RESEARCH ARTICLE

Editorial Process: Submission:02/07/2023 Acceptance:07/02/2023

Development and Evaluation of p16 based Double Antibody Sandwich ELISA for Detection of Cervical Precancer and Cancer

Mayilvahanan Bose[1], Shirley Sunder Singh[2], Selvaluxmy Ganesharaja[3], Aruna S. Chiwate[4], Sanjay Jaydeo Hingmire[4], Thangarajan Rajkumar[1]*

Abstract

Objective: Cervical cancer is the third most common cancer in women, worldwide. This study was designed to develop an affordable, accurate and simpler screening test like Enzyme-linked immunosorbent assay (ELISA) which is low cost and will help in bringing down the disease burden in resource poor countries. **Methods:** In this study, we have raised and evaluated monoclonal antibodies against recombinant p16 using immunohistochemistry (IHC), western blot, immunoprecipitation and ELISA. Double antibody sandwich ELISA (DAS-ELISA) and cytokeratin ELISA was designed for screening women with cervical dysplasia and cancer. **Results:** Cloned, expressed and purified recombinant p16 were used for generation of monoclonal antibodies. After initial screening, six clones were selected, and affinity purified. Except 155D11G10, which was isotype Immunoglobulin (Ig) G1 all the others were found to be IgG2b. 133A6G5 and 151A7B9 were found to be best for p16 IHC, both showed 70 – 80% and 80 – 90% of nuclear staining respectively. All the antibodies positively detected p16 from the HeLa lysates in western blot except 133A6G5. Studies using immunoprecipitation showed 133A6G5, specifically detected p16. DAS-ELISA developed using a combination of our p16 monoclonal antibodies showed sensitivity of up to 2pg. A pilot study using DAS-ELISA and cytokeratin ELISA in cervical samples revealed the assay sensitivity and specificity as 100% and 80%, respectively. **Conclusion:** Using combination of DAS-ELISA and cytokeratin ELISA we have developed an accurate and reliable method for the early detection of cervical cancer in a subject, with minimal false results. In the future after large scale validation, p16 ELISA could be used as a reliable tool for diagnostic purposes.

Keywords: p16 biomarker- Double antibody Sandwich ELISA- Cytokeratin ELISA- Early diagnosis- Cervical cancer

Asian Pac J Cancer Prev, **24 (7)**, 2337-2346

Introduction

Cervical cancer is the third most common cancer in women, worldwide (Ferlay et al., 2021). About 88% of cervical cancer cases occur in developing countries where, in many regions, it is the most common cancer. The higher incidence and mortality in cervical cancer has been mainly associated in countries with lower human development index (Singh et al., 2023). In India, lack of awareness about the symptoms, associated risk factors, availability of screening programmes and knowledge about the preventive measures has contributed to the higher rate of mortality (Kadian et al., 2021). Conventional techniques used for the detection of cervical cancer include pelvic examination, visual inspection with acetic acid / Lugol's iodine and Pap smears. While these techniques can be easily performed even in populations with limited access to healthcare, these cytology based screening has low sensitivity (Malhone and Longatto-Filho, 2019). HPV- DNA is the recommended and widely used marker for cervical cancer detection and it has better sensitivity than PAP smear. However, mere detection of HPV does not mean the subject has cancer or will develop cancer. Expression of p16 is linked to the oncogenic risk of HPV infection resulting in cervical cancer, so p16 over expression is used as a surrogate marker for HPV in cervical cancer detection (Volkova et al., 2021). p16, a tumour suppressor protein inhibits cyclin-dependent kinases (CDK)-4 and -6 and is essential for the regulation of cell cycle. Inactivation of p16 has been detected in various human malignant tumors (Netto and Epstein, 2011; Liu et al., 2015; Venkatesh et al., 2021). In cervical carcinomas, human papillomavirus (HPV) viral DNA integration into the host genome may result in disruption of the E2 open reading frame, resulting in

*[1]Department of Molecular Oncology, Cancer Institute (WIA), Chennai, India. [2]Department of Onco-Pathology, Cancer Institute (WIA), 38, Sardar Patel Road, Guindy, Chennai, India. [3]Department of Radiation Oncology, Cancer Institute, Chennai, 600 036 Tamil Nadu, India. [4]Cervical Cancer Prevention Programme, Nargis Dutt Memorial Cancer Hospital Barshi, Maharahstra, India.*For Correspondence: drtrajkumar@gmail.com*

unregulated overexpression of HPV oncogenes E6 and E7. E7- mediates catabolism of Rb resulting in overexpression of p16 (Munger et al., 2013; Pal and Kundu, 2019). Thus, p16 overexpression is a downstream effect of high-risk HPV infection. The present study was taken up based on our preliminary results from cervical cancer microarray data, where p16 has emerged as a potential biomarker of diagnostic importance (Rajkumar et al., 2011). The current study was designed to develop an ELISA-based assay for p16 which would help screen women for cervical dysplasia and cancer in large-scale screening programmes.

Materials and Methods

Patient sample

This study was performed with approval from institutional review boards at Cancer Institute (WIA), Chennai. All the samples used in the study were collected after obtaining appropriate informed consent from the participants in the study. Samples were collected from patients and volunteers reporting to outpatient department of radiation oncology unit of Cancer Institute (WIA) and screening programme organized by Nargis Dutt Memorial cancer hospital, Barshi, India. Briefly, epithelial cells from ectocervical, endocervical and transformation-zone were collected using Cervex-Brush® and rinsed immediately into the PreservCyt Solution vial by vigorous swirling and stored at 4^{O}C, until further use.

Cloning, expression and purification

Total RNA was isolated from HeLa lysates using RNeasy mini kit (Qiagen, Hilden, Germany) and cDNA synthesis was done using High Capacity cDNA Reverse Transcription Kits, according to the manufacturer's protocol. p16 primers were designed with restriction enzymes flanking the complete coding sequence. (p16 ORF FP5' GGATCCATGGAGCCGGCGGCG 3' and RP5' GCGGCCGCTCAATCGGGGATGTCTG 3'). p16 gene was amplified from the HeLa cell line cDNA by using AmpliTaq Gold DNA polymerase. 25 µl of PCR reaction mix consisted of 2.5 µl of 10x buffer, 0.2 µl of dNTPs, 1.5µl of $MgCl_2$, 10 picomoles of forward and reverse primers, 0.2µl of AmpliTaq Gold DNA polymerase, and 100 ng of cDNA. The cycling conditions included the initial denaturation step of 95°C for 10 mins followed by a cycle of denaturation at 94°C for 1 min, annealing at 80°C for 1 min and extension at 72°C for 2 min for 35 cycles followed by a final extension step at 72°C for 7 mins and finally cooled to 4°C. The PCR product was resolved on ethidium bromide stained 1% agarose gel and documented.

Further, full length p16 was cloned into pCR™2.1-TOPO VECTOR as per TOPO® TA Cloning® Kit protocol. Sub cloning of full length p16 into pET 28a+ expression vector was done using standard techniques. The recombinant pET 28a+ - p16 ligated plasmid was transformed into chemically competent BL21 (DE3) cells (Stratagene, La Jolla, CA). Transformed cells were grown on an LB plate containing kanamycin (50mg/ml) at 37^{o}C overnight. A single colony was then inoculated into 10 ml of LB broth containing 50mg/ml of kanamycin and grown overnight at 37°C with shaking. The overnight culture was diluted 1:50 and grown for 2 more hours at 37°C to get the optical density of 0.6 (log phase) and then the culture was equilibrated at 25°C. Protein production was induced by adding 0.4 mM isopropyl β-D-1- thiogalactopyranoside (IPTG), and bacteria were cultured for an additional 3 hrs at 25°C, and cells were pelleted. Cell pellet corresponding to 100 ml of induced bacterial culture was resuspended in 10 ml of Lysis buffer (50 mM Tris - HCl, 150 mM NaCl, 0.1% NP40 (pH 8) containing lysozyme and protease inhibitors. To facilitate lysis, the sample was sonicated for 10 min and debris removed by centrifugation at 13000 g for 15 min at 4°C. The supernatant was applied to Ni-NTA affinity column (Invitrogen) and incubated at room temperature with constant rocking for 1 hr, after the incubation period column was settled under gravity. The column was washed sequentially with 5 ml of wash buffer containing 25, 50, and 100 mM imidazole. Protein was then eluted from the column using elution buffer containing 250mM imidazole (50 mM Tris - HCl, 150 mM NaCl, 0.1% NP40 (pH 8). His tagged – p16 fusion protein was cleaved at the thrombin site to remove the His-tag from the full length recombinant p16 using THROMBIN CleanCleave™KITaccording to the manufacturer's instruction. Cleaved full length p16 was further concentrated using Amicon Ultra centrifugal filters (3 KDa) and confirmed by SDS – PAGE and western blotting.

Generation Of Monoclonal Antibodies

The generation of mouse monoclonal antibodies was outsourced to BioKlone Biotech Pvt Ltd, after providing the recombinant p16 protein. Entire selection and evaluation of individual clones were done in our lab. Culture supernatants of the individual parent clones from the hybridoma were screened for their specificity and sensitivity in the detection of p16 by IHC and ELISA. Clones showing high specificity and sensitivity by IHC and ELISA were further selected and subjected to limiting dilution to identify a single specific clone secreting highly specific monoclonal antibodies. Finally, after screening a total of 231 clones, six clones were selected for further affinity purification and isotyping of the monoclonal antibody.

Western blot, Immunoprecipitation and Immunohistochemistry

Affinity purified monoclonal antibodies were further evaluated for their specificity and sensitivity using western blotting, immunoprecipitation, and IHC for p16. Western blotting using our mouse monoclonal antibody was done as described previously (Pauck et al., 2014). Immunoprecipitation of p16 was done as described previously, with slight modifications (Sawicka et al., 2013). Briefly, 500 µl (1mg/ml) of recombinant p16 was incubated along with 100µg of 133A6G5 at 4oC in a blood rotator. 50µl of protein G-Sepharose were washed twice with RIPA buffer and mixed with an overnight incubated antigen-antibody complex and incubated for 3 hours at 4^{o}C in a blood rotator. Beads containing immune complexes were washed 5 times and 50µl of sample loading buffer was added to the bead pellet. Finally, the bead pellet was

heat-denatured at 95°C and the supernatant was spun down and loaded in SDS-PAGE followed by western blotting.

Immunohistochemistry for p16 was done as described previously (Vijayalakshmi et al., 2007), briefly, by using wet autoclaving with a hold time of 5 minutes. Mouse monoclonal E6H4 antibody (MTM, Laboratories) was used at 1 in 200 dilutions as a positive control, and affinity purified monoclonal antibodies were evaluated.

Double Antibody Sandwich – ELISA For P16

Sandwich ELISA for p16 was set up by coating microtiter plates in 0.1 M carbonate buffer with 50µl combination of p16 monoclonal antibody 133A6G5 and 151A7B9 (250ng) overnight at 4° C. Antibody coated plates were then blocked with 2% milk for 1 hour at 37°c followed by three washes with PBS-Tween20 (0.05%). Recombinant p16 protein standards (250pg – 0.12pg) in 2 % BSA were then added and incubated for 2 hours at 37°c. Following wash thrice with PBS-Tween20, 50 µl detecting antibody (biotinylated p16 monoclonal antibody 155E11G3 and 155D11G10 (1:5,000) was added to each well and incubated for 30 minutes at 37° c. Finally, Streptavidin- HRP conjugate was added in 1:500 dilutions to each well and incubated for 30 minutes. The plates were then developed after final wash, using OPD substrate buffer and reaction stopped with 2N H_2So_4. The absorbance of each well at 492 nm was recorded using ELISA plate reader.

ELISA for Cytokeratin

Direct ELISA for Cytokeratin was set up briefly by coating 50µl of HeLa lysates from different cell numbers (standards) overnight at 4°C in 0.1 M carbonate buffer. Antigen coated plates were then blocked with 2% milk for 1 hour at 37°c followed by three washes with PBS-Tween20 (0.05%). 50µl (1:150) of mouse Monoclonal (AE1/AE3) antibody (M3515, Dako) in 2 % BSA were added and incubated for 2 hours at 37°C. Following wash thrice with PBS-Tween20, 50 µl (1:5,000) anti-mouse IgG, Biotinylated (RPN1001, Amersham™) was added and incubated for 30 minutes at 37°c. Finally, Streptavidin - HRP conjugate (RPN1231, Amersham™) was added in 1:500 dilutions to each well and incubated for 30 minutes. The plates were then developed after the final wash, using OPD substrate buffer and reaction stopped with 2N H_2SO_4. The absorbance of each well at 492 nm was recorded using ELISA plate reader.

Pilot study

A pilot study to establish a proof of principle was designed using DAS-ELISA and Cytokeratin ELISA. Fifty-five cervical scrape samples with known histological / cytological diagnosis were lysed using sonication. The lysates were cleared by centrifugation at 14000rpm for 15 min at 4°C. 50µl of the supernatant from each sample was used for the ELISA. Samples were run along with p16 standards and HeLa lysates from different cell numbers (standards). The ratio of p16 OD to Cytokeratin OD were used to set the cut-off value based on which the samples were scored positive or negative.

Results

Cloning, Expression, Purification of Full Length Recombinant P16 Protein

The sequences encoding the p16 were amplified using p16 ORF primers and cloned into TOPO TA vector and further subcloned into pET 28a+ vector using standard techniques. Expression in BL21 (DE3) Cells and purification studies revealed a specific band consistent with His – tagged p16 was in the soluble fraction. His tagged – p16 fusion protein was cleaved at the thrombin site to remove the His-tag from full length recombinant p16 using THROMBIN CleanCleaveTMKIT. A single specific band consistent with full length p16 (16 kDa) was observed in the lane containing cut p16 when run along with uncut p16 in SDS – PAGE and purified full length p16 was further confirmed by western blotting (Results not shown).

Screening of Clones After Limiting Dilution

Purified full length recombinant p16 was used for immunization and generation of mouse monoclonal antibodies. Culture supernatants of the individual parent clones from the hybridoma were screened for their specificity and sensitivity in detection of p16 by IHC and ELISA. Clones showing high specificity and sensitivity by IHC and ELISA were further selected and subjected to limiting dilution to identify a single specific clone secreting highly specific monoclonal antibodies.

Evaluation of Mouse Monoclonals Raised Against P16

Finally, after screening a total of 231 clones, six clones were selected for further affinity purification. Isotyping of selected antibodies revealed that except 155D11G10, all of them were of IgG2b. 155D11G10 was found to be isotype

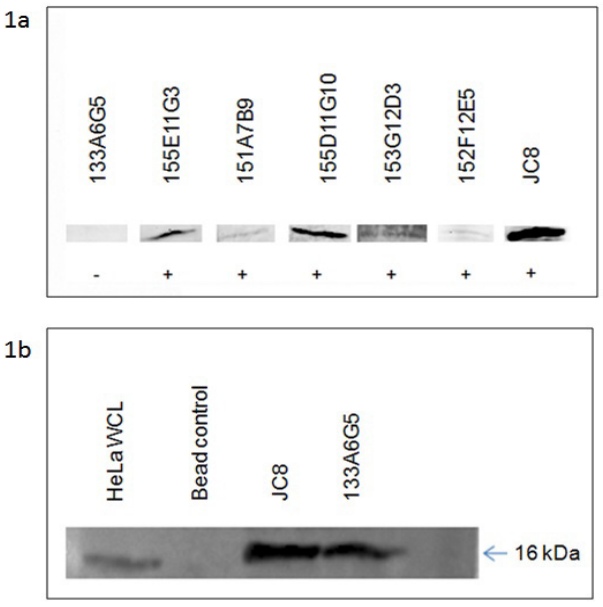

Figure 1. (a), Western blot showing detection of p16 in HeLa lysates by In-house generated mouse monoclonal antibodies (1:200); (b), Immunoprecipitation using 133A6G5 (100µg) confirmed the detection of p16 (Lane1 – HeLa whole cell lysate, Lane2 – Bead control, Lane 3 – JC8 (positive control), Lane 4 – 133A6G5)

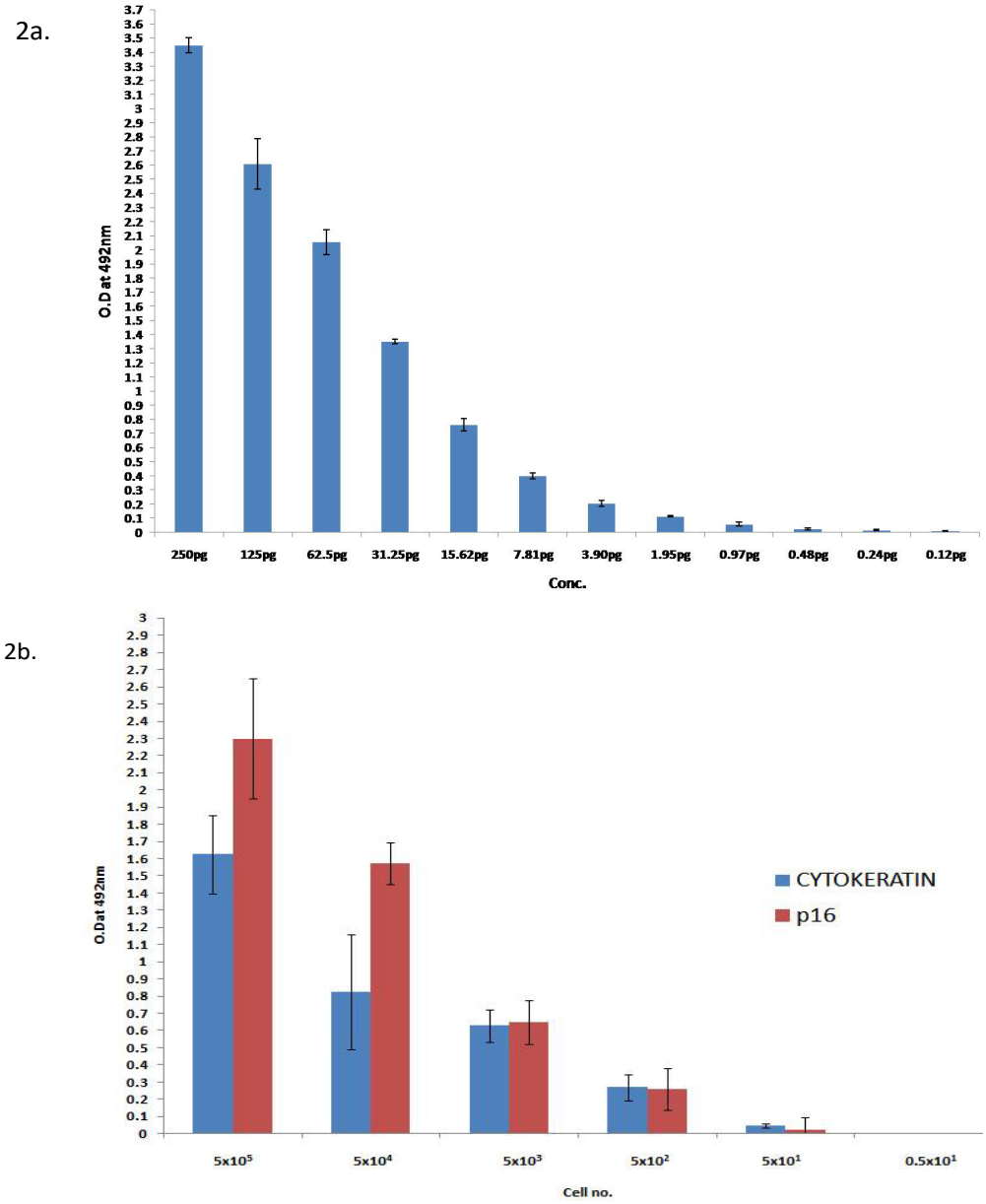

Figure 2. (a), Double antibody - Sandwich ELISA (DAS – ELISA) developed using combination of our p16 antibodies showed greater detecting limits. Sensitivity of the assay was recorded up to 2pg. (b), Direct ELISA for cytokeratin and DAS – ELISA for p16 using HeLa lysates showed detecting limit from lysates up to $5X10^2$ cells.

IgG1 (Table 1). In immunohistochemistry, the staining pattern differed among the selected p16 antibodies, E6H4 antibody was used as a positive control.

Except 133A6G5, all the antibodies positively detected p16 from the HeLa lysates in western blotting (Figure 1.a) and immunoprecipitation using 100 µg of 133A6G5 confirmed a specific p16 band being detected at 16 kDa (Figure 1 b).

Double antibody Sandwich ELISA using combination of 133A6G5 and 151A7B9 as capture antibodies and biotinylated 155E11G3 and 155D11G10 showed better sensitivity in picking up the p16 even at lower concentrations. The detecting limits of this ELISA format was recorded up to 0.1 O.D which was equivalent to 1.95pg of p16 when compared with the standards (Figure 2a).

Evaluation Of Cytokeratin ELISA

Parallel to p16 ELISA, we have also evaluated cytokeratin ELISA using AE1/AE3 antibody and HeLa lysates as standards. Lysates were prepared from serially diluted HeLa cells ranging from $5 X10^5$ to $0. 5 X10^1$. Direct

Table 1. Isotyping and Immunohistochemistry for Affinity Purified p16 Mouse Monoclonal Antibodies

Clones	Isotype	Immunohistochemistry	
		Percentage	Intensity
133A6G5	IgG2b	70- 80 %	+++
155E11G3	IgG2b	10-15%	++
151A7B9	IgG2b	80 – 90 %	++ - +++
155D11G10	IgG1	20 – 25 %	++
153G12D3	IgG2b	40%	++
152F12E5	IgG2b	60 – 70 %	++

Table 2. DAS-ELISA for p16 and Cytokeratin ELISA Using Lysates from Cervical Cancer, Pre Cancerous and Negative Samples

S.No	Histology /Cytology diagnosis	Sample ID	p16 O.D	cytokeratin 0.D	p16/cytokeratin ratio
1	SCC	T1	0.55	0.129	4.26
2	SCC	T2	0.61	0.234	2.61
3	SCC	T3	0.57	0.168	3.4
4	SCC	T4	0.46	0.029	15.62
5	SCC	T5	0.59	0.123	4.81
6	SCC	T6	0.73	0.151	4.85
7	SCC	T7	0.51	0.288	1.77
8	AC	T8	0.44	0.148	2.98
9	CIN2	T9	1.59	0.787	2.02
10	AC	T10	0.62	0.264	2.35
11	SCC	T11	0.68	0.045	15.19
12	SCC	T12	1.28	1.196	1.07
13	SCC	T13	0.69	0.04	17.25
14	SCC	T14	0.69	0.181	3.82
15	SCC	T15	0.4	0.234	1.71
16	SCC	T16	0.7	0.246	2.85
17	SCC	T17	3.27	0.733	4.46
18	SCC	T18	2.12	0.467	4.54
19	SCC	T19	2.24	0.408	5.49
20	SCC	T20	0.06	0.079	0.76*
21	SCC	T21	2.58	0.768	3.36
22	SCC	T22	3.65	0.098	37.28
23	SCC	T23	0.15	0.028	5.29
24	SCC	T24	0.28	1.647	0.17
25	SCC	T25	0.22	0.5	0.44
26	SCC	T26	0.41	0.107	3.82
27	AC	T27	0.21	0.01	21.19
28	SCC	T28	0.29	0.063	4.64
29	PDC	T29	0.19	0.141	1.35
30	SCC	T30	0.14	0.003	51.69
31	PDC	T31	0.13	0.012	11.09
32	SCC	T32	0.29	0.109	2.67
33	SCC	T33	0.49	0.287	1.71
34	SCC	T34	0.2	0.168	1.19
35	SCC	T35	0.18	0.133	1.35
36	CIS	L-06333	0.72	0.431	1.67
37	AC	L-07136	0.22	0.759	0.29
38	ASC-H	L-06999	0.15	0.008	18.87
39	HSIL	L-07002	0.13	0.003	48.13
40	HSIL	L-07009	0.22	0.239	0.92
41	NEGATIVE	L-05903	0.15	0.288	0.52
42	NEGATIVE	L-06318	0.15	0.577	0.26
43	NEGATIVE	L-06501	0.12	0.11	1.09
44	NEGATIVE	L-06601	0.05	0.5	0.1
45	NEGATIVE	L-06998	0.48	0.539	0.890#
46	NEGATIVE	L-07010	0.51	0.505	1.010#
47	NEGATIVE	L-07025	0.17	0.007	24.286
48	NEGATIVE	L-07049	0.17	0.5	0.34

*, indicates sample inadequate; #, indicates false positive

Table 2. Continued

S.No	Histology /Cytology diagnosis	Sample ID	p16 O.D	cytokeratin 0.D	p16/cytokeratin ratio
49	NEGATIVE	L-07105	0.15	0.5	0.3
50	NEGATIVE	L-07140	0.08	0.121	0.66
51	NEGATIVE	L-07142	0.36	0.07	5.140#
52	NEGATIVE	L-07143	0.18	0.281	0.64
53	NEGATIVE	L-07183	0.16	0.13	1.23
54	NEGATIVE	L-07209	0.09	2.25	0.04
55	NEGATIVE	L-07219	0.18	1.636	0.11

*, indicates sample inadequate; #, indicates false positive

ELISA for cytokeratin using AE1/AE3 antibody showed that signals from lysates as low as 500 cells (HeLacells) were detected using this ELISA format (Figure 2b).

Proof of Principle

Towards establishing a proof of principle in our pilot study we have used fifty-five cervical smear samples with known histological / cytological diagnosis. The results were analyzed by comparing values of p16 OD and cytokeratin OD and the samples scored positive, if the p16 O.D was 0.2 or more or if the ratio of p16 OD to cytokeratin OD is greater than 1.35. If the ratio of p16 OD to cytokeratin OD was less than 1.35 it was scored as negative for precancerous change or cervical cancer and if values of both p16 OD and cytokeratin OD were less than 0.1 it indicates the inadequacy of the sample and demands another fresh sample (Table2). An application for patent for the assay had been submitted (Patent Application number – 475/CHE/2014, Status - Application Awaiting Examination)

Discussion

Cervical cancer is the second most common cancer among Indian women and the mortality due to cervical cancer is the highest as compared to any other countries (Arbyn et al., 2020). Our earlier studies using microarray have revealed several novel genes to be differentially expressed in cervical cancer (Rajkumar et al., 2011). p16 was reported to be one of the potential biomarkers of diagnostic importance. Although several groups have reported p16 as a potential diagnostic marker and as a surrogate marker for detection of high risk HPV in pre - neoplastic lesions, the interpretation of p16 immunohistochemical results remains a matter of debate, due to the discrepancies in scoring p16 positive cells among different authors (Volgareva et al., 2004; Yoshida et al., 2004; Bose et al., 2005; Holladay et al., 2006). To overcome such discrepancies combined scoring method has been proposed by Thai group, which showed good reproducibility and correlation when compared with histology (Vinyuvat et al., 2008). Several researchers have reported that HPV testing with p16-INK4A triage produces a significant increase in sensitivity compared with conventional cytology, with no substantial increase in referral to colposcopy (Carozzi et al., 2008; Tsoumpou et al., 2011; Roelens et al., 2012).

Although p16 as potential biomarker has been well documented for over a decade, its use in routine diagnostics and in cervical cancer screening program is very limited. Some of the reasons include unsolved issues in implementing LAST (lower anogenital squamous terminology) guidelines and the need for judicious interpretation of staining patterns (Reuschenbach et al., 2014; Yang et al., 2018). To this end, the current study was taken up with the objective to develop a sandwich ELISA for detection of p16. We have done the cloning, expression and purification of recombinant p16. Our sequencing analysis from the positive colonies after cloning showed 100 % sequence similarity to Homo sapiens cyclin-dependent kinase inhibitor 2A, transcript variant 1. Analysis of soluble and insoluble fractions after expression and purification from induced culture revealed highly expressed His tagged – p16 fusion protein. Ni-NTA column purification of the soluble fraction showed single specific band of His tagged – p16 fusion protein which was then confirmed with western blot using anti- p16 antibody.

Further, in our quest to generate specific monoclonal antibodies against p16, we tried to purify the full length p16 without the His- tag. To this end, we have exploited the presence of thrombin sites in the His- tagged p16 fusion protein. Thrombin site present after the 6 x histidine tag and before the full length p16 was cleaved and the full length p16 was used for generation of mouse monoclonal antibodies. Initial screening and selection of individual parent clones from the hybridoma were done by IHC and ELISA. Finally, six clones were selected based on their specificity and sensitivity in IHC and ELISA. Isotyping of selected antibodies revealed that except 155D11G10, all of them were of IgG2b. 155D11G10 was found to be isotype IgG1.

We then validated all the mouse monoclonal antibodies generated using our recombinant p16 by Western blotting, IHC and ELISA. Individual affinity purified antibodies were evaluated by its ability to detect p16 from HeLa lysates. Except 133A6G5, all the antibodies detected p16 at 1:200 dilutions by Western blotting. Additionally immunoprecipitation was done using 133A6G5, which confirmed the specificity of this antibody for p16 in HeLa lysates. Interestingly, 133A6G5 which could not detect p16 in western blot at 1:200 dilutions and 151A7B9 which showed a faint band were found to best in p16 IHC. Both 133A6G5 and 151A7B9 showed 70 – 80 % and 80 – 90 % of nuclear staining, respectively, with similar intensities (+++). This result suggests that different epitope is being recognized by these antibodies. We

have also evaluated individual monoclonal antibody as either capture or detecting antibody in sandwich ELISA format. 133A6G5, 155E11G3, 151A7B9 and 155D11G10 showed better sensitivity when used as capture antibody. Average detecting limit of these antibodies were up to 15.62pg.133A6G5 and 155D11G10 monoclonal antibodies were found to better as detecting antibody with their average detecting limit recorded up to 31.25 pg.

Previous studies have shown that a Double-Antibody Sandwich ELISA (DAS – ELISA), using a combination of a monoclonal and a polyclonal antibody as the capture and detecting antibodies respectively, resulted in improved specificity compared to normal ELISA or to a sandwich ELISA using polyclonal as the capture and monoclonal antibody as the detecting antibody(Kolbe and Kubicek, 1990; Riske et al., 1990). Based on the results from validation of our antibodies, we have designed a Double antibody Sandwich ELISA (DAS – ELISA) with 133A6G5, 151A7B9 as capture antibodies and biotinylated 155E11G3 and 155D11G10 as detecting antibodies. Our ELISA using this combination of p16 mouse monoclonal antibodies showed greater detecting limits with sensitivity of up to 2pg.

As of now, only one group has come out with an ELISA Kit for the measurement of solubilized p16 from the lysed cervical samples. They have reported a positive correlation between histologically confirmed high-grade cervical intraepithelial neoplasia (CIN) and level of solubilized p16 protein detected by ELISA (Mao et al., 2007). Evaluation of their prototypic ELISA reported an independent relationship between p16 ELISA level and lesion size and the possible lower sensitivity of the p16 ELISA test in detecting small lesions or lesions that shed few abnormal cells(Balasubramanian et al., 2009). One of the major concerns in the ELISA assay is determining sample adequacy, which can be easily detected in a PAP smear. However, once cells are lysed for the assay and estimating p16 levels alone runs the risk of false negatives due to inadequate number of cells. To overcome this, we have introduced a second Cytokeratin ELISA. If the values of both p16 OD and Cytokeratin OD is less than 0.1 it indicates the inadequacy of the sample and demands another fresh sample.

Although Pap smears are used as the most cost-effective cancer screening test developed to date(Greenberg et al., 1995; Denny et al., 2006), management of ambiguous or low-grade cytological results (ASCUS and LSIL) is very controversial, mainly because of the nature of this morphology-based test, which inevitably leads to interobserver variability and some Pap test discordance with histological follow-up. Meta-analysis of Pap test accuracy has revealed that the mean sensitivity of primary Pap tests is ~58%, and the accuracy of a repeat test is only ~66% (Fahey et al., 1995).

HPV-DNA testing detects high-risk strains of HPV (hr-HPV) which cause almost all cervical cancers. Unlike tests that rely on visual inspection, HPV-DNA testing is an objective diagnostic, leaving no space for misinterpretation of results. However, its positive predictive value of pre-cancerous lesions is less than 10 percent especially in CIN2+ stages (Guzel et al., 2021). Although multiple molecular markers has been studied including, viral markers (E6, E7), cellular markers (Ki67/MIB-1, MYC, Survivin), nucleic acid changes (DNA methylation, miRNAs) their usage in population based screening are limited.Many studies have evaluated p16 as a surrogate marker for HR – HPV infection using IHC(Duncan et al., 2013; Gronhoj Larsen et al., 2014). p16 / Ki-67 dual immunostaining has been reported as better approach for cervical cancer screening compared to conventional methods (Eyituoyo Okoturo, 2023). Some of the drawbacks in using p16 IHC are (i) need for trained cytologists / pathologists. (ii) Scoring is subjective and discordant interpretation among pathologists. To overcome these drawbacks, we have developed an ELISA based assay for detection of p16 and cytokeratin in cervical smear lysates. Such a simple calorimetric based ELISA assays can be highly sensitive and specific, does not require trained pathologist / cytologists and can be cost effective with a possibility of automation.

In summary, we have cloned, expressed and purified full length p16 for generation of monoclonal antibodies. We have also validated individual antibodies for their sensitivity and specificity. We have developed a more sensitive Double-Antibody Sandwich ELISA using our p16 mouse monoclonal antibodies and have shown that the sample inadequacy issue can be addressed by introducing an additional Cytokeratin ELISA. We have also established a proof of principle in our pilot study which showed the sensitivity and specificity of our ELISA to be 100% and 80%, respectively. In future studies, if we could demonstrate similar sensitivity with accuracy and reliability in large number patient samples, the p16 DAS-ELISA could be effectively used as a reliable tool for diagnostic purpose in large scale screening programmes.

Abbreviations

ELISA, Enzyme linked immunosorbent assay; IHC, Immunohistochemistry; DAS-ELISA, Double antibody sandwich - Enzyme linked immunosorbent assay; Ig, Immunoglobulin; BSA, Bovine serum albumin;PBS, Phosphate buffered saline; OPD, o-Phenylenediamine dihydrochloride;OD, Optical density; SDS – PAGE, Sodium Dodecyl Sulfate - polyacrylamide gel electrophoresis;SCC, Squamous cell carcinoma; AC, Adenocarcinoma; CIN, Cervical intraepithelial neoplasia; PDC, Poorly differentiated carcinoma; CIS, Carcinoma In-situ; ASC-H, Atypical squamous cells- cannot exclude HSIL; HSIL, High-grade squamous intraepithelial lesion

Author Contribution Statement

MB carried out all the experiments and was involved in data analysis and drafting of the manuscript. S.S.S did the histopathological and Immunohistochemical assessment. GS was involved in the clinical management of cervical cancer patients. A.C and S.J.H screened and recruited patients for the study. TR designed the study; analyzed & interpreted the data, drafted and revised the manuscript..

Acknowledgements

The authors would like to thank the faculties and staff of Cancer Institute (WIA) and Nargis Dutt Memorial Cancer Hospital, Barshi, for their effective and efficient management and care for patients.

Funding Statement

This work was supported by Dept. of Science and Technology, Govt. of India. Grant No: IR/SO/LU-004/2006 and (Grant no: SR/S9/Z-08/2010 dated 25-06-2010). The funding source had no involvement in study design, analysis and interpretation of data and in decision to submit the article for publication.

Availability of data

All the data generated through this study are presented in the form of figures and tables in the results section.

Conflict of interest

A patent application has been filed in Indian patent office [Patent Application number – 475/CHE/2014] with TR and MB as inventors. All the authors declare that they have no competing interests.

References

Arbyn M, Weiderpass E, Bruni L, et al (2020). Estimates Of Incidence And Mortality Of Cervical Cancer In 2018: A Worldwide Analysis. *Lancet Glob. Health*, **8**, 191-203.

Balasubramanian A, Hughes J, Mao C, et al (2009). Evaluation Of An ELISA For P16ink4a As A Screening Test For Cervical Cancer. *Cancer Epidemiol Biomarkers Prev*, **18**, 3008-17.

Bose S, Evans H, Lantzy L, et al (2005). P16ink4a Is A Surrogate Biomarker For A Subset Of Human Papilloma Virus-Associated Dysplasias Of The Uterine Cervix As Determined On The Pap Smear. *Diagn Cytopathol*, **32**, 21-4.

Carozzi F, Confortini M, Palma PD, et al (2008). Use Of P16-INK4A Overexpression To Increase The Specificity Of Human Papillomavirus Testing: A Nested Substudy Of The NTCC Randomised Controlled Trial. *Lancet Oncol*, **9**, 937-45.

Denny L, Quinn M, Sankaranarayanan R (2006). Chapter 8: Screening For Cervical Cancer In Developing Countries. *Vaccine*, **24**, 71-7.

Duncan LD, Winkler M, Carlson ER, et al (2013). P16 Immunohistochemistry Can Be Used To Detect Human Papillomavirus In Oral Cavity Squamous Cell Carcinoma. *J Oral Maxillofac Surg*, **71**, 1367-75.

Eyituoyo Okoturo KR (2023). Meta-Analysis On The Diagnostic Performance Of P16/Ki-67 Dual Immunostaining For Cervical Cancer Screening. *Asian Pac J Cancer Biol*, **8**, 91-9.

Fahey MT, Irwig L, Macaskill P (1995). Meta-Analysis Of Pap Test Accuracy. *Am J Epidemiol*, **141**, 680-9.

Ferlay J, Colombet M, Soerjomataram I, et al (2021). Cancer Statistics For The Year 2020: An Overview. *Int J Cancer*, **2021**.

Greenberg MD, Sedlacek TV, Campion MJ (1995). Cervical Neoplasia: Are Adjunctive Tests To Cervical Cytology Worthwhile. *Clin Obstet Gynecol*, **38**, 600-9.

Gronhoj Larsen C, Gyldenlove M, Jensen DH, et al (2014). Correlation Between Human Papillomavirus And P16 Overexpression In Oropharyngeal Tumours: A Systematic Review. *Br J Cancer*, **110**, 1587-94.

Guzel C, Van Sten-Van't Hoff J, De Kok I, et al (2021). Molecular Markers For Cervical Cancer Screening. *Expert Rev Proteomics*, **18**, 675-91.

Holladay EB, Logan S, Arnold J, et al (2006). A Comparison Of The Clinical Utility Of P16ink4a Immunolocalization With The Presence Of Human Papillomavirus By Hybrid Capture 2 For The Detection Of Cervical Dysplasia/Neoplasia. *Cancer*, **108**, 451-61.

Kadian L, Gulshan G, Sharma S, et al (2021). A Study On Knowledge And Awareness Of Cervical Cancer Among Females Of Rural And Urban Areas Of Haryana, North India. *J Cancer Educ*, **36**, 844-9.

Kolbe J, Kubicek C (1990). Quantification And Identification Of The Main Components Of The Trichoderma Cellulase Complex With Monoclonal Antibodies Using An Enzyme-Linked Immunosorbent Assay (ELISA). *Appl Microbiol Biotechnol*, **34**.

Liu H, Liu XW, Dong G, et al (2015). P16 Methylation As An Early Predictor For Cancer Development From Oral Epithelial Dysplasia: A Double-Blind Multicentre Prospective Study. *Ebiomedicine*, **2**, 432-7.

Malhone C, Longatto-Filho A (2019). Cervical, Ovarian And Endometrial Tumor Markers: Potential Clinical Value. Semin. *Ultrasound CT MR*, **40**, 350-7.

Mao C, Balasubramanian A, Yu M, et al (2007). Evaluation Of A New P16ink4a ELISA Test And A High-Risk HPV DNA Test For Cervical Cancer Screening: Results From Proof-Of-Concept Study. *Int J Cancer*, **120**, 2435-8.

Munger K, Gwin TK, Mclaughlin-Drubin ME (2013). P16 In HPV-Associated Cancers. *Oncotarget*, **4**, 1864-5.

Netto GJ, Epstein JI (2011). Chapter 16 - Immunohistology Of The Prostate, Bladder, Kidney, And Testis. In 'Diagnostic Immunohistochemistry (Third Edition)', Eds W.B. Saunders, Philadelphia, pp 593-661.

Nobori T, Miura K, Wu DJ, et al (1994). Deletions Of The Cyclin-Dependent Kinase-4 Inhibitor Gene In Multiple Human Cancers. *Nature*, **368**, 753-6.

Pal A, Kundu R (2019). Human Papillomavirus E6 And E7: The Cervical Cancer Hallmarks And Targets For Therapy. *Front Microbiol*, **10**, 3116.

Pauck A, Lener B, Hoell M,et al (2014). Depletion Of The Cdk Inhibitor P16ink4a Differentially Affects Proliferation Of Established Cervical Carcinoma Cells. *J Virol*, **88**, 5256-62.

Rajkumar T, Sabitha K, Vijayalakshmi N, et al (2011). Identification And Validation Of Genes Involved In Cervical Tumourigenesis. *BMC Cancer*, **11**, 80.

Reuschenbach M, Wentzensen N, Dijkstra MG, et al (2014). P16ink4a Immunohistochemistry In Cervical Biopsy Specimens. *Am J Clin Pathol*, **142**, 767-72.

Riske FJ, Eveleigh DE, Macmillan JD (1990). Double-Antibody Sandwich Enzyme-Linked Immunosorbent Assay For Cellobiohydrolase I. *Appl Environ Microbiol*, **56**, 3261-5.

Roelens J, Reuschenbach M, Von Knebel Doeberitz M, et al (2012). P16 ^{INK4a} Immunocytochemistry Versus Human Papillomavirus Testing For Triage Of Women With Minor Cytologic Abnormalities: A Systematic Review And Meta-Analysis. *Cancer Cytopathol,* **120**, 294-307.

Sawicka M, Pawlikowski J, Wilson S, et al (2013). The Specificity And Patterns Of Staining In Human Cells And Tissues Of P16ink4a Antibodies Demonstrate Variant Antigen Binding. *PLoS One*, **8**, 53313.

Singh D, Vignat J, Lorenzoni V, et al (2023). Global Estimates Of Incidence And Mortality Of Cervical Cancer In 2020: A Baseline Analysis Of The WHO Global Cervical Cancer Elimination Initiative. *Lancet Glob Health*, **11**, 197-206.

Tsoumpou I, Valasoulis G, Founta C, Et Al (2011). High-Risk Human Papillomavirus DNA Test And P16ink4a In The

Triage Of LSIL: A Prospective Diagnostic Study. *Gynecol Oncol*, **121**, 49-53.
Venkatesh T, Thankachan S, Kabekkodu SP, et al (2021). Chapter 15 - Emerging Patterns And Implications Of Breast Cancer Epigenetics: An Update Of The Current Knowledge. In 'Epigenetics And Reproductive Health', Eds Academic Press, pp 295-324.
Vijayalakshmi N, Selvaluxmi G, Majhi U, et al (2007). Alterations Found In P16/Rb/Cyclin D1 Pathway In The Dysplastic And Malignant Cervical Epithelium. *Oncol Res*, **16**, 527-33.
Vinyuvat S, Karalak A, Suthipintawong C, et al (2008). Interobserver Reproducibility In Determining P16 Overexpression In Cervical Lesions : Use Of A Combined Scoring Method. *Asian Pac J Cancer Prev*, **9**, 653-7.
Volgareva G, Zavalishina L, Andreeva Y, et al (2004). Protein P16 As A Marker Of Dysplastic And Neoplastic Alterations In Cervical Epithelial Cells. *BMC Cancer*, **4**, 58.
Volkova LV, Pashov AI, Omelchuk NN (2021). Cervical Carcinoma: Oncobiology And Biomarkers. *Int J Mol Sci*, **22**.
Yang J, Elliott A, Hoffa AL, et al (2018). Potential Influence Of P16 Immunohistochemical Staining On The Diagnosis Of Squamous Cell Lesions In Cervical Biopsy Specimens: Observation From Cytologic-Histologic Correlation. *Cancer Cytopathol*, **126**, 1003-10.
Yoshida T, Fukuda T, Sano T, et al (2004). Usefulness Of Liquid-Based Cytology Specimens For The Immunocytochemical Study Of P16 Expression And Human Papillomavirus Testing: A Comparative Study Using Simultaneously Sampled Histology Materials. *Cancer*, **102**, 100-8.