PhD thesis

MOLECULAR MARKERS OF DRUG RESISTANT NON-SMALL-CELL LUNG CANCER

By

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DECLARATION

I hereby declare that I am the author of this thesis and I have performed all the work myself unless otherwise specified below.

This PhD thesis includes a chapter on the role of the sphingolipid metabolism pathway proteins in cancer progression and drug resistance. It was written in cooperation with Olivier Cuvillier, PhD from the Institute of Pharmacology and Structural Biology, the University of Toulouse, France; Tissue microarrays were constructed by Mgr. Mária Janíková from the Laboratory of Molecular Pathology and Department of Clinical and Molecular Pathology, Palacký University, Olomouc. Non-small-cell lung cancer patients treated by Carboplatin and Navelbine were selected in cooperation with Yvona Grygárková, MD and Professor Vítězslav Kolek, MD from the Department of Tuberculosis and Respiratory Diseases, Faculty of Medicine and Dentistry, Palacký University and University Hospital, Olomouc. Contributions to the research by others are acknowledged in the thesis.

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1. INTRODUCTION

1.1. Non-small-cell lung cancer

Non-small-cell lung cancer (NSCLC) is defined as a class of histologically heterogeneous epithelial lung tumors, other than small cell lung carcinoma (SCLC), traditionally classified together because of the similarities to routine diagnostic approaches, staging, prognosis and treatment. Non-small-cell lung cancer (NSCLC) accounts for about 80-85% of all lung cancer cases which is the most commonly diagnosed cancer, as well as the leading cause of cancer related death in males worldwide. Among females, it is the third most commonly diagnosed cancer and the second leading cause of cancer related mortality. In 2012 there were 1.82 million (13.0%) new cases recorded globally, and 1.59 million deaths, representing 19.4% of all deaths from cancer, with a five year prevalence rate of 1.89 million (5.8%) (globocan.iarc.fr). In 2012, lung cancer was the third most commonly diagnosed cancer in both males and females in Czech republic. However it was the leading cause of cancer related deaths in men and second most common cause of cancer related deaths in women. Altogether, 0.67 million (11.6%) new cases and 0.52 million (19.4%) deaths due to lung cancer were recorded in the Czech Republic in 2012, with a five year prevalence rate of 0.2 million (2.9%) (globocan.iarc.fr; www.svod.cz).

Tobacco smoking is considered a major risk factor for lung cancer development and the epidemiology of lung cancer is also closely related to changes in tobacco smoking habits (Kuper, Adami, and Boffetta 2002). Other known risk factors, such as radiation exposure, uranium, asbestos, nickel, chromium, coal, mustard gas, arsenic, beryllium, iron, vinyl chloride, radon radiation, gold mining, emissions from automobiles, factories and power plants, play a great role in the development of lung cancer in never smokers. However, smokers exposed to other known risk factors are at even higher risk. Some studies also report infectious diseases and certain genetic polymorphisms as predisposing factors for lung cancer development in never smokers (Couraud et al. 2012).

The overall five year survival rate varies from 25% to 73% according to pathological stage in patients with resectable NSCLC. The prognosis is dependent on the combination of various factors that can be grouped into three major categories: tumor-related, patient related and environmental (Goldstraw et al. 2011). Patient related factors are performance status, comorbidity and sex. Nutrition and choice and quality of treatment can be considered as environmental factors. Tumor-related factors will be discussed in the following chapters.

Despite continuous efforts to improve treatment modalities and predict patient outcome, intrinsic or acquired drug resistance still represents a major problem in NSCLC patient care. Currently, in addition to genomics, there is an increased need for immunohistochemical data to provide support for treatment response and prognosis in patients with NSCLC (Goldstraw et al. 2011).

1.1.1. Histopathological classification and its prognostic relevance

Internationally accepted world health organization (WHO) classification of lung tumors is mainly based on morphological characteristics, identified by light microscopic analysis of standard hematoxylin & eosin sections (Travis et al. 2004). Three major histopathological subtypes of NSCLC are squamous cell carcinoma (SCC), adenocarcinoma (ADC) and large cell carcinoma (LCC). Tumors of mesenchymal, lymphoid and other origin are extremely rare in the lung.

Squamous cell carcinomas (SCCs) account for nearly one third of all NSCLC cases. Morphological diagnosis of SCC is based on the presence of keratinization, pearl formation and/or intercellular bridges. These characteristics are prominent in well-differentiated tumors and focally present in poorly differentiated tumors. Four main morphologic variants of SCC can be distinguished, named papillary, clear cell, small cell and basaloid carcinomas. The independent prognostic value of SCC is limited and is mainly dependent on other more powerful prognostic indicators such as disease stage and patient performance status. However, some histological variants have been shown to have prognostic significance. Endobronchial SCCs with so-called “creeping pattern”, and “alveolar filling” pattern of SCC growing within the parenchymal lung is associated with good prognosis (Watanabe et al. 2011), while the basaloid variant is characterized by poor prognosis (Brambilla et al. 1992). The significance of the papillary pattern is unclear (Kerr 2012). It has been shown that poorly differentiated SCCs are characterized by marked distant metastatic potential, while well differentiated SCCs tend to spread locally within the chest (Travis et al. 2004).

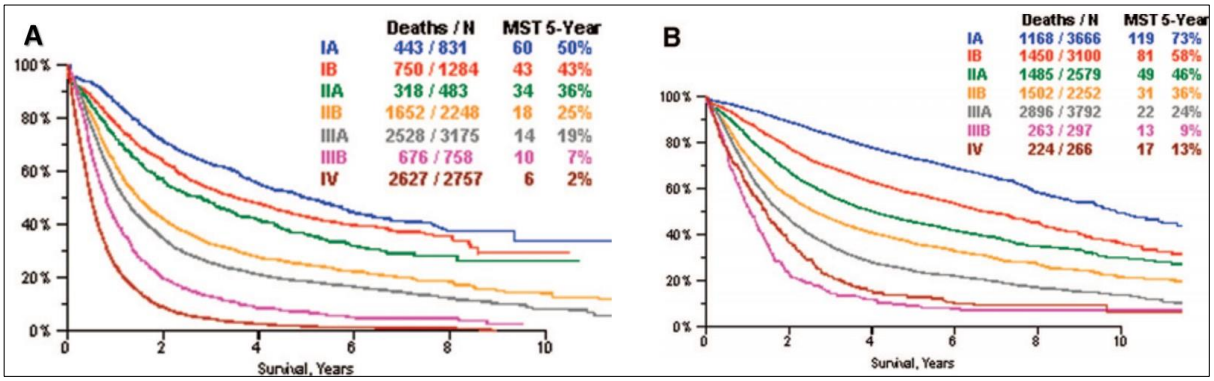
Adenocarcinomas (ADCs) account for the majority of NSCLC cases currently. WHO defines adenocarcinoma as a malignant epithelial tumor with glandular differentiation or mucin production, showing acinar, papillary, bronchioalveolar or solid with mucin growth patterns or a mixture of these patterns, the latter representing ~80% of all ADC cases (Travis et al. 2004). A new lung adenocarcinoma classification was released in 2011 under the sponsorship of the

International Association for the Study of Lung Cancer (IASLC), the American Thoracic Society (ATS), and the European Respiratory Society (ERS). The new classification was based on a multidisciplinary approach which correlated pathological aspects of tumors with clinical, radiologic, and molecular characteristics (Travis, Brambilla, and Riely 2013). Differing from the WHO classification which includes criteria only for resected tumors, a new IASLC/ATS/ERS classification was provided separately for biopsy specimens and resected specimens. The major changes in the IASLC/ATS/ERS classification of ADC in resected specimens, compared to the WHO classification is the elimination of the term "mixed subtype" and classification according to a predominant pattern, elimination of bronchioalveolar subtype, and the introduction of lepidic ADC. Several studies have shown that the classification of ADCs according to predominant pattern carry prognostic information. Tumors with the predominantly lepidic pattern are characterized by relatively good prognosis, while those that are predominantly solid or micro-papillary are associated with relatively poor prognosis (Kerr 2009; Kerr 2012). Poorly differentiated tumors also have generally more local recurrences and lymph node metastases than patients with well or moderately differentiated tumors (Travis et al. 2004).

NSCLCs which cannot be categorized as either SCC or ADC fall under the histological diagnosis of **large cell carcinoma (LCC)**, which mainly represents the diagnosis of exclusion (Travis et al. 2004). These undifferentiated tumors represent about 10% of all NSCLC cases and are characterized by the presence of sheets or nests of large polygonal cells with vesicular nuclei and prominent nucleoli. The morphologic variants include large cell neuroendocrine carcinoma, combined large cell neuroendocrine carcinoma, basaloid carcinoma, lymphoepithelioma-like carcinoma, clear cell carcinoma and large cell carcinoma with rhabdoid phenotype. The basaloid variant of LCC is mainly present in stages I-II disease. However it is characterized by extremely poor prognosis unlike lymphoepithelioma-like carcinomas which present at advanced stages but have better prognosis. Also, large cell neuroendocrine carcinomas show significantly shorter survival in stage I disease, compared to stage I NSCLC (Travis et al. 2004). Overall, the prognostic relevance of histopathological characterization of NSCLC is limited and mainly depends on other more powerful prognostic characteristics, mainly disease stage and patient performance status.

1.1.2. Clinicopathological classification of non-small-cell lung cancer

Clinicopathological classification, based on the tumor node metastases (TNM) staging system is the strongest prognostic indicator of survival in patients with NSCLC. The TNM staging system classifies tumors on the basis of primary tumor size and depth of invasion (T), the presence or absence of metastases in regional lymph nodes (N), and the presence or absence of distant metastases (M). Overall stage of the tumor (stage I to IV) is determined by the combination of T, N and M grades. The currently used TNM staging system represents the 7th edition which has replaced the earlier versions from January 1, 2010. This system was developed by the International Association for the Study of Lung Cancer (IASLC), based on a retrospective analysis of 67 725 NSCLC cases from 46 sources in more than 19 countries, treated between 1990 and 2000 (Goldstraw et al. 2007). Patient survival was estimated by the Kaplan-Meier method and prognostic groups were assessed by Cox regression analysis after adjustment for cell type, sex, age and region. The system was approved by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC). A detailed description is given in tables 1 and 2 and survival rates for each stage are shown in graph 1.



Graph 1. Overall survival, expressed as median survival time and 5-year survival, by clinical stage (A) and pathological stage (B). *Adapted from: Goldstraw P, Crowley J, Chansky K, et al. The IASLC Lung Cancer Staging Project: Proposals for the revision of the TNM stage groups in the forthcoming (seventh) edition of the TNM classification of malignant tumours. J Thorac Oncol 2007; 2:706.*

Table 1. 7th TNM staging system for non-small-cell lung cancer *(Goldstraw et al., 2007)*

Primary Tumor (T)	
T1	Tumor ≤3 cm diameter, surrounded by lung or visceral pleura, without invasion more proximal than lobar bronchus*
T1a	Tumor ≤2 cm in diameter
T1b	Tumor >2 cm but ≤3 cm in diameter
T2	Tumor >3 cm but ≤7 cm, or tumor with any of the following features:
	Involves main bronchus, ≥2 cm distal to carina
	Invades visceral pleura
	Associated with atelectasis or obstructive pneumonitis that extends to the hilar region but does not involve the entire lung
T2a	Tumor >3 cm but ≤5 cm
T2b	Tumor >5 cm but ≤7 cm
T3	Tumor >7 cm or any of the following:
	Directly invades any of the following: chest wall, diaphragm, phrenic nerve, mediastinal pleura, parietal pericardium, main bronchus <2 cm from carina (without involvement of carina)
	Atelectasis or obstructive pneumonitis of the entire lung
	Separate tumor nodules in the same lobe
T4	Tumor of any size that invades the mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, carina, or with separate tumor nodules in a different ipsilateral lobe
Regional lymph nodes (N)	
N0	No regional lymph node metastases
N1	Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension
N2	Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)
N3	Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)
Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Separate tumor nodule(s) in a contralateral lobe; tumor with pleural nodules or malignant pleural or pericardial effusion
M1b	Distant metastasis (in extrathoracic organs)

Table 2. Stage groupings in 7th TNM staging system for non-small-cell lung cancer *(Goldstraw et al., 2007)*

Stage IA	T1a-T1b	N0	M0
Stage IB	T2a	N0	M0
Stage IIA	T1a,T1b,T2a	N1	M0
	T2b	N0	M0
Stage IIB	T2b	N1	M0
	T3	N0	M0
Stage IIIA	T1a,T1b,T2a,T2b	N2	M0
	T3	N1,N2	M0
	T4	N0,N1	M0
Stage IIIB	T4	N2	M0
	Any T	N3	M0
Stage IV	Any T	Any N	M1a or M1b

1.2. Non-small-cell lung cancer treatment and drug resistance

Treatment approaches to NSCLC patients are dependent on the extent of the disease at the time of diagnosis and can be divided into three main groups, according to the latest information from the US National Cancer Institute. These groups are: patients with surgically resectable disease, patients with locally and/or regionally advanced disease and patients with distant metastatic disease.

Stage I, stage II and selected stage III tumors fall into the category of surgically resectable disease. It has been shown that postoperative chemo or radiotherapy does not improve survival in patients with stage IA NSCLC. Complete resection is enough. Postoperative cisplatin-based combination chemotherapy is considered the best therapeutic option in stage IB-III patients (Pignon et al. 2008). A pooled analysis of the five largest trials by the Lung Adjuvant Cisplatin Evaluation (LACE) collaborative group, which included 4,584 patients with cisplatin-based chemotherapy after complete resection of NSCLC, showed 5 – year absolute benefit of 5.4%, without any heterogeneity of treatment effect among trials and there was no significant variation of chemotherapy effect between stages IB and III. It has also been shown that the effectiveness of cisplatin-based chemotherapy does not depend on the other classic clinical factors of stage IB-III NSCLC (Pignon et al. 2008).

Unresectable locally (T3-4) and/or regionally (N2-N3) advanced tumors are treated with radiation therapy in combination with chemotherapy. Selected patients from this group, mainly with T3 or N2 disease can be treated with surgical resection and either with neoadjuvant or adjuvant chemotherapy or chemoradiation therapy.

Metastatic (M1) NSCLC is found in approximately 40% of patients at the time of diagnosis, and these tumors are characterized by the least favorable survival outcomes despite multimodal treatment approaches. Platinum-based chemotherapy is associated with short-term symptom palliation and survival advantage. A meta-analysis of thirty-seven assessable trials, including 7,633 patients with advanced NSCLC, showed a 5% increase in the one year survival rate with platinum-based treatment regimens (D'Addario et al. 2005). Other potentially beneficial treatments include docetaxel, pemetrexed and epidermal growth factor receptor (EGFR) inhibitor, based on EGFR expression status (Scagliotti et al. 2008; R Rosell et al. 2009).

1.2.1. Platinum-based cytotoxic drugs and their mechanism of action

Cisplatin, or cis-diamminedichloroplatinum (II) (CDDP) is a first generation member of platinum-containing anti-cancer drugs, which also includes carboplatin and oxaliplatin. Cisplatin was synthesized in 1844 by Peyrone. However, its cytotoxic properties remained undiscovered until Rosenberg's work in 1965 which showed that cisplatin could inhibit cell division in Escherichia coli. Since then cisplatin's anti-cancer properties had been confirmed for many cancers, including ovaries, testes, head and neck and lung, and in 1978 it became the first cytotoxic drug approved by the Food and Drug Administration (FDA) for the treatment of solid tumors. The second generation cisplatin analog – carboplatin showed the same efficacy in cancer treatment with less toxicity and was approved by the FDA in 1989 for the treatment of ovarian and non-small-cell lung cancer.

Cisplatin and carboplatin are composed of a doubly charged platinum ion surrounded by four ligands, with the amine ligands on the left, forming stronger interactions with the platinum ion, and the chloride ligands or carboxylate compounds on the right forming leaving groups allowing the platinum ion to form bonds with DNA bases (Goodsell 2006). Cisplatin and carboplatin, enter cells by passive diffusion, and by active transport, mainly using copper transporters (Kuo et al. 2007). In humans, cisplatin uptake is shown to be mediated by the copper transporter Ctr1 (I.-S. Song et al. 2004). After entering the cell, cisplatin is activated in the cytoplasm, by displacing the chloride atoms with water molecules, forming a potent electrophile which can react with the sulfhydryl groups on proteins and nitrogen donor atoms in nucleic acids. The end result is the formation of DNA platinum adducts which cause inter- and intra-strand cross-links of purine bases, and hence stalled DNA replication and transcription, activated DNA damage response and finally apoptotic pathways if DNA reparation is unsuccessful. In terms of its structure, carboplatin differs from cisplatin in that it has a bidentate dicarboxylate (CBDCA) ligand in the place of the two chloride ligands which is a more stable leaving group, and causes less toxicity without affecting anti-tumor efficacy. The platinum DNA adducts formed by carboplatin are substantially the same as those formed by cisplatin, but the rate of adduct formation is around 10-times slower and 20–40-times higher concentrations of carboplatin are required (Knox et al. 1986). Besides their effects on DNA, platinum drugs also have the ability to connect N and S-containing phospholipids in the cellular membrane, and cytoplasmic components with nucleophilic sites, such as cytoskeletal microfilaments, thiol-containing peptides, proteins and RNA (Speelmans et al. 1997; Jordan and Carmo-Fonseca 2000; Cepeda et al. 2007).

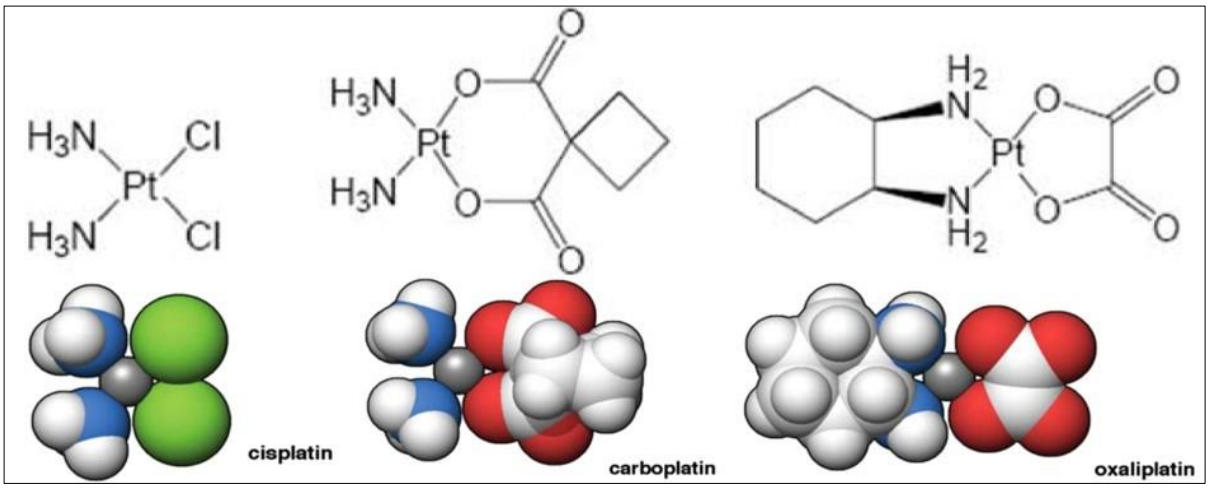


Figure 1. Chemical and computational molecular structures of cisplatin, carboplatin and oxaliplatin. *These platinum compounds are composed of doubly charged platinum ion surrounded by four ligands; with the amine ligands on the left forming stronger interactions with the platinum ion, and the chloride ligands or carboxylate compounds on the right forming leaving groups allowing the platinum ion to form bonds with DNA bases. Adapted from: Goodsell D.S. The Molecular Perspective: Cisplatin, The Oncologist, 2006; 11:316–317*

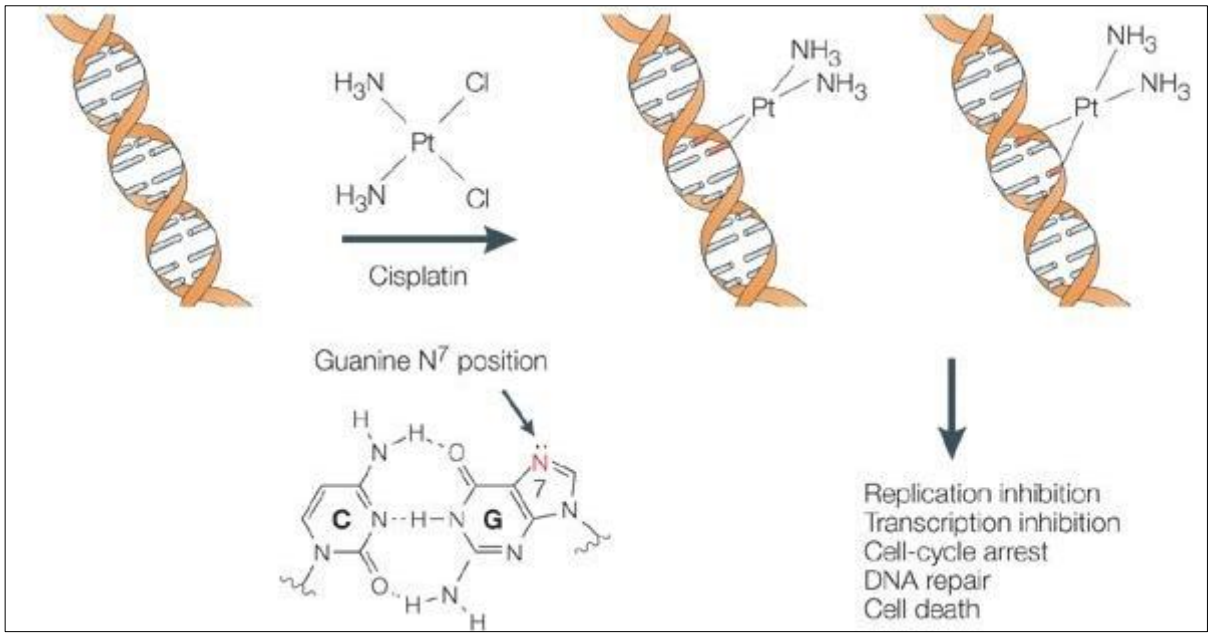


Figure 2. *Mechanism of DNA binding and cleavage by cisplatin. Adapted from: Sangeetha Gowda K.R. et al. Biomedicine and Biotechnology, 2014, Vol. 2, No. 1, 1-9.*

1.2.2. Resistance to platinum-based cytotoxic drugs

Resistance mechanisms to platinating agents are multiple and can be developed at any stage of the intracellular drug delivery. In vitro studies suggest that cisplatin resistance can result from: epigenetic changes at the molecular and cellular levels, including reduced accumulation of the platinum compounds by either active efflux/sequestration/secretion or impaired influx, detoxification by GSH conjugates, metallothioneins and other antioxidants, increased levels of DNA damage repair, changes in DNA methylation status, alterations of membrane protein trafficking as a result of defective organization and distribution of the cytoskeleton, overexpression of chaperones, up- or down-regulated expression of microRNA (miRNA), transcription factors and small GTPases, inactivation of the apoptosis pathway, activation of the EMT (Epithelial Mesenchymal Transition) pathway, and others (Shen et al. 2012; Dasari and Bernard Tchounwou 2014).

In clinical settings, various combinations of platinum-based and other chemotherapeutic agents, mainly taxanes and vinca alkaloids, are used for minimizing the effects of platinum-resistance and increasing the efficacy of chemotherapy. It has also been shown that sequential cisplatin-based chemotherapy given in addition to radical radiotherapy significantly prolonged survival in patients with locally advanced NSCLC. However, the prognosis of these patients still remained poor with a 3-year overall survival of ~14% (Aupérin et al. 2010).

1.2.3. Multidrug resistance

Resistance mechanisms to NSCLC treatment are complex and cannot be attributed to a single factor or molecular pathway. One well-characterized mechanism is called multidrug resistance (MDR) and it is defined as the insensitivity of cancer cells to cytotoxic and cytostatic actions of a number of structurally and functionally unrelated drugs (Škarda et al. 2007; Sharma 2012). Two types of multidrug resistance have been described: The first is mediated by P-glycoprotein (called Pgp multidrug resistance) and the second which is associated with all other mechanisms apart from Pgp (non-Pgp multidrug resistance). These mechanisms include expression of: other ATP dependent transporters such as multidrug resistance related protein (MRP) and lung resistance related protein (LRP). Expression of pro-apoptotic and anti-apoptotic proteins together with DNA repair mechanisms and drug detoxifying systems also play a part in non Pgp mediated MDR (Škarda et al. 2007)

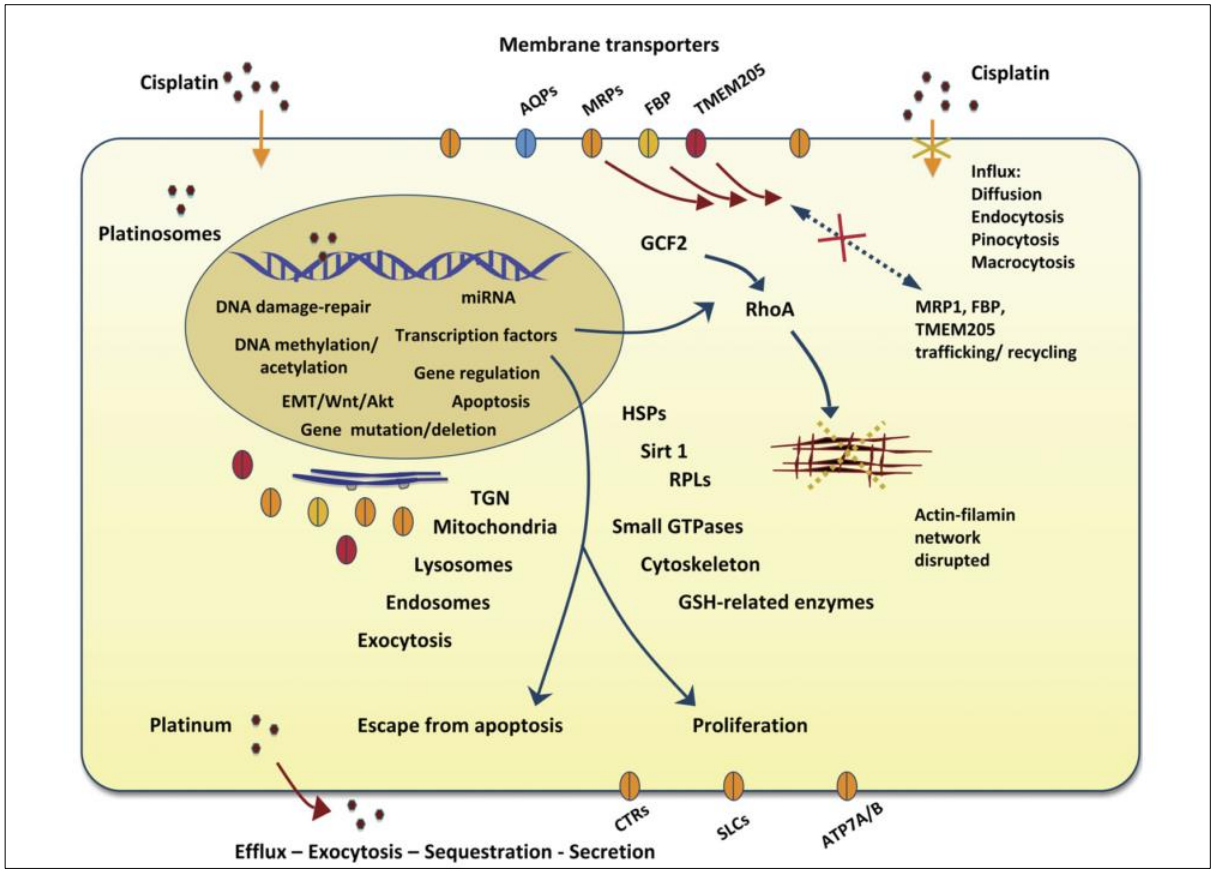


Figure 3. Schematic illustration of cellular self-defense systems that mediate cisplatin resistance. *Cisplatin binds to DNA, triggers the activation or silencing of a number of gene regulatory pathways, such as those related to DNA-damage repair, DNA methylation, histone acetylation, miRNA, EMT, Wnt, transcription factors, and apoptosis, and also inducing gene mutation or deletion. Mislocalization of membrane proteins, such as MRP1, FBP, and TMEM205, largely results from the up-regulation of the transcription factor GCF2, which silences small GTPase rhoA expression, interrupting assembly and or organization of the cytoskeletal actin/filamin network. This in turn results in internalization of several membrane proteins in the intracellular cytoplasm, with decreased influx and accumulation of cisplatin in the cisplatin resistant cells. This could also be due to a defective influx route (i.e., reduced endocytosis) or to other putative proteins that are needed for cisplatin uptake. HSPs, Sirt1, ribosomal proteins (RPLs), and GSH-related enzymes may play roles in regulating cellular response and detoxification of the compound. These cell self-defense mechanisms in CP-r cells serve to allow survival and growth of cancer cells exposed to cisplatin. Adapted from: Ding-Wu Shen, Lynn M. Pouliot, Matthew D. Hall, and Michael M. Gottesman, Pharmacological reviews, 2012; 64:706–721.*

1.2.4. The role of DNA damage repair proteins in resistance to platinum-based cytotoxic drugs

Tumor cells have intrinsically different DNA repair mechanisms compared to normal cells. Many cisplatin-resistant cell lines that are derived from various tumor types have been shown to have increased DNA-repair capacity in comparison to cisplatin-sensitive cell lines (Johnson et al. 1994; Kelland 2007). The highest response rates to cisplatin-containing treatment regimens has been found in patients with testicular cancer, with an increase in cure rate from 5% to 60%, and this success has been attributed to the inherent defects in DNA repair. Parental cell lines derived from testicular cancer show decreased efficacy in removing platinum DNA adducts, compared to cisplatin- resistant cell lines (V. Smith et al. 2002). DNA lesions are repaired by four major DNA repair pathways: nucleotide-excision repair (NER), base-excision repair (BER), mismatch repair (MMR) and double-strand-break repair (DSBR). Bulky DNA adducts by cisplatin are primarily repaired by the nucleotide excision repair (NER) pathway (Matakidou et al. 2007; Zhou et al. 2004).

The Nucleotide excision repair (NER) pathway involves 20 to 30 proteins which recognise DNA lesions, locally unwind the helix, excise the damaged section of DNA and allow for polymerase and ligase to complete the repair process. NER consists of two sub-pathways: global genome nucleotide excision repair (GG-NER) and transcription-coupled nucleotide excision repair (TC-NER). The former recognizes and eliminates bulky DNA damages across the entire genome, while the latter operates in transcriptionally active areas (O'Grady et al. 2014). It is shown that TC-NER is more significant in the repair of cisplatin induced DNA damage (Furuta et al. 2002). The main protein of TC-NER is excision repair cross-complementing 1 (ERCC1) which has been actively studied in cell lines and biopsy specimens in relationship to cisplatin sensitivity. Studies have demonstrated correlation between the low levels of ERCC1 mRNA and increased survival in NSCLC patients treated with cisplatin following tumor resection (Lord et al. 2002; Jian Li et al. 2010). Immunohistochemical expression of ERCC1 protein in resected specimens from NSCLC patients has also been shown to be associated with survival advantages after cisplatin-based adjuvant chemotherapy (Olaussen et al. 2006; Ceppi et al. 2006; Azuma et al. 2007). The study of immunohistochemical expression of ERCC1 in 761 resected tumors from patients included in the International Adjuvant Lung Cancer Trial (IALT) showed that patients with ERCC1 – negative tumors appear to benefit from adjuvant cisplatin-based chemotherapy (Olaussen et al. 2006). Overexpression of another NER protein, XPC, which is necessary for normal functioning of ERCC1 is also shown to be related to poor survival outcomes in lung adenocarcinoma patients

and elevated expression of XPC was associated with increased resistance to cisplatin in adenocarcinoma cell lines (Lai et al. 2011).

Base-excision repair (BER) and mismatch repair (MMR) pathways have also been studied in relationship to cisplatin sensitivity. However, their role in cisplatin mediated DNA damage repair is not yet fully understood and the literature is full of contradictory findings. Base excision repair (BER) pathway mediates the removal of bases damaged by oxidation, alkylation and deamination (A. B. Robertson et al. 2009). The mismatch repair (MMR) pathway recognizes and repairs mismatched base pairs, increasing fidelity up to 1000-fold (Schofield and Hsieh 2003). There is controversial data in the literature on the role of one of the major BER pathway proteins - XRCC1 (X-ray repair cross-complementing protein 1). Wu et al. performed a meta-analysis to evaluate the predictive value of XRCC1 gene polymorphisms on the clinical outcome in patients with advanced NSCLC, treated with platinum-based chemotherapy. These authors found that XRCC1 polymorphisms in Arg194Trp could predict the clinical outcome of this type of chemotherapy in advanced non-small-cell lung cancer (NSCLC). These results were based on studies in Chinese populations. The role of XRCC1 polymorphisms in Arg399Gln was controversial, and its relationship to treatment response was not significant in high-quality studies (Wu et al. 2012). It has been shown that cisplatin adducts can be recognized by the MMR pathway. However, they cannot be repaired efficiently. This causes the repetition of "futile cycles" of MMR and finally leads to double strand breaks, which activates other DNA damage signaling factors, including p53, ATM and ATR (O'Brien et al., 2006). It has been shown that MMR proteins, such as MutL homolog 1 (MLH1), MutS protein homolog 2 (MSH2) and O6-alkylguanine DNA alkyltransferase (MGMT) are frequently decreased in NSCLC (Y. C. Wang et al. 2003; Cooper et al. 2008). Some studies report that low expression levels of hMSH2 and hMLH1 is related to significantly greater survival rates in NSCLC and ovarian cancer patients treated with cisplatin-based chemotherapy (M. Scartozzi et al. 2003; Mario Scartozzi et al. 2006). In contrast, Cooper et al., found no prognostic significance of MLH1, MSH2 and MGMT proteins in NSCLC patients (Cooper et al. 2008).

Double strand breaks (DSB) are considered the most damaging form of genetic mutation. Extensive repair is necessary to correct DSBs and also there is the possibility of developing other mutations through inaccurate pairing of broken ends (O'Grady et al. 2014). There are two main mechanisms of double strand break repair: homologous recombination (HR) and non-homologous end-joining (NHEJ). Recombination is used to repair cisplatin inter-strand cross links that, although forming a small percentage of cisplatin–DNA adducts, are highly toxic and considered essential for the cytotoxic action of cisplatin (Patrick, Tillison, and Horn 2008). It

has been shown that loss of recombination repair in recombination-deficient E. coli strains leads to increased cisplatin sensitivity compared to that found in NER-deficient strains (Zdraveski et al. 2000). Essential proteins in DSB repair via homologous recombination (HR) are RAD51, BRCA1 and BRCA2. However, the last two are also shown to play important roles in nucleotide excision repair, non-homologous end joining and activation of checkpoint responses (Venkitaraman 2001). It has been shown recently that BRCA2 inhibition can enhance cisplatin-mediated alterations in tumor cell proliferation, metabolism, and metastasis (Rytelewski et al. 2014).

1.3. Potential prognostic and/or predictive markers in patients with NSCLC

1.3.1. The role of BRCA1 in non-small-cell lung cancer

BRCA1 (Breast cancer 1) used to be identified as a breast and ovarian cancer susceptibility gene but recently it has acquired scientific interest as a prognostic and predictive marker for various tumors, including non-small-cell lung cancer NSCLC. BRCA1 is a multifunctional tumor suppressor protein, ubiquitously expressed in all tissues and serves as a tumor suppressor in part, as a "caretaker", as well as "gatekeeper" in preserving genomic stability (Rosen, Fan, and Isaacs 2005). BRCA1 plays a key role in essential cellular functions including cell cycle regulation, replication, mitotic spindle assembly, transcription regulation and higher chromatin hierarchical control as well as DNA damage response (DDR) and apoptosis (Yarden and Papa 2006). Hence, besides its tumor suppression function, BRCA1 also modulates the cellular response to cytotoxic chemotherapy. As drug resistance is a major impediment in the successful treatment of NSCLC, BRCA1 is actively investigated as a predictive marker for NSCLC patients.

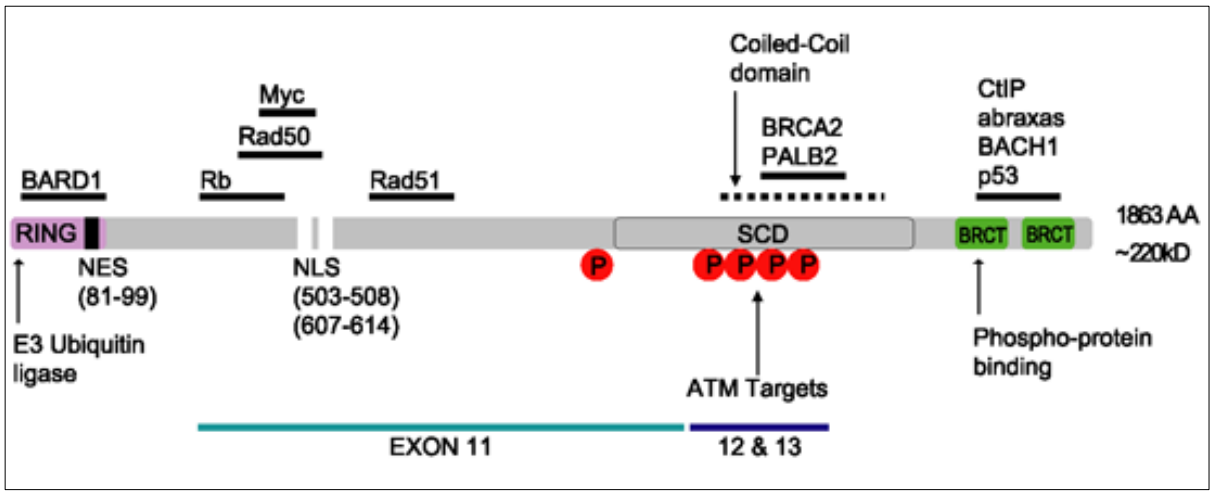


Figure 4. *BRCA1 is large (220kDa) protein with three main structural and functional subunits (N-terminal RING domain, central nuclear localization signal (NLS) domains and C-terminal domain) and multiple binding sites for other proteins of various signaling pathways, mainly related to DNA damage response. BRCA1 also includes several phosphorylation sites for ATM. Horizontal solid black lines indicate protein binding domains for the listed binding partners. Red circles mark phosphorylation sites. Adapted from: Clark, Rodriguez, Snyder, Hankins, & Boehning, Computational and Structural Biotechnology Journal, Volume No: 1, Issue: 1, April 2012*

1.3.1.1. Mutations, Epigenetic changes and genetic variations of BRCA1 in NSCLC

Germline mutations of BRCA1 are associated with increased risk of breast, ovarian, and more recently, prostate cancer but are not found in lung cancer (Kalow, Tang, and Endrenyi 1998; Parmigiani et al. 2004; Potti et al. 2011). BRCA1 mutations and epigenetic changes are present in either hereditary or sporadic forms of breast and ovarian tumors. Decreased BRCA1 mRNA and protein expression due to promoter hypermethylation are found in NSCLC patients. However it is not a frequent event. *Lee et al.* (2007) suggested that promoter hypermethylation is the predominant mechanism for inactivation of the BRCA1 in NSCLC and *Marsit et al.* (2004) suggested that BRCA1 promoter hypermethylation might be specific to some subtypes of NSCLC. The frequency of BRCA1 promoter methylation was found to be only 4% (6/158) in NSCLC, with 5/84 adenocarcinomas and 1/14 large cell carcinomas methylated and no methylation present in lung squamous cell carcinoma (0/60) (Marsit et al. 2004). According to *Lee at al.*, (2007) 30% of NSCLC tumors showed promoter hypermethylation in BRCA1 whereas no or low methylation was found in their matched normal lung tissue. A high concordance was observed between alterations in protein and mRNA expression and promoter hypermethylation of the BRCA1 gene. It is worth mentioning that in this study, low BRCA1 protein expression occurred primarily in patients suffering from adenocarcinoma types of NSCLC (P=0.014), which is somewhat in line with the finding of Marsit et al. Some other studies also found BRCA1 promoter hypermethylation in the normal lung tissue of 2.56% NSCLC patients (Yan Wang et al. 2008). This might be one of the early events in lung carcinogenesis but this concept is the subject of future studies.

The analysis of *single nucleotide polymorphisms (SNPs)* and haplotypes in cancer research have pleotropic implications for clinical and public health issues, as well as cancer biology. Kim et al. (2008) evaluated the associations of four tagging *BRCA1* polymorphisms ([S1613G, IVS13-1893 (A>C), IVS12-1207 (C>T), and IVS12+112 (C>A)]) and their haplotypes (AACC, AACA, GCTC, GATC, and AATC) with treatment outcome in 300 NSCLC patients at stages IIIA (16%), IIIB (31%), and IV (53%). The survival of patients with two copies of the AACC (wild-type) haplotype was significantly shorter than that of patients with zero to one copy (MST, 8.47 *v* 14.57 months; log- rank P=0.0066), even after adjustment for body weight loss, performance status, stage, second-line treatment, and radiation therapy (hazard ratio = 2.097; 95% CI, 1.339 to 3.284). Four other haplotypes (AACA, GCTC, GATC, and AATC) were rare and there was no demonstrated association between any of them and survival time (data not shown).

1.3.1.2. BRCA1 as a modulator of chemotherapy response in NSCLC patients

A number of studies *in vitro*, mouse model and recently from clinical settings, support the concept that BRCA1 mRNA and protein levels, might be a useful biomarker for chemotherapy response in breast, ovarian and also lung cancer. BRCA1 plays a central role in DNA repair and also in mitotic spindle assembly. Hence a lower expression level is predictive of better survival in treatment with DNA-damaging agents (i.e. platinum), whereas a higher level might indicate benefit from anticancer drugs that act on tubulin, such as taxanes (Damsma et al. 2007; Basu and Krishnamurthy 2010; Yi Wang et al. 2000; Yang and Xia 2010).

1.3.1.2.1. BRCA1 mRNA expression studies

The Rosell's group was the first to examine the potential predictive value of BRCA1 mRNA expression in resected specimens from 55 stage IIB, IIIA and IIIB NSCLC patients treated with neoadjuvant gemcitabine/cisplatin followed by surgery. They examined BRCA1 mRNA expression in formalin-fixed paraffin-embedded tissue sections by real-time quantitative polymerase chain reaction (RT-QPCR). BRCA1 was detected in all tumors, although there was a considerable variation in its level of expression, compared with β-actin as internal ranging control. BRCA1 expression was divided into quartiles. Median survival, was not reached for the 15 patients in the bottom quartile (0.28 to 0.61), whereas for the 28 patients in the two middle quartiles (0.65 to 1.20, 1.23 to 2.37) it was 37.8 months (95% CI, 10.6–65), and for the 12 patients in the top quartile (2.45 to 10.43) it was 12.7 months (95% CI, 0.28–28.8) (P 5 0.01)). Five patients who attained a complete pathologic response (T0N0) were all in the bottom quartile. Conversely, in the majority of patients with high BRCA1 levels, no clinical or pathologic down staging was found following chemotherapy and surgery. Also, the comparison with pathologic stage, showed that patients in the bottom quartile had a decreased risk of death compared with those in the top quartile (HR 5 0.294; 95% CI, 0.10–0.83; P 5 0.020), and those in the two middle quartiles also had a decreased risk of death, compared with those in the top quartile. A similar pattern was observed according to clinical stage (Taron et al. 2004). The retrospective analysis of the prognostic value of BRCA1, together with RRM1 and RRM2 mRNA expression in 96 histologically confirmed inoperable stage IIIB and IV NSCLC patients treated with first-line gemcitabine plus docetaxel, showed that the majority of responders had high BRCA1 expression. In contrast to the pattern observed with first-line therapy, low levels of BRCA1 were significantly associated with the lowest risk of progression to second-line therapy

(Boukovinas et al. 2008). BRCA1 mRNA expression levels were inversely correlated with sensitivity to cisplatin in malignant pleural effusions of NSCLC patients (P=0.014, r=0.541), while patients with higher BRCA1 mRNA expression levels had a higher sensitivity to docetaxel compared with those with lower expression levels (NSCLC: P=0.008, r= -0.573) (L. Wang et al. 2008). Two trials confirmed the prognostic relevance of BRCA1 expression. In the first study, 126 chemotherapy-naive patients who had undergone surgical resection had decreased OS associated with increased BRCA1 expression (hazard ratio 1.98; 95% CI 1.11–6.00; P=0.02). In patients with low levels of BRCA1, median survival was not attained while it was 29 months (95% CI, 22.2–35.7 months) for those with high levels (P=0.04) (Wachters et al. 2005). The second study included patients with stage IV adenocarcinoma. Patients with epidermal growth factor receptor (EGFR) mutations received erlotinib, whereas patients without EGFR mutations received customized chemotherapy, including cisplatin/ gemcitabine (low BRCA1 levels), cisplatin/docetaxel (intermediate BRCA1 levels), or docetaxel alone (high BRCA1 levels). Median OS was not attained for patients with EGFR mutations and in patients with low levels of BRCA1 and RAP80 (Rafael Rosell et al. 2009). In contrast to these findings, the Bio-FAST study showed inconclusive results for BRCA1 (Tiseo et al. 2013). Therefore, the role of BRCA1 as a predictive marker for chemotherapy response in patients with NSCLC is still not clear and needs to be evaluated by further studies. Also, for practical purposes study of BRCA1 protein expression, by immunohistochemistry is more valuable for precise evaluation of individual patient prognosis.

1.3.1.2.2. BRCA1 protein expression studies

Protein expression examined using immunohistochemistry has major diagnostic and practical significance in the study of lung cancers. However, there are few studies, using BRCA1 immunohistochemistry. The first report using immunohistochemical staining of BRCA1 was for a total of 33 patients in a randomized phase III trial comparing cisplatin-gemcitabine and epirubicin-gemcitabine as first-line treatment in advanced NSCLC. Patients were selected according to short (<26 weeks) or a longer (>78 weeks) survival times. Anti-BRCA, clone MS110, 1:25 (Oncogene, Boston, MA, USA) was used for the detection of BRCA1 protein. A biopsy with more than 10% of cells with positive nuclear or membrane staining was defined as positive. In ninety percent of patients the tumor showed a positive nuclear BRCA1 staining. Tumor response rate was not significantly different in patient biopsies with positively or negatively scored BRCA1. Also, the percentage of tumor cells positively stained for ERCC1,

hRad51, and BRCA1 was similar in responders versus non-responders. Additionally, no differences in percentage of positively stained cells were found between responders and non-responders treated with cisplatin-gemcitabine or epirubicin-gemcitabine. The percentage of biopsies positive for BRCA1 was similar in patients with a short or long overall survival after chemotherapy. In addition, the percentage of positive cells not correlating with treatment regimen/ was similar in patients with short and long overall survival treated with cisplatin-gemcitabine or epirubicin-gemcitabine (Wachters et al. 2005). Another study of 98 tumors from NSCLC patients using monoclonal antibodies for BRCA1-8F7 (1:500; GeneTex, San Antonio, TX), showed the same results (Rafael Rosell et al. 2007). The quantification of expression of ABC transporter (BCRP, MRP2) proteins and DNA repair proteins (ERCC1, BRCA1) by immunohistochemical staining of tumor biopsy, specimens collected before platinum based chemotherapy, showed no significant associations between BRCA1 expression and response to chemotherapy, or survival. This study used the same clone of BRCA1 antibody (cloneMS110) (Ota et al. 2009). One study evaluated protein expression profiles by immunohistochemistry on surgical specimens of 82 NSCLC patients who underwent platinum- and taxane-based neoadjuvant chemotherapy. The expression levels of proteins were measured semi-quantitatively and the correlations with tumor response, pathologic cell death rate and survival were evaluated. There was no correlation found in this study either (Kang et al. 2010). Unfortunately the BRCA1 clone used was not described, unlike other antibodies used in this study. On the other hand, changes in BRCA1 mRNA expression might be reflected in changes in protein expression and use of appropriate antibody or panel of antibodies could reveal these changes. To the best of our knowledge of the published literature, BRCA1 still remains a potential genomic marker for NSCLC patients. The main weakness of BRCA1 mRNA studies is the small sample size and for protein studies, lack of proper antibody.

1.3.2. The role of RAD51 in non-small-cell lung cancer

RAD51 is eukaryotic homologue of the E. coli RecA protein, which plays an essential role in DNA repair via homologous recombination (HR) (Schmutte et al. 1999). RAD51 functions by forming nucleoprotein filaments in single stranded DNA, mediating homologous pairing and strand exchange reactions between single and double stranded DNA during repair (Vispé and Defais 1997). In response to DNA damage recombination proteins are rapidly relocalized and concentrated into sub-nuclear complexes that are microscopically detected as nuclear foci (Tarsounas, Davies, and West 2004). These nuclear foci usually contain RAD51, which is also used to detect ongoing DNA reparation process. RAD51 foci are also found in undamaged S-phase cells, where they are thought to identify sites where stalled or broken replication forks undergo repair (Tashiro et al. 1996; Haaf et al. 1999). Rad51 protein also interacts with a variety of tumor suppressor proteins including p53 (Arias-Lopez et al. 2006), BRCA1 (Scully et al. 1997) and BRCA2 (Carreira et al. 2009).

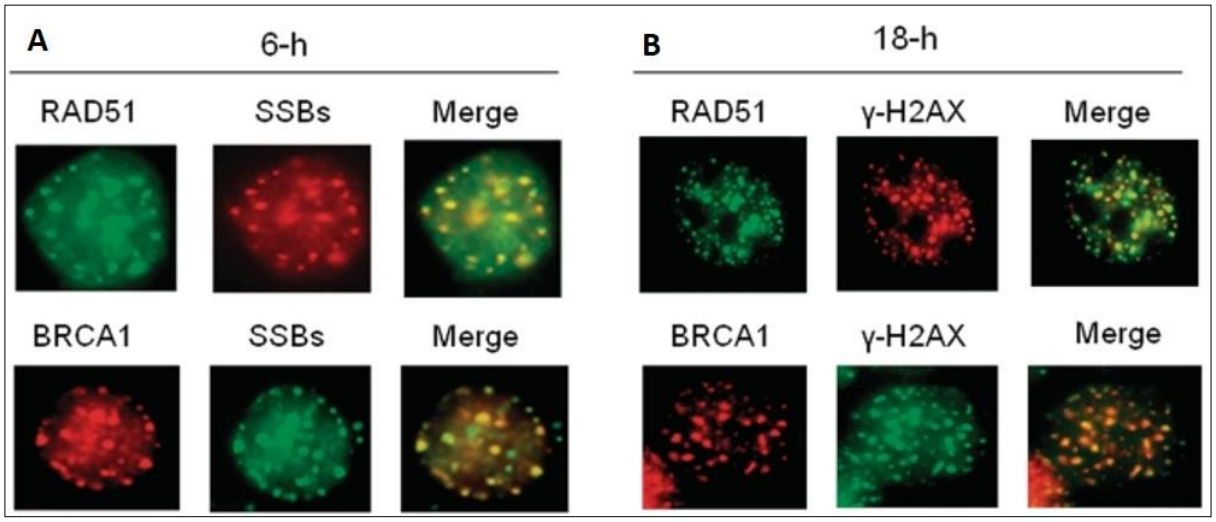


Figure 5. A. BRCA1 and RAD51 colocalizes at the ssDNA region in response to replication fork stalling in MCF7 cells. *The protocol for ssDNA detection has been described elsewhere (Shi et al. 2010; Sartori et al. 2007). Hydroxyurea (HU) has been used to cause either replication fork stalling or collapse by altering periods of HU treatment (Petermann et al. 2010). Cells treated with 6-h HU were co-stained by anti-BrdU antibody and antibody against RAD51 or BRCA1.* ***B.*** *BRCA1 and RAD51 protein colocalizes at DSBs region in response to replication fork collapse. The cells co-stained with anti-γ-H2AX antibodies and antibodies against RAD51 or BRCA1. Adapted from: Zhihui Feng and Junran Zhang, Journal of Nucleic Acids Research, 2012 Jan; 40(2): 726–738.*

It has been shown that targeting of RAD51 by siRNA significantly increases cisplatin sensitivity in vitro (M. Ito et al. 2005). There has been very few number of RAD51 studies in NSCLC patients, as well as *in vitro* cell lines. An analysis of G135C polymorphism of RAD51 in 243 patients with NSCLC showed that 85.4% of cases are characterized by the GG homozygous genotype, 13.8% by the CG heterozygous genotype and 0.8% by the CC homozygous genotype. There was no significant relationship between the RAD51 genotype and tumor stage. However, there was difference is overall survival across different genotypes. Mean survival was higher in carriers of the C allele, compared to other patients (56.0 months vs. 41.7 months; p=0.024) (Nogueira et al. 2010). In NSCLC cell line models, 4 out of 16 (25%) cell lines were defective in RAD51 nuclear foci formation after cisplatin treatment and these cells were characterized by an increased sensitivity to cisplatin (Birkelbach et al. 2013).

Qiao et al. studied the expression of RAD51 in tissue microarrays from patients with resected NSCLC. RAD51 nuclear expression was evaluated as a percentage of positive tumor cells and classified as low (≤10%) versus high (>10%) expression. From 340 NSCLC cases, 100 (29.4%) showed high RAD51 expression as defined by the authors. There was no significant correlation between RAD51 expression, patient characteristics such as age and sex, tumor stage (TNM), histology or tumor differentiation (grade). Although, the univariate analysis of survival (log-rank test) showed that high-level expression of RAD51 was associated with poor survival in all histological subtypes and stage I-III NSCLC. Multivariate analysis showed that RAD51 expression was an independent prognostic factor in this group of patients with NSCLC (Qiao et al. 2005). However, it should be mentioned that there was no exact information available on potential neoadjuvant or adjuvant treatment in this group of patients, and it is not possible to conclude whether the prognostic role of RAD51 is related to patient treatment or not.

Takenaka and colleagues (2007) found a significant relationship between the combined immunohistochemical expression of RAD51 and ERCC1 protein in 41 NSCLC patients and ex vivo chemosensitivity (MTT assay) to cisplatin and carboplatin (p = 0.012 and 0.04 for CDDP, 0.014 and 0.03 for CBDCA). Positive expression of RAD51 and ERCC1 was seen in 17(41%) and 20(49%) cases, respectively. RAD51 expression was also related to squamous-cell carcinoma and poor differentiation. There was no significant relationship between the expression levels of these proteins and sensitivity to paclitaxel, etoposide, vinorelbine, gemcitabine, 5-FU, or irinotecan (Takenaka et al. 2007). Although the literature is full of contradictory findings, all above mentioned data suggest a potential prognostic and predictive role of RAD51 in patients with NSCLC.

1.3.3. Filamin A as a potential prognostic and predictive marker for tumor progression and treatment outcome

Filamin-A (also known as human actin-binding protein 280 (ABP-280) or filamin-1), is a cytoskeletal protein involved in regulation of cell shape and locomotion. The primary function of filamin A protein is to cross-link actin filaments into orthogonal networks and assist the formation of cytoskeleton (Yamazaki, Furuike, and Ito 2002). Filamin A also connects actin networks to plasma membrane and facilitates cell-matrix interactions. This aside, filamin A interacts with more than 60 non-cytoskeletal proteins related to cancer growth and metastasis and, serves as a scaffold for various signaling pathways including DNA damage response (Yue, Huhn, and Shen 2013). The mutations of filamin A gene is related to various developmental malformations, including Periventricular nodular heterotopia (PNH) (Clapham et al. 2012), otopalatodigital syndrome (OPD) (Mariño-Enríquez et al. 2007), frontometaphyseal dysplasia (FMD) (S. P. Robertson et al. 2006) and Melnick–Needles syndrome (MNS) (Foley et al. 2010). Also, missense mutations in filamin A is considered as a genetic cause of familial cardiac valvular dystrophy (Kyndt et al. 2007).

It is known that filamin A interacts with more than 60 proteins related to cancer growth and metastases. However, the role of filamin A in cancer metastatic potential is still uncertain and existing literature is full of contradictory findings. Increased levels of filamin A, identified by comparative proteomic study, is associated with increased metastatic potential in hepatocellular carcinoma (Ai et al. 2011). Also, it has been shown that knockdown of filamin A in melanoma cell lines is associated with reduced metastasis development in xenograft mouse models and also the inhibition of filamin A reduces the invasiveness of breast cancer cell lines (Jiang et al. 2012). A number of studies report different levels of filamin-A expression in cancer tissue, compared to healthy tissue. Increased expression of filamin-A has been found in colorectal (Notterman et al. 2001), pancreatic (C. Li et al. 2009), hepatic (Guedj et al. 2009) and breast (Tian et al. 2013) carcinomas, whilst there is a marked decrease of filamin-A expression in human bladder (S. C. Smith et al. 2007) and nasopharyngeal (Sun et al. 2013) cancer. In colorectal cancer increased immunohistochemical expression of filamin A represents an independent prognostic factor together with lymph node metastases and depth of tumor invasion (HR=3.856, 95%CI [7.326:19. 421], P<0.001) (Notterman et al. 2001). On the contrary, loss of filamin A expression correlates with poor overall survival time by Kaplan–Meier analysis (P <0.05) in patients with nasopharyngeal cancer (Sun et al. 2013).

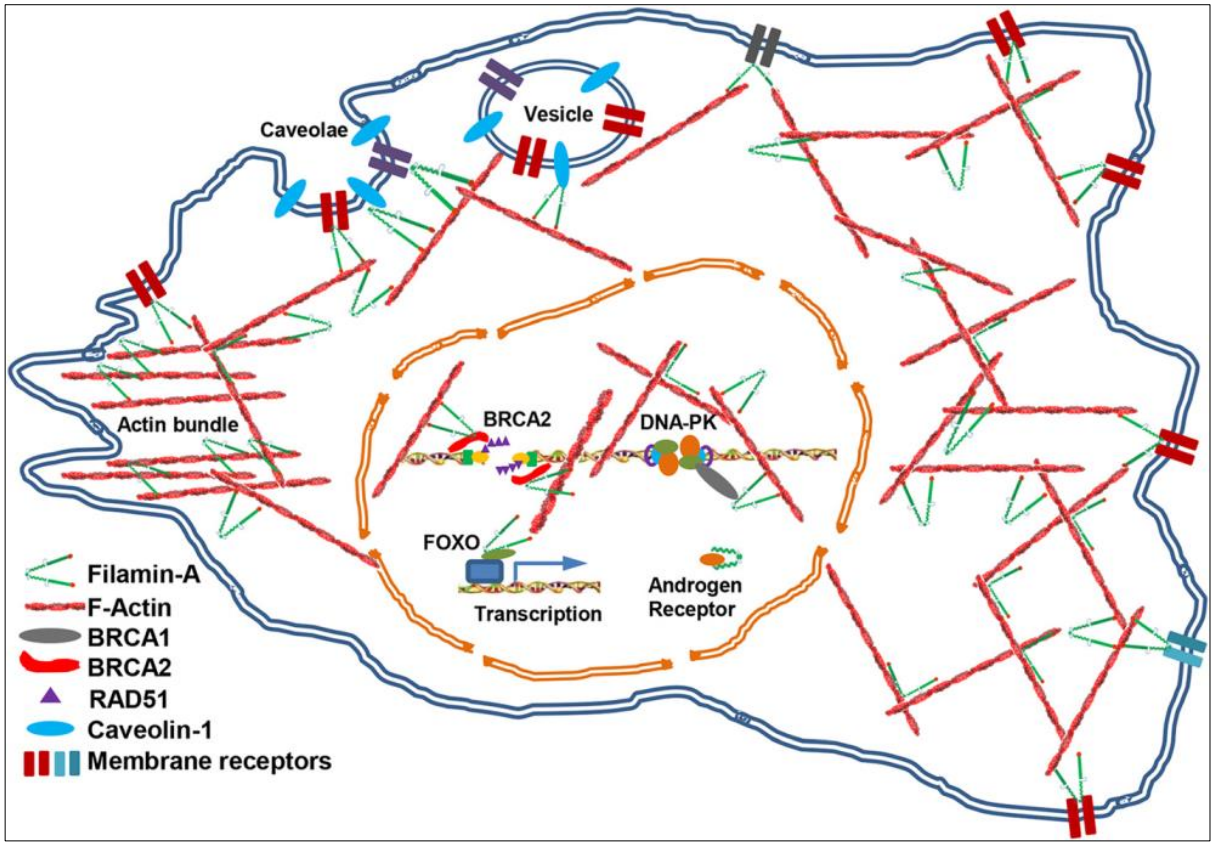


Figure 6. Schematic presentation of filamin A functions. *Through the interactions with its binding partners, filamin-A is endowed with versatile cellular functions, including maintenance of dynamic F-actin networks and regulating cell shape; mediating the communication between cytoskeleton and ECM; acting as a scaffold for cell signaling to regulate cell motility; facilitating intracellular trafficking and promoting membrane protein recycling; regulating RNA transcription through interactions with transcriptional factors and RNA polymerase machinery; modulating nuclear receptor signaling through the binding with androgen receptor; and mediating DNA damage response through interactions with BRCA1, BRCA2. Adapted from: Jingyin Yue, Steven Huhn and Zhiyuan Shen, Cell & Bioscience 2013, 3:7*

Immunohistochemical study of filamin A expression in prostate tissues showed that benign prostate, PIN, and localized prostate cancer had predominantly nuclear filamin A expression, whereas in metastatic prostate cancer, filamin A was found to be primarily in the cytoplasm. This report suggested that the role of filamin A in cancer growth and metastases is depend on its localisation and its specific intracellular localisation, detected by immunohistochemistry, might also serve as useful prognostic marker in cancer patients.

1.3.3.1. The role of filamin A protein in DNA damage response

Shen et al., identified interaction between BRCA2 and filamin A, using yeast two-hybrid system, an in vitro binding assay, and in vivo co-immunoprecipitation in 2001. This was the first study that suggested the potential role of filamin A in DNA damage response, via connection of cytoskeletal signal transduction to DNA damage response pathways (Yuan and Shen 2001). Later, Jingyin et al., showed that cells with lack or decreased expression of filamin A are sensitive to ionizing radiation and also the same cells show reduced RAD51 nuclear foci formation and 2-fold reduction of homologous recombinational repair of DSB (Jingyin et al. 2009). Velkova et al., demonstrated that filamin A acts as a BRCA1 partner and is required for efficient regulation of early stages of DNA repair process. Also, the same author showed that filamin A is required for the stabilization of the components of DNA-PK holoenzyme, DNA-PKcs and Ku86 in BRCA1-independent fashion (Velkova et al. 2010). Another experimental study on melanoma cell lines and mouse models showed that the reduction of filamin-A increases the sensitivity of cancer cells to chemotherapeutic drugs, such as bleomicin and cisplatin, and impairs double strand break (DSB), single strand break (SSBs) and interstrand crosslinks (ICLs) repair, as well as increases chromosome breaks after the treatment. The inhibition of filamin A expression also increases the sensitivity to bleomicin and cisplatin in mouse xenograft models (Yue et al. 2012). Collectively these data suggest an important role of cytoskeleton and particularly filamin A in supporting the DNA repair process and implicates filamin A as a potential prognostic marker in DNA damage based chemotherapy (Jingyin et al. 2009; Yue et al. 2012). However, the clinical relevance of this information is lacking.

1.3.3.2. The role of filamin A in non-small-cell lung cancer

The role of filamin A in non-small-cell lung cancer is not yet clear. Only a few studies have been published up-to-date. One study showed that filamin A is a part of migratory and invasive phenotype in A549 lung adenocarcinoma cells after TGF-beta treatment (Keshamouni et al. 2006). Nallapalli et al., demonstrated that targeting of filamin A reduces K-RAS–induced lung tumor development, reduces the migratory ability of endothelial cells and impedes local tumor growth (Nallapalli et al. 2012). There has been only one study of filamin A protein expression in lung cancer patients, including small cell lung cancer (SCLC), that suggested the possible role of filamin A in angiogenesis, based on the positive association between filamin A and VEGF (vascular endothelial growth factor) expression. Overall 5-year survival rate for

patients with positive and those with negative filamin A expression was 43.7% and 54.9%, respectively (p=0.06). However, univariate and multivariate survival analyses did not show any relationship with filamin A expression.

1.3.4. The role of sphingolipid metabolism pathway proteins in cancer progression and drug resistance

Sphingolipids are a family of membrane lipids that have structural roles in the regulation of the fluidity and the sub-domain structure of the lipid bilayers (Futerman and Hannun 2004). Bioactive sphingolipids such as ceramide, glycosylceramide (GlcCer), sphingosine, and sphingosine 1-phosphate (S1P) play an important roles in various aspects of cancer biology. The generation of endogenous ceramide and sphingosine in response to stress stimuli have been associated with senescence, growth arrest, or apoptosis (Morad and Cabot 2013; Olivier Cuvillier 2002). As opposed to ceramide, S1P plays important roles in mediating cell proliferation, transformation, survival, and migration (Pitson 2011; Maceyka et al. 2012). The balance between the cellular levels of these two lipids has been called the "sphingolipid-rheostat" (O Cuvillier et al. 1996) and is thought to be one of the important mechanisms controlling cell fate.

Sphingosine kinase-1 (SphK1) is a key enzyme(Olivier Cuvillier 2008), which appears to alter the ceramide/S1P balance (O Cuvillier et al. 1996), effectively regulates drug-induced apoptosis, and serves as a chemotherapy sensor both in culture and in animal models of various tumors (Bonhoure et al. 2006; Guillermet-Guibert et al. 2009) including NSCLC (H. Ito et al. 2010; L. Song et al. 2011; Ogretmen et al. 2001).

Several studies have also examined the prognostic and predictive value of SphK1 in solid tumors. In a series of 48 malignant astrocytomas, SphK1 mRNA expression levels correlated with patient survival, with a three-fold increase in median survival in patients with low compared to high-expression (Jun Li et al. 2008).

A recent meta-analysis including thirty-four studies of SphK1 expression in 4,673 patients, showed that there was a significant difference in SphK1 expression between cancer, normal tissue adjacent to cancer and benign tissues, as well as different cancer types, with lowest expression in ER positive breast cancer and highest level of expression in tongue squamous cell carcinoma (Zhang et al. 2014). In addition, SphK1 expression was associated with 5-year and overall survival rates in breast, gastric and other cancers (Zhang et al. 2014). The prognostic value of SphK1 was confirmed in breast cancer where the upper quartile of mRNA SphK1 expression correlated with poor prognosis, irrespective of the estrogen receptor status (Ruckhaberle et al. 2013).

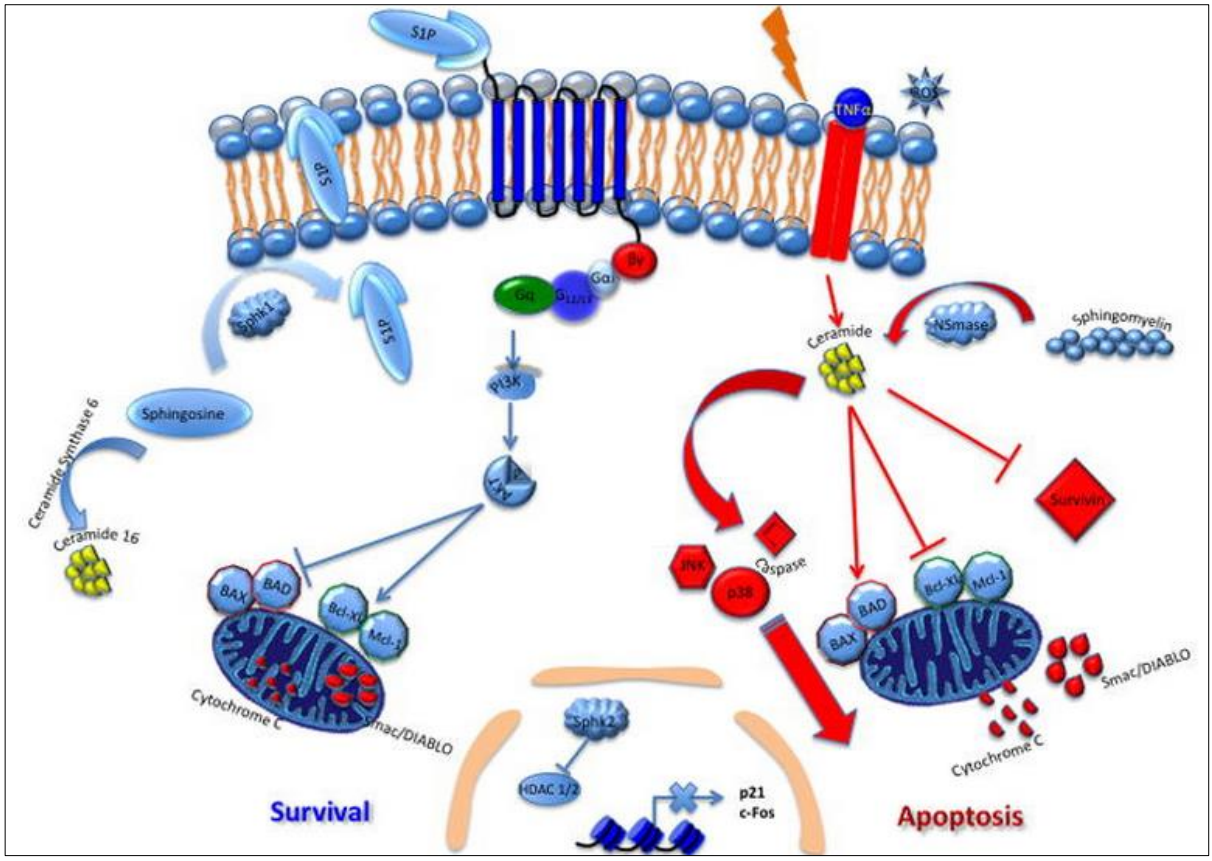


Figure 7. "Sphigolipid-rheostat" model. *Balance of forces between S1P and ceramide, with S1P promoting survival and ceramide promoting apoptosis. Downstream mediators affected by the rheostat include Bad, Bax, Bcl-XL and Mcl-1. Ceramide is also shown here to affect signaling via JNK and p38. Adapted from: Kenneth C. Loh, Dianna Baldwin, and Julie D. Saba, Anticancer Agents Med Chem. 2011 Nov 1; 11(9): 782–793*

Assessing tumor SphK1 activity was also suggested in ovarian cancer to have diagnostic capabilities, as shown by a significant increase of the product of its activity S1P, in ascites (Sutphen et al. 2004). A significant increase in both SphK1 expression and enzymatic activity have also been found to be correlated with aggressiveness in prostate cancer specimens (Malavaud et al. 2010). Increased expression of SphK1 protein and mRNA was also seen in lung cancer tissue, compared to adjacent normal lung and increased SphK1 expression was significantly correlated with tumor progression and poor survival of patients with NSCLC (L. Song et al. 2011). In NSCLC cell cultures, overexpression of SphK1 significantly inhibits doxorubicin- or docetaxel-induced apoptosis, and is associated with upregulation of the antiapoptotic proteins Bcl-xl, c-IAP1, c-IAP2, and TRAF1(L. Song et al. 2011). In contrast, silencing SphK1 expression or inhibiting SphK1 activity with a specific inhibitor, SK1-I, significantly enhanced the sensitivity of NSCLC cells to apoptosis induced by

chemotherapeutics both *in vitro* and *in vivo* (L. Song et al. 2011). Moreover, overexpression of SphK1 is associated with activation of the PI3K/Akt/NF-κB pathway, inhibition of which abrogates the antiapoptotic effect of SphK1 in NSCLC cells (L. Song et al. 2011; Ogretmen et al. 2001).

S1P is irreversibly degraded by the sphingosine 1-phosphate lyase (S1P lyase) which is highly conserved throughout evolution and is required for maintenance of physiological levels of S1P and other sphingolipid intermediates (Serra et al,. 2010). S1P lyase expression potentiates apoptosis in response to DNA damage and other stressful stimuli through a cascading mechanism that involves p53, PIDD, and caspase-2 (Oskouian et al. 2006). HEK293 or A549 human lung cancer cells expressing SPL show increase sensitivity to cisplatin and carboplatin (Min et al. 2005). The first evidence of the loss of S1P lyase expression in a human neoplasm has been recently reported in prostate cancer patients where an inverse correlation was found between SphK1 and S1P lyase expression and activity, suggesting an overall increase in tumor S1P content (Brizuela et al. 2012).

2. THE AIMS OF THE STUDY

Resistance to cytotoxic drugs is a major impediment to the successful treatment of NSCLC. DNA repair genes have been confirmed as predictive markers of treatment response in NSCLC patients. However, detection of these molecular markers by routine diagnostic procedures is complicated. Recently, it has been shown that cytoskeletal protein filamin A plays a key role in DNA damage response and cisplatin resistance in experimental settings, although the clinical relevance of these findings, is not yet fully understood. Interestingly, sphingolipid metabolism pathway proteins have emerged as important players in tumor progression and treatment resistance via regulation of essential cellular processes including apoptosis and DNA damage response. The study includes four main parts, in which we aimed to investigate:

1. The usefulness of BRCA1 protein detection by immunohistochemistry as a prognostic and predictive marker in patients with non-small-cell lung cancer;

2. The immunohistochemical expression of RAD51 protein and its possible prognostic and predictive role in NSCLC patients treated with either neoadjuvant and/or adjuvant platinum-based combination chemotherapy;

3. The immunohistochemical expression of filamin A protein and its relationship with other known clinicopathological prognostic factors, as well as the investigation of its predictive value in NSCLC patients treated with adjuvant platinum-based combination chemotherapy;

4. The prognostic and predictive value of SphK1 and S1P lyase expression in NSCLC patients treated with adjuvant platinum-based combination chemotherapy.

3. MATERIALS AND METHODS

3.1. Patients

The study consisted in all, of 239 NSCLC patients in three different cohorts. These were diagnosed at the University Hospital, Faculty of Medicine and Dentistry, Palacký University, Olomouc, Czech Republic. The first cohort included 115 patients diagnosed from 1996-2000 and treated with neoadjuvant chemotherapy and/or radical surgery alone and/or in combination with adjuvant platinum-based chemotherapy. These patients were from a previous study of molecular markers of NSCLC, which was performed at the Department of Clinical and Molecular Pathology, by my supervisor Josef Škarda, MD, PhD. The second cohort consisted of 80 patients diagnosed at the same hospital during 2005-2011 and treated with radical surgery and adjuvant platinum-based chemotherapy. The third cohort consisted of 44 patients, diagnosed at the same hospital during 2003-2012 and were treated with neo-adjuvant platinum-based chemotherapy. Patient inclusion and exclusion criteria are given in figure 8.

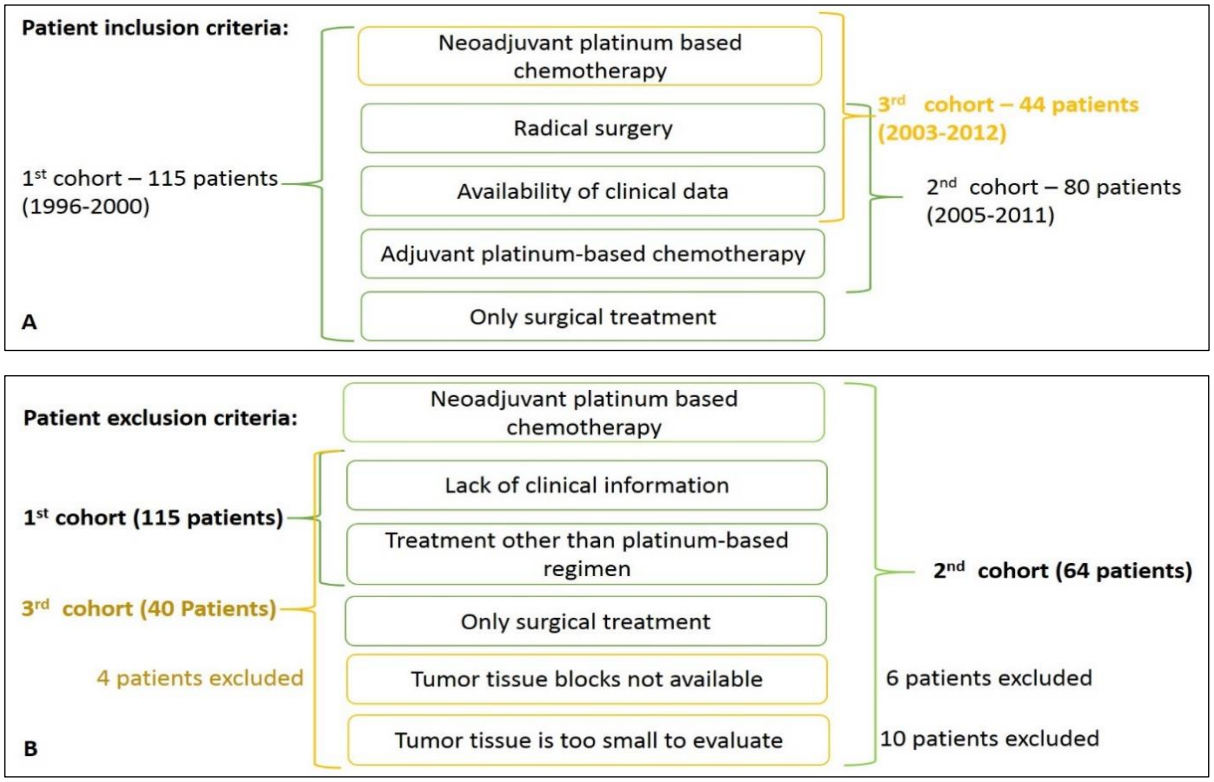


***Figure 8. A**. Patient inclusion criteria for each cohort. Numbers of included patients are given in the brackets for each cohort. **B.** Patient exclusion criteria for each cohort. Patent numbers after exclusion are given in the brackets for each cohort.*

3.2. Samples

Archival formalin-fixed, paraffin-embedded (FFPE) tissue samples and corresponding slides of 219 NSCLC patients were obtained from the Department of Clinical and Molecular Pathology, Faculty of Medicine and Dentistry, Palacký University Olomouc. Resected tumor tissues were fixed in 4% neutral buffered formalin for 24 hours and then embedded in paraffin according to standard procedures. Tissue specimens were cut into 4µ sections using Leica microtome and stained with routine hematoxylin and eosin method.

3.3. Clinicopathological characteristics of non-small-cell lung cancer patients

Patient samples were re-examined and categorized according to WHO classification of tumors of the lung (2004). Clinicopathological parameters, including age, gender, and clinical stage, depth of tumor invasion, lymph node metastasis, distant metastasis and differentiation were evaluated and included in the final patient data base for further statistical analysis. Detailed clinicopathological characteristics of NSCLC patients are given in table 3.

3.4. Survival analysis in different clinicopathological groups of NSCLC patients

All of 219 patients from three different cohorts were included in survival analysis. Patient cohort consisted of 69 stage I, 31 stage II, 109 stage III and 10 stage IV patients. Mean overall survival (OS) was 54.06 months and median OS was 41 months; Mean disease free survival (DFS) was 49.5 months and median DFS was 32 months. Total number of deaths were 124, and total number of patients with NSCLC relapse were 152. One year overall survival rate was 82.5%, three year OS rate was 63.8% and five year OS rate was 50%. One year disease free survival rate was 73.4%, three year DFS rate was 50.4 and five year DFS rate was 39.1%. Table 4. represents the detailed analysis of survival rates in stage I, II and III NSCLC patients.

Table 3. Patients and tumor characteristics

		1st cohort		2nd cohort		3rd cohort	
CHARACTERISTICS	**SUBGROUPS**	**N**	**%**	**N**	**%**	**N**	**%**
SEX	Male	89	77.4	41	64	34	85
	Female	26	22.6	23	36	6	15
AGE	≤65	73	63.5	29	45.3	23	57.5
	>65	42	36.5	35	54.7	17	42.5
HISTOLOGY	ADC	46	40	21	32.8	8	20
	SCC	56	48.7	34	53.1	30	75
	LCC	11	9.6	8	12.5	2	5
	OTHER	2	1.7	1	0.02	0	0
GRADE	G1	8	7	9	14	0	0
	G2	45	39	13	20.3	8	20
	G3	56	49	41	64	32	80
	ANP	6	5	2	3.1	0	0
NODAL STATUS	Negative	40	34.8	31	48.4	0	0
	Positive	61	53	33	51.6	40	100
	Not specified	14	12	0	0	0	0
DISTANT METASTASES	Present	9	7.8	0	0	0	0
	Not present	92	80	64	100	40	100
	Not specified	14	12.2	0	0	0	0
STAGE	I	45	39	24	37.5	0	0
	II	12	10.4	19	29.7	0	0
	III	49	42.6	17	26.6	40	100
	IV	9	7.8	0	0	0	0
ADJUVAN CHT	Yes	29	25.2	64	100	36	90
	No	86	74.8	0	0	4	10
NEOADJUVANT CHT	Yes	24	20.9	0	0	40	100
	No	91	79.1	64	100	0	0
TOTAL N.		**115**		**64**		**40**	

Table 4. Survival rates for stage I-III NSCLC patients

	N	N of Deaths	N of Relapse	One year survival (%)		Three year survival (%)		Five year survival (%)	
				OS	DFS	OS	DFS	OS	DFS
Stage I	69	45	51	83.7	74.9	69.4	56.5	51	41.6
Stage II	29	20	22	75.9	65.5	47.8	41.4	38.6	34.1
Stage III	109	51	71	86.8	73.9	66.1	49	53	38.3

3.4. Tissue microarray construction

Tumor tissue microarrays were constructed using formalin-fixed and paraffin embedded primary lung cancer tissue samples. The tissue area for sampling was based on visual alignment with the corresponding H&E stained section on a slide. Three to five tissue cores (diameter: 2.00 mm; height: 3-4 mm) taken from a donor tumor block were placed in a recipient paraffin block with a manual tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA). A core of normal tissue was punched from each case, and 4 μ sections of the resulting microarray block were used for immunohistochemical analysis.

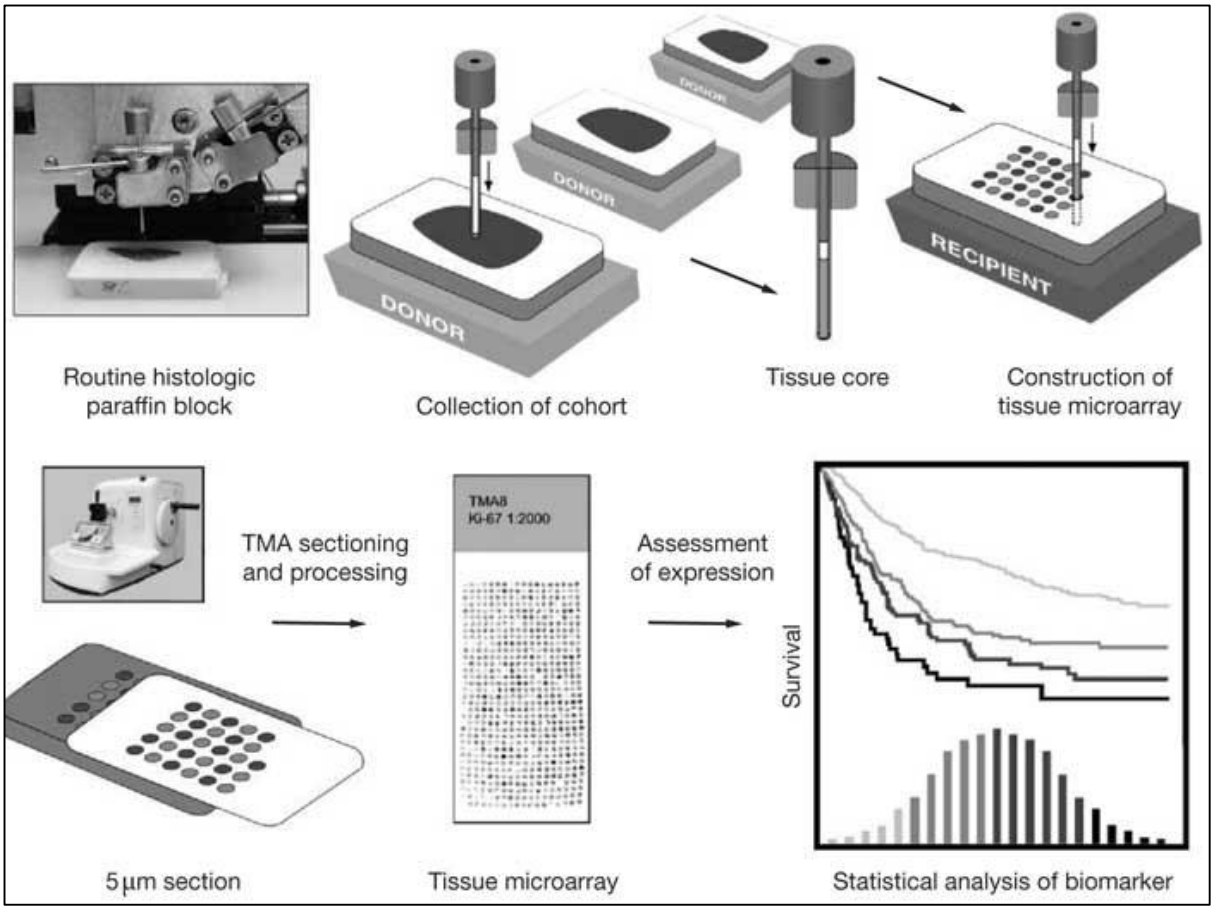


Figure 9. Schematic representation of the main steps of the construction of tissue microarray for biomarker analysis. *Adapted from: Giltnane JM and Rimm DL (2004)*

3.5. Immunohistochemical study

Formalin-fixed and paraffin-embedded tissue sections (4μ) were deparaffinized in xylene and rehydrated by washing in serial dilutions of ethanol (96%, 80% and 70%) and rinsed in deionized water. Detailed information on the antigen retrieval procedures, antibodies and their dilutions is provided in Table 5. After antigen retrieval, slides were rinsed in tap and deionized water. For blocking endogenous peroxidase activity, slides were treated with 0.3% hydrogen peroxide solution for 15 min. Sections were washed in deionized water for 5 min, then twice in 0.05M Tris buffer (pH 7.4-7.6), once in Tris buffer with 0.5% Tween solution and incubated with primary antibody for 1 hour at room temperature in a humid chamber. Slides were washed twice in Tris buffer, once in Tris buffer with 0.5% Tween solution, and incubated with secondary antibody (Dual Link, Dako) for 1 hour. After the last washing step in Tris buffer, slides were incubated in substrate solution (DAB), counterstained in hematoxylin, dehydrated through alcohols and xylene and mounted.

Table 5. Antibody characteristics and dilutions

Antibody	**Clone**	**Species/ clonallity**	**Antigen retrieval**	**Dilution**	**Positive Control**	**Manufacturer**
BRCA1	MS110	Mouse	Citrate, Buffer pH6, MW	1:100	Breast Carcinoma	Abcam
BRCA1	17F8	Rabbit	Citrate, Buffer pH6, MW	1:150	Breast Carcinoma	Abcam
BRCA1	GLK2	Mouse	Citrate, Buffer pH6, MW	1:10	Breast Carcinoma	Abcam
BRCA1 S1524	polyclonal	Rabbit	Citrate, Buffer pH6, MW	1:150	Breast Carcinoma	Abcam
BRCA1 S1423	polyclonal	Rabbit	Citrate, Buffer pH6, MW	1:150	Breast Carcinoma	Abcam
Filamin A	EP2405Y	Rabbit	Citrate, Buffer pH6, MW	1:500	Normal Skin	LSBio
RAD51	EPR4030(3)	Rabbit	Citrate, Buffer pH6, MW	1:400	Normal Testis	Abcam
SphK1	In house	Mouse	Citrate, Buffer pH6, MW	1:200	Normal Colon	In house
SGPL1	polyclonal	Rabbit	Citrate, Buffer pH6, MW	1:1000	Normal Colon	Atlas Antibodies

3.5.1. Selection and validation of antibodies

BRCA1

Five different antibodies were used for the detection of BRCA1 protein: clone MS110 against N-terminal part (1-304aa), clone 17F8 against central part (762-1315aa), clone GLK2 against C-terminal (1832-1863aa) and two antibodies against phosphorylated forms of BRCA1 protein at Ser1423 and Ser1524 residues (figure 10). All antibodies were commercially available and validated by manufacturer.

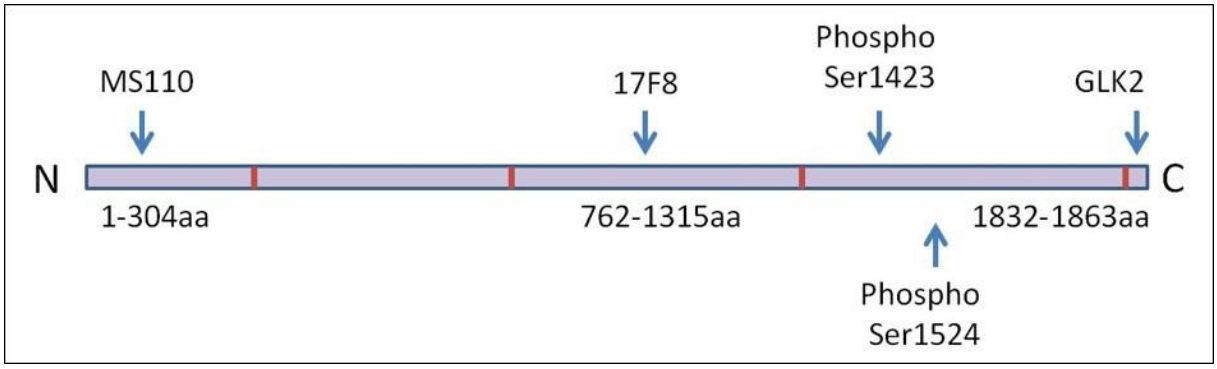


***Figure 10. Schematic representation of used antibodies against different parts of BRCA1 protein*.**

BRCA1 MS110

The MS110 antibody (Abcam, Ab16780) is specifically designed to recognise the N-terminal part (1-304aa) of BRCA1 protein. Formalin-fixed, paraffin embedded breast carcinoma tissue is recommended as positive control. Antibody is validated by manufacturer for flow cytometry, western blot, immunocytochemistry/immunofluorescence, immunoprecipitation, and immunohistochemistry for Formalin-fixed, paraffin embedded and frozen sections. MS110 is the best validated and most frequently used antibody for the immunohistochemical detection of BRCA1 (http://www.abcam.com/brca1-antibody-ms110-ab16780-references.html). We used formalin-fixed, paraffin embedded breast carcinoma tissue as a positive control and optimisation of staining intensity.

BRCA1 GLK2

The GLK2 antibody (Abcam, ab17251) is specifically designed to recognise the C-terminal part (1832-1863aa) of BRCA1 protein. Ovarian carcinoma tissue is recommended as positive control. However, it is known that staining is absent in 100% of ovarian tumors with BRCA1 mutations other than exon 11. We tested the antibody on ovarian and breast carcinoma tissues,

using different antigen retrieval methods (including automatic multifunctional microwave tissue processor (T/T MEGA) at 95 °C, over 5 min, using citrate buffer at pH6 and water bath at 90°C and over 40min, using EDTA, pH8) and different dilutions from 1:10 to 1:100 (low dilution is recommended by manufacturer). We found faint nuclear and cytoplasmic positivity in breast carcinoma tissue at 1:10 dilution.

BRCA1 17F8

The 17F8 antibody (Thermo scientific) is designed to recognise the central part (762-1315aa) of BRCA1 protein. During the optimisation of 17F8 antibody we noticed that the reactivity and the localisation of staining was depend on the antigen retrieval method. After antigen retrieval in citrate buffer at pH6 faint cytoplasmic positivity was detectable in breast carcinoma tissue, whilst nuclear positivity was detected after antigen retrieval in EDTA pH8 in the same tissue at the same dilution (1:150).

BRCA1 Ser1423

Rabbit polyclonal antibody (Abcam, ab47325) detects endogenous levels of BRCA1 only when phosphorylated at serine 1423. Antibody is validated by manufacturer for ELISA, immunocytochemistry/immunofluorescence, and immunohistochemistry for Formalin-fixed, paraffin embedded tissues. We used breast carcinoma tissue as positive control and compared our staining to the picture provided by manufacturer (http://www.abcam.com/prettyPhoto/1/). We further validated the antibody by western blot method, using protein lysates from irradiated DU145 prostate cancer cell lines.

BRCA1 Ser1524

Rabbit polyclonal antibody (Abcam, ab47276) detects endogenous levels of BRCA1 only when phosphorylated at Serine 1524. Antibody is validated by manufacturer for ELISA and immunohistochemistry for Formalin-fixed, paraffin embedded tissues. We used breast carcinoma tissue as positive control and compared our staining to the picture provided by manufacturer (http://www.abcam.com/brca1-phospho-s1524-antibody-ab47276.html). We further validated the antibody by western blot method, using protein lysates from irradiated DU145 prostate cancer cell lines.

Filamin A

Rabbit monoclonal EP2405Y antibody against C-terminus of FLNA (LSBio, LS-C50172) was used for the detection of Filamin A protein. Normal skin tissue was used as a as a positive control and for the optimisation of staining intensity, as recommended by manufacturer. After testing of several antigen retrieval methods, tissue sections were stained by using Benchmark XT automatic tissue stainer from Ventana (USA).

RAD51

Rabbit monoclonal EPR4030 antibody (Abcam, ab133534) against RAD51 is validated by manufacturer for flow cytometry, western blot, immunocytochemistry/immunofluorescence, immunoprecipitation, and immunohistochemistry for Formalin-fixed, paraffin embedded sections. The highest expression is shown in normal testis and thymus, followed by small intestine, placenta, colon, pancreas and ovary. Weak expression is found in breast cancer tissue. RAD51 colocalizes with RAD51AP1 and RPA2 to multiple nuclear foci upon induction of DNA damage. Expected cellular localization is the cell nucleus as well as the cytoplasm (cytoplasm > perinuclear region), and mitochondrial matrix. DNA damage induces an increase in nuclear staining levels. We used normal testis as a positive control and for the optimisation for staining intensity and compared our staining to the picture provided by the manufacturer (http://www.abcam.com/rad51-antibody-epr40303-ab133534.html). The antibody has been recently used by *Shen Y and Rehman FL (2013)*

SGPL1/S1P lyase (Sphingosine-1-phosphate lyase-1)

Commercially available rabbit polyclonal antibody against SGPL1/S1P lyase (Atlas antibodies, HPA023086), was used for the study. Specific reactivity against target PrEST (Protein Epitope Signature) antigen validated on protein array with 384 randomly selected antigens by manufacturer. Colon adenocarcinoma tissue was used as a positive control for staining intensity.

SphK1 (Sphingosine-1 kinase 1)

Antibody against SphK1 was produced and validated at the Sphingolipid metabolism and the cancer research lab. Institute of pharmacology and structural biology, Toulouse, France

3.5.2 Immunohistochemical staining evaluation and scoring

The stained slides were evaluated at least by two independent pathologists (M.G, J.Š) who were blinded to the clinicopathological information of the patients.

BRCA1 nuclear and/or cytoplasmic expression was evaluated quantitatively as the percentage of positive tumor cells. Cases with BRCA1 expression >10% of cancer cells were considered as positive. Typical staining of BRCA1 is shown at figure 11.

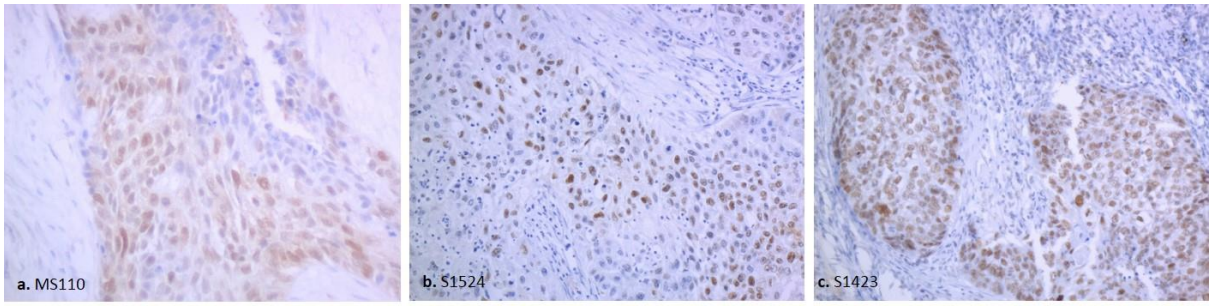


Figure 11. BRCA1 nuclear positivity detected by MS110 (a), S1524 (b) and S1423(c)

RAD51 nuclear expression was also evaluated semiquantitatively and scored by histoscore (Hscore) method. Staining intensity was evaluated as follows: Negative – 0, Weak nuclear positivity with no obvious signs of "nuclear foci" formation – 1+, Moderate nuclear positivity with easily identifiable "nuclear foci" – 2+, Strong nuclear positivity with marked "nuclear foci" formation – 3+. The intensity was multiplied by the percentage of positive cells resulting final histoscore 300.

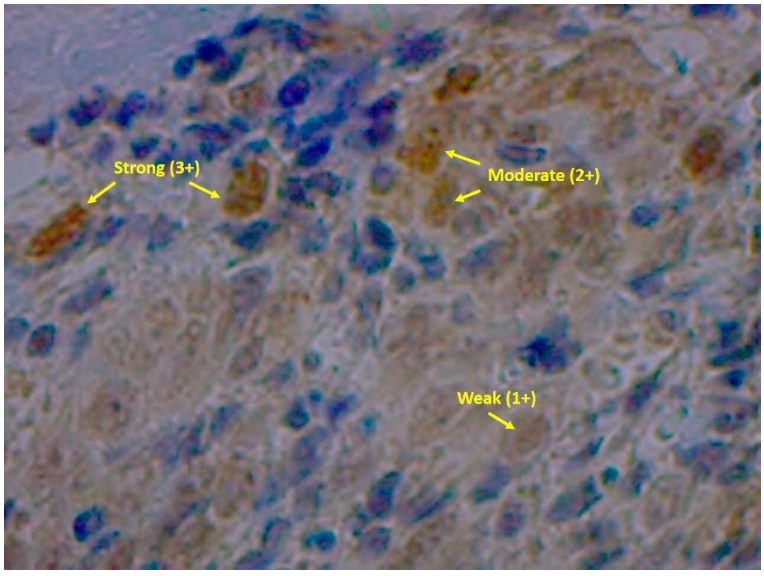


Figure 12. RAD51 staining evaluation

Filamin A, SphK1 and SGPL membranous and cytoplasmic staining was assessed by histoscore method (percentage of positive epithelial cells or of area of interest were counted and multiplied by intensity (categorized as: 0, absent; 1, weak; 2, moderate; and 3, strong), resulting in histoscore ranged from 0 (minimum) to 300 (maximum)). Figures 13 and 14.

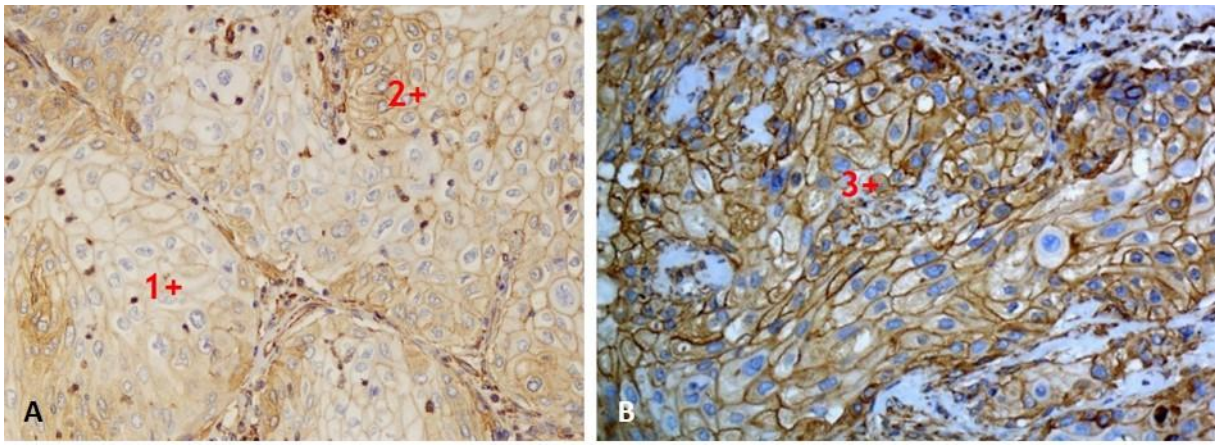


Figure 13. Evaluation of filamin A expression. *Staining intensity varies from weak to moderate (A) and strong (B) in different areas of the same tumor.*

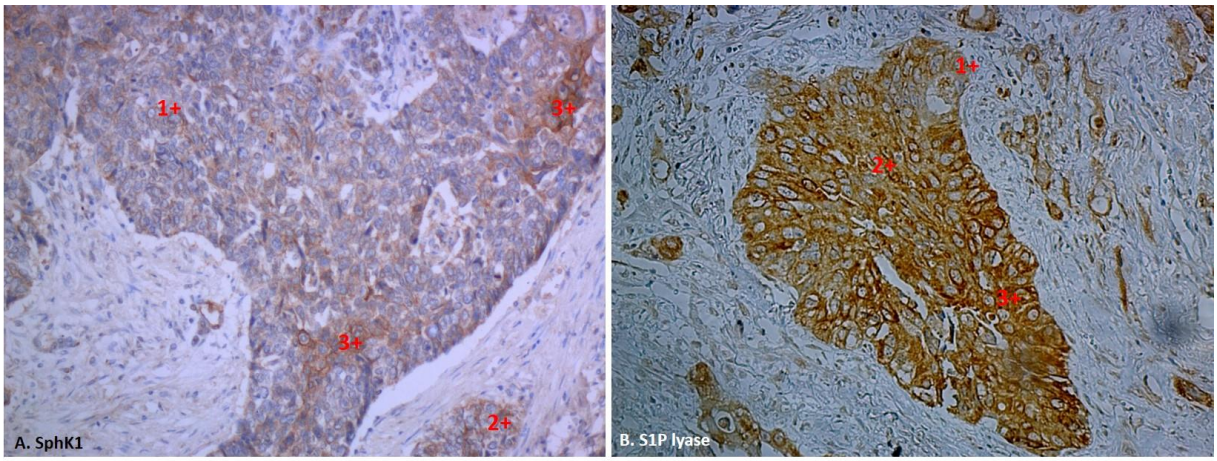


Figure 14. Evaluation of SphK1 (A) and SGPL1/S1P lyase (B) expression. *Staining intensity varies from weak to moderate and strong in different areas of the same tumor.*

3.6. Study Design

The expression and the prognostic value of selected proteins were retrospectively analyzed. Patients were divided into three different treatment groups: (1) patients who had undergone surgery without any chemotherapeutical intervention, (2) patients treated with adjuvant chemotherapy using Carboplatin and Navelbine, (3) patients treated with neoadjuvant chemotherapy using Carboplatin and Navelbine. The primary endpoint of the study was overall survival (OS), the secondary study endpoint was defined as disease-free survival (DFS). OS was defined as the time from the date of tumor surgery until death or to the last date of follow-up; DFS was defined as the time from the date of surgery to the date of relapse or metastasis. Cases lost during follow-up and those ending in death from any cause other than lung cancer were regarded as censored data in the analysis of survival rates. Median follow-up was 38 months (range 2 – 181 months).

3.7. Statistical Analysis

Comparisons between different groups were determined by the Mann-Whitney U test and correlations were assessed by the Spearman rank test. Cox proportion hazards regression model was performed to calculate the prognostic value of the proteins. For survival analysis, Kaplan-Meier curves were calculated, and tests of statistical significance were based on log-rank statistics. In addition, the prognostic value of the protein's expression levels for overall survival was evaluated using univariate and multivariate Cox regression models. The prognostically significant cut off value for RAD51 protein was calculated by online software "Cutoff Finder", from Charite Medical University, Berlin, Germany (Budczies et al. 2012). All other statistical tests were performed using statistical software – SPSS20 (SPSS, Inc., Chicago, IL). In all cases, P-values <0.05 were considered as statistically significant.

4. RESULTS

4.1. Immunohistochemical expression of BRCA1 in relation to survival outcomes of non-small-cell lung cancer patients

4.1.1. BRCA1 protein analysis using different antibodies

First study of BRCA1 protein expression included TMAs from 113 retrospectively selected NSCLC patients. Detailed clinicopathological characteristics of patients are given in table 6. Two antibodies, GLK2 and 17F8, were excluded from the study because of non-satisfactory optimal staining. The staining with GLK2, which specifically recognizes the C-terminal part (1832-1863aa) of BRCA1, was extremely weak to evaluate even at 1:10 dilution, and we did not find any reactivity with 17F8 antibody (against the central part (762-1315aa) of BRCA1) in NSCLC tissue.

BRCA1 MS110 staining (against N-terminal (1-304aa) part of BRCA1) showed mainly nuclear positivity in 28% of NSCLC cases. Only 2 cases were positive in both – nucleus and cytoplasm and none of them in only cytoplasm. BRCA1 phospho S1524 antibody was positive in 27% of NSCLC cases, from which 11 cases were only nuclear, 13 – nuclear and cytoplasmic and only one case showed only cytoplasmic positivity. BRCA1 phospho S1423 antibody showed strong nuclear expression in 75% of cases. Figure 15 shows the typical staining patterns for each antibody.

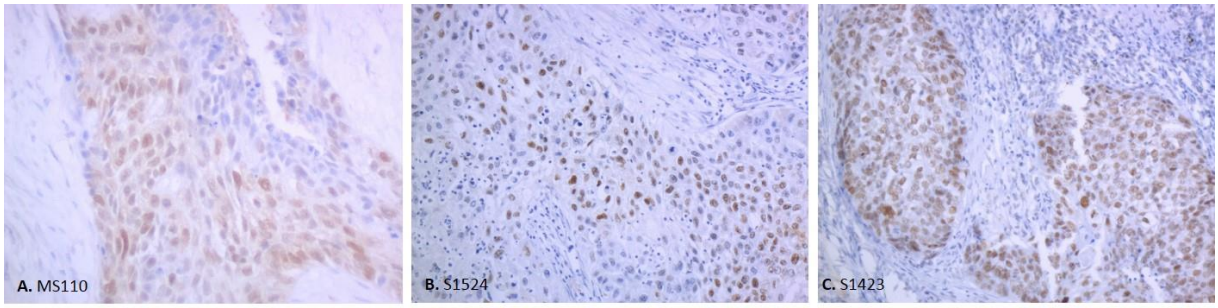


Figure 15. Immunohistochemical expression BRCA1 protein in squamous cell carcinoma of the lung, detected by three different antibodies. *A. MS110 antibody recognizes N-terminal (1-304aa) part of BRCA1 protein, IHC, x200; B. phospho S1524 antibody recognizes the BRCA1 protein only when phosphorylated at 1524 serine residue, IHC, x100; C. phospho S1423 antibody recognizes the BRCA1 protein only when phosphorylated at 1423 serine residue, IHC, x100*

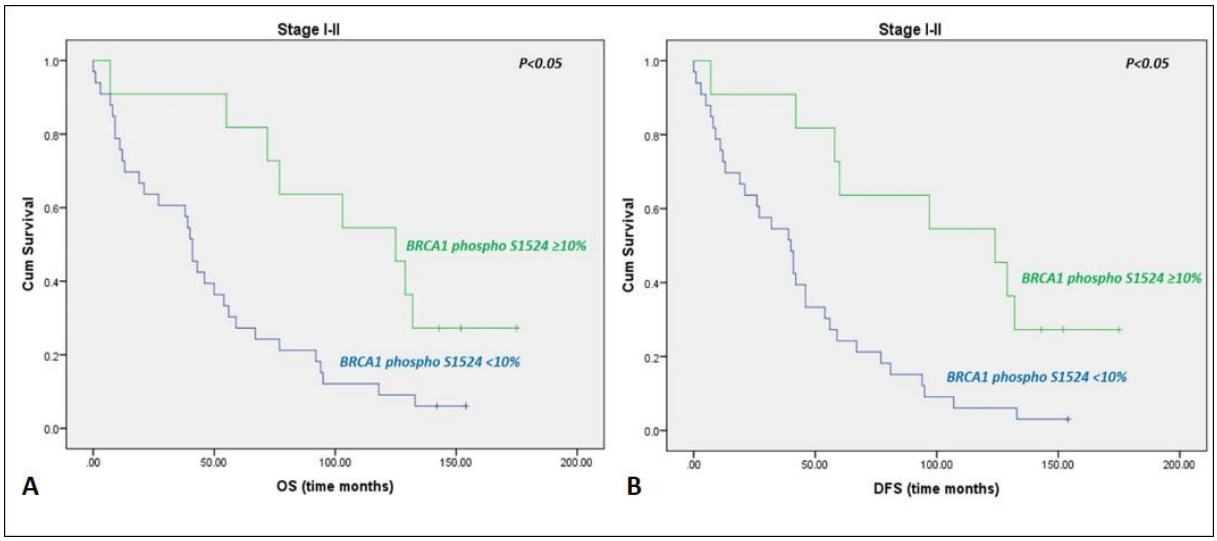
Spearman's correlation and Mann-Whitney U test did not find statistically significant association between the expression of BRCA1 and other clinicopathological factors, including age, sex, histopathological subtype, grade, tumor size, lymph node status, and stage of NSCLC.

Kaplan-Meier survival analysis showed that only nuclear positivity (≥10) with BRCA1 phospho S1524 was significantly associated with higher overall and disease free survival rates in stage I - II patients (P<0.05) (Graph 2), whilst overall and disease free survival rates were significantly lower in phospho S1524 positive stage III - IV patients (P<0.05) (Graph 3). There was no significant association between the expression of BRCA1 MS110 and BRCA1 phospho S1423 and survival outcomes.

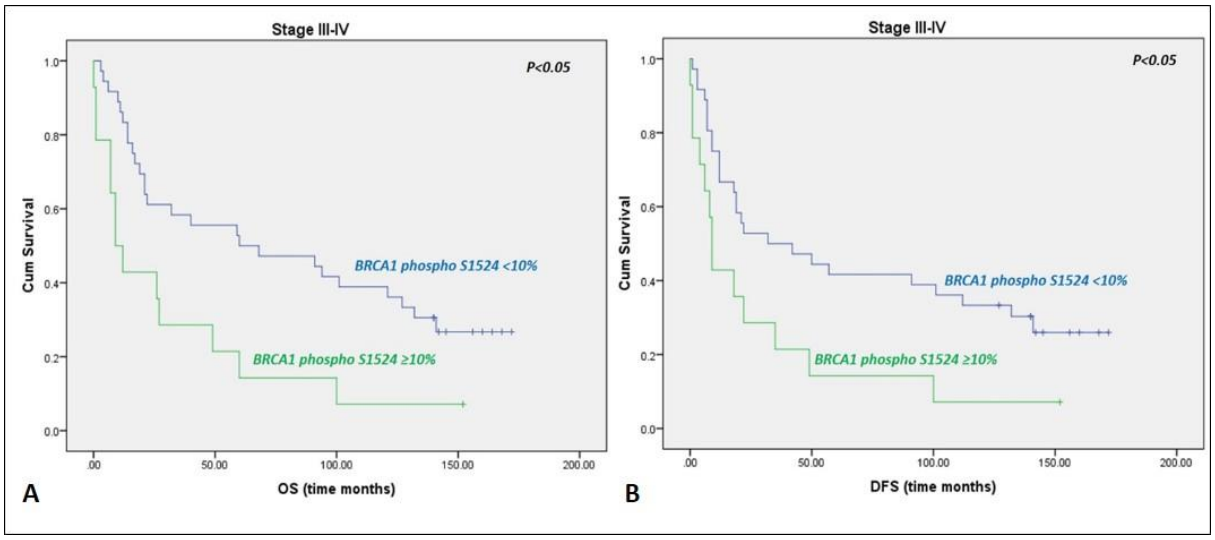
Table 6. Clinicopathological characteristics of NSCLC patients, included in the study of BRCA1 protein expression using different antibodies

	ALL PATIENTS	**I**	**II**	**III**	**IV**
N	113	27	9	52	10
MEDIAN OS (MONTH)	46.03	93.18	40.95	44.03	13.13
MEAN OS (MONTH)	58.62	86.19	48.64	59.86	35.11
MEDIAN DFS	38.82	80.69	31.81	21.71	10.57
MEAN DFS	53.70	81.03	44.90	53.21	31.40
AGE	60	62	57	61.5	54.5
GENDER					
MALE	88	19	8	40	8
FEMALE	25	8	1	12	2
HISTOLOGY					
ADC	43	13	4	16	6
SCC	49	10	3	26	2
Others	21	4	2	10	2
NEOADJUVANT CHT					
CHT	27	2	0	22	3
OBSERVATION	72	25	9	30	6
ADJUVANT CHT	10	1	1	6	2
PT/NVB	0	0	0	0	0
PT/TAX	2	0	0	2	0
OTHER	8	1	1	4	2

NSCLC, non-small-cell lung cancer; ADC, adenocarcinoma; SCC, squamous cell carcinoma; OS, overall survival; DFS, disease free survival; CHT, chemotherapy; NVB, Navelbine; TAX, Taxol.



Graph 2. Kaplan-Meier analysis of survival in stage I-II NSCLC patients. A. *Cumulative overall survival (OS) rate is significantly higher in patients with BRCA1 phospho S1524 positivity (≥10%) (Log-rank, p<0.05);* ***B****. Cumulative disease free survival (DFS) rate is significantly higher in patients with BRCA1 phospho S1524 positivity (≥10%) (Log-rank, p<0.05)*



Graph 3. Kaplan-Meier analysis of survival in stage III-IV NSCLC patients. A. *Cumulative overall survival (OS) rate is significantly lower in patients with BRCA1 phospho S1524 positivity (≥10%) (Log-rank, p<0.05);* ***B****. Cumulative disease free survival (DFS) rate is significantly lower in patients with BRCA1 phospho S1524 positivity (≥10%) (Log-rank, p<0.05)*

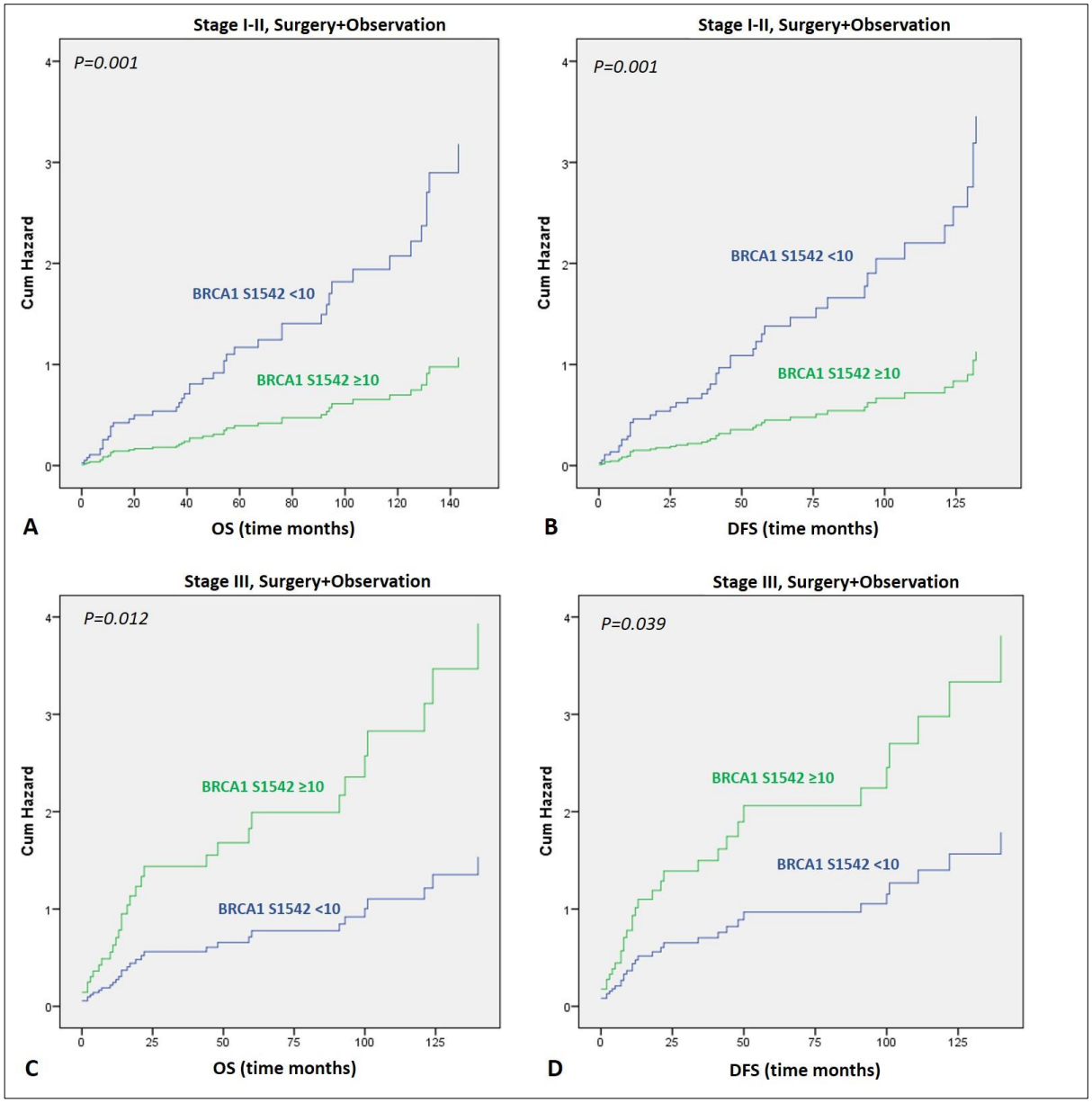
4.1.2. Prognostic role of BRCA1 phospho S1524 expression in NSCLC patients

Study of the relationship between immunohistochemical expression of BRCA1 phospho S1524 and NSCLC patient prognosis included 132 patients with stage I-III disease, treated either by adjuvant platinum based chemotherapy (Carboplatin and Navelbine) or only surgical operation. Clinicopathological characteristics of NSCLC patients are given in table 7.

BRCA1 phospho S1524 nuclear positivity (≥10) was detected in 43 (32%) cases, and was significantly associated with older age (>60) of NSCLC patients (p=0.008).Univariate Cox regression analysis of survival showed that BRCA1 phospho S1524 positivity was significantly associated with the lower risk of death and relapse in stage I-II NSCLC patients, treated with only surgical operation (p<0.01), whilst in stage III patients, treated with only surgical operation BRCA1 phospho S1524 positivity was significantly associated with higher risk of death and disease recurrence (p<0.05) (Graph 4). There was no significant association between BRCA1 phospho S1524 positivity and survival outcomes in patients treated with adjuvant platinum-based chemotherapy. Age adjusted multivariate Cox regression analysis of survival also did not show the independent prognostic value of BRCA1 phospho S1524 positivity in patients with NSCLC.

Table 7. Clinicopathological characteristics of NSCLC patients included in the study of the prognostic role of BRCA1 phospho S1524 expression

CHARACTERISTICS	SUBGROUPS	N	%
SEX	MALE	101	76.5
	FEMALE	31	23.5
AGE	≤60	56	42.4
	>60	76	57.6
HISTOLOGY	Adenocarcinoma	47	35.6
	Squamous cell carcinoma	71	53.8
	Large cell carcinoma	14	10.6
GRADE	G1	10	7.6
	G2	46	34.8
	G3	76	56.6
NODAL STATUS	NEGATIVE	50	37.9
	POSITIVE	67	52.2
	NOT SPECIFIED	13	9.9
STAGE	I	53	40.2
	II	19	14.4
	III	60	45.4
ADJUVANT CHEMOTHERAPY	YES	59	44.8
	NO	61	46.2
SURVIVAL TIMES			
MEDIAN OS	*41.5 months*		
MEAN OS	*56.9 (range 1-164) months*		
MEDIAN DFS	*35 months*		
MEAN DFS	*52 (range1-175) months*		



Graph 4. Univariate Cox regression analysis of survival in different stages of NSCLC patients, treated with only surgical operation. A. *The risk of death, estimated as overall survival (OS) in months, is significantly lower in BRCA1 phospho S1524 positive, stage I-II patients (p=0.001);* ***B.*** *The risk of NSCLC recurrence, estimated as disease free survival (DFS) in months, is significantly lower in BRCA1 phospho S1524 positive, stage I-II patients (p=0.001);* ***C****. The risk of death is significantly higher in BRCA1 phospho S1524 positive, stage III patients (p=0.012);* ***D****. The risk of disease recurrence is significantly higher in BRCA1 phospho S1524 positive, stage III patients (p=0.039).*

4.2. Immunohistochemical expression of RAD51 in relation to survival outcomes of non-small-cell lung cancer patients

The study of the immunohistochemical expression of RAD51 protein included 91 NSCLC patients from two different treatment groups: 35 patients were treated with neoadjuvant chemotherapy, using Carboplatin and Navelbine and 56 patients were without neoadjuvant treatment. All patients with neoadjuvant chemotherapy, were also treated adjuvantely. From 56 patients without neoadjuvant treatment 29 were treated with adjuvant Carboplatin and navelbine, whilst 27 patients were treated by surgery without adjuvant treatment. The detailed clinicopathological characteristics of patients are given in table 8.

Table 8. Clinicopathological characteristics of NSCLC patients included in the study of the immunohistochemical expression of RAD51

CHARACTERISTICS	**SUBGROUPS**	**N**	**%**
SEX	MALE	77	15.4
	FEMALE	14	84.6
AGE	≤60	38	41.8
	>60	53	58.2
HISTOLOGY	Adenocarcinoma	22	24.2
	Squamous cell carcinoma	59	64.8
	Large cell carcinoma	10	11
GRADE	G1	8	8.8
	G2	30	33
	G3	53	57.2
NODAL STATUS	NEGATIVE	23	25.3
	POSITIVE	59	64.83
	NOT SPECIFIED	9	9.9
STAGE	I	26	28.6
	II	14	15.4
	III	51	56
NEOADJUVANT CHEMOTHERAPY	YES	35	38.5
	NO	56	61.5
ADJUVANT CHEMOTHERAPY	YES	64	70.3
	NO	27	29.7
SURVIVAL TIMES			
MEDIAN OS	*37 months*		
MEAN OS	*44 (range 1-168) months*		
MEDIAN DFS	*41 (range 1-153) months*		
MEAN DFS	*28 months*		

Immunohistochemical staining was not satisfactory in 20 cases. From the remaining 71 cases 43 (60.5%) were positive (at least weak expression in >10% of tumor cells) for RAD51. The staining pattern was variable from slight nuclear positivity to moderately and strongly prominent nuclear foci formation (figure 16). RAR51 expression, estimated by Hscore, was significantly higher in large cell anaplstic carcinomas compared to squamous cell (p=0.035) and adenocarcinomas (p=0.015) (figure 17A), as well as in stage III tumors, compared to stage I-II NSCLC (p=0.016) (figure 17B).The expression of RAD51, was higher in patients treated with neoadjuvant platinum-based chemotherapy, however this difference was not statistically significant (figure 17C). We did not find significant relationship between RAD51 and other clinicopathological factors.

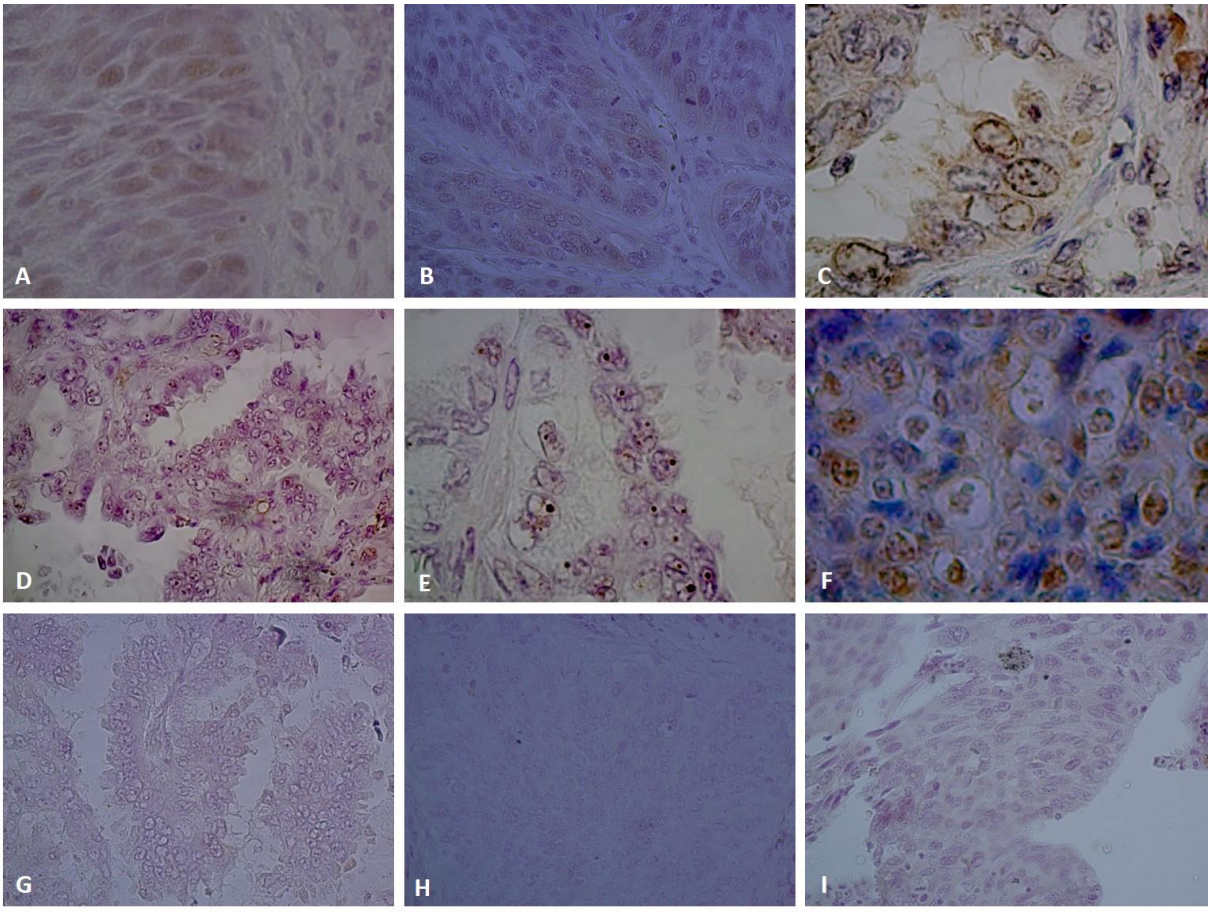


Figure 16. RAD51 protein expression in NSCLC. A*. Weak expression of RAD51, without any prominent nuclear foci, x400;* ***B.*** *Moderate positivity with some nuclear foci formation, X200 and* ***C.*** *the same case at X1000;* ***D****. Strong expression of RAD51 with prominent nucleolar positivity x200,* ***E.F****. Strong expression of RAD51 with marked nuclear foci formation, x400;* ***H,I,J.*** *RAD51 negative cases for comparison, x200*

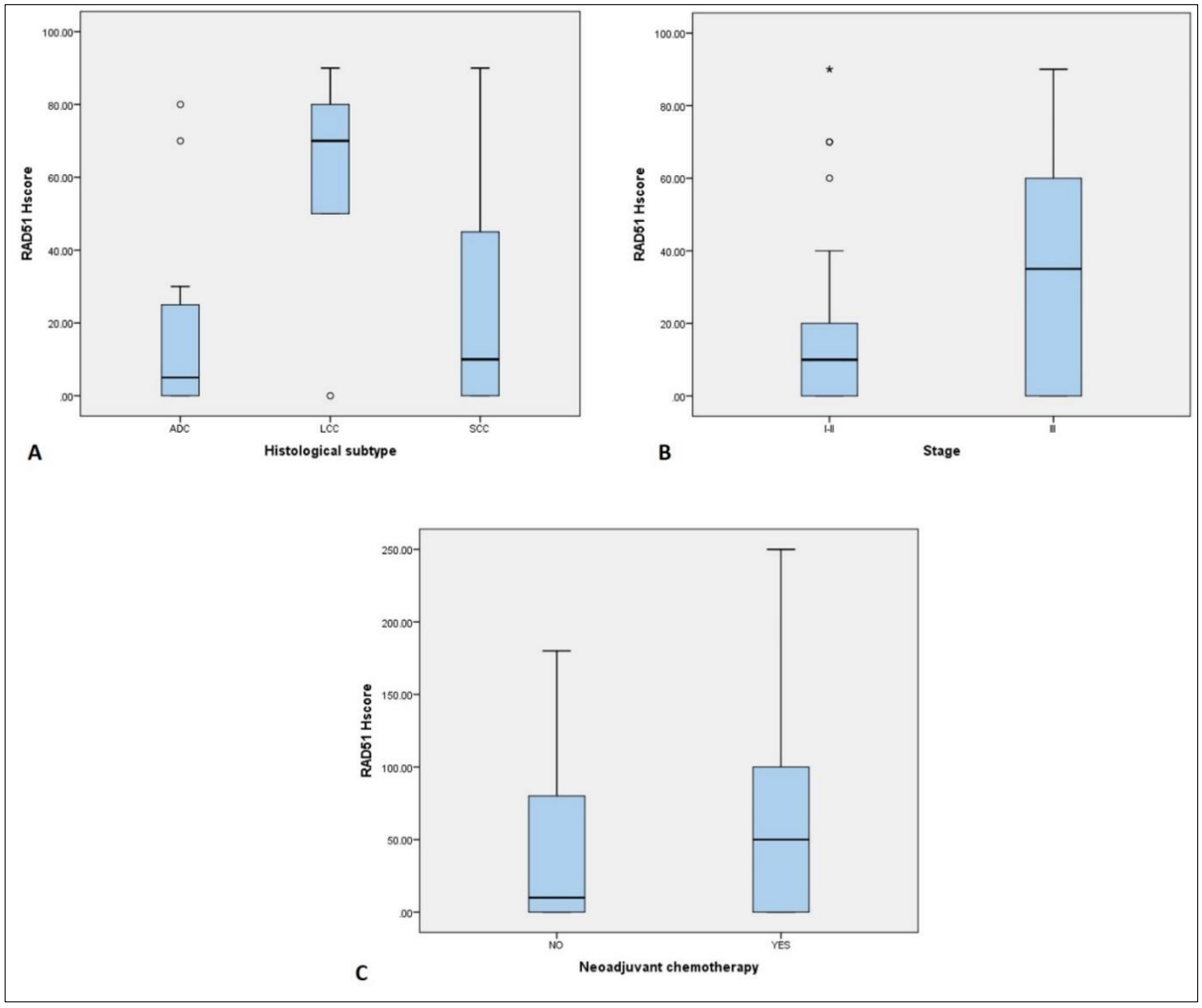
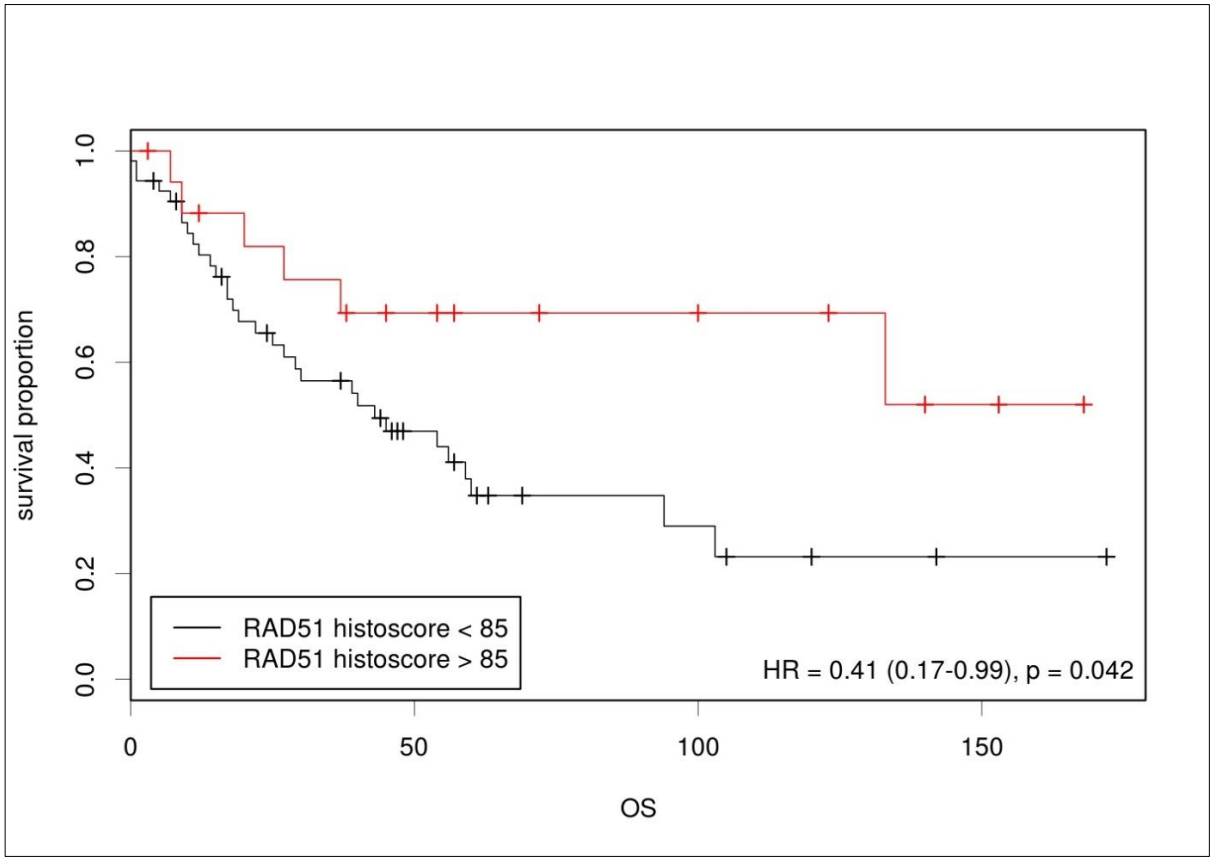


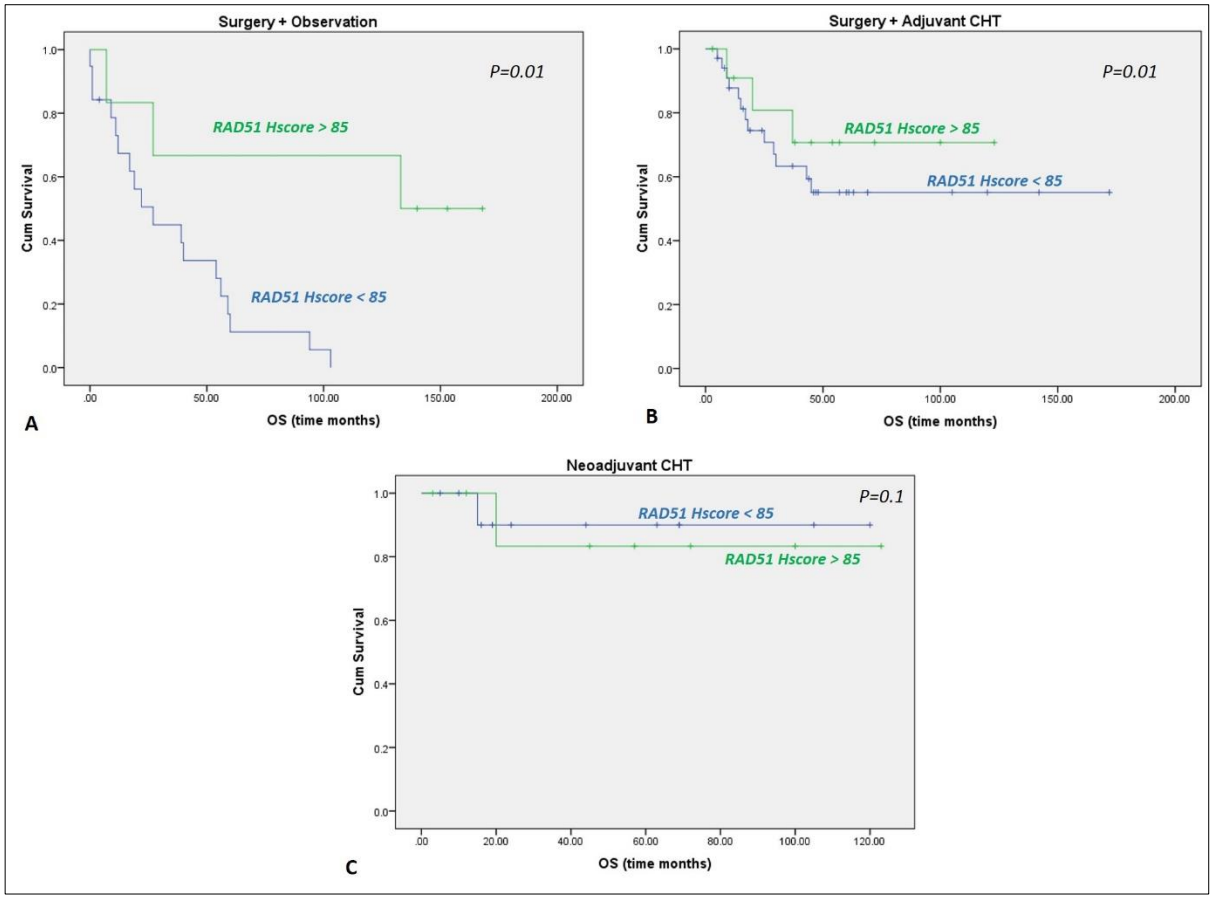
Figure 17. Comparisons of RAD51 expression in different clinicopathological groups (Mann-Whitney U test). A. *Expression of RAD51 is significantly higher in large cell carcinoma (LCC), compared to adenocarcinoma (ADC) and squamous cell carcinoma (SCC) (p=0.015 and p=0.035 respectively);* ***B****. Expression of RAD51 is significantly higher in stage III NSCLC, compared to stage I-II (p=0.016); C. Expression of RAD51 is higher in patients treated with neoadjuvant chemotherapy (Carboplatin+Navelbin), however this difference is not statistically significant (p=0.07)*

Online Cutoff finder from Charite Medical University (Berlin, Germany) identified two prognostically important groups according to RAD51 protein expression. Those with RAD51 Hscore <85 were associated with lower overall survival rate, compared to patients with RAD51 Hscore >85 (p=0.042) (graph 5). The same difference has been seen in disease free survival (not shown). Kaplan-Meier survival analysis in different stages and treatment groups also showed that overexpression of RAD51 (>85) is significantly associated with higher rates of overall survival in all stages of NSCLC, as well as in both treatment groups: patients treated with only surgical operation, and those treated by adjuvant Carboplatin and Navelbine (p=0.01)

(graph 6A,B). In patients treated with neoadjuvant chemotherapy RAD51 overexpression (>85) was associated with lower overall survival rate. However this difference was not statistically significant (p=0.106) (graph 6C).



Graph 5. *Kaplan-Meier survival curve generated by Cutoff finder (Charite Medical University, Berlin, Germany) (Budczies et al. 2012).*



Graph 6. Kaplan-Meier survival analysis in different treatment groups of NSCLC patients*. A. Overexpression of RAD51 (Hscore>85) is significantly associated with better overall survival in patients treated with surgery (p=0.01); B. Overexpression of RAD51 (Hscore>85) is significantly associated with better overall survival in patients treated with surgery and adjuvant chemotherapy, based on Carboplatin and Navelbine (p=0.01); C. Overexpression of RAD51 (Hscore>85) is associated with worst overall survival in patients treated with neoadjuvant chemotherapy, based on Carboplatin and Navelbine (p=0.106)*

4.3. Overexpression of filamin-A protein is associated with aggressive phenotype and poor survival outcomes in NSCLC patients treated with platinum-based combination chemotherapy

Study of the filamin A protein expression included 135 NSCLC patients, from which 73 patients were treated with only surgical operation and 62 patients were treated with adjuvant platinum-based chemotherapy (Carboplatin+Navelbin). Clinicopathological characteristics are given in table 9.

Table 9. Clinicopathological characteristics of patients included in the study of filamin A protein expression in NSCLC

CHARACTERISTICS	**SUBGROUPS**	**N**	**%**
SEX	MALE	103	76.3
	FEMALE	32	23.7
AGE	≤60	62	45.9
	>60	73	54.1
HISTOLOGY	Adenocarcinoma	53	39.3
	Squamous cell carcinoma	66	48.9
	Large cell carcinoma	14	10.4
	Adenosqamous carcinoma	2	1.5
GRADE	G1	15	11.1
	G2	39	28.9
	G3	81	60.0
NODAL STATUS	NEGATIVE	54	40
	POSITIVE	70	51.9
	NOT SPECIFIED	11	8.1
DISTANT METASTASES	PRESENT	9	5.9
	NOT PRESENT	116	85.9
	NOT SPECIFIED	10	8.1
STAGE	I	53	39.3
	II	26	19.3
	III	47	34.8
	IV	9	6.7
ADJUVANT CHEMOTHERAPY	YES	73	54.1
	NO	62	45.9
SURVIVAL TIMES			
MEDIAN OS	***48 months***		
MEAN OS	***51.9 (range 1-164) months***		
MEDIAN DFS	***32 months***		
MEAN DFS	***48 (range 1- 153) months***		

Immunohistochemical expression of filamin A was characterized with marked variability in NSCLC, as well as in lung tissue surrounding the tumor. Bronchial columnar

epithelial cells exhibited mainly moderate apical cytoplasmic and membranous staining, and relatively intensive staining in the attachment to the basal membrane. Submucosal glandular cells exhibited weak membranous positivity (figure 18A). Bronchiolar connective tissue was either negative for filamin A or exhibited a weak cytoplasmic expression (figure 18A,B). Chondrocytes in bronchiolar cartilage were strongly positive (figure 18C). Type I pneumocytes showed weak to moderate cytoplasmic and membranous positivity (figure 18B). NSCLC tissue showed weak to strong cytoplasmic and/or membranous expression of filamin A with marked intratumoral variability. Nuclear staining was present only in 6 cases. Tumor stroma was always moderately or strongly positive (figure 18D) and was served as internal positive control for filamin A negative tumors. Filamin A expression was relatively high in the peripheral parts of tumor tissue, compared to central parts of solid tumors (figure 18E, F). There was no significant difference in filamin A expression between different histological subtypes of NSCLC (figure 20A). Some typical staining patterns are given in figure 18 (G-I). IgG controls and matched NSCLC tissue staining are given in figure 19.

The comparison of filamin A expression in different clinicopathological groups showed that, the highest expression of filamin A is characteristic for grade 2 tumors, followed by grade 3 and grade 1 tumors ($p<0.05$, figure 20 B). Filamin A expression was positively associated with tumor size and invasion (T), particularly it was significantly higher in T3-4 tumors, compared to T1-2 tumors ($p<0.01$, figure 20C). Also, filamin A expression was higher in patients with lymph node (N) and distant metastases (M) ($p<0.05$, figure 20E,F). Overall, filamin A cytoplasmic and membranous expression was significantly higher in stage IV disease, followed by stage III and stage I-II ($p<0.01$, figure 20D). Spearman's rank test also showed a significant positive correlation between filamin A expression and NSCLC stage ($r=0.249$; $P<0.05$), lymph node metastases (N) ($r=0.205$; $P<0.05$) and distant metastases (M) ($r=0.332$; $P<0.01$).

We also found significant positive correlations between the expression of filamin A protein and p53 ($r=0.445$, $p<0.01$), Sphingosine kinase-1 ($r=0.415$, $p<0.0001$) and Sphingosine-1 phosphate lyase ($r=0.337$, $p<0.01$). There was no significant association between filamin A and BRCA1. Overexpression of RAD51 was more frequently seen in patients with filamin A >90 (35% vs. 5.6%). However, this difference was not statistically significant.

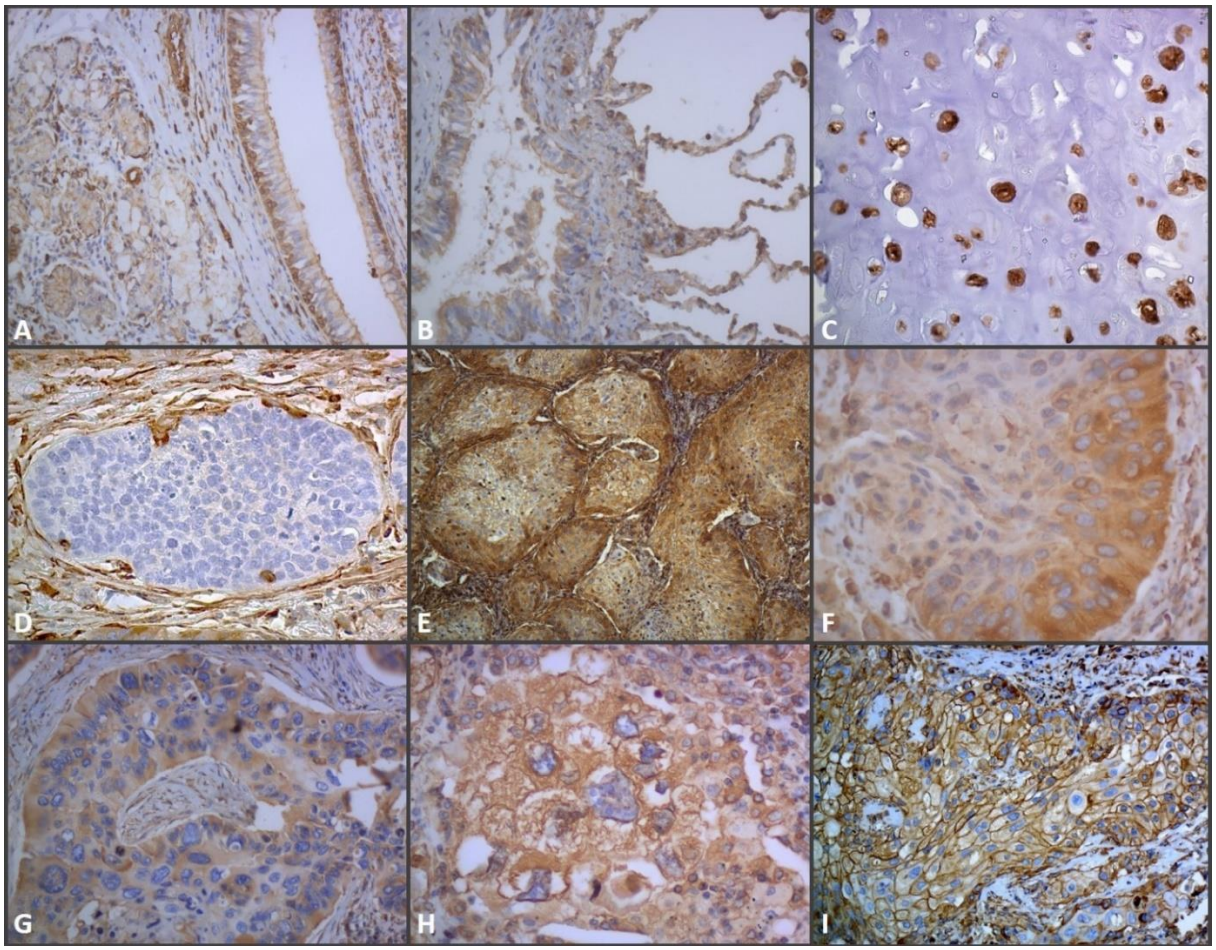


Figure 18. Filamin A protein expression in NSCLC and lung tissue surrounding the tumor. *(**A**) weak to moderate staining of filamin A in the apical parts of bronchial epithelial cells and intensive staining in the attachment to basal membrane, weak membranous staining in Submucosal glands (x200); (**B**) weak to moderate cytoplasmic and membranous expression in type I pneumocytes (x200); (**C**) strong expression in bronchiolar cartilage (x400); (**D**) moderate to strong filamin A expression in tumor stroma, which can be served as inner positive control for filamin A negative tumors (x200); (**E,F**) the increased expression of filamin A protein in the peripheral parts of tumor tissue compared to the center of the tumor in SCC (x200, x400); (**G**) moderate cytoplasmic expression of filamin A in ADC (x200); (**F**) strong membranous and cytoplasmic expression of filamin in LCC (x400); (**I**) strong membranous expression of filamin A in SCC (x200). (*ADC- Adenocarcinoma, LCC – Large Cell Carcinoma SCC – Squamous Cell Carcinoma)*

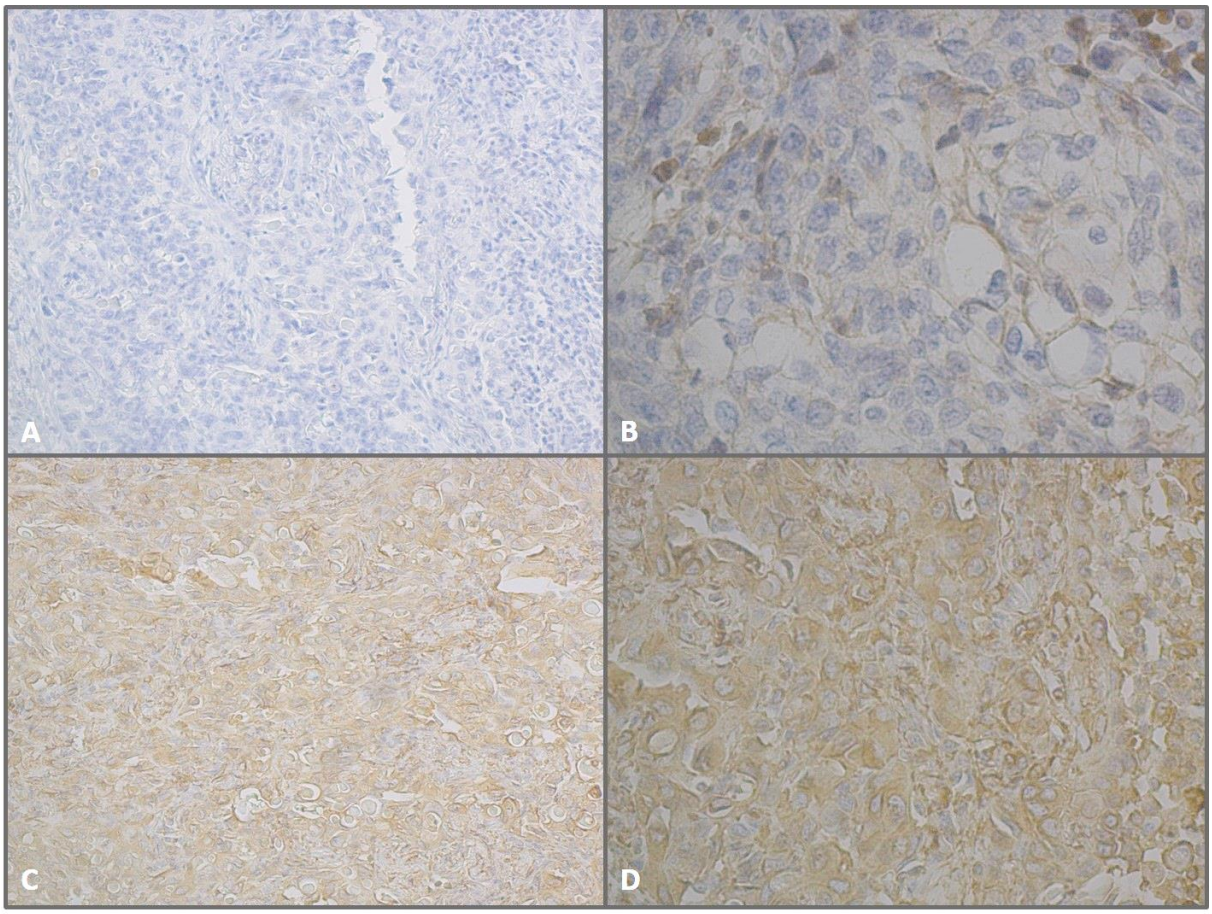


Figure 19. IgG control and filamin A staining of matched NSCLC tissue samples. *(A) IgG control staining shows no reactivity (x200), whilst (C) the same tissue sample is positive for filamin A (x200); (B) IgG control staining shows some background staining (x400), clearly distinguishable from (D) specific filamin A positivity in the matched NSCLC tissue (x400).*

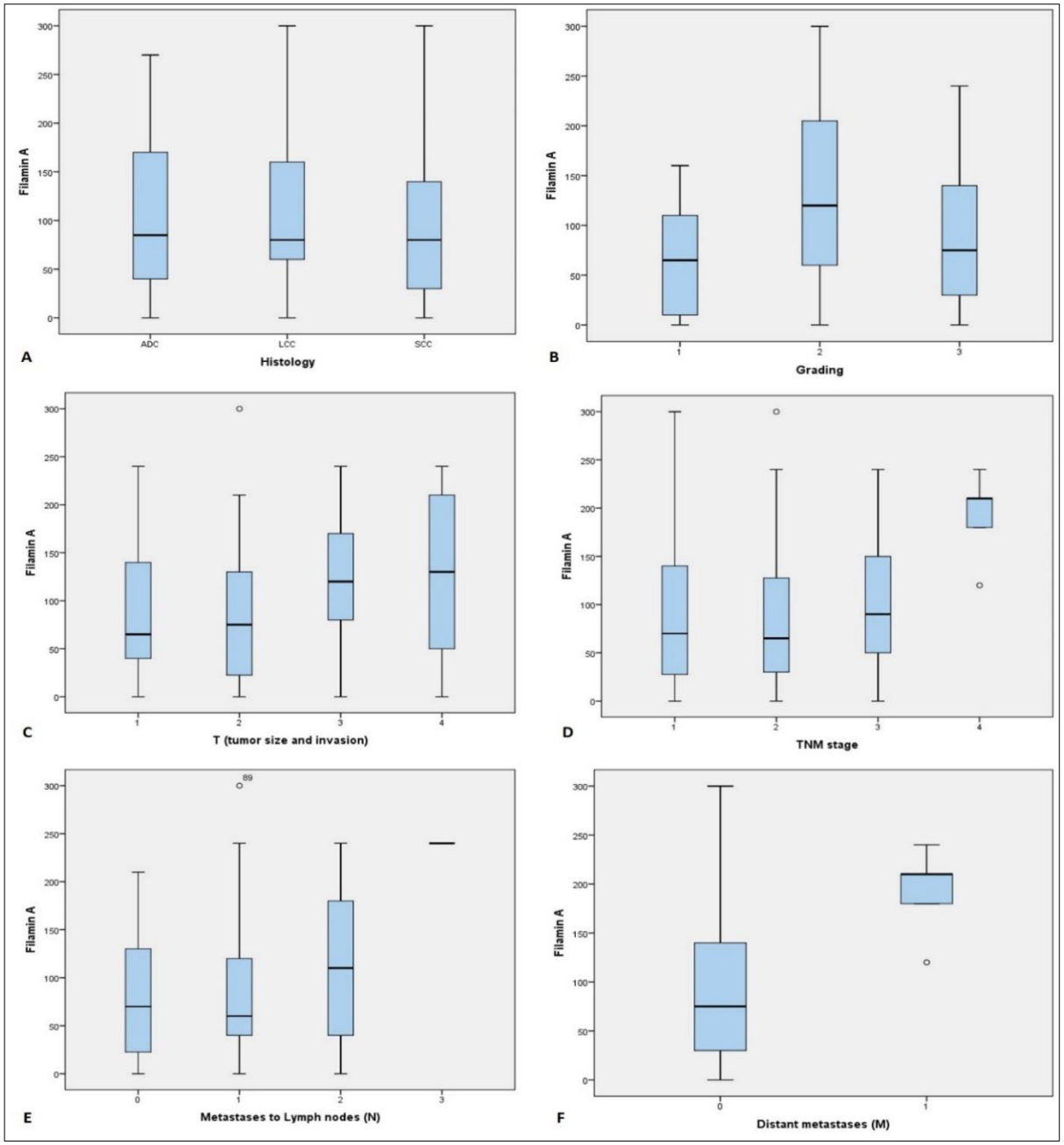


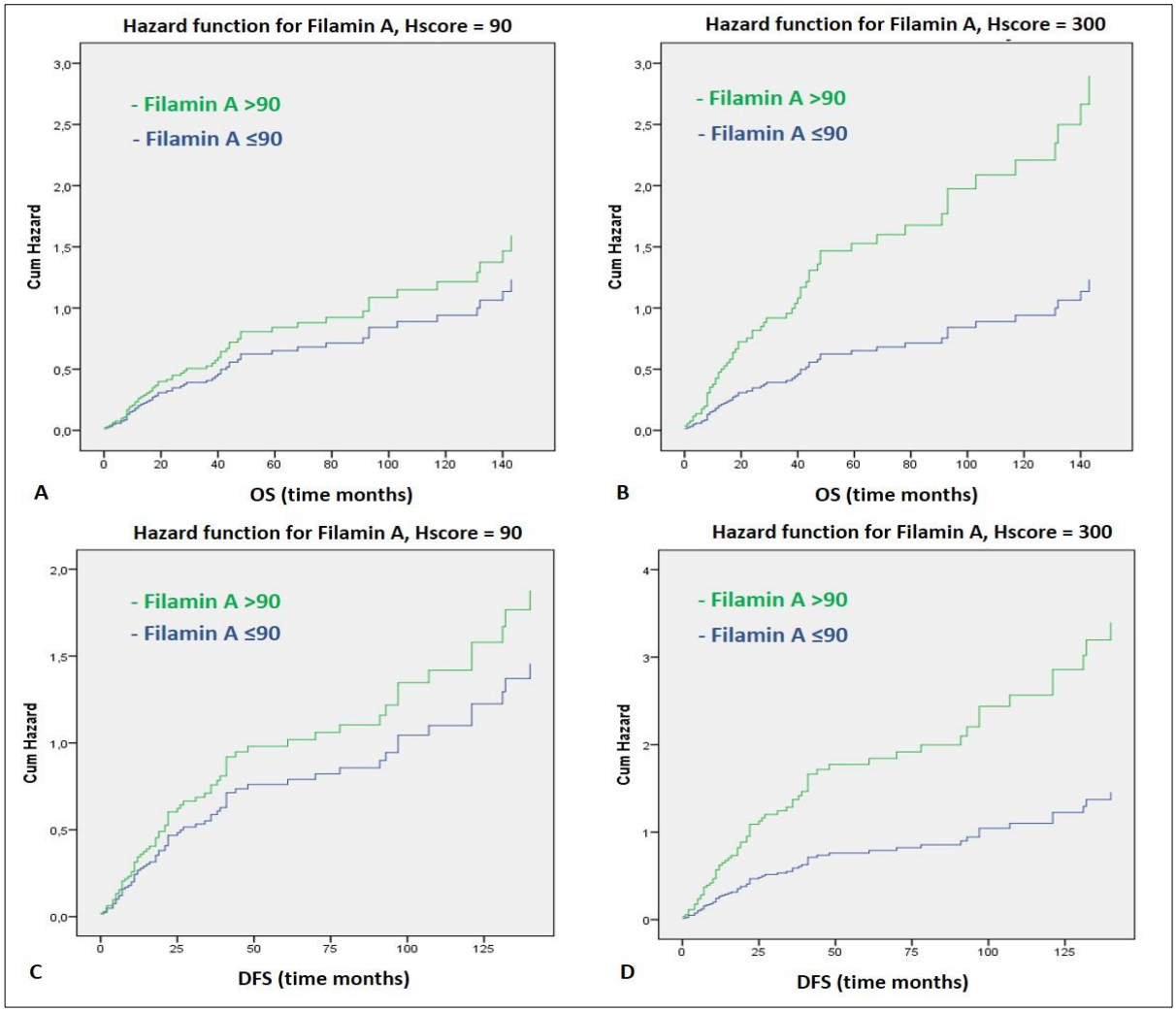
Figure 20. Distribution of filamin A protein expression in different histological and clinicopathological groups. A. *There is no difference in filamin A expression, between different histological subtypes (ADC – Adenocarcinoma, LCC – Large Cell Carcinoma, SCC – Squamous Cell Carcinoma),* ***B.*** *The highest expression of filamin A is present in grade 2 tumors,* ***C.*** *Filamin A expression is markedly higher in T3-4 tumors compared to T1-2,* ***D.*** *The highest expression of filamin A is seen in stage IV NSCLC, followed by stage III and stage I-II,* ***E.*** *Filamin A expression is higher in NSCLC patients with extensive metastatic spread (LN-lymph nodes) and* ***F.*** *with the presence of distant metastases.*

Based on univariate Cox proportional hazards regression model, patients within our cohort were divided into low and high risk groups according to filamin A expression, <=90 and >90 respectively. Particularly, the risk of tumor recurrence was significantly higher in patients with high (>90) filamin A expression, compared to patients with low (<=90) expression, despite the treatment (HR=1.003 95%CI[1.000:1.005], P=0.02, graph 7C,D). Risk of death was also higher in patients with >90 filamin A expression (HR=1.003 95%CI [1.000:1.005], P=0.07, graph 7A,B).

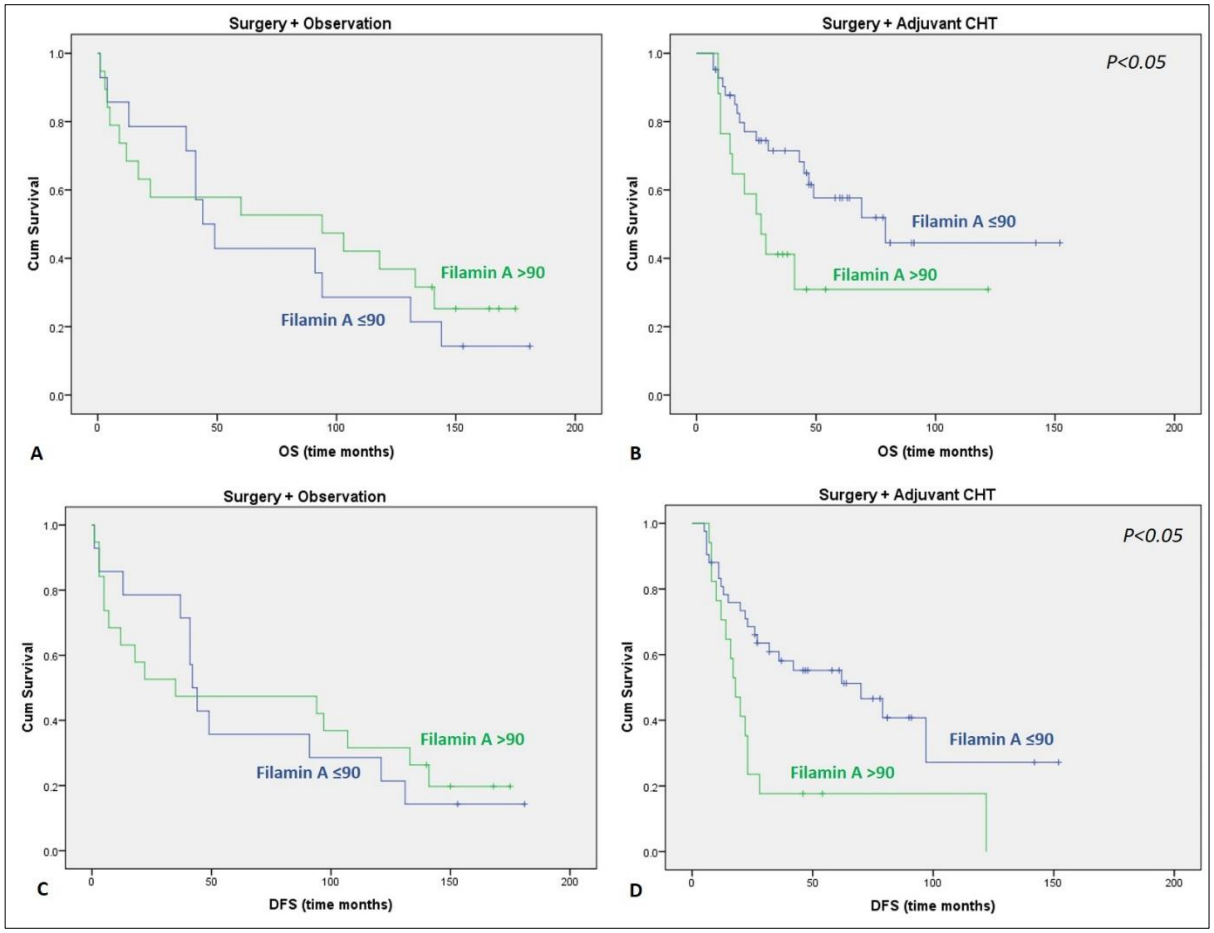
Multivariate Cox regression analysis showed that increased expression of filamin A (>90), represented an additional risk factor for NSCLC recurrence (HR=1.723, 95%CI [1.021:2.909], P<0.05), together with disease stage, tumor size, and the presence of lymph node metastasis. Cox regression analysis of survival in different treatment groups showed that the increased expression of filamin A (>90) in patients, treated with platinum-based combination chemotherapy was significantly associated with shorter OS (HR=1.005, 95%CI [1.000;1.010], P=0.037), as well as with shorter DFS (HR=1.004, 95%CI [1.001:1.008], P=0.017), whilst such association was not seen in patients treated with only surgical operation. The results of survival analysis plotted on Kaplan-Meier curves are shown on graph 8. The quantitative distribution of filamin A expression in different clinicopathological groups is given in table 10.

Table 10. Distribution of Filamin A expression in different clinicopathological groups.

		FILAMIN A HISTOSCORE		TOTAL
		<=90	>90	
EVENT OF DFS	0	22	6	28
	1	35	36	71
EVENT OF OS	0	27	11	38
	1	30	31	61
GRADE	1	8	4	10
	2	13	16	27
	3	37	21	58
ADJUVANT CHT	0	15	25	40
	1	42	17	59
TNM STAGE	1	25	14	39
	2	16	8	24
	3	16	14	30
	4	0	6	6
T	0	2	6	8
	1	8	6	14
	2	38	18	56
	3	6	9	15
	4	3	3	6
N	0	29	19	48
	1	16	9	25
	2	12	13	25
	3	0	1	1
M	0	92	0	92
	1	0	7	7
HISTOLOGY	ADC	19	17	36
	LCC	7	5	12
	SCC	32	19	51



Graph 7. Cox regression hazard function and Kaplan-Meier survival curves in relation to patient death and NSCLC relapse, calculated in months as overall (OS) and disease free survivals (DFS) respectively. (A) *there is a slight difference in the hazard of patient death between lower levels of filamin A expression (histoscore <90 and histoscore =90), whilst* ***(B)*** *the hazard of death is significantly increased in patients with high levels of filamin A (histoscore = 300), compared to patients with lower levels of filamin A (histoscore<90);* ***(C)*** *the hazard of NSCLC relapse is slightly different at lower levels of filamin A expression (histoscore <90 and histoscore =90), whilst* ***(D)*** *there is marked increase in the risk at higher expression (histoscore = 300), compared to lower expression (<90) of filamin A (p<0.05).*



Graph 8. Kaplan-Meier survival curves in relation to filamin A expression and overall and disease free survivals (OS and DFS respectively) in different treatment groups. *graphs* ***A*** *and C show that there is no difference in OS and DFS, based on filamin A expression in patients treated with only surgical operation; graphs* ***B*** *and* ***C*** *show that OS and DFS in patients with >90 filamin A expression is significantly lower compared to patients with <=90 filamin A expression (p<0.05).*

4.4. The prognostic role of sphingosine kinase-1 and S1P lyase protein expression in patients with non-small-cell lung cancer

Study included 120 archival formalin-fixed, paraffin-embedded (FFPE) tissue samples from NSCLC patients, from which 69 patients had received adjuvant chemotherapy (aCHT), mostly based on a combination of platinum with paclitaxel or navelbine. Detailed characteristics of patients are given in Table 11.

Table 11. Patient and tumor characteristics included in the study of sphingolipid metabolism pathway enzymes.

CHARACTERISTICS	**SUBGROUPS**	**N**	**%**
SEX	MALE	90	75
	FEMALE	30	25
AGE	≤60	53	44.2
	>60	67	55.8
HISTOLOGY	Adenocarcinoma	61	50.8
	Squamous cell carcinoma	45	37.5
	Large cell carcinoma + others	14	11.7
GRADE	G1	12	10
	G2	34	28.3
	G3/4	74	61.7
NODAL STATUS	NEGATIVE	46	38.3
	POSITIVE	63	52.5
	NOT SPECIFIED	11	9.2
DISTANT METASTASES	PRESENT	7	5.8
	NOT PRESENT	102	85
	NOT SPECIFIED	11	9.2
STAGE	I	49	40.8
	II	22	18.3
	III	41	34.2
	IV	8	6.7
ADJUVANT CHEMOTHERAPY	YES	68	56.7
	NO	41	34.2
	NOT SPECIFIED	11	9.2
SURVIVAL TIMES			
MEDIAN OS	***36 months***		
MEAN OS	***63.3 (range 1-164) months***		
MEDIAN DFS	***27 months***		
MEAN DFS	***74.3 (range 1- 153) months***		

Immunohistochemical staining of normal adjacent lung tissue and the distribution of SphK1 and S1P lyase were examined in several formalin-fixed paraffin-embedded tissue sections from tumor-free regions of the lung. Normal pseudo-stratified columnar epithelial cells

in bronchiole stained very intensely on the apical surface at the point of ciliary attachment for SphK1 (figure 21A). Figure 21B shows staining of SphK1 in bronchiolar cartilage. Interestingly, SphK1 levels appear to be related to chondrocyte maturation. Although immature chondrocytes showed moderate staining for SphK1, mature chondrocytes were devoid of staining. Alveolar parenchyma and type II pneumocytes exhibited a weak staining for SphK1. Weak to moderate staining of S1P lyase was shown in normal pseudo-stratified columnar epithelial cells and type II pneumocytes (figure 22A,B).

NSCLC samples exhibited various immunostaining patterns for SphK1 (figure 21C,D, E) and S1P lyase (figure 22C,D,E). Staining for both markers were mainly cytoplasmic and membranous and varied from weak to strong. Nuclear positivity of SphK1 was also seen in some cases. Adenocarcinomas showed the strongest expression of S1P lyase (figure 23C), whilst the highest expression of SphK1 was seen in large cell carcinomas, followed by adenocarcinomas and squamous cell carcinomas (figure 23A). Staining for both markers was conspicuously absent in the surrounding stroma. The highest expression of SphK1 was also revealed in grade 2 tumors, followed by grade 3 and 1 (figure 23B). S1P lyase did not show any difference in expression between grades (not shown). IgG control staining is shown in figures 21F and 22F.

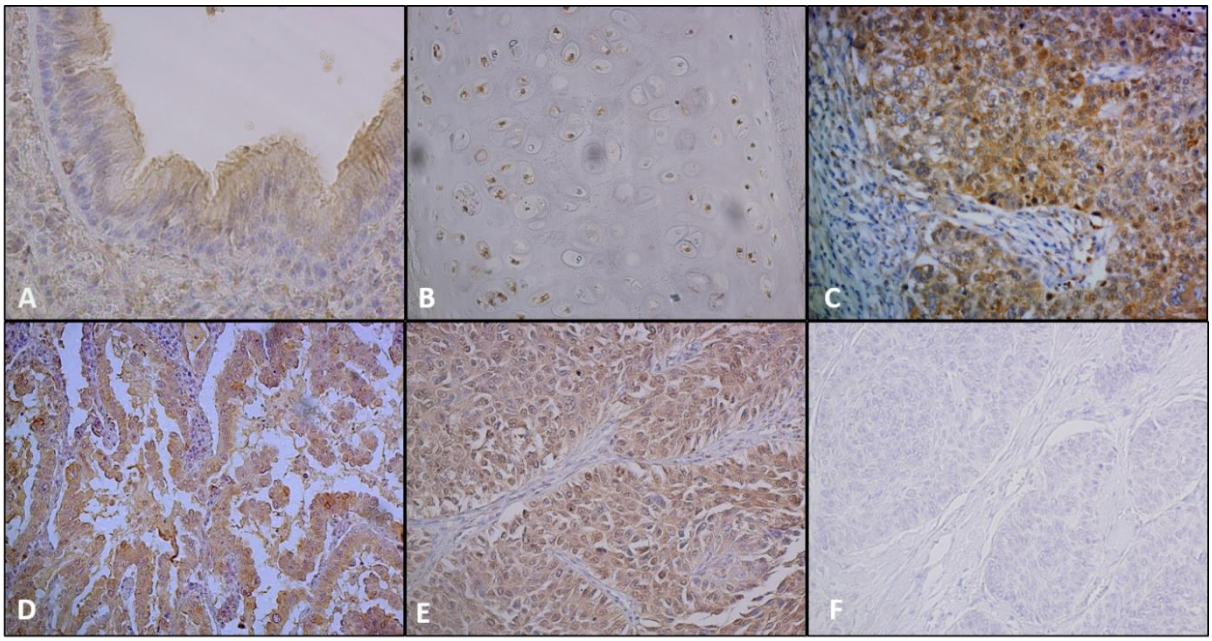


Figure 21. SphK1 staining in normal adjacent and NSCLC tissue. A. *SphK1 staining in normal pseudo-stratified columnar epithelial cells of bronchiole* ***B.*** *bronchiolar cartilage, IHC x200;* ***C.*** *SphK1 staining in large cell carcinoma of the lung,* ***D.*** *and adenocarcinoma of the lung,* ***E.*** *squamous cell carcinoma of the lung and* ***F.*** *matched IgG control, IHC x200.*

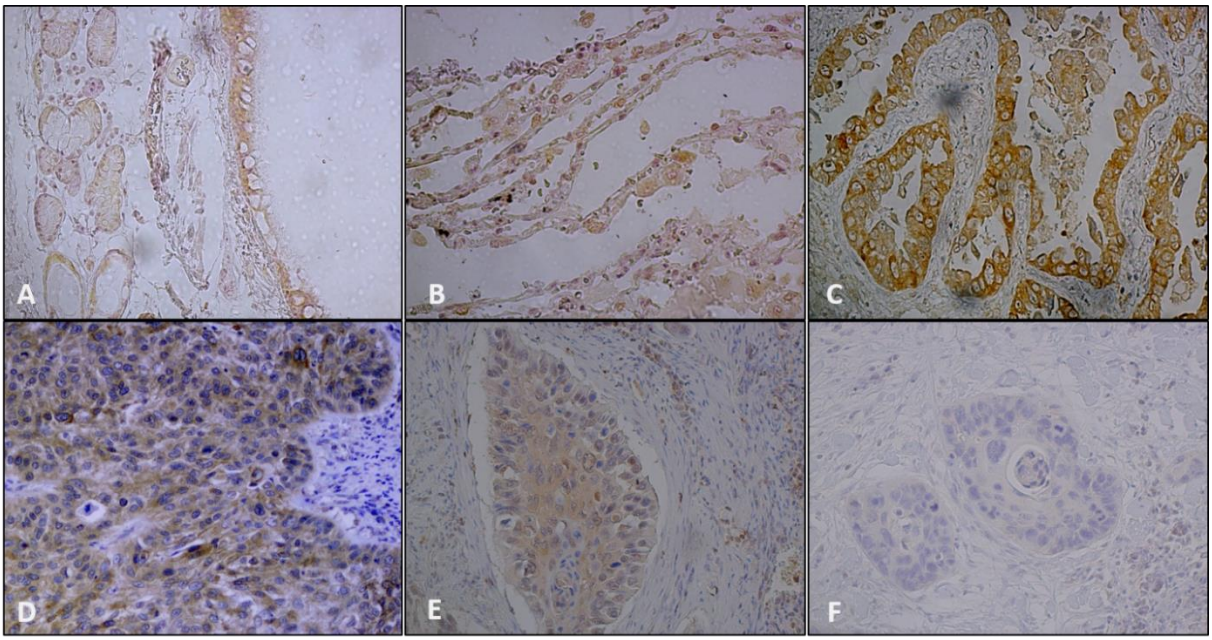


Figure 22. S1P lyase staining in normal adjacent and NSCLC tissue. *A. S1P lyase staining in normal pseudo-stratified columnar epithelial cells and B. type II pneumocytes, IHC x200; C. S1P lyase staining in adenocarcinoma of the lung, D,E. squamous cell carcinoma of the lung and F. matched IgG control, IHC x200*

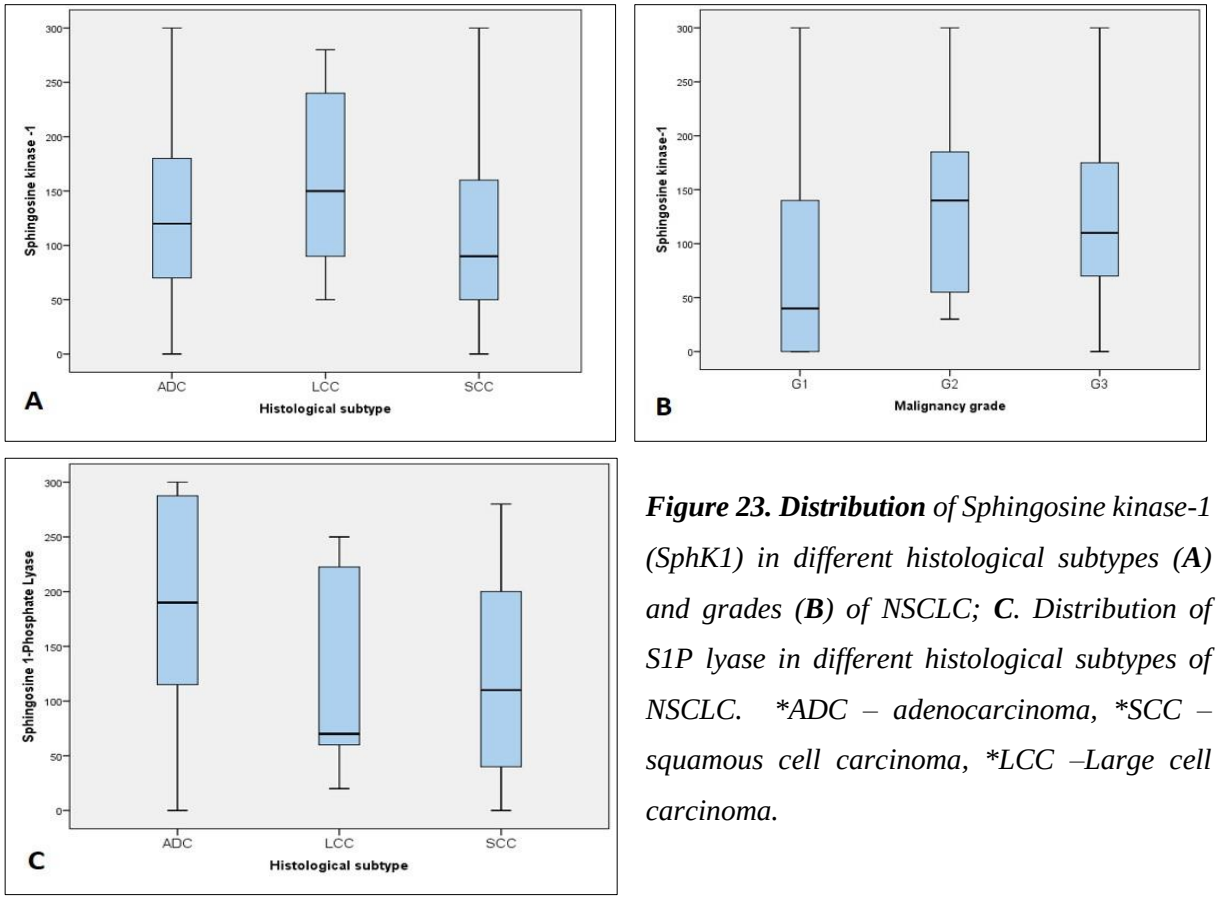
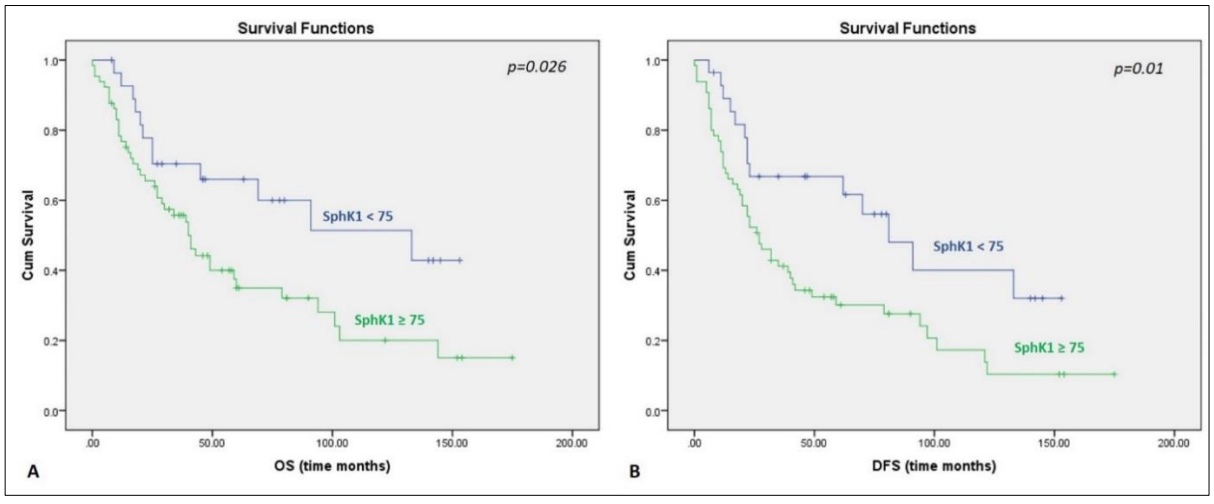


Figure 23. Distribution *of Sphingosine kinase-1 (SphK1) in different histological subtypes (**A**) and grades (**B**) of NSCLC; **C**. Distribution of S1P lyase in different histological subtypes of NSCLC. *ADC – adenocarcinoma, *SCC – squamous cell carcinoma, *LCC –Large cell carcinoma.*

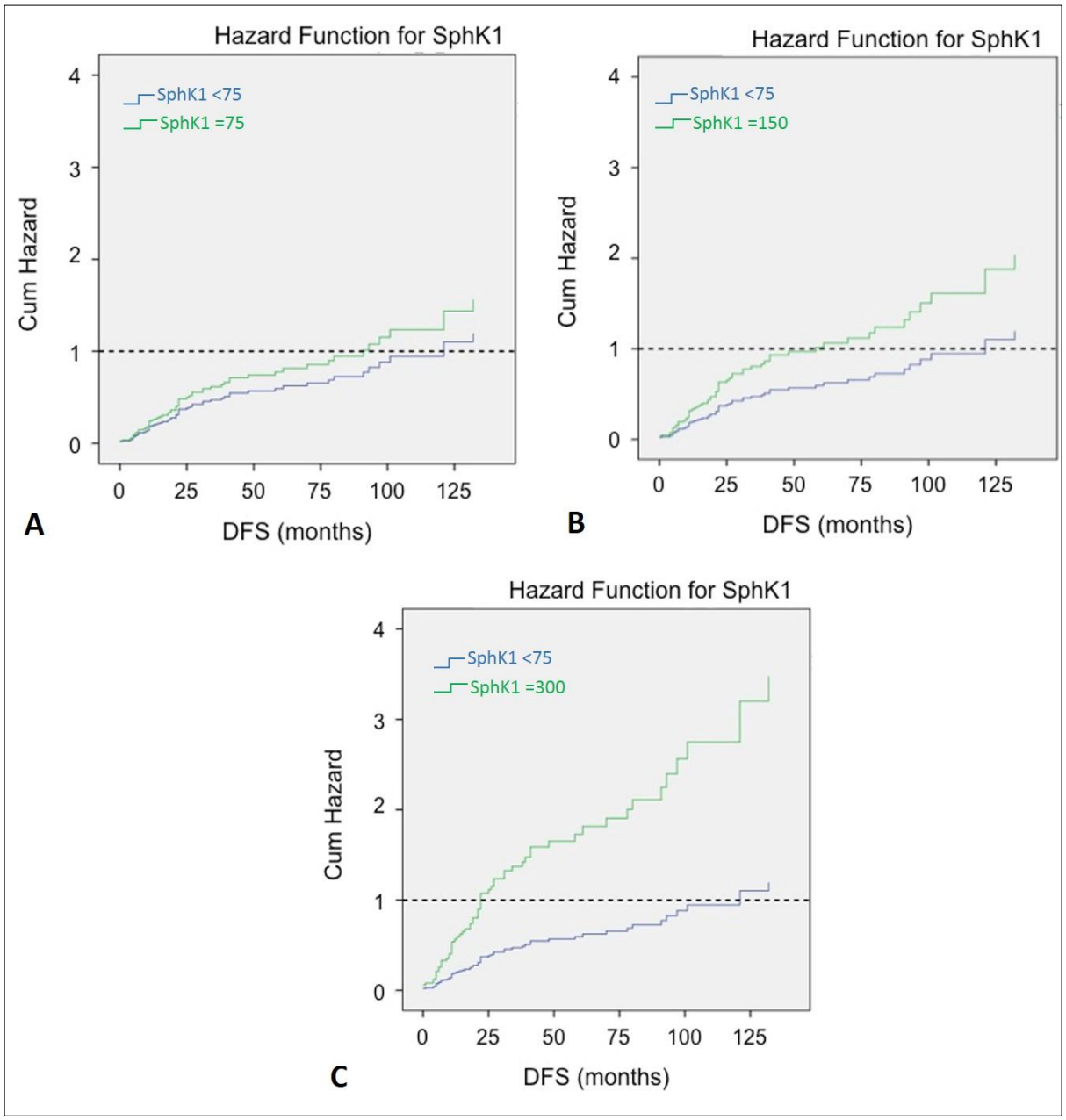
A significant positive correlation was seen between cytoplasmic SphK1 and cytoplasmic S1P lyase expression (r=0.375; p=0.004) and membranous SphK1 expression and membranous S1P lyase expression (r=0.469; p=0.001).

Based on Cox regression analysis patients were divided into low (Hscore<75) and high (Hscore≥75) risk groups according to SphK1 expression. Cross-tabulation of SphK1 expression and survival showed that the risk of relapse is higher in patients with increased SphK1 expression (histoscore ≥75), irrespective of the stage (Odds ratio = 3.333, overall accuracy = 68,8%, P=0.01). Univariate Cox regression model of survival also showed the increase of the risk of relapse in patients with SphK1 ≥75 expression (p=0.006, HR=1.004, 95%CI[1.001;1.006])(graph 11). Kaplan-Meier analysis of survival, using optimal cut off point (Hscore=75), also showed that overexpression of SphK1 protein is significantly associated with lower overall (p=0.026) and disease free (p=0.01) survival rates in all patients, irrespective of disease stage and treatment (graph 10). Multivariate survival analysis, using the Cox regression method also showed that SphK1 represents an independent risk factor for NSCLC recurrence, together with disease grade, stage, tumor size (T) and nodal status (N) (p=0.038, HR=2.048 95%CI [1.039;4.037]).

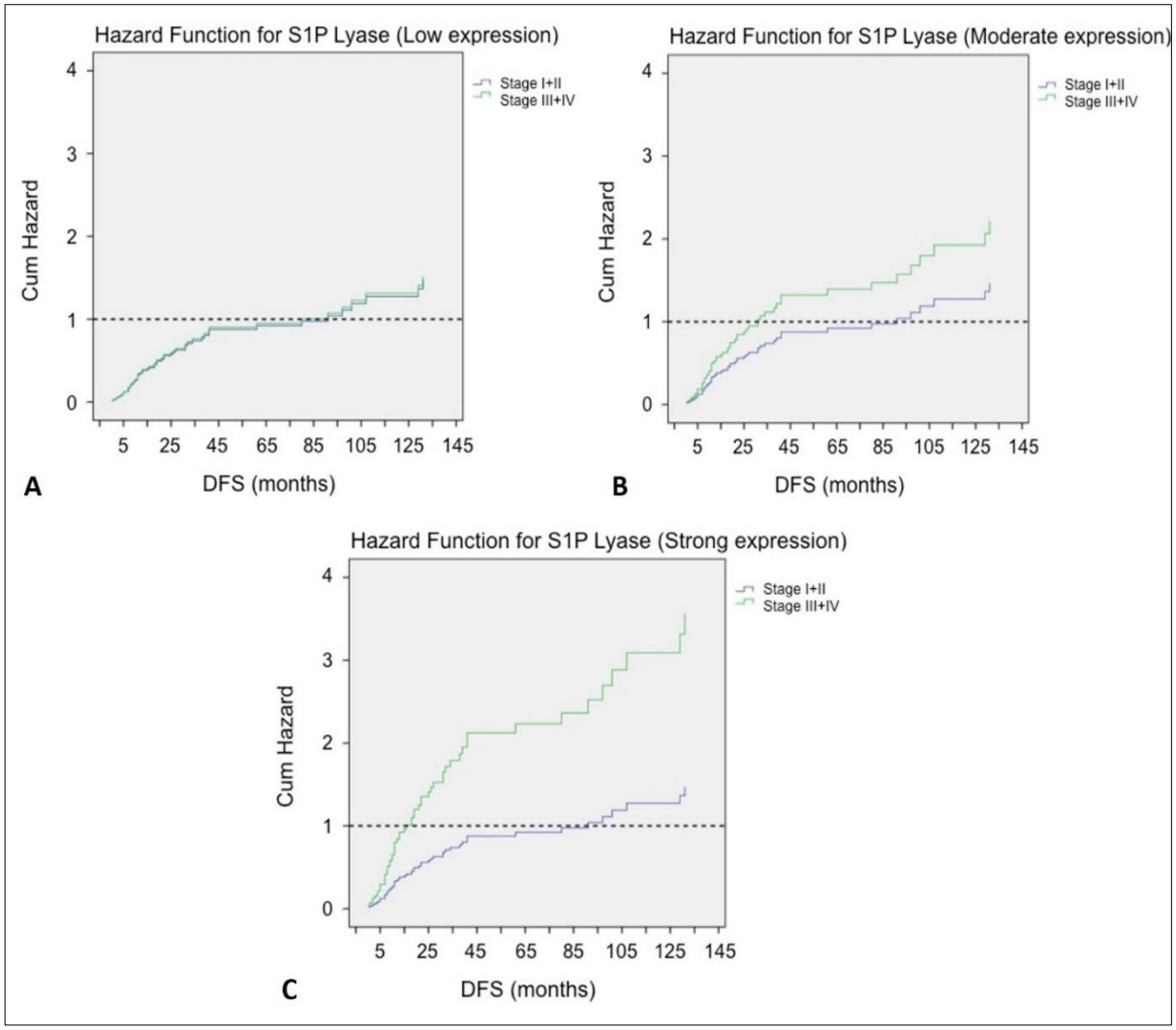
Cox regression analysis of survival also showed that increased cytoplasmic expression of S1P lyase was significantly related to the increased risk of NSCLC progression in patients with stage III-IV NSCLC (p=0.022). Graph 12 illustrates the gradual increase of the risk of NSCLC progression from low (<75) to moderate (75-150) and strong (>150) expression of S1P lyase.



Graph 9. Kaplan-Meier survival analysis. *Overexpression of SphK1 is significantly associated with lower overall (OS) (A) and disease free (DFS) (B) survival rates in NSCLC patients, irrespective of disease stage and treatment modality.*



Graph 10. Cox regression analysis in relation to NSCLC recurrence. A*, the risk of recurrence, shown as DFS time in months is slightly higher at SphK1 Hscore=75 expression level, compared to SphK1 Hscore<75;* ***B.*** *the risk of recurrence at SphK1 Hscore = 150 expression level is increased compared to SphK1 Hscore<75;* ***C****. the risk of recurrence at SphK1 Hscore = 300 expression level is significantly higher, compared to SphK1 Hscore<75.*



Graph 11. Cox regression analysis in relation to disease relapse in different stages of NSCLC. A. *the risk of relapse is similar in early (stage I-II) and advanced (stage III-IV) NSCLC patients with low levels of Sphingosine-1 phosphate lyase expression (Hscore<75); B. The risk of relapse in stage III-IV patients is increased with the increase Sphingosine-1 phosphate lyase expression (Hscore 75-150), whilst it remains the same in stage I-II patients; C. The risk of relapse in stage III-IV patients is significantly increased at high levels of Sphingosine-1 phosphate lyase expression (Hscore >150), whilst it remains the same in stage I-II patients.*

5. DISCUSSION

We analyzed retrospectively the possible prognostic and predictive value of the immunohistochemical expression of DNA repair and sphingolipid metabolism pathway proteins. In particular, two major DNA repair proteins BRCA1 and RAD51, and cytoskeletal protein filamin A which has recently emerged as one of the key players in the DNA damage response. We also examined the prognostic value of two major sphingolipid metabolism pathway proteins, Sphingosine kinase-1 and Sphingosine-1 phosphate lyase. To the best of your knowledge, this is the first immunohistochemical study of BRCA1 phospho protein in relationship to patient survival, the first to examine the potential predictive value of filamin A protein in NSCLC patients treated with carboplatin and navelbine, and the first to study the immunohistochemical expression and possible clinical implication of Sphingosine-1 phosphate lyase in patients with non-small-cell lung cancer.

5.1. Immunohistochemical expression of BRCA1 in relation to survival outcomes of non-small-cell lung cancer patients

Earlier reports found no association between BRCA1 immunohistochemical expression and NSCLC patient survival or other clinicopathological factors. In all these studies, the MS110 antibody was used for BRCA1 evaluation and our results are consistent with previous reports. The BRCA1 gene expands the 100kb region, produces 1863aa full length protein and several alternative splicing isoforms. The MS110 antibody recognizes the N-terminal 1-304 amino acids of protein. This is the only currently proven antibody for BRCA1 protein study, but these amino acid sequences are also retained in alternative forms of BRCA1. This might be one reason for the discrepancy between mRNA and protein study results in lung cancer patients. On the other hand mRNA expression alone does not reflect the presence of the functional protein. From a practical point of view, immunohistochemical detection BRCA1 protein is a more reliable and useful tool for precise evaluation of patient prognosis. To the best of our knowledge, no one has yet examined BRCA1 phospho S1524 expression in NCSLC patients. BRCA1 phosphorylation is an important means by which its cellular functions are regulated. BRCA1 undergoes specific phosphorylation by cell cycle checkpoint kinases in different serine residues, of which ser1524 is known to be specifically phosphorylated by ATM after radiation induced DNA damage (Yi Wang et al. 2000). Due to its various functions in DNA reparation, including nucleotide excision repair (NER) and

double-strand break (DSB) repair, BRCA1 is actively investigated as a resistance marker for cytotoxic chemotherapeutic drugs in lung cancer patients (Gowen et al. 1998; Abbott et al. 1999; Rafael Rosell et al. 2007). In our study, we evaluated phospho-BRCA1 expression in early stage, operable NSCLC cases. It is known that tumor expression of two other DNA-repair genes, RRM1 and ERCC1, signify a survival advantage for patients with early-stage NSCLC (Zheng et al. 2007) given only surgical treatment. In our study, phospho-BRCA1 expression was also significantly correlated with longer OS and DFS in stage I and II NSCLC patients. In advanced stage NSCLC, phospho-BRCA1 expression is associated with shorter OS and DFS. First we attributed this outcome to the fact that patients with advanced NSCLC were treated with adjuvant chemotherapy. However, the extended prognostic study of BRCA1 phospho S1524 expression, showed that the overexpression of BRCA1 phospho S1524 is negatively associated with survival outcomes in stage III patients, treated by surgical operation, without adjuvant chemotherapy, whilst the same has not been seen in patients treated with adjuvant carboplatin and navelbine. Interestingly we found significant positive correlation between the expression of BRCA1 phospho S1524 and patient age. Also, age adjusted multivariate Cox regression analysis did not find the independent predictive value of BRCA1 phospho S1524 expression. Overall, these data suggest that the detection of phosphorylated forms BRCA1, might better reflect the presence of the functional protein and it might be used as a prognostic marker for earlier stages of NSCLC patients. Our study also further emphasizes the complex nature of the BRCA1 protein. We can conclude that the increased phosphorylation of BRCA1 is an accompanying result of other malignant changes in advanced NSCLC and it doesn't represent an independent prognostic factor for this patients. More experimental studies are necessary to decipher the exact mechanisms of activation of BRCA1 at later stages of NSCLC, and that might lead to identification of more reliable prognostic markers. We did not find any relationship with the overexpression of BRCA1 phospho S1524 and survival in NSCLC patients treated with adjuvant chemotherapy, using the combination of carboplatin and navelbine. This might be explained by the complex nature of BRCA1 protein in mediating various chemotherapeutic response. BRCA1 overexpression is associated with increased resistance to platinating agents. However, on the contrary it increases the sensitivity to microtubule poisoning. For further validation of the reliability of this marker, prospective study with customizing chemotherapy or retrospective study including patients with single treatment regimen is necessary.

5.2. Immunohistochemical expression of RAD51 in relation to survival outcomes of non-small-cell lung cancer patients

RAD51 is a key regulator of the homologous recombination pathway for DNA double-strand break repair. High-level expression of RAD51 has been seen in a variety of human malignancies. One study showed overexpression of RAD51 in 29.4% NSCLC cases. High-level RAD51 expression was associated with significantly shorter median survival time of 19 vs 68 months ($p<0.0001$) (Qiao et al. 2005). Also, RAD51 expression was associated with TNM-stage of NSCLC (at the time of diagnosis) in the same study. In the present study, we found RAD51 positivity in 60.5% of cases, and overexpression of RAD51 was significantly associated with higher overall survival rates in both treatment groups, patients with only surgical treatment and those with adjuvant chemotherapy. However, median survival was significantly lower in RAD51-positive patients receiving adjuvant carboplatin and navelbine. Particularly, median overall survival in patients receiving surgery without adjuvant chemotherapy was 22 and 71 months in the subgroups harboring lower and high RAD51 immunoexpression, respectively. In comparison, median overall survival was 30 in 38 months in patients exhibiting low versus high RAD51 immunoexpression, respectively. In patients receiving neoadjuvant platinum-based chemotherapy, overexpression of RAD51 was associated with shorter overall survival, but this was not statistically significant, potentially as a result of the small patient number. High rates of RAD51 expression has have been described in pancreatic (H Maacke et al. 2000) and invasive breast tumors (Heiko Maacke et al. 2000), where it is related to higher grades of disease and poor clinical outcome. On the contrary, Söderlund et al. found that low expression of BRCA1/BRCA2/RAD51 complex is an independent poor prognostic factor for patients with early-stage breast cancer, but is a favorable predictive biomarker for clinical outcome following radiotherapy, in line with the findings of the present study (Söderlund et al. 2007). Yu at al. showed that depletion of endogenous RAD51 is enough to inhibit the growth of A549 lung cancer cells in vitro (Yu et al. 2015). The literature is full of contradictory finding with regard to the prognostic role of RAD51 expression in different tumors. Overall, data suggest that high RAD51 is protective, at least in early-stage malignancies. Based on our and other studies, we can suggest that more in vitro studies are necessary to identify the exact regulation mechanisms of RAD51 in NSCLC and it can be used as one of the useful prognostic markers, in combination with other DNA repair pathway proteins.

5.3. Association of filamin-A protein expression with aggressive phenotype and poor survival outcomes in NSCLC patients treated with platinum-based combination chemotherapy

We found that filamin A expression is characterized by marked inter and intratumoral variability in NSCLC. The strongest expression was observed in the peripheral parts of solid tumors and the expression of filamin A significantly correlated with primary tumor size and, the presence of lymph node and distant metastases. These findings suggest an important role of filamin A in NSCLC growth, invasion and metastases. Evidence for the role of filamin A in tumor progression is controversial in published literature. Some studies report that inhibition of filamin-A reduces cancer metastatic potential, whilst others show the opposite (Yue, Huhn, and Shen 2013). Our finding is supported by two previous experimental studies. It has been shown that filamin A protein is upregulated in A549 cells with high migratory potential (Keshamouni et al. 2006). Also, the targeting of filamin A results in significant decrease in size and invasive potential of lung cancer in mice (Jiang et al. 2012). In clinical studies, a positive relationship between overexpression of filamin-A protein and advanced stage, lymph node metastasis and vascular or neural invasion has been seen in breast and colorectal cancer patients (Tian et al. 2013) whilst the opposite was shown in nasopharyngeal cancer (Sun et al. 2013). This might be explained by the individual nature of each tumor.

Our second finding that filamin A protein represents an additional prognostic factor in NSCLC patients is supported by the multivariate Cox regression analysis, which showed that the overexpression of filamin A (>90) is a negative prognostic factor together with tumor size, disease stage and nodal status (HR=1.723, 95%CI [1.021:2.909], P<0.05). Similar findings were seen in colorectal cancer patients where increased immunohistochemical expression of filamin A represented an independent prognostic factor together with lymph node metastases and depth of tumor invasion (HR=3.856, 95%CI [7.326:19. 421], P<0.001). There has been only one study of filamin A protein expression in lung cancer patients, including small cell lung cancer (SCLC). This study suggested the possible role of filamin A in angiogenesis (Uramoto, Akyurek, and Hanagiri 2010). Similar to our study results these authors found that the overall 5-year survival rate for patients with positive and those with negative filamin A expression was 43.7% and 54.9%, respectively (p=0.06). However, univariate and multivariate survival analyses showed no relation with filamin A expression. This difference might be explained by the comlexity of their study group and different evaluation method.

In our study - we stratified NSCLC patients into two treatment groups – those with and those without chemotherapy. Interestingly, there was a significant relationship between

overexpression of filamin A and worst disease free and overall survival outcomes ((HR=1.004, 95%CI [1.001:1.008], P=0.017) and (HR=1.005, 95%CI [1.000;1.010], P=0.037)) in patients treated with carboplatin and navelbine, whilst such a relationship was not found for patients who underwent surgical treatment only. This finding further emphasizes the idea that filamin A plays a complex role not only in tumor growth and progression, but it also modulates the chemotherapy response, particularly in patients treated with platinum-based chemotherapy. To the best of our knowledge, this is the first clinical study to examine the relationship between filamin A expression and patient survival in two different treatment groups. However, the results are in accord with findings that suggest an important role of filamin A protein in DNA repair and resistance to cytotoxic drugs, including cisplatin (Velkova et al. 2010; Yuan and Shen 2001; Yue et al. 2012). Based on experimental study results on melanoma cell lines and mouse models, Yue et all. (2012) showed that not only endogenous levels filamin A reflect the chemosensitivity of tumor cells and it might be used as a predictive marker, but also filamin A might be used as a therapeutic target to sensitize cells to DNA damaging chemotherapy (Yue et al. 2012). Our study also supports this finding. Moreover, although not statistically significant, we found positive association between the overexpression of filamin A (>90) and RAD51 positivity. However, more *in vitro* and *in vivo* experiments on NSCLC are necessary.

We also found a significant positive correlation between filamin A and other prognostically important proteins. Particularly p53, SphK1 and SPL. In accord to our finding Maceyka et al. (2008) showed that SphK1 is required for filamin A-dependent cell migration in vitro (Maceika et al. 2008). This findings further emphasize the important role of filamin A in the regulation of major cellular pathways, including DNA repair. Moreover, compared to BRCA1, RAD51, SphK1 and SPL, filamin A showed the highest statistically significant association with aggressive features of NSCLC and the negative predictive value for platinum-based treatment outcome.

In conclusion, our study results suggest that filamin A expression may represent an important prognostic marker for NSCLC progression and might help to predict platinum-based treatment response.

5.4. The prognostic role of sphingosine kinase-1 and S1P lyase protein expression in patients with non-small-cell lung cancer

We investigated the prognostic and predictive value of SphK1 and S1P lyase, two key enzymes that control S1P content in cells, in patients with NSCLC treated with adjuvant chemotherapy based on carboplatin and navelbine. NSCLC samples exhibited various immunostaining patterns for both SphK1 and S1P lyase. SphK1 staining was mainly cytosolic and membranous and varied from weak to strong. The highest expression of SphK1 has been seen in large cell carcinomas, followed by adenocarcinomas and squamous cell carcinomas. These observations are in line with the findings of Johnson et al., who originally evaluated the expression of SphK1 in normal and cancerous lung tissue(Johnson KR1, Johnson KY, Crellin HG, Ogretmen B, Boylan AM, Harley RA 2005). Nuclear positivity of SphK1 has rarely been observed. However the biological significance of this expression is not known.

Concerning expression of S1P lyase, the highest expression was unexpectedly observed in adenocarcinomas. In their pioneering work in prostate cancer, Brizuela et al. reported the decrease in S1P lyase enzymatic activity and expression in tumor samples compared to normal adjacent tissues (Brizuela et al. 2012). Importantly, S1P lyase expression and activity were inversely correlated with those of SphK1 implying that the overall increased S1P level commonly observed in cancer would not merely reflect higher SphK1 activity, but could also be a consequence of loss of S1P lyase expression (Brizuela et al. 2012). Thus, the hypothesis that an imbalance in the SphK1/S1P lyase system could play a crucial role in cancer by increasing the cellular levels of S1P, a lipid metabolite involved in cell proliferation and resistance to stresses or therapeutics, was put forward. Interestingly, in line with this assumption, an opposite relationship (low SphK1 and high S1P lyase) was observed in Alzheimer's disease notably characterized by neuronal apoptosis, where S1P is believed to play a critical role as a survival factor for neurons (Edsall et al. 2001; Gomez-Brouchet A, Pchejetski D, Brizuela L, Garcia V, Altié MF, Maddelein ML, Delisle MB 2007; Ceccom et al. 2014). Herein, no inverse correlation between SphK1 expression and S1P lyase expression was observed in NSCLC in contrast to the prostate cancer findings (Brizuela et al. 2012), which might be explained by differences in the nature of these two tumors. In our specimens, S1P lyase staining was variously distributed from negative to strong and did not show a grade dependent pattern in contrast to prostate cancer (Brizuela et al. 2012).

A single study examined the prognostic and predictive role of SphK1 in NSCLC. In 2011, Song et al. observed that immunohistochemical expression of SphK1 was markedly increased in NSCLC, correlating with clinical stage, T classification, N classification and M

classification(L. Song et al. 2011). Importantly, overall survival of patients with high SphK1 expression was found to be shorter than patients with low SphK1 expression (L. Song et al. 2011). In line with these findings, we show here that high SphK1 expression is related to increased risk of disease relapse in patients with NSCLC treated with adjuvant chemotherapy. Although we did not find a statistically significant direct correlation between SphK1 expression and clinical stage, the multivariate Cox regression analysis clearly showed that SphK1 represents an important additional risk factor for NSCLC relapse together with clinical stage, T classification and N classification (p=0.038, HR=2.048 95%CI [1.039;4.037]). The differences between these two studies might be explained with the difference in study groups. For instance, in the study of Song et al., no information about treatment is available and multivariate analysis was not conducted (L. Song et al. 2011). Moreover, we found a positive correlation between cytoplasmic SphK1 and cytoplasmic S1P lyase expression (r=0.375; p=0.004); and between membranous SphK1 and membranous S1P lyase expression (r=0.469; p=0.001). Noteworthy, Cox regression analysis showed that increased S1P lyase cytoplasmic expression clearly represents a determining factor of the risk of relapse at advanced stages of NSCLC.

In conclusion, this study is the first to examine the immunohistochemical expression of both S1P lyase and SphK1 in NSCLC in relationship to survival in patients treated with adjuvant chemotherapy. Our data suggest that S1P lyase quantification might represent a useful companion marker for NSCLC patients together with SphK1. Further investigations are requested to evaluate the role of S1P lyase in cell culture models in order to provide further evidence of this correlation in lung cancer.

6. SUMMARY

Resistance to chemotherapy remains a major impediment to successful outcome for many types of solid tumors, including non-small-cell lung cancer which is the leading cause of cancer related mortality worldwide. Recent technological developments have significantly advanced understanding of the mechanisms of tumor progression and drug resistance. However, the translation of basic research findings into clinical practice remains a challenge.

The objective of this thesis was to investigate the prognostic and predictive value of the immunohistochemical expression of selected potential molecular markers in patients with resected NSCLC. This included proteins related to DNA damage repair and sphingolipid metabolism pathways.

The study included patients divided into three different treatment groups: (1) patients who had undergone surgery without any chemotherapeutical intervention, (2) patients treated with adjuvant chemotherapy using Carboplatin and Navelbine, (3) patients treated with neoadjuvant chemotherapy using Carboplatin and Navelbine.

Immunohistochemical expression of BRCA1 was analyzed using different antibodies. Of the five tested antibodies, only BRCA1 phospho S1524 appeared to reliably detect the functional BRCA1 protein. Univariate Cox regression analysis of survival showed that BRCA1 phospho S1524 positivity was significantly associated with lower risk of death and relapse in stage I-II NSCLC patients treated only surgically, while in stage III patients, BRCA1 phospho S1524 positivity was significantly associated with higher risk of death and disease recurrence in the same treatment group. This finding appears to be dependent on other unknown underlying factors.

Interestingly, the overexpression of RAD51 by immunohistochemistry was also related to longer overall and disease free survival outcomes in two treatment groups of patients: those treated either by surgical operation only or adjuvant chemotherapy. However, the comparison of survival rates in patients with RAD51 overexpression showed that median survival was lower in patients receiving adjuvant Carboplatin and Navelbine. In patients receiving neoadjuvant chemotherapy, overexpression of RAD51 was associated with shorter overall survival but this finding was not statistically significant.

Filamin A protein expression was characterized by marked inter and intra tumoral variability in NSCLC. The strongest expression was observed in the peripheral parts of solid tumors and the expression of filamin A significantly correlated with primary tumor size, the presence of lymph node and distant metastases. Univariate Cox regression analysis of survival

showed that overexpression of filamin A was significantly associated with poor overall and disease free survival in patients treated with adjuvant Carboplatin and Navelbine. The age adjusted multivariate Cox regression analysis also showed an independent prognostic value of filamin A protein, in addition to primary tumor size, nodal status and overall disease stage. There was also a significant positive correlation between filamin A and other prognostically significant proteins, in particular, p53, SphK1 and SPL. RAD51 expression was also higher in patients with filamin A overexpression than those with lower levels of filamin A.

High levels of SphK1 expression was related to lower overall survival rate and increased risk of disease relapse in patients with NSCLC treated with adjuvant chemotherapy. Although we found no direct statistically significant correlation between SphK1 expression and clinical stage, the multivariate Cox regression analysis clearly showed that SphK1 represents an important additional risk factor for NSCLC relapse together with clinical stage, T classification and N classification. Noteworthy is that the Cox regression analysis showed that increased S1P lyase cytoplasmic expression clearly represents a determining factor of the risk of relapse at advanced stages of NSCLC. These data suggest that S1P lyase quantification may be a useful companion marker for NSCLC patients together with SphK1.

Overall, the data suggest that overexpression of BRCA1 and RAD51 is protective at least in earlier stages of malignancy. However, their predictive role in NSCLC patients remains to be elucidated. Interestingly, cytoskeletal protein filamin A had the highest statistically significant independent prognostic and predictive value, as well as a relationship with other studied markers. These findings emphasize the important roles of filamin A in the regulation of major cellular pathways, including DNA damage repair and sphingolipid metabolism, and suggest that targeting cytoskeletal scaffolding proteins, and particularly filamin A might be a key to successful treatment in patients with NSCLC.

7. SOUHRN

Léková rezistence představuje závažnou překážku pro úspěšnou léčbu mnoha typů solidních nádorů, zahrnující nemalobuněčné karcinomy plic, které jsou jedním z hlavních důvodů celosvětové mortality na nádorová onemocnění. Současný vývoj technologií přispěl výrazně k pochopení mechanizmů progrese nádorů a lékové rezistence, přenos základních vědeckých poznatků do klinické praxe však zůstává stále výzvou.

Předmětem práce bylo studium prognostického a prediktivního významu imunohistochemické exprese vybraných potenciálních molekulárních markerů u pacientů s resekovaným NSCLC, zahrnující proteiny spojených s opravami poškozené DNA a s dráhou sfingolipidového metabolismu.

Studie zahrnovala pacienty tří různých skupin: (1) pacienti s prodělanou operací bez chemoterapeutické léčby, (2) pacienti s prodělanou adjuvantní chemoterapií carboplatinou a navelbinem, (3) pacienti s prodělanou neoadjuvantní chemoterapií carboplatinou a navelbinem.

Imunohistochemická exprese BRCA1 byla studována pomocí různých protilátek. Z pěti testovaných protilátek se pouze BRCA1 fosfo S1524 jevila jako spolehlivá pro detekci funkčního proteinu BRCA1. Univariantní Coxova regresní analýza přežití prokázala, že pozitivita BRCA1 fosfo S1524 byla významně spojena s nižším rizikem úmrtí a relapsu ve stádiu I a II pacientů s NSCLC, kteří podstoupili pouze chirurgickou léčbu, zatímco pro stádium III byla pozitivita BRCA1 fosfo S1524 významně spojena s vyšším rizikem úmrtí a rekurence onemocnění u stejné skupiny pacientů. Tyto závěry však závisí na mnoha dalších neznámých příčinách.

Je zajímavé, že nadměrná exprese RAD51 detekovaná imunohistochemicky byla také spojena s delším celkovým přežitím u dvou léčených skupin pacientů, a to buď po chirurgické léčbě, nebo po prodělané adjuvantní chemoterapií. Srovnání stupně přežití pacientů s nadměrnou expresí RAD51 však prokázalo, že medián přežití byl nižší u pacientů s adjuvantní chemoterapií carboplatinou a navelbinem. U pacientů s neoadjuvantní chemoterapií byla nadměrná exprese RAD51 spojena s kratším celkovým přežitím, ale tyto závěry nebyly statisticky signifikantní.

U exprese filaminu A u NSCLC byla pozorována výrazná inter a intratumorální variabilita. Silná exprese byla pozorována v periferních částech solidních nádorů a signifikantně korelovala s velikostí primárního nádoru, přítomností lymfatických uzlin a vzdálených metastáz. Univariantní Coxova regresní analýza přežití prokázala, že nadměrná

exprese filaminu A je signifikantně spojena s špatným celkovým přežitím pacientů léčených adjuvantní chemoterapií carboplatinou a navelbinem, zatímco u pacientů po chirurgickém zákroku nebyly tyto vztahy pozorovány. Multivariantni Coxova regresní analýza také ukázala, že filamin A lze považovat za nezávislý prognostický faktor spolu s velikost primárního nádoru, statut_uzlin a celkové přežití. Rovněž byly prokázány pozitivní korelace exprese filaminu A a dalšími prognosticky významnými proteiny (zejména p53, SphK1 a SPL). Ve srovnání s nižší expresí filaminu A byla u pacientů s nadměrnou expresí filaminu A vyšší exprese RAD51.

Vysoký stupeň exprese SphK1 byl u pacientů s NSCLC s adjuvantní chemoterapií spojen s nižším celkovým přežitím a se zvýšeným rizikem relapsu onemocněním. Ačkoli nebyla prokázána statisticky signifikantní korelace mezi expresí a klinickým stadiem, multivariantní Coxova regresní analýza ukázala, že SphK1 představuje důležitý rizikový faktor pro relaps NSCLC společně s klinickým stádiem a T a N klasifikací. Je zajímavé, že Coxova regresní analýza prokázala, že cytoplasmatická exprese lyázy S1P představuje určující faktor rizika relapsu u pokročilých stádií NSCLC. Tato data ukazují, že kvantifikace lyázy S1P společně s SphK1 mohou představovat vhodné markery u pacientů s NSCLC.

Výsledky prokázaly, že nadměrná exprese BRCA1 a RAD51 jsou protektivní u raných stádií nádorového onemocnění. U pacientů s NSCLC však zůstává jejich prediktivní význam neobjasněn. Je zajímavé, že se jako vysoce statisticky významný nezávislý prognostický znak prokázal cytoskeletální protein filamin A, a to ve vztahu k jiným studovaným znakům. Tyto závěry dále zdůrazňují význam filaminu A v regulaci hlavních buněčných drah, zahrnující opravy poškozené DNA a metabolismus sfingolipidů a dále, že zacílení cytoskeletárních proteinů a zejména filaminu A může představovat klíčový terapeutický nástroj pro pacienty s NSCLC.

8. ABBREVIATIONS

ADC	adenocarcinoma
A549	human alveolar epithelial cells*
ABC	ATP-binding cassette*
AJCC	American Joint Committee on Cancer
Akt	first identified as an oncogene from the AKT-8 thymoma cell line (also known as PKB)*
ATM	ataxia telangiectasia mutated
ATP	adenosine tri-phosphate*
ATR	ataxia telangiectasia and Rad3-related protein*
ATS	American Thoracic Society
Bad	Bcl-2-associated death promoter*
Bax	Bcl-2–associated X protein*
BCL2	B-cell leukemia/lymphoma 2*
Bcl-xl	B-cell lymphoma-extra large*
BCRP	breast cancer resistance protein*
BER	base excision repair
BRCA1	breast cancer 1*
BRCA2	breast cancer 2*
CBDCA	1-cyclo-butane-dicarboxylate platinum (carboplatin)
CDDP	cis-diamminedichloroplatinum (cisplatin)
CHT	chemotherapy
c-IAP1	cellular inhibitor of apoptosis 1*
c-IAP2	cellular inhibitor of apoptosis 2*
CP-r	cisplatin-resistant*
DAB	3,3'-diaminobenzidine*
DDR	DNA damage response
DFS	disease free survival
DNA	deoxyribo nucleic acid*
DNA-PK	DNA-dependent Protein Kinase*
DSB	double strand break
DSBR	double strand break repair
EGFR	epidermal growth factor receptor
EMT	epithelial mesenchymal transition
ER	estrogen receptor
ERCC1	excision repair cross-complementing 1
ERS	European Respiratory Society
FDA	Food & Drug Administration (US)
FFPE	formalin-fixed, paraffin-embedded
FMD	frontometaphyseal dysplasia
GCF2	growth rate controlling factor-2*
GG-NER	global genome nucleotide excision repair
GSH	glutathione

GTP	guanosine 5'-triphosphate*
H&E	hematoxylin and eosin (stain)
H2AX	H2A histone family, member X*
HEK293	Human Embryonic Kidney 293 cell line*
HR	homologous recombination
HR	hazard ratio
hRad51	human Rad51 protein
HSP	heat shock protein*
HU	hydroxyurea
IASLC	International Association for the Study of Lung Cancer
ICLs	interstrand crosslinks
JNK	Jun N-terminal kinase*
K-RAS	kirsten rat sarcoma viral oncogene homolog*
Ku86	DNA repair protein, also known as X-ray repair cross-complementing 5 (XRCC5)*
LACE	Lung Adjuvant Cisplatin Evaluation
LCC	large cell carcinoma
LRP	lung resistance protein
Mcl-1	myeloid cell leukemia-1
MDR	multidrug resistance
MGMT	O6-methylguanine-DNA methyltransferase*
miRNA	micro RNA
MLH1	MutL homolog 1*
MMR	mismatch repair
MNS	Melnick–Needles syndrome
mRNA	messenger ribonucleic acid*
MRP	multidrug resistance-associated protein
MRP1	multidrug resistance-associated protein 1
MRP2	multidrug resistance-associated protein 2
MSH2	MutS homologue 2*
MutL	DNA mismatch repair protein*
MutS	DNA mismatch repair protein*
NER	nucleotide-excision repair
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells*
NHEJ	non-homologous end-joining
NVB	navelbine
OPD	otopalatodigital syndrome
OS	overall survival
Pgp	P-glycoprotein
PI3K	Phosphatidylinositol 3-Kinase*
PIDD	p53-induced death domain protein 1*
PIN	prostatic intraepithelial neoplasia
PK	protein kinase *
PNH	periventricular nodular heterotopia

RAP80	BRCA1-A complex subunit RAP80*
rhoA	Ras homolog gene family, member A*
RNA	ribonucleic acid
RPLs	ribosomal proteins
RT-QPCR	real-time quantitative polymerase chain reaction
S1P	sphingosine-1 phosphate
SCC	squamous cell carcinoma
SCLC	small-cell lung cancer
SGPL1	sphingosine-1-phosphate lyase 1 gene
Sirt1	silent mating type information regulation 2 (Sir2) homolog 1*
SK1-I	sphingosine kinase-1
SphK1	sphingosine kinase-1
SPL	sphingosine-1 phosphate lyase
SSB	single strand break
TAX	taxane
TC-NER	transcription-coupled nucleotide excision repair
TGF-beta	transforming growth factor beta
TMA	tissue microarray
TMEM205	transmembrane Protein 205
TNF	tumor necrosis factor
TNM	tumor, node, metastasis classification
TRAF1	TNF receptor-associated factor 1*
Tris	tris(hydroxymethyl)aminomethane
UICC	the Union for International Cancer Control
VEGF	vascular endothelial growth factor
WHO	world health organization
XRCC1	X-ray repair cross-complementing protein 1

** abbreviations are explained only here*

9. PUBLICATIONS

Publications related to the thesis:

1. **Gachechiladze M,** Skarda J. The role of BRCA1 in non-small-cell lung cancer**.** *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2012 Sep;156(3):200-3. doi: 10.5507/bp.2012.049. Epub 2012 Jun 1.*
2. **Gachechiladze M**, Uberall I, Kolek V, Klein J, Krejci V, Stastna J, Radova L, Fridman E, Skarda J. Correlation between BRCA1 expression and clinicopathological factors including brain metastases in patients with non-small-cell lung cancer. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2012 Nov 1. doi: 10.5507/bp.2012.099*
3. **Gachechiladze M,** Škarda J, M. Janíková M, Mgebrishvili G, Kharaishvili G, Kolek V, Grygárková I, Klein J, Poprachova A, Arabuli M, Kolař Z. Overexpression of filamin-A protein is associated with aggressive phenotype and poor survival outcomes in NSCLC patients treated with platinum-based combination chemotherapy. *Accepted for publication to Neoplasma.*
4. Škarda J, **Gachechiladze M**, Tichý T, Kolek V, Grygárková I, Klein J, Mgebrishvili G, Kharaishvili G, Janíková M, Pitson S, Gomez-Brouchet A, Cuvillier O. Prognostic role of sphingosine kinase-1 and S1P lyase protein expression in patients with non-small-cell lung cancer. *Submitted to Histopathology*
5. **Gachechiladze M**, Škarda J, Kolek V, Grygárková I, Kharaishvili G, Kolař Z, Joerger M, The prognostic role of RAD51 protein expression in patients with non-small-cell lung cancer: immunohistochemical study. *Submitted to Histopathology.*

Other publications:

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