Original Article

The Clinico-Pathological Features in HPV Positive Head and Neck Cancer

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Abstract

Introduction: HPV-16 is the most common viral subtype associated with malignant transformation, ranging between 90% and 95% of all cases [8]. It is present in 86.7% of all HPV-positive oropharyngeal SCCs compared with 68.2% of HPV-positive oral SCCs and 69.2% of HPV-positive laryngeal SCCs [9]. Prevalence of HPV in oral cancers is similar in Europe (16%) and North America (16.1%), but greater in Asia (33%) [9]. Data from multiple studies have indicated that HPV-associated tumors account for 20% to 75% of OPSCC. *Methodology:* Patient who fulfill the inclusion criteria and his/her care takers were explained regarding the nature of the disease, radical treatment with concurrent chemo radiation, its effectiveness and possible side effects. Patient were counseled about the ill effects of tobacco and alcohol consumption and asked to discontinue the same. Patients were also counseled regarding maintenance of good oral hygiene throughout the treatment, good nutritional support, adherence to the planned treatment. *Results:* In arm A, 60%(9/15) of patients had oral cavity cancer, 13.3%(2/15) of patients had oropharyeal cancer and 26.6% (4/15) patients had hypopharyngealcancer. In arm B, 65.7%(23/35) of patients had oral cavity cancer, 8.5%(3/35) of patients had oropharyneal cancer, 5.7% (2/35) patietns had laryngeal cancer, 2.8%(1/35) patients had were in nasopharyeal cancer and 17.1%(6/35) were in hypopharyneal cancer. *Conclusion:* No association found in nodal (N) stage at presentation between HPV positive and negative tumors.

Keywords: HPV; Nasopharyeal Cancer; Head and Neck Cancer.

Introduction

Head and Neck cancer is the seventh most common type of cancer worldwide.[1]They are the most common cancers in developing countries, especially in Southeast Asia. These are more common in males compared to females. These cancers are mainly attributable to the use of tobacco, areca nut, alcohol etc [2]. Head and neck cancer in the developing world differ from those in the Western world in terms of age, site of disease, etiology and molecular biology. Poverty, illiteracy, advanced stage at presentation, lack of access to health care, and poor treatment infrastructure pose a major challenge in management of these cancers in the developing world [2].

According to the International Agency for Research on Cancer, there is a wide geographical variation in the incidence of head and neck cancer. Globally the incidence of overall head and neck cancer is 6.9% with a mortality of 5.15% from these cancers. Estimated incidence in men is 7.9%, where as in women it contributes globally to around 6.1% as per GLOBOCON 2012. In terms of mortality, head and neck cancer rank seventh in the world (4.6% of total deaths). Gender distribution suggest that head and neck cancers are more common in males, both in incidence (6th; 7%) and mortality (6th; 6.3%) than females [1].

According to GLOBOCAN 2012, incidence of cancers in India in 2012 was 1,01,49,00. Lip and oral cavity cancer was 3rd most common cancer contributing to 7.6% of total cancer burden. In men, lip and oral cavity cancers are most common (11.3%) of all cancers. In women, it is 3rd most common cancer after breast and cervix [1].

Overall 54.9% of global head and neck cancers occur in Asia, especially in India and account for 30% of cancers [3]. In India, nearly two-thirds of patients present with advanced stages [3]. The incidence of head and neck cancers varies greatly in differentgeographical regions. Within the same geographical region, there are great differences in distribution between various head and neck sub-sites. This variation is likely due to differences in relative distribution of risk factors [4]. The mean age of patients at presentation of head and neck cancers is the fifth and early sixth decades in Asian populations compared with the seventh and eighth decades in the North American population [5-7].

In addition to the risk factors like tobacco and alcohol, a subset of head and neck cancer are caused by HPV (Human Papilloma Virus), most commonly in the oropharynx [8-10]. HPV-16 is the most common viral subtype associated with malignant transformation, ranging between 90% and 95% of all cases [8]. It is present in 86.7% of all HPV-positive oropharyngeal SCCs compared with 68.2% of HPV-positive oral SCCs and 69.2% of HPV-positive laryngeal SCCs [9]. Prevalence of HPV in oral cancers is similar in Europe (16%) and North America (16.1%), but greater in Asia (33%) [9]. Data from multiple studies have indicated that HPV-associated tumors account for 20% to 75% of OPSCC. The rise in HPV-related cancers has been mainly attributed to thechange in sexual practices in the Western world. These patients are younger, more common in males, have bulky nodes, predominantly oropharyngeal involvement, present at advanced stage and have better survival [10-12].

Methodology

Patient who fulfill the inclusion criteria and his/her care takers were explained regarding the nature of the disease, radical treatment with concurrent chemo radiation, its effectiveness and possible side effects. Patient were counseled about the ill effects of tobacco and alcohol consumption and asked to discontinue the same. Patients were also counseled regarding maintenance of good oral hygiene throughout the treatment, good nutritional support, adherence to the planned treatment. Informed consent same was taken in their own language. The toxicity of the patients undergoing treatment will be evaluated weekly during radiotherapy and the highest toxicities will be recorded using common toxicity criteria version 4.

After completion of treatment, patients were advised to come for first follow up at 2 months for loco-regional response assessment. A criterion for assessing the response to treatment was primarily by clinical examination and if required radiological assessment.

Pre-Treatment Evaluation: This included the following

1. All patients underwent detailed history taking that includes, presenting complaint (duration of symptoms, ulcer, associated pain, dysphagia, oral ulcers, neck swellings, weight loss, dietary habits), past history, associated co-morbidities, family history, personal history including habits of alcohol consumption, tobacco chewing or smoking.

2. General Physical examination including performance status, nutritional status.

3. Systemic examination.

4. Local examination to evaluate the extent of the primary tumor (oral, oropharyngeal, laryngeal examination) and examination of the neck nodes.

5. Biopsy of the primary or FNAC of the metastatic neck nodes.

6. Complete blood count

7. Kidney and liver function tests

8. X-ray chest

9. Dental prophylaxis

10. CT scan of head and neck region (base of skull to clavicle).

Immunohistochemistry

Immunohistochemistry is a technique for identifying cellular or tissue constituents (antigens) by means of antigen-antibody interactions. The antigen-masking effects of formaldehyde fixation are reversible to varying degrees by a process known as antigen retrieval. Antigen retrieval involves exposing the tissue sections to heat or proteolytic enzyme digestion before commencing immunochemical staining. The mechanisms of antigen retrieval are poorly understood but this method is believed to break the methylene bridge cross-links formed between proteins during formaldehyde fixation, thus allowing the proteins to take on a more tertiary-like structure and allowing antibodies access to the epitope.

Therefore, antigen retrieval by pre-treatment during optimization is the vital stage in the technique prior to incubation with the optimal dilution of the antibody. The optimum dilution for an antibody is the highest at which specific immunoglobulin

saturates the available antigen, leaving some unbound antibody in the solution to ensure continued binding.

Immunohistochemistry for p16, a surrogate marker for HPV 16 will be carried out with p16 mouse monoclonal ready to use antibody (CD INK4a) (Catalog no AM540-5M, Biogenex) using the above said epitope retrieval technique. It will be performed according to protocol that is standardized in the Department of Pathology. The protocol includes deparaffinisation, rehydration and incubation with primary antibody and further treatment with high sensitivity peroxidase -DAB system (DAKO Envision detection system).

Tissue Specimen Preparation

A microtome was used to cut 5 μm sections from FFPET (Formalin-fixed, paraffin-embedded tissue) blocks. Later, the multiple sections were gently placed into a water filled electro thermal paraffin section mounting bath (already prewarmed at 55⁰C) to avoid the formation of folding artifacts. An individual section was mounted onto the Polysine slide by placing it in the water bath at an acute angle beneath the floating section and attaching it to approximately the middle of the slide by adhesion. All mounted slides were placed in a metal rack and kept in the oven at 65⁰C for at least 2 hours to remove excess paraffin and to prevent the section floating off during processing.

P16 Immunohistochemistry Staining Protocol

1. The slides containing the sections were deparaffinised and hydrated by washing with xylene for three times which is then followed by alcohol wash and rinsing under tap water.

2. Antigen retrieval-Antigen retrieval was performed in citrate buffer at pH 6 using andecloking chamber at 95⁰C for 30 min. It was checked that the decloking chamber should have 450ml of distilled water. This is then cooled to room temperature for 10-20 minutes before proceeding for immunostaining.

3. The tissues were then processed for incubation with 3% hydrogen peroxide for 5 -10 minutes followed by washing the slides thrice with tris buffered saline (TBS) for 3 minutes each. Appropriately characterized primary antibody p16 (mouse monoclonal IgG, Biogenex,) was applied followed by further incubation for one hour at room temperature.

4. The slides were then washed thrice with tris buffered saline (TBS). Horse radish Peroxidase (HRP) conjugated anti rapid antibody was applied to each section.

5. Freshly prepared DAB (di amino benzidine) substrate was added and incubated until stain developed (DAKO Immunohistochemistry - manual).

6. Sections thus rinsed with distilled water were stained with hematoxylin for 30 seconds.

7. Finally, the stained sections were washed with water followed by dehydration.

8. Cover slips were mounted using permount mounting medium (DPX –Dibutyl Phathalate Xylene) and the slides were evaluated

9. Scoring-All the sections were scored independently, by an experienced pathologist.p16 expression was scored as positive if there was a strong and diffuse brown staining of the nucleus and cytoplasm in 70% of the tumor specimen. For p16, loss of expression was evaluated as negative.

Results

The site of primary tumor was mainly divided as oral cavity, oropharynx, hypopharynx, larynx and nasopharynx.

In arm A, 60% (9/15) of patients had oral cavity cancer, 13.3% (2/15) of patients had oropharyeal cancer and 26.6% (4/15) patients had hypopharyngeal cancer.

In arm B, 65.7%(23/35) of patients had oral cavity cancer, 8.5%(3/35) of patients had oropharyneal cancer, 5.7% (2/35) patietns had laryngeal cancer,

Table 1: Site of primary tumor in Arm A and Arm B patients.

| Site | N=50 | | Chi-Square | P |
	ARM A	ARM B		
Oralcavity	9(60%)	23(65.7%)		
Oropharynx	2(13.3%)	3(8.5%)		
Larynx	0(0%)	2(5.7%)	2.054	0.726
Nasopharynx	0(0%)	1(2.8%)		
Hypopharynx	4(26.6%)	6(17.1%)		
Total	15	35		

2.8%(1/35) patients had were in nasopharyeal cancer and 17.1%(6/35) were in hypopharyneal cancer.

With respect to primary tumor site, the difference between arm A and arm B was not statistically significant. The prevalence of HPV in oral cavity cancer was 28.1% (9/32), in oropharyneal cancers was 40%(2/5) and 40%(4/10) in hypopharyngeal cancer.

In arm A 6.6% (1/15) of patients were presented in T1, 26.6% (4/15) of patients in T2, 46.6% (7/15) in T3 and 20%(3/15) in T4. Where as in Arm B, 2.8% (1/35), 25.7%(9/35), 34.2%(12/35) and 37.1%(13/35) respectively.

High percentage of patients in arm A were presented in T3 stage (46.6%) and T2 stage (26.6%). High percentage of patients in arm B were presented in T3 (34.2%) and T4 (37.1%).

Patients in arm A were presented in early T stage than arm B. The chi square $\chi2$ value for significance of correlation between groups for tumor (T) stage was 1.772 and p value was ≥ 0.05.With respect to tumor (T) stage presentation, the difference between arm A and Arm B was not statistically significant.

In arm A, 3/15 (20%) of patients were node negative and in arm B 5/35 (14.2%) were node negative. In arm A, 5/15 (33.3%) patients presented with N1 stage and 7/15 (46.6%) with N2 stage. In arm B, 12/35 (34.2%) patients were presented in N1, 16/35 (45.7%) were in N2 and 2/35 (5.7%) were in N3.

High percentage of patients in arm A were presented in N1 (33.3%) and N2 (46.6%) stage. High percentage of patients in arm B were presented in N1 (34.2%), and N2 (45.7%) stage.The chi square $\chi2$ value for significance of correlation between groups for nodal stage was 1.076 and p value was ≥ 0.05.

With respect to nodal (N) stage at presentation, the difference between arm A and arm B was not statistically significant.

All patients were staged using the AJCC 7th staging manual and assigned a TNM stage of II to IVB. Stage I & IV C patients were not included in this study as per the protocol. In arm A, 1(6.6%) patient was presented in stage II, 5(33.3%) patients were in stage III, 9(60%) patients were in stage IV.

In arm B, 1(2.8%) patient was presented in stage II, 11(31.4%) were in stage III, 23(65.7%) were in stage IV.

High percentage of patients in arm A were presented in stage IV (60%). High percentage of patients in arm B were presented in stage IV (65.7%).

The chi square ($\chi2$) value for significance of correlation between groups among arm A and arm B for AJCC stage was 0.446 and p value was 0.800. As the p value being ≥ 0.05. With regard to stage at presentation, there was no significant difference between arm A and arm B.

Table 2: Tumor (T) stage distribution in Arm A and Arm B patients, $\chi2$ and p value

| T Stage | N=50 | | Chi-Square $\chi2$ | P |
	ARM A	ARM B		
T1	1(6.6%)	1(2.8%)		
T2	4(26.6%)	9(25.7%)		
T3	7(46.6%)	12(34.2%)	1.772	0.621
T4	3(20%)	13(37.1%)		
Total	15(100%)	35(100%)		

Table 3: B Nodal (N) stage distribution in Arm A and Arm B patients, $\chi2$ and p value

| N Stage | N=50 | | Chi-Square | P |
	ARM A	ARM B		
0	3(20%)	5(14.2%)		
1	5(33.3%)	12(34.2%)		
2	7(46.6%)	16(45.7%)	1.076	0.783
3	0(0%)	2(5.7%)		
Total	15(100%)	35(100%)		

Table 4: TNM stage distribution in Arm A and Arm B patients, $\chi2$ and p values

| Stage | N=50 | | Chi-Square, $\chi2$ | P |
	ARM A	ARM B		
II	1(6.6%)	1(2.8%)		
III	5(33.3%)	11(31.4%)		
IV	9(60%)	23(65.7%)	0.446	0.800
Total	15(100%)	35(100%)		

Table 5: Distribution of grades of primary tumor in Arm A and Arm B patients, $\chi2$ and p value

Biopsy	N=50		Chi-Square	P
	ARM A	ARM B		
GRADE I	13(86.6%)	29(82.8%)		
GRADE II	2(13.3%)	4(11.4%)	1.374	0.712
GRADE III	0(0%)	2(5.7%)		
TOTAL	15(100%)	35(100%)		

All patients had squamous cell carcinoma as their histology with different grades.

In Arm A, out of 15 patients, 13(86.6%) patients had grade I SCC and 2 (13.3%) had grade II SCC . In Arm B, out of 35 patients analyzed, 29 (82.8%) had grade I SCC and 4 (11.4%) had grade II SCC and 2(5.7%) had grade III SCC.

High percentage of patients in arm A had grade I SCC (86.6%) and high percentage of patients in arm B had grade I SCC(82.8%). The chi square $\chi2$ value for significance of correlation for grades of primary tumor was 1.374. As the p value being ≥0.05. With respect to grade of the tumor, the difference between arm A and arm B was not significant.

Discussion

In arm A, 1(6.6%) patients were stage II, 5(33.3%) patients were stage III, 9(60%) patients were stage IV. In arm B, 1(2.8%) patients were stage II, 11(31.4%) in stage III, 23(65.7%) in stage IV. Most common stage at presentation in arm A and arm B was stage IV. But there was no significant difference in TNM stage presentation when compared to HPV negative group.

Lassen P et al analysed the effect of HPV associated p16 expression on response to radiotherapy and survival in squamous cell carcinoma of head and neck. In this study HPV positive tumors were presented in stage III/IV 83% than stage I/II(17%) but it was not statistically significant when comared to HPV negative HNSCC(I/II 20%, III/IV80%) [13].

Smith et al 2010 analyzed the HPV, p16 and p53 expression in association with survival of head and neck cancer. In this study HPV positive tumors were presented in stage IV (63.6%) than stage I-III (36.4%) when comared to HPV negative HNSCC (I-III 51.8% vs. IV 48.2%) [14].

In study by Posner et al on subset analysis from an international trail TAX324 for analysis of survival and HPV in OPSCC HPV positive tumors were presented in stage IV(82%)than 18% in stage III. But when compared to HPV negative (91% vs. 9%) this difference was not significant [15].

In study by Rischin et al on analysis of the prognostic importance of p16 and HPV in patients with OPSCC treated on a phase III concurrent chemoradiation trail found that HPV positive OPSCC were presented in stage IV (94%)than 6% in stage III. But when compared to HPV negative, this difference was not significant [16].

In retrospective analysis of association between tumor HPV status and survival among stage III /IV OPSCC who were enrolled in RTOG 0129 by Ang KK et al found that HPV positive tumor were presented in stage IV (87.9%)than 12.1% in stage IV. But when compared to HPV negative (83.8% vs. 16.2%) this difference was not significant [17].

AM Hong et al 2010 examined the prognostic significance of HPV in patients with locally advanced oropharyngeal squamous cell carcinoma (SCC) treated primarily with surgery or definitive radiotherapy found that HPV positive tumor were presented in stage IV (69% %)than 31% in stage III. But when compared to HPV negative (54% vs. 46%) this difference was not significant [18].

In an Indian study by Nagpal et al reported that, the advanced stages (III, IV) had higher infection rates as compared to earlier stage. But in other studies by Kumar et al 2003 and Bahl et al 2013, significant association was not found between HPV positive and negative tumors with respect to the stage of the tumor.

In this study HPV positive tumors were presented at stage IV than stage III. Results of the present study were in correlation with above studies. But the difference was not significant probably due to small sample size.

In Arm A, out of 15 patients, 13 (86.6%) had grade I SCC (WDSCC) and 2 (13.3%) had grade II SCC (MDSCC). In Arm B, out of 35 patients analyzed, 29 (82.8 %) had grade I SCC and 4 (11.4%) had grade II (MDSCC) and 2 (5.7%) was grade III (PDSCC.)

Several studies suggested that HPV related SCC more likely to associated poorly differentiated tumors

than HPV non related SCC. In a study by Mendelson et al on histopathological features of HPV associated head and neck cancer, found that HPV positive tumors are characterized by distinct histological features such as moderate/poor tumor differention and non keratinizing orbasaloid pathology [19].

AM hong et al 2010 also found significant correlation between HPV and higher grade of the tumor, 52% of HPV positive OPSCC were poorly differentiated comparedto 30% in HPV negative OPSCC which was statistically significant [18].

Fakhry C et al prospective trial to evaluate the association HPV infection andtreatment outcome found that HPV-positive tumors were also more likely than HPV-negative tumors to be poorly differentiated (P = .03) and to have basaloid features (P <.001).

In study by Lassen et al 54% of HPV positive tumors were PDSCC and 46% were well/moderately differentiated SCC, but this results was not significantly different from HPV negative tumors (38% were PDSCC and 62% were WD/MDSCC) [13].

In study by S.heath et al on human papilloma virus in squamous cell carcinoma of the head and neck found that 39% of HPV positive HNSCC were PDSCC than 8% of HPV negative HNSCC were PDSCC, suggested that HPV tumors were significantly associated with PDSCC [20].

In Indian study by Nagpal et al on prevalence of HPV in oral cavity cancers found that moderately differentiated squamous cell carcinoma (MDSCC) showed 41.4% HPV infectivity as compared with 33.8% in cases of well-differentiated squamous cell carcinoma (WDSCC).

In the present study did not find any significant correlation between PDSCC and HPV. This study results were discordant with the above studies. Probable reason for this discordant results was small sample size, majority of patients in this study were oral cavity squamous cell carcinoma.

Conclusion

- Prevalence of HPV in oral cavity cancer was 28.1%, 40% in oropharyngeal cancer and 40% in hypopharyngeal cancer.

- HPV Positive tumor presented in early tumor (T) stage (T2, T3) than HPV negative tumor and this difference was not significant.

- With respect to TNM stage, both HPV positive and negative cancers were presented at advanced stage (stage IV) and difference was not statistically significant.

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