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MOLECULAR DETECTION OF HPV 16 IN SALIVA OF PATIENTS WITH ORAL SQUAMOUS CELL CARCINOMA AND IN THE NORMAL POPULATION OF SOUTHWESTERLY INDIANS

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ABSTRACT
Oral squamous cell carcinoma, one of the most common neoplasms in oral cavity, has been considerably associated with HPV infection. The favourable response of oropharyngeal cancer with HPV positive results towards chemo/radiotherapy has induced the thought for conducting this study to carry out the presence of HPV prevalence and its type specificity among patient and normal population. Salivae of 235 histologically confirmed OSCC patients and 409 randomly selected normal individuals irrespective of age & sex from rural and urban areas were collected and screened for High-risk HPV genotyping by PCR method. Out of 409 individuals in normal population detected for HPV Consensus primer, 193(47.10%) samples were found to be affected from HPV strains. The total no. of samples affected from HPV strains was 149(63.4%) out of 235 histologically confirmed OSCC patients in which 43 samples (18.3%) were identified as HPV 16 positive. While considering HPV consensus primer, significantly less number of HPV affected cases (p=0.000) were recorded in the control population compared to patients suffering from OSCC (Table 2). The data also furnish an impression of HPV being a major causing factor (63.4%) among OSCC patients. When type specific primer HPV 16 was used, the patient samples have significantly more number of High risk HPV 16 +ve cases (p=0.000) than the control population.

KEYWORDS: OSCC, HR-HPV, Consensus primer, Type- specific primer, PCR.

INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC) is considered to be the most common epithelial neoplasm of oral cavity (90%) with malignant effect among all head and neck cancers [1]. It represents almost 5% in men and 2 % in women when comparing all types of malignancies [2,3]. Tobacco use is a well-established risk factor of OSCC which includes chewing tobacco, pipes, cigarettes and cigars. Chewing of Betel quid consisting tobacco is also a common habit found in our study area, Southwest India. Meanwhile, alcohol consumption is another conventional risk factor for causing OSCC, especially when it is combined with cigarette smoking because of their synergistic interaction[4,5]. Additionally, Human Papillomavirus (HPV) infections, serve another factor for the occurrence of oral cancer. OSCC has been found to be considerably associated with HPV infection among both types of patients, with and without addiction of tobacco and alcohol use[6,7]. Conventionally, alcohol and tobacco consumption have been predicted as the main possible factors for the advancement of OSCC. However, it was found that 10 to 20% of patients suffering from OSCC have no history of usage of these substances [8]. In this study, based on current available data, clinical relevance to carry out systematic HPV searches in cases of OSCC is observed. HPV types have been categorized into high and low risk groups according to the extent of expression of concerned virus in malignancies. HPV16 is found to be the cause for several malignancies viz. cervical and oral cancers so it is categorized into the high-risk group [9]. HPV-DNA has been observed in a range of 19%-75% of all squamous cell carcinomas of oropharynx globally, approximately 85%-95% of which are of the HPV-16 type [10-12]. It has been observed that oropharyngeal cancer with HPV positive result is more responsive to chemo/ radiotherapy as well as patients with HPV positive tumors express considerably improved survival results against HPV negative tumors[13]. Several authors also suggested that prognosis for the patients with HPV-DNA positive tumors showed significant improvement irrespective of the mode of treatment[14,15]. Early molecular detection of presence of HPV-DNA would lead to better treatment for the patients. Case-control study is considered to be the most suitable methodology for the exploration of an HPV-OSCC relationship[16]. The testing of OSCC tissue is the most authentic method of research to find out the presence of HPV DNA and activity of HPV[17,18]. Nevertheless, erratic rates of presence of HPV in OSCC tissue, ranging from 15-80% have been observed in various studies, providing conflicting results. Hence, this study is conducted based on saliva samples to evaluate the high risk HPV16 in saliva of OSCC patient's along with the normal population.

MATERIALS & METHODS

Collection of saliva rinse:

Salivae of 235 histologically confirmed OSCC patients were collected in 15ml sterile falcon tubes with the consent of the patients from hospitals such as, Karnataka Cancer Therapy and Research Institute (KCTRI), Hubli and Sri Dharmasthala Manjunatheshwara (S.D.M.) Dental College, Dharwad, India. Salivae samples were also collected from 409 normal individuals randomly irrespective of age & sex from rural and urban (7 villages & 2 taluks) areas. High-risk HPV (HR-HPV) genotyping was carried out by Polymerase chain reaction based method at Karnataka Institute for DNA Research, Dharwad.

Genomic DNA isolation:

Genomic DNA from salivae was isolated by self-standardized protocol. Human saliva samples (2-3ml) were collected in Falcon tubes, they were centrifuged at 4000 rpm for 2 minutes, the supernatant was collected and 250µl of SDS (10%) and 5µl of Proteinase K was added. The mixture was allowed to stand for 45-65 minutes at room temperature. 150µl of Phenol: Chloroform: Isoamylalcohol (25:24:1) was added to this and the mixture was centrifuged at 400 rpm for 5 minutes. After centrifugation two layers were observed, the upper layer was collected into 2ml Eppendorf tube and 3 volumes of pre chilled Isoamyl alcohol was added and centrifuged at 13000 rpm for 5 minutes. The supernatant was drained off and the pellet was dried overnight. To the dried pellet 150µl of $T_{50}E_{20}$ was added and allowed to stand at room temperature for dissolution. The samples were analyzed for the presence of genomic DNA on 0.8% agarose gel (GENEI) and quantified using Biophotometer (Eppendorf, Germany).

Polymerase Chain Reaction

HPV consensus as well as HPV 16 specific primers was procured[19]. The overall presence of HPV DNA was confirmed by performing PCR with consensus primers initially to all the samples (disease and control) to select the HPV positive specimens. Positive (HeLa cell lines) and negative controls were included during each amplification reaction. To identify the specific HPV types in the HPV positive specimens, further PCR was performed using HPV 16 specific primer.

PCR amplification was performed with 20µl reaction volume containing 1.0µl of genomic DNA (80ng/µl to 160ng/µl), 0.3µl of forward and reverse primers (5pmol), 0.4µl of dNTP (10pmol), 0.8µl of Taq DNA polymerases (3units/µl) along with 3.2µl Taq buffer (Bangalore GeNei, India) and the total volume was adjusted to 20.0µl using molecular biology grade water. Amplification was carried out in Thermal Cycler (Veriti, Applied Biosystems). After an initial denaturation at 98ºC for 1 min, 35 cycles were used under following conditions: 98ºC for 10sec (cycle denaturation), primer annealing temperature was set depending on the annealing temperature of each primer (59 for consensus and 59.5 for HPV 16 primer) for 30sec, 72ºC for 15sec (primer extension) and a final 1 cycle extension of 72ºC for 6 min. PCR products were electrophoresed for their respective amplicon size by gel electrophoresis with standard 100bp ladder. The amplified products were identified by UV irradiation of the gels in gel documentation unit (Vilber Lourmat transilluminator). The samples showing PCR amplification products of the expected size were counted to be positive.

RESULTS

The saliva samples collected from 409 individuals, in normal population of different villages and talukas of Dharwad district, were detected for HPV Consensus primer and 193 (47.10%) samples were found to be affected from HPV strains. Type specific primer was used further to find out the prevalence of high risk HPV 16 and total 19 samples (4.76%) were identified to HPV16 positive. The total no. of samples affected from HPV strains was 149(63.4%) out of 235 histologically confirmed OSCC patients in which 43 samples (18.3%) were identified as HPV 16 positive. The normal population has been categorized based on gender, settlement pattern and habits of chewing tobacco, drinking alcohol and smoking (Table 1).

TABLE 1: Depicts the categorized total no. of Controls based on sex, rural, urban area and according to the habits

Age group	No. of controls	Gender		Settlement Pattern				Habits	
		Male	Female	Rural		Urban		Male	Female
				Male	Female	Male	Female		
20-30	176	97	79	58	43	39	36	59	05
31-40	67	32	35	18	19	14	16	23	09
41-50	89	43	46	24	21	19	25	32	11
51-60	28	19	09	10	05	09	04	17	02
61-70	27	09	18	06	11	03	07	07	06
71-80	22	17	05	09	03	08	02	11	03
Total	409	217	192	125	102	92	92	149	36

DISCUSSION

While considering HPV consensus primer, significantly less number of HPV affected cases (p=0.000) were recorded in the control population compared to patients suffering from OSCC (Table 2). The data also furnish an impression of HPV being a major causing factor (63.4%) among OSCC patients. The strikingly similar pattern of prevalence of HPV consensus primer (63%) was reported by Khursid *et al.* (1998) in Japan[20]. The studies conducted by Lambot *et al*., 2000[21] and Guimaraes *et al*., 2001[22] have reported the presence of HPV strains in OSCC patients to be non-significant compared to the control population (p= 0.6188 and p=0.8898 respectively).When type specific primer HPV 16 was used, the patient samples have significantly more

number of High risk HPV 16 +ve cases (p=0.000) than the control population (Table 3) in the present study which is supported by earlier studies conducted by Lyronis *et al*., 2005 [23], Souto Damin *et al*., 2006 [24], Cao *et al*., 2005 [25] and Liu *et al*., 2010 [26]. The data tend to indicate that the low diagnosis of HR HPV 16 (18.3%) in OSCC patients is linked with an actual low prevalence of HPV 16 strain for causing OSCC, independent of anatomical and technical biases. So, it can be concluded that an organized search for HPV in OSCC might be resulted as a misappropriation of health service resources, but may be only relevant in the research environment *viz.* in controlled research protocols. Indeed, advance studies can be continued to examine other infectious agents, including viruses, in OSCC.

TABLE 2: Examining HPV Consensus Primer in Control Population

Reference	HPV Types detected	Positive no. of cases (%)	Positive no. of controls (%)	OR (95% Confidence Interval)	p value	Country	HPV Detection method
Khurshid et al., 1998 [20]	CP,16,18	17/27 (63)	3/12 (25)	5.1 (1.11–23.37)	0.0286	Japan	PCR
Lambot et al., 2000 [21]	CP	1/21 (2)	0/5 (0)	0.80(0.03 –22.63)	0.6188	Belgium	PCR
Guimaraes et al., 2001 [22]	CP	2/32 (6)	4/57 (7)	0.88 (0.15–5.11)	0.8898	China	PCR
Present Study	CP	149/235 (63.4)	193/409 (47.10)	1.94(1.40 – 2.69)	0.0000	India	PCR

OR – odds ratio; CP – consensus primers; PCR – Polymerase chain reaction. [2]Some papers have ORs and p-values deemed as 'incalculable' due to one or more of the four components for OR calculation being a zero value.

TABLE 3: Examining HPV 16 Primer in Control Population

Reference	HPV Types detected	Positive no. of cases (%)	Positive no. of controls (%)	OR (95% Confidence Interval)	p value	Country	HPV Detection method
Lyronis *et al*., 2005 [23]	16,18,other	17/30 (56)	6/27 (22)	4.58 (1.44–14.59)	0.0081	Greece	PCR
Farhadi *et al*., 2005 [27]	16,18	8/38 (21)	5/38 (13)	1.76 (0.52–5.97)	0.3608	Iran	PCR
Souto Damin *et al*., 2006 [24]	16,18	26/165 (16)	0/26 (0)	8.49(0.50 –143.49)	0.0294	Brazil	PCR
Cao *et al*., 2005 [25]	16,18	207/265 (78)	203/357 (57)	2.71 (1.89–3.88)	<0.0001	China	PCR
Koh *et al*., 2008 [28]	16	0/102 (0)	0/40 (0)	0.39(0.00 –20.25)	Incalculable	Korea	PCR
Liu *et al*., 2010 [26]	16	35/69 (51)	2/32 (6)	15.44 (3.42–69.70)	<0.0001	China	PCR
Zhang *et al*., 2010 [29]	16,18,58	35/70 (50)	20/60 (33)	2.00 (0.98–4.08)	0.0552	China	PCR
Antonsson *et al*., 2010 [30]	16, 35	8/222 (4)	0/55 (0)	4.39 (0.25 –77.38)	Incalculable	Australia	PCR
Present Study	16	43/235 (18.3)	19/409 (4.76)	4.60(2.61 –8.10)	0.0000	India	PCR

OR – odds ratio; CP – consensus primers; PCR – Polymerase chain .Some papers have ORs and p-values deemed as 'incalculable' due to one or more of the four components for OR calculation being a zero value.

It is remarkable that studies conducted by Greece and Chinese researchers have found a noticeable percentage (50-80%) of patients affected with HPV 16 strain resulting into OSCC. But, for a country like India with its diverse food habits, temperate and tropical geographical conditions and varied inclination towards different forms of body-abusers (intoxicants) among different parts of country, a single study is not enough to influence the medical interest. The trend of developing of HPV vaccines holds tremendous assurance for developing countries like India where OSCC is gradually becoming a common malignancy. Nevertheless, considering the no. of cases of HPV specific type 16 to be small among patients (18.3%) and the control population (4.76%), it is inappropriate to suggest for the vaccination therapy for specific strains to be promoted as it would reduce the cost-effectiveness of HPV vaccination programmes. Other types of various high risk strains like HPV 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68,73[31] *etc*. might be involving in causing HPV which we have not taken into consideration in this study. Undeniably, infection by HR-HPVs, most frequently type HPV16, has been found to be associated with a growing number of patients suffering with OPSCC [32-35]. Though most of the patients with HPV +ve OSCC confronts with an advanced stage of the disease at the time of diagnosis, these patients express a constructive prognosis irrespective of the therapeutic treatment regimen [36,37]. HPV +ve OSCCs show variances in viral load as well as viral oncogene expression that is closely linked with clinical results [34,36,38,39]. The further research should be sufficiently dynamic and convergent to develop a multipurpose vaccine, able to cure OSCC caused by different strains. Secondly, to enhance the cost effectiveness of the HPV vaccination programmes being conducted in India, it is essential to comprehend the distribution of the major HPV types in different geographical regions which would help in focusing to develop region specific vaccines. The failure of well-known vaccines in the treatment of OSCC in local hospitals has also posed a new challenge in identifying the reasons which may vary from presence of mutations in the genes to the successful administration of vaccines till it reaches different socio-economical groups.

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