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ORIGINAL ARTICLE

Immunohistochemical Expression of Cell Proliferating Nuclear Antigen (PCNA) and p53 Protein in Cervical Cancer

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Abstract
Objective This study was designed to evaluate the immunohistochemical expression of proliferating cell nuclear antigen (PCNA) and p53 protein expression in preneoplastic and neoplastic lesions in uterine cervix.
Study Design A total of 36 cervical biopsies were subjected for immunostaining and the results were correlated with different prognostic parameters. Bivariate and multivariate statistical analyses were done using "STATA" software.
Results PCNA labeling index and p53 expression increased with increasing severity of CIN lesions. PCNA labeling index was maximum (46.0) carcinoma cervix with intense positive staining. In bivariate statistical analysis, p53 and PCNALI were found to be insignificant (0.4184 and 0.4328, respectively). Menopausal stage was significantly associated with CA and CIN groups ($P < 0.166$ and $P < 0.049$), respectively.
Conclusion These markers may be of greater importance in low-grade CIN lesions showing high proliferative index. This will place the low-grade lesions in higher grade indicating the utility of proliferative markers in decision making for intervention. This method is simple and cost effective and may be useful in developing countries where HPVDNA testing is still out of reach because of high cost.

Keywords Cervix · p53 · PCNA · Immunohistochemistry

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Introduction

In developing countries, cervical cancer is a major cause of death in women. In India, cervical cancer is common in the females between 15 and 44 years of age group. It is the first most common malignancy among females. India accounts for one-fifth of the world's burden of cervical cancer [1]. Current WHO findings indicate that every year 132,082 women are diagnosed with cervical cancer and 74,118 die from this disease in India alone [2].

Cervical intraepithelial neoplasia (CIN) is a premalignant (dysplastic) lesion that is characterized by abnormal cellular proliferation, maturation, and nuclear atypia. CIN may regress to normal or progress to invasive cancer if left untreated. Approximately one-third to one-half of cases of CIN 1 and CIN II regress without treatment. Even cases of CIN III have been observed to regress spontaneously. The more severe the abnormality of the lesion, the less likely it is to regress. It is not possible at present to predict which

cases of CIN will progress and which will persist or regress. Histopathological review revealed the conditions which resulted in difficulty in the separation of the low-grade squamous intraepithelial lesions (LSIL), and high-grade squamous intraepithelial lesions (HSIL) included florid koilocytotic change and immature metaplastic squamous epithelium with atypia. Problems also resulted in the separation of basal cell hyperplasia, inflammatory-associated changes, and immature squamous metaplasia from the LSIL. In some cases, there was a full spectrum of diagnoses from normal to HSIL. Reactive/reparative epithelial changes, immature squamous metaplasia, and atrophy are well-recognized mimics of HSIL and frequently cause problems in histological interpretation. Moreover, the morphological criteria assessed do not provide information about the further development of these lesions, which may be the meaning of regression or progression to invasive disease.

Perimenopausal women with gynecological complaint are subjected to Pap smear test which is a time-consuming method and results depend on pathologist opinion. [3]. Early recognition of CIN is difficult because often precancerous lesions and carcinomas are missed in Pap smear [4]. This situation may be due to either sampling problems, i.e., the difficulty to obtain representative cellular material or to diagnostic problems, i.e., the difficulty to distinguish reactive and inflammatory cellular changes from precancerous alterations. In normal human cervical squamous, the expression of biomarkers is limited to the proliferating basal and parabasal cells and these cells normally are not exfoliated in cervical smears. This test is sensitive with limited reproducibility, and many times it showed high rate of false-positive and false-negative results. Women with diagnoses of HSIL, atypical glandular cells of uncertain significance, or malignancy should undergo further investigation (colposcopy). Moreover, colposcopic biopsy subjects the patient to unnecessary surgical intervention. However, in cytosmears, the positivity of immunostaining of atypical cells alone may not be sufficient for diagnosis of the type of the SIL, but a significant difference in proliferative indices among different categories of SILs and carcinoma from normal may be a diagnostic adjunct.

Histopathological evaluation is known as “Gold standard” for the diagnosis of SIL and CIN lesions [5]. In histological sections of CIN and carcinoma in tissue biopsy specimens, these markers show a clear-cut crisp staining in different compartments of the epithelium.

Histopathological diagnosis that directs treatment is also affected by high rates of disconcordance among pathologists. Experienced histopathologists show considerable interobserver variability in grading CIN and more importantly in distinguishing between reactive squamous proliferations and CIN grade. Most pairs of observers can achieve fair interobserver agreement in the reporting of cervical colposcopic biopsies using a modified Bethesda system [6]. Some histopathologists rely on the number and location of mitotic figures in making diagnosis and grading of CIN lesions [7]. The intraepithelial distribution, density, and nature (typical or atypical) of mitotic figures are routinely utilized diagnostic criteria to grade dysplasia and to distinguish high-grade dysplasia from potential histological mimics such as transitional metaplasia, atrophy, or immature squamous metaplasia. This method cannot be used as a diagnostic adjunct in preneoplastic and neoplastic lesions. Several other techniques like thymidine and bromodeoxuridine labeling quantitation of cellular DNA are investigated but they are expensive and cannot be used in routine diagnostic practice. However, the extent of this reduction and the cost-effectiveness of current screening programs remain the subject of debate. Therefore, an objective biomarker will be helpful in the identification of truly dysplastic cells and/or predict disease progression.

Proliferating cell nuclear antigen (PCNA) relates to cell proliferation and it is universally used to evaluate cell proliferation by immunohistochemistry. PCNA gives a rapid and reliable result of cell proliferation and is helpful in understanding of pathogenesis of cervical noeplasia [8].

p53 tumor suppressor gene expression may be a useful marker which can contribute information complementary to morphology, prognosis, and survival outcome of the patients. p53 can inhibit cell proliferation by blocking entry into the S phase of the cell cycle and is also a master regulator of apoptosis. Immunohistochemically, it is difficult to detect wild p53 protein expression, but mutated protein-present intranuclear can be easily detected by immunoperoxidase method [9].

However, the roles of p53 and PCNA expressions in cervical carcinoma and its precursor lesions are controversial. In India, only sporadic reports are published in relation to the roles of p53 and PCNA expressions in non-neoplastic and neoplastic lesions of cervical cancer. Therefore, it is interesting to study the immunohistochemical expressions of p53 and PCNA in different grades of CIN lesions and carcinoma and their correlation with a number of prognostic variables.

Material and Methods

A total of 36 tissue biopsies were collected consecutively from the Histopathology laboratory of the Postgraduate Department of Pathology, Chhatrapati Shahuji Maharaj Medical University Lucknow. Histopathological examination of biopsies for grading and typing of lesions were done according to the FIGO classification. Pap smears for grading and typing was carried out and were divided into

LSIL, HSIL, and carcinoma cervix according to the revised Bethesda classification. Additional Pap smears were obtained from these patients. Only those cases were included in the study in which Pap smear and biopsy were available. Samples were divided into CIN1/LSIL (4/4), CINII/HSIL (2/2), CIN III/HSIL (3/3), and carcinoma cervix ($n = 27/27$), respectively. Tissue sections were selected where sufficient material was present in the block and clinical data were available. From each block, 3–4 μ thick multiple sections were cut. One section was stained with hematoxylin–eosin (HE) staining for observing histological typing, and the rest of the sections were kept for immunostaining (p53 and PCNA). Primary monoclonal antibodies DO7 and PC10 were purchased from Dako Cytomation Ltd. and B Sap universal staining kit (Code No 37101 A) from Span Diagnostics Ltd.

Staining Method for p53 and PCNA [10]

Immunostaining was done by Streptavidin-biotin method. Paraffin sections were rehydrated, kept in citrate buffer (pH 6.0) and processed in microwave oven for antigen retrieval. Sections were kept in 3 % H_2O_2 followed by protein blocking antibody (20 min). After washing with TBS, sections were incubated with primary antibodies (p53 and PCNA) overnight at 4 °C. On the next day, sections were put into biotinylated secondary antibody (30 min). Sections were kept into Streptavidin-peroxidase reagent (45 min) followed by DAB solution for 45 min. These sections were counterstained with hematoxylin and mounted in DPX.

Positive control for p53 staining A histological section of gall bladder adenocarcinoma was used as positive control with each batch of staining.

Positive control for PCNA staining A histological section of reactive lymph node was used as positive control with each batch of staining.

Negative control For negative control, 1 % non-immune serum was used in place of primary antibodies with rest of the steps being the same as for the positive control. No brown color staining was produced in any of the cell.

Criteria for p53 Positivity

Tumors showing 5–10 % cells show brown color staining in the nucleoplasm, which were labeled as positive.

Calculation of PC10 Labeling Index

100 cells were counted in the tumor section, and the PCNA labeling index was calculated as follows:

$$\text{Labeling index} = \frac{\text{No of cells showing positive staining}}{\text{Total no of tumor counted cells}} \times 100$$

Clinicopathological Correlation

Results of p53 and PCNA were correlated with different prognostic parameters including age, menopausal status, clinical stage (FIGO), and tumor type.

Statistical Analysis

Bivariate and multivariate statistical analyses were done by using "STATA" software in Clinical Epidemiology Unit at our institution. Value of <0.05 was considered to be significant. Factors found significant were considered for multiple logistic regressions.

Results

In all positive cases, nuclear staining was expressed as brown color fine or coarse granular dots. Positivity was focal as well as uniformly distributed in the section. p53 and PCNALI were correlated with different prognostic parameters and their clinicopathological correlations are given in Table 1. p53 and PCNA positivities increased from different grades of CIN lesions to cervical carcinoma. P53 positivity was seen in 2/9 (22.2 %) cases of CIN and 11/27 (45.4 %) cases carcinoma, while PCNA was positive in 4/9 (44.4 %) cases of CIN lesions (mean PCNALI—25.0) and 17/27 (63.63 %) cases of carcinoma (mean PCNALI—40.5. As CIN grades increased from I to III, the PCNA positivity also increased from basal to superficial layers. Maximum patients of clinical stage III and IV were positive for p53 and PCNA stainings. PCNALI was higher in the patients of clinical stage of III (40.1).

Bivariate and multivariate statistical analyses were done in different groups to find out any significant correlation between different prognostic parameters (Table 2). The non-squamous malignancies were excluded from statistical analysis due to small numbers. In bivariate statistical analysis, both p53 and PCNA CA as well as CIN were found to be statistically insignificant ($P = 0.4184, 0.4328$). The factors found significant in bivariate analysis at P value <0.25 were considered for multiple logistic regression. It was observed that only menopausal stage was significantly associated with squamous cell carcinoma (SCC).

Discussion

Uterine cervical cancer accounts for 15 % of all cancers in females. Of these, 80 % of cervical cancer is from

Table 1 Correlations of p53 and PCNA in CIN and CA with clinical and histopathological parameters

Prognostic parameters	No. of cases	p53 + cases (%)	PCNA + cases (%)	PCNALI
Age				
<30	15	3 (20.0)	9 (60.0)	32.0
>30	21	10 (47.6)	12 (57.5)	36.0
Menopausal status				
Premenopausal	17	3 (17.6)	10 (58.8)	30.0
Postmenopausal	19	10 (52.6)	11 (58.0)	37.0
Clinical stage				
I	9	4 (44.4)	5 (55.5)	37.6
II	6	1 (16.6)	3 (50.0)	39.0
III	10	5 (50.0)	7 (70.0)	40.0
IV	2	1 (50.0)	2 (100)	32.5
V	9	2 (12.0)	5	27.5
Lymph node metastasis				
Present	3	1 (8.33)	1 (8.33)	35.0
Absent	33	129 (36.3)	20 (60.5)	35.9
Tumor types				
Cervical intraepithelial neoplasia	9	2 (22.2)	4 (44.4)	25.0
CIN I	4	0	1 (25.0)	20.0
CIN II	2	1 (50.0)	1 (50.0)	26.0
CIN III	3	1 (35.0)	2 (66.0)	29.0
Carcinoma cervix	27	11 (45.5)	17 (16.33)	40.5

developing countries while only 20 % are from developed world. P53 abnormalities may be important in the pathology of cervical carcinoma. Studies have shown that point mutations of the p53 suppressor gene are correlated to the malignant transformation. It has also been suggested that complex binding between the p53 protein, and the E6 protein from the human papilloma virus may result in the disturbance of the growth-inhibitory effect of wild-type p53 which in turn results in uncontrolled cell proliferation and malignant transformation [11]. Turkulo [12] suggested that the expression of p53 increased proportionally to the grade of CIN and cervical cancer. Therefore, p53 immunoreactivity can be helpful to decide a neoplastic lesion, but the absence of p53 does not exclude neoplasia. In another study, Cardillo [13] suggested that more than 50 % of neoplastic cells were immunoreactive for p53 protein in 10 % of well-differentiated squamous carcinomas, but no staining was observed in adenocarcinoma, dyaplastic tissue, condylomas, and normal tissue (83.07 %). In our findings, p53 positivity increased from CIN (22.2 %) to carcinoma (45.5 %), but when bivariate statistical analysis was done, p53 expression was found to be statistically insignificant ($P = 0.4184$). This may be due to small sample size. Lool [9] observed that MIB-1 index was higher in high-grade CIN and SCC lesions as compared to normal cervix. They also observed p53 immunoreactivity in 27 % of SCC cases, but it had no significant relationship with SCC staging ($P = 0.791$). This diagnostic method may be helpful in the early detection of intraepithelial squamous neoplasia.

Wang et al. observed negative PCNA expression in normal and inflammatory cases of cervix but increased expression in CIN (63.2 %) and SCC (100 %) groups, respectively ($P < 0.01$, $P < 0.05$). They also reported that PCNA might be a valuable clinical marker to predict the progression of cervical neoplasia [14]. PCNA index may be a predictive indicator for the prognosis of patients with SCC of the cervix treated with radiation therapy alone. These markers may be helpful in the identification of those patients whose CIN lesion will progress and require treatment to be distinguished from those, whose lesion will stay static or regress.

Our findings supported the results of Heatley who observed that PCNALI increased with grades of dysplasia as well as with increase in tumor grade [15]. Same findings were observed by Austidillo [16] who found that PCNA protein expression significantly increased as the grade of cervical lesion becomes higher from normal epithelium to SCC. Statistical analysis showed a positive correlation between p53 and PCNA expressions in CIN I ($r = 0.378$, $P = 0.016$). They suggested that p53 protein expression

Table 2 Bivariate and multivariate analyses of prognostic factors in relation to CA and CIN

Prognostic variables	CA (27)	CIN (9)	Bivariate analysis		Multivariate analysis	
			OR (95 %) Fisher's exact CL	*P* value	OR (95 %) Fisher's exact CL	*P* value
Age > 30 years	21	7	6.00 (0.26, 370.31)	0.1949	2.01 (0.12, 34.91)	0.631
Post menopause	16	2	9.33 (1.2, 107.5)	0.0166	7.26 (1.001, 52.37)	0.049
p53 positivity	10	2	2.92 (0.40, 33.88)	0.4184	1.85 (0.23, 14.96)	0.562
PCNA positivity	14	4	2.19 (0.34, 14.30)	0.4328	2.55 (0.40, 16.33)	0.322
PCNALI Mean ± SD	40 ± 4.31	26 ± 4.32	–	0.0000*		
95 %CL	37.5, 42.5	19.1, 32.9				

* Two-sample *t* test is used to test the level of significance between two means

during cervical tumorigenesis could be playing a pivotal role in cervical tumor progression as a late event. Wang et al. [14] observed the negative PCNA expression in normal or inflammatory cases of cervix but an increased expression of 63.25 % in CIN and 100 % in SCC cases ($P < 0.01$, $P < 0.05$). In our results, PCNA labeling index increased from CIN lesion (26 ± 4.32) to SCC (40 ± 4.31).

Maeda [17] observed that the percentage of PCNA positive cells increased with increasing grades of cervical lesions but p53 expression was weak. Labeling indices of PCNA immunostaining increased with increasing grades of cervical lesions although PCNALI was greater than MIB-1 LI [17]. The reason for higher reactivity may be explained that half-life of PCNA exceeded 20 h which could result in some staining of the nuclei in the G0 phase in the basal cell [18].

The differences in cell proliferation markers found herein further emphasize the progressive loss of epithelial layer organization in the course of the development of preneoplastic changes in cervical squamous epithelium. Altered statuses of p53 and PCNA may be valuable markers to predict to progression of cervical neoplasia.

Therefore, these markers may be useful to study the proliferative activity of epithelial cell which may further help in identifying dysplastic lesions and progression of disease in patients suffering with cervical cancer. This marker in the tissue section can be used as an adjunct to definitively diagnose preneoplastic and neoplastic lesions in the cervix. The use of specific biomarkers of dysplasia in conjunction with histological procedures could greatly improve the accuracy, precision, and sensitivity of cervical screening programs. In a nutshell, it was concluded that PCNA immunostaining might be of greater importance in those cases, which are seen low grade in histology sections but have a high proliferative index. This will place the low-grade CIN lesions in higher grade, thus indicating the utility of proliferative markers in decision making for intervention. These cases should be kept for follow-up studies. These markers may be helpful particularly in developing countries where HPV DNA testing as a screening test is still out of reach. This has to be substantiated by further studies with a larger number of cohorts.

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References

1. Globocan 2008 All Cancers (excluding non-melanoma skin cancer) Incidence and Mortality Worldwide in 2008.
2. WHO/ICO Information Centre on HPV and Cervical Cancer. Available from http://www.who.int/hpvcentre. Cited May 5 2009.
3. Stoler MH, Schiffman M. Interobserver reproducibility of cervical cytologic and histologic interpretations: realistic estimates from the ASCUS-LSIL Triage Study. JAMA. 2001;285:1500–5.
4. Anttila A, Pukkala E, Soderman B, et al. Effect of organized screening on cervical cancer incidence and motality in finland, 1963–1995: recent increase in cervical cancer incidence. Int J Cancer. 1999;83:59–65.
5. Negri G, Vittadello F, Romano F, et al. P16INK4a expression and progression risk of low-grade intraepithelial neoplasia of the cervix uteri. Virchows Arch. 2004;445(6):616–20.
6. McCluggage WG, Walsh MY, Thornton CM, et al. Inter and intra-observer variation in the histopathological reporting of cervical squamous intraepithelial lesions using a modified Bethesda grading system. Br J Obstet Gynaecol. 1998;105(2):206–10.
7. Chi CH, Rubio CA, Lagerlof B. The frequency and distribution of mitotic figures in dysplasia and carcinoma in situ. Cancer. 1977;39(3):1218–23.
8. Herbsleb M, Knudsen UB, Orntoft TF, et al. Telomerase activity, MIB-1, PCNA, HPV16 and p53 as diagnostic markers for cervical intraepithelial neoplasia. APMIS. 2001;109(9):607–17.
9. Lool ML, Dali AZ, Ali SA, et al. Expression of p53, bcl2 and Ki-67 in cervical intraepithelial neoplasia and invasive squamous cell carcinoma of the uterine cervix. Anal Quant Cytol Histol. 2008;30(2):63–70.
10. Goel MM, Mehrotra A, Singh U, et al. MIB-1 and PCNA immunostaining as a diagnostic adjunct to cervical pap smear. Diagn Cytopathol. 2005;33:15–9.
11. Holem R, Skomedal H, Helland A, et al. Immunohistochemical analysis of p53 protein overexpression in normal, premalignant and malignant tissues of the cervix uterine. J pathol. 1993; 169:21–6.
12. Turcuo L, Tezcan S, Kaygusuz G, et al. The role of p53, bcl2 and ki-67 in premalignant cervical lesions and cervical cancer. Eur J Gynaecol Oncol. 2007;28(4):290–3.
13. Cardillo MR, Stamp GW, Pignatelli MN, et al. Immunohistochemical analysis of p53 oncopotein and proliferating cell nuclear antigen (PCNA) in the cervix uteri. Eur J Gynaecol Oncol. 1993;14(6):484–90.
14. Wang JL, Zheng BY, Li XD, et al. Predictive significance of the alteration of p161NK4A, p14ARF, p53 and proliferative cell nuclear antigen expression in the progression of cervical cancer. Clin Cancer Res. 2004;10(7):2407–14.
15. Heatley MK. What is the value of proliferation markers in the normal and neoplastic cervix? Histol Histopathol. 1998;13(1): 249–54.
16. Astudillo H, Perez M, Silva J, et al. p53, Bcl-2, PCNA expression and apoptotic rates during cervical tumorigenesis. Ann N Y Acad Sci. 2003;1010:771.
17. Maeda MY, Simoes M, Wakamatsu A, et al. Relevance of the rates of PCNA, Ki-67 and p53 expression according to the epithelial compartment in cervical lesion. Gynecol Obstet Fertile. 2000;28(1):44–50.
18. Hall PA, Woods AL. Immunohistochemical markers of cellular proliferation: achievements, problems and prospects. Cell Tissue Kinet. 1990;23:505–22.