

Expression of monocyte chemoattractant protein-1 and CC chemokine receptor 2 in non-small cell lung cancer and its significance

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Abstract Expression of monocyte chemoattractant protein-1 (MCP-1) and CC chemokine receptor 2 (CCR2) and its significance has been demonstrated in some cancer cells in recent clinical studies. However, the role of tumor MCP-1 and CCR2 expression in non-small cell lung cancer (NSCLC) remains unknown. The aim of the present study was to investigate the prognostic significance of MCP-1 and CCR2 expression in NSCLC cells. The relationship between MCP-1 and CCR2 expression in NSCLC cancer cells was examined by immunohistochemical staining of surgical specimens from 134 patients. Sixty-five of these patients had follow-up records. Kaplan–Meier analysis and Cox regression model were used to assess overall survival according to the presence or absence of MCP-1 and CCR2 expression in tumor cells. MCP-1 was detected in cancer cells of 107 NSCLC (79.9 %) and CCR2 was detected in cancer cells of 39 NSCLC (29.1 %). MCP-1 expression was correlated with sex, smoking habits, histology, and tumor size. Presence of MCP-1 in tumor cells was associated with better overall survival ($P = 0.018$). By multivariate analysis, MCP-1 expression in cancer cells showed an independent prognostic factor for overall survival ($P = 0.002$, hazard ratio [HR] = 0.256, 95 % confidence

interval [CI] = 0.106–0.616). There was no significant relationship between CCR2 expression in tumor cells and clinical and pathological characteristics. Also, no significant positive correlation between MCP-1 and CCR2 expression was revealed by Spearman correlation analysis. Our data indicate that MCP-1 is overexpressed in NSCLC cells. Its expression in cancer cells is associated with better survival in NSCLC patients.

Keywords Non-small cell lung cancer · Monocyte chemoattractant protein-1 · CC chemokine receptor 2 · Expression · Prognosis

Abbreviations

NSCLC	Non-small cell lung cancer
SCLC	Small cell lung cancer
MCP-1	Monocyte chemoattractant protein-1
CCR2	CC chemokine receptor 2
TAM	Tumor-associated macrophages
UICC	International Union Against Cancer
AJCC	American Joint Committee on Cancer
CCL2	CC ligand 2
MMP-9	Matrix metalloprotease-9
CTL	Cytotoxic T cells
IFN	Interferon
TNF	Tumor necrosis factor
IL-1	Interleukin 1
IL-2	Interleukin 2
SNP	Single-nucleotide polymorphism

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Introduction

Lung cancer is the world's leading cause of cancer-related death and the second most common malignant tumor [1].

Epidemiologically, there has been an marked elevation in morbidity and mortality of lung cancer in China over the past decade [2]. At least 75 % of lung cancer patients have evidence of metastasis at diagnosis [3]. Lung cancer can be divided into two main types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Approximately 85 % of lung cancers are NSCLC and their prognosis is poor [4]. Therefore, surgery plays a critical role in the treatment for clinical stage I–IIIA NSCLC. Because of local recurrence or distant metastasis, even patients with pathologic stage I or patients who have undergone R0 resection do not have good outcomes. Some pathologic stage IA patients may receive adjuvant chemotherapy to minimize the risk of recurrence [5]. Although adjuvant chemotherapy is beneficial to pathologic stage IB–IIIA patients for improving their prognosis, it may not be necessary for all patients [6]. Therefore, new prognostic markers are needed to screen patients at high risk of relapse.

MCP-1 (monocyte chemoattractant protein-1) was first purified by Matsushima et al. [7] from serum-free culture supernatant of human myelomonocytic cells. It is a member of the C–C chemokine family and is a polypeptide containing 76 amino acids. The receptor of MCP-1 is CCR2 and it is expressed in various cell types, such as monocytes and memory T lymphocytes. When MCP-1 combines with CCR2, the allosteric effect and G-protein binding will occur to activate signal transduction pathway. MCP-1 is produced by a variety of activating cells (fibroblasts, endothelial cells, smooth muscle cells, lymphocytes, and macrophages) [8, 9]. Many recent studies have reported that MCP-1 is expressed by several malignant tumor cells, including prostate cancer, breast cancer, esophageal carcinoma, colon cancer, pancreatic cancer, cervical cancer, and ovarian cancer [10–16]. MCP-1 participates in the migration and infiltration of monocytes/macrophages toward inflammatory areas. It also regulates the recruitment and activation of tumor-associated macrophages (TAM) in tumor infiltration, including release of lysosomal enzyme and tumoricidal activity [13]. However, some studies have indicated that MCP-1 could promote tumor invasion and metastasis [12, 17, 18]. The role of MCP-1 in NSCLC is also controversial. It was reported that MCP-1 production was greater in NSCLC than in normal lung tissue, and the level of MCP-1 positively correlated with lung cancer-induced bone resorptive lesions in vivo [19, 20]. However, transfection of NSCLC cell lines by MCP-1 did not change the invasion behavior of lung cancer cell lines or inhibit cancer cell distant metastasis [21, 22]. The aim of the present study was to explore the expression and significance of MCP-1 and CCR2 in NSCLC.

Materials and methods

Tissue specimens

This study was retrospective and was approved by the institutional ethics board of Huadong Hospital, China. A total of 134 patients who underwent surgical resection for NSCLC at Huadong Hospital from January 1999 to December 2009 and who had complete clinicopathologic data were enrolled in the study, of which 65 patients had follow-up data. None of the patients received radiotherapy or chemotherapy before surgery. Histologic type was defined according to World Health Organization classification. TNM classification of NSCLC was made according to the seventh edition published in 2009 [23], with the agreement of both the International Union Against Cancer (UICC) and the American Joint Committee on Cancer (AJCC). Clinicopathological information of the patients is shown in Table 1.

Immunohistochemical analysis

MCP-1 and CCR2 expression was analyzed immunohistochemically on 4- μ m-thick, formalin-fixed, paraffin-embedded specimen sections. Sections were deparaffinized in xylene and rehydrated in a series of grade alcohols. They were then pretreated in ethylenediamine tetraacetic acid antigen retrieval solution (pH 9.0) using heat-induced epitope retrieval technique. After inhibiting internal peroxidase activity with 3 % hydrogen peroxide, the sections were incubated with primary antibodies (MCP-1, Cat No. ab9669, diluted \times 80 [Abcam, USA]; CCR2, Cat No. ab32144, diluted \times 1,100 [Abcam]). Next, EnVision Detection System kit (DAKO, Denmark) was used on sections with diaminobenzidine (DAB) as a chromogen. Finally, sections were counterstained with hematoxylin, then dehydrated and mounted. Positive staining controls were carried out with paraffin-embedded spleen and breast cancer sections using CCR2 and MCP-1 antibodies. Two of the authors reviewed the slides independently twice at two-week intervals.

MCP-1 staining in tumor cells was positive when more than 10 % of cancer cells were stained in each section [12]. Semi-quantitative analysis of CCR2 staining in tumor cells was evaluated as grade 0, 1, 2, or 3 [24]. Grade 0 was defined as CCR2 staining in <1 % of tumor cells, grade 1 as 1–33 %, grade 2 as 34–66 %, and grade 3 as >67 %. Grade 0 corresponded to negative CCR2 expression, and grades 1–3 corresponded to positive CCR2 expression.

Statistical analysis

Overall survival was defined as the time from the date of operation until death or follow-up deadline. Comparisons

Table 1 Clinicopathologic characteristics and frequency of non-small cell lung cancer patients (with or without follow-up records, 134 cases) according to the presence or absence of monocyte chemoattractant protein-1 (MCP-1) or CC chemokine receptor 2 (CCR2) in tumor cells

Characteristics	With follow-up records (<i>n</i> = 65) No.	Without follow-up records (<i>n</i> = 69) No.	MCP-1 (+)	MCP-1 (-)	<i>P</i>	CCR2 (+)	CCR2 (-)	<i>P</i>
<i>Gender</i>								
Male	41	38	55	24	<0.001 ^a	26	53	0.245 ^a
Female	24	31	52	3		13	42	
<i>Age (years)</i>								
≤60	29	33	48	14	0.515 ^a	18	44	0.986 ^a
>60	36	36	59	13		21	51	
<i>Smoking habits</i>								
Yes	26	25	34	17	0.003 ^a	18	33	0.216 ^a
No	39	44	73	10		21	62	
<i>Histological type</i>								
Squamous carcinoma	19	20	23	16	0.001 ^a	15	24	0.305 ^a
Adenocarcinoma	41	47	78	10		22	66	
Others	5	2	6	1		2	5	
<i>Tumor size (cm)</i>								
≤3	38	43	69	11	0.034 ^a	19	61	0.172 ^a
3–7	23	23	31	15		16	30	
>7	4	3	7	1		4	4	
<i>Differentiation</i>								
High-moderate	50	52	84	18	0.197 ^a	31	71	0.558 ^a
Low	15	17	23	9		8	24	
<i>Grade</i>								
T1–2	58	21	97	21	0.065 ^a	31	87	0.05 ^a
T3–4	7	48	10	6		8	8	
<i>Lymph node metastasis</i>								
No	46	53	77	22	0.314 ^a	30	69	0.607 ^a
Yes	19	16	30	5		9	26	
<i>Distant organ metastasis</i>								
No	61	68	104	25	0.259 ^a	39	90	0.321 ^b
Yes	4	1	3	2		0	5	
<i>Tumor stage</i>								
I–II	50	57	87	20	0.402 ^a	28	79	0.136 ^a
III–IV	15	12	20	7		11	16	

MCP-1 (+) was defined as MCP-1-positive expression in NSCLC cells. MCP-1 (–) was defined as MCP-1-negative expression in NSCLC cells. CCR2 (+) was defined as CCR2-positive expression in NSCLC cells. CCR2 (–) was defined as CCR2-negative expression in NSCLC cells

^a Pearson's chi-square test (two-sided), ^b Fisher's exact probability test (two-sided)

between groups based on the presence of MCP-1 and CCR2 in tumor cells were analyzed using Pearson's chi-square tests or Fisher's exact probability tests as required. Spearman correlation analysis was used to detect the association between MCP-1 and CCR2 expression. Kaplan–Meier analyses and log-rank tests were used to evaluate the correlation between the presence of MCP-1 and CCR2 in cancer cells. Log-rank tests were also used for predictors in univariate analysis based on the Kaplan–Meier curves. Cox proportional hazards regression was used for multivariate analysis of overall survival. All predictors with *P* values <0.10 in univariate analysis were used in multivariate analysis, carried out by the forward

stepwise (likelihood ratio) procedure. All analyses were performed using Statistical Package for Social Sciences version 16 (SPSS Inc., Chicago, IL, USA). For all comparisons, two-sided *P* values <0.05 were considered statistically significant.

Results

Expression of MCP-1 and CCR2 in NSCLC

Of the 134 tumor surgical specimens, 107 (79.9 %) expressed MCP-1 in tumor cells. MCP-1 immunostaining

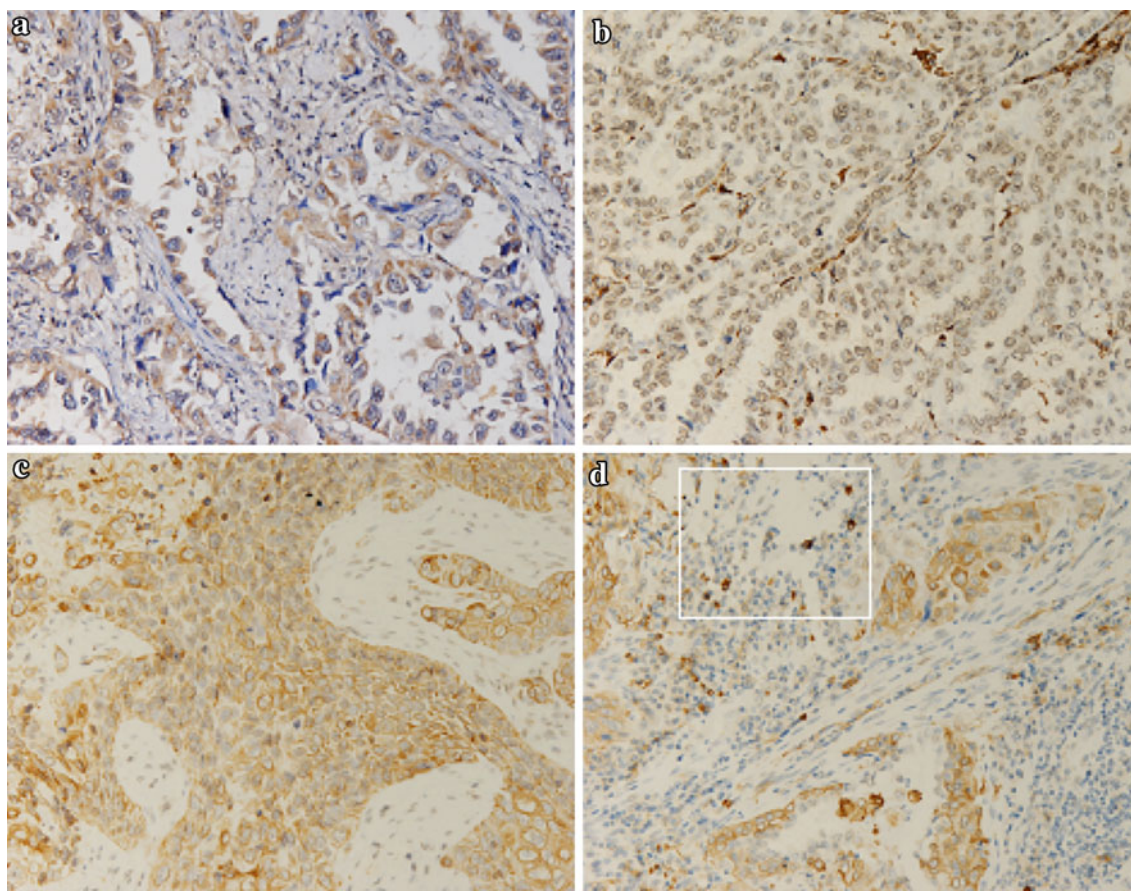


Fig. 1 MCP-1 and CCR2 expression of non-small cell lung cancer ($\times 200$). **a** Positive cytoplasmic staining of MCP-1 in adenocarcinoma of the lung. **b** Positive nuclear staining of CCR2 in

adenocarcinoma of the lung. **c** Positive cytoplasmic staining of CCR2 in squamous carcinoma of the lung. **d** Positive staining of tumor-infiltrating lymphocytes of CCR2

was exclusively observed in cytoplasm of cancer cells. Expression of CCR2 in tumor cells was found in either cytoplasm or nucleus. In total, 39 of 134 cases (29.1 %) showed positive staining: 25 (18.7 %) in cytoplasm and 15 (11.2 %) in nucleus, scarcely in both cytoplasm and nucleus. Infiltrated lymphocytes of CCR2-positive staining were found in NSCLC (Fig. 1).

Association of clinicopathologic characteristics with expression of MCP-1 and CCR2 in NSCLC

MCP-1 expression in NSCLC was associated with gender or smoking habits and histological type of NSCLC. Tumor MCP-1 expression was observed more frequently in men than in women ($P < 0.001$), in never-smokers than in smokers ($P = 0.003$), and in smaller tumors. The presence of MCP-1-positive cancer cells was high in patients with adenocarcinoma of NSCLC relative to tumors of other histological types ($P = 0.001$). There was no significant association between MCP-1 expression and clinicopathological characteristics, including patient age, differentiation, T-grade, stage, lymph node metastasis, and distant

Table 2 Correlation of monocyte chemoattractant protein-1 (MCP-1) or CC chemokine receptor 2 (CCR2) expression in NSCLC tumor cells

MCP-1	CCR2		Total	<i>r</i>	<i>P</i>
	(+)	(-)			
(+)	30	77	107	-0.047	0.592
(-)	9	18	27		
Total	39	95	134		

MCP-1 (+) was defined as MCP-1-positive expression in NSCLC cells. MCP-1 (-) was defined as MCP-1-negative expression in NSCLC cells. CCR2 (+) was defined as CCR2-positive expression in NSCLC cells. CCR2 (-) was defined as CCR2-negative expression in NSCLC cells

organ metastasis. There was no relationship of tumor CCR2 expression with patient age, gender, smoking habits, histologic type of tumor, differentiation, tumor size, T-grade, tumor stage, lymph node metastasis, and distant organ metastasis (Table 1). No significant association was detected between MCP-1 and CCR2 expression in NSCLC tumor cells (Table 2, $r = -0.047$, $P = 0.592$).

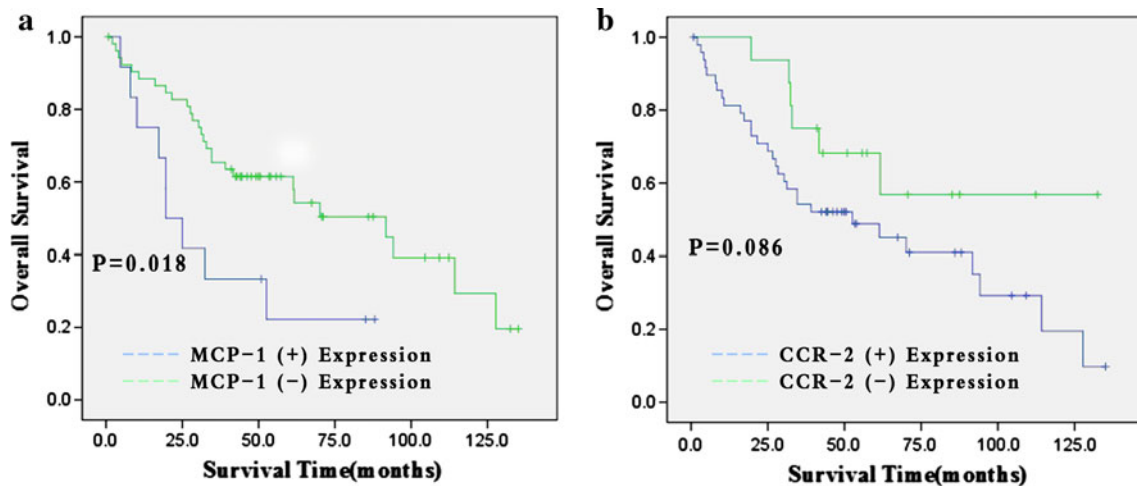


Fig. 2 Kaplan–Meier estimates with log-rank tests for overall survival of 65 non-small cell lung cancer (NSCLC) patients. **a** MCP-1 expression status in NSCLC. **b** CCR2 expression status in NSCLC

Prognostic significance of MCP-1 and CCR2 expression in NSCLC

Expression of MCP-1 in NSCLC correlated with overall survival ($P = 0.018$) in Kaplan–Meier curves, but CCR2 expression of tumor cells had no association with overall survival ($P = 0.086$) (Fig. 2). Univariate analysis of clinicopathological characteristics indicated that histological type of tumor, tumor stage, lymph node metastasis, distant organ metastasis, and MCP-1 expression were significantly related with overall survival ($P = 0.015, 0.012, 0.020, 0.012, \text{ and } 0.018$, respectively). Gender, age, smoking habits, tumor size, differentiation, and T-grade were not associated with increased risk of death (Table 3). Cox proportional multivariate analysis using all predictors with P values < 0.1 in the univariate analysis revealed that histological type of tumor, tumor stage, and presence of MCP-1 were correlated with overall survival (Table 3, $P < 0.001$ HR 3.270 95 % CI 1.758–6.082; $P = 0.021$ HR 2.635 95 % CI 1.156–6.008; $P = 0.002$ HR 0.256 95 % CI 0.106–0.616).

Discussion

Chemokines represent a family of small-molecular-weight chemotactic cytokines with homologous structure and similar function, which bind to G-protein-coupled receptors [25]. According to the arrangement of four conserved amino-terminal cysteine residues in mature proteins, they can be classified into four major subfamilies: CXC, CC, CX3C, and C.MCP-1; this is the most representative member of the CC chemokine subfamily,

also known as CC ligand 2 (CCL2). Besides recruitment of monocytes and memory T cells in peripheral blood, MCP-1 can induce influx of calcium, molecular adhesion, and monocyte secretion of pro-inflammatory factors [26]. Some studies reflected the unfavorable prognostic influence of MCP-1 on certain types of cancer. In one study, the serum level of MCP-1 in breast cancer and ovarian cancer was associated with tumor stage [11, 16]. Koide et al. [12] observed that MCP-1 expression in esophageal carcinoma cells was correlated with venous invasion, distant metastasis, and lymph node metastasis, and theorized that it might attribute to MCP-1 involvement of angiogenesis [17] and production of matrix metalloproteinase-9 (MMP-9) [18]. However, other studies demonstrated that MCP-1 was a favorable prognostic marker in colon cancer and pancreatic cancer. Huang et al. [27] found that MCP-1 could reduce the metastatic potential of colon cancer, and Watanabe et al. [13] found that decreased MCP-1/normal ratio was associated with poor prognosis in colorectal cancer patients. Berencsi et al. [28] theorized that MCP-1 could inhibit the progression of colon cancer cells by regulating tumor-infiltrating cytotoxic T cells (CTL). Similarly, Monti et al. [14] observed that high serum levels of MCP-1 had a significant relation with improved prognosis in resected pancreatic cancer patients and considered that high circulating level of MCP-1 would predict antimalignant activity in pancreatic cancer.

In the present study, we found a striking association between MCP-1 expression and prognosis in lung cancer patients. Namely, positive presence of MCP-1 predicted improved prognosis relative to negative in NSCLC. MCP-1 regulates the progression and invasion of malignant tumors through a series of mechanisms, including infiltration of

Table 3 Univariate and multivariate analyses of variates for overall survival in 65 non-small cell lung cancer patients with follow-up records

Variates	Univariate <i>P</i>	Multivariate		
		Hazard ratio	95 % CI	<i>P</i>
Gender: male versus female	0.261			
Age (years): ≤60 versus >60	0.516			
Smoking habits: no versus yes	0.407			
Tumor size (cm): ≤3 versus 3–7 versus >7	0.925			
Histological type: Squamous carcinoma versus adenocarcinoma versus other types	0.015	3.270	1.758–6.082	<0.001
Differentiation: high-moderate versus low	0.105			
Tumor grade: T1-2 versus T3-4	0.232			
Lymph node metastasis: no versus yes	0.020			
Distant organ metastasis: no versus yes	0.012	2.392	0.539–10.613	0.251
Tumor stage: I–II versus III–IV	0.012	2.635	1.156–6.008	0.021
MCP-1 expression in tumor cells: negative versus positive	0.018	0.256	0.106–0.616	0.002

Log-rank tests were used for predictors in univariate analysis based on the Kaplan–Meier curves. Cox proportional hazards regression was used for multivariate analysis of overall survival. All predictors with *P* values <0.10 in univariate analysis were used in multivariate analysis, carried out by the forward stepwise (likelihood ratio) procedure

inflammatory cells in tumor tissue, elevated cytotoxic activity of monocytes and natural killer cells, and mediation of macrophage-induced angiogenesis. Monti et al. [14] demonstrated that the expression of MCP-1 was significantly increased in a synergistic manner by IFN- γ , tumor necrosis factor (TNF), and interleukin 1 (IL-1). They also found that MCP-1 attracted tumor-associated macrophages in pancreatic tumors in vivo. In a mouse melanoma model, MCP-1 played a dual role in tumor growth [29]: low level of MCP-1 stimulated angiogenesis and promoted tumor

growth, whereas high level of MCP-1 inhibited tumor growth by the recruitment of macrophages. The interaction between macrophages and tumor is very complicated. There were two phenotypes of macrophages: M1 and M2. Classical or M1 macrophages could be activated by interleukin 2 (IL-2) and IFN, and effect tumoricidal activity [30, 31]; M2 macrophages could promote tumor progression by producing various vasogenic and lymphatic factors, cytokines and protease [32]. To explain our findings, we highlighted on the role and contribution to tumor development by tumor-infiltrating lymphocytes and chemokines. We hypothesized that NSCLC tumor cells would produce MCP-1 to amplify monocytes/macrophages recruitment in tumor tissue; then, these monocytes/macrophages might kill tumor cells after activation by secreting pro-inflammatory cytokines, which in turn would stimulate MCP-1 production; meanwhile, cytokines produced by activated macrophages would change macrophage phenotypes. Although MCP-1 exerted angiogenic effects in vivo, it worked at certain doses but not at higher doses [17]. Moreover, chemokines had the potential to activate protease and promoted degradation of matrix regulating by macrophages [33]. This hypothesis might be partly confirmed by our observation since we found the smaller the tumor size, the more positive for MCP-1 expression in tumor cells. We will do more work to clarify the mechanisms in the future studies. Miotto et al. [34] reported MCP-1 plasma levels were similar in patients with NSCLC, healthy smokers, and non-smokers. However, in our study, tumor MCP-1 expression was observed less frequently in smokers than in non-smokers. We could not present definitive explanation for this finding, but we speculate that some possible mechanism dysregulate the expression of MCP-1 in smokers during malignant transformation in the lung induced by smoking.

Some researchers focused on functional single-nucleotide polymorphism (SNP) in the MCP-1 gene. Yang et al. [35] suggested the MCP-1 -2518A/G was correlated with genetic susceptibility to NSCLC in the Han nationality of North China, whereas polymorphism in the MCP-1 distal regulatory region -2518A/G and -2578A/G found to affect the transcriptional activity and regulate the level of MCP-1 expression [36, 37]. Ghilardi et al. [38] and Tse et al. [39] suggested that polymorphisms of MCP-1 SNP -2518 could be used to evaluate the risk of progression for early-staged breast cancer patients and for nasopharyngeal carcinoma patients after treatment. In our studies, tumor MCP-1 expression was observed more frequently in female patients than in males (94.5 and 69 %, respectively), in adenocarcinoma (88.6 %) than in squamous carcinoma (59 %). Although we could not explain this phenomenon, we hypothesized it might relate with polymorphism of MCP-1 gene. Validation researches are warranted in the future.

As a high affinity receptor of MCP-1, CCR2 expression was considered relatively restricted to certain types of cells. According to the difference of C-terminal tails, CCR2 can be divided into two isoforms: CCR2A and CCR2B [40]. Mononuclear cells and vascular smooth muscle cells predominantly express CCR2A isoform, whereas monocytes and activated natural killer cells mainly express CCR2B isoform [41]. CCR2 has dual roles, including pro-inflammatory and anti-inflammatory activities [42]. Several recent studies have detected CCR2 expression in cancer cells, including multiple myeloma and prostate cancer cells [43, 44]. Using immunohistochemistry, we found CCR2 expression in NSCLC cells: 25 of 134 cases (18.7 %) showed positive staining in cytoplasm and 15 (11.2 %) in nucleus; only 1 case showed CCR2 localization in both cytoplasm and nucleus. We also observed CCR2 expression in infiltrating monocytes. Expression of CCR2 in multiple myeloma predicted improved survival [43], while positive CCR2 presence in prostate cancer cells was associated with tumor progression [44]. In our study, however, neither clinicopathological characteristics nor prognosis was correlated with expression status of CCR2 in cancer cells. Spearman correlation coefficient revealed no significant association between MCP-1 and CCR2 expression in NSCLC cells. Interestingly, when Monti et al. [14] demonstrated the potential mechanisms of antimalignant activity for MCP-1 in pancreatic progression, they failed to detect CCR2 expression in pancreatic cancer cells. Therefore, we supposed that MCP-1 might combine with CCR2 in various inflammatory cells, including monocytes/macrophages, memory T cells, natural killer cells, and endothelial cells rather than CCR2 in cancer cells to modulate progression in NSCLC. It is noteworthy that CCR2 mRNA and protein expressions in lung cancer A549 and H1299 cells were significantly higher compared with that in control cells [44]. Potential association between CCR2 expression of tumor cells and malignant transformation in the lung remains unknown and merits further study.

Taken together, in the present study, we demonstrated the expression of MCP-1 and CCR2 in NSCLC by simple immunohistochemistry. We observed the association between MCP-1 expression and prognosis of NSCLC patients. We believe that the expression of MCP-1 is one of the independent prognostic risk factors in NSCLC, suggesting that MCP-1 expression may reflect the malignant potential of a tumor. Nevertheless, this study is restricted by its retrospective nature and the patients gathered in one institute. Additional prospective large sample-sized studies are needed to confirm the role of MCP-1 in progression and invasion of NSCLC. And validation studies on actions of MCP-1 and CCR2 in lung cancer cells by others are awaited.

Conflict of interest The authors made no disclosure.

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