

RESEARCH ARTICLE

Single Nucleotide Polymorphisms in the u-PA Gene are Related to Susceptibility to Oral Tongue Squamous Cell Carcinoma in the Northern Chinese Han Population

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Abstract

Aim: The purpose of this study was to determine whether susceptibility to oral tongue squamous cell carcinoma (OSCC) is related to polymorphisms in the u-PA gene. **Methods:** We examined the rs2227564 C/T and rs2227562 G/A single nucleotide polymorphisms (SNPs) in 196 OSCC patients and 201 age- and gender-matched controls via direct sequencing and PCR-RFLP methods. **Results:** Significant differences were found in allelic and genotypic distributions of the rs2227564 and rs2227562 loci when comparing cases and controls. In addition, logistic analyses indicated that the rs2227564 C/T genotype was related to a 1.52-fold increased risk of developing OSCC (adjusted OR=1.521, 95% CI: 1.144~2.022, P=0.004). Linkage disequilibrium analysis was conducted and no association between the two loci was found (D' =0.031, r^2 =0.000). **Conclusions:** Our findings provide evidence that the rs2227564 C/T SNP in the u-PA gene is associated with the development of OSCC.

Keywords: Oral tongue squamous cell carcinoma - u-PA - polymorphism - linkage disequilibrium

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Introduction

Head and neck cancer is a disease arising in various organs, including oral cavity, tongue, pharynx, and larynx. Oral tongue squamous cell carcinoma (OSCC) is the most common form of head and neck cancer and is the major cause of death among all forms of head and neck cancer globally (Lippman et al., 2005; Parkin et al., 2005; Sudbo and Reith, 2005). In this study, we focused on OSCC, which is significantly more aggressive than other forms of head and neck cancer because of its rapid local invasion and spread (Franceschi et al., 1993).

Genetic factors, such as mutations in the metalloproteinase gene, u-PA gene, IL-8 gene, and SCEL gene, are thought to be associated with carcinoma of the tongue (Zhang et al., 2011). The altered genes might be involved in the changes in cell adhesion or motility in invasive cells (Zhang et al., 2011). The u-PA gene encodes urokinase-type plasminogen activator, which converts plasminogen to plasmin. This system plays an important role in the processes leading to cancer cell invasion and metastasis (Chapman, 1997).

The present investigation comprehensively explored the association between OSCC and single nucleotide polymorphisms (SNPs) of the u-PA gene in the Northern Chinese Han population.

Materials and Methods

Subjects

The study included Northern Han Chinese patients with OSCC living in Shandong Province (n = 196; mean age at onset, 52.22 ± 6.18 yr) and a control group (normal individuals) (n=201; mean age at onset, 52.38 ± 6.15 yr) from the same area. We divided the OSCC patients into early onset subgroup (n = 108; age at onset ≤ 65 yr) and late onset subgroup (n = 88; age at onset > 65 yr). All family members of patients who donated their blood gave informed consent.

The study protocol was approved by the institute's Ethics Committee. There were no significant gender differences between the two groups (patients and controls); the age differences between groups (patients and controls) were adjusted. All patients were diagnosed and met the criteria for OSCC according to the Chinese Stomatological Association. The tumor stages were determined according to the designated classification from The American Joint Committee on Cancer (AJCC). The whole blood specimens were placed in tubes containing EDTA, and then immediately centrifuged and stored at -80°C for further analyses.

Before the conduction of this study, informed written consent was obtained from each individual.

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Table 1. Distribution of rs2227564 Polymorphism in OSCC Patients and Controls

	Total	Genotype			P value	Allele		P value
		CC (%)	CT (%)	TT (%)		C (%)	T (%)	
rs2227564								
OSCC	196	84 (42.9)	90 (45.9)	22 (11.2)	0.015*	257 (65.6)	135 (34.4)	0.004*
Control	201	64 (31.8)	94 (46.8)	43 (21.4)		221 (55.0)	181 (45.0)	
male								
OSCC	103	47 (45.6)	44 (42.7)	12 (11.7)	0.102	139 (67.5)	67 (32.5)	0.040*
Control	108	34 (31.5)	56 (51.8)	18 (16.7)		124 (57.4)	92 (42.6)	
female								
OSCC	93	36 (38.7)	45 (48.4)	12 (12.9)	0.049*	118 (63.4)	68 (36.6)	0.040*
Control	93	30 (32.3)	38 (40.9)	25 (26.9)		98 (52.7)	88 (47.3)	
<=65								
OSCC	95	44 (46.3)	38 (40.0)	13 (13.7)	0.004*	127 (66.8)	63 (33.2)	0.002*
Control	91	21 (23.1)	50 (54.9)	20 (22.0)		92 (50.5)	90 (49.5)	
>65								
OSCC	101	39 (38.6)	51 (50.5)	11 (10.9)	0.091	130 (64.4)	72 (35.6)	0.290
Control	110	43 (39.1)	44 (40.0)	23 (20.9)		130 (59.1)	90 (40.9)	

* $P < 0.05$

Genomic DNA Extraction

Genomic DNA was extracted by QIAamp DNA blood mini kits (Qiagen, Valencia, CA, USA) according to the instructions of manufacturer. DNA was dissolved in TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA] and then quantified by a measurement of OD260. Final preparation was stored at -20 °C until used for genotyping.

Sequencing of the u-PA gene

Genotypic analyses of the u-PA gene were carried out by standard phenol/chloroform extraction methods, using primers (forward: 5'-GGGGGCAACAAGGACCAAA-3', reverse: 5'-CTTAAAGCGGGGCCTCAGA-3'). The PCR was performed in a 25 μ l volume containing 100 ng of DNA template, 2.5 μ l of 10 \times PCR buffer (Invitrogen, Carlsbad, CA), 0.65 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA), 0.75 mM of dNTPs (Promega, Madison, WI), and 600 nM of each primer (MDBio Inc., Taipei).

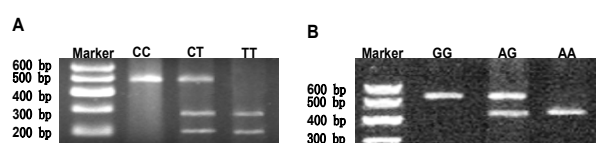
We sequenced PCR products from 10 randomly selected controls and 10 OSCC patients by direct sequencing. Primer syntheses and PCR product sequencing were done by Shanghai Sangon of China (information) and the Gene Center of North China (information).

Genotyping of polymorphisms

Polymorphisms were genotyped by restriction enzyme digestion of the PCR products amplified from genomic DNA. After performing standard PCR with 30 cycles, the PCR products were digested with restriction endonuclease AluI and AlwNI, respectively. Fragments were separated on a 2.5% agarose gel and visualized with an ultraviolet trans-illuminator after ethidium bromide staining.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) was tested using the HWE program as described by Terwilliger and Ott (Terwilliger and Ott, 1994). Allelic and genotypic distributions in patients and controls were compared using the chi-square test in SPSS 11.5. Linkage disequilibrium was checked using the EH program (Xie and Ott, 1993) and D' and r^2 were calculated via an online program

**Figure 1. Two Polymorphisms Found in the u-PA Gene.**

(A) C/T SNP of u-PA. The allele yielded 203 bp and 271 bp products, whereas C alleles yielded a 507 bp product. (B) For G/A SNP of u-PA, G allele yielded 408 bp and 520 bp products, whereas G alleles yielded a 520 bp product (Figure 1B)

(<http://202.120.7.14/analysis/myAnalysis.php>). A haplotype-based case-control study was conducted using the SHEsis software platform (Shi and He, 2005). The strength of association between alleles or genotypes and OSCC was evaluated by odds ratio (OR) presented with 95% confidence intervals (95%CI). Logistic regression analysis was used to stratify gender and age. A p value less than 0.05 was considered significant among all tests.

Results

Sequencing of the u-PA gene

We sequenced the amplified fragment of the u-PA gene and found two polymorphisms: rs2227564 (Figure 1A) and rs2227562 (Figure 1B). As a result, for C/T SNP of u-PA, the allele yielded 203 bp and 271 bp products, whereas C alleles yielded a 507 bp product (Figure 1A). For G/A SNP of u-PA, G allele yielded 408 bp and 520 bp products, whereas G alleles yielded a 520 bp product (Figure 1B).

Genotyping of the rs2227564 and rs2227562 SNPs

Definitive genotyping of the two loci were performed using PCR-RFLP analysis in all OSCC patients and controls (Figure 1). Genotypic and allelic distributions of the two polymorphisms were presented in Tables 1 and Table 2. No deviation from the Hardy-Weinberg equilibrium was observed for any of the polymorphisms in the controls or OSCC patients.

As shown in Table 1, there were statistically significant differences in the allelic and genotypic distributions of the rs2227564 SNP between OSCC patients and controls ($P = 0.004$ and 0.015 , respectively). After stratification by age, only the early onset subgroup (≤ 65 yr) showed

Table 2. Distribution of rs2227562 Polymorphism in OSCC Patients and Controls

rs2227564	Total	Genotype			P value	Allele		
		CC (%)	CT (%)	TT (%)		C (%)	T (%)	P value
rs2227564								
OSCC	196	85 (43.3)	88 (44.9)	23 (11.8)	0.021*	259 (66.1)	133 (33.9)	0.006*
Control	201	62 (30.8)	101 (50.3)	38 (18.9)		220 (54.7)	182 (45.3)	
male								
OSCC	103	45 (43.7)	43 (41.7)	15 (14.6)	0.301	130 (63.1)	74 (35.9)	0.182
Control	108	34 (31.5)	56 (51.8)	18 (16.7)		125 (57.8)	91 (42.2)	
female								
OSCC	93	42 (45.1)	41 (44.1)	10 (10.8)	0.031*	123 (66.1)	63 (33.9)	0.010*
Control	93	28 (30.1)	45 (48.3)	20 (21.6)		98 (52.7)	88 (47.3)	
<= 65								
OSCC	95	36 (37.9)	51 (53.7)	8 (8.4)	0.068	126 (66.3)	64 (33.7)	0.050
Control	91	26 (28.6)	48 (52.7)	17 (18.7)		102 (56.0)	80 (44.2)	
> 65								
OSCC	101	48 (47.5)	36 (35.6)	17 (16.9)	0.056	129 (64.2)	73 (35.8)	0.057
Control	110	35 (31.8)	54 (49.1)	21 (19.1)		120 (54.5)	100 (45.5)	

* $P < 0.05$

significant differences in genotypic and allelic distributions between the OSCC patients and controls ($P = 0.004$ and 0.002 , respectively). However, in the late onset subgroup (> 65 yr), no significant difference was found ($P = 0.091$ and 0.290 , respectively). When stratified by gender, the female subgroup demonstrated significant differences in genotypic and allelic distributions between the OSCC patients and controls ($P=0.049$ and 0.040 , respectively), whereas the male subgroup showed significant difference only in allelic distribution ($P = 0.040$). Logistic analyses indicated that the rs2227564 C/T genotype is related to a 1.52-fold increased risk of developing OSCC (adjusted OR = 1.521, 95%CI: 1.144 ~ 2.022, $P = 0.004$).

As shown in Table 2, there were significant differences in the allelic and genotypic distributions of the rs2227562 SNP between OSCC patients and controls (0.006 and 0.021 , respectively). When stratified by gender, the female subgroup revealed significant differences in genotypic and allelic distributions between the OSCC patients and controls ($P=0.031$ and 0.010 , respectively), whereas the male subgroup showed no significant difference. Analyses of both early and late onset subgroups showed no significant difference of the genotypic and allelic distributions between the OSCC patients and controls. In addition, logistic analyses indicated that the rs2227562 G/A genotype was not related to increased risk of developing OSCC ($P = 0.173$).

Linkage disequilibrium between alleles at both loci were studied using the EH program (Xie and Ott, 1993), and we found no linkage disequilibrium between rs2227564 and rs2227562 ($D' = 0.031$, $r^2 = 0.000$).

Discussion

In recent years, substantial evidence has suggested that variants in certain genes may be relevant to the pathogenic mechanism of particular diseases by altering their own or other genes' transcriptional activity, which may also apply to the genetic susceptibility to OSCC. OSCC is one of the most common forms of head and neck cancer, with a generally poor prognosis due to its tendency toward local invasion and subsequent metastasis (Myoung et al.,

2003). Invasive and metastatic OSCC is also characterized by enhanced expression of u-PA and its receptor u-PAR relative to the normal oral mucosa (Chapman, 1997). To determine the role of the u-PA gene in the pathogenesis of OSCC, we investigated the sequence of the gene and have detected two polymorphisms, rs2227564 C/T SNP and rs2227562 G/A SNP in the Northern Chinese Han population. The current study represents an initial attempt to explore variants in the u-PA gene in this population and, to the best of our knowledge, is the first to describe the genetic characters of rs2227564 and rs2227562.

In this study, we found an association between the two adjacent polymorphisms, rs2227564 and rs2227562, in the u-PA and OSCC in the Northern Chinese Han population. We found that the C allele of the rs2227564 SNP was significantly more common in OSCC patients than in controls, especially in the early onset subgroup and the female subgroup. We also found that the G allele of the rs2227562 SNP was significantly more common in OSCC patients than in controls, especially in the female subgroup. In this respect, the rs2227564 and rs2227562 SNPs were significantly associated with OSCC susceptibility before and after we stratified for gender and age via logistic regression. Moreover, the rs2227564 C/T genotype was found to be related to a 1.52-fold increased risk of developing OSCC.

Cancer invasion and metastasis is a multifactorial process and requires the coordinated action of cell-secreted proteolytic enzymes and their inhibitors. Up-regulation of u-PA expression has been correlated with malignant progression of a wide variety of neoplasms (Conese and Blasi, 1995; Andreassen et al., 1997; Chapman, 1997; Schmitt et al., 1997). The serine protease u-PA, which binds to a specific cell surface receptor uPAR (Stoppelli et al., 1985; Vassalli et al., 1985), facilitates the conversion of plasminogen into the serine protease plasmin. This wide-spectrum protease is able to degrade most components of the extracellular matrix directly or indirectly through activation of metalloproteinases, which subsequently degrade collagens and other matrix proteins (Mignatti et al., 1993). The activity of u-PA can be inhibited by the serpin inhibitors PAI-1 and PAI-2

(Andreasen et al., 1990). In addition, most components of the u-PA system of plasminogen activation have been linked to cell adhesion and migration through both proteolytic and nonproteolytic mechanisms (Conese and Blasi, 1995; Andreasen et al., 1997; Chapman, 1997). Cell migration requires the interaction of cell bound adhesion receptors, such as integrins and u-PAR, with their extracellular matrix associated ligands such as vitronectin (Wei et al., 1994; Deng et al., 1996; Kanse et al., 1996; Wei et al., 1996). Binding of u-PA, or fragments of u-PA containing only the receptor binding domain, enhances binding of uPAR to vitronectin. PAI-1 can inhibit integrin and u-PAR binding to vitronectin, thus directing a stepwise cell migration by allowing tumor cells to be attached or alternatively detached from the extracellular matrix (Deng et al., 1996; Kanse et al., 1996; Stefansson and Lawrence, 1996). We concluded, based on our data, that different genotypes of the u-PA gene may have different influences on the risk of developing OSCC. We presumed that inheritance of the C allele of the rs2227564 SNP and G allele of the rs2227562 SNP might alter u-PA protein levels or even its functions, which might finally affect cell migration and therefore increase the risk of developing OSCC. However, the complex mechanisms relevant to this process await further exploration. In summary, our results indicate that genetic variants in the u-PA gene may have a major influence on OSCC susceptibility in the Northern Chinese Han population.

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