RESEARCH ARTICLE

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Detection of Human Papilloma Virus and Risk Factors among Patients with Head and Neck Squamous Cell Carcinoma Attending a Tertiary Referral Centre in South India

Satheesh K Bandhary[1], Veena Shetty[2], Marina Saldanha[1]*, Priya Gatti[3], Devananda Devegowda[4], Pushkal S R[4], Avinash K Shetty[5]

Abstract

Background: Head and Neck Squamous Cell Carcinomas (HNSCC) is the sixth most common cancer globally. In India, on an average 25-30% of all cancer cases affect the head and neck. The etiological factors associated with HNSCC are tobacco, alcohol and environmental carcinogens. However there are few cases, where there are no obvious risk factors involved. In western counties, there are many reports of human papilloma virus (HPV) association with HNSCC. Hence, we conducted a study to determine the role of HPV infection and risk factors among patients with HNSCC. **Materials and Methods:** A prospective, cross-sectional study was conducted in a tertiary referral centre from January 2014 to March 2016. 88 patients were enrolled in the study. Socio- demographic, behavioural data, site and subsite involvement, histopathology, staging and treatment were documented. Polymerase chain reaction (PCR) was performed to detect the presence of HPV DNA using consensus primers MY 09/11 and GP5+/GP6+ and further the samples were subjected to PCR for detecting HPV type 16 and 18. **Results:** The study included 88 participants with HNSCC. 57 had oral and oropharyngeal squamous cell carcinoma, 11 with laryngeal malignancy and 20 involving hypopharynx. Among the participants buccal mucosa (n=22) was the most common subsite involved, majority (50%) had moderately differentiated squamous cell carcinoma and 53.4% presented in stage IV. 2 (2.6%) cases were positive for HPV consensus and both were positive for HPV 16, one case each in larynx and hypopharynx. There was statistical significance in the association between betel nut chewing, cigarette smoking and alcohol intake as risk factors in the carcinogenesis of HNSCC. **Conclusion:** In our setting in South India, HPV does not play a major role in the carcinogenesis of HNSCC but betel nut chewing, tobacco exposure and alcohol consumption remain major risk factors for HNSCC.

Keywords: Head neck squamous cell carcinoma- risk factors- human papilloma virus- India

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Introduction

Head and Neck Squamous Cell Carcinomas (HNSCC) is the sixth most common cancer globally (Chin et al., 2006; Ferley et al., 2010). Worldwide, an estimated 550,000 new cases of head and neck cancer are diagnosed every year, with 380,000 reported annual deaths (Fitzmaurice et al., 2017); approximately two- thirds of these cancers occur in lower and middle income countries (Shibuya et al., 2000). In India, on an average 25-30% of all cancer cases affect the head and neck (Ferley et al., 2013). Due to the increasing prevalence of cancer in South East Asia, it has been predicted that the death rate will also increase by 75% in 2020 as compared to 2000. India is one among these countries that runs a major risk of producing this cancer burden to South East Asia. (Mishra and Meherota, 2014)

The major risk factors for HNSCC include tobacco (Sturgis et al., 2004; Hashibe et al., 2007; Sturgis and Cincirpini, 2007) cigarette/beedi smoking, betel nut chewing (Balaram et al., 1995) and alcohol. Human Papillomavirus (HPV) whose role in carcinogenesis of cervix was well known is also studied in cancers of head and neck (Bruni et al., 2016). In the last decade, many studies have been published proving the role of HPV as an independent risk factor in head neck cancers (Ragini et al., 2007; Dayyani et al., 2010). The world -wide prevalence of HPV infection in HNSCC is estimated at 20-26% (de Martel et al., 2012) whereas, in India the prevalence of HPV-associated HNSCC varies widely in different geographical regions mainly because of varied methods

*[1]Department of Otorhinolaryngology and Head and Neck Surgery, [2]Department of Microbiology, Research Co-ordinator NUCSReM, K.S.Hegde Medical Academy, [3]Senior Research Fellow, BRNS Project, Nitte University-575018, Mangalore, [4]Department of Biochemistry, Centre of Excellence in Molecular Biology and Regenerative Medicine, JSS Medical College, JSS University, Mysore, Karnataka, [5]Associate Dean for Global Health, Wake Forest University School of Medicine, Attending Physician, Brenner Children's Hospital Winston Salem, NC, India .*For Correspondence: saldanhamarina@gmail.com*

of sample collection and analysis. It has been studied that HPV infections have more affinity to oropharyngeal and oral cancer subsites (Allen et al., 2010; Mallen et al., 2016) and numerous studies from different geographic regions of India show that the presence of HPV is 33.6 % in Eastern India (Nagpal et al., 2002), 31.6% in North- East India (Kumar et al., 2015), 0- 74% positive cases in south India (Balaram et al., 1995; Bijina et al., 2016; Laprise et al., 2016) and 15% Western India (Costa et al., 1998).

The role of HPV in the pathogenesis of HNSCC is an area of great research interest due to its distinct clinical behaviour in terms of risk factors and treatment (Ajila et al., 2015). It has been observed that subjects with HPV associated HNSCC tend to be younger, non-smokers and have a history of multiple sexual partners (Schwatz et al., 2001; Smith et al., 2004, Gillison et al., 2008). Also in terms of treatment, better response to radiotherapy and chemotherapy and improved prognosis is seen among HPV-positive HNSCC compared to HPV-negative tumours (Chen et al., 2013).

The aim of our study was to determine the HPV rate and risk factors in cases of HNSCC in the coastal population of Karnataka, South India.

Materials and Methods

This is a prospective, cross-sectional study carried out from January 2014 to March 2016 involving the Department of Otorhinolaryngology and Department of Microbiology, K.S.Hegde Medical Academy, Mangalore, Karnataka. Ethical clearance was obtained for the study from the Institutional ethics committee. Written informed consent was taken from all the participants.

Newly diagnosed, untreated , histologically proven cases of squamous cell carcinoma(SCC) of head and neck and patients who gave consent to participate in the study were included . Patients with nasal, nasopharyngeal, thyroid and salivary gland malignancy were excluded. A total of 88 cases fulfilled the above criteria , among these 57 subjects were diagnosed with oral and oropharyngeal squamous cell carcinoma, 11 had laryngeal malignancy and 20 with hypopharyngeal malignancy.

Data collection

Data collection was done in a case report format where demographic details, habits such as tobacco chewing, betel quid consumption, smoking and alcohol consumption were documented. Sexual history was taken regarding premarital sex, involvement with multiple partners and oral sex. A detailed head and neck examination, video laryngoscopy, imaging details, cancer staging according to UICC TNM classification and treatment details were noted.

In this study, HNSCC cases were divided into four groups – oral cavity, oropharynx, larynx and hypopharynx which were further classified according to the subsites involved (Table 1). The classification was adopted according to ICD (International classification of disease) coding scheme.

Specimen collection

In proven cases of HNSCC , biopsy was taken from the tumour site , kept in cryovials containing saline and immediately transported and stored at -80 ^{o}C in Remi Ultra Low Quick Freezer (Vasai, India).

DNA extraction

DNA extraction was done from tissue samples using commercially avalaible kit (Dneasy minikit,Qiagen, Valencia, CA) according to manufacturer's instructions. The amount of DNA obtained in the samples ranged from ranged from 20-300ng/ul.

Human Papillomavirus (HPV) detection by PCR

Polymerase chain reaction (PCR) was used to detect the presence of HPV in the extracted DNA. PCR was done at two levels: First a pair of consensus primers such as MY09/11 and GP5+/GP6+ amplifying a 450bp and 150bp length of L1 region respectively was employed in the reaction. Only the positive samples were subjected to type specific PCR to detect the most common high risk HPV subtypes like HPV-16 and HPV-18. A brief master mix was prepared containing 2U/µL of Taq ploymerase (Himedia), 2µM of dNTPs, 10x buffer with 25mM $MgCl_2$ (working = 1x buffer) and 0.2µM of each of forward and reverse primers. DNA concentration ranging from 50-80 ng/reaction was added and the total reaction was made upto 30µL using PCR grade water. Amplification was performed in the Mastercycler gradient (Eppendorf) at 95 ^{o}C for 5min, followed by 32 cycles of 94 ^{o}C for 1 min, 60 ^{o}C for 45 sec, 72 ^{o}C for 1 min and a final extension of 72 ^{o}C for 2 min. GAPDH gene was used as internal control to check the adequacy of PCR reaction and DNA from HeLa cell and SiHa line was used as positive control for HPV 18 and 16 respectively, while HPV-negative C33-A cell line was used as negative control. The amplified PCR products were elecrophoresized on 2% agarose gel with a 100bp ladder (Himedia).

The following are the primers used:

Primer	Sequence	Product length
MY09/11	CGTCCMARRGGAWACTGATC	450
	GCMCAGGGWCATAAYAATGG	
GP5+/GP6+	TTTGTTACTGTGGTAGATACTAC	150
	GAAAAATAAACTGTAAATCATAT	
HPV 16	CACAGTTATGCACAGAGCTGC	467
	CATATATTCATGCAATGTAGGTGTA	
HPV 18	CACTTCACTGCAAGACATAGA	322
	GTTGTGAAATCGTCGTTTTTCA	

Statistical Analysis

The data collected was entered into Microsoft excel spreadsheet and analyzed using IBM SPSS Statistics Version 22 (Armonk, NY: IBM Corp). Descriptive data was presented in the form of frequency and percentage. Chi square test and fishers test were used to test the association between different study variables. P value < 0.05 was considered statistically significant.

Results

Descriptive Data

The study population consists of 88 patients with histologcally confirmed HNSCC. Out of 88 subjects 65 (73.9%) were males and 23 (26.1%) were females. Patients age ranged from 30 to 77 years with a mean age of 55yrs . Majority of the participants, 81(93%) came from rural background and 7(7%) were from urban population.

Site Specific Data

The study population was grouped according to site of malignancy into four groups. Detailed explaination about the site, subsite, stage of presentation and histopathology is given in Table 1.

Oral cavity

37 (42.5%) out of the total 88 participants had oral SCC . The most common site involved in the oral cavity was buccal mucosa (61%) followed by lateral border of the tongue (LBT) (59%). Participants with LBT cancer presented at a earlier stage i.e. Stage II (n=6) in comparison to other sites where majority presented in the III and IV stage. Based on histopathological report 17 (46%) subjects had well differentiated (WD), 12 (33%) had moderately differentiated(MD) and 8 (22%) had poorly differentiated (PD) SCC.

Oropharynx

20 (22.5%) of the 80 subjects had oropharyngeal SCC. Tonsil (80%) was most common site involved. Majority of the patients (95%) presented in the III and IV stage .MD SCC (60%) was the most common hisopathology pattern documented.

Larynx

Out of 88 participants, 11 (12.5%) had laryngeal SCC. Larynx was furthur subclassified into glottis and supraglottis. 5 (45%) patients presented with glottic malignancy and 6 (55%) had supraglottic SCC. In Table 1, it can be observed that only one patient presented in Stage 1 and that patient had glottic SCC. Moderately differentiated SCC was the most common histopathological type noted.

Hypopharynx

20 (22.5%) subjects of the 88 had hypopharyngeal SCC (n=20). Hypopharynx was furthur classified into pyriform fossa, post-cricoid and poterior pharyngeal wall. 11(55%) of the patients pyriform fossa was involved, 7 (35%) had post-cricoid involvement and 2 (10%) had posterior pharyngeal wall involvement. Postcricoid malignancy was only noted in women, all presented in Stage III.

HPV Status

In our study, only 2 cases were positive for HPV consensus and both were positive for HPV 16.

In Larynx SCC group one case was positive. He had Stage III Glottic malignancy. Exposure to alcohol and smoking was present but no history of betel nut/quid chewing. History of oral sex was present. He underwent total laryngectomy with partial pharyngectomy and post-operative radiotherapy. Patient is attending follow up from two years and no signs of recurrence.

One case was noted in Hypopharyngeal group i.e. stage III post-cricoid malignancy . She had no history of alcohol consumption, smoking, betel nut chewing, no history of premarital sex, exposure to multiple sexual partners or oral sex. She was given concurrent chemotherapy and radiotherapy. Patient survived for only 10 months post treatment.

Table 1. Tumour Characteristics of the Study Population

Site	No.	Stage (no.&%)				HPR (no.&%)		
Subsite		1	2	3	4	WD	MD	PD
Oral cavity	37							
BM	22	0	4 (18.2)	4 (18.2)	14 (63.6)	11 (50)	4 (18.2)	7 (31)
LBT	13	0	6 (46.2)	0	7 (53.8)	5 (38.5)	8 (61.5)	0
FM	1	0	0	1 (100)	0	1 (100)	0	0
HP	1	0	0	0	1 (100)	0	0	1 (100)
Oropharynx	20							
BOT	6	0	0	0	6 (100)	0	6 (100)	0
Tonsil	14	0	1 (7.1)	7 (50)	6 (42.9)	6 (42.9)	6 (42.9)	2 (14.3)
Larynx	11							
Glottis	5	1	0	4 (80)	0	0	5 (100)	0
SG	6	0	0	2 (33.3)	4 (66.7)	0	5 (100)	0
Hypopharynx	20							
PC	7	0	0	7 (100)	0	2 (28.6)	3 (42.9)	2 (28.6)
PF	11	0	0	3 (27.3)	8 (72.7)	1 (9.1)	7 (63.6)	3 (27.3)
PPW	2	0	0	2 (100)	0	0	2 (42.9)	0
Total	88	1 (1.2)	11 (12.5)	30 (34%)	46 (52.3%)	26 (29.5)	44 (50)	18 (20.5)

no, number; BM, buccal mucosa; LBT, lateral border of tongue; FM, floor of mouth; HP, hard palate; BOT, base of tongue; SG, supraglottis; PC, post cricoid; PF, pyriform fossa; PPW, posterior pharyngeal wall; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated

Table 2. Risk Factor Characteristics of the Study Population

	Oralcavity	Oropharynx	Hypopharynx	Larynx	Total	Chisquaretest
HPV positive Status	0	0	1 (1.13)	1 (1.13)	2 (2.27)	
Cigarette smoking						0.04*
No	16 (43.2)	4 (20)	10 (50)	1 (9.1)	31 (35.3)	
Yes	21 (56.8)	16 (80)	10 (50)	10 (90.9)	57 (64.8)	
Betelnut chewing						<0.001#
No	8 (21.6)	5 (2.5)	14 (70)	8 (72.7)	35 (39.8)	
Yes	29 (78.4)	15 (75)	6 (30)	3 (27.3)	53 (60.2)	
Alcohol drinking						0.03#
No	15 (40.5)	4 (20)	9 (45)	0	28 (31.8)	
Yes	22 (59.5)	16 (80)	11 (55)	11 (100)	60 (68.2)	
Beedi smoking						0.,06 (NS)#
No	13 (35.1)	2 (10)	8 (40)	1 (9.1)	24 (27.3)	
Yes	24 (64.9)	18 (90)	12 (60)	10 (90.9)	64 (72.7)	
Premarital sex						0.98 (NS)#
No	26 (70.3)	15 (75)	14 (70)	8 (72.7)	63 (71.6)	
Yes	11 (29.7)	5 (25)	6 (30)	3 (27.3)	25 (28.4)	
Multiple sex partner						0.54 (NS)#
No	33 (89.2)	17 (85)	19 (95)	11 (100)	89 (90.9)	
Yes	4 (10.8)	3 (15)	1 (5)	0	8 (9.1)	
Oral sex						0.94 (NS)#
No	25 (67.6)	12 (60)	14 (70)	9 (81.8)	60 (68.2)	
Yes	7 (18.9)	3 (15)	3 (15)	1 (9.1)	14 (15.9)	
DK	3 (8.1)	4 (20)	3 (15)	1 (9.1)	11 (12.5)	

#, Fishers exact test; DK, don't know; *p<0.05, statistically significant; p>0.05, Non Significant, N

Table 3. Relation of Head and Neck Tumour Subsites to Smoking, Alcohol and Betel Nut Chewing

	Oral cavity	Oropharynx	Hypopharynx	Larynx	Total (n=88)
No habit	2 (20.0)	2 (20)	6 (60)	0	10
S	0	1 (33.3)	2 (66.7)	0	3
A	2 (66.7)	0	1 (33.3)	0	3
BN	9 (90)	0	1 (10)	0	10
A+S	4 (21.1)	2 (10.5)	5 (26.3)	8 (42.1)	19
BN+S	4 (80)	1 (20)	0	0	5
BN+S+A	16 (42.1)	14 (36.8)	5 (13.2)	3 (7.9)	38

S, smoking; A, alcohol intake; BN, betel nut chewing

Risk Factors

From Table 2 , it can be noted that the risk factors like betel nut chewing , alcohol intake and cigarrete smoking showed statistical significance (p<0.05). Beedi smoking did not show any statistical significance (p=0.06). Based on the behavioural data of oral sex, multiple partners and premarital sex there was found to be no statistical significance .

In Table 3 it can be observed that in oral cavity SCC group exposure to betel nut chewing(78.4%) is more among the participants. In the oropharyngeal group exposure to beedi and cigarrete smoking is high. In the hypopharyngeal SCC group exposure to betel nut chewing (30%) is less and exposure to alcohol consumption (55%) was more. It was found that the postcricoid subsite involvement in hypopharynx was seen only in women (n=9) and all had no history of exposure to betel nut chewing, cigarrete/beedi smoking and alohol consumption.

In the laryngeal SCC group it was observed that 100% had exposure to alcohol, 90% to beedi and cigarrete smoking and only 3% gave history of betel nut.chewing.

Discussion

The objective of our study was to investigate the role HPV and risk factors in the carcinogenesis of HNSCC in the coastal population of Karnataka.

In our study the mean age of presentation was in the fifth decade and majority of the participants came with oral cavity malignancy (42.5%) at presentation and buccal mucosa was the most common subsite involved.

65 (73.9%) were males and 23 (26.1%) were females. Similar findings were seen in other studies (Mallen et al., 2016; Dikshit et al., 2012).

Majority of the patients presented in the III and IV stage. The reasons for late presentation were – patient thought the oral ulcers would heal, prolonged treatment which would affect the family financially, hospital was located long distance away from their home-town. Similar findings were also reported in an analytical study on delay in presentation of oral cancer cases (Kumar et al., 2001).

On reviewing literature for HPV prevalence in HNSCC or site specific SCC in HNSCC in India and World-wide it was noted that there were numerous articles with varying data. This may be attributed to many factors such as overlap in nomenclature between subsites such as oral cavity and oropharynx, sampling method used (some studies use oral brushing and some use biopsy from tumour site), storage of specimen and type of assay used – immunohistochemistry or PCR. In our study, in 88 participants with HNSCC only 2.27% (n=2) were positive for HPV DNA and both were for HPV 16. One patient had laryngeal SCC another had hypopharynx site involvement. In India, some studies on prevalence of HPV in head and neck cancer show a prevalence of 31.3% (33/106) with HPV 16 having 81.8% and HPV18 is 18.18% (Kumar et al., 2015) and 29% (67/226) in which HPV 16 is 47.7% (Sannigrahi et al., 2016). There are articles on meta-analysis in laryngeal SCC showing 25% HPV prevalence with high-risk HPV type 16 more commonly associated than HPV 18 (Torrente et al., 2010). In the hypopharynx, pyriform fossa has a higher prevalence of HPV than other subsites. (Joo et al., 2013). In our study, we had one HPV positive in the hypopharynx involving the post-cricoid subsite.

In HNSCC, oropharynx subsite especially tonsil and base of tongue show a higher affinity to HPV infections (Gillison et al., 2000). In our study, 57 cases of oral cavity and oropharynx were included but none of the biopsy samples were HPV positive. Another recent study from South India also showed null prevalence (Laprise et al., 2016) in OSCC. In contrast, another study in South India had 40% (19/47) HPV positive oral squamous cell carcinoma cases (Bijina et al., 2016). Studies done specifically on OSCC show that the prevalence in Eastern India is 33.6% (Nagpal et al., 2002), 31.6% in North- East India (Kumar et al., 2015), 0-67% in South India (Balaram et al., 2005, Laprise et al., 2016) and 15% Western India (Koppikar et al., 2005). HPV infection therefore, may not play a major role in the carcinogenesis of HNSCC in the coastal part of South India.

In the present study statistical significance was seen in the association of HNSCC with betel nut chewing, smoking cigarettes and alcohol consumption. Risk factors such as betel nut chewing, tobacco chewing/smoking and alcohol consumption has been attributed to the carcinogenesis of HNSCC (Hashibe et al., 2007; Sturgis and Cincirpini, 2007). Tobacco as an independent risk factor (Mishra and Meherota, 2014) and heavy alcohol consumption as an independent and synergistically with tobacco has been proven to increase the risk of HNSCC (Sturgis and Cincirpini, 2007). The role of betel nut being one of the causative factors in HNSCC especially oral cavity malignancy in South – Central Asia has been reported (Balaram et al., 1995; Laprise et al., 2016; Ko et al 1995).

In our study, in the laryngeal SCC group exposure to alcohol and smoking was seen in 72.7% of the participants. Both alcohol and smoking are accepted risk factors for laryngeal SCC (Lee et al., 2005). Based on our data on hypopharyngeal SCC we observed that in cases with post cricoid subsite involvement all the participants were female and they had no history of betel nut, tobacco smoking/ chewing or alcohol consumption. Peripheral blood smear and haemogram in these participants were normal. One of the well documented etiological factors for post –cricoid malignancy is Patterson- Brown –Kelly syndrome (Axon et al., 1997). Absence of nutritional factors may play a role in post –cricoid malignancy but further studies are required to validate these claims.

The limitation of our study is the sample size is relatively small. Secondly, we recruited patient referred to a single hospital site limiting generalizability of our research findings.

To conclude, from our present data, HPV does not play a major role in the carcinogenesis of HNSCC in coastal region of South India. The risk factors like betel nut chewing, tobacco exposure and alcohol consumption have a greater role in the carcinogenesis of HNSCC. Our results also show HPV 16 was positive in one case of each of laryngeal SCC and hypopharyngeal SCC. None of the cases showed association of HPV among oral cavity and oropharyngeal SCC. Further study is required to support this finding in a larger sample size in patients with HNSCC.

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Conflict of Interest

There is no conflict of interest.

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