Oral Oncology 47 (2011) 594–600

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

CYP26B1 is a novel candidate gene for betel quid-related oral squamous cell carcinoma

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ARTICLE INFO

Article history:
Received 4 January 2011
Received in revised form 25 April 2011
Accepted 30 April 2011
Available online 8 June 2011

Keywords:
Alcohol
Betel quid
Cigarettes
Oral squamous cell carcinoma
CYP26B1

SUMMARY

Substantial epidemiological data suggest a role for environmental factors (for example, the use of alcohol, betel quid (BQ), and cigarettes) in the occurrence of oral squamous cell carcinoma (OSCC), but the evidence for the genes involved has been inconsistent. This study was to investigate the role of *CYP26B1*, together with the use of alcohol, BQ, and cigarettes, on BQ-related OSCC. The association study (247 OSCC cases and 338 controls) was conducted to examine the possible interplay between *CYP26B1* polymorphisms and alcohol, BQ, and cigarettes use. Additional gene expression was evaluated between OSCC tissue and adjacent normal tissue. The genetic polymorphism AA of *CYP26B1* appeared to correlate with the risk of OSCC (OR = 2.26; 95% CI, 1.35–3.80). Chewing BQ multiplicatively interacted with *CYP26B1* AA to increase the OSCC risk (aOR = 70.04; 95% CI, 13.62–360.11). The independent risk of OSCC was observed among BQ chewers with *CYP26B1* AA, and compared with chewers with the *CYP26B1* CC genotype (stratified aOR = 2.88; 95% CI, 1.07–7.74). Increased expression of *CYP26B1* was observed in tumor tissue compared with adjacent normal tissue. The *CYP26B1* gene plays a novel role in the BQ dependent pathogenesis of OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common cancers in the world,[1] especially in Southeast Asia where betel quid (BQ) chewing is prevalent.[2] In Taiwan, chewing BQ is a very popular habit (~2 million chewers in the Taiwan population), particularly among males.[3,4] In addition, Taiwan is a hyperendemic area for oral and pharynx cancer.[2] In 2007, the age-standardized incidence rate, adjusted by world population, of oral/pharynx cancer for Taiwanese males was 36.02 per 100,000, and was ranked as the fourth most prevalent cancer.[5] The age-standardized incidence rate of oral/pharynx cancer of Taiwan males not only ranks very high in the world, but it continues to increase.[2]

In 2004, the International Agency for Research on Cancer (IARC) declared that chewing BQ without tobacco is carcinogenic to humans, based on convincing evidence of increased risk of OSCC.[6] According to the IARC reports, areca nut (AN) is the main component in various forms of BQ chewing, and it has been evaluated to be a group 1 carcinogen for humans.[6] Also, arecoline, the main alkaloid of AN, is known to cause cytotoxicity in mammalian cells *in vivo* and *in vitro*.[7–9]

Abbreviations: OSCC, oral squamous cell carcinoma; BQ, betel quid; ASRW, adjusted by world population; IARC, International Agency for Research on Cancer; OPL, oral precancerous lesions; AN, areca nut; CYP, cytochrome P450; GST, glutathione *S*-transferase; NAT, *N*-acetyl transferase; ANE, areca nut extract; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; SD, standard deviation; SDS, sodium dodecyl sulfate; HGF, human gingival fibroblast; aOR, adjusted odds ratio; CI, 95% confidence interval; IPB, *inflorescence of Piper betel Linn.*; PBL, *Piper betel leaf*; RA, retinoic acid.

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1368-8375/$ - see front matter © 2011 Published by Elsevier Ltd.
doi:10.1016/j.oraloncology.2011.04.024

Environmental carcinogens and genetic polymorphisms, separately or jointly, play an important role in the occurrence of OSCC. Environmental factors alone (such as alcohol consumption, BQ chewing, and cigarette smoking) are significantly associated with the risks for OSCC/oral precancerous lesions (OPL), and a synergistic effect of using these substances has been observed.[6,10,11] The interplay of environmental and genetic factors in the carcinogenesis of OSCC has been demonstrated for phases I and II enzymes, which are involved in the metabolic activation of environmental carcinogens.[12–15] The phase I enzymes, cytochrome P450 (CYP) families, are involved in the metabolic activation of polycyclic aromatic hydrocarbons and nitrosamines. The phase II enzymes, including glutathione *S*-transferase (GST) and *N*-acetyl transferase (NAT), epoxide hydrolase, and others, are predominantly involved in detoxification of active metabolites of carcinogens.[12–15]

Although environmental factors and genes influence metabolizing enzymes have been separately evaluated for OSCC risks in previous studies,[12–15] their combined effect needed to be elucidated. Numerous genes are involved in carcinogen metabolism, and how to select the candidate genes is complicated. Nevertheless, a microarray study showed that BQ and its components (arecoline/areca nut extract (ANE)) modulated the expression of *CYP26B1 in vitro*.[16] This observation was confirmed by real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). There have not been any studies of the association between *CYP26B1* polymorphisms and BQ-related OSCC. Therefore, this association study was designed to provide an insight into the interaction between a novel *CYP26B1* genotype and substances use (particularly the use of BQ, cigarettes and alcohol) in the development of OSCC.

Material and methods

Study subjects and specimens

A total of 247 male patients diagnosed with OSCCs were recruited from the Department of Dentistry, and Department of Otorhinolaryngology, Kaohsiung Medical University Hospital. The 338 control subjects recruited from hospital were ascertained to be unrelated, no cancer history, not previously treated for patients with OSCC, any ambiguity was rejected from study. Standardized questionnaire data included demographic factors, histories of substance use (alcohol, betel, and cigarette use), and more detail information was collected by trained interviewers.

This study was approved by the Human Experiment and Ethics Committee of Kaohsiung Medical University (KMU-IRB-950070 and KMU-IRB-950072), and all participants signed written sheet of informed consent. The questionnaire data, and whole blood sample were obtained from all participants. Cancerous tissues were collected from necessary surgery for patients with OSCC.

For gene expression analysis, pairs of cancer tissue and its adjacent non-cancerous tissue were obtained from OSCC patients. All of the cases have been confirmed histologically to have OSCC by surgeons or pathologists.

Real-time qRT-PCR

The real-time qRT-PCR using the ABI Prism 7900HT System was performed for determining mRNA expression level of the *CYP26B1* gene as described.[16] The gene expression level was normalized, using GAPDH as an internal reference gene. Relative quantification, applying the formula $2^{-\Delta\Delta Ct}$, was used to calculate the average of fold change with SD for triplicate determinations. The mRNA expression was analyzed from 18 OSCC tissues. Compared to the control (its adjacent non-cancerous tissue), an average change greater than 2-fold, was arbitrarily defined as having significant up-regulation.

Western blot analysis

Equal amounts of protein extract were loaded and separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels. After electrophoresis, the gel proteins were transferred to polyvinylidene difluoride membranes (NEN Life Science Products, Boston, MA). After being blocked with nonfat dry milk, the membranes were incubated with the selected primary antibody to *CYP26B1* (Abnova, Taipei, Taiwan). β-actin antibody was used to confirm that equal amounts of protein had been loaded. Horseradish peroxidase-conjugated anti-mouse was used as the second antibody. The bands were visualized by chemiluminescence using an enhanced chemiluminescence kit (NEN Life Science Products, Boston, MA, USA), and exposure to X-ray film for the appropriate time. A total of 14 OSCC tissues was underwent *CYP26B1* protein levels testing. They were quantified by LabWorks 4.6 image acquisition and analysis software (UVP, Upland, CA, USA).

CYP26B1 genotyping

Four single-nucleotide polymorphisms (SNPs) of *CYP26B1* gene (rs707718, located in exon 6, 3′ untranslated region (UTR); rs2241057, located in exon 4; rs2286965 located in exon 5; rs3768641 located in intron 2) were selected with minor allele frequency from the Chinese HapMap-CHB. Genomic DNA was extracted from peripheral blood lymphocytes. The *CYP26B1* genotyping was performed using the TaqMan SNP assay. The reaction plates were then read using the ABI Prism 7900HT Sequence Detection System. The fluorescence results were analyzed and auto called into genotypes using the built-in software of the system.

Cell culture and ANE

Cell culture and ANE were done as previously described.[17] Briefly, in the stage I experiments, for the analysis of arecoline/AN, the primary human gingival fibroblast (HGF) cells were obtained from a healthy subject who did not have the habit of drinking alcohol, chewing BQ, or smoking cigarettes. He had received a surgical crown lengthening in the Department of Dentistry of Chung-Ho Memorial Hospital, Kaohsiung Medical University, with informed consent (KMUH-IRB-950135). In the stage II, for dose–response analysis of arecoline/AN extracts, KB, a cell line from OSCC, was used in this study. The aqueous ANE and saliva-reacted ANE (sANE) were immediately stored until use.

Statistical analysis

The characteristics of questionnaire data regarding the personal information, substance use history and disease status were included in the analysis. Adjusted odds ratio (aOR), 95% confidence interval (CI) and exact *p* value were estimated by an unconditional logistic regression model controlling potential confounders. The aOR was adjusted for demographic factors and substance use (BQ, cigarette, and alcohol). The trends for dose-dependent effects of substance use of alcohol, BQ, and cigarette were calculated by the Cochran–Armitage trend test. The gene-environment interaction analysis was also performed for improving the assessment of the joint effects of both *CYP26B1* genotype and substance use of BQ, cigarette, and alcohol by a multiple logistic regression. All analyses were executed using the SAS Statistical Package (Version 9.1.3, SAS Institute Inc.).