Impact of Genetic Variation in TLR4 3′UTR on NSCLC Genetic Susceptibility

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Toll-like receptors (TLRs) are expressed not only in immune cells but also in a variety of tumor cells. Single-nucleotide polymorphisms (SNPs) located in the TLRs’ promoter or the 3′ untranslated region may affect gene expression by affecting the activity of the promoter or regulating the binding of mRNA to miRNA. This study aimed to investigate the association of the SNPs in TLR genes with the susceptibility to NSCLC. This case-control study involved 700 lung cancer patients and 700 healthy controls. All individuals were genotyped for all selected SNPs in TLR genes using polymerase chain reaction (PCR)-based restriction fragment length polymorphism (PCR-RFLP) and TaqMan SNP genotyping assay. The association of genetic variations in TLRs with the susceptibility to NSCLC was evaluated by unconditional logistic regression with OR (95% CI). After evaluating transcriptional factor or miRNA binding capability by bioinformatics methods, six TLRs were identified for further analysis. We did not find that TLR3 rs5743303, TLR4 rs1927914, TLR4 rs11536891, TLR5 rs1640816, and TLR7 rs3853839 were associated with NSCLC risk (P > 0.05). Our data showed that TLR4 rs7869402 C > T polymorphism reduced the risk of NSCLC with OR (95% CI) of 0.63 (0.45–0.89). When stratified by gender and age, the individuals carrying at least one rs7869402 T allele significantly decreased the NSCLC risk among males (OR = 0.58, 95% CI = 0.38–0.87) and among youngsters (OR = 0.43, 95% CI = 0.27–0.69). Smoking stratification analysis showed that the rs7869402T allele-containing genotype reduced the risk of NSCLC with OR (95% CI) of 0.50 (0.29–0.87) among smokers but not among nonsmokers (P > 0.05). When the individuals were classified by the pathological type, we found that the rs7869402T-containing genotype was associated with the risk of adenocarcinoma (OR = 0.62, 95% CI = 0.41–0.92) but not with that of squamous cell carcinoma (OR = 0.71, 95% CI = 0.44–1.13) and other types (OR = 0.23, 95% CI = 0.03–1.70). Compared with the TLR4 A/rs1927914^-G/rs7869402^-T/rs11536891 haplotype, the G/rs1927914^-T/rs7869402^-T/rs11536891 haplotype was associated with a decreased risk for developing NSCLC with OR (95% CI) of 0.57 (0.41–0.80). These results indicated that the TLR4 rs7869402 variation affects the genetic susceptibility to NSCLC.

1. Introduction

Cancers are major public health problem globally. Worldwide, there are 18.1 million new cancer cases and 9.6 million cancer deaths in 2018, of which lung cancer is the most common one [1, 2]. Non-small-cell lung cancer (NSCLC), as a major type of lung cancer, is the result of interaction of multiple genes and environmental factors, such as smoking, air pollution, and occupational exposure.
DAMPs, TLRs recruit and activate downstream molecules such as TRIF and MyD88 and then activate NF-κB to induce the production of type I interferons and inflammatory factors [3].

TLRs are expressed not only in immune cells but also in a variety of tumor cells. Several studies showed that TLR2, TLR4, and TLR9 are overexpressed in lung cancer tissue compared to normal lung tissue [4–6]. The silencing of TLR4 by siRNA can promote apoptosis and metastasize and inhibit lung cancer cell growth [7, 8]. The elevated TLR5 expression was prone to improved prognosis among NSCLC patients [9]. The expression of TLR7 is also associated with the poor prognosis and resistance to neoadjuvant chemotherapy [10].

Single-nucleotide polymorphism (SNP) is widely found in the genome and is the most common type of genetic variation. SNPs located in the promoter region may affect promoter activity by altering the binding capability of the transcription factor. The SNPs located in the 3′ untranslated region may regulate miRNA binding and further affect the efficiency of mRNA translation.

TLRs play an important role in the pathogenesis of various tumors. By bioinformatic analysis, we found six SNPs which may affect the function of TLR4. In this study, we explored whether these potential functional variants were associated with the risk of NSCLC.

2. Materials and Methods

2.1. Study Population. This group-designed case-control study includes 700 NSCLC patients and 700 healthy controls. The NSCLC cases were collected from 2012 to 2014 in Tangshan Gongren Hospital and Renmin Hospital of North China University of Science and Technology in China. All NSCLC cases were histopathologically confirmed. No radiotherapy or antitumor chemotherapy was performed before blood sampling. At the time of sample sampling, the gender, age, pathological type, and the stage of lung cancer patients were not limited. Patients with a previous history of tumor were excluded. Healthy controls were recruited from a physical examination population in Tangshan area during the same period when the cancer patients were involved. All health controls have no history of cancer and frequency match to cases by sex and age (5 years). All participants provided informed consent. The study was supported by the Institutional Review Board of North China University of Science and Technology.

2.2. SNP Selecting. Based on the data in the dbSNP database and Ensembl database, we screened the SNPs which were located in the promoter region and 3′ untranslated region (UTR) of TLRs (TLR3, TLR4, TLR5, and TLR7). The SNPs with the frequency of minor alleles greater than 0.05 were selected to predict the possible function. For the SNPs in the promoter region of TLRs, transcription factor binding capability was predicted by the online TRANSFAC program. For the SNPs in the 3′ UTR, microRNA binding ability was predicted using the mirSNP and SNPinfo program.

2.3. Genotyping of the TLR Variants. Polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) analysis and TaqMan SNP genotyping assay were applied for genotyping [11, 12]. Genotyping for TLR4 rs1927914 and rs7869402 variants was performed by PCR-RFLP. The primer pairs used to identify TLR4 rs1927914 or TLR4 rs7869402 polymorphisms were 5′-TACTCTGGACCTGAGCCAG-3′ and 5′-TATAACTGGGCTGCTGACTC-3′. PCR was performed in 6 μL PCR reaction mixture with 2 × Taq PCR StarMix, 0.1 μM each primer, and 20 ng genomic DNA. The thermal cycling conditions for TLR4 rs1927914 and rs7869402 variants were 5 min at 94°C followed by 30 cycles (30’s at 94°C, 30’s at 58°C, and 30’s at 72°C) and 5 min at 72°C. PCR amplification products for TLR4 rs1927914 A > G (524 bp) and TLR4 rs7869402 C > T (102 bp) were digested by Nci I (NEB, Ipswich, MA, USA) and Ali I (NEB, Ipswich, MA, USA). DNA sequencing was used to confirm the accuracy of PCR-RFLP results (Figures 1 and 2). A 10% sample was randomly selected and regenotyped, and all results were found to be 100% consistent. Genotyping for other genetic variants (TLR3 rs5743303, TLR5 rs1640816, and TLR7 rs3853839) was performed using TaqMan SNP genotyping assays (C_27310258, C_8812434, and C_2259573) (Thermo Fisher Scientific, Waltham, USA).

2.4. Statistical Analysis. All analyses were conducted with SPSS23.0 (SPSS Inc., Chicago, USA). The basic information (sex, age group, and smoking status) of the subjects was analyzed by χ2 test. Hardy–Weinberg equilibrium (HWE) of all tested SNPs among controls was estimated by χ2 test. After adjusting for possible confounding factors, the association of genetic variations in TLRs with the susceptibility to NSCLC was evaluated by unconditional logistic regression with OR (95% CI).

3. Results

3.1. Subject Characteristics. The basic information of 700 NSCLC patients and 700 health controls is summarized in Table 1. The distributions of gender and age in the case group are consistent with those in the group of controls (P > 0.05). The proportion of smokers in the case group was 44.4%, which was higher than that among health controls (28.1%) (P < 0.01). The distribution of cumulative smoking between the case and control groups has no significant difference (P = 0.773). Among all NSCLC cases, there are 402 patients with adenocarcinomas (57.4%), 279 patients with squamous cell carcinomas (38.6%), and 28 patients with other pathological types (15 adenosquamous carcinoma, 5 large-cell cancer, and 8 cases of bronchoalveolar carcinoma).

3.2. Association of TLR Variants with the Risk of NSCLC. The SNP information is shown in Table 2. The relationship between each genetic variant and the susceptibility to NSCLC is shown in Table 3. The frequency of TLR4 rs7869402 CC, CT, and TT genotypes in the case group and
controls was 90.7%, 9.0%, and 0.3% and 86.0%, 13.3%, and 0.7%. The results showed that individuals with at least one T allele had reduced the risk of NSCLC compared with CC genotype carriers (OR = 0.63, 95% CI = 0.45–0.89). We did not find any statistical difference in the distribution of other SNPs in TLR3, TRL4, and TLR7 between cases and controls.

Figure 1: Analysis of TLR4 rs1927914 polymorphism: (a) partial DNA sequence of PCR products with different TLR4 rs1927914 genotypes; (b) representative gel picture showing PCR-RFLP. M, DNA size markers; lanes 2, 4, and 6, AA genotype; lane 1, GA genotype; lanes 3 and 5, GG genotypes.

Figure 2: Analysis of TLR4 rs7869402 polymorphism: (a) partial DNA sequence of PCR products with different TLR4 rs7869402 genotypes; (b) representative gel picture showing PCR-RFLP. M, DNA size markers; lanes 1, 2, 3, and 5, CC genotype; lane 4, CT genotype; lanes 6 and 7, TT genotypes.
3.3. Stratification Analysis of the TLR4 Variants and NSCLC Risk. To further analyze the relationship between TLR4 rs7869402 genetic variation and NSCLC risk, we performed a stratified analysis by gender, age, smoking status, and pathological type (Table 4). When stratified by gender, males with at least one T allele had a lower risk of NSCLC than those with the CC genotype with OR (95% CI) of 0.58 (0.38–0.87). TLR4 rs7869402 was not associated with NSCLC risk among females with OR (95% CI) of 0.78 (0.43–1.41). In stratified analysis by age, the younger subjects (age ≤ 60) carrying at least one T allele had a decreased risk of NSCLC with OR (95% CI) of 0.43 (0.27–0.69), which was not found among old subjects. When stratified by smoking status, compared with CC genotype carriers, the T allele-containing genotype contributed to a reduced risk of NSCLC (OR = 0.50, 95% CI = 0.29–0.87) among smokers but among nonsmokers (OR = 0.74, 95% CI = 0.49–1.13). Further stratification analysis of smoking levels showed that the T allele-containing genotype contributed to decreased risk of NSCLC among light smokers (≤30 packs/year) with OR (95% CI) of 0.46 (0.23–0.92) but not among heavy smokers with OR (95% CI) of 0.44 (0.17–1.14). A stratified analysis of different pathological types revealed that individuals with at least one T allele were associated with the risk of adenocarcinoma with OR (95% CI) of 0.62 (0.41–0.92) but not with the risk of squamous cell carcinoma with OR (95% CI) of 0.71 (0.44–1.13) and other types of NSCLC with OR (95% CI) of 0.23 (0.03–1.70).

### Table 1: Distributions of selected characteristics of NSCLC cases and control subjects.

| Variables            | Cases (n = 700) | Controls (n = 700) | P valuea |
|----------------------|-----------------|-------------------|----------|
| Gender               |                 |                   |          |
| Male                 | 465 (66.4)      | 469 (67.0)        | 0.821    |
| Female               | 235 (33.6)      | 231 (33.0)        |          |
| Age                  |                 |                   |          |
| ≤50                  | 125 (17.9)      | 124 (17.7)        | 1.000    |
| 51–60                | 261 (37.3)      | 262 (37.4)        |          |
| 61–70                | 225 (32.1)      | 224 (32.0)        |          |
| >70                  | 89 (12.7)       | 90 (12.9)         |          |
| Smoking status       |                 |                   |          |
| Nonsmoker            | 388 (55.6)      | 503 (71.9)        |          |
| Smoker               | 312 (44.4)      | 197 (28.1)        |          |
| Pack year of smoking |                 |                   |          |
| ≤30                  | 184 (59.2)      | 114 (57.9)        | 0.773    |
| >30                  | 127 (40.8)      | 83 (42.1)         |          |
| Histological types   |                 |                   |          |
| Adenocarcinoma       | 402 (57.4)      |                   |          |
| Squamous cell        | 270 (38.6)      |                   |          |
| Other carcinomas     | 28 (4.0)        |                   |          |

aTwo-sided χ² test.

### Table 2: General information of SNPs and Hardy–Weinberg test.

| Gene     | Position | SNP    | Region | Allele gene | MAF   | P value |
|----------|----------|--------|--------|-------------|-------|---------|
| TLR3     | chr4:186067699 | rs5743303 | 5′UTR | A/T         | 0.171 | 0.928   |
| TLR4     | chr9:117702447 | rs1927914 | 5′UTR | G/A         | 0.490 | 0.522   |
| TLR4     | chr9:117715754 | rs7869402  | 3′UTR | C/T         | 0.109 | 0.502   |
| TLR4     | chr9:117717059 | rs11536891 | 3′UTR | T/C         | 0.143 | 0.391   |
| TLR5     | chr1:223145246 | rs1640816  | 5′UTR | G/A         | 0.087 | 0.444   |
| TLR7     | chrX:12889539 | rs3853839  | 3′UTR | C/G         | 0.402 | 0.790   |

### Table 3: Genotype frequencies of SNPs in TLR genes and their association with NSCLC.

| Genotypes | Cases (n = 700) | Controls (n = 700) | OR (95% CI)a | P value |
|-----------|----------------|-------------------|--------------|---------|
| TLR3      |                 |                   |              |         |
| AA        | 514 (73.4)      | 508 (72.6)        | 0.89 (0.69–1.15) | 0.366   |
| GT        | 163 (23.3)      | 177 (25.3)        | 1.46 (0.99–2.46) | 0.270   |
| TT        | 23 (3.3)        | 15 (2.1)          |              |         |
| TLR4      |                 |                   |              |         |
| AA        | 225 (32.1)      | 233 (33.3)        | 0.96 (0.75–1.22) | 0.708   |
| AG        | 351 (50.1)      | 346 (49.4)        |              |         |
| GG        | 124 (17.8)      | 121 (17.3)        |              | 0.841   |
| TLR4      | rs7869402       |                   |              |         |
| CC        | 635 (90.7)      | 602 (86.0)        | 0.65 (0.46–0.91) | 0.013   |
| CT        | 63 (9.0)        | 93 (13.3)         |              | 0.254   |
| TT        | 65 (9.3)        | 98 (14.0)         | 0.63 (0.45–0.89) | 0.008   |
| TLR5      | rs1640816       |                   |              |         |
| TT        | 586 (83.7)      | 605 (86.4)        | 1.22 (0.90–1.65) | 0.203   |
| CT        | 112 (16.0)      | 93 (13.3)         |              | 0.862   |
| CC        | 2 (0.3)         | 2 (0.3)           | 1.19 (0.90–1.65) | 0.200   |
| TLR7      | rs3853839       |                   |              |         |
| Male      | 357 (51.0)      | 352 (50.3)        | 0.85 (0.64–1.19) | 0.263   |
| Female    | 108 (15.4)      | 117 (18.3)        |              | 0.690   |

aAdjusted for age, gender, and smoking status.
3.4. Haplotype Analysis of TLR4 Variants. In order to evaluate the impact of the interaction of multiple SNPs on the risk of NSCLC, we tested the association of statistically inferred haplotypes with the risk of NSCLC using SHE-sis online program. Our results showed that the distribution of the TLR4 haplotype of Grs1927914-Trs7869402-Trs11536891 was statistically different between NSCLC patients and health controls (Table 5). Compared with the TLR4 Ars1927914-Crs7869402-Trs11536891 haplotype, the Grs1927914-Crs7869402-Trs11536891 haplotype was associated with a decreased risk for developing NSCLC with OR (95% CI) of 0.57 (0.41–0.80).

Table 5: Haplotype frequencies of TLR4 among cases and controls and their association with NSCLC.

| Haplotype | Cases (2n = 1400) 2n (%) | Controls (2n = 1400) 2n (%) | OR (95% CI) | P value |
|-----------|--------------------------|-----------------------------|-------------|---------|
| Ars1927914-Crs7869402-Trs11536891 | 788 (56.3) | 787 (56.2) | 1.00 | NC |
| Grs1927914-Crs7869402-Crs11536891 | 99 (7.1) | 86 (6.1) | 1.18 (0.87–1.59) | 0.283 |
| Grs1927914-Trs7869402-Trs11536891 | 430 (30.7) | 414 (29.6) | 1.07 (0.91–1.26) | 0.417 |
| Grs1927914-Trs7869402-Trs11536891 | 59 (4.2) | 100 (7.1) | 0.57 (0.41–0.80) | 0.001 |
| Ars1927914-Trs7869402-Crs11536891 | 16 (1.1) | 11 (0.8) | NC | NC |
| Ars1927914-Trs7869402-Trs11536891 | 7 (0.5) | 3 (0.2) | NC | NC |
| Grs1927914-Trs7869402-Crs11536891 | 0 (0.0) | 0 (0.0) | NC | NC |
| Grs1927914-Trs7869402-Trs11536891 | 1 (0.1) | 0 (0.0) | NC | NC |

Data were calculated by unconditional logistic regression and adjusted for age, gender, and smoking status, where they were appropriate.

4. Discussion

This study explored the relationship between genetic variation in TLR genes and the susceptibility to NSCLC. We screened 6 SNPs in the promoter and 3′UTR of TLRs that may affect the gene expression by bioinformatics prediction. Our finding showed that TLR4 rs7869402 C>T variation decreased the risk of NSCLC. However, TLR3 rs5743303, TLR4 rs1927914, TLR4 rs11536891, TLR5 rs1640816, and TLR7 rs3853839 genetic variants were not associated with NSCLC risk. These studies suggest that TLR4 rs7869402 C>T variation may be involved in the pathogenesis and progression of NSCLC.

Human TLR family included a total of 10 gene subtypes [13]. TLR4 is one of the earliest and most widely studied toll-like receptors. TLR4 is located on chromosome 9 and contains four exons. TLR4 involved in tumor occurrence and development by inducing M2 macrophage infiltration and angiogenesis in tumor microenvironment and participating in the process of apoptosis, MyD88-dependent and independent signal transduction, or other biological processes [14–16]. Studies have shown that TLR4 is overexpressed in lung cancer tissues, and the knockdown of TLR4 can promote apoptosis of A549 cells and inhibit the growth of tumor cells [17]. Therefore, TLR4 may become a susceptibility biomarker for early screening of lung cancer and improve the survival rate of lung cancer patients. In this study, we first demonstrated the relationship between the key variants in the regulation region of TLRs and the risk of NSCLC and found that the TLR4 rs7869402 C>T was associated with the risk of NSCLC. In an ovarian cancer study, researchers found that TLR4 rs7869402 variation reduced...
the overall survival of ovarian cancer [18]. In an oral squamous cell carcinoma research, TLR4 rs7869402 polymorphism had no association with cancer development and progression-free survival [19]. For other SNPs of TLR4, rs1927914 affects the risk of various tumors, including liver cancer [20], prostate cancer [21], gastric cancer [22], and malignant melanoma [23] but not the risk of hepatocellular carcinoma [24]. Shi et al. found no correlation between TLR4 rs11536891 mutation and prostate cancer risk or mortality, while Tsilidis KK et al. showed that TLR4 rs11536891 was associated with susceptibility to colorectal cancer [25].

The development of tumors is related to age and gender. In the gender and age stratification analysis, we found that at least one TLR4 rs7869402T allele in the male and low-age groups had a reduced risk of lung cancer. In addition, environmental factors such as smoking, air pollution, and occupational environment are considered to be risk factors for lung cancer [26]. TLR4 directly interacts with the environment, which may have a contributing effect on lung cancer. Smoking stratification analysis showed that TLR4 rs7869402T allele reduced the risk of NSCLC among smokers but not among nonsmokers. These results suggest that TLR4 rs7869402 genetic variation may affect the risk of lung cancer due to genetic and environmental interactions.

The key SNPs in the regulation region of TLRs may alter the function of genes, which in turn affects on the progress of lung cancer. In conclusion, our results provided new evidence that TLR4 contributed to the progress of lung cancer.

Data Availability

The data used to support the findings of this study are included within the article.

Ethical Approval

All the study procedures were approved by the Ethics Committee of North China University of Science and Technology (Tangshan, China).

Consent

Informed consent was obtained from all individual participants included in the study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors’ Contributions

Hongjiao Wu and Hui Gao contributed equally to this work. All authors agreed the final version of the manuscript for publication.

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