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Association of genetic polymorphisms of *CXCL12/SDF1* gene and its receptor, *CXCR4*, to the susceptibility and prognosis of non-small cell lung cancer

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ABSTRACT

Background: The aim of this study was to evaluate the relations of chemokine *CXCL12*, previously known as stromal cell-derived factor-1 (*SDF1*), and its receptor, *CXCR4*, gene variants on non-small cell lung cancer (NSCLC) risk and disease severity.
Methods: Through a case–control study design, genomic DNA samples of 247 NSCLC patients and 328 age and sex-matched controls were subjected to polymerase chain reaction-restriction fragment length polymorphism analysis. The validity of this technique was proven by direct sequencing of amplified products. Statistical analyses were conducted to explore the contribution of polymorphism of the *CXCL12/SDF1* gene and *CXCR4*, in the susceptibility to and prognosis of NSCLC.
Results: Overall, the genotype frequencies of *CXCL12/SDF1* gene and *CXCR4*, were significantly different between lung cancer patients and controls ($p < 0.0001$), and also different between patients with lung cancers of various stages ($p < 0.0001$). Logistic regression analysis revealed that higher odds ratios (ORs) for lung cancer were seen for individuals with *CXCL12/SDF1* AA (an OR of 1.95, 95% CI 1.08–3.50, $p = 0.018$), or *CXCR4* TT (an OR of 4.71, 95% CI 1.99–11.2, $p < 0.0001$), and for individuals with both *CXCL12/SDF1* AA and *CXCR4* TT genotypes (an OR of 12.4, 95% CI 1.56–98.3, $p = 0.002$). The patients carrying a homologous AA genotype at *CXCL12/SDF1*, or a homologous TT genotype at *CXCR4*, had a tendency to advanced disease and toward poorer prognoses compared with other patients.
Conclusion: A significant association between the polymorphisms of *CXCL12/SDF1* and *CXCR4*, and the susceptibility to and prognosis of NSCLC was demonstrated.

1. Introduction

Lung cancer is one of the most common malignancies worldwide [1] and the leading cause of cancer deaths in industrial countries, including Taiwan [2], with the worst outcome [3]. Cancer is a result of multiple gene–environment interactions occurring over several decades. The major risk factor for lung cancer is an excessive exposure to tobacco smoke. However, only about 11% of tobacco smokers ultimately develop lung cancer, suggesting that genetic factors may influence the risk for lung cancer among those who are exposed to carcinogens. Epidemiological studies revealed an increased risk of approximately 14-fold for lung cancer among regular tobacco smokers. After the effect of tobacco smoke was stratified, an approximately 2.5-fold risk was attributable to a family history of lung cancer [4,5]. Therefore, it is rational to speculate that certain common genetic variants or polymorphisms may have an impact on lung cancer risk.

Invasion, angiogenesis, migration, and metastasis are intertwined processes regulated by overlapping molecular pathways. Chemokines and their receptors compose one such pathway and are involved in cell trafficking, migration, and proliferation [6]. *CXCL12/SDF1* is an important alpha-chemokine that binds to the G-protein-coupled seven-transmembrane span *CXCR4* [7]. The *SDF-1-CXCR4* axis regulates trafficking of normal and malignant cells [8–10]. There are four groups of chemokine receptors: *C*, *CC*, *CXC*, and *CX3C* [11]. *CXCR4* is expressed in dendritic cells, naïve T cells, NK cells, and monocytes and is also the chemokine receptor most commonly expressed in tumors. Within normal

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cells, chemokine receptors are important in immune cell function and migration of stem cells to sites of injury. Within tumor cells, chemokine receptor expression is related to the development of metastases preferentially to sites with expression of the corresponding chemokine [12]. The ligand for *CXCR4* is the chemokine *CXCL12/SDF1* which is expressed in the lung and other sites of metastases. *CXCR4/CXCL12/SDF1* also indirectly promotes tumor metastasis by mediating proliferation and migration of tumor cells and enhancing tumor-associated angiogenesis [13–16]. *CXCL12/SDF1* gene is located on chromosome 10q 11.1 [17,18], and it has been revealed that a single nucleotide polymorphism (SNP), a guanine to adenine (G→A), at position 801 of the 3′-untranslated gene region may affect the expression of *CXCL12/SDF1* chemokine [19]. The *CXCL12/SDF1* A/A homozygotes had been suggested to alter the production of *CXCL12/SDF1* [20,21] and are associated with the risk of carcinogenesis of various origins, including lung cancer [22–24]. Meanwhile, its receptor, *CXCR4*, is located on chromosome 2q2 [25] and a silent SNP of *CXCR4*, a cytosine to thymine (C→T), is found at codon 138 [26]. So far, only the effect of genetic polymorphisms of *CXCL12/SDF1* has been studied in Iranian lung cancer patients [24]. The effect of genetic polymorphisms of *CXCL12/SDF1* and *CXCR4* in NSCLC has not been studied. To clarify the influence of genetic polymorphisms of *CXCL12/SDF1* and *CXCR4* on the susceptibility and clinicopathological development of NSCLC, the relationship between SNPs of *CXCL12/SDF1* and *CXCR4* genes and NSCLC risk as well as the clinicopathological characteristics was investigated in this study.

2. Materials and methods

2.1. Study population

A total of 247 NSCLC patients, consisting of 178 males and 69 females with a median age of 63.8, who were admitted to China Medical University Hospital, Taichung, Taiwan between 2005 and 2009, were included in this study. Of them, 152 patients had adenocarcinomas (AD) and 95 patients had squamous carcinomas (SQ). The histological determination, including tumor types and stages, was performed according to the WHO classification (WHO, 1982) and the TNM system (Mountain, 1986), respectively. Meanwhile, 328 unrelated controls, consisting of 248 males and 80 females with a median age of 64.2, were randomly selected from a pool of healthy volunteers who visited the general health check-up center of China Medical University Hospital during the same period. All controls had no known medical illness or hereditary disorders and were taking no medications.

A detailed questionnaire, included information on the average number of cigarettes smoked daily and the number of years the subjects had been smoking, for each case and control was completed by a trained interviewer. This study was approved by the Research Ethics Committee of China Medical University, and informed consent was obtained from each participant prior to the commencement. Basic characteristics, including age and gender, of all participants are summarized in Table 1 to show no significant differences in the above-mentioned features between patients and controls.

2.2. Sample collection and genomic DNA extraction

Venous blood from each subject was drawn into Vacutainer tubes containing EDTA and stored at 4 °C. Genomic DNA was extracted by QIAamp DNA blood mini kits (Qiagen, Valencia, USA) according to the manufacture's instructions. DNA was dissolved in TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA] and then quantitated by a measurement of OD_{260}. Final preparation was stored at −20 °C and used as templates for polymerase chain reaction (PCR).

Table 1
Clinical features of the study populations.[a]

Variables	NSCLC	Control	*p* value[‡]
Subjects	247	328	
Gender (male/female)	178/69	248/80	0.34
Age (yr)	63.8 ± 8.6	64.2 ± 9.2	0.28
Brinkman index[b]	589.4 ± 58.2	586.8 ± 56.8	0.45
Tumor type			
Adenocarcinomas (AD)	152		
Squamous carcinomas (SQ)	95		
Tumor stage			
I + II	87		
III + IV	160		

[a] Data are presented as no. or mean ± SEM.
[b] Brinkman index = daily cigarette numbers multiplied by smoking years.
[‡] *p* values were calculated using the Mann–Whitney U test.

2.3. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

The *CXCL12/SDF1*-3′A and *CXCR4* polymorphisms were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The sequences of primers used to amplify the *CXCL12/SDF1*-3′A genotype were 5′-CAGTCAACCTGGGCAAA GCC-3′ and 5′-CCTGAGAGTC-CTTTTGCGGG-3′, and those used for the amplification of *CXCR4* genotype were 5′-AACTTCCTATG CAAGGCAGT-3′ and 5′-TATCTGTCATCTGCCTCACT-3′ [22,27]. PCR was performed in a 10 μL reaction mixture containing 100 ng DNA template, 1.0 μL of 10× PCR buffer (Invitrogen, Carslbad, CA), 0.25 U of Taq DNA polymerase (Invitrogen, Carslbad, CA), 0.2 mM dNTPs (Promega, Madison, WI), and 200 nM of each primer (MDBio, Taipei, Taiwan). The PCR cycling started at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min, with a final step at 72 °C for 20 min to allow a complete extension of all PCR fragments. The results are shown in Fig. 1.

2.4. Statistical analysis

Differences in clinical data between the NSCLC patients and the control subjects were examined. All continuous data are expressed as mean ± standard deviation and compared using a two-tailed Student's *t*-test. Categorical variables are reported as a percentage and compared using Chi-square (χ^2) or Fisher's exact test. Hardy–Weinberg equilibrium was assessed using a goodness-of-fit χ^2 test for biallelic markers. The distribution of *CXCL12/SDF1* and its receptor, *CXCR4*, genetic polymorphism between healthy subjects and lung cancer patients was examined by the χ^2 test. Significance was accepted at $p < 0.05$. Odds ratios (ORs) and 95% confidence intervals (CI) for lung cancer of each specific genotype were calculated with logistic regression to quantitatively assess the degree of association observed.

The Kaplan–Meier method was used to estimate the probability of survival as a function of time and median survival. The log rank test was used to assess the significance of the difference between homozygous variant genotype and other genotypes of *CXCL12/SDF1* and its receptor, *CXCR4*, among pairs of survival probabilities. Significance was accepted at $p < 0.05$.

3. Results

The association of the *CXCL12/SDF1* and its receptor, *CXCR4*, genetic polymorphism with certain clinicopathological parameters of lung cancer patients was analyzed and is shown in Tables 2 and 3.

Table 2
The association between the SDF-1 polymorphism and the clinicopathologic parameters of the studied subjects.

Characteristics	Genotypes			Total	*p*-Value	Odds ratio (95% CI) *p*-value[†]
	GG (%)	GA (%)	AA (%)			
Non-cancer control	171 (52)	136 (42)	21 (6)	328		1.00
Lung cancer	99 (40)	112 (45)	36 (15)	247	<0.001[*]	2.49 (1.42–4.39) 0.002
Tumor type						
AD	59 (39)	72 (47)	21 (14)	152	0.004[*]	2.34 (1.42–4.44) 0.009
SQ	40 (42)	40 (42)	15 (16)	95	0.01[*]	2.74 (1.35–5.56) 0.006
Tumor stage						
I + II	52 (60)	33 (38)	2 (2)	87	<0.0001[‡]	11.47 (2.68–49.0) <0.0001
III + IV	47 (29)	79 (50)	34 (21)	160		

[*] *p* value. The frequencies of the genotypes between the cancer and non-cancer control groups were compared with χ^2 analysis.
[‡] *p* value. The frequencies of the genotypes between lung cancers with different tumor stages were compared with χ^2 analysis.
[†] Odds ratios and *p* value were calculated using logistic regression to measure the association of the variant homozygous genotypes AA with lung cancer risk, with that of the GG/GA genotype being referred to as 1.
AD, adenocarcinoma; SQ, squamous cell carcinoma.

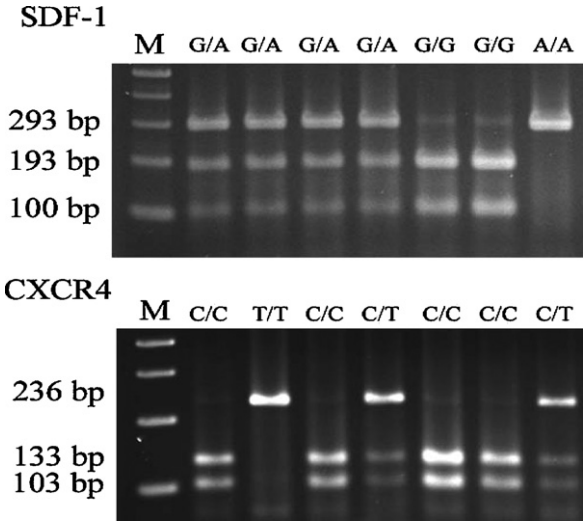

Fig. 1. Polymerase chain reaction-restriction fragment length polymorphism of *CXCL12/SDF1* and *CXCR4* gene. PCR products of *CXCL12/SDF1* and *CXCR4* gene were subjected to enzymatic digestion by incubation with *HpaII* and *BccI* for 4 h at 37 °C and then submitted to electrophoresis in 3% agarose gels. For *CXCL12/SDF1*, wild type homozygous alleles (G/G) yielded 100 and 193-bp products, the heterozygous alleles (G/A) yielded 100-, 193- and 293-bp products, while the mutated type homozygous alleles (A/A) yielded a 293-bp product. For *CXCR4* gene, wild-type homozygous alleles (C/C) yielded 103 and 133-bp products, the heterozygous alleles (C/T) yielded 103-, 133- and 236-bp products, while the mutated type homozygous alleles (T/T) yielded a 236-bp product.

Overall, genotype distributions of the *CXCL12/SDF1* and its receptor, *CXCR4* were significantly different between non-cancer controls and lung cancer patients ($p < 0.0001$). The frequency of *CXCL12/SDF1* variant polymorphic homozygote was low with being 0.15 and 0.06 in the cases and controls, respectively, while that of the wild-type allele was higher (0.71 for cases and 0.63 for controls). The frequency of *CXCR4* variant polymorphic homozygote was low with being 0.12 and 0.02 in the cases and control, respectively, while that of the wild-type allele was higher (0.79 for cases and 0.91 for controls). Results of the χ^2 goodness-of-fit test showed that genotype frequencies were consistent with Hardy–Weinberg equilibrium in the patient population ($\chi^2 = 0.22$; $p > 0.05$) and the control population ($\chi^2 = 0.77$; $p > 0.05$). Logistic regression analysis revealed that higher ORs for having NSCLC (an OR of 4.02, 95% CI 2.39–6.76; $p < 0.0001$), adenocarcinoma (an OR of 3.92, 95% CI 2.23–6.89; $p < 0.0001$) and squamous cell carcinoma (an OR of 4.19, 95% CI 2.19–8.02; $p < 0.0001$), were seen in patients homozygous for *CXCL12/SDF1* variant allele (A/A), as compared with patients with at least one wild-type allele ((G/G) or (G/A)). Also, higher ORs for having NSCLC (an OR of 4.02, 95% CI 2.39-6.76; p < 0.0001), adenocarcinoma (an OR of 3.92, 95% CI 2.23–6.89; $p < 0.0001$) and squamous cell carcinoma (an OR of 4.19, 95% CI 2.19–8.02; $p < 0.0001$), were seen in patients homozygous for *CXCR4* variant allele (T/T), as compared with patients with at least one wild-type allele ((C/C) or (C/T)). Furthermore, patients carrying a variant polymorphic homozygote of *CXCL12/SDF1* and *CXCR4* also had a tendency for advanced disease ($p = 0.001$).

All patients were followed up during the study period. Those patients carrying a variant polymorphic homozygote of *CXCL12/SDF1* and *CXCR4* also had a poorer prognoses compared

Table 3
The association between the CXCR4 polymorphism and the clinicopathologic parameters of the studied subjects.

Characteristics	Genotypes			Total	*p*-Value	Odds ratio (95% CI) *p*-value[†]
	CC (%)	CT (%)	TT (%)			
Non-cancer control	274 (84)	47 (14)	7 (2)	328		1.00
Lung cancer	172 (70)	44 (18)	31 (12)	247	<0.0001[*]	6.58 (2.85–15.2) <0.0001
Tumor type						
AD	108 (71)	29 (19)	15 (10)	152	<0.0001[*]	5.02 (2.00–12.6) <0.0001
SQ	64 (67)	15 (16)	16 (17)	95	<0.0001[*]	6.63 (2.53–17.4) <0.0001
Tumor stage						
I + II	70 (81)	15 (17)	2 (2)	87	0.017[‡]	6.42 (1.47–28.1) 0.005
III + IV	102 (64)	29 (18)	29 (18)	160		

[*] *p* value. The frequencies of the genotypes between the cancer and non-cancer control groups were compared with χ^2 analysis.
[‡] *p* value. The frequencies of the genotypes between lung cancers with different tumor stages were compared with χ^2 analysis.
[†] Odds ratios and *p* value were calculated using logistic regression to measure the association of the variant homozygous genotypes TT with lung cancer risk, with that of the CC/CT genotype being referred to as 1.
AD, adenocarcinoma; SQ, squamous cell carcinoma.

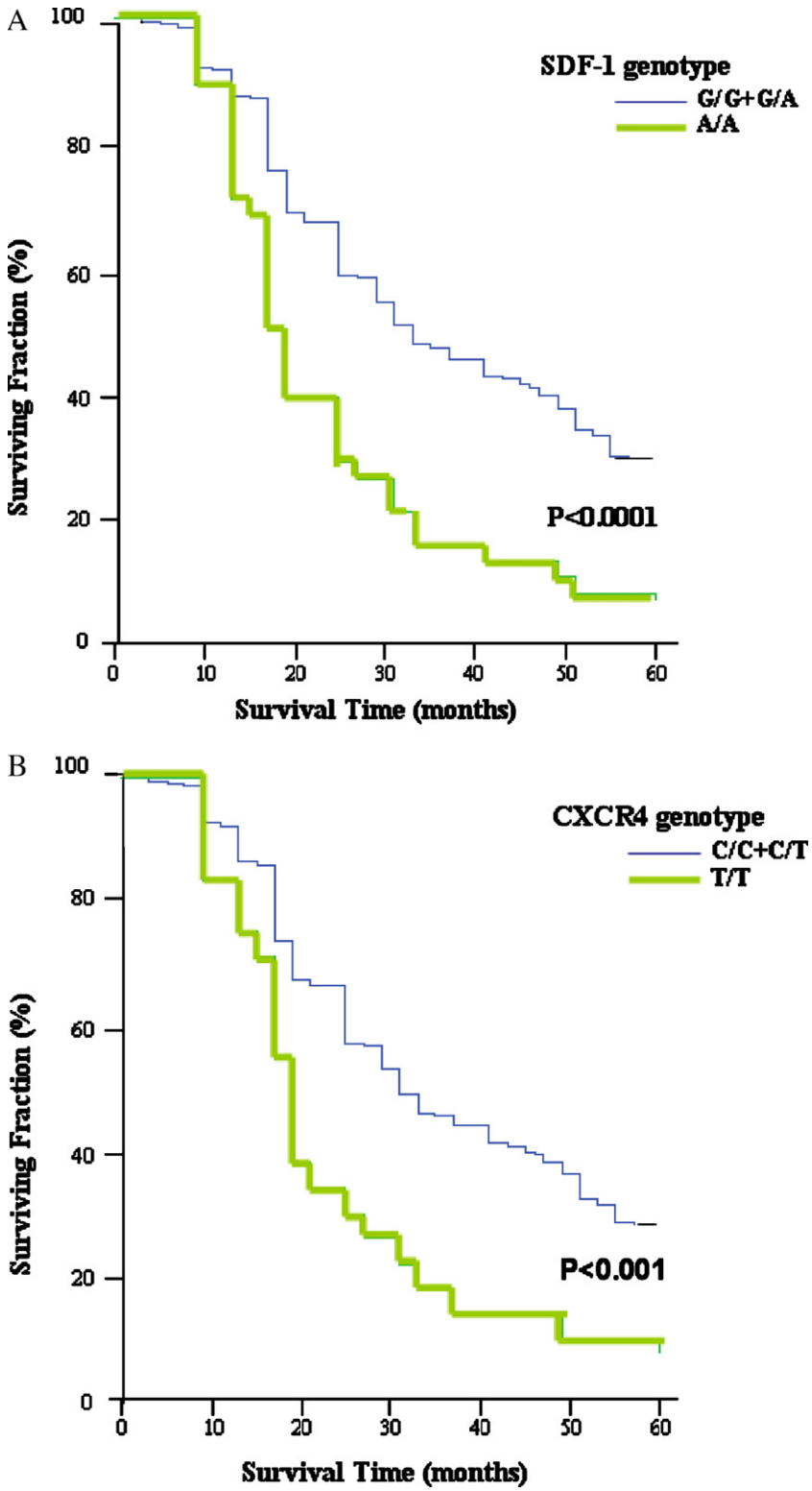

Fig. 2. The Kaplan–Meier survival curves with respect to the *CXCL12/SDF1* G→A and *CXCR4* C→T genetic polymorphisms in relation to non-small cell lung cancer patients. The *p* value for each analysis is indicated.

with other patients ($p < 0.0001$ and $p < 0.001$, by log rank test) (Fig. 2).

4. Discussion

In this study, we provided novel information about the effects of genetic polymorphisms of *CXCL12/SDF1* and its receptor, *CXCR4*, on the susceptibility and clinicopathological development of NSCLC. It has been shown that over-expression of *CXCL12/SDF1* and *CXCR4* or interaction between both chemokines is associated with the development and metastasis of human lung cancer [15,28–30]. The binding of *CXCL12/SDF1* to *CXCR4* induces intracellular signaling through several divergent pathways initiating signals related to chemotaxis, cell survival and/or proliferation,

increase in intracellular calcium, and gene transcription. Homozygous *CXCL12/SDF1*-3′A/A genotype is considered to be associated with an increased level of protein, which is available to bind its exclusive receptor *CXCR4* that contributes to the protective effect of *CXCL12/SDF1*-3′A/A homozygous alleles [21]. In this study, significant difference in genotypic frequencies of *CXCL12/SDF1* gene was found between controls and patients with NSCLC. Individuals with *CXCL12/SDF1*-3′A/A homozygotes had a risk of 2.49-fold to have NSCLC compared with individuals with G/G homozygotes or G/A heterozygotes. As well, individuals with *CXCR4* T/T homozygotes had a risk of 6.58-fold to have NSCLC compared with individuals with C/C homozygotes or C/T heterozygotes.

To the best of our knowledge, only Razmkhah et al. [24] have studied the relationship between *CXCL12/SDF1* G801A gene polymorphism and Iranian lung cancer patients. They investigated 72 patients, included carcinoid tumor, small cell and non-small cell lung cancer, and concluded that AA and AG genotypes of *CXCL12/SDF1* might be considered as factors increasing the susceptibility of Iranian patients to lung cancer. In this study focusing upon NSCLC patients, both *CXCL12/SDF1* and its receptor, *CXCR4*, gene variants were evaluated to reveal that the genetic polymorphisms of the *CXCL12/SDF1* and *CXCR4* increased the susceptibility to NSCLC. Significant differences were found in allele and genotype frequency distribution of *CXCL12/SDF1* and *CXCR4* between NSCLC patients and controls.

To the best of our knowledge, no study had been conducted to investigate the association between *CXCR4* gene polymorphism and NSCLC risk. Although, Hirata et al. [31] reported that A/A or A/G genotype of *CXCL12/SDF1* was associated with prostate cancer. They also demonstrated that A/A or A/G genotype of *CXCL12/SDF1* was significantly associated with higher expression of *CXCL12/SDF1* and *CXCR4*. Subsequently, we investigated the relationships between polymorphisms and clinicopathological status of patients with NSCLC. There was significant association between *CXCL12/SDF1* and *CXCR4* gene polymorphism with advanced stages of NSCLC. Survival analysis was performed and those patients carrying a variant polymorphic homozygote of *CXCL12/SDF1* and *CXCR4* also had a poorer prognosis.

Since the susceptibility to NSCLC is recognized to be affected by multiple genetic factors and genotype, as well as environment interactions, each of which has a certain extent of influence on the development of this disease, it is rational to say that such a relationship between genetic polymorphisms and NSCLC susceptibility may be ethnic-dependent.

Also *CXCL12/SDF1* and *CXCR4* may also play a tissue-specific role, and different regulatory mechanisms may apply to different tumors [31–35]. Nevertheless, our data did demonstrate that the polymorphisms of *CXCL12/SDF1* and *CXCR4* gene had significant associations with susceptibility to and prognosis of NSCLC in our population. However, in this series, the interpretation of our results was limited by sample size. Further study of larger scale on these genotypes and other polymorphisms is required to analyze haplotypes and their association with both the onset of lung cancer and the development of metastases.

In conclusion, by evaluating the polymorphic sites of *CXCL12/SDF1* and *CXCR4*, a significant association of the polymorphisms with NSCLC was revealed. The results of this study support a relation between *CXCL12/SDF1* and *CXCR4* genetic polymorphisms and the susceptibility to and prognosis of NSCLC.

Conflict of interest statement

None declared.

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