CYP2 C9 polymorphism among patients with oral squamous cell carcinoma and its role in altering the metabolism of benzo[a]pyrene

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Objectives. The aim of this study was to evaluate the prevalence of CYP2 C9 polymorphism among healthy controls and patients with oral squamous cell carcinoma (OSCC) and to analyze the risk of disease development. We also investigated the interaction between CYP2 C9 wild type and the polymorphic variants with benzo[a]pyrene by using molecular docking analysis.

Study Design. The study included 46 patients with OSCC and 46 controls. Amplification of the genomic DNA was done by using allele-specific polymerase chain reaction and then analyzed by using agarose gel electrophoresis. Molecular docking was then carried out to determine the interaction of CYP2 C9*1, CYP2 C9*2, and CYP2 C9*3 with benzo[a]pyrene.

Results. In the OSCC group, CYP2 C9*2 and CYP2 C9*3 polymorphisms were 17.4% and 15.2%, respectively, and in the control group, they were 8.7% and 6.5%, respectively. The OSCC group showed a statistically significant ($P = .043$) increase in the prevalence of CYP2 C9 polymorphic variants compared with the control group. The docking analysis showed benzo[a]pyrene to bind specifically to the altered single nucleotide catalytic site in the polymorphic CYP2 C9*3 enzyme.

Conclusions. This study demonstrates that functionally important CYP2 C9 polymorphism exists among patients with OSCC, with a modest increase in the risk of disease development in those individuals who acquire these poor metabolizing variants. The modified docking of CYP2 C9*3 with benzo[a]pyrene signifies altered metabolism in vivo. (Oral Surg Oral Med Oral Pathol Oral Radiol 2020;130:306−312)

Oral cancer accounts for 9.1% of all the cancers in India, according to the World Health Organization/International Agency for Research on Cancer (WHO/IARC) 2018 statistics.[1] Oral carcinogenesis is a multifactorial process with numerous risk factors, such as tobacco, alcohol, betel nut, human papilloma virus, and diet. Worldwide, tobacco and alcohol contribute to 25% and 7% to 19% of oral cancers, respectively, and India is a high-risk country for the incidence of oral squamous cell carcinoma (OSCC) because of the high rate of association with the above risk factors.[2-4] However, the disease does not develop in all of the individuals exposed to these risk factors. Therefore, it can be hypothesized that there are ethnic and individual variations in genetic susceptibility that contribute to the development of OSCC in some people but not in others.[4]

Recently, there has been growing interest in identifying the genes involved in the differential susceptibility to OSCC. Some of the proposed genes are those that encode enzymes, such as cytochrome P450 (CYP450), and that are involved in the activation or detoxification of carcinogenic compounds.[5] Several studies have associated the polymorphisms of the *CYP450* gene family with incidence of cancer. Some of the polymorphisms associated with the incidence of OSCC are CYP1 A1, CYP1 B1, CYP2 A6, CYP2 A13, CYP2 C19, CYP2 C9, CYP2 E1, CYP3A4, and CYP2 6B1.[6-9]

CYP2 C9 is a phase I drug-metabolizing CYP450 enzyme isoform, which plays a major role in the oxidation of procarcinogens, such as polycyclic aromatic hydrocarbons, tobacco-specific nitrosamines, and aromatic amines derived from tobacco, and converts them to intermediate carcinogens.[10−12] Among the

Statement of Clinical Relevance

Genetic polymorphism of CYP2 C9 is found to be more prevalent among patients with oral squamous cell carcinoma (OSCC) compared with the controls. The variant genotypes can modify an individual's response to carcinogens, such as benzo[a]pyrene present in tobacco, making the person more susceptible to OSCC.

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polycyclic aromatic hydrocarbons, benzo[a]pyrene is a potent human carcinogen, which binds to DNA and forms adducts after being metabolically activated by CYP450 enzymes.[13] Studies have shown that CYP1 A1, CYPl A2, CYP2 B6, CYP2 C9, and CYP2 E1 display stereoselectivity, favoring metabolism of benzo[a]pyrene to form 7,8-diol, which is then further oxidized to form the carcinogen diol epoxide I.[14] These diol epoxides are electrophiles, which are stable enough to diffuse and bind to the DNA, resulting in mutagenic activity.[15,16] Of all these enzymes, CYP1 A1, CYP1 A2, and CYP2 C9 showcased the highest metabolic activity to form diol epoxide I from 7,8-diol.[14] However, CYP2 C9 also plays a substantial role in the detoxification of BaP-3-diol, which prevents DNA adduct formation,[11] thus functioning as a double-edged sword.

Thus, genetic polymorphisms of CYP2 C9 can modify an individual's response to these carcinogens and could account for the differential risk of OSCC.[12] The CYP2 C9*2 and CYP2 C9*3 alleles are the most common variants of the CYP2 C9 gene.[17] These variants differ from the wild type CYP2 C9*1 by a single point mutation. CYP2 C9*2 is characterized by a 430 C>T exchange in exon 3, with consequential Arg144 Cys amino acid substitution, whereas CYP2 C9*3 shows an exchange of 1075 A>C in exon 7, producing an Ile359 Leu substitution in the catalytic site of the enzyme.[18] CYP2C9*2 and CYP2C9*3 single nucleotide polymorphism causes the enzyme to have 30% and 80% lower enzymatic activity, respectively.[17]

The present study evaluated the prevalence of CYP2 C9 polymorphism among healthy controls and patients with OSCC. The results of this study will be confirmed further by using molecular docking analysis to check the interaction of CYP2 C9 wild type and its polymorphic variants with the tobacco byproduct benzo[a]pyrene.

MATERIALS AND METHODS

Patient selection

A cross-sectional study was conducted among 92 patients who presented to the outpatient department of Saveetha Dental College and Hospital. The patients were divided into 2 groups, with 46 patients per group. Group I included healthy controls and group II patients with OSCC. All of the patients included in the study belonged to the same ethnic group of South India (Dravidian). Informed consent was obtained from the study patients for inclusion in the study, and it was also ensured that patient anonymity was maintained. All the participants completed a questionnaire covering medical, residential, and occupational history. Information pertaining to family history of the disease, smoking, tobacco chewing, and alcohol drinking was also included in the questionnaire filled by the patients.

In all of the patients with OSCC included in the study, the diagnoses were confirmed with histopathology. Healthy controls were age and gender matched with the cases and were not found to suffer from any chronic diseases, as indicated by findings from clinical examination and past medical history. The protocol of the study was approved by the Scientific Review Board (SRB/SDMDS 160 MP/04), and the study followed the tenets of the Declaration of Helsinki.

Sample collection

Blood samples were obtained from the study patients via venipuncture of the antecubital fossa by using a 5-mL 22-gauge syringe (Nipro, India) and then transferred to an EDTA (ethylenediaminetetraacetic acid) vacutainer tube (BD Vacutainer, UK). Genomic DNA extraction was done for subsequent allele-specific polymerase chain reaction (PCR).

Genomic DNA extraction

For genomic DNA extraction, first, 0.1 mL of peripheral blood was lysed in 100 μL of cell lysis buffer containing 36% to 50% guanidine hydrochloride (Cat# 740951.50, Nucleospin Blood DNA Kit; Machery Nagel, Germany) and incubated at 57˚C for 2 hours (Labline, India) to enable complete lysis of leukocytes. After lysis, an equal volume of 100% ethanol (Jiangsu Huaxi International Trade Co Ltd, China) was added to precipitate the genomic DNA. Subsequently, the entire content was transferred to DNA spin columns containing silica membrane and centrifuged at 8000 rpm for 1 minute (Eppendorf, Germany) at room temperature.

The precipitated DNA was captured in the silica membrane during this step. After DNA capture, the silica columns were washed twice with wash buffer (Machery Nagel, Germany). Degraded proteins and membrane lipid particles were washed off during the wash steps. After the 2 wash steps, the captured DNA from the silica membrane was eluted with 50 μL of elution buffer (Machery Nagel, Germany).

Allele-specific PCR

The reagents—10 μL of Emerald Master mix (cat# RR310 A; Takara, Japan); 2 μL of wild type or mutant forward primer; 2 μL of common reverse primer (10 μM); 1 μL of internal control forward primer (0.2 μM); 1 μL of Internal control reverse primer (0.2 μM); 1 μL of DNA (10 ng); and 3 μL of deionized water—were added to 0.2-mL dome-capped PCR tubes to initiate the PCR. Forward and reverse primers, along with beta actin and internal control primer, are listed in Table I.

Initial denaturation of the sample DNA was carried out at 94˚C for 4 minutes. This was followed by denaturation at 94˚C for 30 seconds. Primer annealing was done at 55˚C for 30 seconds, after which the primer

Table I. Design of the allele-specific primers

Primers to detect CYP2 C9*2 genotype	
CYP2 C9*2 *WT* Forward Primer	GAAGAGGAGCATTGAGGACC
CYP2 C9*2 *Mutant* Forward Primer	GAAGAGGAGCATTGAGGACT
Common Reverse Primer	TCCAAGAATGTCAGTAGAGA
Primers to detect CYP2 C9*3 genotype	
CYP2 C9*3 **WT** Forward Primer	TGCACGAGGTCCAGAGTTACA
CYP2 C9*3 **Mutant** Forward Primer	TGCACGAGGTCCAGAGTTACC
Common Reverse Primer	AATGATACTATGCATTTGGGA CTTCGA
Beta actin primers as internal control	
β-actin Forward Primer	GCATCGCTGGTAACATCCAC
β-actin Reverse Primer	GAGCAGGGTCTAGAGCAGAG

extension was performed at 72˚C for 30 seconds. Denaturation, primer annealing, and primer extension were repeated for 35 cycles in a thermocycler (Takara, Japan). The final extension was carried out at 72˚C for 5 minutes.

Analyzing amplification by using agarose gel electrophoresis

For this step, 10-μL aliquots of amplified PCR products were analyzed by being run in a 1.5% agarose gel (Medox Fine Chemicals, Chennai, India) at 100 V for 15 minutes with 1 X TAE (Tris Acetate EDTA) buffer (Cat# B49; ThermoFisher Scientific, Waltham, MA). The DNA bands were visualized by staining the gel with ethidium bromide (Medox Fine Chemicals, Chennai, India), a DNA intercalating agent that fluoresces when excited by ultraviolet (UV) light in the range of 302 nm to 364 nm, and images were captured with gel documentation unit (Gelstan Mini GS 1012 M, Mediccare, India).

Molecular docking

After wet laboratory analysis, molecular docking was carried out to check for any significant alteration in the interaction of CYP2 C9 wild type and its polymorphic variants with benzo[a]pyrene.

Preparation of receptor (CYP2 C9 wild type and its mutants)

The structure of human CYP2 C9, deciphered by using the x-ray diffraction method, was retrieved from the Protein Data Bank (PDB; http://www.rcsb.org/pdb/home/home.do). Protein structure with a resolution greater than 2.5 Angstrom (Å) was selected for analysis. The pdb structure was downloaded, and the atom coordinates were viewed in a flat file format. Heteroatoms and other bound residues were removed, and the protein molecule was saved as a .pdb file. The obtained pdb structure was optimized 3-dimensionally before docking. We prepared the mutant forms used in the study by manipulating the appropriate amino acids in the flat file format.

Preparation of ligands

The PubChem compound record (http://pubchem.ncbi.nlm.nih.gov/) was used to derive and confirm the structure of ligand benzo[a]pyrene used for the present study. The structure was drawn by using the tools available in ChemSketch (ACD/ChemSketch version 11.0) (http://www.acdlabs.com/resources/freeware/chemsketch/), an advanced chemical drawing tool. This package was also used to generate 2-dimensional (2-D) structures, to clean up the 2-D structure of the ligand, to perform 3-dimensional (3-D) optimization and to view the 3-D structure of the ligand.

Docking simulations of CYP2 C9 wild type and its mutants with the ligands

ArgusLab version 4.0.1 (http://www.arguslab.com/arguslab.com/ArgusLab.html) was used for docking. The binding sites in the receptor were grouped, and the grid size was also calculated. The grid was prepared with the resolution of 0.4 units, and it was centered on the ligand binding site of the protein for docking. Before docking, both the proteins and the ligand were optimized for proper geometry. Docking was performed by using the Lamarckian genetic algorithm. The CYP2 C9 wild type and its mutants with the ligands were simulated by using their optimized structures. Intermolecular flexible docking simulations were then performed, and energy values were calculated from the docked conformations of the ligand−protein complexes. Each run was repeated 5 times to get the best results, and the results of the docking analysis were saved as .pdb files.

Visualization

PyMOL version 2.0 (https://www.pymol.org/), an open-source visualization tool in structural biology, was used to produce high-quality 3-D images of the biologic molecules and the docking simulations of the CYP2 C9 receptor protein. Interaction was measured by the binding energy of the best ligand pose measured in kilocalories per mole (kcal/mol).

Statistical analysis

Statistical analysis was performed by using the SPSS Statistics for Windows, version 23.0 (SPSS Inc., Chicago, IL). The χ^2 test was used in this study to compare the groups, if any expected cell frequency was less than 5, then Fisher's exact test was used. Significance was set at 5% ($\alpha = 0.05$). A P value less than .05 was considered significant and a P value less than .05 was considered not significant. The crude odds ratio was then calculated for groups, and it showed statistical significance.

Table II. Distribution of demographic variables and putative risk factors in healthy controls and patients with oral squamous cell carcinoma

Characteristics		*Controls*	*Oral squamous cell carcinoma*
Number of patients		46	46
Age (mean ± standard deviation)		49.3 ± 13.7	50.6 ± 10
Gender	Male (%)	35 (76.1)	37 (80.4)
	Female (%)	11 (23.9)	9 (19.6)
Betel nut/Pan chewing (%)		0	24 (52.2)
Tobacco users (%)		4 (8.7)	17 (36.6)
Smokers (%)		3 (6.5)	18 (39.1)
Alcoholics (%)		3 (6.5)	14 (30.4)
No habits (%)		38 (82.6)	2 (4.3)

RESULTS

PCR analysis was carried out on samples from 92 participants (mean age for control group, 49.3 years; for OSCC group, 50.6 years). A male preponderance was seen in both groups; smoking, tobacco chewing, betel nut chewing, and alcohol drinking were more prevalent among patients with OSCC compared with the healthy controls. The demographic data of the participants are provided in Table II.

The results of this study showed that the prevalence of the poor metabolizing variant CYP2 C9*2 among patients with OSCC was 17.4% and that among the control group was 8.7%. CYP2 C9*3 was 15.2%among patients with OSCC and 6.5% among the control group. A higher prevalence was found among patients with OSCC compared with the healthy controls.

On considering the genotype, wild type CYP2 C9*1/*1 was found among 40 healthy controls and 32 patients with OSCC (87% and 69.5%, respectively). A higher prevalence was found among the control group compared with the OSCC group. The genotype CYP2 C9*1/*2 was seen among 3 controls and 7 patients with OSCC (6.5% and 15.2%, respectively). Similarly, CYP2 C9*1/*3 was found among 2 controls and 6 patients with OSCC (4.3% and 13%, respectively). The CYP2 C9*2/*3 genotype was found in 1 patient belonging to control group and 1 with OSCC (Figure 1).

The combined prevalence of CYP2 the poor metabolizing variants of CYP2 C9 (CYP2 C9*1/*2, CYP2 C9*1/*3, and CYP2 C9*2/*3) among healthy controls was 13% and among patients with OSCC was 30.4% (Table III). A statistically significant increase was seen among patients with OSCC compared with the healthy controls ($P = .043$).

The odds ratio showcased that the CYP2 C9*1/*1 allele (wild type) showed a statistically significant decreased risk of OSCC. Although individual CYP2 C9*1/*2 and CYP2 C9*1/*3 alleles did not show a consistent relationship with risk of OSCC, the combined poor metabolizing polymorphic variants (CYP2 C9*1/*2, CYP2 C9*1/*3 and CYP2 C9*2/*3) exhibited a statistically significant increased risk of OSCC. The risk was increased 2-fold (see Table III).

The results of the molecular docking analysis revealed that benzo[a]pyrene interacted strongly with both wild-type CYP2 C9 and its polymorphic variants CYP2 C9*2 and CYP2 C9*3. The strong interaction

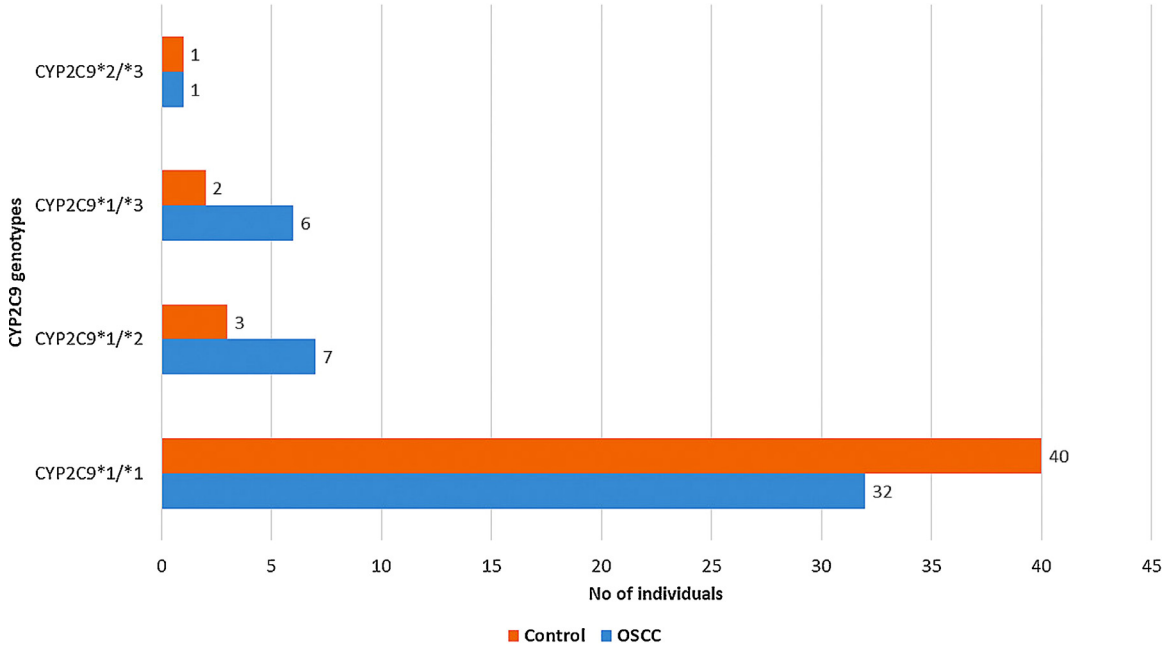

Fig. 1. Graph depicting the prevalence of CYP2 C9*1/*1(wild type), CYP2 C9*1/*2, CYP2 C9*1/*3 and CYP2 C9*2/*3 polymorphisms among healthy controls (control) and patients with oral squamous cell carcinoma (OSCC).

Table III. Combined prevalence of CYP2 C9 poor metabolizing variants (CYP2 C9*1/*2, *1/*3, and *2/*3) among healthy controls and patients with oral squamous cell carcinoma (OSCC) and risk of OSCC among those acquiring the wild-type and polymorphic variants (CYP2 C9*1/*2, *1/*3, and *2/*3)

	Oral squamous cell carcinoma (Cases)	*Healthy controls*	*Odds ratio*	*95% confidence interval*	*P value*
Wild type	32 (69.6%)	40 (87%)	0.3	0.1−0.9	.048
Polymorphic variants	14 (30.4%)	6 (13%)	2.9	1−8.4	.048
Total	46	46			

between CYP2 C9 wild type and benzo[a]pyrene showed a dock score of −15.3262 kcal/mol (Figure 2). Although no hydrogen bond formed during the interaction, benzo[a]pyrene tightly fits into the ligand binding pocket. A similarly strong interaction was also seen with CYP2 C9*2 and CYP2 C9*3 polymorphic variants; CYP2 C9*3 with benzo[a]pyrene showed an interaction at the amino acid residue leucine 359, which is the site of the single nucleotide substitution and is found only in this mutant form (see Figure 2). Thus, there is altered interaction of CYP2 C9 polymorphic variants compared with the wild type.

DISCUSSION

CYP2 C9, a phase I drug-metabolizing enzyme, comprises 20% of the hepatic CYP450 content and plays an important role in the metabolism of drugs and xenobiotic substances, such as polycyclic aromatic hydrocarbons, tobacco-specific nitrosamines, and aromatic amines derived from tobacco.[7,9,19] There are about 33 polymorphic variants of this enzyme, of which CYP2 C9*2 and CYP2 C9*3 are the most prevalent.[20,21] In vitro analyses have shown that these 2 variant alleles have 30% and 80% lower enzymatic activity, respectively, compared with the wild-type, and are called *poor metabolizers.*[22,23] It is hypothesized that individuals with these polymorphic variants have altered metabolism of tobacco products, making them more susceptible to OSCC.

The present study, which was conducted to compare the prevalence of CYP2 C9 polymorphism among healthy controls and patients with OSCC, revealed the presence of functionally important CYP2 C9 polymorphisms among the 2 groups. The frequencies of CYP2 C9*2 and CYP2 C9*3 polymorphisms among the healthy individuals in the control group was 8.7% and 6.5%, respectively. The CYP2 C9*3 polymorphism was comparable with the results obtained from a study conducted among the general Tamil population, where the frequency was 6.7%. However, the same study showed a lower frequency of CYP2 C9*2 polymorphism, with a rate of 2.6%.[24] Jose et al. in another study conducted among the Dravidian population found the frequency of CYP2 C9*3 genotype to be 15%, which was much higher than that in the present study, although the CYP2 C9*2 frequency was similar.[25] On comparing the results with other populations worldwide, the CYP2 C9*2 genotype was found to be much lower than that in Caucasians (11%) and greater than that in the East Asian population (0%). But, the CYP2 C9*3 genotype was comparable with that in Caucasians (8%).[26] The variation in prevalence among different ethnic groups confirms the racial delineation among the groups.

Patients with OSCC showed a higher prevalence of CYP2 C9*1/*2 and CYP2 C9*1/*3 polymorphic genotypes compared with the healthy controls. The combined prevalence of these poor metabolizing variants was 30.4%, which was found to be statistically significant (P = .043) compared with the healthy controls. This is also supported by the odds ratio, which showed a modest increase in the risk of disease development among individuals who acquire these poor metabolizing variants of CYP2 C9. Although the association of

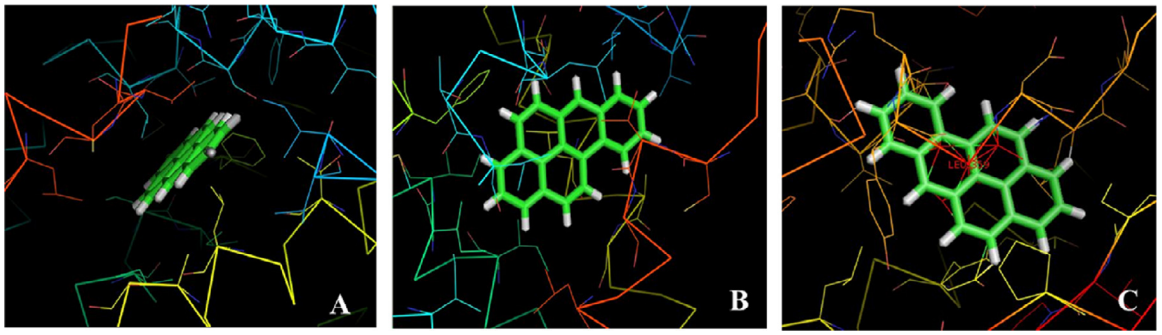

Fig. 2. Docking simulation of CYP2 C9 with benzo[a]pyrene visualized using PyMOL v2.0. **A**, Docking simulation of CYP2 C9 wild type with the ligand benzo[a]pyrene. **B**, Docking simulation of CYP2 C9*2 with the ligand benzo[a]pyrene. **C**, Docking simulation of CYP2 C9*3 with benzo[a]pyrene showing interaction at the amino acid residue leucine 359, which is found only in this mutant form.

CYP2 C9 polymorphisms with OSCC has not been well characterized in the literature, 3 studies conducted among the north Indian population have shown increased prevalence among the case group compared with the healthy controls.[7-9] This is attributed to the altered metabolism of tobacco products, which are the major risk factors for the pathogenesis of OSCC. Benzo[a]pyrene, a procarcinogen derived from tobacco, is considered the most carcinogenic agent among all the polycyclic aromatic hydrocarbons. It makes a substantial contribution to the burden of cancer in humans and is associated with chemically induced cancers, such as lung cancer, oral cancer, esophageal cancer, and colorectal cancer.[7-9,23,27,28]

Benzo[a]pyrene requires metabolic activation before forming DNA adducts, which is an essential mechanism by which it exerts its genotoxic effects. CYP2 C9 polymorphisms are known to play an important role in the activation of benzo[a]pyrene and a substantial role in the detoxification of BaP-3-diol, an oxidation product of benzo[a]pyrene. When individuals acquire the polymorphic variants, they are impaired of CYP2 C9−mediated detoxification, leading to excessive accumulation of the environmental carcinogen, which forms DNA adducts, resulting in carcinogenesis.[11] This theory is also supported by Ozawa et al. who found bulky bronchial DNA adducts in patients with lung cancer associated with CYP2 C9*2 and CYP2 C9*3 genotypes.[29]

In studies conducted among other tobacco-related cancers, Chan et al. and Liao et al. found the polymorphic CYP2 C9 genotypes to be associated with increased susceptibility to colorectal cancer and attributed the elevated risk to the reduced metabolic activity of the enzyme leading to lower intrinsic clearance of many drugs and xenobiotic substances.[11,27] In a study conducted among patients with lung cancer, the marginal increase in polymorphic CYP2 C9 genotype was considered to have occurred by chance.[28] However, Kaur-Knudsen et al., in their study, did not find any association between CYP2 C9 genotype and tobacco-related cancers.[30] Similarly, few other studies conducted among patients with colorectal cancer and those with lung cancer did not show any association.[31-35] The wide variations among studies could be attributed to differences in the ethnic backgrounds of study patients.

Because of the sparse data in the literature to back the results of the present study, a docking analysis was done, and it showed strong interaction of benzo[a]pyrene with both CYP2 C9 wild type and its polymorphic variants. Yadav et al. stated that CYP2 C9 was found to have higher affinity to benzo[a]pyrene compared with other enzymes, such as CYP1 A1 and CYP2 E1.[9] The striking difference seen between the wild-type CYP2 C9 and the variant CYP2 C9*3 was the specific binding of the ligand benzo[a]pyrene to the single nucleotide change region in the enzyme. This alteration in interaction could be a reflection of the poor metabolizer status of the enzyme causing an alteration in the metabolism, detoxification, and clearance of the carcinogen, which is seen in vivo. Because of the paucity of studies on such an interaction, further crystallography studies and nuclear magnetic resonance imaging studies are required to ascertain this bioinformatics information.

Although our study showed promising results, a larger sample size would give a more comprehensive outcome. Another limitation of our study is the lack of similarity between the case and control groups with respect to tobacco and alcohol consumption, and this could be a potential bias. Hence, further studies should be conducted by including healthy individuals with a tobacco habit to make a more conclusive association between the incidence of OSCC and CYP2 C9 polymorphism.

CONCLUSIONS

The results of the present study have demonstrated a high prevalence of CYP2 C9 polymorphism among patients with OSCC compared with the healthy controls, with a modest increase in the risk of disease development among those who acquire these poor metabolizing variants. The CYP2 C9 enzyme coded by variant genotypes could interact with environmental risk factors to modify an individual's susceptibility to OSCC. The docking analysis in our study gave us a glimpse of the interaction between the wild-type and mutant forms of CYP2 C9 and the environmental carcinogen benzo[a]pyrene. This is the first study to investigate this association.

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