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Expression of Molecular Markers and Risk Categorization in Pre-neoplastic and Non-neoplastic Cervical Lesions

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Abstract Worldwide, cervical cancer is the fourth most common cancer in women [1]. It is preceded by many stages of pre-neoplastic lesions classified as Atypical squamous cells of undetermined significance (ASC-US) / Atypical squamous cells cannot exclude HSIL (ASC-H), Low-grade squamous intraepithelial lesion (LSIL), and High-grade squamous intraepithelial lesion (HSIL) (Bethesda, 2001). However, a large proportion of cases of epithelial malignancy arise from apparently normal cell and also from the LSIL where as many HSILs remain static for many years [2]. In this study, many important markers and epidemiological parameters Pap smears were examined to look for high risk cases of cervical non-malignant lesions. Methods: A total of 60 cases and controls were selected by colposcopic examination and smears were collected. Slides were used for immunohistochemical staining for four markers: p53, Ki-67, PCNA and CEA and PAP stain. All experimentally generated data were analyzed by Epi Info (TM) 3.5.3. Results: Expression of PCNA, p53, Ki-67 and CEA were significantly associated with both in Epithelial Cell Abnormalities (ECA) and Negative for Intraepithelial Lesion or Malignancy (NILM). Among HSIL, LSIL and ASCUS, HSIL was more common in Muslim women in comparison to Hindus. Whereas inflammatory lesions like RCC and RCCI were more common in Hindu population. Muslim women have a more normal Cytopathology smear than Hindus. Conclusions: Both the NILM and ECA cases carry risk for cancer conversion. Women with High grade Squamous Intraepithelial lesion or HSIL with PCNA, p53, Ki-67 and CEA strong positivity may have a higher risk of developing cervical cancer.

Keywords: *ASCUS, HSIL, LSIL, cervical cancer, biomarkers, premalignant lesions*

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1. Introduction

At present, cancer is the most serious threat to mankind. It is among the leading causes of death worldwide. In 2012, 14.1 million adults were diagnosed with cancer and there were 8.2 million deaths from cancer [1].

Cervical cancer is the fourth most common cancer in women with an estimated 527,624 new cases and 265,653 deaths in 2012 [1]. According to Cervical Cancer Action: Report Card 2011, in most of the developing countries, cervical cancer is the most common cancer and cause of death in women.

In India it is the second most common cancer among women and current estimate indicates that 122,844 new cases were diagnosed with cervical cancer and there were 67,477 deaths in 2012 [1].

Around the world, cervical cancer has been given the maximum impetus for early diagnosis, treatment as well as for prevention. Even though Pap test has significant subjective false positive (30%) [3] and false negative (15-50%) [4] result, still the risk of cancer attack for a woman who is screened every 3 years with the Pap test is extremely low and similar to the risk of cancer when screened every 5 years with both the HPV test and the Pap test [5].

It is thought that persistent human papillomavirus infection (HPV) along with inflammation, with the involvement of innate immune cells (mast cells and granulocytes) in the tissue, the presence of specific signalling molecules like cytokines and chemokines [6] has been supposed to cause cervical carcinoma and its precursor lesions [7]. It is a multistep disease with several contributing Co factors, including multiple sexual partners, a compromised immune system and cervical inflammation

caused by *Chlamydia trachomatis* or *Neisseria gonorrhea* [8].

Cervical cancer takes more than 10 years to develop and passes through preneoplstic lesions of three stages such as ASCUS, LSIL/koilocytosis (CIN1), HSIL/moderate (CIN2) and HSIL/severe dysplasia (CIN3) [9]. Mild dysplasia often reverses normally after few months, however, moderate and severe dysplasia often persists and progress to carcinoma in situ or cancer (ref). However, it is found that more than 50% cases arise from normal cell [2].

Proliferating cell nuclear antigen (PCNA) plays an essential role in nucleic acid metabolism as a component of the replication and repair machinery [10]. PCNA labeling index and p53 expression increased with increasing severity of CIN lesions [11].

The high risk HPV activates E6 and E7 genes and corresponding E6 and E7 proteins which play a critical role in p53 mutation and p53 tumour suppressor gene inactivation in high grade cervical neoplasia [12]. Immunohistochemical expression of p16INK4a has been associated with dysplastic/neoplastic cells, but not seen in normal cervical epithelium [13]. Though p16 over expression acts as a good marker for an aberrant expression of viral oncogenes, only a minority of high risk HPV-associated lesions progress to uterine cervix carcinoma [14]. Detection of cytology samples either of its own or in combination with the proliferation marker Ki-67, looks very promising as a way of improving the specificity of an HPV positive, cytology low grade samples [15].

Tendler A et al [16] have shown that CEA expression increases in CIN 3 and SCC without elevations in serum CEA and CEA expression may be a useful diagnostic tool and a useful marker for identifying those at risk for progressive cervical neoplasia.

Apart from the PAP smear screening test, histopathological diagnosis of cervical intraepithelial neoplasia (CINS) and cervical carcinoma is considered as the “gold standard” method of diagnosis of cervical neoplasms. However, this can also be biased by interobserver variability as reported before [17]. These factors limit present screening programs and histopathological diagnosis and emphasizes the need for correlating some important biomarkers like p53, Ki-67, PCNA and CEA with the cytopathological diagnosis to identify risk groups for cancer prevention.

Considering the above facts and in order to identify individual risk, we have studied the immunohistochemical expression of proliferative markers like Ki-67, PCNA as well as an expression of the p53 mutant protein along with expression of CEA.

2. Materials and Methods

2.1. Sample Collection and Preparations

In this study, all the Cases and controls were selected through colposcopic evaluation by an eminent gynecologist of our Institute. Initially, 60 cases of cervical lesions barring malignancy along with controls were collected at the Department of Cancer Detection Center (CDC) OPD of CNCI, Kolkata, India. Demographic details of all patients, including age, religion, and personal history about education, addiction, age at marriage, menarche, parity etc. was meticulously recorded. The age of the patients ranged from 20 to 75 years (mean age, 36.55±10.82 years). Among all 60 patients, 42 cases belonged to the Hindu community and the rest 18 cases belong to the Muslim community. All patients were healthy with no systemic disease. Cyto smear was collected on acid alcohol cleaned Poly-L- Lysine coated glass slides from all the 60 patients and controls. At least 8 slides of cytosmears were collected from each subject and fixed in 95% ethyl alcohol for 20 minutes for Pap test and marker studies. The Pap stained slides were evaluated by 2 eminent pathologists separately and classified according to the Bethesda system (2001). All the 60 cases where classified under two broad headings: Epithelial Cell Abnormality (ECA) which included Atypical Squamous Cells of Undetermined Significance (ASCUS), Low Grade Squamous Intra epithelial lesion (LSIL), High Grade Squamous Intra Epithelial lesion (HSIL) and Negative for Intraepithelial Lesion or Malignancy (NILM) comprising of Normal, Reactive cellular changes (RCC) and reactive cellular changes with inflammation (RCCI).

2.2. Papanicolaou Stain of the Study Smears (Modified Pap Procedure)

All the experimental slides for Papanicolaou stain were fixed in 95% Ethanol for 15 minutes. Next, the slides were dipped in double distilled water 10 times and this process was repeated twice. Gill’s Hematoxylin was added and kept for 2 minutes. Slides were then dipped in distilled water 10 times. Next the slides were kept in Scott's tap water for 1 minute and then dipped in distilled water 10 times twice. The slides were then dipped in 95% Ethanol 10 times twice. Next OG-6 stain was added and slides were kept for 1-2 minutes.

The slides were thrice dipped in 95% ethanol (each time 10 dips) and then EA-50 stain was added and incubated for 6-10 minutes. In the next step again the slides were washed thrice with 95% Ethanol (20-30 dips). The slides were then dipped 10 times in absolute ethanol. The slides were then cleared in xylene and mounted with DPX and viewed under light microscope at 40X (Figure 1, Figure 2 and Figure 3).

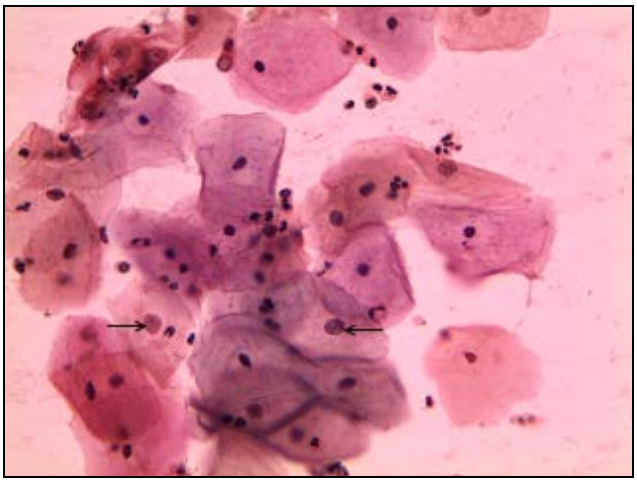

Figure 1. ASCUS (Reactive) x 40X (Black arrows)

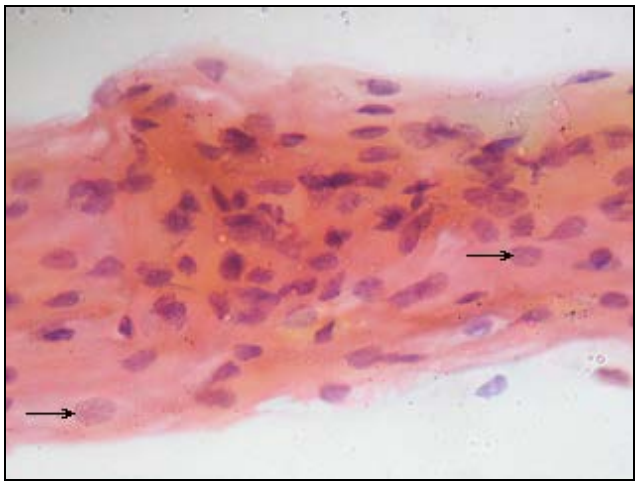

Figure 2. Mild Squamous Dysplasia (LSIL) x 40X (Black arrows)

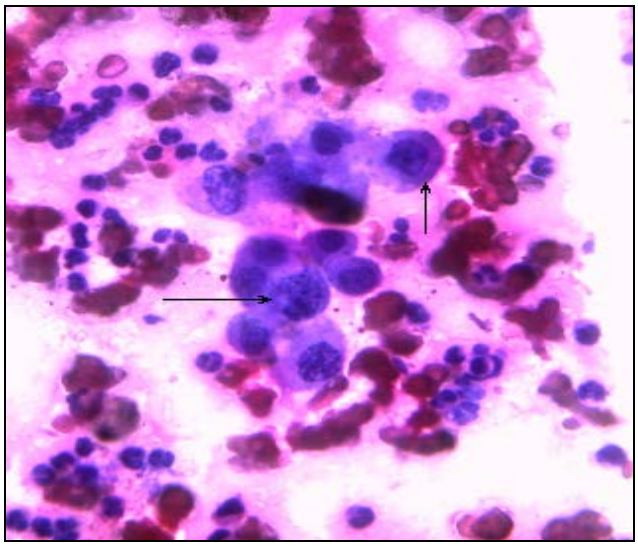

Figure 3. High Grade Squamous Intraepithelial lesion (HSIL) x 40X (Black arrows)

2.3. Immunohistochemical Analysis of the Cases

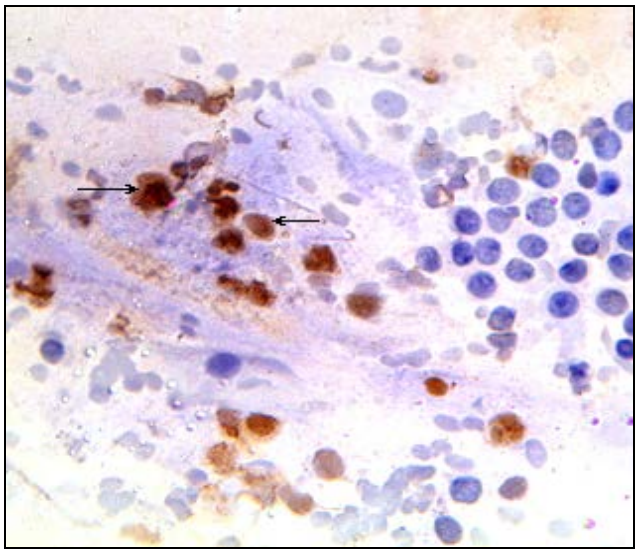

Figure 4. Moderate Squamous Dysplasia (HSIL) expressing p53 x 40X (Black arrows)

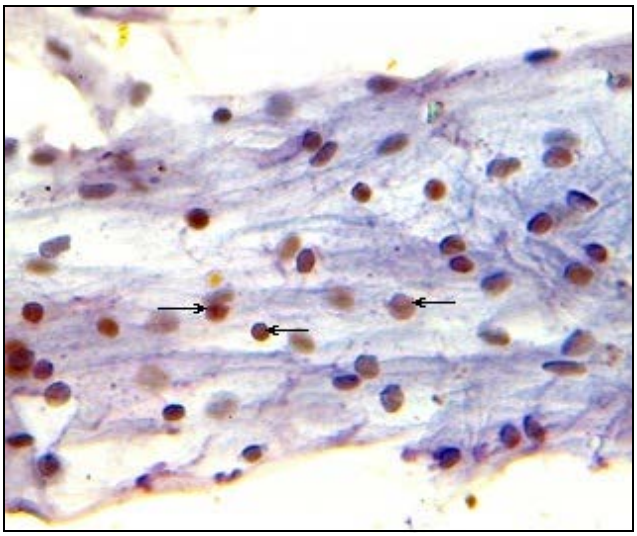

Figure 5. Mild Squamous Dysplasia (LSIL) expressing Ki 67 x 40X (Black arrows)

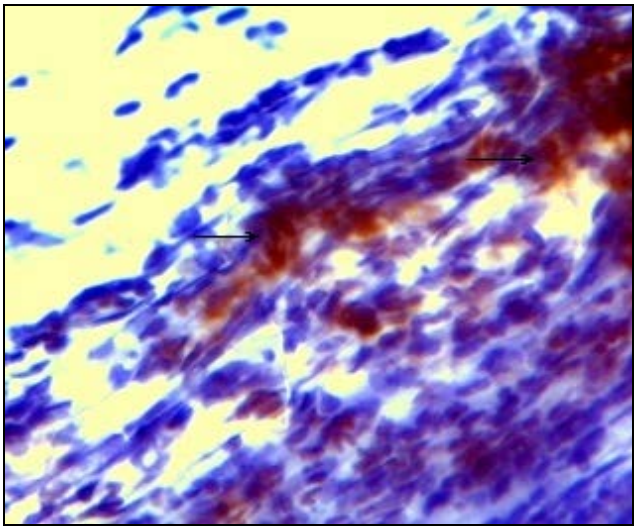

Figure 6. Inflammatory Smear (RCCI) showing strong positivity for PCNA x 40X (Black Arrows)

Protocol: Immunohistochemistry of cervical smears was performed according to the protocol of IHC World with slight modification. The slides were taken with adequate controls and washed in PBS pH 7.6. To avoid nonspecific and background staining slides were incubated with 5% normal goat serum along with 1% BSA and kept for 15 minutes at room temperature. Slides were then washed with wash buffer (PBS, pH 7.6 with 0.5% Tween 20) for 2x3mins and blotted. The primary monoclonal antibody from Sigma USA; p53 (1:400) Clone BP53-12, Ki-67 (1:800) Clone PP-67 protein, PCNA (1:100) Clone PC10 and CEA (1:100) Clone C6G9 were added after proper dilution and then the slides were incubated overnight at 4°C. Next the slides were washed in a similar manner with wash buffer and blotted. Endogenous peroxidase was blocked by incubating the slides in 3% H_2O_2 in methanol for 10 minutes at room temperature. Slides were then washed and blotted in a similar manner. Secondary antibody, biotin conjugated anti-mouse IgG was added after proper dilution (1:400) and incubated for 45 minutes at room temperature. Slides were then washed in a similar manner and blotted. Streptavidin-HRP was added after proper dilution (1:300) and the slides were

incubated for 30 minutes at room temperature. Again the slides were washed in a similar manner. In the next step DAB solution was prepared. 6mg DAB was dissolved in 10 ml 0.05M Tris buffer, pH 7.6.0.1 ml 3% H_2O_2 was added to it and was mixed thoroughly and filtered. The mixture was applied on the smears for experiments and incubated for 3-10 minutes at room temperature depending on the intensity of the color development. The reaction time was stopped by adding distilled water. The slides were blotted with clean tissue paper. The slides were then counterstained with Delafield Haematoxylin and the slides were mounted by coverslip with the help on DPX. Finally the slides were examined under light microscope under the magnification of 20 X and 40X (Figure 4, Figure 5 and Figure 6).

2.4. Statistical Analysis

In this cross sectional observation, we report here, the reflective risk parameters for cancer conversion in 60 cases of pre neoplastic cervical lesions with power 81%, randomly chosen from the patients attended at CNCI, OPD during the period of 2011 – 2013.

Statistical Analysis was performed with the help of Epi Info (TM) 3.5.3. EPI INFO is a trademark of the Centre for Disease Control and Prevention (CDC), NIH, USA.

Descriptive statistical analysis was performed to calculate the means with corresponding standard deviations (s.d). Test of proportion was used to find the Standard Normal Deviate (Z) to compare the difference in proportions. Odds Ratio (OR) with 95% confidence interval (CI) had been calculated to find the risk factors. p≤0. 05 was taken to be statistically significant.

3. Result

60 cases of pre-neoplastic cervical lesions were included in the study. The mean age (mean ± s.d.) of the patients was 36.55±10.82 years with range 20-75 years and the median age was 35.0 years. 43(71.7%) proportion of cases having age ≤ 40 years was significantly higher (p<0.001), only 15% of them were illiterate, proportion of Hindu (70%) were significantly higher than that of Muslim (30%) (p<0.001), 93.3% of them were housewife (p<0.001), only 15% of them had addiction to chewing of tobacco, only 1.7% was unmarried, 71.7% of them had age at marriage less than 20 years (p<0.001), 33.3% had parity more than 2, 40% had at least one abortion in their child bearing period, 68.3% had menarche at the age of 13 years or less, only 10% had menopause.

Cytological diagnosis of 60 cases showed that 28(46.7%) of cases had ECA followed by 32 (53.3%) NILM cases. Out of 28 ECA cases 14(50%) were LSIL, 7(25%) were HSIL and rest 7(25%) were ASCUS and out of 32 NILM cases 12(37.5%) were normal, 11 (34.4%) had RCC Inflammation and 9 (28.1%) had RCC.

Statistical analysis were performed for all the cases.

For ECA cases, statistically it was found that the proportion of ASCUS (100%) and LSIL (64.3%) was significantly higher among Hindu (p<0.01). However, the proportion of HSIL was significantly higher among the Muslim women (p<0.05). It also was found that proportion of ASCUS (57.1%; (p<0.05), HSIL(100%; p<0.01) and LSIL (85.7%; p<0.01) was significantly higher in the cases with age at marriage ≤ 20 years. In case of parity and menarche no significant association was observed in relation to ECA (Table 1).

Table 1. Represents Cytological Parameters, Biomarker Expressions, Epidemiological Parameters And Statistical Significance Of ECA

Epithelial Cell Abnormality (ECA) (28 cases)				
		ASCUS	LSIL	HSIL
Cytological Diagnosis		7 (25%)	14 (50%)	7(25%)
Religion	Hindu	7(100%) **p<0.01**	9(64.3%) **p<0.01**	3(42.9%)
	Muslim	0	5(35.7%)	4(57.1%) **p<0.05**
Parity	P>2	0	6(42.9%)	3(42.9%)
	P≤2	7(100%)	8(57.1%)	4(57.1%)
Age at marriage	≤20	4(57.1%) **p<0.05**	12(85.7%) **p<0.01**	7(100%) **p<0.01**
	>20	3(42.9%)	2(14.3%)	0
Menarch	>13	1(14.3%)	3(21.4%)	4(57.1%)
	≤13	6(85.7%)	11(78.6%)	3(42.9%)
Biomarkers	p53+	4(57.1%) **p<0.05**	7(50%)	5(71.4%) **p<0.01**
	P53-	3(42.9%)	7(50%)	2(28.6%)
	Ki67+	2(28.6%)	5(35.7%)	4(57.1%) **p<0.05**
	Ki67-	5(71.4%)	9(64.3%)	3(42.9%)
	PCNA+	4(57.1%)	5(35.7%)	6(85.7%) **p<0.001**
Biomarkers	PCNA-	3(42.9%)	9(64.3%)	1(14.3%)
	CEA+	6(85.7%) **p<0.001**	5(35.7%)	5(71.4%) **p<0.01**
	CEA-	1(14.3%)	9(64.3%)	2(28.6%)

In relation to marker expression, test of proportion showed that the PCNA positivity was significantly higher in ASCUS (57.1%; p<0.05) and also in HSIL (85.7%; p<0.01) but in case of LSIL it was not significant. It showed that the proportion of p53 positivity was significant in ASCUS (57.1%; p<0.05) along with HSIL (71.4%; (p<0.01). And though the expression of p53 in LSIL cases is almost similar to ASCUS but statistically it was insignificant. Test of proportion showed that Ki-67 positivity was significantly higher in HSIL (57.1%; p<0.05) but ASCUS (28.6%) and LSIL (35.7%) had no significance. Test of proportion showed that CEA positivity was significantly higher in ASCUS (85.7%; p<0.01) and in HSIL (71.4%; p<0.01) but in case of LSIL (35.7%) the proportion was not significant (Table 1) (Figure 7).

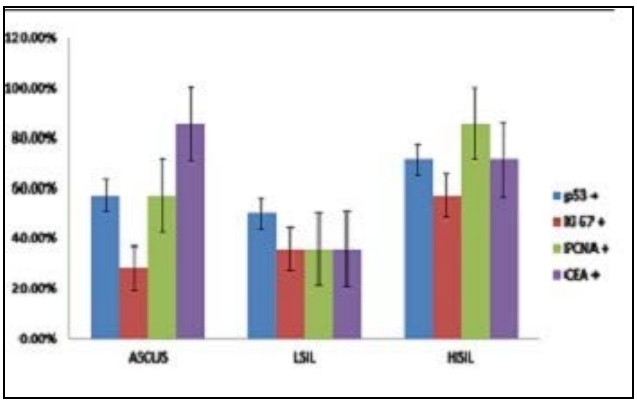

Figure 7. Representative Histogram for comparative analysis of the p53, Ki 67, PCNA and CEA expression among the ECA cases (ASCUS, LSIL and HSIL).

Out of 28 ECA cases, 8 cases were found positive for all the four markers and HSIL (4 cases) was significantly higher among these cases ($p<0.01$). For HSIL condition 1.41 times risk was found for age <40 years with cervical lesion [OR-1.41(0.13, 15.26);p=0.77], 2.27 times risk was found for CEA positivity [OR-2.27(0.35, 14.45);p=0.37], 2.66 times risk was found for Ki-67 positivity [OR-2.66(0.46, 15.35);p=0.26], 2.27 times risk was found for p53 positivity [OR-2.27(0.35, 14.45);p=0.37], 8.00 times risk was found for PCNA positivity [OR-8.00(1.02, 78.73);p=0.04], 5.66 times risk was found for age at menarche ≤13 years [OR-5.66(1.01, 36.08);p=0.05] and 4.26 times risk was found for Muslim [OR-4.26(1.02, 5.87);p=0.01].

All the cases of HSIL condition the age of marriage was < 20 years. So the Odds Ratio could not be assessed and Fishers Exact test showed the proportion of HSIL condition was significantly higher for age of marriage was < 20 years (p=0.02).

For NILM, out of 32 cases the proportion of patients with normal cytopathology (37.5%) was higher followed by RCCI (34.4%) and then RCC (28.1%). The proportion of Muslims with normal cytopathology was higher ($p<0.05$) whereas proportion of Hindu with RCC and RCC inflammation was higher. The proportion of RCC (77.8%) and RCCI (72.7%) were higher for age of menarche ≤ 13 years ($p<0.01$). No significant association was found for age of marriage ≤ 20 years and parity (Table 2).

Table 2. Represents Cytological Parameters, Biomarker Expressions, Epidemiological Parameters And Statistical Significance Of NILM

Negative for Intraepithelial Lesion or Malignancy (NILM) (32 cases)				
		ASCUS	LSIL	HSIL
Cytological Diagnosis		12 (37.5%)	9 (28.1%)	11(34.4%)
Religion	Hindu	5(41.7%)	8(88.9%) **p<0.05**	10(90.9%) **p<0.05**
	Muslim	7(58.3%) **P<0.05**	1(11.1%)	1(9.1%)
Parity	P>2	5(41.7%)	2(22.2%)	4(36.4%)
	P≤2	7(58.3%)	7(%77.8)	7(63.6%)
Age at marriage	≤20	9(75%)	5(55.6%)	6(54.5%)
	>20	3(25%)	4(44.4%)	5(45.5%)
Menarch	>13	6(50%)	2(22.2%)	3(27.3%)
	≤13	6(50%)	7(77.8%) **p<0.01**	8(72.7%) **p<0.01**
Biomarkers	p53+	9(75%) **p<0.05**	5(55.6%) **p<0.05**	9(81.8%) **p<0.05**
	P53-	3(25%)	4(44.4%)	2(18.2%)
	Ki67+	6(50%)	4(44.4%)	7(63.6%) **p<0.01**
	Ki67-	6(50%)	5(55.6%)	4(36.4%)
	PCNA+	4(33.3%)	4(44.4%)	7(63.6%) **p<0.01**
	PCNA-	8(66.7%)	5(55.6%)	4(63.4%)
	CEA+	5(41.7%)	7(77.7%) **p<0.01**	8(72.7%) **p<0.01**
	CEA-	7(58.3%)	3(37.3%)	3(27.3%)

In relation to markers, a proportion of normal cytopathology (75%), RCC (55.6%) and RCCI (81.8%) were higher for p53 positivity ($p<0.05$). The proportion of RCCI (63.6%) were higher for Ki-67 positivity ($p<0.01$) than RCC (44.4%) and normal (50%). The proportion of RCCI (63.6%) were higher for PCNA positivity ($p<0.01$) than RCC (44.4%) and normal (33.3%). The proportion of RCC (77.8%) and RCCI (72.7%) were higher for CEA positivity ($p<0.01$) than the normal (41.7%) (Table 2) (Figure 8).

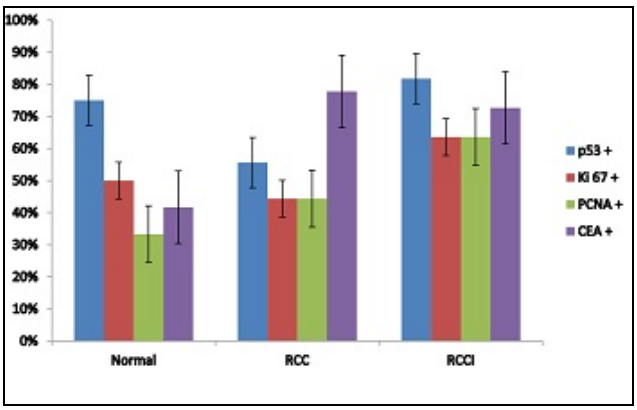

Figure 8. Representative Histogram for comparative analysis of the p53, Ki 67, PCNA and CEA expression among the NILM cases (Normal, RCC and RCCI).

Out of 32 NILM cases, 9 cases were found positive for all the four markers.

4. Discussion

The result obtained from the study and data analysis distinctly shows that there are no clear differences between the Negative for intraepithelial Lesion or Malignancy (**NILM)** and Epithelial Cell Abnormality **(ECA),** categorized according to the Bethesda system 2001, in respect of both the tissue marker expression and the epidemiological parameters. This study includes many parameters and these are compared with the cytological outcome of the cases and evaluated by the statistical data analysis system.

Even after the strict clinical observation of the two gynecologists using Colposcope and acetic acid it is found that a handful of cases fooled the colposcopy observation to identify pathological lesions and again proved the significance of the microscopic interpretation as the gold standard as opined by many authors [18].

In NILM cases, significant result obtained includes the presence of normal smear, which is more common in Muslim women than in Hindus whereas RCC and RCCI lesions are more common in Hindus. Our result has been found to be almost similar to the study of a group of authors from India, where they have found the epithelial cell abnormality was lower in Muslims than non-Muslims in Cytopathological evaluation [19]. Additionally, population-based surveys of cervical cancer in the orient are lacking, cervical cancer is said to be the most common malignant tumor among Hindu women in India, with rates approximately double those among Muslims [20].

This observation is justified by the fact that in the earlier studies conducted in Pune city and greater Bombay with predominant Hindu females revealed a higher incidence of cervical cancer than Muslim females [21].

On the other hand, another study initiated at the Institute of Cytology and Preventive Oncology (ICPO), New Delhi, on uterine cervical dysplasia showed an interesting finding that all malignant lesions were found in Muslim women [22]. And the researchers justified it may be due to the fact, that it was predominantly Muslim

community (76.3 per cent of study population). The above facts, most probably, have been linked to two opinions opposite to each other, however, in our study ASCUS and LSIL cases is significantly higher in Hindus whereas regarding the presence of HSIL cases it is more common in Muslim women than the Hindus population which is more than double than the Muslim population. Therefore, in this part of the world, the Muslim Women may have a higher risk of developing cervical cancer than Hindus with high grade cervical lesion (HSIL).

From the study, it is also observed that parity has got no link with both the NILMs well as ECA lesions indicates that parity carry no or lower risk. However, according to some Authors [23,24] high parity is one of the risk factors for cervical cancer. But there are many studies related to fertility and parity state that the Muslim women are more fertile and multiparous in comparison to other communities of the society [25,26,27] but the incidence of cervical cancer among Muslim women is almost half that of the Hindus [20] or less common [21], more than justifies our observation that parity is insignificant in relation to cervical premalignant and malignant lesion.

Similarly, no significant association is found in relation to diet habit, addiction (only 15% of them had an addiction to chewing of tobacco) and literacy. In this study, it is also found that the early marriage is an important phenomenon both in ECA and NILM cases and has got an intimate relationship with the risk of developing cervical cancer. As well as early menarche is an important phenomenon in the process of the development of cervical cancer as observed by many Authors [28]. In this study, cases having early menarche with ECA (ASCUS and LSIL) are intimately linked to the risk of developing cervical cancer. Whereas, among the NILM both the RCC and RCCI is almost equally important and carries significant risk for cancer conversion. Female adolescents with a shorter duration between the age of menarche and first sexual intercourse are at increased risk of high-grade cervical disease [29]. From this study it appeared that these cases have early marriage and early menarche, which again justify our study of having risk in cases of early marriage and early menarche. A study from Pakistan insights that, early marriage and multiparity were the two most common risk factors seen among the patients [30]. In fact, the presence of high fertility, early marriage is prevalent among the Muslim women, though the incidence of cervical cancer is much less than the Hindus in India with rates approximately double than those of Muslims [20]. The Korean and Chinese women have higher rates of cervical cancer than Muslims, though they have low parity. It is thought that low rate of cervical lesions may be due to some religious practices like circumcision and as well as menstrual abstinence. There is a low rate of cervical cancer in Jewish women and they have included traditional sexual abstinence during and shortly after menses, circumcision of the male, and unspecified genetic factors and on the other hand, Scandinavian countries do not practice circumcision but still have a cervical cancer rate as low as that of Israel And Muslims [20]. There is inconclusive evidence to date that circumcision protects against genital HPV infection. A systematic review of studies published in March 2006 found no evidence of an association between circumcision and genital HPV [31]. Therefore, the different established etiological factors do not significantly predict risk in association with the cytopathological outcome and link with oncogenic HPVs for risk prediction of pre-neoplastic cervical lesion. It is thought that cervical cancer emerges from cervical intraepithelial neoplasia (CIN) induced by high-risk HPV (HR-HPV) infections. The vast majority of CIN lesions regresses spontaneously, and only a few lesions persist or progress to invasive carcinoma. High-risk human papillomavirus (HR-HPV) infection has been recognized as the necessary cause of cervical cancer since the 1990s [7,32,33].

However, HPV infection also is the most common sexually transmitted infection, its prevalence being especially high in young women [34,35].

Thus, the majority of HPV infections are transient, usually cleared in 8–10 months [36,37], and only a few of them will actually lead to invasive cervical carcinoma [38,39]. Therefore**,** on the basis of morphological criteria [40] or HPV infection, it is not possible to differentiate high-grade lesions that will regress or persist from those that inevitably will progress to invasive cancer. Nonetheless, the strong discrepancy between the prevalence of integrate-derived transcripts in high-grade preneoplastic CIN III lesions and invasive carcinomas (15.6% versus 87.5%) [40] suggests the detection of one or more suitable molecular markers for preneoplastic lesions with a high risk for cancer progression.

Keeping the above fact in mind we have, in our study, included more than one tissue and genetic marker for the early risk prediction in this pre-neoplastic cervical lesions and involves inclusion of the study of proliferative and oncogenic markers along with cytopathological or histopathological outcome. The inactivation of p53 compromises the integrity of the cellular genome, causes DNA damage and chromosomal instability, these abnormalities result in increased cell proliferation and tumour development [41]. In our study, we have found that70. 8 % Non-SIL and 57 % LSIL express p53 oncoprotein and there is little difference between the two. Similarly, a group of workers has found no significant differences in p53 expression between Non-SIL and LSIL.There were p53 expression in 42% Non-SIL and 33.3% LSIL samples [42]. In another study for p53 immunostainingCIN showed an increasing percentage positivity of p53 expression with increasing grade of the lesion (CIN I 2/9 cases (22.2%); CIN II 4/8 cases (50%) and CIN III 2/2 cases (100%); $P = 0.110$) [43].

However, statistical analysis of our study shows that PCNA, p53, Ki-67 and CEA show progressive positivity in relation to Cytopathology is most significant in ECA (ASCUS, LSIL, HSIL).There is also increasing marker expression with increase grade of lesion from LSIL to HSIL but ASCUS and LSIL has got almost also similar PCNA,p53 and Ki-67 expression. In NILM, the p53 is the most significant marker and expressed in 81.8% in RCCI and almost half in RCC and p53 showed 75% expressed in normal cytopathological cases ($p<0.01$) whereas Ki-67 and PCNA significantly expressed in RCCI ($p<0.01$). CEA expression in both the RCC and RCCI again raises the question whether progression of cancer occurs directly from normal or from NILM cases.

Our results suggest that PCNA, p53 and Ki-67 overexpression may be involved in the process of neoplastic transformation of the cervical epithelium. In

our study PCNA is the most important proliferative marker followed by Ki-67, p53and CEA. A similar observation was expressed in a study by, Maeda MY et al.2001 [44], wherein the percentage of PCNA and Ki-67 positive cells increased with increasing grade of the cervical lesions, although PCNA immunoreactivity was always greater than the immunoreactivity observed with Ki-67 antigen.

A study by Vasielescu et.al, 2009 [45] also observed that Ki-67 is a proliferative marker, which correlates with the histologic grades of cervical neoplasia.

Another study by Zeng et al. 2002 [46], observed that Ki-67 has to be valued as a complementary tool in cervical cytology, not only in the initial triage of minor abnormalities, but also in the follow up of abnormal Pap smear.

It is justified by the study that the entire marker studied are significantly increases in progressive fashion and strongly increased in HSIL than LSIL and ASCUS. Early marriage and has got an intimate relation in ECA cases. Marriage at an early age specially below 20 years has got higher number of ASCUS, LSIL and HSIL cases therefore it is predicted that early marriage is another very important risk factor for cervical cancer in association with marker expression and cytopathological assessment. Also in general cases having age equal or less than 40 years has higher risk than the rest of the population. Our study indicates that a combination of biological markers along with epidemiological factors like early marriage, religion and cytopathologicalfactor (p=0.013) in combination is a positive approach for risk prediction in preneoplastic cervical lesion.

5. Conclusion

The study concludes that cytopathological assessment alone is insufficient indicator for risk categorization in pre neoplastic and other non neoplastic cervical lesions. The epidemiological factors, cytopathological assessment and biological markers including proliferative markers in combination is the hope for risk prediction in pre neoplastic cervical lesion. A large number of cases and follow up for longer period needs to be studied to come into a definite conclusion.

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