Association of mitochondrial deoxyribonucleic acid mutation with polymorphism in *CYP2E1* gene in oral carcinogenesis

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ABSTRACT

Background: Oral carcinogenesis is a complex process affected by genetic as well as environmental factors. *CYP2E1* gene is involved in metabolism of number of compounds and carcinogens. Its normal functioning is required for homeostasis of free radical. Mitochondrial deoxyribonucleic acid (mtDNA) is 10–100 times more susceptible to damage than nuclear DNA. Mitochondrial DNA large scale deletions are well documented in oral cancer. However, the relationship between *CYP2E1* gene polymorphisms and mtDNA damage is still not documented in literature.

Materials and Methods: Case–control study involving 50 subjects was carried out. Deoxyribonucleic acid extraction was done from study subject tissue samples. Restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) amplification was done to confirm *CYP2E1* gene polymorphisms. The PCR amplification was done for mtDNA 4977 bp deletion. Statistical analysis was carried out using SPSS version 11.5 with χ^2 tests.

Results: c1c1 and DD polymorphisms are prevalent in North Indian population having oral cancer. These polymorphisms are significantly associated with mtDNA 4977 bp deletion.

Conclusion: Mitochondrial DNA damage induced by wild *CYP2E1* forms and imperfect DNA repair in mtDNA may act synergistically to greatly enhance oral cancer risk.

Keywords: *CYP2E1*, DNA, mitochondria, oral cancer.

INTRODUCTION

Oral cancer is the sixth most common cancer globally in terms of numbers.¹ Like other cancers, oral cancer is a result of the sequential accumulation of genetic changes. Nearly, all xenobiotics like carcinogens and procarcinogens require activation by phase I metabolizing enzymes, primarily the cytochrome P450s (CYPs), and detoxification by phase II enzymes, such as glutathione S-transferases (GSTs).² Genetic polymorphisms of these enzymes can modify an individual's response to carcinogens and hence the carcinogenic potential of such exposures. *CYP2E1* polymorphism has been shown to be associated with an increased risk of cancer.³ It has been shown that normal (c1c1) genotype carriers of *CYP2E1**5B are generally more prone to risk of oral cavity, larynx, esophagus, liver, lung, and pharynx

cancer compared with variant genotypes.⁴ The role of mitochondria in energy metabolism, the generation of reactive oxygen species (ROS), aging, and the initiation of apoptosis have implicated their importance in tumorigenesis.^{5,6} Mitochondrial deoxyribonucleic acid (mtDNA) mutations in oral squamous cell carcinoma (OSCC) were first described by Fliss et al in 2000.⁵ Somatic large mtDNA deletions in tumors occur less frequently and usually in a heteroplasmic state that may be age-related rather than tumor-specific.⁷ Mitochondrial DNA with multiple deletions has been reported in oral tumors and surrounding tissues.⁵ Although, most of the mutations reported were in the D-loop region, somatic mutations were also found in the coding regions.⁶ The functional significance of these mutations in tumorigenesis is currently still a mystery. This case–control study was therefore initiated to investigate the association of polymorphisms in

Table 1 Sequence of the primers used for amplification of polymorphic sites

Gene locus	Sequences	References
<i>CYP2E1</i> *5B	FP: 5'-CCAGTCGAGTCTACATTGTCA-3' RP: 5'-TTCATTCTGTCTCTAACTGG-3'	Liu et al (2001)
<i>CYP2E1</i> *6	FP: 5'-AGTCGACATGTGATGGATCCA-3' RP: 5'-GACAGGGTTTCATCATGTTGG-3'	Vidal et al (2004)

the *CYP2E1* gene and mtDNA 4977bp deletion with oral cancer/precancer cases in the North Indian population.

MATERIALS AND METHODS

A hospital-based case-control study comprising 50 male cases with oral cancer and an equal number of healthy male controls was conducted. Patients visiting the outpatient clinic in the Department of Oral and Maxillofacial Surgery, CSM Medical University, for the management of oral cancer were included in this study as cases. The diagnosis, squamous cell carcinoma of the oral cavity, was confirmed by cytological, imaging, and histopathological examinations. An equal number of healthy individuals ($n=50$) of the same ethnic group (Indian-European community) of North India, and drawn from the same geographical and linguistic lineage, served as the controls. Tissue samples were obtained from healthy controls, not suffering from any chronic ailments, based on a routine medical checkup, undergoing third molar extractions with their informed consent. The total genomic DNA was also extracted from the study group and samples were anonymized before processing. Necessary approval was obtained from the local research ethics committee.

Deoxyribonucleic acid extraction

For each sample DNA was extracted from preselected regions of tumor and control tissue using two unstained 20mm sections of formalin fixed, paraffin wax embedded tissue, using the process described by Kim et al.⁸ Briefly, tissue sections were dewaxed in two xylene washes and subsequently dehydrated in 100% ethanol. The tissue was collected by centrifugation, the resultant pellet was dissolved in 140 mL polymerase chain reaction-Tris Ethylene diamine tetra acetic acid (PCR-TE) buffer (0.1 M NaCl, 0.05 M Tris and 0.01 M ethylenediaminetetraacetic acid [EDTA]), 20mL 10% sodium dodecyl sulfate (SDS) (10 g/100mL lauryl sulfate), and 80 mL of 1% proteinase K (100 mg/mL), and incubated overnight at 55°C to digest the tissue. The DNA

was subsequently isolated by phenol/chloroform and re-suspended in a 100 mL filter sterilized H₂O.

Detection of *CYP2E1**5B (*RsaI*) polymorphism

RsaI polymorphic site present in 5'UTR (C-1053T) of *CYP2E1* gene was identified by the methods of Liu et al.⁹ The primer sequences used for identifying *RsaI* polymorphic site in *CYP2E1* are shown in Table 1. The reaction mixture in 50 µL contained 1X buffer (10mM Tris-HCl, pH 8.3, 1.5mM MgCl₂, 25mM KCl), 200 µM of each dNTPs, 200nM of each primers, 1.5 unit of *Taq* polymerase (MBI Fermentas, Germany), 100ng of genomic DNA and sterile milli-Q water. Amplification was performed on Gene Amp PCR system 9700 of Applied Biosystem using the following protocol: 94°C for 5 minutes for initial denaturation followed by 35 cycles of 94°C for 30s, 64°C for 30s, and 72°C for 1 minute and a final elongation step of 72°C for 10 minutes. Polymerase chain reaction resulted in a 410bp product, which was further digested with 10U of *RsaI* restriction enzyme (MBI Fermentas, Germany) for the identification of genotypes. The digested PCR product was separated by electrophoresis using 2% agarose gel containing ethidium bromide and DNA bands were visualized on VERSA DOC Imaging System (Model 1000, Bio-Rad). Genotypes were determined based on the size of the respective DNA fragments. Formation of two fragments of 290bp and 120bp is indicative of wild type genotype while the digestion of PCR product into three fragments of 410bp, 290bp, and 120bp is indicative of the heterozygous genotype. In case of homozygous mutant, a single band of 410bp would be formed following digestion with the *RsaI* (Figure 1).

Detection of *CYP2E1**6 (*DraI*) polymorphism

DraI polymorphic site present in intron 6 (T7632A) of *CYP2E1* gene was identified by the method of Vidal et al.¹⁰ The primer sequences used for identifying *DraI* polymorphism is shown in Table 1. The reaction mixture in 50 µL

Figure 1 Genotyping of *CYP2E1* (*RsaI*) polymorphism by PCR RFLP. Lane 1: PCR product, Lane 2: homozygous wild type, Lane 3: heterozygous mutant, Lane 4: homozygous genotype, Lane 5: 100bp ladder. PCR=polymerase chain reaction; RFLP=restriction fragment length polymorphism.

contained 1X buffer (10mM Tris-HCl pH 8.3, 1.5mM $MgCl_2$, 25mM KCl), 200 μ M of each nucleotides, 200nM of each primers, 1.5 unit of *Taq* polymerase (MBI Fermentas, Germany), 100ng of genomic DNA and sterile milli-Q water. Amplification was performed on a thermal cycler using the following protocol: 94°C for 5 minutes for initial denaturation followed by 35 cycles of 94°C for 30s, 63°C for 30s, and 72°C for 1 minute and a final elongation step of 72°C for 10 minutes. Polymerase chain reaction resulted in the formation of a 375bp product; 20 μ L of the PCR product was digested with 10U of *DraI* (MBI Fermentas, Germany) in a final volume of 30 μ L. This reaction mixture was incubated overnight at 37°C. Electrophoresis was carried out in a 3% agarose gel containing ethidium bromide and analyzed on VERSA DOC Imaging System (Model 1000, Bio-Rad). Digestion of 375bp PCR product into two fragments of 210bp and 165bp indicates the presence of wild type genotype. In case of homozygous mutant, single band of 375bp would be formed while a mixture of all three sizes of fragments (375bp, 210bp, and 165bp) is indicative of the heterozygous genotype (Figure 2).

Mitochondrial deoxyribonucleic acid large-scale deletion analysis

The common 4977bp deletion, from mtDNA nucleotide position (np) 8469 to np 13447, was analyzed by PCR. The mtF8295 forward primer and mtR13738 reverse primer

Figure 2 Genotyping of *CYP2E1* (*DraI*) polymorphism by PCR RFLP. Lane 1: 100bp ladder, Lane 2: PCR product lane, Lane 3: homozygous wild type, Lane 4: heterozygous mutant, Lane 5: homozygous mutant. PCR=polymerase chain reaction; RFLP=restriction fragment length polymorphism.

were used for the detection of deleted mtDNA, which yielded a 466bp fragment.¹¹ The PCR products were separated on a 1.5% agarose gel (Figure 3).

RESULTS

The genotype distribution of the polymorphic *CYP2E1* alleles in controls and cases is shown in Table 2. The genotype frequencies of *CYP2E1* (*RsaI*: Chi-square: 9.756 at 1 d.f. and *DraI*: Chi-square: 7.143 at 1 d.f.). The prevalence of the c1c1 genotype of *CYP2E1*5B* (*RsaI*) was increased in the cases (94%) compared with the controls (70%) (Table 2). On the other hand, the decrease in the frequency of variant genotypes (c1c2+c2c2) was coupled with an approximate 6-fold increase in the risk (odds ratio [OR] 6.714; 95% CI 1.637–31.837) for oral cancer ($P=0.003$). Similarly the decrease in the frequency of variant genotypes (DC+CC) was coupled with an approximate 3.5-fold increase in the risk (OR 3.5; 95% CI 1.247–10.068) for oral cancer ($P=0.013$). Mitochondrial DNA 4977bp deletion was found in 42 of 50 patients and 18 of 50 controls (Figure 1). Mitochondrial 4977bp deletion when charted with *CYP2E1* genotypes (Table 3) *DraI* DD genotype showed significant correlation ($\chi^2=24.667$; $P=0$; OR=60). *RsaI* c1c1 genotype, however, did not show significant correlation though it conferred approximately 3 times risk of having mtDNA 4977bp deletions ($\chi^2=0.713$; $P=0.414$; OR=2.857).

Figure 3 The 4977bp deleted mtDNA in the oral cancer cases. A 466bp PCR product represents the amplification of the junction fragment of 4977bp deleted mtDNA by use of forward and reverse primer. M-100bp DNA ladder, 1–13: oral cancer cases. DNA=deoxyribonucleic acid; mtDNA=mitochondrial deoxyribonucleic acid; PCR=polymerase chain reaction.

Table 2 Genotype frequencies of *CYP2E1* gene and the presence of mitochondrial deoxyribonucleic acid 4977bp deletions in the study group

	<i>RsaI</i>		<i>DraI</i>		<i>mtDNA 4977bp deletion</i>	
	<i>c1c1</i>	<i>c1c2+c2c2</i>	<i>DD</i>	<i>DC+CC</i>	<i>Present</i>	<i>Absent</i>
Cases	47	3	42	8	42	8
Controls	35	15	30	20	18	32
χ^2		9.756		7.143		32.076
<i>P</i>		0.003		0.013		0
OR		6.714		3.5		12.25

mtDNA=mitochondrial deoxyribonucleic acid; OR=odds ratio.

Table 3 Distribution of the study group based on *CYP2E1* genotypes and mitochondrial deoxyribonucleic acid 4977bp deletion

Polymorphisms		RsaI		DraI	
		c1c1	c1c2+c2c2	DD	DC+CC
mtDNA 4977 bp deletion	Present	40	2	40	2
	Absent	7	1	2	6
χ^2		0.713		24.667	
P		0.414		0	
OR		2.857		60	

mtDNA=mitochondrial deoxyribonucleic acid; OR=odds ratio.

DISCUSSION

Tobacco and alcohol are the major environmental causes of oral cancer. The carcinogens and ROS generated by tobacco and alcohol consumption can induce oxidative DNA damage to the buccal mucosa cells.¹² Owing to a particular susceptibility to oxidative damage due to high levels of ROS

generation in mitochondria, inefficient DNA repair system, and a lack of protective histones in this organelle, mutation rate has been reported to be 10–17 fold higher in the mtDNA than in the nuclear.⁶ Recently, somatic mtDNA mutations were found in various types of tumors,^{6–8} and they are now regarded as a general phenomenon in tumorigenesis. These mutations have also been discovered in the paired bodily

fluids of patients with tumors having known mitochondrial alterations.⁶ Human *CYP2E1*, involved in the metabolism of ethanol, low molecular weight solvents, and tobacco-specific nitrosamines, is located on the 10th chromosome, consisting of nine exons and eight introns.¹³ *CYP2E1* possesses a unique ability to reduce molecular oxygen to highly reactive compounds—superoxide anion radical (O_2^-), single oxygen (O_2^1), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^*) even in the absence of substrate. Enhanced synthesis of active oxygen forms leads to intensified lipid and protein peroxidation, DNA damage, and carcinogenesis. Thus, *CYP2E1* functioning may have an important role in the homeostasis of ROS inside mitochondrial and thus any deranging in their relationship may lead to mtDNA damage and subsequent steps of carcinogenesis. In fact, here we have reported for the first time to the best of our knowledge the unique relationship between the polymorphisms of *CYP2E1* and mtDNA large bp deletion.

Genetic polymorphism has been reported for the *CYP2E1* gene. The *RsaI* polymorphism (*CYP2E1*5B*) (rs2031920) is found in the 5'-regulatory region and results in *RsaI* restriction site loss.²⁷ Another replacement polymorphism of *CYP2E1* was identified in intron 6 and was designated as the *DraI* polymorphism (*CYP2E1*6*) (rs6413432).¹⁴ *CYP2E1* polymorphism can be an important link in multi-factor pathogenesis of oral cancer and a genetic marker in the determination of individual susceptibility to this malignancy. Significant risk of oral cavity cancer is associated not only with polymorphic c1/c2 and c2/c2 genotypes in Chinese population (OR=4.7),¹⁵ but also with normal c1/c1 genotype in Caucasians and Afro-Americans²² and with variant CC genotype (*CYP2E1*6*).^{16,17} Our data have provided evidence that *CYP2E1* is polymorphic in the North Indian population and both the *RsaI* and *DraI* polymorphisms of *CYP2E1* are present in the North Indian population. This study has provided evidence that polymorphism in *CYP2E1* modifies susceptibility to oral cancer. An increased association of c1c1 and DD genotypes of *RsaI* and *DraI* polymorphisms, respectively, of *CYP2E1* in cases when compared with the controls is shown with an increase in oral cancer risk. Our study is in conformity with several studies that have reported an association of the wild type genotype (c1c1) of *CYP2E1* with esophageal, liver, and lung cancer.¹⁷ Likewise, Uematsu et al¹⁸ reported that the frequency of the variant allele (C) of the *DraI* polymorphism was significantly lower in lung cancer cases when compared with healthy controls. The role of mitochondrial mutations in carcinogenesis is well documented.⁶ Here, we have shown these genotypes, for the first time in literature, to be significantly correlated with mtDNA 4977 bp deletions and subsequent mitochondriopathy of carcinogenesis. However, in

patients with laryngeal cancer, DD (*CYP2E1*6*) and c1/c1 (*CYP2E1*5B*) genotype frequencies did not differ from that in healthy subjects.¹⁹ Sikdar et al²⁰ found that the *DraI* variant allele is associated with enhanced susceptibility to leukoplakia among tobacco users in an Indian population. A similar decrease in head and neck squamous cell carcinoma (HNSCC) risk associated with wild genotypes of *CYP2E1*, particularly *CYP2E1*5B*, has previously been reported in Orientals and Caucasian case-control studies.^{14,21,22} In contrast, Soya et al²³ reported no association of *RsaI* and *DraI* polymorphisms of *CYP2E1* with the risk of upper aerodigestive tract cancers among the South Indians. The reason for differences in results of these studies with ours is most probably due to c1/c1 genotype distribution frequency in healthy subjects of different ethnic groups and specific exposures associated with these polymorphisms.²⁴ Therefore, *CYP2E1* is presumably a genetically determined factor, which is involved in the early stages of carcinogenesis. This study, as well as previous studies, is based on the assumption that *CYP2E1* polymorphisms may affect the level of enzyme expression. However, to date, there is only little evidence that the variant alleles are related to enhance *CYP2E1* activity in vivo. Studies among Caucasians have demonstrated that heterozygous c1c2 or CD subjects displayed low-level *CYP2E1* activity after chronic ethanol consumption, suggesting a lack of inducibility of the mutated genes.⁴ This is in contrast with other studies performed in the Asian subjects, among whom the frequency of the mutated alleles is higher, where *CYP2E1* mRNA levels tended to be higher in the presence of C or c2 alleles.⁴ Further research is needed to confirm the functional implication of the *CYP2E1* polymorphism, potential ethnic variations, and its relationship with mitochondriopathy.

CONCLUSION

This study demonstrates that polymorphism in *CYP2E1* is significantly correlated to oral cancer in a North Indian population. Several fold higher risk has been found in cases carrying wild alleles of *CYP2E1*. These polymorphisms coupled with mtDNA 4977 bp deletion provide a high-risk factor for oral cancer. It may thus be hypothesized that the presence of *CYP2E1* genotypes, which increase oral cancer risk by activating tobacco-specific carcinogens, results in the accumulation of reactive intermediates in individuals that causes increased mtDNA damage and subsequent steps in early carcinogenesis. Infact, mtDNA damage induced by wild *CYP2E1* forms and imperfect DNA repair in the mtDNA may act synergistically to greatly enhance oral cancer risk.

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