Thesis

Chemokine Receptor Expression Profile in Non-Small-Cell Lung Cancer and Brain-Specific Metastases

submitted by

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Graz, March 15, 2022

Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Graz, March 15, 2022 *Daniel Leitinger m.p.*

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List of Abbreviations

ACTB Beta-actin

AKT Protein kinase B

C Cysteine

cDNA Complementary Deoxyribonucleic acid

EMT Epithelial-mesenchymal transition

FFPE Formalin-fixed paraffin-embedded

GAPD Glyceraldehyde 3-phosphate dehydrogenase

GDP Guanosine diphosphate

GTP Guanosine triphosphate

HE Hematoxylin and eosin

IL22 Interleukin 22

MAPK/ERK mitogen-activated protein kinases/extracellular signal-regulated kinases

MDSC Myeloid derived suppressor cells

MMP2 Matrix metalloproteinase 2

MMP9 Matrix metalloproteinase 9

mRNA Messenger ribonucleic acid

NSCLC Non-small-cell lung cancer

PCR Polymerase chain reaction

PI3K Phosphatidylinositol 3-kinases

RNA Ribonucleic acid

RT-qPCR Reverse transcription-quantitative real-time polymerase chain reaction

SCLC Small-cell lung cancer

TGF-β Transforming growth factor-beta

TH1 Type 1 helper T cell

TH17 Type 17 helper T cell

TH22 Type 22 helper T cell

$\mathbf{T_{reg}}$ Regulatory T cells

WHO World Health Organization

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Zusammenfassung

Hintergrund

Das Lungenkarzinom ist eines der häufigsten Karzinome in Industriestaaten und die häufigste Ursache für tumorbedingte Mortalität. Vor allem das Auftreten von Hirnmetastasen geht mit einer schlechten Prognose einher. Zudem ist das Lungenkarzinom der häufigste Primärtumor von Hirnmetastasen. Daher ist es wichtig, mehr über Ursachen und Vorgänge, die die Metastasierung fördern, herauszufinden. Eine bedeutende Rolle in der Entstehung von Metastasen spielen Chemokine und ihre Rezeptoren. Sie sind bei der Entstehung, aber auch bei der Progression von Tumoren wesentlich beteiligt. Neue Einblicke in die Biologie der Chemokine könnten neue Konzepte für gezielte Therapieansätze liefern.

Methoden

Diese Arbeit setzte sich zum Ziel herauszufinden, ob sich das Muster der Chemokinrezeptorexpression beim nicht-kleinzelligen Lungenkarzinom (NSCLC) zwischen Hirnmetastase und Primum unterscheidet. Mittels RT-qPCR wurde die Expression der mRNA der derzeit am besten charakterisierten Chemokinrezeptoren (CCR1-10, CXCR1-7, XCR1 und CX3CR1) in den Geweben untersucht. Dafür wurden 13 NSCLC Proben und 30 Proben von Hirnmetastasen herangezogen. Die Chemokinrezeptorexpression der Tumoren wurde auch mit jener des umliegenden Normalgewebes verglichen.

Ergebnisse

Die CC-Chemokinrezeptoren CCR4, CCR6, CCR8 und CCR9 und die CXC-Chemokinrezeptoren CXCR4 and CXCR6 waren sowohl in den Primärtumoren als auch in den Metastasen regelmäßig exprimiert. Die CCR6 kodierende mRNA-Expression war in Metastasen signifikant geringer als jene in Primärtumoren (P=0.0429). Dies trifft auch für die mRNA-Expression von CCR9 zu (P=0.0499). Auch die Expressionen von CXCR4 und CXCR6 lagen im Metastasengewebe signifikant unter dem Wert der Expression im Primärtumor (CXCR4: P=0.0107; CXCR6: P=0.034).

Schlussfolgerung

Primärtumor und Metastasen im Gehirn des NSCLC unterscheiden sich hinsichtlich der mRNA-Expression von einigen Chemokinrezeptoren.

Abstract

Background

Lung cancer is one of the most common cancers in industrial countries and the leading cause of cancer-related mortality. Especially the development of brain metastases is accompanied by a poor prognosis. Since lung cancer is the tumor with the highest rate of metastasis into the brain, it is important to elucidate the causes and events in the development of metastases. Chemokines and their receptors play a key role in the formation of metastases, but also in disease progression. New insights in this field may yield new concepts in developing targeted therapies for lung cancer and their metastases.

Methods

This thesis aimed to find out if the chemokine receptor expression in Non-Small-Cell Lung Cancer (NSCLC) is different between the primary tumors and brain-specific metastases. Differences in the expression of the most well-characterized chemokine receptors (CCR1-10, CXCR1-7, XCR1 und CX3CR1) were detected by RT-qPCR. The chemokine receptor expression was analyzed on 13 NSCLC samples and 30 samples of brain-specific metastases from NSCLC. Furthermore, the chemokine receptor expression in tumor tissues was compared to the chemokine receptor expression in the surrounding non-neoplastic tissues.

Results

The CC chemokine receptors CCR4, CCR6, CCR8, and CCR9 and the CXC chemokine receptors CXCR4 and CXCR6 were consistently expressed on both the primary tumor tissues and tissues of metastases. The mRNA expression of CCR6 was significantly lower in metastases compared to the primary tumors ($P=0.0429$), as was the expression for CCR9 ($P=0.0499$). The chemokine receptor expression was also lower in metastases compared to the primary tumors for CXCR4 ($P=0.0107$) and CXCR6 ($p=0.034$).

Conclusion

The mRNA expression profile for some chemokine receptors is different in brain-specific metastases of NSCLC compared to the primary tumors.

1 Introduction

1.1 Lung Cancer

Lung cancer is the second most common cancer and the leading cause of cancer-related mortality worldwide. Lung cancer is responsible for approximately one out of 10 (11.4%) new cancer diagnoses and for 1 out of 5 (18%) cancer deaths, meaning more people die of lung cancer than of colorectal cancer, breast cancer, and prostate cancer combined. Globally, lung cancer is the most common cancer in men and the third most common one in women.[1] About two-thirds of lung cancer deaths worldwide are attributable to smoking.[1] The five-year relative survival rate of lung cancer is only about 21% which reflects that approximately 60% of all patients get their diagnosis in an already advanced stage, i.e. metastatic disease, for which the five-year relative survival rate is only 6%.[2] Two major histologic subtypes of lung cancer can be distinguished: small-cell lung cancer (SCLC) to which approximately 15% of all lung cancers can be attributed to and non-small-cell lung cancer (NSCLC) accounting for approximately 85% of all lung cancers. The most common histological subtypes of non-small-cell lung cancer are adenocarcinoma, squamous-cell carcinoma, and large-cell lung carcinoma.[3] Despite advances in diagnosis, staging, surgical treatment, and chemoradiotherapy, high mortality is still an ongoing problem, even though the advent of targeted therapies, e.g., tyrosine kinase inhibitors, has led to substantial improvement in survival.[4]

1.2 Chemokines

Chemokines, also known as chemotactic cytokines, are a group of small, secreted molecules best known for mediating directional leukocyte trafficking.[5,6] Most chemokines have four conserved cysteines, and they can be divided into four subfamilies, depending on the motif displayed by the first two cysteines adjacent to the N-terminus.[7] These are CXC, CC, C, and CX_3C. The two cysteines in the CXC subfamily are separated by an amino acid whereas in CC chemokines they are adjacent to each other. In the C class, the first and third cysteines are missing, and the sole CX_3C chemokine member has three amino acids between the first two cysteines (**Figure 1**). In 2000 a systematic nomenclature was introduced for chemokines. This nomenclature comprises of the specific subfamily (i.e., CXC, CC, C, CX_3C) followed by the letter L, denoting “ligand”, and finally a number based on when the gene, encoding the chemokine, was first discovered.[6] Approximately 50 chemokines have been discovered so far, making it the largest family of cytokines.[8] **Table 1** gives an overview

of the different chemokines found in humans. Initially known for their role in inflammation, chemokines are now also known to play a significant role in infectious diseases, inflammatory diseases, and cancer.[8,9]

Figure 1. Chemokine structure. Schematic indicating the relationships between conserved cysteine residues (C), together with intrachain disulfide bridges. C-, CC-, CXC-, and CX3C- classes of chemokines are depicted. Modified from Sahingur and Yeudall.[10]

Table 1 Chemokine nomenclature (adapted from Bachelerie et al.[11])

CHEMOKINES	COMMON OTHER NAMES
CXC-CHEMOKINE FAMILY	
CXCL1	GROα, MGSA
CXCL2	Groβ, MIP-2α
CXCL3	Groγ, MIP-2β
CXCL4	Platelet Factor-4
CXCL5	ENA-78
CXCL6	GCP-2
CXCL7	NAP-2
CXCL8	IL-8
CXCL9	Mig
CXCL10	γIP-10
CXCL11	I-TAC
CXCL12	SDF-1α
CXCL13	BLC
CXCL14	BRAK
CXCL16	SR-PSOX
CC-CHEMOKINE FAMILY	
CCL1	I-309
CCL2	MCP-1
CCL3	MIP-1α
CCL4	MIP-1β
CCL5	RANTES
CCL7	MCP-3
CCL8	MCP-2
CCL11	Eotaxin
CCL13	MCP-4
CCL14	HCC-1
CCL15	HCC-2
CCL16	HCC-4
CCL17	TARC
CCL18	PARC
CCL19	ELC
CCL20	MIP-3α, LARC
CCL21	SLC
CCL22	MDC
CCL23	MPIF-1

Table 1 Continued

CHEMOKINES	COMMON OTHER NAMES
CCL24	Eotaxin-2
CCL25	TECK
CCL26	Eotaxin-3
CCL27	CTACK
CCL28	MEC
C-CHEMOKINE FAMILY	
XCL1	Lymphotactin α
XCL2	Lymphotactin β
CX_3C-CHEMOKINE FAMILY	
CX_3CL1	Fractalkine

1.3 Chemokine Receptors

Chemokine receptors, seven-transmembrane-domain G-protein-coupled receptors, have been named based on the chemokine class they can bind (**Table 2**). Most of the chemokine receptors can recognize more than one chemokine, but they are almost always limited to a chemokine subfamily. CXCR1-CXCR6 bind CXC chemokines, CCR1-CCR10 bind CC chemokines, XCR1 binds the XC chemokine and CX3CR1 binds the CX3C chemokine. In addition, chemokines can also bind to different chemokine receptors, giving the chemokine superfamily a complex degree of interaction (**Table 2**).[11,12] When a chemokine binds to his accompanying receptor this leads to a change of the receptor's structure, allowing it to bind to intracellular heterotrimeric G-proteins which consequently activates them. Following the activation, the G-proteins substitute GDP for GTP, enabling the dissociation into α- and βγ-subunits. Chemokine receptors can then couple to various α-subunits leading to the activation or inhibition of downstream effectors such as activation of phospholipase C via G_q or inhibition of adenylyl cyclase by G_i.[13,14] Furthermore, $G_{\beta\gamma}$ can independently or in combination with the α-subunits activate downstream effectors. One effector is the phosphatidylinositol-3-kinase (PI3K), which, when activated, generates phosphatidylinositol 3,4,5-triphosphate (PIP_3) triggering different downstream effectors.[5] Additionally, atypical chemokine receptors (ACKR1-ACKR4) have been described. They act as decoy receptors, competing with other chemokine receptors for their ligands, but are not generating any signaling and therefore alleviate the chemokine response. Chemokine receptors have a specific expression pattern on leukocytes but can be also found on other cells, for example endothelial cells, neurons, or even cancer cells.[11]

Table 2 Chemokine Receptors and their Ligands (adapted from Bachelerie et al.[11])

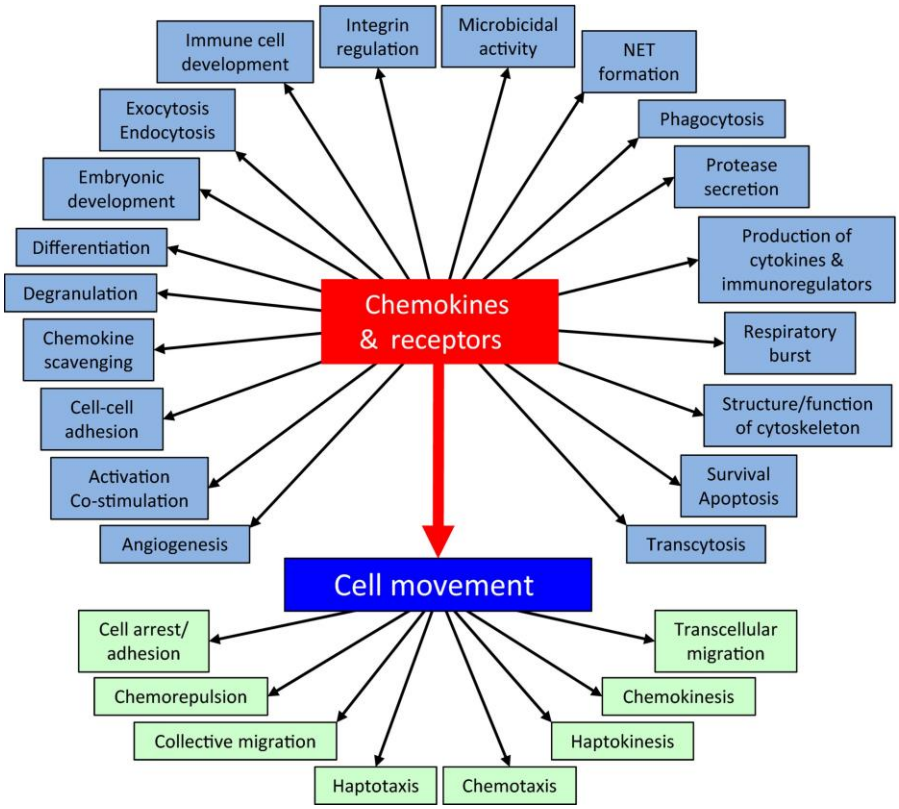
CHEMOKINE RECEPTOR	COMMON OTHER NAMES	LIGAND
G PROTEIN-COUPLED CHEMOKINE RECEPTORS		
CXCR1	IL8RA	CXCL5, CXCL6, CXCL8
CXCR2	IL8RB	CXCL1-3, CXCL5-8
CXCR3	IP10/Mig R	CXCL9-11
CXCR4	Fusin	CXCL12
CXCR5	BLR-1	CXCL13
CXCR6	BONZO, STRL33	CXCL16
CCR1	CC CKR1, MIP-1α/RANTES R	CCL3, CCL4, CCL5, CCL7, CCL8, CCL13, CCL14, CCL15, CCL16, CCL23
CCR2	CC CKR2, MCP-1-R	CCL2, CCL5, CCL7, CCL8, CCL13, CCL16
CCR3	CC CKR3, Eotaxin receptor	CCL4, CCL5, CCL7, CCL11, CCL13, CCL15, CCL24, CCL26, CCL28
CCR4	CC CKR4	CCL17, CCL22
CCR5	CC CKR5	CCL3, CCL4, CCL5, CCL7, CCL14, CCL16
CCR6		CCL20
CCR7	EBI-1, BLR-2	CCL19, CCL21
CCR8		CCL1, CCL18
CCR9		CCL25
CCR10		CCL27, CCL28
XCR1		XCL1, XCL2
CX3CR1	Fractalkine receptor	CX3CL1
ATYPICAL CHEMOKINE RECEPTORS		
ACKR1	DARC, Duffy	CXCL5, CXCL6, CXCL8, CXCL11
ACKR2	D6	CCL2-CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL17, CCL22
ACKR3	CXCR7, RDC1	CXCL11, CXCL12
ACKR4	CCRL1, CCX-CKR, CCBP2, CCR11	CCL19, CCL21, CCL25
CCRL2 (ACKR5)	CKRX, CRAM-A, L-CCR, CRAM-B	CCL19
PITPNM3 (ACKR6)	Nir1	

1.4 The functions of the chemokine network

Based on their expression pattern, chemokines can be divided into either inflammatory or homeostatic chemokines. Inflammatory chemokines guide leukocytes to infected or inflamed tissue, whereas constitutively expressed homeostatic chemokines are vital for the basal migration of leukocytes, e.g., into secondary lymphatic organs, and tissue development.[15] Pro-inflammatory chemokines are released because of an inflammatory

stimulus, i.e., a pathogen, tissue damage, or other pathological conditions. Depending on the specific chemokines secreted, distinct types of leukocytes migrate towards the location of interest.[16] But the function of the chemokine network is not limited to the chemotaxis of cells. The activation of chemokine receptors is involved in a wide spectrum of other processes including survival, endocytosis, proliferation, differentiation, and degranulation of leukocytes. Additionally, some cells move away instead of migrating towards the chemokine source. Moreover, chemokine receptors are not limited to leukocytes, thus many cells, including epithelial cells, neurons, astrocytes, mesenchymal cells, and endothelial cells can express chemokine receptors.[17,18] **Figure 2** gives an overview of the functions in which chemokines and their receptors are involved.

Figure 2. Functions of chemokines and their receptors.[19]



1.5 Chemokines and Cancer

As described by Rudolf Virchow in 1863, a particular feature of tumors is their "lymphoreticular infiltrate". Indeed, tumors are not just bulks of cancer cells. Their tumor microenvironment contains many non-malignant stromal cells.[20] Predominately,

macrophages, lymphocytes, endothelial cells, and fibroblasts constitute the accompanying infiltrate. Additionally, eosinophils, granulocytes, natural-killer cells, and B cells can be found in some tumor tissues as well.[21] Therefore, many leukocytes are infiltrating the tumor and the surrounding tissue and form the leukocyte infiltrate.

The leukocyte infiltrate is regulated by the local production of chemokines by stromal cells or the tumor itself. The chemokine composition affects both the quantity and the type of cells recruited. Moreover, chemokines also directly influence other stromal cells, such as endothelial cells, or even the tumor cells itself. They have been shown to promote proliferation, invasiveness, and metastasis. Consequently, chemokines affect the tumor cell phenotype and hence tumor progression, tumor immunity and could ultimately also affect therapy outcomes.[22,23]

1.5.1 The Chemokine Network determines the Leukocyte Infiltrate

As described above, different leukocyte subsets migrate into the tumor microenvironment depending on the chemokines expressed.

1.5.1.1 Lymphocytes suppressing Tumorigenesis

$CD8^+$ T cells, T_H1 cells, and natural killer cells have potent antitumor activity in an antigen-specific manner. They express CXCR3 and can migrate towards high levels of CXCL9 or CXCL10. $CD8^+$ T cells accomplish antitumor effects by inducing apoptosis and by secreting cytotoxic molecules. Elevated levels of infiltrating $CD8^+$ T cells are associated with reduced metastatic potential and prolonged survival in colon cancer and ovarian cancer. Additionally, T_H17 cells mediate antitumor immune response by recruiting $CD8^+$ T cells, T_H1 cells, and natural killer cells into the tumor microenvironment. T_H17 cells express CC-chemokine receptor 6 (CCR6) and CXCR4 and high levels of CCL20, the ligand for CCR6, and CXCL12, the ligand for CXCR4, are found in the tumor microenvironment, which may attract T_H17 cells into the tumor environment.[23]

1.5.1.2 Lymphocytes promoting Tumorigenesis

T_H22 cells express CCR6 and migrate towards CCL20 and can be found in multiple types of cancer.[24,25] They promote tumorigenesis by Interleukin 22 (IL22). IL22 leads to the upregulation of several genes and results in increased cancer stemness and potential for tumorigenesis.[26] Another important type of cells promoting tumorigenesis are regulatory T (T_{reg}) cells. T_{reg} cells express CCR4 and are recruited into the tumor microenvironment by the chemokine CCL22, which is produced by macrophages and tumor cells.[27] They also

express CCR10 and migrate towards CCL28, a chemokine secreted in hypoxic tissue regions.[28] T_{reg} cells suppress antitumor T cell response and therefore create an immunosuppressive environment leading to tumor growth and cancer progression.[27] Supporting this statement is the fact, that high amounts of T_{reg} cells can be found in the bone marrow.[29] These cells express CXCR4 and are recruited by CXCL12, which is highly synthesized in the bone marrow. Because of the local immunosuppression, they may enable cancer to evade antitumor response, which may be the reason why many cancers metastasize into the bone marrow.

Additionally, B lymphocytes could promote tumorigenesis and tumor progression. B lymphocytes infiltrating the tumor microenvironment express CXCR4 and are attracted by the chemokine CXCL12. In breast cancer, high levels of infiltrating B cells are associated with prolonged survival.[30] In contrast, mouse models have been shown that B cells promote tumor progression, angiogenesis and suppress an antitumor immune response.[23]

1.5.1.3 Antigen-presenting Cells and Chemokines

Antigen-presenting cells, including B cells, dendritic cells, macrophages, and myeloid-derived suppressor cells, have both antitumor activity by interacting with T cells and promote tumorigenesis by interacting with tumor cells and their surrounding stromal cells. These functions depend upon the stage of maturation of dendritic cells. Mature dendritic cells enable potent antitumor activity by activating tumor-specific T cells.[31] However, immature dendritic cells promote T_H2-immune response, which is not effective against tumor cells, and therefore alleviates the antitumor immune response and promotes tumor progression.[32] Consequently, only immature but not mature dendritic cells are found in the cancer microenvironment.[33] Immature dendritic cells express CCR6 and migrate towards CCL20, secreted by tumor cells.[33,34] Interestingly, the overexpression of CCL20 attracts myeloid dendritic cells and promote dendritic cell maturation and therefore inhibits tumor growth, which shows the fragility of the chemokine system.[23]

Plasmacytoid dendritic cells migrate into the tumor environment via the CXCL12/CXCR4 axis.[35] They show protumor activity by promoting the development of regulatory $CD8^+$ T cells which suppress activation of effector T cells by myeloid dendritic cells. Thus, myeloid dendritic cells may promote tumor progression by suppressing antitumor response.

Macrophages express CCR2 and migrate towards CCL2 synthesized by the tumor.[36] These tumor-associated macrophages may inhibit T cell activation and are associated with poor

prognosis in some cancer.[23] They also promote cancer stemness, resilience against chemotherapeutics, tumor progression, and metastasis.[23,37]

Myeloid derived suppressor cells (MDSC) comprise a heterogeneous group of myeloid cells including monocytic and granulocytic cells.[23] Monocytic cells are macrophages in different maturation stages and granulocytic MDSC are neutrophils in different maturation stages. MDSCs are associated with immune suppression and cancer stemness.[38] The chemokine attracting monocytic MDSCs is CCL2.[23] Additionally, the CXCL5-CXCR2 and the CXCL12-CXCR4 axis may be involved in MDSC trafficking.[39] Granulocytic MDSCs express CXCR1 and CXCR2 and migrate towards the chemokine CXCL8, expressed by tumor cells and myeloid cells.[40] The recruitment of neutrophils is leading to angiogenesis and tumor progression.[40,41]

1.5.2 Direct Effects of Chemokines on Malignant Cells

Chemokines promote tumorigenesis and tumor progression in part by trafficking leukocytes into the tumor microenvironment, which then produce growth factors and provide an immunosuppressive environment. However, they also directly affect tumor cells expressing the corresponding chemokine receptor. For example, CCL2, CCL3, and CCL5 promote tumor progression by inducing the secretion of matrix metalloproteinase 9 (MMP9).[42,43] Additionally, CCL2 and CCL5 promote tumor cell proliferation, survival, motility, and epithelial-mesenchymal transition (EMT).[23] CCL18 is another chemokine promoting EMT, invasion, and metastasis. However, these effects vary based on the type of cancer. While CCL18 is associated with the effects stated above in breast cancer, pancreatic cancer, and ovarian cancer, high levels of the chemokine CCL18 are associated with improved outcomes in gastric cancer.[23] Moreover, CCR9, the receptor for CCL25, is highly expressed in many cancers and is responsible for chemoresistance[44] and inducing the expression of matrix metalloproteinases and therefore promoting tumor progression.[45] CCL25 can also facilitate metastasis of malignant cells by attracting $CCR9^+$ tumor cells into tissues with high CCL25 expression levels, e.g., the small intestine. Indeed, it has been shown, that melanoma cells that have metastasized into the small intestine express the chemokine receptor CCR9.[46] Another chemokine is CXCL8, the receptor is CXCR8. On the one hand, it promotes angiogenesis by targeting vascular endothelial cells.[47] On the other hand, it promotes invasion and metastasis by targeting cancer cells expressing CXCR8.[48] Additionally, CXCL12 targets vascular endothelial cells and promotes angiogenesis, but also promotes tumor proliferation, invasion, and metastasis by binding on CXCR4 expressing tumor cells.[23]

CXCR4 expressing cancer cells also show high metastatic potential and reduced sensitivity to radiation.[49]

1.5.3 Chemokines and Tumor Progression

Metastases from tumors are the greatest contributors to cancer-related morbidity and mortality, and distant tumor infiltration often indicates a terminal illness.[50] In order to metastasize, cancer has to overcome several limitations, which inhibit tumor progression. First, tumor cells need the ability to proliferate unlimitedly. In normal tissue, most cells are in a state, which prevents them from entering the cell growth-and-division cycle. This so-called cellular senescence prevents uncontrolled proliferation and subsequent malignant transformation. Chemokines and their receptors may play a role in inducing cellular proliferation arrest. For example, loss of chemokine receptor CXCR2 expression reduces cellular senescence and alleviates DNA-damage response mechanisms, and conversely, the up-regulation of CXCR2 leads to premature cell senescence.[51] Chemokines also exhibit a direct proliferation stimulus to cancer cells by activating the MAPK/ERK signaling pathway.[52] As shown above, the chemokine CXCL12 promotes tumor proliferation and is involved in promoting growth in numerous tumors, including small-cell lung cancer.[53] Additionally, the chemokines CXCL1, CXCL2, and CXCL3 may also contribute to tumor growth in melanoma and lung cancer.[52]

Second, tumor cells have to escape from the primary tumor side and penetrate the basement membrane, the last layer of defense preventing invasiveness of malignant cells. The ability to migrate is lost in polarized epithelial cells. Tumor cells can overcome this opposition by multiple biochemical changes called epithelial-mesenchymal-transformation (EMT). This transformation gives them a mesenchymal cell phenotype, ultimately leading to the acquisition of migrating capability and the production of extracellular matrix components. Tumor cells may then overcome the basement membrane and some of them may ultimately metastasize to distant organs.[54] The upregulation of the chemokine receptor CXCR4 in response to transforming growth factor-beta (TGF-β) may promote EMT.[55] Additionally, another study shows that the CXCL8/CXCR1 axis is crucial for EMT.[56]

Third, transformed tumor cells must leave the primary environment and enter the lymphatic system or the bloodstream. Metastasis is a non-random, finely orchestrated process that allows tumor cells to seed at specific distant tissues. Certain organs, including the lungs, liver, brain, bone marrow, and lymph nodes, are frequently infiltrated by tumor metastases. In contrast, other organs such as the skin or the kidneys are involved rarely.[57] Chemokines

and chemokine receptors are also key players in tumor metastasis.[58] One important contributor to metastasis is the CXCL12/CXCR4 axis. While CXCL12 is particularly expressed in the lung, brain, lymph nodes, and bone marrow, studies show that tumor cells upregulate the expression of the corresponding chemokine receptor CXCR4 and therefore can migrate towards high CXCL12 levels, as leukocytes do in physiological conditions. This axis has an important role in metastasis in various cancers, including breast, ovarian, melanoma, colorectal, gastric, small-cell lung carcinoma, and non-small-cell carcinoma.[52]

Of particular interest is also the axis involving the chemokine receptor CCR7 and its ligands CCL19 and CCL21. In physiological conditions, CCR7 is responsible for the migration of naive T cells and activated dendritic cells into the lymph node where DCs may initiate the adaptive immune response. Tumor cells can use this pathway to metastasize to lymph nodes. Cancers exploiting this axis are for example esophageal squamous cell carcinoma, melanoma, head and neck cancer, gastric carcinoma, and non-small-cell lung cancer.[59-64]

Chemokine receptor CCR9 and the associated chemokine CCL25 are important for mucosal immunity and T cell development because CCL25 is expressed in the thymus and the small intestine.[65] This axis may enable cancer expressing the chemokine receptor CCR9 to metastasize into the small intestine, which is particularly important in the development of metastasis in melanoma.[46]

Another important pathway for metastasis is the CXCR3-CXCL9, CXCL10, CXCL11 axis, which may facilitate metastasis to lymph nodes in malignant melanoma.[66] CXCR3 is also expressed on lung cancer cells.[64]

1.6 Chemokines and Brain Metastases

Brain metastases contribute to a great extent to the cancer burden. They are one major cause of cancer-related morbidity and mortality. However, the pathways for metastasis to the brain are not well understood. The chemokine receptor CXCR4 and the chemokine CXCL12 are of particular interest because studies have shown an overexpression of the chemokine receptor CXCR4 in non-small-cell lung cancer with brain metastases.[67,68] Furthermore, the chemokine receptor CX3CR1 has been associated with metastasis to the brain in breast cancer.[69] Additionally, the chemokine receptor CCR4 is associated with metastasis to the brain in melanoma.[70] These studies suggest that chemokines are also pivotal in the development of brain metastases.

1.7 Chemokines and Non-Small-Cell Lung Cancer

As described above, the chemokine network engages in a variety of different tumors and takes part in nearly every step of tumorigenesis and cancer progression. This is also true for NSCLC.[71]

1.7.1 CC Chemokines and NSCLC

The chemokine CCL2 is responsible for attracting tumor-associated macrophages and angiogenesis. Therefore, it may contribute to cancer progression. But there are conflicting results, and one study even showed a survival benefit when CCL2 is expressed.[72]

There are also conflicting data for chemokine CCL5. While one study suggested better survival, other studies concluded that CCL5 may support cancer progression.[73-75]

Furthermore, the chemokines CCL19 and CCL21 which bind to the chemokine receptor CCR7, lead to the formation of lymph node-like structures. These lymphoid structures are associated with improved antitumor response and survival in NSCLC.[76,77] Additionally, upregulated expression of CCR7 is associated with better survival after surgery.[78]

Another important chemokine/chemokine receptor axis is the CCL25/CCR9 axis. It plays a role in tumor survival, tumor progression, and metastasis, especially in adenocarcinomas of the lung.[79,80] Tumor survival is accomplished by inhibiting chemotherapy-induced apoptosis in a PI3K-/Akt-dependent and focal adhesion kinase-independent manner, whereas increased metastatic potential is achieved by upregulating MMP2 & MMP9.[79]

1.7.2 CXC chemokines and NSCLC

It has been shown that the CXC chemokine family plays a role in regulating angiogenesis in non-small-cell lung cancer.[81]

The chemokine CXCL8, signaling via CXCR1 and CXCR2, has, as shown above, proangiogenic activity and is also involved in cancer progression and NSCLC cells produce high levels of this chemokine.[82] Therefore, increased levels of the chemokine CXCL8 are associated with tumor proliferation, metastasis to lymphatic tissue, and poor survival.[83,84] Additionally, there may be direct growth effects to tumor cells expressing chemokine receptor CXCR1.[82]

Another CXC chemokine, CXCL5, induces angiogenesis via chemokine receptor CXCR2. High levels in NSCLC are associated with increased vascularization.[85] Furthermore, the chemokine CXCL5 attracts neutrophils into the tumor microenvironment and overexpression of CXCL5 in lung cancer is associated with cancer progression, including tumor proliferation, epithelial-mesenchymal transition, migration, and invasion.[86]

The chemokine CXCL12, which binds to the chemokine receptor CXCR4 is one of the most studied chemokines in cancer biology. CXCL12-CXCR4 axis is of particular importance for the formation of metastasis and specifically metastasis to the central nervous system in NSCLC, as numerous studies have shown.[87-95] Likewise, NSCLC cells show high expression of the chemokine receptor CXCR4, which may promote chemotactic metastasis to organs with high levels of CXCL12, such as the bone marrow, the liver, or the brain.[96]

In summary, chemokines and their receptors are involved in a broad spectrum of cancer biology. They guide immune cells into the cancer environment, and they may then promote survival and progression. But chemokines are also important for angiogenesis which is required for cancer survival. Finally, chemokines also have direct effects on cancer cells, for which they promote survival and may help cancer to metastasize into distant organs.
Numerous studies have already shown the effects of chemokines on cancer and specifically on lung cancer. Even though there is already supporting evidence for the CXCL12/CXCR4 axis in brain metastasis, research on differences in the expression pattern of other chemokine receptors in brain metastasis in comparison to the primary tumor in NSCLC is still lacking. This thesis aims on discovering, the specific chemokine expression pattern on NSCLC and their corresponding brain metastases as well as answering if there are significant expression differences.

2 Material and Methods

2.1 Tissue Samples

Tissue samples dating back to 2012 have been obtained from the archives of the Biobank Graz. Lung cancer entities were classified according to the current WHO classification of lung neoplasms.[97] Only adenocarcinomas of the lung and brain metastases from verified adenocarcinomas of the lung have been included in this experiment. Control tissues comprise of lymph node tissue and tonsil tissue, all acquired from autopsies performed at the Diagnostic and Research Institute of Pathology of the Medical University of Graz. Unfortunately, it was not possible to acquire matched samples from the same patients. In total 13 adenocarcinoma of the lung and 30 brain metastases from adenocarcinomas of the lung were retrieved from the Biobank Graz. Additionally, the surrounding lung and brain tissues, acquired from macrodissection as described below, were used in this experiment.

The total number of surrounding tissues was seven for lung tissues and eight for surrounding brain tissues.

Formalin-fixed paraffin-embedded (FFPE) tissues were cut using a microtome with a disposable blade. The first cut was used for HE staining. This has been done to ensure that there is tumor tissue on the slide and to highlight normal tissue for macrodissection. Additionally, the last cut was HE stained as well for tumor presence verification after the serial sections were done. Dr.^{in} med.univ. Marlene Leoni, an experienced pathologist, has performed microscopic verification. Serial sections in between were cut into 10 µm sections. Depending on the tissue size, the total thickness for further processing was approximately 50 µm. When there was no surrounding tissue present, the whole cut was used for RNA isolation and has been put straight into extraction tubes. When normal tissue was present on the slide, it was manually macrodissected by using a single-use toothpick and a sterile, disposable blade. Particular attention was given to the cleaning of the workspace, the microtome, and the tools with Thermo Scientific™ RNase AWAY™ (Thermo Fisher Scientific Inc., Waltham, MA, USA), changing the water between new samples, and always wearing fresh gloves. Ethical approval was obtained from the local ethics committee (31-144 ex 18/19).

2.2 RNA Isolation

Total RNA isolation has been performed with the Relia Prep™ FFPE Total RNA Miniprep System (Promega, Madison, WI, USA) using the manufacturer's protocol (TM353 Revised 12/15). The RNA was diluted in 30 µL nuclease-free water and the RNA quantity was then measured by using the Thermo Scientific™ NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) with the NanoDrop 1000 operating software, version 3.8.1. RNA was stored at -80 °C until further processing.

2.3 cDNA Synthesis

The synthesis of cDNA has been performed by using the Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to revision 11 of the manufacturer's protocol and by using 1 µL random hexamer primer. The total reaction volume was 20µL. The UNO^{96} thermocycler from VWR International GmbH (Darmstadt, Germany) has been used for cDNA synthesis. Cycling

conditions for cDNA synthesis were 25°C for 5 minutes, 42°C for 60 minutes, and the termination of the reaction happened at 70°C for 5 minutes.

2.4 Real-Time PCR

Because of low RNA isolation yield in some samples, a two-step approach was used for reverse transcription-quantitative real-time PCR (RT-qPCR). In a first step, the chemokine receptor expression for all 19 chemokine receptors was tested on one lung cancer sample, one metastasis sample, and on the control tissues. Melt curve analysis showed unspecific amplicons for the chemokine receptors CCR1, CCR5, CCR10, CXCR1, CXCR2, and CX_3CR1, and they were subsequently excluded from the project. The remaining 13 chemokine receptors (CCR2-CCR4, CCR6-CCR9, CXCR3-CXCR7, and XCR1) were tested on five lung cancer samples, on five brain metastasis samples, and the control tissues. Solely these receptors, for which mRNA transcripts could be found in the tumor samples, were further tested on the entire sample size. Primers were ordered from Ingenetix GmbH (Vienna, Austria), except for CCR1, CCR6, CCR8, CCR10, CXCR4, and CX_3CR1, which have been obtained from Eurofins Genomics Germany GmbH (Ebersberg, Germany). **Table 3** shows the primers for the chemokine receptors and their sequence. cDNA was diluted 1:20 with nuclease-free water for PCR. For RT-qPCR, the Luna® Universal qPCR Master Mix M3003 (Applied Biosystems, Foster City, CA, USA) with a modified protocol was used. The total reaction volume was 10 µL, containing 5 µL of Master Mix, 4 µL cDNA, and varying forward and reverse primer concentrations. The optimal primer concentration was determined on tonsil and lymph node tissue before the experiment. **Table 4** shows the concentration used in the experiment for the respective primer. The thermocycling conditions were 50°C for 2 minutes and 95°C for 10 minutes for polymerase activation and 40 cycles at 95°C for 15 seconds and 60°C for 30 seconds. Upon PCR, a melt curve stage, i.e., fluorescence data capturing during gradual temperature increase from 60°C to 95°C, has been carried out to distinguish specific amplicons from nonspecific amplification products. RT-qPCR was performed on the QuantStudio™ 7 Flex Real-Time PCR System and by the usage of the QuantStudio™ Real-Time PCR Software Version 1.3 of Applied Biosystems (Foster City, CA, USA). *GAPD* and *ACTB* were used as reference genes to compare the cycle number in relation to the target genes. Only samples with *GAPD* and *ACTB* C_t values less than 32 were included. The geometric mean of the C_t value of *GAPD* and *ACTB* was used for calculation. FFPE tonsil tissues and lymph node tissues obtained from autopsies

and processed as above served as calibrators. Likewise, the geometric mean of the C_t values has been used for calculation. Relative expression was determined in triplets and calculated based on the $2^{-\Delta\Delta CT}$ method.[98]

Table 3 Nucleotide acid sequence of primers used in the real-time PCR

GENE	PRIMER FORWARD	PRIMER REVERSE
CCR1	GACTATGACACGACCACAGAGT	CCAACCAGGCCAATGACAAATA
CCR2	GATGAATGGGAGTGAGGGATAGTG	GAGCCCTTTGCTTCACCTTTG
CCR3	CAACATCTACCTGCTCAACC	GCCAAAAACCCAGTTATGCC
CCR4	TAATATTGCAAGGCAAAGACTATTCC	GCGATTTACTCCATCAGCCAGTA
CCR5	GATTGATTTGCACAGCTCATCTG	TGTCATAGATTGGACTTGACACTTGA
CCR6	CCTGACTTGCATTAGCATGGA	GCGGTAGTGTTCTGGATCGG
CCR7	GGGCACAGCCTTCCTGTG	CCACCACCAGCACGCTTT
CCR8	CTGTCTGACCTGCTTTTTGTCT	CCACTTTGCACATTACAGTCCC
CCR9	GACTTCACAAGCCCTATTCCTAACA	AAGTCAAGTGAAGTTGAAGTTAACGTAGTCT
CCR10	GCAAACGCAAGGATGTCGC	CGTAGAGAACGGGATTGAGGC
CXCR1	CTCCTACTGTTGGACAC	ACATGTCCTCTTCAGTTTC
CXCR2	AGGTGTCCTACAGGTGAAAAG	AATCTTCAAAGCTGTCACTCTC
CXCR3	CAGCCCAGCCATGGTCCTTG	GGAAGAGCTGAAGTTCTCCAG
CXCR4	GGGCAATGGATTGGTCATCCT	TGCAGCCTGTACTTGTCCG
CXCR5	CAGCCATGAACTACCCGCTAA	CCAATCTGTCCAGTTCCCAGA
CXCR6	AGAGCAGCAGTGAAAACAAG	ACAAAAGTCAAGCCCCAAG
CXCR7	CTACACGCTCTCCTTCATTTAC	TATTCACCCAGACCACCAC
CX3CR1	AGTGTCACCGACATTTACCTCC	AAGGCGGTAGTGAATTTGCAC
XCR1	CCATCGTGGTGGCCTACTTC	CGCAGCTCCGGATGATCT

Table 4 Concentration of the primers used in the experiment

CHEMOKINE RECEPTOR	CONCENTRATION OF FORWARD AND REVERSE PRIMER
CCR1	**100 nM**
CCR2	**250 nM**
CCR3	**250 nM**
CCR4	**500 nM**
CCR5	**125 nM**
CCR6	**250 nM**
CCR7	**500 nM**
CCR8	**125 nM**
CCR9	**250 nM**
CCR10	**125 nM**
CXCR1	**250 nM**
CXCR2	**250 nM**
CXCR3	**125 nM**
CXCR4	**100 nM**
CXCR5	**500 nM**
CXCR6	**500 nM**

Table 4 Continued

CHEMOKINE RECEPTOR	CONCENTRATION OF FORWARD AND REVERSE PRIMER
CXCR7	**500 nM**
CX3CR1	**250 nM**
XCR1	**500 nM**
GAPD	**500 nM**
ACTB	**500 nM**

2.5 Statistical Analysis

Statistical analysis was performed by using SPSS Version 27. The nonparametric Mann-Whitney U test was used to analyze differences in the chemokine receptor expression levels among neoplastic lung tissues, brain metastases, and the surrounding lung and brain tissues. P values less than 0.05 were considered significant. Chemokine receptors expression levels are presented as mean values ± standard deviation (SD).

3 Results

3.1 Chemokine Receptor Expression Pattern

There was inconsistent mRNA expression for the CC Chemokine receptors CCR2, CCR3, CCR5, CCR7, and CCR10, the CXC chemokine receptors CXCR3, CXCR5, CXCR7, the CX_3C chemokine receptor CX_3CR1, and the XCR chemokine receptor XCR1 on tumor cells compared to the calibrator tissue in the preliminary experiment. They were excluded from further testing on the full sample size. The expression of CC chemokine receptors CCR4, CCR6, CCR8, and CCR9 and of the CXC chemokine receptors CXCR4 and CXCR6 was finally tested on the entire sample size. **Table 5** shows the mRNA expression pattern of the tested CC and CXC chemokine receptors on samples of NSCLC, corresponding brain metastases, and the surrounding lung and brain tissues.

Table 5 mRNA expression of CC and CXC receptors in NSCLC, brain-specific metastases of NSCLC and surrounding lung and brain tissues

	CCR4	CCR6	CCR8	CCR9	CXCR4	CXCR6
NSCLC	**13/13**	**11/13**	**6/13**	**10/13**	**13/13**	**12/13**
METASTASIS	**29/30**	**23/30**	**8/30**	**23/30**	**30/30**	**26/30**
SURROUNDING LUNG TISSUE	**7/7**	**7/7**	**1/7**	**6/7**	**7/7**	**7/7**
SURROUNDING BRAIN TISSUE	**6/8**	**7/8**	**3/8**	**7/8**	**8/8**	**8/8**

3.2 Relative expression of CC Chemokine Receptors in NSCLC and Metastases

Calculation of the relative expression of the chemokine receptors showed that the tested CC chemokine receptors CCR4 and CCR9 are highly expressed both in tissues of the primary tumor and tissues of metastases of NSCLC compared to the calibrators. In contrast, the chemokine receptor CCR6 showed high expression levels in the primary tumor and lower expression levels in metastases. The expression of mRNA encoding CCR8 was homogenous in the tumor tissues, but high levels were observed when expressed (**Figure 3 and 5**).

The expression of CCR6 and CCR9 was significantly higher in the primary tumors compared to metastases (P=0.0429 and P=0.0499, respectively). There were no differences in the expression of CCR4 (P=0.0531) and CCR8 (P=0.7546) in metastases, compared to the primary tumors.

3.3 Relative Expression of CXC Chemokine Receptors in NSCLC and Metastases

Both chemokine receptors CXCR4 and CXCR6 showed high expression levels in the primary adenocarcinomas of the lung, whereas they showed lower expression levels in metastases compared to the calibrators (**Figures 4 and 5**).

Expression levels of CXCR4 and CXCR6 were significantly lower in metastases compared to the primary tumor (P=0.0107 and P=0.034, respectively).

3.4 Chemokine Receptor Expression in the Surrounding Tissues

In the surrounding tissues, i.e., lung and brain tissue, all chemokine receptors but CCR8 in lung tissues, showed consistent expression (**Table 5**).

3.4.1 CC Chemokine Receptors in the Surrounding Tissues

The chemokine receptor CCR4 showed a low expression level in lung tissues. In addition, also the chemokine receptor CCR6 showed low expression levels in lung tissues. The expression of mRNA for the chemokine receptor CCR9 was also low compared to the calibrators in the surrounding lung tissues. In the surrounding brain tissues, the chemokine receptors CCR4, CCR6 and CCR9 showed low expression levels compared to the calibrator (**Figures 3 and 5**).

The mRNA expression levels for the chemokine receptor CCR9 were significantly higher in the primary tumor compared to the surrounding lung tissue (P=0.0496). No difference was

observed for the other chemokine receptors in the surrounding lung and brain tissues compared to the primary tumors and brain metastases.

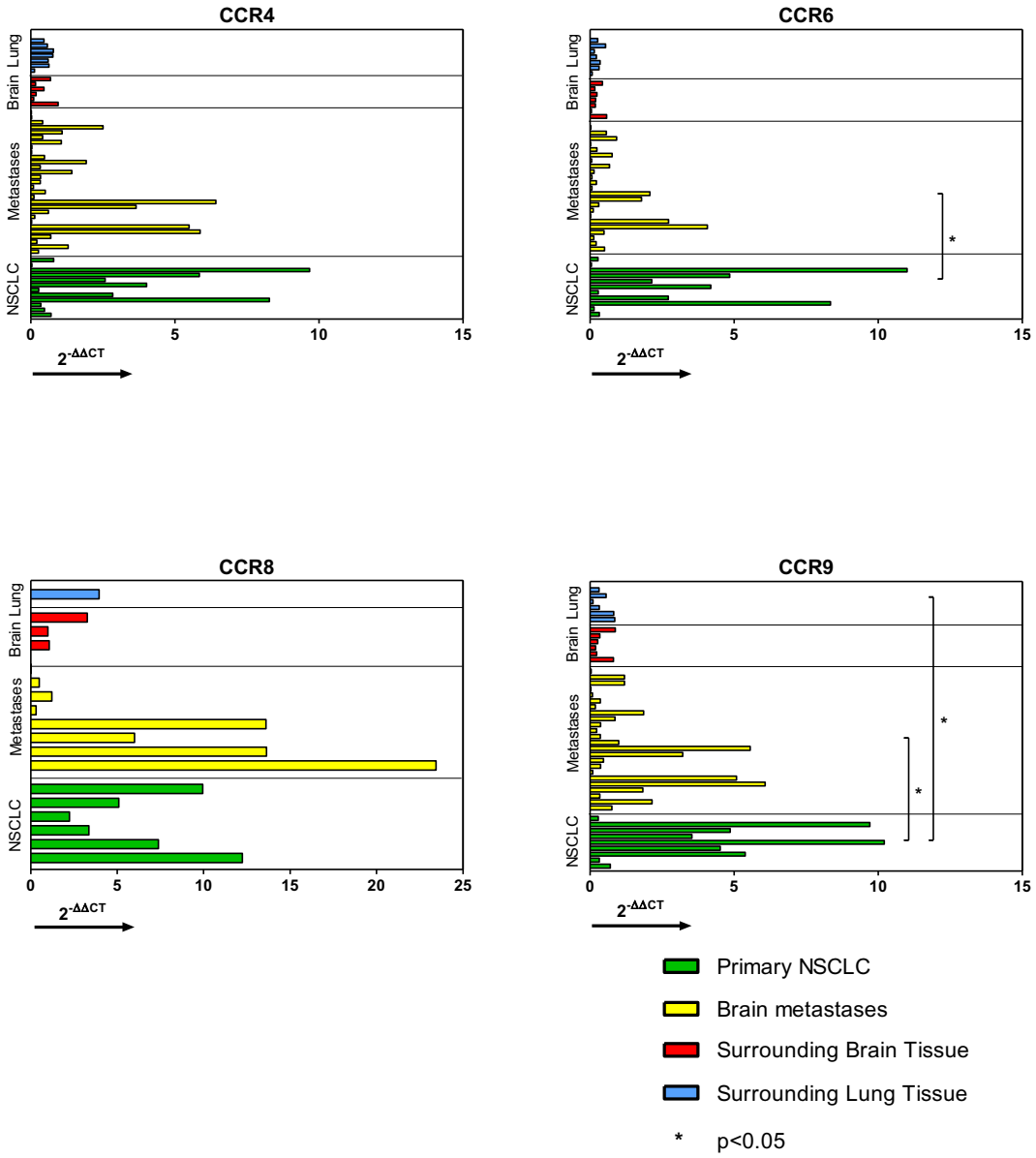


Figure 3 CC chemokine receptor mRNA profile in NSCLC, brain-specific metastases of NSCLC, surrounding lung tissue and surrounding brain tissue. Each bar represents a specimen. Values of gene expression are calculated as relative expression. Asterisk denotes significantly different chemokine receptor expression. NSCLC denotes non-small-cell lung cancer; Metastases, brain-specific metastases of NSCLC; Brain, surrounding brain tissue; Lung, surrounding lung tisue

3.4.2 CXC Chemokine Receptors in the Surrounding Tissues

High mRNA expression levels for the CXC chemokine receptor CXCR4 were detectable in the surrounding lung tissue. The chemokine receptor CXCR6 showed low expression levels in the surrounding lung tissues compared to the calibrator tissues. Both the chemokine receptor CXCR4 and CXCR6 showed a low expression level in the surrounding brain tissue (**Figures 4 and 5**).

There were no significant differences regarding expression levels for CXCR4 and CXCR6 in the surrounding lung and brain tissues compared to the primary tumors and metastases, respectively.

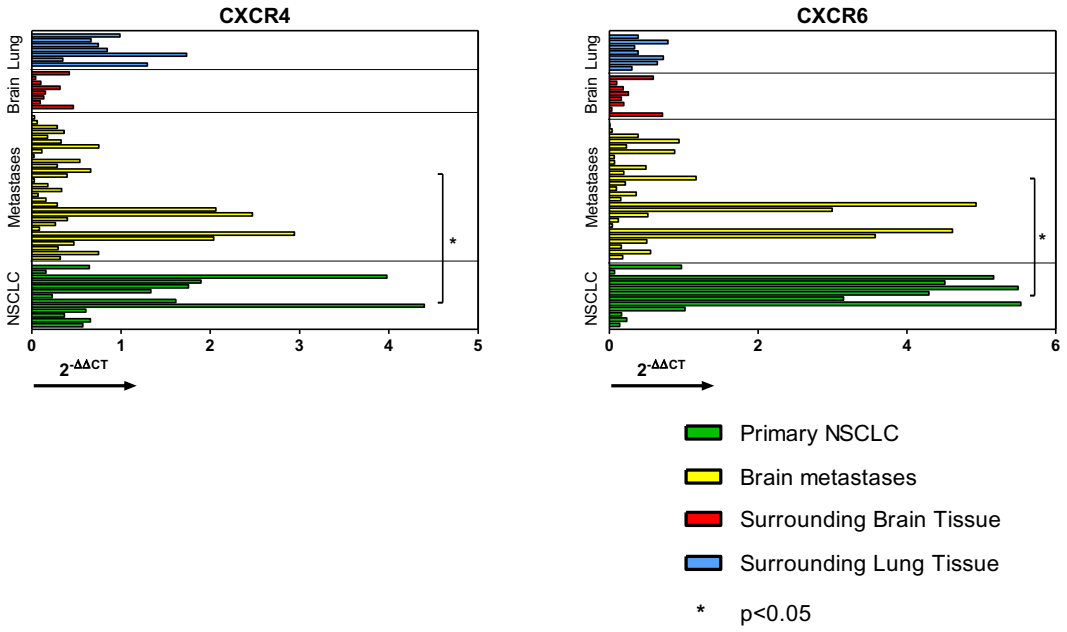


Figure 4 CXC chemokine receptor mRNA profile in NSCLC, brain-specific metastases of NSCLC, surrounding lung tissue and surrounding brain tissue. Each bar represents a specimen. Values of gene expression are calculated as relative expression. Asterisk denotes significantly different chemokine receptor expression. NSCLC denotes non-small-cell lung cancer; Metastases, brain-specific metastases of NSCLC; Brain, surrounding brain tissue; Lung, surrounding lung tisue

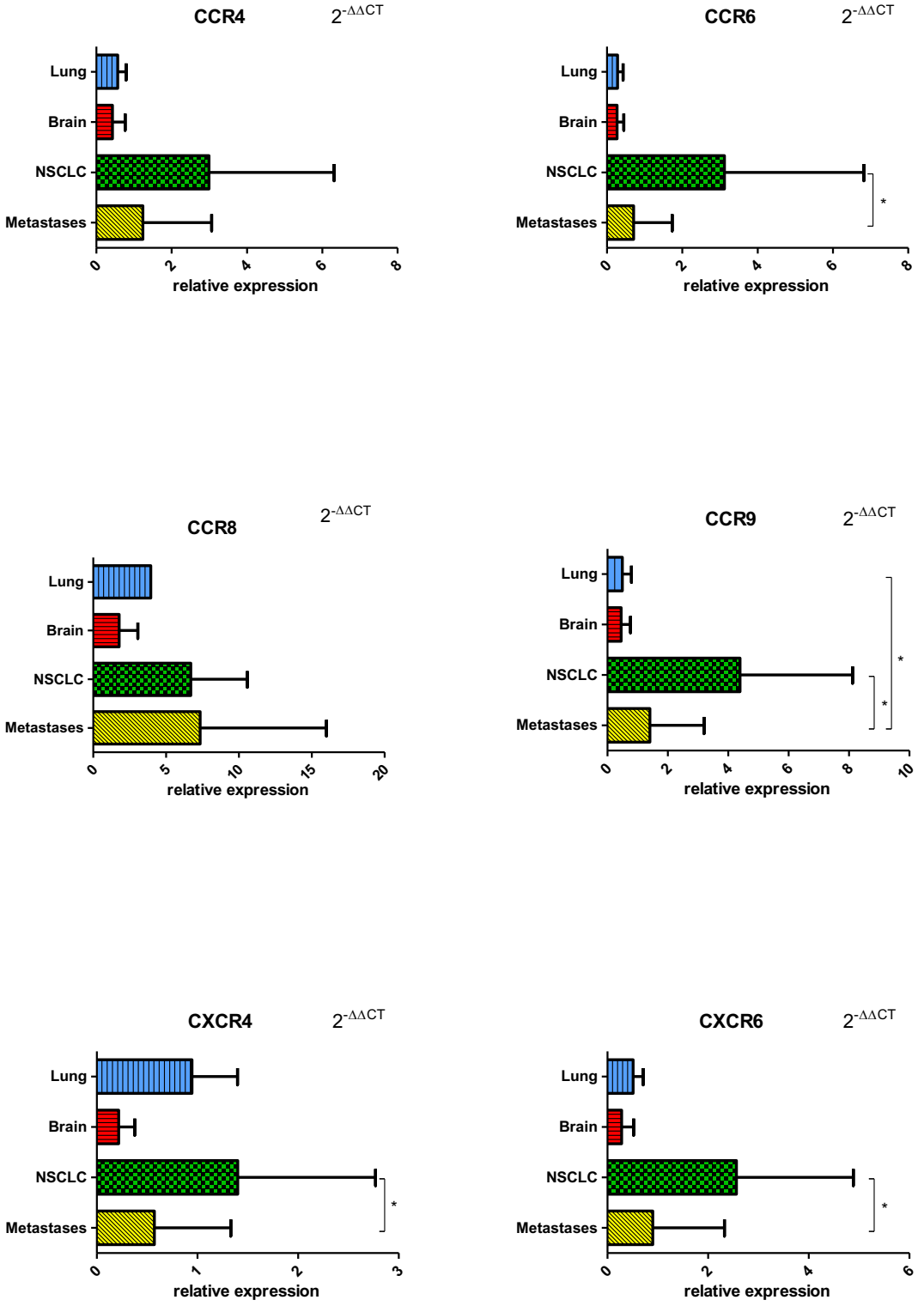


Figure 5 mRNA expression levels of chemokine receptors CCR4, CCR6, CCR8, CCR9, CXCR4 and CXCR6 are presented as mean values ± standard deviation. Asterisk denotes significantly different chemokine receptor expression. NSCLC denotes non-small-cell lung cancer; Metastases, brain-specific metastases of NSCLC; Brain, surrounding brain tissue; Lung, surrounding lung tissue

4 Discussion

Chemokines and chemokine receptors are best known for leukocyte trafficking. But as described above, there is robust evidence, that chemokines and their receptors may play an essential part in the development and progression of cancer including lung cancer. This thesis was designed to analyze the chemokine receptor expression pattern of non-small-cell lung cancer and corresponding brain metastases evolving from NSCLC progression.

The chemokine receptor CCR4 is highly expressed in NSCLC cells and the corresponding brain-specific metastases. Izraely et al. hypothesize that CCR4 may be vital for the development of metastases in melanoma.[70] This may also be an important contributor to cancer progression in NSCLC. Additionally, as described above, T_{reg} cells express the chemokine receptor CCR4 and suppress an effectively antitumor immune response.[27] Indeed, Liu et al. showed that T_{reg} cells migrate into the microenvironment of non-small-cell lung cancer via CCR4 and suppress the antitumor immune response.[99] Unfortunately, no immunohistochemical analysis has been performed in this thesis to allocate chemokine receptor expression to specific cells. However, the high expression of mRNA for CCR4 in the primary tumors and metastases may be attributable to T_{reg} cells in the tumor microenvironment.

The chemokine receptor CCR6 is expressed on T_H22 cells, and these leukocytes promote cancer stemness and tumorigenesis via IL22.[26] High expression levels were observed in NSCLC tissues, which may indicate the presence of T_H22 lymphocytes in the tumor microenvironment.

The presence of mRNA transcripts for CCR8 was high both in tissues of NSCLC and brain metastases. CCR8 and its ligand CCL1 may support cancer metastasis to lymph nodes,[100] a common side for NSCLC metastases. This thesis supports the role of CCR8 in metastatic disease and indicates that the chemokine receptor CCR8 could also facilitate metastasis to the brain.

Prior studies have noted that the chemokine receptor CCR9 is important in cancer progression by promoting survival and metastasis.[79,80] In this experiment, the expression level for CCR9 was high both in the primary tumors and the metastases. Interestingly, CCR9 expression was significantly lower in metastases. Tumors may lose this chemokine axis, because if metastasis already happened it may no longer be a survival benefit in the new environment, i.e., the brain.

This thesis also showed that the chemokine receptor CXCR4 is highly expressed in non-small-cell lung cancer and in brain-specific metastases of non-small-cell lung cancer. These results are in line with those of previous studies.[67,88,91] A recent review supports the thesis that the CXCL12/CXCR4 axis could be crucial for brain-specific metastasis in NSCLC.[94] However, in this experiment the mRNA expression levels for CXCR4 were significantly lower in brain metastases from NSCLC compared to primary NSCLC tissues. This indicates that tumor cells may lose CXCR4 expression once metastasis has occurred. Another explanation may be that loss of the chemokine receptor CXCR4 may facilitate metastasis because physiologically, the expression of CXCL12, the ligand for CXCR4, is high in normal lung tissue,[58] and losing the receptor for this chemokine may enable tumor cells to metastasize to distant organs.

Chemokine receptor CXCR6 expression was high both in the primary tumor tissues and tissues of metastases. This finding is consistent with previous studies.[101,102] Mir et al. hypothesize that the CXCL6/CXCR6 axis may be important for cancer motility and progression by upregulating and modulating matrix metalloproteinases.[101]

Interestingly, four out of the six tested chemokine receptors showed reduced expression in brain-specific metastases. Cancer cells may lose the ability to express chemokine receptors because of mutations as they progress, or they may not depend on chemokines as a growth and survival stimulus any longer.

There was no significant difference, except for CCR9 in NSCLC and the surrounding lung tissue, observable in the surrounding lung and brain tissues compared to NSCLC and brain metastases, respectively. An explanation may be the low sample size of surrounding tissues because there is a trend that the chemokine receptors CCR4, CCR6, CCR9, and CXCR6 show higher expression levels in NSCLC compared to the surrounding lung tissue.

This thesis has some limitations, and their results should be interpreted in line with other studies. As described in the methods part, because of low RNA isolation yield, all 19 well-characterized chemokine receptors were evaluated only on one primary NSCLC tissue, one brain-specific metastasis of NSCLC, and the control tissues to assess the suitability of the chemokine receptor primers. The remaining chemokine receptors were tested on five primary tumors and five metastases in a second step. Because of the low sample size, some chemokine receptors may have been excluded illegitimately from the experiment. As mentioned above, because of the lack of immunohistochemical analysis, no cellular matching to chemokine receptor expression was possible. A further study with more focus on immunohistochemical analysis is currently in the planning stage and could shed more

light on the cell-specific chemokine receptor expression pattern. Further research should also be undertaken to evaluate these results in a larger cohort and correlate them with clinical data.

In recent years, the development of targeted therapies gave hope for a new potent therapy against cancer. Since the chemokine network is involved in inflammation and multiple steps of cancer development, developing drugs targeting chemokines or chemokine receptors may impact future cancer treatment. One promising chemokine axis may be the CXCL12/CXCR4 axis. As described above, this chemokine and the corresponding receptor engage in angiogenesis and tumor progression, and they also may play a vital role in the development of brain metastases. Preclinical studies targeting this axis have shown reduced tumor weight, delayed tumor growth, less invasion, and reduced metastasis.[103-106] Several clinical trials are ongoing to evaluate the safety profile and efficacy of CXCR4 inhibitors.[107]

In summary, this thesis showed that the chemokine receptors CCR4, CCR6, CCR9, CXCR4, and CXCR6 were expressed both in NSCLC and corresponding brain-specific metastases. This indicates that these chemokines may play a role in metastasis to the brain. Furthermore, the expression of the chemokine receptors CCR6, CCR9, CXCR4, and CXCR6 was significantly lower in brain-specific metastases compared to the primary tumor, suggesting that the cancer biology is different in metastases compared to the primary tumor.

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