COMPARISON OF HUMAN PAPILLOMAVIRUS 16 EXPRESSION BY IMMUNOHISTOCHEMISTRY IN CERVICAL CARCINOMAS AND NON-NEOPLASTIC CERVICAL MUCOSA

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ABSTRACT

Objective: To compare the prevalence of Human Papillomavirus 16 in cervical carcinoma and non-neoplastic cervical mucosa by immunohistochemistry.

Methodology: From September 2020 to November 2021, this retrospective cross-sectional study was conducted at the Department of Pathology, Peshawar Medical College, Riphah International University Islamabad, Pakistan. on FFPE blocks of 38 cervical carcinoma cases and 38 non-neoplastic cervical mucosa by immunohistochemistry using a monoclonal antibody against HPV 16 by Bio-SB. SPSS 20 was used for the analysis of data.

Results: The mean age of cervical carcinoma patients was 55.18 ± 11.536. The majority of the Cervical carcinoma cases were more than 50 years of age (n= 28, 73.6%). HPV16 positivity was observed in 44.7 % cases of cervical carcinoma and 5.35 % of normal cervical mucosa. The p-value was < 0.00007 and hence statistically significant. HPV 16 expression was higher in the patients with cervical carcinoma in the fourth and fifth decade of life. Among the subtypes of cervical carcinoma, 60.5 % (n=23/38) were Squamous cell carcinoma out of which 43.5% (n=10/23) were positive for HPV 16 expression, Adenocarcinoma subtype was 34.2% (n=13/38) out of which 46.2% (n=6/13) were positive and the Adenosquamous subtype was 5.3% (n=2/38) out of these 1 was positive for HPV 16 expression. High grade tumors (n=18) showed higher HPV 16 expression (61.1%) as compared to low grade lesions (30.0%).

Conclusion: HPV 16 expression is higher in cervical carcinoma cases as compared to normal cervical mucosa. Although not statistically significant high-grade tumors showed higher expression of HPV 16.

Keywords: Human Papillomavirus; Cervical Intraepithelial Neoplasia; Squamous Cell Carcinoma; Cervical Cancer.

INTRODUCTION

According to the Globocan Global Cancer Statistics of 2020, there will be an expected 604,000 new cases of cervical carcinoma and 342,000 deaths globally. This makes cervical carcinoma the fourth most frequent cancer-related mortality in women.[1] The World Health Organization issued a worldwide call in 2018, leading to enhanced measures to strengthen the management of cervical cancer, which poses a significant risk to the health and lives of women. Cervical cancer is preventable and curable if caught and treated early.[2]

The incidence of cervical carcinoma is always increasing in underdeveloped countries as compared to developed countries. In underdeveloped countries the acceptance of cervical screening programs is low and the majority of the population has no access to cancer care.[3] Cervical cancer ranks second among female cancers in terms of incidence and third among cancers in terms of death in low and middle-income countries, behind only breast cancer and uterine cancer.[4] Countries with the highest Age-specific incidence rates (ASIR) are mainly situated in Sub-Saharan Africa but a few are also found in Oceania and Latin America.[5] The trends of cervical cancer prevalence and mortality are comparatively lower (about 2-4 times lower).[6]

There is very little data on the frequency and prevalence of cervical carcinoma in Pakistan due to a lack of a national screening program.[7,8] The majority of cervical cancer patients being diagnosed at very advanced stages resulting in a higher mortality rate. Cervical cancer continues to be among the top five most frequently diagnosed cancers seen among adult females between the time period of 1994 and 2019 at the Shaukat Khanum Memorial Cancer Hospital and Research Centers (SKMCH&RC).[9]

The primary cause of cervical carcinoma is consid-

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ered to be high-risk human papillomaviruses which are implicated in 90% to 99.7% of cases. Identification of the HPV genome has been made in nearly 95% of cervical carcinoma lesions. Most HPV infections are temporary and are spontaneously cleared by the host immune response. However, continuous infection with certain HPV strains may result in the development of premalignant cervical neoplasia.[10] Infections with types 16, 18, 31, or 33 are at the highest risk of developing into cervical intraepithelial neoplasia 3 (CIN3), before progressing to Invasive Cervical Carcinoma. Furthermore, Types 16 and 18 are the genotypes found most commonly in more than 70% of all cervical tumors.[11]

Vaccination and screening of eligible females can be done to control the adverse health effects of HPV infections in the population.[5, 10, 12-15] To prevent acquiring the most prevalent strains of HPV that cause cancer, there are currently three vaccinations on the market: Cervarix® for HPV 16 and 18, Gardasil® for HPV 6, 11, 16, and 18, and Gardasil® 9 for HPV 6, 11, 16, 18, 31, 33, 45, 52, and 58.[16]

The screening for cervical malignancies can be done using one of two main methods. These include real-time quantitative polymerase chain reaction and molecular hybridization, both of which are based on DNA, and cytological analysis. Only in situations with obvious lesions is the Pap smear test helpful. Patients who have HPV infection but no overt lesion will not benefit from treatment. Cytologic testing cannot confirm or deny the existence of an HPV infection in these situations. In contrast, the molecular HPV test is not ideal for widespread screening programs, especially in poor countries due to its high price, low throughput, and inability to identify all HPV genotypes. Consequently, it is crucial to develop efficient and low-cost assays that may help detect all HPV genotypes and assist in large-scale screening, especially in poor countries.[17]

METHODOLOGY

This retrospective cross-sectional study was conducted in the Department of Pathology, Peshawar Medical College, Riphah international University, Islamabad, Pakistan from September 2020 to November 2021. The study was approved by the Institution Review Board (IRB) of Prime Foundation (letter PRIME/IRB/2020-223). To maintain patient confidentiality, case codes were used instead of the patient's name. Thirty eight cervical carcinoma cases and 38 non-neoplastic cervical mucosa cases were included in the study. Data was retrieved from the archives of Pathology Department, Peshawar Medical College, Riphah international university and Pakistan Institute of Medical Sciences, Shaheed Zulfiqar Ali Bhutto University Islamabad. The inclusion criteria for the study were cases with proper fixation and already diagnosed cases of cervical carcinoma with required variables and the cases with unremarkable lining epithelium at transition zone for the Normal Cervical epithelium group. Those cases were excluded from the study that showed the features of inflammation, polyps, micro glandular hyperplasia and the cases with poor fixation and inadequate antigen retrieval were also excluded from the study.

A 5 µ(micron) section of FFPE block was stained with hematoxylin and eosin and slides were re-examined for tumor type and grade for cervical carcinoma cases and unremarkable lining epithelium at transition zone for Normal cervical epithelium cases. The cases that met the inclusion criteria were selected for IHC staining for HPV.

Immunohistochemistry evaluation was performed using anti-total HPV 16 (CAMVIR-1) mouse monoclonal primary antibody by Bio SB (Catalog No. BSB 2944, Tinto Pre diluted, ready to use 7.0ml diluted). The staining was carried out according to the manufactures' protocol using universal kit by Bio SB.

According to the protocol 3-5 micron FFPE tissue sections were cut and mounted on positive charged slides. Then they were Air dried for 2 hours at 58 ° C. The sections were then Deparaffinized, dehydrated and rehydrated using EDTA (BSB 0175). The tissues were then subjected to heat induced epitope retrieval (HIER) using Immuno DNA Retriever with Citrate (BSB 0020) by placing the slides in a pre-warmed coplin jar containing the Immuno DNA retriever with Citrate at 95-99 ° C for 30-60 minutes after which they were immediately transferred to room temperature and were allowed to stand for 15-20 minutes.

Slides were incubated after adding Peroxidase blocking solution to the sections for 5 minutes at room temperature. The slides were then washed with Phosphate buffered Saline (PBS) and the slides were incubated for 60 minutes in humidity chamber at room temperature after application of Primary Antibody. The slides were washed again with Water. Next the sections were incubated for 20 minutes at room temperature after secondary antibody (a Link of Biotinylated Anti-Mouse and Anti-Rabbit immunoglobulin in 1ml dilution) application. The sections were then washed with Phosphate buffer solution for 6 minutes. Sections were then treated with substrate- Chromogen solution for 10 minutes and the antibody color was revealed which was allowed to develop for 5 minutes and the slides were washed again with water. Counterstaining was performed by immersing slides in hematoxylin for 30 seconds. The slides were rinsed for 15 minutes under the running water faucet. Slides of tissue were dehydrated by passing them through four stages of 95%, 95%, 100%, and 100% alcohol for a total of 20 minutes. At room temperature, slides were cleared in three changes of xylene, mounted using DPX mounting solution, and covered with coverslips.

The HPV 16 staining intensities were evaluated by two independent pathologists at 400x magnification who were blinded to the categories of the slides by using codes. HPV 16 staining was localized primarily in the nuclei. The staining of HPV 16, was identified as positive when there was a clear nuclear staining, even if only one positive nucleus was found. One stained cell in the specimen was interpreted as a positive case.[18]

The documented variables were age, grade and type of Cervical Carcinoma and HPV expression in normal cervical mucosa and cervical carcinoma. SPSS 20 was used for the analysis of data. For age (continuous variable), Mean and Standard deviation were calculated. Frequency and percentage were calculated for categorical variables like grade of Cervical Carcinoma, Type of tumor and HPV expression. Chi square test & Fisher Exact Test were performed to compare HPV expression between normal cervical mucosa and neoplastic cervical mucosa. Similarly, Chi square test and Fisher Exact Tests were used to analyze other categorical variables e.g., Tumor Grade and Tumor Type. Probability value of less than and equal to 0.05 ($P \leq 0.05$) was considered statistically significant.

RESULTS

The study included 38 Cervical Carcinoma cases and 38 normal, non-neoplastic cervical mucosa blocks with identifiable transitional zone that were stained for HPV 16 through IHC using standard protocol. The mean age for cervical carcinoma cases was 55.18 ± 11.536. The Age range was 32 -85 years. For Cervical Carcinoma cases majority were more than 50 years of age (n=28, 73.6%) and a few were less than 50 years of age (n= 10, 26.3%). The Mean age for Non-Neoplastic normal epithelium cases was 44.05 ± 8.48. The Age range was between 30-73 years.

The Cervical carcinoma cases were classified as Squamous Cell Carcinoma (n=23, 60.5%), Adenocarcinoma (n=13, 34.2%) and Adenosquamous Carcinoma (n=2, 5.3%). The cases were further subtyped as Keratinizing (n=10, 26.3%), Non- Keratinizing (n=4, 10.5%), Basaloid (n=8, 21.1%), Papillary (n=2, 5.3%), Microinvasive (n=1, 2.6%) and Not Otherwise Specified – NOS (n=13, 34.2%). The majority of the cases were poorly differentiated (n=18, 47.4%), and the rest were moderately (n=9, 23.7%) and well-differentiated (n=11, 28.9%). Cervical carcinoma cases showed a higher positivity of HPV 16 (44.7%, n=17/38) (Fig 7-9) as compared to Non neoplastic cervical mucosa (5.3%, n=2/38) (Fig 10). The two groups showed the difference statistically significant, (p=0.000071) (Table 1, Fig 1, 2).

The majority of the cervical carcinoma cases were in the 61-70 years age group (34.2%). Although the cases that showed positive HPV 16 expression were in the less than 60-year age group (58.8%). In Non- Neoplastic Group majority of the cases were from the less than 50 years age group (89.5%). Whereas 2 cases showed positive HPV 16 expression in this group (Table 2, 3). The cases of cervical carcinoma were subtyped as Squamous cell Carcinoma (60.5%, n=23/38), out of which 43.5% (n=10/23) were positive for HPV 16 expression (Fig 3-5, Table 4). The other types that were observed were Adenocarcinoma (n=13) and Adenosquamous carcinoma (n=2). 6 (46.2%) out of the 13 Adenocarcinoma cases were positive for HPV 16 expression. While only 1 out of the 2 Adenosquamous carcinoma was positive for HPV 16 expression. The relation of HPV 16 to tumor type was not statistically significant (p = .976).

Table 1: Comparison of HPV 16 expression in cervical carcinoma and non-neoplastic cervical mucosa

	Positive	Negative	Total	P value
Cervical Carcinoma	17 (44.7%)	21 (55.3%)	38 (100%)	.000071
Normal Mucosa	2 (5.3%)	36 (94.7%)	38 (100%)	

Table 2: Distribution of HPV 16 expression in different Age Groups in Cervical Carcinoma Cases

Age Groups (years)	HPV Staining		Total (n, %)
	Positive (n, %)	Negative (n, %)	
30-40	1 (5.9%)	4 (19.0 %)	5 (13.2%)
41-50	5 (29.4%)	4 (19.0%)	9 (23.7%)
51-60	5 (29.4%)	4 (19.0%)	9 (23.7%)
61-70	4 (23.5%)	9 (42.9%)	13 (34.2%)
71-80	1 (5.9%)	0 (0.0%)	1 (2.6%)
81-90	1 (5.9%)	0 (0.0%)	1 (2.6%)

Table 3: Distribution of HPV 16 expression in different Age Groups in Normal epithelium Cases

Age Groups (years)	HPV Staining		Total (n, %)
	Positive (n, %)	Negative (n, %)	
30-40	1 (50.0%)	14 (38.9%)	15 (39.5%)
41-50	1 (50.0%)	18 (50.0%)	19 (50.0%)
51-60	0 (0.0%)	2 (5.6%)	2 (5.3%)
61-70	0 (0.0%)	1 (2.8%)	1 (2.6%)
71-80	0 (0.0%)	1 (2.8%)	1 (2.6%)

Table 4: Relation of HPV 16 expression with Tumor Type

Histological Type	HPV Staining		Total (n, %)	P value
	Positive (n, %)	Negative (n, %)		
Squamous cell Carcinoma	10 (43.5%)	13 (58.5%)	23 (100.0%)	.976
Adenocarcinoma	6 (46.2%)	7 (53.8%)	13 (100.0%)	
Adenosquamous Carcinoma	1 (50.0%)	1 (50.0%)	2 (100.0%)	

Table 5: Relation of HPV 16 expression with Histological Grade

Histological Grade	HPV Staining		Total (n, %)	P value
	Positive (n, %)	Negative (n, %)		
Low Grade	6 (30.0%)	14 (70.0%)	20 (100.0%)	.054
High Grade	11 (61.1%)	7 (38.9%)	18 (100.0%)	

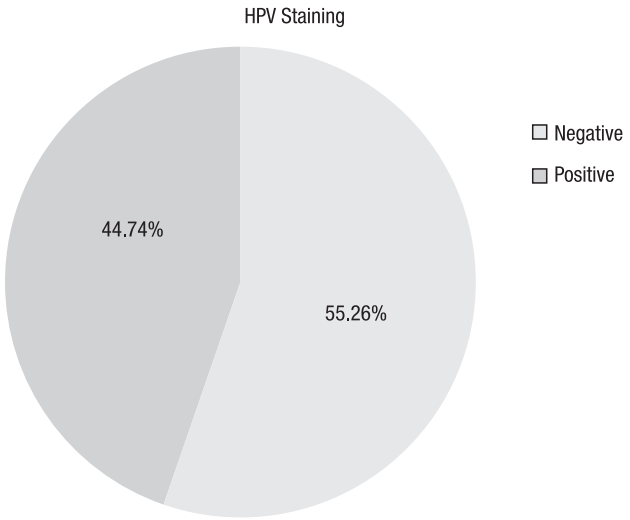

Figure 1: HPV 16 staining results in Cervical Carcinoma

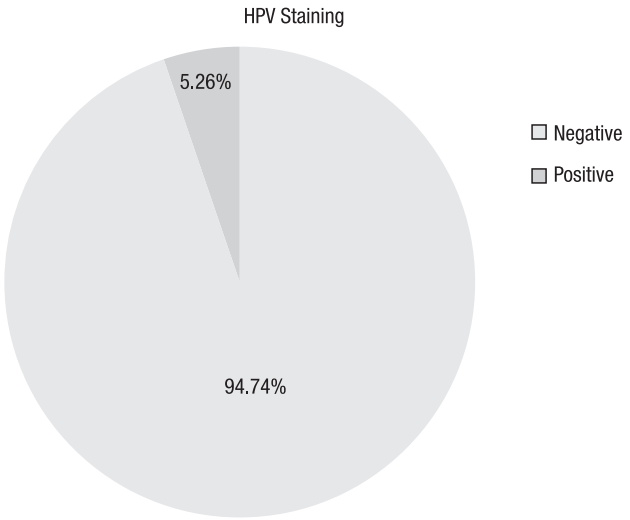

Figure 2: HPV 16 staining results in normal cervical mucosa

Among high grade cervical carcinomas 61.1% (n=11/18) were positive for HPV 16 expressions compared to low grade cervical carcinomas with only 30% (n=6/20) cases with HPV 16 positivity. Although having a higher trend of HPV positivity in higher grades of cervical carcinoma the results were not statistically significant (p= 0.054) (table 5).

DISCUSSION

Human papillomavirus has been studied extensively in order to determine its level of prevalence in different regions of the world. HPV has been implicated in the etiopathogenesis of cervical premalignant and malignant lesions worldwide. However in Pakistan due to a lack of awareness in the masses and absence of an established national cervical cancer screening program there is insufficient data to initiate a national vaccination program.[19]

This study was conducted in an effort to contribute to the data available regarding cervical carcinoma as well as to suggest low-cost and easily available methods such

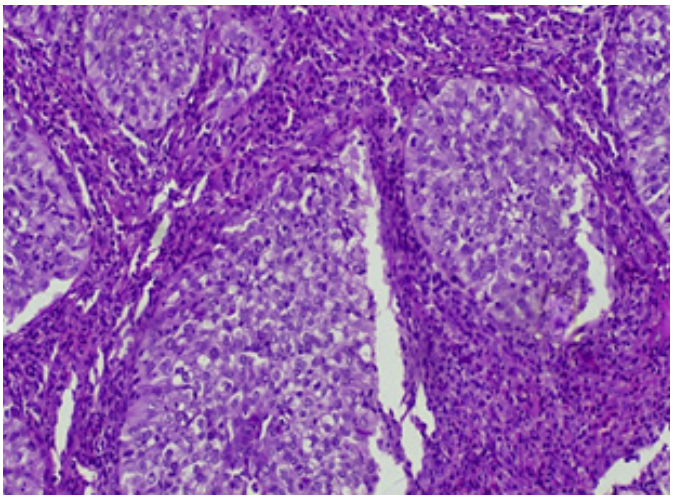

Figure 3: Squamous cell carcinoma (Basaloid Type) - Poorly Differentiated (10x mag)

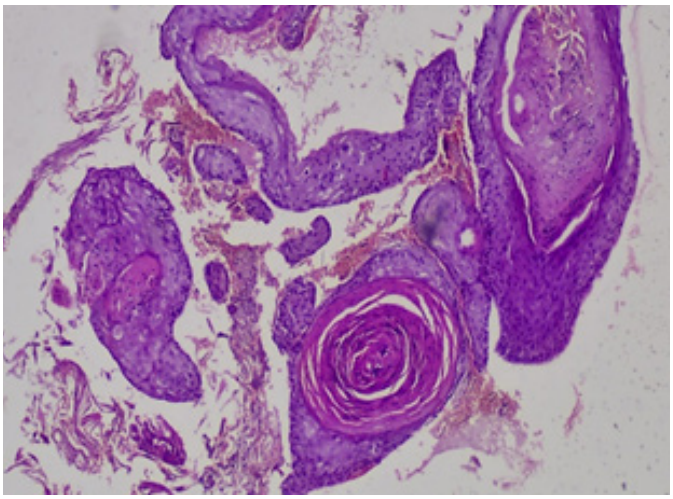

Figure 4: Squamous cell Carcinoma- Keratinizing, Well Differentiated (H & E stain, 10 x Magnification)

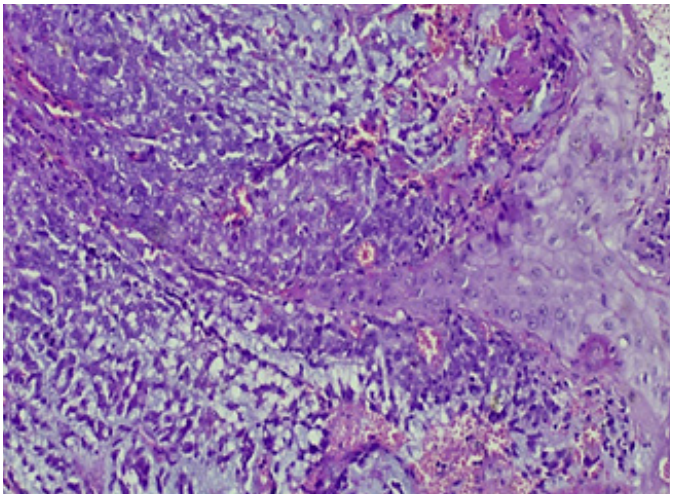

Figure 5: Moderately differentiated Adenosquamous carcinoma (H and E, 10x Magnification)

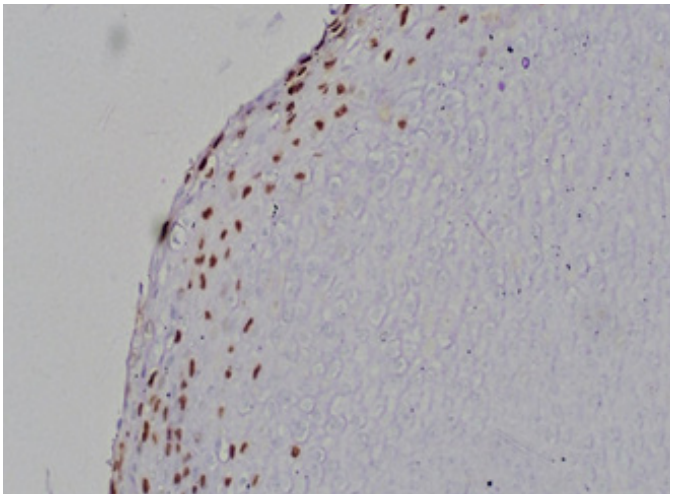

Figure 6: HPV 16 control slide, HPV 16 positive cervical carcinoma- Nuclear brown staining

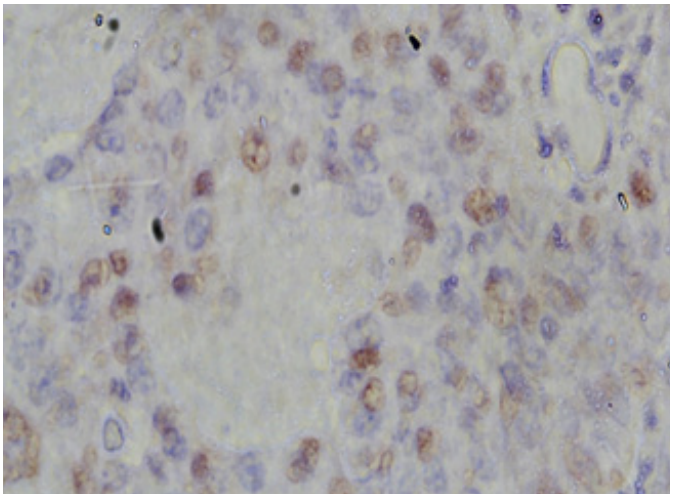

Figure 7: Well Differentiated SCC Keratinizing type showing positive nuclear staining of HPV 16 (IHC stain, 40x)

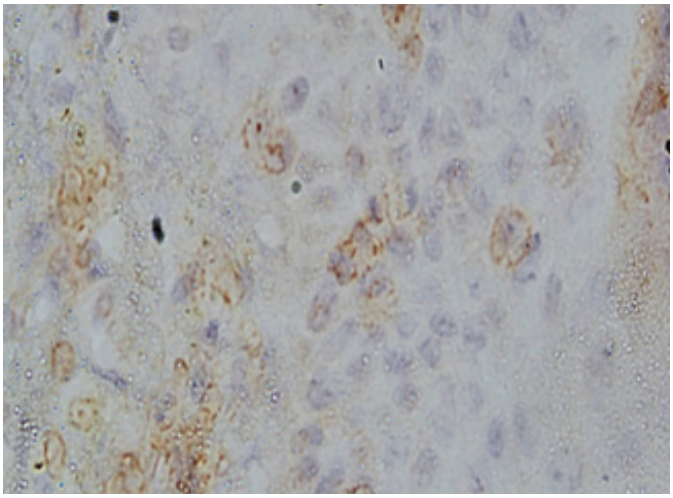

Figure 8: Moderately Differentiated Adenosquamous carcinoma showing positive HPV 16 expression

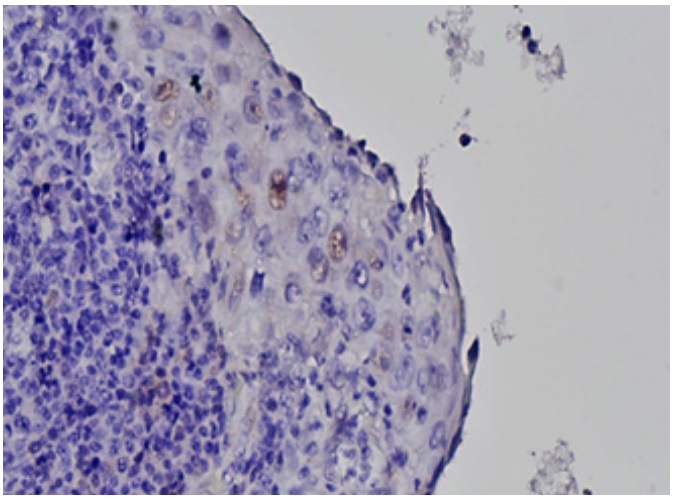

Figure 9: Poorly differentiated, basaloid type Squamous cell Carcinoma showing positive HPV 16 expression

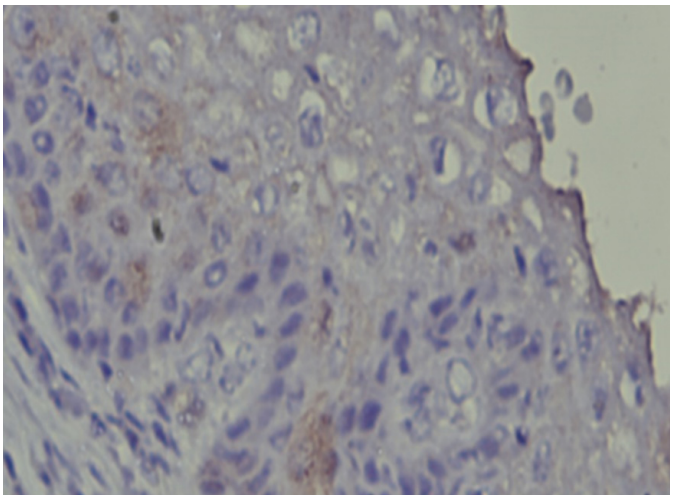

Figure 10: Normal cervical mucosa showing positivity for HPV 16 expression

as immunohistochemistry in the detection of High-Risk HPV types namely HPV16 in cervical carcinomas and normal cervical mucosa.

In our study the frequency of HPV 16 was 44.7 % in cervical carcinoma cases (Table 1). Our results are comparable to Daneshvar et al., 2017[20] from Iran (47.39%), Ogembo et al., 2015[21] from Africa (49.7%) Kaliff et al., 2018[11] from Sweden (43%), Gul et al., 2015[22] from Pakistan reported similar frequency of HPV 16 (44.8%) in cervical carcinoma patients.

Contrary to our results several studies have reported a higher percentage of HPV 16 in cervical carcinoma. Li et al., 2017[23] from China reported a higher HPV 16 frequency 65.2% in High-grade lesions (30 out of 46) and the overall HPV prevalence (combined various genotypes) was 24.1%. Similarly, Salavatiha et al., 2021[16] conducted a large-scale meta-analysis of studies from Iran, reported a higher frequency, the total HPV prevalence to be 81% and HPV 16 in Invasive cervical carcinoma to be 53%. Raza et al., 2010[24] and Loya et al., 2016[25] from Pakistan reported a higher frequency of HPV 16 in cervical carcinoma. Both these studies used the PCR technique to look for multiple HPV genotypes and reported 75.8% and 67.3% HPV positivity respectively. A few studies from various regions of the world have reported a lower frequency of HPV 16 in cervical lesions 17.3%, 19.7% and 33.3%.[26-28]

This wide variation in the frequency of HPV 16 infection from different areas of the world indicates that genetics and other co-factors play an important role in carcinogenesis and not all population may benefit from the current vaccination program.

Globally among women having no clinically apparent disease the HPV infection has a prevalence of 11–12%. The maximum prevalence has been seen in sub-Saharan Africa with a prevalence rate of 24%. It is followed by Eastern Europe and Latin America having 21 and 16% prevalence respectively. Eastern Africa and the Caribbean have a particularly high prevalence rate i.e. >30 %.[29] The frequency of HPV 16 was 5.3 % in the cases with normal cervical epithelium (table 1) which is similar to studies from Pakistan, Iran and Africa with frequency of 4.17%, 8.12% and 4.4% respectively.[20,21,30] Contrary to our results authors from Gulf region, India and Pakistan have reported a higher percentage of Human Papillomavirus expression in normal cervical mucosa i.e., 17.9%, 53.84% and 18.5% respectively.[26,31,32]

The various types of Cervical Carcinoma that were seen in our results were Squamous cell Carcinoma (60.5%), Adenocarcinoma (34.2 %) and Adenosquamous carcinoma (5.3%). Many authors have shown Squamous cell carcinoma to be the dominant histological type in their samples such as studies from India, Sweden, Bangladesh, Pakistan and Portugal.[25,31,33-35]

In our study HPV 16 Positive cases in various types of cervical carcinoma was 43.5% Squamous cell Carcinoma (SCC), Adenocarcinoma (AC) 46.2%, and Adenosquamous Carcinoma (ACS) 50.0% (1/1) (table 4).

Similar to our results Salavatiha et al., 2021 from Iran reported 52% HPV 16 positivity in SCC and 40 % in ADC.[16] Gul et al., 2015 from Pakistan reported 42.42% positivity in SCC and 50% positivity in Adenocarcinomas.[22] Contrary to our findings Kumar et al., 2020 from India reported a much higher frequency of HPV 16 (92.7%) positivity in Squamous Cell Carcinoma.[36]

The majority of the cases were poorly differentiated (n=18, 47.4%) and the rest were moderately (n=9, 23.7%) and well-differentiated (n=11, 28.9%) in our study (table 5). The cases were further grouped as low grade (well differentiated and moderately

differentiated, n= 20) and high grade (poorly differentiated n=18). In our study, 30.0% low-grade lesions were positive for HPV 16 expression and 61.1 % of high-grade lesions were positive for HPV 16.

All of the CIN 3 High-grade lesions in the study by Gul et al., 2015 from Pakistan were positive for HPV 16.[22] A study by Zahid et al., 2016 from Pakistan found that among HPV 16/18 positive cases, 81.2% of the cases were well-differentiated, 82.9% showed moderate differentiation while 69.2% cases showed poorly differentiation.[37] Ilahi et al., 2016 from the northwest region of Pakistan found HPV 16 E6 expression to be higher in well-differentiated samples on PCR.[38] Krashias et al., 2017 from Cyprus reported higher percentage of HPV 16 positivity (19.7 %) in Low-grade lesion (LSIL) as compared to (15.8 %) in High-Grade Lesions (HSIL).[27]

Further multicenter studies will give a clearer picture about the prevalence of High-Risk HPV types in our population. More sensitive and specific tests are needed to investigate the frequency of HPV 16 in Cervical Carcinoma. An Algorithm for the screening and diagnosis of the exact HPV genotype that is implicated in the causation of cervical carcinoma is needed for our population as the available vaccines are highly type specific. A national screening program is highly essential for our population to prevent this deadly disease in our women.

CONCLUSION

We observed a significantly higher expression in cervical carcinoma cases as compared to normal cervical mucosa. The HPV 16 expression was not related to age of the patients and subtype of cervical cancer. The association of HPV 16 expression with histological grade although not statistically significant still demonstrated higher positive percentage in High-grade tumors.

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Author's Contribution

SY conceived the idea, designed the study and contributed in data collection. FR, DD and AKS helped in the analysis and interpretation of data and final write up of the manuscript. Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest

Authors declared no conflict of interest

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None

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.