Original Article

Evaluation of enzyme-linked immunosorbent assay plasma p16INK4a protein in squamous cell carcinoma in uterine cervix: A case–control study

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ABSTRACT

Background: Cervical cancer is one of the common cancers in females. The common method of screening is Pap test which has low sensitivity. Hence, better methods are explored with different biomarkers, of which estimation of P16 protein can be opted in early detection of cervical cancer.

Materials and Methods: Seventy cases and seventy controls were considered for the study. Cases were invasive squamous cell carcinoma (SCC) of cervix confirmed by histopathology. Controls were healthy age-matched females. The blood sample of cases and controls was collected in K2 Ethylenediaminetetraacetic acid vacutainer, and the separated plasma was subjected to estimation of P16 protein by quantitative sandwich Enzyme-Linked ImmunoSorbent Assay method. The data were analyzed for the association between p16 protein in plasma in cases and controls.

Results: The age among cases and controls ranged from 30 to 80 years. The P16 levels among cases ranged from 3.4 to 19.6 ng/ml with a mean of 7.24 ± 2.35 ng/ml. The plasma P16 level in controls ranged between 0.9 and 9.7 ng/ml with mean of 4.1 ± 2.22 ng/ml. At cutoff more than 4.8 ng/ml in cases, the sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy were 92.86%, 72.86%, 77.4%, 91.1%, and 82.86%, respectively. The specificity increased with increase in plasma p16 levels. The P16 levels were maximum in stage IV disease.

Conclusion: This was a pilot study to detect the plasma p16INK4a levels in SCC of cervix. The levels of plasma p16 protein between 3.9 and 5 ng/ml can be considered as the range for the test to be positive. In clinically suspected cases of cervical cancer, levels more than 4.8 ng/ml can be considered for the diagnosis as point of care test.

KEY WORDS: Cervical cancer, enzyme-linked immunosorbent assay, p16INK4a biomarker

INTRODUCTION

Cervical cancer is one of the common cancers in females causing mortality and morbidity. It is the third most common cancer accounting for 7.8% and fourth cancer-related mortality among women worldwide. Human Papillomavirus (HPV) infection has been proved as an etiological factor. About 15%–30% of patients with early stage of cancer present with recurrence after surgical treatment.[1-6]

P16 is a cyclin-dependent kinase inhibitor. In HPV-related cancers, as in cervical cancer, the E7 protein of HR-HPV inactivates pRb protein, resulting in increased synthesis and accumulation of P16 protein in cells and tissue which can be demonstrated by immunocytochemistry or immunohistochemistry techniques, respectively. P16 is markedly positive (90%) in cases of high grade squamous intraepithelial lesion (HSIL) and invasive squamous cell carcinoma (SCC) of cervix.[7-9]

A few studies have reported regarding the quantitative estimation of p16 protein in cervical cancer. Estimation of P16 protein by Enzyme-Linked ImmunoSorbent Assay (ELISA) on lysed samples of

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cervical cells has positive correlation with HSIL. The levels were low in low-grade squamous intraepithelial lesion (LSIL) cases and normal cervix.[8] The aim of this study is to estimate plasma p16 protein by ELISA method in cases of SCC of cervix and evaluate the association of the same with controls.

MATERIALS AND METHODS

This study is a laboratory-based observational study done in department of Pathology in coordination with the department of obstetrics and gynecology. This is a pilot study conducted to detect the p16INK4a levels in seventy cases of SCC of cervix. Seventy age matched healthy female controls were considered for the study. Cases were invasive SCC of cervix diagnosed clinically and confirmed by histopathology. Patients with cervical intraepithelial squamous neoplasia, postchemotherapy cases, postradiotherapy cases, recurrence of the disease, metastatic deposits (secondary deposits) in the cervix and any other primary malignancy in the patient were excluded from the study. Glandular lesions of endocervix as glandular intraepithelial neoplasia and adenocarcinoma of cervix were also excluded from the study.

In clinically suspected cases of cervical cancer, following consent of the patient, cervical biopsy was taken and kept in 10% neutral buffered formalin for routine histopathological diagnosis. At the same time, 6 ml of blood was collected in K2 ethylenediaminetetraacetic acid (EDTA) vacutainer, centrifuged at 1500 rpm for 10 min. The separated plasma was aliquotted in vials and kept at −80°C for the estimation of P16 protein by ELISA method. The clinical case details as hospital number, biopsy number, age, presenting complaints, detailed history, and physical examination findings were entered in the excel sheet. Following confirmation of the case as SCC of cervix by histopathology, the grade and the stage (FIGO staging) of the disease were noted, and the plasma of the cases was subjected to p16 protein estimation.[10,11] Following consent, the blood sample of age-matched healthy 70 controls was collected in K2 EDTA vacutainer, and the separated plasma was subjected to estimation of P16 protein.

The P16 protein estimation was done by quantitative sandwich ELISA method using Human P16 ELISA kit (ImmunoTag, Catalogue No: ITEH01637) as per manufacturer's instruction. A standard curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis, and curve was drawn through the points on the graph. Using the OD of samples, the concentration of the P16 protein was estimated and expressed as ng/ml. The values of both cases and controls were entered in the master chart (excel sheet).

Data were analyzed using IBM SPSS (Statistical Package for the Social Sciences) 22 version software (Bangalore. Karnataka. India). Categorical data were represented in the form of frequencies and proportions. Continuous data were represented as mean and standard deviation. Chi-square/Fisher Exact test was used to find the significance of difference between the categorical parameters. Analysis of variance test was used to find the significance of difference between the continuous data. The $P < 0.05$ was considered as statistically significant. Statistical analysis for association between p16 protein in plasma in cases and controls was done through sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) at different cutoff values of p16 by estimating area under receiver operating characteristics (ROC) curve, z statistics, and Youden index J.

RESULTS

Seventy cases of SCC of cervix were considered for the study. The age ranged from 30 to 80 years with mean of 54.3 ± 12.0. The P16 levels among cases ranged from 3.4 to 19.6 ng/ml with mean of 7.24 ng/ml (standard deviation [SD] =2.35). The mean plasma p16 level in cases was maximum in a case of 80 years and minimum in age range of 50–59 years. Seventy age-matched controls were considered for the study having age range between 30 and 80 years with mean age of 54.3 ± 12.6. The plasma P16 level in controls ranged between 0.9 and 9.7 ng/ml with a mean of 4.1 ± 2.22 ng/ml. The mean plasma p16 level in controls was maximum between in age group of 70 and 79 years and minimum in age group of 30–39 years of age. The plasma p16 levels between cases and controls in different age groups were statistically significant with $P < 0.05$ [Table 1].

Figure 1 shows the area under ROC curve along with sensitivity and specificity between plasma p16 values of cases and controls indicating statistical significance with the $P < 0.0001$.

Table 2 shows the sensitivity, specificity, PPV, and NPV at different cutoff levels of plasma p16 protein in cases. At 95% confidence interval, cutoff levels between 3.9 and 5 ng/ml levels had relatively high sensitivity and specificity. At cutoff more than 4.8 ng/ml in cases, the sensitivity, specificity, PPV, NPV, and diagnostic accuracy or overall positivity was 92.86%, 72.86%, 77.4%, 91.1%, and 82.86%, respectively. The specificity increased with increase in plasma p16 levels [Tables 3 and 4].

Table 5 shows ELISA plasma P16 levels in different stages of the disease where maximum levels of P16 were recorded in Stage IV of the disease. The *P* value was 0.068 between the stages.

Table 6 shows ELISA plasma P16 levels in different histological grades of the disease, maximum was recorded in well-differentiated SCC (WDSCC) and minimum in poorly differentiated SCC PDSCC with gradual decrease in values from WDSCC to PDSCC. The *P* value was 0.018.

DISCUSSION

Cervical cancer is one of the common cancers in females.[2] The average incidence is 25 per one lakh females. In developed

Table 1: Plasma P16 levels in different age groups in cases and controls in the present study

Age range (years)	Cases			Controls			*P*
	n	Plasma P16 range (ng/ml)	Mean plasma P16 (ng/ml)	*n*	Plasma P16 range (ng/ml)	Mean plasma P16 (ng/ml)	
30-39	7	5.5-9.7	7.4±1.3	8	1.3-4.7	2.8±1.25	<0.05
40-49	19	3.4-13.0	7.6±2.5	18	0.9-8.8	3.9±2.32	<0.05
50-59	15	4.4-6.9	5.8±0.7	15	1.5-9.2	4.2±2.23	<0.05
60-69	18	5.2-19.6	8.0±3.1	18	1.2-7.7	4.3±1.96	<0.05
70-79	10	3.8-11.2	6.9±1.9	10	1.0-9.7	4.9±3.00	<0.05
80-89	1	8.4	8.4	1	3.8	3.8	-
Total	70	3.4-19.6	7.2±2.3	70	0.9-9.7	4.1±2.22	<0.05

Area under the ROC curve (AUC)	0.843
SE	0.0353
95% CI	0.772-0.899
Z statistic	9.706
Significance level *P* (area=0.5)	<0.0001

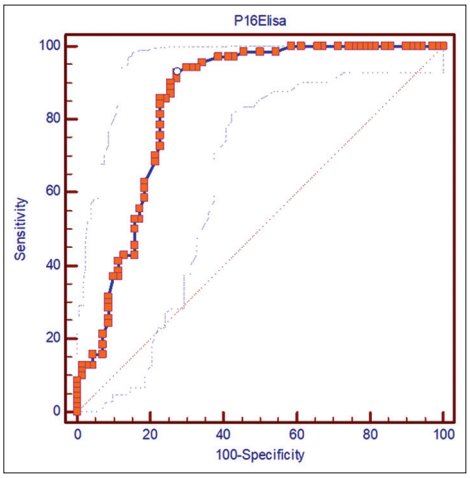

Figure 1: Area under the ROC curve showing the sensitivity and specificity of ELISA plasma p16 protein. ELISA= Enzyme linked immunosorbent assay, ROC=Receiver operating characteristics, AUC=Area under curve, SE=Standard error, CI=Confidence interval

countries, it is the seventh common cause of cancer death. In developing countries, it is the third most common cause of death. It is the major killer disease in developing countries including India. It is the second common cancer in women in India after breast cancer in urban population. The annual estimated global incidence is 500,000 and India contributes approximately 100,000. It gives rise to agonizing morbidity and mortality. However, it is a preventable disease. Early detection and treatment result in decreasing the mortality and morbidity.[3-6] The prevalence of cervical cancer reported in this region is 17% of total cancers in females.[12]

Cervical cancer screening program is conducted since decades worldwide. The common methods of screening are Pap test and detection of HR-HPV DNA. The Pap test, a cytology technique, has low sensitivity, high cost, requires multiple visits and infrastructure. HPV DNA test has low specificity. Hence, better methods are explored with different biomarkers, of which estimation of P16 protein is opted as a better biomarker in early detection of cervical cancer.[7-9]

P16 is a regulator of cell cycle and a tumor suppressor protein. It forms complex with CDK4 and CDK6 and activate pRb which results in arrest of cell cycle at G1-S phase of cell cycle. In non-HPV cancer, P16 gene can be inactivated due to deletion, mutation, hypermethylation, or loss of heterozygosity, resulting in increased CDK activity, inactivation of pRb, and increase in cell proliferation.[7] In HPV-related cancers, as in cervical cancer, the HR-HPV infects the tissue and releases E7 protein which inactivates pRb protein, resulting in increased synthesis and accumulation of P16 protein in cells due to negative feedback mechanism and results in increased cell proliferation.[7-9]

P16 can be demonstrated in tissues and in cells by immunohistochemistry or immunocytochemistry techniques, respectively, in cervical epithelial dysplastic and tumor cells. Expression of P16 protein in precancerous lesion suggests the possibility of progression to malignancy. Studies have shown positive correlation of P16 expression with HSIL and SCC of cervix.[7-9] Studies have shown that the estimation of P16 protein by ELISA on lysed samples of cervical cells had positive correlation with HSIL. The levels were low in LSIL cases and normal cervix. The rate of detection of cervical dysplasia by P16 ELISA and HR-HPV DNA was similar.[7]

In a study by Balasubramanian *et al*., the sensitivity and specificity of P16 ELISA test in cervical samples were similar to cytology, both having low sensitivity and high specificity compared to hybrid capture2 test. P16 ELISA test showed low sensitivity in detecting small lesions using cervical specimens as lesions shed only a few abnormal cells. The rate of positivity with cutoff of 8 U/ml was 90% for ≥CIN3, 77% for CIN2, and 53% for CIN1. The prevalence of screening was 10.4% with P16 ELISA test with cutoff of 8 U/ml. The sensitivity and specificity reported for ≥CIN3 at cutoff of ≥8U/ml was 50.9% and 90.4%, respectively, and at cutoff of ≥6U/ml was 64.1% and 77.5%, respectively. The sensitivity and specificity reported for ≥CIN2 at cutoff of ≥8U/ml was 39.9% and 90.7%, respectively, and at cutoff of ≥6U/ml was 50.1% and 77.7%, respectively.[7]

In a study by Mao *et al*. in cervical samples, the sensitivity and specificity of P16 ELISA at cutoff ≥8U/ml for CIN3 was 90.0% and 46%, respectively, versus 85% and 35.4%, respectively, for Hybrid capture2 test. The sensitivity of P16 ELISA at cutoff of ≥6U/ml was 95.0%. The cutoff between 6 and 12U/ml had relatively high sensitivity and specificity by which the test can

Table 2: Sensitivity, specificity, positive predictive value and negative predictive value at various cut off levels of plasma p16 protein in cases

P16 protein in ng/ml	Sensitivity	95% CI	Specificity	95% CI	PPV	95% CI	NPV	95% CI
>1	100.00	94.9-100.0	2.86	0.3-9.9	50.7	42.1-59.3	100.0	15.8-100.0
>2	100.00	94.9-100.0	20.00	11.4-31.3	55.6	46.4-64.4	100.0	76.8-100.0
>3	100.00	94.9-100.0	32.86	22.1-45.1	59.8	50.4-68.8	100.0	85.2-100.0
>4.1	97.14	90.1-99.7	61.43	49.0-72.8	71.6	61.4-80.4	95.6	84.9-99.5
>4.8	92.86	84.1-97.6	72.86	60.9-82.8	77.4	67.0-85.8	91.1	80.4-97.0
>5	90.00	80.5-95.9	74.29	62.4-84.0	77.8	67.2-86.3	88.1	77.1-95.1
>6	70.00	57.9-80.4	78.57	67.1-87.5	76.6	64.3-86.2	72.4	60.9-82.0
>7	42.86	31.1-55.3	84.29	73.6-91.9	73.2	57.1-85.8	59.6	49.3-69.3
>8	25.71	16.0-37.6	91.43	82.3-96.8	75.0	53.3-90.2	55.2	45.7-64.4
>9.2	12.86	6.1-23.0	98.57	92.3-100.0	90.0	53.0-99.8	53.1	44.1-61.9
>9.4	11.43	5.1-21.3	98.57	92.3-100.0	88.9	48.9-99.8	52.7	43.8-61.5
>10.6	7.14	2.4-15.9	100.00	94.9-100.0	100.0	47.8-100.0	51.9	43.1-60.5
>11.1	5.71	1.6-14.0	100.00	94.9-100.0	100.0	39.8-100.0	51.5	42.8-60.1
>13	1.43	0.04-7.7	100.00	94.9-100.0	100.0	2.5-100.0	50.4	41.8-58.9
>19.6	0.00	0.0-5.1	100.00	94.9-100.0			50.0	41.4-58.6

PPV=Positive predictive value, NPV=Negative predictive value, CI=Confidence interval

Table 3: Validity of enzyme-linked immunosorbent assay plasma p16 protein levels in differentiating Cases and Controls at different cutoff levels

Youden Index	Values
Youden index J	0.6571
95% CI	0.5000-0.7429
Associated criterion	>4.8
95% CI	3.9-5

CI=Confidence interval

Table 4: Sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy at cut off levels of >4.8 ng/ml of plasma p16 protein

Parameter	Estimate (%)	Lower-upper 95% CIs
Sensitivity	92.86	84.34-96.91[†]
Specificity	72.86	61.46-81.88[†]
PPV	77.38	67.35-85.01[†]
NPV	91.07	80.74-96.13[†]
Diagnostic accuracy or overall positivity of P16	82.86	75.76-88.2[†]

PPV: Positive predictive value, NPV: Negative predictive value, CIs: Confidence intervals

Table 5: Shows enzyme linked immunosorbent assay plasma p16 levels in different stages of the disease in cases

Stage of the disease	Number of cases (%)	ELISA plasma P16		*P* value of ELISA plasma p16
		Range (ng/ml)	Mean (ng/ml)	
Stage I	5 (7.1)	3.4-9.4	6.86±2.4	0.068
Stage II	23 (32.8)	3.8-19.6	7.03±3.08	
Stage III	28 (40.0)	4.8-10.6	6.74±1.29	
Stage IV	14 (20.0)	5.1-13.0	8.71±2.26	
Total	70 (100)	3.4-19.6	7.24±2.35	

ELISA=Enzyme linked immunosorbent assay

be considered as positive. The cutoff of ≥ 8U/ml was reported as the reasonable choice. Increased size of the lesion was associated with increased P16 levels.[8]

In a study by Wu *et al*. in cervical samples, it was reported that P16 ELISA had increased specificity in the detection of CIN compared to HPV as screening test. The P16 protein estimated was 32.6U/ml, 38.7U/ml, 63.4U/ml, and 210U/ml in normal, CIN1, CIN2/3, and invasive cervical cancer, respectively, which shows progressive increase in levels of P16 protein with increase in degree of dysplasia. However, the values of P16 protein in each group were quite high compared to other similar studies as the cases were HIV positive women, and in HIV positive women, the viral load of HPV will be increased compared to non-HIV women. In CIN2+ cases, 78.6% showed positive for P16 protein at cutoff level of 9U/ml with sensitivity, specificity, PPV, and NPV of 89.0%, 22.9%, 13.6%, and 93.8%, respectively. The sensitivity and specificity varied in different cutoff levels of P16 protein as; at cutoff 7U/ml, 90.6% and 18.2%, at 8U/ml 89.8% and 20.6%, at 9U/ml 89.0% and 22.9%, and at 10U/ml 85.8% and 26.9%, respectively.[9]

In a study by Lee *et al*. in blood samples for mutation screening in cervical cancer, it was reported that the sensitivity and specificity for the PIK3CA gene as 88.9% and 100%, respectively. The sensitivity and specificity for KRAS gene was 100%. The mutation rates of ZFHX3, KMT2C, KMT2D, NSD1, and RNF213 genes reported to have high frequency in cervical cancer patients. Hence, they concluded that the gene mutation can serve as a prognostic biomarker and mutations in tumor suppressor genes are prevalent in all stages of cervical cancer. Tumor suppressor gene mutations reveal the appropriate treatment modalities in patients. In addition, chemotherapy and radiotherapy affect the allele frequency, which can be utilized for monitoring cancer in blood samples.[1]

In the present study, the blood samples were collected from histologically proved invasive SCC cases and the separated plasma was subjected for estimation for P16 protein by ELISA method. The P16 levels among cases ranged from 3.4 to 19.6 ng/ml with a mean of 7.24 ng/ml (SD = 2.35). There was a statistical significant association of plasma P16 levels between cases and controls at different age groups (<0.05) [Table 1]. In both cases and controls, maximum plasma p16 protein levels were observed in older age group and minimum levels

Table 6: Enzyme linked immunosorbent assay plasma P16 levels in different histological Grades of the disease in cases

Grade of the disease	Number of cases	ELISA plasma P16		P value of ELISA plasma p16
		Range (ng/ml)	Mean (ng/ml)	
WDSCC	38	3.4-13.0	7.1±1.97	0.018
MDSCC	15	5.1-11.4	6.7±1.56	
PDSCC	10	4.4-11.2	6.7±2.01	
NKLCSCC	5	7.7-19.6	5±5.08	
NKSCSCC	2	7.0-7.4	2±0.28	
Total	70	3.4-19.6	7.24±2.35	

ELISA=Enzyme linked immunosorbent assay, SCC=Squamous cell carcinoma, WDSCC=Well differentiated SCC, MDSCC=Moderately differentiated SCC, PDSCC=Poorly differentiated SCC, NKLCSCC= Non-Keratinizing large cell SCC, NKSCSCC= Non-Keratinizing small cell SCC

in younger age group. This may be probably due to majority of cases in older age group in the present study had disease of higher stage compared to younger age group. In controls, the higher plasma p16 levels in older age group may be probably due to senescence changes. The cutoff levels of plasma p16 between 3.9 and 5 ng/ml levels in cases had relatively high sensitivity and specificity by which the test can be considered as positive. At cutoff more than 4.8 ng/ml in cases, the sensitivity, specificity, PPV, NPV, and diagnostic accuracy or overall positivity was 92.86%, 72.86%, 77.4%, 91.1%, and 82.86%, respectively. Hence, cut off at more than 4.8 ng/ml can be considered as reasonable choice for diagnosis of SCC of cervix. The specificity increased with increase in plasma p16 levels in cases. In the present study, ELISA plasma P16 levels between different stages of the disease were not statistically significant, and ELISA plasma P16 levels in different histological grades of the disease from WDSCC to PDSCC were statistically significant.

The ELISA P16 test is a molecular evaluation than the morphological evaluation, and hence estimation of this biomarker will be more objective with increased reproducibility. It will be better indicator of molecular changes associated with carcinogenesis. The test procedure can be improved using enhanced version of ELISA which may offer improved sensitivity in screening. Combined with Hybrid capture2 test, the sensitivity and specificity can be increased. This protein assay biomarker if developed and validated can be more objective, faster, and more affordable with less infrastructure and trained technical personnel. It can be promising potential screening test, especially in low-resource settings and point of care test with low false positivity.[7-9] This study points toward the concept of liquid biopsy which is a minimally invasive technique having potential to revolutionize the treatment of cancer, evaluate the progress, and relapse of the disease unlike biopsy and cytology techniques.[13]

The limitation of the present study is limited number of cases. In addition, we have considered only histopathologically proved cases of SCC of cervix and excluded HSIL and LSIL. We tried to find out the p16 levels in SCC of cervix. The plasma p16 levels in this study showed statistical significant association between cases and controls. The cutoff level of p16 protein level more than 4.8 ng/ml shows statistical acceptable level for diagnosis of disease. The study can be taken forward by conducting it in larger population, standardizing/validating the procedure of the test and accrediting the test with laboratory accredited bodies by which the test can be used for screening and follow-up of the disease.

CONCLUSION

Plasma p16 estimation by ELISA can be considered as test for diagnosis of SCC of cervix with further standardization of procedure. The p16 protein levels between 3.9 and 5 ng/ml can be considered as the range for the test to be positive. In clinically suspected cases of cervical cancer, levels more than 4.8 ng/ml can be considered for the diagnosis as point of care test.

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Conflicts of interest
There are no conflicts of interest.

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