

RESEARCH ARTICLE

Genetic Variation in MicroRNAs and Risk of Oral Squamous Cell Carcinoma in South Indian Population

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Abstract

Background: MicroRNAs (miRNAs) are small non-coding RNA molecules, implicated in several activities like initiation, progression and prognosis of various cancers. Single nucleotide polymorphisms (SNPs) in miRNA genes can lead to alteration in mRNA expression, resulting in diverse functional consequences. The aim of our study was to investigate the association of miR-149C>T and miR-196a2C>T SNPs with susceptibility to development of oral squamous cell carcinoma (OSCC) in South Indian subjects. **Materials and Methods:** 100 OSCC patients and 102 healthy controls from the general population were recruited for the study. Genetic analysis was performed by polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) as per a standard protocol. **Results:** The genotype frequencies in miR-196a2 polymorphism, of TT, CT and CC in the OSCC patients were 69%, 10% and 22% respectively while for control group it was 80%, 15% and 5% respectively. The CC genotype of miR196a2 polymorphism was significantly associated with oral squamous cell carcinoma. The genotype frequencies in miR-149 polymorphisms of CC, CT and TT in the oral squamous cell carcinoma (OSCC) patients were 72%, 22% and 6% respectively and for control group 88%, 12% and 0% respectively. CT and TT genotypes of miR149 polymorphism were found to be significantly associated with OSCC ($p = 0.05$ and 0.07). **Conclusions:** Our study suggests that miR-196a2C>T and miR-149C>T polymorphisms may play crucial roles in the development of OSCC in South Indian subjects.

Keywords: miRNA - polymorphisms - oral squamous cell carcinoma - PCR-RFLP - genotypes - South India

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Introduction

Oral cancer is the sixth most common neoplasm occurring globally and accounts for 30% of all cancers in India (Elango et al., 2006). Oral cancer may arise as a primary lesion and is reported that about 90% of oral cancers are squamous cell carcinomas. In the Indian scenario, oral squamous cell carcinoma (OSCC) is the most common cancer in males and it is the third most common cancer in Indian females (Rai et al., 2004). Oral cancer is a disease induced by environment and genetic factors, and the genetic factors include abnormal changes in protein coding genes and non-coding genes such as miRNAs. It is also reported that more than half of the known miRNAs are located in the cancer-associated genomic regions and play an important role in carcinogenesis (Aqeilan et al., 2010).

MicroRNAs (miRNAs) function as important post-transcriptional regulators of mRNA expression by binding to the 3' UTR and resulting in translational repression (Ambros, 2004; Bartel, 2004). It is estimated that at least 30% of protein coding genes are regulated by miRNAs (Lewis et al., 2005). Alterations of miRNA-

coding genes may be of particular importance since a single miRNA regulates a wide spectrum of target genes, a minor change in the function of a miRNA may result in extensive aberrance of tumor-suppressor genes and subsequently contribute to carcinogenesis (Lovat et al., 2011). It is widely accepted that single nucleotide polymorphisms (SNPs) are associated with cancer risk. SNPs occurring in miRNA sequences can therefore affect processing and binding ability of mature mRNAs. The miRNA-associated SNPs can have direct or indirect effect on the protein synthesis. The direct effects include an impairment of miRNA processing or function. Indirect effects are derived from the SNPs in miRNA promoters that affect transcription (Salzman et al., 2013). Recent studies suggest that functional SNPs occurring in miRNA sequences and binding sites of miRNA influences the susceptibility to different cancers. Functional SNPs of miRNA-149 and miRNA-196a2 have been found to be associated with several cancers like breast, colon, liver etc (Jazdzewski et al., 2008; Xu et al., 2008; Yang et al., 2008). We hypothesized that SNPs in these miRNAs may contribute to the susceptibility of OSCC. Our aim was to study the association between miR-149C>T (rs2292832),

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and miR-196a2 C>T (rs11614913) polymorphisms in oral squamous cell carcinoma (OSCC) in South Indian subjects.

Materials and Methods

The study subjects comprised of 100 patients suffering from OSCC and 102 healthy controls. Patients were recruited from the MNJ Institute of Oncology, Hyderabad for a period of 18 months from April 2013. The controls were age and sex matched during the same time period and comprised of healthy volunteers. Using questionnaire the demographic parameters like history of cancer, consumption of alcohol and tobacco chewing habits were recorded. The clinical characteristics were collected from medical records, including tumor differentiation, tumor size, and chemotherapy given. This study was approved by the institutional ethics committee of MNJ Institute of Oncology, and informed consent was obtained from all the participants. Clinical stage of tumor as well as histopathological evaluation was done for confirmation of tumor type.

DNA extraction and genotyping

Two ml of venous blood was obtained from each participant (OSCC cases and healthy controls) and DNA was extracted from all the samples using the DNA mini kit (Epicenter Technologies, USA, www.epicenter.com), as directed by the manufacturer's instructions. The concentration of DNA and the purity of each sample were measured by Nano drop 2000c (Thermo Scientific, USA). DNA samples were routinely stored at -20°C. Using genomic DNA, miRNA polymorphism was identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Sheng et al., 2012).

Polymerase chain reaction (PCR)

The primers were synthesized at a commercial facility in Hyderabad (Bioserve, Hyderabad, India). The primers used for amplification of miR-196a2 and miR-149 genes (Huang et al., 2013) are listed in Table 1. PCR was carried out using a PCR kit (Bioserve) in a total volume of 50 µl. The PCR mixture contained 2.5 µl of 25 mM MgCl₂, 10 mM dNTP mixture, 160 pmol of each primer (forward and reverse primers), 0.2 µl of Taq (5 U/µl) and a DNA template. The reaction volume was made up to 50 µl with sterile water. The PCR reaction was carried out in a IQ5 thermocycler (Bio-Rad, Hercules, CA, USA) using the following optimal conditions. Initial denaturation was carried out at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 35 sec, annealing at 61°C for 35 sec and extension at 72°C for 40 sec. After

completion of 35 cycles, a final extension step was carried out at 72°C for 5 min. The optimized PCR conditions described above were used throughout the study. PCR amplicon size was identified by electrophoresis using 2% agarose gel containing ethidium bromide under standard electrophoretic conditions. The bands were visualized under UV light, and the gel was imaged using the Gel Dock System (Bio-Imaging System, Hyderabad, India).

Restriction fragment length polymorphism (RFLP)

The restriction enzymes MspI and PvuII were used to distinguish the miR-196a2C>T and miR-149C>T polymorphisms, respectively. In miR-196a2C>T polymorphism wild-type genotype CC generated a single band representing the entire 149bp fragment, the variant genotype TT generated 125bp and 24bp fragment and the heterozygote CT genotype displays 3 bands (149, 125 and 24bp). In miR-149C>T polymorphisms wild-type genotype CC generated a single band representing the entire 254bp fragment, the variant genotype TT generated 194bp and 60bp fragment and the heterozygote CT genotype displays 3 bands (254, 194 and 60bp). The restriction products were analyzed by electrophoresis on a 3% agarose gel containing ethidium bromide.

Statistical analysis

The Fisher's exact test (2x2 only) was performed by using MedCalc software for Windows (version 7.4.1.0; Mariakerke, Belgium) to examine the association of miR polymorphisms between cases and controls and different clinical and pathological parameters. The difference between the groups was considered significant if the P<0.05 was less than 0.05.

Results

Clinical characteristics of the study population

The highest percentage of OSCC patients were identified between 46-65 years. The age range of OSCC male patients was 26-74 years and for female OSCC patients age ranged from 21-63 years. In the control group, among 102 healthy subjects, 64 males were in the age range of 20-70 years and 38 females were within the age range of 20 – 50 years. Regarding the primary tumor site, there was a predominance of buccal mucosa (BM) (37.33%), followed by the tongue (22.0%), mandible and oral cavity at 12%, and 10% respectively. In the present study, clinical stage III showed the highest frequency (40%), followed by stage IV (31.33%), and stage II (22%), while stage I (6.67%), showed very low frequency when compared to other groups (Table-2). All tumor samples were confirmed by histopathological evaluation and included in the study.

Table 1. The Primers used for Amplification of miR-196a and miR-149 Genes

miRs	Primer Sequence	PCR Product
miR-196a2 forward primer:	5'-CCC CTT CCC TTC TCC TCC AGA TA -3'	149bp
miR-196 a2 reverse primer:	5'-CGA AAA CCG ACT GAT GTA ACT CCG-3'	
miR-149 forward primer:	5'-TGTCTTCACTCCCGTGCTTGTC-3'	194bp
miR-149 reverse primer:	5'-TGAGGCCCGAAACACCCGTA-3'	

miR-196a2C-T polymorphism and OSCC susceptibility

The genotype frequencies of TT, CT and CC in the OSCC patients were 69% , 10% and 22% and in control group were 80%, 15% and 5% respectively. No significant difference between the patients and controls for CT genotype was observed (p=0.31). The adjusted P<0.05 between the patients and controls for the CC genotype was significant and this shows that there is a relation in risk of OSCC between cases and controls for the miR196a gene polymorphism (p=0.001) (Table-3).

Table 2. Demographic and Clinical Profile of the Subjects Recruited

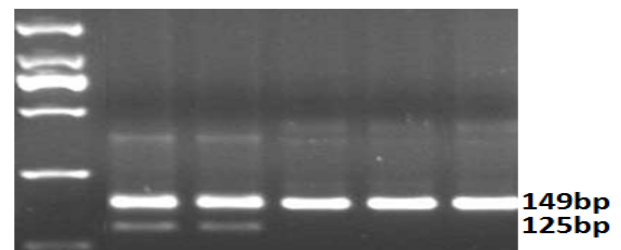
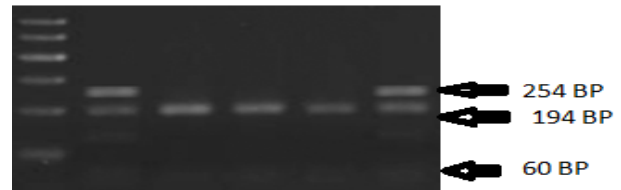
	n = 100 (cases)	n = 102 (Controls)
<i>Demographic characteristics</i>		
<i>Gender</i>		
Males	61(61%)	64(62.74)
Females	39(39%)	38(37.25)
<i>Age distribution</i>		
26-45(years)	19(19%)	29 (28.43)
46-65(years)	62(62%)	58(56.86)
66 and above (years)	19(19%)	15(14.70)
<i>Personal habits</i>		
Alcoholics	16(16%)	-
Smokers	32(32%)	-
Chewing	52(52%)	-
<i>Clinical characteristics</i>		
<i>Site of diagnosis</i>		
Tongue	36(36%)	-
Buccal mucosa (BM)	43(43%)	-
Mandible	12(12%)	-
Oral cavity	9 (9%)	-
<i>Staging</i>		
Stage 1	10 (10%)	-
Stage 2	19(19%)	-
Stage 3	50(50%)	-
Stage 4	21(21%)	-

miR-149C-T polymorphism and OSCC susceptibility

The genotype frequencies of miR-149 showed that CC, CT and TT genotypes in the OSCC patients were 72%, 22% and 6% and for control group were 88%, 12% and 0% respectively. The adjusted p value between the patients and controls for the CT and TT genotypes was significant and this shows that there is a relative risk of OSCC between cases and controls for the miR149 gene polymorphism (p=0.05 and 0.07) (Table-4).

Correlation between genotypes and clinicopathological parameters of OSCC

The association between genotypes and clinicopathological parameters of OSCC was also

**Figure 1. PCR-RFLP Genotyping of SNP miR-196a rs3746444.** Lane: 1 – 100bp Ladder, Lane: 2 & 3 -heterozygous mutations (CT) (149bp, 125bp & 24bp), Lane: 3-5 Homozygous wild type (149bp)**Figure 2. PCR-RFLP genotyping of SNP miR-149 rs3746444.** Lane: 1 – 100bp Ladder, Lane: 2 & 6 - heterozygous mutations (CT) (254bp, 194bp & 60bp), Lane: 3-5 Homozygous (194bp & 60bp)**Table 3. Genotypes Distribution for miR196a2 gene Polymorphism in Oral Squamous Cell Carcinoma (OSCC) and Controls**

miR-196a2 genotyping	Cases (n=100)	Controls (n=102)	Odds Ratio	95% CI	Chi square	p-Value
TT	68(68%)	81(80%)	0.57	0.30-1.09	2.34	0.09
CT	10(10%)	15(15%)	0.64	0.27-1.51	0.64	0.31
CC	22(22%)	6(5%)	4.51	1.74-11.67	9.67	0.001
CT/CC	32(32%)	21(20%)	1.81	0.95-3.34	2.83	0.06
TT/CT	78(78%)	96(94%)	0.23	0.09-0.61	8.7	0.003

p = <0.05 (Significant) OR: Odds Ratio, 95% Confidence intervals, Chi square

Table 4. Genotypes Distribution for miR149 gene Polymorphism in Oral Squamous Cell Carcinoma (OSCC) and Controls

miR-149 genotyping	Cases (n=100)	Controls (n=102)	Odds Ratio	95% CI	Chi square	p-Value
CC	72(72%)	90(88%)	0.34	0.16-0.72	7.39	0.004
CT	22(22%)	12(12%)	2.11	0.98-4.55	3.08	0.05
TT	6(6%)	0(0.0%)	14.1	0.78-253.7	4.39	0.07
CT/TT	26(26%)	12(12%)	2.63	1.24-5.59	5.8	0.01
CC/CT	94(94%)	102(100%)	0.07	0.003-1.25	4.39	0.07

p = < 0.05 (Significant) OR: Odds Ratio, 95% Confidence intervals, Chi square

Table 5. Association between miR-196a2 rs3746444 Genotypes and Demographic & Clinical Characteristics of OSCC Patients

	n=100	TT	CT	CC	P-Value
<i>Demographic characteristics</i>					
<i>Gender</i>					
Males	61(61%)	38(62%)	8(13%)	15(25%)	0.61
Females	39(39%)	30(77%)	2(5%)	7(18%)	0.31
<i>Age distribution</i>					
26-45(years)	19(19%)	16(84%)	1(5%)	2(11%)	0.35
46-65(years)	62(62%)	39(62%)	7(12%)	16(26%)	0.45
66 and Above(years)	19(19%)	13(68%)	2(10%)	4(22%)	0.94
<i>Personal habits</i>					
Alcoholics	16(42%)	11(76%)	2(10%)	3(15%)	0.77
Smokers	32(32%)	28(87%)	1(3%)	3(10%)	0.06
Chewing	52(52%)	34(65%)	5(10%)	13(30%)	0.008
<i>Clinical characteristics</i>					
<i>Site of diagnosis</i>					
Tongue	36(36%)	24(66%)	3(8%)	9(25%)	0.74
Buccal mucosa (BM)	43(43%)	24(56%)	7(16%)	12(27%)	0.04
Mandible	12(12%)	12(100%)	0(0.0%)	0(0.0%)	0.29
Oral cavity	9(9%)	8(89%)	0(0.0%)	1(11%)	0.46
<i>Staging</i>					
Stage 1	10(10%)	10(100%)	0(0.0%)	0(0.0%)	0.37
Stage 2	19(19%)	12(63%)	1(6%)	6(31%)	0.55
Stage 3	50(50%)	33(66%)	6(12%)	11(22%)	0.50
Stage 4	21(21%)	13(62%)	3(14%)	5(24%)	0.43

Table 6. Association between miR-149 rs3746444 Genotypes and Demographic & Clinical characteristics of OSCC Patients

	n=100	CC	CT	TT	P-Value
<i>Demographic characteristics</i>					
<i>Gender</i>					
Males	61(61%)	41(85%)	14(23%)	6(10%)	0.57
Females	39(39%)	31(79%)	8(21%)	0(0.0%)	0.12
<i>Age distribution</i>					
26-45(years)	19(19%)	15(79%)	4(21%)	0(0.0%)	0.78
46-65(years)	62(62%)	45(72%)	12(20%)	5(8%)	0.50
66 and Above(years)	19(19%)	12(62%)	6(33%)	1(5%)	0.27
<i>Personal habits</i>					
Alcoholics	16(42%)	8(50%)	8(50%)	0(0.0%)	0.008
Smokers	32(32%)	26(87%)	6(13%)	0(0.0%)	0.44
Chewing	52(52%)	38(73%)	8(15%)	6(12%)	0.09
<i>Clinical characteristics</i>					
<i>Site of diagnosis</i>					
Tongue	36(36%)	27(75%)	7(19%)	2(6%)	0.62
Buccal mucosa (BM)	43(43%)	28(65%)	11(24%)	4(11%)	0.05
Mandible	12(12%)	9(75%)	3(25%)	0(0.0%)	0.88
Oral cavity	9(9%)	8(88%)	1(12%)	0(0.0%)	0.37
<i>Staging</i>					
Stage 1	10(10%)	10(100%)	0(0.0%)	0(0.0%)	0.16
Stage 2	19(19%)	14(74%)	4(21%)	1(5%)	0.89
Stage 3	50(50%)	35(70%)	12(24%)	3(6%)	0.62
Stage 4	21(21%)	13(62%)	6(28%)	2(10%)	0.34

analyzed in our study (Table-5& Table 6). When miR196a2 and miR149 polymorphisms were correlated with the gender and age, it was noted that there was no significant difference between males, females and age groups. Buccal mucosa (BM) showed a high frequency of cancer development when compared with other sites and we found a relation between carcinoma in buccal

mucosa (BM) and CC genotype (p=0.04) of miR196a2 polymorphism. With reference to the association between the genotype and the clinical staging, no relation between these two polymorphisms and the clinical staging was seen. The observation reveals that patients with CT genotype of miR149 polymorphism showed relationship with personal habits like tobacco chewing (p=0.008).

Discussion

MicroRNAs are small non-coding RNA molecules, which participate in diverse biological processes and may regulate tumor suppressor genes or oncogenes. The dysfunction of miRNAs which target oncogenic or tumor suppressor activity may influence the development of cancer (Gregory et al., 2005; Sassen et al., 2008; Kwak et al., 2010; Yoon et al., 2012). Single nucleotide polymorphisms (SNPs) in miRNA may contribute to diverse functional consequences, including cancer development, by altering miRNA expression (Xu et al., 2011). This type of genetic variant may influence the processing of the mature miRNA (Hu et al., 2008). Numerous studies have shown the association between miRNA SNPs and cancer risk. In our study, we performed a case-control study of the two most commonly studied SNPs in miRNA genes, miR-149C>T (rs2292832), and miR-196a2 C>T (rs11614913) to investigate the risk of OSCC in the South Indian population.

Based on the important role of that rs11614913 polymorphism located in the hsa-miR-196a2 3' mature sequence affects the miRNA maturation and its target mRNA possibility (Ryan et al., 2010 and Chu et al., 2011), it is biologically plausible that genetic variants of hsa-miR-196a2 could modulate the susceptibility to cancer. Several studies reported that 196a2 (rs11614913 cytosine to thymine) polymorphism, was associated with multiple kind of cancers, such as lung cancer (TianTian et al., 2009), breast cancer (Lin et al., 2011), gastric cancer (Okubo et al., 2010; Peng et al., 2010; Daehoahn et al., 2013), liver cancer (Peng et al., 2010; Akkız et al., 2011), glioma (Tonghai et al., 2010), prostate cancer (Ginu et al., 2011), esophageal cancer (Kaiwang et al., 2010, NaWang et al., 2014). When reviewed together, the majority of these studies described significant associations of CC genotype of 196a2 polymorphism and cancer risk. In the present study, our results also show that CC genotype of miR-196a2 polymorphism was significantly associated with OSCC (p=0.001). Similar results were reported in multiple cancers, including squamous cell carcinoma of head and neck (Sheng et al., 2010). We suggest that this polymorphism could play an important role in development and progression of oral squamous cell carcinoma. In addition to cancer risk, this polymorphism may also be associated with poor prognosis (Shen J et al., 2008).

The single nucleotide polymorphism of miR-149 gene was first reported by Hu et al. (2008). A C>T genetic polymorphism was found in the miR-149 gene and was located in the stem region next to the mature miR-149C>T sequence. Regarding miR-149C>T, it is a proapoptotic miRNA to repress the expression of Akt1 and E2F1. Silencing of Akt1 and E2F1 can induce apoptosis in human tumor cell lines (Lin et al., 2011). Previous studies showed that polymorphism in miR-149C>T can change the expression of mature miRNAs or the binding activities to target mRNA, and thus influence cancer risk through various mechanisms (Liu et al., 2010). Our study found that CC genotype of miR-149C>T was associated with

risk of OSCC. Similar results were reported in head and neck cancer by Liu et al. (2010). It was also reported that a combined risk genotype of two SNPs on miR-149, and miR-196a2 was found to be significantly associated with cancer risk. We postulate that this type of genetic variants may influence the processing of the mature miRNA for translational repression.

The association between genotypes and clinicopathological parameters of OSCC was also analyzed in our study. It was noted that there was no significant association between miR196a2 and miR149 polymorphisms with gender or age. The frequency of TT genotypes in miR196a showed higher frequency in advanced stages of OSCC when compared to early stage. We suggest that TT OSCC patients seem to have more advanced tumor progression. Hsi-FengTu, (2012) also reported that TT in miR196a HNSCC patients seems to have a poorer prognosis. We found significant association between TT genotype in miR196a and miR149 and buccal mucosa. The earlier studies also identified the association between TT genotype in pre-miR-149 and the poor survival of HNSCC occurring on buccal mucosa. Hsi-FengTu, et al, also reported that HNSCC patients with TT genotypes are associated with chewing habitual risk. Similarly we also observed that patients with CT genotype in miR149 polymorphism and CC genotype in miR196a2 showed relationship with chewing habit of patients.

As cancer incidence is projected to increase over the decades there is a need for effective research on cancer prevention strategies. Some evidences suggest that altering the personal and dietary habits may be one of the approaches for reducing the cancer risk. Our study demonstrates that miR-196a2C>T and miR-149C>T polymorphisms may play a crucial role in the development of OSCC in South Indian population. These miR SNPs which are associated with the susceptibility to the oral cancer may be useful as biomarkers for the early prediction of the disease and for prevention strategies.

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