their higher sensitivity in comparison to KRAS TS (Roche Diagnostics).

In EQA samples, WGA5 (Sigma-Aldrich) was used on one formalin-fixed paraffin-embedded (FFPE) slice per sample and the whole protocol was run according to manufacturer's instructions, including negative and positive controls. All reagents were before expiration date. However, we observed both false positives and false negatives in those reagents (Table 1).

We stopped using WGA5 (Sigma-Aldrich) and the KRAS SA (ViennaLab Diagnostics), and performed a vertical audit together with a series of tests to determine whether the standard operating procedure had been followed correctly (Fig. 1C). Internal retesting using the KRAS TS (Roche Diagnostics) gave results congruent with the KRAS SA (ViennaLab Diagnostics); conclusive results could not be obtained for two samples due to the low quality of their DNA. The DNA samples were retested externally using the cobas® KRAS Mutation Test (Roche Diagnostics), affirming the KRAS SA (ViennaLab Diagnostics) results in five cases; no result could be obtained for the sixth. These results indicate that the discrepancy originated during the preanalytical or DNA preparation phase.

DNA was therefore extracted from the remaining FFPE slices (if available) using the cobas® DNA Sample Preparation kit and genotyped for *KRAS* using KRAS TS (Roche Diagnostics). The resulting mutation statuses were entirely in concordance with the EQA consensus results. Both WGA5 (Sigma-Aldrich) and cobas-extracted samples were identity checked by STR typing (PowerPlex16, Promega, Madison, WI, USA). Interpretation of the STR results showed that the EQA DNA samples were primarily of low quality, with enhanced stochastic effects. WGA5 (Sigma-Aldrich) generated even more stutters, imbalance, allele dropout, and allele drop-in, but there was no mislabeling or confusion of samples. Open-tube negative control experiments [20] did not reveal detectable contamination by specific gDNA or amplicons. Based on these results, we conclude that the source of the discrepancy is in the very start of analytical phase.

To test whether WGA can introduce bias in controlled experiment, cobas-extracted DNA was amplified according to manufacturer's protocol using the WGA2 kit (Sigma-Aldrich) and then KRAS TS (Roche Diagnostics) genotyped. After WGA2, the incorrect mutation p.Gly12Ser was detected in the wild-type sample; this mutation differs from the p.Gly12Cys mutation produced by WGA5 (Sigma-Aldrich). The difference in mutation type for a single sample when using the WGA5-StripAssay and cobas-WGA2-TheraScreen may be due to a minor contamination and/or preferential WGA amplification of mutated DNA that was already present in the sample but below the detection limit. Also, errors could have been introduced by the stochastic mispriming of oligonucleotides, as encountered in our previous study [21].

To quantitatively elucidate our hypothesis that WGA both increases and decreases the relative number of mutated copies in the sample, three samples of *KRAS* wild-type FFPE slices from one source were amplified with two WGA5 kits (Sigma-Aldrich) of different lots and a WGA technique that uses a different principle: the Repli-g FFPE kit (Qiagen, Hilden, Germany). The amplified DNA was genotyped with KRAS TS (Roche Diagnostics) and sequenced by massively parallel sequencing (also next-generation sequencing, NGS). Namely, DNA was amplified using Thermo-Start Taq DNA Polymerase (Thermo Scientific, Waltham, MA, USA). The primers for *KRAS* genotyping were designed using Primer3 v. 0.4.0. The purified amplicons were tagmented and indexed using Nextera XT DNA Sample Preparation kit (Illumina, San Diego, CA, USA). The pooled samples were sequenced on MiSeq personal sequencer using MiSeq Reagent kit v2—300 cycles (both Illumina) with coverage exceeding 10 000 in every sample tested. The output data were preprocessed using MiSeq Reporter 2.3 (Illumina) and then variants calls were processed using homemade MS Excel VBA script. A false-positive (p.Gly12Cys) rate of 2% was determined for one WGA-amplified wild-type sample using NGS, and qualitatively indicated by KRAS TS (Roche Diagnostics). Samples amplified by Repli-g FFPE were assigned correctly.

Our laboratory has worked under the ISO17025:2005 and ISO15189:2007 standards since 2007 and 2012, respectively,

Table 2. Changes in mutation representation after WGA (Repli-g FFPE kit)

Sample no.	Gene	Mutation	Mutation representation (%)	
			gDNA	WGA
1	*NRAS*	Q61K	46	35
2		Q61L	49	43
3		Q61H	49	91
4		Q61R	74	98
5	*BRAF*	V600K	43	50
6		V600R	45	43
7		V600E	14	0
8		V600E	2	1
9	*KRAS*	G12D	11	1
10		G12S	56	42

and thus participates every year in at least one EQA as well as in several sample exchanges with other laboratories within the Czech Republic for *EGFR*, *KRAS*, and *BRAF* mutation testing. Our participation in these exercises has not suggested any lack of competence or poor performance with these methods. Nor was there any indication of increased background contamination in the laboratory in the time of EQA.

As stated above, we used massively parallel sequencing to verify our results. However, it must be noted that library preparation protocols (nonrandom DNA fragmentation, fragment size selection, and unbalanced PCR amplification due to GC content differences [13]) and bioinformatics algorithms (aligners, alignment parameters, and prealignment data filtering tools [22]) can introduce bias comparable to that associated with WGA. We have observed considerable quantitative changes in the representation of *KRAS* mutation and in other genes, tested in presumably degraded FFPE samples in independent NGS runs (Table 2). FFPE DNA is a problematic source material [23] whose negative properties can adversely affect NGS results. Therefore, WGA bias may have been missed in some papers [17] because high-quality input DNA was used, with fragments of sufficient length, a suitable sequence with a balanced GC content, and no chemical damage induced by formalin.

Bias induced by WGA may also be preamplification method-specific. Both of our tested preamplification methods (adaptor-based WGA5 (Sigma-Aldrich) and multiple displacement based Repli-g) introduced some bias. Other authors have found the multiple displacement principle to be the least bias-producing method [15, 19, 24]. The less known isothermal method, which uses a protein (primase) instead of primers for amplification priming [12, 14, 25], can potentially avoid bias due to primer dimer formation and chimeric DNA rearrangements [26]. On the other hand, it can induce bias due to the dependency of primase on specific sequences, which may present limitations for short template fragments [14].

We excluded all potential sources of error in our EQA tests other than sample heterogeneity and random low

threshold contamination that could not be detected using regular no-template control combined with biased amplification due to stochastic effects during the early phases of amplification by WGA5 (Sigma-Aldrich).

We conclude that the WGA methods should be used in clinical and/or forensic settings only after validation for the appropriate sample types from the beginning to the very end of all procedures. Validation should include EQA samples, and careful consideration should be given to experimental design and choice of the golden standard.

The authors have declared no conflicting interests.

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2.6 Clinical Relevance of KRAS in Human Cancers

Review Article

Clinical Relevance of KRAS in Human Cancers

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The *KRAS* gene (Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) is an oncogene that encodes a small GTPase transductor protein called KRAS. KRAS is involved in the regulation of cell division as a result of its ability to relay external signals to the cell nucleus. Activating mutations in the *KRAS* gene impair the ability of the KRAS protein to switch between active and inactive states, leading to cell transformation and increased resistance to chemotherapy and biological therapies targeting epidermal growth factor receptors. This review highlights some of the features of the *KRAS* gene and the KRAS protein and summarizes current knowledge of the mechanism of *KRAS* gene regulation. It also underlines the importance of activating mutations in the *KRAS* gene in relation to carcinogenesis and their importance as diagnostic biomarkers, providing clues regarding human cancer patients' prognosis and indicating potential therapeutic approaches.

1. Introduction

In 1982 Chang and Der, two postdoctoral fellows working in Geoffrey Cooper's laboratory, discovered Kristen Rat Sarcoma Virus and Murine Sarcoma Virus; retroviral oncogenes related to rodent sarcoma virus genes. The human *KRAS* gene is a homolog of these two oncogenes [1]. A normal form of human c-Ras has been called KRAS or KRAS2 (Kristen Rat Sarcoma Viral oncogene homolog or alternatively Kristen Murine Sarcoma Virus2 homolog). In 1983, Der described an abnormal form of the p21 protein expressed by colon and lung carcinoma cell lines and showed that the gene encoding this protein is able to transform NIH3T3 cells [2]. This finding was later confirmed by Parada and Weinberg [3], who described the transformation of NIH3T3 cells by an activated KRAS oncogene. Aberrant p21 proteins were encoded by the altered *KRAS* gene and their expression in carcinoma tissue was causally linked to an abnormal state of activation [2]. Since then, it has been accepted that KRAS is one of front-line sensors that initiate the activation of an array of signalling molecules allowing the transmission of transducting signals from the cell surface to the nucleus, thus affecting cell differentiation, growth, chemotaxis, and apoptosis. A signal transduction cascade initiated by the activated form of KRAS is depicted in Figure 1. As a result of these effects, KRAS elicits changes in the cytoskeleton and consequently affects cell shape, adhesion and migration [4, 5].

In the following paragraphs, KRAS protein, gene, oncogenesis, and cancer therapy is reviewed.

2. KRAS Protein

2.1. KRAS Protein Structure, Function, and Localization.
KRAS belongs to a group of small GTP-binding proteins, known as the RAS superfamily or RAS-like GTPases. More than 150 RAS-like genes have been identified in mammalian genomes [6]. The entire RAS superfamily is characterised by the presence of a catalytic G domain, but includes members with distinct evolutionary specializations with respect to different cellular process [7]. The RAS subfamily (RAS, RHO, RAB, ARF, RAC, and RAN) includes the most frequently studied proteins, such as Harvey-Ras (H-RAS), neuroblastoma-Ras (N-RAS), and two variants of Kristen-RAS (K-RAS)—one, known as KRAS4A, which is weakly expressed in human cells and the dominant form, known as KRAS4B, which is much more highly expressed.
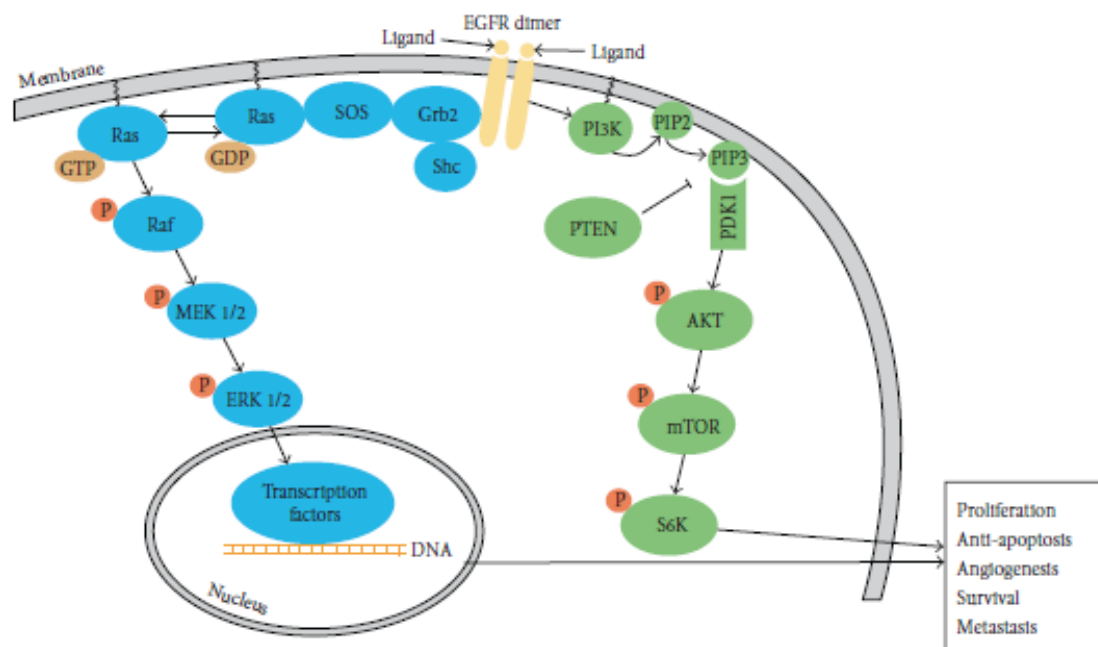
FIGURE 1: Signaling pathway of the KRAS protein. Following EGF binding to its receptor and activation of tyrosine kinases, the KRAS protein becomes activated by binding to GTP, transducing the activation signal to the nucleus by MAPKs and PI3K/AKT-mediated cascades. Specifically, the active state of the KRAS protein is facilitated by binding to the Grb2 protein, which interacts with the SH3 domains of the SOS protein, a member of the nucleotide exchange factor family. In the GTP state, KRAS is able to activate downstream proteins and to regulate cell transformation.

The *KRAS* gene product, KRAS protein, contains 188 amino acid residues with a molecular mass of 21.6 kD and participates in intracellular signal transduction [8]. As mentioned above, the KRAS protein remains inactive until it binds to GTP, as depicted in Figure 2. The switch from an inactive to an active form is regulated by intracellular signals. Once the GTP is bound to the KRAS protein, KRAS undergoes conformational changes that involve two regions of the protein, thus activating it. These two important regions are known as Switch 1 (aminoacids 30–38) and Switch 2 (aminoacids 59–67), which form an effector loop, controlling the specificity of the binding of this GTPase to its effector molecules. This conformational change in the KRAS protein affects its interactions with multiple downstream transducers—GTPase-activating proteins (GAPs)—which amplify the GTPase activity of the RAS protein 100,000-fold [9]. The change also affects interactions with guanine-exchanging/releasing factors (GEFs/GRFs) promoting the release of GTP. The KRAS protein also has intrinsic GTPase activity, stimulated by GAPs, which acts as a timer associated with direct interactions with the effectors [10]. Mutations found in an oncogenic form of the RAS p21 protein impair GTPase activity and make the KRAS protein unresponsive to GAP proteins. Mutated forms of p21 rapidly exchange GDP for GTP, which it prefers as a substrate, thus inducing the active state. Such aberrant forms of KRAS protein deregulate many effectors, thus affecting several important cellular pathways. Many GTP derivatives targeting
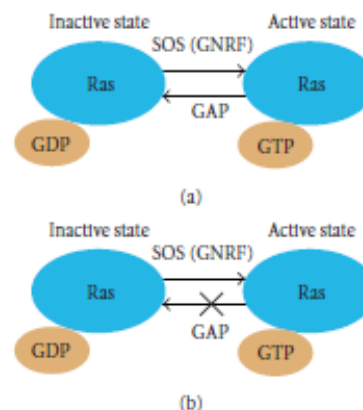

FIGURE 2: Activity states of the KRAS protein. Mutational change in exon 1 of KRAS leads to permanent "on" status.

RAS or RAF effectors have been developed to repair the defective GTPase activity that influences the aberrant RAS signalling [11]. However, little is known about the specificity and transport of compounds modified by GTPs through the plasma membrane.

KRAS contains four domains. The first domain includes 85 amino acids at the N-terminus and is identical in the three forms of RAS (KRAS, NRAS, and HRAS). The second
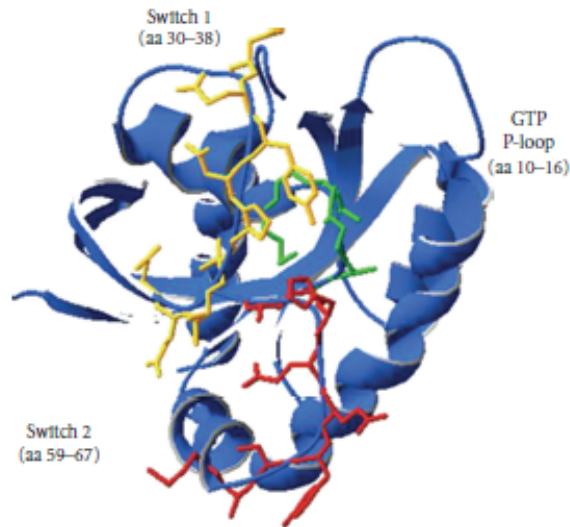
FIGURE 3: Model of the KRAS protein with important domains highlighted. Model of 3GFT molecule was rendered in Swiss-PdbViewer v4.0.1 (http://spdbv.vital-it.ch/). Switch 1, Switch 2, and GTP P-loop domains are highlighted by colour change.

domain contains 80 amino acids, with lower sequence identity (70–80%) among the three forms of RAS protein. These regions are important for the signalling function of the KRAS protein and jointly form the G-domain (amino acids 1–165, Figure 3). The G-domain of the KRAS protein includes the GTP-binding pocket, where P-loop-phosphate binding loops (aminoacids 10–16 and 56–59) interact with the b-phosphate and c-phosphate of GTP. Residues 116–119 and 152–165 interact with the guanine base. The region between amino acids 32 and 40 (the core effector region) is essential for the interactions between the putative downstream effectors and GAPs. RAS protein also contains a hypervariable region (HVR) at the C-terminus (amino acids 165–188/189; the third domain), which guides posttranslational modification and determines plasma membrane anchoring. This region plays an important role in the regulation of the biological activity of RAS protein [12].

Switch regions I and II play important roles in the binding of regulators and effectors. The phosphate binding pocket-P loop permits temporary binding of GTP to the RAS protein. This is also the region of GTPase activity, which negatively regulates the RAS protein via a RAS-GTP hydrolysis reaction and binding of guanosine diphosphate.

The KRAS protein acts like a plasma membrane-localized molecular switch, regulating multiple signal transduction pathways [13]. It is synthesized in the cytosol, where it is farnesylated by farnesyl transferase at the cysteine residue of the carboxy-terminal motif CAAX (where C represents cysteine, A is an aliphatic amino acid, and X is any amino acid). The carboxy-terminal motif forms the last domain of the KRAS protein. The AAX amino acid motif is cleaved by proteases, whereas the C-terminal carboxyl residue of the KRAS protein is methylated. Cleavage of the AXX peptide motif and methylation occur at the cytosolic surface of the endoplasmatic

reticulum and are mediated by the RAS-converting enzyme Rce1 [14]. C-terminal farnesylation plays an important role in membrane localization. In the splice variant KRAS4A, the AXX motif undergoes additional palmitoylation by palmitoyl transferase, resulting in proper targeting of KRAS4A to the membrane. However, there is no detectable palmitoylation of the predominant splice variant KRAS4B, which probably reaches the plasma membrane via a microtubule-dependent mechanism, thus avoiding the Golgi apparatus [13, 15]. Posttranslational farnesylation and carboxymethylation are believed to be important for the oncogenicity of the RAS protein. Treatment with farnesyl transferase inhibitors has been shown to inhibit anchorage-independent growth of both KRAS-transformed mouse fibroblasts and human tumour cells containing KRAS and NRAS mutations. Signal transduction of the KRAS protein does not exclusively occur at the plasma membrane. Activation of downstream signalling pathways by KRAS can also be triggered by signals from subcellular compartments, such as the endoplasmatic reticulum and the Golgi apparatus [16, 17].

2.2. Why Is the KRAS Protein an Important Target to Study? While wild-type KRAS usually promotes cell cycle progression, it can also induce growth arrest, apoptosis, and replicative senescence when increased to abnormal levels. This can be triggered by cellular stress, ultraviolet or ionizing irradiation, heat shock, and some cytokines. In these circumstances, triggering of growth arrest can represent a defence mechanism against inappropriate activation of RAS.

It has been demonstrated that the wild-type *KRAS* gene is a tumour suppressor that is frequently lost during tumour progression in many types of cancer [18]. Once the *KRAS* gene mutates, it acquires oncogenic properties (Table 1) and seems to be causally involved in the development of various human cancers [19, 20]. Loss of the wild-type *KRAS* allele has been observed in both human and mouse tumours, indicating that absence of the normal allele may facilitate transformation by one copy of the oncogenic *KRAS* allele [21]. Oncogenic mutations in the *KRAS* gene prevent the hydrolysis of GTP, thus permanently activating the RAS molecules [22]. Expression of a mutated *KRAS* gene in fibroblasts has been shown to augment metalloproteinase 2 (MMP2) expression in the matrix and enhance the invasion of cancer cells [23]. Overexpression of this mutated form of KRAS also inhibits glycosylation of the integrin β1-chain, resulting in altered polarisation and increased adhesiveness of colon cancer cells. In addition, expression of this oncogenic form of KRAS protein has been shown to be associated with upregulated carcinoembryonic antigen (CEA) expression and disturbance of epithelial cell polarization [24].

3. The *KRAS* gene

3.1. Polymorphism/Structure and Localization of the KRAS Gene. There are two copies of the *KRAS* gene in the human genome, designated KRAS1 and KRAS2. The mRNA encoded by the main KRAS2 is 5.5 kb long, and differs from

TABLE 1: Consequences of oncogenic KRAS on cellular physiology and biology.

RAS effector pathways	Main function of the RAS signalling pathways	Oncogenic RAS perturb the intrinsic biochemical properties of cell pathways
MAPK	proliferation	increase
PI3K/AKT	survival	
NORE1/RASSF1	apoptosis	deregulation
RAL-GDS	membrane vesicle trafficking	
JAK/STAT3	growth arrest and differentiation	
TIAM1/RAC	cytoskeletal organization	
PLCε/PKC	calcium transport signalling	
AF6	cell-cell junctions	
PKCξ	transcription	
PI3K/PDK1	translation	

transcripts of the transforming Kristen murine viral gene by only six codons [8]. Analysis of human placental and embryonic cDNA libraries has revealed that 900 bp of the KRAS1 gene is homologous to the corresponding sequence of the Kristen Murine Sarcoma Virus2 homolog, with one intervening sequence, and 300 bp of the KRAS2 is fully homologous to the viral counterpart. The KRAS1 gene is a pseudogene derived from KRAS2 by alternative mRNA splicing. Therefore, KRAS1 should be officially named KRAS1P [8].

In 1983 McBride and colleagues found that the pro-tooncogenes KRAS1 and KRAS2 are localized at human chromosomes 6 and 12, respectively [25]. Later, KRAS1 and KRAS2 were mapped by *in situ* hybridization to chromosome positions 6p11-12 and 12p11.1-12.1, respectively [26].

Sequencing showed that the KRAS2 gene has six exons. Of these, 2, 3, and 4 are invariant coding exons. Alternative splicing of exon 4 produces two mRNA forms, known as 4A and 4B. Exon 5 can be skipped during alternative splicing, giving rise to isoforms KRASA and KRASB. The 6th exon encodes the C-terminal region in KRASB and is not translated (the 3′untranslated region, 3′UTR) in KRASA. KRASB is the predominant splice variant of KRAS2, and is referred to, briefly, as KRAS [27].

There are indications that allelic losses of chromosome region 12p commonly occur in human cancers, and a frequently deleted region is near the KRAS gene at position 12p12-13 [28]. Further, recent studies on lung adenocarcinoma suggest there is an association between the incidence of allelic losses in the 12p12-13 region and KRAS gene mutation [29].

3.2. Diagnostics of KRAS Mutations in Human Cancers. Diagnostics of KRAS gene mutations in clinical setting is limited by two factors: first, in the time of testing, KRAS mutated tumour cells may be in minority, outbalanced by wild type tumour cells and wild type non-tumour cells present in the sample. Second, analytically preferable snap-frozen tumour samples are rarely available for KRAS mutation testing. Instead, formalin fixed paraffin-embedded (FFPE) tissue is used. There, integrity of DNA may be severely compromised by procedure of formalin fixation (especially by its long duration and low pH).

All the known principles of DNA polymorphism detection are applicable to KRAS mutation detection and demand a dedicated review outside of the scope of this paper. More than 60 methods described can be divided into sequencing methods [30–37], methods based on specific interaction with oligonucleotide, methods based on specific interaction with enzyme [38–40], and conformational methods [41–47]. While many specificity and/or sensitivity enhancement of methods were described as well [48–53], analytical validation, systematic comparison, and assessment of methods side by side is lacking. To authors best knowledge, only Communauté Européene (CE) marked KRAS mutation detection kits are supplied by DxS (mutations in codons 12 a 13 are tested using principle of ARMS-PCR [54] and Scorpion primers [55], Vienna-Lab (reverse dot blot assay format), TIB Molbiol (KRAS LightMix clamped hybridization probes for codon 12), and Invigene (qPCR with sequence suppressor agent StopPrimer for the unwanted excess component, applicable for first two nucleotide positions in codons 12 and 13).

3.3. Regulation of KRAS Gene Expression. KRAS expression is regulated both during the initiation of transcription by the binding of proteins to its promoter and during transcriptional elongation by microRNAs affecting KRAS mRNA stability. Both human and murine KRAS gene promoters contain a nuclease hypersensitive polypurine-polypyrimidine element (NHPPE). The G-rich strand of NHPPE located in the proximal promoter sequence is able to form an intramolecular parallel G-quadruplex, consisting of three G-tetrads and three loops, which recognizes and binds nuclear proteins that are involved in transcriptional repression of KRAS expression. Accordingly, it has been reported that sequestration of nuclear proteins that bind to NHPP by an oligonucleotide mimicking the KRAS G-quadruplex resulted in 40% inhibition of KRAS transcription, compared to controls [56].

The transcription of KRAS is regulated, in part, by an interaction between the promoter region and the 65 kDa ESXR1 protein and, in part, by microRNAs (miRNAs). ESXR1 is a human protein with an N-terminal homeodomain in the nucleus and a C-terminal proline-rich repeat region I in the cytoplasm. The N-terminal fragment of ESXR1 binds to the TAATGTTATTA consensus sequence in exon-1 of the KRAS gene, thus inhibiting its mRNA expression [57]. miRNAs contain a 21-22 nucleotide long noncoding sequence that is able to regulate gene expression [58]. In 2005 it was estimated that there are more than 500 miRNAs, which collectively regulate approximately 30% of all human genes, including the RAS gene family [59].

Regulation of gene expression by miRNAs probably occurs as a result of imperfect hybridization of the miRNA to the complementary sequences located in the 3′untranslated region (3′UTR) of target messenger RNA (mRNA) species. This interaction between miRNA and mRNA both decreases mRNA stability and represses protein synthesis by preventing access to ribosomes [60]. Interestingly, many altered miRNAs have been identified in human cancers [61–63], including some of the most thoroughly analyzed miRNAs—let-7 [64], lin-4 [65], and bentam [66]—all of which regulate cell proliferation and differentiation. Cancer-altered miRNAs can be divided into two main groups. Members of one group, the oncomirs, are upregulated in cancer and can act like oncogenes. The second group, the anti-oncomirs, probably act as tumour suppressors by targeting oncogenes, repressing the cell cycle and division of cancer cells [67]. For example, miRNA-let7 is an oncogene-antioncomir pair that negatively regulates RAS protein levels and decreases cell proliferation rates [68, 69]. KRAS, NRAS, and HRAS harbour multiple let-7 miRNA complementary sites (LCSs) in their 3′UTRs [70]. Zhang et al. [18] found that reducing the activity of let-7 in HeLa cells resulted in a 70% increase in RAS protein levels, while Takamizawa et al. [68] found that let-7 expression was 80% lower in 60% of lung cancer adenocarcinoma and squamous cell carcinoma lines than in normal lung tissue. Moreover, a correlation between low levels of let-7 miRNA and significantly higher RAS protein expression has been found in lung squamous cell carcinomas. These results suggest that let-7 is able to downregulate the expression of RAS in human carcinomas.

These molecular findings provide a strong rationale for developing novel therapeutic treatments aimed at decreasing KRAS protein expression in cancer cells. In many cases KRAS protein expression is dramatically increased due to mutations in the KRAS gene sequence, thus making cells refractory to current therapies, such as those involving use of epidermal growth factor receptor inhibitors [71].

4. KRAS in Oncogenesis

Activating KRAS gene point mutations have been detected in many types of human tumours [72]. Such oncogenic forms of the KRAS gene are prevalent in pancreatic carcinomas (>80%), colon carcinomas (40–50%), and lung carcinomas (30–50%), but are also present in biliary tract malignancies, endometrial cancer, cervical cancer, bladder cancer, liver cancer, myeloid leukemia [73, 74] and breast cancer [75].

Mutations in the KRAS gene have important effects on the process of carcinogenesis, which depend on the cells and tissues involved [76]. The mutations found most frequently in the KRAS gene of cancer cells are located at positions 12 and 13 in exon 1, and less frequently in codons 61, 63, 117, 119, and 146 [77, 78]. These mutations are located near to the GTP binding site. Allelic mutations result in amino acid changes, namely Gly to Asp, Ala, Arg, Ser, Val, or Cys in codon 12 and Gly to Asp in codon 13 [79]. Somatic missense mutations at positions 12, 13, 61, and 63 enable perturbation of the intrinsic GTPase activity of the KRAS
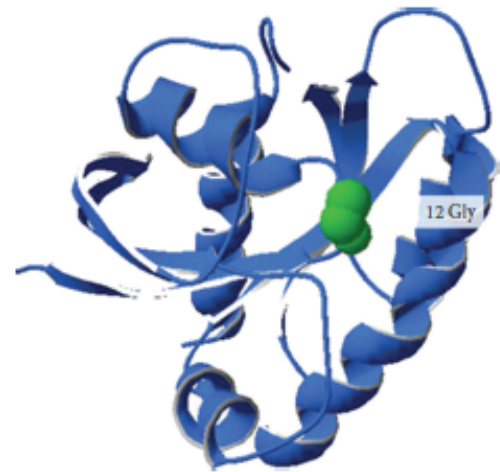

FIGURE 4: Position of codon 12 in KRAS molecule. Wild type aminoacid at codon 12 is shown in green.

protein, resulting in reductions in GTP hydrolysis capacity. Mutations in codons 12 (Figure 4) or 13 are known to lead to conformational changes in the KRAS protein.

Mutation in codon 12 of the KRAS gene causes the encoded KRAS protein to "freeze" in its active state for a much longer duration than its nonmutated counterpart [11]. Mutations resulting in the substitution of amino acids 116, 117, 119, and 146 reduce the nucleotide affinity of the KRAS protein, thereby affecting the rate of GDP/GTP exchange. The oncogenic forms of the RAS protein have a profound effect on the downstream effector pathways, resulting in much higher proliferation rates of cancer cells expressing such forms.

The transforming ability of the KRAS oncogene may result from overexpression of the mutant KRAS allele or from deletion of the wild-type allele [80]. Overexpression of KRAS can also be induced by the loss of p16INK4 (CDKN2A), p19INK4 (CDKN2D), or p53 [81]. However, studies by Zhang et al. (2001) have shown that the wild-type KRAS allele can suppress the oncogenic function of the mutated allele [18].

In addition, the radiosensitivity of tumour cells is altered by oncogenic RAS expression, probably as a result of the effect of the KRAS mutation on several intercommunicating pathways [82].

4.1. Pancreatic Cancer. The prevalence of mutations in the KRAS gene at the time of diagnosis is highest in pancreatic cancers (>80% of cases), notably pancreatic adenocarcinomas predominantly harbour KRAS forms with a guanine to thymine transversion in codon 12 [83]. Wei and colleagues examined samples collected from 30 patients with pancreatic cancer and found that 24 of them showed mutations at codon 12 and only one at codon 13 [84]. However, concurrent KRAS mutations frequently occur in patients with pancreatic cancer [85]. A positive association has been found in patients with pancreatic cancer between tobacco exposure

and mutations in the *KRAS* gene [86]. Similar associations have also been reported for coffee drinking [87], and milk, butter, and alcohol consumption [88, 89]. However, no direct evidence of a causal relationship between these dietary components and mutations in the *KRAS* gene has been presented.

4.2. Colon Cancer. The second highest incidence (about 50% of cases) of mutations in the *KRAS* gene is found in colon cancers [90, 91]. The progression of colon carcinomas can be divided into at least three stages. The first stage is characterized by the development of a small, benign tubular type of adenoma or polyp with sporadically detectable *KRAS* mutation(s) [92]. The second stage is more aggressive and is usually associated with patches of definitive carcinoma cells, which may grow into invasive cancers characterising the third stage. Mutations of the *KRAS* gene have been identified in tissues from both adenoma and carcinoma cases, but at much lower frequencies in colon adenoma tissues than in carcinoma tissues [93, 94]. The incidence of mutation in the *KRAS* gene has been found to be low and to occur mainly in the small adenomas of patients with familial adenomatous polyposis, who have a predisposition to colon cancer [94] in the *KRAS* gene associated with colon cancer appear most often in codons 12 (28%) and 13 (8%) of exon 1 and less frequently in codon 61 [95]. In colorectal cancer, the main substitution (Gly to Asp) has been found to occur in codon 12. Mutation from GGT (Gly) to GTT (Val) in codon 12 has been observed more frequently in primary metastatic carcinoma, suggesting that this mutation may confer a more aggressive phenotype in colorectal carcinoma [96]. A mutation in codon 13, resulting in the substitution of Gly with Asp, observed in colon cancer has been shown to be associated with reduced survival rates [97]. This kind of *KRAS* gene mutation has also been shown to occur more frequently in unstable, than in stable, colon tumours [97, 98].

4.3. Lung Cancer. Losses of *KRAS* wild type alleles in both mouse and human lung adenocarcinomas and squamous carcinomas have been found in many studies, notably in 67% to 100% of chemically induced murine lung adeno-carcinoma cases harbouring a mutant KRAS. In humans, *KRAS* mutations appear in 10–30% of lung carcinoma cases, demonstrating strong associations with a history of smoking [99] and poor prognosis [100, 101]. Among both current and former smokers, *KRAS* gene mutations have been identified in 30% of lung adenocarcinoma cases. Further, although some researchers have found sporadic KRAS mutations in non-smokers with early onset of cancer, smoking history is an important factor and is correlated with increased occurrence of mutations in the *KRAS* gene in lung cancer cases [102]. Mutations in the *KRAS* gene in codons 12 and 13 were detected in 21% of NSCLC (non-small cell lung cancer) tumour samples examined in the TRIBUTE III trial [103]. NSCLC patients have a tendency to accumulate activating mutations in either the EGFR or *KRAS* genes. However, a clinical study has shown that mutations of these two genes are, in general, mutually exclusive. EGFR mutations are more often found in patients who have no history of smoking.

4.4. Breast Cancer. Although higher *KRAS* mutational frequency is primarily found in cancers of the pancreas, colon and lung, possible links between KRAS hyper-activity and human breast cancer have been explored recently. Hollestelle at al. found mutations in 12.5% of cases [104] but the Sanger COSMIC database version 28 (http://www.sanger.ac.uk/genetics/CGP/cosmic/) records only a 5% incidence [105]. The lower frequency of KRAS mutations in breast cancer cell lines suggests that the gene mutation may be less important in breast cancer carcino-genesis than in other forms of cancer, although mutations at a "hotspot" in the *KRAS* gene have been found in a small subset of breast cancers.

5. KRAS and Cancer Therapy

5.1. Tyrosine Kinase Inhibitors of the EGFR1 Gene. Many clinical trials have shown that a poorer response to chemotherapy, a shorter time-to-progression, and worse overall survival are consistently associated with specific mutations in oncogenes. *KRAS* is one of the most frequently mutated oncogenes in many cancers, and it is also one of the most important predictors of resistance to targeted therapy using EGFR1 tyrosine kinase inhibitors (EGFR-TKIs) [106].

Two of the most important EGFR-specific TKIs are gefitinib (Iressa, ZD1839) and erlotinib (Tarceva). The first indications of the predictive strength of the association between the *KRAS* gene and therapeutic responses to the EGFR-TKI gefitinib were originally observed in NSCLC patients with tumours bearing the wild-type form of the *KRAS* gene and constitutively activated EGFR1 gene, due to activating mutations in exons 18 to 21 or high copy number/amplification of the EGFR1 gene. Clinically, better responses to tyrosine kinase inhibitor treatment were observed in patients with adenocarcinomas and well-differentiated tumours, female patients, non-smokers, and people of Asiatic origin [107–109]. Clinical research data show that gefitinib monotherapy is well tolerated and active against a wide range of tumour types, including colon, head, neck, breast, prostate, and lung cancers, especially NSCLCs [110].

EGFR-TKIs are usually used as the second line therapy in patients after failure of chemotherapy. However, gefitinib did not pass the registration procedure in the European Union because insufficient clinical benefit was demonstrated, probably because European clinical trials did not include suf-ficient "good responders" (Asians, women, adenocarcinoma patients and non-smokers). Clinical data also suggest that the drug represents a new therapeutic option for NSCLC patients with brain metastases [111].

After the successful TRIBUTE and TALENT clinical trials, erlotinib (Tarceva) was also approved by the US FDA in 2002 as a second or third line treatment for NSCLC after failure of standard chemotherapy. However, molecular analysis revealed that patients who have activating mutations

in the *KRAS* gene (exon 1: codons 12, 13, or 61) with or without increases in EGFR copy numbers did not derive benefit from this therapy and had about a 96% chance of disease progression [71].

Similarly, Eberhard et al. first observed the relationship between *KRAS* mutations and the outcome of erlotinib therapy in a randomized phase III clinical trial in which the drug was used, in combination with first line "gold standard" chemotherapy (carboplatin and paclitaxel), to treat advanced NSCLC patients [103]. Patients with the *KRAS* mutation exhibited a shorter time to progression (three months) and a shorter overall survival (four months) when treated with a combination of erlotinib and first line chemotherapy, such as treatment with cisplatin, compared to the group with wild-type *KRAS*, for whom the time-to-progression was 12 months. Most NSCLC patients in the erlotinib treatment study had expressed wild-type *KRAS*, and their KRAS status had greater prognostic than predictive value as a biomarker [112]. However, in colorectal cancer, mutations in the *KRAS* gene are important predictive (as well as prognostic) biomarkers, since the effectiveness of treatment with cetuximab and panitumumab is impaired in tumours with the activating mutation. Information regarding the status of the *KRAS* gene allows the selection of appropriate therapies for patients who do not display activating mutations and the selection of alternative therapies for patients with mutations. Although results pertaining to the role of KRAS in the prognosis of clinical outcome or prediction of therapeutic responses to EGFR1 tyrosine kinase inhibitors are interesting, they need to be validated in larger and prospective trials, using standardized and sensitive mutation detection techniques. If the associations are confirmed, knowledge of the mutation status of KRAS in NSCLC tumours could help physicians decide which patients should receive gefitinib and/or erlotinib.

Interestingly, *KRAS* gene mutations also seem to provide strong predictive indication of therapeutic responses to other classes of tyrosine kinase inhibitors, as recently demonstrated for the imatinib mesylate (Glivec). Imatinib is the standard drug for patients with chronic myeloid leukaemia (CML) and patients with gastrointestinal stromal tumours (GISTs), expressing the bcr-abl fusion protein and tyrosine kinase receptor c-kit, respectively. Further, drug resistance to imatinib is usually attributed to mutation of the imatinib-binding sites of these proteins [113], although amplification of the bcr-abl fusion gene or overexpression of multidrug resistance proteins may be involved in some cases [114]. However, a recent study by Agarwal et al. demonstrated that CML patients resistant to imatinib frequently expressed mutated KRAS proteins [115].

5.2. Monoclonal Antibodies as EGFR1 Inhibitors.

Colorectal cancer is another frequent neoplasia that is associated with activation of the EGFR1 pathway, so it is not surprising that novel and successful therapeutic strategies for this condition involve EGFR1 protein kinase inhibition. In contrast to NSCLC, two monoclonal antibodies against EGFR1, rather than small molecular inhibitors of EGFR1, are generally used for treating colorectal cancer: cetuximab (Erbitux®) and panitumumab (Vectibix®). In accordance with the effect of small molecular EGFR1 inhibitors in NSCLC, KRAS alterations play a critical role in the response of colorectal cancer patients to such therapeutic monoclonal antibodies. Indeed, KRAS mutation status is the most important predictor of resistance to cetuximab or panitumumab; both the median progression-free survival of cetuximab-treated patients and overall survival was recently found to be superior in a *KRAS* wild-type group than in a *KRAS* mutant group (31 versus 10 weeks, and 16 versus 7 months, respectively) [116]. On September 27, 2006, the US FDA approved the completely humanized monoclonal anti-EGFR1 IgG2 antibody—panitumumab (Vectibix) for clinical use in the third line treatment of patients with metastatic colorectal carcinoma who had progressed after standard chemotherapy. KRAS Panitumumab therapy was tested in a randomized study involving 463 patients [117], and the results showed that the wild-type *KRAS* gene is essential for its therapeutic activity. Progression-free survival of patients with wild-type versus mutant *KRAS* gene tumours was 12 versus seven weeks, while response rates obtained in another study were 17% versus 0% [118]. These findings strongly indicate that *KRAS* gene status should be routinely tested as a critically important diagnostic biomarker to determine which patients will derive therapeutic benefit from EGFR1 inhibition. Indeed, analysis of the *KRAS* gene status in colorectal cancer cases has become *conditio sinequa non* for deciding whether or not to apply cetuximab or panitumumab therapy in routine clinical practice and FDA has updated Vectibix and Erbitux labels in 2009 to include this information.

5.3. Chemotherapy and Radiation.

Surprisingly, the effects of *KRAS* gene mutations on tumour sensitivity to cytotoxic chemotherapies and radiation have only been explored in a few studies. However, expression of a 12 Val mutated form of KRAS has been shown to increase the resistance of cancer cells to radiation therapy [119]. Similarly, the presence of oncogenic KRAS has been found to significantly increase the sensitivity of cells to a novel class of anticancer agents, cucurbitacins, in a p53- or p21-dependent manner [120]. In contrast, an ovarian cancer cell line TOV-21G bearing a mutant allele of *KRAS* is reportedly significantly more sensitive to cisplatin and radiation, but not to paclitaxel or campthotecin, than the corresponding *KRAS* wild type line [121].

However, results of clinical studies by Rosell and colleagues (1995) showed that patients with a mutation in the *KRAS* gene had poorer clinical responses to paclitaxel monotherapy than wild type controls, suggesting that *KRAS* gene status is a predictive marker of paclitaxel resistance [122]. In a phase III retrospective study on NSCLC patients (TRIBUTE), randomly treated with carboplatin and paclitaxel with erlotinib or placebo, patients with KRAS mutant tumours showed poorer clinical outcomes when treated with erlotinib plus chemotherapy compared to chemotherapy alone [103]. An updated clinical trial (CRYSTAL) involving 540 metastatic colorectal cancer patients demonstrated

that cetuximab in combination with FOLFIRI (folic acid, fluorouracil, and irinotecan) in first line therapy is highly effective against KRAS wild type, but not mutant, tumours. However, further analysis of the data showed that neither the response nor the progression-free survival of patients treated with chemotherapy alone were significantly affected by KRAS gene status, although the overall survival of patients with KRAS mutant tumours was significantly shorter than that of patients with KRAS wild type tumours [123]. Recently we have also shown that EGFR may represent a predictive molecular marker for poor response to preoperative chemoradiotherapy in locally advanced gastric carcinoma [124]. Responses to chemoradiotherapy were found in 60% of EGFR-negative patients, but only 13% of EGFR-positive patients ($P = .044$), and pathologic complete responses were observed in 29% of patients with EGFR-negative staining, but none (of eight) EGFR-positive patients ($P = .16$).

The above findings regarding the role of the KRAS gene in tumour responses to cytotoxic therapies appear to conflict somewhat. The predictive and prognostic significance of oncogenic KRAS seems to have been mixed in many studies, and the contributions of variations in the gene to clinical outcome appear to differ according to tumour types and therapeutic interventions. Clearly, further studies are urgently needed to confirm and clarify the findings in large prospective biomarker-oriented clinical trials.

5.4. Angiogenesis Inhibitors. Other clinical trials have also demonstrated that activating mutations in the KRAS gene can contribute to tumour progression by affecting the expression of vascular endothelial growth factor (VEGF), which plays a critical role in tumour angiogenesis. Inhibition of KRAS expression by selected KRAS antisense oligonucleotides has been shown to be associated with significantly reduced secretion of VEGF-A165 into the medium of colorectal cancer cell cultures [125]. In addition, in a cohort of patients with pancreatic tumours, 25/33 (76%) with KRAS mutations showed higher VEGF expression, and their median survival was shorter, than those with tumours expressing the wild-type allele [126]. Similar findings have also been reported from a study of NSCLCs, in which higher VEGF expression was observed in 50% of tumours bearing a KRAS gene mutation [127]. Although these studies suggest that KRAS gene status could play an important role in responses to anti-VEGF targeted antiangiogenic therapy, a recent study by Hurwitz and Saini [128] showed that groups of patients bearing either KRAS mutant or wild-type tumours derive therapeutic benefit from first line application of the anti-VEGF monoclonal antibody bevacizumab (Avastin). Furthermore, although both groups of patients (i.e., those with wild-type KRAS and mutated KRAS genes) benefited from adding bevacizumab to chemotherapy, both progression-free survival and survival was better for wild-type KRAS patients, both with chemotherapy alone and with chemotherapy plus bevacizumab. Bevacizumab did not increase the percentage of patients with mutated KRAS who responded to treatment.

5.5. Future Therapies. An optimal therapeutic drug should be able to specifically target the mutated KRAS gene or its product, have minimal systemic toxicity and be orally active. Unfortunately, drugs like this remain to be developed and less efficient strategies need to be used in clinical trials. However, in addition to the cancer therapies mentioned above, several therapeutic agents and strategies can directly suppress the activating mutant form of the KRAS gene, and thus improve the efficiency of chemotherapy and biological therapy.

One possible approach for inhibiting KRAS expression is to use antisense oligonucleotides or viral constructs delivering antisense sequences in order to inactivate the mutant oncogene RNA message [125]. In addition, synthesis of mutated KRAS protein has been repressed by applying a small interfering adenovirus-mediated RNA (siRNA), and the specifically designed siRNA was shown to have prolonged anti-proliferative effects against various tumour cancer cell lines expressing mutated KRAS proteins [129]. Another, similar strategy to target mutant KRAS mRNA is based on designing an mRNA ribozyme that specifically interacts with a mutated form of the KRAS mRNA and encodes catalytic RNA molecules that bind to the mRNA substrate by base-pair complementation, leading to translation arrest and/or degradation of the specific mRNA. The KRAS-specific ribozyme strategy has also been shown to suppress successfully the proliferation of KRAS-mutated tumour cells [130].

Recently an interesting novel strategy employing farnesyltransferase inhibitors (FTIs) was shown to inhibit the biochemical transactivation initiated by the mutated KRAS gene. Farnesyl transferase is an enzyme that primarily regulates zinc metabolism by the addition of a farnesyl group to the cysteine residue of a protein. At least 30 proteins (including KRAS) require posttranslational farnesylation to reach their membrane positions and function properly in cell signalling. Farnesyl inhibitors represent a novel class of biologically active anticancer drugs that inhibit cell growth. After the discovery that RAS proteins have to be farnesylated to become functionally active, several farnesyl inhibitors were developed. However, Phase II and Phase III clinical trials conducted to date have found that KRAS FTIs might not be sufficient to inhibit the mutated-overactive forms of KRAS protein. The reason for this is probably incomplete inhibition of farnesylation, because garnesylation of KRAS protein by geranyltransferase I leads to suppression of the effects of farnesyltransferase inhibitors [131, 132].

It should be noted that although KRAS inhibitory strategies have shown promise in preclinical trials and have been partially successful in clinical trials, there are insufficient data on their efficacy in combination with anti-EGFR1 strategies to recommend their routine use as yet.

6. Conclusions

The evidence from various studies summarized in this review demonstrates that the KRAS protein is an important signal transducer involved in the regulation of various

cellular responses during cell proliferation, differentiation, and survival. A pivotal function of KRAS protein in the regulation of the MAPK and PI3K/AKT pathways is its effect on the proliferation rate of both normal and cancer cells. Activating mutations of the KRAS protein, which frequently occur in cancer cells, indicate poorer prognosis and increased resistance to some cancer therapies.

Overall, this review summarizes novel approaches allowing the management of cancers with or without *KRAS* mutations, and highlights the importance of early identification of somatic mutations in the *KRAS* gene in cancer biopsies. The latter assists oncologists to select the best available therapies for their cancer patients.

Acknowledgments

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Summary of the all results

Molecular cancer biomarkers have many applications in clinical oncology including determination of cancer risk, monitoring of cancer progression, and prediction of cancer response to treatment. Targeted therapeutic agents are frequently effective only in a subset of patients who express specific set of genetic biomarkers. For example, wild type status of the *KRAS* gene must be confirmed before administration of biological therapeutics such as panitumumab and cetuximab can be recommended. Therefore, it is very important that reliable detection methods are employed when *KRAS* genotyping is done. These methods should ensure highest possible quality of analytical parameters optimized to achieve high specificity, sensitivity and low detection limit. These methods should also be cost efficient and time efficient and they should have sufficient level of robustness.

First objective of this work was to introduce and compare to each other new strategies for genotyping of oncomarker *KRAS* using heterogeneous clinical tumor samples and to evaluate analytical parameters characteristics of each of the methods, such as their sensitivity, specificity, and detection limit. Five different commercial methods were compared: Sanger sequencing, Pyrosequencing, Real-Time PCR method based on Scorpion primers and allele specific ARMS primers (TheraScreen KRAS kit), Reverse dot-blot hybridization (K-ras Strip Assay), and High Resolution Melting Analysis. Ability of mutation detection using these methods was tested using the 131 DNA samples isolated from frozen archived NSCLC tissue samples. I have concluded that K-ras Strip Assay was a method with the highest likelihood of mutation detection in the *KRAS* gene. However, the most sensitive technology was TheraScreen DxS, followed by K-ras Strip Assay, HRM, Pyrosequencing, and Sanger sequencing, while the most specific technology was TheraScreen DxS, followed by Sequencing, Pyrosequencing, HRM, and K-ras Strip Assay. All five tested methods showed agreement in genotyping results in 78% of tested samples. Excluding HRM method, (that was not used for the analysis of all samples because of insufficient amount of material available for analysis from some tumor samples), genotyping agreement was 82%. In the case of 6.1% of analyzed samples the results generated using the two most appropriate methods, TheraScreen and Strip Assay, were not in agreement. These disagreements could stem from differences in mechanism of action and detection limits of these methods. The thesis contains summary of characteristics and properties of the different methods (including the cost, working time, and the required amount of DNA input).

The second part of the thesis was focused on application of COLD-PCR (modification of conventional PCR amplification) as the first step in Sanger capillary direct sequencing of *KRAS*. We have expected to improve the unfavourable detection limit (25 %) of the Sanger sequencing

technology for the mutated gene in the context of the background of wild-type DNA. Method was tested on cell lines with known *KRAS* mutation using DNA which was mixed with wild-type DNA at different dilution ratios. COLD-PCR was able to change detection limit from 25% to 1.5% (6.25% was detectable by software) in comparison to standard conventional PCR. The results presented in the thesis demonstrated, that COLD-PCR can preferentially amplify minority of mutated DNA in high background of non-mutated DNA, and can be appropriate method for improving detection limit of capillary sequencing on Beckman 8800 Sequence Analyzer.

The third part of thesis was focused on optimization of the Next Generation Sequencing (NGS) methods for testing of *KRAS* and *BRAF* oncomarkers. In our laboratory, two bench diagnostic sequencers are available: MiSeq from Illumina and GS 454 Junior from Roche. These platforms share principle of massively parallel sequencing with high repetition of clonally amplified DNA but differ in their chemistries and configuration. The thesis presents the specific protocols optimized for amplicon sequencing on each of the two platforms. The results obtained using 36 samples demonstrated that optimization of NGS methods allows to achieve improved quality analytical parameters regarding sensitivity and specificity when compared to CE-IVD PCR Real-Time methods (KRAS TheraScreen for mutation detection in *KRAS* gene, PNAClamp and IntellMed for mutation detection in BRAF gene). The analysis of the *KRAS* gene mutations detection by GS Junior-454 (Roche), sensitivity (true positive rate) was estimated to be 95.7%, and specificity (false positive rate) was estimated to be 95.7% as well; NGS MiSeq (Illumina) sensitivity and specificity was estimated to be 100% for both parameters. Based on the results of mutation detection in the *BRAF* gene, sensitivity and specificity were 100% using both NGS methods, while both NGS methods allowed direct quantification of detected mutation. The thesis also presents other workflow performance parameters (i.e. its cost, handling time, maximum number of samples which can be tested simultaneously, and gene coverage) and discusses their importance for daily laboratory routine.

In the last part of thesis, there are presented results generated using IntellMed B-RAF method for genotyping of the *BRAF* V600E to detect potential mutations in the DNA prepared from metastatic lymph nodes of 137 papillary thyroid carcinoma (PTC) patients. *BRAF* mutation is a common genetic change in PTC (average occurrence 45%), which activate MAPK (mitogen-activated-protein-kinase) pathway responsible for constitutive cell proliferation and survival. In the publication we showed that the presence of BRAF mutation in primary tumour tissue of PTC patients have had displayed only tendency towards higher risk of cervical lymph node metastasis (no statistically significant difference was found) and the presence of mutations was not associated with any other clinical and pathological parameters (gender, extra capsular growth, and vascular invasion). *BRAF* mutations appeared significantly more often in classic papillary variant than follicular or papillo-follicular histological variant. We did not confirm *BRAF* mutation to be a reliable marker of node metastasis or other clinico-pathological parameters.

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