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Associations of genetic variations of the endothelial nitric oxide synthase gene and environmental carcinogens with oral cancer susceptibility and development

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ABSTRACT

Oral cancer is a major head and neck cancer that is reported to be causally associated with genetic factors and environmental carcinogens. Endothelial nitric oxide synthase (eNOS) was reported to modulate carcinogenesis and progression through nitric oxide (NO) production. Genetic polymorphisms in the *eNOS* gene can regulate its transcription and further mediate NO production. The purpose of this study was to explore the influences of *eNOS* gene polymorphisms combined with environmental carcinogens on the predisposition for oral cancer. Two single-nucleotide polymorphisms (SNPs) of the *eNOS* gene, −786 T > C (rs2070744) and 894G > T (rs1799983), were genotyped in 1200 controls and 1044 patients with oral cancer using a TaqMan-based real-time polymerase chain reaction (PCR). We found that patients who carried the −786 T > C TC genotype were at higher risk for developing an advanced clinical stage (stage III/IV) compared to those with the −786 T > C TT genotype; however, there was no significant association of the two individual SNPs with oral cancer between patients and the control group. According to behavioral exposure to environmental carcinogens, the presence of these two eNOS SNPs combined with tobacco use and/or betel quid chewing profoundly enhanced the risk of oral cancer. Moreover, carriers with the betel quid-chewing habit who had haplotypes of the two *eNOS* SNPs more easily developed oral cancer. These results indicated an involvement of −786 T > C polymorphisms in the progression of oral cancer and support the interaction between eNOS gene polymorphisms and environmental carcinogens as a predisposing factor of oral carcinogenesis.

1. Introduction

Oral squamous cell carcinoma (OSCC) is one kind of malignant lesion that develops in the oral cavity and accounts for almost 90% of oral cancers [1]. Taiwan has the most rapidly increasing incidence of OSCC, and this cancer is currently the fourth leading cause of cancer-related deaths in males in Taiwan [2,3]. It was reported that OSCC development includes a multistep process of accumulation of multiple genetic alterations, which is affected by an individual's genetic predisposition and by environmental carcinogen exposure, e.g., alcohol and tobacco consumption and betel nut chewing [4,5].

Single nucleotide polymorphisms (SNPs), the most common type of DNA sequence variation in the genome, have become popular markers for association studies related to personalized medicine involving many diseases including cancers [6,7]. SNPs were reported to be located in different regions of genes including promoters, exons, introns, and 3′- and 5′ untranslated regions. Hence, alterations in gene expressions and their effects on cancer susceptibility and progression may rely on the

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location of the SNPs [7]. According to previous studies, it seems likely that genetic polymorphisms alone are unable to elicit clinical manifestations of OSCC, but together with environmental factors, they might increase a person's susceptibility to oral cancer [8,9]. Therefore, SNPs in certain genes might affect an individual's response to stimulation toward tumorigenesis promoted by environmental factors.

There is growing evidence indicating that nitric oxide (NO) plays an important role in modulating cancer formation and progression [10]. Nitric oxide synthase (NOS) is the main source of NO production, which produces NO when converting L-arginine to L-citrulline. Endothelial (e) NOS (or NOS3) is one of three functional classes of NOS which is constitutively expressed by endothelial cells and certain cancer cells, such as head and neck and gastric cancers [11–13]. NO synthesis by endothelial cells was recently reported to show both cancer-promoting and -fighting effects. For example, NO was shown to increase blood flow, induce angiogenesis and metastasis, and impair the tumor-suppressive function of p53 to further promote tumor progression [10,14]. In contrast to the tumor-promoting effects of NO, it was also reported to show antitumor effects by killing tumor cells and reducing tumor cell adhesion to the endothelium [15]. According to these conflicting results, the role of NO in tumor biology remains to be further investigated.

The eNOS gene was mapped onto human chromosome 7q36. It consists of 27 exons, and encodes a protein of 1203 amino acids. More than 168 polymorphisms have been identified in the *eNOS* gene [16]. Among these polymorphisms, *eNOS* −786 T > C (rs2070744) in the promoter region and *eNOS* 894 G > T (rs1799983) in exon 7 have received the greatest attention and have been widely investigated for their associations with risks of different cancer types in different ethnic groups [16–18]. However, results were inconsistent. Previous reports indicated that the *eNOS* 894 G > T polymorphism leads to an amino acid substitution from glutamic acid at codon 298 to aspartic acid (Glu298Asp) that causes reduced eNOS activity and basal NO production in *eNOS* 894 T (298Asp) allele carriers compared to those with the GG homozygote [19]. Actually, a preliminary assessment of the putative functional relevance of *eNOS* 894 G > T in esophageal mucosal tissues from the Genotype-Tissue Expression (GTEx) database showed similar alterations in *eNOS* expression in individuals who carry the polymorphic allele of eNOS 894 G > T (Fig. 1). Moreover, the *eNOS* −786 T > C polymorphism was reported to result in significantly reduced *eNOS* promoter activity and reduced endothelial NO production [20].

Until now, no studies addressing the relationship of these two functional *eNOS* gene polymorphisms with OSCC and their clinical features have been conducted. We therefore examined the genotypic frequencies of the *eNOS* 894 G > T and −786 T > C polymorphisms and their association with OSCC susceptibility and clinicopathological characteristics. The influences of these SNPs combined with betel nut and tobacco consumption, leading to susceptibility of oral cancer, were also evaluated.

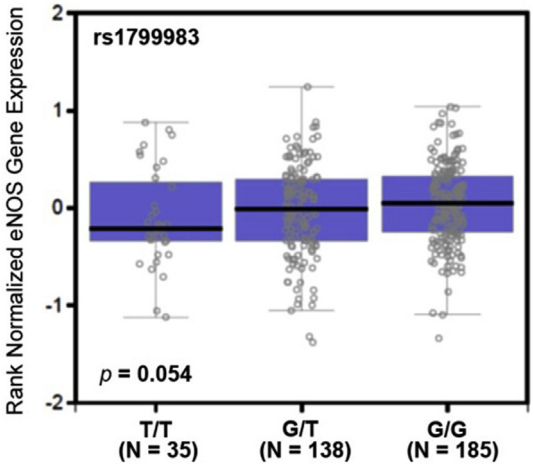

Fig. 1. Expression quantitative trait locus (eQTL) association between rs1799983 and endothelial nitric oxide synthase (eNOS) expression in esophageal mucosal tissues (GTEx dataset). Numbers in parentheses indicate the number of cases.

2. Materials and methods

2.1. Study subjects, ethics, and consent

In the current study, 1044 male patients with OSCC were enrolled from 2008 to 2017, with approval (CSMUH No: CS13214-1) of the institutional review board of Chung Shan Medical University Hospital (Taichung, Taiwan). Informed written consent was obtained from each individual before initiation of the study. We selected 1200 cancer-free male controls without a history of cancer from the Taiwan Biobank. Patients were clinically staged at the time of diagnosis according to the TNM staging system of the *American Joint Committee on Cancer* (AJCC) *Staging Manual* (7th ed.) [21]. Among these tumors, 510 cases were defined as stage I (T1N0M0) + stage II (T2N0M0), and 534 cases were defined as stage III (T3N0M0-T1, -T2, or T3N1M0) + stage IV (any T4 lesion, any N2 or N3 lesion, or any M1 lesion). Cancer-free subjects who had no self-reported history of cancer at any sites were included. Moreover, subjects with oral precancerous disease, such as oral submucosal fibrosis, leukoplakia, erythroplakia, verrucous hyperplasia, etc., were excluded from the control group. For all participants, we used a questionnaire to obtain information on patient exposure to betel quid chewing, tobacco use, and alcohol consumption. Betel quid chewing, tobacco use and alcohol drinking are defined as behavioral use of betel quid (ever-vs. never-user), tobacco use (smoker vs. nonsmoker) and alcoholic drinking (current heavy drinker vs. not current heavy drinker), respectively. Medical information of patients was obtained from their medical records, and included TNM clinical staging, the primary tumor size, lymph node involvement, and histologic grade. In this study, whole-blood specimens collected from all participants were placed in sterile tubes containing ethylenediaminetetraacetic acid (EDTA), immediately centrifuged, and then stored at −80 °C.

2.2. Genomic DNA extraction and eNOS SNP selection

To acquire genomic DNA, preserved blood in EDTA anti-coagulation tubes was extracted using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocols. Briefly, DNA was dissolved in TE buffer (10 mM Tris and 1 mM EDTA; pH 7.8) and then quantified by measuring the optical density at 260 nm. The final preparation was stored at −20 °C and was used to act as templates for the polymerase chain reaction (PCR). In total, two SNPs in *eNOS* gene were selected from the International HapMap Project data for this study. We included the nonsynonymous SNP rs1799983 (Glu298Asp in exon 7) and rs2070744 (−786 T > C in the promoter region) in the coding sequences of the gene. These SNPs were selected for this study since they were reported to affect the enzyme or promoter activity of eNOS and production level of NO [19,20].

2.3. Genotyping of eNOS SNPs by a TaqMan SNP genotyping assay

Assessment of allelic discrimination of the eNOS 1799983 (assay ID: C_ 3219460_20) and rs2070744 (assay ID: C_ 15903863_10) SNPs was performed using the TaqMan SNP Genotyping Assay with an ABI StepOnePlus™ Real-Time PCR System and further evaluated with SDS vers. 3.0 software (Applied Biosystems, Foster City, CA, USA) as previously described [22]. Briefly, the total volume of each reaction was 10 μL including 0.25 μL TaqMan probes, 5 μL Genotyping Master Mix, and 10 ng of genomic DNA. The real-time PCR began with an initial