Detection of P16\textsuperscript{INK4a} in Non-Oro pharyngeal Head and Neck Squamous Cell Carcinoma

1. Dr. S. Sasikala, 2. Dr. K.R. Mohan, 3. Dr. Ravi S., 4. Dr. Johnraj Suresh, 5. Dr. I. Vijay Sathish Kumar, 6. Dr. S. Premalatha, 7. Dr. Devipriya, 8. Dr. Amudhavalli

1. Associate Professor of Pathology, Government Chengalpattu Medical College, Chengalpattu, India
2. Associate Professor of Pathology, Government Chengalpattu Medical College, Chengalpattu, India
3. HOD & Professor of Pathology, Government Chengalpattu Medical College, Chengalpattu, India
4. Final Year Post Graduate in Pathology, Government Chengalpattu Medical College, Chengalpattu, India
5. Associate Professor of Pathology, Government Chengalpattu Medical College, Chengalpattu, India
6. Associate Professor of Pathology, Government Chengalpattu Medical College, Chengalpattu, India
7. Assistant Professor of Pathology, Government Chengalpattu Medical College, Chengalpattu, India
8. Assistant Professor of Pathology, Government Chengalpattu Medical College, Chengalpattu, India

Abstract: The incidence of HPV associated Head and Neck Squamous Cell Carcinoma (HNSCC) is increasing over the past 30 years. It is a growing public health concern. It has been reported that tissues of HPV associated HNSCCs over express p16\textsuperscript{INK4a}. Therefore p16\textsuperscript{INK4a} is used as a surrogate marker to detect HPV associated HNSCC. Immunohistochemical detection of p16\textsuperscript{INK4a} is an easy and simple technique than molecular detection of HPVs. Hence we investigated the presence of p16\textsuperscript{INK4a} in Non-Oro pharyngeal (nasopharynx, hypopharynx and larynx) Squamous Cell Carcinoma (Non-OPSCC).

Aims: The objectives of our study are (1) To study the association of p16\textsuperscript{INK4a} expression with Non-OPSCC, thus with the HPV. (2) To compare the p16\textsuperscript{INK4a} expression in different subsites of the Non-OPSCC. (3) To correlate the level of p16\textsuperscript{INK4a} expression with different grades of Non-OPSCC.

Materials and methods: A total sample of 25 cases were analysed during the period of June 2014 to August 2015. We performed IHC in sections of formalin fixed paraffin embedded tissue of Non-OPSCC cases and correlated the various patterns of p16\textsuperscript{INK4a} positivity with respect to histopathological diagnosis.

Results: In the present study, 80.00% of the Non-OPSCC cases were above 50 years of age. Non-OPSCC was more common in males with male to female ratio of 4:1. 92.00% of Non-OPSCC cases were positive for p16\textsuperscript{INK4a}, of which the most common pattern was diffuse nuclear and cytoplasmic staining (76.00%).

Conclusion: In the present study, increased number of Non-OPSCC cases was seen over expressing p16\textsuperscript{INK4a} (92.00%). Hypopharynx was the commonest site for p16\textsuperscript{INK4a} positivity (92.30%). Of the Non-OPSCC cases, most cases (76.00%) had diffuse pattern of p16\textsuperscript{INK4a} over expression. However, DNA detection based studies are needed to validate the utility of IHC detection of p16\textsuperscript{INK4a} as a surrogate marker for HPV associated HNSCC.

Key words: Head and neck cancer, HPV, Non-OPSCC, p16\textsuperscript{INK4a}, Squamous cell carcinoma

I. Introduction

Head and Neck Squamous Cell Carcinoma (HNSCC) is the fifth most common cancer worldwide with high incidence of more than 600,000 cases every year with high morbidity\textsuperscript{1,2}. It causes 200,000 deaths annually. The incidence is much higher in India, Southeast Asia and Europe\textsuperscript{3}. It is the commonest cancer in males and third most common in females\textsuperscript{4}. HNSCC occurs in 5 anatomical sites namely oral cavity, oropharynx, nasopharynx, hypopharynx and larynx\textsuperscript{5}. There is an association between HPV positive HNSCC and oral sexual behavior, but not in HPV negative HNSCC\textsuperscript{6}. Tobacco use is the main cause of HNSCC with as high as 80% of cases attributed to it. Alcohol usage acts synergistically with tobacco in the increased incidence of HNSCC\textsuperscript{7}. In the past three decades, there is a decrease in the incidence of HPV negative HNSCC due to reduction of tobacco use but there is a remarkable increase in the incidence of HNSCC due to HPV infection\textsuperscript{8}. Prognosis of p16\textsuperscript{INK4a} positive cases has been reported to be better irrespective of histological grade\textsuperscript{9}.

The mechanism of HNSCC in HPV negative patients is frequent DNA mutation. HPV positive HNSCC are due to the genetic alterations. The oncogenic proteins E6 and E7 released by the high risk virus subtypes interrupt the p53 and pRb tumor suppressing pathways respectively, which leads to increased cell proliferation and genomic
stability leading to carcinogenesis. p16\textsuperscript{INK4a} is one of the several cyclin-dependent kinase inhibitors responsible for regulation of normal cell cycle. As pRb is inactivated by E7 protein, cells are released from growth-suppressive stimuli mediated by the p16\textsuperscript{INK4a}. Thus reduced or lost pRb function results in enhanced p16\textsuperscript{INK4a} levels, as a result of a negative feedback control\textsuperscript{9}. p16\textsuperscript{INK4a} is commonly used as a biomarker for transcriptionally active HPV-associated cancers\textsuperscript{10,11,12}.

II. Materials and methods

Study Place: Department of Pathology, Chengalpattu Medical College and Hospital, Chengalpattu.

Study Design: The present cross-sectional study was a prospective study conducted in the Department of Pathology during the period of June 2014 to August 2015. Ethical clearance for the study was obtained from the Institutional Ethics Committee of Chengalpattu Medical College, Chengalpattu.

Study Population: A total sample of 25 cases of Non-OPSCC was analyzed during the period of June 2014 to August 2015.

Inclusion Criteria: Tissue blocks of patients who are diagnosed as Non-Oropharyngeal (nasopharynx, hypopharynx and larynx) Squamous Cell Carcinoma (Non-OPSCC) by biopsy.

Exclusion Criteria: Tissue blocks of patients who are diagnosed as Non-OPSCC by biopsy and underwent Radiotherapy or Chemotherapy.

Materials used:

- Tissue sections prepared from paraffin embedded formalin fixed tissues
- Haematoxylin and eosin stain
- p16\textsuperscript{INK4a} monoclonal antibody kit (Mouse monoclonal, Clone (G175-405) : prediluted)
- Positive control included block sections of known p16\textsuperscript{INK4a} positive cases.
- Negative control included Primary antibody replaced with PBS and normal oral tissue.

Method:

- Blocks and slides of 25 patients in which histopathological examination of hematoxylin and eosin stained sections of biopsy from Non-opharyngeal sites confirmed as SCC were taken up for the study.
- Immunohistochemistry was performed on the tissue sections taken from the blocks of the cases confirmed as SCC.
- Immunostained sections were reviewed and a strong nuclear as well as cytoplasmic staining was considered as positive reaction, as described by Klaes et al\textsuperscript{13}.

Distribution of p16\textsuperscript{INK4a} positivity were scored as negative (<1% cells positive), sporadic (<5% cells positive), focal (<25% cells positive) and diffuse (>25% cells positive) as described by Klaes et al\textsuperscript{13}.

Data Collection:

- H &E stained sections and immunostained sections were assessed using light microscope.

Statistical analysis:

- Datas obtained were coded and entered into the Microsoft excel spread sheet. Datas were compared between groups using Pearson Chi-square or Fisher’s exact tests (p<0.05). All statistical analysis was performed using SPSS statistical software version 11. Charts were prepared using Microsoft excel 2007.

III. Results

In the present study 80% of the cases of Non-OPSCC were above 50 years of age (Table 1.). However none of the cases were observed below 22 years of age. The youngest age for p16\textsuperscript{INK4a} positive Non-OPSCC cases in our study is 22 years and is 60 years for p16\textsuperscript{INK4a} negative cases. Among the 5 Non-OPSCC cases below 50 years of age, all cases (100%) were p16\textsuperscript{INK4a} positive. Among the Non-OPSCC cases above 50 years of age 90.00% were positive for p16\textsuperscript{INK4a}. The range of the age group is much wider (22-80 years) in the p16\textsuperscript{INK4a} positive cases. The mean age for the p16\textsuperscript{INK4a} positive Non-OPSCC cases is lower (58 years) than p16\textsuperscript{INK4a} negative cases. The median age for p16\textsuperscript{INK4a} positive cases is lower (61 years).

In the sex distribution 80.00% of the Non-OPSCC cases were male and 20.00% of the cases were female (Table 1.). All the 5 female cases (100%) were p16\textsuperscript{INK4a} positive. 18/20 male cases (90.00%) were p16\textsuperscript{INK4a} positive. 2/5 female cases are ≤50 years and all are p16\textsuperscript{INK4a} positive. 3/20 male cases are ≤50 years and all (100%) are p16\textsuperscript{INK4a} positive. p16\textsuperscript{INK4a} positive cases are more in the >50 years age groups (78.26%) than in the ≤50 years (Table 1).

1) Hypopharynx was most commonly involved by SCC (52.00%) than Larynx. Majority of Non-OPSCC cases in our study were of histopathological grade 2 (16/25 cases; 64.00%), followed by grade 1 (9/25 cases; 36.00%) (Table 2, Fig.2.). While observing the level of expression of p16\textsuperscript{INK4a} by IHC, 92.00% cases of Non-OPSCC were above 50 years of age 90.00% were positive.
diffuse pattern, followed by focal (12.00%) and sporadic (4.00%) (Table 2, Fig.2.). Diffuse pattern of p16INK4a expression were seen in 88.89% (8/9 cases) of grade 1 and 68.75% (11/16 cases) of grade 2 Non-OPSCC cases. 3/25 cases had focal pattern of expression of p16INK4a and all were of grade 2. (Table 2, Fig.2.).

IV. Discussion

HNSCC continues to be a public health problem with an estimated incidence of 600,000 cases and 200,000 deaths annually\(^1\). The reports implicating specific HPV types in HNSCC were first published in 1985\(^14,15\), p16INK4a over expression can be used as a surrogate marker for detection of HPV association in HNSCC. Our study is a hospital based study and 92.00% cases of Non-OPSCC were positive for the over expression of p16INK4a. According to Caihua Liang et al. 2012, the prevalence of HNSCC based on PCR and p16INK4a detection based studies was 62%.\(^16\). In the present study 80% of the cases of Non-OPSCC are more than 50 years of age. According to Zeyi Deng et al. 2014, 86.67% cases of HNSCC are more than 50 years of age.\(^17\) In the present study, among the 5 cases (25%) of Non-OPSCC which are less than 50 years of age, 100.00% are p16INK4a positive. According to Zeyi Deng et al. 2014, 35% of HNSCC are p16INK4a positive.\(^1\) The mean age for p16INK4a positive Non-OPSCC cases in our study is 58 years. According to Zeyi Deng et al. 2014 it is 61.8 years\(^18\) for HNSCC and according to Caihua Liang et al. 2012 it is 56.4 years.\(^16\) The median age for p16INK4a positive and negative Non-OPSCC cases in our study is 61 years and 70 years respectively. According to GulKanyilmaz et al. 2015, it is 60 and 59 years respectively\(^19\).

**HNSCC.** The youngest age was 22 years among the p16INK4a positive Non-OPSCC cases, while it was 39 years for HNSCC by Zeyi Deng et al. 2014.\(^17\) In our study, male cases among the total Non-OPSCC cases is 80.00% correlating with Zeyi Deng et al. 2014 which is 84.67% for HNSCC.\(^17\)

In our study of Non-OPSCC, 90.00% male patients and 100% female patients are p16INK4a positive. According to GulKanyilmaz et al. 2015, 40.52% male patients and 73.33% female patients are p16INK4a positive\(^19\) for HNSCC. According to Zeyi Deng et al. 2014, 18.9% male patients and 26.1% female patients are p16INK4a positive.\(^4\) According to Caihua Liang et al. 2012, the prevalence of HNSCC among male patients is 27.7% and 11.9% female patients are p16INK4a positive.\(^4\)

In our study, more than 50 years age group constituted the major population with 2 out of which 18 cases (90%) showed p16INK4a positivity. 5 cases were less than 50 years age group, with all cases (100%) showing p16INK4a positivity.\(^1\) In our study, hypopharynx is the most common site followed by larynx which is correlating with Zeyi Deng et al 2014.\(^17\) We did not receive specimens from nasopharynx. Nasopharynx is the least common site involved.\(^1\) The percentage of p16INK4a positive cases is highest (92.30%) in the hypopharynx and lowest (91.67%) in the larynx.\(^1\) According to Zeyi Deng et al. 2014, 7.7% and larynx 12.5% for HNSCC.\(^17\)

In our study, p16INK4a positive cases are highest (64.00%) in the grade 2 Non-OPSCC, followed by grade 1 (36.00%) contradicting with Zeyi Deng et al. 2014, highest (42.1%) in the grade 1 HNSCC, followed by grade 2 (19%) and lowest (14.7%) in the grade 3 HNSCC.\(^17\) In our study, among the Non-OPSCC cases, most (76.00%) are having diffuse pattern of p16INK4a expression, followed by focal (12.00%) and lowest (4.00%) having sporadic pattern of expression.

V. Conclusion

The present study demonstrated increased association of p16INK4a over expression in cases of Non-OPSCC (92.00%). Non-OPSCC was more common in males with male to female ratio of 4:1. Hypopharynx accounted for the most common site (52.00%) than larynx. Also hypopharynx was the most common site for p16INK4a positivity in Non-OPSCC cases (92.30%). Among the p16INK4a positive cases most are Non-OPSCC Grade 2 (65.22%). Of the Non-OPSCC cases, most cases (76.00%) had diffuse pattern of p16INK4a over expression. Diffuse pattern of p16INK4a over expression was most common in Non-OPSCC Grade 2 cases (57.89%).

Further, DNA detection based studies are needed to validate the utility of IHC detection of p16INK4a as a surrogate marker for HPV associated Non-OPSCC. In future, prophylactic vaccination for boys and girls before the starting of sexual activity will prevent HPV infection and thus reduce the incidence of HPV associated HNSCC. Plans to improve public awareness and knowledge of clinical features and risk factors will reduce the disease burden of HPV associated HNSCC.
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Fig. 1. Diffuse pattern of $p^{16\text{INK4a}}$ immunostaining (x400)

![Image of diffuse pattern of p16INK4a immunostaining](image)

Fig. 2. $p^{16\text{INK4a}}$ expression in different grades of Non-OPSCC

![Image of bar chart showing p16INK4a expression](image)

Table 1. Sex distribution of patients with Non-OPSCC in relation to age.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Total</th>
<th>Non-OPSCC Age\leq50 years</th>
<th>Age&gt;50 years</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P16$^{\text{INK4a}+ve}$ n=5</td>
<td>P16$^{\text{INK4a-ve}}$ n=0</td>
</tr>
<tr>
<td>Male</td>
<td>20</td>
<td>3 100</td>
<td>0 0</td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>2 100</td>
<td>0 0</td>
</tr>
</tbody>
</table>

Table 2. Level of $p^{16\text{INK4a}}$ expression in different grades of Non-OPSCC

<table>
<thead>
<tr>
<th>Lesions</th>
<th>Total n=25</th>
<th>Negative n=2</th>
<th>Sporadic n=1</th>
<th>Focal n=3</th>
<th>Diffuse n=19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-OPSCC Grade 1</td>
<td>9</td>
<td>1 11.11</td>
<td>0 0</td>
<td>0 0</td>
<td>8 88.89</td>
</tr>
<tr>
<td>Non-OPSCC Grade 2</td>
<td>16</td>
<td>1 6.25</td>
<td>1 6.25</td>
<td>3 18.75</td>
<td>11 68.75</td>
</tr>
<tr>
<td>Non-OPSCC Grade 3</td>
<td>0</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>2 8.00</td>
<td>1 4.00</td>
<td>3 12.00</td>
<td>19 76.00</td>
</tr>
</tbody>
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