Association of polymorphisms in the genes of the urokinase plasminogen activation system with susceptibility to and severity of non-small cell lung cancer

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Background: Urokinase plasminogen activating (uPA) system is implicated in neoplastic progression. High tissue levels of uPA system components correlate with a poor prognosis in lung cancer. The present study examined the single nucleotide polymorphisms (SNPs) of uPA and the corresponding receptor, uPAR, for exploring their roles in non-small cell lung cancer (NSCLC).

Methods: The allele frequencies and genotype distributions of uPA rs4065 C/T and uPAR rs344781 (−516 T/C) among 375 NSCLC cases and 380 healthy controls were examined using polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) analysis. Putative association between the above SNPs and clinicopathological characteristics of NSCLC were also analyzed.

Results: The genotype frequencies of the variant homozygotes of uPA and uPAR were significantly different between NSCLC and control subjects. Significant association was also observed between the examined genotypes and disease stage of NSCLC. Logistic regression analysis revealed that individuals with uPA rs4065 TT genotype have higher odds ratios (ORs) for lung cancer. Whereas, subjects with uPAR-344781 CC genotype have lower ORs for lung cancer. The patients carrying a homozygous TT genotype at uPA rs4065, or at least a T allele at uPAR-344781 (−516), had a tendency to develop advanced disease.

Conclusions: Our results revealed that genetic polymorphisms of the uPA rs4065 C/T and uPAR rs344781 (−516 T/C) were associated with the susceptibility and severity of NSCLC.

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1. Introduction

Extracellular matrix (ECM) is a complex assembly of proteins and polysaccharides that are secreted, assembled and modeled by cells. The physiological function of ECM is to provide support and organization to tissues. However, alterations in the physical properties and composition of ECM proteinases are divided into three groups: metalloproteinases, cysteine proteinases and serine proteinases. Urokinase plasminogen activator (uPA) system is one of the serine proteinase systems involved in ECM degradation. Members of this system, including uPA and the corresponding receptor uPAR, are overexpressed in several malignant tumors [4]. Clinical studies have shown that overexpression of the uPA/uPAR components correlates with increased proliferation, migration, and invasion of many cancers, including lung cancer [5–7].

Lung cancer is the leading cause of cancer-related mortality around the world. Among various factors, genetic variation is proved to contribute to the development and progression of lung cancer [8–11]. The median levels of uPA and uPAR expression are higher in lung tumor tissues than the adjacent lung parenchyma. Clinically, the association between highly expressed levels of uPA proteins and shorter survival of cancer patients supports the hypothesis that the over-expressed uPA facilitates matrix degradation by promoting uPA activity in the microenvironment [12]. Particularly, uPAR is proved to be significantly correlated with the overall survival of patients with non-small cell lung cancer (NSCLC).

The uPA and uPAR gene is located at chromosome 10q24 and 19q13.2, respectively. Several single nucleotide polymorphisms (SNPs) located within the promoter or other regulatory regions of the genes in the uPA

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system may affect their expression and activity [13–17]. The functional impact of the SNPs of uPA rs4065 and uPAR rs344781 had been reported [18,19]. In addition, genotypes and frequencies of alleles in the uPA and uPAR genes are correlated with tumor progression [20–24], including NSCLC of Han Chinese in Taiwan [25–30]. Nevertheless, the effects of uPA/uPAR genetic polymorphisms on NSCLC have not been explored [31–33] although the genetic polymorphisms in the uPA activation system had been studied in breast cancer, oral cancer, ovarian cancer and colorectal cancer [23,24,34]. In this context, we hypothesized that investigation of the SNPs located in uPA/uPAR genes may be a simple and efficient method to predict the risk and prognosis of lung cancer. To explore the influence of genetic polymorphisms of uPA/uPAR on the susceptibility and clinicopathological development of NSCLC, the present study investigated the relationship between uPA/uPAR SNPs and the clinicopathological characteristics of NSCLC.

2. Materials and methods

2.1. Study population

A total of 375 Han Chinese NSCLC patients (256 male and 119 female; median age 64.3) admitted to China Medical University Hospital between January 2005 and April 2010 were recruited. Among the patients, 245 had adenocarcinomas (AD) and 130 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). In addition, 380 unrelated healthy control subjects (262 male and 118 female; median age 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 the control subjects had no medical illness and hereditary disorders without taking any medications during the study period.

A detailed questionnaire, including information regarding the average number of cigarettes smoked daily and the number of years the subjects had been smoking, was completed by each recruited subject under the instruction of 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. No significant differences in the demographic information between case and control participants were observed (Table 1).

2.2. Samples collection and genomic DNA extraction

Venous blood (5–10 mL) 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 manufacturer’s instructions. DNA was dissolved in TE buffer (10 mM Tris (pH 7.8), 1 mM EDTA) and quantitated by spectrophotometer. The extracted DNA were stored at −20 °C and used as templates for polymerase chain reaction (PCR).

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

Genotypes of the uPA (rs4065, C/T SNP) and uPAR (rs344781, T/C SNP) among the study subjects were determined by PCR-RFLP. The sequences of forward and reverse primers were as follows: 5′-AGTCACACCAAGGAAAAGCAA-3′ and 5′-AGACAAGTGGCTGTCAGTA-3′ for uPA (291 bps); 5′-AATTCGGCTCAGAATGCCTG-3′ and 5′-CAATGCCCTGAATATCGT-3′ for uPAR (308 bps). The PCR was performed in a 10 μL volume containing 100 ng DNA template, 1.0 μL of 10× PCR buffer (Invitrogen, Carlsbad, CA, USA), 0.25 μL of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 0.2 mM dNTPs (Promega, Madison, WI, USA), and 200 μM of each primer (MBio Inc, Taipei, Taiwan). The PCR cycling conditions were 5 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C, and 2 min at 72 °C, with a final step at 72 °C for 20 min to allow a complete extension of all PCR fragments. Then the genotypes of uPA and uPAR genes were determined by HphI and MspAI digestion, respectively. In brief, a 10 μL aliquot of PCR product was subjected to digestion at 37 °C for 4 h in a 15 μL reaction containing 5 U of restriction enzyme (New England Biolabs, Beverly, MA) and 1.5 μL 10× buffer (New England Biolabs). Digested products were separated on a 3% agarose gel and then stained with ethidium bromide. As a result, the PCR fragment containing T allele yielded 187- and 104-bp products for SNP of uPA, while the that containing C alleles yielded a 291-bp product; the PCR fragment containing C allele yielded 200- and 108-bp products for SNP of uPAR, while that containing T alleles yielded a 308-bp product. Representative results were 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 were expressed as mean ± standard deviation and compared using a two-tailed Student’s t-test. Categorical variables were reported as a percentage and compared using Chi-square (χ²) or Fisher’s exact test. Hardy–Weinberg equilibrium was assessed using a goodness-of-fit χ² test for biallelic markers. The genotype distribution of uPA and uPAR between healthy subjects and patients was examined by the χ² test. Significance was reached at p < 0.05.

Table 1

<table>
<thead>
<tr>
<th>Variables</th>
<th>NSCLC</th>
<th>Control</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects</td>
<td>375</td>
<td>380</td>
<td>0.88</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>256/119</td>
<td>262/118</td>
<td>0.46</td>
</tr>
<tr>
<td>Age, y</td>
<td>64.3 ± 8.6</td>
<td>64.2 ± 9.2</td>
<td>0.52</td>
</tr>
<tr>
<td>Brinkman index</td>
<td>510.4 ± 56.2</td>
<td>508.8 ± 54.8</td>
<td>0.52</td>
</tr>
<tr>
<td>Tumor type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinomas (AD)</td>
<td>245</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Squamous carcinomas (SQ)</td>
<td>130</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I + II</td>
<td>73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III + IV</td>
<td>302</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

Fig. 1. Polymerase chain reaction–restriction fragment length polymorphism of uPA and uPAR gene. PCR products of uPA and uPAR gene were subjected to enzymatic digestion by incubation with HphI and MspAI at 37 °C for 4 h and then submitted to electrophoresis in 3% agarose gels. For C/T SNP of uPA, T allele yielded 187- and 104-bp products, while C alleles yielded a 291-bp product; for T/C SNP of uPAR, C allele yielded 200- and 108-bp products, while T allele yielded a 308-bp product.
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.

3. Results

The association of the SNPs in uPA and uPAR genes with clinicopathological parameters of lung cancer patients was analyzed and shown in Tables 2 and 3. Overall, distributions of the uPA and uPAR genotypes were significantly different between non-cancer controls and lung cancer patients ($p < 0.0001$). The frequency of uPA variant polymorphic T/T homozygote was 26% and 5% in the case and control subgroup, respectively, while that of the wild-type allele was higher (58% for case and 74% for control). The frequency of uPAR variant polymorphic C/C homozygote was 15% and 25% in the case and control subgroup, respectively, while that of the wild-type allele was higher (60% for case and 52% for control). Results of the $X^2$ goodness-of-fit test showed that genotype frequencies of uPA and uPAR were consistent with Hardy-Weinberg equilibrium in the population.

Logistic regression analysis revealed that individuals carrying the homozygous uPA variant allele (T/T) had higher ORs for NSCLC (6.28, 95% CI 3.79–10.4, $p < 0.0001$), adenocarcinoma (7.20, 95% CI 4.24–12.2; $p < 0.0001$) and squamous cell carcinoma (4.72, 95% CI 2.54–8.76; $p < 0.0001$), compared with the subjects carrying wild-type allele (C/C or C/T genotype). On the contrary, individuals with homozygous uPAR variant allele (C/C) had lower ORs for NSCLC (0.51, 95% CI 0.36–0.74, $p < 0.0001$), adenocarcinoma (0.55, 95% CI 0.37–0.84; $p = 0.005$) and squamous cell carcinoma (0.44, 95% CI 0.25–0.77; $p = 0.003$), compared with those carrying wild-type allele (T/T or T/C genotype). In addition, subjects carrying homozygous TT genotype at uPA rs4065 ($p = 0.001$) or at least a T allele genotype at uPAR-344781–516 ($p = 0.001$) had a tendency to develop advanced disease.

4. Discussion

The uPA-mediated ECM degradation is an important mechanism in physiological and pathological tissue remodeling [35,36]. The plasminogen pathway plays an important role in the behavior of many tumors including lung cancer [20,24,33,34,37,38]. The roles of uPA expression in tumor occurrence, invasion and prognosis have been established. Hence genetic variants encoding (variants in the genes encoding uPA, uPAR and uPA inhibitor plasminogen activator inhibitor (PAI)) may contribute to cancer prognosis. Our previous study revealed that high plasma uPA levels might be involved in tumor cell invasion and play an important role in NSCLC metastasis [39]. In this context, we hypothesize that genotyping of the SNPs located within the promoter or regulatory regions of the genes in the uPA system may be a simple method to predict the risk and prognosis of cancer because the abovementioned SNPs may affect the expression of the gene or the activities of their corresponding proteins. The present study provides novel information regarding the effects of genetic polymorphisms of uPA and uPAR on the susceptibility and clinicopathological characteristics of NSCLC.

Przybylowska et al. reported the relation between protein levels and gene polymorphisms of uPA in colorectal cancer [40]. The uPAR in proved to play a central role in sustaining the malignant phenotype and promoting tumor metastasis [41]. The interaction between uPA and uPAR mediates various tumor cell activities, including tissue remodeling, chemotaxis, tumor invasion, dissemination, proliferation, and angiogenesis [42].

A significant difference in genotypic frequencies of uPA and uPAR genes between controls and NSCLC patients is demonstrated in this study. Individuals with uPA T/T homozygotes had a 6.28-fold higher risk of having NSCLC, compared with individuals carrying C/C homozygotes or C/T heterozygotes. Whereas individuals with uPAR C/C homozygotes had a 0.51-fold risk of developing NSCLC, compared with those carrying T/T homozygotes or T/C heterozygotes. To the best of our knowledge, the relationship between PAI gene polymorphisms and lung cancer had never been studied except for one report from Di Bernardo et al. [33]. In this study, we focus upon NSCLC patients. Our data revealed that genetic polymorphisms of the uPA and uPAR are associated with the susceptibility of NSCLC. Significant differences were found in the genotype distribution of uPA and uPAR between NSCLC patients and controls. Individuals carrying a homologous uPA rs4065 TT genotype had a higher risk, whereas that carrying an uPAR rs344781 CC genotype had a lower risk for developing NSCLC. In addition, the uPA and uPAR genotypes are associated with the clinicopathological status of NSCLC patients. Significant association between the genotypes of uPA and uPAR genes with advanced stages of

### Table 2

The association between the uPA polymorphism and the clinicopathologic parameters of the studied subjects.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Genotypes</th>
<th>Total</th>
<th>$p$ value</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC (%)</td>
<td>CT (%)</td>
<td>TT (%)</td>
<td></td>
</tr>
<tr>
<td>Non-cancer control</td>
<td>202 (53)</td>
<td>158 (42)</td>
<td>20 (5)</td>
<td>380</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>160 (43)</td>
<td>118 (31)</td>
<td>97 (26)</td>
<td>375</td>
</tr>
<tr>
<td>Tumor type AD</td>
<td>101 (41)</td>
<td>74 (30)</td>
<td>70 (29)</td>
<td>245</td>
</tr>
<tr>
<td>SO</td>
<td>59 (45)</td>
<td>44 (34)</td>
<td>27 (21)</td>
<td>130</td>
</tr>
<tr>
<td>Tumor stage I+II</td>
<td>44 (60)</td>
<td>21 (29)</td>
<td>8 (11)</td>
<td>73</td>
</tr>
<tr>
<td>III+IV</td>
<td>116 (38)</td>
<td>97 (32)</td>
<td>88 (30)</td>
<td>302</td>
</tr>
</tbody>
</table>

AD: adenocarcinoma, SQ: squamous cell carcinoma.

* Odds ratios and $p$ value were calculated using logistic regression to measure the association of the variant genotypes CC with lung cancer risk, with that of the TT/TC genotype being referred to as 1.

b The frequencies of the genotypes between the cancer and non-cancer control groups were compared with chi-square analysis.

c The frequencies of the genotypes between lung cancers with different tumor stages were compared with chi-square analysis.

### Table 3

The association between the uPAR polymorphism and the clinicopathologic parameters of the studied subjects.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Genotypes</th>
<th>Total</th>
<th>$p$ value</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT (%)</td>
<td>TC (%)</td>
<td>CC (%)</td>
<td></td>
</tr>
<tr>
<td>Non-cancer control</td>
<td>109 (20)</td>
<td>174 (46)</td>
<td>97 (25)</td>
<td>380</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>130 (35)</td>
<td>189 (50)</td>
<td>56 (15)</td>
<td>375</td>
</tr>
<tr>
<td>Tumor type AD</td>
<td>80 (33)</td>
<td>126 (51)</td>
<td>39 (16)</td>
<td>245</td>
</tr>
<tr>
<td>SO</td>
<td>50 (38)</td>
<td>63 (49)</td>
<td>17 (13)</td>
<td>130</td>
</tr>
<tr>
<td>Tumor stage I+II</td>
<td>16 (22)</td>
<td>36 (49)</td>
<td>21 (29)</td>
<td>73</td>
</tr>
<tr>
<td>III+IV</td>
<td>114 (38)</td>
<td>151 (51)</td>
<td>35 (11)</td>
<td>302</td>
</tr>
</tbody>
</table>

AD: adenocarcinoma, SQ: squamous cell carcinoma.

* Odds ratios and $p$ value were calculated using logistic regression to measure the association of the variant genotypes CC with lung cancer risk, with that of the TT/TC genotype being referred to as 1.

b The frequencies of the genotypes between the cancer and non-cancer control groups were compared with chi-square analysis.

c The frequencies of the genotypes between lung cancers with different tumor stages were compared with chi-square analysis.
NSCLC is also observed. Patients carrying a homozygous TT genotype at uPA rs4065 or at least 1 T allele at uPAR rs44781 had a tendency to develop advanced disease. Nevertheless, the possibility that the relationship between the uPA/uPAR genotypes with NSCLC susceptibility in the present study is an ethnic-dependent observation cannot be entirely excluded because multiple risk factors and etiology contribute to the pathophysiology of NSCLC development. Besides, uPA and uPAR may have differential expression, functions and regulatory mechanisms in various tissues and tumors [43–47]. Nevertheless, our data demonstrate that the polymorphisms of uPA and uPAR gene are significantly associated with the susceptibility and severity of NSCLC in Taiwanese population. The possible selection bias has been taken into consideration and reduced to as low as possible (lowest level). Given that all lung cancer patients are diagnosed and treated at our hospital, the demographics and clinical characteristics of the cancer patients included in the current study were compatible with those of lung cancer patients in Taiwan in general, and it is reasonable to assume that the case group is representative of the lung cancer patients in our community. In addition, all cases and controls were ethnically Han Chinese in Taiwan with a relatively homogenous genetic background [46]. Therefore, the potential confounding effect of population stratification for genotyping data should not be a major concern.

However, the small sample size may be a limitation of the present study. To enlarge the sample size and to analyze the relationship between NSCLC and uPA haplotypes as well as the relationship between NSCLC and other genotypes are required to further explore and understand the association of the genetic factors with lung cancer and the development of metastases.

In conclusion, our study demonstrates a significant association between uPA/uPAR genotypes and NSCLC. The results of this study uncover the significant relationship between genetic polymorphisms of uPA and uPAR with the susceptibility and severity of lung cancer.

Conflict of interest statement

All authors have no declared conflict of interest.

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