CCL2, CCL8, CXCL12 chemokines in resectable non-small cell lung cancer (NSCLC)

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Background. Complex networks of chemokines are part of the immune reaction targeted against tumor cells. Chemokines influence cancer growth. It is unclear whether the concentrations of chemokines at the time of NSCLC (non-small cell lung cancer) diagnosis differ from healthy controls and reflect the extent of NSCLC.
Aims. To compare chemokine concentrations (CCL2, CCL8, CXCL12) in the plasma of patients with resectable NSCLC to those without cancer. To determine whether the chemokine concentrations differ relative to the stage of disease.
Methods. Sixty-nine patients undergoing surgery for proven/suspected NSCLC were enrolled. They underwent standard diagnostic and staging procedures to determine resectability, surgery was performed. Forty-two patients were diagnosed with NSCLC, while 27patients had benign lung lesions and functioned as the control group. Chemokine concentrations in peripheral blood were assessed using ELISA. Parametric statistics were used for the analysis of results.
Results. There were no differences in plasma chemokine concentrations in NSCLC patients compared to controls. CXCL12 concentrations correlated positively with tumor extent expressed as clinical stage, (mean values: stage I 5.08 ng/mL, SEM 0.59; stage II and IIIA 7.82 ng/mL; SEM 1.06; *P*=0.022). Patients with NSCLC stages II+IIIA had significantly higher CXCL12 concentrations than controls (mean values: stage II+IIIA 7.82 ng/mL; SEM 1.06; controls 5.3 ng/mL; SEM 0.46; *P*=0.017).
Conclusion. CXCL12 was related to tumor growth and could potentially be used as a biomarker of advanced disease.

Key words: NSCLC, resectability, chemokine CCL2, chemokine CCL8, chemokine CXCL12, peripheral blood, ELISA method, biomarker

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BACKGROUND

Lung cancer is the most common type of cancer in the world. Lung cancer is also the most common cause of cancer death worldwide with a fatality rate (the overall mortality ratio divided by the incidence) of 0.87 (ref.[1]). Therefore NSCLC (non-small cell lung cancer), which is the most common type of lung cancer, has been the subject of many studies.

Histopathologic studies have shown that solid tumor tissue consists of not only neoplastic and stromal cells (immune cells, fibroblasts, and endothelial cells (leading to angiogenesis supporting tumor growth)), it also contains extracellular matrix (ECM) components, and inflammatory infiltrates containing tumor-associated macrophages, tumor-infiltrating lymphocytes (T, B, and NK cells), and tumor-associated neutrophils[2].

Local production of chemokines plays a crucial role in the inflammatory process within the tumor, mediating the recruitment of immune cells into the tumor microenvironment, which affects the immune response, and regulates angiogenesis[3,4]. A complex network of chemokines participates in immune system vs. tumor interactions. Different chemokine profiles can influence antitumor responses and thus determine a patient's prognosis as well as predict therapeutic effectiveness.

Like many chemokines, CCL2 (monocyte chemoattractant protein-1 (MCP-1)) is secreted from tumor cells and associated tumor stromal cells. It influences tumor progression and metastasis caused by angiogenesis[5].

CCL8 (monocyte chemoattractant protein 2 (MCP-2)) displays chemotactic activity relative to monocytes, lymphocytes, basophils, and eosinophils. By recruiting leukocytes to sites of inflammation, this cytokine contributes to tumor-associated leukocyte infiltration[6].

CXCL12 chemokine expression appears modulated through interactions between the tumor cells and infiltrating macrophages. The macrophages that infiltrate the tumor induce the lung cancer cells to elaborate chemokine CXC that promotes angiogenesis[7].

We chose to investigate the above-mentioned chemokines because CCL2 and CCL8 are crucial with respect to the composition of the tumor microenvironment and influence tumor progression. To date, their role in NSCLC

has not been wholly or clearly described. In contrast, the chemokine CXCL12 axis is the best-studied chemokine axis in lung cancer. CXCL12 is highly expressed in primary lung cancer and metastasis[8]; however, the correlation between CCL2, CCL8, and CXCL12 profiles and the prognosis for operable NSCLC stages is still unclear.

Our present study compared chemokine concentrations (CCL2, CCL8, and CXCL12) in the plasma of patients with resectable NSCLC to those without cancer. We sought to determine whether the concentrations of these chemokines differed relative to the stage of the disease.

METHODS

Study design

After signing an informed consent form (approved 2016 Ethics Committee of Thomayer Hospital and Institute of Clinical and Experimental Medicine, Prague, Czech Republic), 69 patients (Table 1) undergoing surgery for proven/suspected NSCLC were prospectively enrolled in the study (start of study 6/2016 – end of study 6/2019).

All patients provided a past medical history including smoking and asbestos exposure, and underwent a physical examination, blood tests, and a computed tomography of the chest to assess the intra-thoracic extent of the disease. Bronchoscopy was done (when possible) to obtain cytology and histology samples for a histopathologic evaluation[9].

Flowchart of study

Pre-operative and Operative Protocol

A) When NSCLC was suspected but not confirmed/excluded, the staging was based on abdominal ultrasound, brain CT, bone scintigraphy, chest CT or 18-fluorodeoxyglucose positron emission tomography (in indicated cases). Patients underwent lung function tests (spirometry and lung diffusing capacity for carbon monoxide), and operability was assessed. A multidisciplinary team was consulted regarding operability (relative to the patient) and tumor resectability, as well as determining the extent of the surgical procedure. Perioperative tissue histology was mandatory in all cases. When NSCLC was confirmed and perioperative findings indicated surgical treatment, a radical surgical procedure was performed.

Table 1. Basic patient characteristics.

	NSCLC	Control group (benign lesions)
Number of patients	42	27
Number of males/females	24/18	13/14
Sm./former sm./non-sm.	22/18/2	8/9/10
NSCLC stage I	22	NA
NSCLC stage II+IIIA	14 (stage II) + 3 (stage IIIA)	NA
NSCLC stage >IIIA	3	NA

sm., smokers; NA, not applicable.

B) When NSCLC was confirmed, staging procedures were completed, and lung functions were tested; the case was then further discussed within the multidisciplinary team. Suitable patients underwent lung surgery. Appropriate resection was performed (preferably anatomical resections), including dissection of the mediastinum and extirpation of all accessible lymph nodes.

Peripheral blood samples (to determine chemokine concentrations) were collected on the morning of surgery. Chemokine concentrations in peripheral blood were estimated using ELISA LSBio Kits (LSBio, Seattle, Washington, USA).

A total of 42 patients were diagnosed with NSCLC (3 patients had advanced NSCLC, stage >IIIA; radical surgery was not indicated, and they were excluded from the study); 27 patients had benign lung pathologies and acted as the control group.

NSCLC staging

We used the pathological classification found in the 8th Edition of the TNM classification for lung cancer to determine the stage of the disease[10]. We included patients with radical surgery for NSCLC and a final pathological staging up to stage IIIA (i.e., up to pT4 pN1 M0 included).

Peripheral blood-ELISA (enzyme-linked immunosorbent assay): The patient's peripheral blood was collected on the morning of surgery – EDTA (ethylenediaminetetraacetic acid) was used as the anticoagulant. We centrifuged samples for 15 min at 1000xg at 2–8 °C, which was done within 30 min of collection. We collected the supernatant for assaying. Supernatant samples were stored at −80 °C until analyzed (however, samples were never stored for more than six months).

Samples were brought to room temperature before performing assays. CCL2, CCL8, and CXCL12 concentrations were assessed using ELISA LSBio Kits according to the manufacturer's recommendations and using good laboratory practice. The concentrations of CCL2 and CCL8 are reported in pg/mL, while those of CXCL12 are reported in ng/mL.

Analysis: Data are expressed as mean ± standard error of the mean (SEM) unless otherwise stated; parametric statistics were used for analyses. Differences in chemokine concentrations across various groups (e.g., exposed vs. control patients, disease stage) were examined using a one-way analysis of variance (ANOVA). *P* values below 0.05 were considered statistically significant. Statistical analysis was performed using statistical software SPSS version 25 (SPSS Inc., Chicago, IL, USA). Stages II+ IIIA were evaluated together because of the low number of patients with stage IIIA (3 patients).

RESULTS

There was no difference between the concentrations of chemokines in the plasma of NSCLC patients and control patients (Fig. 1, 2, and 3). The CXCL12, but not CCL2 and CCL8 concentrations, were significantly

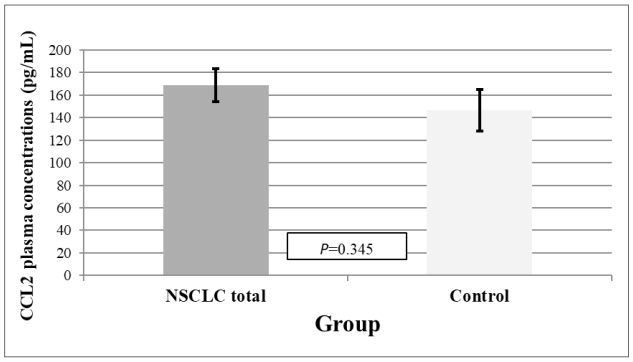

Fig. 1. Difference in CCL2 plasma concentrations between NSCLC patients total and control group (benign lesions).
NSCLC, non-small cell lung cancer.

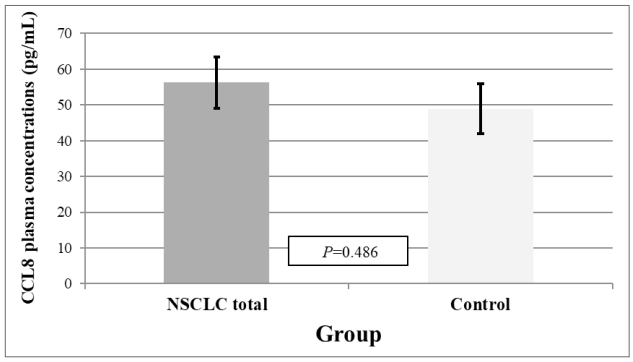

Fig. 2. Difference in CCL8 plasma concentrations between NSCLC patients total and control group (benign lesions).
NSCLC, non-small cell lung cancer.

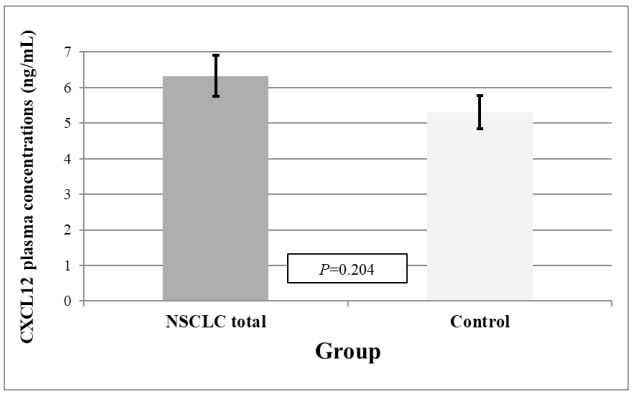

Fig. 3. Difference in CXCL12 plasma concentrations between NSCLC patients total and control group (benign lesions).
NSCL, non-small cell lung cancer.

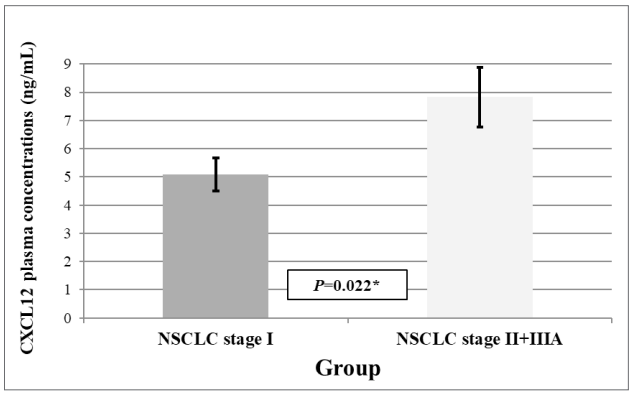

Fig. 4. Difference in CXCL12 plasma concentrations within NSCLC patients.
NSCLC,non-small cell lung cancer; *, statistically significant.

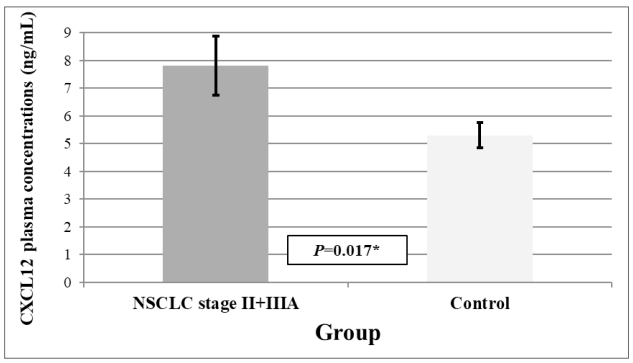

Fig. 5. Difference in CXCL12 plasma concentrations between NSCLC stage II+IIIA patients and control group (benign lesions).
NSCLC, non-small cell lung cancer stage, *, statistically significant.

positively related to the extent of the tumor as expressed by clinical stage with pathological TNM classification (i.e., stages II+IIIA had significantly higher concentrations of CXCL12 than stage I patients; mean values: stage I 5.08 ng/mL, SEM 0.59; stage II+IIIA 7.82 ng/mL, SEM 1.06; *P*=0.022), (Fig. 4). Even patients with NSCLC stages II+IIIA had significantly higher CXCL12 concentrations than the control group (mean values: stage II+IIIA 7.82 ng/mL, SEM 1.06; control group 5.3 ng/mL, SEM 0.46; *P*=0.017), (Fig. 5).

DISCUSSION

The study goal was to compare chemokine concentrations (CCL2, CCL8, and CXCL12) in the plasma of patients with resectable NSCLC to those without cancer. We also wanted to determine whether the chemokine concentrations differed relative to the stage of the disease.

We expected to find higher concentrations of the investigated chemokines in the plasma of patients with NSCLC compared to those without cancer. Furthermore, patients with higher NSCLC stages were expected to have higher concentrations of the studied chemokines compared to patients with lower stages.

Our hypothesis was based on the following findings from previous studies. CCL2 has been reported to be involved in the progression and metastasis linked to angiogenesis[5,11,12]. The role of CCL8 in NSCLC remains unclear, but it was shown that the CCL8 gradient drives breast cancer cell dissemination[13]. The elevated levels of CXCL12, as assessed by ELISA and immunohistochemistry, have been observed starting in stage IA through stage IIB of human NSCLC tumor samples[14].CXCL12 is highly expressed in primary lung cancer as well as during

metastasis[7]. It has been shown that CXCL12 levels are related to greater invasiveness and a higher potential for metastasis[15].

Our study did not find any significant difference in the concentrations of chemokines in the plasma of NSCLC patients relative to controls. This might be explained by chemokines also playing a role in benign lesions, especially in the inflammation process (some patients in our control group had final diagnoses such as pneumonia, abscesses, and tuberculosis). So far, little has been published regarding benign pulmonary lesions and the concentrations of chemokines, except for tuberculosis cases. Chemokines and cytokines are critical for initiating and coordinating organized and sequential recruitment and activation of cells into lung tissue infected with Mycobacterium tuberculosis, as well as disease development[16]. We also know that chemokines, together with adhesion molecules, cytokines, and proteases, are essential for the directed migration of leukocytes during normal and inflammatory processes[17]. As such, we believe that it would be helpful to assess (1) chemokine concentration gradients (differences in circulating chemokine concentrations between systemic and tumor vascular beds), which can vary[18], (2) the presence of chemokine receptors, and (3) the role of tumor- infiltrating cells to further understand tumor microenvironments.

Our study found that the concentrations of chemokine CCL2 did not differ relative to disease stage. A possible explanation for this is that we know that CCL2 influences tumor progression and metastasis caused by angiogenesis[5,11,12] and that CCL2 is overexpressed in NSCLC cells. However, we also know that its expression in cancer cells is associated with better survival in NSCLC patients[19]. A different study showed that the CCL2 content in plasma and CCR-2 expression in PBMC (peripheral blood mononuclear cells), assessed using cytospin techniques, were similar in patients with early-stage lung cancer compared to those with late- stage lung cancer[20]. Hence, the role of CCL2 in lung cancer is at least controversial, having both pro-tumorigenic and anti-tumorigenic effects, which might explain why plasma concentrations measured in our study did not differ.

Concentrations of chemokine CCL8 did not differ relative to disease stage in our study. CCL8, in NSCLC, has not been broadly studied, although it has been studied in breast cancer. A previous study showed that CCL8 gradients are associated with breast cancer cell dissemination[13]. A different study failed to find any significant differences in CCL8 serum levels between healthy individuals and breast cancer patients[21]. A possible explanation for why we did not find any difference in concentrations might be that the role of CCL8 is not a critical factor in NSCLC, or it might also be that differences exist but only relative to specific histological subtypes of NSCLC.

Patients with NSCLC stages II+ IIIA had significantly higher CXCL12 plasma concentrations than controls, with even greater differences compared to stage I, which confirms previous findings. Previous studies found that abnormal expression of CXCL12, together with chemokine receptor CXCR4, contributed to more advanced TNM stage, larger tumor size, poorer differentiation, lymph node metastasis, and distant metastasis in NSCLC (ref.[7,15,22]). The challenges and potential benefits of incorporating drugs that target CXCL12/CXCR4 into immune-based lung cancer therapeutic protocols are currently a topic of discussion[23]. Our data suggest that chemokine CXCL12 is related to tumor growth and could potentially be used as a biomarker of advanced disease, even before surgical treatment, and might indicate a more radical approach to treatment. We intend to continue our study by enrolling more patients and extending the protocol of regular follow-ups with peripheral blood collection to monitor chemokines CCL2, CCL8, and CXCL12, as well as including a clinical evaluation of patients using the RECIST criteria. Our goal is to correlate chemokine concentrations (over time) with patient prognosis. We would like to confirm the role of CXCL12 as a predictive biomarker.

CONCLUSION

The present data suggest that chemokine CXCL12 is linked to tumor growth and could potentially be used as a biomarker of advanced disease prior to surgical treatment. It might also be useful in making earlier lung cancer diagnoses as well as detecting a disease relapse after surgery.

We believe that a longitudinal follow-up of patients undergoing surgical treatment could provide valuable and currently missing data regarding the role of chemokines in tumorigenesis. Additionally, we hope it opens doors for use as biomarkers.

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Ethics approval: The study was approved by the Ethics Committee of the Institute of Clinical and Experimental Medicine and Thomayer Hospital (2016).

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Supplemental Material:

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