Aberrant methylation of *CXCL12* in non-small cell lung cancer is associated with an unfavorable prognosis

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Abstract. Chemokines play an important role in the pathogenesis of non-small cell lung cancer (NSCLC). However, aberrant methylation of *CXCL12* has not been examined in NSCLC. CXCL12 mRNA expression and methylation were examined in 17 NSCLC cell lines by RT-PCR and methylation-specific PCR (MSP). MSP was performed on 236 tumor specimens from NSCLC patients who received curative intent surgery. CXCL12 and CXCR4 protein expression was examined in 90 of the 236 NSCLC specimens by immunohistochemistry. Down-regulation of *CXCL12* expression was found in 10 of 17 (59%) NSCLC cell lines compared with normal bronchial cells. Treatment of 8 expression-negative cell lines with a demethylating agent restored expression in all cases. Twelve cell lines (71%) showed aberrant methylation, and good concordance between methylation and expression was present. Aberrant methylation occurred in 85 out of 236 (36%) primary NSCLCs in a tumor-specific manner. In multivariate analysis, *CXCL12* methylation correlated strongly and independently with prognosis both in all patients with NSCLCs and in those with stage I NSCLCs (hazard ratio=1.68, P=0.015 and hazard ratio=3.58, P=0.017). Secreted protein CXCL12 and its receptor CXCR4 were abundant in NSCLC cells (72 out of 90, 80%; 57 out of 90, 63%) and correlated with the progression of NSCLCs. In conclusion, epigenetic silencing of *CXCL12* is a frequent event in NSCLCs, and could be an independent and powerful prognostic marker in patients with NSCLCs and those with stage I disease. Analysis for *CXCL12* may provide novel opportunities for prognosis and therapy of resected NSCLCs.

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Introduction

Lung cancer is the leading cause of malignancy-related death worldwide. According to the American Cancer Society, the 5-year survival rate for all stages combined is only 16%. The survival rate is 49% for cases detected even when the disease is localized and amenable to curative surgical resection. Although recent studies have shown that adjuvant chemotherapy improves survival in completely resected non-small cell lung cancer (NSCLC) (1,2), improvement of long-term survival is still low and only an additional 5-15% of treated individuals ultimately benefit (3). Therefore, new approaches are needed to individualize treatment by selecting the subset of patients, especially stage I, whose 5-year survival rate is 76% (4) and who are most likely to benefit from a given adjuvant therapy when only micrometastasis remains. To this end, the study of prognostic/predictive factors in addition to post-surgical stage is a promising approach to improve survival rates.

Chemokines are small pro-inflammatory chemotactic cytokines that play an important role in the regulation of cellular trafficking. In many types of tumors, several chemokines may play a critical role in the establishment and in autocrine or paracrine regulation of progression and metastasis (5). One of the well known chemokine-chemokine receptor pairs is CXCL12 (formerly known as stromal cell-derived factor-1) and its cognate receptor CXC chemokine receptor 4 (CXCR4) (6). Phillips *et al* examined the role played by the CXCL12-CXCR4 system in the regulation of NSCLC metastasis (7). They showed that NSCLC cell lines (A549 and Calu-1) and lung adenocarcinomas do not express CXCL12. However, CXCL12 protein levels were present in both tumor and normal lung tissues by ELISA analysis. Other authors also showed that CXCL12 expression was detectable in 42 of 46 NSCLC samples using immunohistochemistry (8). Down-regulation of CXCL12 expression in tumor cells was present in other tumor cell lines including breast, cervix, colon, duodenal, gastric, liver, lung, and pancreatic tumor as well as leukemia and melanoma lines (9). The mechanism and role of down-regulation of CXCL12 expression in NSCLC cells has not been clarified to date.

Aberrant methylation of CpG islands specific to tumor cells plays important roles in carcinogenesis (10,11). The shift

to a metastatic cellular phenotype by the epigenetic down-regulation of *CXCL12* was reported in colon cancer (12). In this study, we first examined *CXCL12* expression and found that its down-regulation is common in NSCLC cell lines. We hypothesized that down-regulation is due to aberrant methylation. Therefore we examined the aberrant methylation of *CXCL12* in NSCLC cell lines. Next, we examined the aberrant methylation in 236 primary NSCLCs and then expression of CXCL12 and CXCR4 in 90 primary NSCLCs by immunohistochemistry. These data were analyzed together and correlated with clinicopathological features.

Materials and methods

Cell lines. Seventeen non-small cell lung cancer cell lines were used in this study. These cell lines were established and provided by Dr Adi F. Gazdar of the University of Texas Southwestern Medical Center. Cell cultures were grown in RPMI-1640 medium (Life Technologies Inc., Rockville, MD) supplemented with 5% fetal bovine serum and incubated in 5% CO_2 at 37˚C. Cell lines established at the National Cancer Institute have the prefix NCI and those established at the Southwestern Medical Center have the prefix HCC. Normal bronchial epithelial cells (NHBECs) were cultured as reported previously (13), and normal trachea RNA was obtained from Clontech (Palo Alto, CA).

Patients. Surgically resected samples were obtained from 236 unselected patients with NSCLC who had received neither any treatment prior to resection nor adjuvant chemotherapy from 1995 to 2000 at the Chiba University Hospital, Chiba, Japan. The study protocol was approved by the Institutional Review Board of our hospital, and written informed consent was obtained from all participants. All patients received curative intent surgery. Resected samples were immediately frozen and stored at -80˚C until use. Methylation assays were performed for 236 cases, and immunohistochemistry assay for 90 of the 236 cases.

Reverse transcriptase-PCR assay for CXCL12. A reverse transcriptase-PCR (RT-PCR) assay was used to examine *CXCL12* mRNA expression. Total RNA was extracted from samples with Trizol (Life Technologies Inc.) following the manufacturer's instructions. The RT reaction was performed on 4 μg of total RNA using deoxyribonuclease I and the SuperScript II First-Strand Synthesis System with the oligo(dT) Primer System (Life Technologies Inc.), and aliquots of the reaction mixture were used for subsequent PCR amplification. Primer sequences for *CXCL12* amplification were: forward, 5'-TGA GCT ACA GAT GCC CAT GC-3'; reverse, 5'-TTC TCC AGG TAC TCC TGA ATC C-3' (product size 178 bp; GenBank accession number U16752; forward nucleotides 157-176; reverse nucleotides 313-334). We confirmed that genomic DNA was not amplified with these primers, which cross an intron. The amplification program for *CXCL12* transcript was 1 min at 94˚C, 1 min at 62˚C, and 1 min at 72˚C for 28 cycles. The housekeeping gene *GAPDH* was used as an internal control to confirm the success of the RT reaction. Primer sequences for *GAPDH* amplification were: forward, 5'-CAC TGG CGT CTT CAC CAC CAT G-3'; reverse, 5'-GCT TCA CCA CCT TCT TGA TGT CA-3' (NM_002046). These primer sequences were identical to those of the endogenous human target genes as confirmed by a BLAST search. PCR products were analyzed on 2% agarose gels. NHBECs and normal trachea were used as normal controls for RT-PCR.

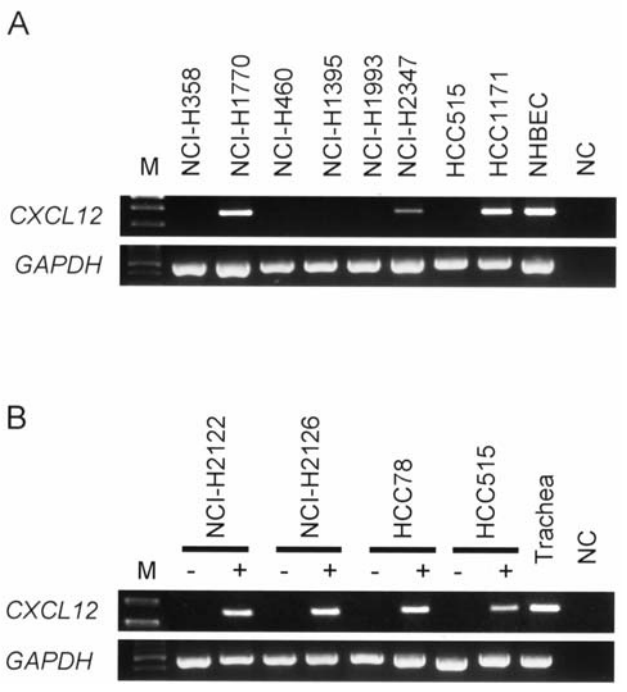

Figure 1. Representative examples of RT-PCR results for *CXCL12* expression in NSCLC cell lines (A) and the effect of 5-aza-CdR treatment on *CXCL12*-negative cell lines (B). Treatment with 5-aza-CdR restored expression of *CXCL12* in four cell lines. Expression of the housekeeping gene *GAPDH* was run as a control for RNA integrity. M, size marker; NEG, negative control (genomic DNA); -, before 5-aza-CdR treatment; +, after 5-aza-CdR treatment.

5-aza-2'-deoxycytidine (5-aza-CdR) treatment. Eight tumor cell lines with negative gene expression were incubated in culture medium with 1 μM of the demethylating agent 5-aza-CdR (Sigma-Aldrich, St. Louis, MO) for 6 days, with medium changes on days 1, 3 and 5. Cells were harvested and RNA was extracted at day 6 (14).

DNA extraction and MSP. Genomic DNA was obtained from primary tumors and non-malignant tissues by digestion with proteinase K (Life Technologies Inc.), followed by phenol/chloroform (1:1) extraction (15).

DNA was treated with sodium bisulfite as described previously (16). PCR amplification was conducted with bisulfite-treated DNA as the template, using specific primer sequences for the methylated and unmethylated forms of the genes (12). DNA methylation patterns in the CpG island of these genes were determined using the method of methylation-specific PCR (MSP) as reported by Hermann *et al* (17). Universal methylated and unmethylated DNAs (Chemicon, Temecula, CA) after bisulfite treatment were used as positive controls for the methylated and unmethylated alleles. Also, DNA from peripheral blood lymphocytes (n=5) from healthy

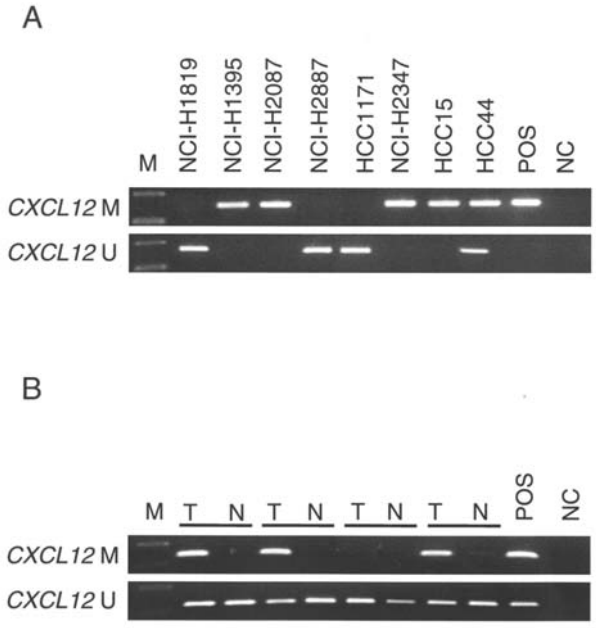

Figure 2. Representative examples of MSP assays for *CXCL12* in cell lines (A) and primary tumors (B). T, Lung cancer; N, matched non-malignant lung tissue. *CXCL12* M, CXCL12-methylated form; *CXCL12* U, CXCL12-unmethylated form; M, size marker; POS, positive control (artificially methylated DNA); NEG, negative control (water blank). A visible band indicates amplification of methylated form. Because of contamination of normal tissues, either the unmethylated band only or both the methylated and unmethylated bands were present in primary samples. Each underlined 'T' and 'N' is patient matched.

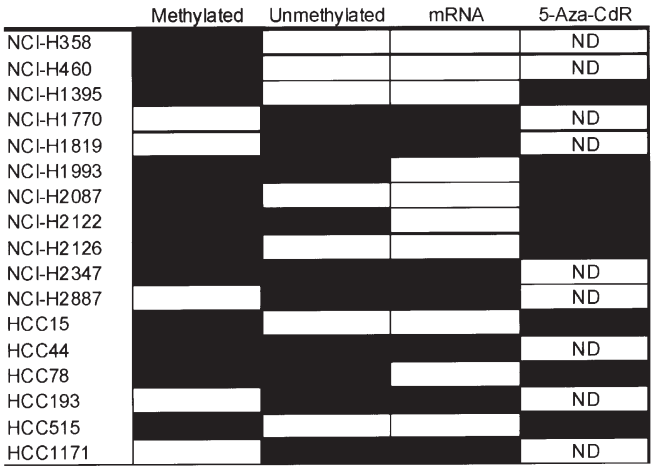

Figure 3. *CXCL12* expression and methylation in NSCLC cell lines. Closed box, positive band detected; open box, negative band detected; ND, not done.

subjects (nonsmoking) was used for negative controls for MSP assays. Nine microliters of each PCR product was loaded on 2% agarose gels stained with ethidium bromide. Results were confirmed by repeating bisulfite treatment and MSP for all samples.

Immunohistochemistry. Three serial 5-μm sections of 90 formalin-fixed, paraffin-embedded lung cancer samples were stained either by standard H&E or by the biotin streptavidin-peroxidase method. CXCR4 and CXCL12 protein expression was determined using goat polyclonal antibody (sc-6279, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:250 and mouse monoclonal antibody (mAb; clone 79018, R&D Systems, Minneapolis, MN) diluted to 8 μg/ml. All primary antibodies were incubated overnight at room temperature. Tumor cells with CXCR4 or CXCL12 cytoplasmic and/or membrane immunohistochemical expression were considered to be positive cells. Macrophage positivity was used as an adequate internal positive control for each case to validate the technical procedure. Staining was assessed using three semi-quantitative categories based on the percentage of stained (positive) tumor cells: absence of staining or <10% positive cells (low), 10-50% positive cells (moderate), and >50% positive cells (high). In particular, cases were considered positive when >10% of the tumor cells showed cytoplasmic and/or membrane expression. These staining and scoring methods have been widely used to evaluate the results of immunohistochemical staining for CXCR4 and CXCL12 (18,19).

Statistical analysis. The Fisher's Exact test and Mann-Whitney U test were applied to assess the association between categorical variables. Overall survival curves were calculated with the Kaplan-Meier method and were compared by the log-rank test. The Cox Proportional Hazards Regression model was used for multivariate analyses. Statistical significance was defined as a P-value <0.05. All P-values were two-sided.

Results

Expression and aberrant methylation of CXCL12 in the cell lines. Expression of *CXCL12* was examined by RT-PCR, and representative examples of the results are shown in Fig. 1A. *CXCL12* expression was present in NHBECs and normal trachea. However, no *CXCL12* expression was observed in 59% of the NSCLC cell lines (10 out of 17). To confirm that aberrant methylation was responsible for silencing *CXCL12* expression, 8 of the 10 cell lines that showed no *CXCL12* expression were treated with the demethylating agent 5-aza-CdR. *CXCL12* expression was restored after the treatment in all 8 cell lines tested (Figs. 1B and 3).

Representative examples of aberrant methylation of *CXCL12* in cell lines are illustrated in Fig. 2A, and the results of our analysis are detailed in Fig. 3. Aberrant methylation was absent in normal lymphocytes and NHBECs. Aberrant methylation was found in 71% of NSCLC cell lines (12 out of 17). Only 2 cell lines had methylation and expression of *CXCL12*. The overall concordance between loss of gene expression and aberrant methylation of *CXCL12* was 88% in NSCLC cell lines.

Aberrant methylation of CXCL12 in primary tumors. *CXCL12* methylation in primary tumors and normal tissues obtained by MSP are detailed in Table I, and representative examples are illustrated in Fig. 2B. CXCL12 methylation was present in 11 (7%) of 163 resected cases where corresponding non-malignant lung tissues were available. A comparison of malignant (85 out of 236) with corresponding non-malignant lung tissues (11 out of 163) indicated that *CXCL12* methylation was a tumor-specific event (P<0.0001, Table I). Tumor tissues

Table I. Methylation of *CXCL12* in NSCLCs.

No. of cases	No. methylated (%)
NSCLC cell lines (17)	12 (71)
Tumors (236)	85 (36)
Non-malignant lung tissue (163)[a] and NHBECs (1)	11 (7)
Clinical characteristics of patients/primary tumors	
Gender	
Male (167)	68 (41)[c]
Female (69)	17 (25)
Age[b]	
≤65 (114)	44 (39)
>65 (122)	41 (34)
Smoking	
Smoker (170)	67 (39)
Never (66)	18 (27)
Histology	
Adenocarcinoma (134)	46 (34)
Squamous cell carcinoma (87)	33 (38)
Large cell carcinoma (12)	6 (50)
Adenosquamous carcinoma (3)	0 (0)
p-Stage	
I (84)	30 (36)
II, III, IV (152)	55 (36)

NHBECs, normal human bronchial epithelial cells (methylation negative). [a]Adjacent to resected tumors. [b]Divided into 2 groups by median age. [c]P=0.025 (Fisher's exact test); other comparisons were not significant.

consist of mixtures of tumor cells (i.e. malignant cells) and non-malignant cells, and the unmethylated forms of all the genes were present in all of the tumor samples.

Methylation was found in 85 (36%) of 236 NSCLCs. Next we correlated these results with clinical factors. The methylation frequency was higher in males (P=0.025). There were no significant associations with age, smoking history, or histologic types. The 5- and 10-year survival rates were 58.5 and 53.9%, respectively, for all patients (n=236); 81.2 and 76.9%, respectively, for stage I patients (n=84), by the Kaplan-Meier method. *CXCL12* methylation was significantly associated with a poor prognosis for all and stage I patients, as estimated using the log rank test (P=0.0033 and 0.0063, respectively) (Fig. 5A and B). Cox proportional hazard regression analysis was performed to determine whether *CXCL12* methylation is an independent prognostic factor (Table II). Prognosis was significantly poorer in *CXCL12* methylation than non-methylation cases for all and stage I patients (hazard ratio=1.68, P=0.015 and hazard ratio=3.58, P=0.017).

Protein expression of CXCL12 and CXCR4. Typical immunostaining patterns for CXCL12 and CXCR4 in NSCLCs are shown in Fig. 4. Using the criteria described in Materials and methods, high, moderate, and low expression scores of CXCL12 were found in 42 (47%), 30 (33%), and 18 (20%) tumors, respectively. Those of CXCR4 were found in 22 (24%), 35 (39%), and 33 (37%) tumors, respectively. CXCL12-positive expression (i.e., high and moderate expression) was not significantly related to gender, age, and histology. CXCL12-positive expression (n=72) was present more frequently in cases with lymph node involvement (N0, 36 out of 50; N1/2, 36 out of 40, P=0.038). Also, CXCR12-positive expression was present more frequently in cases with advanced stage tumors (stage I, 20 out of 31; stage II/III/IV, 52 out of 59, P=0.0120). Similarly, CXCR4-positive expression was not significantly related to gender, age, and histology. CXCR4-positive expression (n=57) was present more frequently in cases with advanced than early stage tumors (stage I, 14 out of 31; stage II/III/IV, 43 out of 59, P=0.0121). There were no relationships between positive expression of CXCR4/CXCL12 and prognosis. However, high expression of CXCL12 correlated with poor prognosis in adenocarcinomas (P=0.015, Fig. 5C). Also, high expression of CXCR4 correlated with poor prognosis in NSCLCs (P=0.023, Fig. 5D). Next we compared these protein expression levels with *CXCL12* methylation. There was no relationship between CXCR4/CXCL12 and *CXCL12* methylation.

Discussion

In this study, we demonstrated that inactivation of *CXCL12* mRNA expression occurs in NSCLC cell lines and is correlated with methylation of the gene. *CXCL12* expression was restored after treatment with a demethylating agent. These results indicate that methylation was the likely mechanism by which *CXCL12* mRNA expression was suppressed. Although there are other possible mechanisms for down-regulation of *CXCL12* expression, the concordance between methylation and loss of gene expression strongly supports this concept.

However, protein expression of CXCL12 is not correlated with methylation of the gene in primary tumors. This contradiction needs to be addressed. One explanation is that CXCL12 is a secreted protein and immunohistochemistry does not discriminate between both endogenous and exogenous proteins. Even if endogenous CXCL12 is down-regulated by aberrant methylation in tumor cells, exogenous CXCL12 from other cells may enter tumor cells. In fact, this contradictive phenomenon was reported as an 'attractive force' of metastasis in breast and colon cancer (12,20,21). Liotta (20) hypothesized that the CXCL12 moves to sites of low concentration in a gradient-like manner, and according to a study by Muller *et al* (21) CXCL12 movement directs metastasis along a CXCL12-CXCR4 axis in breast cancer. Also, Wendt *et al* proposed that colon carcinoma cells (which do not produce their own CXCL12) can respond to exogenous stimulation by chemokines produced at distal sites (12). *CXCL12* methylation may therefore cause a density gradient of CXCL12 protein, metastasis along the CXCL12-CXCR4 axis, and thereby poor prognosis.

In this study, positive expression of CXCL12 and CXCR4 correlated with progression of NSCLC, and high expression of CXCL12 and CXCR4 are prognostic factors in lung

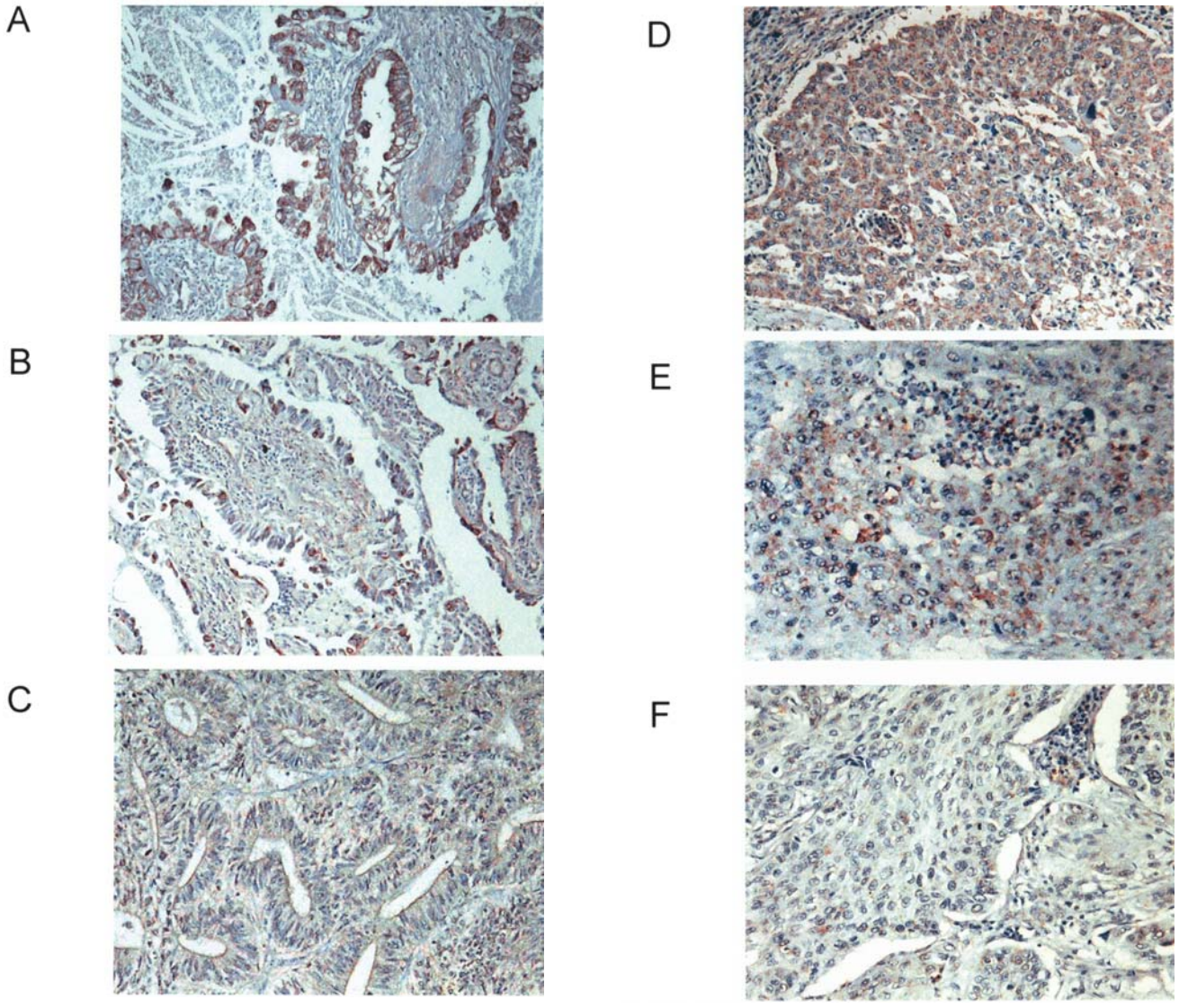

Figure 4. Immunohistochemical staining patterns for CXCL12 and CXCR4 in resected NSCLCs. CXCL12: (A) An adenocarcinoma with a high score (cytoplasmic and/or nuclear immunostaining in >50% of tumor cells; strong staining intensity); (B) an adenocarcinoma with a moderate score (cytoplasmic and/or nuclear immunostaining in >10% of tumor cells; moderate staining intensity); and (C) an adenocarcinoma with a low score (cytoplasmic and/or nuclear immunostaining in <10% of tumor cells; weak staining intensity). CXCR4: (D) An adenocarcinoma with a high score; (E) an adenocarcinoma with a moderate score; and (F) an adenocarcinoma with a low score.

adenocarcinoma and NSCLCs, respectively. Notably, immunohistochemical analysis of CXCL12 expression in 46 patients with NSCLC found that disease recurrence rates in a subgroup of adenocarcinoma patients showed a tendency to correlate with high CXCL12 expression in the tumor (8). Also, immunohistochemical analysis of CXCR4 expression in 36 patients with NSCLC found that patients with high CXCR4 tumors were more prone to clinical metastasis than patients with low expression tumors (22). Thus, previous reports suggest that CXCL12 and CXCR4 expression might influence the progression of lung adenocarcinoma (8) and NSCLC (22), respectively. While these studies were limited to relatively small populations, we provide statistically significant evidence that strengthens and clarifies the importance of the observations in these studies.

In summary, we demonstrated novel findings. Secreted protein CXCL12 and its receptor CXCR4 were abundant in NSCLC cells, and high accumulation of these proteins in tumor cells correlated with progression of NSCLCs. Furthermore, *CXCL12* methylation was common in NSCLC and has been associated with patient outcome strongly and independently. In our judgment, our survival analysis was reliable since the number of patients including stage I was large and all patients were treated at a single institution and received a long-term follow-up of more than 10 years after surgery. The association between methylation of *CXCL12* and poor survival rates suggests that *CXCL12* is a useful marker for selection of candidates for adjuvant chemotherapy among patients with resected NSCLC. This may be more useful in stage I NSCLC because 76% of patients in a previous study (4) and 81.2% in this study survived 5 or more years, and the selection of patients who receive benefit from adjuvant chemotherapy is important. Additional basic and clinical studies are required to investigate the role of *CXCL12* methylation in NSCLC, to validate the prognostic role of *CXCL12* and to explore possible therapeutic approaches.

Table II. Univariate and multivariate statistics of the prognostic value of gender, age, stage, and *CXCL12* methylation for overall survival of the NSCLC cases.

A, All NSCLCs (n=236).

Variable	Univariate	Multivariate		
	P	Hazard ratio	95% Confidence interval	P
Gender (male/female)	0.8000	0.563	0.316-1.005	0.0520
Age	0.3500	1.005	0.983-1.028	0.6700
Smoking status +/-	0.0307	1.901	0.962-3.759	0.0600
Stage III, IV/I, II	<0.0001	3.891	2.242-6.711	<0.0001
CXCL12 methylation	0.0038	1.681	1.107-2.551	0.0148

B, Stage I NSCLCs (n=84).

Variable	Univariate	Multivariate		
	P	Hazard ratio	95% Confidence interval	P
Gender (male/female)	0.6900	0.483	0.135-1.726	0.2600
Age	0.1200	1.061	0.997-1.130	0.0600
Smoking status +/-	0.1600	3.333	0.687-16.129	0.1400
Stage IB/IA	0.5200	1.307	0.459-3.717	0.6200
CXCL12 methylation	0.0109	3.584	1.261-10.204	0.0166

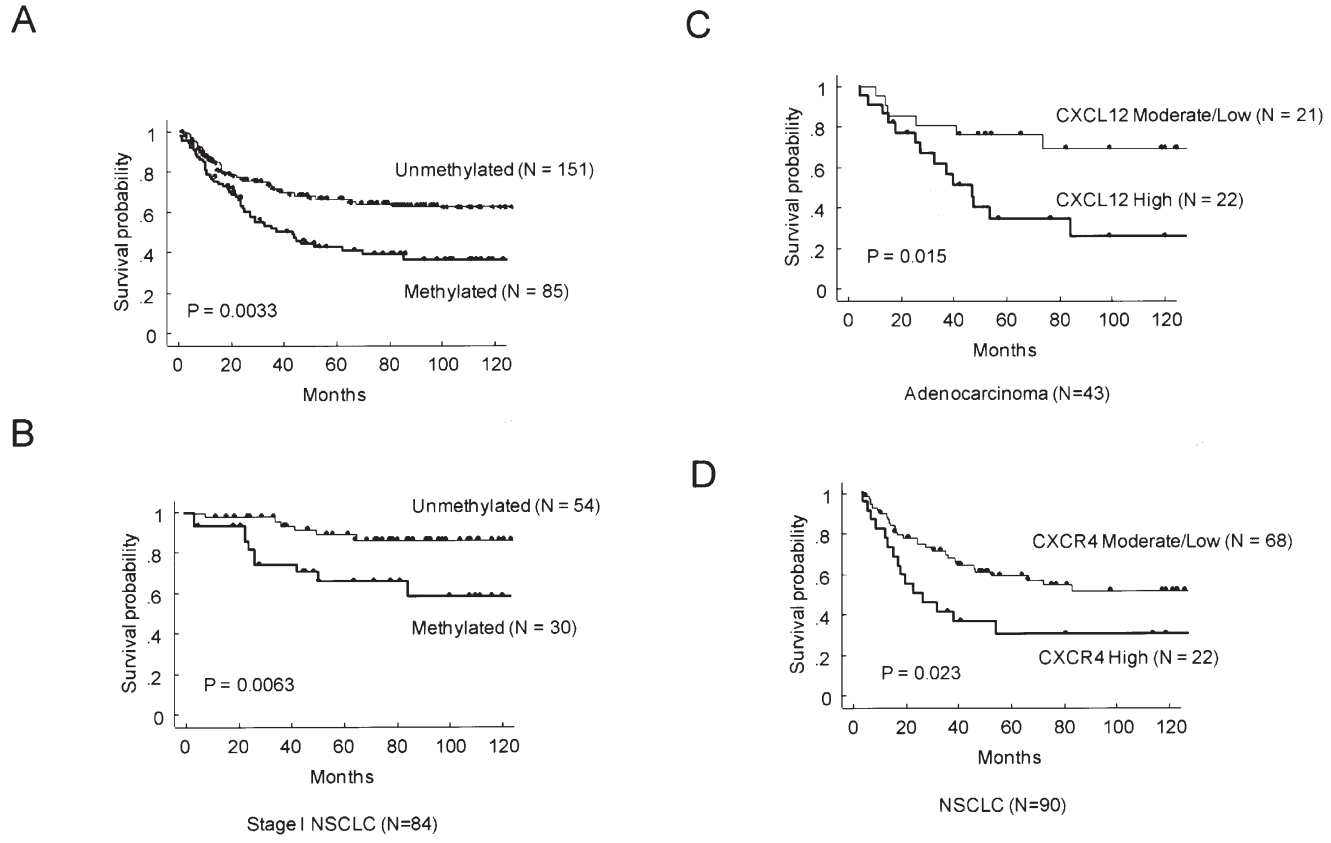

Figure 5. The rates of overall survival of the patients with NSCLC correlated with methylation and protein expression. (A) The 5- and 10-year survival rates were 66.4 and 62.8%, respectively, for patients with unmethylated *CXCL12*; 44.7 and 38.5%, respectively, for patients with methylated *CXCL12*. (B) The 5- and 10-year survival rates were 89.4 and 86.7%, respectively, for patients with stage I disease and unmethylated *CXCL12*; 66.0 and 58.7%, respectively, for patients with stage I and methylated *CXCL12*. (C) The 5- and 10-year survival rates were 76.2 and 69.3%, respectively, for adenocarcinoma patients with moderate/low CXCL12 expression; 34.2 and 25.7%, respectively, for adenocarcinoma patients with high CXCL12 expression. (D) The 5- and 10-year survival rates were 58.6 and 51.0%, respectively, for NSCLC patients with moderate/low CXCR4 expression; 29.8 and 29.8%, respectively, for NSCLC patients with high CXCR4 expression.

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