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Original Research Article

Histopathological Study of Head and Neck Squamous Cell Carcinoma with Special Reference to P53 and P16

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Abstract

Background and Aim: In this study, the prevalence of head and neck squamous cell carcinoma (HNSCC) is examined, as well as any potential associations between it and factors like HPV infection, tobacco usage, and alcohol intake. The purpose of the study is to determine whether the expression of p53 and p16 in these tumors correlate with one another.

Material and Methods: This is a cross-sectional retrospective study. In this study, it is being investigated if head and neck squamous cell carcinoma specimens can be used to detect the presence of p53 and p16 proteins using immunohistochemistry. In the investigation, 50 samples from June 2019 to May 2021 were evaluated. The chi-square test was used to assess the percentage of p53 and p16 staining for squamous cell carcinoma of the head and neck region. A value of <0.05 was regarded as statistically significant.

Results: According to the study, the male-to-female ratio is 1.5:1. 80% of the patients had cancers in their mouths, with 10% having tumors in their oropharynx. 18% also smoked while they were drinking, joined by 52% who had a history of smoking. It was seen that 68% of them had well-differentiated carcinomas. However, higher-grade tumors had higher levels of p53 and p16 expression ($p<0.05$).

Conclusion: Our research has found that high levels of p53 and p16 are present in patients with head and neck squamous cell carcinomas and that a relationship between these markers and tumor grade exists. This information might be useful in the early detection and treatment of head and neck squamous cell carcinoma.

Keywords: Clinicopathological correlation, immunohistochemistry, p53, p16, head and neck squamous cell carcinomas.

Introduction

Head and neck squamous cell carcinomas are the most common type of cancer in the head and neck region, and they develop from the mucosal epithelium in the mouth, throat, and larynx. Each year, over 650,000 people worldwide are diagnosed with this cancer, and 350,000 die from it. The National Cancer Registry Programme found that the East-Khasi Hills district had the highest incidence rate of head and neck cancers, followed by Kamrup urban and then the Cachar district.[1]

HNSCC is common in different parts of the world, and is related to tobacco, alcohol, or a combination of both. HPV-18 and other HPV strains are progressively associated with oropharyngeal tumors.[2] HPV-16 is associated with the incidence of OSCC in the tonsils, implying that the next risk for HPV infection is in this area.[3]

Oral cancer is a common type of cancer, and it often develops in areas that have been affected by a premalignant lesion. Leukoplakia, erythroplakia, and oral submucous fibrosis are all common premalignant lesions of the mouth[4]. Laryngeal squamous cell carcinoma (SCC) is also a common type of cancer, and it accounts for around 1.5% of all cancers [5].

The p53 factor product is important for the cell cycle and helps to prevent the progression of the cell cycle when DNA damage occurs. It is a growth suppressor that keeps an eye on the stability of the genome and helps to encourage DNA repair. Mutations within the genome can lead to the formation of an oligomeric complex with wild-type p53 that appears to be functionless and mutant p53 acquires a new oncogenic function that overcomes negative regulation by trace amounts of wild-type p53[6].

The human papillomavirus (HPV) is a small, non-enveloped, circular DNA virus that is part of the papilloma-viridae family [6]. High -risk strains such as HPV 16 and 18, are associated with head and neck squamous cell carcinoma (HNSCC). HPV alters the genes that regulate apoptosis, the cell cycle, and DNA repair, which encourages the development of cancer. By binding to and disrupting the p53 and Rb tumor-suppressing pathways, respectively, the oncogenic proteins E6 and E7 are produced, which promote cell proliferation, genomic instability, and ultimately cancer. Loss or reduction of the Rb function causes a rise in p16 levels, which is a tumour suppressor gene[7]. Immunohistochemistry is able to quickly identify p16 in HPV-infected tumour cells due to the E7 oncogene's transforming activity[8].

Several studies have found that the p16 protein, which is associated with the over-expression of tumor suppressor genes, can be a reliable alternative or complementary test for identifying HPV in head and neck cancers. The American Joint Committee on Cancer (AJCC) 8th Edition recommends using p16 as a standard biomarker for HPV[8].

This study is investigating how histopathologically invasive head and neck squamous cell carcinomas are characterized, and whether there are any biomarkers that can indicate HPV-associated tumors. The study also looks at how tumor status is related to the presence of p53 and p16, two commonly used biomarkers for HPV-associated tumors.

Materials and Methodology

Study design and participants

The current hospital-based cross-sectional retrospective study, "Histopathological Study Of Head And Neck Squamous Cell Carcinoma With Special Reference To p53 and p16," was carried out at the Department of Pathology, Silchar Medical College and Hospital, Silchar. Between June 2019 and May 2021, there was a 2-year study period.

The study was approved by the Institute's Ethics Committee. (SMC/15,124). According to the Helsinki Declaration's ethical guidelines, the study is compliant.

The pathology department at Silchar Medical College and Hospital received 50 biopsy/resection specimens of head and neck squamous cell carcinoma patients. These specimens were analyzed for histopathology using immunohistochemistry and the results were compared with p53 and p16 expression levels.

Inclusion Criteria: Invasive Primary Squamous Cell Carcinoma of the Head and Neck that was diagnosed histologically.

Exclusion Criteria

1. Any secondary cases.
2. Primary Squamous Cell Carcinomas in sites other than the head and neck.
3. All carcinoma in-situ cases.

After obtaining the patients' consent, a clinical history is gathered and all routine investigations are carried out. Histopathologically identified cases are used for immunohistochemistry on paraffin-embedded tissue.

According to established protocols, all of the biopsied tissue samples were immersed in paraffin wax after being fixed in 10% neutral buffered formalin for 24-48 hours. In order to make a histological diagnosis in each case, the biopsied tissues were divided into tissue sections with a thickness of 3 μm, and hematoxylin and eosin staining was performed. To conduct an immunohistochemistry (IHC) research, further consecutive sections were produced.

Immunohistochemistry protocol [8,9].

High-quality IHC staining was performed on all 50 HNSCC cases. Tissues that had been formalin-fixed and paraffin-embedded were sectioned at a 3 m thickness and placed on frosted slides. After being deparaffinized three times in xylene for five minutes each, the sections on frosted slides were rehydrated in various ethanol concentrations

A target retrieval solution with a high pH and Tris-EDTA buffer was used to retrieve the antigen for 30 minutes in an autoclave. After 20 minutes of Tris-buffered saline solution with Tween 20, the slides were blocked with peroxidase blocking reagent, which is composed of phosphate buffer with 15 mmol/L hydrogen peroxide, sodium azide, and detergent. Following a 20-minute H2O2 blocking step, the sections were incubated for an hour at room temperature in the humidity chamber with the primary antibodies for the p53 antigen(clone: DO7) and for the p16 antigen(clone: JC2).

The immunostaining was done with an HRP goat secondary antibody against rabbit and mouse immunoglobulins conjugated with peroxidase molecules. The sections were then washed with wash buffer for 5 minutes and then rinsed with distilled water for 5 minutes. After 1 hour of incubation with the secondary antibody, the sections were stained with DAB + Chromogen. The slides were then washed with distilled water and counterstained with hematoxylin for 2–3 minutes. The slides were dried, then dipped in 100% ethanol and then xylene to clean the sections. The slides were seen through a microscope after being mounted with DPX mountant. The degree of immunostaining and the proportion of stained cells were examined for 100 cells.

Scoring system

1. P53: Shows nuclear positivity

1. Grade 0- 0-10% cells showing nuclear positivity (Negative).
2. Grade 1- 10-30% of cells showing nuclear positivity (Mild expression).
3. Grade 2- 31-50% nuclear expression (Moderate expression).
4. Grade-3- Greater than 50% nuclear expression (Intense expression).

2. P16: Cytoplasm, nucleus, or both staining strongly

1. Grade 0- No staining
2. Grade 1+- Rare singly dispersed cells
3. Grade 2+-Patchy
4. Grade 3+-Diffuse

Statistical analysis

Statistical Package for the Social Sciences version 21 was used to enter and analyse data (SPSS 21, IBM Corporation, United States). The percentage of p53 and p16 protein staining for head and neck squamous cell carcinoma was evaluated using the chi-square test. Statistical significance was defined as a "p" value <0.05.

Results

In our study, "Histopathological study of head and neck squamous cell carcinoma with special reference to p53 and p16", various clinico-pathological parameters are analyzed and are presented as under.

The study shows that punch biopsy was the most common biopsy performed(70%) followed by incisional and excision biopsy in 12 (24%) and 2 (4%) cases respectively. Resection biopsy was performed in only 2% of cases. The mean age of the patients having head and neck squamous cell carcinoma was 52.14 ± 11.75 years and the majority of the patients belonged between 40 to 49 years of age (40%). This was followed by 20% of cases each belonging to the age range of 50 to 59 years and 60 to 69 years of age.

Male predominance was observed for head and neck squamous cell carcinoma with a male: female ratio of 1.5:1. About 60% of patients were males and 40% were females. This proportion may be the cause of increased consumption of alcohol, cigarette smoking, and chewing tobacco. Among the 50 cases, 52% had a positive history of smoking, followed by 18% consuming alcohol along with smoking. 10% had a history of pan or gutkha consumption, 4 patients (8%) chewed tobacco whereas 2 patients (4%) had a history of smoking + pan. 8% of patients did not have any risk factors or any habit.

We found that majority of the patients (80%) had tumor in the oral cavity followed by 10% in the oropharynx, 8% in the larynx, and 2% in the laryngopharynx. There was no incidence of squamous cell carcinoma in the nasopharynx during this study period. Cervical lymph node metastasis was present in 8% of cases whereas, in 4% of cases, the bone invasion was documented. 88% of patients had no history of cervical metastasis or bone invasion.

Table 1: Distribution according to histological grade of HNSCC

HNSCC grading	Frequency (n = 50)	Percentage
Well differentiated SCC	34	68
Moderately differentiated SCC	10	20
Poorly differentiated SCC	6	12

In Table 1, histopathology showed that 68% of the cases of squamous cell carcinoma were well-differentiated, while 20% were moderately differentiated and 12% were poorly differentiated.

p53 immunohistochemistry:

Table 2: Table showing p53 grading of cases

Grade of P53 Staining	No. of Cases	Percentage of Cases
NEGATIVE	19	38
GRADE 1+	3	6
GRADE 2+	12	24
GRADE3+	16	32

31 out of 50 cases (62%) showed p53 positive expression whereas 19 cases (38%) had negative p53 expression.

The percentage of p53 nuclear staining, 19/50 cases showed negative staining (0-10% cells showing positivity), 3/50 cases showed grade 1+ (10-30% of cells showing positivity), 12/50 cases had grade 2+ score (31-50% expression) and 16/50 cases showed grade 3+ (Greater than 50% expression) according to table 2.

Table 3: Table showing p53 grading of cases according to histologic grade

Histologic Grade	Total Case	Number Of P53 Positive Cases	Number Of P53 Negative Cases	Percentage Of P53 Positive Cases According To Histologic Grade
Well-Differentiated	34	17	17	50
Moderately Differentiated	10	8	2	76
Poorly Differentiated	6	6	0	100

In table 3, The p-value is 0.028, this is significant at $p<0.05$.

There is a correlation between histologic grade and p53 expression which was statistically significant. 17 cases out of 34 well-differentiated carcinomas (50% of all well-differentiated carcinoma cases), 8 cases out of 10 moderately differentiated carcinoma (76% of all moderately differentiated carcinoma cases), and 6 cases out of 6 poorly differentiated carcinoma (100% of all poorly differentiated carcinoma cases) are positive for p53 expression. This implies that there is increased p53 expression in higher-grade tumors.

***p16* immunohistochemistry:**

Out of 50 patients with HNSCC, p16 immune expression was positive in 27 patients (54%) and negative in 23 patients (46%).

Table 4: Distribution according to intensity of p16 expression

Intensity of p16 expression	Frequency (n=50)	Percentage
0	23	46
1+	14	28
2+	8	16
3+	5	10

Out of 50 cases, p16 expression of varying intensity was observed in 27 cases as in table 4. Among the p16 positive cases, 28%, 16% and 10% cases had an intensity of 1+, 2+, and 3+ respectively.

Table 5: Association between grading of HNSCC and intensity of p16 expression

HNSCC grading	p16 expression			
	0 (n=23)	1+ (n=14)	2+ (n=8)	3+ (n=5)
Well differentiated SCC	16 (69.6%)	13 (92.9%)	4 (50%)	1(20%)
Moderately differentiated SCC	5 (21.7%)	1 (7.1%)	3 (37.5%)	1(20%)
Poorly differentiated SCC	2(8.7%)	0(0)	1 (12.5%)	3(60%)
χ^2	23.7			
P value	0.01			

According to table 5, majority of well-differentiated SCC cases had either no or low intensity of p16 expression (1+) whereas the majority of cases with poorly differentiated cancer had a high

intensity of p16 expression (3+). We noticed that the higher the grading of carcinoma, the higher is that the intensity of p16 expression. The association between grading of HNSCC and intensity of p16 expression was found to be statistically vital ($p<0.05$).

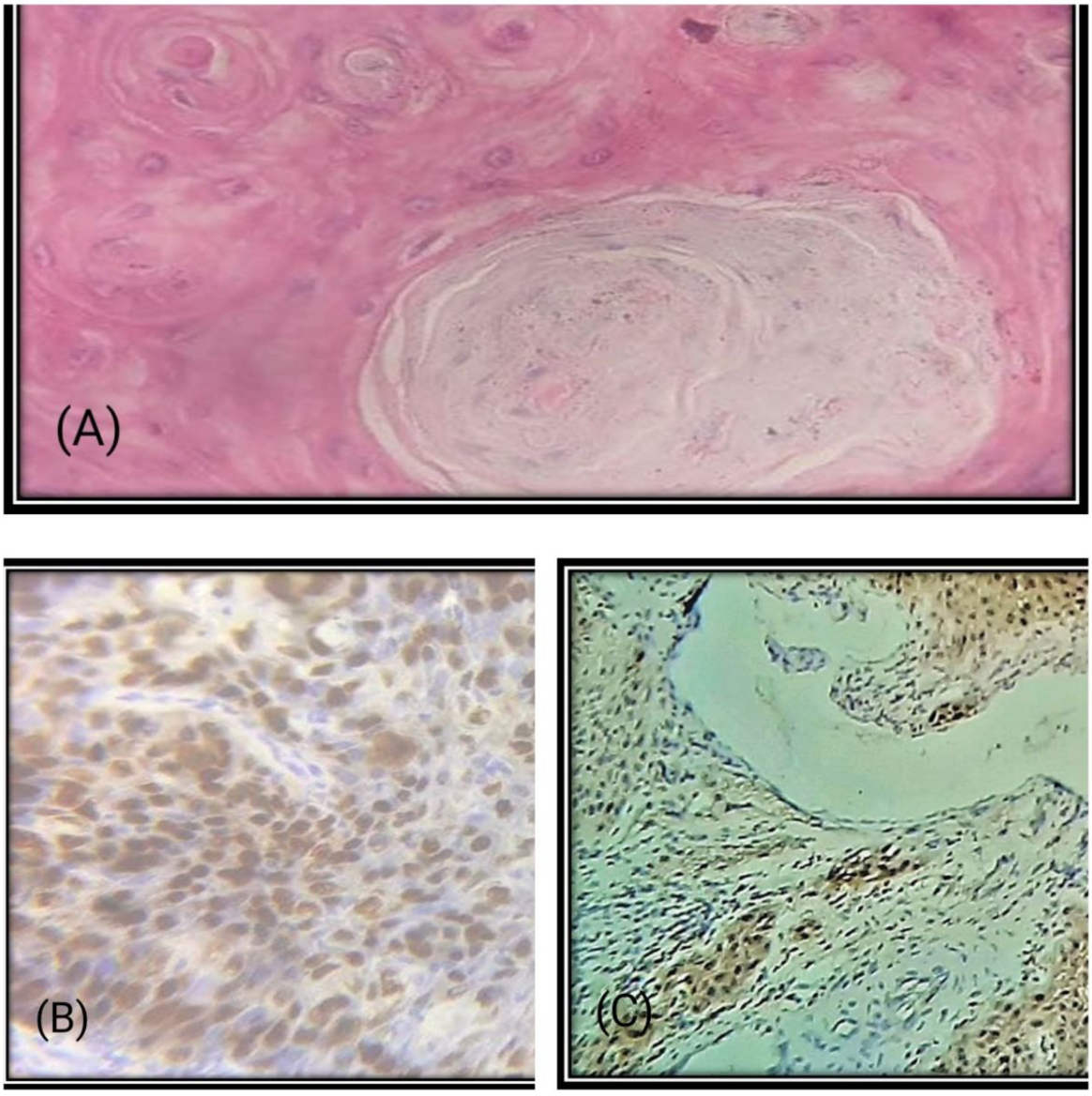

Figure 1: Photomicrograph showing well differentiated squamous cell carcinoma in H&E (A), p53 (B), p16 immunohistochemistry (C).

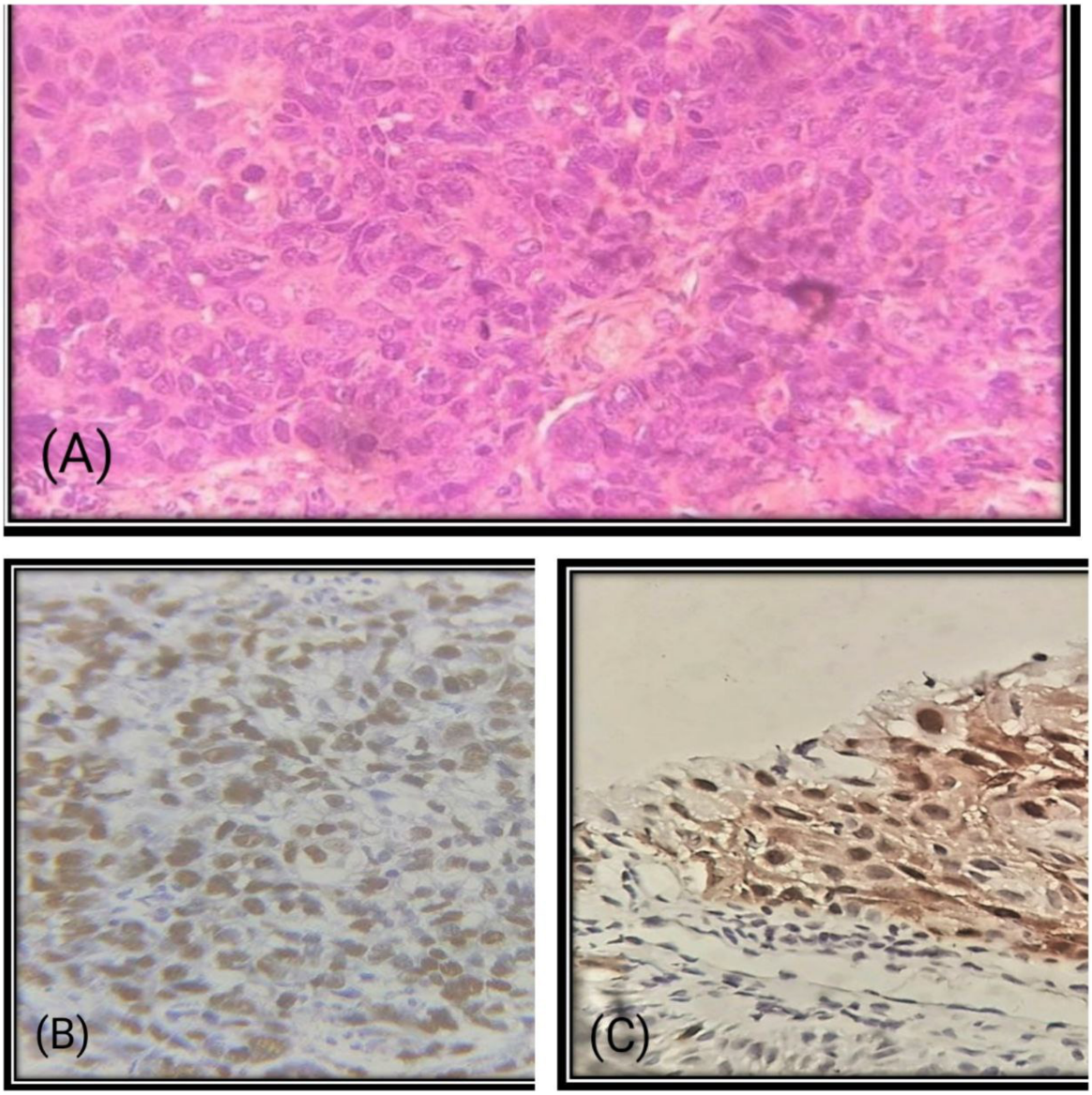

Figure 2: Photomicrograph showing moderately differentiated squamous cell carcinoma in H&E (A), p53 (B), p16 immunohistochemistry (C).

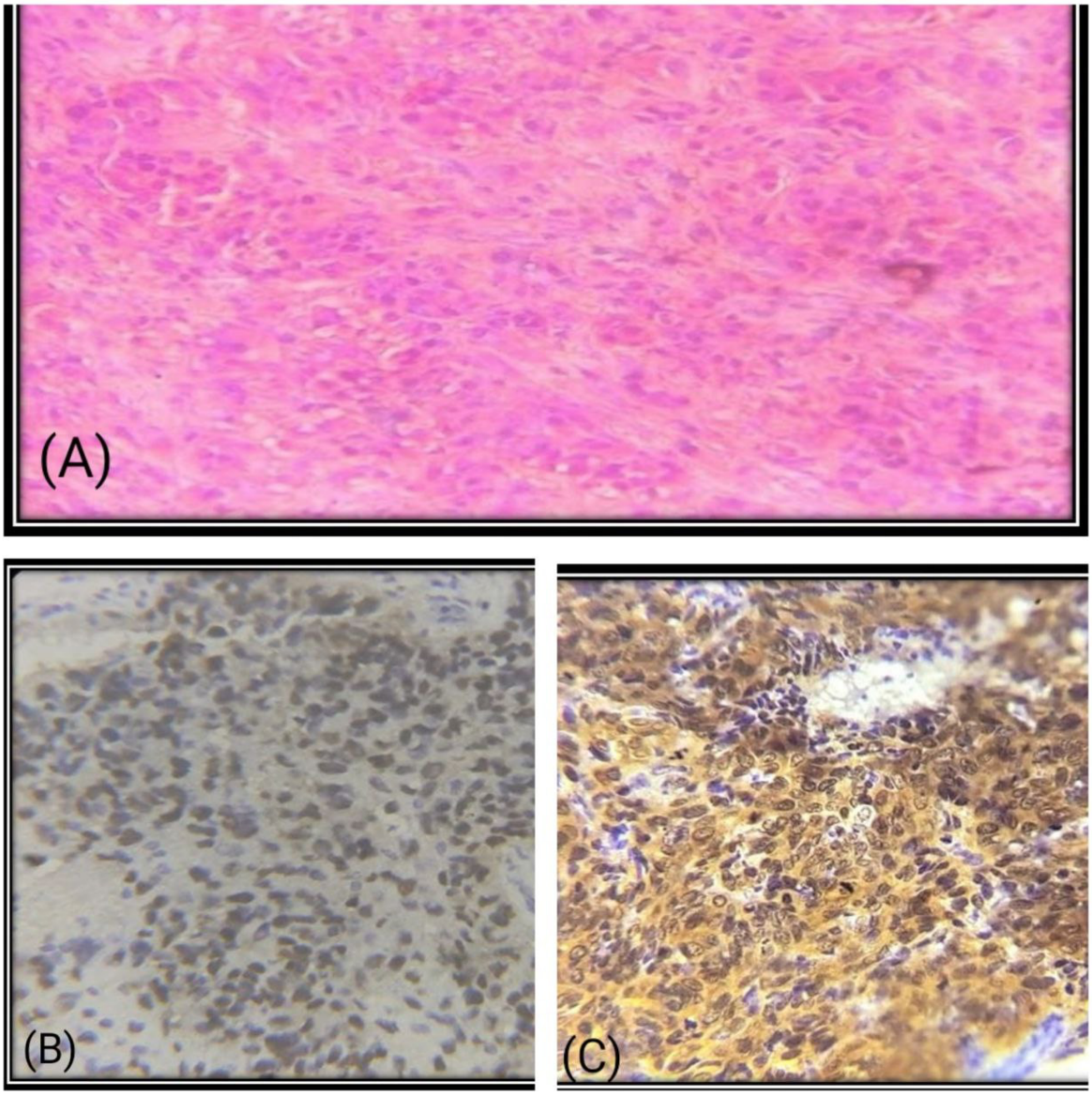

Figure 3: Photomicrograph showing poorly differentiated squamous cell carcinoma in H&E (A), p53 (B), p16 immunohistochemistry (C).

Discussion

This study found that the average age of patients with head and neck squamous cell carcinoma is 52.14 years, with a standard deviation of 11.75 years. The peak incidence of this cancer is seen between 40 and 49 years of age, with 40% of patients having this condition at that point in their lives. The male-to-female ratio is 1.5:1. Smoking and drinking are both associated with increased odds of head and neck squamous cell carcinoma, with 80% of patients having the tumor in the oral cavity. Cervical lymph node metastasis was found in 8% of patients, and 4% of patients showed bone invasion. Histological differentiation showed that 68% of cases were well-differentiated carcinoma, while 20% of cases were moderately differentiated and 12% of cases were poorly differentiated.

These observations are similar to the studies conducted by K. Boslooper *et al* (2008) and Ruchi Dhuria et al (2020) [10,11].

P53:

We found that 38% of patients with cancer had negative p53 expression, while 62% had positive p53 expression. The proportion of p53 nuclear staining was related to the histologic grade or tumour differentiation. P53 expression was positive in 100% of poorly differentiated cancers, 80% of moderately differentiated cancers, and 50% of well-differentiated cancers. This suggests that higher-grade cancers express p53 more frequently.

The results of the current study are similar to those of the other studies:

According to K. Boslooper *et al*. (2008), p53 is found in 63% of tumors, with the most common being grade 1. Higher-grade tumors in head and neck squamous cell carcinoma are more likely to have p53.[10]

Jenny K. Peltonen *et al*. (2010), found that in individuals with head and neck squamous cell carcinoma, p53 is positive in 50% of well-differentiated carcinoma, 48.3% of moderately-differentiated carcinoma, and 71.4% of poorly-differentiated carcinoma [12].

There is a strong correlation between p53 overexpression and histological grading in studies by Erber *et al*(p=0.021) [8] .

P16:

According to the study, 54% of squamous cell carcinomas showed p16 expression, while 46% did not. Of the studied cases, 18 of the 34 (52.94%) were found to be positive for p16, with most of these cases demonstrating a +1 staining pattern. Out of the 10 moderately differentiated cases, 5 (50%) were p16 positive, with 3 of these cases demonstrating +2 staining. Out of the 6 poorly differentiated cases, 4 (66.6%) were p16 positive, with 3 of these cases demonstrating a +3 staining pattern. Variations in p16 expression between histological grades was found to be statistically significant ($p<0.05$). In poorly and moderately differentiated squamous cell carcinomas, diffuse and robust p16 expression (+2/+3) was more common, which may be indicative of an increased viral load. In contrast, single spread cell (1+) staining or no staining was most commonly seen in well-differentiated squamous cell carcinomas.

The results of the current study are similar to those of the other studies:

p16 positivity was determined to be 68% and 52% in the studies by Gonzales-Moles *et al*. (2002) [13] and Ramshankar *et al*.[14]

According to studies by Balaram *et al*.(1995)[15] and Rali M *et al*. (2016)[16], high-grade cervical lesions have a high proportion of cancer cells, which is associated with histological grades.

According to the 8th edition of the AJCC, p16 overexpression is a reliable and long-lasting surrogate biomarker of HPV status[17]. In the current study, 26% of cases of p16 overexpression, which implies HPV positive, were found, mostly in tonsil and tongue cancers. This frequently aligns with research done by Bahl *et al*.(2013)[18], Jitani *et al*.(2015)[19], and Verma *et al*. (2017)[20], who reported 22.8%, 29%, and 22.9% HPV positive, respectively.

Conclusion

In developing nations, squamous cell carcinoma of the head and neck is a severe health concern. A study found that p53 and p16 expression is correlated with histological differentiation and that only nearly half of the lower-grade lesions had positive p53 and p16. This suggests that p53 and p16 expression is increased as cancer develops and can be used to assess the disease's

proliferative activity and propensity for progression. The prognosis for individuals with advanced head and neck malignancies remains poor, and these diseases are challenging to treat.

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