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CYP2C19 Polymorphism in Dravidian Subjects with Oral Squamous Cell Carcinoma- A Hospital Based Study

Deepa Gayathri, Jeevitha Gauthaman, Krithika. C. L., Anuradha. G., Yesoda Aniyan. K., Swathi. K.V.

Department of Oral Medicine and Radiology, SRM Dental College, Ramapuram, Chennai: 600 089

Abstract

Oral squamous cell carcinoma is one of the most radically spreading diseases in India and other South-Asian countries. CYP2C19 is a subfamily of CYP450 that contributes to 20% of the total CYP450 enzyme. The *CYP2C19* gene undergoes mapping onto chromosome 10 (10q24.1-q24.3) and encodes a 490-amino-acid protein. Various cytochrome in the gene were responsible in reduced enzyme activity. Poor metabolizer (PM) and extensive metabolizer (EM) phenotypes have been determined based on the capacity to metabolize (*S*)-mephenytoin and other CYP2C19 substrates. The current study was conducted to determine the prevalence of CYP2C19 gene in Dravidian subjects and to determine its functional significance. A total of 30 Non cancer patients and 30 Oral squamous cell carcinoma patients (n=60) fulfilling the inclusion and exclusion criteria were recruited for the study. Blood samples from the patients with clinically and histopathologically diagnosed oral squamous cell carcinoma and normal individuals were taken. The total genomic DNA extracted was amplified by polymerase chain reaction with allele specific primers for CYP2C19 gene. Descriptive analysis was performed to estimate the percentage occurrence of CYP2C19 mutations in 60 samples of both OSCC & Non- OSCC patients. Mean DNA concentration difference between OSCC and Non- OSCC sample is compared by using independent t- test. Pearson correlation & coefficient analysis was used to calculate the relationship of DNA concentration between the groups. Chi- square test was performed to calculate the association of CYP2C19 (#2, #3) between the groups. Data analysis was performed and the percentage obtained from the mutation of CYP2C19*2 genotype was 63.3% in OSCC samples and CYP2C19*3 genotype was 6.7% in OSCC samples. The study did not find any significant difference between the frequency of alleles between the subjects and controls. This study has set a platform to enlighten the role of Genetics polymorphism of CYP2C19 allele in Dravidian population.

Keywords: CYP2C19, allele, oral squamous cell carcinoma, Dravidian population

Full-length article **Corresponding Author*, e-mail: *drjeevitha28@gmail.com*

1. Introduction

Oral squamous cell carcinoma is one of the most radically spreading diseases in India and other South-Asian countries. The lifestyle modifying factors along with certain genetic factors lead to the development of oral squamous cell carcinoma (OSCC). OSCC has a 3-9 times higher occurrence in individuals who use tobacco products and consume alcohol. Genetic polymorphisms of CYP2C19 gene plays an important role in chemotherapeutic drug metabolism thus worsening disease prognosis [1, 2]. Yadav et al in 2008 stated that CYP2C19 enzyme was considered as a predominant CYP isoform which involved in the process of forming active metabolite following clinical medication to liver cells [3].

The polymorphism of CYP genes may cause any of the following: absence of enzyme diminished enzyme expression, enzyme with altered substrate specificity or increased enzyme expression. CYP2C19 is a subfamily of CYP450 that contributes to 20% of the total CYP450 enzyme. Based on the construction of this specific gene, the acquired subjects may be grouped into 4 major phenotypes- poor metabolizers, having two Non-functional genes, intermediate metabolizers being deficient on one allele, extensive metabolizers having two copies of normal genes and ultrarapid metabolizers having three or more functional active gene copies [4, 5]. Mutation of CYP genes may affect the drug metabolism of chemotherapeutic drugs and the availability of their active metabolites. As a result, the therapeutic drugs may not reach effective concentration in patients carrying loss of function polymorphisms in CYP2C19 [6].

The *CYP2C19* gene undergoes mapping onto chromosome 10 (10q24.1-q24.3) and encodes a 490-amino-acid protein. Various cytochrome in the gene were responsible in reduced enzyme activity. Poor metabolizer (PM) and extensive metabolizer (EM) phenotypes have been determined based on the capacity to metabolize (*S*)-mephenytoin and other CYP2C19 substrates. These

cytochromes *CYP2C19*2* were categorized by a 681G→A substitution in exon 5 leading to a splice-defective site, and *CYP2C19*3* which has a wild type mutation in exon 4, obtaining to a premature stop codon. The existence of the specific gene is normally concerned to be adequately predictive for the phenotypes to be concluded [7, 8].

The distribution of polymorphisms of CYP2C19 allele was 0–13% in Japanese and 2–5% in people from Thailand. Goldstein and de Morais et al distinguished genetic defects in CYP2C19 (CYP2C19*2 and CYP2C19*3) that are important for the EM genotype CYP2C19*2 mutation shows in exon 5 of CYP2C19 which processes a splice site in Thai subjects. CYP2C19*3 made of a mature stop codon (G–A) in exon 4 and is resulted in both Japanese and Chinese but no variant seen in Thai population. Thus, individuals heterozygous for the *CYP2C19*2* and *CYP2C19*3* cytochrome are reviewed to be extensive metabolites, when the samples were mutated at least one CYP2C19*2 or *3 gene are categorized as extensive metabolizers (EM) [9]. As far the status of CYP2C19 is concerned, there is not much data available. The current study was conducted to determine the prevalence of CYP2C19 gene in Dravidian subjects and to determine its functional significance.

1.1. Aim

The aim of the current study was to investigate the occurrence of CYP2C19 polymorphism in oral Squamous Cell Carcinoma patients relative to age and gender controls.

2. Materials and Methods

The study was conducted in the Department of Oral Medicine and Radiology, SRM Dental College Ramapuram, Chennai from September 2015 to October 2017. The study was approved by the Institutional Review Board and Ethical Committee (SRMDC/IRB/2015/MDS/NO-902), SRM Dental College Ramapuram, Chennai. All the participants were explained about the details of the research and formatted "informed consent" was acquired from all the subjects before entering into the research. The study was conducted in the department of oral medicine and radiology of SRM dental college, Ramapuram, Chennai: 600089. A total of 30 Non cancer patients and 30 Oral squamous cell carcinoma patients (n=60) fulfilling the inclusion and exclusion criteria were recruited for the study.

2.1. Inclusion criteria

Patients with clinically and histopathologically confirmed oral squamous cell carcinoma, male female ratio were not included as a criterion because the distribution of CYP2C19 polymorphisms is not known to differ among the gender. A broad age range of 30 years and above was added as an inclusion criteria.

2.2. Exclusion criteria

Patients with medical (systemic) complication, unrelated to cancer like infectious diseases, depression, and other neurological conditions were excluded. Terminally ill patients and those patients who cannot be communicated due to the extent of carcinoma induced discomfort and pain were excluded. Patients who were not willing to participate were also excluded from the study. Blood samples from the patients with clinically and histo-pathologically diagnosed oral squamous cell carcinoma and normal individuals were taken. 0.5ml of blood was collected in EDTA coated tubes and stored in refrigerator. Blood samples was collected in EDTA tubes and stored in refrigerator. The blood samples were processed for DNA extraction by silica column-based protocol, which is capable of extracting DNA even from very small amount (less than 0.1ml) of blood samples. The total genomic DNA extracted was amplified by polymerase chain reaction with allele specific primers for CYP2C19 gene. The primers for amplification were optimized after sample collection has been initiated. The PCR protocol used was as follows: 94deg/4mins followed by 35 cycles of 94deg/45 sec, 55deg/45sec, and 72deg/45sec, applied. 'PCR amplicons' was run by agarose gel electrophoresis to identify the CYP2C19 alleles. The study design is given as a flowchart in table 1.

2.3. Genomic DNA Extraction

0.1ml of peripheral blood was lysed in 100µl 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 to enable complete lysis of leucocytes. Following lysis, an equal volume of 100% ethanol 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 1min at room temperature. The precipitated DNA gets captured in the silica membrane during this step. Following DNA capture, the silica columns were washed twice with wash buffer (supplied by the manufacturer Machery Nagel). Degraded proteins and membrane lipid particles get washed off during the wash steps. After the two wash steps, the captured DNA from the silica membrane was eluted with 50µl of elution buffer (supplied by the manufacturer Machery Nagel).

2.4. Allele Specific PCR

Each of the forward primer specifically binds only to its respective allele. The reverse primer was designed as a universal primer and was used with both wild type and mutant forward primers. PCR was performed by combining either 2Af or 2Gf and 2R and 3Af or 3Gf and 3R. If a sample carries A genotype at CYP2C19*2 region, then amplification will be seen only with 2Af and 2R. If a sample carries G genotype at CYP2C19*2 region, then amplification will be seen only with 2Gf and 2R. The designing of primers was done as given in tables 2 and 3. Denaturation at 94°C for 4 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds primer annealing at 55°C for 30 seconds primer extension at 72°C for 30 seconds with a final extension at 72°C for 5 minutes.

2.5. Gel Electrophoresis

10µl aliquots of amplified PCR products was obtained by "TAE (Tris Acetate EDTA) buffer". The

DNA bands were visualized by staining the gel with ethidium bromide (a DNA intercalating agent that fluoresces when excited by UV in the range of 302nm to 364nm), and images were captured with gel documentation unit.

2.6. Statistical Analysis

Descriptive analysis was performed to estimate the percentage occurrence of CYP2C19 mutations in 60 samples of both OSCC & Non- OSCC patients. Mean DNA concentration difference between OSCC and Non-OSCC sample is compared by using independent t- test. Pearson correlation & coefficient analysis was used to calculate the relationship of DNA concentration between the groups. Chi- square test was performed to calculate the association of CYP2C19 (#2, #3) between the groups. All the statistical analysis was done by using SPSS software 22.0.

3. Results

In order to determine the CYP2C19 genotypes in the blood sample of patients with carcinoma, total genomic DNA was extracted using silica column-based protocol as mentioned in the methods section. An equal number of age matched normal individuals were also included as control group. DNA was extracted from control group subjects in a similar manner. To determine the concentration of DNA, a 2µl aliquot of DNA from each of the sample was quantified with fluorescence-based QUBIT fluorometer. The fluorometer expresses DNA concentration in nanogram per microliter.

Data analysis was performed, and the percentage obtained from the mutation of CYP2C19*2 genotype was 63.3% in OSCC samples and CYP2C19*3 genotype was 6.7% in OSCC samples. (Table 4, 5). It should be noted that the concentration (quantity) of the DNA extracted depends largely on the number of leucocytes in the blood. Hence, the variation of the concentration observed among samples indicates that the cell count varied in samples. Nevertheless, as these samples are amplifiable in PCR reactions, all samples were included in the study.

4. Discussion

Oral cancer, a subdivision of Head and Neck Cancers, includes cancers of the lips, tongue, buccal mucosa, gingiva, retromolar trigone, and hard palate [10]. It is the most common type of cancer among men in the Indian subcontinent. Recently, there has been growing interest in identifying the genes involved in the differential susceptibility to OSCC. Several studies have associated the polymorphisms of the CYP450 gene family with incidence of cancer. The enzymatic transformation of therapeutically important compounds into active, inactive or more soluble forms is catabolized by cytochrome P450 system [11]. CYP2C19 and 2D6 are the most studied genetic polymorphisms among different populations, including North Indians. CYP2C19 metabolizes several therapeutically important drugs, namely, omeprazole, lansoprazole, propranolol, imipramine, mephenytoin, chloroguanide, diazepam, mephobarbital, and hexobarbital.

The standard treatment modality for squamous cell carcinoma is chemotherapy, radiotherapy, surgical excision or combination of both. When the combination of multiple drugs is taken as a single dosage, it is necessary to monitor the metabolism of each drug [12]. Enzyme P450 CYP2C19 and its sub-family *2, *3 cytochrome has prime concern in role of therapeutic medicine activation. Thus, the range of activated enzyme levels differs in each patient which subsequently alters the drug dosage level accordingly [13]. Many studies were conducted to examine the polymorphism of sub- types like CYP2C19*17 and *1 from blood samples of patients having Hypertension and cardio vascular diseases using normal PCR assay [14, 15]. However, the ability of normal PCR assay in identifying differential frequency alleles is comparatively lesser than specific allele PCR assays. Thus, in our study we performed Specific allele PCR assay which is an advanced method used to amplify DNA accurately. The strategy in the current research was adapted from previous literature that suggested that CYP2C19 polymorphism occurred in Dravidian population. In the current study, the distribution of CYP2C19*2 was 63.3% and the prevalence of CYP2C19*3 was 6.7%. Our studies were similar to study results done in various population. Previous studies have shown that CYP2C19 occurs with the frequency of 18% to 23% in Japanese subjects, 2% to 5% in white subjects, 11% to 20% in Chinese subjects, 12% in Korean subjects, and as high as 70% in the residents of Vanuatu island in Melanesia.

Lamba et al conducted a study in 2000 to determine the CYP2C19*3 polymorphisms among a north Indian population. The authors performed phenotyping of 100 unrelated healthy subjects and omeprazole used as a probe drug. However, they could not identify CYP2C19*3 polymorphism in those subjects. But in the present study although a smaller sample size was used, CYP2C19*3 polymorphism was seen in the Dravidian population though the prevalence was minimal [16]. Another research was conducted to examine the occurrence of CYP2C19 polymorphism in north Indian subjects were the result shows 12 % was occurred among the population. Similar results were obtained in earlier studies that were conducted in north Indian population. Yadav et al stated that the frequency of CYP2C19*2 was found to be almost similar to that observed in Oriental and other Asian populations, though relatively higher when compared to the Caucasians and Africans. He further suggested that a high occurrence of CYP2C19*2 allele is an earlier indicator for development of head and neck cancer. In our study the levels of CYP2C19*2 and CYP2C19*3 alleles in OSCC samples were 63.3% and 6.7% respectively [3].

Our study did not find any significant difference between the frequency of alleles between the subjects and controls. These results were similar to earlier studies. Sukasem et al in 2013 conducted a study to evaluate the distribution of CYP2C19 polymorphisms among Thai population suffering from cardiovascular disease. A total of 1,051 Thai patients participated in the study. Genotypes for CYP2C19 polymorphisms were detected by the microarray-based technique.

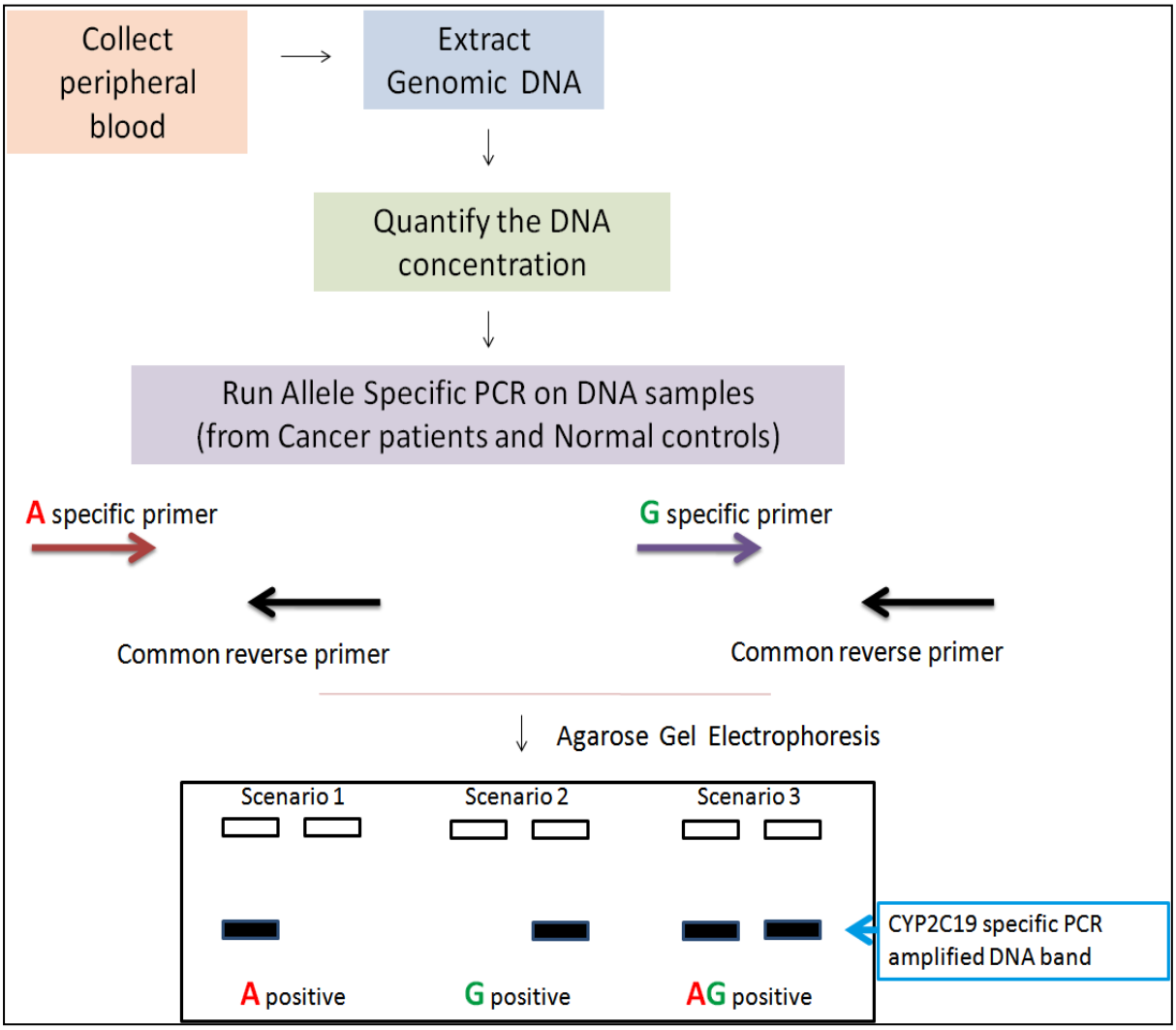

Figure 1. Flowchart of the Protocol

Table 1: Designing primers

GENE	PRIMER CODE	PRIMER SEQUENCE
CYP2C19*2 **A** SPECIFIC	2Af – MUTANT	ATCATTGATTATTTCCC**A**
CYP2C19*2 **G** SPECIFIC	2Gf – WILD TYPE	ATCATTGATTATTTCCC**G**
CYP2C19*2	2R	GTAAACACACAACTAGTCAATG

Table 2: Designing primers

GENE	PRIMER CODE	PRIMER SEQUENCE
CYP2C19*3 **A** SPECIFIC	3Af – MUTANT	GTAAGCACCCCCTG**A**
CYP2C19*3 **G** SPECIFIC	3Gf – WILD TYPE	GTAAGCACCCCCTG**G**
CYP2C19*3	3R	ACTTCAGGGCTTGGTCAATATAGA

Table 3: Comparison of mean DNA concentration between the group

	Group	N	Mean	Std .dev	t-value	p-value
DNA Concentration (nano Gram)	CA	30	27.93	8.017	-0.732	0.467
	Normal	30	29.40	7.5	-0.732	0.467

Table 4: Comparison of "CYP2C19*2" and "CYP2C19*3" genotype between the groups

Genotype	Group		
CYP2C19*2	CA	Normal	Total
	19 63.3%	15 50%	34 56.7%
CYP2C19*3	02 6.7%	01 3.3%	03 5.01%

Thai population are heterogenous in origin and so the authors evaluated the distribution of polymorphs among Asians and Caucasians separately. The authors concluded that there was no difference in frequency of the CYP2C19*2 allelic variants among Asians in this study, but there was a difference in Caucasians [17]. In the present study, the protocol was standardized by the following earlier studies that stated the importance of using allele specific PCR and the same was followed in our study. Also, an enzyme actively involved in therapeutic drug metabolism namely, P450, allele (CYP2C19) was selected. In spite of all this, the study result has not shown significant variation between the research subjects and the controls. Our results can be attributed to the small sample size as the current study was of an 'exploratory' one. Further studies with larger sample size can be initiated to evaluate the prevalence of P450 alleles among Indian population.

5. Conclusion

This study has set a platform to enlighten the role of Genetics polymorphism of CYP2C19 allele in Dravidian population. This exploratory study can be directed in future towards the application of CYP2C19*2,*3 in therapeutic drug- dosage alteration and also, by increasing large sample size it can be assessed in cancer patients undergoing radiation therapy and chemotherapy.

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