KWAME NKRUMAH UNIVERSITY OF SCEIENCE AND TECHNOLOGY

COLLEGE OF HEALTH SCIENCES

SCHOOL OF MEDICAL SCIENCES

COMPARATIVE ANALYSIS OF THE QUANTITY OF RETINOIC ACID-INDUCED PROTEIN 3 (RAI3) BETWEEN TRIPLE AND NON TRIPLE NEGATIVE BREAST CARCINOMAS AMONG GHANAIAN WOMEN

A thesis submitted to the Department of Molecular Medicine, College of Health Sciences, in fulfillment of a

Master of Philosophy Degree in Immunology

By

Abigail Kwakye-Fosu

June 2016

DECLARATION

I hereby declare that the research work conducted in this project was solely undertaken by me at the Department of Molecular Medicine and Department of Pathology, Komfo Anokye Teaching Hospital, Kumasi (KATH). The work has not been presented for a degree to any University. Acknowledgement has been given to all sources of help.

…… ……

Abigail Kwakye-Fosu Date
(Student)

…… ……

Prof. (Mrs.) Margaret T. Frempong Date:
(Supervisor)

…… ……..

Dr. Ernest Adjei Date

(Co - Supervisor)

…… ……..

Prof. F.A. Yeboah Date
(Head of Department)

ABSTRACT

Introduction Retinoic acid-induced protein3 (RAI3) belongs to the family of G-protein coupled receptors (GPCRs). It is expressed in normal human tissue and is also likely to play an important role in breast cancer cell proliferation although the exact mechanism remains unclear. This study of RAI3 was carried out to provide evidence of its expression on breast cancer cells as well as normal breast tissues and also to compare its expression in breast cancer based on their hormone receptor status.

Methods Women of all ages visiting the Breast Clinic for the first time following the development of a self-detected breast condition, and upon further suspicion by the medical doctor through clinical examination were recruited in this research. A total of 100 women formed the study population. Tru cut biopsies were performed by surgeons and ER, PR and Her 2 immunohistochemistry was performed on the formalin fixed paraffin embedded breast cancer tissues. RAI3 immunohistochemistry was performed on some selected triple negative, estrogen receptor positive as well as normal breast tissues.

Results The mean age was 48.15 with the youngest age being 26 and the oldest age of 78. Estrogen receptor positive and triple negative breast tumours accounted for 44.18% and 43.02% respectively. A total of 36 tissue blocks were analysed for RAI3 expression and they showed various expression levels of RAI3.The immunohistochemical staining of normal and breast cancer cells with anti RAI3 antibody revealed the presence of RAI3 in both the cytoplasm and the cell membrane making it a potential biomarker and a therapeutic target. Very low levels were found in normal tissues. Higher expressions of RAI3 were found more in triple negative than non-triple negative. The mean

immunoreative score (IRS) in TNBC was 6.3 as compared to the entire non-TNBC which was 4.2 There was however no significant statistical relationship between RAI3 expression and ER (P=0.228) PR (P=0.732) and Her2 (P=0.758) status.

Conclusion This study confirms the expression of retinoic acid induced protein 3 (RAI3), in some selected normal, triple negative, and non-triple negative breast tissues at different Intensities. To the best of knowledge of this investigator this is the first analysis of RAI3 being carried out at the protein level among indigenous black people, albeit involving small sample size. There is clearly the need to work on a large cohort of normal breast tissues as well as different malignant tissues among indigenous black people and other race to better understand the role this protein plays in breast cancer growth and its potential use as a biomarker and a novel therapeutic option in the management and treatment of breast cancer.

ACKNOWLEDGEMENTS

The Lord Almighty has been gracious unto me to bring this research to a successful end. Lord I am very grateful. I am also grateful to my thesis supervisors Prof Mrs. Margaret Frempong for the knowledge I have acquired, her guidance and patience and Dr Ernest Adjei for his assistance. I would also wish to thank Dr Torsten Klockenbring and his colleagues at Fraunhofer IME, Germany for supplying the anti RAI3 antibodies.

I would also wish to thank the surgeons and the nurses at the Breast Care unit at Komfo Anokye Teaching Hospital (KATH) especially Dr. Francis Atipilla for his assistance. To Dr. Nicholas Titiloye and other pathologists as well Mr. Emmanuel Asiamah and other histo-technologists at the histopathology department at KATH, I owe so much gratitude for your immense assistance.

DEDICATION

This work is dedicated to the memory of my late mother and all breast cancer victims and survivors in Ghana.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PICTURES

CHAPTER ONE

1.1 Introduction

Breast cancer is one of the commonest cancer the world over, affecting mainly women, with one million cases being reported each year (Parkin *et al.*, 2005).It is a clinically and genetically heterogeneous disease, varying substantially in incidence and mortality according to race/ethnicity (Chlebowski *et al.*, 2005). Black women have higher risk of death after breast cancer diagnosis than white women and are more likely than white women to have an aggressive subtype of breast cancer that is associated with a higher mortality (Ma et al., 2013). The etiology of the widening racial disparity is not fully understood. Some studies have attributed these widening racial disparities to differences in healthcare, quality or access, different responses to new medical interventions or alterations in risk factors such as nutrition, physical activity, obesity or child bearing practices (Jatoi et al., 2005).

In Ghana, breast cancer is the leading malignancy (Badoe and Baako, 2008). In 2007 it accounted for 15.4% of all malignancies and this number increases annually (Clegg-Lamptey et al., 2009).Studies done in Ghana and among other black populations suggest more Estrogen receptor(E R) negative tumors and a higher prevalence of triple negative breast cancer (Stark et al., 2010, Fregene and Newman 2005, Gukas et al., 2005).

Triple Negative Breast Cancer (TBNC) refers to any breast cancer that does not express the gene for estrogen receptor, progesterone receptor or Human epidermal growth factor receptor 2 also known as Her 2 /neu (Lehmann et al., 2011). Treatment of TNBC is

challenging due to the absence of well-defined molecular target and the heterogeneous nature of the disease (Carey L A et al., 2007, Wiggans RG et al., 1979, Pegram M D et al., 1998). Attempts are being made to identify selective molecular targets for development of strategies for earlier diagnosis and treatment of breast cancer. Targeted therapies being investigated include tyrosine kinase inhibitors (Okabe et al., 2004, Gee et al., 2003) and Poly ADP-ribose polymerase (PARP) inhibitors (Farmer et al., 2005). However there still remains no acceptable molecular therapeutic targets for Triple negative breast cancer (Tan et al., 2008).

Retinoic acid-induced protein3 (RAI3) has been identified in the course of some of these studies as one of the proteins that could play a role in breast carcinogenesis and therefore could be used as a molecular target for the treatment of breast cancer, as RAI3 was overexpressed in human breast cancer in a study by Nagahata et al., 2005. Joriben et al., 2009 also found weaker expression of RAI3 in normal breast epithelial cells as compared to invasive breast carcinoma cells.

Retinoic acid-induced protein3 (RAI3) belongs to the family of G-protein coupled receptors (GPCRs) and among the receptor family in mammals they are the largest consisting of more than a thousand members (Joriben et al., 2009).They are integral plasma membrane proteins that transduce signals from extracellular ligands to signals in intracellular heterotrimetric GTP binding (G proteins).They activate numerous signal transduction cascades and thus play pivotal role in the regulation of many physiological and pathological processes including cell growth and differentiation, a reason that makes them valuable and interesting drug targets for the numerous human diseases that have been associated with dysfunctional GPCRs(Li et al., 2005).

1.2 HYPOTHESIS

If RAI3 molecules are associated with breast carcinogenesis, anti-RAI3 monoclonal antibodies could be used for the treatment of breast cancers or the selective suppression of signal from RAI3 might hold promise for development of a new strategy for treating breast cancers.

1.3 STATEMENT OF THE PROBLEM

Hormone receptor positive breast cancers generally have a better prognosis because they benefit from hormonal therapy (Nabholtz *et al.*, 2000). However the major limitation of endocrine therapy remains the nearly universal development of chemoresistance. Most estrogen receptor positive breast cancers that respond initially to endocrine therapies may develop resistance to anti-estrogen therapy and convert to estrogen negative tumours which tend to be more aggressive and unresponsive to hormonal therapy (Goldhirsch *et al.*, 2003).Studies have shown that breast cancer in African women are less likely to express ER and higher proportions of triple negative breast cancer (Stark et al., 2010).

Previous studies done on RAI3 has found higher levels of its expression in breast cancer cells and likely to have an important role in growth and survival of breast cancer cells, that could suggest that RAI3 represents a potential target for the development of novel therapeutic drug against breast cancer (Wu et al., 2005)

No study of RAI3 in breast cancer has been done in Ghana or among the indigenous black population in general. The study of RAI3 in breast cancer patients based on their hormonal

receptor status would provide findings that could add on to the knowledge required to understand RAI3 expression among indigenous black population.

1.4 AIM OF THE RESEARCH

To investigate comparatively, the level of expression of the protein, RAI3 in triple negative breast cancers, non-triple negative breast cancer and normal breast cells.

1.5 OBJECTIVES OF RESEARCH

- To study the distribution of various histologic types of breast cancer and to determine their hormone receptor status as well as HER 2/neu expression.
- To assess the therapeutic potential of RAI3 in breast cancer cells
- To study RAI3 expression in breast cancer cells according to their hormone receptor status.

1.6 JUSTIFICATION AND BENEFITS OF THE RESEARCH

The abundant expression of RAI3 on the surface of cancer cells would make it an interesting target for the development of antibody- based therapy for triple negative breast tumours (Joriben *et al.,* 2009). Identification of molecular signatures that allow detection of the transition from normal breast epithelia cells to malignant invasive cells is a critical component in the development of diagnostic, therapeutic and preventive strategies for human breast cancer (Cha et al., 2010).Thus the development of new anticancer agents to target molecules that are highly specific to malignant cells will be most effective.

Work done on RAI3 molecules focused on the systematic analysis of RAI3 expression in normal and cancerous human breast tissue but this work will go further to analyse its expression in TNBC as compared to its expression in non TNBC and normal breast cells. The study will put to test the diagnostic value of the highly specific RAI3 antibodies recently produced and characterized by JoriBen et al., 2009.

According to Stark et al., 2010, the study of breast cancer in women with African ancestry offers the promise of identifying markers for risk assessment and treatment of triple negative breast cancer, a point which explains why the study population will be African.

CHAPTER TWO

LITERATURE REVIEW

2.1 BREAST CANCER: EPIDEMIOLOGICAL AND DISEASE BURDEN

Breast cancer is the most commonly diagnosed invasive cancer among women with an average of I million cases detected worldwide (Nagahata *et al.,* 2004) and is a leading cause of cancer related deaths. It is predominantly a disease of females with male breast cancer accounting for only about 1% of all breast cancers (Achampong, 1990). An estimated 12% of women worldwide are at risk of developing breast cancer at some time in their lives. According to the World Health Organization report, an estimated 508,000 women in 2011 died due to breast cancer. In sub Saharan Africa an estimated 44,000 women die each year from breast cancer (Ly et al., 2012). Africans (Black women) have lower incidence of the disease, yet highest mortality from breast cancer. The ratio of the mortality rate to incidence is 0.69 in Africa, as compared with 0.19 in North America (Porter 2008). The lower incidence of the disease could be largely due to late menarche, early menopause, high parity with prolonged breastfeeding, irregular menses, and fewer ovulatory cycles that is common among black women (Fregene and Newman, 2005; Anyanwu 2000; Muguti 1993; Agyei-Frempong et al., 2008).These reproductive behaviors lead to lower exposure to endogenous estrogen over a lifetime period which may cumulatively decrease breast cancer risk. Despite this protective reproductive history, black (including Ghanaian) women develop breast cancer some 10 to 15 years earlier than Caucasians(white) with the peak incidence between the ages of 35 and 45 (Fregene and Newman, 2005) and also have lower survival rate. About 55% of breast cancer deaths

occur in developing countries (Curado et al., 2007). In developing countries, increase in urbanization and life expectancy coupled with the adoption of western lifestyles could be contributing factors to the increase in the incidence of the disease. Western lifestyle include decline in fertility rates, changes in diet and decreased physical activity. Inadequate or lack of quality healthcare and different responses to medical interventions could also account for this widening racial disparity. The problem is compounded by the late presentation of the disease. The reasons for the delayed presentation are multi factorial and include a lack of breast screening services combined with socioeconomic, cultural and sometimes religious beliefs which makes women present with advanced breast cancer.

It is clear that breast cancer will continue to be an increasing burden and therefore effective strategies need to be developed to reverse the increasing trend of breast cancer mortality especially in developing countries including Ghana. Early detection through screening programs is one surest way of decreasing disease related deaths.

2.2 What is Cancer?

Cancer arises from a loss of normal growth control. In normal tissues, the rate of new cell growth and old cell death are kept in balance. In cancer this balance is disrupted. This disruption can result from uncontrolled cell growth or loss of cell's ability to undergo cell suicide by a process called "apoptosis" i.e. the mechanism by which old or damaged cells normally self-destruct. Apoptosis can take place during embryonic development, during organogenesis, in several physiological processes and pathophysiological conditions during adult life. The gradual increase in the number of dividing cells creates a growing mass called tumor. The normal organization of the tissue gradually becomes disrupted as

more and more of the dividing cells accumulate and the tumour increase in size because new cells are being produced than needed. Most cancers originate from epithelial tissues in which oncogenes are activated and tumor suppressor genes lose their functions (Wu et al., 2005).During the tumor formation processes, proteases, inhibitors and other regulatory bodies are produced by normal stromal cells that support the genetically abnormal epithelium (Wu et al., 2005).Cancers including breast cancer are capable of spreading throughout the body by two mechanisms: invasion and metastasis. Invasion refers to the direct migration and penetration by cancer cells into neighboring tissues. In metastasis the cancer cells are able to break through tissue boundaries, through the lymphatic and blood vessel and circulate through the bloodstream and then invade normal tissues elsewhere in the body and this is frequently the cause of cancer related deaths.

2.3 Anatomy of the breast

The normal female breast consists of milk producing glands that are connected to the surface of the skin at the nipple by narrow ducts. The milk producing glands are organized into lobes which contain smaller structures called lobules where milk is produced. Fatty, fibrous connective tissues support the glands and ducts and give the breast shape. Blood vessels, nerves, lymph and lymph nodes make up most of the rest of the breast tissue. Breast cancer cells can travel in lymphatic vessels and begin to grow in lymph nodes. If cancer cells spread to lymph nodes, there is a greater chance that the cells have also spread to other places in the body.

Breast cancer develops primarily in the breast milk ducts (ductal carcinoma) and fewer breast cancers start in the lining of the lobules (lobular carcinoma).

Breast cancers fall into these two main categories:

i. In situ breast cancers, also called noninvasive breast cancers: These are cancers confined to breast ducts (milk tubes) and lobules (milk-producing glands).
ii. Invasive breast cancers, also called infiltrating breast cancers: These cancers can start in the ducts or lobules, but then they spread into surrounding breast tissue. Invasive cancers may eventually become metastatic, meaning they find their way to other organs, like the bones, lungs, liver, and brain.

2.4 Invasive ductal carcinoma (IDC)

This is the most common type of breast cancer accounting for about 80% of all breast cancer cases (Adachi et al., 2007). IDC starts in the breast's milk ducts and invades surrounding breast tissue. If not treated at an early stage, IDC is capable of moving to other parts of the body through the bloodstream or lymphatic system. More patients with IDC are likely to express estrogen and progesterone receptors (Petricevic et al., 2011).

2.5 Invasive lobular carcinoma.

This accounts for about 5-15% of invasive breast cancer (Arpino et al., 2004). ILC starts in the lobules or milk glands and then spreads into the surrounding breast tissues. It is characterized by large tumor size, low histologic grade, has high incidence in older women and are biologically distinct and has better survival than invasive ductal carcinoma (Toikkanen et al., 1997).Patients with ILC are more likely to be ER/PR positive and negative for HER2, P53 and epidermal growth factor receptor(EGFR) and early diagnosing

can be challenging due to their inability to form distinct masses that can make diagnosing by palpation or mammography difficult (Arpino et al., 2004)

2.6 Ductal carcinoma in situ (DCIS)

This is also called intraductal carcinoma and is the breast cancer in the lining of the milk ducts that has not yet invaded nearby tissues. Ductal carcinoma in situ is a proliferation of malignant epithelial cells confined to the ductolobular system of the breast (Bane 2013). This is considered non - nvasive or pre invasive breast cancer. It may progress to invasive cancer if untreated. Studies have shown that 30% of DCIS will progress to invasive cancer over a 30 year time period (Page et al., 1982) with the rate of progression likely to be high in high grade DCIS (Collins et al., 2005). Approximately 75% of low grade DCIS lesions express estrogen receptor compared to only 30% of the high grade DCIS lesions (Dobrescu et al., 2011) Risk factors for the development of DCIS are similar to those for invasive breast cancer such as increasing age (Kerlikowske et al., 2008).

2.7 Lobular carcinoma in situ (LCIS)

This is not a true cancer but is a marker for an increased risk of invasive cancer in the same or both breasts. It involves the abnormal growth of cells that start in the lobules.

2.8 Other types of breast cancers

There are other types of breast cancer though some of them quite rare. These include

2.8.1 Inflammatory breast cancer

This accounts for 1% to 3% of all breast cancers. Usually there is no single lump or tumor. Instead, inflammatory breast cancer (IBC) makes the skin on the breast look red and feel warm. This type of breast cancer tends to have a higher chance of spreading and a worse outlook (prognosis) than typical invasive ductal or lobular cancer.

2.8.2 Papillary Carcinoma

This accounts for 0.5% of all breast cancer and both invasive and in situ forms seem to be more prevalent in postmenopausal women and in males as well (Sumanta et al., 2010).They are usually small and positive for estrogen and/or progesterone receptors and negative for Her 2.They are less likely to involve the lymph nodes and are more responsive to treatment and may have a better prognosis compared to other types of breast cancer.

2.8.3 Adenoid Cystic Breast Cancer (ACBC)

This accounts for less than 0.1% of all breast cancer (Louwman et al., 2007).Like other types of invasive ductal cancers, ACBC begins in the milk duct of the breast before spreading to the tissues around the duct. Histologically, the cells look like cancer cells more commonly found in the salivary glands and saliva. (Ro et al., 1987). They are often estrogen and progesterone receptor negative (Arpino et al., 2002). They are less likely to involve the lymph nodes and are more responsive to treatment and may have a better prognosis (Cadoo et al., 2012).

2.8.4 Apocrine Breast Cancer

This is another uncommon type of breast cancer accounting for less than 0.4% of all invasive breast cancer with the mean age of presentation to be 51 to 62 yrs. (Matsuo et al., 2002).Some studies have reported it to be less likely to involve lymph node and a lower histological grade (Matsuo et al., 2002., Japaze et al., 2005).They are often triple negative and more responsive to treatment and may have a better prognosis than other types of invasive ductal cancer

2.8.5 Medullary Breast cancer

This is also a rare subtype of invasive ductal carcinoma accounting for less than 5% (Reinfuss et al., 1995) and more common in carriers of BRCA 1 mutations. The tumor is a soft fleshy mass but the cells are of higher grade. They are less likely to involve lymph node and are of higher grade. They are often triple negative. Data on the prognosis of MBC is conflicting. Whiles some studies report of favorable prognosis despite its association with unfavourable biological features which usually characterize a more aggressive subtype (Reinfuss et al., 1995) others do not confirm this observation (Vu-Nisihino et al., 2005).

2.8.6 Paget's Disease

This is a rare form of breast cancer which accounts for less than 3% of all breast cancers. It is usually characterised by skin changes in the nipple area leading to itching, flaking and nipple discharge. They are often found in high grade and about half are found to be positive for estrogen and progesterone receptors

2.9 Breast cancer grade

Breast cancer grades are based on their microscopic appearance and arrangement of tumour 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).The modified Bloom-Richardson-Elston grading system also known as the Nottingham grading system is now frequently being used (Elston and Ellis 1991, Genestie et al., 1998). The grade can help predict a patient's prognosis. In general, a lower grade number indicates a slower-growing cancer that is less likely to spread, while a higher number indicates a faster-growing cancer that is more likely to spread. The grade is as follows:

Grade 1 (well differentiated) cancers have relatively normal-looking cells that do not appear to be growing rapidly and are arranged in small tubules.

Grade 2 (moderately differentiated) cancers have features between grades 1 and 3.

Grade 3 (poorly differentiated) cancers, the highest grade, lack normal features and tend to grow and spread more aggressively.

The tumor grade can help decide if further treatment is needed after surgery.

Staging on the other hand is the process of determining the size of the tumor and whether it has spread within the breast or to other parts of the body. The stage of the cancer is a key factor in making treatment recommendations and estimating a patient's chances of recovery.

There are two main systems for determining the stage of breast cancer:

I. TNM staging: TNM is an acronym of the words tumor, nodes, and metastasis. This system describes breast cancer in terms of the size of the original tumor, whether any lymph nodes are involved, and whether the cancer has metastasized, or spread from the breast to another part of the body.

II. Overall stage grouping: The following terms are used loosely to refer to these stages:

- Non invasive cancers are at stage 0.
- Early-stage cancers are at stages I and II, and sometimes III.
- Advanced cancers are at stages III and IV.

2.10 RISK FACTORS OF BREAST CANCER

2.10.1 Age

The incidence of breast cancer increases with age particularly above 40 yrs (Berg 2010). About 1 out of 8 invasive breast cancers develop in women younger than 45. About 2 out of 3 invasive breast cancers are found in women 55 or older. The aging process poses a big risk for breast cancer. The longer one lives the more one is prone to genetic damage (mutations) and the body becomes less capable of repairing genetic damage.

2.10.2 Family History

Women with a positive family history of breast cancer in a first-degree relative have a higher risk of developing breast cancer than women without a family history of the disease.

The increased risk is further elevated if two first-degree relatives have the disease. First degree relative is a mother, sister or daughter. It is also greater among women with a single first-degree relative, particularly if they were diagnosed at an early age (Pharoah et al., 1997).

2.10.3 Hereditary

Genetic factors breast cancer account for 5-10% of all breast cancer cases and about 90% of hereditary breast cancer involves mutations of BRCA1 and BRCA2 genes (Sakorafas and Tsiotou 2000). These are located on the long arms of chromosomes 17 and 13 respectively and mutations can occur at almost any position. BRCA functions to repair cell damage and keep breast cell growing normally. Cancer risk is increased when these genes contain abnormality or mutations and are passed from generation to generation. Breast cancers linked to these mutations occur more often in younger women and more often affect both breasts than cancers not linked to these mutations.

2.10.4 Previous benign breast disease

A person diagnosed with previous benign breast conditions has a higher risk of breast cancer. In these conditions the cells growing in the milk duct or lobules grow faster than normal but they look normal. These include

- Ductal hyperplasia
- Complex fibroadenoma
- Sclerosing adenosis
- Papilloma or papillomatosis
- Radial scar

The risk is even higher when diagnosed with atypical ductal hyperplasia and atypical lobular hyperplasia. In these conditions the cells in the ducts and lobules grow faster than normal and look abnormal

2.10.5 Age at menarche and menopause

Women who start menstruating early (before age 12) or who have late menopause (after age 55) have an increased risk of developing breast cancer. This could probably be due to increased endogenous estrogen and progesterone exposure. Women who have late menopause (after age 55) are twice as likely to develop breast cancer as women who experience menopause before the age of 45.

2.10.6 Age at first pregnancy

Women who have had no children or who had their first child after age 30 have a slightly higher breast cancer risk. Having many pregnancies and becoming pregnant at a young age reduce breast cancer risk. The reason could be due to the fact that pregnancy reduces a woman's total number of lifetime menstrual cycles.

2.10.7 Having dense breast

The breast is made up of fibrous tissue, connective tissue and fat cells .Having a high breast density increases one's risk of developing breast cancer. The reason for this phenomenon remained previously unclear but a new study has revealed how dense breast tissue drives the early stages of cancer. The study revealed that structural cells called fibroblasts from high density breast tissue activates a cellular communication network

called JNK1.This is known to instruct cells to release a chemical that create an inflammatory environment that drives tumor formation (Lisanti et al., 2014).

2.10.8 Lifestyle:

2.10.8 (a)Alcohol Consumptions

Alcohol consumption increases a woman's risk of breast cancer particularly hormone receptor positive breast cancer. Alcohol can increase levels of estrogen and other hormones associated with hormone receptor positive breast cancer. Alcohol also may increase breast cancer risk by damaging DNA in cells.

2.10.8 (b) Smoking

Smoking may slightly increase one's risk of developing breast cancer. While some studies suggest that smoking of long duration, smoking before a first full-term pregnancy and passive smoking may probably increase breast cancer risk (Terry and Rohan 2002), other studies also found no link between smoking before a first full-term pregnancy and breast cancer risk (Lowlor et al., 2004). More research is therefore needed to make solid conclusions on the potential link between smoking and breast cancer.

2.10.8 (c) Being Overweight

Being overweight increases one's risk of breast cancer compared to maintaining a healthy weight especially after menopause. There is also the risk of disease recurrence with women who have had the disease with being overweight. More estrogens are available from the extra fat cells which fuel the cancer cells to grow.

2.10.8 (d) Oral Contraceptives

Oral contraceptives (OCs) may produce a slight increase in breast cancer risk among long-term users, but this appears to be a short-term effect. Some studies have found a slightly greater risk of breast cancer in users of oral contraceptives than in non-users (Collaborative group 1996). This risk seems to go back to normal over time after the cessation of the oral contraceptive use. There is no significant increase in breast cancer risk after ten years of cessation of oral contraceptive user. Duration of use, age at first use, dose and type of hormone within the contraceptive appear to have no significant effect on breast cancer.

2.10.8 (e) Hormone Replacement Therapy

Estrogen is often combined with progesterone in hormone therapy to help relieve symptoms of menopause and to help prevent osteoporosis (thinning of the bones). Using combined hormone therapy after menopause increases the risk of getting breast cancer. The risk increases with increasing duration of use. This risk is reduced after cessation of use of HRT and seems to disappear after about 5 years. The use of hormone for treatment of menopausal symptoms for a short time may have little or no effect on breast cancer (Collaborative group 1997). A study conducted among Asian-American women attributed increase in breast cancer rates among these group of people to changes in modifiable lifestyle such as high body weight and use of menopausal hormone therapy(Wu et al., 2006).In the western populations these modifiable lifestyles are important risk factors for post-menopausal breast cancer which reflect the critical etiological role of estrogen.

2.11 SCREENING FOR BREAST CANCER

Breast cancer screening is a method of detecting breast cancer at a very early stage. It results in prompter treatment which enhances better diagnosis as it detects breast cancer early. Thus breast cancer treatment and management is easier when detected at an early stage than a late stage. There are various screening methods employed in breast cancer. These include the following:

2.11.1 Breast Self-Examination (BSE)

This involves examining one's own breasts by looking at, and feeling each breast for possible lumps, distortion or swelling. Breast self-examination was aimed at detecting breast cancer in early stages but large randomized controlled studies have found it to be ineffective in reducing breast cancer mortality but rather increase the number of biopsies performed because of false positives (Thomas et al., 2002). Breast self-examination is therefore not recommended as a screening method but may be appropriate and help create awareness among women at higher risk. These studies have been done in countries where there are screening programmes and women are aware of breast cancer. In many developing countries such as Ghana however, screening mammography programme is not common and so breast self-examination may be useful as it has not been shown to be ineffective (Clegg-Lamptey et al., 2009).

2.11.2 Clinical Breast Examination (CBE)

This is the physical examination done by a health care provider. Clinical breast examination increases case finding of breast cancer, however, evidence of CBE in reducing

breast cancer mortality is not fully known. The effectiveness of CBE has not been proven in large, well designed trials. The sensitivity of CBE is higher in women 50 and younger (Green and Taplin, 2003). It finds breast cancer missed on mammography. Randomized controlled studies indicate that five percent of breast cancer is detected by CBE (Doug and Steiner, 2007) and independently contributes to breast cancer detection when combined with mammography (Barton et al., 1999).

2.11.3 Mammography

It is an x-ray of the breast taken whiles carefully compressing the breast. It can detect small changes in breast tissue that may need further investigations to check for cancer. It is the only screening method that has proven to be effective. It can detect cancer that could be treated through lumpectomy instead of mastectomy and decrease the need for chemotherapy.

Mammogram screening is recommended for women 50 and older. For women aged 40-49 mammogram has been shown to be less effective (Carney et al., 2003). Women in this age group usually have lower incidence of the disease, have denser breast which can lower the sensitivity of the mammography and affect interpretation of the results and on average faster growing cancer. (Carney et al., 2003, Elmore et al., 2005). However results from other randomized trials present the effectiveness of screening women in their 40s (Moss et al., 2006).Some clinical trials indicate that mammography screening reduces breast cancer mortality by 25% in post-menopausal women but it takes about 7 to 9 years to see this effect after the initial screening(Kerlikowski et al., 1995).

There are some disadvantages associated with mammography. It could result in false positive or false negatives. This risk also increases with women with mammographically dense breast (Laya et al., 1996).One study reported independent risk factors associated with false positive mammogram which include, younger age, increasing number of breast biopsies, a positive family history of breast cancer, estrogen use (Christainsen et al., 2000). According to Carney et al, about 20%-40% of false negative mammography occur in women with breast cancer (Carney et al., 2003). The procedure could also cause pain, anxiety, distress and other psychological responses.

Other studies have also reported of the possibility of over diagnosis and over treatment of clinically insignificant lesions by mammography especially ductal carcinoma in situ (Green and Taplin 2003). Concerns have also been raised about the possible radiation-induced breast cancer from exposure to repeated mammography but the potential benefits are thought to outweigh the possible risk of radiation (Feig and Henderick 1997).

2.11.4 Breast Ultrasound

Breast ultrasound is a diagnostic aid to mammography. It is a valuable tool to use along with mammography because it is widely available and less expensive. Usually, breast ultrasound is used to target a specific area of concern found on the mammogram. It is also helpful for women with dense breast. It helps distinguish between solid masses and also between benign and malignant masses (Stavros et al., 1995).It is usually not recommended for screening the general population.

2.11.5 Magnetic Resonance imaging (MRI)

MRI is helpful in screening women at high risk for breast cancer such as those with strong family history of breast cancer and BRCA1 and BRCA2 mutation carriers. It has a higher sensitivity than that of mammography but has a lower specificity (Elmore et al., 2005). MRI can diagnose benign findings at a glance, thereby eliminating the need for costly and unnecessary biopsies or surgical procedures. It is however a relatively expensive procedure and not recommended for screening general population. The impact of MRI on breast cancer mortality is not fully known.

2.12 BIOMARKERS OF CLINICAL IMPORTANCE IN BREAST CANCER

Molecular biomarkers are used as an indicator for identifying particular disease state which helps in diagnosis of that particular disease. There are several types of molecular biomarkers for breast cancer. Whiles prognostic biomarkers provide information regarding patient clinical outcome independent of treatment, predictive factors aim to foretell the response of a patient to a specific therapeutic intervention and are associated with tumor sensitivity or resistance to that therapy. Estrogen receptors(ER) and progesterone receptors (PR) are the two most widely used predictive markers which are used to select breast cancer patients who are likely to benefit from endocrine therapy.

These can also serve as prognostic markers as well. The human epidermal growth factor receptor 2 (HER2) is not only a prognostic factor but also a predictor of response to HER2 targeting therapy such as trastuzumab (Heceptin). Breast cancer may be defined as ER positive, PR positive or hormone receptor negative. A few of them are considered below:

2.12.1 Estrogen Receptors

Estrogens are hormones and belong to a family of related molecules that stimulate the development and maintenance of female characteristics and sexual reproduction. The natural estrogens are steroid molecules and the most prevalent forms of human estrogens are estradiol and estrone. Both are produced and secreted by the ovaries, although estrone is made in the adrenal glands and other organs. Estrogen functions as signaling molecules travelling through the bloodstream and interacting with cells in a variety of target tissues. The breast and the uterus which play important roles in sexual reproduction are the main target tissues of estrogen. The brain, bone, liver and heart are also acted on by estrogen molecules. Estrogen exerts its influence in target tissues and cause cells to divide in a process called proliferation.

Estrogen interacts with cell in target tissues with the help of estrogen receptors. The estrogen receptors are activated when an estrogen molecule enters the cell and passes into the nucleus. The estrogen binds to its receptor causing a conformational change. The estrogen-receptor complex then binds to specific DNA sites called estrogen response elements. These are located near genes and are controlled by estrogen. After becoming attached to estrogen response elements, the estrogen receptor complex binds to co-activator protein and more nearby genes become active. The active gene produces molecules of messenger RNA which guide the synthesis of specific proteins. These proteins can then influence cell behaviour in different ways depending on the cell type involved. Estrogen receptors trigger gene activation.

There are two different forms of the estrogen receptors, commonly referred to as alpha(α) and beta (β), each encoded by a separate gene, ESR 1 and ESR 2. Both forms of receptors show significant sequence homology and are composed of five domains. They also bind to estrogen response element with similar affinity due to a high homology of their DNA-binding domains. ERβ is thought to mediate sometimes opposite effects to ERα due to different binding regions (Kastner et al., 1990).

The growth of ER positive tumor is fueled by estrogen which makes ER the direct target of endocrine therapies. Studies suggest that ER is over expressed in about 70% of breast cancer cases (Anderson et al., 2002). Earlier studies done on ER, reports of a direct correlation between ER expression levels and response to endocrine therapy. Higher levels of ER are associated with a lower risk of recurrence (Nadji et al., 2005). Similar studies have found higher levels of ER being related to improved outcome of endocrine therapy such as tamoxifen (Byar et al., 1979). According to Nadji et al., estrogen receptors are weak prognostic markers of outcome and strong predictive markers of response to endocrine therapy (Nadji et al., 2005). ER status is strongly influenced by tumor grade and histology (Bardou et al., 2003).

Estrogen receptors can be assessed in tissues using immunohistochemistry which has become a method of choice and has been shown to be superior to that of biochemically based assays. ER status was until the late 1990s assessed using ligand binding assay. Studies have however demonstrated that immunohistochemistry is a good assay for predicting response to hormonal therapy (Elledge et al., 2000).

ER determination by immunohistochemistry as with all other biomarkers requires accurate and quantitative assessment of the results but several factors can affect the apparent ER and PR status of breast cancer. These include choice of ER/PR antibody, fixation and determination of threshold for reporting positive results.

Inadequate fixation may lead to false negative. At least 6-8 hrs. of formalin fixation time for breast biopsies is required to obtain reliable ER determination by IHC as demonstrated by Golsttein et al., 2003.

For best results antibodies that are robust and clinically validated should be the preferred choice. A cut off score for positivity that is reproducible, clinically validated and standadized should be employed by the laboratory. It is important to follow guidelines set by multidisciplinary panels such as the American Society of clinical Oncology and the College of American Pathologist in the reporting of results.

2.12.2 Progesterone receptors

Progesterone receptors belong to a member of the steroid-receptor superfamily of nuclear receptors. They are activated by progesterone hormone. This hormone stimulates and regulates various functions and plays an important role in maintaining pregnancy. As found in other steroid receptors, the progesterone receptors consist of N-terminal regulatory domain, a DNA binding domain, and a C-terminal ligand binding domain. Two forms of human progesterone receptors, known as PR-A and PR-B exist and are encoded by a single gene but are controlled by separate promoters (Conneely and Lydon 2000).The main structural difference between the two forms is that PR B has additional 165 amino acids which is present in the N-terminus.

The expression of the PR is strongly dependent on the presence of ER. According to Viale et al, almost all tumor expressing PR express ER and tumors expressing PR without ER account for less than one percent of all breast cancer cases in some large series (Viale et al., 2007). While some studies have shown that PR provides additional predictive value independent of ER values (Ogawa et al., 2004), other studies rather suggest that the predictive role of PR may not be as clinically important as ER (Viale et al., 2007).

2.12.3 HER 2

The human epidermal receptor protein-2 (c-erbB-2; HER2) oncogene protein is a transmembrane glycoprotein in the epidermal growth factor receptor family. It is expressed at low levels in a variety of normal epithelia, including breast duct epithelium and plays important role in growth and development. Some breast cancer cells have higher levels of Her2 on their surfaces which stimulate them to grow.

Overexpression and amplification of HER2 can be detected in about 15% of all primary breast cancers, and this group of patients benefit significantly from anti-HER2 therapies such as Trastuzumab (Herceptin). It is strongly associated with increased disease recurrence and a poor prognosis (Tan and Yu 2007). Over expression and gene amplification of Her2 can be measured using IHC and FISH respectively. The IHC results is scored on the scale of 0 to 3+.Tumors with 0 to 1+ are reported as negative while tumors with 2 to 3+ are considered positive but for tumors with the score of 2+, guidelines recommend for florescence in situ hybridization (FISH). FISH measures the amount of a gene called the HER2/neu gene, which is responsible for the overproduction of HER2 protein in each cell. This helps to identify people with a level of 2+ who will benefit from

treatment with Herceptin. The expression of HER2 is regulated by signaling through estrogen receptors. Normally, estradiol and tamoxifen acting through the estrogen receptor down-regulate the expression of HER2.

2.13 TRIPPLE NEGATIVE BREAST CANCER

Triple negative breast cancer (TNBC) is defined by a lack of expression of both estrogen (ER) and progesterone (PgR) receptors as well as human epidermal growth factor receptor 2 (Lehmann et al., 2011).Triple negative breast cancer account for nearly 15% of all breast cancers (Carey et al., 2010). Epidemiologically TNBC is more common in younger women than African American women (Newman and Martin 2007). The tumours of TNBC are generally larger in size and are of higher grade (Hafty et al., 2006). More than 90% of TNBC tumors fall within the basal-like subgroup (Livasy et al., 2006), meaning the cells look like the basal cells that line the breast ducts but does not necessarily mean that they come from these cells. Basal like breast cancer are less responsive to endocrine therapy, have aggressive metastatic behavior and worse prognosis than BRCA-1 related breast cancer (Foulkes et al., 2003, Sorlie et al., 2001).Using gene expression profile, basal like subgroup are noted for expression of proliferative gene such as Ki 67 and the epidermal growth factor receptor(EGRF). The receptor tyrosine kinase EGFR is frequently (30%-52%) expressed in TNBC (Rakha et al., 2007), Basal cytokeratin (particularly 5, 14 and 17) are also expressed in TNBC. Other studies however suggest that TNBC tumours are heterogeneous, exhibit distinct phenotypes and not limited to basal-like phenotype as evidenced by gene expression patterns. (Lehmann et al., 2011). Bidard et al., 2007 also reported that triple negative phenotype indeed includes a mixture of basal-like tumours together with some luminal-B tumours characterized by a decreased ER expression.

A recent study of gene expression profile on 587 TNBC cases identified 6 subtypes of TNBC with each subtype displaying distinct ontologies, having different sensitivities to a variety of agents (Lehmann et al.,2011).These subtypes include two basal-like, an immunomodulatory, a mesenchymal stem-like and a luminal androgen receptor. This could provide biomarkers that can be used for patient selection in the design of clinical trials for TNBC.

Patients with TNBC do not respond effectively to hormonal therapies and HER2 targeted agents because of lack of expression of these therapeutic agents in tumour cells. A number of clinical trials are currently underway which could hold promise for the treatment of TNBC. This includes the use of DNA damaging agents, Poly ADP-ribose polymerase (PARP) inhibitors in BRCA 1 mutation carriers. PARP is involved in DNA double strand contributing to its stability. Another therapeutic mechanism being proposed is the use of anti-epidermal growth factor receptor (EGFR) antibodies and EGFR tyrosine kinase inhibitor in triple negative cancer with EGFR gene amplification.

Chemotherapy is the single most effective treatment for triple-negative breast cancer. The reason is that chemotherapy works better than other treatments at killing cancer cells that divide quickly, which is very common in triple-negative disease. When triple-negative breast cancers are found early, response rates to chemotherapy are high. Treatment for TNBC is based on whether the cancer has travelled into the lymph nodes near the breast, the size of the primary tumor and the details of pathology tests such as the tumor grade which shows how quickly the cancer cells are dividing. The heterogeneous nature of the disease and the absence of well-defined molecular targets make treatment of TNBC a challenging one (Carey et al., 2010).Despite having higher response rate to chemotherapy,

there is a higher rate of disease recurrence and poorer prognosis than women with other subtypes of breast cancers (Dent et al., 2007). Majority of the patients with metastatic TNBC die within 5 years after initial treatment. A substantial number of women are cured if they remain recurrence-free for the first several years after a diagnosis (Dent et al., 2007).

Like other types of breast cancers chemotherapy may be given before (neo adjuvant therapy) or after surgery. Chemotherapy might not be given in very rare cases such as very low or small tumours or if the risk to chemotherapy outweighs the benefits. After surgery radiation may be given to kill any cancer cells where the cancer was found. This helps prevent the cancer from coming back to the same place.

2.14 RETENOIC ACID INDUCED PROTEIN 3 (RAI3)

Retinoic acid-induced protein 3 (RAI3) belongs to the family of G-protein coupled receptors (GPCRs), (Cheng and Lotan, 1998). They can also be referred to as RAIG1 (retinoic acid-induced gene 1) or GPRC5A, (G protein-coupled receptor family C, member 5, group A). GPCRs are the largest and most abundant receptor family in mammals consisting of more than a thousand members. For more than 200 GPCRs, the physiological ligands are known and about 150 “orphan” GPCRs with no known endogenous ligand or function (Lee et al., 2003, Nagahata et al., 2004).

All cells communicate with each other and receive information from the extracellular stimuli like hormones, sensory stimuli and neurotransmitters because of the possession of transmembrane signaling (Gilman 1987). All transmembrane signaling systems share two basic elements, a receptor which is able to recognize an extracellular stimulus as well as an

effector which is controlled by the receptor and which can generate an intracellular signal. In contrast, the G protein-mediated signaling system is relatively complex consisting of a receptor, a heterotrimeric G protein, and an effector. Each component i.e. the receptor, the G protein as well as the effector can be regulated independently by additional proteins, soluble mediators, or on the transcriptional level. The relatively complex organization of the G protein-mediated transmembrane signaling system provides the basis for a huge variety of transmembrane signaling pathways that are tailored to serve particular functions in distinct cell types. Two principal signal transduction pathways involved in GPCR Camp signal and the phosphatidylinositol signal pathways (Gilman 1987).

The heterotrimeric G protein consists of α, β- and γ-subunits that are involved in intracellular signaling activities. When a receptor is activated by its endogenous ligand, it causes a conformational change in the receptor which facilitates the coupling of the receptor to the G protein. Coupling of the activated receptor to the heterotrimer promotes the exchange of GDP for GTP on the G protein α-subunit. The GTP-bound α-subunit dissociates from the activated receptor as well as from the βγ-complex, and both the α-subunit and the βγ-complex are now free to modulate the activity of a variety of effectors like enzymes. GPCRs activate numerous signal transduction cascades and thus play pivotal role in the regulation of many physiological and pathological processes including cell growth and differentiation, a reason that make them valuable and interesting drug targets for the numerous human diseases that have been associated with dysfunctional GPCRs (Lis et al., 2005). All GPCRs have a membrane topology with seven α-helical transmembrane segments. The presence of this characteristic motif and the location of RAI3-GFP chimeric protein at the plasma membrane, support a conclusion that RAI3 is a member of the GPCR

superfamily (Cheng and Lotan 1998). Because the seven transmembrane domains are common to all members of GPCRs, most GPCRs bear sequence similarity with one another, primarily in the transmembrane regions (Probst et al., 1992).

RAI3 is thought to regulate cell proliferation but the exact molecular mechanism is not fully known (Hirano et al., 2006). Research has shown that higher levels of RAI3 expression promote tumour growth as seen in cDNA microarray study which saw RAI3 up regulated in breast cancer specimens. In a further study, RAI3 overexpression was seen using quantitative reverse transcription-PCR (RTPCR), in 19 of 25 breast cancer samples compared to matched normal mammary gland tissues and 6 of 11 breast cell lines. The results also suggested that RAI3 is likely to have an important role in cell proliferation, i.e. growth/survival of breast-cancer cells. (Nagahata et al., 2004).

RAI3 is expressed in several normal human tissues with the highest level found in fetal and adult lung (Cheng and Lotan, 1998). Other studies also find low expression of RAI3 in heart, placenta, kidney and other tissues with the normal mammary tissue also expressing it (Nagahata et al., 2004). Another study on RAI3, suggested that p53 interacts with the promoter of RAI3 and repressed its expression at the onset of apoptosis. The expression of RAI3 is elevated in most tumor cell lines expressing mutant p53, whereas RAI3 mRNA is relatively repressed in the tumor cell lines expressing wild-type p53(Wu et al., 2005).

2.15 IMMUNOHISTOCHEMICAL (IHC) ASSAY OF ESTROGENS, PROGESTERONE RECEPTORS, HER 2/NEU IN PARRAFIN EMBEDDED BREAST CANCER SAMPLES

Immunohistochemistry which is a method for demonstrating the presence and localization of proteins in tissue sections has become the medium of choice for most clinical and research studies because of the superior morphology provided by formalin fixed paraffin embedded tissue. Immunohistochemistry has been helpful in the diagnosis, prognosis and treatment of diseases such as cancer. With the help of the microscope, IHC makes it possible to visualize the distribution and localization of specific cellular components within cells. The main processes involved in immunohistochemistry include the following:

2.15.1 Tissue fixation and embedding

Fixation is done to prevent cellular components from degradation or other modifications that occur in normal unfixed tissue sample. The most common fixative is 10% neutral buffered formalin (NBF).Chemically fixation can cross link proteins which can mask the antigen of interest. Both over fixation and under-fixation can affect the antigen of interest and therefore the right fixation time must be achieved depending on the target antigen to be stained. For most common applications the time for fixation is between 18-24 hrs.

Tissue samples which are fixed are embedded in paraffin. This is done to maintain the natural shape and architecture of the sample when it is stored for a long time or sectioned.

2.15.2 Sectioning and mounting

Formalin fixed, paraffin embedded tissues are cut into thin slices of 4- 5 microns with a microtome. These sections are then mounted onto glass slide that are coated with adhesive. The adhesive is commonly added by treating the surface of the glass slide with APES (amino-propyl-tri-ethoxy-saline).After mounting on the positively charged slides, they are

dried in an oven (58-60^{0}C ± 5^{0}C) for at least 2 hours (but not longer than 24 hours) in preparation for deparaffinization.

2.15.3 Deparrafinization

Formalin fixed, paraffin embedded tissues commonly require a treatment to unmask the antibody epitopes. Incomplete removal of paraffin can cause poor staining of the section.

2.15.4 Antigen retrieval

During fixation methylene bridges can occur and cross link proteins in tissue samples. These bridges can mask antigenic sites and prevent antibody binding. The two methods of antigen retrieval are heat- induced epitope retrieval, or HIER and enzymatic degradation. Both methods break the methylene bridges and expose the antigenic sites in order to allow the antibodies to bind.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

The study was an observational case control one and ethical approval was obtained from the Committee on Human Research Publication and Ethics of Kwame Nkrumah University of Science and Technology and Komfo Anokye Teaching Hospital. The study was done to investigate comparatively the level of expression of retinoic induced protein 3 (RAI3) between triple negative breast cancer and non-triple negative breast cancer as well as in normal breast tissues. Women visiting the hospital with various breast conditions were recruited in the study. Tru-cut biopsy was performed by surgeons to obtain breast tissue samples after results of mammography, ultrasonography and other tests gave suspicion to breast cancer. The tissue samples were immediately fixed in 10% neutral buffered formalin and embedded in paraffin wax to form tissue blocks. Histologic categorizations of the tissue specimen were made by pathologists and immunohistochemical analysis of estrogen receptor, progesterone receptor and HER2 were done on those with histologically confirmed breast cancer. The Analysis of the expression of RAI3 was done on a selected number of breast cancer tissues as well as normal breast tissues using immunohistochemistry. Demographic and reproductive information were also obtained from the patients' medical records or by interaction with the patients where available.

3.2 Study Site

The study was done at the Breast clinic and the Histopathology Department of the Komfo Anokye Teaching Hospital, Kumasi in the Ashanti Region of Ghana. This hospital serves as a referral center for patients in the northern sector of the country and second largest teaching hospital in the country. On a regular working day, the breast clinic attends to an average of 30 clients with various breast conditions including men.

3.3 Study Population

Women of all aged between 26 and 78 years visiting the Breast Clinic for the first time following the development of a self-detected breast condition, and upon further suspicion by the medical doctor through clinical examination were recruited for this research from October 2012 to December 2013. A total of 100 subjects formed the study population.

3.4 Inclusion and exclusion criteria

Women between the ages of 26 and 78 presenting with a breast condition at the breast clinic for the first time and had given their consent to take part were included. Women who had already been diagnosed with breast cancer and those that had started treatment were excluded.

3.5 Sample Collection

Tru cut biopsy was performed by surgeons to obtain breast tissue samples after results of mammography; ultrasonography and other tests gave suspicion to breast cancer. The samples were immediately fixed with 10% neutral buffered formalin which was the

fixative of choice for 24 hours. Each sample was assigned a specific identification number starting B/001 to B/100.

3.5.1 Tissue Processing

All samples were subsequently processed using automated tissue processor (MICROM STP-120, Madrid, Spain). This was run on 18hrs schedule, subjecting the tissues to ethanol of different concentration in ascending order, ending with 3 changes of absolute ethanol for complete dehydration, 2 changes of xylene for optimum clearing and 2 changes of molten paraffin wax (60^0C) for adequate infiltration of the tissue specimen. Processed samples were then embedded into paraffin blocks using the modular tissue embedding centre (Leica EG1150; Leica Biosystems, Nussloch,GmbH) according to its standard operating protocol.

The tissue blocks were sectioned at 4 microns with a microtome. The sections were mounted onto a glass slide and stained with Haematoxylin and Eosin (H and E stain) and visualized by light microscopy.

3.5.2 Histologic Diagnosis

Pathologists made histologic categorization of the specimen as either breast cancer or non-breast cancer (either normal or benign tissue).The breast cancer cases were then assigned histologic type and grade. Immunohistochemistry assay of estrogen receptor, progesterone receptor and Her2/neu were performed on the breast cancer cases. RAI3 immunohistochemistry was done by randomly selecting cases each of ER/PR positive, Her 2 positive and triple negative as well as some of the non-breast cancer cases to serve as control.

3.6 IMMUNOHISTOCHEMICAL (IHC) ASSAY OF ESTROGENS, PROGESTERONE RECEPTORS, HER 2/NEU IN PARRAFIN EMBEDDED BREAST CANCER SAMPLES

The tissue blocks, already processed as previously described were cut at 4 microns using rotary microtome (Leica RM2235) and placed on super frosted coated slides (Fisher Scientific, Pittsburgh, PA, U.S.A). The slides were dried in hot air oven at 60 ^{0}C for 2 hrs. They were deparafinized in xylene and then rehydrated in graded alcohol by performing the following washes:

1. Xylene: 2× each for 3 minutes
2. 1: 1 concentration of xylene and 100% ethanol for 3 minutes
3. 100% ethanol: 2× for 3minutes
4. 95% ethanol for 3 minutes
5. 70% ethanol for 3 minutes
6. 50% ethanol for 3 minutes

The slides were rinsed under gently running tap water and kept in the tap water until ready to perform antigen retrieval. At no time from this point were the slides allowed to dry as drying would cause non-specific antibody binding and therefore high background staining.

3.6.1 Antigen Retrieval

Heat-induced epitope retrieval was carried out using temperature controlled pressure cooker referred to as Decloaker from Biocare, U.S.A. This was filled with distilled water to the graduated mark of 500mls. This was usually programmed at different temperatures for different boiling times. The process was carried out by placing the tissue sections into a 200ml plastic jar which is resistant to heat and filled with 10mM Sodium Citrate, pH 6.0. This was then immersed in the distilled water. The lid was tightly closed and switched on to set at a temperature of 95℃ for 40 minutes. Optimal retrieval was performed at this set temperature and time. The completion of retrieval by the pressure cooker was indicated by the beeping of alarm from the device. After retrieval, the lid was opened and the plastic jar with the citrate buffer and tissue sections were carried into the sink. The slides were allowed to run under tap water for 20 minutes to cool down the sections and wash the citrate buffer.

3.6.2 Immunohistochemistry Procedure

The immunohistochemical staining protocol was continued as follows:

1. The slides were placed in phosphate buffered saline (PBS) wash buffer for 5 minutes and blotted to remove excess buffer.
2. Hydrogen peroxide (3%) was applied on the slides and incubated for 5 minutes and washed with PBS.
3. Protein block/background snipper was applied and incubated for 10 minutes and slides washed 3 times with PBS.

4. The primary antibody (anti-ER or anti-PgR or anti HER2/neu) from Biocare Medical, U.S.A (Please see Appendix 1) was applied and incubated for 60 minutes and slides were washed with PBS.
5. MACH 1 probe was applied on slides ER and PgR (excluding slide for HER2/neu) and incubated for 15 minutes and washed 3 times with PBS.
6. MACH 1 HRP polymer was applied on slides (including ER, PgR and HER2/ nueu) for 40 minutes and washed 3 times with PBS.
7. Benzoid DAB chromogen was applied and incubated for 5 minutes and slides washed 3 times with PBS.
8. The slides were washed in distilled water and counterstained with hematoxylin for 1 minute and washed with distilled water.
9. The slides were dehydrated in ascending ethanol series, cleared in xylene before mounting under coverslip.

3.6.3 Assessment of ER, PR and Her2

For ER and PR immunohistochemistry, the percentage of cells with nuclear immunoreactivity was semi-quantitatively assessed. Tumors were categorized as ER or PR negative if no nuclear staining was observed. Tumors were classified as ER or PR positive if nuclear staining was observed in at least 10% of nuclei.

For Her2 protein expression tumours were also classified as IHC score 0 (negative) if no membrane is stained at all, an IHC score 1+ (negative) if a faint staining was observed in more than 10% of tumour cells, an IHC score 2+ if weak or moderate complete membrane staining was observed in more than 10% of tumour cells. This is interpreted as

inconclusive and further analysis of Flourescence in situ hybridization (FISH) would have to be done to determine Her2/*neu* amplification. However this could not be done due to the inability to carry out FISH. An IHC score 3+ (strongly positive) was given if strong complete membrane staining was observed in more than 10% of tumour cells.

3.6.4 RAI3 Immunohistochemistry

RAI3 immunohistochemistry was done on randomly selected formalin-fixed paraffin embedded tissue blocks each from ER/PR positives, triple negative, Her2 positives as well as non-cancer breast tissues. The tissue blocks were cut at 4 microns and mounted on a slide. After paraffin removal, rehydration, and antigen retrieval done as previously described, the immunohistochemistry protocol were carried out as follows:

1. Endogenous peroxidase was blocked by applying peroxidase blocking solution for 5 minutes and rinsed with wash buffer.
2. Background snipper was applied for 10 minutes and rinsed with wash buffer.
3. The anti-RA13 antibody Mab 24 2.3 supplied by Frahaufer Institute, Germany was applied in a dilution of 1: 50 and incubated at room temperature for 60 minutes and rinsed with wash buffer.
4. HRP Probe was applied for 15 minutes and rinsed with wash buffer.
5. HRP polymer detector was applied and incubated for 40 minutes and rinsed with wash buffer.
6. DAB chromogen was added and incubated for 5 minutes and rinsed with wash buffer.

7. The slides were washed in distilled water and counterstained with hematoxylin for 1 minute and washed with distilled water.
8. The slides were dehydrated in ascending ethanol series, cleared in xylene before mounting under coverslip.

3.6.5 Assessment of RAI3

The immunohistochemical expression of RAI3 staining was scored by an experienced pathologist by light microscopy according to the scoring suggested by Remmele and Stegner (Remmele and Stegner, 1987).The immunoreactive score (IRS) was achieved by multiplying intensity of cytoplasmic/membrane staining and the percentage of tumour cells stained as presented in the table below:

Percentage of positive Cells	X Intensity of staining	= IRS (0-12)
0 = no positive cells 1= <10% positive cells 2 = 10-50% positive cells 3 = 51-80% positive cells 4 = >80% positive cells	0 = no colour reaction 1 = mild reaction 2 = moderate reaction 3 = intense reaction	0-1 = negative 2-3 = mild 4-8 = moderate 9-12 = strongly positive

CHAPTER FOUR

RESULTS

4.1 Demographic and reproductive information

A total of 100 cases were investigated. The mean age was 48.15 with the youngest being 26yrs and the oldest 78yrs.There were more married women (53%) than single (26%), widowed (14%) or divorced (7%).Reproductive information regarding the 100 women who consented for their samples to be taken were obtained from the patient's medical records which was kept at the breast clinic. The mean menopausal age was 50.75. All of them had children ranging between 1 and 9 with their mean age of having the first full term pregnancy to be 23.38.

4.2. Pathological Diagnosis

Microscopic diagnosis of the 100 tissue samples by the pathologist confirmed 86 breast cancer cases while 14 were not breast cancer (10 normal and 4 benign tissues). Seven of the normal breast tissues were selected and used as control in RAI 3 analysis. The mean age of the breast cancer patients was 48.15 with the youngest age being 26 and the oldest age of 78.(Fig 4.1.a). Invasive ductal carcinoma (IDC) was the most predominant histological type diagnosed (85/86; 98.8%) and one case (0. 01%) of Paget's (Fig 4.1.b). High grade lesions/ tumours i.e. grade 2 and 3, accounted for 40.70% (35/86) and 46.5% (40/86) respectively whiles grade 1 was 12.79% (11/86). Approximately 29.1% (25/86) of grade 3 and 27.9% (24/86) of grade 2 IDC were ≤50 years whiles 6.98%(6/86) of grade 1 were≤ 50 years. (Table 4.1.a).

4.3 Expression of ER, PR and Her-2 in breast cancer cells

All 86 breast cancer cases were stained for the three receptors ER, PR and Her-2. ER expression i.e. ER positive (more than 10% nuclear staining) was observed in 44.18% (38/86) of the tumor cells whiles PR positive was 18.6% (16/86). All cases that showed expression for progesterone also showed expression for estrogen, that is no PR+/ER- was observed but ER+/PR- was 22.09% (19/86). Tumour cells that showed expression for both ER and PR was i.e ER+/PR+ was 10.4% (9/86).

A high percentage of 82.6% (71/86) of Her 2 negative and 81.40% (70/86) of PR negative were observed. Approximately 55.2% (48/86) of the samples analysed were ER negative. Exactly 43.02% (37/86) were negative for all the three receptors (i.e triple negative). Less than a quarter were either ER+/PR-/Her2-, ER+/PR+/Her2+, ER-/PR-/Her2+, ER+/PR-/Her2+.

Her2 over expression (immunohistochemical score of 3+) was observed in 11.62% (10/86). Her2 amplification was inconclusive in 5 cases since they had immumohistochemical score of 2+ but Flourescence in situ hybridization (FISH) could not be carried out.

Exactly 56.25% (27/48) of ER negative subjects were 50yrs or below. More than half (58.33%; 28/48) were of grade 3 IDC. The expression of ER/PR was decreased significantly in Her2+ in comparison with Her2- tumors. Table 4.2.b gives the summary. In triple negative breast cases, exactly 59.45% (22/37) were 50yrs or below with grade 3 IDC also occurring in 51.35% (19/37) and grade 2 IDC seen in 35.14% (13/37) and just a few were grade 1 IDC (10.8%; n=4) (Fig 4.2.b).

4.4 Expression of RAI3 in breast cancer and non- breast cancer cells

A subset of thirty six (36) tissue specimen including ten triple negative, twelve ER+/PR±/Her2-, seven ER-/PR-/Her+ and seven normal breast tissues (from the initial 100 cases) were randomly selected for RAI3 immunohistochemistry. The mean age was 49.03 with the minimum age of 29 years and maximum age 70 years.

Whiles there was abundant cytoplasmic/membrane expression of RAI3 in some tissues, others also showed weak expression. More triple negative tissues expressed RA13 and actually higher mean RA13 score than non-triple negative breast tissues. (Table 4.3 g and h).The mean RAI3 score in triple negative was 6.3 whiles mean score in all the non-triple negative combined was 4.2. (Table 4.3.g). In Her 2+ breast carcinomas there was abundant expression of RAI3 in 3 out of the seven tissue samples, the highest being 7 whiles the rest showed weak expression (Table 4.3.c). In ER+/PR+ tissues, RAI3 expression was abundant in 2 out of the 12 samples analysed with the highest IRS of 7 and those with weak expression had IRS ranging between 4 and 0 (Table 4.3.b). In triple negative breast carcinomas however, either RAI3 was abundantly expressed (Picture 1), or no staining was observed at all i.e. IRS of 0. Six out of the 10 samples analysed had RAI3 score ranging from 5 to 8 and the rest had RAI3 score of 0. One case of Paget disease which was triple negative also expressed RAI3 abundantly in the cytoplasm (IRS 5).Table 4.3.a gives the summary. All normal breast tissue had an IRS of either 2 or 0 (table 4.3.d).

Attempt was made to establish individual statistical relationship between RAI3 expression and histology grade, ER expression, PR expression, as well as Her 2 status. Pearson chi square is significant at $p < 0.05$. Correlation between histology grade and RAI3 was

statistically non-significant (p=0.155).Statistical relationship between RAI3 and ER expression was also non-significant (p=0.228), likewise between RAI3 and PR expression (p=0.732). Correlation between Her 2 expression was also statistically non-significant (p=0.758).Table 4.3.g.

4.5 Statistical Analysis

Microsoft excel and SPSS version 16.0 (SPSS Inc, Chicago, IL) were used for statistical evaluation. Patients' age at diagnosis, histology type and grade, ER, PR and Her-2 expression were computed by excel. Chi square test used to examine the association between RAI3 expression and histology grade, ER expression, PR expression as well as Her-2 expression were done using SPSS. Differences were considered statistically significant at $p < 0.05$.

Fig 4.1.a Frequency distribution of the ages of women with histologically confirmed breast cancer

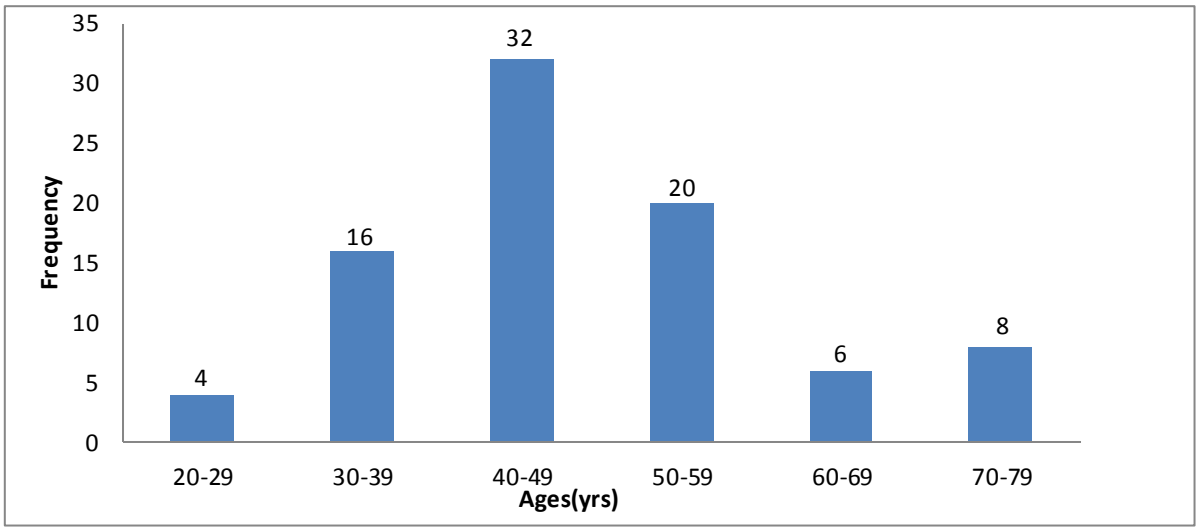


Fig 4.1.b Frequency distribution of the Histology type

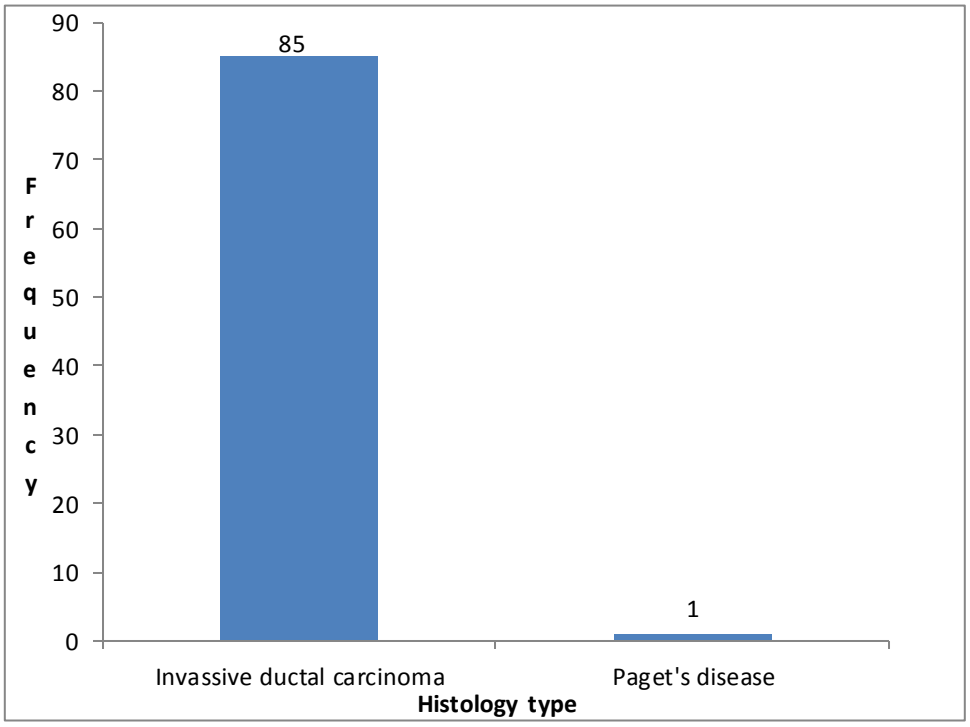


Fig 4.1.c Frequency distribution of the histology grade seen in breast cancer patients

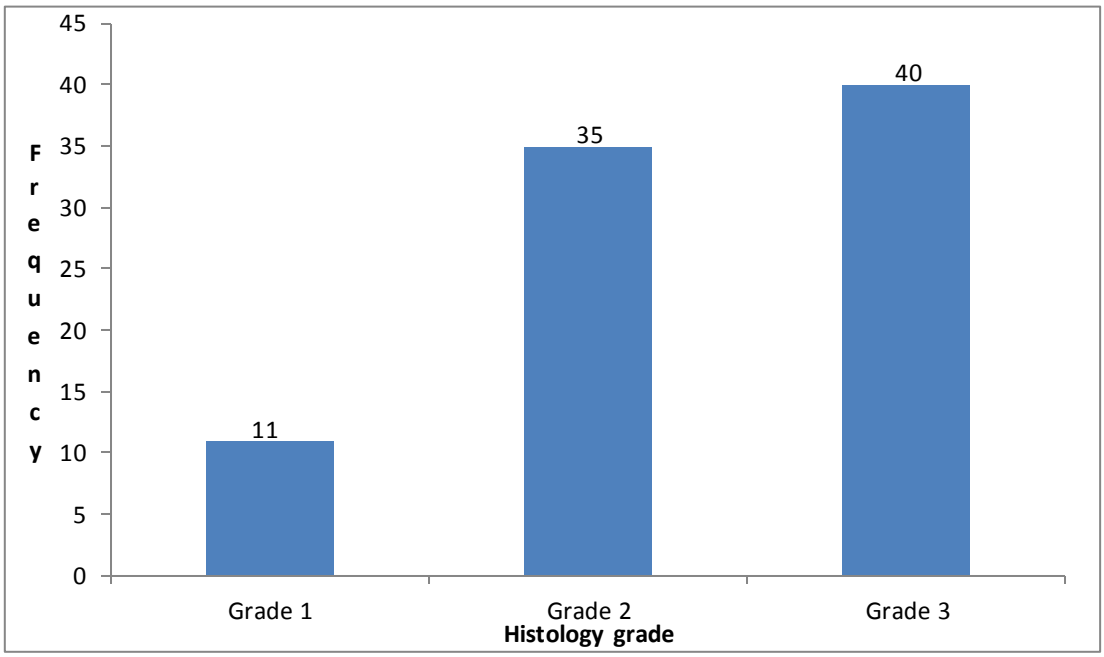


Fig 4.2.a Frequency distribution of ER/PR/Her 2 expression in histologically confirmed breast cancer cases

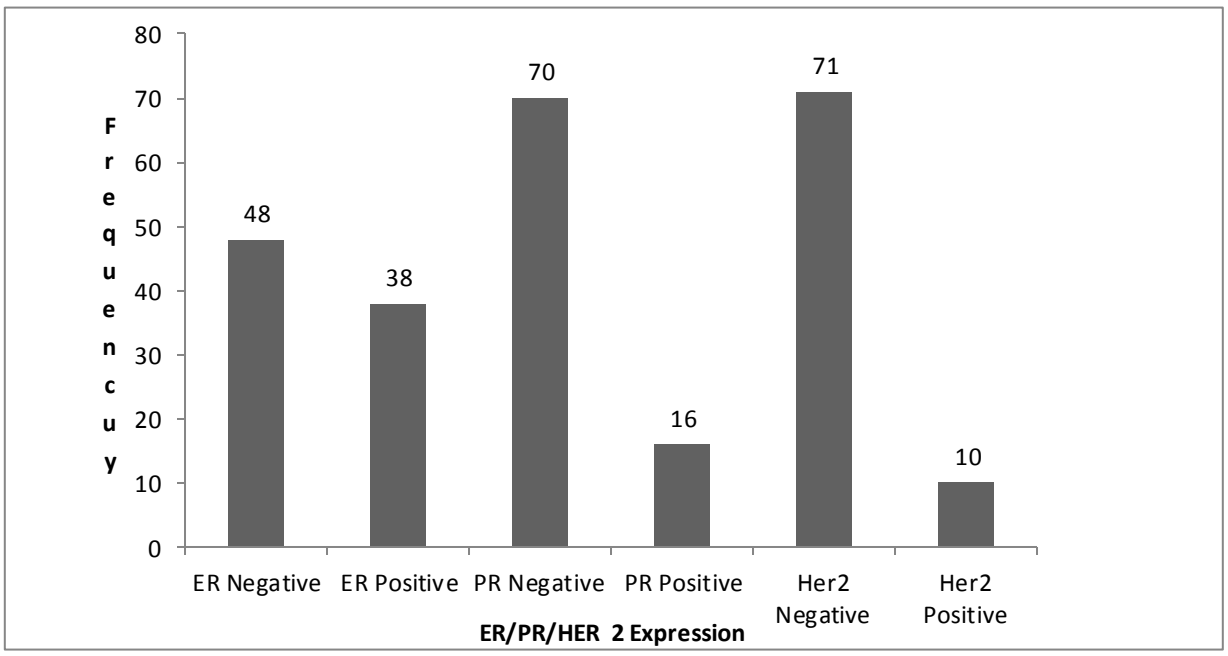


Fig 4.2.b Frequency distribution of invasive ductal carcinoma grade seen in triple negative cases

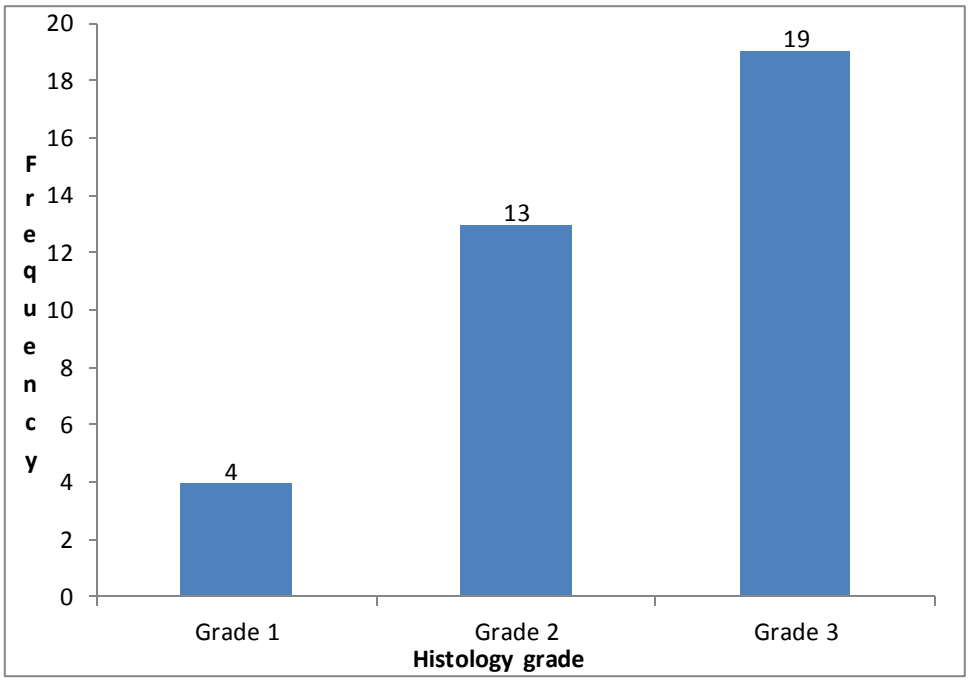


Table 4.1. Distribution of histology grade of invasive ductal carcinoma (IDC) seen in women with histologically confirmed breast cancer and also below 50 yrs

Histology Grade	Number (%)	50 yrs and below
1	11(12.79%)	6 (6.98 %)
2	35(40.70%)	24 (27.90%)
3	40(46.51%)	25 (29.1%)
Total number =	86 (100%)	56 (63.98%)

Table 4.2 Distribution of ER/PR/Her 2 expressions in breast cancer cases investigated

ER/PR Her2 Expression	Frequency(N=86)	Percentage (%)
ER+/PR+	9	10.47
ER+/PR-	19	22.09
ER-/PR-	48	55.81
ER-/PR-/Her2-(triple negative)	37	43.02
ER+/PR+/Her2-	15	17.44
ER+/PR-/Her2-	19	22.09
ER+/PR+/Her2+	1	1.16
ER-/PR-/Her2+	8	9.30
ER+/PR-/Her2+	1	1.16

Fig 4.3.a Frequency distribution of the RAI3 scores of randomly selected histologically confirmed breast cancer

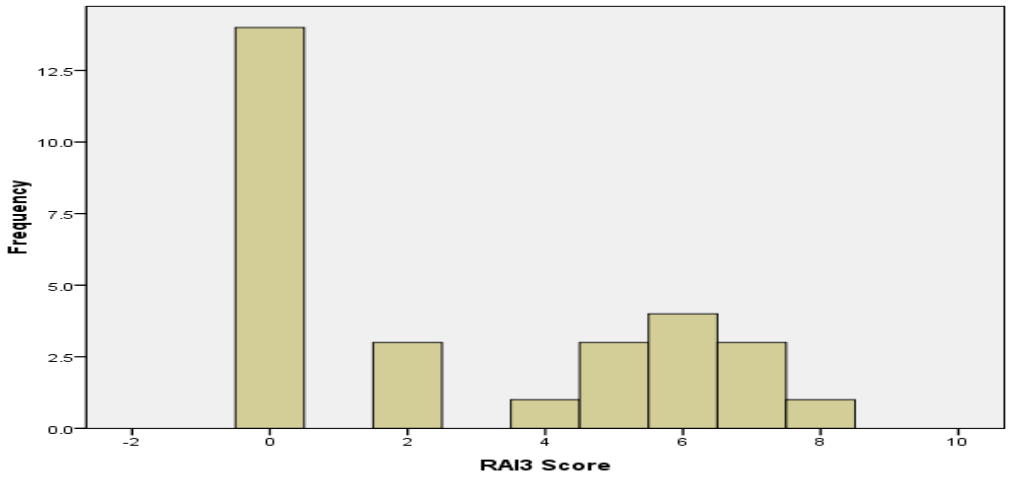


Table 4.3.a Age, diagnosis, and RAI3 scores of randomly selected triple negative breast cancer specimen.

Age	Diagnosis	ER Expression	PR Expression	Her2 Expression	RAI3 Score	RAI3 Expression
70	G 3 IDC	Negative	Negative	Negative	6	abundant
58	G 3 IDC	Negative	Negative	Negative	0	low
46	G 3 IDC	Negative	Negative	Negative	6	abundant
40	Pagets disease	Negative	Negative	Negative	5	abundant
51	G 2 IDC	Negative	Negative	Negative	6	abundant
29	G 3 IDC	Negative	Negative	Negative	7	abundant
65	G 3 IDC	Negative	Negative	Negative	8	abundant
37	G 3 IDC	Negative	Negative	Negative	0	low
45	G 3 IDC	Negative	Negative	Negative	0	low
63	G 2 IDC	Negative	Negative	Negative	0	low

For statistical analysis, RAI3 score of ≤ 4 was considered low while RAI3 score of ≥ 5 was considered abundant.

Table 4.3.b Age, diagnosis, and RAI3 scores of randomly selected histologically confirmed ER+/PR± breast cancer specimen

Age	Diagnosis	ER Expression	PR Expression	Her2 Expression	RAI3 Score	RAI Expression
39	G 2 IDC	Positive	Positive	Negative	0	low
66	G 2 IDC	Positive	Positive	Negative	0	low
50	G 1 IDC	Positive	Positive	Negative	2	low
40	G 3 IDC	Positive	Positive	Negative	0	low
35	G 2 IDC	Positive	Negative	Negative	0	low
53	G 1 IDC	Positive	Negative	Negative	0	low
37	G 2 IDC	Positive	Positive	Negative	7	abundant
70	G 1 IDC	Positive	Positive	Negative	0	low
56	G 3 IDC	Positive	Negative	Negative	0	low
47	G 2 IDC	Positive	Positive	Negative	5	abundant
55	G 2 IDC	Positive	Positive	Negative	4	low
57	G 3 IDC	Positive	Positive	Negative(0)	2	low

For statistical analysis, RAI3 score of ≤ 4 was considered low while RAI3 score of ≥ 5 was considered abundant

Table 4.3.c Age, diagnosis, and RAI3 scores of randomly selected histologically confirmed Her2+ breast cancer specimen.

Age	Diagnosis	ER Expression	PR Expression	H2N Expression	RAI3 Score	RAI3 Expression
51	G 3 IDC	Negative	Negative	Positive (3+)	7	abundant
54	G 3 IDC	Negative	Negative	Positive (3+)	2	low
45	G 3 IDC	Negative	Negative	Positive (3+)	5	abundant
38	G 2 IDC	negative	Negative	Positive (3+)	2	low
42	G 3 IDC	Negative	Negative	Positive (3+)	6	abundant
42	G 3 IDC	Negative	Negative	Positive (3+)	0	low
41	G 3 1DC	Negative	Negative	Positive (3+)	0	low

For statistical analysis, RAI3 score of ≤ 4 was considered low while RAI3 score of ≥ 5 was considered abundant

Table 4.3.d Age, diagnosis, and RAI3 scores of randomly selected histologically confirmed Her2+ breast cancer specimen.

Age	RA13 Score	RAI3 Expression
31	0	low
28	0	low
38	2	low
38	2	low
41	0	low
29	2	low
35	2	low

Table 4.3.e Frequency distribution of RAI3 scores of some ramdomly selected breast cancer specimen

Score	Frequency	Percent	Valid Percent	Cumulative Percent
0	14	48.3	48.3	48.3
2	3	10.3	10.3	58.6
4	1	3.4	3.4	62.1
5	3	10.3	10.3	72.4
6	4	13.8	13.8	86.2
7	3	10.3	10.3	96.6
8	1	3.4	3.4	100.0
Total	29	100.0	100.0	

Table 4.3.f Mean age and RAI3 score of the randomly selected breast cancer specimen

		Ages	RAI3 Score
Total Number		29	29
Mean		49.03	2.55
Std. Error of Mean		2.039	.508
Median		47.00	1.00
Std. Deviation		10.982	2.733
Minimum		29	0
Maximum		70	7
Percentiles	25	40.00	.00
	50	47.00	1.00
	75	56.50	5.50

Table 4.3.g. Comparison of mean RA13 score of triple and non- triple negative breast tissues

Cancer Tissue	Total Number expressing RA13	Mean RA13 score
Triple Negative	6	6.3
ER+/PR+	5	4
Her 2	5	4.4
Normal Breast	4	2
Non triple negative combined	10	4.2

Table 4.3.h. ER/PR/Her 2 and RA13 expressions in both triple and non-triple negative breast tissues

	Triple -ve specimen (n=10)		ER+/PR+ (n=12)		Her2+ (N=7)		Non-triple -ve specimen (n=19)	
	Positives	Negatives	Positives	Negatives	Positives	Negatives	Positive	Negative
ER	0(0.0)	10(100.0)	12(100.0)	0(0.0)	0(0.0)	7(100.0)	12(63.2)	7(36.8)
PR	0(0.0)	10(100.0)	9(75.0)	3(25.0)	0(0.0)	7(100.0)	9(47.4)	10(52.6
Her2+	0(0.0)	10(100.0)	0(0.0)	12(100.0)	7(100.0)	0(0.0)	7(36.8)	12(63.2)
RAI3	**6(60.0)**	4(40.0)	**5(41.7%)**	(7(58.3)	**5(71.4)**	2(38.6)	**10 (52.6)**	9(47.4)

Values are presented as frequency proportion

Table 4.3.g Clinicopathological and immunohistochemical parameters in relation to RAI-3 immunoreactivity

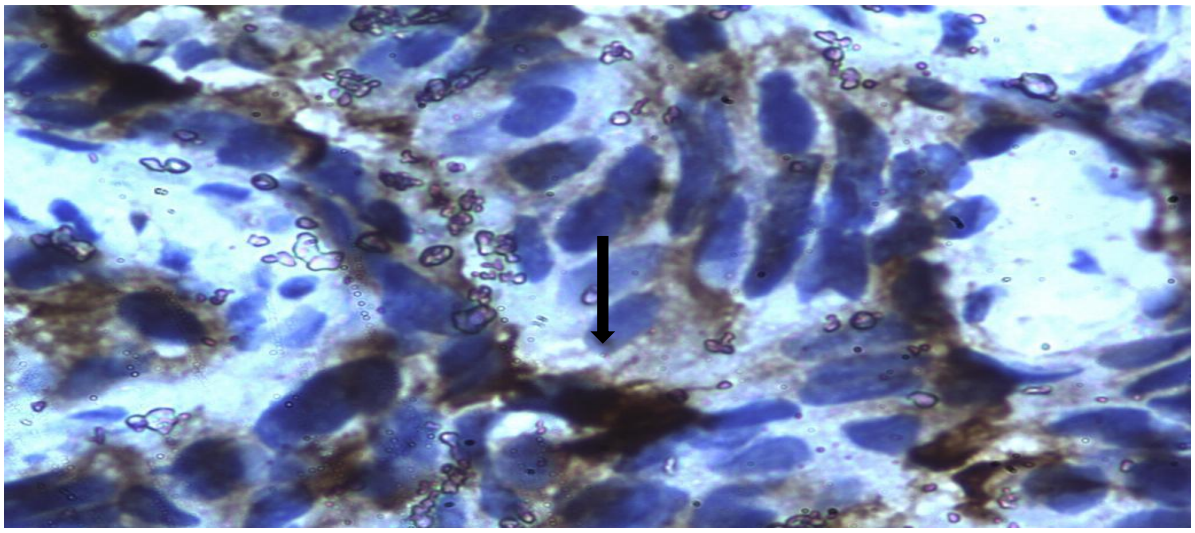
	RAI 3 Expression			Pearson test(two sided)
Clinicopathological data	low	Abundant	Total	*p value
Histology Grade				
Grade 1	5	0	5	0.155
Grade 2	4	3	7	
Grade 3	9	8	17	
Total	18	11	29	
ER expression				
Negative	9	8	17	0.228
Positive	10	2	12	
Total	19	10	29	
PR expression				
Negative	12	8	20	0.732
Positive	6	3	9	
Total	18	11	29	
Her 2				
Negative	14	8	22	0.758
Positive	4	3	7	
Total			29	

* Significant value ($p < 0.05$)

Some Pictures of immunohistochemistry results of formalin-fixed paraffin embedded tissues during the study

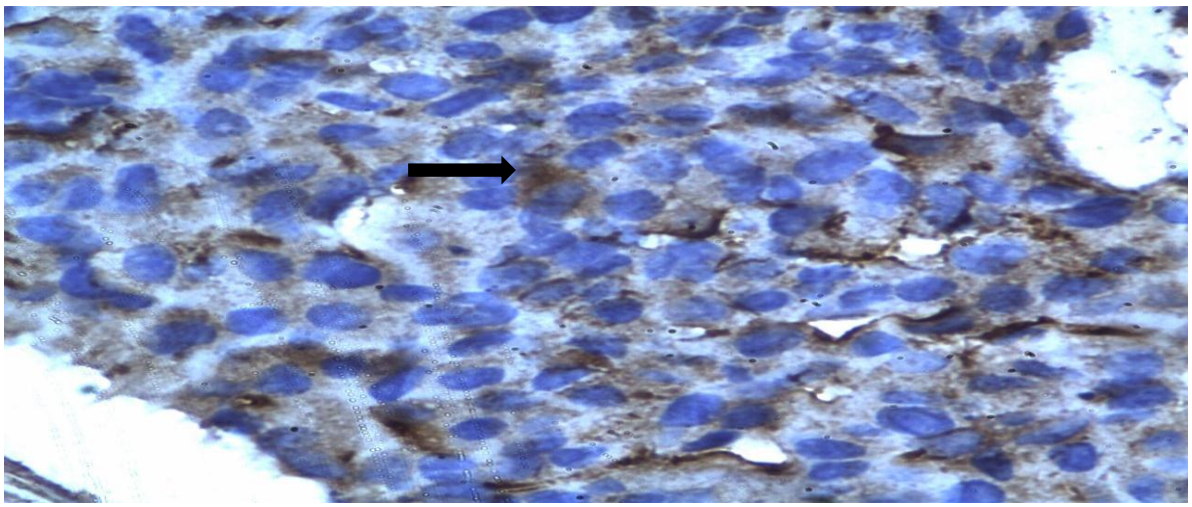
Picture 1

Arrow showing abundant cytoplasmic expression of RAI3 in grade 3 invasive ductal carcinoma from triple negative patient



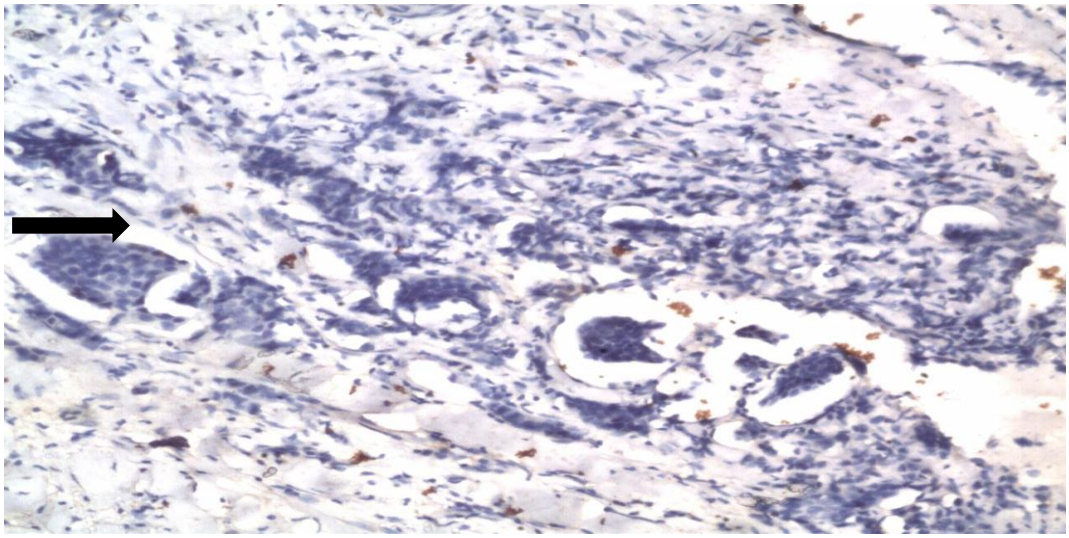
Picture 2

Grade 2 invasive ductal carcinoma from ER/PR positive patient showing abundant cytoplasmic expression of RAI3



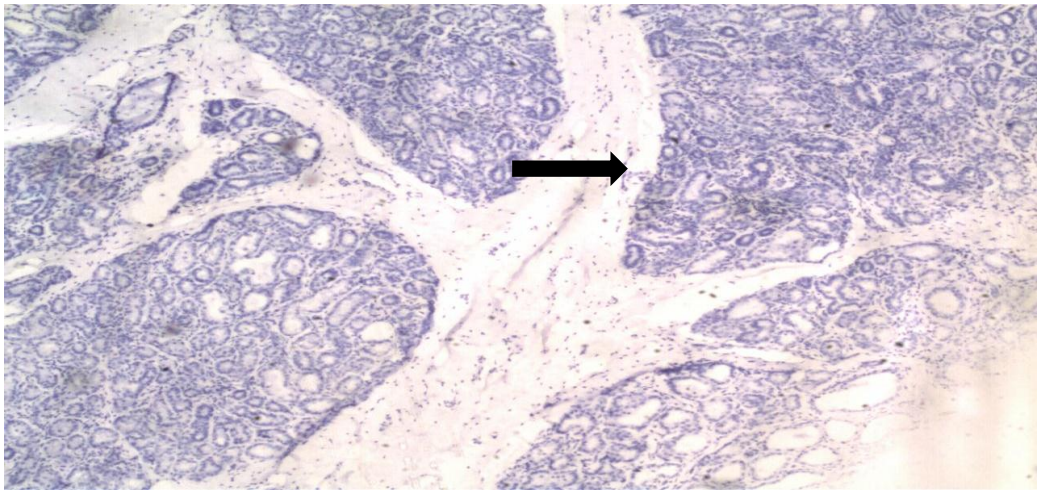
Picture 3

ER/PR positive specimen showing negative expression of RA13



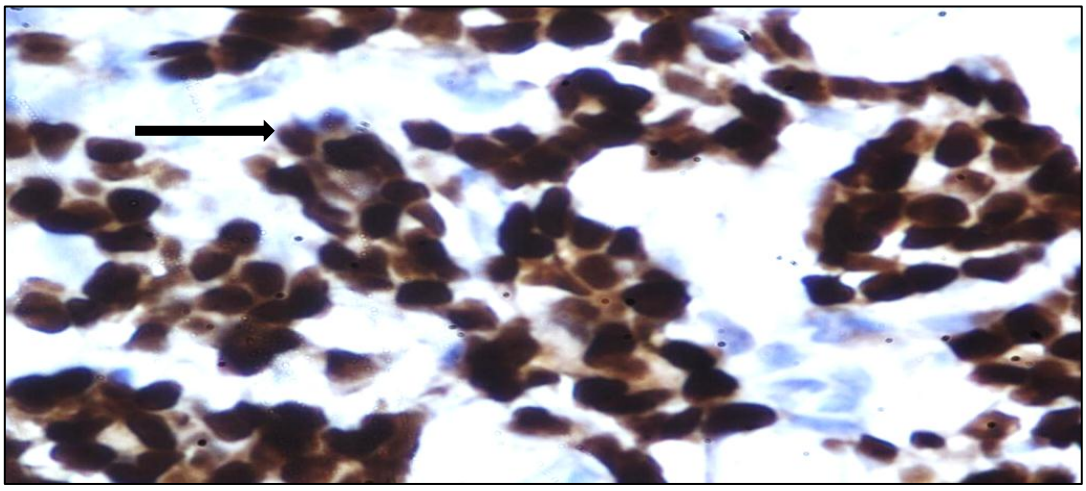
Picture 4

Normal breast specimen showing negative expression of RAI3



Picture 5

Breast carcinoma showing abundant nuclear expression of estrogen receptor



CHAPTER FIVE

DISSCUSSION

The mean age at diagnosis for the 86 cases was 48.15 with the peak age at diagnosis to be 40-49 yrs. Other studies in Ghana report of similar findings 0f peak age (Ohene Yeboah and Adjei 2012, Clegg-Lamptey et al., 2007). Out of the 86 sample breast cancer cases analysed for immunohistochemistry, ER negative and triple negative breast tumours accounted for 55.2% and 43.02% respectively whiles ER positive accounted for 44.418%. This result on triple negative is certainly lower than that of Stark et al., 2010 who reported a much higher incidence among Ghanaian women. The five year survival period after initial treatment tends to be low as most of the deaths occur within this period with the peak risk of recurrence occurring in the first 3 yrs. However when compared to endocrine positive tumours, the risk of recurrence decreases by 50% after the five years of initial treatment (Ovcaricek et al., 2011).The characteristic features observed in TNBC in this study i.e. high grade invasive ductal histology, young age at presentation are also similar to other studies done in Ghana and elsewhere (Ly et al., 2012, Stark et al., 2010., Ohene-Yeboah and Adjei 2012., Ovcaricek et al., 2011). The reason for the poor prognosis is not certain. It is not clear whether the poor prognosis is as a result of the inherent aggressive behavior or lack of targeted therapy. The heterogeneous nature of the disease and the treatment failure for some patients require that efforts should be made to identify selective molecular targets for development of strategies for earlier diagnosis, management or treatment of breast cancer.

The main work involved the study of a potential novel biomarker RAI3 among indigenous black women based on their hormonal receptor status. Retinoic acid induced protein 3 (RA13) belongs to a family of G-protein coupled receptor (Cheng and Lotan, 1998).The pharmaceutical industry regards members of the GPCR superfamily as interesting drug targets since they are known to be involved in many physiological processes including cell growth and differentiation (Li et al., 2005, Lagerstrom and Schioth, 2008).However the exact molecular mechanism of RAI3 in regulating cell proliferation remains poorly understood (Hirano et al., 2006).

This study confirms the expression of RA13 in normal breast tissue and breast cancer tumours but at different levels based on their hormone receptor status. For statistical analysis, an immunoreactive score (IRS) of 5 or more was considered abundant whiles IRS 4 or less was considered weak. If no staining was observed an IRS of 0 was given (negative). In normal breast tissues, RAI3 expression was less abundant compared to the breast carcinomas. The very low levels of expression found in the normal breast is consistent with previous study. (Nagahata et al 2004). The immunohistochemical staining of normal and breast cancer cells with anti RAI3 antibody in the analysis revealed the presence of RAI3 in the cell membrane. This raises the possibility that RAI3 can be used as a potential biomarker and therapeutic target. Joriben et al., 2009 were the first to analyse the expression of RAI3 protein by immunohistochemistry and also observed similar findings. This study to the best of my knowledge thus remains the second immunohistochemical study on RAI3. All other previous studies on RAI3 have been at the molecular level/mRNA levels. Cheng and Lotan 1998, found RAI3 being expressed in several normal tissues with the fetal and adult lung having the highest level whiles

Nagahata et al., 2004 found very low levels being expressed in normal mammary tissues and also sited RAI3 as being implicated in the growth of breast cancer cells. In a work done by Wu et al., which involved interaction of p53 tumour suppressor gene with RAI3, RAI3 expression was elevated on most cell lines expressing mutant p53 while RAI3 mRNA was repressed in tumor cell line expressing wild type p53, which further suggests that it is a p53 transcriptional target gene (Wu et al., 2005). Some studies have however considered RAI3 as a tumour suppressor gene, because of its association with retenoids, a vitamin A metabolite known to suppress carcinogenesis (Ye et al., 2009). In an analysis carried by Tao et al., using knock-out mouse model, mice lacking the RAI3 gene were more likely to develop lung cancer than the wild type mice at 1-2 yrs (Tao et al., 2007).

This study observed that patients with triple negative breast tumours exhibited a trend quite different from non-triple negative tumours. Either RAI3 was expressed abundantly (RAI3 score $\geq$5) in TNBC tumors or negative expression was made (RAI3 score of 0). Six out of the 10 TNBC samples analysed showed abundant expression of RAI3. However in ER+ and/or PR+ as well as Her+ samples, few abundant expressions were seen. The rest showed intermediate to very low expressions (Table 4.3.b and c). More triple negative patients expressed RA13 and the mean immunoreactive score (IRS) was higher in triple negatives (6.3) than the entire non-triple negative combined (4.2).Despite these observations, correlation between cytoplasmic/membranous RAI3 protein level and the clinicopathological characteristics were not significant. This could mean that the expression of RAI3 may not be associated with the presence or absence of these receptors (Table 4.3.g).This would have to be considered in relation to the fact that only about 34% of samples analysed initially for ER/PR and Her2 immunohistochemistry were randomly

selected for this analysis. In a study elsewhere to measure mRNA levels by quantitative reverse transcriptase (RT)-PCR in 2 normal mammary cells and 8 malignant breast epithelial cells, upregulation of mRNA (50%) was observed in the estrogen receptor negative cell lines compared to the normal mammary cell lines (Wu et al., 2005). In a study that led to the production of anti-RAI3 antibody, a large cohort of 157 breast cancer specimen and 44 normal breast tissue specimen were used and the investigators could not detect a significant correlation between RAI3 and the hormone receptor status (Joriben et al., 2009).

Breast cancer is certainly a heterogeneous group of tumours and disease presentation among indigenous black population is quite different from the Caucasians as previously stated. Carrying out this work in normal and cancerous breast tumors among indigenous black population and Ghana to be precise, who are more likely to be hormonal receptor and Her2-enriched negative is therefore of great interest.

The abundant expression of this membrane-bound protein in breast cancer cells at different levels suggest that RAI3 could be a potential biomarker and as a target for antibody-based cancer therapy most probably in triple negative breast cancer. The interaction of an antibody with its target inhibits pathways that are essential for cell proliferation or metastasis or activates pathways that culminate in cell death or apoptosis (Muss Hyman 2006). In Her 2 positive breast cancer for instance, the most predominant therapeutic monoclonal antibody is Herceptin (trastuzumab).This humanized monoclonal antibody works best by targeting the extracellular domain of Her2 (Muss Hyman 2006). Triple negative breast cancer (TNBC) patients have worst prognosis and do not benefit from endocrine therapy. It is no doubt that studies on breast cancer in identifying and

understanding the molecular pathways and mechanisms have led to the improved management of the disease. Specific and effective systemic therapies have led to improvement in the clinical outcomes of endocrine sensitive and Her2+ positive patients. It appears from this study that few Ghanaians could be beneficiaries to these therapies considering the high proportion of patients who are hormonal receptor negative, as chemotherapy remains the only therapeutic option for triple negative breast cancer patients. There is also scanty data on which to base TNBC treatment selection (Ovcaricek et al., 2011).

Though endocrine and Her 2 therapies have been successful, there are limitations to these interventions. More ER positive breast cancer patients that respond initially to endocrine therapy later acquire resistance and convert to ER negative tumours which is more aggressive (Goldhirsh et al., 2003). Most patients also develop resistance to Herceptin (Nahta and Esteva 2006). So the additional discovery of other biomarkers and therapeutic targets will be of great interest. RA13 may be such a candidate molecule and anti RA13 antibody could be used in the diagnosis and treatment of breast cancer. This may add on to some of the targeted therapies that are being investigated such as the use of tyrosine kinase inhibitors (Okabe et al.,2004, Gee et al., 2004) and Poly ADP ribose polymerase(PARP) inhibitors (Farmer et al., 2005).

More of such work on RAI3 representing a large cohort of both normal and malignant breast tumours should be carried out among indigenous black people and other groups of people to gain better understanding on the role of RAI3 in breast carcinogenesis.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

Retinoic acid induced protein 3(RAI3) is expressed in a number of most types of breast cancer. However it is expressed in a significant number of triple negative breast cancer which could indicate that RAI3 could be a new target for the treatment of this group. The mean immunoreactive score (IRS) of RA13 in triple negative tissues was 6.3 as compared non-triple negatives which was 4.2. It is a membrane/cytoplasmic bound protein. To the best of my knowledge this is the first analysis of RAI3 being carried out at the protein level among indigenous black people, albeit involving small sample size.

6.2 RECOMMENDATIONS AND SUGGESTIONS

There is clearly the need to work on a large cohort of normal breast tissues as well different malignant tissues among indigenous black people as well as other race of people to better understand the role this protein plays in breast cancer growth and its potential use as a biomarker and a novel therapeutic option in the management and treatment of breast cancer. Further studies could also be done to study expression of RAI3 in relation to the various risk factors associated with breast cancer.

REFERENCES

Achampong E.Q (1990). Breast Cancer in Ghana, National Cancer Foundation.

Adachi K., Fijioka T., Fujitomi Y and Uco H (2007). Psychosocial factors affecting the therapeutic decision-making and postoperative mood states in Japanese breast cancer patients who underwent various types of surgery: Body image and sexuality. *J. clin. Onco*; **37**:412-418.

Agyei-Frempong M.T., Ghartey F.N., Asare Poku S and Wiafe-Addai B (2008). A cross sectional view of estrogen receptors in 32 human breast cancer tissues. *J. Med Sci*; **8**(4): 420-424.

Anderson W.F., Chatterjee N., Ershler B.W and Brawley W.O (2002*)*. Estrogen receptor breast cancer phenotypes in the surveillance, epidemiology and results database. *Breast Cancer Res Treat*; **76:** 27-36.

Anyanwu S.N (*2000)*. Breast cancer in eastern Nigeria: a ten year review. *West Afr J Med* ; **19**: 120–125.

Arpino G., Bardou V.J., Clark G.M and Elledge R.M (2004). Infiltrating lobular carcinoma of the breast: tumor characteristics and clinical outcome. *Breast Cancer Res*; **6**: 149–156.

Arpino G., Clark G.M., Mohsin S., Bardou V.J and Elledge R.M (*2002)*. Adenoid cystic carcinoma of the breast: Molecular markers, treatment, and clinical outcome. *Cancer*; **94**:2119–2127.

Badoe E.A and Baako B.N (2008): The breast. In Badoe E A, Archampong E Q, J.T da Rocha-Afodu (editors).Principles and practice of surgery including pathology in the tropics(Third Edition).Accra: Department of Surgery. University of Ghana Medical School. pp 488-515.

Bane Anita (2013). Ductal carcinoma *in-situ:* what the pathologists needs to know and why. *International Journal of Breast Cancer*; **2013**: 1155-1159.

Bardou V.J., Arpino G., Elledge R.M., Osborne C.K and Clark G.M (*2003).*Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer database. *J.Clin Oncol*; **21:** 1973-1979.

Barton M.B., Harris R and Fletcher S.W (1999). The rational clinical examination. Does this patient have breast cancer? The screening clinical breast examination: should it be done? How? *JAMA*; **282**: 1270–80.

Berg A Wendie (*2010).*Benefits of Screening Mammography. *JAMA*; **303**(2): 168-169.

Bidard F.C., Conforti R., Boulet T., Michiels S., Delaloge S and Andre F (2007). Does triple negative phenotype accurately identify basal-like tumour? An immunohistochemical analysis based on 143 'triple-negative' breast cancers. *Annals of Oncology*; **18**(7):1285-1286.

Byar D.P., Sear M.E and McGuile W.L *(1979*). Relationship between estrogen receptor values and clinical data in predicting the response to endocrine therapy for patients with advanced breast cancer. *European Journal of Cancer*; **15:** 299-31.

Cadoo K.A., McArdle O., O'Shea A., Power C.P and Hennesey B.T (2012). Management of unusual histological types of breast cancer. *Oncologist*; **17**(9): 1135–1145.

Carey L., Winer E., Viale G., Cameron D and Gianni L (2010).Triple-negative breast cancer: disease entity or title of convenience? *Nat Rev Clin Oncol*; **7**: 683-692.

Carey L.A., Dees E.C., Sawyer L., Gatti L., Moore D .T., Collichio F., Ollila D .W., Sartor C.I., Graham M.L and Perou C.M (2007). The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes. *Clin Cancer Res*; **13**(8): 2329-2334.

Carney P.A., Miglioretti D.L., Yankaskas B.C., Kerlkowske K., Rosenberg R., Rutter C.M., Geller B.M., Abraham L.A., Taplan S.H., Dignan M., Cutter and Ballard-Barbash R(2003). Individual and combined effects of age, breast density, and hormone replacement therapy use on the accuracy of screening mammography. *Ann Intern Med*; **138**:168–175.

Cha S., Imielinski M. B., Rejtar T., Richardson E.A., Thakur D., Sgri D.C and Karger B.L (2010). In situ proteomic analysis on human breast cancer. *Mol Cell Proteomics*; **9**(11):2429-2544.

Cheng Y and Lotan R (1998). Molecular cloning and characterization of a novel retinoic acid-inducible gene that encodes a putative G protein-coupled receptor. *Journal of Biological Chemistry*; **273**: 35008–35015.

Chlebowski T.R., Chen Z., Anderson L.G., Rohan T., Aragaki A., Lane D., Dolan D., Paskett D., McTiernan., Hubbell F., Adams-Campbell L and Prentice R (2005).Ethnicity

and Breast Cancer: Factors Influencing Differences in Incidence and Outcome. *J. Natl Cancer Inst*; **97** (6):439-448.

Christainsen C.L., Wang F., Barton M.B., Kreuter W., Elmore G.J., Gelfand A. E and Fletcher W.S *(2000*). Predicting the cumulative risk of false positive mammograms. *J Natl Cancer Inst*; **92**: 1651-1666.

Cleater S., Heller W and Coombes R.C (2007). Triple negative breast cancer: therapeutic options. *Lancet Oncol*; **8**: 235-44.

Clegg-Lamptey J., Dakubo J and Attobra Y.N (*2009*). Why do breast cancer patients report late or abscond during treatment in Ghana? A pilot study Ghana. *Med J*; **43**(3): 127–131.

Clegg-Lamptey J.N.A. and Hodasi W. *(*2007*)*."A study of breast cancer in Korle Bu teaching hospital: assessing the impact of health education". *Ghana Medical Journal;* **41** (2): 72–77.

Collaborative Group on Hormonal Factors in Breast Cancer (1996).Breast cancer and hormonal contraceptives: collaborative reanalysis of individual data on 52,297 women with breast cancer and 100,239 women without breast cancer from 54 epidemiological studies Lancet; **347**(9017):1713-1727.

Collaborative Group on Hormonal Factors in Breast Cancer **(**1997). Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. *Lancet*; **350** (9084):1047-59.

Collins L.C., Tamini R.M., Baer H.J, Connolly J. L., Colditz G. A and Schnitt S. J (2005). "Outcome of patients with ductal carcinoma *in situ* untreated after diagnosis biopsy: results from the nurses' health study". *Cancer*; **103**(9): 1778-1784.

Conneely O.M and Lydon J.P (2000). Progesterone receptors in reproduction: functional impact of the A and B isoforms. *Steroids*; **65**:571-7.

Curado M.P., Edwards B., Shin H.R., Storm J., Ferlay M., Heanue M and Boyle P (2007). Cancer incidence in five continents. Vol. IX. Lyon, France: International Agency for Research on Cancer.

Dobrescu A., Chang M.,Kirtani V.,Turi G., Hennawy R., and Hindenburg A.,(2011).Study of estrogen receptor and progesterone receptor expression in breast ductal carcinoma in situ by immunohistochemical staining in ER/PgR-negative invasive breast cancer. ISRN Oncol; 673790.

Dent R., Trudeau M., Prictchard K.I., Hanna W.M., Kahn H.K., Sawka C.A., Lickley L.A., Rawlinson E., Sun P and Narod S.A (2007). Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin Cancer Res*; **13**: 4429–34.

Doug K and Steiner E (2007). Screening for Breast Cancer: Current Recommendations and Future Directions, *Am Fam Physician* ; **75** (11):1660-1666.

Elledge R. M., Green S., Pugh R., Allred D.C., Hill J., Ravdin P., Silvana M., and Kent O (2000). Estrogen receptor(ER) and progesterone receptor(PR), by ligand binding assay compared with ER, PgR, a p52 by immunohistochemistry in predicting response to

tamoxifen in metastatic breast cancer: a Southwest Oncology Group Study. *Int J Cancer*; **89**: 111-117.

Elmore G.J., Armstrong K., Lehman D.C and Fletcher W.S (2005). Screening for breast cancer. *JAMA*; **293**(10): 1245–1256.

Elston C.W and Ellis I.O (1991). "Pathologic prognostic factors in breast cancer. The value of histological grasdes in breast cancer.Experience from a large study with long-term follow up". *Histopathology*; **19**(5): 403-10.

Ernster V.L and Barclay J (1997). Increases in ductal carcinoma in situ (DCIS) of the breast in relation to mammography: a dilemma: *J Natl Cancer Inst Monogr*; **22**: 151-6.

Farmer H., McCabe N., Lord C.J., Tutt A.N., Johnson D.A., Richardson T.B., Santarosa M., Dillon K.J., Hickson I., Knights C., Martin N.M., Jackson S.P., Smith G.C and Ashworth A (2005).Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*; **434**(7035): 917-921.

Feig S .A and Hendrick R.E (1997). Radiation risk from screening mammography of women aged 40-49 years. *J Natl Cancer Inst Monog* ; **22**:119-24.

Fisher E.R., Kenny J.P., Sass R., Dimitrov N.V., Siderits R.H and Fisher B (1990).Medullary cancer of the breast revisited. *Breast Cancer Res Treat*; **16**:215-229.

Foord S.M., Bonner T.I., Neubig R., Rosser E.M., Pin J.P., Davenport A.P., Spedding M and Harmar A.G (2005). Protein-coupled receptor list. *Pharmacol International Union of Pharmacology*; **57**:279-88.

Foulkes W.D., Stefansson I.M., Chappuis P.O., Begin L R., Goffin J R., Wrong N., Trudel M and Akslen L.A *(*2003). Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. *J Natl Cancer Ins*; **95**: 1482–5.

Fregene A. and Newman L. A (*2005)*. Breast cancer in sub-Saharan Africa: How does it relate to breast cancer in African-American women? *Cancer*; **103**: 1540–1550.

Genestie C., Zafrani B., Asserlain B., Fourquest A., Rozan S., Validire P., Vincent-Salomon A and Sastre-Garau X (1998). "Comparison of the prognostic value of Scarff-BloomRichardson and Nottingham histological grades in a series of 825 cases of breast cancer.Major importance of the mitotic count as a compared to grading systems". Anticancer Research; 18 (1B): 571-6.

Gilman A.G *(1987)*. G proteins transducers or receptor-generated signal. *Annual Ref of Biochem*; **56:** 615 649.

Goldhirsch A., Wood W.C., Gerber R.D., Coates A.S., Thurlimann B and Senn H.J (2003). Meeting and highlights: Updated international expert consensus on the primary therapy of early breast cancer. *Journal of Clinical Oncology*; **21**: 3357-3365.

Goldstein N.S., Ferkowioz M., Odish E., Mani A and Hastah F (*2003)*. Minimum formalin fixation time for consistent estrogen receptor immunohistochemical staining on invasive breast carcinoma. *Am J Clin Pathol*; **120**: 86-92.

Green B.B and Taplin S.H (2003). Breast cancer screening controversies. *Journal of the American Board of Family Medicine*; **16**(3): 233-241.

Gukas I.D., Jennings B.A., Mandong B.M., Igun G.O., Manasseh A.N., Ugwu B.T., Leinster S.J (2005). Clinicopathololological features and molecular markers of breast cancer in Jos Nigeria. *West Afr J Med* ; **24**:209–213.

Haffty B.G., Yang Q., Reiss M., Kearney T., Higgins S.A., Weidhaas J., Harris L., Hait W and Toppmeyer D (2006). Locoregional relapse and distant metastasis in conservatively managed triple negative early-stage breast cancer. *J Clin Oncol*; **24**(36):5652–5657.

Hirano M., Zang L., Oka T., Ito Y., Shimada Y., Nihsimura Y and Tanaka T (2006). Novel reciprocal regulation of cAMP signaling and apoptosis by orphan G-protein-coupled receptor GPRC5A gene expression. Biochemical and biophysical research communications; **351**(1): 185-191.

Japaze H., Emina J., Diaz C., Schwam R.J., Gercovich N., Demonty G., Morgenfeld E., Rivarola E., Gil D.E and Gercovich F.G (2005). 'Pure' invasive apocrine carcinoma of the breast: A new clinicopathological entity? *Breast* ; **14**:3–10.

Jatoi I., Anderson W.F., Rao S.R and Devessa S.S (*2005).* Breast cancer trends among black and white women in the United States. *Journal of clinical Oncology*; **31** (25): 7836-7841.

Joriben H., Bektas N., Dahl E., Hartmann A., Haaf A., Fiore S., Kiefer H., Thess A., Barth S and Klockenbring T(2009). Production and characterisation of monoclonal antibodies against RAI3 and its expression in human breast cancer. BMC Cancer; **9**: 200.

Kastner P., Krust A., Turcotte B., Stroop U., Tora L., Gronemeyer H and Chambon P (1990). Two distinct estrogen-regulated promoters generate transcripts encoding the two

functionally different human progesterone receptor forms A and B" . *EMBO J;* **9** (5): 1603–14.

Kerlikowske K., Grady D., Rubin S.M., Sandrock C and Ernster V.L (1995). Efficacy of screening mammography: A meta-analysis. *JAMA*; **273**: 149-154.

Kerlikowske K., Walker R., Miglioretti D.L., Desai A., Ballard-Barbash R and Buist D.S (2008).“Obesity, mammography use and accuracy, and advanced breast cancer risk]. *Journal of the National Cancer Institute*; **100** (23): 1724–1733.

Lagerstrom M.C and Schioth H.B (2008). Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat Rev Drug Discov*; **7**(4): 339-357.

Laya M.B., Larson E.B., Taplin S.H and White E (*1996).* Effect of estrogen replacement therapy on the specificity and sensitivity of screening mammography. *J Natl Cancer*; **88**: 643-649.

Lee D.K., George S.R and O'Dowd B.F (*2003).* Continued discovery of ligands for G protein-coupled receptors. *Life Sci; **74**: 293–297.*

Lehmann B.D., Bauer J.A., Chen X., Sanders M.E., Chakravarthy A.B., Shyr Y and Pietenpol J. A (2011). Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapy. *The Journal of Clinical Investigation*; **121**(7): 2750-2767.

Li S., Huang S and Peng S.B (2005). Overexpression of G protein-coupled receptors in cancer cells: involvement in tumour progression. *Int J Oncol*; **27** (5):1329-1339.

Lisanti M.P., Tsirigos A., Pavlides S., Reeves K.J., Peiris-Pages M., Chadwick A.L., Sanchez-Alvarez R., Lamb R., Howell A., Martinez-Outschoorn U.E and Sotgia F (*2014).* JNK1 stress signaling is hyperactivated in high breast density and the tumor stroma: Connecting fibrosis, inflammation and stemness for cancer prevention. *Cell Cycle;* **13** (4): 580-599.

Livasy C.A., Karaca G., Nanda R., Tretiakova M.S., Olopade O.I., Moore D.T and Perou C.M (2006). Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. *Mod Pathol*; **19**:264–71.

Louwman M.W., Vriezen M., van Beek M.W., Nolthenius-Puylaert C., van der Sangen M.J., Roumen R.M., Lambertus A.L., Kiemeney M and Coebergh W(2007). Uncommon breast tumors in perspective: Incidence, treatment and survival in the Netherlands. *Int J Cancer*; **121**: 127–135.

Lowlor D.A., Ebrahim S and Davey Smith G (2004).Smoking before the birth of a first child is not associated with increased risk of breast cancer: findings from the British women cohort study and meta-analysis.*Br J. Cancer*; **9**(3): 512-8.

Ly M., Antoine M., Dembele A.K., Levy P., Rodenas A.,Toure B.A., Badiaga Y., Dembele B K., Bagayogo D.C., Diallo L., Kone A.A., Callard P., Bernaudin J.F and Diallo D(2012). High Incidence of Triple-Negative Tumors in Sub-Saharan Africa: A Prospective Study of Breast Cancer Characteristics and Risk Factors in Malian Women Seen in a Bamako University Hospital. *Oncology*; **83**:257–263.

Ma H., Lu Y., Malone K.E., Marchbanks P.A., Deapan D.M., Spirtas R, Burkman R.T., Strom B L., McDonald J.A., Folger S.G., Simon M.S., Sullivan-Harlley J., Press M.F and Bernstein L (2013). Mortality risk of black women and white women with invasive breast cancer by hormone receptors, HER2, and P 53 status. *BMC Cancer*; **13**: 25.

Matsuo K., Fukutomi T., Hasegawa T., Akashi-Tanaka S., Nanasawa T and Tsuda H *(2002)*. Histological and immunohistochemical analysis of apocrine breast carcinoma. *Breast Cancer*; **9**:43–49.

Mayo R and Hunter A (2003). Fatalism towards breast cancer among the women in Ghana. *Health Care for Women International*; **24** (7): 608-616.

Moss S.M., Cuckle H., Evans A., Johns L., Waller M and Bobrow L (2006). Trial Management Group. Effect of mammographic screening from age 40 years on breast cancer mortality at 10 years' follow-up: a randomised controlled trial. *Lancet*; **368** (9552):2053-60.

Muguti G.I (1993). Experience with breast cancer in Zimbabwe. *J R Coll Surg Edinb*; **38**: 75–78.

Muss B Hyman (2006).Targeted therapy for matastic breast cancer. *N Engl J Med;* **335**:2783-2785.

Nabholtz J.M., Buzdar A., Pollak M., Harwin W., Burton G., Mangalik A., Steinberg M., Webster A and von Euler M (2000). Anastrozole is superior to tamoxifon as first-line therapy for advance breast cancer in postmenopausal women: result of a North American

multicenter randomized trial. Arimidex Study Group. *Journal of Clinical Oncology*; **18**: 3758-3767.

Nadji M., Gomez F.C., Ganjei-Azar P and Morales R.A (*2005).* Immunohistochemistry of estrogen and progesterone receptors reconsidered experience with 5993 breast cancer. *A J Clin Pathol*; **123:** 21-23.

Nagahata T., Onda M., Nagai H., Twumdari K., Fujimoto T., Hirano A., Sato T., Nishakawa K and Ayiyama F(2004). Expression profiling to predict postoperative prognosis for estrogen receptor-negative breast cancers by analysis of 25,344 genes on cDNA microarray. Cancer science; **9**(3): 218-225.

Nahta R and Esteva F.J (2006). HER2 therapy: molecular mechanisms of trastuzumab resistance. *Breast Cancer Res*; **8**(6)**:**215.

Newman L.A and Martin I.K (2007). Disparities in breast cancer. *Curr Probl Cancer*; **31**:134-156.

Ogawa Y., Moriya T., Kato Y., Oguma M., Ikeda K., Takashima T., Nakata B., Ishikawa T and Hirakawa K (2004). Immunohistochemical assessment of estrogen receptors and progesterone receptor status in breast cancer .Analysis for cut-off point as the predictor for endocrine therapy. *Breast Cancer*; **11:** 267-275.

Ohene-Yeboah M and Adjei E (2012). Breast cancer in Kumasi. *Ghana Medical Journal*; **46**(1):8-13.

Okabe H., Furukawa Y., Kato T., Hasegawa S., Yamoaka Y and Nakamura Y(2004). Isolation of development and differentiation enhancing factor-like 1 as a drug target for hepatocellular carcinomas. *International Journal of Oncology*; **24**: 43-48.

Ovcaricek T., Frkovic S., Matos E., Mozina B and Borstnar S (2011). Triple negative breast cancer-Prognostic factors and survival. *Radiol Oncol*; **45** (1): 46-52.

Page D.L., Dupont W.D., Rogers L.W and Landenberger M (1982). "Intraductal carcinoma of the breast: follow up after biopsy only". *Cancer*; **49** (4): 751-758.

Parkin D.M., Bray F and Ferlay J.P (*2005).* Global Cancer Statistics, 22. *Cancer J Clin*; **55**: 74-108

Parkin D.M., Vizcain A.P., Skinner M.E.G and Ndhlovu A (1994). Cancer patterns and risk factors in the African population of Southwestern Zimbabwe 1963–1977. *Cancer Epidemiology, Biomarkers and Prevention*; **3**: 537–547.

Payne D.K., Sullivan M.D and Massie M.J (1996). Women's psychological reactions to breast cancer. *Semin Oncol*; **23**: 89-86.

Pegram M.D., Lipton A., Hayes D.F., Weber B.L., Baselga J.M., Tripathy D., Blay D., Baughman S.A., Twadell T., Glaspy J.A and Slaman D.J (1998). Phase II study of receptor enhanced chemosensitivity using recombinant humanized anti-p185 HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-over expressing metastatic breast cancer refractory to chemotherapy treatment. *J Clin Oncol*; **16**(8): 2659-2671.

Petricevic J., Petkovic M and Jonjic Nives(2011). Expression of estrogen and progesterone receptors in human ductal invasive breast carcinoma not otherwise specified: is there any difference between premenopausal5 and postmenopausal women? *Acta Clin Croat*; **50**:169-175.

Pharoah P.D., Day N.E., Duffy S., Easton D.F and Ponder B.A (1997). Family history and the risk of breast cancer: a systematic review and meta-analysis. *Int J Cancer* ;**71** (5):800-9.

Porter Peggy (2008). "Westernizing" Women's Risks? Breast Cancer in Lower-Income Countries. *N Engl J Med*; **358**:213-216.

Probst W. C., Snyder L. A and Schuster D. I (1992). Sequence alignment of the G -protein coupled receptor super family. DNA Cell Bio; **11**:1–20.

Rakha E.A., El-Sayed M.E., Green A.R., Lee A.H., Robertson J.F and Ellis I .O (2007). Prognostic markers in triple-negative breast cancer. *Cancer*; **109**:25-32.

Reinfuss M., Stelmach A., Mitus J., Rys J and Duda K (1995). Typical medullary carcinoma of the breast: a clinical and pathological analysis of 52 cases. *J Surg Oncol*; **60**:89-94.

Remmele W and Stegner H.E (*1987)*.Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection in breast cancer tissue. *Der pathologe*; **8** (3): 138-140.

Ro J.Y., Silva E.G and Gallager H.S (1987). Adenoid cystic carcinoma of the breast. *Hum Pathol*; **18**:1276–1281.

Roseman D.L., Straus A.K and Shorey W. (1990). A positive family history of breast cancer: does its effect diminish with age? *Arch Intern Med*; **150**: 191-4.

Sakorafas G.H and Tsiotou A. G (2000). Genetic predisposition to breast cancer. A surgical perspective. *Br J Surg*; **87**: 149-162.

Sorlie T., Perou C.M., Tibshirani R., Aas T., Geisler S., Johnson H., Hastie T., Eisen M.B.,van de Rijn M., Jeffery S.S.,Thorsen T., Quist H.,Matese J.C., Brown P.O., Botstein D., Lonning P.E and Borresen-Dale A.L (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl AcadSci U S A*; **98**: 10869–74.

Stark A., Kleer C.G., Martin I., Awuah B., Nsiah-Asare A., Takyi V., Braman M., Quayson S., Zarbo R., Wicha M and Newman L(*2010).* African ancestry and higher prevalence of triple negative breast cancer: findings from an international study. *Cancer*; **116**:4926–32.

Stavros A.T., Thickman D., Rapp C.L., Dennis M., Parker S and Sisney G (1995). Solid breast nodules: use of sonography to distinguish between benign and malignant lesions. *Radiology*; **196**(1):123-34.

Sumanta K., Sean K and Laura K (2010).Papillary Carcinoma of the Breast: An Overview Breast *Cancer Res Treat*; 122(**3**): 637–645.

Tan M and Yu D (2007). "Molecular mechanisms of erbB2-mediated breast cancer chemoresistance". *Adv. Exp. Med. Biol*; **608**: 119–29.

Tao Q., Fujimoto J., Men T., Ye X., Deng J., Lacroix L., Clifford J.L., Mao L., Van Pelt CS., Lee J.J., Lotan D and Lotan R (2007). Identification of retinoic acid-inducible Gprc5a as a new lung tumor suppressor gene. *Journal of the National Cancer Institute*; **99**(22)**:**1668-1682.

Terry D.P and Rohan T. E (2002).Cigarette smoking and the risk of breast cancer in women: a review of the literature. *Cancer Epidemiology, Biomarkers and Prevention*; **11**: 953-971.

Thomas D. B., Gao D.L., Ray R.M., Wang W.W., Allison C.J., Chen F.L., Porter P., Hu Y W., Zhao G.L., Pan L.D., Li W., Wu C., Coriaty Z., Evans I., Lin M.G., Stalsberg H and Self S.G(2002).Randomized trial of breast self-examination in Shangai .Final results. *J. Natl Cancer Inst*; **94**: 1445-1457.

Toikkanen S., Pylkkagnen L., and Joensuu H (1997). Invassive lobular carcinoma of the breast has a better short and long term survival than invasive ductal carcinoma. Br J Cancer ; 76(9): 1234–1240.

Viale G., Meridith M., Maiorano E., Mastropasqua., Dell P., Rasmussen B.B., Raffoul J., Neven P., Orosz Z., Braye S.,Ohlschlegal C.,Thurlimann B., Gelber D. R., Castiglione M., Price N.K., Goldhirsch A., Gusterson B.A and Coates A.S (2007). Prognostic and Predictive value of centrally reviewed expression of estrogen and progesterone receptors in

a randomized trial comparing tetrozole and tamoxifen adjuvant therapy for post menopausal early breast cancer. *Journal of Clinical Oncology*; **25:** 3846-3852.

Vu-Nishino H., Tavassoli F.A., Ahrens W.A and Haffty B.G (2005). Clinicopathologic features and long-term outcome of patients with medullary breast carcinoma managed with breast-conserving therapy (BCT). *Int J Radiat Oncol Biol Phy* ; **62**: 1040-1047.

Wiggans R.G., Wooley P.V., Smith T., Hoth D., McDonald J.S., Green L and Schein P S (1979). Phase-II trail of tamoxifen in advanced breast cancer. *Cancer Chemother Pharmacol*; **3**(1): 45-48.

Wu A.H., Yu M.C., Tseng C and Pike C.M (2006). Body size, hormone therapy and risk of breast cancer in Asian-American women. *Int J.Cancer*; **120**: 844-852.

Wu Q., Ding W., Mirza A., Arsdale T., Wei I., Robert W., Basso A., McClanahan T., Luo L., Kirschmeier P., Gustafson E., Hernandez M and Liu S (2005).Integrative Genomes Revealed RAI3 is a cell growth-promoting gene and a novel P53 transcriptional target. *The Journal of Biological Chemistry*; **280**: 12935-12943.

Ye X., Tao Q., Wang Y., Cheng Y and Lotan R (2009). Mechanisms underlying the induction of the putative human tumor suppressor Gprc5a by retinoic acid. *Cancer Biology & Therapy*; **8**(10):951-962.

APPENDIX 1

ANTIBODIES

Primary antibodies used

(1) Rabbit Monoclonal Anti-ER (Catalogue No. APA 308AA, H)

Rabbit Monoclonal Anti-PR (Catalogue No.PM 34AA, H)

Rabbit Monoclonal Anti-HER2 (Catalogue No. PME 342AA) all pre dilute, ready to use from Biocare Medical, 4040 Pike Lane Concord, CA 94520 U.S.A.

(2) Mab MK 24 2.3 mouse monoclonal anti RA13 antibody from Franhuafer Institute, Germany.