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Original Articles

Serum chemokine network correlates with chemotherapy in non-small cell lung cancer

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ABSTRACT

Objective: Inflammation plays an important role in the microenvironment of lung cancer. The present study aimed to evaluate the association of inflammatory biomarker networks with chemotherapies for patients with non-small cell lung cancer (NSCLC).
Methods: The sera of healthy non-smokers (n = 14) and patients with NSCLC (n = 50), 36 with adenocarcinoma and 14 with squamous cell carcinoma, were collected. Healthy patients were untreated, while those with NSCLC were either chemotherapy-naïve or had received one and two courses of chemotherapy. The cytokine concentrations were measured using multiplexed cytokine immunoassays. The clinical informatics was scored with a Digital Evaluation Score System (DESS) to assess the severity of the patients. All patients completed follow-up for up to 2 years.
Results: Among the 40 mediators measured, 13 significantly differed between patients with lung cancer and healthy controls, while 18 differed between untreated patients and those with stage IV adenocarcinoma who had undergone the first and second chemotherapy courses. The protein network of cytokines in NSCLC after multiple courses of chemotherapy was similar to that of normal persons. MIP-3α is the most crucial biomarker for predicting survival rates in NSCLC patients.
Conclusions: Our data identify an NSCLC-specific profile of inflammatory mediators that may be useful for cancer sub-classification, as well as the evaluation of therapeutic effects and overall survival.

Introduction

Lung cancer is the most common cancer and the leading cause of cancer-related death worldwide. This cancer has become the top killer among malignant tumors over the past three decades [1–3], and the mortality of lung cancer has increased five-fold in China. The mortality rate of lung cancer is approximately 23 times higher in current male smokers and 13 times higher in current female smokers than in lifelong non-smokers [2]. Currently, 301 million adults in China are smokers [3]. The increasing incidence of lung cancer has made this malignancy the leading cause of death in China in 2008. The mortality rate of lung cancer in China in 2008 was 28 per 100,000 people, and the incidence was 35 per 100,000 people [4]. An estimated 0.86 million people will be newly diagnosed with lung cancer per year in China by 2025 [4].

Chemokines and chemokine receptors play important roles in the development of malignant tumors as signaling molecules that recruit inflammatory cells into the tumor microenvironment [5,6]. Chemokines released by tumor and stromal cells can induce the expression and distribution of tumor-associated leukocytes, trigger angiogenesis and generate fiber keratinocytes [6,7]. Chemokines released into the matrix can also directly contribute to the growth of malignant cells [5,6]. Chemokine receptors expressed only on malignant cells may be responsible for the migration of cancer cells toward a ligand gradient, such as the CXCR4/CXCL12 axis [8,9]. CXCR4 is expressed on malignant cells to promote tumor metastasis, and its expression is lost on the surrounding, healthy cells [10]. In a lung cancer study, chemokine receptors, such as CXCR2, CXCR3 and CCR1,

Abbreviations: NSCLC, non-small cell lung cancer; DESS, Digital Evaluation Score System; CEPIN, co-expressed protein interaction network; MIP-3α, macrophage inflammatory protein 3α; MMPs, matrix metalloproteinases.

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Table 1
Variables for different types of lung cancer (mean ± SD).

	Health Control	NSCLC	SQ	ADK
Cases	14	50	14	36
Total	0.00	65.62 ± 19.06	65.61 ± 19.42	65.64 ± 18.81
C1 Clinical manifestations	0.00	7.82 ± 4.75	10.14 ± 5.38	6.92 ± 4.23*
C1-1 Primary tumors caused symptoms	0.00	4.26 ± 2.87	6.29 ± 2.67	3.47 ± 2.57**
C1-2 Local tumor extension caused symptoms	0.00	1.32 ± 1.80	1.21 ± 1.72	1.36 ± 1.85
C1-3 Systemic symptoms	0.00	2.08 ± 2.72	2.64 ± 2.98	1.86 ± 2.62
C1-4 Metastasis caused symptoms	0.00	0.26 ± 0.78	0.00 ± 0.00	0.36 ± 0.90
C1-5 Paraneoplastic symptoms	0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C2 Physical examination	0.00	4.72 ± 6.47	4.79 ± 7.15	4.69 ± 6.30
C3 Past medical history	0.00	3.86 ± 3.41	4.57 ± 2.53	3.58 ± 3.69
C4 Lung cancer assessment imaging	0.00	27.02 ± 9.71	25.00 ± 9.32	27.81 ± 9.88
C5 Past therapy	0.00	13.16 ± 6.88	11.21 ± 7.37	13.92 ± 6.63
C5-1 Surgery	0.00	0.50 ± 1.79	1.07 ± 2.90	0.28 ± 1.09
C5-2 Non-surgical patients	0.00	12.66 ± 7.46	10.14 ± 8.14	13.64 ± 7.06
C6 Laboratory tests	0.00	8.90 ± 5.96	9.93 ± 5.15	8.50 ± 6.26

Note: NSCLC: non-small cell lung cancer; SQ: squamous cell carcinoma; ADK: adenocarcinoma. * and ** represent p values less than 0.05 and 0.01, respectively, compared with healthy controls.

were up-regulated in tumor tissues and proposed to serve as prognostic biomarkers of poor outcome [11], whereas CXCR4 up-regulation was an independent predictor of better prognosis [12,13].

The present study aimed to characterize non-small cell lung cancer (NSCLC)-specific biomarkers to monitor disease management by integrating targeted proteomics with clinical phenotypes that describe clinical informatics in patients with lung cancer. We

Table 2
Change of cytokine protein profiling in patients with lung comparison to health control (p value).

Type	NSCLC	SQ					ADK				
Cytokine	All	All	M0	M1	<IIIB	≥IIIB	all	M0	M1	<IIIB	≥IIIB
Cases	50	14	11	3	7	7	36	7	29	4	32
6Ckine	**0.006**		0.044				**0.005**	**0.001**	0.027	**0.002**	0.014
Axl	0.017	0.031				**0.006**	0.031	0.017		**0.003**	
BTC	0.012		**0.006**		**0.002**		0.012	0.031	0.023		0.018
CCL28	0.014						**0.005**	0.021	0.011		**0.008**
CTACK	0.026		0.037				0.034		0.040		0.038
CXCL16										0.023	
ENA-78											
Eotaxin-3			0.021					0.025			
GCP-2	0.016*	0.032*	0.010*		0.009*		0.023*		0.029*		0.024*
GRO	0.024						**0.008**		**0.007**		**0.005**
HCC-1		0.037	0.036		0.039						
HCC-4		0.015		0.023		0.017					
IL-9	0.018						**0.007**	0.005*	0.018	**0.008**	0.017
IL-17F				0.032							
IL-18-BPa	**0.002**	0.014*	**0.007***		**0.003***		**0.002**	0.013*	**0.005**		**0.002**
IL-28A										**0.006**	
IL-29											
IL-31				0.023							
IP-10											
I-TAC											
LIF	0.032						0.036			0.012	
LIGHT	0.040						0.033			0.032	
Lymphotactin											
MCP-2											
MCP-3	**0.003**						**0.001**	0.028	**0.002**		**0.002**
MCP-4	**0.000**	**0.004**	**0.002**		**0.008**	0.044	**0.000**	**0.001**	**0.001**	**0.005**	**0.000**
MDC	**0.005**	**0.001**	**0.005**	0.012	0.011	**0.005**	0.028			0.015	
MIF											
MIP-3α											
MIP-3b											
MPIF-1											
MSPa		0.048			0.030						
NAP-2	**0.009**	0.010	**0.009**		0.017		0.037	0.039			
OPN	0.027						0.037		0.028		0.023
PARC	0.017	0.016	0.013		0.017			0.039			
PF4		0.013	0.024			**0.003**					
SDF-1α	0.014						**0.006**	0.038	**0.009**		**0.005**
TARC											
TECK							0.036				0.030
TSLP											

Note: Mann–Whitney U test was applied to compare between different groups, except for applying T-test in * marked groups (in red: higher expression in patients than control; in blue: lower expression in patients than control). NSCLC: non-small cell lung cancer; SQ: squamous cell carcinoma; ADK: adenocarcinoma.

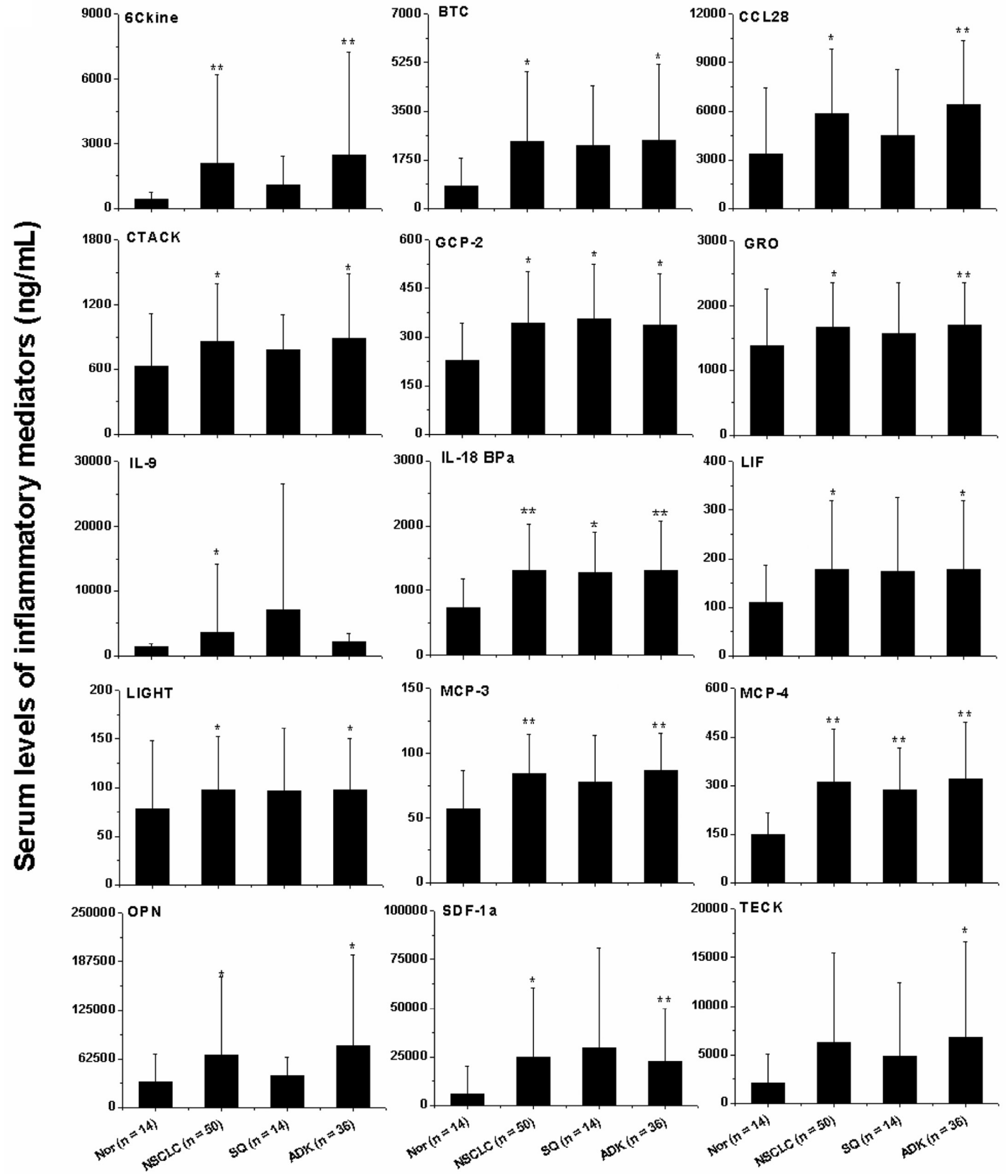

Fig. 1. Serum level of chemokines for different pathological patient groups. The serum levels of chemokine (C–C motif) ligand 21 (6Ckine), betacellulin (BTC), C–C motif ligand 28 (CCL28), cutaneous T-cell attracting chemokines/CCL27 (CTACK), granulocyte chemotactic protein 2 (GCP-2), growth-regulated oncogene (GRO), interleukin (IL)-9 and -18 BPa, leukemia inhibitory factor (LIF), lymphotoxin-like inducible protein that competes with glycoprotein D for herpes virus entry on T cells (LIGHT), monocyte chemoattractant protein (MCP)-3 and -4, osteopontin (OPN), stromal cell-derived factor-1α (SDF-1α) and thymus expressed chemokine (TECK) in healthy controls, patients with non-small cell lung cancer (NSCLC), patients with squamous cell carcinoma (SQ) and patients with adenocarcinoma (ADK). * and ** represent *p* values less than 0.05 and 0.01, respectively, compared with healthy controls.

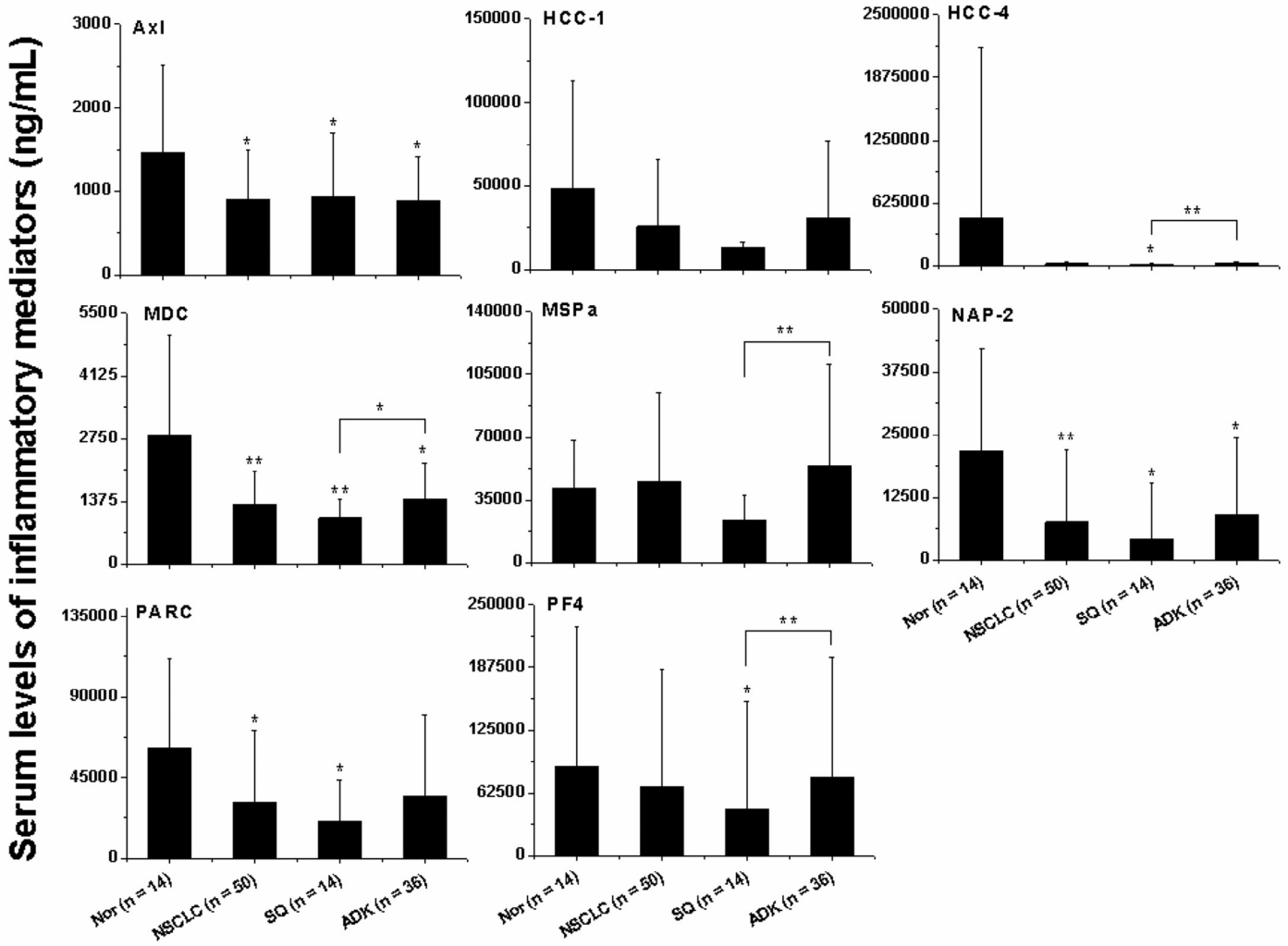

Fig. 2. Serum level of chemokines for different pathological patient groups. Serum levels of tyrosine-protein kinase 7 (Axl), hemofiltrate CC-chemokine (HCC)-1 and -4, macrophage-derived cytokine (MDC), macrophage stimulating protein a (MSPa), neutrophil-activating protein-2 (NAP-2), recombinant human MIP-4 (PARC) and platelet factor 4 (PF4) in healthy controls, patients with non-small cell lung cancer (NSCLC), patients with squamous cell carcinoma (SQ) and patients with adenocarcinoma (ADK). * and ** represent p values less than 0.05 and 0.01, respectively, compared with healthy controls.

compared the serum cytokine profiles of 40 serum biomarkers between healthy individuals and chemotherapy-naïve or post-chemotherapy NSCLC patients.

Materials and methods

Patients

In the training set, thirty-six patients with adenocarcinoma, fourteen patients with squamous cell carcinoma and fourteen non-smoking healthy volunteers (serving as controls) were enrolled at the Department of Pulmonary Medicine, Zhongshan Hospital, Fudan University. In the validation set, we randomly enrolled fifty-four patients with adenocarcinoma and ninety non-malignant patients (serving as controls) from the Risk Stratification of Patients using the Lung Cancer Biomarker Panel in China Study (ClinicalTrails.gov Identifier: NCT01928836). Histological and cytological diagnoses were performed according to the criteria of the WHO classification [14], and stage classifications followed the 7th edition staging criteria [15]. The patient demographics are presented in Supplementary Table S1. The study was conducted with the approval of the local ethics committee of our institution. We obtained consent for participation in the study from each patient.

Sample preparation

Serum samples were intravenously collected for diagnosis upon the first hospital admission and again upon the second and third admissions following one and two courses of chemotherapy, respectively. The aliquots of serum were collected in serum tubes, centrifuged at 1000 *g* for 10 minutes and then stored at –80 °C until analysis. The study protocol was approved by the ethics board of Zhongshan Hospital at Fudan University.

Cytokine multiplex microarray and ELISA Kit

In the training set, serum samples were analyzed using a SilverQuant multiplex quantitative antibody array kit (Gentel Biosciences, Fitchburg, WI), a microplate-based antibody array that measures up to 40 cytokines with a 7-point standard curve and 1 blank in a single well. The biological mediators measured in the present study are listed in Supplementary Table S2. In the validation set, the serum samples were analyzed using an ELISA Kit (Cloud-Clone Corp., Houston, TX).

Digital Evaluation Score System

The Digital Evaluation Score System (DESS) is a score index that translates clinical descriptions and information into clinical informatics, which takes into account patient symptoms, signs, medical history, biochemical analyses and radiology evaluations in patients with lung cancer (see Supplementary Tables S3–S7). To assess severity, each component is then assigned different weights: 0, 1, 2 and 4. A value of 3 was not applied in this scoring system. The use of exponential values highlights differences between severity stages. In this study, patients were scored on the day that the serum was collected.

Statistical analysis

All values are expressed as mean ± SD. Statistical analyses were performed using the SPSS software (SPSS 18.0, SPSS Inc., Chicago, IL). A multiplex assay was analyzed using 4-parameter regression curves to determine the concentration of each analyte following data acquisition. The amount of cytokine was measured and statistically compared (Student's t-test and Mann–Whitney U test). For cytokine concentrations marked as 'over' or 'under,' the concentration was replaced by the lowest or highest concentration in the standard curves. Cox's proportional hazards

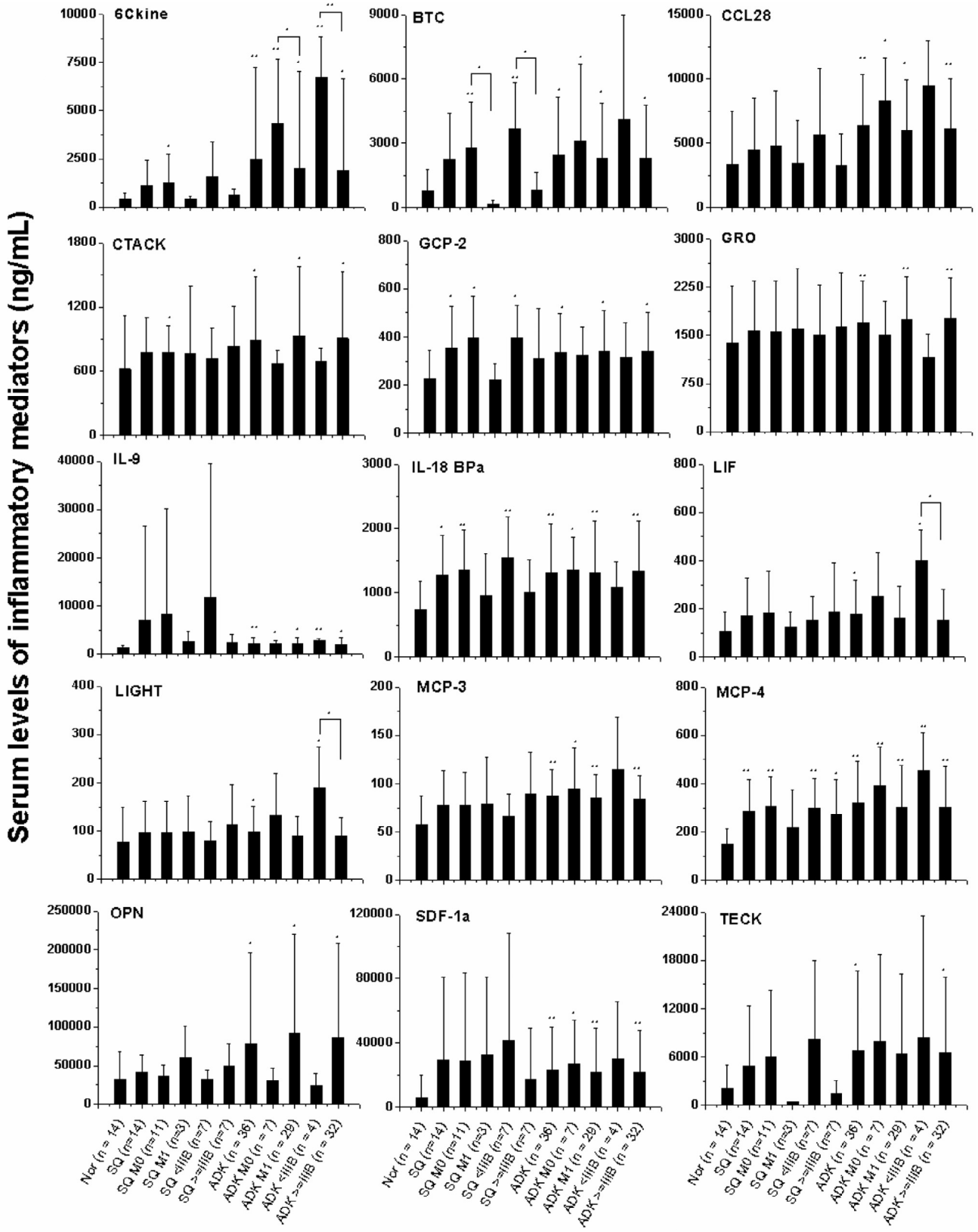

Fig. 3. Serum level of chemokines by cancer stage. The serum levels of chemokine (C–C motif) ligand 21 (6Ckine), betacellulin (BTC), C–C motif ligand 28 (CCL28), cutaneous T-cell attracting chemokines/CCL27 (CTACK), granulocyte chemotactic protein 2 (GCP-2), growth-regulated oncogene (GRO), interleukin (IL)-9 and -18 BPa, leukemia inhibitory factor (LIF), lymphotoxin-like inducible protein that competes with glycoprotein D for herpes virus entry on T cells (LIGHT), monocyte chemoattractant protein (MCP)-3 and -4, osteopontin (OPN), stromal cell-derived factor-1α (SDF-1α) and thymus expressed chemokine (TECK) in healthy controls, patients with squamous cell carcinoma (SQ) (all, stage M0, stage M1, stages I–IIIA and stages IIIB–IV) and patients with adenocarcinoma (ADK) (all, stage M0, stage M1, stages I–IIIA and stages IIIB–IV). * and ** represent *p* values less than 0.05 and 0.01, respectively, compared with healthy controls.

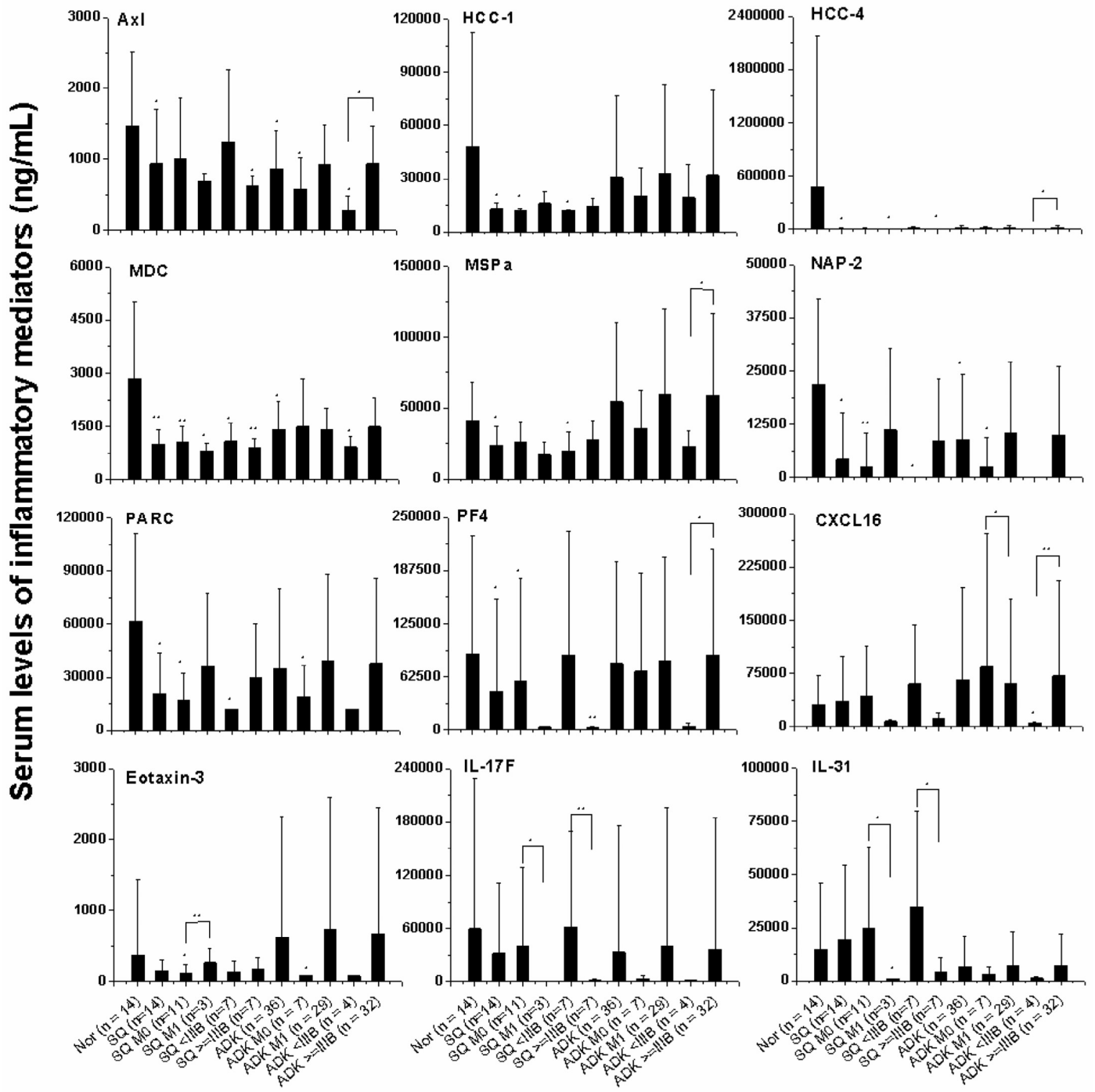

Fig. 4. Serum level of chemokines by cancer stage. Serum levels of tyrosine-protein kinase 7 (Axl), hemofiltrate CC-chemokine (HCC)-1 and -4, macrophage-derived cytokine (MDC), macrophage stimulating protein a (MSPa), neutrophil-activating protein-2 (NAP-2), recombinant human MIP-4 (PARC), platelet factor 4 (PF4), C–X–C motif chemokine ligand 16 (CXCL16), Eotaxin-3, interleukin (IL)-17F and -31 in healthy control, patients with squamous cell carcinoma (SQ) (all, stage M0, stage M1, stages I–IIIA and stages IIIB–IV) and patients with adenocarcinoma (ADK) (all, stage M0, stage M1, stages I–IIIA and stages IIIB–IV). * and ** stand for *p* values less than 0.05 and 0.01, respectively, compared with healthy control.

regression [16] was used to evaluate the hazard ratios of selected chemokines. A p-value < 0.05 was considered significant.

Bioinformatics analysis

The feature selection method SVM-RFE (10-cv) was used to select potential chemokines, and their performances were evaluated by AUC, F-measure, MCC and accuracy. The serum chemokine networks of the four different NSCLC stages were constructed using their expression correlation with a cutoff of PCC > 0.8 to analyze the evolution of their interactions. The inferred co-expressed protein interaction network of these protein markers was used to distinguish patients before and after one and two courses of chemotherapy.

Results

Various scores of clinical indices among different subtypes and stages

The score of 'primary tumor-induced symptoms' was significantly higher in patients with squamous cell carcinoma than in

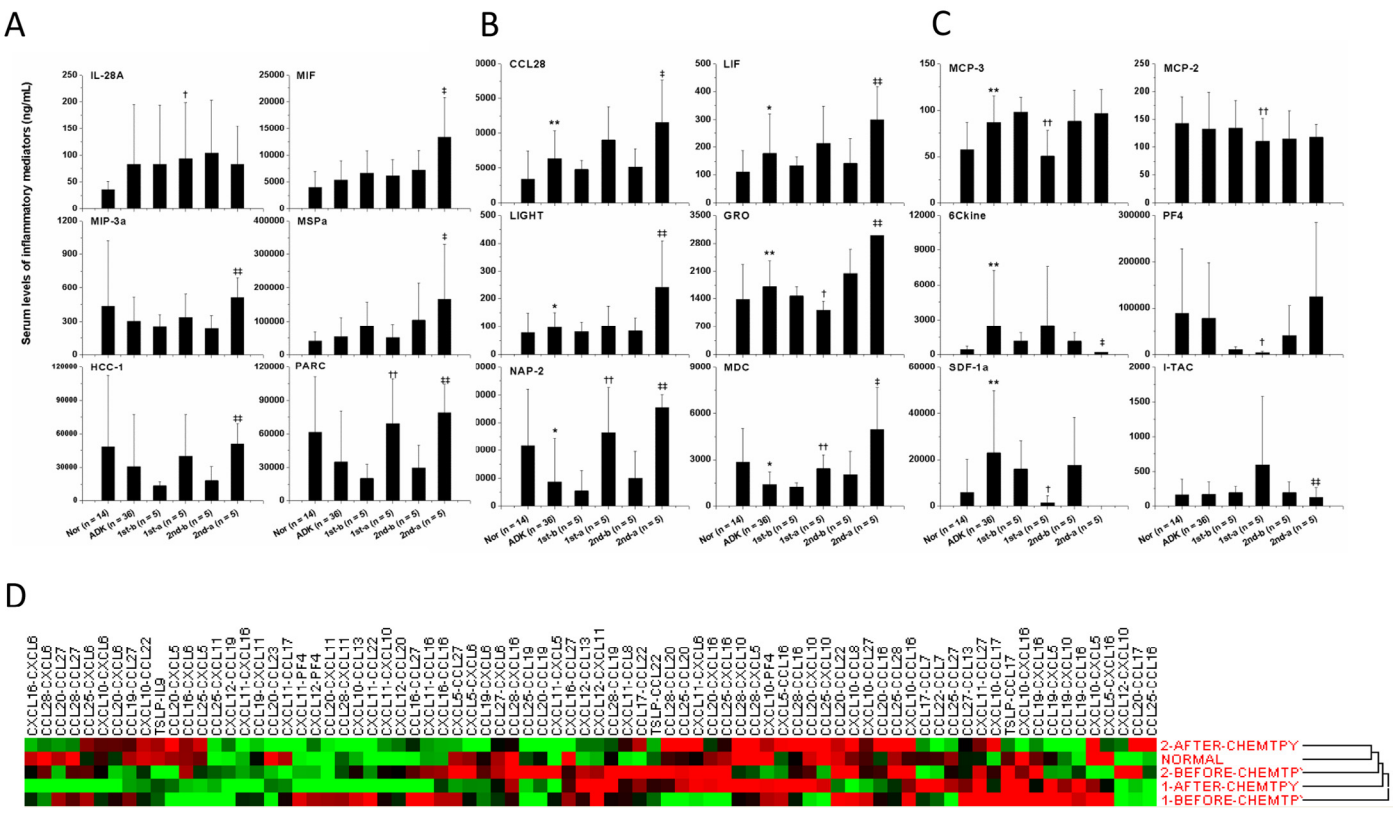

Fig. 5. Serum levels of chemokines related to chemotherapy. (A) Serum levels of interleukin (IL)-28A, macrophage migration inhibitory factor (MIF), macrophage inflammatory proteins (MIP)-3α, macrophage stimulating protein a (MSPa), hemofiltrate CC-chemokine (HCC)-1 and -4 and recombinant human MIP-4 (PARC) in healthy controls and patients with adenocarcinoma. (B) The serum levels of C–C motif ligand 28 (CCL28), leukemia inhibitory factor (LIF), lymphotoxin-like inducible protein that competes with glycoprotein D for herpes virus entry on T cells (LIGHT), growth-regulated oncogene (GRO), neutrophil-activating protein-2 (NAP-2) and macrophage-derived cytokine (MDC) in healthy controls and patients with adenocarcinoma. (C) Serum levels of monocyte chemoattractant protein (MCP)-3 and -2, chemokine (C–C motif) ligand 21 (CCL21) (6Ckine), platelet factor 4 (PF4), stromal cell-derived factor-1a (SDF-1α) and interferon-inducible T-cell alpha chemoattractant (I-TAC) in healthy controls and patients with adenocarcinoma (all, before and after the 1st course of chemotherapy and before and after the 2nd course of chemotherapy). * and ** represent p values less than 0.05 and 0.01, respectively, compared with healthy controls. † and †† represent p values less than 0.05 and 0.01, respectively, compared with patients with adenocarcinoma before the 1st course of chemotherapy. ‡ and ‡‡ represent p values less than 0.05 and 0.01, respectively, compared with patients with adenocarcinoma before the 2nd course of chemotherapy. (D) Hierarchical clustering of serum chemokine expression at different time points during chemotherapy and from healthy controls.

patients with adenocarcinoma (Table 1, Supplementary Fig. S1). The scores of 'sputum' and 'hemoptysis' were significantly higher in squamous cell carcinoma than in adenocarcinoma patients (see Supplementary Fig. S2). The scores of 'imaginary' and 'local tumor extension-induced symptoms' significantly differed by lung cancer stage (see Supplementary Figs. S3 and S4).

Chemokines dynamically differ among subtypes

The levels of fifteen chemokines were significantly increased in the non-small cell lung cancer group. The levels of GCP-2, IL-18-BPa and MCP-4 were significantly higher in patients with squamous cell carcinoma and adenocarcinoma than in the healthy volunteers (Fig. 1 and Table 2, $p < 0.05$), while the levels of BTC, CCL28, CTACK, GRO, IL-9, LIF, LIGHT, MCP-3, OPN, SDF-1α, 6Ckine and TECK were significantly altered in patients with adenocarcinoma. Eight chemokines were significantly decreased. The levels of Axl, MDC and NAP-2 were significantly lower in cancer patients than in healthy volunteers (Fig. 2 and Table 2, $p < 0.05$), while the levels of HCC-1, HCC-4, MSPa, PARC and PF4 were significantly altered in patients with squamous cell carcinoma.

Chemokines correlate with advanced stage disease and chemotherapy efficacy in adenocarcinoma

Twelve chemokines were significantly increased in the metastasis group at stages M1 or IIIB–IV. The levels of CTACK, GCP-2, GRO and OPN were significantly higher in advanced disease patients than in healthy patients (Fig. 3 and Table 2 , $p < 0.05$). The levels of six chemokines were significantly decreased in patients with stage M0 or I–IIIA disease. The levels of Eotaxin-3, NAP-2 and PARC were significantly lower in patients with stage M0 disease compared with healthy volunteers and patients with stage M1 disease (Fig. 4 and Table 2 , $p < 0.05$). The efficacy of chemotherapy was based on the Response Evaluation Criteria in Solid Tumors (RECIST). All enrolled patients exhibited stable disease (SD) during the first two courses of chemotherapy. The 1st course of chemotherapy significantly improved the GRO level relative to the basal levels (Fig. 5 and Table 3). The 1st and 2nd courses of chemotherapy also significantly improved the levels of NAP-2 and PARC compared with the untreated group (Fig. 5 and Table 3).

Computational analysis framework of the chemokine panel

The protein biomarkers (PBT) used to classify adenocarcinoma and squamous cell carcinoma samples are shown in Supplementary Fig. S5. All potential biomarkers were obtained using the feature selection method SVM-RFE (10-cv), and their performances were evaluated based on the AUC, F-measure, MCC and accuracy (Supplementary Fig. S6). In addition, the protein biomarker PBTN1 was used to distinguish adenocarcinoma samples from normal ones, while the protein biomarker PBTN2 was used to distinguish squamous cell carcinoma from normal samples. The protein biomarkers for the binary classification of cancer stages I–III vs. IV (PBS) and

Table 3
Cytokines with statistically significance between different groups.

Cytokine	NSCLC (n = 50)	SQ (n = 14)	ADK (n = 36)	Post 1st chemotherapy (n = 5)	Post 2nd chemotherapy (n = 5)
6Ckine	↑		↑		↓
Axl	↓	↓	↓		
BTC	↑		↑		
CCL28	↑		↑		↑
CTACK	↑		↑		
GCP-2	↑	↑	↑		
GRO	↑		↑	↓	↑
HCC-1		↓			↑
IL-18BPa	↑	↑	↑		
IL-28A				↑	
IL-9	↑		↑		
I-TAC					↓
LIF	↑		↑		↑
LIGHT	↑		↑		↑
MCP-2				↓	
MCP-3	↑		↑	↓	
MCP-4	↑	↑	↑		
MDC	↓	↓	↓	↑	↑
MIF					↑
MIP-3α					↑
MSPa		↓			↑
NAP-2	↓	↓	↓	↑	↑
OPN	↑		↑		
PARC	↓	↓		↑	↑
PF4		↓		↓	
SDF-1α	↑		↑	↓	
TECK			↑		

Note: ↑ up, ↓ down. NSCLC, SQ and ADK vs. health control; post 1st chemotherapy and post 2nd chemotherapy vs. before 1st chemotherapy. NSCLC: non-small cell lung cancer; SQ: squamous cell carcinoma; ADK: adenocarcinoma.

the protein biomarkers for the binary classification of short-term survival (<6 months) and long-term survival (>16 months) (PBD) are shown in Supplementary Fig. S5. We identified three co-expression networks between PBD with PBT, PBTN1 and PBTN2. By combining these findings with the clinical index scores (DESS), we found clinical indicators (CPBD) assisting PBD for advanced NSCLC prognosis, which are shown in Fig. 6.

Network of protein biomarkers responding to chemotherapy

Interestingly, the co-expressed protein interaction network (CEPIN) of cytokines that are specific to adenocarcinoma showed dynamic rewiring during chemotherapy (Fig. 7). This finding suggests that the sub-system protein network of cytokines in NSCLC is similar to that of normal persons after multiple courses of chemotherapy, which is supported by the hierarchical clustering of five CEPINs that correspond to different time points before and after chemotherapy (Fig. 5).

Survival time analysis

We applied univariate and multivariate analyses to the relationship between chemokines and the survival time. The hazard ratios of the 15 chemokines calculated with Cox's proportional hazards regression analysis are shown in Supplementary Table S9. MCP2 had the highest hazard ratio of 5.691, which indicates that the risk rate would amplify 5.691 times if the MCP2 concentrations in NSCLC patients increased by one unit concentration (100 pg/mL). In a further validation, we found that the serum level of MCP-2 is significantly higher in patients with adenocarcinoma, especially in advanced cancer, than in non-malignant patients (Supplementary Fig. S7). Macrophage inflammatory protein 3α (MIP-3α, also called CCL20) had the lowest risk rate with a significant *p*-value, which suggests that MIP-3α may be a cancer suppressor protein.

Network analysis of NSCLC stage

The network of each stage is shown in Fig. 6. Interestingly, a large chemokine group in the healthy stage (S1) strongly correlated with the cancer stages (S2, S3, S4), which may suggest that the subsequent tumorigenesis disrupted the close connection between these chemokines. We found that the edges of the network decreased from S1 to S4, which suggests that the consistency of chemokine expression was gradually lost as the disease progressed.

Discussion

The present study divided patients into squamous cell carcinoma and adenocarcinoma subgroups based on the histopathological classification of NSCLC. We found that the levels of NSCLC-specific chemokines in the circulation differed between specific pathological subgroups, which may suggest that the chemokine-mediated pathophysiological processes differ between the adenocarcinoma and squamous cell carcinoma microenvironments. Alternatively, a spectrum of specific serum chemokine changes could provide an additional tool for the clinical diagnosis of NSCLC via serum diagnostics.

In our study, we discovered three different patterns of chemokine expressions based on lung cancer stages: (1) changes in only the M0 or stage I–IIIA groups, such as Eotaxin-3, HCC-1 and PARC, which were only expressed in the early stages of NSCLC compared with healthy controls and advanced NSCLC groups; (2) changes in only the M1 or IIIB–IV groups, such as GRO, HCC-4 and OPN, which were only expressed in advanced NSCLC. In the previous reports, OPN expression in the lung tissue was found to be closely related to lymph node metastasis [17,18]. The result of our study suggests that high levels of serum OPN may be associated with the metastatic potential of NSCLC; (3) significant differences in the chemokine profiles were found in both the early and advanced stages of lung cancer. These findings suggest that different chemokine patterns were involved in regulating distinct mechanisms during lung cancer development. The early protein spectrum may be primarily involved in lung cancer growth and survival-related pathways, and the late protein spectrum may be involved in migration-related lung cancer pathways [19]. A further pathway enrichment analysis of different protein markers showed that "Granulocyte Adhesion and Diapedesis" and "Agranulocyte Adhesion and Diapedesis" are involved in the subtype and therapy of NSCLC. These two pathways may be up-regulated in adenocarcinoma but down-regulated in squamous cell carcinoma (Supplementary Fig. S8). Moreover, we selected key proteins based on the topological structure of the protein network. We identified CXCL10, CXCL11 and CCL20 as core proteins because they are hubs of the network that connect most other proteins. CCL8 and CCL23 are partners of core proteins because they are only connected to core proteins (Fig. 8).

The 40 evaluated serum cytokines can be used to classify cancer and healthy samples in clinical applications. These 40 proteins can also be used to estimate cancer stage. The PBD scores were high for AUC, F-measure and accuracy and acceptable for MCC; thus, proteins in the PBD can be used to predict NSCLC prognosis. When comparing the overlap between the different biomarkers, PBD contained more proteins than PBS, and PBS covered more proteins than PBT. Therefore, from the standpoint of a genotype–phenotype relationship, the prognosis of cancer patients is tightly related to cancer type and stage. Twenty-one of the 40 evaluated serum cytokines belong to protein association networks according to the STRING database, and 14 of the 21 proteins belong to PBD. Therefore, the rewiring of the co-expression network of these 14 proteins needs to be considered. For example, the positive correlation between CCL19 and CXCL5 was strong in adenocarcinoma samples but weak in normal or squamous cell carcinoma samples. Finally, clinical

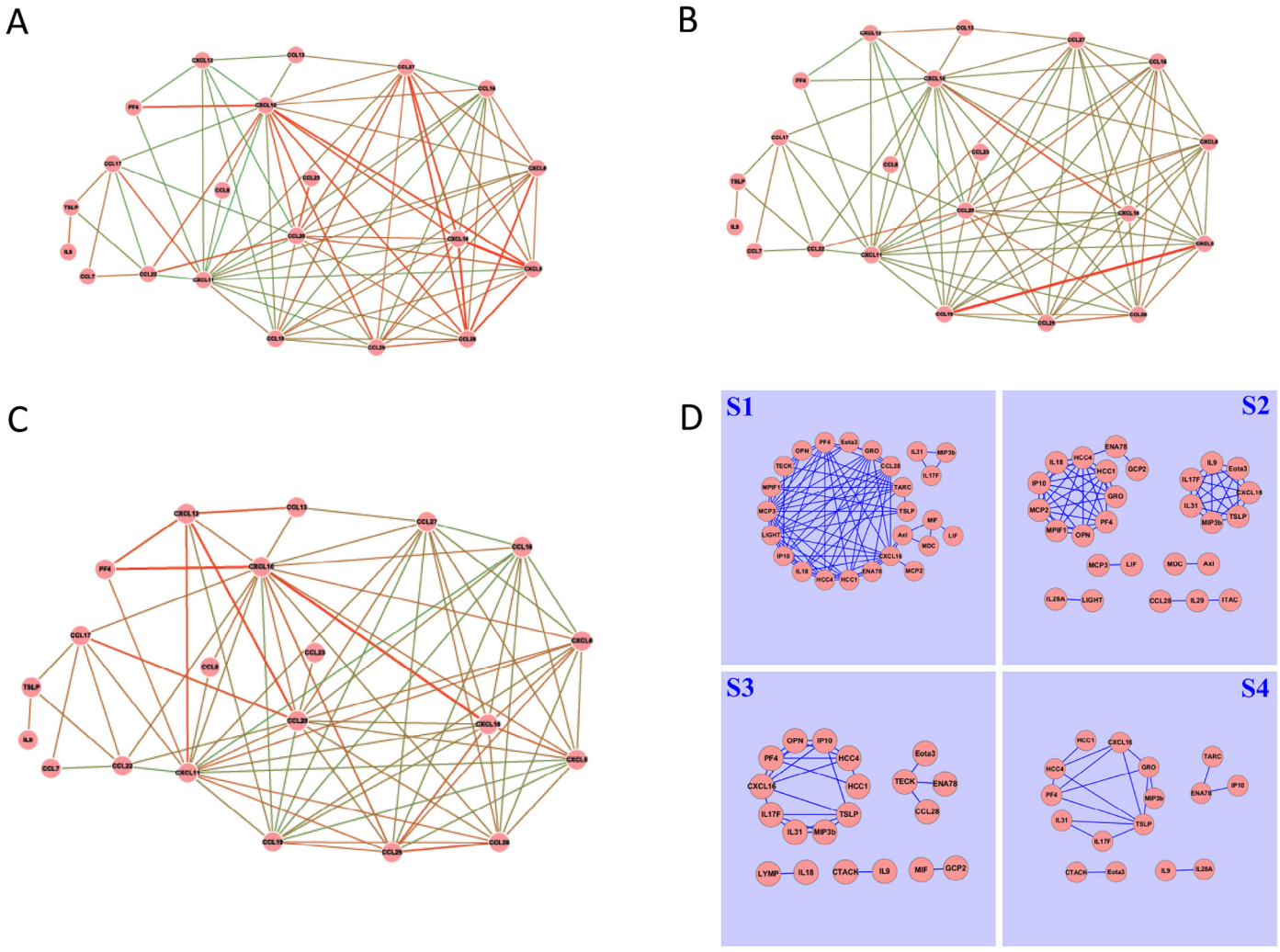

Fig. 6. Co-expression network analysis of chemokines by patient group. (A) The co-expression network based on normal samples. (B) The co-expression network based on adenocarcinoma samples. (C) The co-expression network based on squamous cell carcinoma samples. (D) The chemokine correlation network of each stage. S1: healthy persons; S2: stages IB, IIB and IIIA; S3: stages IIIB and IVA; S4: stage IVB.

indicators are commonly used as prognostic indicators in the biomedical field. Therefore, the combination of PBD and clinical indicators (CPBD) was further filtered, and the new biomarker CPBD yielded performed better in various computational evaluations than PBD. At this time, 13 proteins in the original PBD and 7 clinical indicators were used. Six proteins in CPBD remain evident in the known protein association network, and most of these proteins are hub-nodes, such as CXCL6, CXCL10, CXCL11 and CXCL16. Thus, these relevant proteins are likely important on a molecular level and cannot be replaced by the clinical indicators at the phenotype level.

In the survival time analysis, MCP2 had the highest hazard ratio, whereas MIP-3α had the lowest. MCP2 is a 109-amino acid cytokine that is overexpressed in HeLa cells and can prevent cancer metastasis in vivo [20]. MIP-3α is a chemokine involved in many types of cancers and was reported as a potential therapeutic target for NSCLC patients [21]. In the stage network analysis, we constructed the cytokine network and identified 80, 59, 27 and 18 edges in the S1, S2, S3 and S4 stages, respectively. Three edges (HCC1-HCC4, IL17f-IL31, HCC4-PF4) were present in all stages, and these edges may be relatively important in normal and cancer stages.

In the present study, we found that the serum levels of SDF-1α were significantly increased in patients with adenocarcinoma and recovered after the first course of chemotherapy. SDF-1 is secreted by fibroblasts in a variety of organs and tissues, including the bone marrow, lymph nodes, lung, liver and muscle [22,23]. Hypoxia-inducible factor-1α and nuclear factor kappa B upregulated the expression levels of CXCR4 and SDF-1 in tumor cells under hypoxia [24,25]. The activation of the epidermal growth factor receptor can increase the expression of CXCR4 in lung cancer cells [24]. The phosphatidylinositol 3-kinase and mitogen-activated protein kinase signaling pathways can activate the pathway of the CXCR4/SDF-1 axis [26,27], resulting in chemotaxis, cell migration [28], and the secretion of large amounts of matrix metalloproteinases (MMPs), including MMP-2 and MMP-9 [29,30]. This activation also participates in the infiltration of tumor cells into the basement membrane [31]. In addition, the activation of the pathway downstream of CXCR4/SDF-1 can also induce the expression of different cell surface integrins, including very late antigen-4 (VLA-4) and VLA-5 [32–34], and the secretion of VEGF [35].

The limitations of this study include the small patient sample size and the selected range of chemokines for profiling, which could not cover the entire network of inflammatory mediators. Most patients randomized in our database had developed advanced stages of lung cancer since the time of initial diagnosis. To further select sensitive and specific biomarkers for the early diagnosis of lung cancer, chemokine protein profiling studies of earlier stages of lung cancer (stages I and II) are needed.

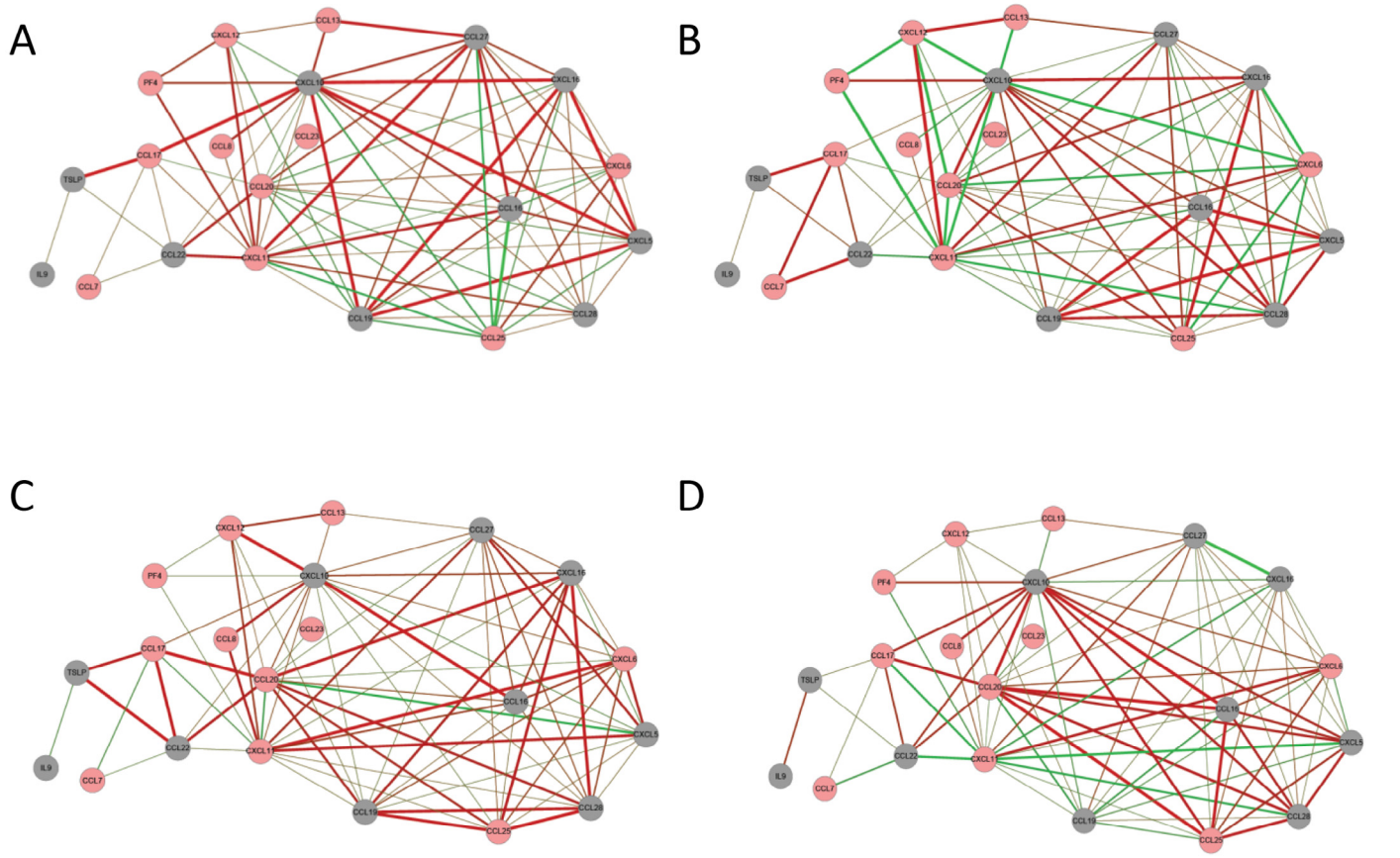

Fig. 7. Network of protein biomarkers responding to chemotherapy. (A) Before the 1st course of chemotherapy; (B) after the 1st course of chemotherapy; (C) before the 2nd course of chemotherapy; (D) after the 2nd course of chemotherapy.

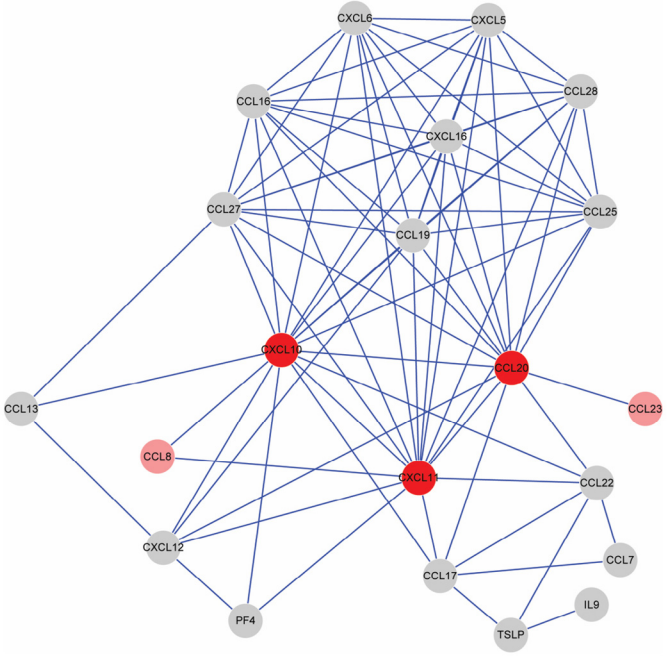

Fig. 8. Key protein selection based on topological structure of protein network. CXCL10, CXCL11 and CCL20 are core proteins because they are hubs of the network that connect most other proteins.

In this study, we developed a new set of tumor markers in an assessment method by comparing inflammatory mediators in different groups of lung cancer types based on lung cancer staging systems and prognosis characteristics. We further combined these findings with clinical informatics and bioinformatics methods to study the signaling networks of various types of cytokines in lung cancer tumorigenesis and development. Future studies will examine the large-scale verification of this new evaluation system and the potential biomarker panel of chemokines in lung cancer diagnosis and prognosis prediction.

Author's contributions

Xiangdong Wang and Chunxue Bai supervised the conduct of the whole project and they had full access to all of the data in the study and taken responsibility for the integrity of the data and the accuracy of the data analysis. Dawei Yang, Jian Zhou, Tao Zeng and Zhiyuan Yang performed the experiment, analyzed data and drafted the manuscript. Wang Xun and Jie Hu recruited the subject and approved the submitted article. Yuanlin Song, Luonan Chen and Dan Peer contributed to the study design, intellectual discussion of the results and approval of the submitted article.

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Conflict of interest

The authors declare that they have no competing interests.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.05.001.

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