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PREVALENCE OF HUMAN PAPILLOMAVIRUS IN HEAD & NECK SQUAMOUS CELL CARCINOMA IN KASHMIR

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ABSTRACT **Introduction:** Head and neck squamous cell cancer (HNSCC) is the sixth most common cancer worldwide and eighth by death. It represents almost 3.5% of all tumors. Data supporting human papilloma virus as a causative agent in the development and progression of head and neck cancer, particularly that of oropharynx has accumulated during past three decades. **Aim:**The aim of this study was to assess the prevalence of Human papilloma virus (HPV) in head and neck squamous cell carcinoma in Kashmir. **Materials & Methods**: All patients with head and neck squamous cell carcinomas were enrolled in the study and were evaluated in detail. Clinical examination was done and investigations were performed wherever deemed necessary. Tissue sample was collected and put in sterilized labelled plastic vials containing 05 ml of normal saline and transported from the theatre to the biochemistry laboratory on ice and stored at -80°C for further analysis. Tissue samples were divided into two parts, one part was sent to histopathological diagnosis, other half was stored at -80°C for molecular investigations. Only histopathologically confirmed HNSCC cases were included for molecular analysis. Polymerase chain reaction analysis was done and results were obtained. **Results:** The HPV DNA detected by PCR was seen in 6.7% of carcinomas only. Highest percentage of HPV DNA was seen in patients of oral cavity carcinomas (25%) followed by laryngeal carcinomas (6.25%). **Conclusion:** This study shows a low prevalence of HPV 16 and 18 among head and neck squamous cell carcinoma patients in Kashmiri population. In this study, HPV-positive tumors were more commonly observed in young males, non- smokers, early stages of disease and well differentiated tumors.

KEYWORDS : Head and neck squamous cell cancer (HNSCC), Human papilloma virus (HPV).

INTRODUCTION:

Head and neck squamous cell cancer (HNSCC) is the sixth most common cancer worldwide,[1] representing almost 3.5% of all tumors. Among the 120 sub types of HPV identified, about 20 are known to be carcinogenic, with HPV16 being primarily associated with head and neck cancer. The overall HPV incidence varies depending on tumour location.[2,3]

The etiological role of HPV in these cases gains significance. During the past three decades , data supporting human papilloma virus as a causative agent in the development and progression of head and neck cancer, particularly that of oropharynx has accumulated. The incidence rates of the virus in oral cancers ranges from 10% to 51% as observed in studies from different parts of world.[4] Studies in India have revealed incidence rates of 15%-31% in oral cancers.[5,6] Recently, several reports of increasing incidence of head and neck cancers, specifically oral cancers in atypical population groups of females or young adults, with no history of smoking or alcohol abuse, have been published.[7-11] It seems highly probable that at least in a part of these cases, human papilloma virus (HPV) played an important etiological role.

The association of HPV with head and neck cancer is documented by many studies; the likelihood of viral infection in oral carcinoma is placed at 4.7 times more than normal mucosa while in premalignant lesions it is between 2-3 times.[12] The rates of associations have varied from 4-48% in oral carcinoma patients[13,14] and from 12.5% in nodular leukoplakias to 60% in verrucous lesions.[15,16]

MATERIALS AND METHODS

All patients with head and neck squamous cell carcinomas who were histopathologically confirmed, irrespective of stage, age, and gender were enrolled in the study. General examination was done and ENT examination was performed. Fibro-optic laryngoscopy, sinonasal endoscopy, Direct-Laryngoscopy examination, Computed Tomography and Magnetic Resonance Imaging was done as and when required. (Figure 1,2,3)

Collection and storage of tissue samples: Tissue samples were put in sterilized labeled plastic vials (10 ml volume) containing 05 ml of normal saline and transported from the theatre to the biochemistry laboratory on ice and stored at -80°C for further analysis.

HNSCC Tissue samples were divided into two parts

- One part was sent to histopathological diagnosis
- Other half was stored at -80°C for molecular investigations
- Only histopathologically confirmed HNSCC cases were included for molecular analysis.

MOLECULAR ANALYSIS

Extraction of Genomic DNA

High Molecular Genomic DNA from the histopathologically confirmed fresh HNSCC tissue samples were extracted by kit based method. The kit used was Quick- g DNA™ MiniPrep supplied by ZYMO RESEARCH.

The DNA eluted was stored at 4°C for a short time but the vials were kept at -20°C for longer duration storage for further investigation.

Qualitative and Quantitative Analysis

The integrity of the genomic DNA was examined by gel electrophoresis using 0.8% agarose gel to which 10 µl/ 50 ml (of gel solution) of fluorescent dye ethidium bromide was added during its cooling and then gel was cast and 20 µl wells were cast into it by usage of suitable combs. 2 µl of each DNA sample was mixed with 1 µl of 1X DNA loading dye and was loaded in the gel. Electric current was applied at 50V until DNA entered into the gel and was raised to 70V for rest of the run. Run was stopped when the dye had travelled nearly 2/3rd of the gel. DNA in the gel was visualized with the help of Gel documentation system (Alphaimager™ 2200, Alpha Innotech Corporation) under UV light and picture was captured by using CCD camera system.

The quantity of the above isolated Cancerous genomic DNAs were determined by measuring optical density (OD) at 260 nm and 280 nm by double beam spectrophotometer and the concentration was

determined by using the fact that 1 absorbance unit equals to 50 µg/ cm^3 and therefore, the concentration of DNA sample is given by the following equation.

DNA (µg/ml) = A_{260} x 50 x dilution factor

The ratio of 260/280 nm was calculated and the DNA samples for which the ratio was 1.7-1.9 was considered for the future use. The DNA was stored at 4°C for a short time but the vials were kept at -20°C for longer duration storage.

HPV (High Risk) Detection

All tumors were evaluated for the presence of oncogenic HPV-16 and HPV-18 DNA by using Polymerase Chain Reaction (PCR). Prepare the PCR reaction as per Kits protocol (3B Black Bio Biotec HPV Kit).

Reagent Preparation

a) Prepare the PCR Mix as follows:

Name of the reagent	For "3" reaction	For "n" reaction
HPV Mastermix	108 µl	36 x n µl
50mM $MgCl_2$	4.5 µl	1.5 x n µl
DNA polymerase 1U/ µl	7.5 µl	2.5 x n µl

b) Aliquot 40 µl of the reaction mixture in each amplification vial, in the laminar flow cabinet.
c) Remove vials from laminar flow cabinet.
d) Add 50-100ng (3 µl) templates DNA from the purified samples to each amplification vial.
e) 3µl of Positive Control DNA was added to the Positive Control vial.
f) Complete up to 50 µl final volume with sterile distilled water.

Amplifications were performed with the following cycling profiling:

Taq DNA polymerase activation was performed by incubation at 94°C for 10 min, followed by 40 cycles of denaturation for 30 sec at 94°C, 1 min of annealing at 55°C, and 1 min of elongation at 72°C. The last cycle was followed by a final extension step of 10 min at 72°C. Amplification was performed in an Eppendorf thermal cycler system. A positive control was also amplified by PCR. 10 µl of the amplicons was analysed by electrophoresis on 2% agarose gels and ethidium bromide staining. The HPV genotypes in the samples were identified by the sizes of the amplicons.

RESULTS

In this study, 88.9% of patients were males and 11.1% were females. Most of the patients (73.3%) belonged to rural areas. The maximum of patients presented in 7th decade of life. We found that risk factors were present in 82.2% of patients. The most common type of risk factor seen was tobacco. Other risk factors seen are given in table 1. 75.6% of patients presented within 6 months and 13.3% presented after 1 year period. The most common site of disease in our study was larynx with 71.1% percentage followed by pharynx (17.8%), oral cavity (8.9%), external ear (2.2%). The most common laryngeal subsite involved in our study was glottis with 87.5% followed by supraglottis with 9.37% and subglottis was involved in 3.12% cases. The most common pharyngeal subsite involved in our study was hypopharynx with 87.5% followed by oropharynx with 12.5% . 68.9% of patients had moderately differentiated squamous cell carcinoma followed by well differentiated squamous cell carcinoma (22.22%) and poorly differentiated squamous cell carcinoma (8.9%). The HPV DNA detected by PCR was seen in 6.7% of carcinomas only(figure 4). Highest percentage of HPV DNA was seen in patients of oral cavity carcinomas (25%) followed by laryngeal carcinomas (6.25%). HPV16 was found in 4.44% of patients and HPV18 was found in 2.22% of patients (table 2).

HPV detected by PCR was more commonly associated with well differentiated squamous cell carcinomas (20%) and among these it was seen more commonly with those of larynx (14.3%) . HPV detected by PCR was found in 50% of patients with well differentiated squamous cell carcinomas of oral cavity.

While correlating the prevalence of non smokers showing HPV**,** it was positive in 12.5% patients who were non-smokers.

While correlating the gender with HPV positivity it was seen HPV was present in 7.5% of male patients and absent in female patients. The mean age of HPV- positive patients was 47 years and mean age of HPV- negative patients was 63.83.

With increase in T, N, M stage of tumor the percentage of HPV positivity decreases by PCR method. HPV is positive in 20% of T1 patients and 6.67% of T3 patients. For N0 patients HPV positivity is 8.1% and in M0 patients HPV is positive in 6.97% of patients.

DISCUSSION:

This cross-sectional study was done in the Postgraduate Department of Otorhinolaryngology Head and Neck surgery, in collaboration with Department of Biochemistry Government Medical College and Associated Hospitals Srinagar. Our study group comprised total of 45 histopathologically documented Head and Neck Squamous Cell Carcinoma patients recruited from outpatient clinics of otorhinolaryngology department of SMHS hospital and associated hospitals of GMC Srinagar.

We believe that male predilection of HNSCC found in this study is because of them being addicted to tobacco. Many other studies have found this male predilection in head and neck squamous cell carcinomas like the study by **Yanan Xu et al., 2015**[17] from **Chinese patients**. **Carole Fakhry et al., 2008**[18] from **USA,** included in their study 96 patients of head and neck squamous cell carcinomas with male to female ratio of 77/19 (8:2).

We found that most of our patients 24(53.4%) out of 45 presented to us in 7th and 8th decade of their life, which is consistent with the *study of* ***Maura L. Gillison et al., 2000***[19] *from* ***USA,*** *and* another study of ***Elaine M. Smith et al., 2004***[20] *from* ***USA.***

In this study we noted tobacco exposure in 37(82.2%) patients out of 45 and 8(17.8%) patients had no tobacco exposure, which is consistent with the study of **Yanan Xu et al., 2015,**[17] from **Chinese patients.**

Contrary to the literature we did not find any positive history of alcohol consumption in our patients. A study by **Elaine M. Smith et al., 2004**[20] from **USA,** has found history of alcohol consumption in 75% of total patients of head and neck cancer.

The most common squamous cell carcinoma in our study was laryngeal carcinoma (71.1%)(figure 1,2,3) followed by pharyngeal tumors (17.8%) and oral cavity tumors (8.9%). These results are comparable to a study of **Gudleviciene Zivile et al., 2014**[21] from **Lithuanian patients**. In another study of **Sankaranarayanan et al., 1998**[22] Oral cavity cancers were more common in the Indian subcontinent, nasopharyngeal cancer was more common in Hong Kong, and pharyngeal and/or laryngeal cancers were more common in other populations. Contrary to rest of Indian subcontinent, in our study laryngeal cancers are more common. This can be due to the fact that tobacco chewing is not common in Kashmiri population, while as history of heavy smoking is found to be common.

In this study HPV infection was detected by PCR in 2 (6.25%) out of 32 kashmiri patients of laryngeal carcinoma. Out of the these two positive cases, HPV-16 was found in one case and HPV-18 in another case. Similar finding were seen in the study of **Fouret et al., 1995**[23] from **France.**

In this study it was found that none of the 8 pharyngeal cancer specimens from Kashmiri patients were HPV DNA-positive. This is in contrast to the study of **Gudleviciene Zivile et al., 2014**[21] from **Lithuanian patients and Belarusian patients.** Our result in pharyngeal cancer patients could be related to the small number of cases included in the study group.

In our study HPV-16 was detected in 1(25%) out of 4 patients of oral cavity carcinomas. It is consistent with the study of **Seye Abogunrin et al., 2014**[24] on the prevalence of human papillomavirus in head and neck cancers in **European populations.**

HPV was detected by PCR in 3(6.7%) out of 45 patients and out of these 3 patients HPV16 was detected in 2(66.7%) patients and HPV18 in 1(33.33%) patient. (Table 2)

In this study low Prevalence of Human Papillomavirus in Head and Neck Squamous Cell Carcinoma was found. This can be related to the fact that small number of patients were taken in our study group, differences in populations and detection methods used.

Traditionally, tobacco smoking and excessive alcohol consumption

have been considered the main risk factors for head and neck cancer. Individuals with HPV-positive head and neck cancer tend to be white, male, non-smokers, and non-drinkers when compared with those with HPV-negative tumors (**Gillison et al., 2008).**[25] In the present study, it was seen that HPV was positive in 1(12.5%) out of 8 patients who were non-smokers and 2(5.4%) patients out of 37 patients who were smokers.

In our study it was also found that HPV was positive in 3 (7.5%) out of 40 male patients and absent in all female patients.

SUMMARY

Our study showed a low prevalence of HPV 16 and 18 among head and neck squamous cell carcinoma patients in Kashmiri population.

CONCLUSION

The prevalence of HNSCC varies among various population groups and association of HPV with HNSCC also depends on the population group examined and various other factors.

LMITATIONS: Less number of patients were included in this study.

Table – 1: Type of risk factors in Head and Neck Squamous cell Carcinoma Patients (n=37)

Risk factors	No of Patients	Percentage
Tobacco(cigarettes)	21	56.76
Tobacco(pipe)	11	29.73
Tobacco(smokeless)	5	13.5
Alcohol	0	0
Total	**37**	**100.0**

Table – 2: Subtype of HPV extracted in Head and Neck Squamous cell Carcinomas (n=45)

HPV type	No. of Patients	HPV (Positive)	Percentage
HPV16	45	2	4.44
HPV18	45	1	2.22
Total	**45**	**3**	**6.67**

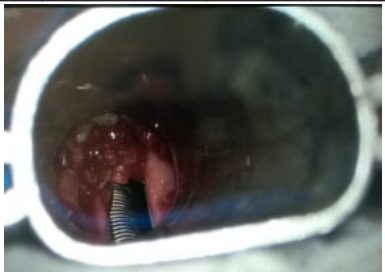

Figure 1. Direct laryngoscopy picture of T2 glottic squamous cell carcinoma

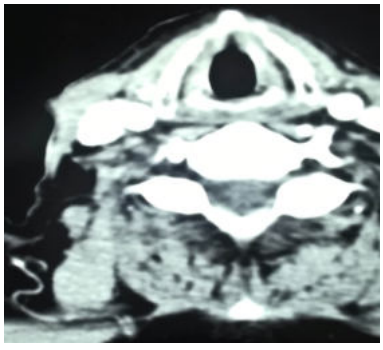

Figure 2: CECT picture of patient with T2 glottic squamous cell carcinoma

Figure 3: 70 degree hopkin rod picture of T3 hypopharyngeal squamous cell carcinoma

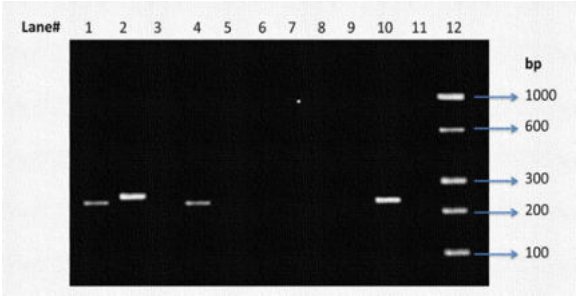

Figure 4: Representative gel picture of the PCR amplified products run on 2.0 % agarose gel

Lane 1& 4: Sample Positive for oncogenic HPV-16 (bp 238)
Lane 2: Sample Positive for oncogenic HPV-18 (bp 268)
Lane 3, 5, 6, 7, 8 & 9: Sample Negative for oncogenic HPV
Lane 10: Positive control
Lane 11: Negative control
Lane 12: 100bp DNA Molecular Marker

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