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Polymorphism of angiotensin I-converting enzyme gene is related to oral cancer and lymph node metastasis in male betel quid chewers

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SUMMARY

Objectives: Angiotensin I-converting enzyme (ACE), a type I cell surface zinc metallopeptidase, is differentially expressed in several malignancies and plays a role in tumor cell proliferation, tumor cell migration, angiogenesis, and metastatic behavior. We aimed to investigate the effects of *ACE* gene (rs1799752) variants on oral cancer risk.

Materials and Methods: Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) 32 was used to measure ACE gene polymorphisms in 88 patients with oral precancerous lesion (OPL), 186 33 patients with oral cancer, and 120 control subjects without any oral lesions. All study subjects were male 34 betel quid chewers.

Results: Patients with oral cancer or OPL had a higher frequency of the DD genotype than the control patients did. Oral cancer patients with the DD genotype had a significantly higher prevalence of lymph node metastases than patients with the II/ID genotype did. After adjusting for age, smoking, drinking, and betel quid chewing status, we found that individuals with the DD genotype of the *ACE* gene had a 5.46-fold and 3.13-fold higher risk of developing oral cancer or OPL, respectively, than those with the II genotype did. Furthermore, oral cancer patients with the DD genotype of the *ACE* gene had a 2.16-fold higher likelihood of lymph node metastasis.

Conclusion: Our data suggest that the *ACE* gene polymorphisms may be associated with increased susceptibility to OPL and oral cancer and lymph node metastasis from oral cancer.

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Introduction

Oral cancer is the most common malignancy observed in the head and neck area. In more than 90% of cases, oral cancer is characterized by oral squamous cell carcinoma (OSCC),[1] and more than 90% of OSCCs develop from oral precancerous lesions (OPL) and oral precancerous conditions.[2,3] Areca nut/betel quid chewing as well as occupational and environmental exposures are the primary risk factors in oral cancer development.[4,5] However, many studies have shown that multiple genetic polymorphisms play roles in the development of OPL and oral cancer, implying that genetic factors, in combination with specific environmental factors, may predispose individuals to OPL and oral cancer.[6–8]

Angiotensin-converting enzyme (ACE) is expressed in a wide range of tissues,[9–12] and it converts angiotensin I to the potent vasoconstrictor, angiotensin II. Angiotensin II, the main peptide of the renin–angiotensin system, is strongly implicated in chronic inflammatory diseases such as hypertension, cardiovascular disease, and diabetes mellitus.[13–16] There is mounting evidence that ACE also participates locally in the pathology of carcinomas.[17,18] ACE is differentially expressed in several malignancies[17] and influences tumor cell proliferation, migration, angiogenesis, and metastatic behavior.[18–20] In humans, a polymorphism in the *ACE* gene—consisting of insertion (I) or deletion (D) of a 287-bp DNA fragment in intron 16—accounts for 20–50% of the variance in the expression of the ACE protein and its activity[21–24]; the highest ACE levels were found in DD homozygotes, the lowest in II

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homozygotes, and intermediate values in heterozygotes.[21] Furthermore, previous studies have shown that ACE is expressed locally in gastric cancer[25,26] and that I/D gene polymorphism influences metastatic behavior.[27] Patients with the DD genotype had greater lymph node metastases and an advanced Union International Contre le Cancer (UICC) tumor stage than carriers of the ID or II genotype did.[27] Another study found that ACE is differentially expressed in colorectal cancers and that gene polymorphism is associated with gender-specific differences in primary tumor size and patient survival.[28] Our previous study also found that the *ACE* polymorphism increases the risk of OPL in a Taiwanese aboriginal population with a high prevalence of the betel quid chewing.[29] On the basis of these observations, we aimed to further substantiate the influence of genetic polymorphisms of *ACE* on the susceptibility and clinicopathological development of oral cancer and the relationship between SNPs of the *ACE* gene with oral cancer risk and the clinicopathological characteristics.

Materials and methods

Patient populations and samples

Male patients with oral cancer (205) and 88 male patients with OPL were recruited from the Dental and Plastic Department of Kaohsiung Medical University and E-Da Hospital, I-Shou University, Taiwan between January 2003 and December 2010. Among patients with oral cancers, 15 showed veruccous carcinoma, four showed carcinoma *in situ*, and 186 showed OSCC. All patients were betel quid chewers. Oral cancer and OPL (including 19 persons with oral submucous fibrosis [OSF], 62 persons with oral leukoplakia lesions, and seven persons with OSF accompanied by oral leukoplakia lesions) were diagnosed on the basis of the patient's medical history, oral examination, and the WHO criteria for pathologic confirmation of lesions.[30] Clinicopathologic stags of the oral cancer patients were determined by the TNM classification criteria defined by the American Joint Committee on Cancer (AJCC). In addition, male betel quid chewers (120) without any oral lesions were recruited from a local prison to serve as controls. All study subjects lived in the same region at the time of the study. This study was approved by the Human Research Ethics Committee of our hospital, and written informed consent was obtained from each participant before enrollment.

Each subject was interviewed in person to obtain demographic information, and information on occupation, betel quid chewing, smoking history, alcohol drinking habit, and personal and family history of various cancers. Detailed information was obtained regarding each subject's betel quid chewing, cigarette smoking, and alcohol drinking habits; we specifically queried at what age the habit began, what the average daily consumption quantity was, and at what age the habit stopped. Cumulative exposure to betel quid chewing was derived by multiplying the daily consumption of betel quid chewing (in quid/day) by the duration of betel quid chewing (in years). Valid questionnaires were examined further by a structured questionnaire. Blood was also collected; serum and white blood cells (WBCs) were separated on the day of blood collection and frozen at −80 °C until further analysis.

Determination of ACE genotype (rs1799752)

Genomic DNA was extracted from whole blood samples collected from study subjects by using QIAamp DNA Blood Mini kits (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. DNA was resuspended in TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA] and quantitated on the basis of OD_{260}. Prepared DNA was stored at −20 °C until used as a template in polymerase chain reaction (PCR). The *ACE* I/D polymorphisms were determined using PCR-restriction fragment length polymorphism (PCR-RFLP) assays. The sequences of the primer pairs, which amplify the intron 16 region where the I/D fragment is located as follows: forward, 5′-CTGGAGACCACTCCCATCCTTTCT-3′ and reverse, 5′-GATGTGGCC ATCA CATTCGTCAGAT-3′. PCR amplification was performed in 25-μL reactions (0.5 pg genomic DNA; 500 pmol each primer; 0.5 mmol/L each of dATP, dGTP, dCTP, dTTP; 1.5 mmol/L $MgCl_2$; 0.5 units Taq DNA polymerase (Takara Tag™; Takara Shuzo Co., Ltd, Otsu Shiga, Japan); 50 mmol/L KCl; 0.001% gelatin; 10 mmol/L Tris–HCl, pH 8.3) with 4 min of denaturation at 94 °C, followed by 35 cycles of 15 s at 94 °C, 5 s at 67 °C, and 30 s at 74 °C in a thermal cycler (Gene Amp PCR System 9700; Perkin-Elmer, Foster City, CA, USA). Reaction was terminated at 72 °C for 2 min. To avoid ID/DD mistyping of a heterozygote as a DD-homozygote,[31] all DD genotype samples were confirmed with the following primer pair that produced an amplification product only if the insertion was present: forward, 5′-TGGGACCACAGCGCCCGCCACTAC-3′ and reverse, 5′-TCGCCAG CCCTCCCATGCCCATAA-3′.[32] PCR reaction conditions were the same as for I/D detection, except that the annealing temperature was 62 °C. All PCR products were visualized by electrophoresis on a 2% agarose gel followed by ethidium bromide staining (Fig. 1). All genotyping was performed blindly. In addition, we also randomly selected 23 PCR products for direct sequencing to confirm the correctness of the PCR procedure. Briefly, the method used for sequencing is as follows: PCR products of *ACE* I or D allele were collected and purified from agarose gel by using the commercial available FavorPrep™ Gel/PCR Purification Kit (Favorgen Biotech Corp. Ping-Tung, Taiwan) and sequenced in an automated sequencer (ABI 3730 XL DNA Analyzer System; Applied Biosystems). The sequences were analyzed using LALIGN (http://www.ch.embnet.org/software/LALIGN_form.html) and Chromas Version 2.33 software. The nucleotide sequences of the PCR I products ($n = 11$) matched those of GenBank rs1799752. In addition, the nucleotide sequences of PCR D products ($n = 12$) completely matched GenBank sequences that lack the Alu repetitive element in the intron of the *ACE* gene.

Statistical analyses

Data are shown as the mean ± SD or as percent values. All statistical analyses were performed using SAS statistical software (Version 8.2, SAS Institute Inc., Cary, NC, USA). The two-sample *t* tests, *chi*-square, and Fisher's exact tests were used to compare means and proportions between the control, oral cancer, and OPL

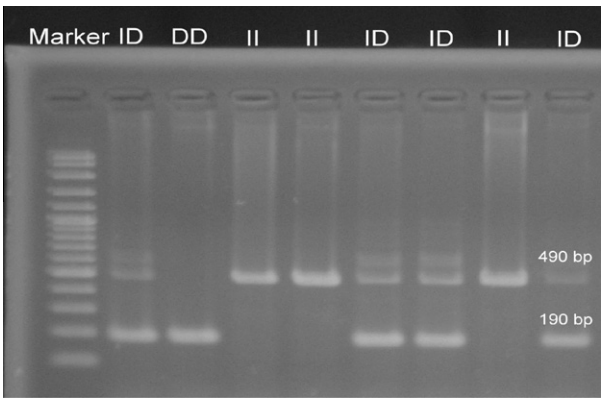

Figure 1 Direct visualization of *ACE* I/D PCR products by electrophoresis on a 2% agarose gel, followed by ethidium bromide staining. A 490-base pair *ACE* I allele and a 190-base pair *ACE* D allele are visible. Results from 8 patients are shown. Lane 1 is the marker, lanes 2, 6, 7, and 9 are ID heterozygotes, lane 3 is DD homozygote, and lanes 4, 5, and 8 are II homozygote.

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