TUMOR SUPPRESSOR GENE EXPRESSION IN HPV POSITIVE AND NEGATIVE ORAL SQUAMOUS CELL CARCINOMA: CORRELATION WITH CLINICOPATHOLOGICAL VARIABLES

SUMMARY
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Oral squamous cell carcinoma (OSCC) is the most prevalent malignancy in India accounting for about 30% of all types of cancers. Approximately 83,000 new cases and more than 46,000 deaths have been reported each year in India. It ranks number one in terms of incidences among men and third among women.

Though the widely accepted etiologic factor for OSCC is the use of tobacco in various forms, an increased involvement of human papilloma virus (HPV) in the head and neck squamous cell carcinoma (HNSCC) has been reported in past 10 years. However, the presence of HPV was firmly established with oropharyngeal and tonsillar carcinoma. The role of HPV in the etiopathogenesis of sites of HNSCC other than oropharynx and tonsil is still ambiguous and need more solemn attention of researchers in this direction.

HPV is one of the most common causes of sexually transmitted diseases in both men and women around the world and associated with 90-100% of cervical cancer cases. In India a wide variation in HPV associated OSCC has been reported, ranges from 15-37% in majority of studies. Based on oncogenic potential HPVs are divided into high-risk (HR-HPV) and low-risk (LR-HPV) types. LR-HPV can cause common genital warts or benign hyper proliferative lesions. HR-HPV, highlighting HPV 16 and 18, are associated with the occurrence of pre-malignant and malignant cervical lesions, penile, vulvar, anal and HNSCC and contribute to over 40% of oral cancers.

HPV is a small, non-enveloped, DNA virus. The viral genome organized into three segments; early region (E), late region (L) and genomic regulatory region. Through wounds or abrasions HPV infect the actively dividing basal epithelial cells. The HPV oncoproteins E6 and E7 have the ability to deregulate the tumor suppressor function of p53 and pRb proteins. E6 binds to E6AP along with p53, resulting in ubiquitination and

proteasomal degradation of p53 that ultimately cause abrogation of p53 function. E7 attaches with pRb preventing its binding to E2F transcription factor, thereby leaving E2F available to promote the cell to S-phase and cause cell-cycle progression and malignant transformation. Moreover functional inactivation of pRb results in a reciprocal over-expression of p16 protein.

p53 is a tumor suppressor gene (TSG), plays a role in cell cycle progression, DNA repair, cellular differentiation and apoptosis. Mutation of p53 is the most common event occurs in over 50% of all cancers.

p16 is an another important TSG which regulates gene expression at different levels by modifying functional equilibrium of transcription factors. In normal oral epithelium, p16 is detected merely in the basal and supra basal cell layers where the cells are actively proliferative. It has been postulated that HPV presence can be connected to an overexpression of p16.

HPV positive tumors constitute a distinct subgroup clinopathologically. These are usually poorly differentiated, non-keratinized and have a basaloid appearance in contrast to the HPV negative tumors that are well differentiated and keratinized**.** Furthermore, patients with HPV positive tumors tend to be younger at time of diagnosis. HPV positive tumors also represent a distinct molecular phenotype with a unique mechanism of tumorigenesis independent of the mutagenic effect of tobacco and alcohol. HPV positive cancers are characterized by loss of expression of pRb, cyclin D1 and over expression of p16. In contrast, HPV negative tumors consistently show over expression of pRb and cyclin D1 and loss of p16. Mutations in the p53 gene are common in HPV negative cancers whereas the inactivation of p53 gene is more commonly seen in HPV positive cancers. The

carcinogenic role of HPV in different sites of head and neck cancer except oropharynx and tonsil is still ambiguous and need more consideration for optimizing better treatment of HPV associated patients. Keeping the above facts in mind we undertook the present study.

The Study Objectives were:

- To check frequency of Oral Squamous Cell Carcinoma in Indian population associated with Human Papilloma Virus (HPV) and to identify the different subtypes of HPV.
- To analyze phenotypic as well as gene expression of p53 and p16 genes in HPV positive versus HPV negative OSCC.
- To study difference of any in the clinical parameters of HPV positive versus HPV negative Oral Squamous Cell Carcinoma in terms of tumor size, nodal status, age, stage, tumor grade and gender.

This was an observational case series study conducted at the Department of Pathology, Dr. Ram Manohar Lohia Institute of Medical Sciences (RMLIMS), Lucknow, U.P., India. A total of 369 clinically and histopathologically proven cases of OSCC were included prospectively in this study. Biopsies and surgical removal tissues were collected from the Department of Surgical Oncology, King George's Medical University (KGMU), Lucknow, U.P., India. HPV detection was carried out by Real time PCR using 13 HIGH RISK HPV REAL TIME PCR KIT and Conventional PCR with PGMY09/11 primers. HR- HPV types (HPV 16 and HPV 18) were detected in all HPV positive OSCC cases by type-specific conventional PCR. The diagnostic criteria implemented, considered only those cases as HPV positive which were positive by any of two methods. Immunohistochemistry (IHC) was performed on all HPV positive and equal number of

age and sex matched HPV negative OSCC cases to understand the distribution and localization of p16 and p53 in tumor tissue. Relative m-RNA expression of p16 was quantified with real-time PCR using SYBR Green assay and β actin was used as internal control.

p16 staining was evaluated by two different criterion: Criteria I and criteria II. According to criteria I, p16 was considered as positive when cytoplasmic as well as nuclear staining was present in >10% of tumor cells. As per criteria II, p16 was scored as positive for strong cytoplasmic and nuclear staining in 50-70% and >70% tumor cells. Tumors showing >50% moderate and strong nuclear staining was considered as having mutant-type p53 while tumor with <50% p53 protein expression considered positive for wild-type p53. Fold change expression of p16 was calculated by using relative gene quantification method (ΔΔCT method).

The results of present study were obtained as:

- Basic characteristics of total 369 OSCC patients are summarized. The age of patients ranged from 22-80 years with mean (± SE) age 47.40 ± 0.63 years. Among patients, mostly middle age group (41-60 years) (49.9%), mostly male (81.3%), married (96.5%) and from rural areas (67.2%). Maximum patients were attributed with tobacco chewing (84%), followed by betel quid chewing (68%), tobacco smoking (46.9%) and alcohol consumption (26.6%). Multiple habits were presented in 78.6% OSCC cases and only 7.9% cases were not had any risk habit. In patients, buccal mucosa was the most prevalent site (45%). Most of the patients diagnosed in stage IV (43.9%) and least in stage I (8.7%). Histological grade of majority of patients diagnosed as well differentiated (62.2%) and least was poorly

differentiated (3.5%). Large cell keratinized was most prevalent cellular morphology (99.5%) and only 0.5% cases presented basaloid morphology.

- Out of 369 OSCC cases, 31 cases were HPV positive. So the prevalence of HPV was 8.4%.
- Association of HPV with demographic and clinicopathological characteristics of OSCC patients showed that Mean (± SE) age of HPV positive cases was lower as compare to HPV negative cases (44.45.±2.28 years vs. 47.67±0.66 years). Females were more prone for HPV infection (OR=2.25, 95% CI=1.0-5.03, p=0.048). HPV presence was significantly associated with habit of betel quid chewing, tobacco chewing and multiple habits (p=0.017, 0.043 and 0.036 respectively). Buccal mucosa was the most affected site in both HPV positive as well as HPV negative cases. Most of HPV positive and negative cases were diagnosed for well differentiated grade with keratinized morphology. Only one case in both groups had basaloid morphology of carcinoma cell.
- Subtyping of cases revealed that Out of 31 HPV positive OSCC cases, 13 (41.9%) cases had HPV 16 infection while 6 (19.4%) were positive for HPV 18. Six cases (19.4%) showed co-expression of both HPV16/18. While 6 (9.4%) cases were negative for both 16 and 18.
- p16 protein expression according to criteria I, showed that positive staining for p16 was presented in 10 HPV positive and 7 HPV negative cases. However, by more stringent criteria II, p16 expression was seen in 6 HPV positive and 4 HPV negative cases. Majority of OSCC cases by both criterion showed negative expression of p16 irrespective of HPV presence.

- Association of p16 protein expression (by both criterion) with the risk habits of OSCC patients showed that p16 expression obtained by criteria I was found to be significantly associated with habit of smoking (p=0.013) but not by criteria II.
- p16 expression by criteria I was significantly associated with histological grade of HPV negative cases (0.021) but not correlated with any other variables of OSCC cases. p16 expression by criteria II did not associate with demographic and clinicopathological variables among HPV positive and negative OSCC cases. However one HPV positive case with basaloid morphology showed positive expression of p16 by both criterion.
- Mutant-type p53 was present in 54.8% OSCC cases while wild-type p53 was present in 45.2% OSCC cases. Further mutant-type p53 was distributed equally in HPV positive vs. HPV negative cases (17 vs. 17) with no significant difference in both groups (1.00).
- Analysis of p53 protein expression with risk habits of HPV positive and negative groups showed maximum patients of both wild-type and mutant-type p53 were addicted with multiple risk habits.
- p53 wild-type and mutant-type expression did not associate with demographic and clinicopathological variables among HPV positive and negative OSCC cases. One HPV positive case with basaloid morphology showed mutant-type p53.
- Analysis of co-expression showed that out of total p16 positive cases (n=10), most cases had mutant-type p53 expression (n=8) and theses 8 cases distributed equally in HPV positive and negative groups (4 vs. 4). However all p16 positive/p53wild-type co-expressed cases (n=2) belonged to HPV positive group.

p16 protein expression in 50-70% tumor cells along with wild-type p53 was present in only 2 cases of HPV positive group. Mutant-type p53 with 50-70% expression of p16 was present in five cases. All cases with >70% p16 expression (n=3) showed only mutant-type p53 and belonged to HPV positive group.

- The mean m-RNA fold change of p16 in HPV negative cases was slightly lower (1.16 ± 0.53) as compare to HPV positive cases (1.32 ± 0.70) and this difference was statistically insignificant (p=0.367).
- A significant positive correlation (correlation coefficient r=0.69, P<0.001) was observed between p16 protein and m-RNA expression in total OSCC patients. Among HPV positive OSCC group, p16 m-RNA expression level strongly correlated with p16 protein expression (r=0.87, p=<0.001). p16 m-RNA and protein correlation in HPV negative OSCC group showed an average positive correlation (r=0.463, p=<0.05).

Following conclusions were drawn from present study:

- Our findings illustrated that 8.4% OSCC cases harbor HPV in North Indian population which is slightly lower than that observed in previous Indian studies. HPV 16 was the most prevalent HPV sub type found in this population.
- Tobacco was the major risk factor in both HPV negative as well as positive cases. The presence of HPV is only transient and opportunistic among the subgroup we have studied and this might be due to the unhealthy lifestyle or a consequence of genetic instability contributed by mutagens, but the independent, specific and strong carcinogenic effect of HPV is difficult to corroborate because of strong confounding influence of tobacco.

- Negative expression of p16 in majority of OSCC cases, however p16 positive expression in few HPV positive cases along with high percentage staining of p16 in HPV negative cases also, depicted that p16 over expression was not associated with HPV infection in this case series.
- In association with p53 we found, equal distribution of mutant-type and wild-type p53 among HPV positive and negative OSCC cases.
- In addition, all p16 positive cases were addicted with tobacco/alcohol related risk habits. Risk habits distribution was not associated with mutant-type p53 expression.
- Presence of mutant-type p53 along with majority of p16 positive cases in this case series suggests the co-existence of HPV related and tobacco related pathways of carcinogenesis as the squamous epithelia of oral cavity exposed to both mutagens and HPV.
- Further the use of p16 over expression is not a reliable marker in oral cancer to assess the potential etiologic role of HPV in North Indian scenario.

The result of present study contribute information about HPV status in oral cancer patients from north Indian population and can be useful for the implication of standard pathological and clinical evaluation with planning of different type of treatment strategies for HPV associated oral cancer patients.