IJISET - International Journal of Innovative Science, Engineering & Technology, Vol. 8 Issue 5, May 2021
ISSN (Online) 2348 – 7968 | Impact Factor (2020) – 6.72
www.ijiset.com

The Predictive Significance of P16INK4A, Ki-67 and Ck7 Expressions in the Progression to SCC of the Cervix

Ekundina Victor Olukayode[1], Oladele Abraham Ayowole[1], Nwachukwu Elizabeth Obichukwu[1], Ogazi MacDonald[1], and Sanya Joseph Olurotimi[2]

Departments of [1]Medical Laboratory Science (Histocytopathology Unit) And [2] Department of Physiology, college of medicine and health sciences, Afe Babalola University, Ado-Ekiti, Ekiti, Nigeria.

Corresponding author address: ekundinav@abuad.edu.ng, +2348038836691

ABSTRACT

Expression of P16INK4A, Ki-67, and CK7 IHC markers wasevaluated to determine theirpredictive significance in progression to SCC of the cervix.IHC analysis of the expression of P16INK4A, Ki-67 and Ck7 was performed on 50 formalin fixed, paraffin-embedded tissue blocks retrieved from pathology archives. The tissue blocks were divided into normal cervical tissue (5 cases), CIN I (10 cases), CIN II&III (15 cases) and SCC (20 cases) of the cervix according to histologic diagnosis. Expression of P16INK4A was significantly higher in CIN I (50%), CIN II&III (75%) and SCC (95%) of the cervix than in the normal cervical tissue (20%). The positivity rate of Ki-67 expression was upregulated in the transition from normal cervical tissue (20%) to CIN I (60%), CIN II&III (86.7%) and SCC of the cervix (90%). There was a gradual increase in Ck7 staining intensity in the cases ranging from normal cervical tissue (20% expressing strong staining intensity) to SCC of the cervix (65% expressing strong staining intensity). A positive relationship between the degree of expression of P16INK4A, Ki-67 and Ck7, and the severity of the lesions in the progression to SCC of the cervix was observed through the course of this study. While these markers have proven to be effective in predicting the progression of normal cervical tissue or CIN to cervical SCC based on their staining patterns, none of these markers can stand on its own to give a fully definitive result and should be used in concordance with each other to compensate for their limitations and obtain relevant results.

Keywords:IHC, SCC, P16INK4A, Ki-67, Ck7

INTRODUCTION

Cervical cancer is a disease in which malignant cells proliferate within the tissues of the cervix (Hasan, 2009). Although the disease is regarded as preventable, it has proven to be a significant public health issue; a significant number of deaths are recorded each year globally (Bisi-Onyemaechi *et al*., 2018). The uterine cervix is positioned anatomically below the cavity of the uterus and forms a canal with an internal and external os (Tiran, 2010). The squamocolumnar junction (SCJ) is the point where the endocervical and ectocervical epithelium meet. The SCJ is particularly susceptible to HPV infection and most squamous cell carcinoma develops at this point(Reich*et al*., 2017) (Ganesan *et al*., 2015). The cervical epithelium undergoes precancerous changes before it progresses to cancer, these changes are called cervical intraepithelial neoplasias (CINs). These classifications are; very mild to mild (CIN I), moderate dysplasia (CIN II) and severe in situ carcinoma dysplasia (CIN III) depending on their level of dysplasia. Most mild dysplastic lesions disappear spontaneously, but a significant proportion of the CIN III lesions

may progress into squamous cell carcinoma (SCC) of the cervix if left untreated (Marth *et al*., 2017).Cervical cancer that develops from squamous epithelial cells within the cervix is called squamous cell carcinoma (SCC). Most cervical cancers are squamous cell carcinomas. SCC is responsible for about 80 to 90% of all cervical cancer (Hasan, 2009). Predicting which cases of CIN will progress, regress or persist is not possible with regular histological and cytological techniques. Although the screening through the cytological techniques has aided in the reduction of mortality and morbidity rates, the success of this screening method is limited with respect to sensitivity and specificity. Interpretation of the cytology screening relies on subjective, morphological evaluation. Interpretation of histopathological techniques is affected by interobserver discordance (Murphy *et al*., 2003). Immunohistochemistry (IHC) is a method for localizing specific antigens in tissues primarily based on antigen-antibody recognition; it seeks to take advantage of specificity furnished through the binding of an antibody with its antigen at a light microscopy level (Dabbs, 2013). IHC can be employed to study the molecular changes in tissues, identifying cellular modifications not generally seen with H&E, providing a relatively rapid and simple method to better determine the origin of neoplastic tissue or investigate the behaviour or progression of a given neoplasm (Painter *et al*., 2010).

Studies have proven that HPV plays an etiologic role in cervical cancer (Wang *et al*., 2004). Integration of high risk HPV genome into the human chromosome will result in the stabilization oncogenes E6 and E7 through the disruption of viral E2 gene. E7 binds to retinoblastoma protein leading to loss of cell cycle control. Functional inactivation of retinoblastoma results in overexpression of P16INK4A, as retinoblastoma inhibits P16INK4A transcription. Recent studies have shown IHC expression of P16INK4Ais relevant in the prediction of the transition of cervical tissue to SCC of the cervix (Lee *et al*., 2017).

Ki-67 is a nuclear antigen that is a cellular marker for proliferation (Shi *et al*., 2019). An increase in cell proliferation will cause a significant rise in expression of Ki-67.Expression of Ki-67 can be said to have a direct relationship with the progression of to SCC of the cervix as cell proliferation increases in concordance to the severity of a lesion (Bullwinkel *et al*., 2006).

Cytokeratin (CK) 7 is an SCJ marker. Ectocervical cells that appear normal but are positive for CK7 have been found to express HPV E2 which is an early marker of HPV infection. These cells are also positive for HPV E6/E7 thus supporting the role of these cells in cervical carcinogenesis. Most CIN and carcinomas express CK7, CIN I cells that have a significant CK7 expression are more likely of progressing to SCC of the cervix than CIN I cells that do not express CK7. This finding implies that CK7 is a predictive marker for progress to SCC of the cervix (Lee *et al*., 2017).

The potential of P16INK4A, Ki-67 and CK7 as IHC markers that can predict squamous cell carcinoma of the cervix from samples of the normal and various transitional stages of CIN was evaluated by correlating their IHC expression to the lesion severity in the progression to SCC of the cervix. These IHC markers may prove to be valuable in clinical practice if they are found to be predictors of SCC of the cervix.

MATERIALS AND METHODS

Case Selection: Cervical tissue blocks will be selected from the pathology files of Obafemi Awolowo University Teaching Hospital Complex (OAUTHC). All samples were fixed in formalin and embedded in paraffin wax by conventional techniques. Haematoxylin and eosin stained slides of all samples were reviewed and classified. Confirmed cervical tissue blocks of non-malignant, CIN and invasive squamous cell carcinoma (SCC) were selected. In total, 50 biopsy samples were taken. Among these, 5 cervical tissue blocks were non-malignant, 10 cervical tissue blocks had CIN I diagnosis, 15 cervical tissue blocks were diagnosed with CIN II and III, and 20 cervical tissue blocks were diagnosed with SCC of the cervix.

PREPARATION OF SECTIONS FOR IMMUNOHISTOCHEMISTRY

All of the specimens were formalin-fixed and paraffin-embedded. 4µm thick serial sections were cut, and the end sections were stained with H&E to ensure that the lesions were still present in the serial sections. The sections were processed for immunohistochemical analysis as followed;

Deparaffinization was carried out with xylene followed by rehydration through graded alcohols. Epitope retrieval was performed by heating the sections for 10 min in citrate buffer (pH6.0) at 121°C. The sections were incubated in 3% hydrogen peroxide (H_2O_2) in methanol for 5 min to block endogenous activity, followed by blocking of nonspecific binding of primary antibodies to epitopes by a preincubation step with 5% normal goat serum for 10 min at 37°C. The primary antibodies used in this study are p16INK4A (Dako, P16INK4A kit, 1:100), Ki-67 (Dako, M7240, 1:100) and CK7 (Dako, Carpinteria, CA, 1:100). Incubation with antibodies was done for 30 min at room temperature. Colour development will be carried out with diaminobenzidine (DAB). The slides was counterstained with haematoxylin, dehydrated in ascending grades of alcohol, cleared in xylene and mounted in Dibutylphtalate polystyrene xylene (DPX) mountant (Lee *et al*., 2017). Staining expression was evaluated optically using the light microscope at ×100 magnification. Photomicrographs of fields of relevance were taken at × 100 magnifications with the use of a light microscope and camera. Expression of P16INK4A, ki-67 and CK7 was determined through a semi-quantitative method.

IMMUNOSTAINING ASSESSMENT

The immunoreactivity of these markers was determined by accessing the cytoplasmic staining intensity and percentage of stained cell nuclei per field. The cytoplasmic staining intensity of P16INK4A and Ck7 was graded as mild, moderate and strong. The percentage of stained nuclei per field of P16INK4A and Ki-67 was graded as follows: Staining in 0-10%of cells = negative (-), staining in 10-50% of cells = +, Staining in 50-80% of the cells = ++; and staining in 80-100% of the cells = +++ (Izadi-mood *et al*., 2012).

PHOTOMICROGRAPHY

The Stained sections were examined under a LEICA research microscope (LEICA DM750, Switzerland) interfaced with digital camera (LEICA ICC50). Digital photomicrographs of stained sections for the histomorphology, immunohistochemistry on the organs studied were taken at various magnifications, and reported for Morphological changes.

DATA ANALYSIS

Statistical analysis of obtained results was carried out using Graph pad prism software program.

RESULTS

Table 1:immunochemistry staining expression of P16INK4A in normal cervical tissue, CIN I, CIN II&III and SCC of the cervix and their percentage positivity rates

Groups	N(50)	-	+	++	+++	Positivity rate (%)
Normal	5	4	1	0	0	20
CIN I	10	5	5	0	0	50
CIN II&III	15	4	3	4	4	75
SCC	20	1	5	6	8	95

Table 1 depicts the grading of the expression of P16INK4A observed in various tissue sections which have been grouped into the various stages of the progression to SCC of the cervix according to their confirmed histologic diagnosis. 1 out of 5 (20%) cases diagnosed as normal cervical tissue was positive

for P16INK4A, 5 out of 10 CIN I cases were positive for P16INK4A, 75% of the CIN II&III cases showed significant P16INK4A expression, whereas 95% of the 20 cases diagnosed to be SCC of the cervix were P16INK4A positive.

Table 2: shows the correlation between histological diagnoses of the progression to SCC of the cervix and cytoplasmic staining intensity of P16INK4A

	Normal	**CIN I**	**CIN II&III**	**SCC**
Intensity				
Negative	4(80%)	3(30%)	-	-
Weak	1(20%)	5(50%)	4(26.7%)	1(5%)
Moderate	-	2(20%)	3(20%)	5(25%)
Strong	-	-	8(53.3%)	14(70%)
Total	5	10	15	20

Table 2 shows the increasing staining intensity from cases with normal cervical tissue to SCC. A positive correlation between lesion severity and staining intensity of P16INK4A was noticed, the cytoplasmic staining intensity of P16INK4A deepened as the condition progressed to SCC of the cervix with a greater percentage of cases having moderate-strong staining intensity in CIN II&III and SCC of the cervix whereas CIN I cases had a higher percentage of weak cytoplasmic staining intensity and the normal cervical tissue was mostly negative for cytoplasmic expression of P16INK4A.

Table 3: alterations of Ki-67 expression in the transition from normal cervical tissue, CIN I, CIN II&III and SCC of the cervix and their percentage positivity rates

Groups	**N(50)**	**-**	**+**	**++**	**+++**	**Positivity rate (%)**
Normal	5	4	1	0	0	20.0
CIN I	10	4	6	0	0	60.0
CIN II&III	15	2	6	4	3	86.7
SCC	20	2	3	7	8	90.0

Table 3 shows the positivity rates observed between normal cervical tissue, CIN I, CIN II&III and SCC of the cervix as 20% (1 of 5), 60% (6 of 10), 86.7% (13 of 15) and 90% (18 of 20) respectively.

Table 4: represents the comparison between the percentage positivity rates of P16INK4A and ki-67 in the various stages in the progression to cervical squamous cell carcinoma.

Groups	**P16INK4A**	**Ki-67**
Normal	20.0%	20.0%
CIN I	50.0%	60.0%
CIN II&III	75.0%	86.7%
SCC	95.0%	90.0%

Table 4 illustrates the correlation between the percentage positivity rate of tumour biomarkers P16INK4A and Ki-67 in the various stages in the progression to cervical squamous cell carcinoma. It was observed that there was an up regulation in the percentage positivity rate as the condition progressed from a state of pre-malignancy to malignancy in the tissues stained with both markers.

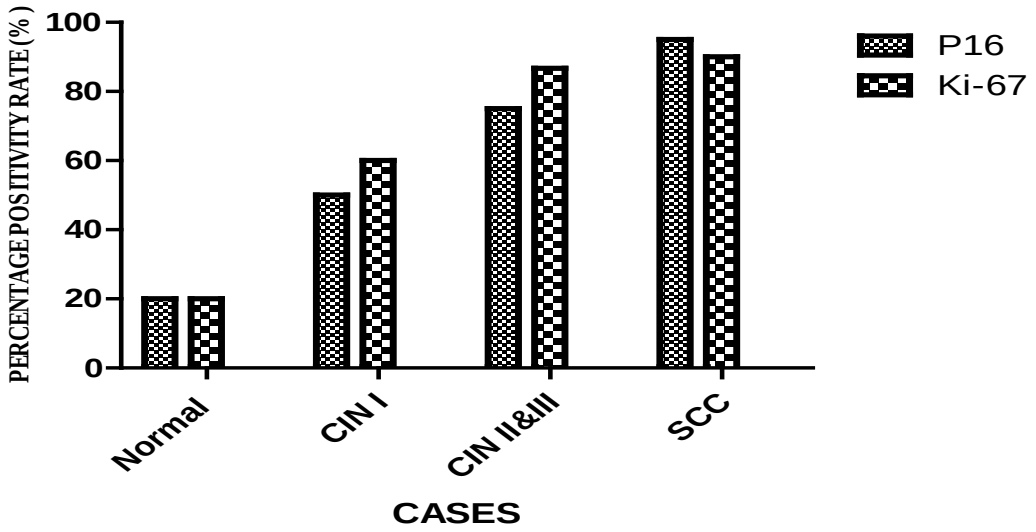

Figure 1: a graph illustrating the comparison between the percentage positivity rates of p16INK4A and ki-67 in the various stages in the progression to cervical squamous carcinoma.

Table 5: portrays the cytoplasmic staining intensity profile of ck7 in histologically confirmed normal cervical tissue, CIN I, CIN II&III and SCC of the cervix.

	Normal	CIN I	CIN II&III	SCC
Intensity				
Weak	3(60%)	2(20%)	2(13.3%)	2(10%)
Moderate	1(20%)	7(70%)	4(26.7%)	5(25%)
Strong	1(20%)	1(10%)	9(60.0%)	13(65%)
Total	5	10	15	20

Table 5 shows the increasing cytoplasmic staining intensity in cases ranging from normal cervical tissue to SCC that have been treated with CK7. Relevant expressions of the cytoplasmic staining intensity in cases histologically diagnosed to be normal cervical tissue (60% weak, 20%moderate, 20% strong), CIN I (20% weak, 70% moderate, 10% strong), CINII&III (13% weak, 26.7% moderate, 60% strong) and SCC (10% weak, 25% moderate, 65% strong). A positive correlation between lesion severity and cytoplasmic staining intensity was noticed.

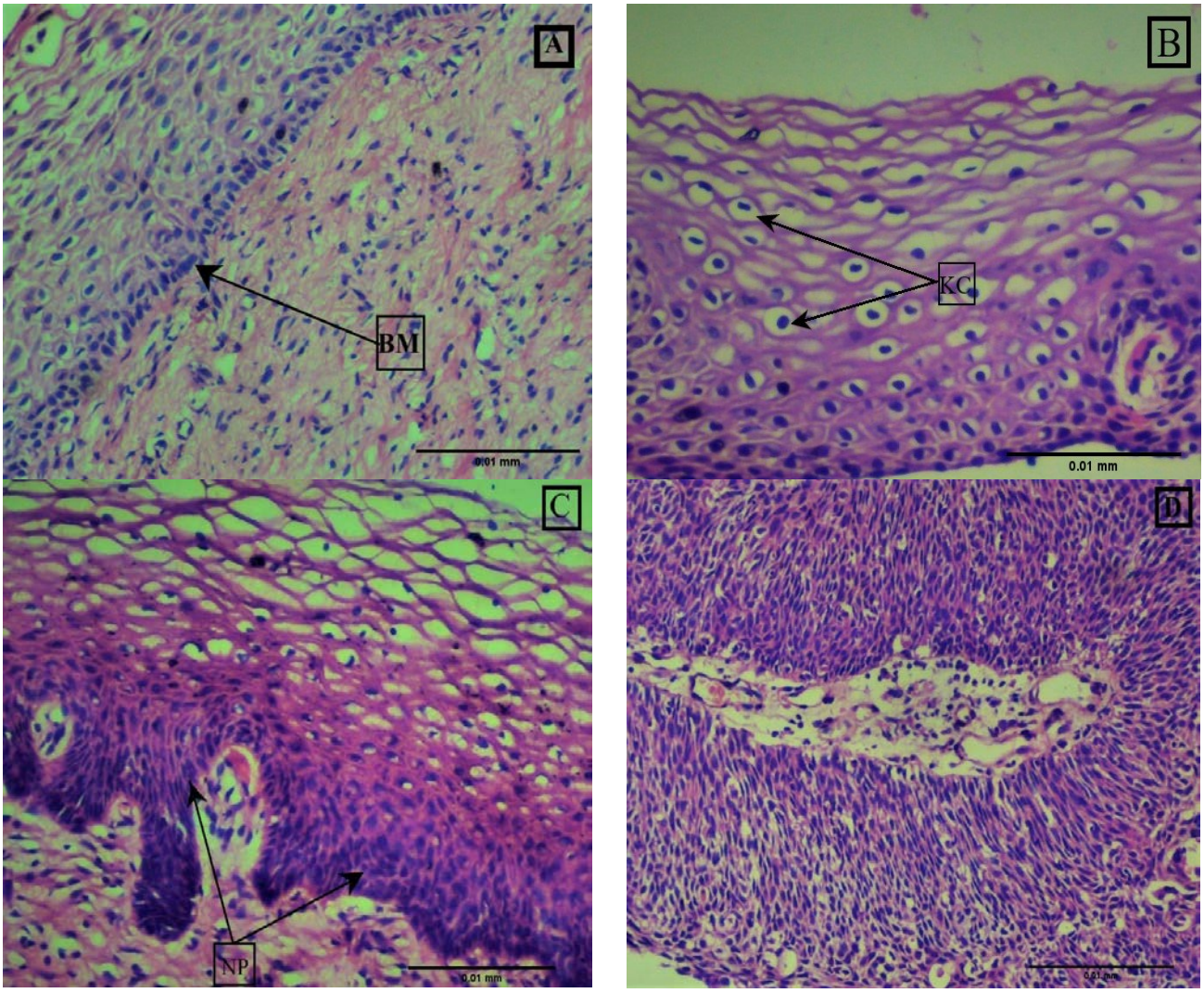

Figure 2: micrographs of cervical sections stained with H&E showing: (A) (H&E ×100) showing stratified squamous epithelium, the basement membrane (BM), in normal cervical tissue, CIN I (B) (H&E ×100) showing cells with hyperchromatic nuclei and Koilocytosis (KC), (C) (H&E ×100) CIN II&III showing marked dysplasia and nuclear polymorphism (NP) in the basal and parabasal cells and SCC (D) (H&E×100) proliferating cells have completely infiltrated the basement membrane.

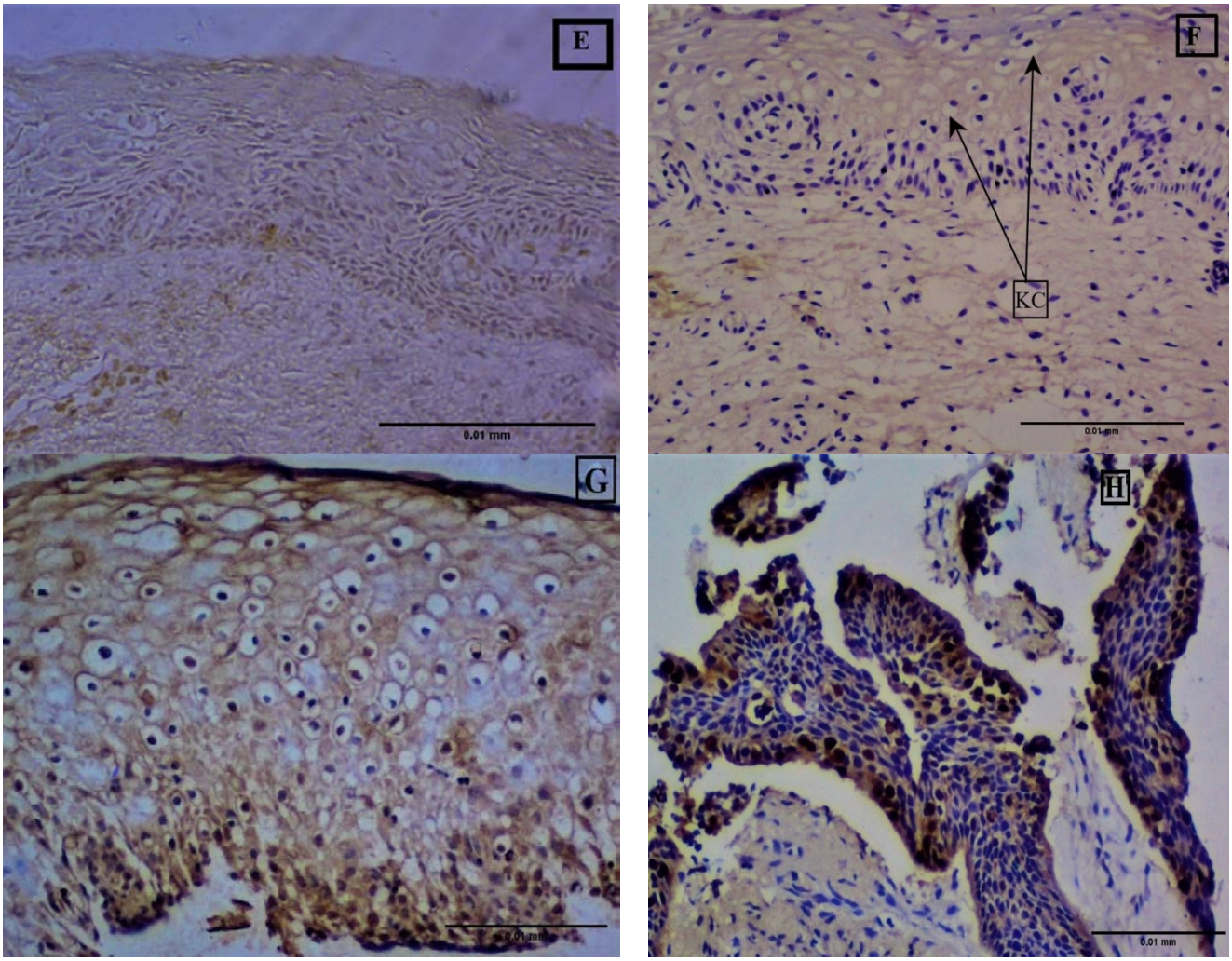

Figure 3: micrographs of cervical sections stained with P16INK4A showing: (E&F) ectocervical squamous epithelium strata (E) (P16INK4A ×100) illustrating the basement membrane (BM) in normal cervical tissue with few cells expressing weak staining intensity, CIN I (F) (P16INK4A ×100) showing koilocytosis (KC) expressing weak to moderate staining intensity, (G) (P16INK4A ×100) CIN II&III showing dysplasia and nuclear polymorphism (NP) in the basal cells. The cells in this section expressed nuclear and cytoplasmic staining of majorly moderate and strong staining intensity and SCC (H) (P16INK4A ×100) showed cells having marked dysplasia that have invaded the basement membrane. Strong nuclear and cytoplasmic expression of P16INK4A was observed.

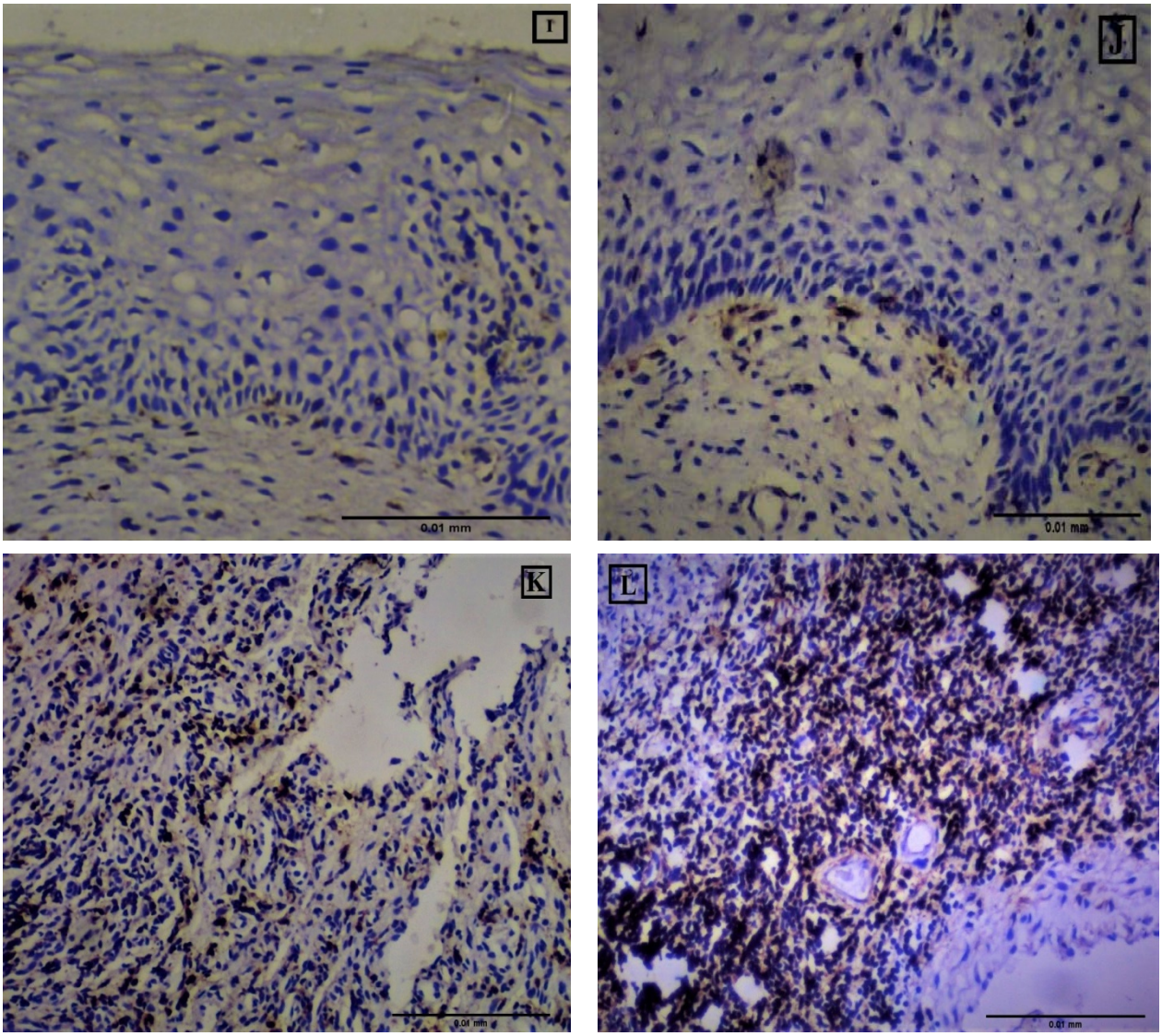

Figure 4: micrographs of cervical sections stained with Ki-67 showing: (I&J) ectocervical squamous epithelium strata (I) (Ki-67×100) illustrating the basement membrane (BM) in normal cervical tissue with cells having nuclear expression Ki-67 being <10% and CIN I (J) (Ki-67×100) showing koilocytosis (KC) and about 10% of cells showing nuclear expression of Ki-67, (K) (Ki-67 ×100) CIN II&III showing marked dysplasia and invasion of the basement membrane by the proliferating cells. >50% of the cells in this section expressed nuclear staining by Ki-67. SCC (L) (Ki-67 ×100) showed cells having marked dysplasia that have invaded the basement membrane, populating the connective tissue stroma. >80% the cells expressed nuclear Ki-67 staining.

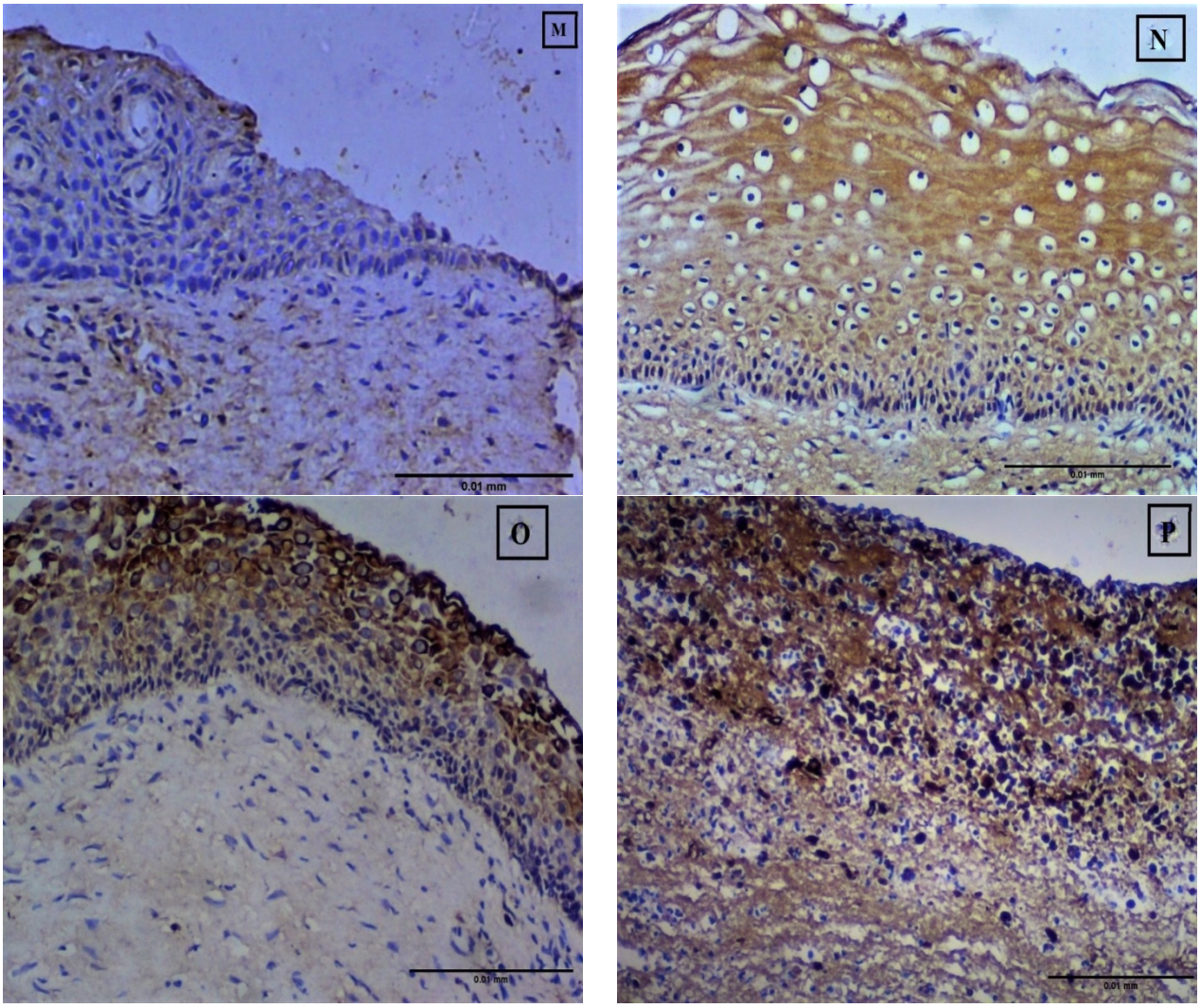

Figure 5: micrographs of cervical sections stained with Ck7 showing: (M&N) ectocervical squamous epithelium strata (M) (Ck7×100) illustrating the basement membrane (BM) in normal cervical tissue with few cells expressing weak staining intensity and CIN I (N) (Ck×100) showing mild dysplasia with koilocytosis (KC) expressing moderate staining intensity, (O) (Ck7 ×100) CIN II&III showing dysplasia and nuclear polymorphism (NP) in the cells. The cells in this section expressed cytoplasmic staining of moderate and strong staining intensity across the epithelium. SCC (P) (Ck7 ×100) showed cells having marked dysplasia that have invaded the basement membrane completely. Strong cytoplasmic expression of Ck7 was observed.

DISCUSSION

Cervical cancer is one of the most prevalent malignant tumours affecting the female gynaecological tract (FGT) worldwide, staying one of the top causes of mortality from cancer in women worldwide, with clinical studies showing that the incidence of cervical cancer in young women is rising yearly. There is therefore a need for enhanced diagnostic techniques to investigate the potential usefulness of tumour-related antigen markers as an adjunct to these conventional techniques in screening, diagnosis and potentially predicting whether a case will progress to SCC of the cervix. HPV is an important causal factor although other genetic and epigenetic factors may be involved in tumour progression (Wang *et al*.,

2004). In this study the expression of several specific immunohistochemistry markers in histologically confirmed normal cervical tissue, CIN and SCC of the cervix was observed and evaluated in order to determine if these markers were relevant in predicting whether a case will progress to SCC of the cervix.

P16INK4A is a tumour suppressor gene that participates in the regulation of the normal cell cycle and is of significance to researchers in early detection of cervical cancer. (Cioffi-Lavina *et al*., 2010) (Tozawa-Ono *et al*., 2012).In this study, amongst the five (5) normal cervical tissues stained with P16INK4A antibody one (1) case displayed up 10% nuclear expression of the marker which enabled it to be classified as positive, the same section exhibited a weak cytoplasmic staining intensity. In the CIN I cases, 50% of the ten (10) cases exhibited significant positive nuclear reactions with 70% of the positive cases exhibiting weak to moderate staining intensity. Whereas CIN II&III cases, an almost uniform nuclear and cytoplasmic staining was observed along most of the squamous epithelium strata, an elevated percentage nuclear positivity was discovered in eleven (11) of the fifteen(15) selected cases, giving it a 75% positivity rate. The staining intensity notably deepened with more cells having moderate to strong staining intensity. The selected SCC cases a marked positive nuclear expression of P16INK4A, 95% of the twenty (20) SCC cases were positive, 5% of the cases displayed moderate staining intensity while 70% showed strong staining intensity. From the results obtained, the percentage positivity rate of each case distribution was calculated and it was discovered that there was a direct relationship between the lesion severity, percentage positivity and staining intensity. There was a significant increase in the values of these parameters as the condition progressed from the premalignant phases to a state of malignancy. The findings in this study are in concurrence with the studies ofSilva*et al* and Murphy *et al* that also established that there was a significant increase in the expression ofP16INK4Awhich was directly proportional to the lesion severity. Significant positive expression of P16INK4A in the normal cervical tissue can be said to be resulting from a possible infection and genomic integration with HPV in tissues that are yet to undergo the dysplastic changes in the squamous epithelium. This coincides with a study by Murphy *et al* in which all cases positive for HPV expressed the P16INK4A IHC marker, but not all cases positively expressing P16INK4A were HPV positive. The suggested principle by which these findings can be explained was expressed in the work by Lee*et al* who stated that the presence of HPV proteins E6 and E7 will cause an increase in p53 degradation or inactivation of the retinoblastoma protein respectively which in turn causes P16INK4A overexpression.

Ki-67 is a gene that monitors cell proliferation;hence irregular Ki 67 protein expression usually implies abnormal proliferation of cells. (Yu *et al*., 2015) (Gertych *et al*., 2012). Results of this study showed that Ki-67 expression was minimal in normal cervical tissues with only 20% of the distribution having a significant positive expression, but significant expressions were detected in the progression from CIN I (60%), CIN II&III (86.7%) and SCC (90%), the percentage positivity rate of Ki-67 expression was significantly elevated as the degree of cervical lesions in the progression to SCC increased. These findings correspond with Shi *et al* who observed an increased positivity rate with an increase in increased degree of severity of the cervical lesions. The exact role of Ki-67 has not been established, however, Ancuta *et al* stated in a study that level of Ki-67 expression is used to determine the cell proliferation status which is elevated in cases of malignancy.

Related literature has focused on IHC for SCJ markers, CK7 in particular. Herfs and colleagues were among the first to demonstrate CK7 IHC's position as a critical tool for CIN I biopsies. In this study, Ck7 cytoplasmic staining intensity in normal cervical tissue was weak in the squamous epithelial cells at the transformation zone with very few cells having moderate-strong staining. This staining pattern was mostly consistent in the 5 normal cervical tissue sections. In the CIN I distribution, all cells expressed Ck7, the cells closer to the basement membrane displaying a weaker staining intensity which gradually transitioned to a moderate staining intensity towards the superficial layer of the squamous epithelium. About 70% of the CIN I distribution had a moderate staining intensity. The superficial layer of the CIN II&III distribution was stained the most intensely and the strength of the intensity gradually decreased in

the transition to the basement membrane. 60% of cells in this distribution conveyed strong staining intensity which was predominantly located in the superficial layer. 13 out of 20 (65%) cases histologically diagnosed to be SCC of the cervix exhibited a predominantly homogeneous strong staining intensity.It is to be noted that the staining intensity has a direct relationship with the progression of the condition. Khan *et al* disclosed a principle that viral proteins seem to reorganise and regulate the cytokeratin (CK) network. It is believed that the viral E4 protein is involved in CK disruption, release of viruses and transmission.

CONCLUSION

The data obtained from this study has enabled the discovery that P16INK4A is expressed in normal cervical tissue, CIN and SCC of the cervix, and the percentage positivity rate and the staining intensity of the IHC marker both exhibit a positive correlation to the progression of the disease. Ki-67 positivity rate was also found to have a positivity rate that was directly proportional to the progression of the conditions leading up to SCC of the cervix. It was found that the rate at which the staining intensity of Ck7 increased in accordance to the rate of advancement of the lesions to SCC of the cervix. These findings suggest that variations in the expression of P16INK4A, Ki-67 and Ck7 are valuable markers for predicting the progression towards squamous cell carcinoma of the cervix but P16INK4A and Ki-67 used in conjunction with one another has proven to be of greater predictive and clinical value. Although these markers have proven to be significant in predicting the progression of a normal cervical tissue or cervical squamous intraepithelial lesion to SCC of the cervix, none of these markers can stand alone to give a completely definitive result and as such they should be used in concordance to one another in order to make up for their limitations and obtain pertinent results.

ACKNOWLEDGEMENT

The authors express profound gratitude to Mr.AremuAdegokeOlalekan of the Department of Morbid Anatomy and Forensic Sciences, Obafemi Awolowo University Teaching Hospital Complex (OAUTHC) for the assistance rendered in the immunohistochemistry (IHC) study.

FUNDING

This research was privately funded.

COMPETING INTERESTS

The authors declare that they have no competing interests

REFERNCES

Ahmed, S. A., Obaseki, D. E., Mayun, A. A., Mohammed, A., Rafindadi, A. H., & Abdul, M. A. (2017). The role of biomarkers (P16INK4a and Ki-67) in cervical cancer screening: An appraisal. *Annals of Tropical Pathology*, *8*(1), 1.

Ancuta, E., Ancuta, C., Cozma, L. G., Iordache, C., Anghelache-Lupascu, I., Anton, E., ...&Chirieac, R. (2009). Tumor biomarkers in cervical cancer: focus on Ki-67 proliferation factor and E-cadherin expression. *Rom J MorpholEmbryol*, *50*(3), 413-8.

Arbyn, M., Weiderpass, E., Bruni, L., de Sanjosé, S., Saraiya, M., Ferlay, J., & Bray, F. (2020). Estimates of incidence and mortality of cervical cancer in 2018: a worldwide analysis. *The Lancet Global Health*, *8*(2), e191-e203.

Beauséjour, C. M., Krtolica, A., Galimi, F., Narita, M., Lowe, S. W., Yaswen, P., &Campisi, J. (2003). Reversal of human cellular senescence: roles of the p53 and P16INK4A pathways. *The EMBO journal*, *22*(16), 4212-4222.

Bisi-Onyemaechi, A. I., Chikani, U. N., &Nduagubam, O. (2018).Reducing incidence of cervical cancer: knowledge and attitudes of caregivers in Nigerian city to human papilloma virus vaccination. *Infectious agents and cancer*, *13*(1), 1-6.

Bullwinkel, J., Baron-Lühr, B., Lüdemann, A., Wohlenberg, C., Gerdes, J., & Scholzen, T. (2006). Ki-67 protein is associated with ribosomal RNA transcription in quiescent and proliferating cells. *Journal of cellular physiology*, *206*(3), 624-635.

Buza, N., & Hui, P. (2017). Immunohistochemistry in gynecologic pathology: An example-based practical update. *Archives of pathology & laboratory medicine*, *141*(8), 1052-1071.

Cardoso, F. A., Campaner, A. B., & Silva, M. A. L. G. (2014). Prognostic value of P16INK4A as a marker of clinical evolution in patients with cervical intraepithelial neoplasia grade 3 (CIN 3) treated by cervical conization. *Apmis*, *122*(3), 192-199.

Cheah, P. L., Looi, L. M., Mun, K. S., AbdoulRahman, N., &Teoh, K. H. (2011). Implications of continued upregulation of P16 (INK4a) through the evolution from high-grade squamous intraepithelial lesion to invasive squamous carcinoma of the cervix. *Malays J Pathology*, *33*(2), 83-7.

Cioffi-Lavina, M., Chapman-Fredricks, J., Gomez-Fernandez, C., Ganjei-Azar, P., Manoharan, M., &Jorda, M. (2010).P16INK4A expression in squamous cell carcinomas of cervix and bladder. *Applied Immunohistochemistry & Molecular Morphology*, *18*(4), 344-347.

Cuschieri, K., & Wentzensen, N. (2008).Human papillomavirus mRNA and P16INK4A detection as biomarkers for the improved diagnosis of cervical neoplasia. *Cancer Epidemiology and Prevention Biomarkers*, *17*(10), 2536-2545.

Dabbs, D. J. (Ed.).(2013). *Diagnostic immunohistochemistry e-book*.Elsevier Health Sciences. (page 1-3).

Egawa, N., Egawa, K., Griffin, H., &Doorbar, J. (2015).Human papillomaviruses; epithelial tropisms, and the development of neoplasia. *Viruses*, *7*(7), 3863-3890.

Ganesan, K., Morani, A. C., Marcal, L. P., Bhosale, P. R., &Elsayes, K. M. (2015). Cross-Sectional Imaging of the Uterus.In *Cross-Sectional Imaging of the Abdomen and Pelvis* (pp. 875-936).Springer, New York, NY.

Gertych, A., Joseph, A. O., Walts, A. E., & Bose, S. (2012). Automated detection of dual P16INK4A/Ki-67 nuclear immunoreactivity in liquid-based Pap tests for improved cervical cancer risk stratification. *Annals of biomedical engineering*, *40*(5), 1192-1204.

Glenn McCluggage, W. (2007).Immunohistochemistry as a diagnostic aid in cervical pathology. *Pathology*, *39*(1), 97-111.

Hasan, H. (2009). *Cervical Cancer*.The Rosen Publishing Group, Inc.

Izadi-Mood, N., Asadi, K., Shojaei, H., Sarmadi, S., Ahmadi, S. A., Sani, S., &Chelavi, L. H. (2012). Potential diagnostic value of P16INK4A expression in premalignant and malignant cervical lesions. *Journal of research in medical sciences: the official journal of Isfahan University of Medical Sciences*, *17*(5), 428.

Khan, J., Davy, C. E., McIntosh, P. B., Jackson, D. J., Hinz, S., Wang, Q., &Doorbar, J. (2011). Role of calpain in the formation of human papillomavirus type 16 E1^ E4 amyloid fibers and reorganization of the keratin network. *Journal of virology*, *85*(19), 9984-9997.

Kim, T. H., Han, J. H., Shin, E., Noh, J. H., Kim, H. S., & Song, Y. S. (2015). Clinical implication of P16INK4A, Ki-67, and proliferating cell nuclear antigen expression in cervical neoplasia: improvement of diagnostic accuracy for high-grade squamous intraepithelial lesion and prediction of resection margin involvement on conization specimen. *Journal of cancer prevention*, *20*(1), 70.

Koay, K., Schofield, P., &Jefford, M. (2012).Importance of health literacy in oncology. *Asia-Pacific Journal of Clinical Oncology*, *8*(1), 14-23.

Lee, H., Lee, H., & Cho, Y. K. (2017).Cytokeratin7 and cytokeratin19 expression in high grade cervical intraepithelial neoplasm and squamous cell carcinoma and their possible association in cervical carcinogenesis. *Diagnostic pathology*, *12*(1), 18.

Lin, Z., Gao, M., Zhang, X., Kim, Y. S., Lee, E. S., Kim, H. K., & Kim, I. (2005). The hypermethylation and protein expression of P16INK4A and DNA repair gene O 6-methylguanine-DNA methyltransferase in various uterine cervical lesions. *Journal of cancer research and clinical oncology*, *131*(6), 364-370.

Marth, C., Landoni, F., Mahner, S., McCormack, M., Gonzalez-Martin, A., Colombo, N., & ESMO Guidelines Committee. (2017). Cervical cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of Oncology*, *28*(suppl_4), iv72-iv83.

Mills, A. M., Paquette, C., Terzic, T., Castle, P. E., &Stoler, M. H. (2017).CK7 Immunohistochemistry as a Predictor of CIN1 Progression. *The American Journal of Surgical Pathology*, *41*(2), 143-152.

Munhoz, N. G., Rodrigues, D. A., Takase, Y., Pedregosa, J. F., Rodrigues, J. O., Miyazaki, K., ...&Uezato, S. (2009). The use of molecular markers (P16INK4a, Ki-67 and E-Cadherin) in uterine cervical biopsies. *The Open Pathology Journal*, *3*(1).

Murphy, N., Ring, M., Killalea, A. G., Uhlmann, V., O'Donovan, M., Mulcahy, F., ...&Sheils, O. (2003). P16INK4A as a marker for cervical dyskaryosis: CIN and cGIN in cervical biopsies and ThinPrep™ smears. *Journal of clinical pathology*, ***56***(1), 56-63.

Nicol, A. F., Golub, J. E., Silva, J. R., Cunha, C. B., Amaro-Filho, S. M., Oliveira, N. S., ... &Grinsztejn, B. (2012). An evaluation of P16INK4A expression in cervical intraepithelial neoplasia specimens, including women with HIV-1. *Memórias do InstitutoOswaldo Cruz*, *107*(5), 571-577.

Painter, J. T., Clayton, N. P., & Herbert, R. A. (2010).Useful immunohistochemical markers of tumor differentiation. *Toxicologic pathology*, *38*(1), 131-141.

Reich, O., Regauer, S., McCluggage, W. G., Bergeron, C., & Redman, C. (2017). Defining the cervical transformation zone and squamocolumnar junction: can we reach a common colposcopic and histologic definition?. *International Journal of Gynecological Pathology*, *36*(6), 517-522.

Roncaglia, M. T., Fregnani, J. H. T., Tacla, M., De Campos, S. G. P., Caiaffa, H. H., Ab'Saber, A., ...&Baracat, E. C. (2013). Characterization of P16INK4A and E6 HPV-related proteins in uterine cervix high-grade lesions of patients treated by conization with large loop excision. *Oncology letters*, *6*(1), 63-68.

Sano, T., Oyama, T., Kashiwabara, K., Fukuda, T., & Nakajima, T. (1998). Expression status of P16INK4A protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions. *The American journal of pathology*, *153*(6), 1741-1748.

Shi, Q., Xu, L., Yang, R., Meng, Y., &Qiu, L. (2019).Ki-67 and P16INK4A proteins in cervical cancer and precancerous lesions of young women and the diagnostic value for cervical cancer and precancerous lesions. *Oncology letters*, ***18***(2), 1351-1355.

Silva, D. C., Goncalves, A. K., Cobucci, R. N., Mendonca, R. C., Lima, P. H., &Júnior, G. C. (2017). Immunohistochemical expression of p16, Ki-67 and p53 in cervical lesions–A systematic review. *Pathology-Research and Practice*, *213*(7), 723-729.

Tiran, D. (2010). Structural reflex zone therapy in pregnancy and childbirth: A new approach. *Complementary therapies in clinical practice*, *15*(4), 234-238.

Tozawa-Ono, A., Yoshida, A., Yokomachi, N., Handa, R., Koizumi, H., Kiguchi, K., ...& Suzuki, N. (2012). Heat shock protein 27 and P16INK4a immunohistochemistry in cervical intraepithelial neoplasia and squamous cell carcinoma. *Human cell*, *25*(1), 24-28.

Wang, C. W., Wu, T. I., Yu, C. T., Wu, Y. C., Teng, Y. H., Chin, S. Y., ... & Chen, T. C. (2009). Usefulness of P16INK4A for differentiating primary pulmonary squamous cell carcinoma from cervical squamous cell carcinoma metastatic to the lung. *American journal of clinical pathology*, *131*(5), 715-722.

Wang, J. L., Zheng, B. Y., Li, X. D., Ångström, T., Lindström, M. S., &Wallin, K. L. (2004).Predictive significance of the alterations of P16INK4A, p14ARF, p53, and proliferating cell nuclear antigen expression in the progression of cervical cancer. *Clinical cancer research*, *10*(7), 2407-2414.

Xue, Y., Bellanger, S., Zhang, W., Lim, D., Low, J., Lunny, D., & Thierry, F. (2010). HPV16 E2 is an immediate early marker of viral infection, preceding E7 expression in precursor structures of cervical carcinoma. *Cancer research*, *70*(13), 5316-5325.

Yu, J. Q., Zhou, Q., Zheng, Y. F., &Bao, Y. (2015). Expression of vimentin and Ki-67 proteins in cervical squamous cell carcinoma and their relationships with clinicopathological features. *Asian Pac J Cancer Prev*, *16*(10), 4271-4275.