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Research Article
Lack of Association of CYP2C9 genetic polymorphism with oral squamous cell carcinoma

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Abstract
There is increasing evidence for the role of polycyclic aromatic hydrocarbons and heterocyclic aromatic amines in carcinogenesis, including oral squamous cell carcinoma (OSCC). Several of these mutagenic substances are cytochrome (CYP)2C9 enzyme substrates. In this study we examined the association of CYP2C9*2 and *3 genetic polymorphisms in 58 OSCC patients and 174 healthy, age and sex-matched controls. Genotyping was done with allele-specific polymerase chain reaction followed by agarose gel electrophoreses while selected samples were sequenced for confirmation of genotyping. Results show that wild type genotype (CYP2C9*1*1) was observed at 83%, *1*3 at 8%, *1*2 at 5%, *2*2 at 2% and *2*3 at 2% in combined case and control groups. On further analysis, however, our results did not reveal association of these variants with OSCC samples (Odds ratio: 0.608, 95% Confidence Interval: 0.289 - 1.281, p-value: 0.190). While larger studies are needed to confirm or refute these results, they show a lack of association of CYP2C9*2 and *3 polymorphisms with OSCC in this population.

Keywords: Oral carcinoma, CYP2C9, genetic polymorphism, head and neck cancers

Introduction
Approximately 5% of all cancers worldwide are head and neck squamous cell cancers (HNSCC). Among the HNSCC, approximately 90% are squamous cell carcinomas of the oral cavity, pharynx, and larynx (Curado and Hashibe 2009). The main risk factors include tobacco and alcohol use (Hunter, Parkinson, and Harrison 2005). The risk is further enhanced by polymorphisms in genes involved in the metabolism of alcohol and tobacco (Bartsch et al. 2000). These factors strongly influence a patient’s vulnerability to cancer. Specifically, polymorphisms in cytochrome P450 (CYP) genes such as CYP2E1 and CYP1A1 are associated with HNSCC (Hashibe et al. 2003).

This interaction involving potent carcinogen-metabolizing enzymes may help to determine individuals that are at greater risk of tumor development. Gattás et al. reported that Brazilian patients carrying the CYP1A1*2A genotype presented increased HNSCC risk (Gattás et al. 2006). Furthermore, Hashibe et al. conducted a meta-analysis associated with pooled-analysis in a total of 6969 cases and 8536 controls, demonstrating a modest association of the GSTM1 and GSTT1 genotypes with HNSCC risk (Hashibe et al. 2003). These data support the notion of higher risk when genotypes at multiple GST loci are considered in a multigenic model. In a similar analysis, Varela-Lema et al. observed a joint effect of...
GSTM1 homozygous deletion and the CYP1A1*1A/*2A genotype on cancer risk (Varela-Lema et al. 2008). A significant interaction between CYP1A1*2A and GSTM1 homozygous deletion was reported in the development of HNSCC in Polish population groups (Reszka et al. 2008).

CYP2C9 is one of the most important drug-metabolizing enzymes in humans and carries out the metabolism of ifosfamide, cyclophosphamide, tamoxifen, warfarin, and etoposide (van Schaik 2005). CYP2C9*2 and CYP2C9*3 genotypes result in a ‘poor-metabolizer’ phenotype and consequently slow down the metabolism of drugs and other substrates metabolized by CYP2C9 (Higashi et al. 2002). In a yeast expression system, CYP2C9*2 and CYP2C9*3 were associated with a significant increase in cyclophosphamide clearance (Griskevicius et al. 2003). Apart from metabolizing various drugs, many mutagens and carcinogens are detoxified by CYP2C9 (Shou et al. 1996; Bauer et al. 1995). The frequencies of CYP2C9*2 and CYP2C9*3 have been reported for various populations and display significant variations between Caucasian and Oriental populations (Wang et al. 1995). A study has previously reported a higher frequency of CYP2C9*2 polymorphism in lung cancer patients (London et al. 1996). The risk of distal colorectal adenoma is also reported to be increased with polymorphisms in CYP2C9 (Chan et al. 2004). However, the association of CYP2C9 genetic polymorphisms with HNSCC patients has not been established yet. The present study was started to find out if these polymorphisms in CYP2C9 gene are associated with the risk of OSCC in a Pakistani cohort.

**Materials & Methods**

The present study comprised a group of 58 patients with a diagnosed and histopathologically-confirmed OSCC who were hospitalized at the Dow University Hospital, Karachi during January 2015-December 2017. The patients had neither previous radiotherapy nor chemotherapy. For control arm, 174 age and sex-matched, unrelated healthy individuals were recruited. Informed written consent was obtained from all participating individuals. This research was approved by the Ethics Committee and is per the GCP regulations. 5-6 micron sections of formalin-fixed, paraffin-embedded (FFPE) tissues were used for the extraction of DNA by using QiaAmp FFPE tissue kit (QIAGEN, Germany) as per manufacturer’s instruction. Isolated genomic DNA was stored at -20°C until further processing.

**Genotyping**

CYP2C9*2 and *3, were genotyped using ARMS-PCR (Allele Refractory Mutation System- Polymerase Chain Reaction) using a pair of outer primers and a pair of inner primers. PCR for both the SNPs was performed in a single tube with a total reaction volume of 25µl containing 12.5µl of 2X Dream Taq Master mix (ThermoScientific), 0.5 pM of 2C9*2 wild type reverse primer, 1.5 pM of 2C9*2 mutant reverse primer, 3.0 pM of common forward primer, 1.0 pM of 2C9*3 wild type forward primer, 2.0 pM of 2C9*3 mutant forward primer, 3.0 pM of common reverse primer and 3 μl of template DNA (20-50 ng/µl). Thermal profile was as follows: initial denaturation at 95°C for 10 minutes followed by 37 cycles with denaturation at 95°C for 45 seconds, 45 seconds of primer annealing at 58°C, initial extension at 72°C for 45 seconds and a final extension at 72°C for 7 minutes. For visualization 12μl of PCR product was directly loaded onto 4% agarose gel. The PCR products for 2C9*2 had 105 bp fragment for the wild type allele and 114 bp fragment for the mutant allele. Whereas, 2C9*3 had 159 bp fragment for the wild type allele and 168 bp fragment for the mutant allele. Selected samples were sent for sequencing to further validate the results obtained through ARMS-PCR.

**Statistical Analysis**

Allelic and genotype frequencies are determined from the observed numbers for each allele and genotype in our experiments. Data were recorded and analyzed using SPSS version 23.0. The association between disease status and genotype was investigated using the chi-square and Fisher exact test. A logistic regression model was run to determine the odds ratio and 95% confidence interval for estimating the risk of OSCC. P-value of less than 0.05 was considered to be statistically significant.

**Results**

This study included 58 patients with OSCC and 174 healthy controls. The mean age of the patients was 42.64 years (SD ±12 years) in cases while 40.25 years (SD ± 11.64) in controls. Males were 82% and female 18% in cases while in the control group 75.9% were male and 24.1% were female. Of the 58 cases, 24 (41.37%) were localized to buccal cavity, 17 (29.3%) to tongue, 11 (18.96%) to lips and 6 (10.34%) to the palate (Figure 1).
Figure 1: Locations of lesions in the oral cavity in OSCC patients.

Figure 2: Relative distribution of different CYP2C9 genotypes in samples obtained from cases and controls.

In cases, *1*1 genotype was found at a frequency of 77.6%, *1*2 at 5.2%, *1*3 at 7.5%, *2*2 at 5.2% and *2*3 at 1.7% while in control group, *1*1 genotype was found at a frequency of 85.1%, *1*2 at 3.4%, *1*3 at 12.1%, *2*2 at 0.6% and *2*3 at 1.7% (Figure 2). Overall, wild type genotype was observed at 83%, *1*3 at 8%, *1*2 at 5.5%, *2*2 and *2*3 at 2% each in combined case and control groups (Figure 3).
Figure 3: Overall distribution of CYP2C9 genotypes in cases and controls combined.

In patients with OSCC localized to buccal cavity, 16.7% had genotypes *1*3, 4.2% had *2*3 while remaining (79.2%) had *1*1 genotype. Of the 17 cases affecting the tongue, *1*3 and *2*2 genotypes were 11.8% each while the remaining 76.5% were *1*1 genotype (Figure 4). In OSCC patients in which lesions were localized to lips, genotypes *1*2, *1*3 and *2*2 were found in 9.1% cases each. In 6 cases in which lesions were localized to the palate, 5 had wild type genotype while only one had *1*2 genotype.

Figure 4: Relative distribution of different CYP2C9 genotypes in OSCC samples obtained from various anatomic sites in the oral cavity.
To determine whether polymorphisms in the CYP2C9 gene were able to predict the presence of OSCC, genotyping data were compared (Table 1). The distribution of variant alleles did not show a greater incidence in HNSCC than in control subjects. A statistically insignificant difference was detected between these groups for CYP2C9*1*1 and CYP2C9*1*2, *1*3, *2*3, *2*2 combined (P =0.190). Similarly, no statistically significant association was found for these genotype groups when samples were compared from various anatomic sites (Buccal, p= 0.459, Tongue, p=0.358, Lip, p=0.285, Palate= 0.907) with controls (Table 1).

**Discussion**

Cytochrome P450 is a family of enzymes involved in the metabolism of many environmental agents, including tobacco and alcohol, and evidence exists suggesting their association with increased risk of HNSCC (Bethke et al. 2007; Bolufer et al. 2006; Kiyohara 2000). It is presumed that metabolic gene polymorphisms modulate cancer susceptibility via their interaction with carcinogens (Boccia et al. 2008). Therefore, in this case-control study, two SNPs polymorphisms in CYP2C9 gene were analyzed in HNSCC samples.

Apart from the effects of external chemical carcinogens, the risk of developing different types of cancer is dependent on the interindividual variability in sensitivity towards carcinogens. However, the distribution of the evaluated polymorphisms revealed in our study that the variant alleles did not show a greater incidence in HNSCC patients. Similarly, while evaluating CYP1A1, CYP2E1, GSTM1, GSTT1, EPHX1, and NAT2 polymorphisms in 210 HNSCC cases compared to 245 control subjects, Boccia et al. demonstrated a lack of interaction between the polymorphisms studied and environmental exposure (Boccia et al. 2008). In contrast, in a separate investigation, genetic variants of CYP1A2 and CYP2E1 showed a greater incidence in HNSCC patients, thus confirming their association with cancer risk (Olivieri et al. 2009).

**Table 1: Association among genotype groups from various anatomic sites.**

<table>
<thead>
<tr>
<th>Location</th>
<th>Categories</th>
<th>Controls/Cases</th>
<th>OR</th>
<th>95% CI</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSCC (Overall)</td>
<td><em>1</em>1</td>
<td>148/45</td>
<td>1</td>
<td>ref</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>1</em>2+<em>1</em>3+ <em>2</em>2+ <em>2</em>3</td>
<td>26/13</td>
<td>0.608</td>
<td>0.289 - 1.281</td>
<td>0.190</td>
</tr>
<tr>
<td>Buccal</td>
<td><em>1</em>1</td>
<td>148/19</td>
<td>1</td>
<td>ref</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>1</em>2+<em>1</em>3+ <em>2</em>2+<em>2</em>3</td>
<td>26/5</td>
<td>0.668</td>
<td>0.229 - 1.946</td>
<td>0.459</td>
</tr>
<tr>
<td>Tongue</td>
<td><em>1</em>1</td>
<td>148/13</td>
<td>1</td>
<td>ref</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>1</em>2+<em>1</em>3+ <em>2</em>2+<em>2</em>3</td>
<td>26/4</td>
<td>0.571</td>
<td>0.173 - 1.887</td>
<td>0.358</td>
</tr>
<tr>
<td>Lip</td>
<td><em>1</em>1</td>
<td>148/8</td>
<td>1</td>
<td>ref</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>1</em>2+<em>1</em>3+ <em>2</em>2+<em>2</em>3</td>
<td>26/3</td>
<td>0.468</td>
<td>0.117 - 1.887</td>
<td>0.285</td>
</tr>
<tr>
<td>Palate</td>
<td><em>1</em>1</td>
<td>148/5</td>
<td>1</td>
<td>ref</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>1</em>2+<em>1</em>3+ <em>2</em>2+<em>2</em>3</td>
<td>26/1</td>
<td>0.878</td>
<td>0.099 - 7.826</td>
<td>0.907</td>
</tr>
</tbody>
</table>

The behavior of tumors arising at various sites differs significantly, suggesting different intrinsic tumor properties (dos Reis et al. 2002). In this study, a comparison was made between the genotypes and their relationship to the four major sites of HNSCC: buccal cavity, lip, tongue, and palate. Analysis showed that the CYP2C9 genotypes were observed in equal frequency in all the anatomic areas observed (Table 1). These data suggest that the CYP2C9 variants are not involved in oral tumorigenesis in the Pakistani population. In the majority of squamous cell cancers, CYP1A1, CYP2A6, CYP2E1, and CYP3A genotypes are not...
frequently expressed in the early stage, but in non-small cell lung cancer, they do appear in early-stage and differentiated adenocarcinoma (Oyama et al. 2007) It is speculated that independent of tumor initiation, CYP levels affect various signaling transduction pathways, which alter cell cycle and cause apoptosis or aberrant cell growth and therefore might be correlated with carcinogenesis and/or tumor progression (Bolt, Roos, and Thier 2003; Rossini et al. 2006). However, our study could not find any association of CYP2C9 genetic polymorphisms with OSCC. There are a large number of studies that have reported a similar lack of association of various CYP genetic variants with OSCC as reviewed by Vukovic and colleagues (Vukovic et al. 2016) and Qiu and colleagues in excellent meta-analyses (Qiu et al. 2010). However, this was the first study attempting to find out the association of CYP2C9 genetic polymorphisms with OSCC. These findings suggest that the genetic polymorphisms in CYP2C9 genes (CYP2C9*2 and *3) are not predictors of risk and are not associated with OSCC. This indicates a lack of a role for CYP2C9 in the pathogenesis of OSCC in this population of patients.

Conflict of interest
The authors declare that they have no competing interests.

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Ethics approval
Yes. The study was approved by the Institutional Review Board & Ethics Committee of Dow University of Health Sciences, Karachi.

Consent forms
Yes. Consent forms were obtained from the participating patients.

Authors contribution
SK, HA, and Ai were involved in conceptualization of the study, ZZ, and SUN did sample collection and experimentations, ZZ, and KJ performed the data analysis, SK, HA, and MM wrote the manuscript. All authors read and approved the final version of the manuscript.

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