Paradigm Academic Press
Journal of Innovations in Medical Research
ISSN 2788-7022
JUN. 2025 VOL.4, NO.3

The Risk Assessment for Malignant Conversion of Cervical Pre-Neoplastic and Other Lesions by High Risk HPV and Relevant Markers

Dipanwita Ghosh[1], Avirup Roy[2] & Asoke K. Roy[3]

[1] Ex. Senior Research Fellow, Department of Pathology and Cancer Screening, Chittaranjan National Cancer Institute, Kolkata, West Bengal, India

[2] Ex House Physician, Institute of Medicine and Sagar Dutta Hospital, Kolkata, West Bengal, India

[3] Ex Head Dept. of Pathology and Cancer Screening, Chittaranjan National Cancer Institute, Kolkata, West Bengal, India

Correspondence: Asoke K. Roy, Ex Head Dept. of Pathology and cancer Screening, Chittaranjan National Cancer Institute, Kolkata, West Bengal, India.

doi:10.63593/JIMR.2788-7022.2025.06.005

Abstract

Objective is to identify high risk cases of cervical pre -neoplastic lesions through the study of p16 (HR-HPV), Ki-67, DNA ploidy & Apoptosis. Western Blot analysis was performed to identify the high-risk HPV association. Immunohistochemistry was done to study Ki-67; DNA content study was performed with the help of FACS & Apoptotic index was studied by Tunnel Assay. The association of p16 with the cervical pre-neoplastic pathology was not only strong but also very significant. In this study, 72% cases were positive, and 28% cases were negative for p16 study by Western Blot analysis. Only 2 cases of other associated conditions (Koilocytosis) showed positivity for p16. The study of Ki-67 was also highly significant & showed positivity in 73% cases and has similarity with other studies. The test of proportion showed that patients with positive TUNEL assay (63%) were significantly higher than patients with negative TUNEL assay (37%) ($p<0.00024$). To conclude, in India, Cervical cancer is the second most common cancer among women. It is preceded by cervical pre neoplastic lesions (CINS). Apart from HR HPV expressed in CIN's, condyloma, cervicitis, reactive cellular and koilocytotic changes are also caused by HR HPV. Therefore, study of HR HPV association along with identification of DNA status, Ki67 expression and Apoptosis play a very important and crucial part for risk assessment and should be done at the earliest for cervical cancer prevention.

Keywords: HR-HPV, pre-neoplastic lesion, cervical cancer, Western Blot, immunohistochemistry, DNA ploidy, Tunnel assay

1. Introduction

Worldwide, among all cancer cases, cervical cancer has been placed in the fourth position with approximately 600400 new cases and 34200 deaths[1].

It is classified into two major histopathologic types, Squamous cell carcinoma (72.6%) and adenocarcinoma (21.8%) (S. Nagase, T. Ohta, F. Takahashi et al., 2022). Almost all the Squamous cell carcinomas (95%) and more than 80% of cervical adenocarcinoma are HPV associated. But 10–15% of them, such as gastric-type adenocarcinoma and clear cell carcinomas, are HPV independent (The Cancer Genome Atlas Research Network,

[1] WHO 2020.

2017; S. Stolnicu, L. Hoang, R.A. Soslow et al, 2019).

Majority of cervical cancer preceded by pre-neoplastic lesions designated as cervical intra- epithelial neoplasia and classified as mild (CIN I), moderate (CIN II) and severe (CIN–III) lesions. Annually, approximately 1.5 per 1000 women in developed countries is diagnosed with CIN II/CIN III (K. Tainio, A. Athanasiou, K.A.O. Tikkinen et al., 2018). Most of the cervical cancer occurred in the squamocolumnar junction (F. Bray, J. Ferlay, L. Laversanne et al., 2015).

Human papillomavirus (HPV) infection and integration of cervical epithelium has been considered as the key factor for the development of cervical cancer (K. Munger, A. Baldwin, K.M. Edwards, et al., 2004). All-over the world, in general, HPV infection among women is about 9 to 13% and in Indian women it varies between 7.5% to 16.9% (A. Sridevi, R. Javed, A. Dinesh et al., 2015).

Papillomaviruses are small, non-enveloped, icosahedral DNA viruses with a diameter of 52–55 nm. The viral particles consist of a double-stranded DNA molecule of about 8000 base-pairs that is bound to cellular histones and contained in a protein capsid composed of 72 pentameric capsomers. Apart from general population it is also widely distributed in the animal kingdom.

So far, more than 200 genotypes of HPVs have been identified (I. Lesnikova, M. Lidang, S. Hamilton-Dutoit, et al., 2009). HPVs are classified as High Risk HPVs (HR HPV) and Low Risk HPVs (LR HPV) (D. Uyar, J. Radar., 2014). Most of the HPVs are low risk HPV and produce benign warts (Münger, K., Werness, B. A., Dyson, N., Phelps, W. C., Harlow, E., & Howley, P. M., 1989). The International Agency for Research on Cancer (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans Biological Agents, 2012). IARC working group classified HPV as Groups 1–4 based on the risk of carcinogenesis. Group 1 or Group 2A with a high risk of carcinogenesis includes 13 types of HPV; HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. Particularly, HPV types 16 and 18 are responsible for 70% of the HPV genotypes detectable in cervical cancer (Y. Azuma, R. Kusumoto-Matsuo, F. Takeuchi, et al., 2014).

However, immune protection clears 90% of all HPV infection spontaneously but some cases have persistent viral infection and leads to the development of cervical cancer (Hu, Z., et al., 2015).

The key event of carcinogenesis is the integration of HPV genome with the host genome(chromosome). It often occurs at the common fragile sites of host genome (Thorland, E. C., et al., 2003). HPV integration follows more specific pattern with relation to the HPV genome and as well as clonal proliferation of epithelial cells.

Expression of viral E6 & E7 genes leads to constant presence of E6, E7 proteins where as other portions of the viral DNA are deleted (V.V. Baker., 2008). Viral E6 protein binds to P53 tumour suppressor protein causing degradation of P53 protein and E7 protein inactivates retinoblastoma (Rb) protein resulting inhibition of apoptosis and leading to abnormal cell cycle and proliferation. In addition to this fact, E7 also triggers upregulation of CDKN2A tumour suppressor gene resulting marked accumulation & overexpression of p16 genes as p16 protein (Aupperle, K. R., et al., 1998; M. Narisawa-Saito & T. Kiyono, 2007).

Though, histopathology is considered as ‘gold Standard,’ it fails to assess cancer conversion risk of cervical pre-neoplastic lesions. This fact accentuates the importance for biomarker study in pre neoplastic lesions.

Currently, high risk HPV marker, CDKN2A (M. Narisawa-Saito & T. Kiyono, 2007), proliferative marker ki 67 (R. SalesGi & P. Vagnarelli, 2018), DNA ploidy analysis (Melsheimer, P., et al., 2004), and apoptosis are getting importance for the risk assessment of cervical pre-neoplastic lesions.

2. Materials and Methods

Study Design:

The study took Place under the Dept. of Pathology and Cancer Screening, and the samples were collected at Gynaecology OPD and CDC OPD of Chittaranjan National Cancer Institute, Kolkata, India.

Both the cervical smear samples and punch Biopsy specimens were taken from 100 pre- diagnosed cases. The samples were processed according to the standard protocol.

P16 Estimation–Western Blot

(i) Sample collection: Cervical punch biopsy specimen was collected, in I X PBS in Eppendorf tube separately for100 cases and the whole protein was extracted from each of the sample within 24 hours.

(ii) Protein extraction: Tissue samples were washed with 1 x PBS thoroughly and transferred to a 2mL Eppendorf tube. Next, 250 uL of lysis butter added to the tube and kept on ice for 15 to 20 minutes and homogenised thoroughly. Homogenized tissue was sonicated for 1.5 minutes and kept on a rocker at 4° C for 2 hours and centrifuged at 13500 rpm for 15 minutes at 4°C and the supernatant was placed in a clear and sterile Eppendorf tube for further experiment.

(iii) Protein estimation: 1 ml of supernatant was added to 200 uL of Bradford reagent mixed with 49 ul of distilled water. The soln. was placed in a ELISA plate & mixed well. The reagent turned blue. The protein estimation was done with the help of ELISA Reader at an absorbance of 595 nm & the reading was taken. Rest of the supernatant (extracted protein, was stored at –70°c for further use).

(iv) Western Blot Analysis: For identification of P16 protein — the experiment was performed using SDS — PAGE following standard protocol.

Immunohistochemistry (IHC) for Ki- 67

Ki-67 IHC was performed in 100 cases of deparaffinised tissue sections and positive control according to the protocol of IHC World (IHC World, 2008) with slight modification. Commercially available kit (IHC Select-HRP/DAB Kit Millipore) was used for detection.

Briefly, antigen retrieval was carried out by boiling the slides in 0.01 M citrate buffer, pH 6.0 in a microwave oven at 900 watts for 10 minutes allowed to cool down at room temperature, washed with rinse buffer and blotted. Endogenous peroxidase was blocked by 3% hydrogen peroxide in water for 10minutes.Sections were washed with rinse buffer and blotted. Non -specific binding was blocked by blocking reagent (provided in the detection kit, Millipore) for 5 minutes in a humid chamber and excess reagent was blotted. Next primary monoclonal antibody (Sigma, USA, clone PP 67, dilution 1:50) were added to the sections and incubated overnight in 4 degree centigrade. Slides were washed thoroughly with rinse buffer to remove excess primary antibody and blotted. Secondary antibody (Millipore) was added to the sections and incubated in a humid chamber for 10 minutes, washed and blotted. Next, Streptavidin HRP was applied on the tissue sections and incubated for 10 minutes. After washing and blotting freshly prepared DAB solution was added to the tissue sections and incubated for 10 minutes then sections were washed for 5 minutes and blotted. Counter staining was done by Meyer's haematoxylin for 1 minute. The slides were then passed through a series of graded alcohol and xylene and mounted with DPX mountant. Primary anti-body was omitted from the negative control slides.

Scoring method:

Ki 67 expression were analysed semi quantitively by scoring 1000 cells at 40x under light microscope. Ki 67 immunoreactivity was considered in cases having 10% positive nucleus (Qin, L. X., et al., 2002).

DNA ploidy Analysis:

The DNA content study was done following the protocol of University of Virginia[1].

Method: From a total of 100 previously diagnosed pre-neoplastic cases of cervix along with controls, cells were collected using cytobrush in 12x75 mm tube containing 4.5 ml of 70% ethanol kept on ice. The collected samples were immediately dipped in 5 ml of PBS pH-7.6.

Samples and controls were put to test tubes containing PBS PH 7.6 and centrifuged for 5 minutes in 300 g. The pellet was re-suspended in PBS PH 7.6. A total of 10^6 to 10^7 cells were taken from it in 5 ml PBS PH 7.6 and suspension was made. The suspension again centrifuged at 300g for 5 minutes and the supernatant was discarded, and cells were re-suspended as single cell suspension in 0.5 ml PBS. The cells were then transferred into a test tube containing chilled 70% ethanol and kept for 2 hours. The ethanol fixed cells were again centrifuged for 5 minutes at 300g and the supernatant was discarded thoroughly. The cell pellet was suspended in 5 ml PBS for 1 minute and again centrifuged for 5 minutes in 300 g. The supernatant was discarded, and the cells were suspended in 1 ml PI solution and kept under dark for30 minutes at room temperature.

Flow cytometry study was carried out with the help of FACS calibre Flow cytometer of Becton Dickinson make using an ergon laser, wavelength 485 nm run at 15 mV. Calibration was carried out using latex beads. Maximum permitted error was 3%. Histogram data was generated, and DNA index was calculated according to the following formula.

DNA Index=Mean Go/G1peak of test sample/Mean Go/G1 peak of control. Histogram interpretation was done according to the method of Bergers, E., van Diest, P. J., & Baak, J. P., (1997).

Apoptosis:

From 100 study cases and controls, tissue sections were deparaffinised following standard protocol. Sections were rehydrated with PBS PH 7.6, blotted and were treated with proteinase K at 37degree centigrade for 12 minutes and rinsed with 2 changes in PBS for 10 minutes. The sections were incubated with 3% H2O2 in PBS for 10 minutes for blocking endogenous peroxidase. The tissue sections were then rinsed with PBS for 3 times, 2 minutes each. The sections were then treated with reaction buffer for 10 minutes followed by incubation in TdT reaction mixture for1.5 hours at 37-40 degree centigrade in humidified chamber. The reaction was stopped by

[1] http://hsc.virginia.edu/intrnet/cytometry/protocols/dnacontent.efm

adding stop wash buffer and incubated for 10 minutes. The sections were thoroughly washed with PBS thrice for 2 minutes each. Streptavidin HRP antibody (ultrasensitive S2438, Sigma, USA) was added to the sections and incubated for10 minutes then rinsed 3 times with PBS. The sections were treated with DAB solution for 2 minutes and rinsed in running tap water for 5 minutes. Slides were then dehydrated through graded alcohol and dried and cleaned in 2 changes of xylene for 5 minutes each and mounted with DPX and visualised under light microscope.

Statistical Analysis

Statistical Analysis was performed with the help of Epi Info (TM) 7.2.2.2. EPI INFO is a trademark of the Centres for Disease Control and Prevention (CDC). Descriptive statistical analyses were 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 proportions and Chi-square ($\chi 2$) test was performed to find the associations. In the cases where one of the cell frequencies were less than 5 corrected Chi-square ($\chi 2$) was used to find the association between variables. t-test was used to compare the means. $p<0.05$ was taken to be statistically significant.

3. Results

Status of p16 expression by Western Blot of the patients

The positivity for p16 expression by western blot is 72.0%, which was significantly higher than that of negativity (28.0%) ($p<0.00001$).

Table 1. Status of p16 expression by Western Blot

Status of p16 by Western Blot	Number	%	p-value
Positive	72	72.00%	
Negative	28	28.00%	p<0.00001*
Total	100	100.00%	

* Significant level ($p<0.05$), >0.050 -not significant.

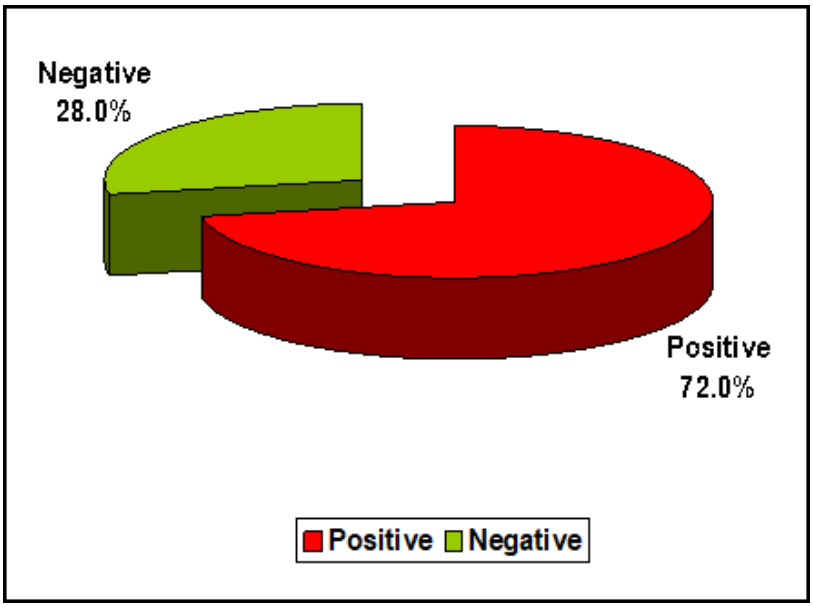


Figure 1. Pie chart showing Status of p16 expression by Western Blot

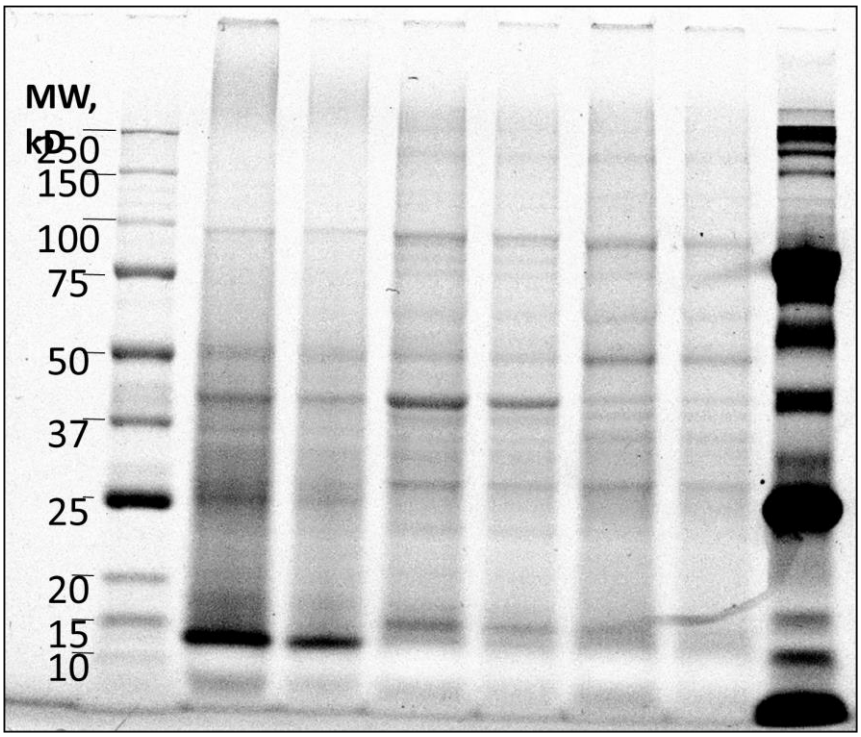


Figure 2. Stain-Free Gel image of Tissue protein samples

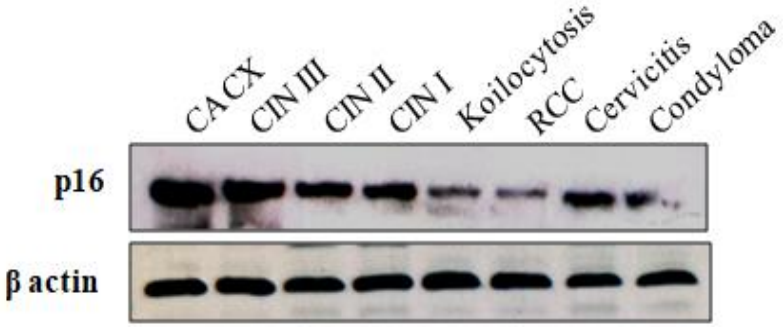


Figure 3. Western Blot of p16 protein in different cervical lesions

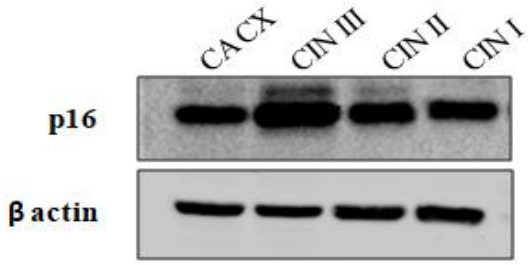


Figure 4. Western Blot of p16 protein in different grades of cervical intraepithelial neoplasia

Status of Ki-67 expression

Out of 100 cases, Ki67 immunopositivity (73%) was significantly higher than negative cases (27%) ($p<0.001$). CIN I (72.2%), CIN-II (77.8%) and CIN-III (67.6%) all were significantly prevalent among patients with Ki67 immunopositivity ($p<0.00001$). The expression for Ki67 increased from CIN I to CIN II (CIN I – 72.2%, CIN II

– 77.8%) and slightly decreased in CIN III (67.6%). 8 cases (88.9%) of other associated lesions showed Ki67 positivity (condyloma 1, cervicitis 2, RCC 1, Koilocytosis 4). Ki67 positivity with strong intensity (58.9%) was significantly higher than moderate intensity and mild intensity staining (26.0% and 15.1%, respectively) ($p<0.001$). Ki67 expression was significantly seen in the whole of the epithelium (87.67%) ($p<0.001$).

Table 2. Status of Ki-67 expression

Biomarkers	Result of IHC	Number	%	p-value
Status of Ki67	Positive	73	73.0%	p<0.001*
	Negative	27	27.0%	
	Total	100	100.0%	

* Significant level ($p<0.05$), >0.050 -not significant.

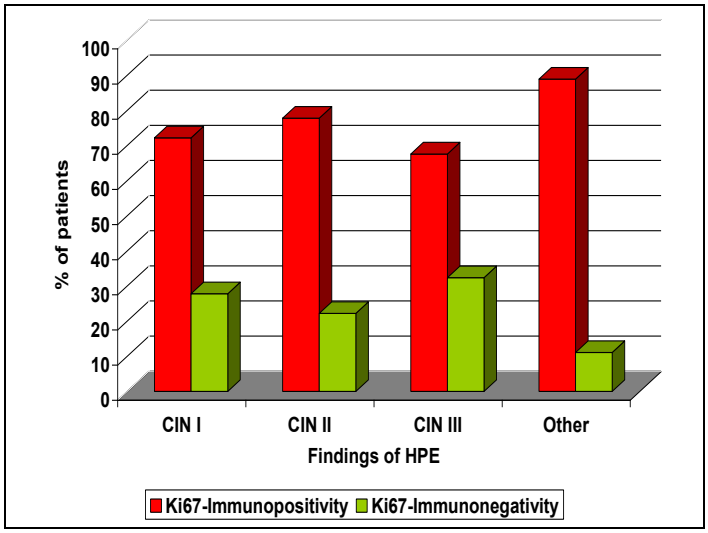
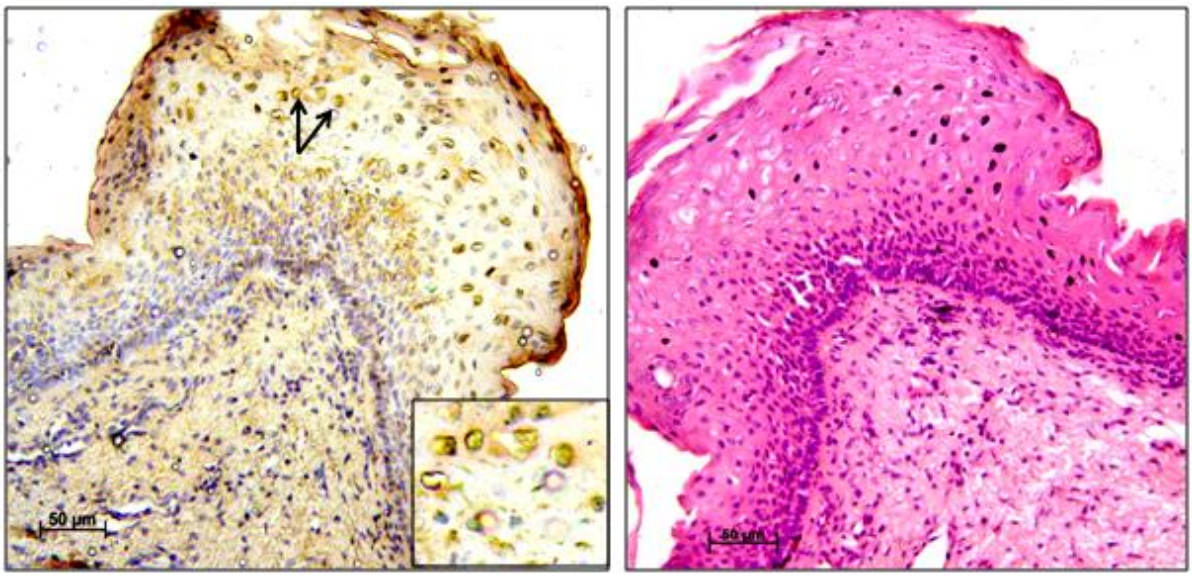


Figure 5. Bar diagram showing the association between Ki67 Immunohistochemical result and findings of HPE of the patients



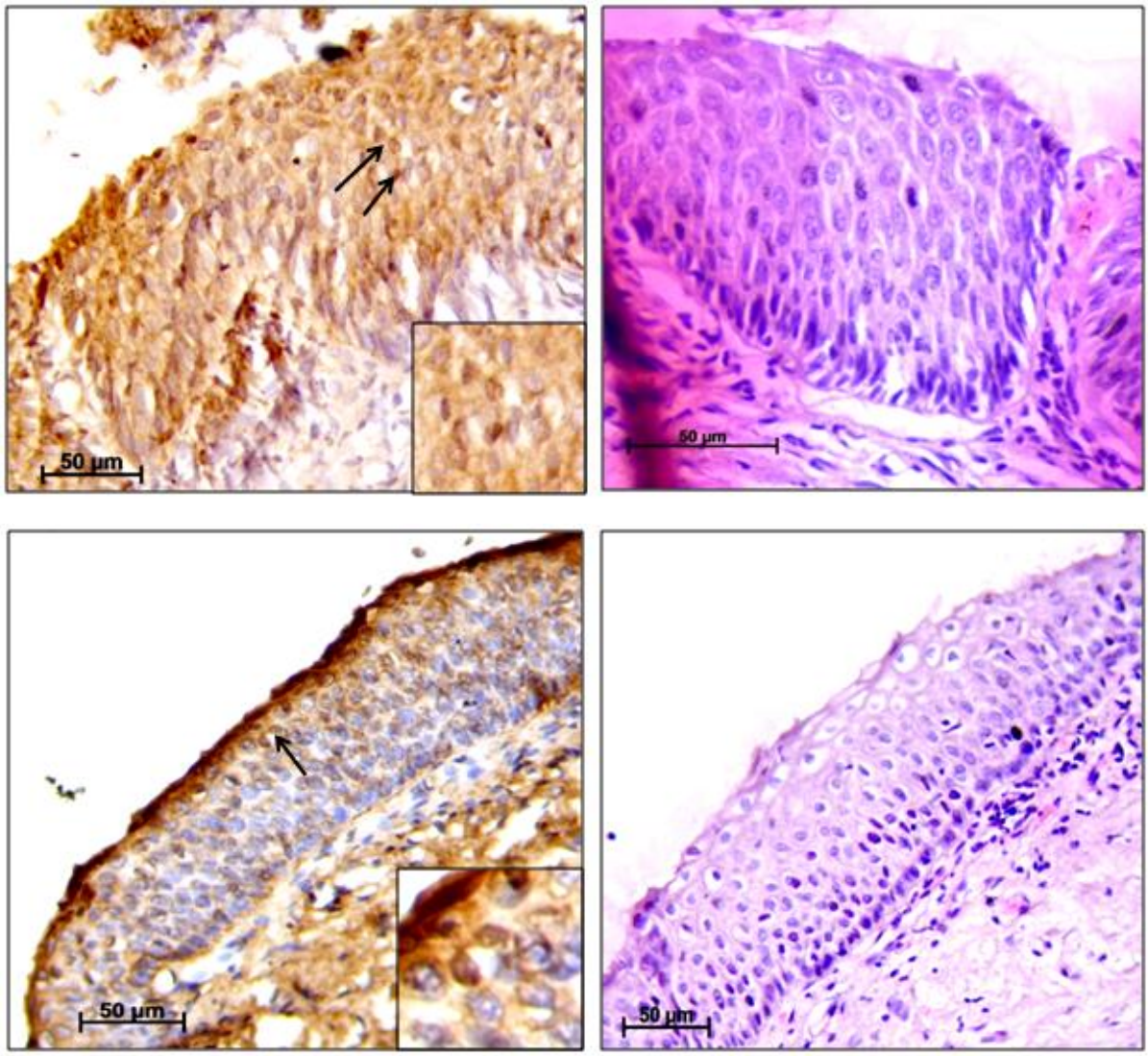


Figure 6. A(a) Ki67 immunopositivity in Cervical Intraepithelial Neoplasia I (CIN I), A(b) Corresponding Histopathology (HP) image (H/E stain); B(a) Ki67 immunopositivity in CIN II, B(b) Corresponding HP image (H/E stain); C(a) Ki67 immunopositivity in CIN III, C(b) Corresponding HP image (H/E stain). The magnification of the main and inset image is 20X and 40X, respectively. Black arrows denote Ki67 immunopositive cells.

Status of TUNEL assay in patients

The test of proportion showed that patients with positive TUNEL assay (63%) were significantly higher than patients with negative TUNEL assay (37%) ($p<0.00024$).

Table 3. Status of TUNEL assay of the patients

Status of TUNEL assay	Number	%	p-value
Positive	63	63.00%	
Negative	37	37.00%	p<0.00024*
Total	100	100.00%	

* Significant level ($p<0.05$), >0.050 -not significant.

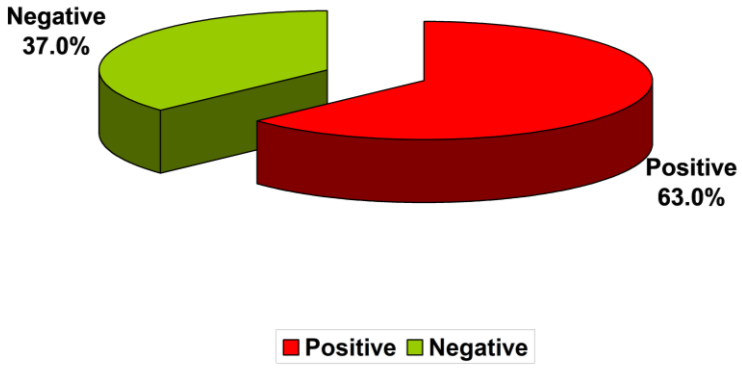


Figure 7. Pie chart showing the status of TUNEL assay of the patients

Association between the status of TUNEL assay and Histopathological findings

CIN I (58.3%), CIN II (61.1%), and CIN III (83.8%) were significantly prevalent among patients with positive for TUNEL assay (p=0.023; p=0.002; p<0.00001).

Apoptotic Index significantly increased from CIN I (58.3%) to CIN II (61.1%) to CIN III (83.8%). 1 case (11.1%) with other pathological conditions (condyloma) showed positive for TUNEL assay.

Univariate analysis showed that CIN III had a significant risk for apoptosis. However, no risk of apoptosis was found in CIN I and CIN II cases.

Table 4. Association between Result of TUNEL Assay and findings of HPE of the patients

Result of TUNEL Assay	**Histopathological Grades**				**TOTAL**
	CIN I	CIN II	CIN III	OTHER	
Positive	21 (58.3%) p=0.023*	11 (61.1%) p=0.002*	31 (83.8%) p<0.00001*	1 (11.1%)	63 (63.0%)
Negative	15 (41.7%)	7 (38.9%)	6 (16.2%)	8 (88.9%)	37 (37.0%)
TOTAL	36 (100.0%)	18 (100.0%)	37 (100.0%)	9 (100.0%)	100 (100.0%)

*Significant level (p<0.05), >0.050 -not significant.

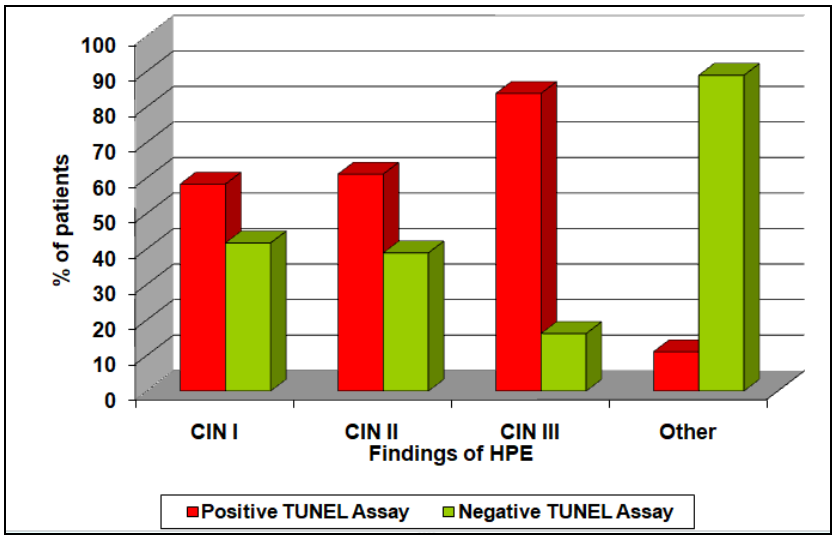
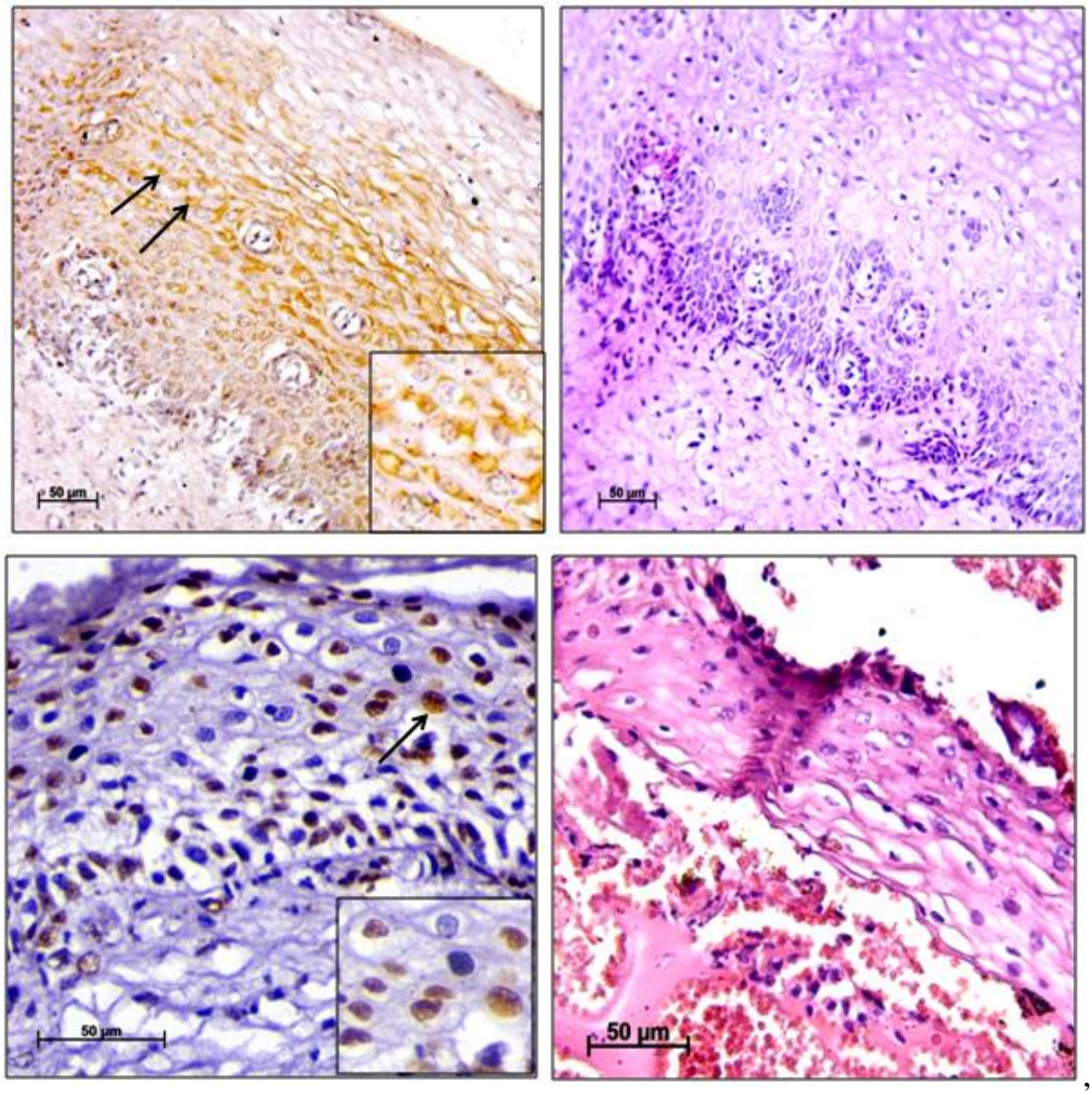


Figure 8. Bar diagram showing association between Result of TUNEL Assay and findings of HPE of the patients

Table 5. Risk factors associated with Apoptotic Index content and Histopathological grades

Biomarkers	**Histopathological grades**					
	CIN-I		CIN-II		CIN-III	
	OR with 95% Confidence Interval	p-value	OR with 95% Confidence Interval	p-value	OR with 95% Confidence Interval	p-value
Positive TUNEL assay	0.733(0.316,1.698)	0.468	0.859(0.300,2.475)	0.777	5.005(1.833,13.661)	<0.0001*

* Significant level (p<0.05), >0.050 -not significant.



,

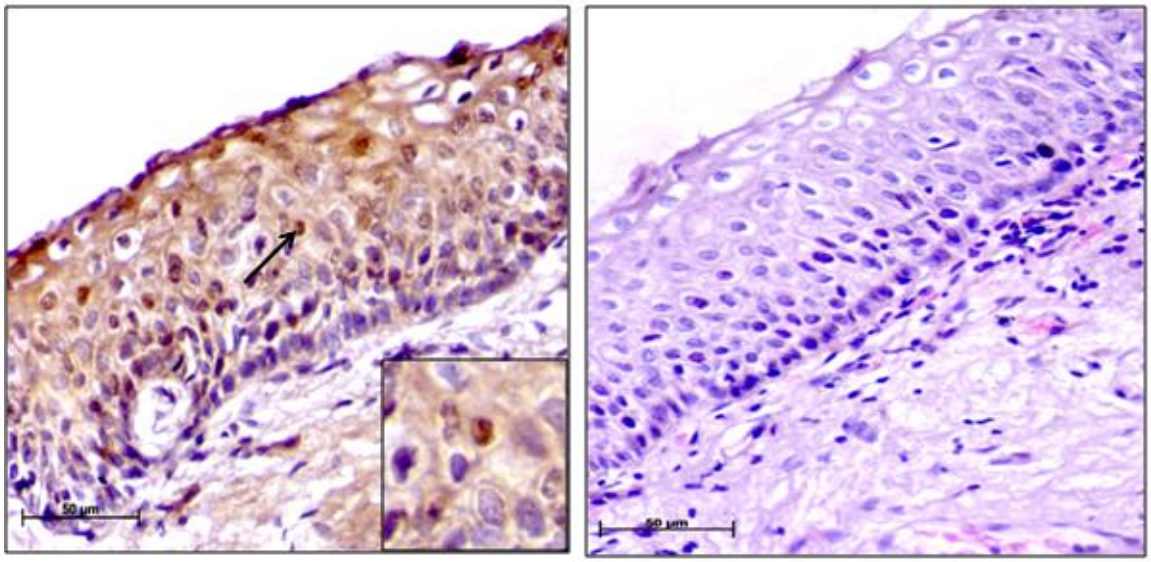


Figure 9. A(a) Cervical Intraepithelial Neoplasia I (CIN I) with TUNEL positive cells, A(b) Corresponding Histopathology (HP) image (H/E stain); B(a) CIN II with TUNEL positive cells, B(b) Corresponding HP image (H/E stain); C(a) CIN III with TUNEL positive cells, C(b) Corresponding HP image (H/E stain). The magnification of the main and inset image is 20X and 40X, respectively. Black arrows denote TUNEL positive cells.

Status of DNA content in patients

All 100 cases were included in this study, and DNA content was detected by flow cytometry. 57% of the cases showed aneuploid DNA content, and the remaining 43% had diploid DNA content. Thus, Patients with aneuploid DNA content (57.0%) were significantly higher than patients with diploid DNA content (43.0%%) ($p<0.047$).

Aneuploid DNA content was significantly prevalent among patients with CIN II (66.7%) and CIN III (70.3%) ($p<0.00001$) and significantly increased from CIN I (50.0%) to CIN II (66.7%) to CIN III (70.3%). 1 case (11.10%) with other pathological conditions (condyloma) showed aneuploid DNA content.

Univariate analysis showed that CIN III had a significant risk for aneuploidy. The risk for aneuploidy was also found among CIN II patients, but it was not significant. However, no risk of aneuploidy was found in CIN I cases.

Table 6. Status of DNA content (FACS) of the patients

Status of DNA content	Number	%	p-value
Aneuploid	57	57.00%	
Diploid	43	43.00%	p=0.047*
Total	100	100.00%	

* Significant level ($p<0.05$), >0.050 -not significant.

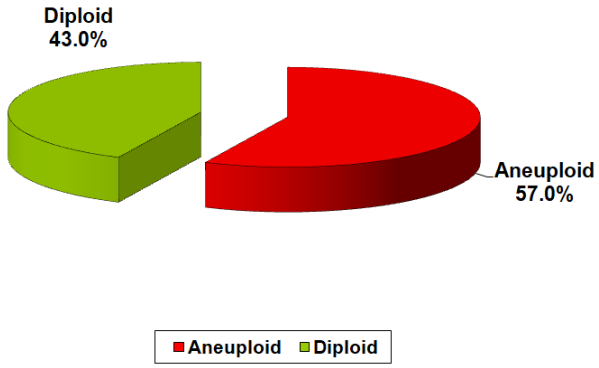


Figure 10. Pie chart showing the status of DNA content (FACS) of the patients

Table 7. Association between DNA Content Status and findings of HPE of the patients

DNA Content Status	Histopathological Grades				TOTAL
	CIN I	CIN II	CIN III	OTHER	
Aneuploid	18 (50.0%)	12 (66.7%) p<0.00001*	26 (70.3%) p<0.00001*	1 (11.1%)	57 (57.0%)
Diploid	18 (50.0%)	6 (33.3%)	11 (29.7%)	8 (88.9%)	43 (43.0%)
TOTAL	36 (100.0%)	18 (100.0%)	37 (100.0%)	9 (100.0%)	100 (100.0%)

* Significant level (p<0.05), >0.050 -not significant.

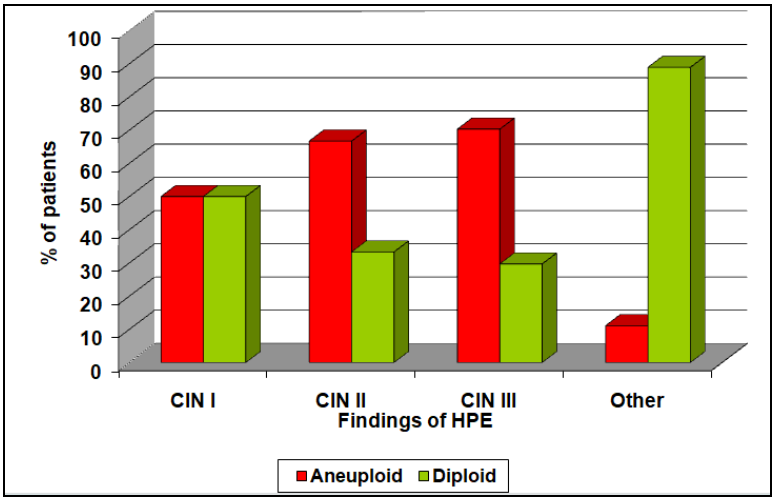


Figure 11. Bar diagram showing the association between DNA Content Status and findings of HPE of the patients

Table 8. Risk factors associated with Aneuploid DNA content and Histopathological grades

Biomarkers	Histopathological grades					
	CIN-I		CIN-II		CIN-III	
	OR with 95% Confidence Interval	p-value	OR with 95% Confidence Interval	p-value	OR with 95% Confidence Interval	p-value
Aneuploid DNA content	0.641[0.281,1.461]	0.288	1.644[0.562,4.804]	0.360	2.439[1.031,5.770]	0.039*

* Significant level (p<0.05), >0.050 -not significant.

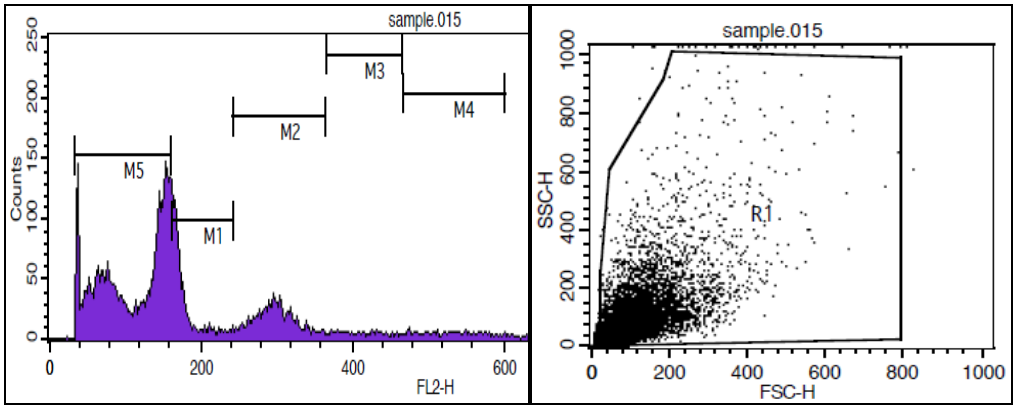


Figure 12. A. Histogram and Dot plot of CIN I showing diploid DNA content [DNA Index (DI) - 0.88]; B. Histogram and Dot plot of CIN II showing diploid DNA content (DI- 0.84); C. Histogram and Dot plot of CIN III showing diploid DNA content (DI- 0.97)

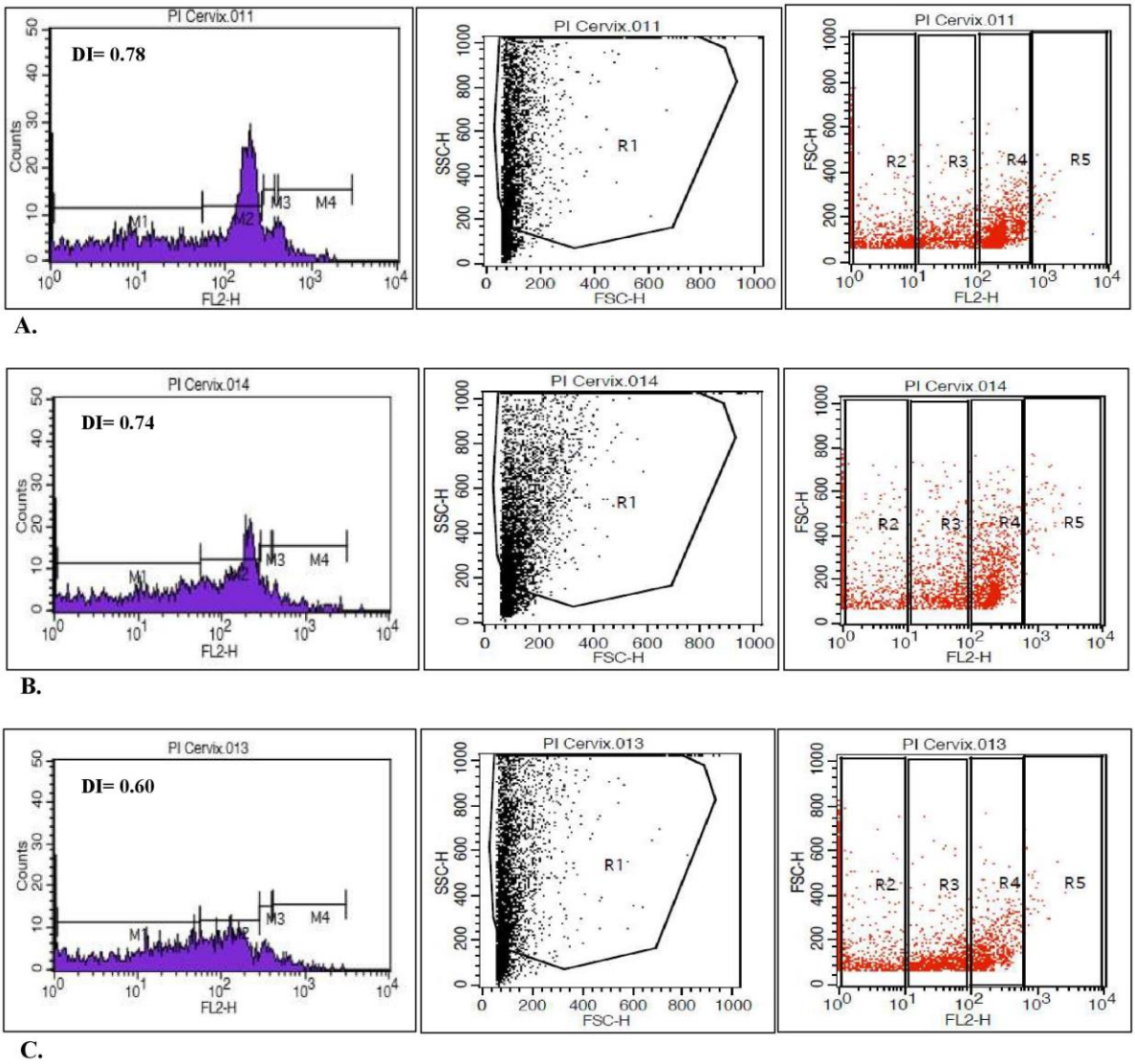


Figure 13. A. Histogram and Dot plot of CIN I showing aneuploid DNA content (DI - 0.78); B. Histogram and Dot plot of CIN II showing aneuploid DNA content (DI- 0.74); C. Histogram and Dot plot of CIN III showing aneuploid DNA content (DI- 0.60)

4. Discussion

p16 overexpression has been considered as a surrogate marker for high risk HPVs and discriminates it from non -integrated low grade HPV infection (I. Lesnikova, M. Lidang, S. Hamilton-Dutoit, et al., 2009; Sarma, U., Biswas, I., Das, A., Das, G. C., Saikia, C., & Sarma, B., 2017). Thus, it is considered as specific and sensitive biomarker of cervical pre neoplastic and non-neoplastic lesions and high-risk HPV association.

In this study, the high-risk HPV (HR HPV) association was studied, by western -Blot Analysis. The study shows that 72% cases of pre neoplastic lesions expressed HR HPV association. Two cases of Koilocytotic changes were also found to be positive for HR HPV infection. These two cases were negative for p16 immunohistochemistry. The above fact shows the higher sensitivity and specificity of Western Blot analysis over Immunohistochemistry.

Many CIN III cases expected to regress spontaneously (I. Lesnikova, M. Lidang, S. Hamilton-Dutoit, et al., 2009). The rate of regression of CIN III is almost threefold in comparison to the progression of invasive carcinoma. Therefore, the decreased conversion of p16 in high grade cervical lesions justifies the theory of regression. On the other hand, many research articles expressed view that the p16 expression increases progressively with change of histopathological grade such as CIN I has the lowest expression and CIN III has the highest (Tsoumpou, I., et al., 2009; von Knebel Doeberitz, et al., 2012; Wu, J., et al., 2014), which subsequently progress to carcinoma cervix.

Ki67, a cell cycle associated protein, is necessary for cellular proliferation and is also associated with ribosomal RNA transcription. It is expressed in all active phases of the cell cycle except resting cells (Go). According to many studies Ki 67 considered as gold standard for proliferative marker to measure the proliferative capacity of almost all malignancies (L.L. Mee, H.Z. Ahmad, D. Mohd, et al., 2008; K. Raju, S. S. Punnayanapalya & N. Mariyappa, 2015).

Normal cervical squamous epithelium shows proliferation within physiological limit and is expressed in paranasal cells. However, with increasing grades of cervical lesions, Ki67 expression increases which may be due to inactivation of p53 and Rb by E6 and E7 viral oncoproteins of HPV respectively (W. Feng, J. Xian, Z. Zhang, et al., 2007; Hwang, S. J., & Shroyer, K. R., 2012) and Ki67 expression becomes strong and more diffused involving the whole length of the epithelial layer (W. Feng, J. Xian, Z. Zhang, et al., 2007).

In the present study, Ki67 expression was highly significant and showed positivity in 73% of the cases which was similar to the finding of “Vasilescu et al., (2009)” (76.92%) and “Godoy et al., (2014)” (72.6%). Most of the studies reported that Ki67 expression increased with increasing grades of lesion (C. Isacson, T. D. Kessis, L. Hedrick, et al., 1996; J.M. Kim, D.M. Shin, A El-Naggar, et al., 2001; M.L. Looi, A. Z. Dali, S. A. Ali. et al., 2008; K. Gupta, K. Alam, V. Maheshwari, et al., 2013). This study also found that Ki67 expression increased from CIN I to CIN II but the expression slightly reduced in CIN III (CIN I 58.3%, CIN II 83.3%, CIN III 50%), which was again comparable with the study conducted by “Nam et al.” (E.J. Nam, J.W. Kim, J.W. Hong, et al., 2008).

This decrease in expression may be due to increase in host defence mechanism. It was also found that the strong intensity of Ki67 positivity was significantly higher than moderate and mild intensity, and also the Ki67 expression was significant in all layers of squamous epithelium for positive (CIN) cases similar to the findings of “Feng et al., (2007) & Looi et al.” (2008). Study of “Looi et al.” (2008), also stated that overexpression of Ki67 confirms the progression and aggressive behaviour of dysplastic and tumour cells. Whereas “Carreras et al.”, (2007) in their study has shown that Ki67 expression is useful in distinguishing the different grades of dysplasia but is unable to predict their behaviour.

In 1869, “Friedrich Miescher”, a Swiss physiological chemist, first isolated and identified DNA. The basal genome size of an organism is defined as the content of DNA, which is measured by weight or number of base pairs in a single copy of the entire sequence of DNA found within cells of that organism (T. R. Gregory, P.D.N. Hebert, 1999). It is the most frequently measured entity of the cell (Z. Darzynkiewicz, 2011) and its quantification serves to assess DNA ploidy level, cell position in the cell cycle and may also reveal the presence of apoptotic cells.

DNA content measured by cytometry is termed as DNA ploidy or DNA index (T.R. Gregory, P.D.N. Hebert, 1999; Z. Darzynkiewicz, 2011).

Persistent infection with high-risk HPVs is considered to be the strongest independent risk factor for cervical pre-neoplastic lesions and cervical cancer and chromosomal aberrations have been reported in pre-neoplastic lesions and cervical cancer (M. Singh, S. Mehrotra, N. Kalra, et al., 2008). Chromosomal aneuploidy is considered as an early key event in tumorigenesis caused by genomic instability (Melsheimer, P., et al., 2004; M. Singh, S. Mehrotra, N. Kalra, et al., 2008). Thus, DNA ploidy or DNA content assessment by flow cytometry technique can be used as a significant prognostic marker in cervical pre-neoplastic lesion and cervical cancer as aneuploid cell clones are more likely to survive and progress to more advanced dysplasia as compared to diploid cell clones. High-grade dysplasia, along with aneuploidy, has a 66% risk of developing cancer within five years

(J.M. Dunn, G.D. Mackenzie, D. Oukrif, et al., 2010).

Another study has reported the incidence of aneuploidy to vary between 14% and 75.5% in CIN I, 55% and 64.3% in CIN II and 83.3% and 95.2% in CIN III (M. Singh, S. Mehrotra, N. Kalra, et al., 2008; J.M. Dunn, G.D. Mackenzie, D. Oukrif, et al., 2010) in a study have shown that aneuploid DNA content could be used as a biomarker of progressive cervical changes toward malignancy and have found progressive increase in aneuploidy with the increase in severity of the lesion (49.36% for mild dysplasia, 77.77% for moderate dysplasia versus 91.66% for severe dysplasia) (M. Singh, S. Mehrotra, N. Kalra, et al., 2008; D. Demirel, N. Akyürek & I. Ramzy, 2013) in their study also reported abnormal DNA content in 65% of cases with cervical pre-cancerous lesions (D. Demirel, N. Akyürek & I. Ramzy, 2013). However, Melsheimer et al., 2004 reported the percentage of aneuploidy ranged from 20% in low-grade lesions (CIN I) to 32% in high-grade lesions, but they also stated that DNA aneuploidy was significantly linked to the degree of neoplastic progression (Melsheimer, P., et al., 2004). DNA ploidy can be used as a valid clinical test used in the assessment of prognosis of the cancer patients. (C.E. Duarte, et al., 2014; Aziz, D. C., & Peter, J. B., 1991).

In this study, 57% of the cases showed aneuploid DNA content, and the remaining 43% had diploid DNA content. The results of this study also found a progressive increase in aneuploidy from CIN I (50.0%) to CIN II (66.7%) to CIN III (70.3%) which was similar with the findings of “Singh et al., (2008) & Demirel et al., (2013)”. “Duesberg et al, (2004)” stated that lesions with aneuploid DNA content are considered more dangerous and aggressive. “Bollmann et al., (2001)” suggested that ASCUS with abnormal DNA content represented similar biological features as HSIL and had elevated risk to develop cancer (Bollmann, R., et al., 2001). However, univariate analysis of this study showed that CIN I with aneuploid DNA content does not carry risk, but CIN II with aneuploid DNA content carries risk, whereas CIN III with aneuploid DNA content carries a significant risk for cancer conversion.

By measuring cellular DNA content, patients at risk could be identified earlier, and treatment regimens could be given selectively to improve the current poor survival rate of cervical cancer. Thus, flow cytometric analysis of DNA ploidy may provide a strategic tool for early detection of carcinoma of uterine cervix.

Therefore, the present study supports the fact that the measurements of DNA content by flow cytometry provide important information, and the presence of aneuploidy could serve as a useful biomarker for assessing risk in cervical pre-neoplastic lesions.

Apoptosis or programmed cell death involves genetically determined elimination of damaged cells with defective repair process (Kerr J. F., 2002; J.F. Kerr, et al, 1972). It is same as an inherent anti-malignant programme of the host cells during the course of carcinogenesis (S.C. Tan & R. Ankathil, 2015).

In HPV infected cells apoptosis is a part of host defense mechanism for production of new virus particles and replication (S.C. Tan & R. Ankathil, 2015). Apoptosis is also responsible for maintaining number of cells by tight regulation and tumour formation (S.C. Tan & R. Ankathil, 2015). This pathway justifies the non-conversion of most of the CIII for malignancy. Apoptosis can be a useful biomarker for evaluating proliferative activity and progressive potential of the cervical pre-neo-plastic lesions and cervical cancer.

Studies have shown that inhibition of apoptosis plays a role in carcinogenesis since evasion of apoptosis allows accumulation of cells with damaged and unchecked genetics alteration and may also lead to unbalanced proliferative activity of tumours (M.M. Garrity, L.J. Burgart, D.L. Riehle, et al., 2003).

In this study, 63% cases were positive for Tunnel assay and the rate of apoptosis progressively increased from CINI (58%), CINII (61.1%), and CINIII (83.8%). Similar results were also observed by “Chauhan et al., (2016), Mysoekar, et al., (2008), Bharadaj. et al., (2015)”.

Univariate analysis revealed that CINIII had significant relation with apoptosis. In a study by “Gupta et al.,” also reported that apoptotic index increased progressively with increased grades of dysplasia. However apoptotic index between CIN II and CIN III was not significant but apoptotic index between CIN I and CIN II, and between CIN I and CIN III were statistically significant (K. Gupta, K. Alam, V. Maheshwari, et al., 2013).

From the above discussions, it can be stated that apoptosis is a useful and reliable biomarker for risk categorization of cervical pre neoplastic lesions that have high risk for malignant conversion.

5. Conclusions

Cervical cancer causes enormous health, social and economic problem in India and around the world. Apart from HR HPV expressed in CIN’s, other lesions such as koilocytotic changes are also caused by HR HPV.

The High-risk HPV association1 and cervical cancer is a well-established fact. Preventive vaccination for it is an important step. However certain percentage of cervical adenocarcinoma are HPV independent.

Therefore, early diagnosis of pre-neoplastic lesions, risk categorization by marker study and treatment are the

most important steps needed for stopping malignant conversion. The early diagnosis and risk assessment are not only lifesaving but also reduce the treatment cost and incidence of cervical cancer.

Ethics Approval and Consent to Participate

The study design (Project) was ethically approved by the Ethical committee of Chittaranjan National Cancer Institute (CNCI), Kolkata. CNCI is an autonomous Institute under the Ministry of Health and Family Welfare, Govt. of India.

List of Abbreviation

HR-HPV, CDKN2A, Ki-67, P53, DNA-PLOIDY, FACS, TUNEL, CINS, IARC, CDC-OPD, CDS-PAGE, ELISA, HRP, DAB.

Data Availability

To guarantee the confidentiality of personal and health information, only the authors have had access to the data. The data set may be available for research upon reasonable request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Funding Statement

The Chittaranjan National Cancer Institute provided all the required help by supplying fine and essential chemicals as well as made an easy access to use important instruments for the study.

Authors' Contributions

All the authors including AKR, DG, AR, contributed to either conception or design of the study, acquisition of data, or analysis and interpretation. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The corresponding author had final responsibility for the decision to submit for publication.

Acknowledgments

We like to thank friends, well-wishers and Authority for their encouragement and frequent selfless help for pursuing the study at Chittaranjan National Cancer Institute, Kolkata.

References

A. Godoy, K. Bazzo, L. Moura, et al., (2014). Expression Analysis of p53, Ki-67 and bcl-2 in pre malignant Lesions of the Cervix. *OJOG*, *4*, 462-469.

A. Sridevi, R. Javed, A. Dinesh et al., (2015). Epidemiology of cervical cancer with special focus on India. *Int J Womens Health*, *16*(7), 405-14. doi: 10.2147/IJWH.S50001.eCollection.

Aupperle, K. R., Boyle, D. L., Hendrix, M., Seftor, E. A., Zvaifler, N. J., Barbosa, M., & Firestein, G. S., (1998). Regulation of synoviocyte proliferation, apoptosis, and invasion by the p53 tumor suppressor gene. *The American journal of pathology*, *152*(4), 1091-1098.

Aziz, D. C., & Peter, J. B., (1991). DNA ploidy and cell-cycle analysis: tools for assessment of cancer prognosis. *Journal of clinical laboratory analysis*, *5*(6), 422-438. https://doi.org/10.1002/jcla.1860050611

Bergers, E., van Diest, P. J., & Baak, J. P., (1997). Reliable DNA histogram interpretation. Number of nuclei requiring measurement with flow cytometry. *Analytical and quantitative cytology and histology*, *19*(4), 277-284.

Bhardwaj, S. and Wani, F., (2015). Evaluating the Importance of Apoptotic Index, Mitotic Index and Turnover Index in Premalignant and Malignant Lesions of Cervix. *Open Journal of Pathology*, *5*, 29-37. doi: 10.4236/ojpathology.2015.52005.

Bollmann, R., Bollmann, M., Henson, D. E., & Bodo, M., (2001). DNA cytometry confirms the utility of the Bethesda system for the classification of Papanicolaou smears. *Cancer*, *93*(3), 222-228.

C.E. Duarte, C.R. Carvalho, A.L. Silva-Filho, (2014). Adaptation of image cytometry methodology for DNA ploidy analysis of cervical epithelium samples: a pilot study. *Taiwan J Obstet Gynecol*, *53*(2), 227-31.

C. Isacson, T.D. Kessis, L. Hedrick, et al., (1996). Both cell proliferation and apoptosis increase with lesion grade in cervical neoplasia but do not correlate with human papillomavirus type. *Cancer research*, *56*, 669-67. https://pubmed.ncbi.nlm.nih.gov.

D. Demirel, N. Akyürek, I. Ramzy, (2013). Diagnostic and prognostic significance of image cytometric DNA

ploidy measurement in cytological samples of cervical squamous intraepithelial lesions. *Cytopathology*, *24*(2), 105-12.

D. Uyar, J. Radar., (2014). Genomics of cervical cancer and the role of human papilloma virus Pathobiology. *Clin chem*, *60*, 144-146.

Duesberg, P., Li, R., & Rasnick, D., (2004). Aneuploidy approaching a perfect score in predicting and preventing cancer: highlights from a conference held in Oakland, CA in January, 2004. *Cell cycle (Georgetown, Tex.)*, *3*(6), 823-828.

E.J. Nam, J.W. Kim, J.W. Hong, et al., (2008). Expression of the p16 and ki 67 in relation to the grade of cervical intraepithelial neoplasia and high risk human papillomavirus Infection. *J Gynecol Oncol*, *19*(3), 162-168. doi: 10.3802/jgo.2008.19.3.162.Epub.

F.Bray, J. Ferlay, L.Laversanne et al., (2015). Cancer Incidence in Five Continents: Inclusioncriteria, highlights from Volume X and the global status of cancer registration. *Int J Cancer*, *137*(9), pp. 2060-71. doi: 10.1002/ijc.

F. Vasilescu, M. Ceausu, T. Cristina, et al., (2009). p53, p63 and ki-67 assessment in HPV induced cervical neoplasia. *Romanian Journal of Morphology and Embroylog*, *50*(3), 357-361.

Hu, Z., Zhu, D., Wang, W., Li, W., Jia, W., Zeng, X., Ding, W., Yu, L., Wang, X., Wang, L., Shen, H., Zhang, C., Liu, H., Liu, X., Zhao, Y., Fang, X., Li, S., Chen, W., Tang, T., Fu, A., … Ma, D., (2015). Genome-wide profiling of HPV integration in cervical cancer identifies clustered genomic hot spots and a potential microhomology-mediated integration mechanism. *Nature genetics*, *47*(2), 158-163. https://doi.org/10.1038/ng.3178.

Hwang, S. J., & Shroyer, K. R., (2012). Biomarkers of cervical dysplasia and carcinoma. *Journal of oncology*, *2012*, 507286. https://doi.org/10.1155/2012/507286

I. Lesnikova, M. Lidang, S. Hamilton-Dutoit, et al., (2009). p16 as a diagnostic marker of cervical neoplasia: a tissue microarray study of 796 archival specimens. *Diagn Pathol*, *4*(22).

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans Biological Agents, (2012). IARC Monogr Eval Carcinog Risks Hum, Article ID 100b441.

IHC World, (2008). http://www.ihcbworld.com/smf/index.php.topic=2604.0V9230.

J.F. Kerr, A.H. Wyllie, A.R. Currie, (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*, *26*, pp. 239-57.

J.M. Dunn, G.D. Mackenzie, D. Oukrif, et al., (2010). Image cytometry accurately detects DNA ploidy abnormalities and predicts late relapse to high-grade dysplasia and adenocarcinoma in Barrett's oesophagus following photodynamic therapy. *Br J Cancer*, *102*(11), 1608-7161. doi: 10.1038/sj.bjc.6605688.

J.M. Kim, D.M. Shin, D. A El-Naggar, et al., (2001). Chromosome polysomy and histological Characteristics in oral premalignant lesions. *Cancer Epidemiol Biomarkers Prev*, *10*, 319-351.

K. Raju, S.S. Punnayanapalya, N. Mariyappa, (2015). Significance of p53, pRb and Ki-67 markers in Cervical intraepithelial lesion and Malignancy. *Biomed Res Ther*, *2*, 374-384.

K. Gupta, K. Alam, V. Maheshwari, et al., (2013). Apoptotic Index and Mib-1 Antibody Expression in Premalignant and Malignant Lesions of Uterine Cervix. *Gynecol Obstet*, *3*, 173.

K. Munger, A. Baldwin, K.M. Edwards, et al., (2004). Mechanisms of human papillomavirus-induced oncogenesis. *J. Virol*, *78*(21), 11451-11460.

K. Tainio, A. Athanasiou, K.A.O. Tikkinen et al., (2018). Clinical course of untreated cervical intraepithelial neoplasia grade 2 under active surveillance: systematic review and meta-analysis. *BMJ*, *360*, k499.

Kerr J. F., (2002). History of the events leading to the formulation of the apoptosis concept. *Toxicology*, *181-182*, 471-474. https://doi.org/10.1016/s0300-483x(02)00457-2.

L.L. Mee, H.Z. Ahmad, D. Mohd, et al., (2008). Carcinoma of the uterine cervix. Analytical and quantitative cytology and histology Expression of p53, bcl-2 and Ki-67 in cervical intraepithelial neoplasia and invasive squamous cell. *Anal Quant Cytol Histol*, *2*, 63-70.

M. Narisawa-Saito, T. Kiyono, (2007). Basic mechanisms of high-risk human papillomavirus-induced carcinogenesis: roles of E6 and E7 proteins. *Cancer Sc*, *98*(10), 1505-1511.

M. Singh, S. Mehrotra, N. Kalra, et al., (2008). Correlation of DNA ploidy with progression of cervical cancer. *J Cancer Epidemio*. doi: 10.1155/2008/298495. Epub.

M.L. Looi, A.Z. Dali, S.A. Ali.et al., (2008). Expression of p53, bcl-2 and Ki-67 in cervical intraepithelial

neoplasia and invasive squamous cell carcinoma of the uterine cervix. *Analytical and quantitative cytology and histology*, *30*, 63-70.

M.M. Garrity, L.J. Burgart, D.L. Riehle, et al., (2003). Identifying and quantifying apoptosis: navigating technical pitfalls. *Mod Pathol*, *16*(4), 389-94.

Melsheimer, P., Vinokurova, S., Wentzensen, N., Bastert, G., & von Knebel Doeberitz, M., (2004). DNA aneuploidy and integration of human papillomavirus type 16 e6/e7 oncogenes in intraepithelial neoplasia and invasive squamous cell carcinoma of the cervix uteri. *Clinical cancer research: an official journal of the American Association for Cancer Research*, *10*(9), 3059-3063. https://doi.org/10.1158/1078-0432.ccr-03-0565

Münger, K., Werness, B. A., Dyson, N., Phelps, W. C., Harlow, E., & Howley, P. M., (1989). Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *The EMBO journal*, *8*(13), 4099-4105. https://doi.org/10.1002/j.1460-2075.1989.tb08594.x

Qin, L. X., Tang, Z. Y., Ma, Z. C., Wu, Z. Q., Zhou, X. D., Ye, Q. H., Ji, Y., Huang, L. W., Jia, H. L., Sun, H. C., & Wang, L., (2002). P53 immunohistochemical scoring: an independent prognostic marker for patients after hepatocellular carcinoma resection. *World journal of gastroenterology*, *8*(3), 459-463. https://doi.org/10.3748/wjg.v8.i3.459.

R. Chauhan, N. Verma, S.P. Sharma, et al, (2016). Role of apoptotic index, mitotic index and MIB 1 antibody expression as biomarkers in preneoplastic and neoplastic lesions of uterine Cervix. *Int J Res Med Sci*, *4*, 2093-100.

R. SalesGil, P. Vagnarelli, (2018). Ki-67: More Hidden behind a Classic Proliferation Marker. *Trends Biochem Sci*, *43*(10), pp. 747-748. doi: 10.1016/j.tibs.2018.08.004.

R.A.F. Carreras, G.Mancebo, (2007). A study of Ki-67, c-erbB2 and cyclin D-1 expression in CIN-I, CIN-III and squamous cell carcinoma of the cervix. *Histol Histopathol*, *22*(6), 587-592.

S. Nagase, T. Ohta, F. Takahashi et al., (2022). A report of the committee on gynecologic oncology, the Japan society of obstetrics and gynecology: annual patient report for 2018 and annual treatment report for 2013. *J Obstet Gynaecol Res*, *48*(3), pp. 541-552. doi: 10.1111/jog.15134.Epub.

S. Stolnicu, L. Hoang, R. A. Soslow et al, (2019). Recent advances in invasive adenocarcinoma of the cervix. *Virchows Arch*, *475*, 537-549. DOI:10.1007/s00428-019-02601-0.

S.C. Tan, R. Ankathil, (2015). Genetic susceptibility to cervical cancer: role of common polymorphisms in apoptosis-related genes. *Tumour Biol*, *36*(9), 6633-44.

Sarma, U., Biswas, I., Das, A., Das, G. C., Saikia, C., & Sarma, B., (2017). p16INK4a Expression in Cervical Lesions Correlates with Histologic Grading — a Tertiary Level Medical Facility Based Retrospective Study. *Asian Pacific journal of cancer prevention: APJCP*, *18*(10), 2643-2647. https://doi.org/10.22034/APJCP.2017.18.10.2643.

T.R. Gregory, P.D.N. Hebert, (1999). The Modulation of DNA Content: Proximate Causes and Ultimate Consequences. *Genome Res*, *9*, 317-324.

The Cancer Genome Atlas Research Network, (2017). Integrated genomic and molecular characterization of cervical cancer. *Nature*, *543*, 378-384. https://doi.org/10.1038/nature21386

Thorland, E. C., Myers, S. L., Gostout, B. S., & Smith, D. I., (2003). Common fragile sites are preferential targets for HPV16 integrations in cervical tumors. *Oncogene*, *22*(8), 1225-1237. https://doi.org/10.1038/sj.onc.1206170

Tsoumpou, I., Arbyn, M., Kyrgiou, M., Wentzensen, N., Koliopoulos, G., Martin-Hirsch, P., Malamou-Mitsi, V., & Paraskevaidis, E., (2009). p16(INK4a) immunostaining in cytological and histological specimens from the uterine cervix: a systematic review and meta-analysis. *Cancer treatment reviews*, *35*(3), 210-220. https://doi.org/10.1016/j.ctrv.2008.10.005

V.V. Baker., (2008). Molecular Biology of Gynecologic Cancer. *Glob. libr. women's med*., (ISSN: 1756-2228), DOI: 10.3843/GLOWM.10275.

V.V. Mysorekar, S. David, S.G. Rao, (2008). Proliferative and Apoptotic Indices in Squamous Epithelial Lesions of the Cervix. *Bahrain Medical Bulletin*, *30*, 156-9.

von Knebel Doeberitz, M., Reuschenbach, M., Schmidt, D., & Bergeron, C., (2012). Biomarkers for cervical cancer screening: the role of p16(INK4a) to highlight transforming HPV infections. *Expert review of proteomics*, *9*(2), 149-163. https://doi.org/10.1586/epr.12.13

W. Feng, J. Xian, Z. Zhang, et al., (2007). Senescence and apoptosis in carcinogenesis of cervical squamous

carcinoma. *Mod Pathol*, *20*, 961-6.

Wu, J., Li, X. J., Zhu, W., & Liu, X. P., (2014). Detection and pathological value of papillomavirus DNA and $p16^{INK4A}$ and p53 protein expression in cervical intraepithelial neoplasia. *Oncology letters*, *7*(3), 738-744. https://doi.org/10.3892/ol.2014.1791

Y. Azuma, R. Kusumoto-Matsuo, F.Takeuchi, et al., (2014). Human papillomavirus genotype distribution in cervical intraepithelial neoplasia grade 2/3 and invasive cervical cancer in Japanese women. *Jpn J Clin Onco*, *44*(15), 910-7. Article ID doi: 10.1093/jjco/hyu112.

Z. Darzynkiewicz, (2011). Critical aspects in analysis of cellular DNA content. *Curr Protoc Cytom*, Chapter 7, 7.2.1-7.2.8. doi: 10.1002/0471142956.