

Expression of P16 biomarker in squamous cell carcinoma of uterine cervix and its association with clinico-pathological parameters: A cross-sectional study

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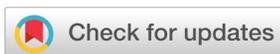
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ABSTRACT

Introduction: Cervical cancer is the most common cancer among females. P16 is the surrogate marker for cervical carcinoma. This study aimed to evaluate the association of P16 marker with clinic-pathological parameters in squamous cell carcinoma of uterine cervix. **Methods:** This was a cross-sectional study. Histological confirmed cases of squamous cell carcinoma (SCC) of cervix were considered. All cases were evaluated for IHC P16 expression as per lower anogenital squamous terminology (LAST) criteria and correlated with clinico-pathological parameters. The data was analyzed by SPSS software version 22. **Results:** Out of 75 cases, P16 biomarker expression was block positive, ambiguous and negative in 67 (89.3%), 5 (6.6%), and 3 (4%) cases, respectively. There was a significant association between P16 expression and age ($p = 0.005$). All cases between 30-59 years of age showed block positivity. There was no significant association between P16 expression and age at marriage ($p = 0.951$), age at menopause ($p = 0.311$), parity ($p = 0.554$), clinical symptoms/signs, stage of disease ($p = 0.28$), or histopathological grade ($p = 0.877$). Maximum expression was seen between 40-44 years. Moreover, all cases having 1 & 2 parity showed block positivity and all stage I cases showed block positivity. **Conclusion:** P16 biomarker was significantly expressed in cervical cancers of the relatively younger age group and those with early stage of disease.

Key words: Cervix, clinico-pathological parameters, P16 biomarker, squamous cell carcinoma of cervix

INTRODUCTION

Cervical cancer is the third most common cancer in women worldwide with a global prevalence of 11.7%, and accounts as the 5th most common cause of cancer-related deaths. Its annual estimated global incidence is 500,000 cases, with India accounting for approximately 100,000 cases¹⁻³. Cervical cancer is the second common cancer in underdeveloped countries among females¹. The Human Papilloma Virus (HPV) is a proven etiological factor^{1,3,4}. The age range for cervical cancer is reported to be 27-80 years with mean age of 54.2 years; the maximum cases have been noted between 41-60 years of age¹. The most common symptom is vaginal bleeding. Many times the colposcopic appearance is non-specific². Histologically, squamous cell carcinoma is common, and classified as non-keratinizing or keratinizing variants¹. Histomorphological diagnosis of cervical biopsy can result in under- or over-treatment and low diagnostic agreement rates⁵. Use of P16 as an adjuvant biomarker has improved diagnostic agreement⁶. P16 expression indicates infection with high risk HPV (HR-HPV) and integration of viral genome with host

genome⁴. Different pathologists use different criteria and threshold for interpretation of P16 expression^{7,8}. Lower anogenital squamous terminology (LAST) criteria defines P16 immunoreactivity as block positive, ambiguous or negative, based on the consideration of P16 expression in the nucleus with or without cytoplasmic staining. The LAST project has been co-sponsored by the American Society for Colposcopy and Cervical Pathology (ASCCP) and the College of American Pathologists (CAP). LAST gives standard guidelines for the precise utility of P16 biomarker, decreases interobserver's variation, and increases accuracy^{8,9}.

In this study, we have evaluated the association of immunohistochemistry (IHC) P16 biomarker expression and clinico-pathological parameters in our patient population.

MATERIALS — METHODS

This was a cross-sectional study from October 2017 to September 2018. Ethical clearance was obtained from the Institutional Ethics Committee before the start of the study. A total of 75 clinically suspected and

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histopathologically confirmed cases of SCC of cervix, without radiotherapy or chemotherapy, was considered for the study. The case details such as hospital number, biopsy number, age, presenting complaints, menstrual history, personal history, past history, family history, per-abdominal examination findings, per-speculum examination findings, per-vaginal examination findings, and stage was taken from hospital records. Staging was done per the FIGO staging¹⁰. Paraffin blocks and the corresponding slides were retrieved from the Department of Pathology. The slides were screened by two pathologists and histological typing was conducted. Histologically, the cases were classified as keratinizing or non-keratinizing types. Keratinizing squamous cell carcinomas was further classified as well-differentiated (WDSCC), moderately differentiated (MDSCC) and poorly differentiated (PDSCC). The non-keratinizing squamous cell carcinomas were further classified as small cell type (NKSCSCC) or large cell type (NKLCSCC)¹⁰.

Tissue sections were cut from paraffin blocks and evaluated by IHC for P16 marker using a mouse monoclonal anti-p16^{INK4} clone G175-405 as primary antibody (Biogenex, USA); tissue from all 75 cases were evaluated with positive and negative controls. The IHC procedure was carried out per the manufacturer's instructions. The P16 expression on the tissue sections was classified as block positivity, ambiguous staining, or negative per the LAST criteria. "Block" pattern staining corresponded to strong, continuous, nuclear positivity- with or without cytoplasmic staining extending from basal layers upwards for at least 1/3rd thickness of the epithelium (basal & parabasal layers)- which can be further graded as 1/3rd, 2/3rd, more than 2/3rd, and laterally over a significant distance with diffuse staining of > 25% of cells. "Ambiguous" staining corresponded to strong, basal, diffuse and continuous staining (involving only lower 1/3rd without upward extension) or weak, diffuse and discontinuous staining (involving at least 2/3rd of the epithelium), or strong, focal and discontinuous staining (located at any level of the epithelium). "Negative" staining meant a total absence or weak, focal and discontinuous, or only cytoplasmic staining⁹.

All data were entered in Microsoft Excel, and the data analyzed using SPSS version 22. The categorical data were presented as frequency and proportions. Continuous data were presented as mean, standard deviation and confidence intervals. Significance of difference between the groups was estimated using standard 't' test and chi-square test; p value < 0.05 was considered as statistically significant.

RESULTS

Seventy-five cases of SCC of cervix were considered for the study. The ages of the cases ranged from 30-80 years with a mean of 54.3 ± 12.0 . **Table 1** shows the age distribution of the cases. Postmenopausal cases and pre- or peri-menopausal cases accounted for 74.7% and 25.3%, respectively. The majority of women had attained menopause between 40-57 years of age with a mean of 46.3 ± 3.9 years. Age range at marriage was 12-23 years with a mean of 15.7 ± 2.1 years; maximum cases were at 15 years (26.6%) followed by 14 years (16%) of age. Parity ranged from 1-11 with a mean of 3.6 ± 1.6 ; the cases had a maximum parity of ≥ 5 (28%) followed by 4 (26.6%).

Table 1: Age distribution in cases in present study

Age Range (years)	Cases
30 - 39	8 (10.6%)
40 - 49	19 (25.3%)
50 - 59	18 (24%)
60 - 69	19 (25.3%)
70 - 79	10 (13.3%)
80 - 89	1 (1.3%)
Total	75 (100%)
Total pre and perimenopausal cases	19 (25.3%)
Total postmenopausal cases	56 (74.7%)

Clinical presentations of the cases are shown in **Table 2**, where bleeding per vagina was the common symptom. Per-speculum findings of cases are shown in **Table 3**, with growth and bleeding as the most common findings. Per-vaginal findings of cases are shown in **Table 4**. Stages of the disease of the cases are shown in **Table 5**, with the maximum number of cases in stage III (40%). The histological grade of disease in the cases are shown in **Table 6**, with keratinizing SCC as the maximum (90.6%) compared to non-keratinizing type (9.2%). Among the keratinizing SCC, the maximum cases were WDSCC (56%) followed by MDSCC (21.3%).

Out of 75 cases, P16 expression was block positive, ambiguous or negative in 67 (89.3%), 5 (6.6%), and 3 (4%) cases, respectively. Block positivity was maximal in pre- & peri-menopausal women (94.7%) compared to post-menopausal women (87.5%); the differences were not statistically significant ($p = 0.55$) (**Table 7**). A statistically significant association was observed between age of cases and P16 expression

Table 2: Clinical presentation in cases in present study

Clinical Presentation	Total no of cases presented n = 75 (%)	Pre & Peri-menopausal cases n = 19 (%)	Post-menopausal cases n = 56 (%)
Bleeding per vagina	60 (80%)	13 (68.2%)	47 (83.9%)
WDPV	51 (68%)	10 (52.6%)	41 (73.2%)
Others	48 (64%)	11 (57.8%)	37 (66.0%)
Pain Abdomen	40 (53.3%)	12 (63.1%)	28 (50.0%)
Post-coital bleeding	4 (5.3%)	1 (5.2%)	3 (5.3%)
Mass per vagina	2 (2.6%)	1 (5.2%)	1 (1.7%)
No symptoms	2 (2.6%)	2 (10.5%)	00

Table 3: Per-speculum findings in cases in present study

Per-speculum findings	Total No. of cases	Pre & Peri-menopausal cases	Post-menopausal cases
Growth	49 (65.3%)	11 (57.8%)	38 (67.8%)
Bleeding	8 (10.6%)	2 (10.5%)	6 (10.7%)
Erosion	6 (8%)	3 (15.7%)	3 (5.3%)
Ulcer	5 (6.6%)	2 (10.5%)	3 (5.3%)
Unhealthy	3 (4%)	1 (5.2%)	2 (3.5%)
Mass	2 (2.6%)	00	2 (3.5%)
Stenosis	1 (1.3%)	00	1 (1.7%)
WDPV	1 (1.3%)	00	1 (1.7%)
Total	75 (100%)	19 (100%)	56 (100%)

Table 4: Per-Vaginal findings in cases in present study

Per vaginal examination	Total No. of cases	Pre & Peri-menopausal cases	Post-menopausal cases
Friable Growth	45 (60.0%)	11 (57.8%)	34 (60.7%)
Induration	28 (37.3%)	7 (36.8%)	21 (37.5%)
Erosion	1 (1.3%)	00	1 (1.7%)
Stenosis	1 (1.3%)	1 (5.2%)	00
Total	75 (100%)	19 (100%)	56 (100%)

Table 5: Stages of the disease in cases in present study

Stage of the disease	Total No of Cases	Pre & Peri-menopausal cases	Post-menopausal cases
Stage I	6 (8%)	4 (21.0%)	2 (3.5%)
Stage II	24 (32%)	7 (36.8%)	17 (30.5%)
Stage III	30 (40%)	3 (15.7%)	27 (48.2%)
Stage IV	15 (20%)	5 (26.3%)	10 (17.8%)
Total	75 (100%)	19 (100%)	56 (100%)

Table 6: Histological grade of the disease in cases in the present study

Grade of the disease	No of cases (%)	Pre & Peri-menopausal cases	Post-menopausal cases
WDSCC	42 (56%)	13 (68.4%)	29 (51.7%)
MDSCC	16 (21.3%)	3 (15.7%)	13 (23.2%)
PDSCC	10 (13.3%)	1 (5.2%)	09 (16.1%)
NKLCSCC	5 (6.6%)	2 (10.5%)	3 (5.3%)
NKSCSCC	2 (2.6%)	–	2 (3.5%)
Total	75 (100%)	19 (100%)	56 (100%)

($p = 0.005$) (Table 8). All cases (100%) between 30-59 years of age showed block positivity. There was no significant association between age at marriage of cases and P16 expression ($p = 0.951$) (Table 9). The p16 expression was maximum among females with age of marriage between 15 and 18 years. There was no statistically significant association between age at menopause of cases and P16 expression ($p = 0.311$) (Table 10). Among the post-menopausal women, P16 expression was maximal between 40-44 years. There was no significant association ($p = 0.554$) between parity and P16 expression. However, all cases (100%) with parity one and two showed block positive P16 expressions (Table 11).

There was no significant association between P16 expression and clinical presentation ($p = 0.135$), or per-speculum examination findings ($p = 0.217$), or per-vaginal examination findings ($p = 0.982$). There was no significant association between P16 expression and stage of the disease ($p = 0.28$) (Table 12). However, all stage I cases (100%) showed block positive P16 expression. There was no significant association between histopathological grade and P16 expression ($p = 0.877$) (Table 13). However, most of the cases of WDSCC (88.0%) and MDSCC (93.7%) showed block positive P16 expression, though there was no statistical significance.

DISCUSSION

Cervical cancer is one of the most common cancers in women worldwide, especially in women from developing countries. It is the most common cause of cancer-related deaths in females. The prevalence of cervical cancer in the Southeast part of Karnataka (India) at a tertiary health care center is 17% of the total cancers of females¹¹. The prevalence reported at Bangalore by the Kidwai Memorial Institute of Oncology

between 2004 – 2005 was 15.9%¹². The National Cancer Registry Program newsletter has reported the incidence to be 6.2% – 22.6% between 2001 - 2011 in India and 21.1% in Bangalore¹³.

Pap smear test as a screening test has helped decrease the incidence of cervical cancer by 75%, especially in developed countries. However, it has some limitations such as low sensitivity, false negative results and low reproducibility. To overcome this, the P16 biomarker has been used as an alternative and has emerged as a surrogate marker for *in-situ* as well as advanced cervical cancer. The P16 biomarker test has better reproducibility. The P16 gene undergoes alterations like amplification following HPV infection and integration of viral genome with host genome. P16 may also undergo mutations^{14,15}. Normally, the expression of P16 increases with age which results in a decrease of the renewal activity of stem cells. In conditions of inhibition / low P16 expression, there will be high expression of cancer stem cells which results in increased ability of self-renewal of cancer stem cells¹⁶. The sensitivity, specificity and accuracy of P16 biomarker to diagnose SCC of cervix is 96.0%, 88.2% and 91.7%, respectively¹⁷.

In the present study, IHC P16 expression per the LAST classification showed block positivity in 67 cases (89.3%), ambiguity in 5 cases (6.6%) and negativity in 3 cases (4%). In a study by Sarwath H *et al.*, block positivity was seen in 92.2% of cases and negativity in 7.8% of cases, with sensitivity, specificity, PPV and NPV of 79.2%, 46%, 83.9% and 27.2%, respectively. The absence of P16 expression in SCC of cervix may be due to absence of HPV infection, improper IHC technique, mutations in promoter region, epigenetic mechanisms and hypermethylation¹. Stoler MH *et al.* classified P16 expression as diffuse, focal and negative per the LAST criteria and found that P16 expression was diffuse in 100% of invasive cancers⁶. Amaro-Filho has reported P16 expression as diffuse, focal and negative in 85.5%, 9.9% and 4.6%

Table 7: P16 expression as per LAST criteria in cervical biopsy by IHC in the present study

P16 expression	No of Cases (%)	Pre & Peri-menopausal cases	Post-menopausal cases
Negative	3 (4%)	00	3 (5.3%)
Ambiguity	5 (6.6%)	1 (5.2%)	4 (7.1%)
Block Positive	67 (89.3%)	18 (94.7%)	49 (87.5%)
Total	75 (100%)	19 (100%)	56 (100%)

P value of P16 expression between pre & peri-menopausal and post-menopausal cases was 0.555

Table 8: Association between age distribution in cases and P16 expression in the present study

Age range of cases	Expression of P16 (n)			Total cases
	Negative	Ambiguous	Block positivity	
30 - 39	0	0	8	8
40 - 49	0	0	19	19
50 - 59	0	0	18	18
60 - 69	2	3	14	19
70 - 79	1	1	8	10
80 - 89	0	1	0	1
Total cases	3	5	67	75

P value between age of cases and P16 expression was 0.005

Table 9: Association between age at marriage in cases and P16 expression in the present study

Age at menopause	Expression of P16 (n)			Total cases
	Negative	Ambiguous	Block positivity	
12 - 14 years	1	1	17	19
15 to 18 years	2	4	46	52
> 18 years	0	0	4	4
Total cases	3	5	67	75

P value between age of cases and P16 expression was 0.951

Table 10: Association between age at menopause in cases and P16 expression in the present study

Age at menopause	Expression of P16 (n)			Total cases
	Negative	Ambiguous	Block positivity	
Peri & Post-menopausal	0	1	18	19
40 - 44	0	1	15	16
45 - 49	2	1	22	25
50 - 54	1	1	11	13
55 - 59	0	1	1	2
Total cases	3	5	67	75

P value between age at menopause of cases and P16 expression was 0.311

Table 11: Association between parity in cases and P16 expression in the present study

Para of cases	Expression of P16 (n)			Total cases
	Negative	Ambiguous	Block positivity	
Para 1	0	0	6	6
Para 2	0	0	11	11
Para 3	0	1	16	17
Para 4	1	3	16	20
Para ≥ 5	2	1	18	21
Total cases	3	5	67	75

P value between parity of cases and P16 expression was 0.554

Table 12: Association between stage of disease and P16 expression in the present study

Stage of disease	Expression of P16 (n)			Total cases
	Negative	Ambiguous	Block positivity	
Stage I	0	0	6	6
Stage II	2	1	21	24
Stage III	1	1	28	30
Stage IV	0	3	12	15
Total cases	3	5	67	75

P value between stage of disease and P16 expression was 0.285

Table 13: Association between histopathology grades in cases and P16 expression in the present study

Para of cases	Expression of P16 (n)			Total cases
	Negative	Ambiguous	Block positivity	
NKSCCC	0	0	2	2
NKLCSCC	0	1	4	5
PDSCC	0	1	9	10
MDSCC	1	0	15	16
WDSCC	2	3	37	42
Total cases	3	5	67	75

P value between parity of cases and P16 expression was 0.877

cases, respectively, in SCC of cervix; the data were statistically significant ($p < 0.001$)¹⁸.

In the present study, there was a statistically significant association between age and P16 expression, where all cases (100%) between 30-59 years showed block positivity. Pre- and peri-menopausal women showed maximal (94.73%) block positivity over post-menopausal women; the data was not statistically significant. Among the post-menopausal women, women between 40-44 years showed maximal (93.75%) block positivity, although this data was not statistically significant. Sarwath *et al.* stated that

there was a significant correlation between P16 expression and age group between 41–60 years. This was thought to be due to active transforming precancerous lesions in younger age group women. Hence, P16 is an appropriate surrogate marker for use in early screening of cervical cancer¹.

In the present study, parity and P16 expression did not show a statistically significant association. However, all cases (100%) with parity one and two showed block positivity. There was no statistically significant association between stage / histological grade of the disease and P16 expression. However, all stage

I cases (100%) showed block positivity and a majority of cases of WDSCC (88.0%) and MDSCC (93.7%) showed block positivity. Fu HC *et al.* in their study stated that P16 expression was not found to have an association with tumor stage, tumor size, histological grade, vascular invasion, CEA levels, SCC Ag levels, or non-squamous cell carcinoma. The independent prognostic factors for cervical cancer regarding disease-free survival (DFS) is high-grade SCC, non-SCC and low P16 expression¹⁶. Sarwath *et al.* stated that P16 expression did not correlate with tumor grade and size of the tumor¹. Huangfu M *et al.* in their study reported that auto-antibodies against P16 protein (tumor-associated antigen) are released in cases of cervical cancer, and they are found to be at the highest levels in serum of stage I cervical cancer patients, with sensitivity of > 90% and specificity of 20.3%. Hence, P16 auto-antibody can be used as one of the parameters for early diagnosis and assessment of prognosis¹⁹.

Cervical cancer with P16 expression has better prognosis^{16,20}. High P16 expression in cervical cancer is reported to have high five-year overall survival and DFS, both of which were statistically significant. Five-year overall survival in high and low P16 expression was 62.0% and 35.2%, respectively. DFS in high and low P16 expression was 60.0% and 31.2%, respectively^{16,20}. In the present study, the cases were not followed up for prognosis. P16 is also an indicator for radiosensitivity. Thus, due to its anti-cancer activity, P16 can be exploited for development of targeted chemotherapy in cervical cancer¹⁶.

The limitation of the present study was that we did not follow up with the cases to assess their prognosis. However, P16 block positivity was high in young females and was statistically significant. P16 expression was maximal in pre- and peri-menopausal females, post-menopausal females between 40-44 years of age, females with one/two parity and in stage I disease, although these were not statistically significant. The study can be conducted in a larger population to further confirm the above findings.

CONCLUSION

Significant p16 biomarker expression was observed in cervical patients of younger age and early stage of the disease. Therefore, P16 biomarker may have a beneficial use in screening or early diagnosis leading to better prognosis of SCC of cervix. This findings from the study can also be used as a concept for targeted therapy as P16 protein has anti-cancer properties.

ABBREVIATIONS

- Ag:** Antigen
ASCCP: American Society for Colposcopy and Cervical Pathology
CAP: College of American Pathologists
CEA: Carcinoembryonic Antigen
DFS: Disease Free Survival
FIGO: International Federation of Gynaecology and Obstetrics
HPV: Human Papilloma Virus
HR-HPV: High Risk Human Papilloma Virus
IHC: Immunohistochemistry
LAST: Lower Anogenital Squamous Terminology
MDSCC: Moderately Differentiated Squamous Cell Carcinoma
NKLCSCC: Non-Keratinizing Large Cell Squamous Cell Carcinomas
NKSCC: Non-Keratinizing Small Cell Squamous Cell Carcinomas
PDSCC: Poorly Differentiated Squamous Cell Carcinoma
SCC: Squamous Cell Carcinoma
WDSCC: Well Differentiated Squamous Cell Carcinoma

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Nil

AUTHOR'S CONTRIBUTIONS

Kalyani Raju: Concept, Study design, data collection, literature search, statistical analysis, manuscript writing, manuscript editing. Raghuveer CV: Concept, Manuscript editing, manuscript review. Sheela SR: Data collection, manuscript editing.

FUNDING

Nil

AVAILABILITY OF DATA AND MATERIALS

Data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was conducted in accordance with the amended Declaration of Helsinki. The institutional review board (The Institutional Ethics Committee of Sri Devaraj Urs Medical College, Tamaka, Kolar) approved the study, and all participants provided written informed consent.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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