Journal of Advanced Scientific Research

Available online through http://www.sciensage.info

ISSN
0976-9595
Research Article

ASSOCIATION OF HIGH RISK HUMAN PAPPILOMA VIRUS SUBTYPES 16 AND 18 WITH P53 GENE POLYMORPHISMS IN ORAL SQUAMOUS CELL CARCINOMA

Sonal Mayur Chitroda*[1], Menka Shah[2], Piyush Chudasama[3]

[1]*Smt. L. P. Patel Institute of Medical Laboratory Technology, Karamsad, Anand, Gujarat India*

[2]*Department of Pathology, Pramukhswami Medical College, Karamsad, Anand, Gujarat, India*

[3]*Sat Kaival hospital Pvt Ltd, Anand, Gujarat, India*

**Corresponding author: sonalmc@charutarhealth.org*

ABSTRACT

HPV infections particularly the HPV types 16 and 18 have found associated with the mutations of p53 tumor suppressor gene in oral carcinoma. The present study is aimed to evaluate the role of HPV infections with the commonly found mutations of p53 gene in oral squamous cell carcinoma. The study includes 113 of control samples from healthy individuals & 102 of test samples from the confirmed cases of oral squamous cell carcinoma. All the test and control blood samples were analysed for HPV type 16 & 18 infections and also for the polymorphisms of p53 gene by using PCR-RFLP method. HPV type 18 infection was not detected in any of the control and test samples. Out of all the cases, 22.55% (23cases) of test samples & 19.47% (22 cases) of control samples were found positive for HPV type 16 infection. HPV type 16 infections were found more prevalent in the polymorphism of codon 249 at exon 7 of p53 gene in OSCC. The result suggested that there is a significant association of HPV type 16 infections with the polymorphisms of codon 249 at exon 7 of p53 gene in OSCC.

Keywords: Human Papilloma Virus, tumor suppressor gene, p53 polymorphisms, oral squamous cell carcinoma, PCR-RFLP.

1. INTRODUCTION

Oral cancer is the sixth most common cancer worldwide [1]. It includes a group of neoplasms affecting any region of the oral cavity, pharyngeal regions, and salivary glands. The most important risk factors for oral squamous cell carcinoma are use of tobacco or betel quid and regular drinking of alcohol beverages. Apart from that, infection with high risk human papilloma virus (HPV) genotypes, and a diet low in fresh fruits and vegetables have also recently been implicated in the aetiopathogenesis of oral squamous cell carcinoma [1,2].

However, as not all persons who practice these high risk habits like tobacco chewing and drinking alcohol will develop oral squamous cell carcinoma, as OSCC may be idiopathic, there must be person specific genetic characteristics and environmental factors which may either afford protection against the development of OSCC or may predispose to or even promote the development of OSCC. The development of OSCC is a multistep process requiring the accumulation of multiple genetic alterations, influenced by a patient's genetic predisposition as well as by environmental influences. Such genetic alterations consist of two major types: tumor suppressor genes, which promote tumor development when inactivated; and oncogenes, which promote development when activated. Tumor suppressor genes can be inactivated through genetic events such as mutation, loss of heterozygosity, or deletion, or by epigenetic modifications [3].

The loss of activity of the p53 protein can be achieved either by a mutation of the p53 gene [4] or by binding to the HPV encoded E6 proteins [5]. In oral cancer, both mechanisms of inactivating p53 may play a role in carcinogenesis. Several studies have shown high frequencies of p53 mutations in HPV-positive oral cancers, analyzing the relationship between p53 mutations and HPV infection in malignant oral tumors [6, 7].

The role of HPV in the development of anogenital cancers has been widely studied, and current evidence shows that HPV infection is necessary for the development of most cervical cancers.[8] Approxi-mately 80-90% of cervical carcinomas contain HPV

DNA and the predominant or high risk types appear to be HPV 16, 18 and 33 [9]. HPV E6 and E7 proteins, consistently expressed in HPV transformed and HPV positive tumours can exert their oncogenic potential by inactivating the products of p53 gene [8, 10] and the retinoblastoma (Rb) gene [11]. In oral malignant lesions the state of HPV infection has been reported to be as high as 76% [12], although there are still conflicting results in infection rate and role of HPV in oral carcinogenesis.

The most common genetic polymorphisms found in oral cancer are single nucleotide polymorphism at codon 72 in exon 4, codon 249 in exon 7, 16 bp duplication in intron 3 and G>A transition in intron 6 that are found to alter the structure and function of the p53 protein. Genetic polymorphisms at the gene involved in carcinogenesis may determine individual susceptibility of cancer. Storey *et al*, [13] studied the probable association between p53 polymorphisms and HPV associated cervical carcinogenesis. They found that population homozygous for Arg-72 (arginine) is more susceptible to develop cervical cancer than heterozygous population. Another study from eastern India also found codon 72 polymorphism to be more susceptible to HPV infection and oral carcinogenesis [14]. Balderas-Loaeza *et al,* [15] reported the association of HPV-16 with oral squamous cell carcinoma and found that carcinogenic processes are under the influence of HPV-16 in most of the oral malignant lesions. So the present study is aimed to evaluate the role of high risk HPV subtypes HPV type 16 and HPV type 18 in oral squamous cell carcinoma.

2. MATERIAL AND METHODS

2.1. Study subjects

This study was approved by the Institutional Human Research Ethics Committee, Pramukhswami Medical College, Karamsad. Patients that were histopathologically diagnosed for oral squamous cell carcinoma were included as test subjects for the study. Control subjects were the voluntary healthy individuals genetically unrelated to the patients and had no previous history of any type of cancer. Informed concern and brief history of consumption of alcohol and tobacco chewing habit was taken from all the test and control subjects.

2.2. Methodology

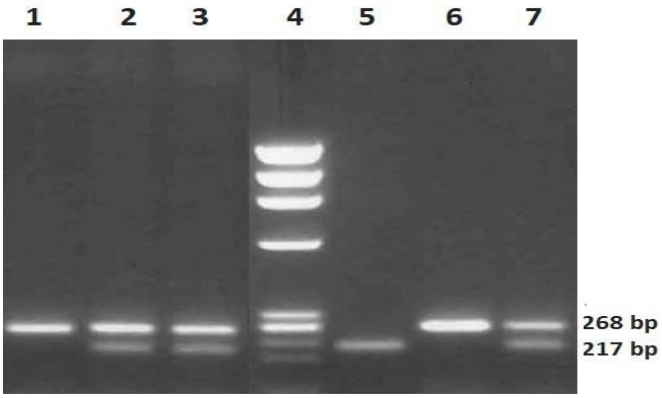
Blood samples from all the study subjects were collected in EDTA vacutte and stored at 4°C until analysis. All the collected blood samples were then processed for DNA isolation by using HiPurA™ Multi-sample DNA Purification Kit manufactured by HIMEDIA. Isolated DNA was analysed for the detection of HPV type 16 & HPV type 18 and also for p53 genotyping by polymerase chain reaction followed by Restriction Fragment Length Polymorphism (PCR-RFLP) method. Exon 4, exon 7, intron 3 and intron 6 were amplified within which the polymorphisms fall, using primers as mentioned in earlier studies [16, 17]. For detection of HPV type 16 DNA were amplified by using the Forward primer: 5'ACC CAG TAT AGC TGA CAG T 3' and Reverse primer: 5' CTC GTT TAT AAT GTC TAC ACA 3'. For detection of HPV type 18 DNA were amplified by using the Forward primer: 5'ATA GCA ATT TTG ATT TGT C 3' and Reverse primer: 5' AAA CTC ATT CCA AAA TAT G 3'. The PCR products were resolved by 2% gel electrophoresis and 15% polyacrylamide gel electrophoresis and then visualized after staining with ethidium bromide.

The result for 16 bp duplication in intron 3 was directly interpreted from 15% PAGE analysis of the PCR products. PCR products of intron 3 were result in either 432 or 448 bp DNA fragments depending on the presence of 16 bp duplication in intron 3 of template genomic DNA. Homozygotes for the absence of duplication (A1/A1) produced band of 432 bp DNA fragment; heterozygotes produced both the bands of 432 & 448 bp (A1/A2) and homozygotes for the presence of 16 bp duplication (A2/A2) produced band of 448 bp DNA fragment.

The PCR product for intron 6 was 913 bp DNA fragment which was then further digested with enzyme *Ncil* (Thermoscientific, Banglore), followed by 15% PAGE and visualized after staining with ethidium bromide. Homozygotes for the absence of *Nci*1 restriction site produced band of 563 bp DNA fragment (A/A); heterozygotes for *Nci*1 restriction site produced bands of 563 bp, 286 bp and 277 bp DNA fragments (G/A) and homozygotes for the presence of *Nci*1 restriction site produced two bands of 286 bp and 277 bp DNA fragments (G/G). All the three types of individuals mentioned above contained band of 350 bp DNA fragment due to presence of nonpolymorphic *Nci*1 site in the amplicon. Reproducibility of the assay was confirmed by repeating the samples two times.

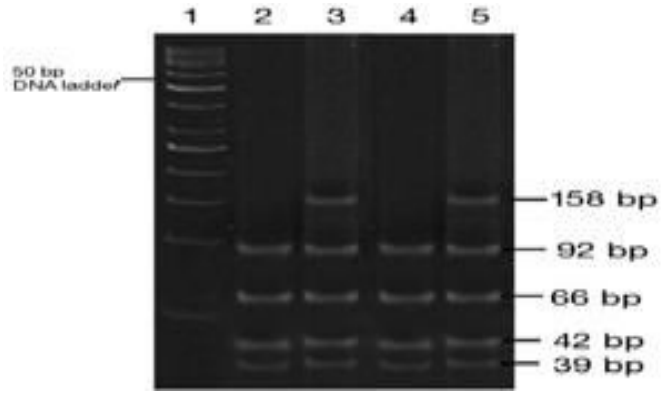
The expected size of the PCR product for codon 72 located in the exon 4 of the p53 gene was 309 bp, which was then cleaved by the enzyme *BstU*1

(Thermoscientific, Banglore). After being cleaved by restriction enzyme it gave two DNA fragments having size of 174 bp and 135 bp according to the presence and absence of restriction site for the enzyme.



Lane 4 represents 100 bp DNA ladder. Lane 2,3,7 represents HPV type 16 positive bands of 217 bp. Lane 1 & 6 represents bands for HPV 16 negative cases. Lane 5 represents positive control of HPV type 16 DNA.

Fig. 1: HPV type 16 detection by PCR.



Lane 1 represents 50 bp DNA ladder; Lane 2, 4 represent homozygous for the presence of restriction site (Arg/Arg) ; Lane 3,5 represent heterozygous for the Arg/ Ser allele at exon 7 of p53 gene.

Fig. 2: Amplified products of exon 7 of p53 gene after HaeIII digestion.

The expected size of the PCR product for exon 7 was 286 base pairs and this fragment was then cleaved by the enzyme *Hae*III (Thermoscientific, Banglore) and gave 92 bp, 66 bp, and several small fragments from the 286 bp DNA product of the PCR reaction as shown in figure 2. If there is a polymorphism at codon 249, it results in an uncleaved 158 bp fragment and this feature will be distinguished from that of normal samples on 15% PAGE. Absence of the band at 286 bp (full-length PCR products) provides a control for complete digestion of the PCR product.

2.3. Statistical analysis

The statistical analysis was done by using SPSS software version 17. Chi-square was used to analyse categorical variables and the association between HPV infection and p53 polymorphisms. Comparisons were made between different genotypes and P-values less than 0.05 were considered statistically significant.

3. RESULTS

The present study included 102 cases of oral squamous cell carcinoma and 113 cases of control or healthy individuals. As shown in the Table 1, out of 102 cases of test samples, HPV type 16 was detected in 23 cases (22.55%) and out of 113 cases of control samples it is found in 22 cases (19.47%). HPV type 18 was not detected in any of the test or control blood samples. So, the present study did not found any association of HPV type 18 infections with OSCC.

As shown in the Table 2, out of 23 positive cases of HPV type 16 infections, codon 249 polymorphism in exon 7 of p53 gene found more prevalent *i.e.*, in 9 cases (39.13%) as compared to that of in control sample *i.e.*, 2 cases (9.09%). Other polymorphisms like 16 bp duplication in intron 3, G>A transition in intron 6 and codon 72 at exon 4 of p53 gene did not found any significant association with HPV type 16 infections in OSCC. Polymorphism of codon 72 in exon 4 of p53 gene found more prevalent *i.e.*, in 10 cases (43.48%) out of 23 cases positive for HPV type 16 infection compared to that of only 3 cases (13.64%). But it did not found statistically significant association with HPV type 16 infections in OSCC.

Table 1: Prevalence rate of Human Papilloma Virus type 16 & 18 in OSCC

TYPE OF HPV	RESULT	TEST SAMPLE (n = 102)	CONTROL SAMPLE (n = 113)
HPV 16	Positive	23 (22.55%)	22 (19.47%)
	Negative	79 (77.45%)	91 (80.53%)
HPV 18	Positive	00	00
	Negative	102	113

Table 2: Association of HPV type 16 with p53 polymorphisms in OSCC

POLYMORPHISMS		P53 GENOTYPE			Chi ^{2} Value P - value
		(1-1)*	(1-2)*	(2-2)*	
INTRON 3	Test	16 (69.56%)	05 (21.74%)	02 (08.69%)	0.3113/
16 bp duplication	Control	14 (63.64%)	05 (22.73%)	03 (13.64%)	0.905
INTRON 6	Test	04 (17.39%)	10 (43.48%)	09 (39.13%)	0.3113/
G>A Transition	Control	05 (22.73%)	08 (36.36%)	09 (40.90%)	0.928
EXON 4	Test	02 (08.69%)	11 (47.82%)	10 (43.48%)	7.3506/
Codon 72	Control	08 (36.36%)	11 (50.00%)	03 (13.64%)	0.026
EXON 7	Test	07 (30.43%)	07 (30.43%)	09 (39.13%)	5.8177/
Codon 249	Control	12 (54.55%)	08 (36.36%)	02 (09.09%)	0.062

**Here in Table 2, 1 = A1 at intron 3, A at intron 6, Proline at exon 4 and Arginine at exon 7, 2 = A2 at intron 3, G at intron 6, Arginine at exon 4 and Serine at exon 7*

4. DISCUSSION

Although there have been several studies reported to evaluate the role of HPV infection in cervical cancer, oesophageal cancer and oral cancer, the role of this virus in development of cancer is still not clearly understood. The present study reported the prevalence of high risk HPV subtypes in association with commonly found p53 polymorphisms in oral squamous cell carcinoma. HPV DNAs have been found in various locations in the human body and there is some certainty that HPV infections play an important role in carcinogenesis, especially in genital lesions [18]. As oral mucosa is covered by squamous epithelium that resembles cervical epithelium, it is important to investigate the relationship between HPV infections and oral cancers. Many researchers have reported frequent association of the HPV infections in oral cancers, but the exact nature of its relationship to oral carcinogenesis still remains undetermined [19, 20].

The present study analyzed 102 of test blood samples, 113 of control blood samples for the presence of HPV type 16 and HPV type 18 infections. We did not find any significant association of HPV infections with oral cancer risk. From the present study it is found that HPV infections are important but may not be sufficient to develop malignancies and that synergistic actions with other carcinogenic agents may be required. There is also a question regarding a singular role for HPV infections in oral carcinomas as few authors reported high prevalence of HPV infections in a normal control population also [21, 22].

In population from Eastern India prevalence of HPV infections is 33.6% [14] as compared to with 67% in South India [23] & 15% in Western India [24]. The HPV infection is more prevalent in OSCC in India compared to 23% of the prevalence in the Japanese population [25]. However, HPV plays an important role in the development of OSCC in the Indian population. Nagpal *et al*, [14] studied the prevalence of HPV type 16 and 18 infections in OSCC in the population of Eastern India and found the prevalence of HPV type 16 infection 22.7%, HPV type 18 infection 14.5% and 10% of prevalence for HPV type16&18 coinfection. This shows that HPV type 18 infections is not as prevalent and significant in the development of oral cancer, which is in agreement with the present study and in disagreement with the study of Balaram *et al*, [23] in the south Indian population.

From the present study we found that codon 249 polymorphism in exon 7 of p53 gene is more prevalent compared to the other commonly found polymorphisms in OSCC. Previous study by Hamel *et al,*[26] in head and neck squamous cell carcinoma cases in Canada found no association between codon 72 polymorphism of p53 gene & risk of oral cancer. This is in agreement with the present study that did not found any significant prevalence of codon 72 polymorphism of p53 gene with HPV infections in OSCC.

4.1. Strength and limitation of the study

The strength of this study is the large sample size and it includes combined analysis of the four polymorphisms commonly found in the p53 gene in the high risk subtypes of HPV in OSCC. However, the issue is still a matter of controversy. This study has a limited power and includes the limited population from the Gujarat, India. So additional studies including larger cohorts and with different geographical populations with other risk factors of cancer are warranted to confirm the biological effect of genetic variation in oral cancer.

5. ACKNOWLEDGEMENTS

The authors are grateful to all the participants of the study for their valuable support and are also thankful to CHARUSAT University, Changa to permit the molecular work to be done at their institute.

6. REFERENCES

1. Shah JP and Gil Z. *Oral Oncology*, 2009; **45 (4):**394-401.
2. Petti S. *Oral Oncology*, 2009; **45 (4):**340-350.
3. Choi S, Myers JN. *J Dent Res*, 2008; **87**:14-32.
4. Crook T, Wrede D, Vousden KH. *Oncogene*, 1991; **6**:873-875.
5. Scheffner M, Werness B, Huibregtse J et al. *Cell*, 1990; **63**:1129-1136.
6. Brachman DG, Graves D, Vokes E et al. *Cancer*, 1992; **52**:4832-4836.
7. Barten M. Ostwald C, Milde-Langosch K et al. *Virchows Arch*, 1995; **427**:153-157.
8. Zur Hausen H. *Curr Topics Microbiol Immunol*, 1994; **186**:131-156.
9. Yoshikawa H, Kawana T, Kitagwa K et al. *Jpn J Cancer Res*, 1991; **82**:524-531.
10. Levine AJ. *Virology,* 1990; **177**:419-426.
11. Dyson N, Howley PM, Munger K, Harlow E. *Science*, 1989; **243**:934-937.
12. Snijders PJF, Van den Brule AJC, Meijer CJLM, Walboomers JMM. *Curr Topics. Microbiol Immunol.*, 1994; **186**:177-198.
13. Storey A, Thomas M, Kalita A, et al. *Nature (Lond)*, 1998; **393**:229-234.
14. Nagpal JK, Patnaik S and Das BR. *Int. J. Cancer*, 2002; **97**:649-653.
15. Balderas-Loaeza A, Anaya-Saavedra G, Ramirez Amador V, et al. *Int. J. Cancer,* 2007; **120**:2165-2169.
16. Mitra S, Chatterjee S, Panda CK, et al. *Annals of Human Genetics*, 2003; **67**:26-34.
17. Vijayaraman KP, Veluchamy M, Murugesan P et al. *Asian Pacific Journal for Cancer Prevention*, 2012; **13(2):**511-516.
18. Herrington CS. *Journal of Clinical Pathology*, 1995; **48**:1-6.
19. Brachman DG, Graves D, Vokes E et al. *Cancer Research,* 1992; **52:**4832-4836.
20. Barten M, Ostwald C, Milde-Langosch K et al. *Virchows arch*, 1995; **427**:153-157.
21. Jenison SA. Yu XP. Valentine JM et al. *Journal of Infectious Diseases*, 1990; **162**:60-69.
22. Jalal H. Sanders CM. Scully C, Maitland NJ. *Journal of Oral Pathology and Medicine*, 1992; **21**:465-470.
23. Balram P, Nalinakumar KR, Abraham B, et al. *Int J Cancer*, 1995; **61:**450-454.
24. Costa DJ, Saranath D, Dedhia P, et al. *Oral Oncol Eur J Cancer*, 1998; **34**:413-420.
25. Shindoh M, Chiba I, Yasuda M, et al. *Cancer,* 1995; **76**:1513-1521.
26. Hamel N, Black MJ, Ghadirian P, et al. *Br J Cancer,* 2000; **82**:757-759.