INTRODUCTION

The retinoblastoma (RB) tumor suppressor protein plays a pivotal role in the control of cell cycle, terminal differentiation, and various other biological events (Dyson, 2016). RB is genetically or functionally inactivated in numerous human cancers, including retinoblastoma, small cell lung cancer (SCLC), prostate cancer, and breast cancer (Shunsuke *et al.,* 2020). The retinoblastoma susceptibility gene (*RB1*) was the first tumor suppressor gene to be molecularly defined and the retinoblastoma protein (pRb) which is its gene product regulates transcription and is a negative regulator of cell proliferation (Dyson, 2016). Major task of *RB1* is to prevent unwarranted cell growth by inhibiting cell cycle advancement until a cell is ready to divide. When the cell is ready to divide, *RB1* is phosphorylated to pRb, driving to the inactivation of the action of pRb. This manner permits cells to pass in into the cell cycle state (Shunsuke *et al.,* 2020). RB belongs to the pocket protein family, whose members have a pocket for the functional binding of other proteins (Korenjak and Brehm, 2005). Should an oncogenic protein, such as those shaped by cells infected by high-risk types of human papillomaviruses, bind and inactivate pRb, this can lead to cancer. The canonical pathway whereby RB exerts its tumor suppressive activity entails the formation of a transcriptional repression complex with E2F transcription factors and various chromatin modifiers, such as histone deacetylases (HDACs). This complex orchestrates the G1/S transition during cell cycle progression primarily by controlling E2F target genes (Dyson, 2016; Kitajima and Takahashi, 2017).

Over four decades of work have revealed that the Rb protein (pRb) is a master regulator of biological pathways influencing virtually every aspect of intrinsic cell fate including cell growth, cell-cycle checkpoints, differentiation, senescence, self-renewal, replication, genomic stability and apoptosis. While these many processes may account for a significant portion of *RB1*'s potency as a tumor suppressor, a small, but growing stream of evidence suggests that *RB1* also significantly influences how a cell interacts with its environment, including cell-to-cell and cell-to-extracellular matrix interactions (Engel *et al.,* 2015).
The retinoblastoma tumor-suppressor gene (*Rb1*) is centrally important in cancer research. Mutational inactivation of *Rb1* causes the pediatric cancer retinoblastoma, while deregulation of the pathway in which it functions is common in most types of human cancer. The *Rb1*-encoded protein (pRb) is well known as a general cell cycle regulator, and this activity is critical for pRb-mediated tumor suppression (Goodrich, 2006). The molecular mechanisms underlying pRb functions are based on the cellular proteins it interacts with and the functional consequences of those interactions. Better insight into pRb-mediated tumor suppression and clinical exploitation of pRb as a therapeutic target will require a global view of the complex, interdependent network of pocket protein complexes that function simultaneously within given tissues (Goodrich, 2006). Most mitogenic signals commonly merge on the transcriptional upregulation of D-type cyclins and then stimulate cyclin-dependent kinases, including CDK4/6. D-type

cyclin-CDK4/6 complexes have been proposed to promote mono-phosphorylation on RB, which allow it to exert early G1 functions by starting the release of E2Fs. E2F target genes, including cyclin E and A, in cooperation with CDK2 or CDK1, are responsible for full phosphorylation of RB at 13 remaining sites. This allows cells to enter the S and M phases (Rubin, 2013; Dyson, 2016; Sanidas *et al.,* 2019). Since uncontrolled cell proliferation is a hallmark of cancer cells, it has been postulated that genes inhibiting RB function, including CCND1 and CDK4, act as oncogenes. Conversely, genes activating RB function, including cyclin-dependent kinase inhibitors (e.g., CDKN1A, CDKN1B, and CDKN2A), are well-known to act as tumor suppressor genes (Witkiewicz *et al.,* 2011; Shunsuke *et al.,* 2020). The fact that genetic and/or epigenetic aberrations of the components in the RB pathway tend to be mutually exclusive in the patients might implicate a linearity of the RB pathway (Shunsuke *et al.,* 2020).

Cervical cancer is a major gynaecological cancer which encompasses hysterical cell division and tissue invasiveness of the female uterine cervix (Dasari *et al.,* 2015). Cervical cancer is the second most common cause of cancer-related death among women worldwide, with over 500,000 new cases diagnosed annually and 50% mortality rate in Asia (Daniyal *et al.,* 2015). Among the female population in Nigeria, it is the most common cancer (Mohammed *et al.,* 2006). In 2007, it was reported that 36.59 million women aged ≥15 years in Nigeria are at risk of developing cervical cancer. Cell division requires cell cycle checkpoints (CPs) that are used by the cell to both monitor and regulate the progress of the cell cycle. Tumor-suppressor genes (TSGs) or antioncogenes are genes that protect the cell from a single event or multiple events leading to cancer. When these genes mutate, the cell can progress to a cancerous state (Ana and Howard, 2015). Several unique classes of checkpoint proteins exist, and among these are the TNFR superfamily (Locksley *et al.,* 2001) and B7 family (Collins *et al.,* 2005), which can influence immune cells differently based on their expression patterns. Cancer cells can disrupt the immune response through the overexpression of inhibitory molecules like PD-L1 (Patel and Kurzrock, 2015) or the loss of expression of stimulatory molecules like CD40L (Dimberg *et al.,* 2006). Tumors can evade immune surveillance even in the presence of tumor antigens because the immune cells may not receive adequate signals for activation and proliferation or are suppressed by inhibitory checkpoint proteins (Whiteside, 2006). The decision to commit to a new round of cell division occurs when the cell activates cyclin-CDK-dependent transcription which promotes entry into S phase (*Bertoli et al., 2013*). During early G1, the transcriptional repressors Rb (retinoblastoma), p107 and p130, known as pocket proteins, bind to the E2F transcription factors to prevent G1-to-S transition. Rb binds and represses activator E2F transcription factors (E2F1-3), while p107 and p130 bind E2F4 and E2F5 respectively to form complexes which repress transcription of G1-to-S promoting factors (proteins) (*Bertoli et al., 2013*). Upon the decision to progress past the G1 checkpoint, cyclin D levels rise, and cyclin D forms a complex with CDK4 and CDK6, which in turn phosphorylate the pocket proteins. Phosphorylation of the pocket proteins causes the release of their bound targets, thereby relieving the repression of the E2F1-3 activators and translocating repressors E2F4 and E2F5 from the nucleus to the cytoplasm. This results in the transcriptional activation of downstream targets, which promote the G1-to-S transition,

including another cyclin, known as cyclin E, which forms a complex with CDK2 (*Bertoli et al., 2013*). This study therefore investigate the expression of retinoblastoma protein **(**pRb) in grades of cervical lesion.

MATERIALS AND METHODS

Study Population

This study was conducted on tissue blocks from women that had CIN I, II, III and cancer of the cervix in Lagos University Teaching Hospital, Lagos. The age of the patients ranged from 28 to 64years. The duration of the tissue blocks used was between year 2002-2016.

Ethical Consideration

Ethical approval for this study was obtained from the Ethics and Research Committees of Lagos University Teaching Hospital, Lagos

Specimen collection

Two hundred (200) cases of archived formalin fixed paraffin embedded (FFPE) tissues of already diagnosed cervical lesions and cervical carcinoma tissue blocks were selected for this study.

Histopathological Procedures

Each formalin fixed paraffin wax embedded tissue was sectioned to harvest tissue areas with the highest lesion. Sections of about 3µ thick were cut for immunohistochemical staining technique. Four sections were obtained from each block of CIN 1, CIN 2, CIN 3 and cervical carcinoma; from which one section was used for haematoxylin and eosin staining technique, one section was treated with pRb while the other section served as negative and positive controls. Haematoxylin and eosin method was carried out to confirm earlier diagnosis before proceeding to the immunohistochemical analysis.

Haematoxylin and Eosin Staining Technique

The sections were taken to water, stained using Cole's haematoxylin for 10min, washed in tap water then differentiated in 1% acid alcohol for few seconds. The slides were rinsed in water and blued in running tap water for 10min. Stained sections were counterstained in 1% aqueous eosin for 2min. They were then washed in tap water, dehydrated, cleared and mounted in DPX (Avwioro, 2014).

Immunohistochemical Technique

Monoclonal antibodies for pRb were purchased from ABCAM life science Plc, UK. The Mouse and Rabbit Specific horseradish peroxidase/ diaminobenzidine (HRP/DAB) detection IHC kit from ABCAM life science Plc, UK were used for immunostaining.

The method that was used is the Avidin Biotin Complex (ABC) method and the antibodies/Proteins used were manufactured by Novocastra. The antibody dilution factor used was 1:100 dilutions for all the antibody markers.

The processed tissues were sectioned at 3µ on the rotary microtome and dried on the hot plate at 70^{o}C for over 1hr.

Sections were taken to water by passing them through 2 changes of Xylene, 3 changes of descending grades of alcohol and then to water. Hydrated sections were heated in a citric acid solution of PH 6.0 using the microwave at 100^{o}C for 15min to retrieve the antigen and equilibrated gradually with cool water to displace the hot citric acid for 5min. Peroxidase blocking was done on the sections by covering them with 3% hydrogen peroxide (H_2O_2) for 15min and washed with Phosphate buffer saline (PBS) while protein blocking was performed in avidin for 15min. Sections were washed in PBS and endogenous biotin in tissue was blocked using biotin for 15min. Following washing in PBS, sections were incubated with the diluted (1:100) primary antibody/Proteins (pRb) for 60 min. Excess antibody were washed off with PBS and a secondary antibody (link) was applied on sections for 15min. Sections were washed and Horseradish Peroxidase (HRP) was applied on the sections for 15min. A working DAB solution (3, 3'-diaminobenzidine) was prepared by mixing 1 drop (20µl) of the DAB chromogen in 1ml of the DAB substrate and a drop applied on sections after washing off the Horseradish Peroxide (HRP) in PBS for 5min. The brown reaction began to appear at this moment especially for a positive target. Excess DAB solution and precipitate was washed with distilled water. Sections were counterstained in Mayer's haematoxylin solution for 2min and blued briefly, dehydrated in ascending grades of alcohol, cleared in xylene, and mounted in DPX (Marc, 2009).

Control for Immunohistochemistry (IHC):

Positive control sections for the marker was obtained from tissues that are known to express the antigen. In negative control, tissues that are kown not to express the antibody marker was used, while in the reagent negative controls, pRb antibody that is being tested was omitted.

Reading of Immunohistochemistry results

Cells with specific brown colours in the cytoplasm, cell membrane or nuclei depending on the antigenic sites were observed. The haematoxylin stained cells without any form of brown colours were scored negative. Non-specific binding/brown artifacts on cells and connective tissue was disregarded (Marc, 2009).

Immunoreactive Scoring System Used by Klein *et al*. (1999).

The immunohistochemical staining was semi-quantitatively scored based on percentage of cells that stained positive and the intensity of the staining. 'A' is the percentage (%) or the proportion of the tumour cells stained which is graded as '0' when it is 0% and '1' when is <30%, '2' when it is between 30%-60% and '3' when it is >60%. 'B' is the intensity of the tumour cells stained which is graded as '0' when there is no reaction (negative) and '1' when there is a weak reaction, '2' when there is a mild reaction and '3' when there is a strong reaction. The final score is the addition of 'A' and 'B' which range from 0 to 6.

Final interpretation of the IHC scoring:

0/6= Negative reaction

1/6, 2/6 and 3/6 = Low expression

4/6, 5/6 and 6/6 = High expression

Statistical Analysis

Photomicrograph was basically used for analyzing the expression and comparative analysis of the data generated from the expression of the pRb protein were statistically analyzed by

the statistical data software SPSS version 16 (SPSS Inc. Chicago, Illinois) for windows. Descriptive statistical analysis was also used to analyse some of the data. Frequency of data was calculated using chi-square (x^2).

RESULTS

The tables below summarizes the expression of pRb protein in CIN 1, CIN 2, CIN 3, and Cervical squamous cell carcinoma (SCC).

pRb was highly expressed in 48.9% of the overall CIN diagnosis but in 10.1% of SCC cases. Low expression of pRb was observed in 46.5% and 50.7% of overall CIN and SCC cases respectively. pRb expression was significantly reduced as the dysplasia progresses ($p<0.05$) (Table 1A).

pRb was most expressed among participants diagnosed of CIN II (53.2%), followed by CIN I(48.8%), CIN III (43.9%) whereas those diagnosed of SCC had the least expression of pRb (10.1%). The expression of pRb in CIN I, II, and III as well as SCC significantly reduced as the CIN cases matured to malignancy ($p<0.05$) with CIN III been observed with the least expression of pRb among CIN cases. Generally, the SCC revealed the least expression of pRb (10.1%) in all dysplasia cases (Table 1B).

Table 1A: Expression of pRb in Overall CIN and SCC

Case	No. tested	**Positive Expression**		**Negative Expression (No expression)(%)**
		High(%)	Low(%)	
CIN	**131**	64(48.9)	59(46.5)	8(4.6)
CSCC	**69**	7(10.1)	35(50.7)	27(39.2)

*p<0.05

CIN - Cervical Intraepithelial Neoplasia; SCC - Squamous cell carcinoma of the Cervix

Table 1B: Expression of pRb in CIN I, CIN II, CIN III, and Squamous cell carcinoma of the Cervix

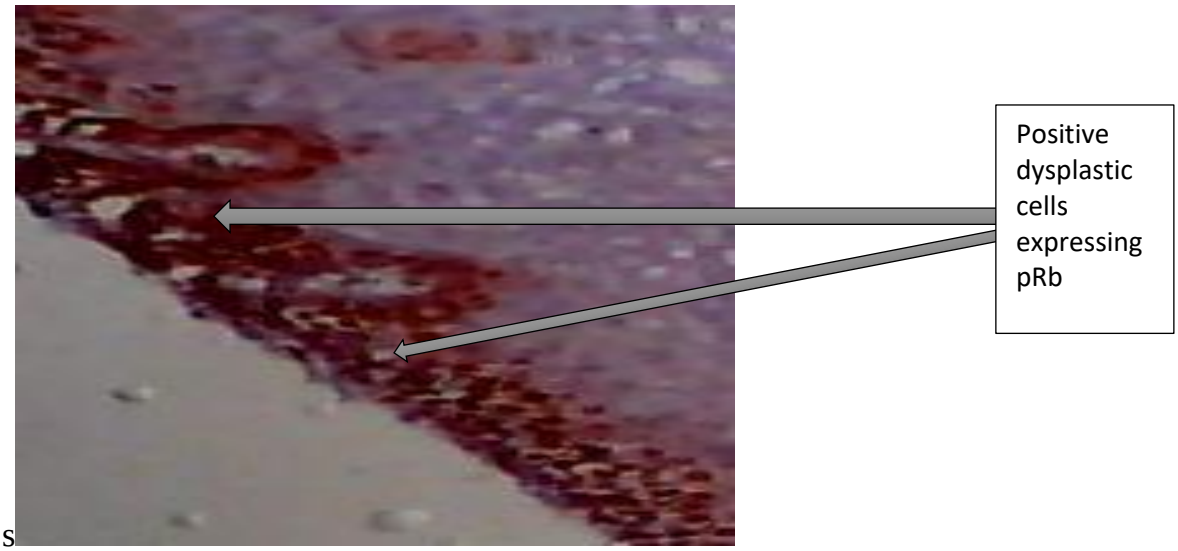
Case	**No. tested**	Positive Expression		Negative Expression
		Low(%)	High(%)	(No expression)(%)
CIN I	**43**	20(46.5)	21(48.8)	2(4.7)
CIN II	**47**	22(46.8)	25(53.2)	0
CIN III	**41**	17(41.5)	18(43.9)	6(14.6)
SCC	**69**	35(50.7)	7(10.1)	27(39.2)

*p<0.05

CIN - Cervical Intraepithelial Neoplasia; SCC - Squamous cell carcinoma of the Cervix

In this study, strong expression of pRb was observed in CIN I which was demonstrated endogenously with the brownish pigmentation on the section (Plate 1).

Moderate expression of pRb was observed in CIN II that was demonstrated endogenously with the brownish pigmentation on the section (Plate 2).
Mild expression of pRb was observed in CIN III which was demonstrated endogenously with the brownish pigmentation on the section (Plate 3).
Low expression of pRb was indicated in SCC which was demonstrated endogenously with the brownish pigmentation on the section (Plate 4).
There was no expression of pRb in SCC (Plate 5).



S

Plate 1: Immunohistochemistry of Strong Expression of pRb in CIN I (mag x400)

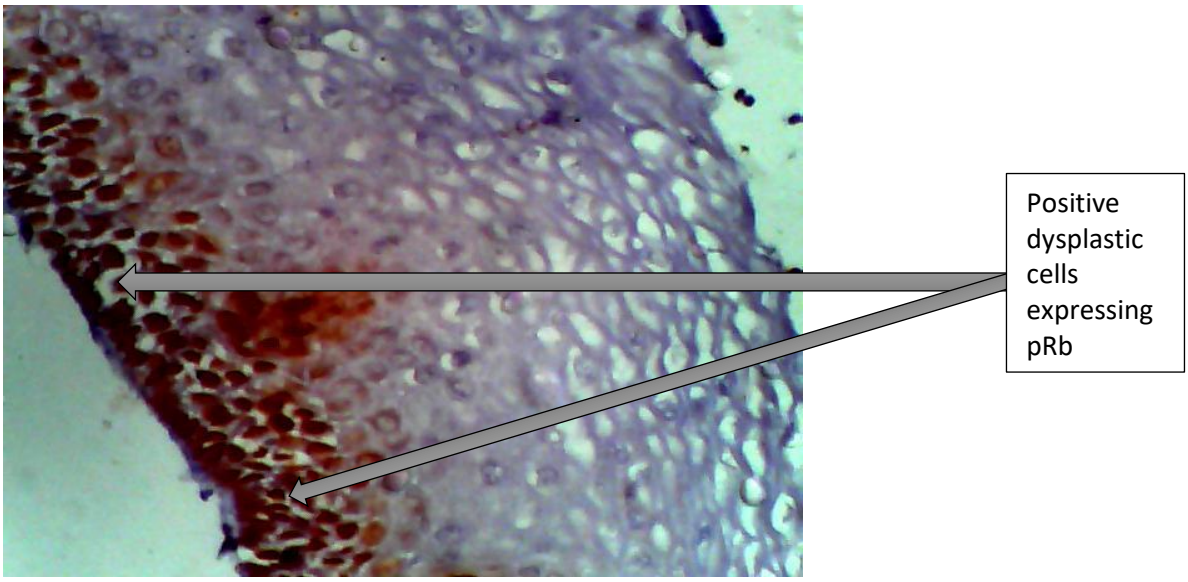


Plate 2: Immunohistochemistry of Moderate Expression of pRb antibody in CIN II (mag x400)

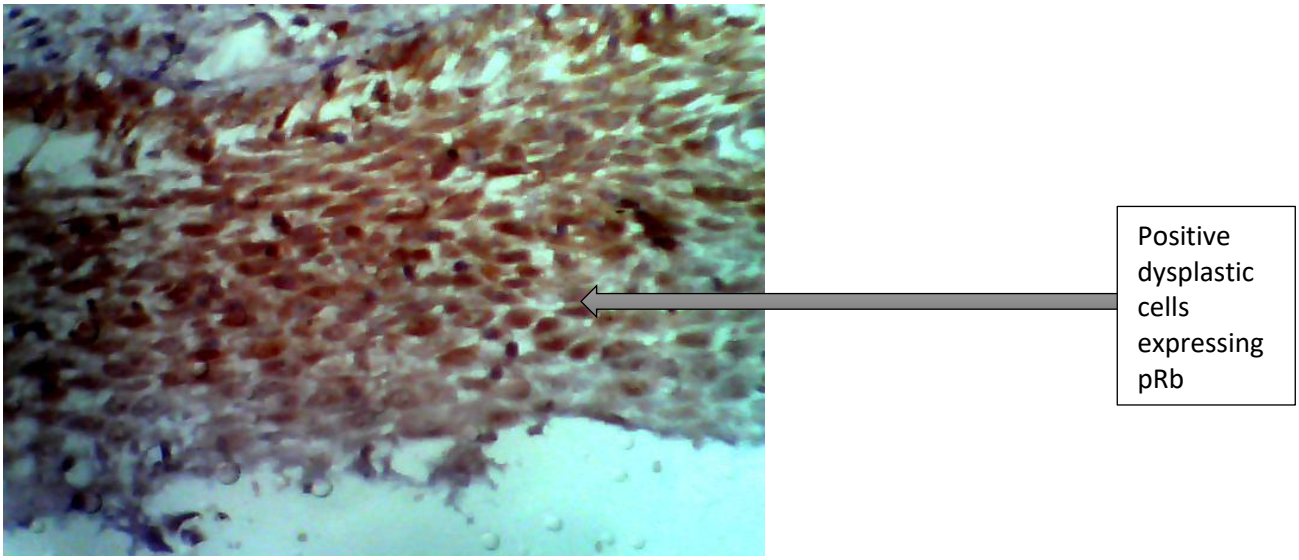


Plate 3: Immunohistochemistry of Mild Expression of pRb antibody in CIN III (mag x400)

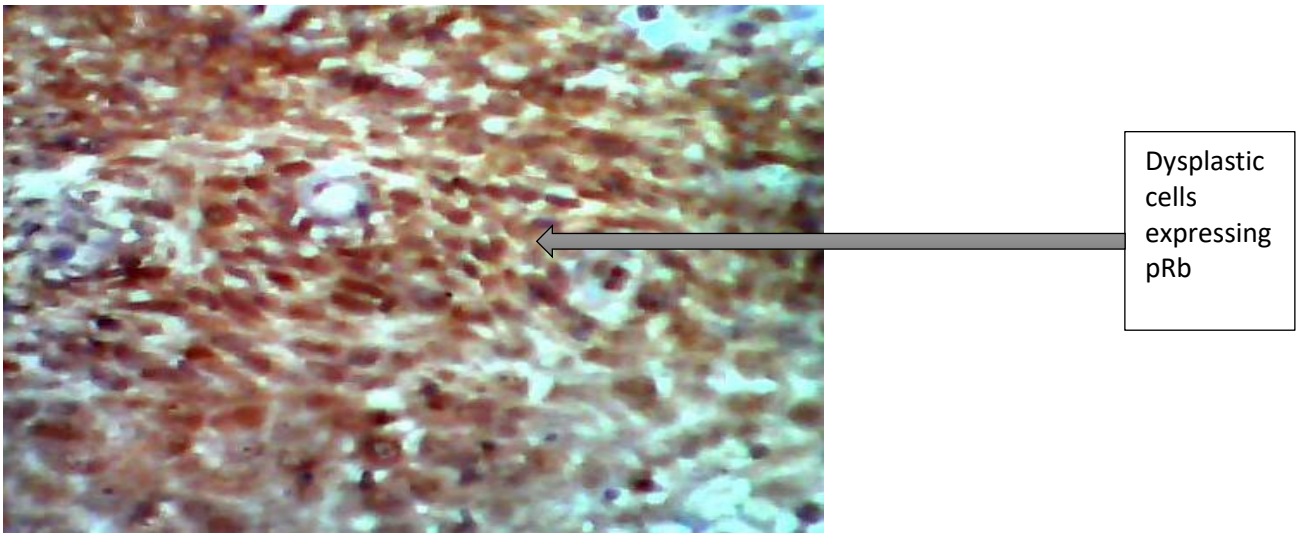


Plate 4: Immunohistochemistry of Low Expression of pRb antibody in SCC (mag x400)

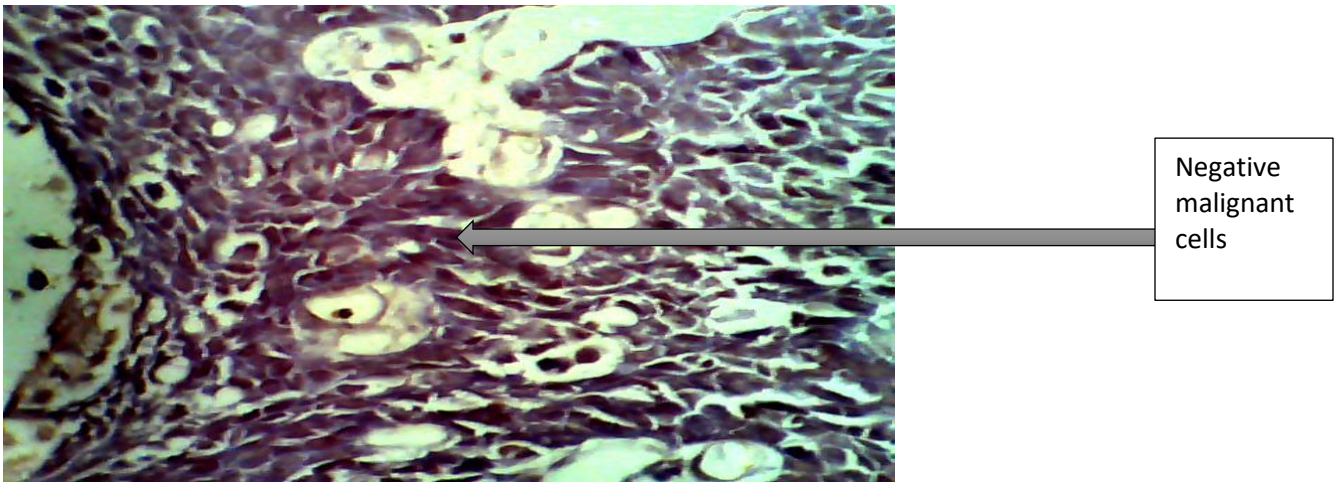


Plate 5: Immunohistochemistry of Negative Expression of pRb antibody in SCC (mag x400).

DISCUSSION

The RB tumor suppressor pathway has been extensively studied (Dick *et al.*, 2018). The retinoblastoma protein (pRb) is a cell cycle regulator predominantly known as cell cycle repressor deactivated in most human cancers. Loss of pRb function results from mutations in the gene coding for pRb or for any of its upstream regulators (Sosa-García *et al.*, 2010).

The inactivation of RB1 through somatic mutation, deletion, or epigenetic silencing in various cancers has been reported. Hypophosphorylated pRB binds to and represses the transcriptional activity of E2F family members, which control the expression of genes necessary for cell cycle progression (Wang *et al.,* 2018).

In this study, the expression of retinoblastoma Protein (pRb) was observed to be 95.3% in CIN I cases, 100% in CIN II cases, 85.4% in CIN III cases, and 60.8% in SCC cases. Low level expression of pRb was observed to be more frequent in SCC cases (50.7%) followed by CIN II (46.8%), CIN I (46.5%), and CIN III (41.5%). This study showed pRb immunoreactivity to be frequent in majority of the CIN and SCC cases which is comparable to the findings by several authors that reported ubiquitous expression of pRb in normal or cancerous tissues (Horowitz *et al.* 1990; Furukawa *et al.,* 1991; Cordon-Cardo and Richon, 1994; Salcedo *et al.,* 2002); Some studies that were also done on normal uterine-cervix tissue shows that pRb is expressed in mature and differentiated cells, in the basal third epithelium in 90% of normal/reactive atypia or in the scattered nuclei of normal cells in all cases tested (Benedict *et al.,* 1990; Cance *et al.,* 1990; Cordon-Cardo *et al.,* 1992). This study observed that pRb immunoreactivity in SCC was frequently lower than in CIN which is in agreement with the report of some workers (Ludlow *et al.,* 1993; Boyer *et al.,* 1996) suggesting that the downregulation of retinoblastoma (RB) gene could be involved in cervical carcinogenesis. The low expression of pRb may also be linked to inactivation of pRb which is as a result of complex formation with high risk HPV E7 oncoprotein and its degradation, downregulation mechanisms (Boyer *et al.,* 1996; Whyte *et al.,* 1988; Dyson *et al.,* 1989). The heterogeneous pRb immunostaining observed in CIN stages as compared to SCC in this study is similar to that reported by Salcedo *et al.* (2002) which also observed heterogeneous pRb immunostaining during the different stages of cervical carcinogenesis and suggest that this staining pattern could be a common feature implicated in pathological process of uterine-cervix carcinoma. This study also showed a positive statistical correlation between the degree of dysplasia and the degree of pRb expression in all the cases which indicates that as degree of cervical dysplasia increases (from premalignant to malignant lesion), there will be gradual decrease in pRb expression; this findings on CIN and SCC can be related to the suggestion of some authors that pRb negative tumours might be clinically more aggressive and with a poorer prognosis than those tumours containing a variable pRb expression (Benedict *et al.,* 1990; Hanson *et al.,* 1994; Harbour *et al.,* 1988). In support of this possibility, some studies also reported that high RB gene expression inhibits tumour cell invasion *in vitro* (Li *et al*., 1996).

CONCLUSION

Retinoblastoma protein (pRb) expression revealed that as the degree of cervical dysplasia progresses (premalignant to malignant lesion), there was gradual decrease in pRb expression. The pRb immunoreactivity in SCC is frequently lower than the overall CIN cases. The downregulation of retinoblastoma (RB) protein may indicate mutation of RB gene in cervical lesions and it could be involved in cervical carcinogenesis.

CONFLICT OF INTEREST

Authors declare no conflicts of interest

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