ORIGINAL RESEARCH

Prognostic Impact of P16 and EGFR Overexpression in Patients with Cervical Cancer

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ABSTRACT
Background: Cervical cancer is the second most prevalent cancer, both in terms of incidence and mortality, after breast cancer in regions with lower Human Development Index (HDI). However, it holds the distinction of being the most frequently diagnosed cancer in 28 countries and the primary cause of cancer-related deaths in 42 countries. The majority of these countries are located in Sub-Saharan Africa and South Eastern Asia. Cervical cancer stands as a prominent contributor to cancer-related fatalities in women. Based on estimates from the World Health Organization (WHO) and the International Agency for Research on Cancer (IARC), there were approximately 529,000 new cases of cervical cancer worldwide in the year 2008. **Methods:** This study was done by retrieving information of 101 patients from records of Pathology Department in whom cervical malignancy was suspected by per speculum and per vaginal examination and confirmed by biopsyorhysterectomy. Standard streptavidin-biotin peroxidase method of IHC (immunohistochemical) was followed. **Results:** Increased expression of EGFR was seen in moderately differentiated squamous cell carcinoma than in adenocarcinoma and poorly differentiated SC carcinoma. P-value of EGFR over expression in MdSCC is <0.05, hence there is a significant association of EGFR over expression in MdSCC. **Conclusion:** The study revealed a noteworthy presence of the p16 biomarker in cervical cancer patients who were younger in age and at early stages of the disease. Consequently, the utilization of the p16 biomarker in screening or early diagnosis may contribute to improved prognosis for squamous cell carcinoma of the cervix.
Keywords: Cervical Cancer, EGFR, P16, Radiotherapy, Chemotherapy

INTRODUCTION
Cervical cancer is a prevalent and serious malignancy that poses a significant threat to women's health worldwide. In 2018, total 570,000 new cases are reported and 311,000 deaths. It stands as the fourth most commonly diagnosed cancer and the fourth leading cause of cancer-related deaths among women[1]. Notably, approximately 85% of cervical cancer deaths worldwide are concentrated in underdeveloped or developing countries, where the mortality rate is 18 times higher compared to wealthier nations[2]. In India, cervical cancer ranks as the second most common malignant tumor affecting women. [3-5].
In May 2018, the World Health Organization (WHO) made a global plea for the eradication of cervical cancer. This call to action received a positive response from over 70 countries and international academic societies, who promptly initiated efforts in that direction[6, 7].Subsequently, on November 17, 2020, WHO unveiled a comprehensive global strategy aimed at expediting the elimination of cervical cancer as a public health issue. This milestone marked the first collective commitment of 194 countries to eliminate cervical cancer[8, 9]. Multiple studies have consistently identified various risk factors associated with the development of cervical cancer. These factors include low economic status, inadequate personal and sexual hygiene, smoking, early onset of sexual activity, and engaging in multiple sexual partnerships[10, 11].

The primary causal factor in the process of carcinogenesis is human papillomavirus (HPV). However, it is important to note that not all HPV infections in women lead to cervical cancer. The progression from a normal cell to a precancerous lesion and eventually an invasive lesion is triggered by high-risk HPV genotypes. The pathogenesis of HPV infection involves the over expression of viral onco-proteins, which can hinder various cellular proteins and impact biological processes such as cell proliferation, the cell cycle, and apoptosis. The viral and host cellular changes that contribute to cervical carcinogenesis provide valuable insights into the nature of the disease and serve as a source of inspiration for the development of targeted molecular therapies[12-15].

Cervical cancer arises in the cervix, the narrow passage that connects the uterus to the vagina through the endocervical canal (Fig. 2) [16].Early-stage cervical cancer is often asymptomatic and may be diagnosed during a routine screening or pelvic examination. The most common symptoms include heavy or abnormal vaginal bleeding, in particular following intercourse[17, 18].

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein receptor with a molecular weight of 170 kDa. It undergoes dimerization to activate a tyrosine kinase domain, which plays a crucial role in regulating various cellular functions such as cell differentiation, growth, gene expression, and development[19, 20]. In cervical cancer, high expression of the epidermal growth factor receptor (EGFR) is associated with tumor development, as EGFR expression increases with increasing malignant stage and subsequent EGFR activation leads to cell growth, differentiation, resistance to apoptosis, cellcycle progression and angiogenesis. EGFR-based therapy has established efficacy in selected patients with head and neck squamous cell carcinoma, colorectal carcinoma and non-smallcell lung carcinoma[21-23].

The over expression of EGFR has been linked to decreased survival rates in patients with cervical cancer, and this relationship has been the subject of investigation for several years[24]. The effectiveness of EGFR inhibition as a treatment for cervical cancer is still uncertain. Although several clinical trials investigating EGFR inhibitors in cervical cancer patients have been conducted, the outcomes and impact of these drugs are not firmly established. It has been observed through numerous clinical trials that only a specific subgroup of patients shows a response to EGFR inhibitors. However, conflicting findings have been reported by various research groups. [25-27].
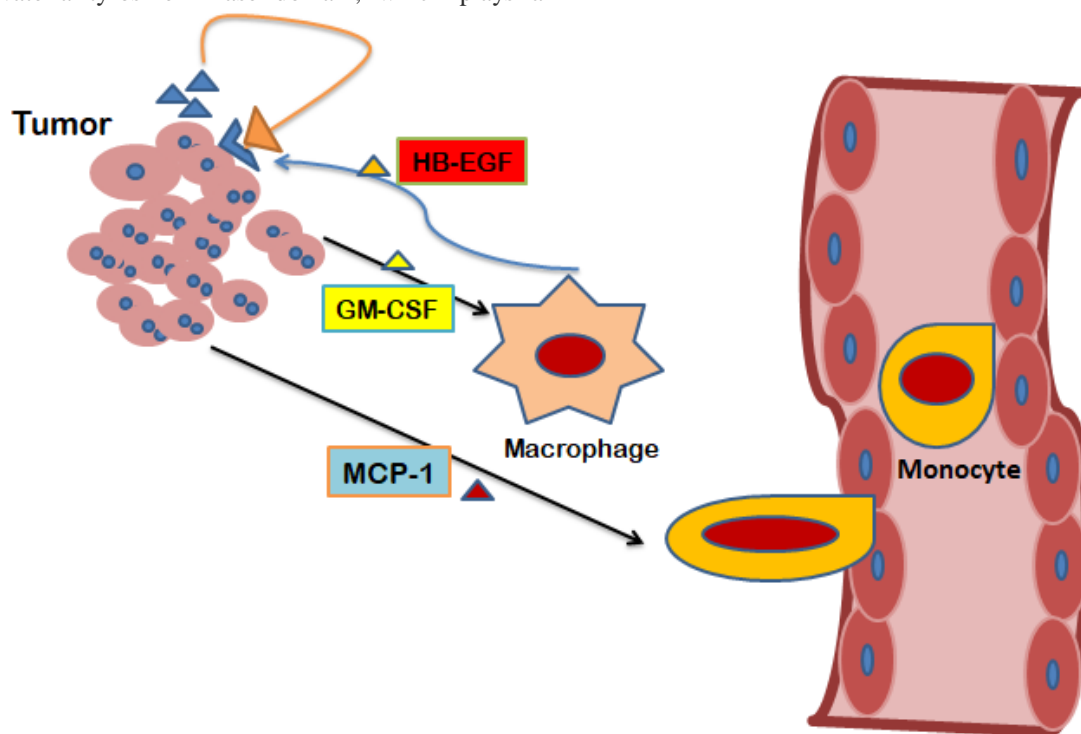

Figure 1: Proposed mechanism of heparin-binding EGF-like growth factor (HB-EGF) expression and macrophage recruitment in cervical cancer patients

It has been suggested that HB-EGF is expressed by cervical cancer-associated stromal fibroblasts, thus promoting cancer cell proliferation in a paracrine manner. In vitro experiments with head and neck squamous cell carcinoma cells show that this HB-EGF autocrine loop is associated with invasive processes through the EGFR-Src-cortactin cascade[22, 28-30]. Cervical cancer cells are shown to express GM-CSF to attract macrophages to the tumor environment. Although macrophages and other stromal cells express the primary EGFR ligand HB-EGF, they do not appear to be the major source of HB-EGF, as the

relative amount of HB-EGF measured in the tumor cell compartment was on average four times higher than the relative amount of HB-EGF in the stromal compartment **(Figure 1)**[22, 30, 31].
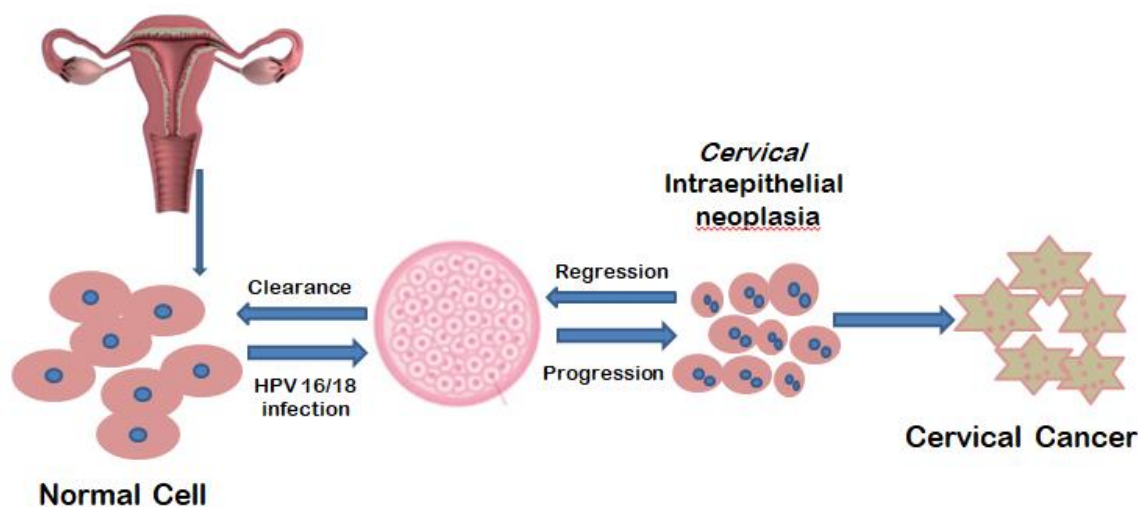
Figure 2: Sequence of events leading to the development of HPV-related cervical carcinoma.

Cervical intraepithelial neoplasia (CIN) occurs when squamous cells exhibit abnormal growth due to persistent HPV infection. While many of these changes naturally resolve over time, in some cases, the integration of viral particles leads to the transformation of cells and their progression into cervical cancer[30, 32]. As demonstrated in **Figure 2**, pre-cancerous changes can occur in cervix cells infected with high-risk HPV, and most of these changes resolve over time, but some do progress to cervical cancer. HPV is responsible for almost 100% of cervical cancers[30, 33].The genome of HPV encodes gynaecological cancer-related oncogenes, which include E5, E6 and E7.These oncogenes encode proteins that are implicated in carcinogenesis, a process that has been demonstrated in both in vitro and in vivo models[34, 35].

Most of HPV infections are asymptomatic and partners can unknowingly pass it on to one another through sexual contact. Majority of HPV-related cancers are prevented by using commercial HPV vaccines; however, these vaccines are ineffective in eradicating persistent HPV infections, and have not been demonstrated to slow down the HPV-related progression of malignant tumours [36-38]. To facilitate the development of cervical cancer, human papillomavirus (HPV) disrupts the cellular regulatory mechanisms by inducing the expression of viral proteins. These proteins, in turn, promote cellular transformation by modulating the expression of p53. It should be noted that the expression of p53 varies depending on the stage of cervical cancer[39, 40].

Cervical cancer cases exhibiting P16 expression have shown a more favorable prognosis, as reported in previous studies. High P16 expression in cervical cancer has been associated with a significant improvement in both five-year overall survival and disease-free survival rates. It is worth noting that the present study has a limitation in terms of not conducting follow-up assessments to evaluate the long-term prognosis of the cases. Nevertheless, it was observed that P16 block positivity was particularly high among young females and this finding was statistically significant [41, 42]. The utilization of P16 as an additional biomarker has significantly enhanced diagnostic consensus. The expression of P16 is indicative of high-risk human papilloma virus (HR-HPV) infection and the integration of the viral genome into the host genome. However, it is important to note that different pathologists may employ varying criteria and thresholds for interpreting P16 expression[43, 44]. The Lower Anogenital Squamous Terminology (LAST) criteria categorize P16 immuno-reactivity into three groups: block positive, ambiguous, or negative. This classification is determined by evaluating the presence of P16 expression in the nucleus with or without cytoplasmic staining[45].

Further research is necessary for prognostic significance of EGFR and P16 in cervical cancer. In present study utilize immune-histochemical analysis of P16 and epidermal growth factor receptor (EGFR) to establish a diagnosis of cervical carcinoma in all cervical biopsies and hysterectomy specimens received.

MATERIAL AND METHODS

A retrospective study was carried in the department of pathology at G.S.V.M. Medical College, Kanpur from January 2020 to January 2021 and all relevant clinical details and finding were revised on the requisition form. This study was done by retrieving information of 101 patients from records of Pathology Department in whom cervical malignancy was suspected by per speculum and per vaginal examination and confirmed by biopsy or hysterectomy. Standard streptavidin-

biotin peroxidase method of IHC (immunohistochemical) was followed[46, 47]. Sections were immunohistochemically stained for mouse monoclonal antibody to p16 manufactured by BioGenex p16 and EGFR. So, all adequate cervical biopsies and hysterectomy specimen of all age group were included in the study and Exclusion criteria for the study was patients who were already treated for cervical cancer,Inadequate biopsies**.**

RESULT

Total 101 case were included in this study, maximum case (57.14%) lie between the age group of 41-60 years. The mean age was 55 years. Well Differentiated carcinoma was the commonest grade.40% of patients in this study was smokers suggesting that carcinoma of cervix were more common in smokers. Patients who have a history of use of contraceptive pills for contraception, shows higher grades of malignancy. However, duration of use was not taken into consideration.

It was observed that highest parity 5 was found in female with poorly differentiated SCC. Parity didn't have any significance effect on other grade of malignances.
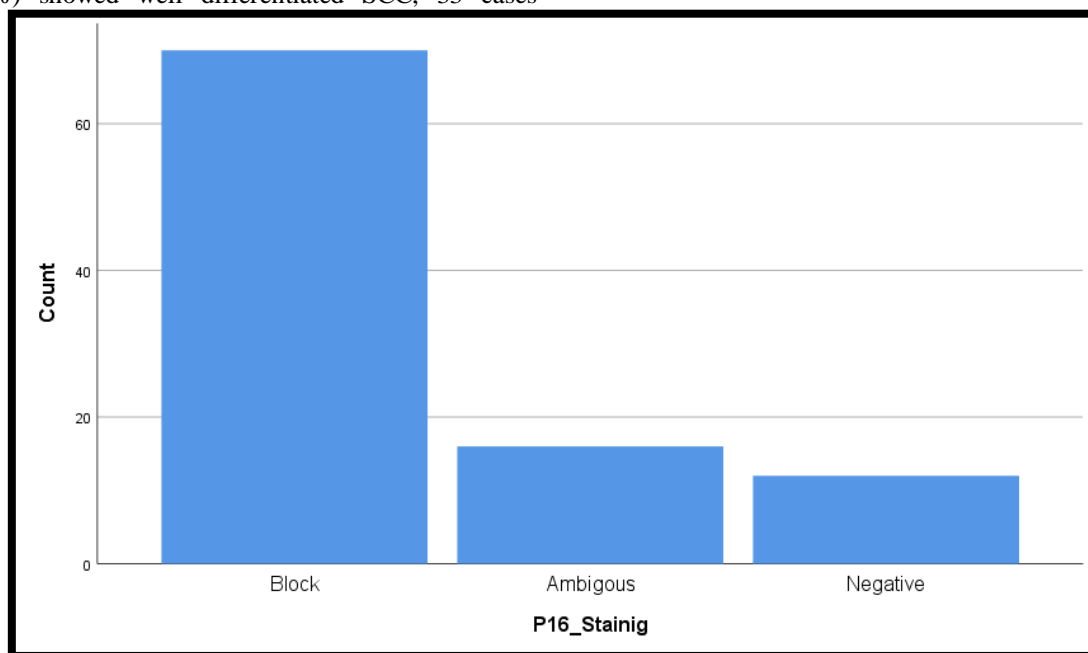
It was observed that carcinoma cervix was more common in postmenopausal women. A total of 114 cases (54%) were post-menopausal of which 24 cases (40%) showed well differentiated SCC, 33 cases (57.90%) showed moderately differentiated SCC and 21 cases (58.33%) showed poorly differentiated SCC. It was also observed that carcinoma cervix was more common in women who gave history of irregular periods. The grade of malignancy did not show any specific relationship to menstrual status.

The well-differentiated squamous cell carcinoma exhibited diffuse block positivity when stained with P16 and well-differentiated squamous cell carcinoma, there was observed an over expression of EGFR in **figure 5 & 6** respectively.

Bleeding was comments symptom in all the cases with maximum frequency in poorly differentiated SCC (93%) followed by well differentiated SCC (75%). Lower abdominal pain was seen in a significant percentage of women with both well differentiated SCC and moderately differentiated SCC (85%), but slightly higher frequency of pain was found in women having adenocarcinoma (87%). Discharge per vaginal was seen only in a small percentage cases, mostly in poorly differentiated malignancy.The other findings on examination in order of frequency were bleeding on touch and necrosis. The presentation did not alter with the grade of malignancy.

In the current study the total number of cases showing P16 block,ambiguous and no positivity were 70, 13 and 9 respectively as shown in **Figure 3** below.

Figure 3: Showing P16 staining pattern in cervical carcinoma cases

It was observed that increased block positivity of p16 was seen in moderately differentiated squamous cell carcinoma than in adenocarcinoma and poorly differentiated carcinoma SCC. P-valueofp16 block staining in MdSCC is<0.05, hence there is a significant association ofp16block positivity in MdSCC as shown in **Table1&2.**

Table1: Showing correlation of intensityof P16stainingwithWDSCC & MDSCC

			WdSCC			MdSCC		
			No	Yes	Total	No	Yes	Total
P16_Stainig	Block	Count	52	18	70	23	47	70
		% within P16_Stainig	74.3	25.7	100	32.9	67.1	100
	Ambigous	Count	14	2	16	8	8	16
		% within P16_Stainig	87.5	12.5	100	50.0	50.0	100
	Negative	Count	8	4	12	9	3	12
		% within P16_Stainig	66.7	33.3	100	75.0	25.0	100
Total		Count	74	24	98	40	58	98
		%within P16_Stainig	75.5	24.5	100	40.8	59.2	100

Table2: Showing correlation of intensity of P16 Staining with PDSCC & Adeno CA

			Poorly differentiated SCC			Adeno CA		
			No	Yes	Total	No	Yes	Total
P16_Stainig	Block	Count	65	5	70	70	0	70
		% within P16_Stainig	92.9	7.1	100	100	0.0	100
	Ambigous	Count	13	3	16	13	3	16
		% within P16_Stainig	81.3	18.8	100	81.3	18.8	100
	Negative	Count	9	3	12	10	2	12
		% within P16_Stainig	75.0	25.0	100	83.3	16.7	100
Total		Count	87	11	98	93	5	98
		% within P16_Stainig	88.8	11.2	100	4.0	5.1	100

In the current study the total number of cases showing EGFR expression as absent and over expression are 23(22.8%) and 78(77.2%) respectively as shown in **Figure 4**.
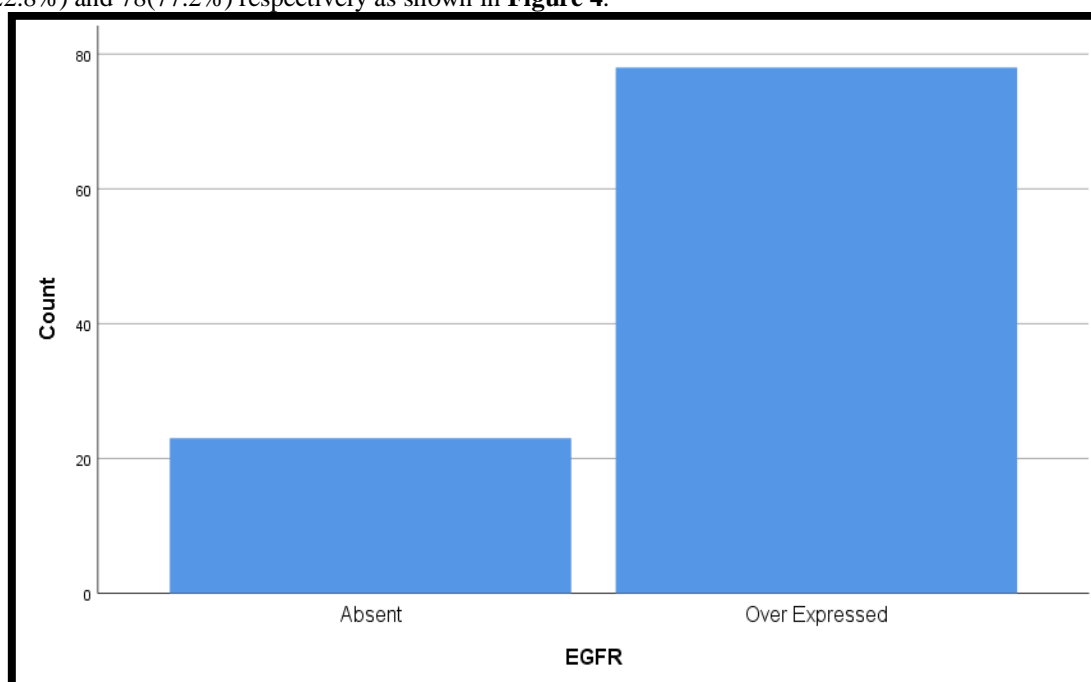
Figure 4: Showing EGFR expression in total number of cases

It was observed that increased expression of EGFR was seen in moderately differentiated squamous cell carcinoma than in adenocarcinoma and poorly differentiated SC carcinoma. P-value of EGFR over expression in MdSCC is <0.05, hence there is a significant association of EGFR over expression in MdSCC as shown in **Table3 &4**.

Table3: Showing correlation of EGFR expression with WdSCC & MdSCC

			WdSCC			MdSCC		
			No	Yes	Total	No	Yes	Total
EGFR	Absent	Count	16	7	23	17	6	23
		% within EGFR	69.6	30.4	100	73.9	26.1	100
	Over Expressed	Count	58	20	78	26	52	78
		% within EGFR	74.4	25.6	100	33.3	66.7	100
Total		Count	74	27	101	43	58	101
		% within EGFR	73.3	26.7	100	42.6	57.4	100

Table4: Showing correlation of expression of EGFR with PDSCC

|			Poorly differentiated SCC			Adenocarcinoma		
			No	Yes	Total	No	Yes	Total
EGFR	Absent	Count	16	7	23	20	3	23
		% within EGFR	69.6	30.4	100	87	13	100
	Over Expressed	Count	74	4	78	76	02	78
		% within EGFR	94.9	5.1	100	97.4	2.6	100
Total		Count	90	11	101	96	5	101
		% within EGFR	89.1	10.9	100	95	5	100

In the current study there is no significant correlation between P16 and EGFR especially in squamous cell carcinoma cases as shown in **Table 5.**

Table5: Showing correlation of P16stainingwithEGFR

			EGFR		
			Absent	Over Expressed	Total
P16_Stainig	Block	Count	10	60	70
		% withinP16_Stainig	14.3%	85.7%	100.0%
	Ambigous	Count	6	10	16
		% withinP16_Stainig	37.5%	62.5%	100.0%
	Negative	Count	6	6	12
		% withinP16_Stainig	50.0%	50.0%	100.0%
Total		Count	22	76	98
		% withinP16_Stainig	22.4%	77.6%	100.0%
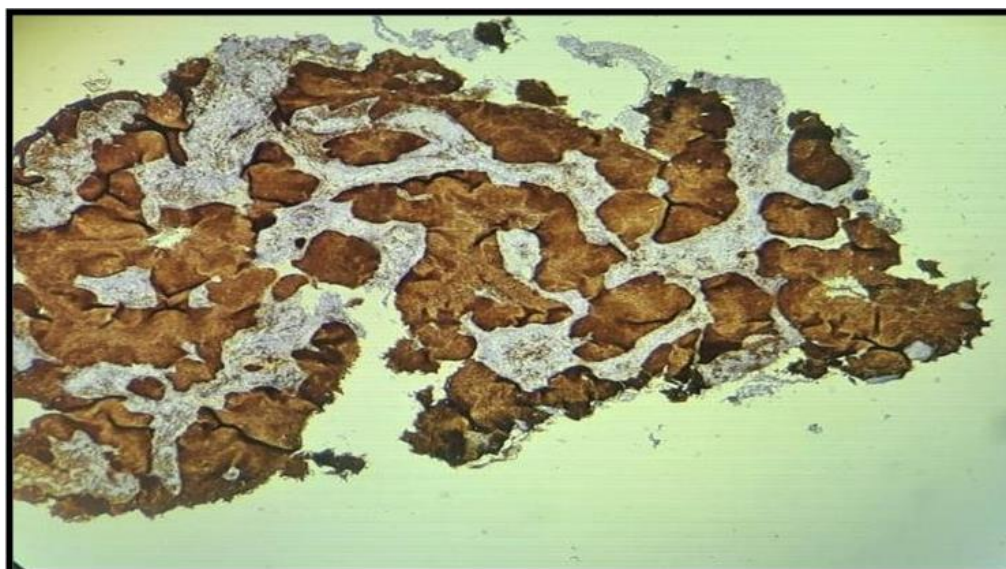
Figure5: Showing P16 staining in well differentiated squamous cell Carcinoma showing diffuse block positivity(IHC; 400X magnification)
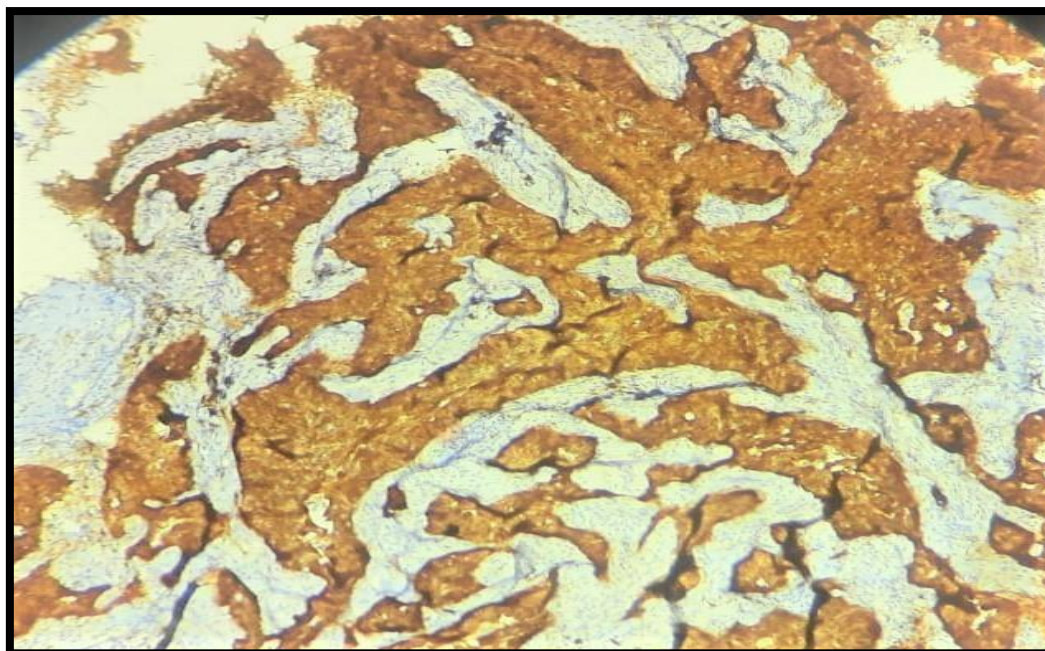
Figure 6 : EGFR over expression in well differentiated Squamous cell carcinoma (IHC; 400 X magnifications).

DISCUSSION

Cervical malignancy is the 2nd most common malignancy of the female[48]. This retrospective study was done in the department of pathology; GSVM Medical College, Kanpur and a total of 101 cases were involved in this study. In this study it was observed that mean age for cervical carcinoma was ≥55 years and region for higher incidence of cervical carcinoma in this age group were effect of hormonal change and increase susceptibility to genital tract infection. Strategies to prevent cervical cancer, both primary and secondary, play a crucial role in reducing the impact of this disease. There has been extensive literature addressing these prevention strategies [49, 50].

In some cases, women may exhibit a vaginal discharge that can vary in consistency from watery to mucoid or purulent, accompanied by an unpleasant odor. However, it is important to note that this symptom is rarely observed in isolation and is often accompanied by other symptoms. In advanced stages of the disease, patients may experience additional symptoms such as lower limb edema, flank pain, as well as pelvic or lower back pain[51].In patients exhibiting any symptoms of cervical cancer, a pelvic examination is conducted, which includes the visualization of the cervix and vaginal mucosa. If any abnormalities are observed, a biopsy is performed[52].

Radiotherapy, which involves the use of high-energy X-rays, is a primary treatment modality in the management of cervical cancer[53].Chemotherapy plays a crucial role in the standard treatment protocol for cervical cancer. It is commonly used as an adjuvant therapy following surgery, particularly when poor prognostic tumor characteristics elevate the risk of disease recurrence. Additionally, chemotherapy is administered in combination with radiotherapy, as mentioned earlier, and as a standalone treatment for cases of locally advanced disease[54].

The use of immunotherapy, specifically targeting HPV oncoproteins, has emerged as a promising approach in the treatment of cervical cancer. This novel treatment method offers the advantage of specifically targeting dysplastic precancerous and malignant cervical epithelial cells that express HPV oncoproteins [55].

Chemotherapy agents are known to have a cytotoxic effect on both cancer cells and normal rapidly dividing cells, leading to various side effects such as anemia and alopecia. In contrast, targeted therapies are designed to specifically inhibit molecules, typically proteins,that are specifically expressed by cancer cells. These targeted therapies aim to disrupt the growth, proliferation, and metastasis of cancer cells[56].

As targeted therapies have a higher specificity for cancer cells compared to normal cells, it is expected that they will exhibit increased efficacy and reduced adverse effects compared to current chemotherapies. This anticipation stems from the fact that targeted therapies selectively target cancer cells, resulting in a more precise and focused treatment approach[57].

In the current study it was observed that highest parity 5 was found in female with Poorly Differentiated Squamous cell carcinoma and that parity have significance role in cervical carcinoma which is in accordance to the study of Natthakan Vanakankovitetal(2008)[58].In our study it was observed that carcinoma cervix was more common inmost postmenopausal women. It was also observed that carcinoma cervix more common in women who

gave history of irregular periods which was quiet similar to the study of Gloria YFetal(1998)[59]. InourstudyalsoshowsbleedingwaspresentedinallthecaseswithmaximumfrequencyinpoorlydifferentiatedSquamouscellcarcinoma(93%)followedbywelldifferentiatedSquamouscellcarcinoma(75%)lowerabdominalpainin significantpercentageofwomen which is similar to the study of Louise A et al (1987)[60].It was observed that mean count increased with increasing grade ofmalignancy.Lowestcountobtainedin*InSitu*Squamous cellcarcinomatoModeratelyDifferentiatedSquamousce llcarcinomaandhigherinpoorlydifferentiatedSquamous cellcarcinoma which is similar to the results observed by Singh Uma etal (2006)[61].

In the current study, increased block positivity of p16 was seen in moderately differentiated squamous cell carcinoma in comparison to adenocarcinoma and poorly differentiated carcinoma. P-value of p16 staining in MdSCC is <0.05, hence there is a significant association of p16 block positivity in MdSCC.

Kalyani et al (2020) showed that there is significant association between age and p16 expression, where all cases between 30-59 years showed block positivity. Among the post-menopausal women, women between 40-44 yrs showed maximum block positivity(93.75%)[62]. Sarwath et al (2017) stated that there was a significant correlation between p16 expression and age group between 41-60 years. Hence, p16 is an appropriate surrogate marker for use in early screening of cervical cancer [63].Fu HC et al (2018) stated that p16 expression was not found to have an association with tumor stage, tumor size, histological grade, vascular invasion[42].

T. Soonthorn thumetal(2011) stated that EGFR over expression is associated with poor prognostic factors such as increased tumor size, lymphnode metastases and recurrence of disease. The average percentage of squamous cell carcinoma over expressing EGFR is 51%compared with 23% for adenocarcinoma and 38% for adenosquamous carcinoma[64].According to D Pfeiffer et al (1998), who suggested that EGFR expression may be indicative of the biological aggressiveness of cervical squamous cell carcinoma[65].

In the current study, we found that compared to EGRF expression in squamous cell carcinoma versus adenocarcinoma and poorly differentiated carcinoma, the squamous cell type always stains more strongly, that means strong positivity for squamous cell carcinoma as compared to other type of carcinoma like adenocarcinoma.

CONCLUSION

The study revealed a noteworthy presence of the p16 biomarker in cervical cancer patients who were younger in age and at early stages of the disease. Consequently, the utilization of the p16 biomarker in screening or early diagnosis may contribute to improved prognosis for squamous cell carcinoma of the cervix. Additionally, this finding suggests the potential application of targeted therapies based on the anti-cancer properties of the p16 protein. The importance of p16 in cervix is that it is seen maximum in moderate grade lesion of SCC more than any other type of SCC or adenocarcinoma. Therefore, p16 biomarker may have a beneficial use in screening or early diagnosis leading to better prognosis of squamous cell carcinoma of cervix. It has been showed that squamous cell carcinoma of the cervix frequently express EGFR, often at high concentration and least frequently in adenocarcinoma. Current study showed that immuno-histochemical staining of elevated expression of EGF receptor in cervical squamous epithelium was associated with its neoplastic transformation and emerged as a tumor marker for cervical squamous neoplasia.

CONFLICT OF INTEREST

All the authors declare that they have no conflict of interest.

ETHICAL STATEMENT

All the necessary permissions are obtained from the institution.

AUTHOR'S CONTRIBUTIONS

Anjana Trivedi: Concept, Study design, data collection, literature search, statistical analysis, manuscript writing. Vandana Mishra Tiwari: Manuscript writing and editing, Dr. Suman Lata Verma: manuscript editing, Anita Omhare: manuscript review

ABBREVIATIONS

DFS: Disease Free Survival
HPV: Human Papilloma Virus
IHC: Immunohistochemistry
MDSCC: Moderately Differentiated Squamous CellCarcinoma
SCC: Squamous Cell Carcinoma
WDSCC: Well Differentiated Squamous Cell Carcinoma
EGFR: Epidermal growth factor receptor
HB-EGF:Heparin-binding epidermal growth factor
IARC: International Agency for Research on Cancer
CIN: Cervical intraepithelial Neoplasia

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