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Research Article

High-Risk Human Papillomavirus & P16INK4A Detection in Oral Cavity Squamous Cell Carcinoma and Severe Dysplasia Among Patient Attending Tertiary Care Hospital

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Abstract

There are variability in data of High-Risk Human Papillomavirus (HR-HPV) origin and progression in Oral Cavity Squamous Cell Carcinoma (OCSCC) among Indian population [1,2]. Evidence suggested that High-Risk Human Papillomavirus (HR-HPV) 16/18 is risk factor for Oropharyngeal squamous cell carcinoma (posterior one third of tongue, soft palate and tonsils). HPV infection in India estimates that 25.6% of all oropharyngeal cancer and 22.2% oral cavity squamous cell carcinoma are attributable to HPV infection but Role of High-risk HPV (16,18) in Oral cavity squamous cell carcinoma (anterior 2/3rd tongue, lateral side of tongue, Buccal mucosa, floor of the mouth, gums, Alveolus, retromolar trigone, Hard palate) is not consistent [3,4]. The objective of this study was to assess the proportion of High-risk HPV DNA and p16 immuno histochemical marker in oral cavity squamous cell carcinoma and severe dysplasia. This was cross-sectional study, in which included histopathological proven 58 oral cavity squamous cell carcinoma and severe dysplasia. All the 58-oral brush liquid-based biopsy sample were tested negative for HPV 16 and HPV 18 genotypes by TaqMan-based Realtime Multiplex PCR. Our results do not show any significant association between the clinical presentation and the risk factors (tobacco, alcohol and HPV) for oral cavity SCC but High-risk HPV DNA type associated with Histomorphological grade of OCSCC (Table 1). The proportion of High-risk HPV DNA was detected in 9 of 58 (15.5%) and p16 was expressed in 8.6% of oral cavity squamous cell carcinoma & severe dysplasia.

Keywords: HPV; Human Papillomavirus; Oral Cavity Squamous Cell Carcinoma; P16INKA4A; Polymerase Chain Reaction

Introduction

Cancer of lip & oral cavity squamous cell carcinoma is the 16th rank and the incidence of Oral cavity squamous cell carcinoma, globally represented are 355,000 new cases and 177,000 death estimated in 2018 but in India the scenario is much worse having 2nd rank with almost 119,992 (10.4%) new cases and death 72,616 (9.3%) [5,6] Oropharyngeal (1,40,000,) cases and Oral cavity squamous cell carcinoma (2,80,000) cases of which 44,000 and 5900 are HPV attribute infection in India (GLOBOCAN 2018) [7] .Tobacco chewing, smoking and alcohol intake are considered the primary risk factors in the pathogenesis of Head and neck cancer. Evidence also suggest HPV infection of the head and neck region is associated with 25% of Head And Neck Cancer (HNC) cases worldwide but in India estimates that 25.6% of all Oropharyngeal cancer and 22.2% oral cavity squamous cell carcinoma are attributable to HPV infection [8]. Unlike Oropharyngeal cancer, there is uncertainty whether high risk HPV has a significant role in the pathogenesis of Oral cavity squamous cell carcinoma. Based on International Agency for Research on oral cancer recognized that High-Risk Human Papillomavirus (HR-HPV) 16/18 is risk factor for Oropharyngeal carcinoma (posterior one third of tongue, soft palate and tonsils), P16INK4a can also be in tumors, overexpression in head and neck cancer and it is used as diagnostic tool and have direct association with infection by high risk genotypes of HPV. Recent studies showed that Expression of the tumour suppressor P16INK4A has been proposed as surrogate marker for HPV infection its overexpression is thought to reflect the presence of biologically active HPV infection given that functional inactivation of pRb by viral E7 induces P16INK4A upregulation [9-13] but Role of HPV DNA Marker in Oral cavity squamous cell carcinoma (anterior 2/3rd tongue, lateral side of tongue, Buccal mucosa, floor of the mouth, gums, Alveolus, retromolar trigone, Hard palate) is not clear [14,15].

Material and Methods

After obtaining ethical clearance from Institutional ethics committee, oral brush biopsy sample collected using cytobrush with 10 gentle strokes from clinically evident area until pinpoint bleed likely to occurs then detached cytobrush was kept in 10 ml vial containing transport medium and container stored at 4-degree Celsius temperature up to 1 week, simultaneously tissue biopsy were collected in 10% buffered formalin at room temperature for histological diagnosis. The demographic, exposure and clinical details of the patients were recorded on questionnaire at the time of sample collection.

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Sample Preparation

Specimen processing was done only inside the biosafety cabinet. Oral brush cytology sample was transferred to 15 ml falcon tube centrifuged at 4000 rpm for 10 min then discard the supernatant pellet is obtained and washed with buffer, take 2ml Eppendorf tube labelled with test sample id no and mixed with 1000 µl molecular water, centrifuge 8000 rpm for 5 minutes then discard supernatant, repeat this process 3-4 times, completely remove aqueous solution from 2 ml Eppendorf tube add 200 µl water and stored -20 degree until DNA extraction.

DNA Extraction

DNA from oral brush cytology was isolated by commercially available QIA DNA Mini Kit (QIAGEN) as per manufacturer protocol. The extracted DNA was eluted in 60µl of elution buffer and it can be stored at 2-8 degree for short term storage (< 2days) beyond that these samples are stored at -80 degree Celsius until PCR was done.

DNA Quantification

Nucleic acid quantification was done by using a spectrophotometer to check the quantity and purity of DNA in terms of A260/A280. All the eluted DNA A260/280 ratio was between 1.75 to 1.8.

High-risk HPV Polymerase chain reaction

All the sample were subjected to Real-time multiplex PCR to amplify up to E7 region of HPV genome of HPV type (16,18) and other 12 types as pool. Samples were tested in human β-globin gene and individual type-specific real-time multiplex HPV PCR assays directed against the E7 gene of 15 high-risk HPV types that uses fluorescent probes for the detection of one or more accumulating PCR Product. During each PCR cycle the fluorescent signal increases in a logarithmic manner resulting in an amplification curve. As soon as the amplification curve of the target comes above the threshold, the sample is considered positive for the target. This assay allows the simultaneous detection of four different fluorescent dyes per reaction, with each fluorescent dye representing different targets. The four different targets are 1. HPV 16, 2. HPV18, 3. Others HPV types as pool and 4. The human beta globin gene as internal control determining both the quality of the sample DNA and the presence of potentially inhibitory substances. The assays were performed in a72 tubes per Rotor gene Q MDx run, reaction setup was done at room temperature. Each well contained 5µl of sample DNA, PCR master mix 15 µl including primer & probe (Qiagen) and positive and negative control, Place a 72 well rotor on the rotor holder. The cycling conditions used for the PCR were 50°C for 2 min, 95°C for 15 min, followed by 40 cycles of 94°C for 15 s and 60°C for 1 min, pre-established thresholds were set, and the data were exported to a Microsoft Excel workbook for compilation and analysis. A sample was considered PCR positive for a specific HPV type.

Analysis of Real-Time PCR Data

Targets in the QIA screen positive control Ct value that were lower than 29 for B-globin, lower than 30 for HPV16 and HPV 18, and lower than 32 for HPV other.

Immunohistochemistry

4µm thin section placed on oraganosilane coated slide. Fix the section by microwave at power at 5-60°C for 3 minutes, deparaffinized by using dewax solution for 3 min, Antigen retrieval was done by immersing the section in 10 mmol sodium citrate buffer, PH 6.5 and boiled in pressure cooker for 5 whistles (Standardized) respectively, coupling jar with slide & buffer was kept at room temperature to cool down. Then slide were washed with Tris buffer and sections were incubated with Hydrogen peroxide block (HK111-50K) for 5 minutes to inactivated endogenous peroxidase when Horseradish peroxide is used for staining. Then power block (HK083-50K) one drop of vial used for 5 min which contains buffer, casein, preservative and reduces background staining due to nonspecific binding of antibodies. Without washing after power block, step sections were incubated with pre-diluted with specific monoclonal primary antibody against p16 (clone; G175-405, Mouse monoclonal, Biogenix, CA) for overnight incubation (standardized) at 4°c temperature in humidification chamber. After that sections were washed twice with Tris buffer at pH 7.2 for about 5 minute each. The Super enhancer was applied to cover section & incubated for 20 minutes at room temperature. The sections were incubated in specific secondary antibody (Super sensitive polymer HRP Kits, Biogenix, CA) for 30 minutes at room temperature in humidification chamber and washed twice with tris buffer at pH at 6.5 for about 5 min each. Tissue sections were covered with DAB (3, 3-diaminobenzidine tetrahydrochloride) substrate solution & incubated for 5 min at room temperature and rinsed with Tris buffer solution. Mayer's haematoxylin was used for counter staining the sections for 3 minute and cervical squamous cell carcinoma section was used as a positive control in each run.

Evaluation of p^{16} expression

P16INK4a protein expression evaluated by use of an antibody against P16 and evaluated the immunostaining in five areas of the tissue section under a microscope using 400x original magnification. Nuclear ± cytoplasmic staining is considered as immunopositively, positively stained cells (intensity score) group according to following categories: 0 (no staining), +1 (week, but detectable), +2 (moderate) +3 (intense). H-SCORE value was calculated by multiplying the percentage of cells with intensity scores in each slide using the formula H-SCORE= Pc×I where I represent the intensity score and pc is the corresponding percentage of the cells.

Results

A total of 58 cases were obtained for analysis of High-risk HPV DNA and p16ink4a molecular marker in the known case of squamous cell carcinoma and severe dysplasia of oral cavity by Real-time Polymerase chain reaction and Immunohistochemistry. In this present study total of 58 cases were included in which 43 (74.3%) male and 15 (25.8%) female patients with histopathologically confirmed Oral cavity squamous cell carcinoma (Table 1). Mean age of patients were 59.9 (39-82) years. In this study we found 5 cases less than 45 years age, 37 cases in between 45-69 years age and 16 cases more than 70 years of age. Based on histopathology grade majority of cases of oral squamous cell carcinoma were found mostly of tongue (N=18) followed by Buccal mucosa (N=28), Alveolus (N=5), Floor of the mouth (N=4), Hard palate (n=1), Retromolar trigone (N=2), Commissure of lip (N=1) and tonsillar fossa (N=1). In our analysis High-risk HPV DNA was detected in 9 of 58 (15.5%) in OCSCC and severe dysplasia and expression of p16 was detected in 5 (8.6%)

Table 1: Demographic, Exposure & clinical characteristics of Patient with Oral cavity squamous cell carcinoma & severe dysplasia.

Characteristics	HPV Positive (N= 9)	HPV Negative (N=49)	P value
Gender			
Male	**6 (14.0%)**	**37(86%)**	
Female	**3 (20%)**	**12 (80%)**	**0.682**
Age, Years			
<45	**0 (0%)**	**5 (100%)**	
45-69	**4 (10.8%)**	**33 (89.2%)**	
>70	**5 (31.3%)**	**11 (68.8%)**	**0.102**
Tobacco user			
Ever user	**6 (15%)**	**34 (85%)**	
Never user	**3 (16.7%)**	**15 (83.3%)**	**1**
Alcohol use			
Ever user	**6 (20.7%)**	**23 (79.3%)**	
Never user	**3 (10.3%)**	**26 (89.7%)**	**0.47**
Smoking History			
Ever user	**6 (16.2%)**	**31 (83.8%)**	
Never user	**3 (14.3%)**	**18 (85.7%)**	**1**
Diet			
Vegetarian	**1 (7.1%)**	**13 (92.9%)**	
Non vegetarian	**8 (18.2%)**	**36 (81.8%)**	**0.431**
Anatomical Site			
• Tongue	**4 (22.2%)**	**14 (77.8%)**	
• Alveolus	**0 (0%)**	**5 (100%)**	
• Floor of the Mouth	**1 (25%)**	**3 (75%)**	
• Hard palate	**1 (100%)**	**0 (0%)**	
• Buccal mucosa	**2 (7.7%)**	**24 (92.3%)**	
• Retromolar trigone	**0 (0%)**	**2 (100%)**	
• Commissure of Lip	**0 (0%)**	**1 (100%)**	
• Other tonsillar fossa + Buccal mucosa	**1 (100%)**	**0 (0%)**	**0.431**
Histopathology grade			
• Well differentiated SCC	**2 (5.9%)**	**32 (94.1%)**	
• Moderate differentiated SCC	**6 (46.2%)**	**7 (53.8%)**	
• Poorly differentiated SCC	**0 (0%)**	**7 (100%)**	
• Severe dysplasia	**1 (33.3%)**	**2 (66.7%)**	
• Descriptive	**0 (0%)**	**1 (100%)**	**0.044**

Table 2: Cross tabulation of P16ink4a and High-risk human papillomavirus infection.

p16_IHC * High-risk HPV Crosstabulation					P value (Fisher exact test)
		High-risk HPV infection		Total	
		no	yes		
p16ink4a	no	47	6	53	**0.023**
	yes	2	3	5	
Total		49	9	58	

The expression of p16 significant associated with HPV DNA infection in Oral cavity squamous cell carcinoma and severe dysplasia

of lesions and associated with HPV DNA (Table 2). All the 58-oral brush liquid-based biopsy sample were tested negative for HPV 16 and HPV 18 genotypes by TaqMan-based Realtime Multiplex PCR. Our results do not show a significant association between the clinical presentation and the risk factors (tobacco, alcohol and HPV) for oral cavity SCC but High-risk HPV & other were genotypes associated with histopathology grade. Of intraepithelial lesion (Table1). Using Immunohistochemistry,specific immunoreactivity for p16 was found to be diffusely present in both nuclear as well as cytoplasm of malignant cells. (Figure 1,2) showed that p16 expression were found in Severe dysplasia, well differentiated SCC followed by moderate differentiated oral cavity squamous cell carcinoma.

Discussion

All the sample were tested to TaqMan based real-time PCR assay specific for HPV 16, HPV 18 and others genotypes (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 67, and 68), were confirmed negative for high-risk HPV 16 and 18, in oral cavity squamous cell carcinoma and severe dysplasia but other genotypes were found 9 (15.5%). Positive for high-risk HPV associated with histopathology grade of squamous cell carcinoma. About 65.5% participants belongs to age group 45-69 years and 27.5 % above 70 years. The majority of the cases (34)58.8% presented as well differentiated squamous cell carcinoma followed by (13)22.4% moderate differentiated squamous cell carcinoma and (7)12% poorly differentiated squamous cell carcinoma and (3)6.8% dysplasia and leukoplasia cases. Majority of the participants had history of alcohol consumption, smokeless tobacco and tobacco users. The overall prevalence of HPV associated OCSCC globally varies from 0-30%,In Indian studies have reported wide variation in prevalence of HPV associated OCSCC between 15-70% in northeast, eastern and Sothern part [16,17] Laprise et al (2016) and Paul sebestian et al (2014) noted that there is no role of HPV in oral cavity squamous cell carcinoma [3-18]. HPV prevalence differ according to the geographical distribution, ethnicities, and sexual behaviour of the cohorts. Diverse sample such as tissue sample, frozen section, Formalin fixed paraffin embedded, and oral rinses were subjected for

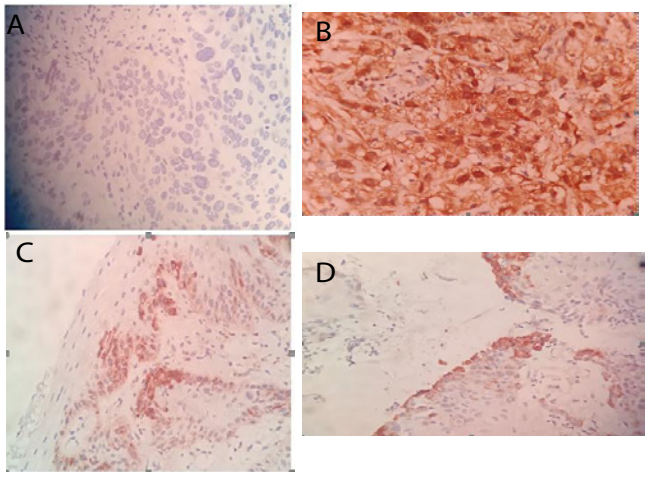

Figure 1: p16ink4a expression in positive control and negative control of Oral cavity squamous cell carcinoma and severe dysplasia. **A**- Positive control. **B**- Negative control. **C**- Severe dysplasia showing cytoplasmic staining. **D**- Well differentiated SCC cytoplasmic staining.

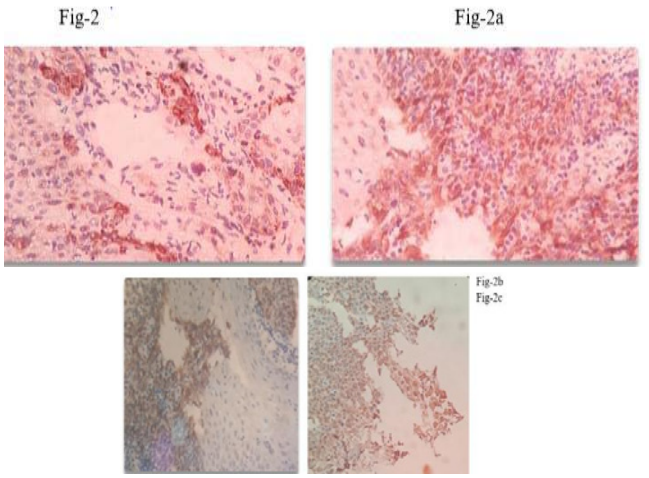

Figure 2: a,b,c: Overall, p16INK4a was expressed in oral cavity tumours characterized by mild, moderate or strong staining of the cytoplasm and nucleus in of tumour cells. Figure-2 well differentiated squamous cell carcinoma showing weak staining nuclear/cytoplasm positivity for p16 expression (40X, IHC). Fig-2a Mod. Differentiated squamous cell carcinoma showing strong nuclear/cytoplasm positivity for p16 expression (40X, IHC) Fig-2b: well differentiated squamous cell carcinoma showing weak staining nuclear/cytoplasm positivity for p16 expression (40X, IHC).Fig-2c: Mod. Differentiated squamous cell carcinoma showing strong nuclear/cytoplasm positivity for p16 expression (40X, IHC)

DNA amplification in the studies carried out globally. but There is no uniformity in the methods employed for the detection and most of the studies used assays like Polymerase chain reaction, Hybrid capture-2, Chromogenic Insitu Hybridization (CISH) Immunohistochemistry (ISH), In PCR commonly used primer set are PGMY09/11, GP5+/GP6+ and SPF/SPF2 which target the conserved sequence in the L1 region [19,20]. Laprise and Sebastian stated that there is no role of HPV in oral squamous cell carcinoma in South Indian Population [18-21]. Priscila Marinho de Abreu et al stated that low frequency of high-risk HPV DNA, supports the findings that HPV is not involved in the genesis of oral cavity SCC in Brazilian population [22]. In this Study we found 9 cases of high-risk HPV associated oral cavity squamous cell carcinoma but none positive for HPV 16 and 18 Genotypes, This study shows similia results with the studies conducted by Laprise , Sebastian, and Priscila Marinho de Abreu [3-22] . P16 protein expression was found in various grades of squamous cell carcinoma and there was increased and decreased expression of p16 have reported. Some of the previous study evaluated only nuclear staining whereas other utilized both cytoplasm as well as nuclear staining to study the p16 expression. In present study nucleur positively expression were seen higher in moderate differential squamous cell carcinoma than well differentiated squamous cell carcinoma and dysplasia cases.

Conclusion

In this study, we observed an overlapping pattern of both tobacco and alcohol as risk factor and the low frequency of other Genotypes of HPV DNA, this supports the evidence that HPV16 and HPV 18 Genotypes was not involved in the development of oral cavity Squamous cell carcinoma and severe dysplasia among south Indian population. Larger sample size and wider geographical area required to understand the association of High-risk HPVDNA & p16INK4A and gold slandered molecular oncogene E6/E7 m RNA Marker needs to be considered for HPV associated Oral cavity squamous cell carcinoma and severe dysplasia.

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