IMMUNOHISTOCHEMICAL EVALUATION OF THE DIFFERENTION-RELATED GENE-1 EXPRESSION IN AND SURVIVAL RATES OF ER BREAST CANCER WOMEN PATIENTS ATTENDING MOI TEACHING AND REFERRAL HOSPITAL IN KENYA

BY

BOR HILLARY

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CELL AND MOLECULAR BIOLOGY

DEPARTMENT OF ZOOLOGY

MASENO UNIVERSITY

DECLARATION

I declare that this is my original work and has not been presented in any other institution or university for any award.

Signature …… Date…….

Bor Hillary (PG/MSC/) 60/011)

Department of Zoology,

Maseno University

This thesis has been submitted for examination with our approval as supervisors:

Prof. Eluid N. Waindi,

Department of Zoology,

Maseno University, Kenya.

Signature…… Date……

Dr. Benson Nyambega,

Department of medical Biochemistry,

School of Medicine

Maseno University, Kenya.

Signature……. Date……..

Dr. Kirtika Tushar Patel,

Immunology Department,

School of Medicine,

Moi University, Kenya.

Signature…… Date……..

ACKNOWLEDGMENT

All my appreciation goes to Almighty God the creator of our universe for sustaining me up to this far. I am grateful to National Commission of Science Technology and Innovation (NACOSTI) for sponsoring my studies at Moi Teaching and Referral Hospital (MTRH). I am also grateful to Dr. Benson Nyambega, who has been my supervisor and academic mentor, has encouraged me to peruse this topic and spend extra time helping me to achieve clear structure. Equally much grateful to Prof. Eliud Waindi who has been my supervisor, for his steadfast encouragement to complete this study and for the valuable insights, he has shared. Not forgetting Dr. Kirtika Patel who is my supervisor by providing me with her expertise on Immunohistochemistry and space to study at her lab and also allowing me use her facilities without limits during my study.

I sincerely express my gratitude to staffs of MTRH starting with Dr. Francis Ndiangui Dr. Walter Nalynya, Zainabu Jerotich, Janet Tuimising, Benjamin Kiprotich, for guidance on clinical and laboratory procedures and Mr. Alfred Keter for his guidance during early stages of data analysis. In addition, I am grateful to chairman Zoology Department Prof. G.Z.O. Jura for securing a place for me at MTRH, ensuring my study goes on smoothly, and the staffs of Maseno University for all their efforts they gave me during this study. Last but not least, I wish to thank my parents, brothers, sister and all people who in one way or the other have made this study successful.

DEDICATION

I dedicate this thesis to my Mother Alice Biegon, My brother Ken Bor, Geoffrey Bor and My Sister Anneth Chepkemoi for their immense support throughout the study period.

ABSTRACT

Breast cancer is a disease that affects both men and women and currently the leading type of cancer in women globally, commanding a huge social and health impact. Approximately 52.5% of breast cancer cases express estrogen receptors (ER). ER is a receptor proteins type of breast cancer and it fuels growth of breast cancer. ER breast cancer which has been shown to be the most aggressive and misdiagnosed thus, leading to overtreatment. However, it has not been study in Africans Kenyan women. Age is one of the factors that contribute to breast cancer conditions. Women of 40 years and above have been shown to be the most vulnerable group to breast cancer among the Caucasian women. However, it has not been shown in sub-Saharan African women. Metastasis is the spread of primary tumor to the secondary site and it is the major cause of mortality among breast cancer patients. Differential-Related Gene-1 (DRG1) is a suppressor gene that prevents the spread of tumor to the secondary site without affecting primary tumor. Poor prognosis, prediction of recurrence and management of breast cancer in clinics is a major challenge. However, studying the expression of DRG1as a biomarker in tissue sections in predicting metastasis and recurrence is necessary. This study evaluate the viability of *DRG1*gene as a prognosis biomarker in breast cancer using cancer tumor blocks and determine, the distribution of age, ER and survival rate of breast cancer patients at Moi Teaching and Referral Hospital (MTRH). Using Cochran (1963) formula, a sample size of 37 tumor blocks was used in this study. The tumor blocks were subjected to histological grading to ascertain the presence of a tumor. Immunohistochemistry technique was used to determine the expression of DRG1 and ER. Rabbit polyclonal anti-DRG1 and rabbit monoclonal anti-ER was used in this study. Data were recorded in a form and images captured on a camera. The most affect age group was between 35 and 50 years and vulnerable to breast cancer due to effects of estrogen hormone. Of the total percent breast cancer, 50% were in grade 2 a second stage of breast cancer. 56.8% were ER positive and all the tumor sections tested for DRG1 were all positive. Even though, all expressed DRG1 and clinically proofed to have metastasis, it was not significant statistically as sample size tested did reach calculated sample size. In addition there was no association between age and DRG1 (p value 0.493). Survival rate of breast cancer patients is 2.18 years thus, it's poor. This study guides clinicians in prognosis, treatment and management of breast cancer patients.

TABLE OF CONTENTS

ACRONYMS AND ABBREVIATIONS

ATM	Ataxia telangiectasia mutated
BC	Before Christ
BRAC1	Breast cancer type 1 susceptibility protein
BRAC2	Breast cancer type 2 susceptibility protein
CALLA	Common acute lympholastic leukemia antigen
CCI	Columbus chemical industries
CD	cluster of differentiation
CDH1	Cadherin-1
CHECK2	Checkpoint homolog
CK	Cytokeratin
CPG	CG doublets
DRG1	Differention-Related Gene-1
E_2	Estradiol
E-Cadherin	Epidermal cadherin
EGFR	Epidermal growth factor receptor
EPCAM	Epithelial cell adhesion molecule
ER	Estrogen receptor
ESA	Epithelial surface antigen
HER2	Human epidermal growth factor receptor 2
IREC	Institutional Research and Ethics Committee

KDHS	Kenya Demographic Health Survey
MTRH	Moi Teaching and Referral Hospital
MUCI	Mucin 1
PAM50	50-genes set
PBS	Phosphate buffers
PR	Progesterone receptor
PTEN	Phosphatase and tensin homologue
TGFβ	Transforming factor beta

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1 Background

Globally, breast cancer affects both men and women. Currently, it is the most common cause of mortality in women, accounting for 16% of cancer deaths in adult women (Campbell 2008). Cancer refers to transformed cells that have undergone genetic mutation in their pro-oncogenes and/or tumor suppressor genes (Ince *et al.* 2007). Such mutations arise from specific cancer-causing agents e.g. radiation, chemicals, hormones, viruses and genetic factors (Shavers and Brown 2002). When transformation occurs, cancer cells lose their intrinsic ability of regulating cell division but maintain certain characteristics of the original cells. About 5% of the transformed cancers are due to hereditary genes while the rest are due to somatic mutations. Somatic mutations may arise from internal factors such as hormones or the metabolism of nutrients within cells, or external factors e.g. tobacco, chemicals, and sun radiation (Shavers and Brown 2002). Another major factor is age which has been shown by some studies to be more aggressive among the Chinese women between the age of 40 and 50 years old. (Su *et al*. 2011). In addition, age affects cell growth and development hence affecting every tissue, organ and generally all the body system. African-American woman has been shown to be more predispose to breast cancer than Caucasian as from 18 years old (Ferlay *et al*. 2008). However, age prevalence's and distribution among Africans Kenyans women age group has not been shown.

Breast cancer starts as a malignant tumor in the cells of the breast with a complex and heterogeneous variety of histopathology and molecular sub-forms based on clinical and established risk factors (Weigelt *et al*., 2009). In all breast cancer cases, approximately 70%

express estrogen (ER) and progesterone (PR) receptors. The two hormones are known for fueling growth of breast cancer cells. In addition 52.5% of hormone receptor breast cancer express ER and depend on estrogen for growth and survival (Sorlie *et al*. 2003). In all types of breast cancer ER is the most aggressive causing high mortality rate (Riza *et al.*2014). Furthermore, ER breast cancer is the most misdiagnosed and over treated type of breast cancer resulting in poor management. However, no studies have been carried out to ascertain the distribution of ER breast cancer in Kenya.

Based on this ER characteristic, breast cancer has been classified on gene expression profile as luminal-type. Luminal-type has two sub-type "A" and "B", luminal-subtype-A tumor expresses high level of ER than luminal-subtype-B tumors (Kobayashi *et al*. 2013). Luminal-subtype-A has a better prognosis than luminal-subtype-B based on proliferation level of ER expression. Tumors of luminal subtype-A have lower rate of DRG1 a suppressor gene mutation than those of luminal subtype-B. However, no study has been done to evaluate the association between suppressor gene *DRG1* and breast profile gene marker (ER) in Kenya.

DRG1, a metastasis suppressor gene, controls metastasis dissemination without affecting growth of primary tumor (Baig *et al*. 2012). *DRG1* which has been reported in several studies has yielded good results as molecular biomarker in determining the level of metastasis in several *in vitro* cell-lines (Baig *et al*. 2012 ; Fotovati *et al*. 2006; Guan *et al*. 2000; Bandyopadhyay *et al*. 2004). Currently clinics in Kenya use only clinicopathological characteristics for prognosis, prediction of metastasis and recurrence of breast cancer. However, studying DRG1 as a biomarker to predict metastasis and recurrence of breast cancer will give more accurate information. In addition, it has not been used before in Africa and will reproduce as good result

as reported in cell-lines via a simple immunohistochemistry technique. Low level of DRG1 expression in breast cancer cells has shown correlation with poor survival period (Baig *et al* 2012). In addition, cell-lines research unlike tumor sectioning research is high technology, and hence requires expensive facilities and is not easily available in sub-Saharan Africa. Therefore, using tumor blocks from breast cancer patients is necessary. ER breast cancer prevalence data in Kenya is still scanty thus; the study evaluated breast cancer distribution of *DRG1* among patients visiting MTRH facility.

1.2 Problem statement

Breast cancer is one of the leading diseases that are currently claiming many lives in Kenya (Kenya Demographic Health Survey report 2013). Age is one of the major risk factor for breast cancer in women globally (Frelay *et al.* 2008). It has been shown that breast cancer incidence starts at the age 18 and increases rapidly to the age of 55 when it starts to decline. Breast cancer incidences are high within the age group of 40-50 years in Chinese women. African-American woman have been shown to be more predispose to breast cancer than Caucasian woman as from the age 18 years. However, no study has been done to determine the most predispose age group and distribution of breast cancer in African Kenyan women.

Normal breast cells and some breast cancer cells have estrogen receptors (ER), which have been found to fuel cancer cell growth. It does it by increasing proliferation and differentiation rate thus contributing to metastasis in breast cancer. Approximately 70% express hormone receptor (HR) and 52.5% of these express ER in all reported breast cancer cases. In all types of breast cancer ER is the most aggressive causing high mortality rate. In addition, ER breast cancer is the most

misdiagnosed and over treated type of breast cancer resulting in poor management. However, no studies have been carried out to ascertain the distribution of ER breast cancer in Kenya.

DRG1 gene is an important prognosis biomarker in determining the level of breast cancer metastasis. Currently clinics in Kenya use only clinicopathological characteristics for prognosis, prediction of metastasis and recurrence of breast cancer which has several short comings. Although DRG1 has been shown to work in such cell-lines, it has not been applied in cancer tumor sectioning. Cell-lines studies are high technology that requires high-level facilities and are not easily applicable in this region. In addition, there has been no study on the association of the expression level of ER and DRG1 in tumor sections. Therefore, the prognosis potentiality of *DRG1* has not been demonstrated anywhere in this region.

1.3 Justification

Moi Teaching and Referral Hospital (MTRH) is the only clinical facility where patients are referred to in western Kenya region. Western Kenya is cosmopolitan region with many ethnical populations' groups' hence have diverse population genetics. Facts and figures on the most predispose age group to breast cancer will guide clinicians in prognosis of breast cancer. Furthermore, having information on the most prevalent age group will entice Kenyan women to visit clinics for early detection and treatment. ER breast cancer is the most prevalent and aggressive type of breast cancer with poor prognosis. Poor prognosis of ER cancer has resulted in over treatment and poor management. In addition, clinicians only depend on clinicopathological characteristics to prognoses; predict metastasis and recurrence of breast cancer with its short comings. Therefore, DRG1 as a biomarker will give informative and accurate results thus, proper medication administration and management of patients.

DRG1 gene has only been studied in cell-lines and not in breast cancer tissue blocks. Consequently, monitoring *DRG1* gene expression products (protein) via a simple immunochemistry by determining both metastasis level and recurrence in tissue sections and ER assay is warranted. Tumor sections are easily prepared and processed in resource strain health facilities. Furthermore, *DRG1*gene prevalence in breast cancer patients visiting MTRH for medical assistance and by extension, in the region and the country in general has not been ascertained. This study provides an easy and affordable method to undertake studies to predict metastasis in women breast cancer.

1.4 Main objective

To evaluate immunohistochemistry prognosis potential of *DRG1* gene and survival rate in ER breast cancer patients.

1.4.1 Specific objectives

1) To determine age groups distribution of ER breast cancer women.
2) To determine frequencies of ER expression in breast cancer tumor sections.
3) To determine the expression levels of *DRG1* gene and survival rate in ER breast cancer patients.

1.4.2 Hypothesis

There is no significant difference between the expression of DRG1 in cell-lines and tumor sections.

1.4.3 Study limitation

1**.** Difficulties in following up patients after treatment to understand how ER expresses after hormonal therapy.

2. Follow up of patients to understand how DRG1 expressed after therapy.

3. Difficulties to perform DNA expression of DRG1 as DNA integrity was compromised by formaldehyde during preparation of tumor blocks.

4. Lack of adequate resources to test for both ER and DRG1 using other quantitative technique.

CHAPTER TWO

LITERATURE REVIEW

2.1 History of Cancer and distribution

Cancer refers to transformed cells that have undergone genetic mutation in their pro-oncogenes and/or tumor suppressor genes (Ince *et al.* 2007). When transformation occurs, cancer cells lose their intrinsic ability to regulate cell division but maintain certain characteristics of its original cells. About 95% of the transformed cancers are due to somatic mutation and the rest are due to hereditary genes (Ince *et al*. 2007). Somatic mutation may arise from internal factors such as hormones or the metabolism of nutrients within cells or external factors e.g. tobacco, chemical and sun radiation (Shavers and Brown 2002). Cancer has been in existence in human history and was first recorded in Egypt in 3000 BC by Edwin Smith Papyrus (Hajdu *et al*., 2011) and he identified breast cancer. From that time to date over 200 types of cancer have been recorded with the most prevalent cancers globally being: Lung-12.7%, Breast10.9%, Colorectal-9.8%, Stomach-7.8%, Prostrate-7.1%, Liver-5.9%, Cervix and Uterus-4.2%, Oesophagus-3.8%, Bladder-3.0%, Non-Hodgkin lymphoma-2.8%, Leukemia-2.8%, Corpus uteri (endometrium)-2.3%, Pancreas-2.2% and Kidney-2.2% (Ferlay *et al*. 2008). The same statistics showed that 12.7 million-cancer cases in the world during the year 2008. In which 6.6 million were males and 6.0 million females. These translate to 13% of all death every year and half of the total death from developing world. In Sub-Sahara Africa, about 530,000 new cancer cases are reported annually in which 251,000 are males and 279,000 are females (Campbell 2008).

According to Age-standard Rate per 100,000 (world) ranking the first top ten countries with cancer cases; Denmark-326.1, Ireland-317.0, Australia-314.1, New Zeland-309.2, Belgium-

306.8, France (metropolitan)-300.4), United states of America-300.2, Norway-299.1, Canada-296.6 and Czech Republic-295.0 (Ferlay *et al*.2008). From this report, 60% of cancer patients are below 70 years and 70-80% of them are diagnosed at the late stage. Based on sex of the individual, in females, - breast leads with 33.5 per 100,000; cervical is the second with 25 per 100,000. While in males prostate cancer leads with 17 per 100,000 and esophageal 9 per 100,000. In terms of race cancer incidence are prominent in whites followed by Asians and then Africans.

2.1.1 Risk factors

There are several risk factor associated with breast cancer this include age, family history, early menarche, late menopause, use of combined estrogen and progestin menopausal hormones, alcohol consumption and physical inactivates. Age is one of the major risk factor for breast cancer in women globally. It has been shown that breast cancer incidence starts at the age 18 and increases rapidly to the age of 55 when it starts to decline (Akarolo-Anthony *et al*. 2010). Breast cancer incidences are high within the age group of 40-50 years in Caucasian women (Su *et al*. 2011). However, no study has been done to indicate the incidences of breast cancer in African Kenyan women age groups. Moreover survival period, an African-American has low survival period of less than 5 years after infliction with cancer, while Asian and the white women with an average of 15 years (Ferlay *et al*.2008). However, no study has been done to ascertain the most prevalent age group in western Kenya region.

2.1.2 Metastasis in cancer

Metastasis is the spread of cancer cells from one organ or part to another non-adjacent organ or part in a non-random manner. Metastasis is a process whereby malignant cells undergo

intravasation in the circulation followed by extravasations in a second site. At the second site they re-penetrate the vessel and multiply forming a secondary tumor (Klein 2008). Several soluble molecules that include chemokines and transforming factor beta (TGFβ) are involved in this mechanism. In normal human cells 3 kinds of cell migration are involved amoeboid movement, collective motility and mesenchymal-movement (Chiang *et al*. 2008). Cancerous cells have been shown to use this opportunistic migration to metastasize. Generally, metastasis start by first spreading in lymphatic node, transcoelomic, transplantation or implantation, haematogenous (Drabsch *et al*. 2011).

Metastasis of cancer in human body is usually suppressed by protein referred to as suppressor proteins which include P^{53}, and DRG1. In breast cancer metastasis usually starts from lymph nodes and then to bone marrow, lung, liver and brain (Muller-Hocker, *et al.* 2001). ER is the major molecule that fuel growth of breast cancer by stimulating E-cadherin which is important in controlling cell division. ER in general is among the contributing factors in metastasis of breast cancer cells in women and it influences survival of the patients. Therefore, studying the level of expression of ER and a suppressor protein DRG1 will elucidate their association in breast cancer metastasis.

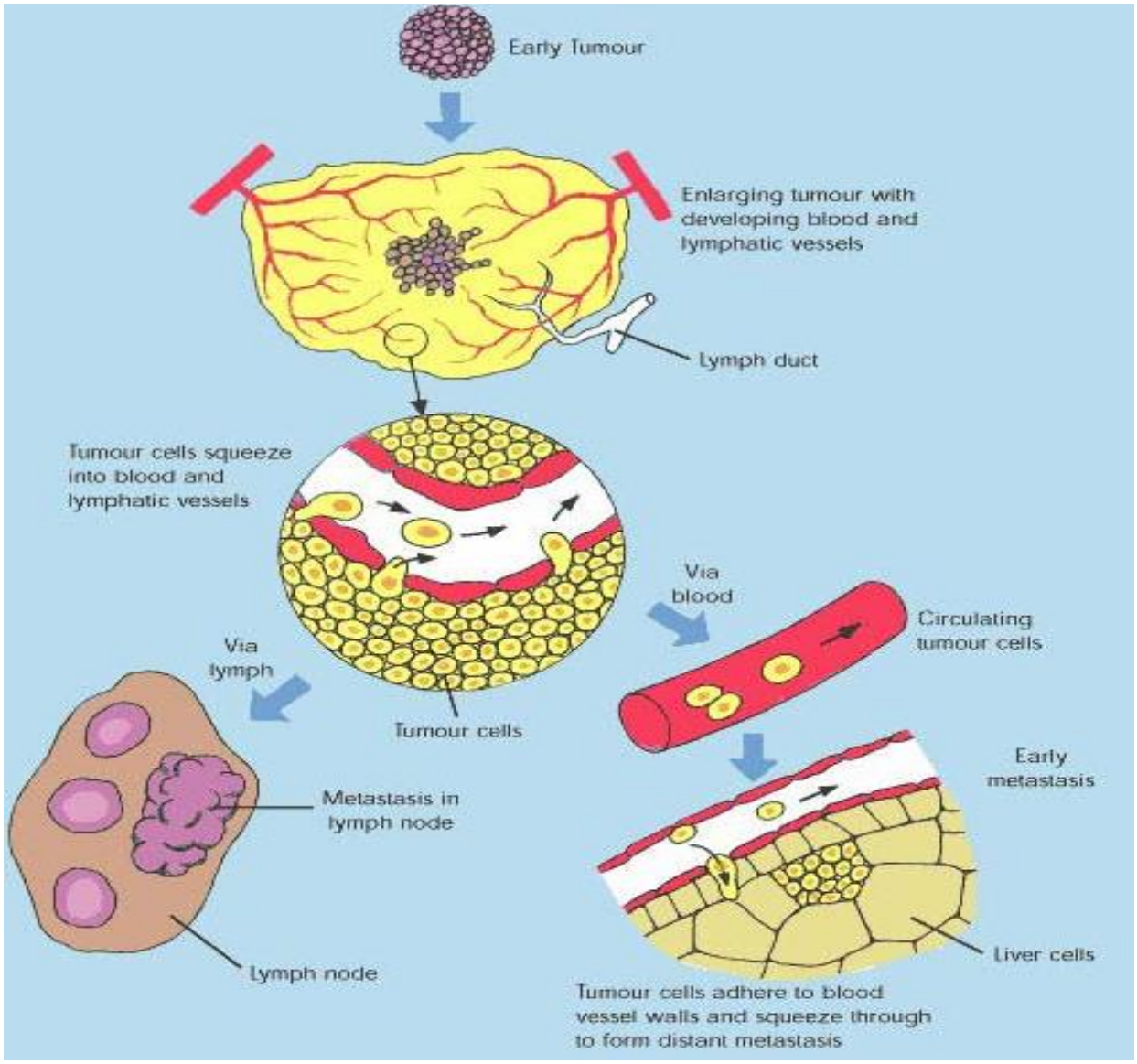


Figure1: General diagram on tumor metastasis. First tumor enlarges at the primary organ then attaché to the lymph node as well as undergoes angiogenesis. This helps in the supply of oxygen and nutrients; it squeezes itself into blood vessels where it circulates in the body. While circulating it evades immune mechanism until it reaches the secondary site. In the secondary site it attaches to an organ by squeezing in and start to enlarge as it was in the primary organ. *htt/www.drugdevelopment-technology.com/projects/bmsanticancer/bmsanticancer.html*.

2.1.3 Breast cancer staging

Breast cancer staging is the description of how cancer has spread or extend at which the disease at the time of diagnosis (Campbell 2008). This is important as it determines the choice of therapy and assessing prognosis. Breast cancer staging is based on clinicopatholagical characteristics; the primary tumor size, location and if it has metastasis. This method is called TNM staging system and it assesses tumors in three ways: extent of the tumor (I), absence or presence of the lymph node involvement (N) and absence or presence of distant metastasis (M). Based on this a stage of I,II, III or IV is assigned, stage I is an early breast cancer followed by II which is moderate with little difference from stage I. Stage III is in transition to advanced i.e.to stage IV of breast cancer. It has been shown and adopted in clinical pathology labs that a tumor with measurement ≥5cm has metastasis (Elena *et al.* 2005). This method of describing how breast cancer has metastasis based on TMN has been shown to have short comings (Cronin *et al*. 2007). However, using a biomarker that predicts metastasis at molecular level during prognosis is warranted.

2.2 Breast pathology

Female breast comprises lobules, ducts and stroma that are the fatty tissue, connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels. Breast a rises from the

invagination of the epidermis into the underlying mesenchymal tissue during 10-24 weeks after conception and the process gives rise to epithelial ducts that develops to rudimentary lactiferous ducts (Morrison *et al*. 2008). This organ keeps undergoing morphological and functional changes up to adulthood, with secondary changes at puberty and greatest differentiation during pregnancy and lactation.

Estrogen hormone is responsible for the elongation of the breast at puberty and stem cell activity at the terminal end bud. Prolactin and progesterone drive ductal branching and the formation of acini, giving rise to mature breast tissue. During this development, two major subclasses of cells are formed: the outer myoepithelial (or basal) cells and the inner luminal epithelial cells. Myoepithelial cells are known by the expression of common acute lymphoblastic leukemia antigen (CALLA) or CD10, Ty-1, alpha-smooth muscle actin, Vimentin, and cytokeratin (CK) 5 and CK14. Furthermore, there are contractile cells that form a sheath around the ductal network of the breast. While the luminal epithelial cells are known for the expression of mucin 1 (MUCI), epithelial surface antigen (ESA) also referred to as EpCAM (epithelial cell adhesion molecule), and CK7, CK18 and CK19 as well as ER and PR (Morrison *et al*. 2008).

On the other hand, breast cancer has been shown to express ESA in both primary tumor and secondary metastasis. In physical appearance, breast cancer tissue has a new lump or mass; painless, hard mass with irregular edges. In addition, it can also be tender, soft or rounded, painful. While some other, appearance could be swelling of all or part of a breast, skin irritation or dimpling. Other signs include breast or nipple pain, nipple retraction (turning inwards), redness, scaliness or thickening of the nipple or breast skin, nipple discharge other than breast milk (Lewis *et al.* 2006).

2.2.1 Estrogen Receptor (ER) and Breast cancer

Normal breast cells and some breast cancer cells have estrogen receptors, which have been found to fuel cancer cell growth. It does it by increasing proliferation and differentiation rate of tumor hence contributing to metastasis in breast cancer (Tabatabai *et al.*2012). Approximately 70% express hormone receptor (HR) and 52.5% of these express ER in all reported breast cancer

cases (Sorlie *et al*. 2003). In all types of breast cancer ER is the most aggressive causing high mortality rate (Riza *et al.*2014). Depending on ER characteristic breast cancer has been classified on gene expression profile which is luminal-type. Luminal-type has two sub-types "A"and "B", luminal-subtype-A tumors express high level of ER than luminal-subtype-B tumors (Kobayashi *et al*.2013). However, no studies have been carried out to ascertain frequency of ER breast cancer in Kenya.

Alteration of ER expression plays an important role in development and progression of hormone-related cancers. The level of ER in a cell is maintained by dynamic balance between ER synthesis and ER breakdown (Nonclercq *et al*. 2004). Synthesis of ER is repressed by methylation of ER promoters, hypermethylation of promoter that is associated with the loss of ERα and ERβ in majority of cancers and cancer cell lines (Swedenborg *et al.* 2009). Phosphorylation of ERs and interaction of receptors with several proteins, among them ubiquitin ligases and ubiquitin-binding proteins cause changes on ER status. However, proteasome-mediated degradation of ERs controls the level of endogenous ligands in tumor microenvironment like in hypoxia where it down regulates chaperons and increase expression of ubiquitin ligases (Richter and Buchner 2001).

2.2.2 Breast cancer classification

Breast cancers have been classified according to different prognosis. Two broad categories of prognosis factors that currently exist are those indicating the level of tumor advancement and that indicating tumor aggressiveness. From 1960 to 1990, breast cancer has been categorized according to three receptor proteins: ER, PR and HER2. These proteins receptor were determined via immunohistochemistry in combination with tumor size and a number of nodes as

a way of classifying them. In the year 2000, it was proposed that breast cancer could be better classified than previously, by measurement of RNA expression of large number of genes. Through this measurement five or more categories were proposed, this includes luminal A and basal-type cancers e.g. 50-genes set (PAM50) an intrinsic subtypes though it has not replaced receptor-based categories in cancer clinics (Nielsen *et al*.2011).

In 2012, a fourth generation was proposed depending on genetics: inherited (germ line) or/and acquired (somatic) variation. Germ line variation consist of small point mutation such as single nucleotide polymorphism and copy number variant that is either increased or decreased in the modal copy number of two in a given chromosomal segment. Therefore, tumor variability could be identified by incorporation of both genetic mutation status (tumor genome) and or RNA expression level of the tumor.

2.2.3 ER Breast cancer therapeutic approaches

Several approaches are being used to treat breast cancer patients depending on the type of breast cancer and the stage it is. Early ER positive breast cancer, the first line used is adjuvant endocrine therapy (Stephen 2010). The therapy is given for 5 years after primary surgery to delay local and distance relapse thus prolonging survival. ER breast cancer women that are past menopausal, aromantase inhibitor (AI) therapy are given to improve disease-free survival (Osborne et *al*. 2005). Patient with ER positive that has metastasis has been shown to have poor response to endocrine treatment therefore; AI is the standard first-line therapy (Smith *et al.* 2003).

2.2.4 Genetics of Breast cancer

Breast cancer has been shown to express several genes and from these genes, it has been used to classify (Cronin *et al*.2007). The general broad classification is based on hereditary genes and somatic genes. Hereditary genes in breast cancer are *BRAC1* and *BRAC2*, these genes helps to prevent cancer from making proteins that keep the cells from growing abnormally. Other genes that can be inherited are *ATM* that helps to repair damaged DNA. Inheriting 1 mutated copy of this gene has been shown to increase high rate of breast cancer in some families. *TP53* is another gene that helps in production of p53 protein and the protein help in stopping growth of abnormal cells. On the other hand, *PTEN* gene, which is involved in regulating cell growth, has been shown to increase risk for both benign and malignant cancer in the breast. Other studies have shown that *CDH1* gene cause invasive lobular breast cancer and can be inherited with another gene *DRG1* and control metastasis.

Diffentiation-related gene-1 (*DRG1*) is a metastasis suppressor gene in a number of malignancies (Guan *et al.*, 2000). *DRG1* encodes a 43kDa protein with 394-amino acids. This protein is expressed both in the nucleus and in the cytoplasm (Chen *et al*.2006). Its mRNA is expressed universally with highest level of expression in kidney, prostrate, ovary and intestine. In breast tissue, it is highly expressed in the epithelial cells and basal cell layers in normal mammary duct-lobular units but low in tumor cells and not expressed in the stroma (Bandyopadhyay *et al*. 2004).

Expression level of DRG1 gene is controlled by hypermethylation of CG doublets (CPG islands) i.e. the promoter site (Guan *et al*. 2000). *In vitro* study has shown that DRG1 is a metastasis suppressor in breast cancer by affecting the step of invasion through extracellular matrix. *DRG1*

mRNA levels of expression is low in lymph node and bone metastasis resulting in a poor survival of breast cancer patients. Furthermore, *DRG*1 inhibit polyploidy by causing cell arrest when disruption of spindle checkpoint happen in several p53-deficient tumor cell lines. This conclusion was reached when *DRG1* knock-down in normal human mammary epithelial cells resulting in the disappearance of astral microtubule. *DRG1*, which is localized in the centrosome, binds to microtubule by ensuring cell division fidelity (Kim *et al.* 2004). DRG1 inhibits tumor cell growth by regulating microtubule disruption. This shows that *DRG1* regulates microtubule dynamics and maintain genomic euploidy, and with it causing genomic instability in cancer cells resulting in metastasis. In proteomic analysis, DRG1 protein is more concentrated in the cytoplasm. However, in response to p53 and DNA damage it is redistributed to the nucleus (van Belzen *et al.* 1997). In study (Baig *et al*.2012) on cell-line, both expression and prognostic potential of DRG1 as a biomarker in predicting metastasis level of breast cancer has been demonstrated. However, no study has been done to demonstrate its potentiality in predicting breast cancer metastasis and recurrence using tumor sections hence influencing survival rate.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

The study was conducted at the Moi Teaching and Referral Hospital (MTRH) with geographical coordinates $0^0$31’0"North, 35^0 17’ 0" East. It is the largest referral Hospital in western Kenya region that serve several counties; Uasi Gishu, Vihiga, Nandi, Elgeyo Marakwet, Baringo, Pokot, Trans Nzoia, Kakamega, Bungoma,Kisumu, Kericho, Bomet, Nyamira and Kisii. MTRH is the only government facility in the western region of Kenya with oncology clinic. In addition, it covers an area with high percentage breast cancer patients reporting late for diagnosis and treatment as per MTRH records which agree with Kenya Demographic Health Survey (KDHS) report 2013.

3.2 Study design

This was a retrospective study, on fixed paraffin embedded tumor tissue blocks. Tumor tissue blocks were retrieved from archives at MTRH histopathology laboratory. Participants for this study comprised of patients who had visited MTRH facility for treatment. Tumor blocks were sectioned in histopathology laboratory of MTRH. The samples were processed for both histological and immunohistochemistry staining of breast cancer proteins ER and DRG1.

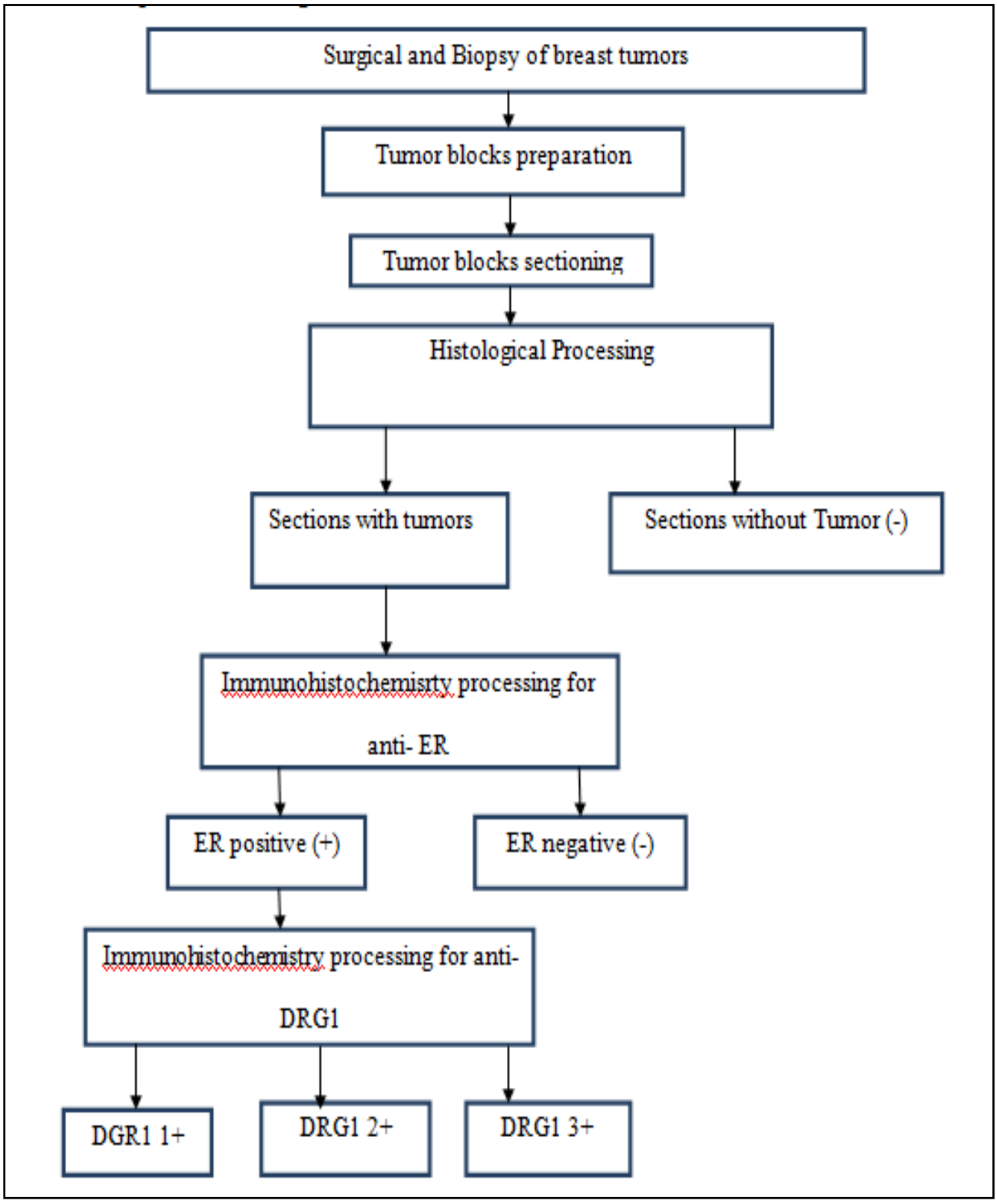


Figure 2: Flow chart

3.3 Study population and sample size

Eldoret town where MTRH is situated has a population of 289,380 with diverse population genetics since it is cosmopolitan town in western Kenya. Tumor blocks of patients that were in the archive and there records were available were use in this study.

The study size of 37 was arrived at using (Cochran 1963) formula, with 5% margin of error in a population that has a proportion of 90% with breast cancer at grade 2 or 3.

$$n = \left(\frac{Z_{1-\alpha/2}}{\delta} \right)^2 P(1-P)$$

Where

P- Is population of those that have started to metastasize.

δ- Is margin of error (5%)

$Z_{1-\alpha/2}$- is the $(1-\alpha/2) \times 100\%$ quartile of the standard normal distribution.

It gives a sample size 139 but correcting this to finite size gives

$$\left(n \Big/ 1 + n \Big/ N \right) = \left(139 \Big/ 1 + 139/50 \right) = 37$$

3.3.1 Inclusion criteria

Breast tissue sections from women of age 18-55 years that was confirmed histologically and clinically were used for this study. Slides that expressed ER positive after staining were chosen and tested for DRG1 expression. Patients that were suffering from other diseases and were on medication of the disease or taking pills were excluded.

3.3.2 Ethical Considerations

Study review and approval was secured from Ethical Review Board IREC-MTRH Eldoret (IREC NO 0001203) see appendix 1. All the samples collected were coded for identification using identification number. The investigators controlled access of data and no sample had the patient name.

3.4 Sample collection

Tissues that were harvested both surgical and by biopsy were rinsed in PBS Dako (Agilent technologies Inc., California, USA) to remove blood. The tissue blocks used were retrieved from pathological lab archives based on clinical records and controls used were donated by anatomy laboratory of MTRH. Tissue blocks were picked from the archives after picking blocks numbers in histopathology registry book on the ER positive one only. The tissues were cut into slices of 5 microns and fixed in buffered formalin (3.7% formaldehyde: 10mM phosphate buffer, pH 7.4) (Agilent technologies Inc., California, USA) and incubated for 24 hours at room temperature, rinsed in PBS and stored in 70% ethanol (Agilent Technologies Inc., Glostrup, Denmark) and H_2O. Tissues were then trimmed and inserted into labeled histology cassettes, clamped, closed and immersed in 70% ethanol (Agilent Technologies Inc., Glostrup, Denmark) in the storage bucket. Dehydration was performed in three ethanol baths of increasing concentrations 70%, 80% and 90% (Agilent Technologies Inc., Glostrup, Denmark) for 1 hour each so as to prevent tissue damage. Clearing was performed, three xylene (Agilent Technologies Inc., Glostrup, Denmark) baths for 1 hour each to replace ethanol trapped inside tissues and to be a miscible solvent with wax. Wax filtration followed in hot wax bath at 60^0C for 1 hour to solidify the tissue. All these stages were performed by the STP120-3 machine (Especlaldades Meldlcas

MYRS.L.43700.Elvendrel.Spain EC). This was embedding: the tissue was oriented inside a mold filled with hot paraffin and left to cool at room temperature and after solidifying the block was stored in cabins ready for the next stage: histological and Immunochemistry (IHC) processing.

3.4.1 Sample processing

Tumor blocks were retrieved form cabins as indicated in section 3.4.0 above and cooled down for easy cutting in a microtome. The refrigerated blocks were arranged in a tray with respective histological and IHC slides labeling. Tumor blocks were cut using rotary microtome Lerts Leica 1512 (W. NUHSBAUM, INC, McHenry, Illinois) set to cut 5µm. Sections were put to float on distilled water at room temperature for easy selection of the best section. Sections were transferred to glass slide and allowed to dry overnight at room temperature in slide holder.

For histological technique, slides were deparaffinized in 2 charges of xylene (Agilent Technologies Inc., Glostrup, Denmark) at 95% and 95% for 5 minutes each. Slides were transferred into 3 baths of ethanol (Agilent Technologies Inc., Glostrup, Denmark) at 80%, 95% and 100% respectively for 3 minutes each. The slides were rinsed in tap water and Hematoxylin (Merck KGart, 64271 Darmstadt Germany) was applied for 5 minutes. The slides were washed in tap water and eosin (LOBA Chemical DVT.LTD Mumbai 400005- India) was applied for 1 min and rinsed in tap water. The slides were placed in racked, dipped into 3 baths of 100% ethanol for 40 seconds each respectively and also in 3 baths of xylene (Agilent Technologies Inc., Glostrup, Denmark). Cover slips were placed using glass rod and permount were applied and slides were dried overnight in a hood. Slides were viewed in Olympus BH-2 microscope (Olympus Inc., Tokyo, Japan) at ×400 for grading of tumor. Results was recorded, presented in a graph and captured in a camera (figure 4 and 5) respectively.

For Immunohistochemical test, slides were deparaffinized in xylene (Agilent Technologies Inc., Glostrup, Denmark) for 5 minutes and dipped twice in 100% ethanol (Agilent technologies Inc., California, USA), once in 95%, 70%, and 50% ethanol (Agilent Technologies Inc., Glostrup, Denmark) respectively for 3 minutes each. Blocking followed by incubating slides in 3% H_2O_2 solution in ethanol at room temperature for 10 minutes to block endogenous peroxidase activity. The slides were then rinsed in 300 ml PBS 2 changes, for 5 minutes each and blocking buffer was drained off. Primary antibody was added anti-ER Dako (Agilent Technologies Inc., Glostrup, Denmark). Anti-DRG1 Sigma life science (Sigma-Aldrich Inc., St. Louis, Missouri, USA) to their respective slides and incubated in humidified chamber at room temperature for 1 hour followed by washing in 300ml PBS Dako (Agilent Technologies Inc., Glostrup, Denmark), 5 minutes and all antibody was dilution in 1:100. 100µl Sav-HRP conjugate Dako (Agilent technologies Inc., Glostrup, Denmark) was applied and incubated in a humidified chamber at room temperature for 30 minutes while protecting from light. Slides were washed in 300 ml PBS Dako (Agilent technologies Inc., Glostrup, Denmark) for 2 changes, 5 minutes each. Followed by an addition of 100µl DAB substrate Dako (Agilent technologies Inc., Glostrup, Denmark) for 5mins, slides were placed in a rack washed in 300 ml PBS for 3 changes 2 minutes each. After that, slides were immersed in counter stain Hematoxylin Dako (Agilent technologies Inc., Glostrup, Denmark) for 5 minute and slides were rinsed in running tap water for 15 minutes. Dehydration followed in 3 changes of ethanol (Agilent Technologies Inc., Glostrup, Denmark) (50%, 70%, and 95%) 5 minutes each. Slides were cleared in 3 changes of xylene and cover slip using permount. Color of antibody ER and DRG1 were observed in Olympus BH-2 microscope and recorded (Olympus Inc., Tokyo, Japan) at ×400.

3.4.2 Histological tumor grading

Talukder *et al*., (2007) grading follows the arrangement of the cells in relation to each other: whether they form, tubules; how closely they resemble normal breast cells (nuclear grade) and how many of the cancer cells are in the process of dividing (mitotic count). In this features it was graded into three, where by grade 1 had relatively normal-looking cells that appear to be growing slowly and were arranged in small tubules. Grades 2 had unlike normal cells that that appear to be growing quickly than normal and were arranged in lager tubules. Grade 3 was the highest grade, looked abnormal cells that appeared growing very quickly. These were arranged in the largest tubules (Talukder et al. 2007) and all results is shown in an histogram in figure 5.

3.4.3 Immunohistochemistry interpretation

The stain intensity on the slides was used to categorize how DRG1 was expressed in ER breast cancer cells in relation to their controls. The scores for DRG1 were as follows base in reference to controls: score 0 which was regarded as negative i.e. had no stain on the specific ligand and were recorded as ≤ 10% and 1+ to 3+ was recorded as positive. Score 1+, faint >10% of tumor cells; score 2+ weak to moderate staining of the entire nuclei in > 10%, score 3+, strong staining of the entire nuclei in > 10% of the tumor cells (Fotovati et al. 2006). The interpreted results were tabulated in table 1.

For the ER receptor was defined by percentage with reference to controls; of cells with strong stained nuclei ≥ 10% was defined a gland as positive for ER and ≤ 9% defined as negative and the classified cells was tabulated in table 1 and observed in high power Olympus BH-2 microscope (Olympus Inc., Tokyo, Japan) ×400

3.4.4 Data analysis

Clinical records on tumor grades, level of metastasis, and recurrence of breast cancer was obtained from the hospital records. Data analysis was done using software for statistical computing (R core Team, 2015). Categorical variables were summarized as frequencies and the corresponding percentages. Continuous variables that assumed the Gaussian distribution were summarized as mean and the standard deviation (SD), i.e. mean ±SD. Those that violated the Gaussian assumptions were summarized as median and the corresponding inter quartile range (IQR). Gaussian assumptions were assessed using Shapiro Wilk test. Association between age and DRG1 gene was assessed using two samples Wilcox on rank sum test. Association between Histological tumor grade and DRG1 was analyzed using Fisher's exact test. A Kaplan-Meier survival curve was used to characterize the survival from the date of admission. Results were presented using tables and graphs.

CHAPTER FOUR

RESULTS

4.1 Age groups distribution of breast cancer patients

A total of 37 breast cancer patients (cases) files were retrieved for this study. The demographic distribution was summarized as mean and the standard deviation (SD), i.e. mean ±SD using Shapiro Wilk test (figure 3). The results show that the mean age of the participants was 41.8 ± 7.7 years (range 26-55).

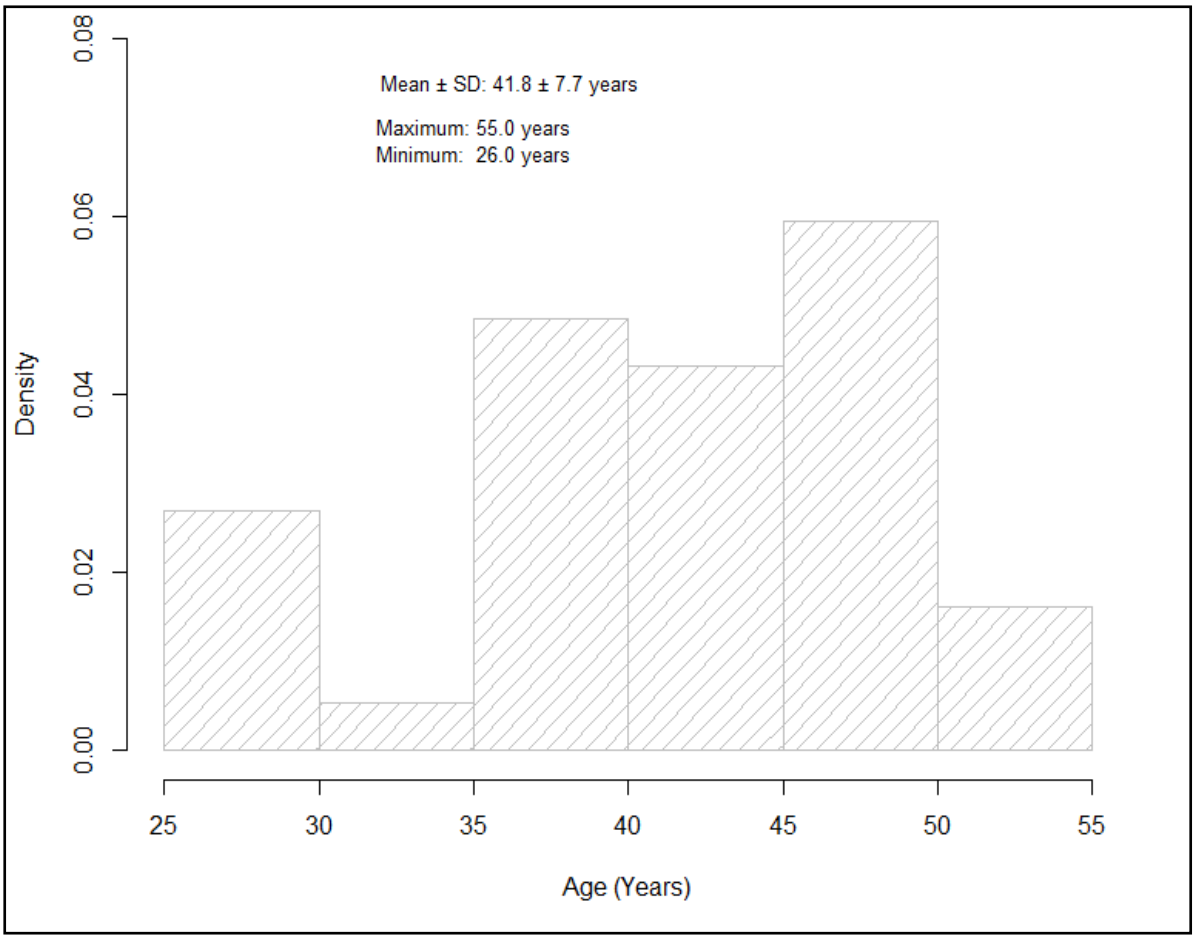


Figure 3: Age distribution of breast cancer patients**.** A bigger percentage of women with breast cancer are between the ages of 35years to 50 years. As there population density is above 0.05 thus more than half statistically. The lowest percentage of breast cancer women are between the age of 30 years and 35 years.

Table 1: Ethnic distribution

Variable		Sample size	n (%)
Tribe	Kalenjins		11 (29.7%)
	Luos		11(29.7%)
	Luhya	37	7 (18.9%)
	Kikuyus		6 (16.2%)
	Gusii		2(5.5%)

Table 1: Ethnic group's distribution of Breast cancer: Kalenjins and luos are the leading with 29.1% and are both Nilotic. Therefore, they could be having the same genetics that predispose them to breast cancer.

4.2 Histological tumor grading

Tumor blocks grading are illustrated in figure 2 and the majority are in grade II with 51.4% followed by grade I with 35.1% and the least being grade III with 13.5%.

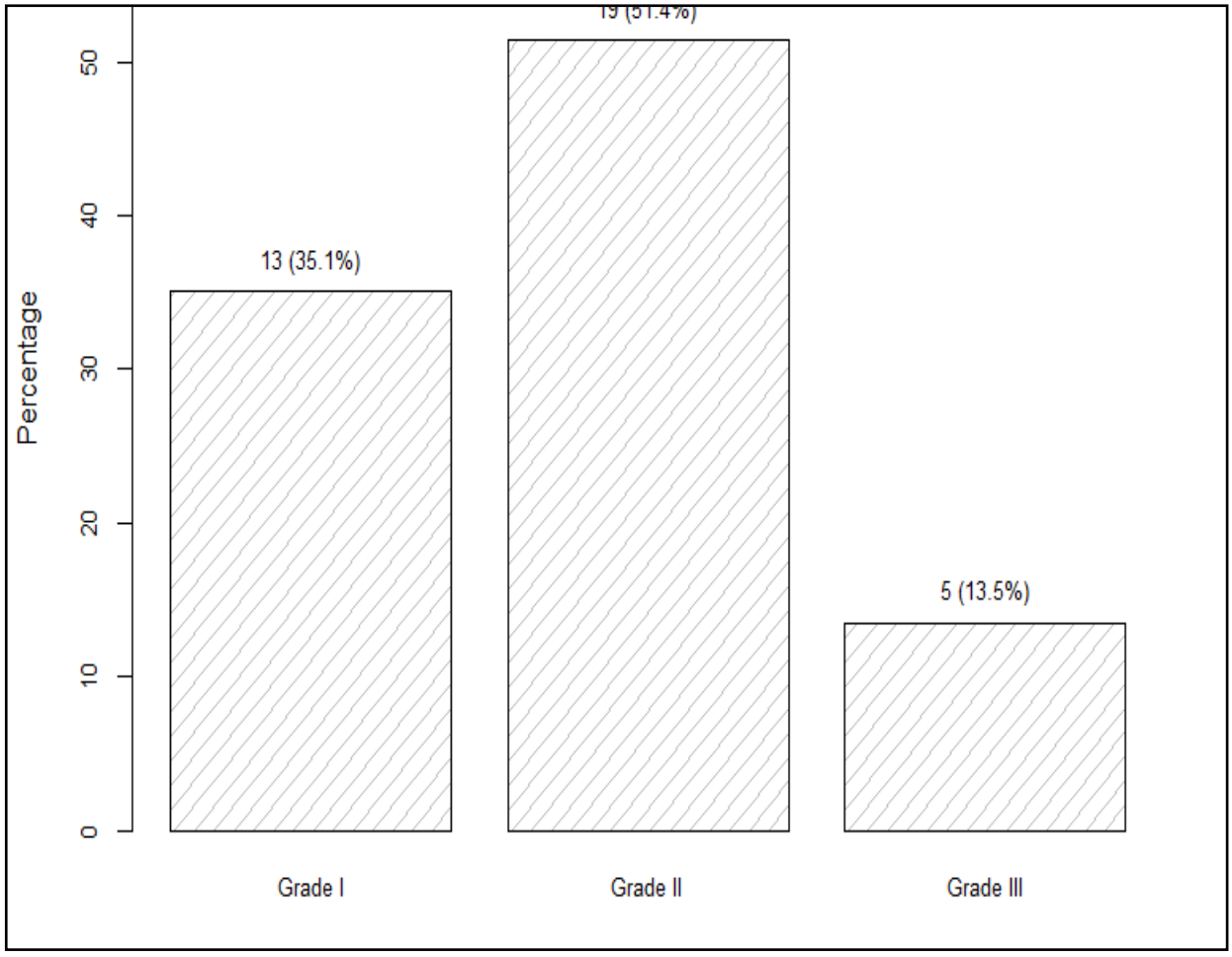


Figure 4: Histological tumor grades. Breast cancer women are mostly in grade II stage which the most hard to manage since there are in the transition stage to poorly manage grade III.

Tumor slides based on grades were captured and recorded in a camera as shown in figure 5.

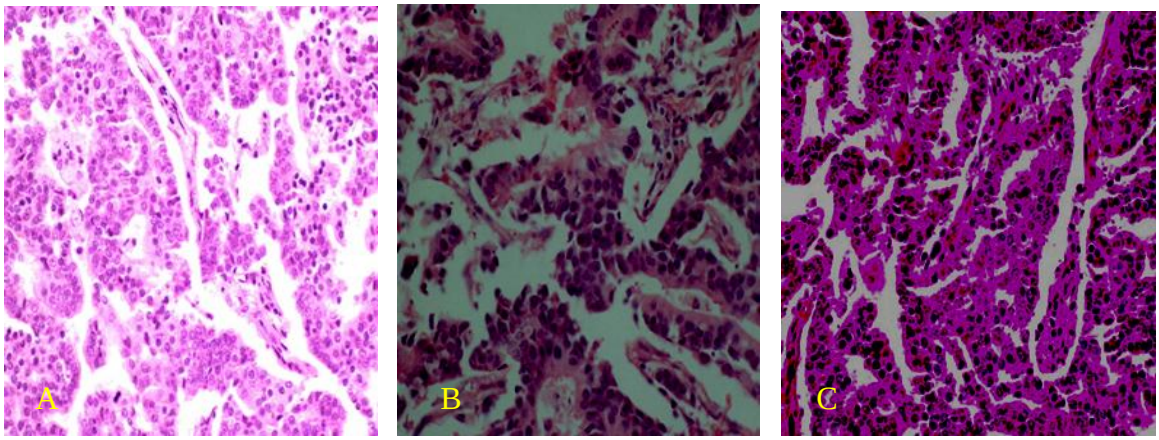


Figure 5: Histological grading**.** These are tumor grades (A) I, (B) II, (C) III respectively captured in a light microscope using a camera. These were graded based on the average of tubule formation, mitotic count and nuclear pleomorphism following Nottingham grading system. ×400.

4.3 Lymph-node metastasis and Tumor size

Breast cancer patient's records showed that 75% of the samples had lymph-node metastasize while 25% had not (figure 6). Tumor size was measured as <5cm or ≥ 5cm and it were found out that 44.8% had <5cm and 55.2% had ≥ 5cm (figure 7).

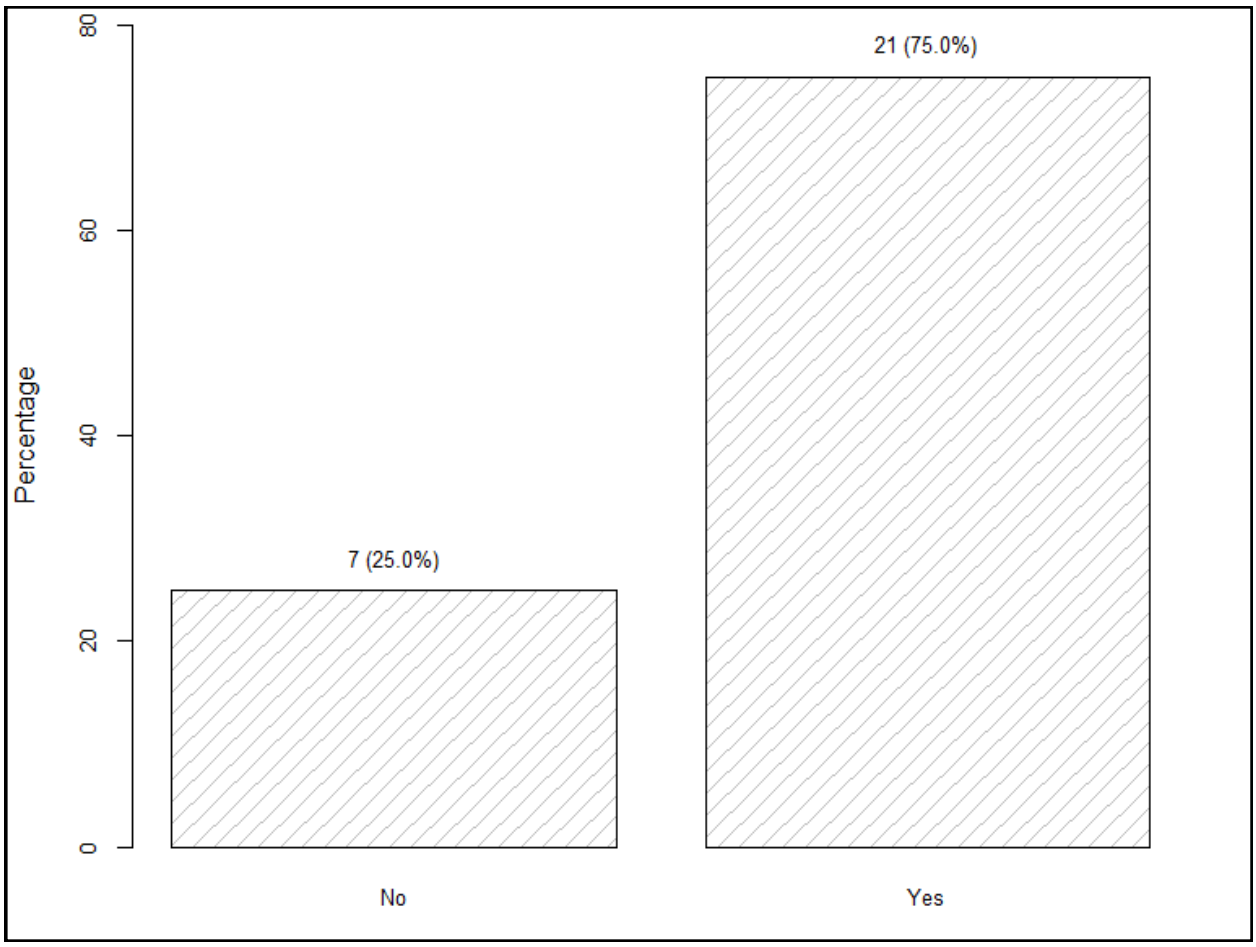


Figure 6: Lymph-node metastasis**.** Larger percentage of 75% had metastasized to secondary organs and is related to tumor size as described Weigelt *et al* 2005. 25% had not metastasis clinically; this is one of the clinicopathological characteristics in predicting breast cancer metastasis.

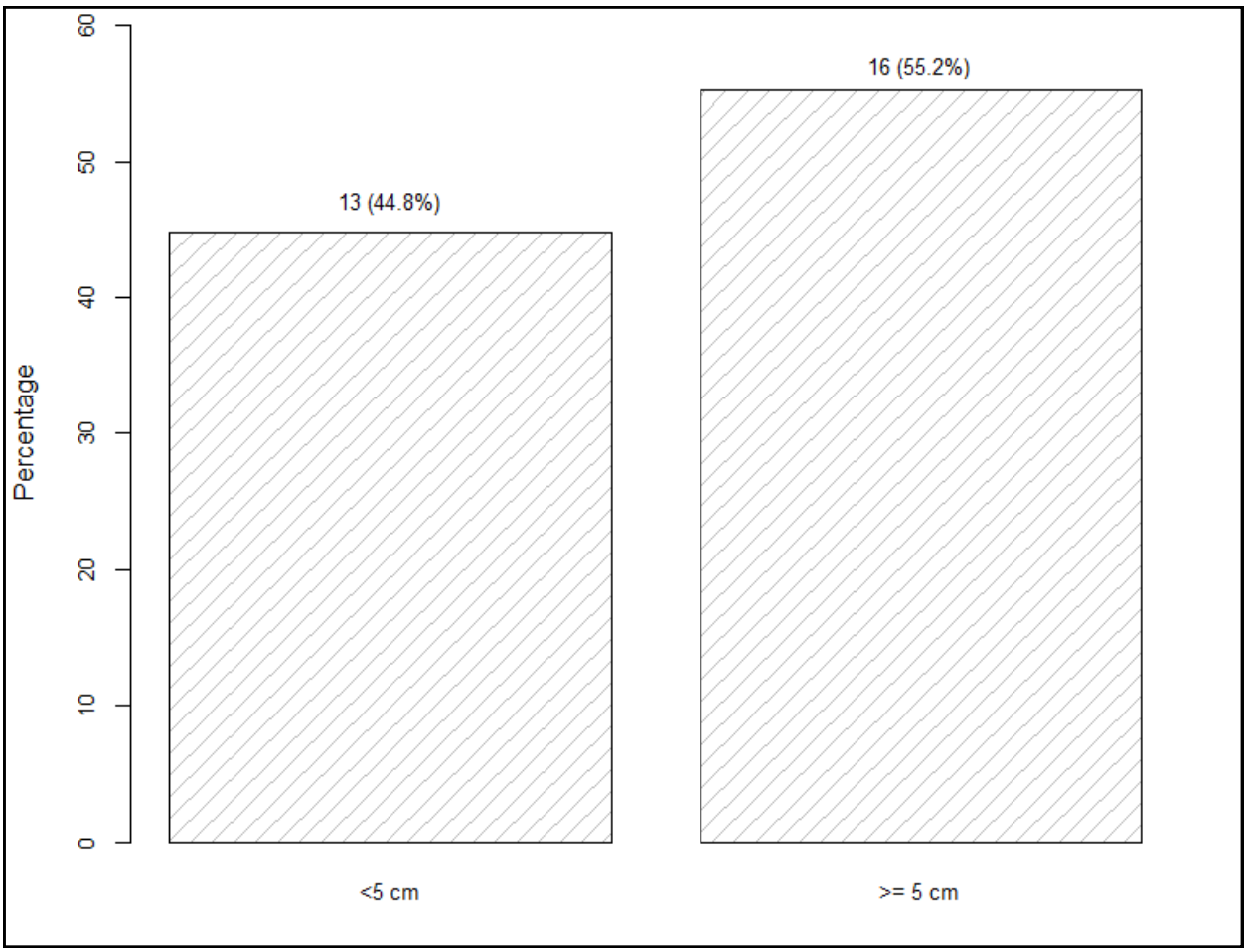


Figure 7: Tumor size**.** A larger percentage of 55.2% had tumor size ≥ 5cm and 44.8% of ≤ 5 cm and this is another clinicopathological characteristic of predicting breast cancer metastasis.

4.4 DRG1 Expression

ER expression was determined in all the samples and their frequencies reported 56.8% expressed ER (table 1). A total of 26 samples were used for DRG1 expression in which 3.8% scored 1+, 50.0% scored 2+ and 46.2% scored`3+ as presented (table 1). The expression levels of DRG1 antibodies were based on the intensity of staining while for ER was either positive or negative (Figure 8 and 9 respectively).

Table 2: ER and DRG1 expression of tumor slides

Variable		Sample size	n (%)
ER	Negative		2 (5.4%)
	Positive		21 (56.8%)
	Folded	37	1 (2.7%)
	No tissue		2 (5.4%)
	No tumor		8 (22.2%)
	Too small		3 (8.1%)
DRG1	1+	26	1 (3.8%)
	2+		13 (50.0%)
	3+		12 (46.2%)

Table 2: General expression of ER and DRG1: ER positive had the highest percentage of 56.8%. 37.8% of the slides couldn't give meaningful scientific information. DRG1 +2 had the highest percentage of 50% and were followed closely by DRG1+3 with 46.2%.

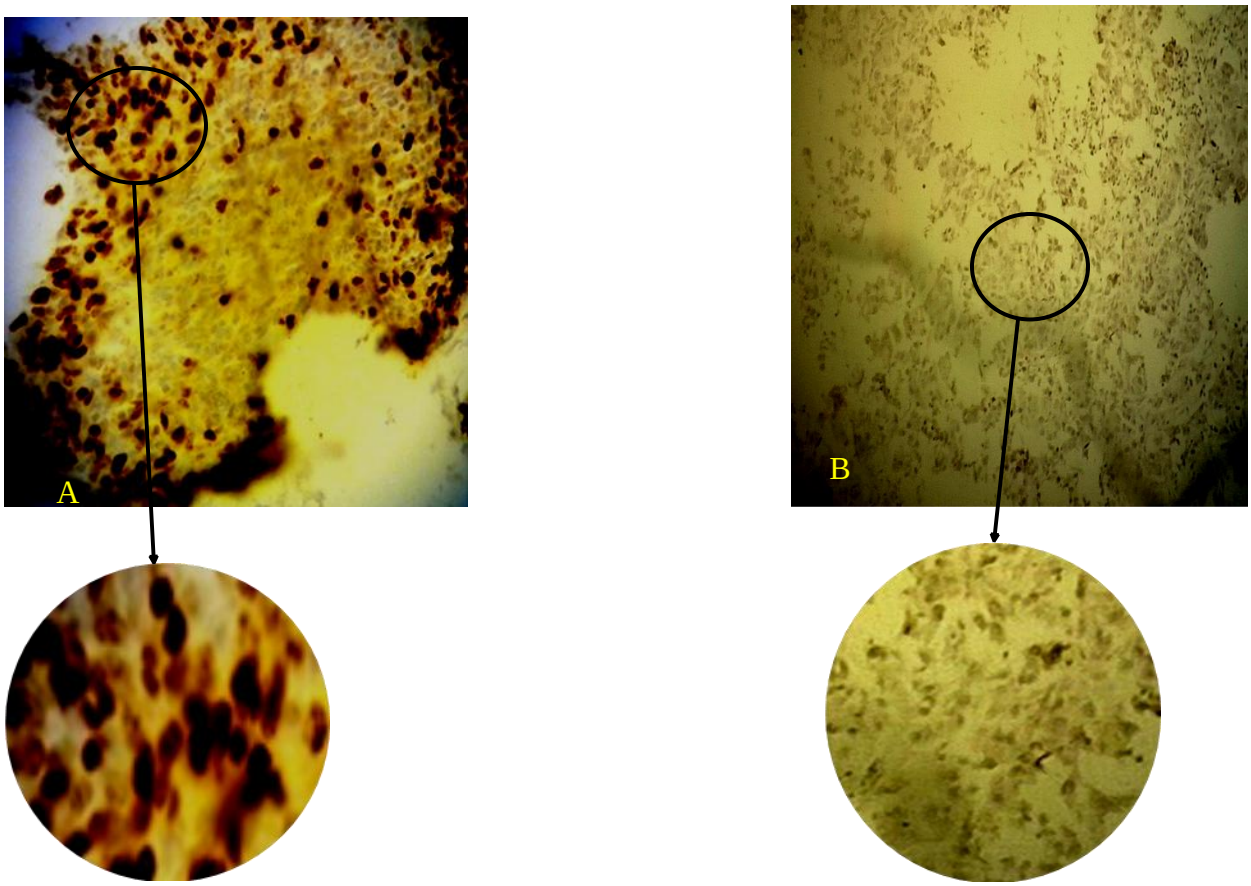


Figure 8: ER expression of**:** (A) ER positive, the marked side with a circle represent how ER positive appears and the arrow point the enlarged circulated section by 269%. (B) ER negative, the circulated represent how ER negative look like and the arrow point the enlarged circulated section by 269%. Anti-Rabbit monoclonal were used in the ratio 1:100 and staining intensity of ≥ 10% was positive and with ≤ 9% was rated to be negative. The arrow points at the more stained nucleus. ×400

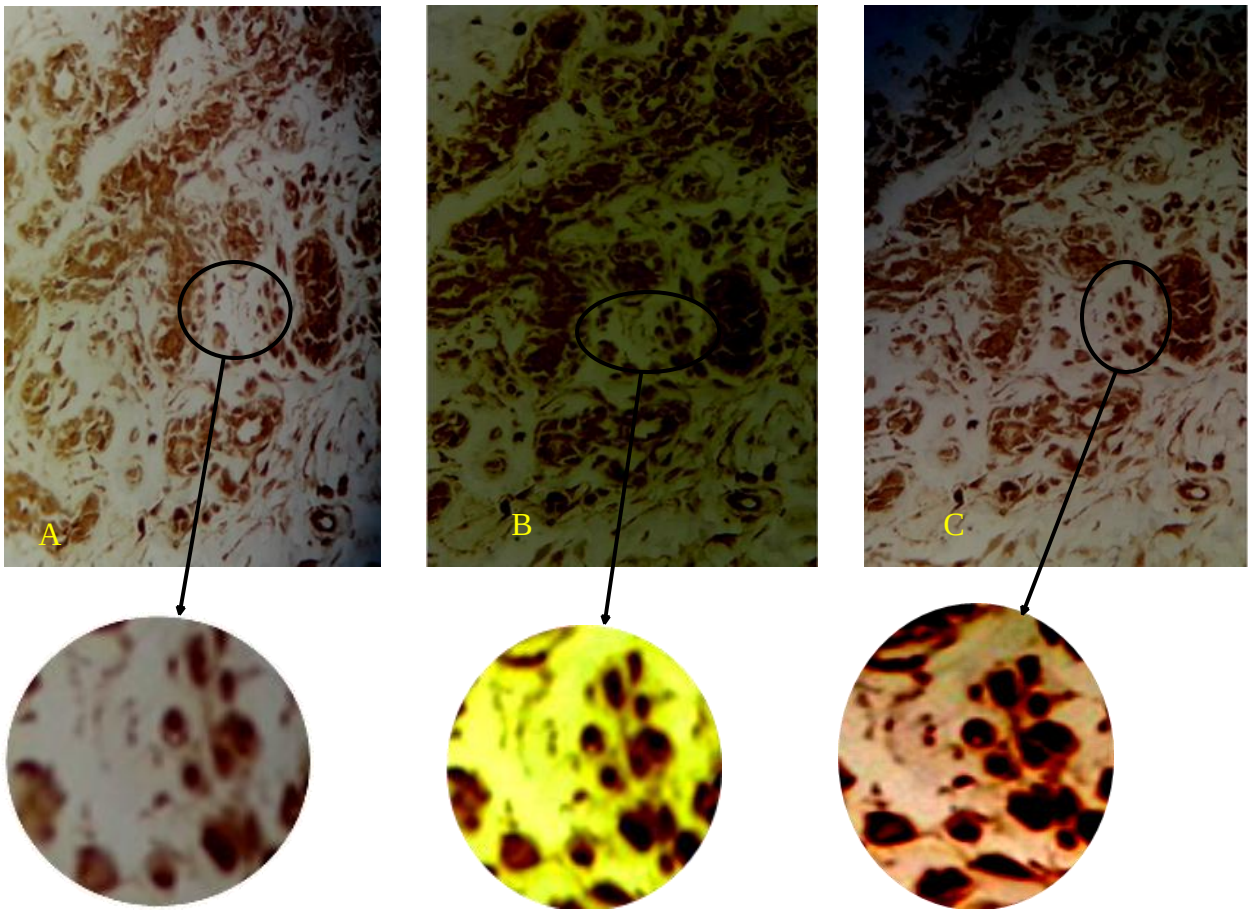


Figure 9: DRG1expression. (A) Intensity 1+, circulated part of the cytosol shows the intensity and the arrow points enlarged one with 269%. (B) Intensity 2+, circulated part of the cytosol shows the intensity and the arrow points enlarged one with 269% (C) Intensity 3+, circulated part of the cytosol shows the intensity and the arrow points enlarged one with 269%. Anti-DRG1 Rabbit polyclonal was used in the ratio 1:100. The stain intensity of DRG1 protein as seen in the cytoplasm of the breast tumor was used to classify into classes. 3+ had the highest stain intensity followed by 2+ and lesser in 1+.

4.5 Association between Histological grades and DRG1 Expression

Prior to determining the association between histological grade and DRG1, the distribution was analyzed using Fisher's exact test (table 2). The results reveals that a higher proportion of those in tumor grade III were in DRG1 3+ (75.0%) and a higher proportion of those in DRG1 intensity 2+ were in tumor Grade 1 (46.2%). The test therefore, shows that there was no association between the two variables (P=0.313).

Table 3: Association between Histological grades and DRG1 Expression

	DRG1 : Frequencies of DRG1 classes in respective tumor grades			
Tumor Grade	1+	2+	3+	Total
I	0 (0.0%, 0.0%)	3 (33.3%, 25.0%)	6 (66.7%, 46.2%)	9 (34.6%)
II	1 (7.7%, 100%)	8 (61.5%, 66.7%)	4 (30.8%, 30.8%)	13 (50.0%)
III	0 (0.0%, 0.0%)	1 (25.0%, 8.3%)	3 (75.0%, 23.1%)	4 (15.4%)
Total	1 (3.8%)	12 (46.2%)	13 (50.0%)	26 (100%, 100%)

4.6 Association between DRG1 and Age

Prior to determining the association between DRG1 and age, the distribution in the cases was analyzed using Wilcox on rank sum test (table 3). Results reveals that there is no significant association between DRG1 classes with age (P=0.493).

Table 4: Association between Age and DRG1

		Number	Median Age (IQR)	P value
DRG1	1+	1 (3.8%)	46.0	
	2+	13(50.0%)	41.0 (40.0, 50.0)	
	3+	**12 (46.2%)**	**42.0 (36.0, 48.0)**	**0.493**

4.7 Survival rate and outcome

Prior to determining the general outcome, distribution of the cases that were analyzed and presented according to their frequencies (Figure 10). A frequency of 32.4% succumbed to the diseases within and out of MTRH facility which is relatively high while, 5.4% are still alive. In addition, Kaplan-Meier survival curves were used to characterize the survival rate as from the date of admission (figure 11). Furthermore, the average survival time for those who succumbed to breast cancer was 1.5 ± 1.6 years while the average survival time before going out of the health facility was 1.0 ± 0.7 years. The overall median survival time from the graph is 2.18 years as indicated in the curve.

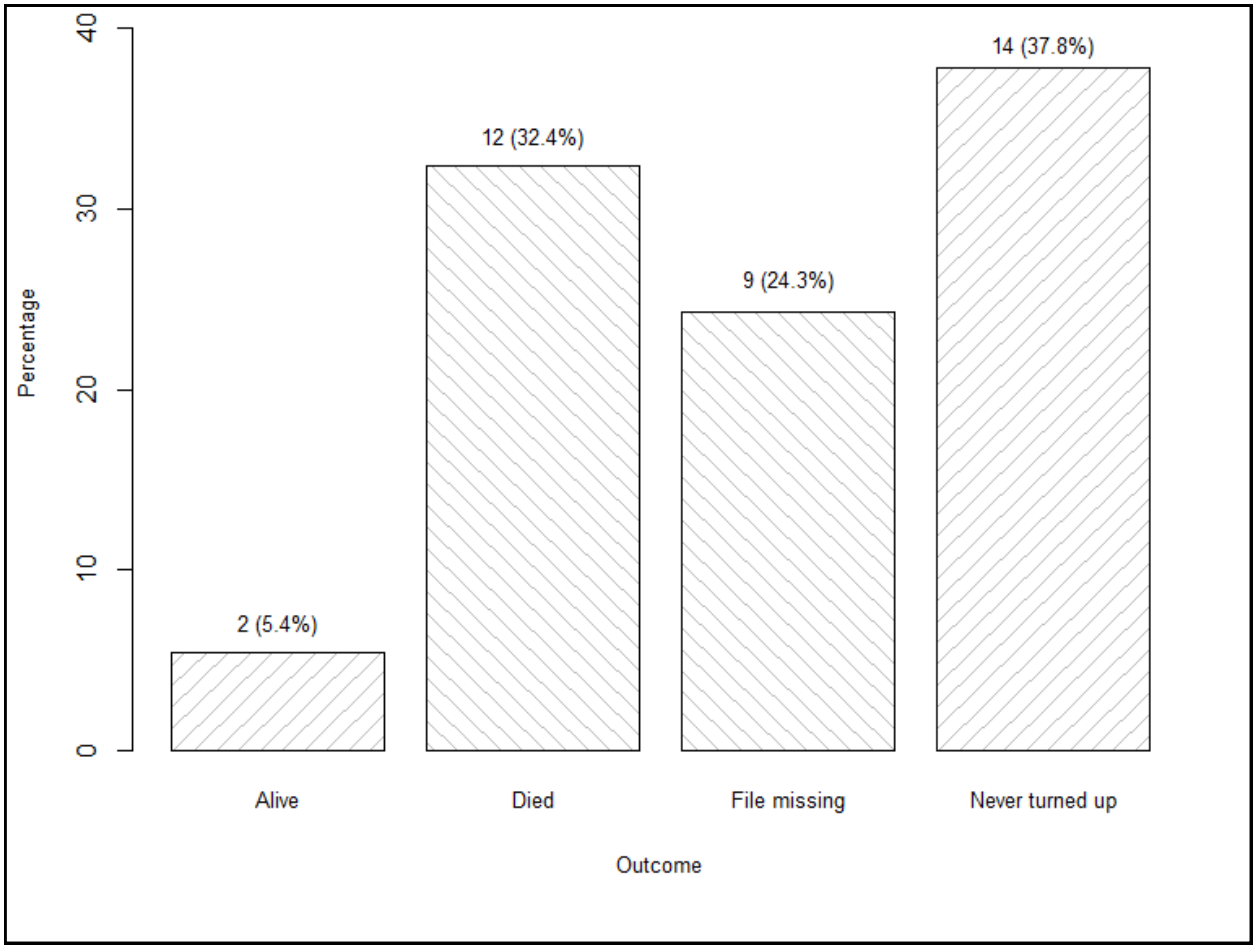


Figure 10: General frequency distribution of breast cancer patient based on the date of admission to the day of discharge: 37.8% were lost to follow up while 32.4% succumbed to breast cancer. This frequency distribution in percentages gives us an inside understanding on how patients are after being diagnosed with breast cancer.

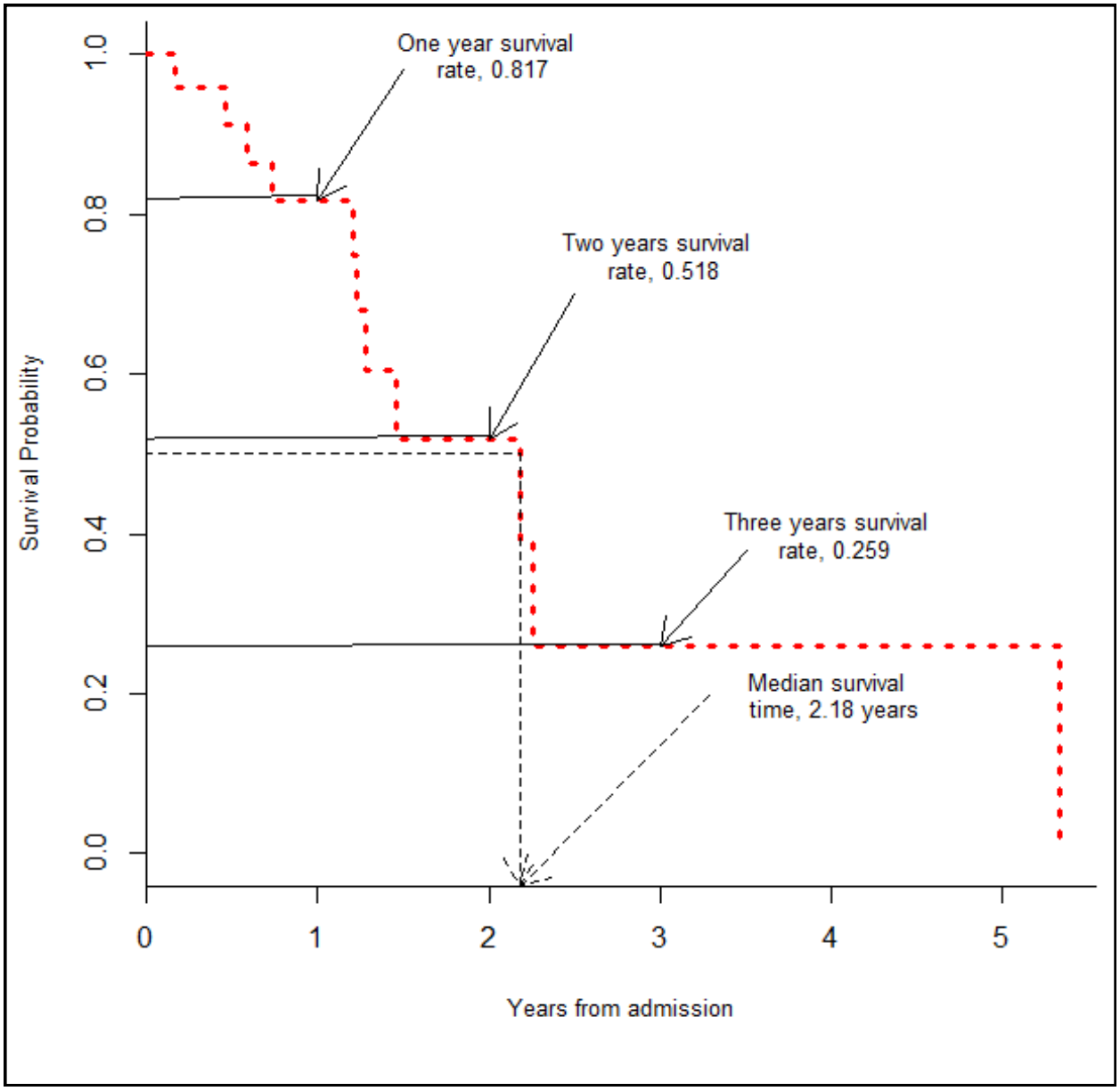


Figure 11: Survival period of patients as from the date of admission to the date of discharge**.** Overall survival curve for breast cancer patients admitted to the facility. The general survival median is 2.18 and three year survival period of 0.259 years.

CHAPTER FIVE

DISCUSSION

Prior to determination of the age, it was shown that breast cancer affects women of mean age 41.8±7.7 years (figure 3).This finding agrees with earlier findings by (Sawe *et al.* 2016) that mean age was 51.9±7.7 years. The age group 35 to 50 years were the most affected, this could be attributed to estrogen circle as they approach menopause. Other factor could also contribute to this e.g. birth control pills. Many women are known to use pills to control pregnancies and this could be the reason. A study should be carried out to point out the real cause of breast cancer in this age group. Caucasian women have been shown to have high prevalence of breast cancer in the age group of 40-50. Therefore, this finds agrees with (Su et al. 2011) therefore, age is a factor that increases the risk of breast cancer in women.

The most vulnerable age is productive thus, has both negative health and social impact in our country. The mean age has an active estradiol which control cell proliferation and differentiation (Tabatabai *et al*. 2012). Nevertheless, 4th-Global Breast Health Global Initiative (BHGI) affirm that age is one of the contributing factors to poorer outcome of breast cancer condition, control and management (Cazap *et al.* 2010). In addition kalenjins and Luos had the highest perevalence of breast cancer with 29.7%. This could be genetic factor since the two are Nilotic. The other communities had lesser prevalence though we can conclude it since MTRH is situated in Rift valley which is mostly dominated by kalenjins. Therefore, a study should be conducted to ascertain this at genetic level.

All patients' samples were subjected to histological grading and it was found that grade I 35.1%, II 51.4% and III 13.5% (Figure 4). Grade II had the highest frequency showing that most breast

cancer patients get to health facility at grade II. This findings shows that patients are diagnosed at the middle stage of cancer and it agree with previous findings (Joensuu *et al*. 2013) thus, it require proper treatment and management so as to reduce breast cancer mortality. Furthermore, clinical report showed that 75% of the patients' had metastasis. Tumor metastasis is a major clinical problem resulting in high mortality to breast cancer. Thus, these findings agree with 32.5% mortality of breast cancer patients as compared to those surviving of 5.4% (figure 10).

Tumor size is another clinicopathological characteristic that shows aggressiveness of the disease. In this study it was found that 55.2% of the tumors had ≥ 5cm and 44.8% were <5 cm. Breast cancer patients that have advanced cancer have larger tumor than those at initial stages (Kobayashi *et al*., 2013). This high percentage of tumors size ≥5cm agrees with high mortality of 32.5% as a number of breast cancer women visit MTRH facility when it has already metastasized.

Gene expression is one of the current powerful tools used for prognosis of breast cancer as it's a more precise tool. Immunohistochemisrty is of one the methods used to detect expression of protein mostly in tumors thus, showing the level of the gene that codes the protein in question. Immunohistochemistry technique is more sensitive to specific ligands compared to histopathology technique (Dalto *et al.* 2000). On the other hand, immunohistochemistry gives qualitative results as compared to PCR that gives quantitative results. Last but not least it's cheaper, easy to perform even in resource strain facility than PCR. Breast cancer that expresses ER has been shown to be more aggressive than other types of breast cancer (Riza *et al.*2014). Results revealed that ER positive was 56.8% therefore; a larger number of ER breast cancer women attend MTRH. This could be attributed to human genetics that African women are more

prone to ER breast cancer. To understand it better why ER breast cancer is the more prevalent than ER negative among Africans women a study should be done.

ER which fuel proliferation and differentiation of tumor has also been shown to contribute to metastasis in breast cancer (Tabatabai *et al.*2012). High percentage of death among breast cancer patients is attributed to ER expression (Stephen 2010). Furthermore ER breast cancer is the most over treated type of cancer since it is hard to predict metastasis and recurrence.

In this study 26 cases were subjected to DRG1 test using Immunohistochemistry. The intensity 1+ was 3.8%, of 2+ was 46.2% and 3+ was 50.0%. The results revealed that there is high expression of DRG1 in intensity 3+ which disagree with the earlier findings (Baig *et al*.2012). Intensity 2+ and 3+ are considered to be having metastasis since the protein that express the gene will have been translated and synthesized thus, its activity in the nuclear for maintaining cell division fidelity will have been lost and moved to the cytosol. Many studies have shown that DRG1 in the cytosol correlates with metastasis in human cell-lines (van *et al.* 1997; Baig *et al*.2012). This study also showed that DRG1 expression in the cytosol could be correlated to metasatasis. Thus, suggesting that this is a good biomarker in determining metastasis level in breast cancer women.

Prior to determination of association between histological tumor grade and DRG1 it was revealed that DRG1 intensity 2+ and tumor grade II had the highest frequencies of 50% compared to the others of 34.6% for grade I and DRG1 intensity 1+ 15.4% for grade III and 3+ (table 3). When Fisher's exact test was performed to ascertain the association it was noted that there is no association between DRG1 and tumor grade (P=0.313).This affirms that DRG1 biomarker is independent from tumor grading in predicting breast cancer metastasis. However,

this finding was not in agreement to early findings by Fotovati *et al*. 2006. This could be attributed to small number of DRG1 in this study hence further study should be conducted to ascertain it.

Prior to determination of association between age and DRG1 it was found out that there was no significant difference between the two (p=0.493). This shows that age also affects the expression of DRG1 as those in intensity 1+ had median 46.0, 2+ 41.0 (40.0,50.0) and 3+ 42.0 (36.0, 48.0). Those in intensity 1+ had the highest median of 46.0 suggesting that as age advances the expression level also reduces. This could be due to ER expression which agrees with the findings ofFotovati *et al.* (2006). This is due to the common phenomena that ER activity reduces as women approach menopause. However, association between ER and DRG1 expressions could not be calculated statistically due to experimental design. Therefore, further studies should be conducted using appropriate experimental design that gives quantitative results especially for ER expression.

Prior to determination of survival rate it was found out from survival curve that the medium survival rate is 2.18 year. Kenyan women have low survival rate as compared to African-American women as their survival rate is 5years (Ferlay *et al*.2008) The survival period for those admitted in MTRH is 2.18 years as compared to those in Netherlands of 5.05 years (Tabatabai *et al*.2012). Therefore, more studies should be done to ascertain the exact cause of poor survival rate of patients visiting MTRH. However, survival rate for DRG1 biomarker in predicting breast cancer metastasis couldn't meet statistical requirement due to small number of samples. Therefore, further studies should be done to determine this association.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSION

In summary, these results demonstrate that:

i. Breast cancer incidences are high between the ages of 35-55 years.

ii. ER positive is the most prevalent type of breast cancer in western Kenya than ER negative.

iii. DRG1 is highly expressed in breast cancer tumor sections.

iv. The survival rate for breast cancer women patients is 2.18 year and it's very low.

6.2 RECOMMENDATIONS

i. Women between the ages of 35-55 years should regularly be examined for breast cancer.

ii. Before oncologist make decision on the type of breast cancer they should first test ER positive and give proper medication and management to ER positive patients.

iii. DRG1 is the right biomarker for predicting breast cancer metastasis and recurrence.

iv. Because of the low level of survival rate, proper prognosis, treatment and management of breast cancer patients should be improve to reduce high motility.

REFERENCES

Akarolo-Anthony S.,Temidayo O. Ogundiran, Clement A. Adebamowo (2010) Emerging breast cancer epidemic: evidence from Africa. *Breast cancer Research*, 12 (suppl4):S8.

Baig, R. M., A. J. Sanders, M. A. Kayani & W. G. Jiang (2012) Association of Differentiation-Related Gene-1 (DRG1) with Breast Cancer Survival and in Vitro Impact of DRG1 Suppression. *Cancers (Basel),* 4**,** 658-72.

Bandyopadhyay, S., S. K. Pai, S. Hirota, S. Hosobe, Y. Takano *et al*., (2004) Role of the putative tumor metastasis suppressor gene Drg-1 in breast cancer progression. *Oncogene,* 23**,** 5675-81.

Campbell, G. (2008) Statistics in the world of medical devices: the contrast with pharmaceuticals. *J Biopharm Stat,* 18**,** 4-19.

Cazap, E., S. R. Distelhorst & B. O. Anderson (2010) Implementation science and breast cancer control: a Breast Health Global Initiative (BHGI) perspective from the 2010 Global Summit. *Breast,* 20 Suppl 2**,** S1-2.

Chen, B., D. M. Nelson & Y. Sadovsky (2006) N-myc down-regulated gene 1 modulates the response of term human trophoblasts to hypoxic injury. *J Biol Chem,* 281**,** 2764-72.

Chiang, A. C. & J. Massague (2008) Molecular basis of metastasis. *N Engl J Med,* 359**,** 2814-23.

Cochran, W. G. (1963) Methodological problems in the study of human populations. *Ann N Y Acad Sci,* 107**,** 476-89.

Cronin, C. Sangli, M. L.Liu, M. Pho, D. Dutta, A. Nguyen *et al*.,(2007) Analytical validation of the oncotype DX genomic diagnostic test for recurrence prognosis and therapeutic response predition in node-negative, estrogen receptor-positive breast cancer. *Clin Chem,* 53, 6, 1084-91.

Dalto, M.D., S.E.Pinder, M.R.C.Path, C.E. Elston,E.R.C.Path *et al*., (2000) Histological grading of breast cancer: Linnkage of patient outcome with level of pathologist agreement. *Pathology Inc,*13,7,730-735.

Drabsch, Y. & P. ten Dijke (2011) TGF-beta signaling in breast cancer cell invasion and bone metastasis. *J Mammary Gland Biol Neoplasia,* 16**,** 97-108.

Ferlay, J., H. R. Shin, F. Bray, D. Forman, C. Mathers & D. M. Parkin (2008) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer,* 127**,** 2893-917.

Fotovati, A., T. Fujii, M. Yamaguchi, M. Kage, K. Shirouzu *et al*., (2006) 17Beta-estradiol induces down-regulation of Cap43/NDRG1/Drg-1, a putative differentiation-related and metastasis suppressor gene, in human breast cancer cells. *Clin Cancer Res,* 12**,** 3010-8.

Guan, R. J., H. L. Ford, Y. Li, L. M. Shaw & A. B. Pardee (2000) Drg-1 as a differentiation-related, putative metastatic suppressor gene in human colon cancer. *Cancer Res,* 60**,** 749-55.

Hajdu, S.I (2011) A note from history; landmarks in history of cancer, part 1 *Cance*r 117,1097-102.

Ince, T. A., A. L. Richardson, G. W. Bell, M. Saitoh, S. Godar *et al*., (2007) Transformation of different human breast epithelial cell types leads to distinct tumor phenotypes. *Cancer Cell,* 12**,** 160-70.

Joensuu, K., M. Leidenius, L. C. Andersson, K. B. Horwitz & P. Heikkila (2013) ER, PR, HER2, Ki-67 and CK5 in Early and Late Relapsing Breast Cancer-Reduced CK5 Expression in Metastases. *Breast Cancer (Auckl),* 7**,** 23-34.

Kim, K. T., P. P. Ongusaha, Y. K. Hong, M. Nakamura, S. K. Kurdistani *et al.,* (2004) Function of Drg1/Rit42 in p53-dependent mitotic spindle checkpoint. *J Biol Chem,* 279**,** 38597-602.

Klein, C. A. (2008) Cancer. The metastasis cascade. *Science,* 321**,** 1785-7.

Kobayashi, T., K. Iwaya, T. Yamasaki, H. Tsuda, J. Yamamoto & O. Matsubara (2013) A simple immunohistochemical panel comprising 2 conventional markers, Ki67 and p53, is a powerful tool for predicting patient outcome in luminal-type breast cancer. *BMC Clin Pathol,* 13**,** 5.

Lewis, J. T., L. C. Hartmann, R. A. Vierkant, S. D. Maloney, V. Shane Pankratz *et al.,* (2006) An analysis of breast cancer risk in women with single, multiple, and atypical papilloma. *Am J Surg Pathol,* 30**,** 665-72.

Morrison, B. J., C. W. Schmidt, S. R. Lakhani, B. A. Reynolds & J. A. Lopez (2008) Breast cancer stem cells: implications for therapy of breast cancer. *Breast Cancer Res,* 10**,** 210.

Muller-Hocker, J., C. H. Zietz & A. Sendelhofert (2001) Deregulated expression of cell cycle-associated proteins in solid pseudopapillary tumor of the pancreas. *Mod Pathol,* 14**,** 47-53.

Nielsen, B. S., S. Jorgensen, J. U. Fog, R. Sokilde, I. J. Christensen *et al.,* (2011) High levels of microRNA-21 in the stroma of colorectal cancers predict short disease-free survival in stage II colon cancer patients. *Clin Exp Metastasis,* 28**,** 27-38.

Nonclercq, D., F. Journe, J. J. Body, G. Leclercq & G. Laurent (2004) Ligand-independent and agonist-mediated degradation of estrogen receptor-alpha in breast carcinoma cells: evidence for distinct degradative pathways. *Mol Cell Endocrinol,* 227**,** 53-65.

Osborne CK., Schiff R. (2005) Estrogen-receptor biology: continuing progress and therapeutic implication. *J Clin oncol* 25:1616-22

Pietro Riza, I. Barone, D. Zito, F. Giordono, M. Lanzino, F. De Amicis *et al.,* (2014) Estrogen receptor beta as anovel target ofandrogen receptor action in breast cancer cell lines. *Breast cancer Research,* 16:12

Richter, K. & J. Buchner (2001) Hsp90: chaperoning signal transduction. *J Cell Physiol,* 188**,** 281-90.

Sawe, M. Kerper, S. Badve, J. Li, M. Sandoval-Cooper, Z. Shi *et al.,* (2016) Aggressive breast cancer in western kenya has onset, high proliferation and immune cell infiltration. *BMC cancer* 16:204.

Shavers, V. L. & M. L. Brown (2002) Racial and ethnic disparities in the receipt of cancer treatment. *J Natl Cancer Inst,* 94**,** 334-57.

Smith IE.,Dowsett M. (2003) Aromatase inhibitors in breast cancer. *N Engl J Med*, 348:2431-42.

Sorlie, T., R. Tibshirani, J. Parker, T. Hastie, J. S. Marron, A. Nobel *et al*., (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A,* 100**,** 8418-23.

Stephen R.D. Johnston (2010) New strategies in Estrogen Receptor-positive breast cancer. *Clin cancer Res* 16:1979-87

Su, Y. Zheng, W. Zheng, K. Gu, Z. Chen, G. Li,W.Lu *et al.*,(2011) Distinct distribution and prognostic significance of molecular subtypes of breast cancer in Chinese women: a population-base cohort study *BMC cancer* 11:292.

Swedenborg, E., K. A. Power, W. Cai, I. Pongratz & J. Ruegg (2009) Regulation of estrogen receptor beta activity and implications in health and disease. *Cell Mol Life Sci,* 66**,** 3873-94.

Tabatabai, M. A., W. M. Eby, N. Nimeh, H. Li & K. P. Singh (212) Clinical and multiple gene expression variables in survival analysis of breast cancer: analysis with the hypertabastic survival model. *BMC Med Genomics,* 5**,** 63.

Talukder, S. I., M. A. Haque, M. H. Huq, M. O. Alam, A. Roushan, Z. Noor *et al.*,(2007) Histopathological analysis of hysterectomy specimens. *Mymensingh Med J,* 16**,** 81-4.

van Belzen, N., W. N. Dinjens, M. P. Diesveld, N. A. Groen, A. C. van der Made, Y. Nozawa *et al.,* (1997) A novel gene which is up-regulated during colon epithelial cell differentiation and down-regulated in colorectal neoplasms. *Lab Invest,* 77**,** 85-92.

Weigelt, B. & J. S. Reis-Filho (2009) Histological and molecular types of breast cancer: is there a unifying taxonomy? *Nat Rev Clin Oncol,* 6**,** 718-30.

Weigelt, J.L.Peters, L.J.Van't veer (2005) Breast cancer metastasis: Markers and models. *Nat Rev Can,* 5, 591-602.

APPENDICES

Appendix 1: IREC approval

INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC)

MOI TEACHING AND REFERRAL HOSPITAL
P.O. BOX 3
ELDORET
Tel: 33471//2/3

MOI UNIVERSITY
SCHOOL OF MEDICINE
P.O. BOX 4606
ELDORET

Reference: IREC/2014/79
Approval Number: 0001203

24th June, 2014

Mr. Bor Hillary,
Maseno University,
School of Medicine,
Department of Zoology,
P.O. Box 333,
MASENO-KENYA.

INSTITUTIONAL RESEARCH & ETHICS COMMITTEE
24 JUN 2014
APPROVED
P. O. Box 4606-30100 ELDORET

Dear Mr. Bor,

RE: FORMAL APPROVAL

The Institutional Research and Ethics Committee has reviewed your research proposal titled:-

"Immunohistochemical Evaluation of Biomarker for Metastasis Differention-Related Gene – 1 in Breast Cancer Women in Western Kenya".

Your proposal has been granted a Formal Approval Number: ***FAN: IREC 1203*** on 24th June, 2014. You are therefore permitted to begin your investigations.

Note that this approval is for 1 year; it will thus expire on 23rd June, 2015. If it is necessary to continue with this research beyond the expiry date, a request for continuation should be made in writing to IREC Secretariat two months prior to the expiry date.

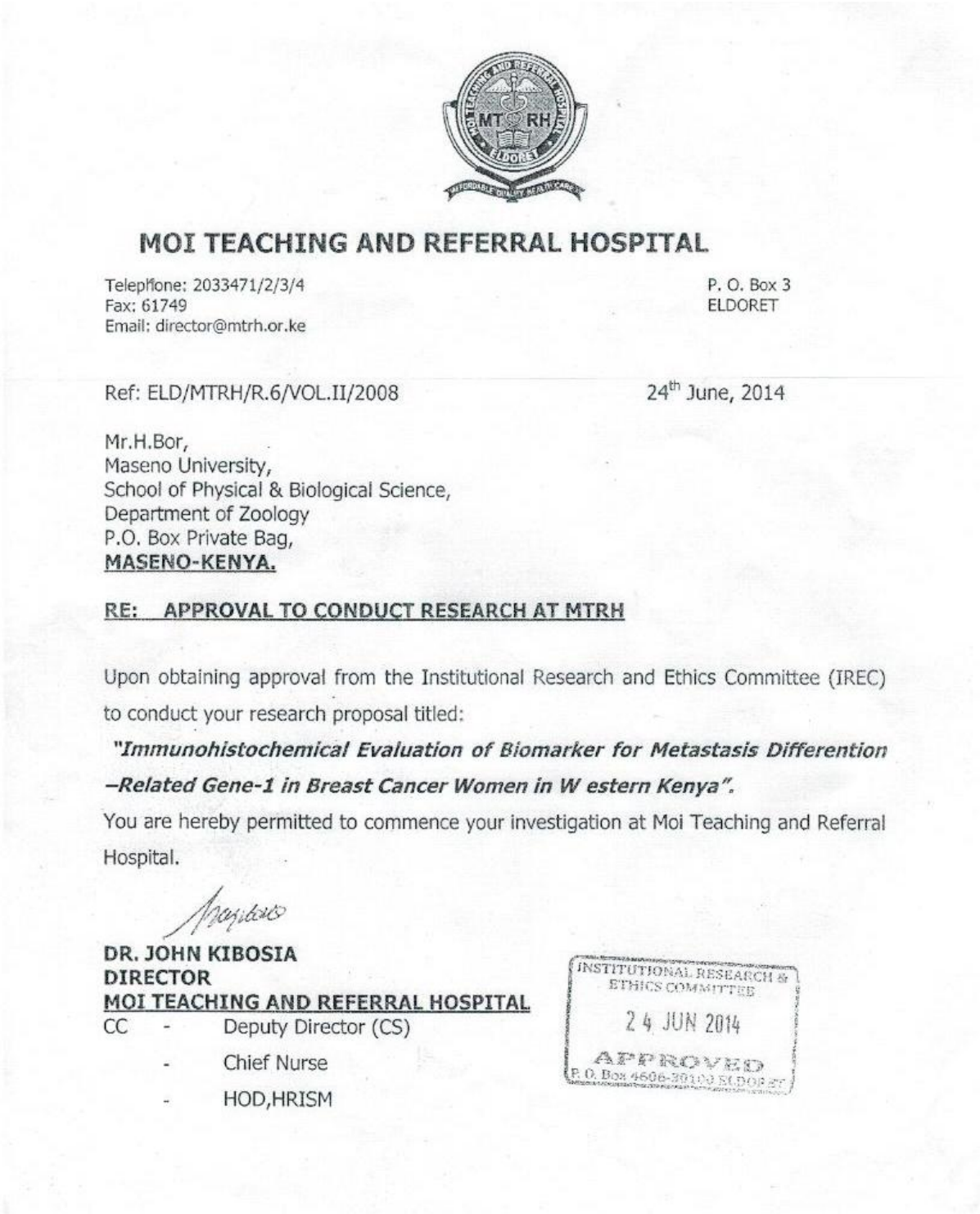
You are required to submit progress report(s) regularly as dictated by your proposal. Furthermore, you must notify the Committee of any proposal change (s) or amendment (s), serious or unexpected outcomes related to the conduct of the study, or study termination for any reason. The Committee expects to receive a final report at the end of the study.

Sincerely,

PROF. E. WERE
CHAIRMAN
INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE

cc	Director	-	MTRH	Dean	-	SOP	Dean	-	SOM
	Principal	-	CHS	Dean	-	SON	Dean	-	SOD

Appendix 2: Facility Approval



MOI TEACHING AND REFERRAL HOSPITAL

Telephone: 2033471/2/3/4
Fax: 61749
Email: director@mtrh.or.ke

P. O. Box 3
ELDORET

Ref: ELD/MTRH/R.6/VOL.II/2008

24th June, 2014

Mr.H.Bor,
Maseno University,
School of Physical & Biological Science,
Department of Zoology
P.O. Box Private Bag,
MASENO-KENYA.

RE: APPROVAL TO CONDUCT RESEARCH AT MTRH

Upon obtaining approval from the Institutional Research and Ethics Committee (IREC) to conduct your research proposal titled:

"Immunohistochemical Evaluation of Biomarker for Metastasis Differention –Related Gene-1 in Breast Cancer Women in W estern Kenya".

You are hereby permitted to commence your investigation at Moi Teaching and Referral Hospital.

DR. JOHN KIBOSIA
DIRECTOR
MOI TEACHING AND REFERRAL HOSPITAL

CC - Deputy Director (CS)
- Chief Nurse
- HOD,HRISM

INSTITUTIONAL RESEARCH & ETHICS COMMITTEE
24 JUN 2014
APPROVED
P. O. Box 4606-30100 ELDORET