Impact of CCL4 gene polymorphisms upon the progression of lung cancer in a Han Chinese cohort

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Abstract
Lung cancer is the most common malignancy in China and has a low survival rate amongst Han Chinese. The high mortality is largely attributed to late-stage diagnosis, when treatment is largely ineffective. Identification of genetic variants could potentially assist with earlier diagnosis and thus more effective treatment. Chemokine C–C motif ligand 4 (CCL4) plays a critical role as a chemotractant in tumor development, metastasis and angiogenesis. In this study, we explored three CCL4 single nucleotide polymorphisms (SNPs) (rs1634507, rs1719153, and rs10491121) in 538 patients with lung cancer and 370 healthy, cancer-free controls. Carriers of the GT + TT heterozygote at rs1634507 had a lower risk of lung cancer than wild-type (GG) carriers, while the presence of the AG + GG heterozygote at rs10491121 was associated with a higher risk of lung cancer compared with having the AA genotype. The G/A/G + TT heterozygote of rs1634507 had a lower risk of lung cancer than wild-type (GG) carriers, while the presence of the AG + GG heterozygote at rs10491121 was associated with a higher risk of lung cancer compared with having the AA genotype. The G/A/G and T/A/A CCL4 haplotypes significantly reduced and increased the risks for lung cancer, respectively. Our study is the first to document correlations between CCL4 polymorphisms and lung cancer development and progression in people of Han Chinese ethnicity.

Abbreviations: AJCC = American Joint Committee on Cancer, AOR = adjusted odds ratio, CCL4 = Chemokine C motif ligand 4, CI = confidence interval, CIs = confidence intervals, HWE = Hardy–Weinberg equilibrium, IASLC = International Association for the Study of Lung Cancer, MIP-1 = inflammatory protein-1, OR = odds ratios, PCR = polymerase chain reaction, SNP = single nucleotide polymorphisms, TNM = tumor node metastasis.

Keywords: CCL4, gene polymorphism, Han Chinese, lung cancer

1. Introduction
As a result of aging populations, increasing air pollution, cigarette smoking, environmental carcinogens, and genetic predisposition, lung cancer is now the most common malignancy worldwide, constituting around 13% of all cancer cases globally.[1–4] In 2012, worldwide age-standardized mortality rates for males and females with lung cancer were 30.0/100,000 and 11.1/100,000, respectively, reflecting poor 5-year relative survival rates.[5] In 2014, 782,000 new lung cancer cases and 626,000 lung cancer deaths occurred in China.[6] The specific genetic risks underlying lung cancer development and progression remain unclear. Genetic variants and epigenetic changes are known to influence the development and progression of non-small cell lung cancer.[7–9] Thus, specific information about genetic aberrations could potentially assist with risk prediction and earlier diagnosis of lung cancer in individual patients.

Chemokine C–C motif ligand 4 (CCL4), also referred to as macrophage inflammatory protein-1 (MIP-1B), is a chemotractant that plays a critical role in immune response, inflammation and tumor development.[10,11] Increasing evidence indicates that CCL4 controls several tumor-related functions such as proliferation, invasion, metastasis, and angiogenesis.[10,12,13] It has been suggested that high CCL4 concentrations predict unfavorable survival in lung adenocarcinoma.[14] Genetic factors and single nucleotide polymorphism (SNP) genotyping are pivotal in investigations into the risk and prognosis of tumorigenesis.[15–17] SNPs in the CCL4 chemokine gene are known to be associated with breast cancer, oral cancer, and hepatocellular carcinoma,[18–20] while a recent study has reported...
a protective effect against the risk of developing rheumatoid arthritis in individuals carrying the T-containing genotype of the CCL4 rs1719153 polymorphism. Thus, the evidence suggests that it is worth seeking associations between CCL4 gene polymorphisms and lung cancer diagnosis. Our case-control study therefore sought to determine possible associations between three SNPs (rs1634507, rs1719153, and rs10491121) in the CCL4 gene and lung cancer.

2. Materials and methods

2.1. Patients and blood samples

We collected blood specimens from 538 patients (cases) diagnosed with lung cancer at Dongyang People’s Hospital between 2014 and 2018. All patients with lung cancer were diagnosed and were undergoing primary surgical treatment at Affiliated Dongyang Hospital of Wenzhou Medical University (Dongyang, Zhejiang, China). We included normal participants from physical examination center of Dongyang People’s Hospital. A total of 370 healthy participants without any history of cancer served as controls. All participants provided written informed consent, and this study has used consecutive or convenient enrollment of subjects. A total of 370 healthy participants without any history of cancer served as controls. All participants provided written informed consent. The study was approved by the Ethics Committee of Dongyang People’s Hospital Ethics Committee and Institutional Review Board (2016-YB003). Medical records were reviewed for clinico-pathological characteristics. Detailed clinical data on age, sex, smoking history, and alcohol consumption were obtained at the Revised International System for Staging Lung Cancer.

2.2. Selection of CCL4 polymorphisms

All three CCL4 SNPs (rs1634507, rs1719153, and rs10491121) selected for this study had minor allele frequencies of greater than 5%.

2.3. Genomic DNA extraction and genotyping by real-time PCR

Total genomic DNA was isolated from peripheral blood leukocytes using a QIAamp DNA blood mini kit (Qiagen, CA), according to the manufacturer’s instructions. DNA was dissolved in TE buffer (10 mM Tris, pH 7.8, 1 mM EDTA) and stored at −80°C for subsequent DNA extraction.

2.4. Statistical analysis

Differences between the two groups were considered to be statistically significant if P values were <.05. Hardy–Weinberg equilibrium was assessed using Chi-square goodness-of-fit tests for bi-allelic markers. As the data were independent and normally distributed, Fisher’s exact test was used to compare differences in demographic characteristics between healthy controls and patients with lung cancer. As the data were independent and normally distributed, Fisher’s exact test was used to compare differences in demographic characteristics between healthy controls and patients with lung cancer. Odds ratios (ORs) combined with 95% confidence intervals (CIs) calculated associations between genotype frequencies and the risk of lung cancer. To further exclude the impact of confounding variables, adjusted odds ratios (AORs) with 95% CIs were estimated by multiple logistic regression models that controlled for confounding covariates. A P value of <.05 was considered statistically significant. Data were analyzed using SAS statistical software (Version 9.1, 2005; SAS Institute Inc., Cary, NC).

3. Results

We enrolled 538 patients with lung cancer and 370 healthy, cancer-free subjects; all were of Han Chinese ethnicity. Between-group differences in general demographic characteristics are listed in Table 1. The mean age was 60.20 ± 10.91 years for the lung cancer cohort and 42.23 ± 19.11 years for the controls (P <.001). Significantly higher proportions of lung cancer patients compared with controls were either current or ex-tobacco smokers, or consumed alcohol (both P <.001). As according to the American Joint Committee on Cancer (AJCC) tumor/node/metastasis (TNM) classification and staging system,

### Table 1

| Variable                        | Controls (n = 370) | Patients (n = 538) | P value |
|---------------------------------|-------------------|-------------------|---------|
| Age, y                          | 42.23 ± 19.11     | 60.20 ± 10.91     | <.001  |
| Gender                          |                   |                   |         |
| Male                            | 161 (43.5%)       | 274 (50.9%)       | <.001  |
| Female                          | 209 (56.5%)       | 264 (49.1%)       |         |
| Alcohol consumption             |                   |                   |         |
| No                              | 326 (86.1%)       | 396 (73.6%)       | <.001  |
| Yes                             | 44 (11.9%)        | 142 (26.4%)       |         |
| Cigarette smoking               |                   |                   |         |
| No                              | 325 (87.8%)       | 335 (62.3%)       | <.001  |
| Yes                             | 45 (12.2%)        | 203 (37.7%)       |         |
| Tumor size (T)                  |                   |                   |         |
| ≤T2                             | 478 (88.8%)       | 437 (81.2%)       |         |
| >T2                             | 60 (11.2%)        | 101 (18.8%)       |         |
| Lymph node status (N)           |                   |                   |         |
| N0/N1                           | 437 (81.2%)       | 101 (18.8%)       |         |
| N2/N3                           | 101 (18.8%)       | 101 (18.8%)       |         |
| Distant metastasis (M)          |                   |                   |         |
| MO                              | 466 (86.6%)       | 72 (13.4%)        |         |
| MI                              | 72 (13.4%)        | 72 (13.4%)        |         |
| Clinical stage                  |                   |                   |         |
| I/II                            | 418 (77.7%)       | 418 (77.7%)       |         |
| III/IV                          | 120 (22.3%)       | 120 (22.3%)       |         |

All values are given as the mean±SD (standard deviation).

* P < .05 versus controls, by Mann–Whitney test.

* P < .05 versus controls, by Pearson’s Chi-squared test.
we identified 418 patients (77.7%) with clinical stage I/II and 120 patients (22.3%) with clinical stage III/IV disease (Table 1). To reduce the possible interference of confounding variables, AORs with 95% CIs were estimated by multiple logistic regression models after controlling for age, gender, alcohol consumption and cigarette smoking.

The results of genotyping for the three CCL4 SNPs (rs1634507, rs1719153, and rs10491121) in the cases and controls are shown in Table 2. In both study groups, the alleles with the highest odds ratios (ORs) and their associated 95% confidence intervals (CIs) were estimated by Pearson’s Chi-squared test. The adjusted odds ratios (AORs) with their 95% CIs were estimated by multiple logistic regression modeling that controlled for age, gender, alcohol consumption and cigarette smoking.

Table 2

| rs10491121 | Controls (n = 370) | Patients (n = 538) | OR (95% CI) | P value | AOR (95% CI) | P value |
|------------|-------------------|-------------------|-------------|---------|--------------|---------|
| rs1634507  |                   |                   |             |         |              |         |
| GG         | 94 (25.4%)        | 213 (39.6%)       | 1.00 (reference) |        | 0.608 (0.417–0.888) | <.01 |
| GT         | 175 (47.3%)       | 242 (45.0%)       | 0.610 (0.447–0.833) | <.002  | 0.395 (0.254–0.616) | <.01 |
| TT         | 101 (27.3%)       | 83 (15.4%)        | 0.363 (0.248–0.529) | <.001  | 0.537 (0.378–0.762) | <.01 |
| rs1719153  |                   |                   |             |         |              |         |
| AA         | 161 (43.5%)       | 228 (42.4%)       | 1.00 (reference) |        | 1.994 (1.383–2.867) | <.01 |
| AT         | 164 (44.3%)       | 240 (44.6%)       | 1.033 (0.779–1.372) | .220   | 1.032 (0.729–1.461) | .857 |
| TT         | 45 (12.2%)        | 70 (13.0%)        | 1.089 (0.718–1.691) | .665   | 1.080 (0.658–1.807) | .738 |
| rs10491121 |                   |                   |             |         |              |         |
| AA         | 160 (43.2%)       | 133 (24.7%)       | 1.00 (reference) |        | 3.348 (2.072–5.411) | <.01 |
| AG         | 165 (44.6%)       | 272 (50.0%)       | 1.983 (1.468–2.678) | <.001  | 1.994 (1.383–2.867) | <.01 |
| GG         | 45 (12.2%)        | 16 (3.0%)         | 3.556 (2.362–5.351) | <.001  | 3.348 (2.072–5.411) | <.01 |
| AG+GG      | 210 (56.8%)       | 405 (75.3%)       | 2.320 (1.748–3.082) | <.001  | 2.317 (1.640–3.275) | <.01 |

All values are given as the mean ± SD (standard deviation). The odds ratios (ORs) with their associated 95% confidence intervals (CIs) were estimated by Pearson’s Chi-squared test. The adjusted odds ratios (AORs) with their 95% CIs were estimated by multiple logistic regression modeling that controlled for age, gender, alcohol consumption and cigarette smoking.

Table 3

| rs10491121 | Patients (n = 538) | OR (95% CI) | P value | AOR (95% CI) | P value |
|------------|-------------------|-------------|---------|--------------|---------|
| rs10491121 |                   |             |         |              |         |
| AA         | 110 (20.4%)       | 23 (4.3%)   | 1.00 (reference) |        | 1.308 (0.751–2.277) | .444 |
| AG         | 205 (38.1%)       | 67 (12.5%)  | 1.563 (0.923–2.648) | .095   | 1.245 (0.647–2.437) | .512 |
| GG         | 103 (19.1%)       | 30 (5.6%)   | 1.393 (0.760–2.554) | .283   | 1.275 (0.759–2.168) | .369 |
| AG+GG      | 308 (57.2%)       | 97 (18.0%)  | 1.506 (0.910–2.493) | .110   | 1.375 (0.750–2.505) | .369 |
| GT         | 239 (44.4%)       | 111 (2.0%)  | 3.135 (2.072–5.013) | .241   | 1.195 (0.559–2.558) | .646 |
| GG         | 117 (21.7%)       | 42 (7.8%)   | 3.757 (2.362–5.351) | .109   | 1.297 (0.537–3.040) | .569 |
| AG+GG      | 290 (59.2%)       | 137 (25.1%) | 1.527 (0.769–3.035) | .224   | 1.224 (0.593–2.520) | .585 |
| AT         | 122 (22.7%)       | 11 (2.0%)   | 1.00 (reference) |        | 1.461 (1.031–2.083) | .224 |
| AG         | 394 (43.4%)       | 33 (6.1%)   | 1.541 (0.974–2.408) | .154   | 1.260 (0.706–2.251) | .434 |
| GG         | 109 (20.3%)       | 24 (4.5%)   | 4.244 (2.650–6.918) | .049   | 1.059 (0.528–2.124) | .872 |
| AG+GG      | 324 (60.2%)       | 81 (15.1%)  | 1.412 (0.826–2.410) | .204   | 1.192 (0.683–2.080) | .536 |

All values are given as the mean ± SD (standard deviation). The ORs with their associated 95% CIs were estimated by Pearson’s Chi-squared test. The adjusted odds ratios (AORs) with their 95% CIs were estimated by multiple logistic regression modeling that controlled for age, gender, alcohol consumption and cigarette smoking.
A reconstructed linkage disequilibrium plot of the genotyped polymorphisms in the study population is depicted in Figure 1. CCL4 rs10491121 and rs1719153 displayed 95% linkage disequilibrium, while rs1634507 and rs10491121 expressed 88% linkage disequilibrium. The most common haplotype, G/A/G in healthy controls, was used as the reference for analysis distribution frequencies of all 3 polymorphisms examined in this study (rs1634507, rs1719153, and rs10491121). The G/A/G CCL4 haplotype significantly reduced the risk of developing lung cancer by 0.440-fold (95% CI: 0.016–0.120), while the T/A/A CCL4 haplotype significantly enhanced the risk by 7.156-fold (95% CI: 4.526–11.315) (Table 4).

4. Discussion

It is recognized that several SNP genes affect an individual’s susceptibility to lung cancer.[7,28,29] Correlations between CCL4 gene polymorphisms and lung cancer have rarely been documented; most of the evidence is associated with breast cancer, oral cancer and hepatocellular carcinoma.[18–20] A comprehensive understanding about genetic polymorphisms is crucial for the successful identification of novel therapeutic strategies for lung cancer.[7,30] To the best of our knowledge, our study provides the first clinical evidence on associations between CCL4 polymorphisms (rs1634507, rs1719153, and rs10491121) and lung cancer susceptibility, as well as on interactions between these polymorphisms and clinical characteristics in a Han Chinese population. In this study, we increased our overall numbers of participants by incorporating the patients and controls included in our previous study.[8] Our results show that CCL4 gene polymorphisms only slightly affect the susceptibility for lung cancer. This might be because most of our study participants (cases and controls alike) were nonsmokers and because the cigarette smoker/nonsmoker ratios for both patients (37.7:62.3) and controls (12.2:87.1) were relatively normally distributed, as were the proportions of those who consumed alcohol. In this study, we included all the cases and controls without matching age and gender, so that we analyzed the multiple logistic regression modeling statistics by controlled for confound variables. The statistic results between OR and AOR which controls for age and gender were approaching though controls group is younger than cases group, suggesting that CCL4 SNPs were high risks in lung cancer patients. Although our results demonstrated that tobacco smoking and alcohol consumption were significant risk factors for lung cancer (Table 1; both P < .001), the significance of this association did not persist in CCL4 SNPs genotyping analyses that controlled for smoking consumption (Supplementary Table S1, http://links.lww.com/MD/D654). These results suggest that smoking and alcohol consumption would not be the primary factors for CCL4 SNPs risks in LC patients, and whether there are other factors, such as air pollution or circumstances, it requires to be further considerate in the future.
Accumulating evidence indicates that CCL4 plays a critical role in different tumor cell types. For example, CCL4 enhances prostate cancer progression via STAT3-dependent signaling. A previous report has suggested that the AT haplotype and T alleles of rs1719153 occurring in CCL4 SNPs are more frequently expressed in healthy controls than in patients with HIV-1 infection. To the best of our knowledge, this current study is the first to investigate the distribution of the CCL4 SNPs rs1634507, rs1719153 and rs10491121 and their possible association with susceptibility to lung cancer. In analyses adjusting for confounding factors, the rs1634507 GT + TT heterozygote polymorphism was associated with a significantly lower risk of developing lung cancer compared to the presence of the rs10491121 GG homozygous polymorphism. Moreover, individuals carrying the AG + GG heterozygote at rs10491121 had a higher risk of lung cancer compared with individuals carrying the wild-type AA polymorphic allele.

Linkage disequilibrium can be used for the genetic mapping of adjacent variants that participate in the detection and treatment of disease, while haplotype analyses clarify genetic contribution to disease susceptibility. Here, our results indicated that CCL4 rs10491121 and rs1719153 displayed 95% linkage disequilibrium, whereas rs1634507 and rs10491121 expressed 88% linkage disequilibrium. We also show that the G/A/G CCL4 haplotype significantly reduced the risk of lung cancer development, while the T/A/A CCL4 haplotype significantly enhanced the risk, suggesting that these CCL4 haplotypes play an important role in lung cancer progression.

In summary, our results demonstrate significant associations between CCL4 SNPs rs1634507 and rs10491121 and the risk of lung cancer in a Han Chinese population. This study is the first to report any such correlation between CCL4 polymorphisms and lung cancer. Our evidence indicates that CCL4 could be developed as a genetic prognostic marker for lung cancer prognosis.

Author contributions

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