Association of IHC p16INK4a Expression and ELISA Plasma p16INK4a Protein in Squamous Cell Carcinoma of Uterine Cervix: A Concept of Liquid Biopsy

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ABSTRACT

Introduction: Cervical cancer is the 3rd most common cancer among women worldwide. p16 biomarker is the surrogate marker in cervical cancer and can be detected by Immunohistochemistry (IHC).

Aim: To evaluate the association of IHC p16INK4a expression in tissue sections and Enzyme-linked Immunosorbent Assay (ELISA) p16INK4a protein in plasma of paired samples in Squamous Cell Carcinoma (SCC) of uterine cervix.

Materials and Methods: This laboratory-based observational, pilot study was conducted at tertiary care centre of Sri Devaraj Urs Medical College in South India from June 2020 to May 2021. Total 17 new cases of SCC of cervix diagnosed by histopathology were considered for the study. The cases were staged as per International Federation of Gynaecology and Obstetrics (FIGO) staging system and classified by histopathology as keratinising and non keratinising types. IHC p16INK4a evaluation was done on tissue sections and classified as block positivity, ambiguity and negative. ELISA p16INK4a estimation was done using plasma from Dipotassium Ethylenediaminetetraacetic Acid (K2-EDTA) blood sample of same cases. Statistical analysis was done using Statistical Package for the Social Sciences (SPSS) version 22.0 software.

Results: The age ranged from 30-80 years with mean of 54.3±12.0 years. Plasma ELISA p16INK4a level ranged from 3.4-19.6 ng/mL with a mean of 7.2±2.35 ng/mL in SCC of cervix. The plasma p16INK4a ELISA levels of 5.1 to 6.2 ng/mL, 6.0 to 6.6 ng/mL and 5.5 to 9.7 ng/mL predicts negative, ambiguity and block positivity of IHC p16INK4a expression respectively in corresponding tissue biopsy. Plasma ELISA p16INK4a levels were maximum in Non Keratinising Squamous Cell Carcinoma (NKSCC) followed by Well Differentiated Squamous Cell Carcinoma (WDSCC), Moderately Differentiated Squamous Cell Carcinoma (MDSCC) and Poorly Differentiated Squamous Cell Carcinoma (PDSCC).

Conclusion: The plasma ELISA p16INK4a levels and IHC p16INK4a expression were maximum in higher disease stage compared to lower stage. This was a pilot study to evaluate the association between tissue IHC p16 expression and plasma ELISA p16 levels. Further study has to be done on larger study population with standardised procedure to prove the hypothesis.

INTRODUCTION

Cervical cancer is the 3rd most common cancer among women worldwide with 11.7% of global prevalence. It ranks 5th in cancer related deaths in women. The annual estimated global incidence is 500,000 and India contributes approximately 100,000 [1-3]. Cervical cancer is the second most common cancer in developing countries among females [1]. Human Papilloma Virus (HPV) is proved aetiological factor [1,3].

p16 is a cyclin dependent kinase inhibitor. In cervical cancer, the High Risk-HPV (HR-HPV) E7 protein inactivates pRb protein resulting in increased synthesis and accumulation of p16 protein in tissues and cells which can be demonstrated by IHC or immunocytochemistry techniques respectively. p16 shows marked positivity (90%) in High grade Squamous Intraepithelial Lesion (HSIL) and invasive SCC of cervix [4-6]. Lower Anogenital Squamous Terminology (LAST) criteria in 2012 classifies p16 immunoreactivity as block positive, ambiguous and negative considering p16 biomarker expression in the nucleus with or without cytoplasmic staining [7,8].

Liquid biopsy is a technique where the liquid sample as blood, body fluids and urine are used to isolate Circulating Tumour Cells (CTC), circulating tumour Deoxyribonucleic Acid (ctDNA), Ribonucleic Acid (RNA), exosomes and proteins which are shed by tumour cells [9,10]. Studies have reported that estimation of p16 protein by ELISA on lysed samples of cervical cells had positive association with HISL and SCC of uterine cervix [4,5].

The aim of this study was to evaluate the association of IHC p16 expression with Enzyme-linked Immunosorbent Assay (ELISA) plasma p16 protein levels in Squamous Cell Carcinoma (SCC) of cervix cases and derive the significance of plasma p16 protein levels in cervical cancer. A part of this study of IHC p16 expression correlating the clinical features is already published [11].

MATERIALS AND METHODS

This laboratory-based observational, pilot study was conducted in Department of Pathology in co-ordination with Department of Obstetrics and Gynaecology at Sri Devaraj Urs Medical College (Tertiary Health Care Centre) Kolar, Karnataka, India, from June 2020 to May 2021. Ethical clearance for the study has been obtained from Institutional Ethics Committee (SDUMC/KLR/IEC/45/2020-21 dated:09-05-2020).

Sample size calculation: Sample size was calculated using the formula:

$$Z^2 \times pq/d^2$$

Considering the prevalence (p) of p16 expression as 95% in cervical cancer [12], an absolute error (d) of 5% and confidence level of 95%. The estimated sample size for the study was 60. Considering 10% non responsive case, total sample size of 70 was considered.

Inclusion and Exclusion criteria: Total 70 new cases diagnosed as SCC of cervix clinically and confirmed by histopathology were included in the study. Patients with Cervical Intraepithelial Neoplasia (CIN),

postchemotherapy cases, postradiotherapy cases, relapse cases, Cervical Glandular Intraepithelial Neoplasia (CGIN), adenocarcinoma of cervix, metastatic deposits in the cervix and any other primary malignancy in the patient were excluded from the study.

Procedure

Following informed consent, in clinically suspected cases of cervical cancer, cervical biopsy was taken and fixed in 10% buffered formalin for routine histopathological diagnosis and IHC p16 biomarker expression. A 6 mL of blood was collected in Dipotassium Ethylenediaminetetraacetic Acid (K2-EDTA) vacutainer and centrifuged at 1500 rpm for 10 minutes. The separated plasma was kept in vials at -80° centigrade for quantitative estimation of p16 protein by ELISA sandwich method. The case details including age, hospital number, biopsy number, presenting complaints, personal history, menstrual history, family history, past history, per abdominal examination findings, per speculum examination findings, per vaginal examination findings and per rectal examination findings were entered in the excel sheet. Following histopathology report confirmation, staging of the disease as per International Federation of Gynaecology and Obstetrics (FIGO) staging was noted [13]. The slides were screened by two Pathologists and classified histologically as keratinising and non keratinising types. Keratinising SCC was further analysed [14]:

- Poorly Differentiated Squamous Cell Carcinoma (PDSCC)
- Moderately Differentiated Squamous Cell Carcinoma (MDSCC)
- Well Differentiated Squamous Cell Carcinoma (WDSCC)
- Non Keratinising Squamous Cell Carcinoma (NKSCC)

    -Large Cell Type (NKLCSCC)

    -Small Cell Type (NKSCSCC)

Tissue sections were cut from paraffin blocks and subjected to IHC p16 biomarker (Mouse monoclonal anti-p16INK4 clone G175-405, Biogenex, USA), primary antibody, on all cases with positive and negative control. The procedure followed was as per the manufacturer's instruction. The p16 expression on tissue sections were classified as block positivity, ambiguous staining and negative as per LAST criteria 2012.

- "Block" pattern staining means strong, continuous, nuclear positivity with or without cytoplasmic staining extending from basal layers upwards for atleast 1/3rd thickness of the epithelium (basal and parabasal layers) which can be further graded as 1/3rd, 2/3rd and more than 2/3rd and laterally over a significant area with diffuse staining of >25% of cells.

- "Ambiguous" staining means strong and basal (strong, diffuse, continuous, involves only lower 1/3rd without upward extension) or weak, diffuse and discontinuous staining, involving atleast 2/3rd of the epithelium or strong, focal and discontinuous located at any level of the epithelium.

- "Negative" staining means total absence or weak or focal and discontinuous or only cytoplasmic staining [Tables/Fig-1-3] [8].

The separated plasma of the cases were subjected to p16 protein estimation by quantitative sandwich ELISA method using Human p16 ELISA kit (ImmunoTag, Catalogue No: ITEH01637) and performed as per manufacturer's instruction. A standard curve was constructed by plotting the average Optical Density (OD) for each standard on the vertical axis (Y) against the concentration on the horizontal axis (X) 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 were entered in the master chart (excel sheet).

STATISTICAL ANALYSIS

Data was analysed using Statistical Package for the Social Sciences (SPSS) version 22.0 software. Categorical data was represented in the form of frequencies and proportions. Continuous data was 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 (ANOVA) test was used to find the significance of difference between the continuous data. The p-value <0.05 was considered as statistically significant. Statistical analysis for association between IHC p16 expression in tissue sections and ELISA p16 protein in plasma was done through sensitivity, specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) and diagnostic accuracy at different cut-off values of plasma p16 protein levels.

RESULTS

Total 70 cases of cervical cancer of SCC type were considered for the study. The age ranged from 30-80 years with mean of 54.3±12.0 years. Maximum cases were seen in age group of 40 to 49 years (n=19) followed by 60 to 69 years (n=18). Bleeding per vagina was the most common presentation (n=40) followed by white discharge per vagina (n=30), pain in lower abdomen (n=40), postcoital bleeding (n=4), mass per vagina (n=2) and other constitutional symptoms (n=48). The p-value between age of cases and IHC p16 expression was 0.008. All cases between 30-59 years of age showed block positivity for p16 IHC (i.e., 100%) [Table/Fig-4].

The ELISA plasma p16 levels ranged from 3.4 to 19.6 ng/mL with mean of 7.24±2.35 ng/mL. Plasma p16 levels were maximum in a case of 80 years and minimum in age range of 50-59 years. There was no statistical significant association (p-value=0.294) between stage of the disease and IHC p16 expression. However, all stage I cases (100% cases) showed block positive p16 expression. Regarding ELISA plasma p16 levels in different stages of the disease, maximum levels of p16 was recorded in stage IV of the disease. The p-value was 0.068 [Table/Fig-5]. The p-value between IHC p16 expression and ELISA p16 values in stage II, III and IV was 0.975, 0.917 and 0.652, respectively.

There was no statistical significant association (p-value=0.887) between histopathological grade and IHC p16 expression of the
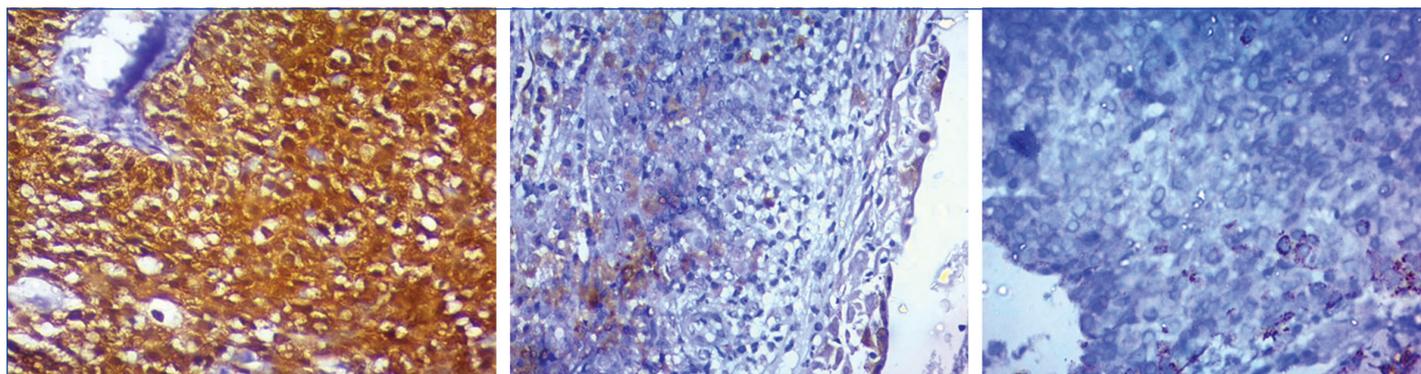
[Table/Fig-1]: Microphotograph showing block positive staining of immunohistochemistry p16 (IHC p16: 400X); [Table/Fig-2]: Microphotograph showing ambiguity staining of immunohistochemistry p16 (IHC p16: 400X); [Table/Fig-3]: Microphotograph showing negative staining of immunohistochemistry p16 (IHC p16: 400X). (Images from left to right)

| Age range | Expression of p16 (n) | | | |
	Negative	Ambiguous	Block positivity	Total cases
30-39	0	0	7	7
40-49	0	0	19	19
50-59	0	0	15	15
60-69	2	3	13	18
70-79	1	1	8	10
80	0	1	0	1
Total cases	3	5	62	70

[Table/Fig-4]: Shows association between age distribution in cases and IHC p16 expression in the present study.

disease. Total 33 (86.8%) of the cases of WDSCC and 14 (93.3%) of MDSCC cases showed block positive expression p16 IHC but it was not statistically significant (p-value=0.887). Regarding, ELISA plasma p16 levels in different histological grades of the disease, maximum was recorded in NKLCSCC followed by NKSCSCC/ WDSCC and minimum in MDSCC/PDSCC. The p-value was 0.018. The p-value between IHC p16 and ELISA p16 in WDSCC, MDSCC and PDSCC was 0.682, 0.406 and 0.008, respectively [Table/Fig-6].

| Stage of the disease | n, % | Immunohistochemistry p16 expression | | | p-value of IHC p16 | ELISA plasma p16 | | p-value of ELISA plasma p16 |
		Negative (n, %)	Ambiguous (n, %)	Block (n, %)		Range (ng/mL)	Mean (ng/mL)	
Stage I	5 (100%)	0 (0)	0 (0)	5 (100%)		3.4-9.4	6.86±2.4	
Stage II	23 (100%)	2 (8.7%)	1 (4.3%)	20 (87%)		3.8-19.6	7.03±3.08	
Stage III	28 (100%)	1 (3.6%)	1 (3.6%)	26 (92.8%)	0.294	4.8-10.6	6.74±1.29	0.068
Stage IV	14 (100%)	0 (0)	3 (21.4%)	11 (78.6%)		5.1-13.0	8.71±2.26	
Total	70 (100%)	3 (4.3%)	5 (7.1%)	62 (88.6%)		3.4-19.6	7.24±2.35	

[Table/Fig-5]: Shows IHC p16 expression and ELISA plasma p16 levels in different stages (FIGO stages) of the disease in present study [13].
Statistical test: Chi-square test

| Grade of the disease | n, % | Immunohistochemistry p16 expression | | | p-value of IHC p16 | ELISA plasma p16 | | p-value of ELISA plasma p16 |
		Negative (n, %)	Ambiguous (n, %)	Block (n, %)		Range (ng/mL)	Mean (ng/mL)	
WDSCC	38 (100%)	2 (5.3%)	3 (7.9%)	33 (86.8%)		3.4-13.0	7.1±1.97	
MDSCC	15 (100%)	1 (6.7%)	0 (0)	14 (93.3%)		5.1-11.4	6.7±1.56	
PDSCC	10 (100%)	0 (0)	1 (10.0%)	9 (90.0%)	0.887	4.4-11.2	6.7±2.01	0.018
NKLCSCC	5 (100%)	0 (0)	1 (20.0%)	4 (80.0%)		7.7-19.6	10.6±5.08	
NKSCSCC	2 (100%)	0 (0)	0 (0)	2 (100%)		7.0-7.4	7.2±0.28	
Total	70 (100%)	3 (4.3%)	5 (7.1%)	62 (88.6%)		3.4-19.6	7.24±2.35	

[Table/Fig-6]: Shows IHC p16 expression and ELISA plasma p16 levels in various histological grades of the disease in present study.
Statistical test: Chi-square test; Poorly Differentiated Squamous Cell Carcinoma (PDSCC); Moderately Differentiated Squamous Cell Carcinoma (MDSCC); Well Differentiated Squamous Cell Carcinoma (WDSCC); Non Keratinising Squamous Cell Carcinoma (NKSCC); Large Cell Type (NKLCSCC); Small Cell Type (NKSCSCC); p-value <0.05 considered significant

Among 70 cases, IHC p16 biomarker expression showed block positive, ambiguity and negative in 62 (88.6%), 5 (7.1%) and 3 (4.3%) cases, respectively. Regarding ELISA plasma p16 levels in different groups of IHC p16 expression as per LAST criteria, the levels were maximum in ambiguity group followed by block positivity and then negative cases with p-value of 0.598 [Table/Fig-7]. Validity of plasma p16 ELISA in predicting negative expression of IHC p16 by LAST criteria is showed in [Table/Fig-8] with plasma p16 ELISA range of 5.1 to 6.2 ng/mL. Validity of plasma p16 ELISA in predicting ambiguity expression of IHC p16 by LAST criteria is showed in [Table/Fig-9] with plasma p16 ELISA range of 6.0 to 6.6 ng/mL. Validity of plasma p16

IHC p16 expression	n, %	Plasma p16 range (ng/mL)	Mean plasma p16 (ng/mL)	p-value
Negative	3 (4.3%)	5.1-6.2	6.6±1.30	
Ambiguity	5 (7.1%)	6-6.6	8.2±1.88	
Block positive	62 (88.6%)	5.5-9.7	7.1±2.42	0.598
Total	70 (100%)	5.1-9.7	7.24±2.35	

[Table/Fig-7]: Shows ELISA plasma p16 levels in different groups of IHC p16 expression as per Lower Anogenital Squamous Terminology (LAST) criteria in present study.
Statistical test: Pearson Correlation, r=-0.120

ELISA in predicting Block positive expression of IHC p16 by LAST criteria is showed in [Table/Fig-10] with plasma p16 ELISA range of 5.5 to 9.7 ng/mL. [Table/Fig-11] shows the sensitivity, specificity, PPV, NPV and diagnostic accuracy at different cut-off levels of ELISA plasma p16 protein in predicting negative, ambiguity and block positive IHC p16 expression by LAST criteria, respectively.

DISCUSSION

Cervical cancer is the common cancer in women worldwide. The incidence and mortality has decreased by 75% to 80% in developed countries by well-organised screening programs. Papanicolaou test (pap) test for screening cervical cancer has markedly decreased the incidence of cervical cancer. Inspite of well-developed screening procedure, women in developing countries are diagnosed at late stage of the disease resulting in high mortality [14-16]. High Risk Human Papillomavirus (HPV) is the proved aetiological factor and HPV vaccine has been implemented in many countries [1,3,17].

The common age range of cervical cancer reported is 27 to 80 years of age with mean age of 54.2 years and maximum cases are noted between 41 to 60 years of age [1]. In this study, the age ranged from 30-80 years with mean of 54.3±12.0, maximum cases ranged from 40 to 49 years followed by 60 to 69 years. The most common symptom reported is vaginal bleeding [2]. In the present study, bleeding per vagina was the most common presentation followed by white discharge per vagina. Histologically, squamous cell carcinoma has been classified as non keratinising and keratinising variants constituting 68.8% and 26% of cases respectively with 5.2% having no data [1]. In the present study, non keratinising and keratinising variants constituted 10% and 90%, respectively.
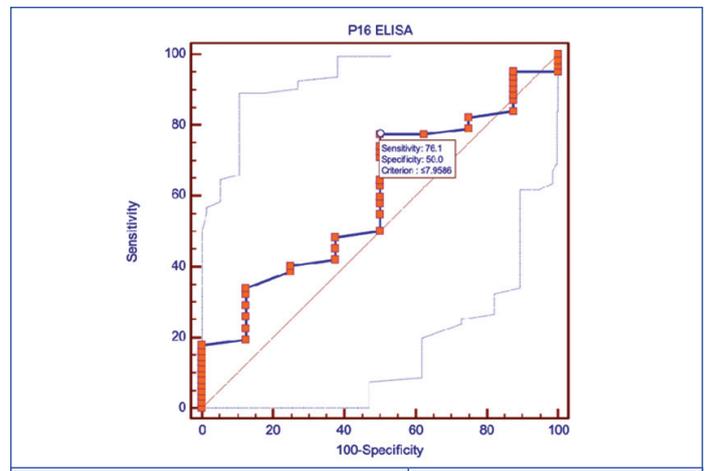
p16 is a regulator of cell cycle and a tumour suppressor protein. It forms complex with Cyclin Dependent Kinase 4 (CDK4) and CDK6 and activate pRb which results in arrest of cell cycle at G1-S phase of cell cycle. In non HPV cancer, p16 gene can be inactivated due to deletion, mutation, hypermethylation or loss of heterozygosity resulting in increased CDK activity, inactivation of pRb and increase in cell proliferation [4]. 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 [4-6].

There is improvement in diagnostic agreement when histomorphological diagnosis of cervical biopsy is correlated with IHC p16 expression as an adjuvant biomarker [4,15]. Studies have reported
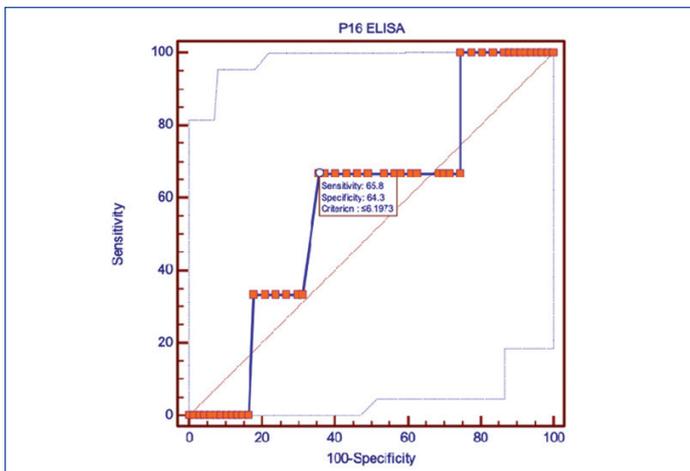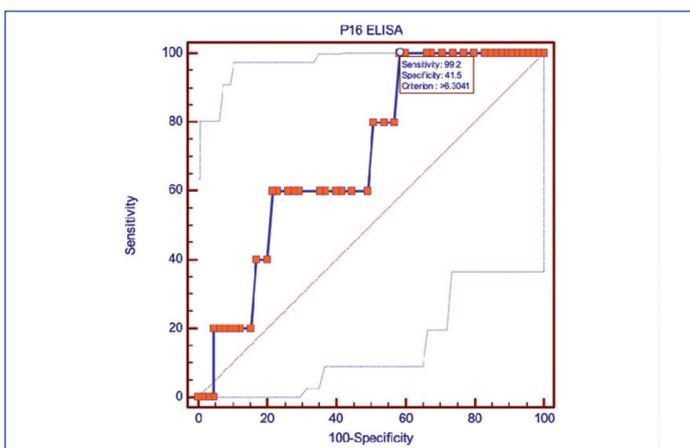
Variables	Values
Area under the ROC curve (AUC)	0.582
Standard error	0.176
95% confidence interval	0.458 to 0.699
z statistic	0.467
Significance level p (Area=0.5)	0.6407
Youden index J	0.3085
95% confidence interval	0.1940 to 0.3433
Associated criterion	≤6.2
95% confidence interval	5.1 to 6.2

[Table/Fig-8]: ROC curve showing validity of p16 ELISA in predicting negative IHC p16 expression by LAST criteria.

Variables	Values
Area under the ROC curve (AUC)	0.702
Standard error	0.109
95% confidence interval	0.580 to 0.805
z statistic	1.843
Significance level p (Area=0.5)	0.0653
Youden index J	0.4154
95% confidence interval	0.2769 to 0.5538
Associated criterion	>6.3
95% confidence interval	6 to 6.6

[Table/Fig-9]: ROC curve showing validity of p16 ELISA in predicting Ambiguity IHC p16 expression by LAST criteria.

Variables	Values
Area under the ROC curve (AUC)	0.599
Standard error	0.104
95% confidence interval	0.475 to 0.714
z statistic	0.950
Significance level p (Area=0.5)	0.3420
Youden index J	0.2742
95% confidence interval	0.1331 to 0.4227
Associated criterion	≤8
95% confidence interval	5.5 to 9.7

[Table/Fig-10]: ROC curve showing validity of p16 ELISA in predicting block positive IHC p16 expression by LAST criteria.

Parameter	≤6.2 ng/mL predicting negative IHC	>6.3 ng/mL predicting ambiguity IHC	≤8 ng/mL predicting block positive IHC
Sensitivity	66.67%	100%	77.42%
Specificity	64.18%	41.54%	50%
Positive predictive value	7.692%	11.63%	92.31%
Negative predictive value	97.73%	100%	22.22%
Diagnostic accuracy	64.29%	45.71%	74.29%

[Table/Fig-11]: Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value and Diagnostic accuracy at cut-off levels of ≤6.2 ng/mL, >6.3 ng/mL and ≤8 ng/mL of ELISA plasma p16 protein in predicting negative, ambiguity and block positive IHC p16 expression by LAST criteria respectively.
Statistical test: Z statistics with Youden index J

present study, IHC p16 expression of block positivity, ambiguity and negative constituted 88.6%, 7.1% and 4.3%, respectively.

Tumour diagnosis is conventionally done by radiological findings and invasive surgical biopsy. In surgical or tissue biopsy a small chunk of tissue is taken from the cancer tissue for histopathological examination and diagnosis. Of late non invasive technique where blood sample, urine and body fluids are used to extract CTC and genetic material for cancer diagnosis and treatment which is called as "Liquid Biopsy" [20-22]. In this technique the liquid sample is used to isolate CTC, circulating tumour DNA (ctDNA), RNA, Exosomes and proteins which are shed by tumour cells into blood circulation, body fluids or urine in most of the cancers depending on the site of the cancer. This technique enables non invasive profiling of solid tumours, the results which can be comparable with that of tissue biopsy [9,10]. Studies have shown that estimation of p16 protein by ELISA on lysed samples of cervical cells had positive correlation with HSIL and SCC of cervix. The levels were low in Low grade Squamous Intraepithelial Lesion (LSIL) cases and normal cervix. The rate of detection of cervical dysplasia by p16 ELISA and HR-HPV DNA were similar [4,6]. In the present study, p16 protein was estimated in the plasma of cases of SCC of cervix by ELISA method which showed a range of 3.4-19.6 ng/mL with a mean of 7.2±2.35 ng/mL.

positive association of p16 expression with HSIL and SCC of cervix [4-6]. p16 expression in cervical tissue infection with HR-HPV along with integration of viral genome with host genome [17,18]. The criteria for interpretation of p16 expression across globe is not uniform [7,19]. LAST criteria (2012) define p16 expression as block positive, ambiguous and negative. Lower Anogenital Squamous Terminology (LAST) project is co-sponsored by College of American Pathologists (CAP) and American Society for Colposcopy and Cervical Pathology (ASCCP). LAST gives standard guidelines for utility of p16 biomarker [8,9]. In the

There was significant association between age distribution among cases and IHC p16 expression with younger age (30 to 59 years) showing 100% block positivity probably because of evolving phase of the disease. However, association between age distribution and plasma ELISA p16 levels was not significant. There was no significant association between stage of the disease with IHC p16 expression and plasma ELISA p16 levels. However, all stage I cases showed block positivity and maximum cases of block positivity were in stage III followed by stage II indicating increased expression at higher stage of the disease. Plasma ELISA p16 levels was maximum in stage IV followed by stage II indicating plasma ELISA. p16 increases with stage of the disease. There was no significant association between histological type and IHC p16 expression. However, there was significant association between plasma ELISA p16 levels and histological type where the levels were maximum in WDSCC, followed by MDSCC and PDSCC.

There was no statistical significant association between IHC p16 expression and plasma ELISA p16 levels. However the plasma ELISA p16 levels were maximum in ambiguity and block positive cases compared to negative cases. In addition this study shows that the plasma p16 ELISA levels of 5.1 to 6.2 ng/mL, 6.0 to 6.6 ng/mL and 5.5 to 9.7 ng/mL predicts negative, ambiguity and block positivity of IHC p16 expression in corresponding tissue biopsy.

Limitation(s)

The limitation of this study was, all the cases were SCC of cervix and Cervical Intraepithelial Neoplasia (CIN) as High grade Cervical Intraepithelial Neoplasia (CIN) or Low grade Cervical Intraepithelial Neoplasia (CIN) was not considered for the study as authors thought of proving the hypothesis in frank cases of SCC of cervix and then consider in CIN. There is limitation in numbers of cases, hence, further studies should be done on larger study population and the procedure has to be standardised to prove the hypothesis of the concept of liquid biopsy. However, as far as author's knowledge, the concept of this study with liquid biopsy is first of its kind in English literature. This is only a pilot study to find the association between tissue IHC p16 expression and plasma ELISA p16 levels.

CONCLUSION(S)

Plasma ELISA p16 protein level predicts negative, ambiguity and block positivity of IHC p16 expression respectively in corresponding tissue biopsy. Plasma ELISA p16 levels were maximum in NKSCC and WDSCC, followed by MDSCC and PDSCC. The plasma ELISA p16 levels and IHC p16 expression were maximum in higher disease stage compared to lower stage. This observation suggests the possibility of using the blood sample to diagnose and predict the prognosis of SCC of cervix, the concept of liquid biopsy.

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