p16 INK4A EXPRESSION AS A MARKER FOR HPV INFECTION IN WOMEN INVASIVE BREAST CARCINOMAS

PRINCIPAL INVESTIGATOR:
DR. EMILE KARINGANIRE
H58/83552/2012
DEPAERTMENT OF HUMAN PATHOLOGY

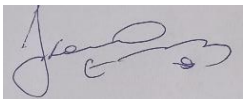
A DISSERTATION SUBMITTED IN PARTIAL FULLFILMENT FOR THE AWARD OF DEGREE MASTER OF MEDICINE, DEPARTMENT OF HUMAN PATHOLOGY, FACULTY OF HEALTH SCIENCES, UNIVERSITY OF NAIROBI.

2022

DECLARATION

I, **DR. EMILE KARINGANIRE**, declare that this is my original work and has not been presented in any other university or learning institution.

DR. EMILE KARINGANIRE

Signature*:**  ***Date: 24th October, 2022

SUPERVISORS; APPROVAL

DR. WAIRIMU WAWERU. MBChB, MMed (Path), FRCPath (ECSA)

Senior Lecturer, Anatomic pathology Unit, Department of Human Pathology,

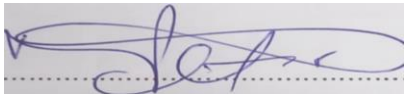
Faculty of Health Sciences, University of Nairobi.

Signature:  **Date: 2nd October,2022**

DR. DANIEL ZURIEL, MBChB, MMed (Path)

Senior Lecturer, Anatomic pathology Unit, Department of Human Pathology,

Faculty of Health Sciences, University of Nairobi.

Signature*:**  ***Date: 3rd October 2022

DEDICATION

To the loving memory of my parents, my wife UMUGWANEZA Marie Rosine, my daughters Chekina, Chientha and my brothers and sisters.

ACKNOWLEDGEMENTS

First I think God for wisdom and perseverance that has been given to me during this period.

I would like to express my sincere gratitude to my supervisors; Dr. DANIEL ZURIEL and Dr. WAIRIMU WAWERU for continuous support, patience and motivation.

I would like to thank the government of Rwanda, and Regional Alliance for sustainable Development (RASD-Rwanda) support through Albert Einstein College of Medicine, Yeshiva University, NY (Einstein), particularly Dr. Kathryn Anastos, Lynn Murchson and Dr. Eugene Mutimura.

I acknowledge the assistance I received from Rwandan Ministry of Defense and Rwanda Military Hospital.

My sincere thanks to MrOtieno Willis for technical assistance, colleagues and friends, my wife and daughters for the unconditional support in different ways.

TABLES OF CONTENTS

Contents **Pages**

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATION

ACS: American Cancer Society

BRCA1: Breast Cancer gene type 1

BRCA2: Breast Cancer gene type 2

BLBC: Basal Like Breast carcinoma

CDK: Cyclin D Kinase

CI: Confidence Interval

DNA: Desoxyribonucleic acid

ER: Estrogen Receptor

EBV: Epstein Barr Virus

H&E: Hematoxylin and Eosin

HPV: Human Papilloma Virus

HSV: Human Simplex Virus

IARC: International Agency for Research on Cancer

IHC: Immunohistochemistry

ISH: In Situ Hybridization

p16: protein 16

p16 INK4A: protein 16 as member of **INK4**proteins (Inhibitor of CD**K4)** and classified as **p16INK4a**

PCR: Polymerase Chain Reaction

Rb: Retinoblastoma

SPSS: Statistical Package for the Social Sciences

USA: United State of America

WHO: World Health Organization

ABSTRACT

Background

Breast cancer is the most commonly diagnosed cancer among women in both developed and developing countries. Studies have shown several risk factors including viral agents especially high risk HPV which can be diagnosed by studying p16 INK4A expression on formalin fixed, paraffin wax embedded tissue blocks. p16Immunohistochemistry is considered the best candidate for initial diagnosis of high risk HPV related lesions because of its availability, easy interpretation and its high sensitivity and specificity. This study examined p16 INK4A expression in series of invasive breast carcinoma at Kenyatta National Hospital in order to determine its utility in identifying high risk HPV related invasive breast carcinomas.

Objectives

The primary objective was to establish the p16 INK4A expression as a marker of high risk HPV infection in women invasive breast carcinomas at Kenyatta National Hospital.

Design: Our study was retrospective laboratory based.

Setting: The study was conducted at University of Nairobi, anatomic pathology laboratory at Kenyatta National Hospital.

Study population: Ninety-six (96) formalin fixed, paraffin wax embedded tissue blocks from breast biopsies reported as invasive breast carcinomas in women who attended KNH from January 2013 to February 2016.

Results: Our study demonstrated that 25 years old patient was the youngest while the oldest patient was 96 years old. The mean age was 46 years. The most affected age group was 41-50 age group (37.5%) followed by 31-40 age group (27%). The predominant histological type was invasive ductal carcinoma NOS (82.2%). Invasive breast carcinoma grade II were predominant (47.3 %) and majority of the patients presented with stage III invasive breast carcinomas (65.6 %).

Positivity was considered when there is nuclear and cytoplasmic staining or expression for p16INK4A. Positivity was noted in 11.4% of our cases and majority of the positive cases were noted among grade III breast carcinoma cases.

There was no statistically significant association between invasive breast carcinoma stage or grade to p16INK4A expression.

Conclusion: High risk HPV infection has been identified in a series of high grade and advanced stageinvasive breast carcinomas in Kenyatta National Hospital. Women aged from 40-50 years are the most affected by invasive breast carcinoma. Mastectomy was most common specimen. P16INK4A expression is not associated with breast tumor stage or grade.

Recommendation: Further scientificstudies are recommended to confirm if there is an associationor causal relationship between high risk HPV infection and invasive breast carcinoma among women in our setting.

1.INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

1.1.1. BREAST CANCER EPIDEMIOLOGY

Breast cancer is commonly diagnosed cancer among women bothin developed and developing countries. Worldwide in 2012, a total of 1.7 million women were diagnosed with breast cancer and the number of women living with breast cancer was 6.3 million. Breast cancer isamong the most common cause of cancer deaths (522.000 deaths in 2012) and the most frequently diagnosed cancer in women worldwide (1).Breast cancer development is associated with numerous internal and external factors such as genetic predispositions (mutations in BRCA1/2 gene and other genes), ethnicity, and family history of breast cancer, lifestyle, dense breast tissue, obesity, endogenous and exogenous estrogen exposure. Biological agents are responsible of 18-20% of cancer cases including breast cancer (2).

A total of232,670 new cases of breast cancer in women and 40.000related deaths were estimated in united States of America in 2014. (3). In Africa, breast cancer isthe mostdiagnosed malignancy in women and second leading cause of death among cancer patients. In 2008, African countries registered 92.600 cases of breast cancer with 50.000 deaths recorded. In general breast cancer prevalence in sub-Saharan Africa is estimated at 6.7% (4). Breast cancer in African women occur in young women compared to Western Europe, majority of the patients presenting late with advanced stage and sometimes terminal stages and are predominantly triple negative. Available reports indicate that epidemiological and clinical data are incomplete in most Africans countries. The number of cancers in Africa is increasing because of aging, increased prevalence of risk factors and growth of the population(4), (5).

In Kenya, cancer is 3rd cause of morbidity following infectious diseases in 1st position and cardiovascular diseases as 2nd cause. Each year, Kenyaregisters 39.000 new cases of cancer and more than 27.000 deaths annually are estimated; 70 to 80% of patients with cancer are diagnosed in late stage due to lack of awareness, high cost of treatment, inadequate diagnostics and treatment facilities. Frequently diagnosed cancer in women is breast cancer (34 100.000) followed by cervical cancer (25 100.000)(6).

Breast cancer survival rate varies greatly worldwide; 80% survival rate is observed in developed countries, 60% survival rate in middle income countries and less than 40% survival rate in low-income countries (7).

The low incidence in less developed countries is explained by poor statistic national programs and documentation procedures. Also lack of early detection programs, poor health infrastructures, lack of qualified health professionals results in high proportions of women presenting with advanced disease, as well as lack of good and timely diagnosis and treatment facilities (8). Breast cancer risk factors have been well known. Family history of breast cancer among 1st degree relatives increase the risk by a factor of 2 to 3. Genetic mutations particularly in BRCA1 and BRCA2 are also recorded among breast cancer risk factors. Reproductive factors associated with prolonged exposure to endogenous estrogens such as early menarche, late menopause and advanced age at 1st pregnancy are also documented as breast cancer risk factor (9). Exogenous hormones such as oral contraceptive and hormonal replacement therapy users are at high risk of developing breast cancer than non-users (10). Modifiable risk factors such as alcohol, smoking, physical inactivity, overweight and obesity contribute to development of breast cancer andcause around 25% of all breast malignancy cases (11).

1.1.2. HPV AND BREAST CANCER

Various studies have shown association of viral infectious agents and malignancies including breast cancer. Viruses associatedwith breast cancer are especiallyMouse mammary tumor virus (MMTV),Herpesviruses (CMV, EBV, HSV) and Human papilloma viruses (HPV) (12). Human Papilloma Viruses are non-enveloped DNA viruses belonging to the family of papillomavirus which have tropism to squamous epithelium (13). They can cause benign growth (papilloma), and malignant tumors and transient infections. HPV that infect the genital mucosa is classified as low risk and high risk type (13) (14). Low risk HPV types are associated with benign conditions and include HPV types 6,11,40,42,43,44,54,61,72,73 and 81. High risk HPV types are associated with malignant conditions (cervical, anogenital, oral and nasopharyngeal carcinomas) especially HPV 16, 18. High risk HPV types include types 16,18,31,33,35,35,39,45,51,52,56,58,59 and 82 (13)(14) (15).

People at risk of developing HPV infections are young, people with multiple sexual partners, immunosuppressed,female with early age of 1st sexual intercourse considered as sexual

intercourse done at 16 years of age or before and having male partner who has multiple sex partners(15) (16). Genetically HPV has early (E) and late (L) genes which cause malignant transformations by integrating into host cell genome. The early gene (E6) gene inhibits apoptosis by binding p53 which is tumor suppressor gene and late gene (E7) binds to pRb gene to induce S phase entry in the cell cycle) (17). This results in p16/Rb gene abnormalities leading to abnormalcell proliferationand causing malignant transformations. Scientific studies have proved that high risk HPV are involved in other cancers in addition to cervical cancers such as head and neck and breast carcinomas (17) (18).

HPV DNA found in breast malignant neoplasm specimens is similar to HPV DNA found in cervical malignant tumor biopsies, koilocytic changes have been identified in some malignant breast tissues associated with HPV infection (19). The theories that predict how high risk HPV reach breast andcause malignant tumors is not well explained but some authors suggest that is similar to cervical cancer pathology. Mode of transmission of HPV in breast malignant neoplasms is thought to be direct to the breast through the skin or nipple during sexual intercourse or by hematogenous spread (20).Viral associated breast malignancies including HPV positive breast cancer have been reported in sexually activeyounger women compared to HPV negative breast cancers suggesting a different etiology for younger women involving a causal role for HPV (20). Certain types of breast cancer such Basal Like Breast Carcinomas, Triple negative (ER, PR, HER2 neuro negative) are most likely associated with high risk HPV infections, also aggressive, high grade breast malignant neoplasmsmay be associated with viral agent especially HPV (20) (21). The diagnosis of HPV related breast cancer is molecular using PCR, FISH to detect HPV DNA sequencing or immunohistochemistry using p16INK4A and studying its expression and staining characteristics (22).

2. RATIONALE AND STUDY JUSTIFICATION

Viruses causes with many types of cancers such as cervical, anal, penile, head and neck malignancies. Hepatocellular carcinoma and lymphomas are also associated with viral agents. With advanced technology, some viruses have been discovered as causative agents of malignant transformations in human body. Those viruses are HPV (Human Papilloma Virus), EBV (Epstein Barr Virus), MMTV (Mouse Mammary Tumor Virus) and BLV (Bovine Leukemia Virus). Recent

studies suggest that those viruses might have an association with malignant transformation in the breast (23).

Globally, the most frequent malignant neoplasm in womenis breast cancer, responsible for the highest number of cancer deaths. The number of new cases continuing to rise. Breast cancer is most commonly diagnosed cancer in Kenyan womens; mostly diagnosed in younger patients compared to Western countries and presenting in late stage (24). Breast cancer is associated with internal, external factors and biological agents.Human Papillomavirus DNA has been identified in series of breast carcinoma using different laboratory techniques (25). Studies have shown that around 18-20% of cancer cases are linked to biological carcinogens worldwide including breast malignancies. Now a significant number of studies have shown that almost 23% of breast carcinomas are associated withhigh risk HPV subtypes especially 16, 18, and 33 (26).

However some studies have shown no evidence of association between HPV and breast carcinoma (27), the disparities are thought to be linked to lack of standardized techniques among laboratories, cross-contamination during sample collection and processing as well as geographic differences in HPV prevalence (high positivity correspond with HPV high prevalence area) and storages conditions as some positive specimens become negative after being frozen at -70C) for 3 months.(28). The diagnosis of high riskHPV associated tumors is done using Immunohistochemistry techniques (use of p16 INK4A and study of nuclear and cytoplasmic staining) (29)and use of Molecular techniques such PCR and FISH (30) (31).

The strong association of HPV infection with female breast cancer is seen in BLBC(Basal Like Breast Carcinomas), Triple Negative (ER, PR, HER2 negative), high grade invasive breast carcinoma (ductal,lobular or mixed), breast carcinomas diagnosed among sexually active young women and patients with both cervical and breast cancer (32). Around 89% of BLBC, triple negative is associated with HPV related breast carcinomas and 55% of high - grade invasive breast carcinomas in young women are high risk HPV related and show positive reactions to p16 antibodies and those breast carcinomas are associated with poor prognosis.

Considering the high prevalence of HPV infection in Kenyan population and high number of HPV associated malignancies, there is a possibility that also malignant breast tumors may be associated with HPV infection. Comparing IHC technique to molecular laboratory techniques(PCR, FISH, E6/E7 messenger RNA) which is expensive and not available in all health facilities,

theimmunohistochemistry approach by studying staining pattern for p16INK4A in formalin fixed, paraffin wax embedded tissue blocks from breast biopsies diagnosed as invasive carcinomacan be suitable to diagnose HPV (Human Papilloma Virus) presence in breast tissues. The IHC technique is simple, suitable, cost - effective method to screen and diagnose high risk HPV related breast malignancies all over the world. The p16 INK4A is highly specific and highly sensitive (33) (34).

Considering that IHC p16 INK4A is an ideal marker of high risk HPV subtypes especially 16 and 18 which are also the most common in Kenya, study of its expression on formalin fixed, paraffin wax embedded histological blocks from confirmed breast malignant lesions may be useful to determine whether HPV infection is associated with breast malignant lesions among Kenyan women. The study of IHC (p16 INK4A) staining pattern on paraffin fixed, paraffin embedded tissue blocks from histologically confirmed breast malignant lesions will give us useful information on the association of HPV infection and breast malignant transformation among Kenyan women. The study is conducted in order to identify p16INK4A expression in invasive breast carcinoma tissues and determine whether there is an association between breast cancer development and high risk Human Papilloma virus infections.

3. RESEARCH QUESTION AND OBJECTIVES

3.1. RESEARCH QUESTION

Is HPV associated with female invasive breast carcinomas in Kenyatta National Hospital?

3.2. BROAD OBJECTIVES

To determine the prevalence of female invasive breast carcinomas associated with HPV infection in Kenyatta National Hospital.

3.3. SPECIFIC OBJECTIVES

1.To determine p16INK4A expression in female invasive breast carcinoma.

2. To determine morphological changes associated with HPV infection in female invasive carcinomas of the breast.

3. To determine the age group most affected by female breast carcinomas associated with HPV.

4. STUDY DESIGN AND METHODOLOGY

4.1. TYPE OF STUDY

The study was laboratory based retrospective study.

4.2. STUDY AREA DESCRIPTION

Cases were recruited from KNH (Kenyatta National Hospital) Histology laboratory. Processing of specimens (retrieved tissue blocks) was carried out at University of Nairobi, Unit of Anatomic Pathology Laboratory.

4.3. STUDY POPULATION

All cases of breast biopsies (mastectomy, lumpectomy and core biopsies) seen and diagnosed as invasive breast carcinoma, obtained from women processed at Kenyatta National Hospital during a period from January 2013 to February 2016.

4.4. STUDY ELIGIBILITY CRITERIA

4.4.1. INCLUSION CRITERIA

1. Female patients diagnosed with invasive carcinomas of the breast, grade 1,2 and 3.

4.4.2. EXCLUSION CRITERIA

1. All cases diagnosed as Carcinoma in situ.

2. All malignant tumors of the breast that are not carcinomas

3. Technically poorly processed tissues.

4.4.3. Sample size determination

Sample size was calculated using the prevalence of breast cancer in Sub-Saharan Africa (6.7%) (4) and Fisher's formula was used.

$$n = \frac{z^2 \times p(1-p)}{d^2} = \frac{1.96^2 \times 0.067(1-0.067)}{0.05^2} = 96$$

n: sample size

p: known prevalence.

Z: normal standard deviate that correspond to 95% Confidence Interval.

d: margin of error degree of precision (+/_ 5%).

The sample size was 96 cases of invasive breast carcinomas.

4.5. SAMPLING METHOD

Cases meeting inclusion criteria were recruited into the study. Inclusion criteria is all formalin fixed, paraffin wax embedded histological blocks belonging to female patients diagnosed with invasive carcinoma of the breast of all grades. The histology blocks retrieval started following ethical review committee approval on 23rd February 2016.

4.6. RECRUITMENT PROCEDURE

Patients files containing histology report at KNH in order to identify all patients diagnosed with invasive breast carcinoma were retrieved. This started after ethical review committee approval in February 2016. The name, gender, ward, patient's hospital number, hospital name and laboratory number were noted from the histology report as the cases were identified. This information was used to retrieve the complete case notes and the archival formalin fixed paraffin embedded blocks.

4.7. METHODS

4.7.1. SAMPLE RETRIEVAL AND PROCESSING

Tissue blocks which are formalin fixed, paraffin wax embedded were retrieved from histology archives (KNH histology laboratory) using the laboratory number as mentioned in pathology reports. For confidentiality purpose, slides were labeled with study number as E001 /2015, E002/2015 up to E096/2015. The test name was indicated on the slide. The routine H&E histological staining was done at University of Nairobi histology laboratory as shown in appendix 4.p16INK4AImmunohistochemistry staining was done for all cases that meet inclusion criteria.

The staining was performed using p16 INK4A monoclonal antibody detecting high risk HPV sub types (16, 18) following staining protocol for manual use(Appendix 2). IHC (p16INK4A) interpretation was done according to 3 tier system based on nuclear cytoplasmic staining reactivity. The grading was done from 0 (negative), +1 and +2. The lesion is qualified as negative when there are 0 – 5 % reactive cells. The lesion is graded +1 when there is focal or scattered reactivity

representing 5% and less than or equal to 80% reactive cells. The lesion is qualified +2 when there is diffuse positivity characterized by more than 80% reactive cells showing both nuclear and cytoplasmic positivity.

The slides were processed in different batches. Dry slides were stored serially in atray according to the study numbers ready to be reported first by the principal investigator, slide review was done by both principal investigator with supervisors. The blocks were used for IHC processing for determination of p16INK4A expression after which they were serially arranged in a tray to await reporting by the principal investigator first then together with supervisors.

4.7.2. SPECIMEN PROCESSING

A 5-micron section was cut from each of the block. The sections were mounted on the H&E labeled slides, stained using standard H&E staining procedure. When dry, they were stored serially in a tray according to study numbers ready for reporting first by principal investigator, then together with supervisors (Appendix 1). This was to confirm the initial diagnosis and classify the findings as invasive breast carcinoma grade 1, 2 and 3.

The formalin fixed, paraffin wax embedded were used in Immunohistochemistry processing for determination of p16INK4A expression after which they were serially arranged in a tray to await reporting by principal investigator first then together with the supervisors. The information was recorded into the data sheet. The blocks were transported for IHC staining to University of Nairobi, histology laboratory. A 5-micron section was cut from the formalin fixed, paraffin embedded tissues blocks of cases meeting inclusion criteria and mounted on two Poly-Lysine treated slides well labeled with study number and type of test. The slides were air dried and processed for p16 INK4A staining and expression in cytoplasm and nucleus. A positive control was included in each batch. The p16 expression was analyzed and entered into data collection sheet.

4.8. QUALITY ASPECTS

Reagents were prepared according to the manufacturer's instructions. SOPs were followed during the procedure. The reagents expirations date, turbidity, odor and precipitates were checked and unqualified reagents were not used. Reagents storage conditions were observed. Positive controls were used for IHC staining interpretation. The slides were labeled and then arranged in order to avoid mix up of the slides. Scores were independently reviewed.

4.9. DATA COLLECTION INSTRUMENT

Data collection instrument usedwas data collection sheet (Appendix 1).

5. DATA ANALYSIS AND PRESENTATION

Data were entered in Microsoft Excel (Ms 2007), password protected and were analyzed using SPSS (Statistical Package for the Social Sciences) software and charts.

6. ETHICAL CONSIDERATION

Permission for records and specimen retrieval of use in this study was obtained from the KNH/UON ethical research committee. No consent was required from the patients because of retrospective nature of the study. Patient identifiers were protected to maintain confidentiality. The slides were labeled study number as E001/15…. E096/16. Nonames ward or laboratory number appeared on data collection sheet. All data was entered and saved as soft copy, password protected. Study numbers were used instead of original laboratory numbers to maintain confidentiality.

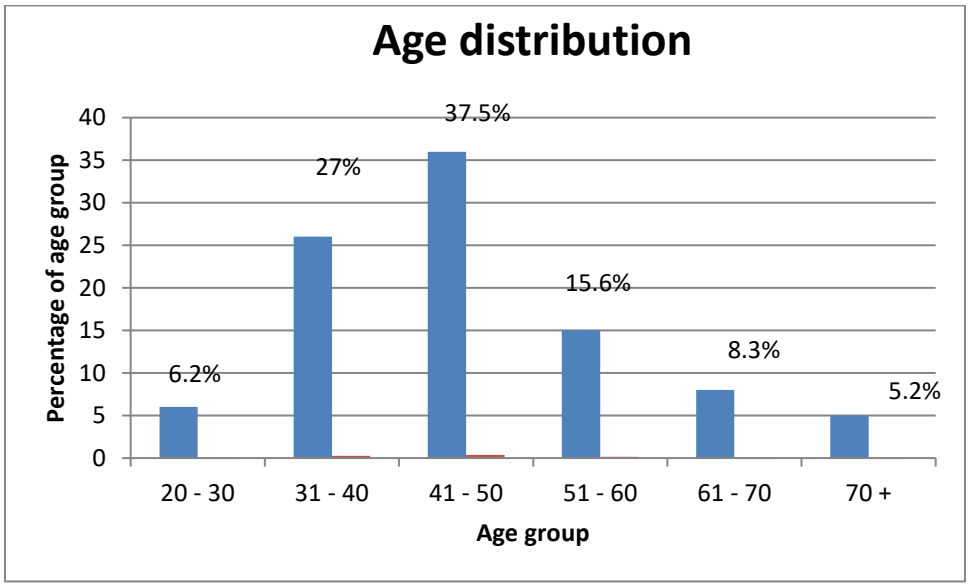
7. RESULTS

7.1. Age distribution.

The present study was carried out on total number of 96 patients diagnosed with invasive breast carcinomas. Among our patients, the younger was 25 years old, while the oldest was 96 years. The mean age was 46.8 (SD +/-5). Majority of our patients were in 41-50 age grouprepresenting 37.5% followed by patients belonging to 31-40 age group representing 27 %.

The 3rd group was composed of patients belonging to 51-60 age group representing 15.63%, while the 4th group was composed of patients belonging to 61-70 age group representing 8.30%. The 5th group was composed of patients belonging to 20- 30 age group representing 6.2%. The last group was composed of patients who are 70 years old and above representing 5.2%.

Figure 1: Age distribution (n=96)



7.2. Histological types.

We identified seven (7) histological types including ductal carcinoma NOS, mucinous type,cribriform type, lobular type, papillary type, medullary type and anaplastic carcinoma of the breast. The predominant histological type was ductal carcinoma with NOS with 79 cases followed by both lobular and papillary carcinomas with 5 cases each. Less histological types are represented by ductal carcinoma cribriform type, medullary and anaplastic carcinoma with 3 cases and 1 cases respectively. Ductal carcinoma NOS histological type represent 79%, lobular and papillary

carcinomas represent 5.2% each. Ductal carcinoma cribriform type represents 3.1%, medullary and anaplastic carcinoma histological types represent 1% each.

Table 1: Breast carcinoma: histological types

Variable	Frequency (n:96)	%
Histological Type		
Ductal Carcinoma NOS	79	82.2
Mucinous Carcinoma	2	2
Ductal Carcinoma Cribriform type	3	3.1
Lobular Carcinoma	5	5.2
Papillary Carcinoma	5	5.2
Medullary Carcinoma	1	1
Anaplastic Carcinoma	1	1

7.3. Histological grade.

Reference to Modified Bloom - Richardson breast cancer grading system which is based on three (3) morphological features: degree of tumor tubules formation, mitotic activity and nuclear pleomorphism as presented in annex IV. This study showed that majority of patients (45 out of 96) presented with breast carcinoma grade II representing 47.3% of the total cases followed by 36 out of 96 patients presented with breast carcinoma grade III representing 37.8% of the total cases. The least common grade was grade I breast carcinoma seen in 15 out 96 patients representing 14.7%.

Table 2: Breast carcinoma: Histological grade

Variable	Frequency (n: 96)	%
Carcinoma Grade		
Grade 1	15	14.7
Grade 2	45	47.3
Grade 3	36	37.8

7.4. Breast carcinoma: histological stage.

The staging was done using TNM WHO staging system as shown in annex IV going from stage I up to stage IV. Our study shows that majority of patients (63 out of 96) presented with breast carcinoma stage III representing 65.6% of total cases followed by 18 out of 96 patients who presented with stage II breast carcinoma representing 18.7% of total cases. 8 out of 96 patients presented with stage IV breast carcinoma representing 8.3% of total cases while 7 out of 96 patients presented with stage I breast carcinoma representing 7.2% of total cases.

Table 3: Breast carcinoma histological stage.

Variable	Frequency (n:96)	%
Carcinoma Stage		
Stage I	7	7.2
Stage II	18	18.7
Stage III	63	65.6
Stage IV	8	8.3

7.5: Breast carcinoma types of specimen.

Our study shows that mastectemy was the most frequent type of specimen. Among 96 patients, mastectomy was performed on 88 patients representing 91.6% and less common type of specimen was lumpectomy performed on 8 pationts out of 96 representing 8.3% of total cases.

Table 4: Breast carcinoma: type of specimens

Variable	Frequency (n: 96)	%
Type of specimen		
Lumpectomy specimen	8	8.3
Mastectomy specimen	88	91.6

7. 6: Breast carcinoma, p16INK4A expression.

Our study shows that 11 out of 96 cases representing 11.4 % stained positive to p16INK4A while 85 out of 96 cases representing 88.5% were negative for p16INK4A.

Table 5: Breast carcinoma: p 16INK4A expression

Variable	Frequency (n : 96)	%
P 16 Expression		
Negative	85	88.5
Positive	11	11.4

7.7. Association between p16INK4A expression and histological type.

This study demonstrated that p16INK4A IHC positivity was predominantly observed in ductal carcinoma NOS histological types as 8 out of 71 cases of ductal carcinoma NOS stained positive. All cases (2 out of 2) of mucinous carcinoma of the breast stained positive to p16INK4A IHC while 1 case out of 2 ductal carcinomas cribriform subtype stained positive for p16INK4A with P value of 0.005 and Cramer's value of 0.44. All cases of lobular, papillary, medullary and anaplastic carcinoma of the breast stained negative for p16INK4A IHC.

Table6: p16INK4A expression and histological type.

P16 Expression	Negative	Positive	Cramer's V	P-value
Histological Type			0.44	0.005
Ductal Carcinoma NOS	71	8		
Mucinous	0	2		
Cribriform	2	1		
Lobular carcinoma	5	0		
Papillary	5	0		
Medullary	1	0		
Anaplastic	1	0		

7.8. p16INK4A expression and breast carcinoma grade.

This study shows 6 grade III breast carcinomas (6 out of 30 cases) stained positive for p16INK4A, 3 out of 42 grade II breast carcinomas cases stained positive to p16INK4A IHC while 2 out of 12 grade I breast carcinoma cases stained positive for p16 INK4A IHC with p value of 0.148 and Cramer's value of 0.355.

Table 7: p16INK4A expression and breast carcinoma grade.

P16 Expression	Negative	Positive	Cramer's V	P-value
Breast carcinoma Grade			0.355	0.148
Grade 1	12	2		
Grade 2	42	3		
Grade 3	30	6		

7.9. p16 expression and breast carcinoma stage.

The study shows that majority of stage III breast carcinoma (8/63) stained positive for p16INK4A IHC followed by stage II breast carcinoma with 2 cases out of 18 which stained positive for p16INK4A IHC with p value of 0.179 and Cramer's value of 0.382. None of stage I or stage IV breast carcinoma case stained positive for p16INK4A**.**

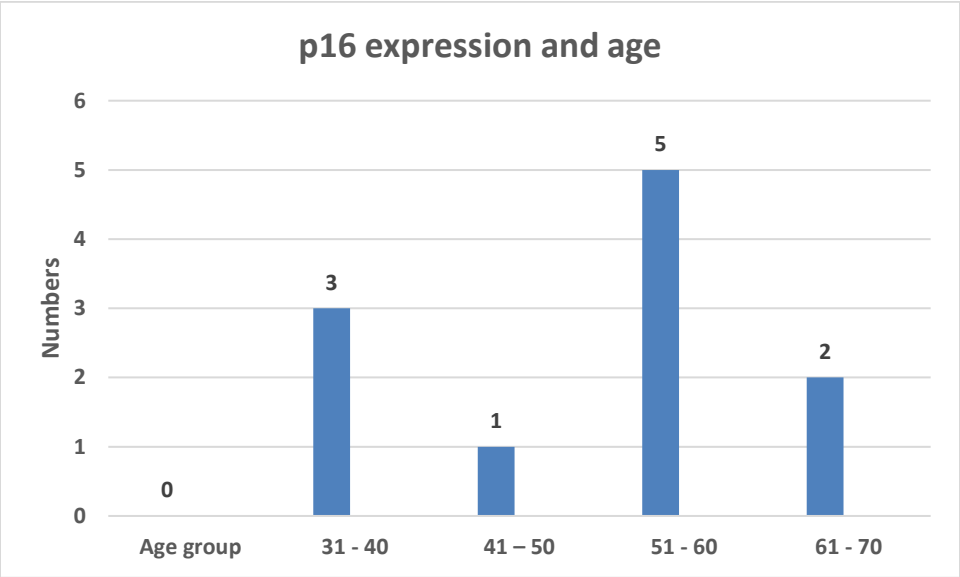
Table 8: p16 INK4A expression and breast carcinoma stage.

P16 Expression	Negative	Positive	Cramer's V	P-value
Carcinoma Stage			0.382	0.179
Stage I	7	0		
Stage II	18	2		
Stage III	63	9		
Stage IV	80			

7.10. p16INK4A expression and age

This study showed that 3 patients out of 11 belonging to 31 – 40 age expressed p16INK4A while 1out of 11 patients belonging to 41 – 50 age group expressed p16INK4A. Majority of patients who expressed p16INK4A (5 out of 11) belongs to 51 – 60 age group. Two (2) out of 11 patients who expressed p16INK4A belongs to 61-70 age group.

Figure 2: p16INK4A expression and age



8. DISCUSSION

This study analyzed 96 cases of invasive breast carcinomas, youngest patient was25 year old and oldest was 96 years old; the mean age was46.8 years.The high number of invasive breast cancer was noted in the age group ranging from 41-50 years. These findings are consistent with study done in Kenya by Makanga et al. (35) done at Agha Khan Hospital whereby the youngest patient was 26 and the oldest patient was 94 years old, the mean age of developing invasive breast cancer was 50 and highest proportion was seen in age group ranging from 45 to 49 years old. Our study was in agreement with the study done in Central African Republic by Augustin Balekouzu et al (36)whereby 16 year old was the youngest and oldest patient was 90 years old. During their study, they found that the highest proportion of invasive breast carcinoma was between 45 and 54 years old and represented 29.3%. The results are also in agreement with study done by Lopes et al in Angola (37) where they found that the youngest patient who developed invasive breast carcinoma was 16 years old and oldest patient was 87 years old. The highest proportion of invasive breast carcinoma was 47 years.

In our study population, the highest proportion of invasive breast carcinoma was noted in younger age group ranging from 41 – 50 years. Generally, patients with breast malignant lesions in sub-Saharan Africa are younger compared to American and European women, the reason of early occurrence of breast cancer is not certain but is partly attributed to low life expectancy in African countries. Hormonal imbalance might be a contributing factor as black women have high levels of estrogen compared to white women which predispose them to high rate of cell division and DNA copying errors leading to high risks of early breast cancer development(38)

The study showed that the predominant histological type was invasive ductal carcinoma NOS (82.2%) followed by lobular carcinoma and papillary carcinoma of the breast representing 5.2% each and less common histological types are invasive medullary type and anaplastic invasive breast carcinoma representing 1% each. Our findings are consistent with findings of the study done in Norway by Grethe et al (38) where the most common histological type was invasive ductal carcinoma NOS representing 81,4%, lobular carcinoma representing 6.3%, mucinous carcinoma of the breast representing 1.5% and medullary carcinoma of the breast representing 1.1%. Our results are in agreement with results of the study done by Peter Rambau in Tanzania (39) where they found that the predominant histological type was invasive ductal carcinoma NOS of the breast

representing 91.5% followed by invasive mucinous carcinoma representing 5.2% and invasive lobular carcinoma of the breast representing 3%.

Majority of our patients presented with high grade invasive breast carcinoma. Our study shows that he predominant invasive breast carcinoma grade was grade 2 (47.3%) followed by Grade 3 (37.8 %) and Grade 1 breast carcinoma (14.7%). The grading was done using Modified Bloom – Richardson grading system. Our results are comparable to those of a study done in Ghana by S.E Quayson et al ((40) which found that majority of patients presented with high grade breast carcinoma (grade 2 and 3) whereby grade 3 represented 37.5%, grade II represented 36.5% and grade I breast carcinomas represented 26%. Our results are also consistent with study done by GD Forae (41). In this study, majority of patients (78.7%) presented with grade 3 invasive breast carcinoma followed by 19.6% of patients who presented with grade II invasive breast carcinoma and 1.7% who presented with grade I invasive breast carcinoma. Considering the advanced tumor stage and grade at the time of presentation at health facilities, the majority of our patients presented late because of poverty, low awareness and weak health systems. The difference between the number of grade III breast lesions in Forae study and ours might be linked to the difference in sample size as we analyzed 96 samples while he had much larger samples of 905.

Our study demonstrated that most of patients consult medical facilities with breast cancer stage II and IV. Majority of our patients presented with stage III breast carcinomas (65.6%) followed by stage II(18.7%), stage IV breast carcinomas (8.3%) and stage I breast carcinomas represented 7.2%. Our results are consistent with study done in Cameroun by Charlotte Nguefack et al (42)whereby they found that more than half of patients (54%) presented with stage III, followed by stage II (28%), 14% of patients presented with stage IV and only 2% presented with breast cancer stage I.

Considering the advanced tumor stage and grade at the time of presentation at health facilities, the majority of our patients presented late for many reasons including inability to pay medical services, ignorance, misdiagnosis at primary health care and cultural beliefs.

Almost allof analyzed specimen were mastectomy specimen (91.6%) followed by lumpectomy specimen. Majority of the patients presenting late with advanced stage explain the high number of mastectomy specimen as surgical treatment is the only option to these particular patients. It is also

possible that since KNH is a referral hospital, the diagnosis had been made elsewhere, then they were referred for definitive surgery to KNH.

This study shows that 11.4% of cases expressed diffuse nuclear and cytoplasmic staining considered positive for high risk HPV type 16 or 18 as our kit was detecting only 2 HPV subtypes (16 and 18). These results are consistent with those of the study done by B. Heng et al. (43) in 2009 suggesting that the presence of HPV in breast carcinoma worldwide has a prevalence varies from 4-86% and the study done by Fernandes et al. saying that the prevalence of HPV in invasive breast carcinomas in Latin America ranges between 5 and 40%.Our results are also in agreement with one done by James Lawson et al in 2015 (44) showing that HPV types 16 was present in breast cancer at a proportion of 10% and HPV type 18% at a proportion of 50%. The results of our study however shows a disagreement in terms of frequency or proportion. This difference may be linked with the small sample size in our study (96) compared to Lawson sample size of 855 cases. The difference may also be explained by differences in method used. Our method (p16INK4A IHC) used to detect only high risk HPV type16 and type 18 whereas Lawson's study used PCR techniques which detect more subtypes of high risk HPV thus increasing the finding of a higher the prevalence of HPV infection in breast invasive malignant lesions.

Considering p16INK4A expression and breast carcinoma grade, our study showed that 2 grade I, 3 grade 2 and 6 grade 3 cases of invasive breast carcinoma expressed p16INK4A with p value of 0.148 and Cramer's value of 0.355. There was therefore no association between p16INK4A expression and breast carcinoma grade.

Considering p16INK4A expression and breast carcinoma stage, our study shows that 2 and 9 cases of invasive breast carcinoma stage II and stage III respectively showed nuclear and cytoplasmic diffuse p16INK4A expression with p value of 0.179 and Cramer's value of 0.382. This shows that the association between p16INK4A and invasive breast carcinoma stage is not statistically significant.

9. LIMITATIONS

During our study, the limitations were due to poorly preserved blocks, poorly processed tissues and lack of appropriate clinical information on the request forms.

10. CONCLUSION

Using High Risk Human Papilloma Virus IHC detection kit (p16INK4A CINTEC), around 11.4% of invasive breast carcinoma diagnosed at KNH (Kenyatta National Hospital) express p16INK4A which is a marker of HPV subtype 16 and 18.

p16INK4A was mostly expressed in high grade invasive ductal carcinoma NOS.

There are no specific morphological changes demonstrated in association with p16INK4A expression.

There was no specific age group associated with p16 expression among female patients diagnosed with invasive breast carcinoma of the breast at KNH (Kenyatta National Hospital).

The expression of p16INK4A which is a marker of high risk HPV (Human Papilloma Virus) in breast tissues diagnosed as invasive breast carcinoma might be associated with theory that High Risk HPV infection can cause breast cancer. However further studies using different diagnostic platforms are advised to rule out possible association between Human Papilloma Virus infection and invasive breast carcinoma in women.

11. RECOMMENDATIONS

We recommend further studies to rule out any possible association between HPV infection and invasive breast carcinomas in our setting.

Molecular studies using Polymerase Chain Reaction (PCR) detecting all High Risk Human Papilloma Virus subtypes in breast tissue diagnosed as invasive breast carcinoma is recommended.

12. CONFLICT OF INTEREST

The author declares that there is no conflict of interest relevant to this study.

12. REFERENCES

1. Alibek K, Kakpenova A, Mussabekova A, et.al. Role of virus in the development of breast cancer. Infectious Agents and cancer. 2013 September 2; 8(32): p. 1750-9378.

2. Key TJ, Verkasalo PK, Banks E. Epidemiology of breast cancer. Lancet Oncol. 2001 Marchr; 2(3): p. 133-140.

3. Rebecca S, Jiemin M, Zhaohui Z, et.al. Cancer statistics 2014. infectious agents and cancer. 2014; 64(1): p. 9-29.

4. Ahmedin J, Freddie B, David F, et.al. Cancer burden in Africa and opportunities for prevention. American Cancer Society. 2012 September 15; 118(18): p. 4372-4384.

5. Katrina R, Rosemery D, Monica B, et.al. Descrptive analysis of Estrogen receptor(ER)-negative, Progesterone receptor(PR)-negativeandHER2-negative invasive breast cancer, the so called triple-negative phenotype. Cancer. 2007 May; 109(9): p. 1721-1728.

6. Opiyo A, William KM, Geoffrey M, et.al. National Cancer Control Strategy 2011-2016. National Cancer Control Strategy. 2011; 1(1): p. 8-10.

7. Coleman PM, Manuela Q, Franco B, et.al. Cancer survival in 5 continents: a worldwide popuatin-based study (CONCORD). The lancet oncology. 2008 August; 9(8): p. 730-756.

8. Nagi S, Mazen KK, Ali IS. Trends in epidemiology and management of breast cancer in developing Arab countries: a literature and registry analysis. International Journal of Surgery. 2007 August; 5(4): p. 225-233.

9. McPherson K, Steel CM, Dixon JM. ABC of breast diseases: epidemiology, risk factors and genetics. British Medical Journal. 2000 September 9; 321(7261): p. 624-628.

10. Linda KW, Ronald TB, Kara LC, et.al. Hormone replacement therapy regimens and breast cancer risk. Obstetrics & Gynecology. 2002 December; 100(6): p. 1148-1158.

11. Hellmann SS, Thygesen LC, Tolstrup JS, et.al. Modifiable risk factors and survival in women diagnosed with primary breast cancer: results from a prospective cohort study. European Journal of Cancer Prevention. 2010 september; 19(5): p. 366-373.

12. Wang T, Ling W. Role of Papillomavirus infection in breast cancer. Medical Oncology. 2011 February; 29(1): p. 48-55.

13. Eileen MB. Human Papilloma Virus and cervical cancer. Clinical Microbiology Reviews. 2003 January; 16(1): p. 1-17.

14. Veronique B, Robert B, Kurt S, et.al. A review of human carcinogens-Part B: biological agents. The lancet Oncology. 2009 April; 10(4): p. 321-322.

15. Clifford MG, GS, HR, et.al. Worldwide distribution of human papillomavirus types in cytologically normal women in the International Agency for Research on Cancer HPV prevalence surveys: pooled anaysis. The Lancet. 2005 September 17-23; 366(9490): p. 991-998.

16. Moscicki AB, Ellenberg JH, Farhat S, ea. Persistence of Human Papillomavirus Infection in HIV-Infected and -uninfected Adolescents Girls: Risk factors and differences , by Phylogenetic types. The Journal of Infectious Diseases. 2004 July 1; 190(1): p. 37-45.

17. Eun KY, Jong SP. The role of HPV E6 and E7 Oncoproteins in HPV-associated Cervical Carcinogenesis. Cancer Research and Treatment. 2005 December; 37(6): p. 319-324.

18. Heng B, Glenn WK, Ye Y, et.al. Human Papilloma virus is associated with breast cancer. British Journal of Cancer. 2009 October; 101(8): p. 1345-1350.

19. Glenn WK, HB, Whitaker NJ, et.al. Reply: Koilocytes indicate a role for human papilloma virus in breast cancer. British Journal of Cancer. 2010 February 16; 102(4): p. 788.

20. Bodaghi S. Could Human Papillomaviruses Be Spread through Blood? J Clin Microbol. 2005 November; 43(11): p. 5428-5434.

21. Akil N, Kassab A, Yasmeen A, et.al. Human breast cancer and sexual activity. British Journal of Cancer. 2008 January 29; 98(2): p. 508-509.

22. Fabiana RR, Rochael MM. The possible involvement of HPV in breast cancer. J bras Doencas Sex Transm. 2011; 23(4): p. 182-185.

23. Amany AA, Hany IE. p16 expression correlates with basal-like triple- negative breast carcinoma. ecancermedicalscience. 2013 may 14; 7(317).

24. James L, Heng B. Viruses and breast cancer. Cancers (Basel). 2010 June; 2(2): p. 752-772.

25. David EW, George OO, Margaret O, et.al. Predictors of breast cancer treatment outcomes in Kenyan women. African Journal of Pharmacology and Therapeutics. 2013; 2(4): p. 109-105.

26. Andrea PD, Rashid K, Claudio GZ, et.al. Evidence for an association of human papillomavirus and breast carcinoma. Breast Cancer Research and treatment. 2004; 84: p. 131-137.

27. Simoes PW, Medeiros LR, Simoes Pires PD, et.al. Prevalence of human papilloma virus in breast cancer. Int J Gynecol Cancer. 2012 March; 22(3): p. 343-7.

28. Suresh H, Umesh K, Showket H. Breast cancer and Human papillomavirus: No evidence of HPV etiology in Indian women. BMC Cancer. 2011; 11(27): p. 1471-2407.

29. Ava K, Candy PL, Vivian YS, et.al. No evidence of Human Papillomavirus in Patients with Breast Cancer in Hong Kong, Southern China. International Scholarly Research NoticesVirology. 2013; 2013(2013): p. 4.

30. Milde-Langosch K, Bamberger AM, Rieck G, et.al. Expression of the p16 cell cycle inhibitor in breast cancer is associated with a more malignant phenotype. Breast Cancer Research and Treatment. 2001 May; 67(1): p. 61-70.

31. Khan NA, Castillo A, Koriyama C, et.al. Human Papillomavirus detected in female breast carcinomas in Japan. British journal of Cancer. 2008 August 5; 99: p. 408-414.

32. Ana L, Pereira S, Mario AL, et.al. Presence of Human Papilloma virus in a Series of Breast Carcinoma from Argentina. PLosOne. 2013; 8(4): p. 1370-1371.

33. Andrea PS, Ty S, Hind N, et.al. Most Basal-like Breast Carcinomas Demonstrate the same Rb-/p16+ Immunophenotype as HPV- related poorly differentiated Squamous Cell Carcinomas which they ressemble morphologically. Am J Surg Pathol. 2009 February; 33(2): p. 163-175.

34. Kong CS, Balzer BL, Troxell ML, et.al. P16 Immunohistochemistry is superior to human papillomavirus in situ hybridization for the detection of high rrisk human papillomavirus in atypical squamous metaplasia. Am J Surg Pathol. 2007 January; 31(1): p. 33-43.

35. Wang SS, Trunk M, Schiffman M, et.al. Validation of p16INK4A as a marker of oncogenic human papillomavirus infection in cervical biopsies for a population based cohort in Costa Rica. Cancer Epidemiol Biomarkers Prev. 2004 August; 18(8): p. 1355-1360.

36. Makanga W WRSH. A Profile of Female Breast Cancer Patients in Kenyan Urban Private Hospital. The Annals of African Surgery. 2013 January; 10(1).

37. Augustin Balekouzou PYCPeal. Epidemiology of breast cancer: retrospective study in the Central African Republic. BMC Public Health. 2016 December; 16(1230).

38. Lygia Vieira Lopes fMHFeal. Stage at presentation of breast cancer in Luanda, Angola - a retrospective study. BMC Health Services Research. 2015; 15(471).

39. Linnenbringer E. Black-White Disparities in Breast Cancer Subtype: The Intersection of Socially Patterned Stress and Genetic Expression. AIMS Public Health. 2017 November 24; 4(5): p. 526-556.

40. Grethe Albrektsen IHST. Histological type and grade of breast cancer tumors by parity, age at birth, and time since birth: a register-based study in Norway. BMC Cancer. 2010; 10(226).

41. Peter Rambau PCMMea. Pathological features of Breast Cancer seen in Northwestern Tanzania: a nine years retrospective study. BMC Resaerch Notes. 2011; 4(214).

42. S E Qyayson EKWDAea. Breast cancer in Accra, Ghana. Journal of Medical and Biomedical Sciences. 2014; 3: p. 21-26.

43. GD Forae FNAI. Histopathological profile of breast cancer in an African population. Ann Med Health Sci Res. 2014 May - June; 4(3): p. 369-373.

44. Charlotte Nguefack MBAMea. Epidemiology and surgical management of breast cancer in gynecological department of Douala General Hospital. Pan Afr Med J. 2012; 13(35).

45. B Heng. W K Glenn YYea. Human papilloma virus is associated with breast cancer. Br J Cancer. 2009 September; 101(8).

46. James S lawson WKGDSea. Human Papilloma Viruses and Breast Cancer. Front Oncol. 2015; 5(277).

47. WHO. latest cancer statistics (press release 223). 2013 December 12.

48. Steven SC, Donatus UE. Breast cancer as global health concern. 2009 october 6; 33(2009): p. 315-318.

49. Eilleen MB. Human Papilloma Virus and cervical cancer. Clinical Microbiology Reviews. 2003 Januar; 16(1): p. 1-17.

13. APPENDIX

APPENDIX I: DATA CAPTURE SHEET

UTILITY OF p16INK4A EXPRESSION BY IMMUNOHISTOCHEMISTRY IN DIAGNOSIS OF FEMALE HPV ASSOCIATED BREAST CARCINOMA

DATE……

1. IN PATIENT NUMBER……
2. LABORATORY NUMBER……..
3. STUDY
   NUMBER……
4. STUDY
   SITE……
5. AGE (specify in completed years)…….
   20-30
   31-40
   41-50
   51-60
   61-70
   71 and above
6. **REVIEW OF MORPHOLOGY**
   GRADE I
   GRADE II
   GRADE III
7. **IMMUNOHISTOCHEMISTRY**
   NEGATIVE
   POSITIVE +1
   POSITIVE +2
   Each lesion will be graded according to 3-tier system: brown nuclear and cytoplasmic reactivity will be scored from negative to 2+:
   - Negative: representing 0-5% reactive cells

- +1: focal/scattered positivity, representing greater than 5% and less than or equal to 80% reactivity.
- +2: diffuse positivity, representing greater than 80% reactivity.

8. HISTOLOGICAL TYPES

- Invasive ductal carcinoma
- Invasive lobular carcinoma
- Mixed carcinoma
- Mucinous carcinoma
- Metaplastic carcinoma
- Papillary carcinoma
- Medullary carcinoma
- Signet ring cell carcinoma
- Pleomorphic carcinoma
- Cribriform carcinoma – Neuroendocrine carcinoma

9. TYPES OF SPECIMEN

- Mastectomy
- Lumpectomy
- Core biopsies

APPENDIX II:

p16INK4A IHC MANUAL PROCEDURE (CINTEC HISTOLOGY KITS)

1. **Deparaffinization and rehydratation**

Prior to deparaffinization, place slides in drying oven at a temperature of no more than 60 Celcius centigrade for at least 20 minutes but no more than one hour to uantitavely remove water thereby improving adherence of tissue to the glass slide (“ backing”) and to melt the paraffin. Tissue slide must be deparaffinized to remove embedding medium and must be then rehydrated before the staining procedure can be performed. It is crucial to avoid incomplete removal of paraffin as residual embedding medium will result increase non-specific staining.

Incubate the slides at ambient temperature (20-25 Celcius centigrade) according to the following steps:

- 5(+/-1) minutes in Xylene bath
- Repaet this step once with fresh bath
- Remove excess liquid
- 3(+/-1) minutes in 95% ethanol
- Repeat this step once in fresh bath
- Remove excess liuid
- Minimum of 30 seconds in distilled or deionized water.

2. **Staining protocol for manual use**

Step1. Epitope retrieval

- Fill staining jar eg. Plastic Coplin jar, with diluted epitope retrieval solution
- Place staining jar containing epitope retrieval solution in water bath and heat water bath and epitope retrieval solution to 95-99 Celcius centigrade. At this step it is important to adjust the level of water in the water bath to make sure that the jarsare immersed in water to a level of 80%. To stabilize the temperature and avoid evaporation, cover jars with lids.
- Immerse deparaffinized sections into the preheated epitope retrieval solution in the staining jars; this step will usually lower the temperature in jars to less than 90 Celcius centigrade.

- Bring the temperature of the water bath and the epitope retrieval solution in the jars back to 95-99 Celcius centigrade; check the temperature of epitope retrieval solution in the jars;
- Incubate for 10(+/-1) minutes at 95-99 Celcius centigrade; start count down only after the temperature of epitope retrieval solution have been verified to hane reached a temperature of 95-99 celcius degree centigrade.
- Remove the entire jar with slides from the water bath;
- Allow the slides to cool in the epitope retrival solution for 20 (+/-)minutes at room temperature;
- Decant the epitope retrieval solution and rince sections in diluted wash buffer;
- For optimal performance, soak sections in wash buffer for 5 (+/-) after epitope retrieval and prior to staining .

Step2. Peroxidase-Blocking reagent

- Apply 200 Micro liter Peroxidase – Blocking reagent to cover the specimen.
- Incubate for 5(+/-) minutes;
- Tap off excess liquid and place slides in fresh wash buffer for 5 (+/-1) minutes

Step 3: Primary antibody or negative Reagent Control

- Remove excess buffer;
- Cover specimen with 200 micro liter of primary antibody (Mouse Anti-Human p16INK4A or negative reagent control)
- Incubate for 30 (+/-1) minutes;
- Tap off excess liquid and place slides in fresh wash buffer for 5(+/-1) minutes;

Step 4: Visualization reagent

- Remove excess water
- Cover specimen with 200 micro liter of visualization reagent
- Tap off excess liquid and place slides in fresh buffer bath for 5 (+/-1) minutes;
- Repeat this step twice with afresh wash buffer bath;

Step 5: substrate-chromogen solution (DAB)

- Cover specimen with 200 micro lliter of substrate-chromogen (DAB)
- Incubate 10 (+/-1) minutes;
- Tap off excess liquid and rince gently with distilled or deionized water
- Collect substrate-chromogen solution (DAB) waste in hazardous materials containers for proper disposal.

Step 6: Counterstain (Instructions are for Hematoxylin)

- Immerse slides in a bath of hematoxylin, Incubate for 2-5 minutes depending on the strength of hematoxylin used;
- Place slides in a tap water bath and rince gently with tap running water. Ensure all residual hematoxylin has been cleared.

Step 7: Mounting

- Non-aqueous, permanent mounting medium is recommended. For Xylene- based mounting media a dehydration procedure is necessary e.g
  3 minutes 70% Ethanol
  3 minutes 70% Ethanol
  3 minutes 96% Ethanol
  3 minutes 96% Ethanol
  5 minutes Xylene
  5 minutes Xylene
  Otherwise, aqueous mounting medium is also acceptable. Adhere to instruction of use of the supplier for mounting medium.

  NOTE: To minimize fading, protect slides from light and store at ambient temperature (20-25 Celcius centigrade).

APPENDIX III: HARRIS HEAMATOXYLIN AND EOSIN PROCEDURE

Principal of the stain

The mordant forms a lake on the tissue. It is on the lake that the stain attaches thus coloring the cell nuclei. The nuclei having the affinity for the basic radical in the dye retain the coulor even after treatment with 1% acid alcohol. Eosin stain the cytoplasm as a counter stain.

Staining technique

1. Bring section to water.
2. Stain in Harris Heamatoxylin for 5 minutes.
3. Rinse in tap water.
4. Differentiate in1% acid alcohol, 3 dips.
5. Rinse in tap water.
6. Blue in Scott tap water for 30 seconds or in running tap water for 10 minutes.
7. Counter stain in Eosin for 5 minutes.
8. Rinse in tap water excess eosin followed by 70% ethanol to obtain the desired shades of red and pink.
9. Dehydrate in 3 changes of absolute alcohol.
10. Clear in 3 changes of Xylene.
11. Mount with D.P.X.

APPENDIX IV: GRADING OF BREAST CANCER (Modified Bloom Richardson grading system).

This grading scheme is based on 3 morphological features:

Degree of tumor tubules formation

Tumor mitotic activity

Nuclear pleomorphism

Total score and each of the 3 components should be reported based on invasive degree only.

1.

Tubules formation	score
>75% of tumor cells arranged in tubules	1
10-75% of tumor cell arranged in tubules	2
< 10% of tumor cells arranged in tubules	3

2.

Nuclear pleomorphism (anaplastic area)	score
Small, regular, uniform nuclei, uniform chromatin	1
Moderate variability in size and shape, vesicular with visible nucleoli	2
Marked variation, vesicular, often with multiple nucleoli	3

3.

Mitotic activity	score
< 10 mitosis in 10 HPF	1
>10 and < 20 mitosis in 10 HPF	2
>20 mitosis in 10 HPF	3

3-5 points: GRADE I: Well differentiated

6-7 points: GRADE II: Moderately differentiated

8-9 points: GRADE III: Poorly differentiated.

APPENDIX V

BREAST CANCER STAGING

T: for primary tumor

Tx: Primary tumor cannot be assessed

To: No evidence of primary tumor

Tis: Carcinoma in situ

Tis (DCIS): Ductal carcinoma in situ

Tis (LCIS): Lobular carcinoma in situ

Tis (Paget): Paget diseases of the nipple with no tumor

T1: Tumor measuring 2 cm or less in its greatest diameter

T1mic: Microinvasion not larger than 0.1 cm in greatest dimension

T1a: tumor measuring 0.1-0.5 cm in greatest dimension

T1b: tumor measuring 0.5-1.0 cm in greatest dimension

T1c: tumor measuring 1.0- 2.0 cm in greatest dimension

T2: Tumor measuring more than 2 cm and less than 5 cm in diameter

T3: Tumor measuring more than 5 cm in diameter

T4: tumor of any size with direct extension to the skin or chest wall

T4a: Tumor extension to the chest wall not involving pectoralis muscle

T4b: edema (including peaud'orange) , skin ulceration, satellite skin nodules confined to the breast

T4c: both of the above

T4d: Inflammatory carcinomas

REGIONAL LYMPNODE

Nx: Regional lymph node cannot be assessed

N0: no regional lymph node metastasis

N1: Metastasis to movable ipsilateral lymph node

METASTASIS

M0: No distant metastasis

M1: Distant metastasis

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
P O BOX 19676 Code 00202
Telegrams: varsity
Tel:(254-020) 2726300 Ext 44355

KENYATTA NATIONAL HOSPITAL APPROVED 23 FEB 2016 KNH/UoN-ERC P.O. Box 20723-00202 NRB.

KNH-UON ERC
Email: uonknh_erc@uonbi.ac.ke
Website: http://www.erc.uonbi.ac.ke
Facebook: https://www.facebook.com/uonknh.erc
Twitter: @UONKNH_ERC https://twitter.com/UONKNH_ERC

KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

Ref: KNH-ERC/A/72

23rd February, 2016

Dr. Emile KARINGANIRE
H58/83552/2012
Dept.of Human Pathology
School of Medicine
College of Health Sciences
University of Nairobi

Dear Dr. KARINGANIRE

Revised research proposal: Utility of p16 INK4A Expression in Diagnosis of HPV Associated Female Invasive Breast Carcinomas (P780/12/2015)

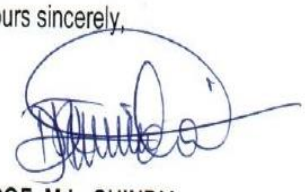
This is to inform you that the KNH- UoN Ethics & Research Committee (KNH-UoN ERC) has reviewed and **approved** your above proposal. The approval period is from 23rd February 2016 – 22nd February 2017.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH-UoN ERC before implementation.
c) Death and life threatening problems and serious adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH-UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH- UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH- UoN ERC for each batch of shipment.
g) Submission of an *executive summary* report within 90 days upon completion of the study. This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/ or plagiarism.

For more details consult the KNH- UoN ERC website **http://www.erc.uonbi.ac.ke**

Yours sincerely,



PROF. M.L. CHINDIA
SECRETARY, KNH-UoN ERC

c.c. The Principal, College of Health Sciences, UoN
The Deputy Director, CS, KNH
The Chair, KNH-UoN ERC
The Assistant Director, Health Information, KNH
The Dean, School of Medicine,UoN
The Chair, Dept.of Human Pathology,UoN
Supervisors: Dr. D.Zuriel, Dr.W.Waweru