

p53 polymorphism and association of human papillomavirus in oral submucous fibrosis and oral squamous cell carcinoma: A case–control study

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Abstract

Introduction: The tumor-suppressor p53 protein is inactivated by the human papillomavirus (HPV) E6 oncoprotein, causing polymorphism of the p53 at codon 72 of exon either proline (Pro) or arginine (Arg). Specific allele predisposition has been reported in the literature. The association between the p53 allele and HPV types has been reported. We analyzed the association between p53 polymorphism at codon 72 and HPV 16 and 18 genotypes in control, oral submucous fibrosis (OSF) and oral squamous cell carcinoma (OSCC).

Materials and Methods: Of the total 90 cases, biopsy tissues of all groups (30 cases of OSF, OSCC and control each) were collected to extract DNA. Polymerase chain reaction was used to detect HPV 16 and 18 and alleles of codon 72 in p53 were evaluated in all the samples.

Results: In control, OSF and OSCC samples showed the presence HPV 63.3%, 33.3% and 60%, respectively. In OSF, HPV 16 and 18 was detected in four and four cases, respectively, whereas in OSCC, HPV 16 and 18 was detected in ten and nine cases, respectively. In all three groups, predominantly, Arg/Arg protein was present followed by Pro/Pro and Arg/Pro. Among the control, Arg/Arg type protein was frequently seen followed by Arg/Pro, Pro/Pro in the presence of HPV. OSF and OSCC were associated homologous genes in the presence of HPV.

Conclusion: The definite association between p53 codon 72, polymorphism and HPV 16 and 18 was seen in OSCC with low frequency in OSF. Frequency of homozygous genotype is at high risk in the presence of HPV 16 and 18 in developing OSCC.

Keywords: Human papillomavirus, oral squamous cell carcinoma, oral submucous fibrosis, p53

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is a major disease, and it constitutes the sixth most common malignancy in the world. OSCC is often preceded by preexisting potentially

malignant disorders such as oral submucous fibrosis (OSF).^[1] The prevalence of OSF reported by Gupta *et al.* is 28.3%, which is much higher than that of leukoplakia (13.4%).^[2] The disease is prevalent in the 2nd and 3rd decades with

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malignant transformation rate as high as 7.6%.^[3] The etiology associated is betel nut either in the form of betel quid or gutkha, and its etiopathogenesis is multifactorial. In addition, the high-risk human papillomavirus (HPV) is also been detected in OSF and OSCC.^[1]

HPV detection in potentially malignant and OSCC showed many discrepancies, but HPV 16 and 18 genotypes were most frequently found virus. HPV 16 associated carcinogenesis is mediated by expression of viral E6 and E7 oncoproteins which cause deregulation of the cell cycle by inactivating p53 and pRb response. Loss of function of p53 gene, that is, the tumor suppressor gene can occur by mutations by viral oncoproteins, such as E6 of HPV. HPV is a small DNA virus with over 70 different types. It plays an important role in the pathogenesis of cervical, oral and pharyngeal carcinoma. The percentage of recorded virus varies from 0% to 100%, the possible reasons for such a large variation are the use of different detection methods and differences in the populations studied. One of the major difficulties in the detection of HPV infection in oral cancer is the presence of the virus in only a subpopulation of cells and at low copy number in the infected cells, highly sensitive and controlled methods are therefore required.^[1,4,5]

To the best of our knowledge, this will be the first study to examine high-risk HPV and p53 genotypes in OSF, OSCC and control using tissue in the North Karnataka population. Our aim is to screen for high-risk HPV genotypes in tissue samples of OSF, OSCC and controls. Moreover, explore the association between p53 and HPV in OSF and OSCC patients. Further analyzed the correlation between the habit, site and clinical staging of the disease with the presence of HPV.

MATERIALS AND METHODS

Study population

All the 60 patients (30 OSFs and 30 OSCCs) included randomly from rural and urban areas have visited Oral Medicine department. Among all 30 healthy controls were age- and sex-matched individuals. The procedure and the significance of the study was explained to the patients. After thoroughly explained to them, a written consent form was obtained from the patients who were willing to participate in the study. The study was approved by the Institutional Ethical Committee Board (IRB.No. 2013/S/OP/12).

Sample collection, fixation and storage

Tissue

Punch biopsy tissue sample (20 mg) is collected, preserved and fixed in a 2 ml of Eppendorf tube containing RNA later (DNA-/RNA-stabilizing solution) from all the above

referred and identified individuals. The Eppendorf tube containing tissue samples were preserved and stored at -80°C deep freezer. HeLa cell lines were obtained from the National Center for Cell Sciences, Pune, India. Further, these cell lines were used as a positive control in all the polymerase chain reaction (PCR).

Genomic DNA isolation

DNeasy Tissue Kit was used to isolate the genomic DNA from OSF, OSCC and control were done and was confirmed by 0.8% of agarose gel electrophoresis; and the DNA bands were observed by gel documentation. DNA quantification was done on microvolume ultraviolet spectrophotometer (Quawell Q3000).

Primer design

Specific primers were designed for HPV consensus sequences and HPV 16 and 18 genotypes.

Electrophoresis

After the PCR amplification, the amplicons are run through 4% agarose gel electrophoresis and the DNA bands were observed in gel documentation.

Polymerase chain reaction

PCR-based detection of HPV sequences in clinical samples is done as per the standardized protocol.

Polymerase chain reaction-restriction fragment length polymorphism

- The forward primer and reverse primer are used to amplify the region containing p53: Arg72Pro polymorphism (rs1042522)

n	Primer sequence	5'-3'
1	p53 F p53 R	TGCTCTTTTCACCCATCTAC ATACGGCCAGGCATTGAAGT

- PCR product is digested with 2 U of BstUI at 60°C about 4 h. DNA fragments are run on a 2% agarose gel stained with ethidium bromide. (i) The arginine (Arg) allele is cleaved by BstUI, yielding two fragments of 213 and 140 bp. (ii) The proline (Pro) allele not cleaved by BstUI and has a single 353 bp band. (iii) Heterozygotes contain all three bands, i.e., 353, 213 and 140 bp.

Following the clinical examination, the demographic details of patients were recorded in the preprepared case pro forma, and data were entered in the excel sheet for the further analysis.

RESULTS

In the present study, we noted OSF patients below 40 years of age were 80% and the patients above 40 years of age

were only 20% in comparison, and in contrast, OSCC patients above 40 years of age were 70% and the patients below 40 years were 30%. In both the groups, male predominance was observed [Table 1].

HPV presence was frequent in the control group. When the association between the individual variants and the three groups were assessed, HPV 16 and 18 combined ($P = 0.01$) and other type ($P = 0.004$) showed a statistically significant association.

In the control group, the predominantly type of virus seen was others (36.7%), followed by HPV 16 (23.3%) and HPV 18 (10%). In OSF, only 33.3% of cases showed the presence of HPV, of which HPV 16 and 18 were 13.3% and 13.3%, respectively. In contrast, OSCC samples showed 60% presence of HPV, with HPV 16, HPV 18 and both HPV 16 and 18, 33.3%, 30.0% and 13.3%, respectively. Other types of HPV were seen more in the control group, followed by OSCC and OSF. There was a statistically significant association between the presence/absence of HPV among the three groups ($P = 0.04$) Table 2.

Protein levels in tissue of healthy control, OSF and OSCC showed similar findings. In tissue A/A was 60% followed by A/P was 26.7% and P/P was 13.3%. Whereas in OSF and OSCC A/A was 50%, 60% followed by A/P was 26.7% and 33.3% and P/P was 23.3% and 6.7%, respectively. The difference in the proportions of protein levels among different groups was not statistically significant ($P > 0.05$ in both saliva and tissue) Table 3.

Table 3 depicts statistically significant association between the presence/absence of HPV and the presence of a particular type of protein in controls and OSCC patients. In OSF, the frequency distribution was not statistically significant. Among the healthy controls A/A type protein was frequently seen followed by A/P, P/P irrespective of presence or absence of HPV. In the OSCC group, in the absence of HPV A/A was common followed by P/P and A/P, and in the presence of HPV P/P was common followed by A/A and A/P Table 4.

No significant association between age, gender, habit, site and clinical stage were seen with the presence or absence of HPV in tissue and distribution of proteins in the tissue.

DISCUSSION

In the present study, we studied the distribution of TP53 codon 72 genotypes in 30 OSF and 30 OSCC patients and 30 controls, to assess the amino acid variability and associated risk of developing the tumor. Genetic polymorphism of

Table 1: The descriptive data of the groups

Characteristics	Control, n (%)	OSF, n (%)	OSCC, n (%)
Age (years)			
Below 40	23 (76.7)	24 (80)	9 (30)
Above 40	7 (23.7)	6 (20)	21 (70)
Sex			
Female	4 (13.3)	0	4 (13.3)
Male	26 (86.7)	30 (100)	26 (86.7)
Habit			
Habit	-	30 (100)	18 (60)
No habit			12 (40)
Site			
BM	-	-	17 (56)
Tongue			13 (44)
HPV in tissue			
Positive	19 (63.3)	10 (33.3)	18 (60)
Negative	11 (36.7)	20 (66.7)	12 (40)
Types of protein in tissue			
Arginine/arginine	18 (60.0)	15 (50.0)	18 (60.0)
Arginine/proline	8 (26.7)	7 (23.3)	2 (6.7)
Proline/proline	4 (13.3)	8 (26.7)	10 (33.3)

OSF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma, HPV: Human papillomavirus, BM: Buccal mucosa

Table 2: The distribution of human papillomavirus in tissue and its variants among different groups

Groups	HPV in tissue, n (%)	HPV 16, n (%)	HPV 18, n (%)
Control			
Absent	20 (36.7)	23 (76.7)	27 (90.0)
Present	10 (63.3)	7 (23.3)	3 (10.0)
Total	30 (100)	30 (100)	30 (100)
OSF			
Absent	20 (66.7)	26 (86.7)	26 (86.7)
Present	10 (33.3)	4 (13.3)	4 (13.3)
Total	30 (100)	30 (100)	30 (100)
OSCC			
Absent	12 (40.0)	20 (66.7)	21 (70.0)
Present	18 (60.0)	10 (33.3)	9 (30.0)
Total	30 (100)	30 (100)	30 (100)
χ^2	6.502	3.35	4.71
P	0.04	0.18	0.09

HPV: Human papillomavirus, OSF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma

gene may determine the individual susceptibility for cancer. The p53 gene contains single-nucleotide polymorphism that encodes either Arg or Pro at amino acid codon 72 of p53. Individual inherits either homozygous, either (Arg/Arg) or (Pro/Pro) or heterozygous (Arg/Pro) at p53 codon.^[6] Homozygosity at p53 amino acid might represent a risk factor for carcinogenesis, the authors have reported that homozygous for Arg-72 is about seven times more susceptible to development of cervical cancer for than heterozygous.^[7] In addition, correlation between distribution of p53 genotype and prognosis of the lung, cervix and esophagus cancer has been reported. Studies in oral cancer are limited and controversial. In the present study, homozygous Arg/Arg and Pro/Pro genotypes were associated with OSCC compared to heterozygous type. Furthermore, control and OSF cases were associated

Table 3: The distribution of tissue proteins based on presence or absence of human papillomavirus among different groups

Groups	Type of tissue proteins			Total	χ^2	P
	Arginine/arginine, n (%)	Arginine/proline, n (%)	Proline/proline, n (%)			
Control						
HPV in tissue						
Absent	10 (55.6)	1 (12.5)	0 (0.0)	11 (36.7)	7.09	0.02
Present	8 (44.4)	7 (87.5)	4 (100.0)	19 (63.3)		
Total	18	8	4	30		
OSF						
HPV in tissue						
Absent	9 (60.0)	6 (85.7)	5 (62.5)	20 (66.7)	1.50	0.47
Present	6 (40)	1 (14.3)	3 (37.5)	10 (33.3)		
Total	15	7	8	30		
OSCC						
HPV in tissue						
Absent	10 (55.6)	1 (50.0)	1 (10.0)	12 (40.0)	5.64	0.05
Present	8 (44.4)	1 (50.0)	9 (90.0)	18 (60.0)		
Total	18	2	10	30		

OSF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma, HPV: Human papillomavirus

Table 4: Distribution of different types of proteins in tissue in different variants of human papillomavirus among different groups

Groups	Proteins in tissue			
	HPV 16		HPV 18	
	Present	Absent	Present	Absent
Control				
Arginine/arginine	2	16	1	17
Arginine/proline	5	3	2	6
Proline/proline	2	2	0	4
OSF				
Arginine/arginine	4	11	2	13
Arginine/proline	0	7	1	6
Proline/proline	0	8	1	7
OSCC				
Arginine/arginine	4	14	4	14
Arginine/proline	0	2	1	1
Proline/proline	6	4	4	6

OSF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma, HPV: Human papillomavirus

with Arg/Arg homozygotes. Similar to our findings, homozygous type (Arg/Arg) is predominantly reported in control and OSCC.^[6,8-11] Very few authors have reported with heterozygous type (Arg/Pro) association OSCC.^[12-15] Brief review of distribution of the p53 codon 72 genotype among control and OSCC is given in Tables 5 and 6.

Further, we assessed the p53 codon 72 genotype and association with HPV 16 and 18 in control, OSF and OSCC. Several investigators have studied the prevalence of HPV in potentially malignant disorder, hoping to find a similar HPV prevalence as in malignant diseases. In the present study, in control group, 63.3% cases showed the presence of HPV, but they were other type than the HPV 16 and 18 types. Other authors have observed a high percentage of HPV in normal mucosa which lies in accordance with the present study.^[16,17] In contrast, many others have not found the presence of HPV.^[18-21] An increasing prevalence of HPV in premalignant disorder suggests that it may play

a role in malignant transformation. Ha *et al.* reported 1% prevalence of HPV 16 and 18 in premalignant oral lesions using the quantitative PCR.^[22] On the other hand, Bagan *et al.* did not find any association between HPV DNA and proliferative verrucous leukoplakia using PCR.^[23] Another study analyzed the high-risk HPV DNA in OSF and to determine the role of HPV infections. A total of 105 cases, 33 (31.4%) patients were positive for high-risk HPV, while 72 (68.6%) patients were negative.^[24] Chen *et al.* reported the positivity of HPV 16 as 52.6% and HPV 18 as 25% in OSF by the PCR method.^[11] Vidal *et al.* reported 72.5% negative for low- and high-risk HPV DNA, 22.5% positive for low- and high-risk HPV DNA, 2.5% positive for low-risk HPV DNA and 2.5% positive for high-risk HPV DNA in 40 oral carcinoma cases by the Hybrid Capture (HC) technique.^[25]

In the present study, 33.3% HPV presence were detected in OSF using PCR including both HPV 16 and 18. Chaudhary *et al.* studied and compared between the HC II test and PCR-based assay for the detection of HPV DNA in OSF and OSCC. It was noted that the overall prevalence of HPV 16 E6 DNA positivity was nearly 26% by PCR and 27.4% by the HC II assay in OSF and, 32.4% HPV 16 E6 positive by PCR, and 31.4% by the HC II assay in OSCC.^[26]

Elamin *et al.* detected HPV DNA in 14/28 (50%) carcinomas and 4/12 (33%) precancerous lesions using nested PCR. The detection procedure ensured sensitivity and consistency of the detection of low copy numbers of the virus DNA. It was concluded that the presence of HPV found in 33% of premalignant tissues.^[1]

In India, the presence of HPV association with OSCC seen ranged from 15% to 67%.^[27] About 67% is highest reported from Southeast India,^[28] whereas in the Japanese

23%, USA 8%–20% and 19% Dutch population.^[29–32] In the present study, the frequency of HPV 16 and 18 is 33.3% and 13.3%, respectively, did not find any statistical association. The prevalence of HPV 16 is seen associated with risk of development of OSCC than HPV 18; similar findings are reported by various authors Perrone *et al.*, D'Costa *et al.* and Bouda *et al.*^[12,27,29] Review of the studies on association between HPV and p53 codon 72 genotypes among oral cancer is detailed in Table 7.

Various studies have shown the distribution of p53 genotype and its correlation with predisposition and prognosis of cancer of cervix, lung and esophagus. The

allele frequency in the present study was homozygous allele's, i.e., AA and PP, respectively. Higher frequency of Pro allele is found in the Indian population.^[14,19,22]

The frequency of A/A, P/P and A/P genotypes in HPV-positive cases as compared to frequency for HPV-negative cases. Hamel *et al.* did not find any association between p53 codon 72 polymorphism of oral cancer.^[8] However, Storey *et al.* now reveal that the Arg form of p53 is more susceptible to degradation by the HPV E6 protein than in the Pro form.^[7] Moreover, patients with HPV-associated cervical cancer are much more likely to contain the Arg form of p53 compared with the rest

Table 5: Distribution of p53 codon 72 genotypes among healthy controls

Control sample	Arginine/arginine (%)	Arginine/proline (%)	Proline/proline (%)	Author
163	95 (58.3)	61 (37.4)	7 (4.3)	Hamel, 2000
308	168 (54.54)	112 (36.36)	28 (9.09 s)	Summerrgill, 2000
333	175 (52.6)	134 (40.2)	24 (7.2)	Shen H, 2002
20	5 (25)	12 (60)	3 (15)	Katiyar, 2003
77	84 (59.6)	47 (33.3)	10 (7.1)	Perrone, 2007
342	179 (52.4)	140 (40.9)	23 (6.7)	Jix, 2008
72	14 (19)	6 (8)	52 (72)	Tandle, 2001
26	13 (50)	11 (42.3)	2 (7.6)	Nagpal, 2002
349	181 (51.9)	144 (41.2)	24 (6.9)	Chen
94	21 (22)	51 (54)	22 (24)	Chakraborty
30	18 (60)	8 (26.7)	4 (13.3)	Present study

Table 6: Distribution of p53 codon 72 genotypes among oral squamous cell carcinoma cases

OSCC	Arginine/arginine (%)	Arginine/proline (%)	Proline/proline (%)	P	Author
163	88 (54.0)	68 (41.7)	7 (4.3)	0.50	Hamel, 2000
190	102 (53.68)	70 (36.84)	18 (9.4)	0.77	Summersgill, 2000
304	158 (52.0)	125 (41.1)	21 (6.9)	0.970	Shen H, 2002
153	22 (14)	100 (65)	31 (20)		Tandle, 2001
110	3 (28.18)	58 (52.72)	21 (19.09)		Nagpal, 2002
44	10 (22.72)	24 (54.54)	10 (22.72)	-	Katiyar, 2003
77	63 (81.8)	8 (10.4)	6 (7.8)	0.005	Perrone, 2007
188	103 (54.8)	74 (39.4)	11 (5.8)	0.837	Jix, 2008
326	183 (56.1)	121 (37.1)	22 (6.8)	0.519	Chen
82	20 (25)	38 (46)	24 (6.9)	0.647	Chakraborty
30	18 (60)	2 (6.7)	10 (33.3)		Present study

OSCC: Oral squamous cell carcinoma

Table 7: Association between human papillomavirus and p53 codon 72 genotypes among oral cancer in cases and controls

	Number of cases	p53 arginine/arginine, n (%)	p53 arginine/proline, n (%)	p53 proline/proline, n (%)	Author
HPV 16 +ve	25	8 (32)	13 (52)	4 (16)	Nagpal
HPV 18 +ve	16	6 (37.5)	7 (43.75)	3 (18.75)	
HPV -ve	69	17 (24.63)	38 (55.07)	14 (20.28)	
HPV 16 +ve	16	11 (80)	1 (10)	4	Perrone
HPV 16 -ve	61	52 (85.24)	7 (11.47)	2 (3.27)	
HPV +ve	13	2 (15.4)	10 (76.9)	1 (7.7)	
HPV -ve	31	8 (25.8)	14 (45.2)	9 (29.0)	Katiyar
HPV +ve	46	26 (56.5)	15 (32.6)	5 (10.9)	
HPV -ve	154	76 (52.8)	65 (38.2)	13 (9.0)	
HPV 16 +ve	101	42 (40.8)	45 (52.9) (combined)		Jix
HPV 16 -ve	87	NA	NA		
HPV +ve	93	48 (26.2)	52 (36.4) (combined)		
HPV -ve	226	135 (52.8)	91 (63.6)		Chen
HPV 16 +ve	10	4 (40)	0	6 (60)	
HPV 18 +ve	6	4 (66.66)	1 (16.66)	1 (16.66)	
HPV -ve	14	10 (71.42)	1 (7.41)	3 (21.42)	Present study

NA: Not available

of the population. The authors conclude that patients with two copies of the Arg form have a sevenfold higher risk of developing cervical cancer than people with the Pro form. Shen *et al.* and Vidal *et al.* reported Pro/Pro patients reported with younger age group than Pro/Arg and Arg/Arg among oral cancer.^[6,25]

Nagpal *et al.* observed A/P genotype in HPV-positive infection as compared to P/P genotype, indicating A/P genotype is highly susceptible to HPV infection and oral carcinogenesis.^[14] Sixteen similar findings have been noted by Tandle *et al.*, Katiyar *et al.* and Chakraborty *et al.*^[15,13,16] Summergill *et al.* did not see any association between p53 codon 72 polymorphism and HPV.

Although Makni *et al.* reported with interlaboratory variation in the proportion of A/A, A/P and P/P.^[33] Perrone *et al.* speculated the protective effect of the RP genotype in oropharyngeal squamous cell carcinoma with combination of R72 and P72. R72 has a greater capacity to induce apoptosis, whereas P72 has greater transcriptional transactivation ability and induce cell cycle arrest at G1 phase. Greater frequency of PP in HPV-positive SCC indicates the risk factor of oropharynx carcinoma, whereas other study HPV-negative tumors were associated with PP genotype.^[12]

Summergill *et al.* (2000) evaluated the association between p53 polymorphism at codon 72 and HPV infection in the oral cavity as well as its association with oral cancer. Oral squamous cells from 202 patients with oral cancer and 333 age-sex frequency-matched controls were evaluated by PCR for the presence and type of HPV and for alleles of codon 72 in p53. It was noted that p53 codon 72 polymorphism is not associated with HPV infection. In addition, no association was found with the codon 72 polymorphism and oral cancer.^[9]

No statistical significant correlation was seen with age, sex, habit, distribution of site, clinical stage and grade of OSCC, indicating HPV infection is not influenced by these factors. Similar findings have been recorded by various authors,^[29,30] but Paz *et al.* noted advanced tumors with lymph node metastasis had high HPV infection.^[32] Ji and Li *et al.* studies showed HPV 16 seropositivity among squamous cell carcinoma of oropharynx (SCCOP) was higher among men and younger individuals.^[10,34]

CONCLUSION

High-risk HPV infection 16 and 18 is observed in patients with homozygous genotypes including Arg/Arg and

Pro/Pro in OSCC, apparently suggesting homozygous genotypes are at high risk than heterozygous type. We also observed potential malignant disorder, i.e., OSF did not find higher frequency of homozygous genotype is at high risk in the presence of HPV 16 and 18 in developing OSCC.

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Conflicts of interest

There are no conflicts of interest.

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