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CXC GROUP CHEMOKINES AS BIOMARKERS OF NON-SMALL CELL LUNG CANCER

DOCTORAL THESIS

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Abstract

Involvement of CXC chemokines and receptors in the process of carcinogenesis was proven in several studies, but particular role of CXC chemokines in lung cancer including clinical application remains unclear.

Aim of the study was to assess biomarker potential of CXC chemokines and correlate CXC chemokines, tumor cells, immune cells and microenvironment associated parameters with clinical data from lung cancer patient cohorts.

Author used novel biomarker discovery approach to confirm CXC chemokine involvement in carcinogenesis, particularly CXCL1, CXCL4, CXCL5, CXCL7, CXCL8, CXCL9 and CXCL10.

CXCL9, CXCL10 and CXCL11 appeared to be powerful diagnostic biomarker panel. Besides CXCL4 showed prognostic biomarker properties and correlated with tumor angiogenic activity. Author has also found that CXC chemokine levels and gradients correlate with CXC receptor expression and number of tumor infiltrating immune cell subpopulations.

Key words: CXC chemokines, lung cancer, tumor angiogenesis, tumor immune cell infiltrate, tumor microenvironment

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Abbreviations

CXCL1	C-X-C motif chemokine ligand 1, growth-related oncogene protein-α, melanoma growth stimulatory activity factor
CXCL2	C-X-C motif chemokine ligand 2, growth-related oncogene protein-β, macrophage inflammatory protein 2α
CXCL3	C-X-C motif chemokine ligand 3, growth-related oncogene protein-γ, macrophage inflammatory protein 2β
CXCL4	C-X-C motif chemokine ligand 4, platelet factor 4
CXCL5	C-X-C motif chemokine ligand 5, epithelial cell-derived neutrophil-activating peptide
CXCL6	C-X-C motif chemokine ligand 6, granulocyte chemotactic protein 2
CXCL7	C-X-C motif chemokine ligand 7, neutrophil-activating peptide-2
CXCL8	C-X-C motif chemokine ligand 8, interleukin 8
CXCL9	C-X-C motif chemokine ligand 9, monokine induced by gamma interferon
CXCL10	C-X-C motif chemokine ligand 10, interferon gamma-induced protein 10
CXCL11	C-X-C motif chemokine ligand 11, interferon-inducible T-cell alphachemoattractant
CXCL12	C-X-C motif chemokine 12, stromal cell-derived factor 1
CXCR1	C-X-C motif chemokine receptor 1, interleukin 8 receptor-α, CD181
CXCR2	C-X-C motif chemokine receptor 2, interleukin 8 receptor-β, CD182
CXCR3	C-X-C motif chemokine receptor 3, Gαi protein-coupled receptor, CD183
CXCR4	C-X-C motif chemokine receptor 4, fusin, CD184
ELR	amino acid sequence (or motif) of glutamic acid-leucine-arginine
NSCLC	non-small cell lung cancer
TIL	tumor infiltrating lymphocytes

INTRODUCTION

Lung cancer is a highly aggressive and challenging disease and is the leading cause of cancer mortality worldwide (Banat et al., 2015). Involvement of CXC chemokines and receptors in the process of carcinogenesis was proven in several studies, but particular role of CXC chemokines in lung cancer including clinical application remains unclear. CXC chemokines are promising cancer biomarker candidates while the importance of the characterization of diagnostic and prognostic biomarkers is increasingly recognized in oncology research and practice. To date, there has not been a validated biomarker shown to have adequate sensitivity, specificity, and reproducibility to be used as a biomarker for the early detection and treatment of lung cancer. CXC group chemokines could be candidate biomarkers for diagnostic and prognostic purposes.

Understanding the role of CXC chemokines in lung cancer development and progression requires a comprehensive analysis of the interactions between three highly complex systems – the tumor cells, the immune response and the tissue microenvironment. Aim of the study was to analyze CXC chemokine's biomarker potential and integrate CXC chemokines, tumor cell, immune cell and microenvironment associated parameters with clinical data from lung cancer patient cohorts.

Aim of the study

The purpose of this study was to investigate the role of CXC group chemokines in non-small cell lung cancer (NSCLC) with a major focus on NSCLC patients undergoing surgery. Primary aims of the study were to evaluate CXC chemokine's involvement in the process of cancerogenesis, to explore clinical application of CXC chemokines as diagnostic and prognostic biomarkers, and to assess CXC chemokine's network involvement in tumor microenvironment.

Objectives

1. To develop and to verify new approach for lung cancer biomarker research, to introduce and incorporate novel method into surgical practice, to verify method's safety and to evaluate intraoperative and postoperative complications.
2. To measure and to compare CXC chemokine ligand levels in peripheral blood samples of early stage and advanced NSCLC patients and healthy individuals.
3. To assess CXC chemokine ligand levels in NSCLC surgical patient's blood samples representing systemic and tumor microenvironment; and subsequently calculate

chemokine concentration gradients in blood after passing through tumor vessel bed in order to determine chemokine involvement in the process of cancerogenesis.

4. To evaluate diagnostic and prognostic value of CXC chemokine ligands as NSCLC biomarkers.
5. To assess additional factors related to CXC chemokine ligand levels and process of cancerogenesis – expression of CXC chemokine receptors, tumor angiogenesis activity and tumor immune cell infiltrate.

List of manuscripts

1. **Spaks A**. Role of CXC group chemokines in lung cancer development and progression. *Journal of Thoracic Disease* 2017, 9(Suppl 3): 164-71.

2. **Spaks A**, Svirina D, Spaka I, Jaunalksne I, Breiva D, Tracums I, Krievins D. CXC chemokine ligand 4 (CXCL4) is predictor of tumourangiogenic activity and prognostic biomarker in non-small cell lung cancer (NSCLC) patients undergoing surgical treatment. *Biomarkers* 2016, 21(5):474-8.

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2. **A. Spaks,** D. Breiva, I. Tracums, I. Jaunalksne, A. Bistrova, D. Krievins. Evaluation of tumor infiltrating immune cells for the development of non-small lung cancer immunoscore. 24th European Conference on General Thoracic Surgery, Naples, Italy, 2016.

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4. **A. Spaks.** Novel strategies in lung cancer biomarker research. 6th Baltic Congress of Oncology "Multidisciplinary Approach in Cancer Care". Vilnius, Lithuania, 2014.

   Published: Congress abstract book

5. **Spaks A,** Jaunalksne I, Pirtnieks A, Tracums I, Basko J, Krievins D. CXCL4 chemokine as novel diagnostic biomarkers in early stage non-small cell lung cancer. Riga Stradins University Conference, Riga, Latvia, 2014.

   Published: Conference abstract book

6. **Spaks A,** Pirtnieks A, Basko J, Jaunalksne I, Krievins D. Plasma platelet factor 4 as predictor of angiogenic activity: diagnostic and prognostic tool for early stage non-small cell lung cancer. 21st European Conference on General Thoracic Surgery, Birmingham, UK, 2014.

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7. **A. Spaks,** D. Isajeva, I. Spaka, A. Pirtnieks, I. Jaunalksne, J. Grusina-Ujumaza, J. Basko, I. Taivans, D. Krievins. Role of CXCL4 chemokine in angiogenic activity of non-small cell lung cancer (NSCLC). European Multidisciplinary Conference in Thoracic Oncology, Lugano, Switzerland, 2013.

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10. **A. Spaks,** J. Basko, I. Jaunalksne, A. Pirtnieks, U. Kopeika, I. Spaka, A. Babjoniseva. Potential association of platelet factor 4 (PF4) with angiogenesis in non-small cell lung cancer (NSCLC). 3rd European Lung Cancer Conference, Geneva, Switzerland, 2012.

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1. LITERATURE REVIEW

1.1. Chemokines

Chemokines are a family of chemoattractant, cytokine-like proteins that bind to and activate a family of chemokine receptors. Chemokines or chemotactic cytokines, and their receptors have been discovered as essential and selective mediators in leukocyte migration to inflammatory sites. Besides their functions in the immune system, they also play a critical role in tumor initiation, promotion and progression (Vandercappellen et al., 2008).

Over 50 chemokines have been identified and can be divided into four families - CXC, CX3C, CC and C, according to the positions of four conserved cysteine residues. CXC chemokines are characteristically heparin binding proteins. On a structural level, they have four highly conserved cysteine amino acid residues, with the first two cysteines separated by one non-conserved amino acid residue, hence the name CXC (Taub et al., 1994) (Figure 1.1.1.).

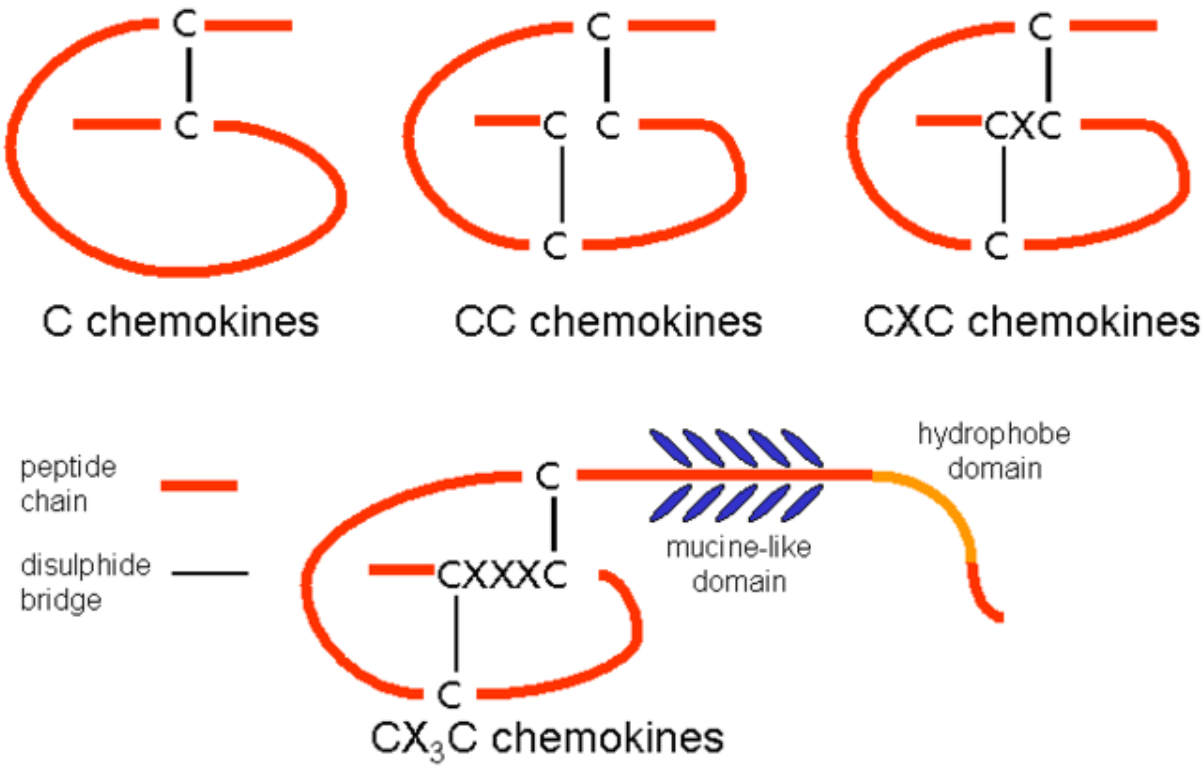

Fig. 1.1.1. **Structure of chemokine classes (Yung et al., 2013)**

Chemokines, which are structurally and functionally similar to growth factors, bind to G protein-coupled receptors on leukocytes and stem cells and process guanine nucleotide-binding proteins to initiate intracellular signaling cascades that promote migration towards the chemokine source (Muller et al., 2001).

Most, if not all chemokines activate leukocytes through binding to G protein-coupled seven transmembrane receptors (GPCR) designated CXCR or CCR (Figure 1.1.2.). Illustration explains the ligand-binding patterns of the seven-transmembrane domain G-protein-coupled human chemokine receptors. Receptors CXCR1, CXCR2 and CXCR3 bind several chemokines. By contrast, CXCR4, CXCR5 and CXCR6 bind only one ligand each. Duffy is considered to be 'deceptor', as it binds ligands but do not signal, thereby acting as a negative feedback for chemokine responses. The binding of a chemokine to its receptor results in the migration of immune cells by interactions with selectins and integrins. Subsequently, leukocytes infiltrate the tissue in response to a gradient of chemokines, produced at the site of inflammation. In addition, these GPCRs may account for the angiogenic or angiostatic action of chemokines.

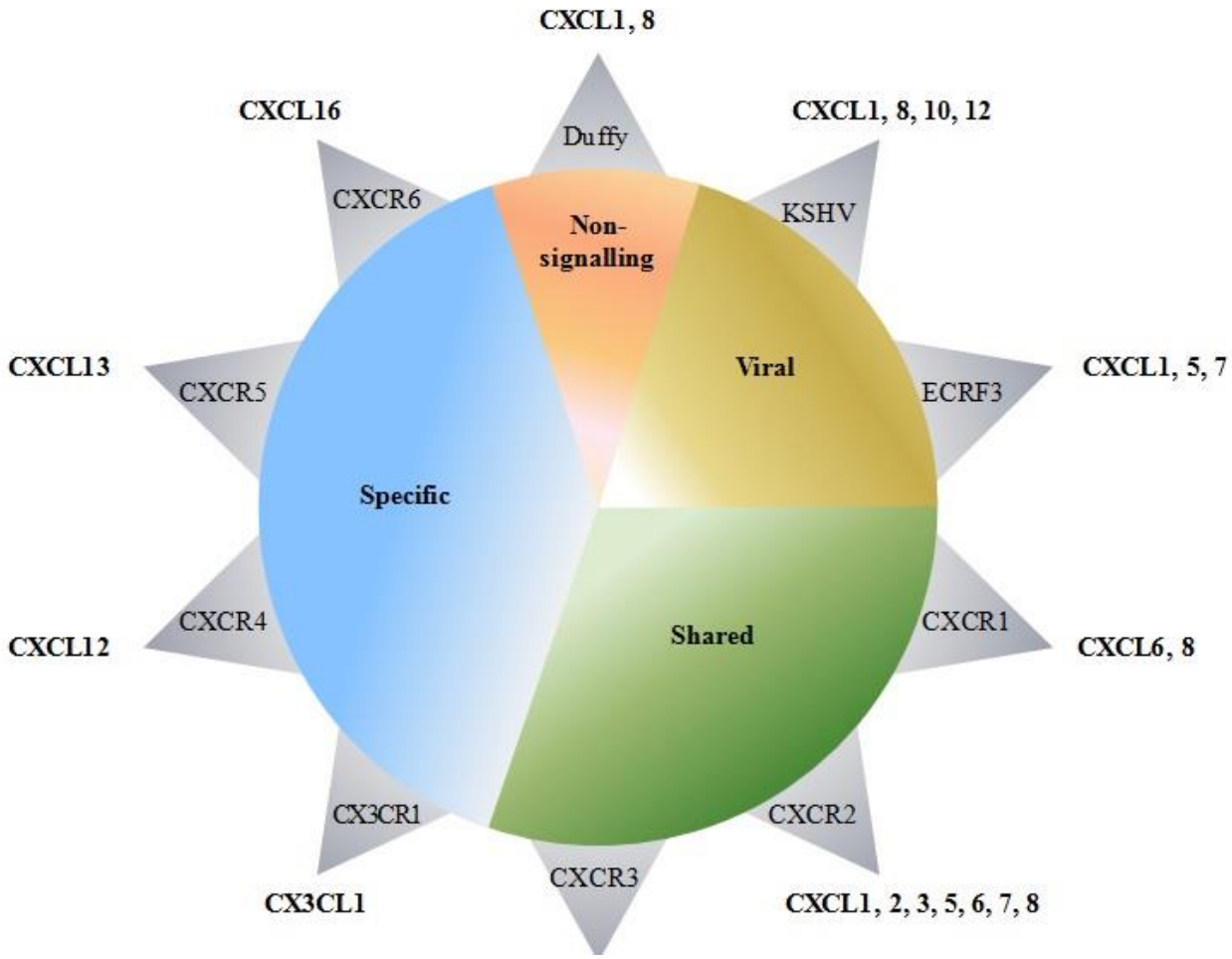

Fig. 1.1.2. **The chemokine wheel**

The binding of a chemokine to its receptor results in the migration of immune cells by interactions with selectins and integrins. Subsequently, leukocytes infiltrate the tissue in response to a gradient of chemokines, produced at the site of inflammation. In addition, these GPCRs may account for the angiogenic or angiostatic action of chemokines.

Chemokines are best known for inducing directional cell migration, particularly of leukocytes during inflammation. Prolonged inflammation is thought to facilitate carcinogenesis by providing a microenvironment that is ideal for tumor cell development and growth. Chemokines can stimulate or inhibit tumor development in an autocrine fashion by attracting cells with pro- and anti-tumoral activities. Chemokines affect tumor development indirectly by influencing angiogenesis, tumor-leukocyte interactions, as well as directly by influencing tumor transformation, survival and growth, invasion and metastasis. The role played by chemokines is rather complex as some chemokines may favor tumor growth and progression, while others may enhance anti-tumor immunity. Solid tumors contain in addition to tumor cells, also various types of stromal cells, such as fibroblasts and endothelial cells. Moreover, tumors are infiltrated by inflammatory cells, including neutrophils, macrophages and lymphocytes. Tumor cells, stromal cells, as well as the tumor-associated leukocytes contribute to the local production of chemokines inside the tumor.

Although the CXC motif distinguishes this family from other chemokine families, a second structural domain within this family dictates their angiogenic potential. The NH2-terminus of the majority of the CXC chemokines containing three amino acid residues (Glu-Leu-Arg: the „ELR" motif) precedes the first cysteine amino acid residue of the primary structure of these cytokines. The family members that contain the ELR motif (ELR+) are potent promoters of angiogenesis (Strieter et al., 1995). In contrast, members that lack the ELR motif (ELR-) are potent inhibitors of angiogenesis. This difference suggests on a structural/functional level that members of the chemokine family are unique cytokines in their ability to behave in a disparate manner in the regulation of angiogenesis (Sun et al., 2008).

1.2. CXC chemokine ligands

1.2.1. CXCL1, CXCL2 and CXCL3

CXCL1, also known as growth-related oncogene protein-α (GRO-α) or melanoma growth stimulatory activity factor (MGSA), is a polypeptide which was initially isolated from Hs294 human melanoma cells. GRO chemokines – CXCL1/GRO-α, CXCL2/GRO-β and CXCL3/GRO-γplay a role in chemotaxis and metastasis of several tumor cell lines and act as autocrine growth factors for melanoma and other tumors (Luan et al., 2007).

1.2.2. CXCL4

The first described chemokine, CXCL4/PF-4 (platelet factor-4), is a tetrameric heparin-binding chemokine released from the α-granules of activated platelets. This ELR- chemokine inhibits endothelial cell migration, proliferation and angiogenesis both in vitro and in vivo (Sharpe et al., 2010). CXCL4 binds to vascular endothelium, selectively in tissues with regions of active angiogenesis (Hansell et al., 1995). Recently, elevated expression of platelet CXCL4 was found by proteomic analysis after implantation of human tumors, like liposarcoma, osteosarcoma or adenocarcinoma in mice, which suggests that CXCL4 can serve as a biomarker for early tumor growth (Cervi et al., 2008).

1.2.3. CXCL5

CXCL5/ENA-78 is an epithelial cell-derived neutrophil-activating peptide that binds to CXCR2. Recently, it was demonstrated that strong CXCL5 expression is correlated with late stages of gastric cancer (Park et al., 2007). In addition, CXCL5 contributes to enhanced proliferation and invasion of head and neck squamous cell carcinomas (Miyazaki et al., 2006). CXCL5 is an important angiogenic factor in NSCLC – levels of CXCL5 are associated with the degree of tumor vascularity and passive immunization of NSCLC tumor-bearing mice with neutralizing anti-CXCL5 antibodies resulted in reduced tumor growth, vascularity and metastases (Arenberg et al., 1998).

1.2.4. CXCL6

CXCL6/GCP-2 is a granulocyte chemotactic protein originally identified from human osteosarcoma cells in parallel with CXCL8 (Proost et al., 1993). Subsequent studies have shown that this chemokine is also induced in other tumour cell lines and in mesenchymal cells (Wuyts et al., 2003). CXCL6/GCP-2 displays angiogenic activities in different in vitro and in vivo models (Van Coillie et al., 2001). This ELR+ chemokine binds to CXCR1 and CXCR2 which are responsible for the chemoattraction of neutrophils by CXCL6, whereas CXCR2 is associated with the angiogenic activity (Addison et al., 2000). Moreover, the angiogenic effect of CXCL6 correlates with the attraction of tumor-associated neutrophils and with the intratumoral expression of matrix metalloproteinase (MMP) – 9/gelatinase-B. The release of this enzyme as a consequence of neutrophil degranulation, promotes tumor invasion through the degradation of extracellular matrix components and allows the migration of tumor cells toward the blood circulation. In addition, CXCL6 is up-regulated by IL-1β and hypoxia in lung cancer cell lines (Zhu et al., 2006). Because these same lung cancer cell lines also

express CXCR1 and CXCR2 and respond to CXCL6 treatment by enhanced proliferation, CXCL6 may act as an autocrine mitogen in the growth and metastasis of lung cancer.

1.2.5. CXCL7

CXCL7/NAP-2 (neutrophil-activating peptide-2) is a cleavage product of the platelet α-granule component platelet basic protein (PBP) and its derivate, connective tissue-activating peptide III (CTAP-III) (Walz et al., 1990). CXCL7 is a CXCL2 agonist that shows chemotactic activity for neutrophils and endothelial cells. The role of CXCL7 in cancer is expected to be similar to that of other CXCR2 agonists, with the exception that this angiogenic chemokine is predominantly released within the vascular lumen. CTAP-III and NAP-2 from platelets have previously been reported to demonstrate heparanase activity and degrade heparan sulfates (Hoogewerf et al., 1995), which are major components of basement membranes and cell surfaces, but the specific consequences in cancer pathology are still not well elucidated. CXCL7 expression was found to be increased in breast cancer cells and conferred invasive properties in CXCL7 transfected breast cells. These cells demonstrated elevated heparanase enzymatic activity which could alter the extracellular matrix and thus facilitate cancer metastasis (Tang et al., 2008). CXCL7 and its receptor CXCR2 contribute important roles in tumor growth and development as well as tumor angiogenesis in lung cancer and other cancer cell types (Keane et al., 2004; Gabellini et al., 2009). Increased expression of the CXC chemokine connective tissue-activating peptide (CTAP)-III has been reported in plasma specimens of lung cancer patients (Lee et al., 2011)

1.2.6. CXCL8

CXCL8 was the first described angiogenic chemokine and is the prototype of ELR+ CXC chemokines (Elner et al., 1994; Strieter et al., 1992). CXCL8 is a strong inducer of angiogenesis mediating endothelial cell chemotaxis and proliferation in vitro and angiogenic activity in vivo (Koch et al., 2002). CXCR2 is implicated in the angiogenic effects of CXCL8 (Heidemann et al., 2003; Mestas et al., 2005). This chemokine exerts its angiogenic activity in part by the up-regulation of MMP-2 and MMP-9 (Opdenakker et al., 2001). Indeed, degradation of the extracellular matrix by these proteases is required for endothelial cell migration. In addition, endothelial cells are good producers of angiogenic CXCL8 and CXCL6. CXCL8 is secreted by a variety of human normal and tumoral cells exposed to pro-inflammatory cytokines, such as IL-1 and TNF-α (Kamohara et al., 2007) and promotes proliferation of pancreatic carcinoma cells. A direct correlation between the expression levels

of CXCL8 and CXCR2 in melanoma, and aggressive growth and metastasis of these tumors has been found (Varney et al., 2006). Further, neutralizing antibodies to CXCL8 have shown to inhibit proliferation of NSCLC (Zhu et al., 2004).

1.2.7. CXCL9, CXCL10 and CXCL11

The angiostatic IFN-γ-inducible CXCR3 ligands, monokine-induced by IFN-γ (CXCL9/Mig), IFN-γ-induced protein-10 (CXCL10/IP-10) and interferon-inducible T cell α chemoattractant (CXCL11/I-TAC), are produced in vitro by a variety of cells, including endothelial cells, fibroblasts, mononuclear cells and tumour cells (Loos et al., 2006), and their presence has been confirmed in several types of tumor tissues (Furuya et al., 2007). These ELR- CXC chemokines inhibit the angiogenic activity of the ELR+ CXC chemokines in the endothelial cell chemotaxis assay in vitro.

Inhibition of adenocarcinoma growth and impaired metastasis was demonstrated in mice after treatment with murine CXCL10, as well as reduced tumour growth and vascularity in Lewis lung carcinoma after administration of CXCL9 (Struyf et al., 2007).

1.2.8. CXCL12

The homeostatic chemokine stromal derived factor-1 (CXCL12/SDF-1) regulates many essential biological processes, including cardiac and neuronal development, stem cell motility, neovascularisation and tumorigenesis (Barbieri et al., 2006; Hattori et al., 2001; Petit et al., 2007). It binds to the widely expressed cell surface receptor CXCR4 (Bleul et al., 1996; Nagasawa et al., 1996). The CXCL12 interaction with CXCR4 plays a prominent role in tumorigenesis. CXCL12-CXCR4 axis promotes angiogenesis and migration of tumour cells into metastatic sites in many cancers (Smith et al., 2004).

1.3. CXC chemokine receptors

1.3.1. CXCR1

This protein mainly serves as a receptor for CXCL8/interleukin 8. It binds to CXCL8 with high affinity, and transduces the signal through a G-protein-activated second messenger system. Stimulation of CXCR1 in neutrophils by its primary ligand interleukin 8, leads to neutrophil chemotaxis and activation (Bergin et al., 2014). CXCR1 is expressed on a wide variety of cell types, including neutrophils, monocytes, CD8 T cells, mast cells, basophils, natural killer cells, keratinocytes, fibroblasts, neurons, endothelial cells, and melanocytes. In malignant melanoma expression of CXCR1 at the cell surface is present, independent of the

cancers stage. It is thought to have a role in the cell growth and angiogenesis required for tumor survival. In this way it has been identified as a potential therapeutic target (Sharma et al., 2010). Blocking CXCR1 inhibits some human breast cancer stem cells in vitro and in mice (Ginestier et al., 2010).

1.3.2. CXCR2

The angiogenic receptor for ELR+ CXC chemokines is CXCR2 (Addison et al., 2000). The protein encoded by this gene is a member of the G-protein-coupled receptor family. This protein is a receptor for CXCL8/interleukin 8 which is a powerful neutrophil chemotactic factor. It binds to CXCL8 with high affinity, and transduces the signal through a G-protein activated second messenger system. This receptor also binds to CXCL1, a protein with melanoma growth stimulating activity, and has been shown to be a major component required for serum-dependent melanoma cell growth. This receptor mediates neutrophil migration to sites of inflammation. Blockade of this receptor leads to a decrease in angiogenesis in pancreatic cancer (Wente et al., 2006), and a significant inhibition of human melanoma tumor growth and experimental lung metastases in CXCR2 negative mice, as well as a reduction of angiogenesis (Singh et al., 2009). A point mutation in CXCR2 results in constitutive signaling; promoting pre-neoplastic to neoplastic cellular transformation (Burger et al., 1999). CXCR2 was reported to play a critical role in a range of cancers, such as colon cancer (Heidemann et al., 2003), oral squamous cell cancer (Qian et al., 2014), esophageal cancer (Wang et al., 2006) and breast cancer (Halpern et al., 2011). CXCR2 had been found to be the primary functional chemokine receptor in mediating endothelial cell chemotaxis (Murdoch et al., 1999). All ELR+ CXC chemokine ligands, binding to CXCR2, mediated angiogenic activity, which was crucial for cancer cells proliferation.

1.3.3. CXCR3

There are at least three splice variants of CXCR3, named CXCR3-A, CXCR3-B and CXCR3-alt (Strieter et al., 2005). CXCR3-A is involved in the chemotactic activity of the IFN-γ-induced CXC chemokines on activated T lymphocytes and NK cells. Classically, the recruitment of these cells facilitates anti-tumor immunity resulting in tumor regression (Wenzel et al., 2005). CXCR3-B is postulated to mediate the angiostatic activity of CXCL4, CXCL9, CXCL10 and CXCL11 on endothelial cells (Yang et al., 2004). Some tissues, like heart, kidney, liver, and skeletal muscle express both CXCR3 splice variants. Other tissues, such as placenta, express only CXCR3-A, whereas human microvascular endothelial cells selectively express CXCR3-B (Lasagni et al., 2003). The relative expression of these two

splice variants on breast cancer cells is suggested to be important in regulating their proliferation in response to CXCL10. Indeed, after down-regulation of CXCR3-B, CXCL10 rather promotes proliferation of breast cancer cells, which confirms the role of CXCR3-B in cell growth inhibition and suggests the involvement of CXCR3-A in promoting cell proliferation (Datta et al., 2006). Constitutive expression of CXCR3 in melanoma, colon and breast carcinoma accelerated tumor metastasis to lymph nodes (Kawada et al., 2007a, 2007b). CXCR3 ligands have opposing effects on cells of the tumor microenvironment (anti-malignant) and on the tumor cells (pro-malignant).

1.3.4. CXCR4

CXCR4 has previously been highlighted for its role in cancer metastasis. In most, but not all, of the cancer types studied, CXCR4 is co-expressed with other CC or CXC chemokine receptors on malignant cells and some of these receptors have also been implicated in cancer progression (Balkwill, 2004a). The CXCL12/CXCR4 axis is important for activating a plethora of phenomena, including chemotaxis, invasion, tumorigenicity and angiogenesis and proliferation in cancer, particularly in the process of metastasis (Liang et al., 2010; Xie et al., 2014), demonstrating that tumor cells expressing a high level of CXCR4 exhibit metastasis to target tissues. The target tissues express high levels of CXCL12, allowing tumor cells to directionally migrate to target organs via the CXCL12-CXC4 chemotactic axis. CXC4 is hypothesized to be involved in cancer invasion and metastasis, and higher levels of this receptor are associated with higher grades and poor prognosis of cancer (Torregrossa et al., 2012). Several retrospective studies have also examined the role of CXCR4 in NSCLC by investigating the association between CXCR4 expression with clinical outcome; NSCLC patients with greater CXCR4 expression on the surface of tumor cells have been observed to be more likely to have metastatic disease (Spano et al., 2004). There is increasing evidence to suggest that the CXCL12/CXCR4 chemokine axis is important for the cell invasion and migration of several types of tumor, particularly lung cancer. It has been shown that a number of NSCLC cell lines express high levels of CXCR4, which is associated with aggressive behavior, and that CXCL12-activated CXCR4 promotes migration and invasion of these cell lines in vitro (Phillips et al., 2004).

Tumor cells from at least 23 different types of human cancers of epithelial, mesenchymal and haematopoietic origin express CXCR4 (Balkwill, 2004b). Not all cancerous cells in the primary tumor are CXCR4 positive. In ovarian and non-small-cell lung cancer, for instance, only a sub-population of cells expresses this receptor (Scotton et al., 2001; Kijima et al., 2002). Activation of CXCR4 stimulates directed migration of cancer cells and increases

their invasion through Matrigel and monolayers of endothelial cells, bone marrow stromal cells and fibroblasts (Balkwill, 2004b; Scotton et al., 2002; Koshiba et al., 2000; Libura et al., 2002). If CXCR4 is associated with metastatic activity *in vivo*, expression of CXCR4 and/or its receptor CCL12 might be higher in metastases compared with primary tumors. When NSCLC cells were grown in severe combined immunodeficiency (SCID) mice, 99% of the cells in metastases expressed CXCR4, compared with only 35% of the cells in the primary tumor (Phillips et al., 2003). Taken together, these experiments indicate that cancer cells with high levels of CXCR4 are more likely to form metastases. However, they do not prove that CXCR4-expressing cancer cells in primary tumors spread only because they migrate towards gradients of CXCL12 in other organs. The ability of tumor cells to use CXCR4–CXCL12 during the process of metastasis might depend on chemokine gradients in the primary tumor, as well as common sites of spread, levels of functional receptor, and the presence of other cytokines and proteases that can cleave ligand and receptor.

1.4. Immune system and carcinogenesis

A fundamental role of the immune system is maintenance of tissue homeostasis by continuous immunosurveillance and initiation of inflammatory reactions that involve the coordinated activation of innate and adaptive immune cells (Demaria et al., 2010). Neoplastic transformation alters the orderly structure of tissues and induces immune responses that can eliminate incipient tumors. In situations where elimination is incomplete, neoplastic transformation of cells is able to escape immune control. This process has been best conceptualized by the cancer immunoediting theory, which is supported by a large body of experimental data and clinical evidence (Mittal et al., 2014). Immunoediting defines malignant progression on the basis of tumor and immune cell interactions in three phases: elimination, equilibrium and escape (Figure 1.4.1.).

While patients are most frequently diagnosed in the escape phase, this relationship between the tumor and host immunity continues to evolve and sometimes with it the magnitude of the antitumor immune response. Even at advanced disease stages, immune parameters have now been recognized as directly or indirectly influencing patient survival (Galon et al., 2006). As a consequence of constant immune selection pressure on genetically unstable tumor cells, tumor cell variants may emerge that: are no longer recognized by adaptive immunity; become insensitive to immune effector mechanisms; induce an immunosuppressive state within the tumor microenvironment.

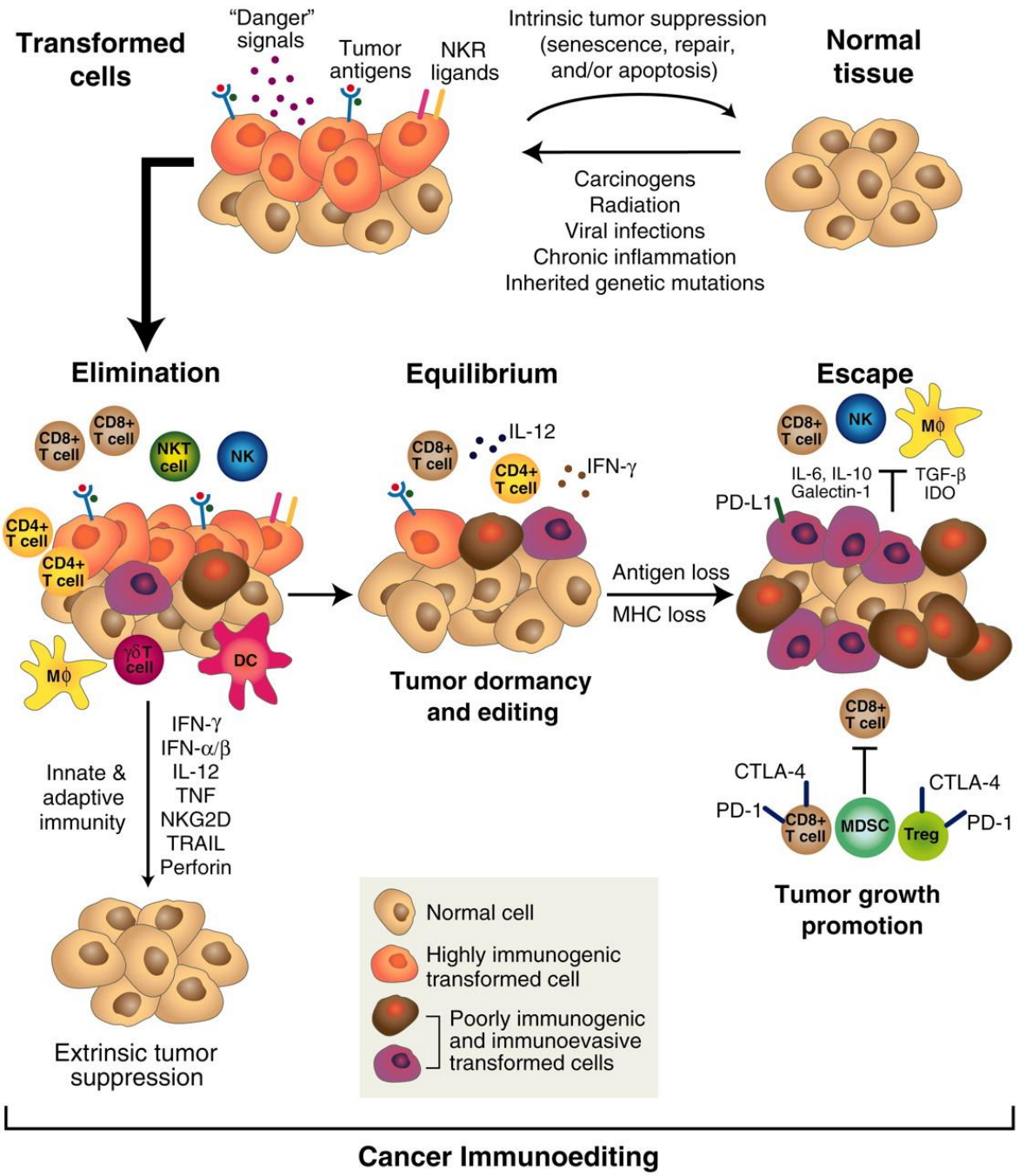

Fig. 1.4.1. **The cancer immunoediting concept (Schreiber et al., 2011)**

Several recent clinical studies have evaluated the prognostic and predictive importance of tumor-infiltrating lymphocytes. Immune cells-infiltrating tumors are frequently observed, but the composition of cells involved in innate and adaptive immunity varies between tumor types or organ sites (Galon et al., 2013). Cumulative data from murine and human studies have associated most leukocyte subsets with a predominant contribution to either pro- or

antitumor activities. Murine models have identified myeloid lineage leukocytes, including tumor-associated macrophages, dendritic cells and myeloid-derived suppressor cells as playing a central role in shaping the microenvironment via the factors they produce, towards either an immunostimulatory antitumor milieu or a wound healing tumor-promoting microenvironment. Antitumor T cells migrating into these contrasting settings can therefore either be activated or suppressed (Coussens et al., 2011).

1.5. Chemokines and tumor immune cell infiltrate

Tumors grow within an intricate network of epithelial cells, vascular and lymphatic vessels, cytokines and chemokines, and infiltrating immune cells (Fridman et al., 2012). During carcinogenesis, tumor cells interact with a complex microenvironment that is composed of extracellular matrix and non-neoplastic host cells, including mesenchymal cells, vascular endothelial cells and inflammatory or immune cells. Inflammatory cells and immune cells are present to varying degrees (from absent to intense) in the tumor microenvironment, which can be observed routinely in pathology practice. The tumor microenvironment provides cancer cells with nutrients, oxygen, growth factors, cytokines, and other chemical mediators that support tumor proliferation, survival, invasion, and metastasis. Persistent inflammatory reactions may be an important contributor to tumor progression. On the other hand, immune responses to neoplastic cells may inhibit disease progression, as indicated by prolonged survival of patients with cancer who exhibit a strong immune response to their tumor.

Tumor microenvironment is increasingly recognized as contributing directly to cancer initiation, progression and metastasis (Swartz et al, 2012; Hanahan et al., 2011). The tumor microenvironment, depending on the tumor location, is composed of stromal cells including fibroblasts, immune and inflammatory cells, adipocytes, glial cells, smooth muscle and resident and recruited vascular cells along with the extracellular matrix, growth factors/cytokines and other proteins that are locally and/or systemically produced. They may either simulate or inhibit cancer cell proliferation/malignancy depending on the tumor microenvironment and the various interactions they may have with the cancer cells (Hanahan et al., 2012; Remerk et al., 2015).

Immune cells-infiltrating tumors are frequently observed, but the composition of cells involved in innate and adaptive immunity varies between tumor types or organ sites. Cumulative data from murine and human studies have associated most leukocyte subsets with a predominant contribution to either pro- or antitumor activities. Murine models have identified myeloid lineage leukocytes, including tumor-associated macrophages, dendritic

cells and myeloid-derived suppressor cells as playing a central role in shaping the microenvironment via the factors they produce, towards either an immunostimulatory antitumor milieu or a wound healing tumor-promoting microenvironment. Antitumor T cells migrating into these contrasting settings can therefore either be activated or suppressed. In turn, macrophage polarization toward protumorigenic M2 or antitumor M1 functional phenotypes are regulated by T lymphocytes (DeNardo et al., 2009), highlighting the importance of cellular cross-talk in shaping the tumor microenvironment.

Histopatholoical analyses of human tumors have provided evidence that variable numbers of infiltrating immune cells are found in different tumors of the same type, and are found in different locations within and around a tumor. Macrophages, mast cells, granulocytes and myeloid-derived suppressor cells are found in most cases infiltrating or surrounding tumor beds both in the core and at the invasive front of the tumor. It is well established that chronic inflammation and the presence of M2 macrophages favor tumor growth and spreading (Mantovani et al., 2008). Lymphocytes are not randomly distributed but are located in specific areas. Therefore, NK cells are found in the stroma and are not in contact with tumor cells. B cells are mostly found in the invasive margin of growing tumors and tertiary lymphoid structures that are adjacent to tumor beds (Dieu-Nosjean et al., 2008). T cells, particularly CD8+ T cells, may be located in the invasive margin but can also be in the tumor core. Few CD8+ T cells are seen in tertiary lymphoid structures, which are similar to secondary follicles in lymph nodes that contain naive T cells and memory T cells, B cells and mature dendritic cells. The distribution of immune cells varies between tumor types. All subsets of T cells are present at the core and at the invasive margin of the tumor in colorectal cancer, non-small cell lung cancer, melanoma, and head and neck cancers. In colorectal cancer, the proportion of tumors with high densities of CD4+ memory T cells and CD8+ memory T cells decreases with local tumor invasion, as assessed by the T stage of the TNM classification. Conversely, the proportion of primary tumors with high infiltrates of CD4+ memory T cells, particularly in the core, is lower in patients with tumors that recur. It has also been reported that T cells are found only in the invasive margin in liver metastases of colon cancer (Halama et al., 2011). Recent studies (Sica et al., 2012) suggest that during carcinogenesis, macrophages may polarize to M1 (anti-tumorigenic) and M2 (tumorigenic) subtypes and thus can exert differential effects.

Correlations between the levels of immune cell infiltration of tumors and clinical outcome have been investigated in many cancers – summary of 124 published articles showed a strong lymphocytic infiltration has been reported to be associated with good clinical outcome in many tumor types; high densities of CD3+ Tcells, CD8+ cytotoxic T cells and

CD45RO+ memory T cells were clearly associated with a longer disease-free survival; in contrast to the effects of cytotoxic T cells and memory T cells, analysis of the effect of CD4+ T cell populations on clinical outcome has resulted in apparent contradictory results (Fridman et al., 2012). Previous studies which analyzed how T cells affect clinical outcome in lung cancer have also yielded contradictory results (Kilic et al., 2011; Kawai et al., 2008). CD8+T cells recognize and destroy cancer cells while CD4+ cells aid CD8+ in tumor rejection. Therefore their number and localization in tumor tissue may influence tumorigenity (Bremnes et al., 2011). Although high in number, CD8+ T cells that infiltrate lung tumors may be dysfunctional due to tumor microenvironmental factors which may subsequently lead to reduced numbers of effector CD8+ T cells. These altered CD8+ T cells may even release compounds that promote tumor progression.

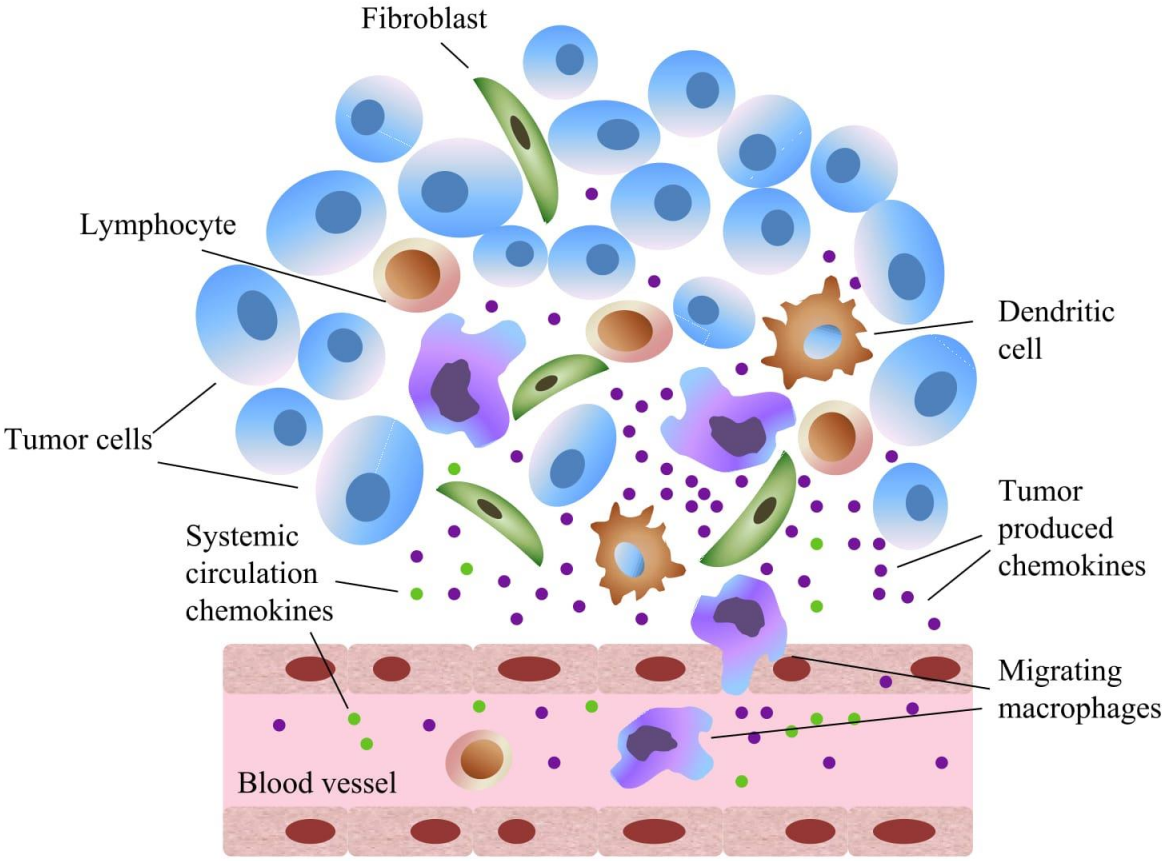

Fig. 1.5.1. **Tumor model.** Tumor model including immune cell infiltrate and tumor vascular bed. Chemokine gradients in systemic circulation and tumor microenvironment determine cell composition in immune cell infiltrate as well as chemokine concentrations.

Tumor-host interactions are mediated indirectly through extracellular matrix molecules and soluble bioactive molecules (including CXC chemokines) released from host or neoplastic cells, and directly mediated through cell-surface molecules on host and neoplastic cells. As such, tumor-host interactions are likely influenced by the genome and epigenome of both the neoplastic and non-neoplastic cells (Figure 1.5.1.)

Various chemokines are associated with generating an anticancer immune contexture (Kondo et al., 2006). In fact, a fine balance is generated in the tumor microenvironment between the chemokines that are involved in attracting the relevant immune cells into the core and the invasive margin of the tumor and those chemokines that are that are involved in generating immune responses in secondary lymphoid structures adjacent to the tumor. The active recruitment of naive and memory T cells from blood into tertiary immune structures via endothelial venules is due to the local production of CCL19, CCL17, CCL22, CXCL13 and IL-16 (Chaisemartin et al., 2011). It is hypothesized that, as in secondary lymphoid organs, the interaction of T cells with mature dendritic cells generates central memory and effector T cells in tertiary lymphatic structures. B cells interact with follicular dendritic cells to generate affinity maturation of immunoglobulins, and B cells locally produce antibodies, some of which react with tumor-associated antigens. The CD4+ and CD8+ memory T cells that are generated migrate out through lymphatic vessels expressing CCL21 and migrate into the tumor or to the periphery where memory T cells may patrol for long periods of time to eventually target circulating malignant cells or nascent metastases (Mlecnik et al., 2011; Sautes-Fridman et al., 2011). Therefore, a complex interplay of immune cells that express receptors for different chemokines produced at selected locations of the tumor environment builds the architecture of the immune contexture, the coordination of which is an essential trait for effective control of tumor. Subtle modifications of this architecture that are provoked by changes in the tumor cells, in the host immune system owing to infections, or in the chemokine milieu of the local microenvironment, will result in a loss of coordination and inefficiency of immune control of the tumor, even if high densities of memory T cells are present (Tartour et al., 2013).

1.6. Chemokines and angiogenesis in cancer

Angiogenesis is a pervasive, normal biological event related to critical new blood vessel growth under both physiologic and pathologic conditions. Angiogenesis is crucial for tumor growth and metastasis; the angiogenic switch refers to a phenotypic change that occurs early in a tumor's development that is necessary for growth beyond 2-3 mm in size (McClelland et al., 2007). Hypoxia is the trigger for many of the mechanisms by which a

tumor induces angiogenesis. Angiogenesis is a complex process in which numerous stimulatory and inhibitory signals, such as integrins, angiopoetins, chemokines, oxygen sensors, growth factors, extracellular matrix proteins, and many other molecules are involved. The regulation of angiogenesis depends on a dual, yet opposing, balance of local factors that promote or inhibit neovascularization (Mittal e al, 2014). Tumor growth occurs when the equilibrium between angiogenic and angiostatic factors is disturbed in favor of angiogenic factors (Figure 1.6.1.). Tumor-derived angiogenesis during tumorigenesis is determined, in part, by an imbalance in favor of the overexpression of angiogenic compared with angiostatic CXC chemokines (Table 1.7.1.). Other important factors that promote tumor angiogenesis either directly or indirectly include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), macrophage migration inhibitory factor (MIF), angiopoietin 1, and epidermal growth factor (EGF), among others (Zhan et al., 2009).

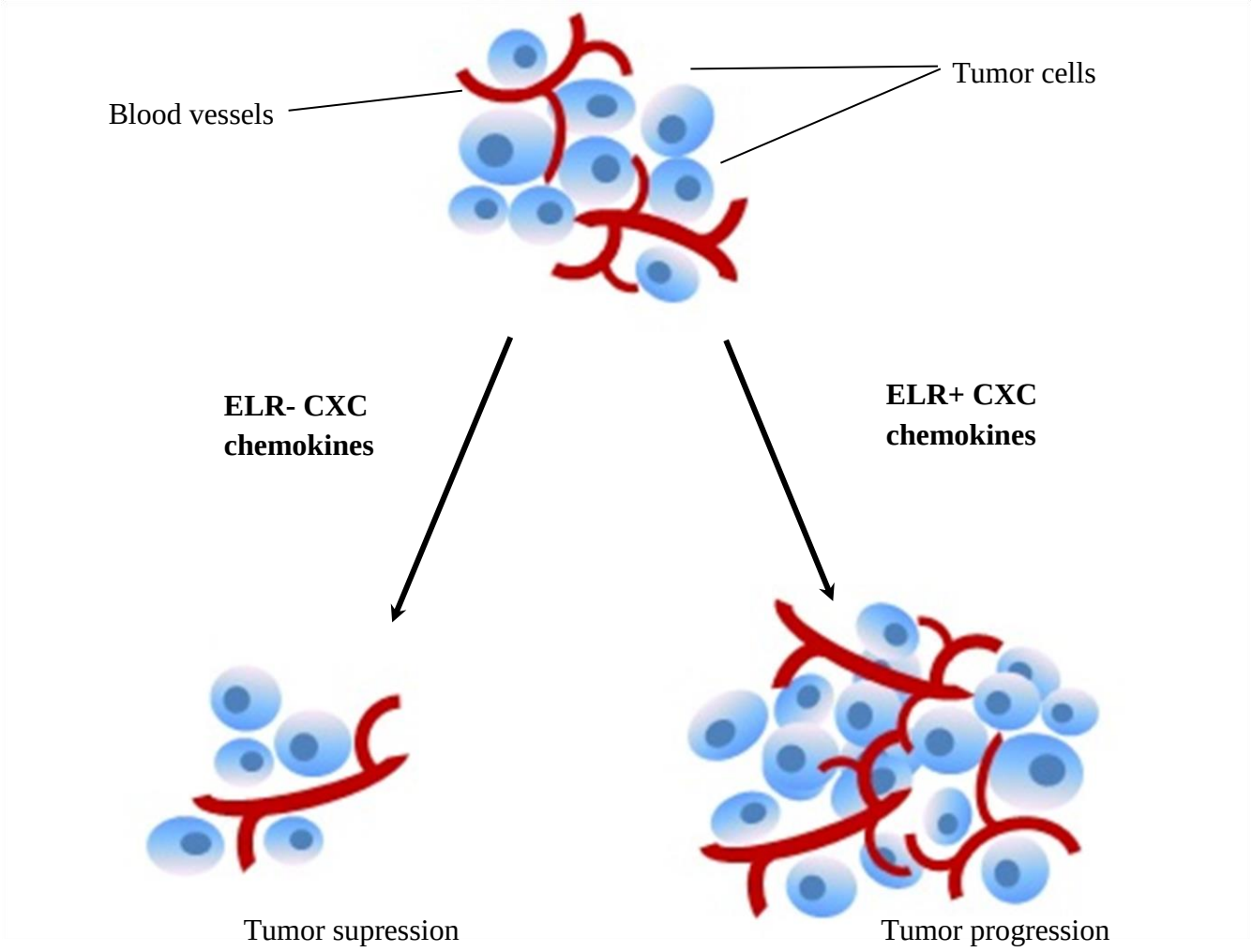

Fig. 1.6.1. **CXC chemokines and angiogenesis in cancer**

CXCL4 was the first CXC chemokine reported to regulate angiogenesis. CXCL4 was found to inhibit bFGF-induced angiogenesis and attenuate growth of melanoma and colon carcinomas in a murine model of tumorigenesis (Hansell et al., 1995). In contrast, CXCL8 was the first CXC chemokine found to induce angiogenesis. CXCL8 was shown to mediate both in vitro endothelial cell chemotactic and proliferative activity as well as in vivo angiogenesis in the absence of preceding inflammation (Hu et al., 1993). These findings have been substantiated by other investigations, which have shown that CXCL8, similar to bFGF or VEGF, induces endothelial cell tube formation in vitro and angiogenesis in vivo (Yoshida et al., 1997). These experiments prove that CXCL8 has direct effect on the endothelial cell and that this angiogenic activity is distinct from its ability to induce inflammation. These findings suggest that members of the CXC chemokine family function in a disparate manner and can behave as either potent angiogenic or angiostatic factors in regulating neovascularization (Strieter et al., 1999). Studies in melanoma support that CXCL1, CXCL2 and CXCL3 play a significant role in promoting tumorigenesis related to both their mitogenic and angiogenic activities. Although CXCL8 may represent an important angiogenic CXC chemokine, CXCL5 may be more important angiogenic CXC chemokine than CXCL8 in NSCLC. Human surgical specimens of NSCLC tumors were found to display a direct and significant correlation of CXCL5 protein levels with tumor neovascularization. The biological relevance of this finding was confirmed in a SCID mouse model using human NSCLC cell lines (Arenberg et al., 1998; Murakami et al., 2003; Murakami et al., 2002).

Generally CXC chemokine group members that contain the ELR motif are angiogenic. In contrast, most of the CXC chemokines without ELR motif are angiostatic (Vandercappellen et al., 2008). An exception of the relation between the ELR motif and angiogenesis is CXCL12, an angiogenic ELR- CXC chemokine. All angiogenic ELR+ CXC chemokines mediate their angiogenic activity through CXCR2. Initial candidate CXC chemokine receptors for the angiogenic activity of ELR+ CXC chemokines were CXCR1 and CXCR2. However, only CXCL8 and CXCL6 specifically bind and activate CXCR1, whereas, all ELR+ CXC chemokines activate CXCR2 (Addison et al., 2000). In the human system, CXCL8 and CXCL6 signal through both CXCR1 and CXCR2, whereas the other ELR+ CXC chemokines are agonists for CXCR2 only (Wuyts et al., 2007). ELR+ CXC chemokines bind neutrophil receptors CXCR1 and CXCR2 to each other (Baggiolini et al., 2001). The ELR+ chemokines are primarily chemotactic for endothelial cells and neutrophils. These chemokines are potent promoters of angiogenesis, as the recruited neutrophils are known to synthesize and store angiogenic molecules like vascular endothelial growth factors (Nannuru et al., 2011). Studies highlight the importance of angiogenic pathways that show the

importance of ELR+ CXC chemokines in the promotion of tumor-associated angiogenesis (Strieter et al., 2008). A dense vascular network may allow potential metastatic cells to escape the primary tumor but, conversely, can also favor the infiltration by immune cells provided that the relevant chemokines are produced.

1.7. Summary

An overview of the characteristics of CXC chemokines in terms of receptor binding, chemotactic, ELR motif and angiogenic/angiostatic activity is given in Table 1.7.1.

Table 1.7.1.

Properties of CXC chemokines

Chemokine	Receptor	ELR motif	Chemotaxis	Angiogenesis
CXCL1	CXCR2	+	Neutrophils, endothelial cells	+
CXCL2	CXCR2	+	Neutrophils, endothelial cells	+
CXCL3	CXCR2	+	Neutrophils, endothelial cells	+
CXCL4	CXCR3	-	Activated T cells	-
CXCL5	CXCR2	+	Neutrophils, endothelial cells	+
CXCL6	CXCR1, CXCR2	+	Neutrophils, endothelial cells	+
CXCL7	CXCR2	+	Neutrophils, endothelial cells	+
CXCL8	CXCR1, CXCR2	+	Basophils, monocytes, neutrophils, endothelial cells	+
CXCL9	CXCR3	-	Natural killer cells, activated T cells	-
CXCL10	CXCR3	-	Natural killer cells, activated T cells	-
CXCL11	CXCR3, CXCR7	-	Natural killer cells, activated T cells	-
CXCL12	CXCR4, CXCR7	-	B cells, basophils, dendritic cells, monocytes, natural killer cells, T cells, endothelial cells	+

2. METHODS

2.1. Patient selection

Between June 2010 and September 2011, 50 patients with NSCLC (preoperative clinical and radiological stage IA – IIB, lung cancer TNM staging 7th edition) were enrolled in a single center prospective study. All patients underwent radical pulmonary resection – open lobectomy with mediastinal lymph node dissection. All 50 patients formed an initial cohort (Tab. 2.1.1.)

Postoperative staging of the patients was based on the seventh edition of international TNM classification for lung cancer (Goldstraw et al., 2009). Histologic classification was performed according to the World Health Organization histologic typing of lung tumors (Travis et al., 1999). We excluded patients with a high probability of micrometastatic disease (postoperative pathological stage IIIA and above) and rare histologic subtypes of NSCLC other than squamous carcinoma and adenocarcinoma. The final cohort included 30 asymptomatic patients, diagnosed with stage IA – IIB NSCLC (Table 2.1.2.). Patients were followed-up every 6 months after surgery for up to five years. General status of the patient and the information about disease outcome and relapse was obtained during follow-up visits, and confirmed with the National Cancer registry. The final collection of survival data was in September 2016.

After formation of final patient cohort from June 2010 to November 2012 we have enrolled two control groups. First control group consisted of 30 sex, age, tumor histology and smoking history matched metastatic NSCLC patients (Table 2.1.2.). Second control group consisted of 30 sex, age and smoking history matched healthy volunteers (Table 2.1.2.).

This study has been approved by Pauls Stradins Clinical University Hospital Foundation ethical committee, and was conducted in accordance with the ethical standards of the Helsinki Declaration of the World Medical Association. The informed consent was obtained from all patients.

Table 2.1.1.

Primary cohort of surgical patients

Characteristics	**Value**	
	n	%
Age (years)		
Median	65.4	N/A
Range	50 - 79	N/A
Gender		
Male	32	64
Female	18	36
Smoking		
Never	1	2
Former	22	44
Current	20	54
Stage		
IA	8	16
IB	14	28
IIA	12	24
IIB	8	16
IIIA	6	12
IIIB	1	2
IV	1	2
Histology		
Adenocarcinoma	15	30
Squamous cell carcinoma	26	52
Large cell carcinoma	4	8
Pleomorphic carcinoma	2	4
Non-differentiated carcinoma	3	6

Table 2.1.2.

Final cohort of surgical patients and control groups

Characteristics	Patients with early stage NSCLC		Patients with metastatic NSCLC		Healthy volunteers	
	Value					
	n	%	n	%	n	%
Age (years)						
Median	62.5	N/A	64.1	N/A	59.8	N/A
Range	50 - 78	N/A	52 - 76	N/A	49 - 65	N/A
Gender						
Male	17	56.6	16	53.4	18	60
Female	13	43.4	14	46.6	12	40
Smoking						
Never	0	0	1	3.4	3	10
Former	10	33.4	12	40	12	40
Current	20	66.6	21	56.6	15	50
Stage						
IA	8	26.6	N/A	N/A	N/A	N/A
IB	12	40	N/A	N/A	N/A	N/A
IIA	6	20	N/A	N/A	N/A	N/A
IIB	4	13.4	N/A	N/A	N/A	N/A
IIIA	N/A	N/A	N/A	N/A	N/A	N/A
IIIB	N/A	N/A	N/A	N/A	N/A	N/A
IV	N/A	N/A	30	100	N/A	N/A
Histology						
Adenocarcinoma	13	43.4	15	50	N/A	N/A
Squamous cell carcinoma	17	56.6	15	50	N/A	N/A

2.2. Blood sample collection and processing

Peripheral blood samples were taken from cubital vein into 5 ml vacutainer tubes using standard phlebotomy technique. In surgical patients during operations paired blood samples were obtained from the lobar pulmonary vein draining lung part containing tumor (represents tumor vascular bed), and from the peripheral cubital vein (represents the systemic circulation). Tubes were centrifuged at room temperature for 10 minutes at 1300g. Following centrifugation plasma was immediately archived at -70^{o}C pending utilization.

2.3. Measuring circulating CXC chemokine levels

Prior to ELISA assay samples were defrosted at room temperature and processed according to ELISA kit manufacturer protocol (Raybiotech, USA). Each standard or sample was assayed in duplicate. The CXC chemokine concentrations in plasma were calculated from the standard curve. Levels of CXC chemokine ligands are given as mean ± SD. Chemokine gradients were calculated for paired blood samples. Difference in circulating chemokine concentrations between systemic and tumor vascular beds can be described as chemokine concentration gradient. Chemokine gradient was calculated according to the formula: ((TCV-PCV)/ PCV) ×100, where TCV—tumor circulation representing value, PCV—peripheral circulation representing value.

2.4. Measuring standard lung cancer biomarker levels

CEA, CA125 and CYFRA 21-1 levels were measured in the plasma of early-stage NSCLC patients by immunoassay in a certified clinical laboratory.

2.5. Tumor sample preparation

Resected tumor tissue samples were fixed for 24 hours in 10% neutral buffered formalin and embedded in paraffin. Samples were cut into 3–4 μm sections and placed on clean electrostatically charged Histobond+ (Marienfeld, Germany) glass slides. Sections were dried overnight in a 37°C incubator and dewaxed in xylene and brought to absolute alcohol followed by rehydration in 90% and 70% ethanol and distilled water. The slides were then incubated in fresh hematoxylin for 20 minutes and washed in distilled water, followed by incubation in acidified eosin solution for 1 minute and washing. Finally, the slides were dehydrated in 90% and 100% ethanol, air dried, and mounted (Gamble et al., 2012). The hematoxylin and eosin stained tumor sections were assessed by two independent pathologists to determine histological subtype of the tumor and for differentiating the tumor cells from tumor stroma. Verified tumor specimens containing representative tumor and tumor stroma

tissue were further processed using immunohistochemistry. To assess the process of angiogenesis we have selected for analysis the invasive edge in the tumor area.

2.6. Immunohistochemistry

Tumor tissue samples were cut into 3–4 μm sections and placed on clean electrostatically charged Histobond+ (Marienfeld, Germany) glass slides. Sections were dried overnight in a 37°C incubator and dewaxed in xylene and brought to absolute alcohol. Trypsin was used for enzymatic antigen retrieval. Slides covered with 0.5% trypsin solution were placed in a humidified container and then into the 37^{o} C incubator for 10 minutes. Slides were washed in water/tris-buffered saline (TBS) and blocked in 10% normal serum with 1% bovine serum albumin (BSA) in TBS for 2 hours at room temperature. We followed the specific standardized protocol supplied by the manufacturer (Santa Cruz Biotechnology, US). Omission of the primary antibody served as a negative control. Primary antibody (Table 2.6.1.) diluted in TBS with 1% BSA applied and slides incubated for 30 minutes. Slides were washed and secondary antibody diluted to the concentration recommended by the manufacturer in TBS with 1% BSA was applied to the slides, and incubated for 1 hour at room temperature. Slides were rinsed in TBS and developed with chromogen diaminobenzidine (DAB) for 10 min at room temperature, rinsed in distilled water, counterstained with hematoxylin. Slides were dehydrated, cleared and mounted (Jackson et al., 2012).

Table 2.6.1.

Immunohistochemistry details

Parameter of interest	**Cluster of differentiation (CD)**	**Antibody dilution**
CXCR1 expression	CD181	1:500
CXCR2 expression	CD182	1:250
CXCR3 expression	CD183	1:500
CXCR4 expression	CD184	1:500
T helper cells (Th)	CD4	1:1000
T cytotoxic cells (Tc)	CD8	1:500
B cells	CD20	1:200
Macrophages	CD68	1:1000
Plasma cells	CD138	1:500
Angiogenesis	CD34	1:200

2.7. Tumor immune cell infiltrate assessment

Tumor immune cell infiltrate was defined as composition of immune cells including granulocytes, macrophages (CD68), plasma cells (CD138), T helper cells (CD4), T cytotoxic cells (CD8) and B cells (CD20) in tumor stroma and tumor tissue. One section (4–5 μm, magnification x400) per patient is currently considered to be sufficient (Salgado et al., 2015). The average number of positively stained cells per four high power fields was counted in tissue sections. In combination with an immunohistochemical stain, we also relied on a histomorphological assessment by pathologist. The total number of immune cells forming immune cell infiltrate was calculated.

2.8. CXC chemokine receptor expression assessment

In order to provide an overview of protein expression patterns, all images of immunohistochemically stained tissues were manually annotated by specially educated personnel followed by review and verification by a second qualified member of the staff. Basic annotation parameters included an evaluation of staining intensity subdivided into four categories - negative, weak, moderate or strong; fraction of stained cells also subdivided into four categories - rare, <25%, 25-75% or >75%.

2.9. Tumor microvessel density assessment

To determine microvessel density (MVD) twenty high-power fields (400x magnification) with total area of 4.8 mm^2 were examined per tumor tissue sample after tumor sample staining with CD34 antibody. MVD values are given as mean number of microvessels per mm^2 and SD.

2.10. Statistical analysis

Prior to analysis, data normality was assessed using Kolmogorov-Smirnov test, parametric or non-parametric tests were applied accordingly. Peripheral blood CXC chemokine ligand levels were statistically evaluated for median differences in concentration by Kruskal-Wallis test and the p value under 0.05 was considered significant. Paired and unpaired two-tailed Student’s t-test was used where applicable. Sensitivity and specificity were calculated using receiver-operating characteristics (ROC) curve analysis. The area under the ROC curve (AUC) was measured to assess discriminatory power of test.

Correlation analysis (Pearson and Spearman) was used to study the strength of a relationship between numerically measured continuous variables. Values between -0.4 and 0.4 were considered non-significant.

Survival time was measured from the date of surgery. Disease-free survival is defined as the interval from anatomic resection to clinical or radiographic demonstration of the recurrence or censorship. Overall survival is defined as the interval from treatment initiation to death or censorship. Survival curves are estimated by the Kaplan-Meier method and are compared across groups by the log rank test.

All p values considered are two-sided, and p values less than 0.05 are marked as significant. Microsoft Excel Analysis Toolpack and MedCalc software was used for data analysis.

3. RESULTS

3.1. Introduction to the results

The results are presented here as original publications. Each publication corresponds to one or several study objectives.

Manuscript I describes development and verification of new lung cancer biomarker discovery approach, method's safety, reproducibility and limitations.

Manuscript II focuses on CXC chemokine ligand levels in peripheral blood samples of lung cancer patients and control individuals, describes diagnostic and prognostic value of CXC chemokines as NSCLC biomarkers and compares them to standard cancer biomarkers.

Manuscript III describes tumor angiogenesis and production of CXC chemokines related to it. Manuscript III also focuses on prognostic value of CXC chemokines.

Manuscript IV summarizes previous research results and focuses on holistic approach for better understanding of the role of CXC chemokines in lung cancer. CXC chemokine ligand gradients, CXC chemokine receptor expression and tumor immune cell infiltrate assessed and described.

Some results were not included in publications due to limited importance or being out of the scope of published manuscripts, and are covered in "Unpublished results" section.

3.2. Manuscript I

Application of novel methods for non-small cell lung cancer (NSCLC) biomarker discovery

Acta Chirgica Latviensis 2013, 13(1): 13–6.

ORIGINAL ARTICLE

Application of Novel Methods for Non-Small Cell Lung Cancer (NSCLC) Biomarker Discovery

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Summary
Introduction. Research in NSCLC biomarker field and application of new methods is essential as it is promising strategy to reduce cancer mortality.
Aim of the study. The aim of the study was to develop new approach for lung cancer biomarker research and introduce novel method into surgical practice, verifying method's safety and evaluate intraoperative and postoperative complications.
Materials and methods. 50 patients with early stage NSCLC undergoing lobectomy were randomized into two groups – experimental (n = 25) and control group (n = 25). In experimental group at the time of thoracotomy and resection of the lung, paired blood samples were obtained from the lobar pulmonary vein draining lung segment containing tumor and from the peripheral vein which represents the systemic circulation. Safety of the procedure and its influence on surgery was evaluated.
Results. New approach for lung cancer biomarker research was developed. Injuries of major anatomical structures as pulmonary blood vessels, bronchi, trachea and pericardium due to blood sample collection were not observed. There was no difference in hospital stay and chest tube drainage duration. There were no perioperative deaths in both groups. Morbidity rate in experimental group was similar to control group.
Conclusions. Application of new method for developing multimodal lung cancer model is safe and does not increase the risk of intraoperative and postoperative complications.
Key words: biomarkers, lung cancer, lung resection.

INTRODUCTION

Lung cancer is the most common cancer worldwide with the number of incident cases around 1.6 million annually (14). Overall, survival rate at 5 years is less than 20% and the search for prognostic factors has led to extensive research and publication of an impressive number of papers. There are plenty of publications in the literature about lung cancer biological markers which can facilitate early detection of lung cancer in such a way improving results of treatment and improving 5-year survival. A blood based biomarkers are attractive targets because blood is easily accessible and measurements may be repeated over time (5, 9, 16). Blood is a medium which carries information about cellular processes, tumor progression and growth, signaling and much more (20). It is enormous amount of data which cannot be processed without building strategy allowing targeted approach. Isolating blood draining tumor gives insight into metabolism of cancer cells. Combination of surgical practice with new research strategies could help to achieve progress in NSCLC biomarker discovery. We describe a novel approach to biomarker discovery that used the same subject as control to identify elevated proteins in the pulmonary venous effluent draining the tumor vascular bed compared to systemic blood. This approach allows the differentlially present proteins to be identified against a complex and variable background of proteomic profile. The analytic issue is reduced to determining what has changed in an individual pre- and post- passage through the affected lung to get around the problem of finding specific biomarkers in blood.

AIM OF THE STUDY

The aim of the study was to develop new approach for lung cancer biomarker research, introduce and incorporate novel method into surgical practice, verify method's safety and evaluate intraoperative and postoperative complications.

MATERIAL AND METHODS

This study was designed as a randomized, case-control study. Development of novel approach was incorporated in a study design of Pauls Stradins Clinical University Hospital Department of Thoracic Surgery scientific project "Investigation of CXC group chemokines – novel diagnostic biomarkers for early stage lung cancer".
Patients with early stage (IA – IIB, 7th edition of TNM in Lung Cancer of the International Association for the Study of Lung Cancer) NSCLC undergoing lung resection with curative intent were involved in the study (n = 50) from January 2010 till December 2012. All patients participating in the study signed informed consent form.
A simple randomization was used. Patients were randomized into two groups – experimental (n = 25) and control group (n = 25). In all patients extent of resection was radical and restricted to lobectomy. Open lobectomy and nodal dissection was performed

in all patients. Steps of standard procedure were as follows - lateral thoracotomy, mobilization of lung, verification of tumor location, division of mediastinal and interlobar pleura, exposure and identification of hilar structures, dissection of pulmonary artery and vein, ligation and division of pulmonary vessels, dissection of lobar bronchus, division and closure of bronchus, haemostasis, drainage and wound closure. In experimental group at the time of thoracotomy and resection of the lung, paired blood samples were obtained from the lobar pulmonary vein draining lung segment containing tumor, and from the peripheral cubital vein which represents the systemic circulation. After dissection of pulmonary vein 5 mL of blood were aspirated with a syringe with 21-gauge needle prior to blood vessel ligation (Fig.1).

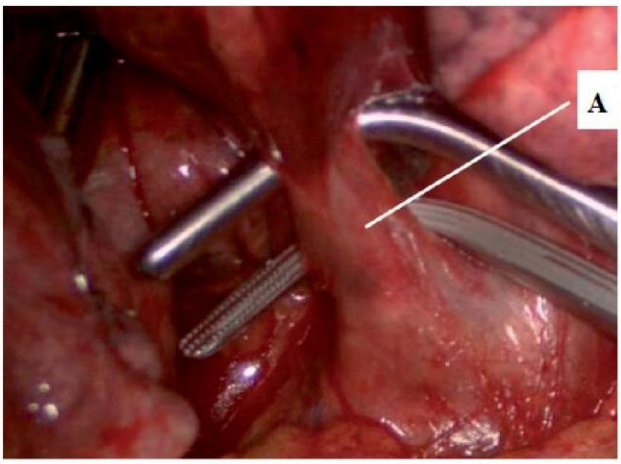

Fig. 1. Anterior view of dissected middle lobe vein of right lung before blood sample aspiration and vein ligation. Pulmonary vein puncture site (A).

Afterwards pulmonary vein was ligated proximally to puncture site. Blood samples were processed during next two hours after collection – centrifuged at 3000 g for 10 minutes and stored at -70°C for further analysis. Safety of procedure and its influence on surgery was evaluated. Various parameters were assessed including duration of surgery, intraoperative blood loss, postoperative hospital stay, duration of chest tube drainage, volume of postoperative fluid drained per day (postoperative day 1 to 3), haemoglobin concentration in blood and pleural fluid on the first postoperative day. Cardiac events were assessed intraoperatively and postoperatively – episodes of arrhythmia and miocardial ischemia were recorded. Data of two groups were compared.

Descriptive statistics and Student's paired t-test were used for the comparison of the means of numerical data.

RESULTS

Duration of surgery in experimental group was similar to control group (148.8 ± 37.1 minutes versus 141 ± 38 minutes, p = 0.47). Intraoperative blood loss was slightly higher in experimental group, but difference was not statistically significant (482 ± 177 ml (ranged 250 – 900 ml) versus 432 ± 146 (ranged 260 – 780 ml), p = 0.28). Volume of postoperative fluid drainage per day was similar in both groups with minor difference on the first day after surgery (Fig. 2).

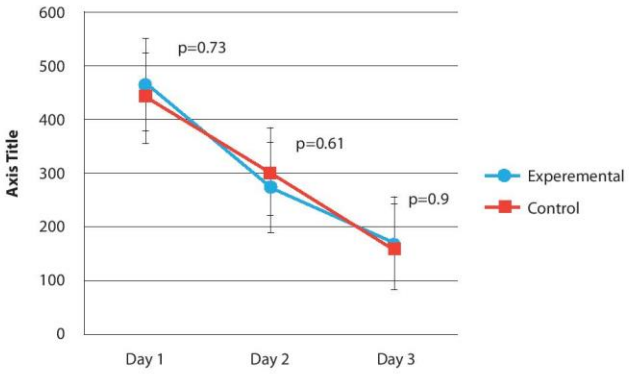

Fig. 2. Volume of postoperative fluid drainage per day (day 1 – 3). Values are expressed as the mean drainage (in milliliters) ± SE.

Haemoglobin concentration in blood and pleural fluid on the first postoperative day was similar in experimental and control groups (Fig. 3).

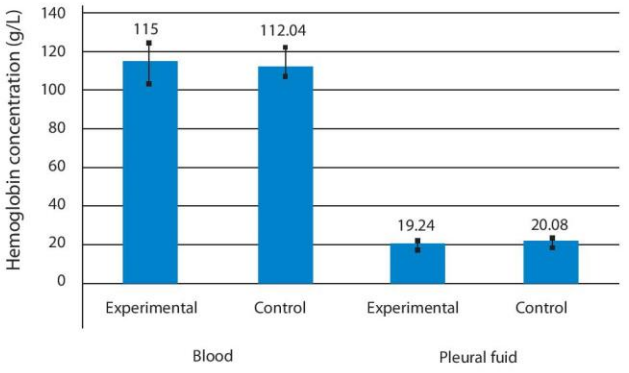

Fig. 3. Haemoglobin concentration in blood and pleural fluid on the first postoperative day. Values are expressed as the mean hemoglobin concentration (g/L) ± SE.

Injuries of major anatomical structures as pulmonary vessels, bronchi, trachea and pericardium due to blood sample collection were not observed. Minor hemorrhage from pulmonary vein needle puncture site was observed in 5 patients (20 %). Duration of bleeding was restricted to several minutes (ranged 1 – 5 minutes) and related to the time necessary for pulmonary vein ligation.

There was no difference in hospital stay duration (7 ± 2 days in experimental group versus 8 ± 3 days in control group, p > 0.05). Chest tube drainage was longer in experimental group (6 ± 4 days) than in control group (5 ± 2), p = 0.04 (Fig. 4).

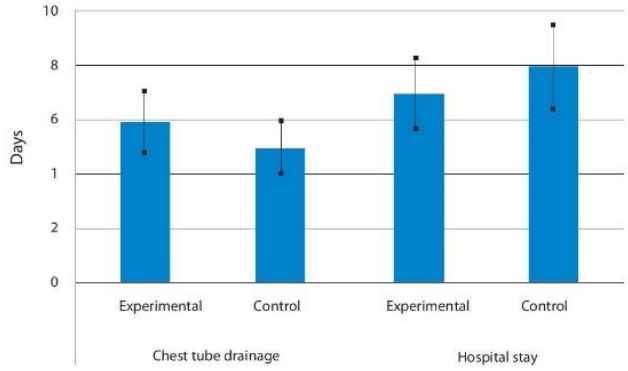

Fig. 4. Hospital stay and chest drainage duration. Values are expressed as the mean number of days ± SE.

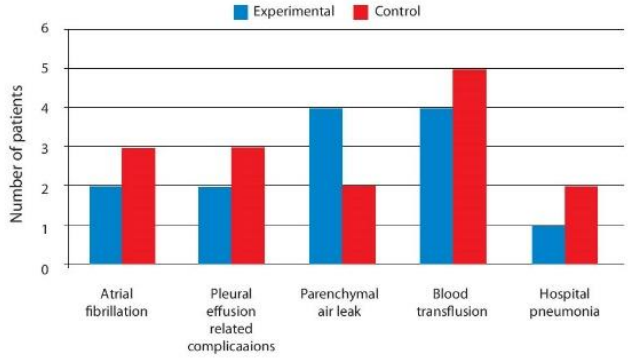

Fig. 5. Postoperative morbidity.

There were no perioperative deaths. Cardiac complications were noted in one of the cases in experimental group (4 %) and three of the cases in control group (12 %) – atrial fibrillation developed and required treatment. In all cases arrhythmia successfully resolved during hospital stay. Four patients from experimental group (16 %) and five patients from control group (20 %) were given blood transfusion.

In two experimental cases (8 %) and three control cases (12 %) effusion-related complications developed that necessitated antibiotic treatment and prolonged drainage. Four patients from experimental group (16 %) and three patients from control group (12 %) had parenchymal air leaks persisting for more than 7 days. There were two cases of hospital pneumonia in control group (8 %) (Fig. 5.).

DISCUSSION

Research in biomarker field and discovery of novel potential biomarkers of NSCLC is essential and important as it is promising strategy to reduce lung cancer mortality. Single peripheral blood sample or *in vitro* tumor models not always adequately reflect complex metabolic processes and cellular interaction which can be found *in vivo*. Molecular theories based on experimental findings *in vitro* have limited impact on progress in cancer research (12, 15). On the other hand many clinical studies fail to isolate particular pathways in cancer cell biology due to complexity of molecular mechanisms, versatile function of receptors and ligands, as well as individual nature of every tumor based on unique genetical information. Up to date there is limited range of methods which make detailed analysis of cancer biology in vivo possible (2). As a rule with few exceptions these methods are invasive and ethical issues narrow spectrum of methods to be applied in human in clinical setting. Applied method allowed us to collect valuable material for further analysis with no impact on morbidity and minimal impact on course of surgical procedure.

Presence of pleural adhesions can sufficiently prolong the time of procedure, but what is more important – excessive tissue trauma during mobilization of lung can cause metabolic changes and affect potential lung cancer biomarker profile (3, 18). One of the main goals during surgery was careful handling of lung tissue, maximally short time interval between skin incision and collecting blood samples to minimize systemic response to general anesthesia and surgical trauma.

Variations of pleural fluid drainage can reflect extent of surgical trauma (4, 6, 11). Chest tube drainage duration was longer in experimental group, but further analysis revealed prevalence of lower lobectomies in experimental group. It is known, that due to anatomical features absorption of pleural fluid is more impaired in case of lower lobectomy than middle or upper lobectomy (8, 13).

Careful handling of pulmonary vessels during surgery is essential (10, 17), because minor tear in blood vessel wall can change course of operation or even affect extent of pulmonary resection. Major risk associated with novel approach is bleeding and injury of hilar structures which did not occur in any of 25 experimental cases. According to our results cardiac complications were not related to experimental procedure and occurred more often in control group, indicating possible relation to surgery itself.

One of main issues is use of analytic methods that do not provide precise and accurate determination of potential tumor specific proteins that are expressed in very low concentrations resulting in false negative results (1) – gaining access to tumor microcirculation and its unique environment holds promise to solve this problem.

CONCLUSIONS

Combination of surgical practice with new research strategies can help to achieve progress in NSCLC biomarker discovery. Pulmonary resection remains a procedure containing a high risk of postoperative complications by itself, but application of new method for developing multimodal lung cancer model is safe and does not increase the risk of intraoperative and postoperative complications.

Conflict of interest: None

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3.3. Manuscript II

Diagnostic value of circulating CXC chemokines in non-small cell lung cancer

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Diagnostic Value of Circulating CXC Chemokines in Non-small Cell Lung Cancer

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Abstract. *Background/Aim: To evaluate the diagnostic value of circulating CXC chemokines as biomarkers for non-small cell lung cancer and compare them against a standard panel of already existing cancer biomarkers. Materials and Methods: A total of 90 individuals were enrolled in the study. We analyzed 30 patients with stage IA-IIB carcinoma of the lung who underwent pulmonary resection, 30 patients with metastatic NSCLC, and 30 healthy volunteers. The biomarkers levels were measured in plasma blood samples, by ELISA and immunoassays. Results: The levels of circulating CXCL4, CXCL8, CXCL9, CXCL10 and CXCL11 were higher and those of circulating CXCL1 were lower in patients with early-stage NSCLC compared to metastatic NSCLC patients and controls ($p<0.05$). CXCL4, CXCL9 and CXCL11 were included in the panel that showed a sensitivity of 100% versus 60% for CEA, CA125 and CYFRA21-1 ($p<0.001$). Conclusion: Combination of CXCL4, CXCL9 and CXCL11 has a high diagnostic value.*

Lung cancer is by far the leading cause of cancer-related mortality globally, with an estimated 1.3 million new cases diagnosed worldwide each year, accounting for nearly 12% of all cancers and an estimated 1.1 million deaths each year (1). A blood-based biomarker is an attractive, non-invasive modality that could complement technologies, such as helical CT scans, in facilitating early detection and treatment of lung cancer. There is currently no single clinical biomarker that meets the sensitivity and specificity criteria required for screening or stratification purposes (2). Other biomarkers, such as carcinoembryonic antigen (CEA) (3), carcinoma antigen 125 (CA125) and cytokeratin-19 fragment (CYFRA 21-1) have been used in clinical practice for many years (4, 5) and there exist many molecules currently undergoing validation processes.

Numerous targets have been identified for research in the field, one of which is a group of C-X-C motif (CXC) chemokines. In the last two decades, studies have demonstrated that CXC chemokines and chemokine receptors can directly enhance or inhibit tumor-associated angiogenesis, promote tumor-related immunity, and enhance organ-specific metastases (6, 7). Malignant cells from different cancer types have different profiles of chemokine ligand and receptor expressions. Unlike other cytokines, chemokine proteins can be detected at picogram levels in the blood, which makes them more attractive targets for investigation in lung cancer biomarker research. There exists strong scientific evidence demonstrating direct or indirect involvement of CXCL4, CXCL7, CXCL8 and CXCL10 in the lung cancer development process (8-11).

The aim of the present study was to evaluate the diagnostic value of circulating CXC chemokines: CXCL1, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11 and CXCL12 as biomarkers in non-small cell lung cancer and compare them against a standard panel of cancer biomarkers including CEA, CA125 and CYFRA 21-1. We hypothesized that NSCLC patients could have a distinct CXC chemokine profile, and levels of circulating CXC chemokines are characteristic for discrete processes of carcinogenesis.

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Key Words: Chemokines, CXC, NSCLC biomarkers, lung cancer.

Materials and Methods

Patients. From June 2010 to June 2011 total of 90 individuals were enrolled in the study. The cohort consisted of 30 asymptomatic patients diagnosed with stage IA–IIB adenocarcinoma and squamous cell carcinoma of the lung who underwent pulmonary resection, 30 patients with metastatic NSCLC, and 30 healthy volunteers. Surgical patients underwent pulmonary resection at our Institution. Postoperative staging of patients was based on the seventh edition of international TNM classification for lung cancer. Histological classification was performed according to the World Health Organization histologic typing of lung tumors (12). The final cohort included 30 asymptomatic patients, diagnosed with stage IA-IIB NSCLC. Two age-, sex-and smoking history-matched control groups were established; 30 patients with metastatic NSCLC and 30 healthy blood donors. Patients' demographics and cancer characteristics are provided in Table I.

This prospective study was conducted in accordance with the ethical standards of the Helsinki Declaration of the World Medical Association and with full approval by the Pauls Stradins Clinical University Hospital Foundation Ethics committee. All patients' data were obtained after informed consent by the patient.

Measurement of plasma CXC chemokine concentrations. Double blood samples of subjects were drawn from the cubital vein into 5-ml vacutainer tubes using a standard technique. In the surgical group, blood samples were obtained prior to surgery. Tubes were centrifuged at room temperature for 10 min at 1,300 × *g*. Following centrifugation plasma was immediately archived at –70˚C pending utilization. Prior to ELISA assay samples were de-frosted at room temperature and processed according to the ELISA kit manufacturer (Raybiotech, Norcross, GA, USA) protocol (CXCL1/growth regulated oncogene-alpha (GRO-α), CXCL4/platelet factor 4 (PF4), CXCL5/epithelial-derived neutrophil-activating peptide 78 (ENA78), CXCL6/granulocyte chemotactic protein-2 (GCP2), CXCL7/ neutrophil-activating protein-2 (NAP2), CXCL8/interleukin-8 (IL-8), CXCL9/monokine induced by gamma interferon (MIG), CXCL10/ interferon gamma-induced protein 10 (IP10), CXCL11/interferon-inducible T-cell alpha chemoattractant (I-TAC) and CXCL12/stromal cell-derived factor 1 beta (SDF-1). Measurements were obtained twice per each sample. Measurements outside the working range for any given assay were excluded and may result in minor fluctuations in the cohort numbers. The CXC chemokine concentrations in plasma were calculated from the standard curve.

CEA, CA125 and CYFRA 21-1 levels were measured in the plasma of early-stage NSCLC patients by immunoassay in a certified clinical laboratory (normal range=CEA <5 ng/ml, CA 125 <35 U/ml, CYFRA 21-1 <2.08 ng/ml).

Statistical methods. Prior to analysis, data normality was assessed using the Kolmogorov-Smirnov test that indicated necessity for non-parametric test application. Biomarker levels were statistically evaluated for median differences in concentration by the Kruskal-Wallis test and a *p*-value under 0.05 was considered significant. Sensitivity and specificity of CXC chemokine levels were calculated using receiver-operating characteristics (ROC) curve analysis. The area under the ROC curve (AUC) was measured to assess discriminatory power of test. Test results with an AUC less than 0.7 were considered not clinically-useful. Test results with an AUC more than 0.85 were considered of high clinical value. Based on AUC values, a panel of three most clinically significant CXC chemokines were selected, and compared with standard lung cancer biomarker panel consisting of CEA, CYFRA 21-1 and CA 125 in the early-stage NSCLC group. The associations of clinical variables with individual biomarker levels were assessed by the Spearman's correlation coefficient, values between –0.4 and 0.4 were considered non-significant.

Table I. *Patients' demographics and tumor characteristics.*

Characteristics	Early stage NSCLC*		Metastatic NSCLC		Healthy donors	
	n	%	n	%	n	%
Age (years)						
Median	62.5	N/A	64.1	N/A	59.8	N/A
Range	50-78	N/A	52-76	N/A	49-65	N/A
Gender						
Male	17	56.6	16	53.4	18	60
Female	13	43.4	14	46.6	12	40
Smoking						
Never	0	0	1	3.4	3	10
Former	10	33.4	12	40	12	40
Current	20	66.6	21	56.6	15	50
Stage						
IA	8	26.6	N/A	N/A	N/A	N/A
IB	12	40	N/A	N/A	N/A	N/A
IIA	6	20	N/A	N/A	N/A	N/A
IIB	4	13.4	N/A	N/A	N/A	N/A
IV	N/A	N/A	30	100	N/A	N/A
Histology						
AC	13	43.4	15	50	N/A	N/A
SCC	17	56.6	15	50	N/A	N/A

*Early-stage NSCLC defined as clinically diagnosed at stage IA, IB, IIA and IIB. AC, Adenocarcinoma; SCC, squamous carcinoma.

Results

Results of the present study indicating that ELISA kits provided reproducible results and the observed biomarker levels are presented in Table II.

We observed significant differences in levels of circulating CXCL1, CXCL4, CXCL8, CXCL9, CXCL10 and CXCL11 in patients with early-stage NSCLC compared to metastatic NSCLC patients and controls. Interestingly there was no difference in CXCL9, CXCL10 and CXCL11 levels between patients with metastatic NSCLC and healthy controls. Representative box-and-whisker plots of CXC chemokine levels are shown in Figure 1. No statistically significant correlation between CXC chemokine levels and clinical parameters, such as age, sex, smoking, T stage, N stage and histological sub-type of NSCLC was noted ($p>0.05$).

ROC curve analysis showed that CXCL1, CXCL4, CXCL8, CXCL9, CXCL10 and CXCL11 are statistically significantly, sensitive and specific with the area under the ROC curve more than 0.7 (Table III).

Table II. *Levels of CXC chemokines in the plasma of patients.*

	Early-stage NSCLC		Metastatic NSCLC		Healthy donors	
Chemokine	Median (pg/ml)	Range	Median (pg/ml)	Range	Median (pg/ml)	Range
CXCL1	25	6-130	42.5	22-127	40	21-98
CXCL4	12,899.1	9307.5-17,500	11000	8,030-15,512.5	9,495	7884-11680
CXCL5	700	160-4,000	950	280-1900	880	380-2100
CXCL6	142	19-2,500	120.5	39-395	153	42-490
CXCL7	3525.5	2,520-4,277.5	3721.3	2,972.5-3,960	3315	3,030 –3,812.5
CXCL8	2.2	0.5-26	7.1	0.5-26	4.5	0.5-19.5
CXCL9	505	120-3,800	937.5	50-3,900	165	88-450
CXCL10	10	3-41	11	7-40	8.6	2-12
CXCL11	5.5	0.1-39	4	0.1-75	0.1	0.1-4.9
CXCL12	27.2	14-172	28.4	24.3-39.5	27.4	26-30

Table III. *ROC curve analysis.*

Chemokine	Sensitivity	Specificity	Criterion	AUC	95% CI	*p*-Value
CXCL1	48.4	92.8	≤24	0.72	0.56-0.84	<0.01
CXCL4	80.6	92.8	>11,059	0.91	0.79-0.98	<0.001
CXCL5	25.8	100	<340	0.59	0.44-0.74	0.212
CXCL6	29.1	92.8	<80	0.56	0.41-0.71	0.607
CXCL7	58.1	85.7	>3,450	0.61	0.45-0.75	0.302
CXCL8	70.9	78.6	≤3	0.74	0.59-0.86	<0.05
CXCL9	61.3	100	>450	0.86	0.73-0.95	<0.001
CXCL10	35.5	100	>12	0.75	0.58-0.86	<0.001
CXCL11	51.6	100	>4,9	0.8	0.65-0.91	<0.001
CXCL12	41.9	92.8	≤26	0.57	0.41-0.72	0.403

Based on the AUC values, we selected the three CXC chemokines with the highest values – CXCL4 (AUC=0.91), CXCL9 (AUC=0.86) and CXCL11 (AUC=0.8) to include in our biomarker panel.

CXC chemokine levels were interpreted as positive and negative based on criterion value corresponding with the Youden index. CEA, CA125 and CYFRA 21-1 levels were also interpreted as positive and negative based on the given normal range. All three CXC chemokines were positive in 9 patients (30%), two chemokines were positive in 13 patients (43.3%), and a single biomarker was positive in 8 patients (26.7%). There were no negative cases in the CXC chemokine panel. In contrast, application of the standard biomarker panel (CEA, CA125 and CYFRA 21-1) showed that all three biomarkers were positive in 3 cases (10%), two biomarkers were positive in 2 cases (6.7%), and a single biomarker was positive in 13 patients (43.3%). There were 12 negative cases (40%). The sensitivity of the CXC chemokine panel was calculated as 100% *versus* 60% for CEA, CA125 and CYFRA 21-1 ($p<0.001$).

Discussion

To date, there has not been any validated biomarker to show adequate sensitivity, specificity, and reproducibility to be used for the early detection and treatment of lung cancer. Cancers have a complex chemokine network that influences the immune-cell infiltration of a tumor, as well as tumor cell growth, survival and migration, and angiogenesis. CXC levels might be elevated in different cancer types, but one would speculate a chemokine profile or molecular fingerprint specific to cancer type. Tumor-related proteins may be secreted into the peripheral circulation of patients with cancer and are detectable by protein analysis.

Our findings suggest the most sensitive and specific diagnostic biomarkers in the early-stage NSCLC patient group, were CXCL4, CXCL9, CXCL10 and CXCL11. Results were statistically significant despite the small patient cohort. Interestingly, all four chemokines belong to the sub-group of ELR- CXC chemokines. The NH_2-terminus of the majority of the CXC chemokines contain three aminoacid residues (Glu-

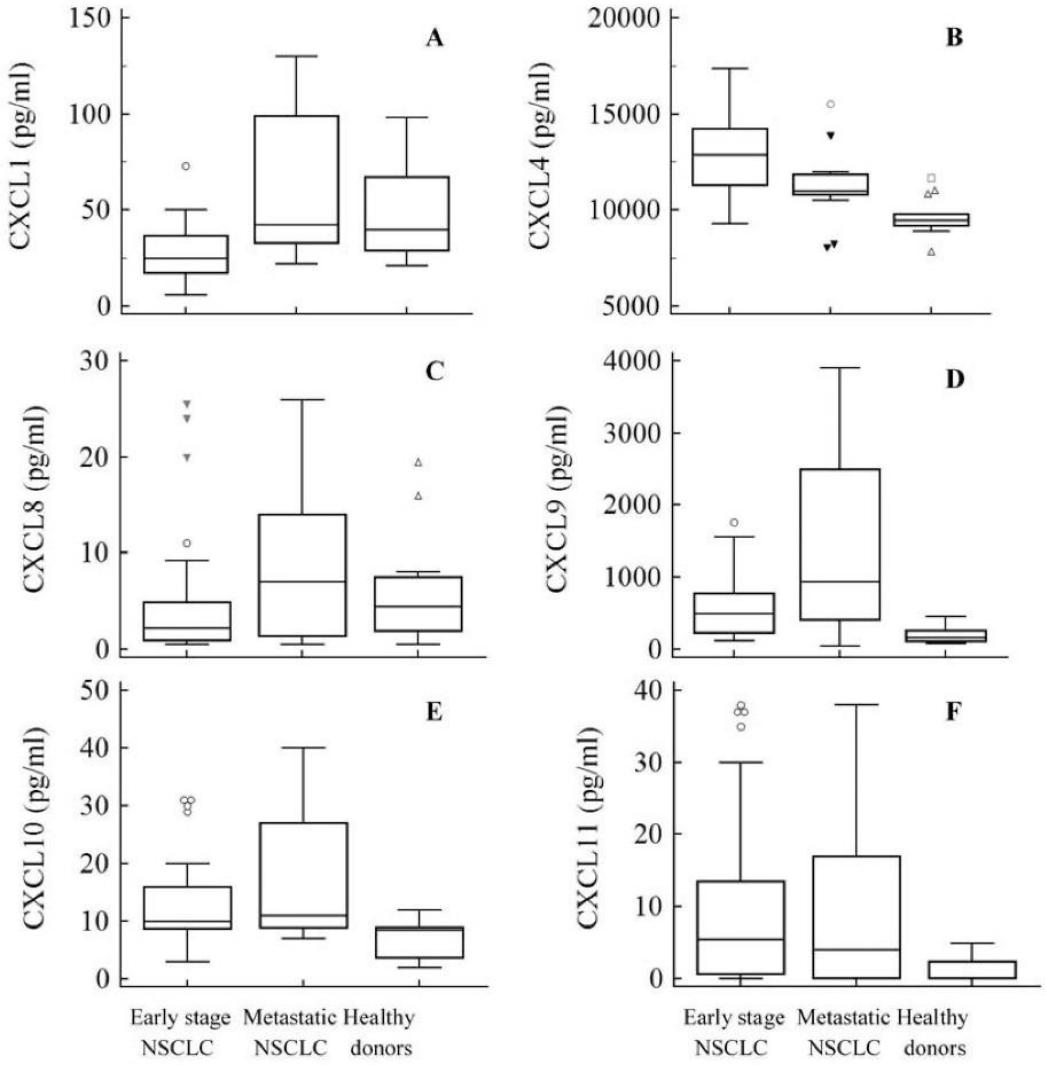

Figure 1. *Multiple comparison box and whisker plots representing CXC chemokine levels – plot A - CXCL1 ($p<0.01$), plot B - CXCL4 ($p<0.001$), plot C - CXCL8 ($p<0.05$), plot D - CXCL9 ($p<0.001$), plot E - CXCL10 ($p<0.01$), and plot F - CXCL11 ($p<0.01$). (Circles and triangles represent outside and far out values).*

Leu-Arg: the "ELR" motif) preceding the first cysteine amino-acid residue of the primary structure of these cytokines (13, 14). The family members that contain the ELR motif (ELR^+): CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8 are potent promoters of angiogenesis (15). In contrast, members that lack the ELR motif (ELR^-):CXCL4, CXCL9, CXCL10 and CXCL11 are potent inhibitors of angiogenesis. This difference suggests, on a structural and functional level, that members of the chemokine family are unique cytokines regarding their ability to behave in a disparate manner in the regulation of angiogenesis.

There exists limited scientific evidence on the diagnostic value of CXC chemokines. Previous reports have shown that CXCL4 (16) and CXCL7 contribute in tumor growth and development as well as in tumor angiogenesis in lung cancer (17) and other cancer cell types (18, 19), but CXCL8 levels can also be indicative of lung cancer risk several years before diagnosis (20). Our results did not confirm the diagnostic value of CXCL1, CXCL5, CXCL7, and CXCL12.

Recent studies have indicated that CEA and CYFRA 21-1 are reliable serum tumor markers for the diagnosis of lung cancer in addition to CT scans when combined or used individually at twice the standard cut-off level in high-prevalence rate groups (21). Despite that fact, standard biomarkers including CEA, CA125 and CYFRA 21-1 that are still widely used in clinical practice (22), did not show high

sensitivity and were negative in 40% of cases, when only single biomarkers were positive in 43% of cases. The panel of CXC chemokines was much more sensitive and reliable than the panel of standard biomarkers, but further larger multi-institutional studies are required for validation of our findings. CXC chemokine profile assessment may be effective in high-risk patients and patients undergoing diagnostic investigations for possible NSCLC.

A limitation of the study is reporting on a single-Centre experience and therefore on a relatively small number of patients. We found an increased expression of CXCL4, CXCL8, CXCL9, CXCL10 and CXCL11 in plasma specimens of early-stage NSCLC patients compared to control subjects. The findings also demonstrated that a combination of CXCL4, CXCL9 and CXCL11 has a better diagnostic value in NSCLC than each marker separately, as NSCLC may have a specific molecular fingerprint. Further studies are required to determine if these chemokines can be utilized in a blood-based biomarker panel for the diagnosis of lung cancer.

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Conflicts of Interest

None.

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3.4. Manuscript III

CXC chemokine ligand 4 (CXCL4) is predictor of tumour angiogenic activity and prognostic biomarker in non-small cell lung cancer (NSCLC) patients undergoing surgical treatment

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RESEARCH ARTICLE

CXC chemokine ligand 4 (CXCL4) is predictor of tumour angiogenic activity and prognostic biomarker in non-small cell lung cancer (NSCLC) patients undergoing surgical treatment

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ABSTRACT

Objective: To evaluate the association of CXC chemokine ligand 4 (CXCL4) plasma levels with tumour angiogenesis in non-small cell lung cancer (NSCLC) and to assess association of CXCL4 with clinical outcomes.

Patients and methods: Fifty patients with early stage NSCLC who underwent pulmonary resection. CXCL4 levels were analysed by ELISA. Angiogenesis was assessed by immunohistochemistry, and microvessel density (MVD) count.

Results: There was positive correlation between MVD and CXCL4 levels. Patients with higher CXCL4 levels had worse overall and disease-free survival.

Conclusions: Plasma levels of CXCL4 are associated with tumour vascularity. Increased CXCL4 levels in NSCLC patients undergoing treatment may indicate active cancer-induced angiogenesis associated with relapse and worse outcome.

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Angiogenesis; chemokines; CXC chemokine ligand 4; non-small cell lung cancer; platelet factor 4

Introduction

Lung cancer is the most common malignant tumour and the leading cause of cancer death worldwide, with approximately 1.6 million new cases and 1.38 million deaths annually (Jemal et al., 2011). Treatment outcomes for non-small cell lung cancer (NSCLC) patients are in general disappointing.

One of the promising approaches which could facilitate NSCLC management is assessment of tumour angiogenic activity. There is a considerable evidence associating angiogenesis with tumour growth and metastasis (Jain et al., 2009a,b), and efforts are on-going to identify the best method for evaluation of tumour angiogenic activity. Direct assessment of angiogenesis requires measuring of microvessel density (MVD) in tumour tissue which is invasive, complicated and a time-consuming technique. At the same time, the process of angiogenesis can be assessed indirectly – by measuring levels of specific blood-based biomarkers involved in the process of microvessel formation, maturation and remodelling (Maeda et al., 2013).

Previous studies carried out by our group suggested the potential role of CXC chemokine ligand 4 (CXCL4) as a useful diagnostic and prognostic biomarker in NSCLC (Spaks et al., 2012a,b,c). The aim of our study was to evaluate whether measuring CXCL4 plasma levels can adequately reflect angiogenic activity of tumour and replace invasive techniques, broadening clinical application of the method. A secondary objective for the study was to evaluate the associations of CXCL4 with clinical outcomes, including disease-free survival and overall survival.

Methods

Patients

Between June 2010 and September 2011, 50 patients with NSCLC (preoperative clinical and radiological stages IA–IIB, lung cancer TNM staging 7th edition) were enrolled in a single centre prospective study. All patients underwent radical pulmonary resection – open lobectomy with mediastinal lymph node dissection without neoadjuvant and/or adjuvant therapy. We excluded patients with a high probability of micrometastatic disease (postoperative pathological stage IIIA and above). Patients were followed-up every six months after surgery for up to four years. General status of the patient and the information about disease outcome and relapse was obtained during follow-up visits, and confirmed with the

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National Cancer registry. The final collection of survival data was in November 2014.

This study has been approved by Pauls Stradins Clinical University Hospital Foundation ethical committee, and was conducted in accordance with the ethical standards of the Helsinki Declaration of the World Medical Association. The informed consent was obtained from all patients.

Methods

Prior to surgery peripheral blood samples were taken from cubital vein into 5 ml vacutainer tubes using a standard phlebotomy technique. Tubes were centrifuged at room temperature for 10 min at 1300*g*. Following centrifugation plasma was immediately archived at −70 °C pending utilization. Prior to ELISA samples were defrosted at room temperature and processed according to ELISA kit manufacturer protocol. CXCL4 levels were determined by ELISA kit with sensitivity 20 pg/ml and detection range 20–15,000 pg/ml (Raybiotech, Norcross). Each standard or sample was assayed in duplicate. The CXC chemokine concentrations in plasma were calculated from the standard curve. CXCL4 levels are given as mean ± SD.

Resected tumour tissue samples were fixed in formalin and embedded in paraffin. Paraffin-embedded tissue sections were dewaxed in xylene and rehydrated through graded concentrations of ethanol. Tumour tissue samples were stained with haematoxylin and eosin, histological classification was performed according to the World Health Organization histological typing of lung tumours (Travis et al., 1999). After reviewing the haematoxylin- and eosin-stained slides of the tumour specimens, we selected blocks of the invasive edge in the tumour area. Antigen retrieval was performed with 0.1% trypsin/CaCl2 for 30 min at 37 °C. Samples were then stained with primary antibody for CD34. Primary antibody used in the present study was rabbit polyclonal antibody to CD34 (Santa Cruz Biotechnology, Dallas) diluted 100-fold. Antigens were visualized with Dako EnVision kit and diaminobenzidine (DAB) as the chromogen (Dako, CA) as suggested by the manufacturer.

To determine MVD 20 high-power fields (400× magnification) with total area of 4.8 mm^2 were examined per tumour tissue sample. MVD values are given as mean number of microvessels per $mm^2$and SD.

Survival time was measured from the date of surgery. Disease-free survival is defined as the interval from anatomic resection to clinical or radiographic demonstration of the recurrence or censorship. Overall survival is defined as the interval from treatment initiation to death or censorship. Survival curves are estimated by the Kaplan–Meier method and are compared across groups by the log rank test. The Student's *t*-test for differences in mean values was used for comparison of nominal data. The effects of CXCL4 plasma levels, MVD of tumour, pathology type on overall survival and disease-free interval were assessed. Associations between variables were analysed with Pearson correlation test. All *p* values considered are two-sided, and *p* values less than 0.05 are marked as significant.

Results

All the 50 patients were restaged after surgery. Postoperative pathologic TNM stage confirmed the preoperative staging in 44 (88%) cases, stage IIIA disease was due to unexpected N2 disease in 6 (12%) cases which were excluded from the cohort. There were four main tumour histological subtypes – adenocarcinoma (AC), squamous cell carcinoma (SCC), large cell carcinoma (LCC) and pleomorphic carcinoma (PC). SCC and AC were the most frequent histologic tumour subtypes (45.5% and 34%). The basic clinical and pathologic features of patients are given in Table 1.

Mean level of CXCL4 observed in plasma samples was 12.7 ± 2.5 pg/ml. Patients with no relapse had lower CXCL4 levels (mean 11.8 ± 1.8 pg/ml, range 9.4–14.2) at the time of surgery than patients who relapsed (mean 14 ± 2.8 pg/ml, range 9.8–22.6) ($p < 0.05$) (Figure 1).

Average MVD values for tumour tissue samples were 14 ± 7.5 microvessels per mm^2 of tumour tissue. More active angiogenesis was found in tumour tissue samples from patients with relapse (16.3 ± 2.3 blood vessels per mm^2) com-

Table 1. Patient demographics, postoperative staging and histology.

	Value	
Characteristics	*n*	%
Age (years)		
Median	62.5	N/A
Range	50–78	N/A
Gender		
Male	36	82
Female	8	18
Smoking		
Never	0	0
Former	10	22.7
Current	34	77.3
Stage		
IA	14	31.8
IB	16	36.4
IIA	9	20.5
IIB	5	11.3
Histology		
AC	15	34
SCC	20	45.5
LCC	5	11.4
PC	4	9.1

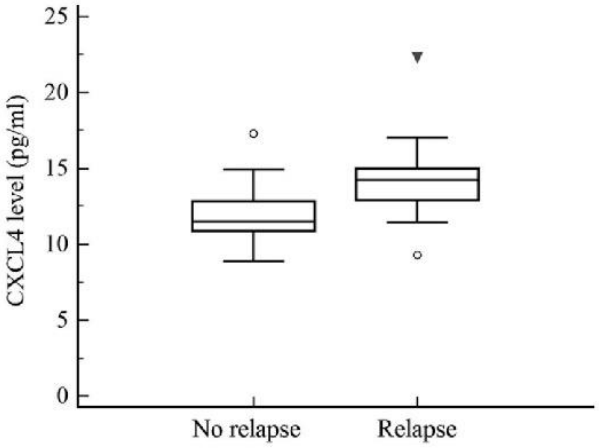

Figure 1. Levels of CXCL4 in blood samples.

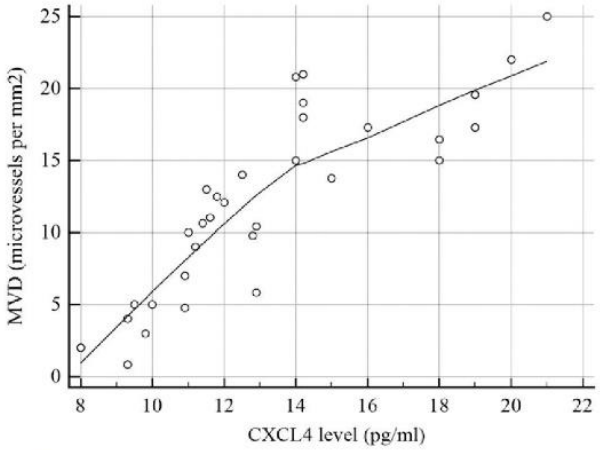

Figure 2. MVD and CXCL4 correlation.

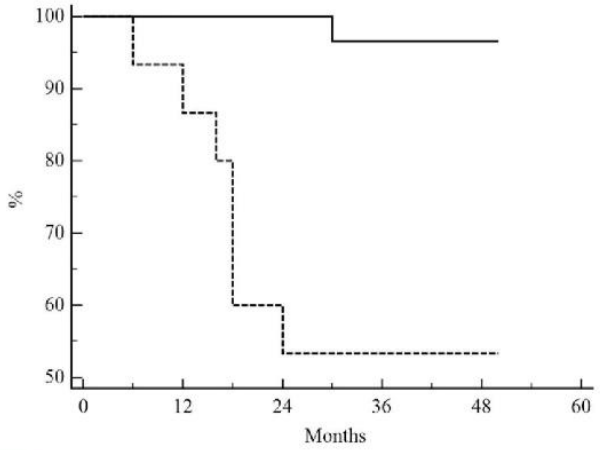

Figure 3. Overall survival curve (solid line – CXCL4 < 12.5 pg/ml; dotted line – CXCL4 ≥ 12.5 mg/ml); $\chi^2 = 13.7$, $p < 0.001$).

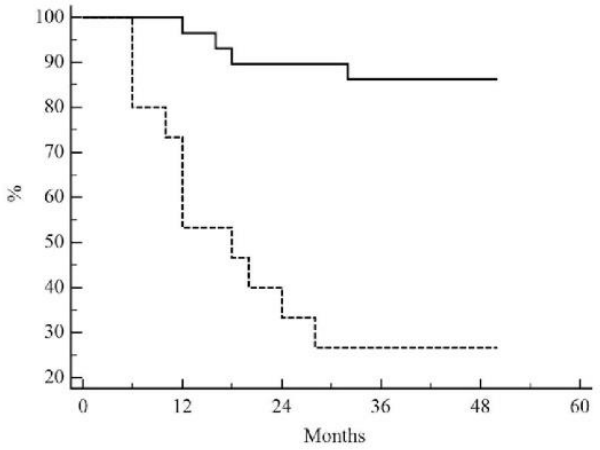

Figure 4. Disease-free survival curve (solid line – CXCL4 < 12.5 pg/ml; dotted line – CXCL4 ≥ 12.5 mg/ml); $\chi^2 = 13.7$, $p < 0.001$).

pared to patients with no relapse (12.4 ± 0.9 blood vessels per mm^2) ($p < 0.05$).

There was positive correlation between MVD rate and CXCL4 plasma levels: $r = 0.6$ ($p < 0.01$). The analysis of histological subtypes showed stronger correlation in cases of AC – $r = 0.84$ ($p < 0.01$), correlation was weaker in other histological subtypes – SCC ($r = 0.57$, $p < 0.01$), LCC ($r = 0.46$, $p > 0.05$) and PC ($r = 0.7$, $p > 0.05$). Higher CXCL4 plasma levels were closely associated with higher MVD (Figure 2) rates and increased risk of relapse ($p < 0.01$). There was no gender correlation with CXCL4 levels ($r = -0.09$, $p = 0.55$) and MVD rates ($r = 0.25$, $p = 0.12$).

Median follow-up time was 40 months (range 34–50 months). There was no postoperative mortality. At the time of final collection of survival data, 36 (82%) patients were alive: 29 patients (66%) were alive and disease free, 15 patients (34%) had disease recurrence, and eight deaths. The deaths were cancer related in five (62.5%) patients. Average time to relapse was 27.7 ± 12.4 months. Actuarial overall survival at one and three years was 97.7% and 79.5%, respectively. In terms of disease-free survival, we found high levels of CXCL4 and increased MVD to be significantly associated with poor outcomes. Figures 3 and 4 illustrate the Kaplan–Meier survival curves for patients with high and low levels of CXCL4, the cut-off value for CXCL4 was 12.5 pg/ml. Associations of CXCL4 levels with clinical parameters were weak and not statistically significant.

Discussion

Angiogenesis is the formation of new blood vessels from pre-existing ones. There is an active process of angiogenesis in a number of diseases, including cancer. Many solid tumours depend on an extensive newly formed vascular network to become nourished and to expand (Maeda et al., 2013; Piuda & Parkinson, 1996). Angiogenesis is an important factor that has been shown to correlate with tumour malignancy and was demonstrated as a prognostic indicator for a wide range of cancers, including NSCLC. The formation of a new vascular bed around the tumour maintains tumour growth and also facilitates the escape of tumour cells, a small number of which may develop into metastases and dictate a more aggressive course of the disease. Our particular interest in this study was assessment of angiogenic activity of tumour using direct and indirect methods. MVD can be evaluated to assess angiogenic activity of tumour directly (Kreuter et al., 2009; Meert et al., 2002). Intratumoral blood vessels can be observed by immunohistochemical detection of specific endothelial markers such as CD34, CD31, D2-40/Podoplanin and Factor VIII. After immunohistochemical analysis, microscopy can quantify the density of blood vessel networks (Choi et al., 2005; Jain et al., 2009a; Mineo et al., 2004; Pathak et al., 2008). At the same time measuring, MVD is difficult and a time-consuming technique which requires invasive methods for obtaining adequate tumour tissue samples. We found that measuring CXCL4 plasma levels is simple and less invasive technique which can adequately reflect angiogenic activity of tumour.

Many clinical studies showed a positive association between tumour angiogenesis and tumour aggressiveness in the carcinoma of the breast, lung, and prostate, malignant melanoma and other solid tumours (Donnem et al., 2008; Oldenhuis et al., 2008). Several studies have demonstrated

an association between MVD and patient outcomes in NSCLC. In a retrospective study of 223 patients with operable NSCLC (stages IA–IIIA), higher MVD was a significant prognostic factor by univariate (hazard ratio 2.34; $p = 0.0001$) and multivariate analysis (hazard ratio 2.080; $p = 0.039$) (Han et al., 2001; Jain et al., 2009b; Kadota et al., 2008; Mineo et al., 2004; O'Byrne et al., 2000). A meta-analysis of published literature demonstrated that a high microvessel count within lung tumours was a poor prognostic factor for survival in patients with NSCLC. There are on-going debates about the usefulness of MVD for evaluation of angiogenesis, this method still lacks standardization. One of the greatest disadvantages of MVD measurement is invasiveness of method – it can be applied only when sufficient tissue specimen is available – in practice it limits employment of methods to surgically treated groups of patients which is rather small in the case of NSCLC. A less invasive and much more accessible method is required to determine the activity of tumour angiogenesis in whole groups of patients with NSCLC eligible for treatment. Although angiogenic biomarkers in NSCLC are not yet validated or used in clinical practice, several measures and mediators of angiogenesis are under investigation (Han et al., 2001; Jain et al., 2009b; Kadota et al., 2008) and one potential candidate is a group of CXC chemokines. Members of the CXC chemokine family function in a disparate manner and can behave as either potent angiogenic or angiostatic factors in regulating neovascularization (Strieter et al., 1999). CXCL4 was the first CXC chemokine reported to regulate angiogenesis. CXCL4 was found to inhibit bFGF-induced angiogenesis and attenuate growth of melanoma and colon carcinomas in a murine model of tumorigenesis (Hansell et al., 1995). Blood biomarkers like CXCL4 are easier accessible targets. Measurements may be made before and after treatment and repeated during follow-up periods. CXCL4 levels can be used for monitoring response to antiangiogenic therapy. Prognostic biomarkers like CXCL4 could guide personalized use of antiangiogenic agents for NSCLC.

However, the limitation of this study is reporting a single centre experience and therefore a relatively small number of patients, therefore statistical significance of results for rare subtypes of NSCLC was not sufficient. Higher incidence of NSCLC in males makes gender analysis less applicable in small cohorts like ours.

In conclusion, there are no reliable prognostic or predictive angiogenic markers in the NSCLC population. Our study showed that CXCL4 could be useful prognostic biomarker in NSCLC which reflects angiogenic activity of tumour. Increased CXCL4 plasma levels in NSCLC patients undergoing treatment may indicate active cancer-induced angiogenesis associated with worse long-term outcome. Large prospective clinical trials are needed to evaluate candidate biomarkers, and additional confirmatory studies will be necessary for validation.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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3.5. Manuscript IV

Role of CXC group chemokines in lung cancer development and progression

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Original Article

Role of CXC group chemokines in lung cancer development and progression

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Background: Clinical and translational research on lung cancer patients undergoing surgical treatment can provide valuable scientific data and unique opportunity to study tumor microenvironment. CXC chemokines, which are members of a big family of cytokines, are undoubtedly involved in tumor growth regulation and metastasizing pathways. For better understanding of CXC chemokine involvement in the process of carcinogenesis we have studied the cohort of early stage non-small cell lung cancer patients undergoing surgery with curative intent. Our aim was to assess CXC chemokine ligand (CXCL) levels in patient blood samples representing systemic circulation and tumor microenvironment; assess CXC chemokine receptor (CXCR) expression in tumor tissue; and measure tumor infiltrating immune cell subpopulations.

Methods: A total of 54 patients with NSCLC had radical lung resection were enrolled in a single center prospective study and were followed-up annually for up to six years. During surgical procedure peripheral and tumor draining blood samples were taken. CXCL1, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11 and CXCL12 levels were determined by ELISA, and chemokine concentration gradient was calculated. Tumor infiltrating immune cells (T helper cells, T cytotoxic cells, macrophages, B cells, plasma cells) and expression of CXCR1, CXCR2, CXCR3 and CXCR4 in tumor tissue were assessed by immunohistochemistry.

Results: Statistically significant decrease in chemokine concentration was found for CXCL4 (P=0.002) and CXCL5 (P=0.011), and statistically significant concentration increase was found for CXCL7 (P=0.001) in total cohort. We have found statistically significant CXC chemokine concentration change for majority of chemokines—CXCL1 (P=0.002), CXCL4 (P=0.001), CXCL5 (P=0.013), CXCL7 (P=0.036), CXCL8 (P=0.026), CXCL9 (P=0.034) and CXCL10 (P=0.032) in a group of patients who had good clinical result after surgery with no evidence of relapse, on the other hand patients with cancer recurrence including local and systemic cancer spread did not show any change of chemokine concentration in blood except for CXCL1 (P=0.041). We have also found that chemokine levels and gradients correlate with CXC receptor expression and number of tumor infiltrating immune cell subpopulations

Conclusions: Assessment of tumor microcirculation is useful for evaluation of different types of circulating biomarkers and application of our method can be very wide, integrating thoracic surgeons into translational cancer research.

Keywords: CXC chemokines; lung cancer; immune cell infiltrate; cancer biomarkers

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Introduction

Lung cancer is by far the leading cause of cancer death among both men and women; about one out of four cancer deaths are from lung cancer (1). Understanding of cancerogenesis requires a holistic approach including, analysis of epidemiological and clinical data, molecular testing of blood biomarkers and thorough analysis of tumor cells and immune cell infiltrate of the tumor. Data acquired over the last few decades has confirmed heterogeneity of cancer as a disease (2) which indicates there is no single perfect biomarker for lung cancer, but a panel of multiple parameters (3,4) could be a much more objective diagnostic and prognostic tool.

Solid tumors like lung cancer contain in addition to tumor cells, also various types of stromal cells, such as fibroblasts and endothelial cells. Moreover, tumors are infiltrated by inflammatory cells, including neutrophils, macrophages and lymphocytes (5). Tumor cells, stromal cells, as well as the tumor-associated leukocytes contribute to the local production of chemokines inside the tumor and affect systemic circulating chemokine levels (*Figure 1*).

In our study we have focused on a group of CXC chemokines, which are members of a big family of cytokines undoubtedly involved in tumor growth regulation and metastasizing pathways (6). Previous research by our group has confirmed diagnostic and prognostic value of CXC chemokines as biomarkers (7,8).

Clinical and translational research on lung cancer patients undergoing surgical treatment can provide valuable scientific data and unique opportunity to study tumor microenvironment. For better understanding of CXC chemokine involvement in the process of carcinogenesis we have studied the cohort of early stage non-small cell lung cancer patients undergoing surgery with curative intent. Our aim was to assess CXC chemokine ligand (CXCL) levels in patient blood samples representing systemic and tumor microenvironment; and subsequently calculate chemokine concentration gradients in blood after passing through tumor vessel bed in order to determine chemokine involvement in the process of cancerogenesis. To ensure holistic approach our additional goal was to determine CXC chemokine receptor (CXCR) expression intensity in tumor tissue, and assess composition of tumor immune cell (TIC) infiltrate.

Methods

Patients

Between June 2010 and December 2011, patients with NSCLC (n=54) who had radical lung resection (open lobectomy with lymphadenectomy) and postoperative clinical, radiological and pathological stage IA–IIB adenocarcinoma (n=26) or squamous lung cancer (n=28) were enrolled in a single center prospective study. There were 18 women in the cohort. All patients had no neoadjuvant and/or adjuvant therapy. Patients were followed-up annually after surgery for up to six years. General status of the patient and the information about disease outcome and relapse was obtained during follow-up visits, and confirmed with the National Cancer registry. The final collection of data in September 2016 showed relapse in 24 patients (44%).

Methods

During surgical procedure peripheral blood samples were taken from cubital vein into 5 mL vacutainer tubes using standard phlebotomy technique, simultaneously tumor draining blood samples were collected from pulmonary vein of lung lobe being resected (9). Tubes were centrifuged at room temperature for 10 minutes at 1,300 g. Following centrifugation plasma was immediately archived at –70 °C pending utilization. Prior to ELISA assay samples were defrosted at room temperature and processed according to ELISA kit manufacturer protocol. CXCL1, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11 and CXCL12 levels were determined by ELISA kit with sensitivity 1 pg/mL and detection range 1–15,000 pg/mL (Raybiotech, USA, USA). Each standard or sample was assayed in duplicate. The CXC chemokine concentrations in plasma were calculated from the standard curve. Chemokine levels are given as mean with 95% confidence intervals.

Difference in circulating chemokine concentrations between systemic and tumor vascular beds can be described as chemokine concentration gradient. Chemokine gradient was calculated according to the formula: ((TCV-PCV)/PCV) ×100, where TCV—tumor circulation representing value, PCV—peripheral circulation representing value.

TCV and PCV values were compared using two-tailed paired *t*-test. CXC chemokine level gradients were compared between patients with remission and patients with relapse using unpaired two-tailed *t*-test.

Expression of CXCR in tumor tissue and TIC infiltrate were assessed by immunohistochemistry. Resected tumor tissue samples were fixed in formalin and embedded in paraffin. Samples were cut into 3–4 μm sections, dried and dewaxed. The slides were then incubated in hematoxylin and

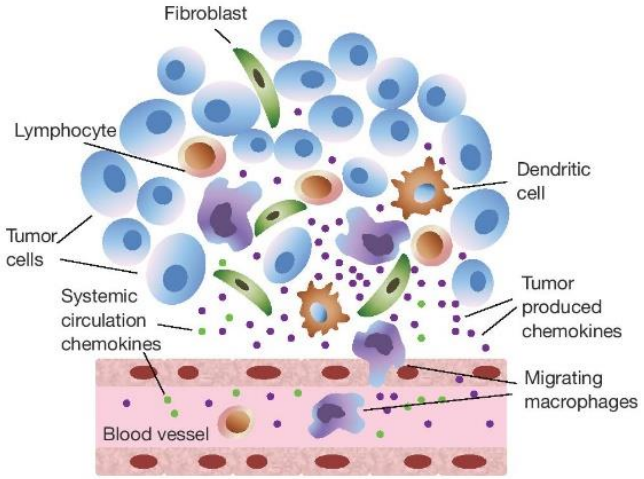

Figure 1 Tumor model including immune cell infiltrate and tumor vascular bed. Chemokine gradients in systemic circulation and tumor microenvironment determine cell composition in immune cell infiltrate as well as chemokine concentrations.

Table 1 Tumor sample immunohistochemistry details

Parameter of interest	Cluster of differentiation (CD)	Antibody dilution
CXCR1 expression	CD181	1:500
CXCR2 expression	CD182	1:250
CXCR3 expression	CD183	1:500
CXCR4 expression	CD184	1:500
T helper cells (Th)	CD4	1:1000
T cytotoxic cells (Tc)	CD8	1:500
B cells	CD20	1:200
Macrophages	CD68	1:1000
Plasma cells	CD138	1:500

washed in distilled water, followed by incubation in acidified eosin solution and washing. The hematoxylin and eosin stained tumor sections were assessed by two independent pathologists to determine histological subtype of the tumor and for differentiating the tumor cells from tumor stroma. Verified tumor specimens containing representative tumor and tumor stroma tissue were further processed—dewaxed slides covered with 0.5% trypsin solution were placed in a humidified container and then into the 37 °C incubator for 10 minutes. Slides were washed in water/tris-buffered saline (TBS) and blocked in 10% normal serum with 1% bovine serum albumin (BSA) in TBS for 2 hours at room temperature. We followed the specific standardized protocol supplied by the manufacturer (Santa Cruz Biotechnology, USA). Omission of the primary antibody served as a negative control. Primary antibody (*Table 1*) diluted in TBS with 1% BSA applied and slides incubated for 30 minutes. Slides were washed and secondary antibody diluted to the concentration recommended by the manufacturer in TBS with 1% BSA was applied to the slides, and incubated for 1 hour at room temperature. Slides were rinsed in TBS and developed with chromogen diaminobenzidine (DAB) for 10 minutes at room temperature, rinsed in distilled water, counterstained with hematoxylin. Slides were dehydrated, cleared and mounted.

TIC infiltrate was defined as composition of immune cells including granulocytes, macrophages, plasma cells, T helper cells, T cytotoxic cells and B cells in tumor stroma and tumor tissue. The average number of positively stained cells per four high power fields was calculated. In order to provide an overview of CXCR expression patterns, staining intensity of tumor cells and tumor stromal cells was assessed using semi-quantitative score (negative =0, weak =1, moderate =2 or strong =3).

Associations between variables were analyzed with Pearson correlation test, and P values less than 0.05 marked as significant.

This study has been approved by Pauls Stradins Clinical University Hospital Foundation ethical committee (Nr. 500210-4L), and was conducted in accordance with the ethical standards of the Helsinki Declaration of the World Medical Association. The informed consent was obtained from all patients.

Results

Initially we have compared chemokine concentration in paired blood samples (n=54) representing systemic and tumor bed circulation; chemokine concentration gradients were calculated accordingly and showed in *Table 2*. Statistically significant decrease in chemokine concentration was found for CXCL4 (P=0.002) and CXCL5 (P=0.011), and statistically significant concentration increase was found for CXCL7 (P=0.001).

For further analysis we have divided patients according to the follow-up data into two groups based on cancer recurrence. Chemokine levels and concentration gradients

Table 2 Concentration of chemokines in peripheral and tumor draining blood samples with chemokine gradient

Chemokine	Concentration in peripheral blood (pg/mL) ± SD	Confidence interval	Concentration in tumor blood (pg/mL) ± SD	Confidence interval	Concentration gradient ± SD	Confidence interval	P value
CXCL1	118.01±365.51	16.69–219.31	123.31±332.32	31.19–215.41	–62.69±76.72	–83.95–41.42	0.223
CXCL4	12,932.61±2,699.86	12,182.13–13,683.07	11,560.59±2,343.96	10,903.87–12,217.32	9.71±12.87	6.13–13.27	0.002
CXCL5	882.74±783.41	665.61–1,099.89	728.28±547.46	576.53–880.02	11.13±27.56	3.48–18.79	0.011
CXCL6	237.17±441.56	114.78–359.56	240.81±440.67	118.66–362.94	–70.97 ± 348.61	–167.61–25.66	0.408
CXCL7	3,417.08±517.77	3,273.56–3,560.61	3,634.31±389.11	3,526.46–3,742.16	–8.81±11.68	–12.05–5.56	0.001
CXCL8	6.03±10.67	3.07–8.99	10.34±22.45	4.11–16.56	–403.27±1,285.07	–759.47–47.08	0.173
CXCL9	993.91±1,668.04	531.56–1,456.25	1,024.14±869.81	783.04–1,265.23	–70.44±175.05	–118.96–21.92	0.489
CXCL10	27.51±58.81	11.21–43.81	26.08±30.91	17.52–34.65	–77.36±170.15	–124.52–30.19	0.445
CXCL11	12.15±17.75	7.22–17.07	12.81±15.54	8.51–17.11	–558.77±1,494.84	–973.11–144.42	0.468
CXCL12	33.24±27.05	25.74–40.73	29.24±8.04	27.01–31.47	1.73±33.19	–7.47–10.93	0.215

are showed in *Table 3* for patients with no recurrence (n=30) and *Table 4* for patients with relapse (n=24) accordingly.

Tumor tissue samples from 40 patients undergone full immunohistochemical analysis after initial evaluation by pathologists, other samples were excluded due to staining artifacts and excessive tumor heterogeneity. Further analysis comprised of quantification of immune cells infiltrating tumor revealing prevalence of T cytotoxic cells and macrophages followed by T helper cells, B cells and plasma cells (*Table 5*).

Immunohistochemistry showed moderate to strong expression intensity of CXCR1 (2.71±0.73) and CXCR3 (2.23±1.3); however expression intensity of CXCR2 (0.71±0.92) and CXCR4 (0.8±0.91) was weak. Dividing patients into subgroups according to recurrence status did not show any statistically significant difference, except for CXCR4. Patients with relapse had higher CXCR4 expression in tumor tissue (1.09±1.14), than patients with no relapse (0.57±0.65), P=0.045.

CXCR1 expression intensity correlated with tumor infiltration by macrophages (r=–0.63, P=0.044) and total number of tumor infiltrating immune cells (r=–0.37, P=0.049). CXCR2 expression intensity correlated with levels of systemic CXCL1 (r=0.7, P=0.025) and CXCL5 (r=–0.39, P=0.05); tumor infiltration by plasma cells (r=–0.52, P=0.018). CXCR3 expression intensity correlated with levels of systemic CXCL11 (r=–0.41, P=0.049); tumor infiltration by B cells (r=–0.45, P=0.01), T helper cells (r=–0.52, P=0.01) and T cytotoxic cells (r=–0.4, P=0.03).

CXCL1 gradient correlated with absolute number of B cells (r=–0.41, P=0.048); CXCL4 gradient correlated with absolute number of macrophages (r=–0.44, P=0.02) and total number of tumor infiltrating immune cells (r=0.59, P=0.01); CXCL5 gradient correlated with absolute number of T helper cells (r=0.53, P=0.008), percentage of T helper (r=0.5, P=0.01) and T cytotoxic (r=–0.46, P=0.038) cells in infiltrate. CXCL6 gradient correlated with absolute number of B cells (r=–0.54, P=0.02); CXCL7 gradient correlated with absolute number of T helper cells (r=0.49, P=0.03); CXCL8 gradient correlated with absolute number of T cytotoxic cell (r=0.43, P=0.035) and total number of tumor infiltrating cells (r=0.4, P=0.05). There was no statistically significant correlation between CXCL gradients and immune cell subpopulations

Discussion

Chemokines or chemotactic cytokines, and their receptors

Table 3 Concentration of chemokines in peripheral and tumor draining blood samples with chemokine gradient for patient subgroup with no cancer recurrence

Chemokine	Concentration in peripheral blood (pg/mL) ± SD	Confidence interval	Concentration in tumor blood (pg/mL) ± SD	Confidence interval	Concentration gradient ± SD	Confidence interval	P value
CXCL1	25.53±11.89	21.28–29.79	41.61±20.44	34.28–48.92	−80.89±82.98	−110.59–51.21	0.002
CXCL4	12,630.83±2,197.85	11,844.35–13,417.31	11,438.19±2,557.55	10,522.99–12,353.38	10.11±8.35	7.11–13.09	0.001
CXCL5	658.33±388.65	519.26–797.41	496.79±313.21	384.71–608.87	23.25±31.77	11.88–34.62	0.013
CXCL6	130.53±102.96	93.69–167.38	148.47±99.56	112.84–184.09	−134.89±489.79	−310.16–40.37	0.304
CXCL7	3,426.51±572.66	3,221.58–3,631.42	3,673.39±406.39	3,527.97–3,818.82	−2.88±31.92	−14.31–8.55	0.036
CXCL8	7.38±13.98	2.38–12.38	8.11±9.93	4.56–11.67	−199.04±309.24	-309.71–88.38	0.026
CXCL9	587.33±382.21	450.56–724.11	1,031.07±772.63	754.59–1,307.55	−99.71±228.23	−181.37–18.03	0.034
CXCL10	21.75±36.07	8.84–34.66	34.65±37.58	21.21–48.11	−129.65±210.39	−204.94–54.35	0.032
CXCL11	13.68±18.10	7.21–20.16	14.66±17.83	8.27–21.04	−859.38±1,960.77	−1,561.02–157.74	0.979
CXCL12	38.26±38.66	24.43–52.11	28.43±6.41	26.14–30.72	10.87±39.76	−3.36–25.11	0.371

Table 4 Concentration of chemokines in peripheral and tumor draining blood samples with chemokine gradient for patient subgroup with relapse

Chemokine	Concentration in peripheral blood (pg/mL) ± SD	Confidence interval	Concentration in tumor blood (pg/mL) ± SD	Confidence interval	Concentration gradient ± SD	Confidence interval	P value
CXCL1	273.09±587.52	15.61–530.58	262.45±534.48	28.21–469.69	−50.42±70.47	−81.31–19.53	0.041
CXCL4	13,334.97±3,331.21	11,875.02–14,794.91	11,723.91±2,192.78	10,762.78–12,684.81	9.62±17.78	1.83–17.42	0.131
CXCL5	1,213.33±1,111.94	726.01–1,700.653	1,022.27±689.97	719.88–1,324.66	14.67±39.56	−2.66–32.02	0.225
CXCL6	403.54±705.03	94.56–712.53	396.72±710.79	85.21–708.23	2.09±28.38	−10.34–14.53	0.815
CXCL7	3,345.41±404.45	3,168.16– 3,522.67	3,574.31±348.12	3,421.75–3,726.88	−0.08±32.33	−14.08–14.08	0.985
CXCL8	3.73±3.82	2.05–5.41	15.06±35.14	−0.34–30.465	−695.34±1,981.73	−1,563.86–173.16	0.251
CXCL9	1,514.66 ±2,607.41	371.93–2,657.39	929.54±1,088.65	452.44–1,406.64	−2.62±54.75	−26.62–21.36	0.868
CXCL10	41.76±88.33	3.05–80.47	16.07±20.94	6.89–25.24	−0.087±54.67	−24.04–23.87	0.991
CXCL11	13.65±19.94	4.19–22.39	14.64±14.13	8.45–20.84	−267.44±730.59	−587.64–52.74	0.499
CXCL12	29.34±10.04	24.94–33.74	29.83±10.59	25.19–34.47	3.41±38.98	−13.67–20.49	0.765

Table 5 Immune cells infiltrating tumor tissue

Cell type	Mean number ± SD	Confidence interval	%	Confidence interval
T helper cells	50±36	37–63	22±10	18.6–25.7
T cytotoxic cells	66±41	51.3–80.8	31±15	25.1–36.3
B cells	24±27	14.6–34.2	10±8	7.12–13.1
Macrophages	63±28	53.2–73.5	31.6±15	26.3–37
Plasma cells	11±8	8–14.2	5.3±4.4	3.8–6.9
Total number of immune cells	215.6±85.5	185–246.2	N/A	N/A

have been discovered as essential and selective mediators in leukocyte migration to inflammatory sites. Besides their functions in the immune system, they also play a critical role in tumor initiation, promotion and progression.

CXC chemokines are characteristically heparin binding proteins. On a structural level, they have four highly conserved cysteine amino acid residues, with the first two cysteines separated by one non-conserved aminoacid residue, hence the name CXC (10). Chemokines, which are structurally and functionally similar to growth factors, bind to G protein-coupled receptors on leukocytes and stem cells and process guanine nucleotide-binding proteins to initiate intracellular signaling cascades that promote migration towards the chemokine source (11). Although the CXC motif distinguishes this family from other chemokine families, a second structural domain within this family dictates their angiogenic potential. The NH2-terminus of the majority of the CXC chemokines containing three aminoacid residues (Glu-Leu-Arg: the ELR" motif) precedes the first cysteine aminoacid residue of the primary structure of these cytokines (12). The family members that contain the ELR motif (ELR+) are potent promoters of angiogenesis (13). In contrast, members that lack the ELR motif (ELR–) are potent inhibitors of angiogenesis. This difference suggests on a structural/functional level that members of the chemokine family are unique cytokines in their ability to behave in a disparate manner in the regulation of chemotaxis and angiogenesis. Most, if not all chemokines activate leukocytes through binding to G protein-coupled seven transmembrane receptors (GPCR) designated CXCR or CCR. The binding of a chemokine to its receptor results in the migration of immune cells by interactions with selectins and integrins. Subsequently, leukocytes infiltrate the tissue in response to a gradient of chemokines, produced at the site of inflammation. In addition, these GPCRs may account for the angiogenic or angiostatic action of chemokines. Chemokine characteristics and effects are summarized in *Table 6*.

Chemokines are best known for inducing directional cell migration, particularly of leukocytes during inflammation. Prolonged inflammation is thought to facilitate carcinogenesis by providing a microenvironment that is ideal for tumor cell development and growth. Chemokines can stimulate or inhibit tumor development in an autocrine fashion by attracting cells with pro- and anti-tumoral activities. Chemokines affect tumor development indirectly by influencing angiogenesis, tumor-leukocyte interactions, as well as directly by influencing tumor transformation, survival and growth, invasion and metastasis (14). The role played by chemokines is rather complex as some chemokines may favor tumor growth and progression, while others may enhance anti-tumor immunity.

Biomarker gradient measurement is an important part of comprehensive analysis of the interactions between three highly complex systems—the tumor cells, the immune response and the tissue microenvironment. Our approach allows the differentially present proteins to be identified against a complex and variable background of proteomic profile. The analytic issue is reduced to determining what has changed in an individual pre- and post- passage through the affected lung to get around the problem of finding specific biomarkers in blood. Circulating CXC chemokine ligands produced in other tissues may get bound by CXCR expressed by tumor or tumor infiltrating immune cells thus decreasing concentration of CXCL in blood draining tumor. On the other hand CXCL can be produced by tumor or tumor infiltrating immune cells with following release of CXCL into blood draining tumor thus increasing concentration.

Interestingly, we have found statistically significant CXC chemokine concentration change for majority of chemokines—CXC1, CXC4, CXC5, CXC7, CXC8,

Table 6 Chemokine characteristics and effect on chemotaxis and angiogenesis

Chemokine	Receptor	ELR motif	Chemotaxis	Angiogenetic effect
CXCL1	CXCR2	+	Neutrophils, endothelial cells	Positive
CXCL2	CXCR2	+	Neutrophils, endothelial cells	Positive
CXCL3	CXCR2	+	Neutrophils, endothelial cells	Positive
CXCL4	CXCR3	-	Activated T cells	Negative
CXCL5	CXCR2	+	Neutrophils, endothelial cells	Positive
CXCL6	CXCR1, CXCR2	+	Neutrophils, endothelial cells	Positive
CXCL7	CXCR2	+	Neutrophils, endothelial cells	Positive
CXCL8	CXCR1, CXCR2	+	Basophils, monocytes, neutrophils, endothelial cells	Positive
CXCL9	CXCR3	–	Natural killer cells, activated T cells	Negative
CXCL10	CXCR3	–	Natural killer cells, activated T cells	Negative
CXCL11	CXCR3, CXCR7	–	Natural killer cells, activated T cells	Negative
CXCL12	CXCR4, CXCR7	–	B cells, basophils, dendritic cells, monocytes, natural killer cells, T cells, endothelial cells	Positive

CXC9 and CXC10 in a group of patients who had good clinical result after surgery with no evidence of relapse, on the other hand patients with cancer recurrence including local and systemic cancer spread did not show any change of chemokine concentration in blood except for CXCL1. These findings could be explained by immunoediting theory (15,16) where patients with no recurrence are going through tumor elimination or equilibrium phase with an active involvement of immune cells and chemokines bound or produced by tumor or immune cell infiltrate. Patients going through the next tumor development phase, which is escape phase, experience immunosuppression which allows tumor spread.

We have also found that chemokine levels and gradients correlate with CXCR receptor expression and number of tumor infiltrating immune cell subpopulations, which subsequently can facilitate development of immunoscore for lung cancer.

Major study limitations were performing the study in single institution and effect of surgical intervention on chemokine levels. At the moment of patient enrolment in the study in 2010 the main surgical technique for anatomical lung resection used in our institution was open approach via muscle sparing lateral thoracotomy which was rapidly replaced by VATS approach over the following 5 years. Any surgical intervention unavoidably causes release of chemokines and cytokines into systemic circulation from damaged tissue (17). Taking into account minimal trauma to soft tissue due to small incisions in case of VATS it could be hypothesized that VATS could cause less prominent tissue trauma cytokine influx into systemic circulation with potentially less influence on target chemokine concentrations.

Conclusions

Biomarker discovery strategy can be more productive when testing biomarker panels which are designed based on our knowledge about the process of carcinogenesis rather than randomly testing human proteome with preference given to quantity over quality.

Assessment of tumor microcirculation is useful for evaluation of different types of circulating biomarkers and application of our method can be very wide, integrating thoracic surgeons into translational cancer research.

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Footnote

Conflicts of Interest: The author has no conflicts of interest to declare.

Ethical Statement: The study was approved by Pauls Stradins Clinical University Hospital Foundation ethical committee (Nr. 500210-4L) and written informed consent was obtained from all patients.

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3.6. Unpublished data

3.6.1. Tumor immune cell infiltrate

In this study, employing tissue arrays, we comprehensively analyzed tumor infiltrating cell composition in different lung cancer types. Importantly, we observed extensive immune and inflammatory cell infiltration in tumor tissue samples. We characterized and quantified T lymphocytes, cytotoxic T cells (CD8+), T helper cells (CD4+), B cells (CD20+), macrophages (CD68+), plasma cells and neutrophilic granulocytes.

Infiltration of T lymphocytes into human lung tissue was assessed by immunohistochemical analysis using CD4 antibody. Mean number of T helper cells per high power field was 50 ± 36 cells; 95% CI 37 – 63. The proportion of T helper cells of total TIL number was 22% ± 10%, 95% CI 18.6 – 25.7. There was no statistically difference between histological subtypes.

Infiltration of T cytotoxic lymphocytes into human lung tissue was assessed by immunohistochemical analysis using CD8 antibody. Mean number of T cytotoxic cells per high power field was 66 ± 41 cells; 95% CI 51.3 – 80.8. The proportion of T helper cells of total TIL number was 31% ± 15%, 95% CI 25.1 – 36.3. There was no correlation between the prevalence of cytotoxic T cells and cancer type, tumor size or nodal status.

Infiltration of macrophages into human lung tissue was assessed by immunohistochemical analysis using CD68 antibody. Mean number of macrophages per high power field was 63 ± 28 cells; 95% CI 53.2 – 73.5. The proportion of macrophages of total TIL number was 31.6% ± 15%, 95% CI 26.3 – 37.

Infiltration of B cells into human lung tissue was assessed by immunohistochemical analysis using CD20 antibody. Mean number of B lymphocytes per high power field was 24 ± 27 cells; 95% CI 14.6 – 34.2. The proportion of B cells of total TIL number was 10% ± 8%, 95% CI 7.12 – 13.1.

Infiltration of plasma cells into human lung tissue was assessed by immunohistochemical analysis using CD138 antibody. Mean number of plasma cells per high power field was 11 ± 8 cells; 95% CI 8 – 14.2. The proportion of plasma cells of total TIL number was 5.3% ± 4.4%, 95% CI 3.8 – 6.9.

The number of infiltrating neutrophil granulocytes was determined based on hematoxylin and eosin staining. Mean number of granulocytes per high power field was 29 ± 27 cells; 95% CI 19.2 – 38.8. The proportion of granulocytes of total TIL number was not calculated as there was no immunohistological verification of granulocyte subpopulations.

Results of tumor immune cell infiltrate analysis are summarized and represented in Table 3.6.1.1.

Table 3.6.1.1.

Tumor immune cell infiltrate composition

Cell type	Number of cells per high power field	SD	Range	95% CI	Percentage of cells in infiltrate (%)	SD	95% CI
T helper cells	50.6	36.5	4 - 118	37.5 - 63.6	22.2	10	18.6 - 25.7
T cytotoxic cells	66.1	41.2	11 - 148	51.3 - 80.8	30.7	15.6	25.1 - 36.3
B cells	24.4	27.5	1 - 91	14.5 - 34.2	10.1	8.3	7.1 - 13.1
Plasma cells	11.2	8.6	2 - 28	8.1 - 14.2	5.4	4.4	3.8 - 6.9
Macrophages	63.3	28.4	17 - 145	53.1 - 73.5	31.6	14.7	26.3 - 36.8

Correlation of subpopulations of immune cells with multiple clinical parameters and follow-up results was assessed and represented in Table 3.6.1.2. and Table 3.6.1.3..

Table 3.6.1.2.

Correlation of immune cell subpopulations with clinical parameters

Cell type	Relapse		Survival		Time to relapse	
	r	p value	r	p value	r	p value
B cells	-0,27	0,18	-0,15	0,45	-0,2	0,62
Th cells	0,17	0,41	0,11	0,57	-0,25	0,52
Tc cells	-0,13	0,53	-0,01	0,95	-0,21	0,61
Macrophages	-0,1	0,61	0,02	0,9	0,13	0,74
Plasma cells	-0,06	0,77	-0,14	0,48	0,16	0,68
Neutrophils	0,1	0,59	-0,04	0,84	**-0,74**	**0,02**
B cells %	-0,31	0,12	-0,16	0,41	-0,33	0,41
Th cells %	**0,44**	**0,02**	0,25	0,21	-0,3	0,46
Tc cells %	-0,04	0,82	0,01	0,97	-0,2	0,63
Macrophages %	-0,06	0,74	-0,05	0,82	0,35	0,38
Plasma cells %	-0,003	0,98	-0,14	0,5	0,15	0,7
Total number of TIL	-0,11	0,57	-0,01	0,95	-0,14	0,73

Table 3.6.1.3.

Correlation of immune cell subpopulations with clinical parameters

Cell type	T status		N status		Tumor size		NSCLC stage	
	r	p value	r	p value	r	p value	r	p value
B cells	0,005	0,98	0,1	0,63	-0,17	0,4	-0,01	0,93
Th cells	0,002	0,98	-0,24	0,23	-0,01	0,97	-0,22	0,27
Tc cells	0,29	0,14	0,05	0,8	0,08	0,7	**0,34**	**0,08**
Macrophages	-0,02	0,95	-0,22	0,27	-0,21	0,3	-0,2	0,33
Plasma cells	0,05	0,78	-0,03	0,89	0,13	0,5	-0,11	0,58
Neutrophils	0,28	0,15	0,12	0,54	0,11	0,55	0,19	0,33
B cells %	-0,08	0,67	0,23	0,24	-0,24	0,24	-0,01	0,94
Th cells %	-0,08	0,69	-0,27	0,18	0,09	0,66	**-0,34**	**0,09**
Tc cells %	**0,3**	**0,13**	0,17	0,41	0,24	0,23	**0,52**	**0,006**
Macrophages %	-0,23	0,25	-0,12	0,56	-0,22	0,26	-0,26	0,19
Plasma cells %	0,05	0,78	-0,03	0,87	0,14	0,48	-0,16	0,42
Total number of TIL	0,15	0,46	-0,12	0,54	-0,07	0,71	-0,01	0,95

3.6.2. CXC chemokine receptor expression

Immunohistochemistry showed moderate to strong expression intensity of CXCR1 (2.71 ± 0.73) and CXCR3 (2.23 ± 1.3); however expression intensity of CXCR2 (0.71 ± 0.92) and CXCR4 (0.8 ± 0.91) was weak. Dividing patients into subgroups according to recurrence status did not show any statistically significant difference, except for CXCR4. Patients with relapse had higher CXCR4 expression in tumor tissue (1.09 ± 1.14), than patients with no relapse (0.57 ± 0.65), p=0.045. Stromal and tumoral expression of CXC chemokine receptors represented in Figure 3.6.2.1.

Additionally we assessed correlation of CXC chemokine receptor expression with CXC chemokine ligand levels in peripheral and tumor draining blood, and CXC chemokine ligand gradients (Table 3.6.2.1.)

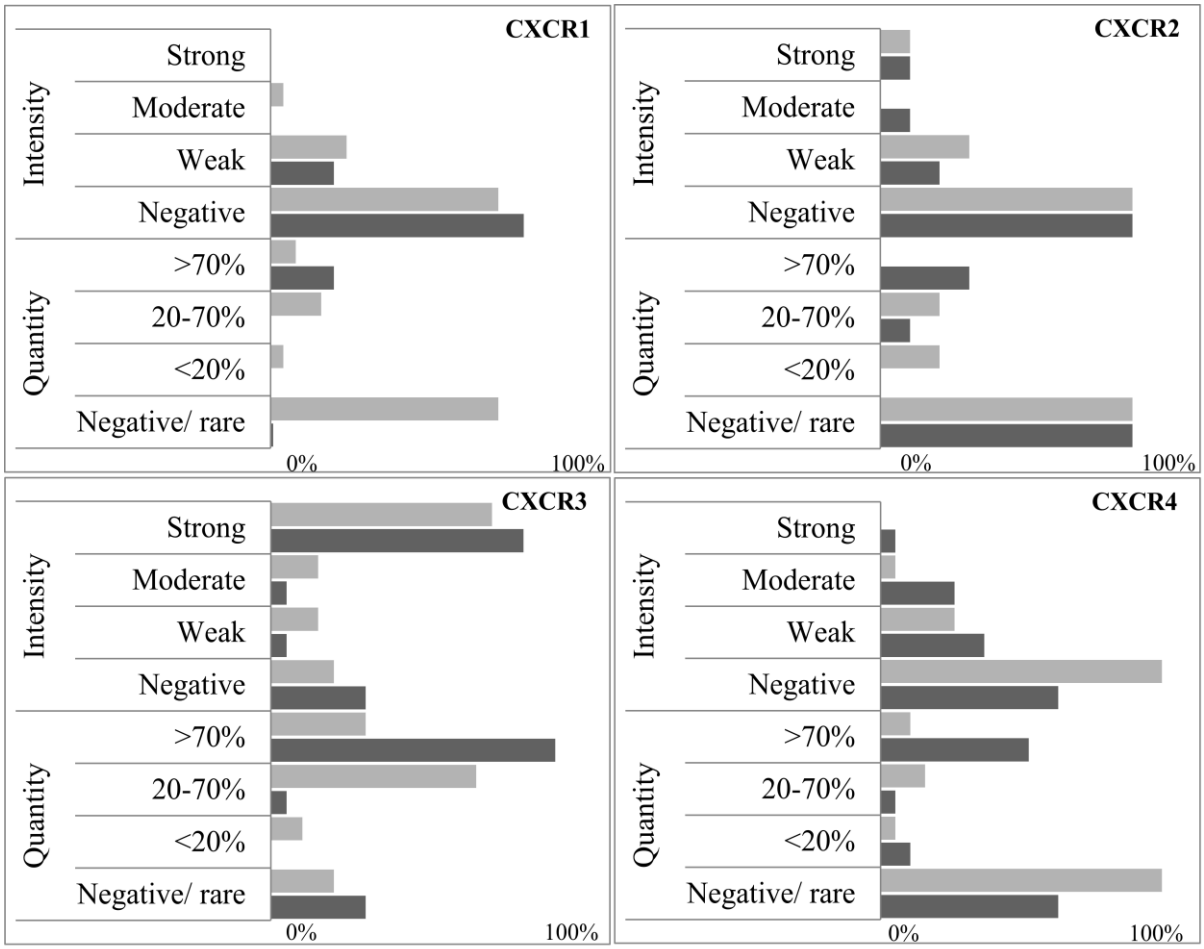

Figure 3.6.2.1. CXC chemokine receptor expression in tumor tissue (light grey – expression in tumor stroma; dark grey – expression in tumor cells)

Table 3.6.2.1.

Correlation of CXC chemokine receptor expression with CXC chemokine ligand levels

(Values were calculated for specific receptor-ligand pairs)

		CXCR1 expression in tumor cells		CXCR1 expression in tumor stroma		CXCR2 expression in tumor cells		CXCR2 expression in tumor stroma		CXCR3 expression in tumor cells		CXCR3 expression in tumor stroma		CXCR4 expression in tumor cells		CXCR4 expression in tumor stroma	
		r	p value	r	p value	r	p value	r	p value	r	p value	r	p value	r	p value	r	p value
Peripheral	CXCL1					**0.35**	**0.07**	0.27	0.18								
	CXCL4									0.12	0.55	0.13	0.51				
	CXCL5					-0.3	0.18	**-0.4**	**0.05**								
	CXCL6	-0.1	0.73	-0.1	0.7	0.04	0.81	-0.1	0.81								
	CXCL7					0.06	0.76	0.11	0.57								
	CXCL8	0.06	0.76	0.14	0.49	0.3	0.13	0.26	0.2								
	CXCL9									-0.3	0.2	0.03	0.88				
	CXCL10									0.02	0.91	0.29	0.15				
	CXCL11									-0.2	0.47	**-0.4**	**0.05**				
	CXCL12													-0.2	0.36	0.11	0.58
Tumor microcirculation	CXCL1					0.04	0.84	0.15	0.48								
	CXCL4									0.23	0.26	0.15	0.46				
	CXCL5					-0.1	0.58	-0.1	0.81								
	CXCL6	0.14	0.51	0.12	0.57	-0.1	0.59	-0.1	0.61								
	CXCL7					-0.1	0.62	-0.3	0.21								
	CXCL8	-0.1	0.57	-0.1	0.55	-0	0.99	-0.1	0.72								
	CXCL9									**-0.5**	**0.03**	**-0.5**	**0.04**				
	CXCL10									-0.2	0.39	-0.2	0.32				
	CXCL11									-0.1	0.77	-0	0.87				
	CXCL12													0.08	0.73	-0.1	0.81
Gradient	CXCL1					0.33	0.11	0.25	0.23								
	CXCL4									0.09	0.65	0.05	0.81				
	CXCL5					-0.1	0.62	-0.2	0.24								
	CXCL6	0.07	0.72	-0.1	0.72	0.04	0.81	0.04	0.83								
	CXCL7					0.14	0.51	0.19	0.36								
	CXCL8	0.02	0.92	0.25	0.23	0.3	0.14	0.25	0.23								
	CXCL9									-0.1	0.68	0.1	0.63				
	CXCL10									0.1	0.65	**0.42**	**0.05**				
	CXCL11									-0.2	0.48	**-0.4**	**0.05**				
	CXCL12													-0.3	0.24	0.19	0.37

3.6.3. Additional data on correlation of CXC chemokine levels with clinical parameters

Correlation analysis revealed CXC chemokine ligands which levels correlate with basic clinical oncology parameters like relapse, time to relapse and cancer stage (Table 3.6.3.1.)

Table 3.6.3.1.

Correlation of clinical parameters with CXCL levels

		Relapse		Time to relapse		Cancer stage	
		r	p value	r	p value	r	p value
Systemic circulation	CXCL1	**0.42**	**0.02**	-0.26	0.49	-0.03	0.85
	CXCL4	**0.6**	**0.01**	0.08	0.8	0.02	0.91
	CXCL5	0.24	0.19	0.37	0.28	-0.11	0.56
	CXCL6	**0.35**	**0.04**	**-0.61**	**0.01**	0.07	0.72
	CXCL7	0.23	0.21	0.53	0.11	-0.04	0.83
	CXCL8	0.25	0.16	0.07	0.83	-0.18	0.34
	CXCL9	**0.35**	**0.05**	-0.33	0.35	0.007	0.97
	CXCL10	0.12	0.53	-0.26	0.48	0.08	0.67
	CXCL11	0.03	0.85	**0.62**	**0.01**	0.27	0.17
	CXCL12	-0.17	0.36	-0.01	0.97	0.27	0.17
Tumor microcirculation	CXCL1	0.34	0.08	-0.25	0.51	-0.03	0.85
	CXCL4	0.12	0.52	0.08	0.8	0.019	0.92
	CXCL5	**0.48**	**0.01**	0.31	0.37	-0.11	0.56
	CXCL6	0.25	0.2	-0.53	0.14	0.056	0.78
	CXCL7	0.002	0.99	**0.69**	**0.007**	0.13	0.52
	CXCL8	0.16	0.41	0.16	0.64	-0.057	0.78
	CXCL9	-0.04	0.84	-0.2	0.56	**0.4**	**0.044**
	CXCL10	-0.24	0.22	-0.02	0.95	**0.41**	**0.032**
	CXCL11	0.06	0.76	0.11	0.74	0.08	0.67
	CXCL12	0.17	0.4	0.52	0.12	0.022	0.91
Gradient	CXCL1	0.23	0.25	-0.15	0.69	0.04	0.82
	CXCL4	-0.24	0.2	-0.04	0.9	0.078	0.69
	CXCL5	-0.02	0.89	0.18	0.6	-0.088	0.66
	CXCL6	0.21	0.29	-0.24	0.52	-0.18	0.35
	CXCL7	0.08	0.68	-0.11	0.76	-0.26	0.17
	CXCL8	0.03	0.88	-0.07	0.84	-0.16	0.42
	CXCL9	**0.36**	**0.05**	-0.28	0.71	**-0.42**	**0.025**
	CXCL10	0.32	0.1	-0.21	0.57	-0.31	0.11
	CXCL11	-0.08	0.71	0.58	0.13	0.23	0.27
	CXCL12	-0.09	0.63	-0.28	0.43	0.04	0.82

4. DISCUSSION

4.1. General discussion

Clinical and translational research on lung cancer patients undergoing surgical treatment can provide valuable scientific data and unique opportunity to study tumor microenvironment. CXC chemokines, which are members of a big family of cytokines, are undoubtedly involved in tumor growth regulation and metastasizing pathways.

Although chemokine biology was originally felt to be restricted to only recruitment of populations of leukocytes, it has become increasingly clear that these cytokines can display pleiotropic effects in mediating biology that go beyond their originally described function. Chemokines have autocrine, paracrine, and hormonal roles related to tumor growth and to metastasis. CXC chemokines can play a critical role in mediating the full development of immunity to tumor-associated antigens. Chemokine receptors are up-regulated on tumor cells, allowing the tumor to take advantage of chemokine rich environments, promoting tumor growth and vasculature. In addition, chemokines can recruit macrophages and neutrophils, which detect the hypoxic environment within the tumor and subsequently secrete pro-angiogenic factors (Raman et al., 2007). Initially chemokines were thought to only play a role in attracting specific leucocytes to a site of injury; nevertheless it has now been shown that they are involved in the neoplastic transformation of a cell, promotion of angiogenesis, tumor clonal expansion and in particular mediate organ specific metastases in cancer (Lazzenec et al., 2010). Although it seems that chemokine production in cancers has a direct relationship with the nature and extent of the leukocyte infiltrate, we do not have a clear picture of the overall chemokine repertoire of an individual human cancer type. There is even less information on the chemokine-receptor profile of the infiltrate. Infiltrating leukocytes are not the only cells that respond to chemokine gradients in cancers; cancer cells themselves can express chemokine receptors and respond to chemokine gradients (Murphy et al., 2001). In fact, organ-specific metastasis might be governed, in part, by interactions between chemokine receptors on cancer cells with metastatic potential and chemokine gradients in target organs. There are similarities, for instance, between the transport of dendritic cells to lymph nodes, which is regulated by chemokine gradients, and the lymphatic spread of cancer cells (Allavena et al., 2000). Malignant cells from different cancer types express different profiles of CC and CXC chemokine receptors. Some chemokines produced by tumor cells and infiltrating stromal cells in cancer were previously described in different studies (Anderson et al., 2000; White et al., 2001). Current study focuses on group of CXC chemokines in lung cancer in holistic way: from molecular features to clinical parameters and survival analysis.

4.2. Clinical application and perspectives

Circulating CXC chemokines are blood-based biomarkers which are attractive, relatively non-invasive modality that could facilitate early detection and treatment of lung cancer. Biomarkers may help in the identification of individuals with radiographic abnormalities that should undergo further invasive testing. In addition to tumor markers, host factors that include the immune response to the tumor might determine tumor behavior or serve as informative biomarkers (Ogino et al., 2011). As tumors are heterogeneous and show distinctive genetic and epigenetic profiles, there may not be a single biomarker that will prove sufficient information. Every person has a unique set of genomic and epigenomic variants and any given tumor arises as a result of interactions between these unique host and transformed cells. The carcinogenic process that gives rise to an individual tumor is unique; and each tumor pathway is unlikely to be exactly recapitulated by any other tumor. Literature data support the uniqueness concept of carcinogenesis process of an individual tumor (Ogino et al., 2008).

The pathological examination of immune-cell infiltrates in a tumor tissue section provides a powerful approach to assess host antitumor reactivity. Other methods such as measurements of plasma biomarkers in peripheral blood may serve as surrogates of the host immune response. Antitumor immune response may lead to proliferation of lymphocytes and the enlargement of lymph nodes, resulting in an increased number of detectable lymph nodes in a resection specimen. Thus, the immune response and the node count are inter-related, and the immune-cell infiltrate may be a confounder in a survival analysis based on the lymph-node count. Further research of tumor immune cell infiltrate could lead to development of Immunoscore for lung cancer in order to improve prognostic function of TNM classification, as limited predictive accuracy of TNM is related to assumption that tumor progression is largely a cell-autonomous process, focusing only on cancer cells and without considering the host immune response. Even at advanced disease stages, immune parameters have now been recognized as directly or indirectly influencing patient survival (Galon et al., 2006).

It is clear that chemokines and their receptors are involved in malignant progression and that a better understanding of chemokine signaling in this process could lead to new diagnostic and therapeutic strategies for cancer. With this in mind, it is possible that drugs that are being tested in inflammatory and autoimmune disease that target the chemokine network (Proudfoot et al., 2002) could also be useful as cancer biotherapies. Chemokine and cytokine antagonists have the potential to inhibit tumor-promoting leukocyte infiltrate, tumor angiogenesis, metastatic spread and angiogenesis (Szlosarek et al., 2003; Belperio et al.,

2000). As the chemokine network is complex, it is unlikely that an individual chemokine antagonist would have a powerful action in cancer.

We can implement CXC chemokine profile and immune cell evaluation to guide clinical decision making, and take a step closer to our ultimate goal of personalized cancer medicine. The purpose of personalized medicine is to identify the optimal treatment for each individual patient to maximize treatment benefit and minimize adverse effects. To achieve this goal, informative biomarkers need to be identified to stratify patients for specific therapies.

4.3. Study limitations

The importance of multicenter large-scale studies cannot be overemphasized. Notably, the number of events, but not the total sample size, is the determinant of statistical power in survival analysis. Adequate statistical power requires larger sample size, because many studies require subset analyses. In addition to consideration on study sample size, investigators should examine important variables as patient age, sex, tumor location, disease stage and tumor molecular variables for potential confounding. Due to small sample group multivariate analysis was not possible.

Samples from cancer are derived from surgical material. Due to subgroups and heterogeneity of tumors within each cancer type, included cases represent a typical mix of specimens. Lung cancer includes both squamous cell carcinoma and adenocarcinoma - tumor heterogeneity and inter-individual differences may be reflected in diverse production and expression of proteins resulting in variable immunohistochemical staining patterns. NSCLC tumors differ in their expression of CXC receptors and immune cell infiltrates which form their unique phenotype or signature.

The immunohistochemical and pathological evaluation of immune cells in cancer tissue has been a challenge, and no standardized method exists. There exist not only general challenges in pathological evaluations of tumor tissue markers, but also challenges specific for immune cell evaluations. General challenges in pathological evaluations of tumor tissue markers include pre-analytical variables, such as tissue fixation and processing, and may have considerable impact on the antigenicity of proteins in the tissue. Immunoreactivity of tissue antigens may be substantially influenced by subtle differences in the conditions of the immunohistochemical procedures. Inter-observer variability among pathologists is a continuing issue in a pathology testing, and an even harder challenge in immune cell evaluation because of its complexity. A detailed examination of immune cells in different compartments of the tumor mass is desirable. Sensitive and robust methods of detecting

molecular alterations are needed to avoid correlative errors in cancer tissue analysis owing to the complex inter-relationships between molecular features and the immune reaction to tumors.

At the moment of patient enrolment in the study in 2010 the main surgical technique for anatomical lung resection used in our institution was open approach via muscle sparing lateral thoracotomy which was rapidly replaced by VATS approach over the following 5 years. Any surgical intervention unavoidably causes release of chemokines and cytokines into systemic circulation from damaged tissue. Taking into account minimal trauma to soft tissue due to small incisions in case of VATS it could be hypothesized that VATS could cause less prominent tissue trauma cytokine influx into systemic circulation with potentially less influence on target chemokine concentrations. Collection of multiple blood samples at the different time points before, during, and after surgery could improve understanding of chemokine's secretion dynamics.

5. CONCLUSIONS

1. Novel biomarker discovery and verification approach was developed and applied - during lung resection procedure peripheral and lung tumor draining blood samples can be collected easily and safely allowing further analysis of circulating biomarkers and tumor microcirculation. Assessment of tumor microcirculation is useful for evaluation of different types of circulating biomarkers and application of our method can be very wide, integrating thoracic surgeons into translational cancer research.

2. Significantly increased levels of CXCL4, CXCL8, CXCL9, CXCL10 and CXCL11 were found in plasma specimens of lung cancer patients compared to control subjects.

3. Lung cancer cells and tumor microenviroment produce and bind different CXC chemokines confirmed with measuring circulating CXC chemokine gradients. Statistically significant CXC chemokine concentration change was found for majority of chemokines – CXCL1, CXCL4, CXCL5, CXCL7, CXCL8, CXCL9 and CXCL10.

4. Findings demonstrated diagnostic value of CXC chemokines. Particularly CXCL9, CXCL10 and CXCL11 can be used as diagnostic biomarkers for NSCLC. There is no single unique biomarker for NSCLC, but combination of several chemokines allows achieving 100% sensitivity. Levels of CXC chemokines have prognostic value. Particularly CXCL4 can be applied as prognostic biomarker.

5. CXC chemokines appear to be important endogenous factors that regulate angiogenesis in association with tumorigenesis in NSCLC. CXCL4 levels reflect angiogenic activity of tumor serving as prognostic biomarker. CXC chemokine levels and gradients correlate with CXC receptor expression and number of tumor infiltrating immune cell subpopulations, which subsequently can facilitate development of Immunoscore for lung cancer.

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