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Analysis and Determination of the Proliferative Biomarker Potential of Ki-67 in Cervical Lession/Cervical Cancer using Immunohistochemical Method.

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Christy Chinyere Fredrick
Department of Pathology and Forensic Medicine, College of Health Sciences, University of Abuja, Nigeria.
Christy.fredrick@uniabuja.edu.ng

Obum-Nnadi Charity Ndidi
Department of Medical Laboratory Science, Faculty of Health Sciences, Veritas University, Abuja, Nigeria
Email: n_charitez@yahoo.co.uk

Kelechukwu Sylvester Nwokorie
Department of Medical Laboratory Science, Faculty of Health Sciences, Veritas University, Abuja, Nigeria.

Dennis Amaechi
Department of Biochemistry, Faculty of Natural and Applied Sciences, Veritas University, Abuja, Nigeria
amaechitoexcel@yahoo.com

Jonathan U. Madukwe
Department of Medical Laboratory Science, Baze University, Abuja, Nigeria

Nnawuihe Chika Winnifred
Department of Medical Laboratory Science, Imo State University, Owerri, Nigeria

Ravi Kanna
Cachar Cancer Hospital and Research Center, Silchar India.

Rajeeve Kumar
Cachar Cancer Hospital and Research Center, Silchar India.

Anurdha Talukdar
Cachar Cancer Hospital and Research Center, Silchar India.

Litika Vermani
Cachar Cancer Hospital and Research Center, Silchar India.

Poulome Mukherjee
Cachar Cancer Hospital and Research Center, Silchar India.

Humphery B. Osadulor
Department of Medical Laboratory Science, Ambrose Ali University Ekpoma, Benin, Edo State, Nigeria.

Keywords
Immunochemistry, histopathology, cervical neoplasm, intraepithelial, cervical cancer.

Abstract
Background: Cervical cancer is believed to be one of the most widespread cancers worldwide with the utmost burden prevalent among women in areas where resources are scarce.
Methods:The study population was from 10 years retrospective archival tissue blocks of cervical cancer among women in

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Abuja metropolis from 2005 to 2015 Histopathological diagnosis by H and E was made, from the 80 cases examined 20(25%) Cases were cervicitis (CC), 15(18.8%) were cervical intraepithelial neoplasia (CINI), 15(18.8%) were cervical intraepithelial neoplasia (CIN2) while 30(37.3%) cases were squamous cell carcinoma SCC They were examined immunohistochemically by Avidin Biotin Complex (ABC) method. Ki67 (proliferation marker)

Results: A statistically significant rate of positivity was found in the expression of Ki-67 among all the categories of the studied cases.

Conclusion: This study showed that most of the immunological markers examined can be used for identification of different stages involved in cancer progression, also for diagnosis of cancer, predictive prognosis and can be explore as therapy targets for cervicitis and Squamous cell carcinoma.

1. Introduction

Cervical cancer is believed to be one of the most widespread cancers worldwide with the utmost burden prevalent among women in areas where resources are scarce especially in developing country. A protein, which it is located in the nucleus called ki-67 is only produce in actively dividing cells. Ki-67 antigen described by Gerdes, is a maker of proliferation controlling the cell cycle. The immunohistochemistry (IHC) analysis of ki-67 is a reference test to access tumor cell proliferation in cervical tissue embedded in paraffin wax according to international ki-67 assessment, this study showed that ki-67 overexpression was present in 100% in invasive cervical cancer cases studied. The positivity rate of ki-67 expression was significantly higher in cervical cancer cells than in cervical lesion; cervicitis, cervical intraepithelial1, (CIN1), cervical intraepithelial 2 (CIN 2). ki-67 is a proliferative marker useful in grading of cervical cancer intraepithelial neoplasia by giving uniform and reliable outcome independent of inter and intra-observer differences.The cervix is the lower, narrow part of the uterus of a woman, or womb. The cervix connects the main body of the uterus to the vagina, or birth canal. The female reproductive system contains the cervix [1]. The female reproductive system generally comprises internal organs such as the vagina, uterus, ovaries and fallopian tubes. The female reproductive system also comprises the external genital organs such as the parts that make up the vulva (the clitoris, vaginal lips and the opening to the vagina). The pelvis which is the lower part of the abdomen between the hip bones contains all the internal organs. The glands that make and release mucus are contained in part of the lining of the cervix [1]. The mucus is usually thick during pregnancy and menstrual cycle, and this helps to prevents sperm from entering the uterus during this period. The thick mucus equally helps to prevent harmful bacteria from entering the reproductive organs. However, during ovulation, the mucus becomes thinner. This allows the passage of sperm from the cervix into the uterus [2].

Cervical cancer is a cancer that emanates from the cervix. Cervical cancer is commonly caused due to abnormal growth of cells that could also invade or spread to other parts of the body[3].Symptoms are generally not noticeable during the early stage of cervical cancer. However, at later stage after the invasion of the cancer, symptoms such as abnormal vaginal bleeding, pelvic pain, or pain during sexual intercourse may begin to occur. Another noticeable symptom could be bleeding after sexual intercourse, even though it may not be serious [4].

Ki-67, a proliferative marker is useful in grading of cervical cancer and cervical intraepithelial neoplasia by giving uniform and reliable outcome independent of inter and intra-observer differences. Ki-67 is a multiplying marker identified as prognostic element for the development of tumors. Ki-67 can be described as a nuclear antigen (connected with hetero- and euchromatin) which is usually expressed during all active levels of the cell cycle and the cell proliferation status is determined by the stage of Ki-67 [5]. Ki-67 can be identified through the means of several qualitative and quantitative analyses comprising monoclonal antibody in immunohistochemical assays, electron microscopy, ELISA, flow cytometry, immunocytochemistry [6]. Ki-67 is generally found only in dividing cells, either normal or tumor, but absent in resting cells. This is because Ki-67 is present only in dividing cells, either normal or tumor, but absent in resting cells. In other words, Ki-67 can only assess cells that over-express p53 or p21. Ki-67 is detected essentially in parabasal epithelial layers and some basal layers in some cases in normal cervical squamous mucosa. Ki-67 is identified basically in parabasal epithelial layers (the main source for cell renewal) in normal cervical squamous mucosa. In addition, Ki-67 is also detected in certain basal layers [7]. A study has demonstrated

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significant indirect correlation between Ki-67 proliferation index and E-cadherin is maintained among women with invasive squamous cervical lesion and those at the clinical stage. Thus, there is an indirect statistically important moderate correlation between Ki-67 expression and E-cadherin [8].

2. Methodology

Study area

The research was carried out in these health Institutions: University of Abuja Teaching Hospital (UATH), Mataima General Hospital, Gwarinpa General Hospital, National Hospital in FCT Abuja and in Cachar cancer hospital and Research Centre India where the analysis will be done. Data wase collected to enhance effective and credible results from the research.

ANALYTICAL SITE

Figure 1 Showing the Map ofIndian (mapsofindian.com)

Cachar Cancer Hospital and Research Center is a DSIR SIRO (Govt. of India) recognized not for profit comprehensive cancer care centre situated in the outskirts of Silchar on land provided by the Govt. of Assam. It is administered by a non-profit society The Cachar Cancer Hospital Society. Cachar Cancer Hospital and Research Centre (CCHRC), established (in 1996) and administered by the Cachar Cancer Hospital Society, a non-profit NGO registered under the Societies Registration Act, is located in the outskirts of Silchar town in the Barak Valley of Assam in India. The society came into existence in 1992 as a result of a desperately felt need for a cancer hospital (since the only cancer hospital in the entire north east was in faraway(Guwahati) with three principal objectives:

(1) To make people aware of cancer, adopt preventive measures and seek early detection

(2) To establish a full-fledged cancer hospital to provide meaningful services to all suffering people and

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(3) To set up a cancer research Centre.

SAMPLE TECHNIQUE

The study samples shall be grouped into four study groups into four groups and the control group. They study group is made up of one those diagnosed for squamous cells carcinoma, cervicitis, CIN and control(Taro Yamane, 1967 updated 2008).

Taro-yamane formula shall be applied to calculate sample size.

When n signifies sample size

N signifies the population under study

e signifies the margin error

$n= \frac{N}{1+(e)2}$

$= \frac{160}{1+0.0025}$

n = 160/(1+1)

n = 160/2

n =80

Sample collection

Sections of about 3 – 5 microns are cut from the selected samples (blocks) using microtome and microtome blade, it will be picked on IHC special slides and preserved for IHC staining. Prior to staining slides are placed in oven at 60º for 1 hour to avoid wash-off-during the IHC Staining.

Sample analysis

1. IHC analysis for Ki-67 was carried out using Dako antibodies Immune reactivity target is in the nucleus.

Statistical analysis:

The results of case and control was collated and manage statically using EPI info, statically package for social science (SPSS) and Microsoft access soft wares. The SPSS shall be used for data analysis; descriptive statistic was used to analyze all variable in order to access association of independent variables within the study.

Summary of ethical issue involved in the research

The potential subjectsmustbeadequately informed of the aim, methods, anticipated benefits and potential hazards of the study and the discomfort it may entail. The right of every subject mustberespected. The subjectshall also beinformed that they have the right to opt out of the study if they want to do so. The interest of the subjects was placed above that of the research work.

Consequences of the Study for The Local Community, Environment and Participants: There was no adverse consequences for the local community; environment and participants since safety measures was put in place to ensure that any waste generated are adequately disposed of by the health facilities.

Dissemination of results of study: The results of study was for academic purpose; however, the findings of the results will be used to prefer solution for the management, prevention, treatment of cancer patient and awareness creation to the general public on issues relating to cervical cancer.

Confidentiality and privacy: To ensure confidentiality and privacy, the consent forms bearing the bio data of the participants shall bedestroyed immediately after the collection of the results of the analysis. Participants' names will not be usedonly the age, sex and the nature of the sample.

The cost and sources of funding of research: This research work was funded from personal effort and financial support from my Employee University of Abuja.

Ethical consideration

Study was approved by the Federal Capital Territory Health Research Ethics Committee, Abuja with reference number FHREC/2019/01/93/07.10.19. A copy is attached at the appendix.

Inclusion criteria:

All Haematoxylin and Eosin-stained slides were reviewed to confirm diagnosis of cervical cancers. Furthermore, all tumorcases that were selected were ensured to have adequate tumor tissue representation.

The age range must befrom 17 years and above.

Exclusion criteria:

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i) Inadequate tissue sections and cases not diagnosed as cervical lesion or cervical cancers were excluded from the study.
ii) Cases with no clinical information in the records or for which the tissue blocks are missing or damaged were excluded.

PROCEDURE FOR HANDLING AND TREATMENT OF ARCHIVED TISSUE BLOCKS

The archived tissue blocks representing the cancer diagnosis were retrieved from the archive storage room. Proper selections were made by evaluating the existing state of the tissue, which consists of checking the gross tissue adequacy on the paraffin blocks, the orientation of the tissue, the presence or absence of dust particles and molds. Re-embedding was done for the tissues that required it.

All samples selected were sectioned at 3 microns using a rotary microtome and stained with standard Haematoxylin and Eosin staining method. The slides were reviewed independently by two pathologists and diagnosis were classified into Cervicitis, CIN1, CIN2 and squamous cell carcinoma.

Four sections of 3microns were further cut for each sample to represent the four antibody markers that were used in this study. Also, sections were cut 3microns for specify positive and negative control for the four different antibodies.

The tissue sections were brought down to water through deparaffinization in xylene, hydration in descending grades of alcohol and finally washed in water. Antigenic sites of tumor cells were retrieved using heat mediated antigen retrieval method that required pressure cooker and citric acid of PH 6.0. Tissue sections were cooled in water and ready for further steps of the immunohistochemical technique

Principle of Hematoxylin and Eosin staining (Titford 2005; Chan, 2014)

Hematoxylin and Eosin stain is the most widely used stain in histology and Histopathology Laboratories for the purpose of demonstrating a wide range of normal and abnormal cells and tissue components. The Hematoxylin component stains the cell nuclei blue-black showing good intra-nuclear details while eosin stains cell cytoplasm and most connective tissue fibers in varying shades and intensities of pink, orange and red.

The principle is based on the acidic component of the cell which has the affinity to basic dye and the basic component of the cells which have the affinity to acidic dye. In Hematoxylin and Eosin stains the acidic part of the cell is the nucleus. Therefore,hematoxylin is called a nuclear stain while eosin act as an acidic stain and bind with the basic part of the cell – the cytoplasm and staining pink.

THE IMMUNOHISTOCHEMISTRY METHOD (David, 2010)

The method is the Avidin Biotin Complex (ABC) method also referred to as the Avidin biotin Immunoperoxidase method microns thick of formalin fixed and paraffin embedded tissue was cut for the IHC. Tissue antigenic sites were retrieved using citric acid solution PH 6.0 and pressure cooker. Peroxidases, protein and biotin blocks were done using Hydrogen peroxide, avidine and biotin respectively. Sections were incubated with the different antibodies for the study. These were followed by the biotylinated secondary antibody, streptavidine, DAB/substrate reaction and hematoxylin counterstain.

The antibody dilution factor used were as follows:1:150 for E-cadherin, 1:50 for p16 while ready to used mouse monoclonal antibody was used for ki-67 and beta-catenin antibody markers

Below is the detail of the IHC protocol

The processed tissue was sectioned at 2micons on the rotary microtome and placed on the hot plate at 70 degrees for at least 1hour, sections were brought down to water by passing the on 2 changes of xylene, then 3 changes of descending grades of alcohol and finally to water. Antigen retrieval was performed on the sections by heating them on a citric acid solution of PH 6.0 using the Microwave at power 100 for 15minutes. The sections were equilibrated gradually with cool water to displace the hot citric acid for at least 5min for the section to cool.

Peroxidase blocking were done on the sections by simply covering section with 3% hydrogen peroxide (H_2O_2) for 15min, sections were washed with PBS and protein blocking were performed using avidin for

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15min, sections were washed with PBS and endogenous biotin in tissue were blocked using biotin for 15min, after washing with PBS sections were incubated with the respective diluted primary antibody for example E-cadherin antibody diluted 1:100 for 60 min, excess antibody were washed off with PBS and a secondary antibody (LINK) were applied on section for 15min. Sections were washed and the (LABEL) which is the horseradish peroxidase (HRP) were applied on the sections for 15min.

A working DAB solution is made up by mixing 1 drop (20microns) of the DAB chromogen to 1ml of the DAB substrate. This working solution is applied on sections after washing off the HRP with PBS for at least 5min. The brown reactions begin to appear at this moment especially for a positive target. Excess DAB solution and precipitate are washed off with water. Sections were counterstained with Hematoxylin solution for at least 2min and blued briefly. Sections are dehydrated in alcohol, cleared in xylene and mounted in DPX

Cells with specific brown colors in the cytoplasm, cell membrane or nuclei depending on the antigenic sites are considered to be positive. The hematoxylin-stained cells without any form of brown colors are scored negative. Non-specific binding/brown artifacts on cells and connective tissue are disregarded.

IMMUNOHISTOCHEMICAL METHODS

The immonohistochemistry analysis for Ki67 was done using the IHC technique which was based on the principle of Avidin biotin immunoperoxidase reaction with all markers exhibiting similar nuclear immunoreactivity target ;
Ki67 antibody by Ventana antibody, anti Ki67 (30-9) WITH Lot number F-10365

3.10 CONTROL FOR IHC:

Positive control for all markers was obtained from tissues that are known to express the antigen.In negative control, tissues that are known not to express spicific antibody markers were used, while in the reagent negative controls, various antibodies that are being tested were omitted.

SCORING SYSTEM FOR CIN (Klein *et al*., 1999)

A =% of IHC	**B intensity of IHC reaction**	**Final score**
0 = 0%	0 = no reaction	A + B = range from 0 to 6
1 = <30%	1 = weak	
2 = 30-60%	2 = mild	
3 = >60%	3 = strong	

where A is the % or the proportion of tumour cells stained, and B is the intensity of the tumour cells stained

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

CLINICAL INFORMATION OBTAINED

a. Type of cancer diagnosis

b. Site of the cancer

c. Sex of patients

3. 3.Result:

This study involved Eighty (80) female subjects including Cervicitis 25%, CIN1,18.8%, CIN2,18.8% and 37.50% for SCC as shown in figure 4.1

There was significant ($P<0.05$) high degree of expression of K-i67 in SCC, while a significant ($p<0.05$) low degree expression of Ki67 in Cervicitis and CIN, while no statistically significant ($p>0.05$) difference in the expression of Ki-67 was found in CIN2.

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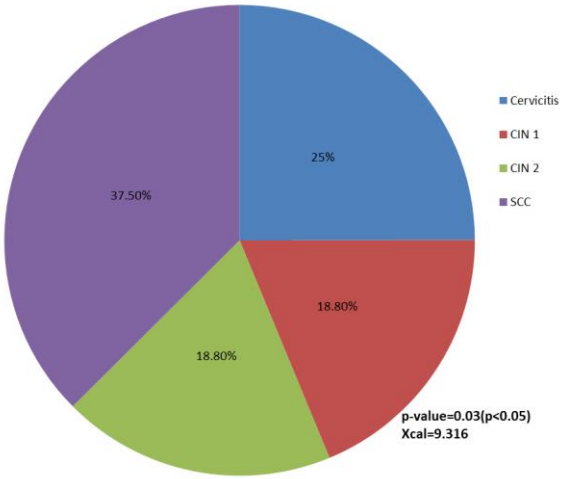

Figure 1: Classification of the Cases of the Subjects Examined in this study.

Table 1: Rate of Expression of Ki-67 among the difference cases in the study

Diagnosis	Number examined	Positive Expression n(%)	Negative Expression n(%)	p-value
Cerviticitis	20	17(85.0)	3(15.0)	<0.0001
CIN 1	15	15(100)	0(0.0)	<0.0001
CIN 2	15	13(86.7)	2(13.3)	<0.0001
SCC	30	30(100)	0(0.0)	<0.0001
Total	80	75(93.8)	5(6.2)	<0.0001

KEYS: CIN intraepithelial neoplasm

SCC; squamous cell carcinoma

% ; percentage of expression

P-value: significant level

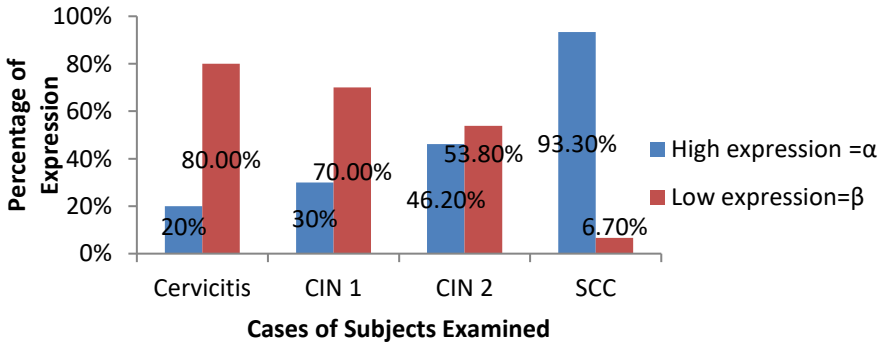

Figure 2: Degree of Expression of Ki-67 across the difference cases

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KEY

Cervicitis β>α(P<0.05)

CIN 1 β>α (P<0.05)

CIN 2 β>α (P>0.05)

SCC α>β (P<0.05)

LIN AND EOSIN

STAINED SLIDES

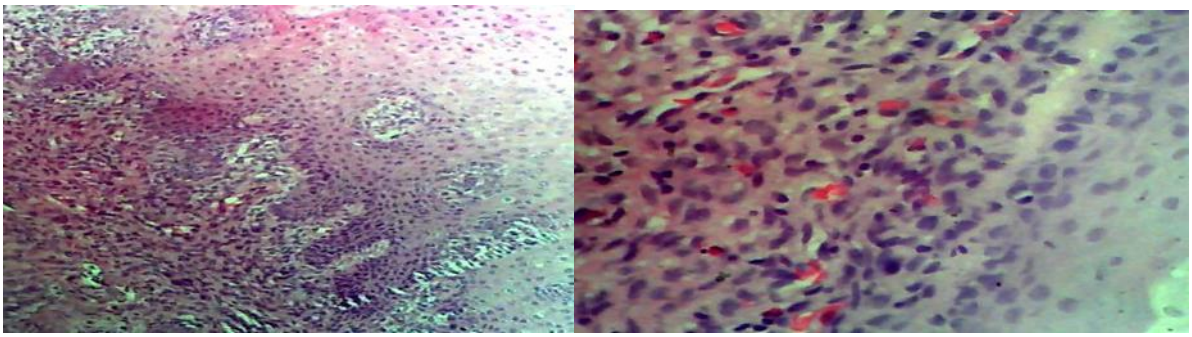

Plate. 1 CERVICITIS X10 CERVICITIS x40

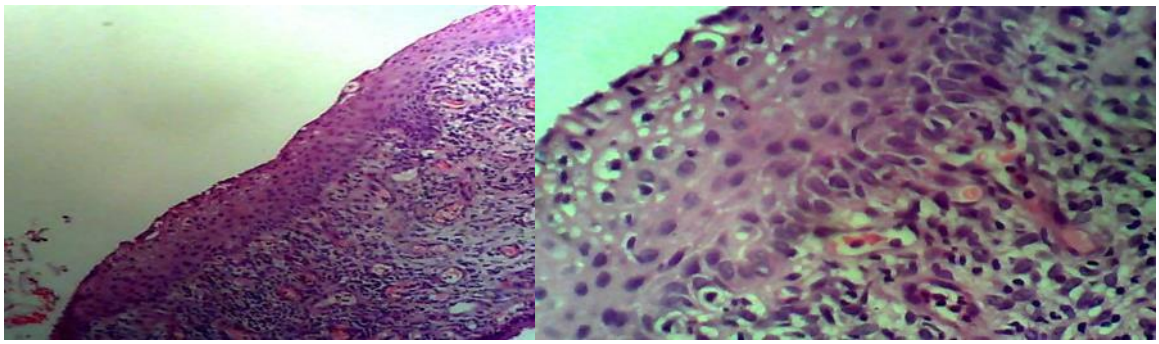

Plate 2: CIN2 x10 C1N2 x40 Objectives

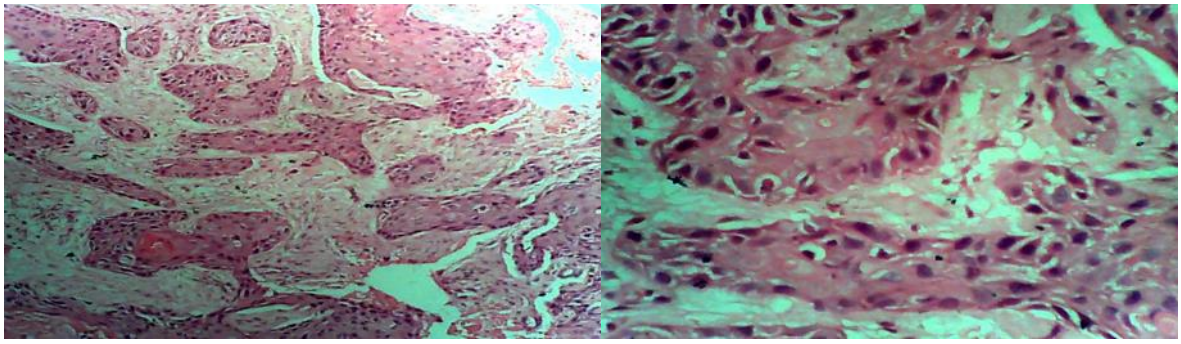

Plate 3: SCC X10 SCC x40 Objectives

Diagnostic photos of expression of biomarkers

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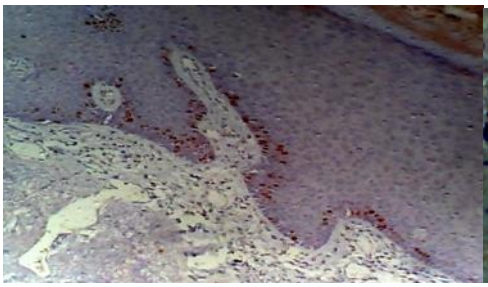
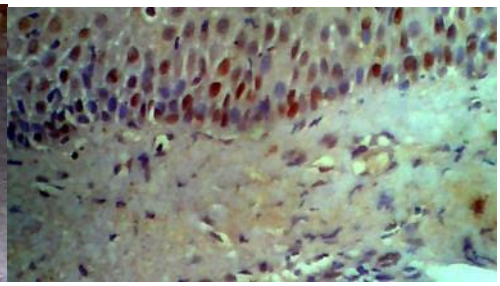
The expressions of KI67

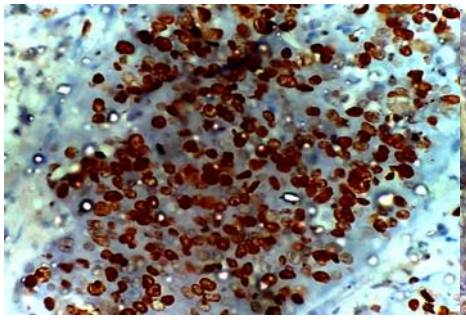
CIN 1 **CIN2**

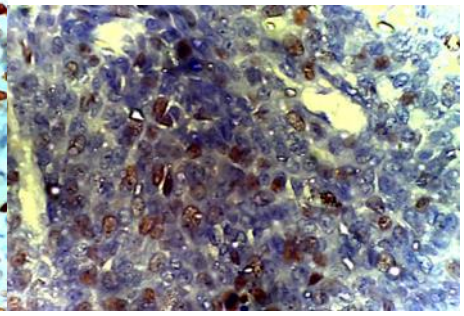




Low expression ki67 **High expression**

Plate 4: SCC SCC





High expression Low expression

Plate 5

4. Discussion

Ki-67 plays an important role as a tumor marker in cancers due to its close correlation with cell proliferation. It was reported that overexpression of K-i67 had significant association with poor prognosis in cervical cancer [9]. Our results confirm that an increase of Ki-67 expression was closely correlated with the prognosis of cervical cancer, demonstrating that it might be used as a potential predictor of prognosis in cervical cancer. Uncontrolled cell proliferation and malignant transformation are the basic elements in the development of malignant disease including cervical cancer and its precursors. Cell proliferation can be followed up by the expression of some cell proteins such as Ki-67 antigen. Ki-67 is a nuclear antigen expressed during G1, S, M and G2 periods of cell cycle. The expression level of Ki-67 indicated the status of cell proliferation. Some studies have showed that Ki-67 protein could be a biomarker in the evaluation of the proliferative activity and progressive potential of normal, dysplastic and neoplastic changes [10]. Our results showed that there was significant relation between the proliferative Ki-67 activity and CIN grade. Statistical difference between CIN1 and CIN2 group was significant. We have also found differences of the Ki-67 positive cell distribution according to the CIN and cancer grades Cervicitis 20%, CIN1 30%, CIN2 46.20% and SCC 93.30%. There was a clear trend for increasing number of cases with Ki-67 positive cells in the middle and upper third of the epithelial layer with increasing CIN and cancer grades, although there was statistically significant difference only between CIN1 and CIN2 grade.

Studies have shown a correlation between Ki67 expression and poor prognosis in several types of cancer, such as ovarian cancer [11], breast cancer,[12], esophageal squamous cell carcinoma [13], lung cancer, [14], and carcinoma of the bile duct and gallbladder [15], which indicated that Ki-67 might

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be used as a potential predictor for the survival of patients with some cancers. Similarly, to the aforementioned cancers, the finding from the available research demonstrates that high expression of Ki-67 can predict poor prognosis in cervical cancer, [9].

Ki-67 proliferative marker is very important for predict the value for progression of CIN and carcinoma lesions but according to recent studies it is not enough[16] showed that other markers are also important such as(Retinoplastoma protein) Rb-positive nuclei in the deeper half of the epithelium and the proportions of CK13 and CK14-positive cells [9].

5. Conclusion:

The prognostic relationship was explored in the carcinogenesis of cervical cancer by the sequential or increasing proliferative indices of Ki67 marker. Higher proliferation of Ki67 were established in cervical cancer when compared with dysplastic cervical lesions and not in cervicitis.

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