INVESTIGATION INTO DEATH-ASSOCIATED PROTEIN KINASE ONE
(DAPK1) AS A NON-INVASIVE MARKER FOR BREAST CANCER

BY

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA,
LEGON IN PARTIAL FULFILMENT FOR THE AWARD OF MSC
MEDICAL LABORATORY SCIENCE DEGREE.

DEPARTMENT OF MEDICAL LABORATORY SCIENCES

JULY 2017
DECLARATION

This is to declare that this thesis is as a result of an independent research undertaken by me, Bright Afriyie Owusu under the supervision of Dr. Benjamin Arko-Boham, Dr. Nii Armah Adu-Aryee and Prof. Richard Kwasi Gyasi towards the award of Master of Science Degree in Medical Laboratory Sciences, Department of Medical Laboratory Sciences, School of Biomedical and Allied Health Sciences, College of Health Sciences, University of Ghana.

I also declare that this work has not been submitted to any other institution for any award but to only the above mentioned institution.

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ABSTRACT

**Background:** Malignant tumour that begins in the breast with the capability of spreading is called Breast Cancer. Breast cancer is still the single most common malignancy in women worldwide. In 2012 an estimated 1.7 million women were diagnosed with breast cancer all over the world and 6.3 million women were diagnosed five (5) years ago. The fight against breast cancer seems difficult because of cancer’s ability to proliferate and metastasize and cause damage from primary site to other areas such as the lungs and the liver. Halting proliferation is daunting because of biological capabilities of cancer cells during multistep development of cancer.

Death associated protein kinase 1 (DAPK1) is part of the proteins involved in cancer proliferation. DAPK1 plays a role in apoptosis in wild-type p53 cell environment preventing cell growth but changes this helpful role to promoting the growth and aggressiveness of cancer in the mutant p53 environment.

**General Aim:** The study sought to investigate into DAPK1 as a non-invasive marker for breast tumour aggression and progression.

**Methodology:** Blood samples of participants were drawn after consent forms were signed. Data on age, treatment, and diagnosis and pathology numbers were retrieved from folders. The pathology numbers retrieved from patents folders were used to retrieve the slides and tissue blocks clients at the pathology department. Slides were screened by pathologist to indicate those particular blocks that contained a lot of malignant cells. Sections were cut and hematoxylin and eosin stains were applied. Slides were screened to confirm malignant diagnosis. Sections from confirmed malignant blocks were taken from selected archival tissue blocks for
immunohistochemistry using DAPK1 antibodies. The expression levels of the protein were determined by using colour intensity and scores were generated. DAKP1 levels in blood serum were quantified using commercial anti-DAPK1 ELISA kit. The data were analyzed using Statistical Package for the Social Sciences (SPSS) version 20.0. Data were presented as mean ±SEM (Standard Error) and one-way ANOVA were used as the post hoc analysis to confirm significance between DAPK1 in blood samples of breast cancer and non-breast cancer patients. Chi-square was used to test for significance in the expression of protein in tumour tissues.

RESULTS: Breast cancer slides showed variable staining intensities of brown colouration but non-breast cancer tissues showed no staining intensity. There was a statistical value of 0.000008 which was significant compared to our $P$ value $\leq 0.05$. There were traces of DAPK 1 protein in sera of breast cancer patients but there were no traces of DAPK 1 in the sera of non-breast cancer patients with a statistical value of 0.11 which was higher than my $P$ value $\leq 0.05$.

CONCLUSION: DAPK 1 protein was overexpressed in malignant breast cancer patients than non-malignant breast cancer patients.
DEDICATION

I dedicate this dissertation to God Almighty; it has all been by Your faithfulness. This work is also dedicated to my parents Mr and Mrs Owusu and to my sister Abigail Owusu Afriyie and a wonderful person Dorothy Agyeiwaa, thanks so much for the motivation and support.
ACKNOWLEDGEMENT

Thank You, God Almighty for your goodness and mercies in the land of the living.

I am most grateful to my pathology unit head, Dr. Seidu Mahmood, and my supervisors Dr. Benjamin Arko-Boham, Dr. Nii Armah Adu-Aryee and Prof. Richard Kwasi Gyasi for all the guidance you gave me. I say God bless you for the assistance given.

Also to the staff of Department of Pathology, University of Ghana School of Biomedical and Allied Health Sciences, especially Mr Billy Brobbey (Chemotherapy unit), Mr Daniel Potakey, Mr Emmanuel Ametepe, Mr Andrews Hooper, Big Joe, Mr Maxwell Akanburichaab, Mrs Cecelia Krampah, Mrs Dinah Neequaye, Mr. Abdul Rashid Adams and Mr Jonathan Kofi Adjei. I want to say a big thank you for all the help you gave me in this research.

Also to my family, I appreciate all the support and inspiration. I am glad to belong to this family.

And to everyone who has in diverse ways offered support, I say a big thank you. God bless you.
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LIST OF ABBREVIATION

DNA – Deoxyribonucleic acid
RNA – Ribonucleic acid
TNM – Tumour Lymph Node and Metastasis
mRNA – messenger Ribonucleic Acid
AFP – Alta Feto Protein
B-HCG – Beta-Human Chorionic Gonadotropin
LDH – Lactate Dehydrogenase
AJCC – Armenian Journal for Cancer Committee
DAPK1 – Death Associated Protein Kinase One
ZIP – Zipper Interacting Protein
DRP – DAPK 1 Interacting Protein
ZIPk – Zipper Interacting Protein Kinase
PDB – Protein Data Bank
CaM – Calcium Modulin
Arg – Arginine
Lys – Lysine
TSC – Tuberous Sclerosis
cDNA – Complementary DNA
JNK – Janus Kinase
PDK – Protein Kinase D
MRI – Magnetic Resonance Imaging
CI – Computed Tomography

DET – Diethyltryptamine

FFPE - Formalin-Fixed paraffin-embedded

IHC – Immunohistochemistry

AR – Antigen Retrieval

DAB – Dimethyl amino benzidine tetra hydrochloride

HRP – Horseradish per oxidase

PBS – Phosphate buffered Saline

TMB – Tetramethylbenidine

ERK- Extracellular-signaling regulated kinase

JNK- c-Jun NH$_2$-terminal Kinase

MADIB- Mitotic Spindle Assembly Check Point Protein

MAB 21-Monoclonal Antibody 21

PIN- Prostatic Intraepithelial Neoplasia

mTORC1- Mammalian Target of Rapamycin Complex 1.

MyD88- Myeloid Differentiation Primary Response gene 88
CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

The abnormal division of cells that invades through the basal membrane boundaries and metastasizes into areas of the body far from the initial sites of growth is called Hallmarks of cancer (Aktipis et al., 2015). Challenges with the mitotic division at various phases of the cell cycle and failure of checkpoints to halt abnormal genes before mitosis can lead to cells dividing uncontrollably to cause cancer (Grivennikov et al., 2010). Cancer is a major global public health challenge (Siegel et al., 2012). Malignant neoplasms emerge from normal cells that change through dysplastic to neoplastic cells due to accumulated genetic and epigenetic changes.

The challenge has been identifying the cells from which malignant tumour originates. Stem cells have been suggested to be the target cells from which cancer originates because they have many features as cancer cells. These features include; the ability to regenerate themselves, give rise to varied progeny and colonize surrounding tissues (Kyprianou, 2010). Cancer cells are part of tumour cells which possess stem cell features and are capable of growth, proliferation and recurrence of the tumour (Clark & Gomez, 2016). Cancer cells have many different pathways that promote their growth, proliferation and immortalization. The hallmarks of cancer cells include proliferative signal sustenance, attacking growth suppressors, invasion and growth activation, that evasion of the immune system the formation of blood vessels and refusing to die are the six hallmarks of cancer (Hanahan & Weinberg, 2011).

All over the world, the most common cancer among women is breast cancer. Breast cancer is a derivation of diseases, which the growth of cells in the breast is uncontrollable. The
uncontrolled proliferation of cells in the breast forms a lump and they can either be malignant or benign (Adhikari et al., 2017). Breast cancer incidence is on ascendancy, especially in developing countries. Most breast cancer survival rates are lower than 30% especially metastatic cancers (Watson, 2008).

Breast cancers can be categorized into three forms namely; invasive breast cancer, non-invasive breast cancers and metastatic breast cancer (American cancer society, 2007). Non-invasive breast cancers do not spread beyond ducts or lobules where it originates. Breast cancers such as Ductal Carcinoma In situ (DICS) which grow inside of the milk ducts do not spread to nearby tissues. Non-invasive cancers have the propensity to develop into invasive breast cancer. Invasive breast cancers; occurs when cancerous cells spread from milk duct to other tissues within the breast (Casey et al., 2008). Metastatic breast cancers; are invasive cancers that spreads from origin (breast) to other tissues with in the body resulting in hepatocellular carcinoma in liver, bronchogenic carcinomas in lung, Osteosarcoma in bones and Glioblastoma multiforme in brain (Veljkovic et al., 2010).

Breast cancer worldwide claimed as many as 458,503 which is 13.7% deaths in women as at 2008 whiles 6.0% of cancer deaths are were recorded in both men and women (Liu et al., 2012). There are clear variations in incidents of breast cancer around the world. It is lowest in developing countries and greatest in developed countries. In twelve (12) regions in the world, the standardized age for breast cancer incidence rates per 10,000 women are; East Africa, 18; South Central Asia, 22; Sub-Saharan Africa, 22; South-Eastern Asia, 26; North Africa and Western Asia, 28; South and Central America, 42; Eastern Europe, 49; Southern Europe, 56; Northern Europe, 73; Oceania, 74; Western Europe, 78; and in North American, 90 (Laurance, 2006: University of Ghana  http://ugspace.ug.edu.gh
Olson, 2002). There has been a quantum upsurge in breast cancer cases worldwide since 1970 because of modernized life styles.

The protein of relevance to our study of breast cancer is Death Associated Protein Kinase One (DAPK 1). Death Associated Protein Kinase One (DAPK1) is a calcium/calmodulin-dependent serine/threonine kinase involved in multiple cellular signaling pathways that triggers death, autophagy, and cell survival (Zhao et al., 2015). Other names of DAPK1 include DKFZp781I035 and EC2.7.11.1 with locus ID 1612. Programmed cell death mediated by DAPK1 is induced by interferon-gamma. DAPK1 encodes structurally unique 160kD and it is made up 8 ankyrin repeats and 2 putative P-loop consensus positions. It is a tumor suppressor or candidate (Cohen & Kimchi, 2001).

DAPK1 locus type gene is with the product; 9 and is located on the long arm of chromosome 9, region 3, sub-region 4.1. (9q34.1). DAPK1 is restricted to the first helix of the N-terminal CaM lobe, two residues (Arg 53, Arg 54) of DAPK1 family-specific basic loop, and two additional residues (Arg 23, Lys 222) from other catalytic domain surface loops. The CaM-binding domains are Lys 304, ser 308, Lys 298 and Arg 310 (Inbal et al., 2002). One of the most up-regulated members of the s6 kinase family is the death associated protein kinase one (DAPK1). DAPK1 is part of a group of kinases like DAPK2, DAPK3, DAP kinase-related apoptosis-inducing protein kinase 1 (DRAK1) and DRAK2 (Zhao et al., 2015). DAPK1 is protein modulated by calcium through the p53 dependent pathways for the instigation of death signals (Raval et al., 2007; Raveh et al., 2001). Substrates of DAPK1 include p21 and p53 (Dryden et al., 2014).
Physical, chemical and biological carcinogens which are stimuli to DAPK1, increases expression of the protein which activates dephosphorylation of ser 308 and p53 through the p14/p19<sup>ARF</sup> pathway, which results in programmed cell death. Not only is DAPK1 activated for apoptosis, studies have shown that DAPK1 is also involved in autophagy, immune response and proliferation (Cohen <i>et al.</i>, 1997; Bradley, 2008). The roles of DAPK1 may change depending on the cell setting. Cell growth is regulated by DAPK1 through the modulation of TSC1/TSC2 complex formation in the mTOR pathway. Phosphorylation of TSC1 and TSC2 leads to up regulation in cell growth and protein synthesis and thereby ensuring a homeostatic equilibrium between survival and death signaling. In contrast, p53 mutant cells show elevated expression of the protein DAPK1. The dysfunction of p53 the cells prevent DAPK1 from inducing apoptosis, thereby ending in a shift of function from apoptosis towards activation of growth pathway. In p53 wild type, DAPK1 undergo apoptosis but immediately changes role and proliferate in in a p53 mutant setting, hence the elevation of its expression in different cancers. Studies done on DAPK1 suggest the sensitivity of cancers like ovarian and pancreatic cancers to DAPK1 inhibitors (Zhao <i>et al.</i>, 2015) But less is known about DAPK1 and its usefulness even as a screening tool in breast cancer.

Tools for screening tumours should neither be invasive or expensive. This is to prevent further harm and also allow many people to have access to it. Substances secreted by malignant tumour tissues mostly contain traces of elements or proteins which is identifiable to such tumours (Greenwald, 1982).

Biomarkers however can indirectly inform screening. Hormonal changes and immune response impact the growth and profile of a tumour. Biomarkers need to be highly specific for the kind of cancer example, prostate specific antigen (PSA) are highly sensitive to prostate cancer (Margaret
et al., 2001). The search for biomarkers need to begin with preclinical studies comparing tumour tissues with non-tumour tissues. Immunohistochemistry has been extensively used for biomarker studies (Schummer et al., 1999; Swets & Picket, 1982).

Biomarkers are molecular proteins and their presence in blood, or tumor can be indicative of a particular type of tumour, severity of the disease and the site released from. Studies evidently suggest unbound nucleic acids, which are DNA markers in supernatants of urine. These DNA markers are better used in the detection of cancer of the urothelial. They are more sensitive than most markers found in sediments for the diagnosis of urothelial carcinomas (Thakor & Gambhir, 2013) and differentiating tumor grades (Zhao et al., 2015).

Detectable antigens such as Alpha-feto protein is released into the blood and tissues by germ tumour cell and hepatocellular carcinomas. Calretinin a calcium binding protein is released from tumour types such as mesothelioma, stromal tumors, adrenal cortex and synovial sarcoma. Sarcomas, renal cell, endometrial and lung cancers release the protein vimentin which helps in their diagnosis. Tumor m2-pk which is an enzyme for tumor metabolism is released by colorectal cancers, breast cancer, esophageal cancer, stomach cancer, cervical cancer and ovarian cancer (Shastri et al. 2006; Kumar et al., 2007).

Biomarkers potentially have many applications and clinical significance in oncology. These include screening, diagnosis, risk assessment, determination of response to treatment and monitoring of disease progression (Henry & Hayes, 2012). Other use of biomarkers includes guiding antibiotic therapy, gaging the response to treatment and recovery from diseases (Zambon et al., 2008). Biomarkers can be categorized into DNA, RNA and Protein biomarkers.
DNA biomarkers; the early markers evaluated for the staging of cancers are circulating DNA and tumour cells. Metastatic cancers and other conditions like autoimmune disease and sepsis are associated with high levels of serum DNA (Sidransky, 2002; Leo et al., 1977). Some studies suggest promulgating malignant tumour cells in the blood (Kahn et al., 2004; Hoon, 2000) or bone marrow (Nishizuka et al., 2003) as markers for systemic metastases, the clinical sample sizes for DNA markers have mostly been small (Meng et al., 2004).

RNA biomarkers; the development of high technologies have led to the comprehensive assessment of mRNA expression whiles previously most DNAs were evaluated singly (Paweletz, 2001). Most RNA-based biomarkers part taking in clinical evaluations comprises of multi-gene molecular framework (Weigelt et al., 2008). The accuracy patterns of RNA markers are better compared to single-molecule markers, considering which genes to include in the patterns, additional layers of statistical complexity, provoking new developments in biostatistics, bioinformatics and data visualization. Molecular biomarkers and their frameworks have been analyzed by innumerable SUPERVISED ALGORITHMS (Dudsky et al., 2013).

Protein biomarkers; protein malignant tumour biomarkers are single proteins and mostly serum derived. Alpha-Feto Protein (AFP), Beta-Human Chorionic Gonadotropin (B-HCG) and Lactate Dehydrogenase (LDH) are patronized by the American Joint Committee on Cancer (AJCC) system in diagnosing testicular cancer. Proteins which are not formally used for staging are important for prognosis and selection of therapy. Protein function is predominantly dependent on phosphorylation, glycosylation, post-translational modifications, location in the cell and the location in tissue (Dudsky et al., 2013: Carol and Caggiano, 2014).
1.2 PROBLEM STATEMENT

Breast cancer is a public health challenge to the nations of the world. This is because breast cancer is the malignancy in women that claims many lives. In 2012, an estimate of 1.7 million women was cancer diagnosed and 6.3 million have been living with the disease for the past five (5) years. Nationwide deaths in Ghana revealed 3659 deaths annually with a male to female ratio 1.2:1 (Wiredu and Armah, 2006). Despite the numerous approaches to cancer therapy and prevention, Cancer remains a prime cause of death worldwide (Loginov et al., 2017).

Currently, the best approach to cancer treatment is surgery but surgical approach to cancer treatment still does not guarantee non-recurrence (Hamilton & Sharp, 2004). Most non-surgical approaches like chemotherapy or radiotherapy targeted at metastatic cells also end up in necrosis of normal cells which mostly results in anemia. Other side effects include peeling and/or blistering of the skin, stiffness of jaws, lymphedema, tooth decay, vomiting, depression, weight loss, impotency in men amongst several others limit treatment (Henderson & Finn, 1996; Disis & Cheiver, 1996). Patients have suffered recurrence and/or died from cancer (Smith et al., 2010) because of the challenge of early detection.

Early detection is the main challenge of cancer diagnosis. Simple and easy way of detecting cancer at a very early stage will reduce cancer deaths and will reduce some of the side effects of treatment. A protein that is indicative of the presence of breast cancer in the body can be useful in cancer diagnosis, therapy, and prognosis.

1.3 JUSTIFICATION

The recurring ability of breast cancer is as a result of breast cancer stem cells. Breast cancer is lethal because of its ability to metastasize to far way locations causing pain and harm to
neighboring tissues and organs. The negative effect of cancer which most importantly is claiming lives has placed a huge burden on the nations of our world. Late detection has played to the advantage of cancer. Regardless of the medical community’s most meticulous attempt at early detection before metastatic capability is attained, many malignancies either simply may not be identified effectively at an early stage or frequently metastasize at early stages of development.

A protein which plays major role in cancer development and progression is of importance if we are considering early detection in cancer. It is believed that DAPK1 has a role to play in cancer aggression and progression. Clearly defined role and contribution to cancer growth or metastasis will help in therapeutic measures against the disease.

1.4 SIGNIFICANCE OF STUDY

This study is to identify DAPK1 as a biomarker that can be used for early diagnosis of breast cancer and to monitor treatment of cancer. This will help solve the problem of early detection of cancer and escape of the disease from being monitored. Protein diagnostic markers are less expensive compared to other forms of diagnostic tools; hence DAPK1 if proven a biomarker will reduce the financial burden on breast cancer clients.

1.5 AIM

This study sought to investigate DAPK1 as a non-invasive marker for breast tumour aggression and progression.

1.6 SPECIFIC OBJECTIVES.

1. Determine DAPK1 expression levels in breast tumour and non-breast tumour tissue samples.
2. Determine DAPK1 levels in sera from blood samples of breast cancer and non-breast cancer patients.

3. Determine DAPK1 levels among breast cancer patients at different grades of breast cancer development
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 CANCER

Cancer represents the collapse of the multicellular cooperation. Cancer cells spread to the tissues and organs leading to devastating effects for the organism’s level of fitness. Cancer is the leading life threatening disease in the world (Sources, 2008). In 2012 it was estimated that about 12.7 million new cancer cases were diagnosed and about 7.6 million deaths estimated worldwide (Ferlay et al., 2012). Include inherited factor, the World Health Organization (WHO) categories four different groups of the external agent as carcinogens which are capable of causing cancer. These carcinogens include physical, biological, chemical, and dietary components like cured meat (World cancer report, 2014).

The various cancer types can be grouped based on their diagnosis as single primary cancer or multiple primary cancer (Elena et al., 2012). Single primary cancers might be identified through a shared etiological factor whiles multiple primary cancers are as a result of treating a disease or increased disease surveillance (Bajdik et al., 2006). Cancers disposed to early detection, include carcinoma of the prostate or colon. With early detection, few patients continue to develop metastatic disease that ultimately proves to be fatal (Diaz-cano, 2015). Increased fatigue, increased muscle weakness and reduced system and physical function are associated with progressiveness of disease and unresponsiveness to treatment of cancers, all having a negative impact on the life of patients (Lowe et al., 2016).
2.1.1 BREAST CANCER

Malignancy most common amongst women is breast cancer. Breast cancer is a derivation of
diseases, in which the growth of cells in the breast is uncontrollable. The uncontrolled
proliferation of cells in the breast forms a tumour and they can either be malignant or benign
(Adhikari et al., 2017). Extent of breast cancer is on the increase, especially in developing
countries. Most breast cancer survival rates are lower than 30% especially metastatic cancers
(Watson, 2008).

Breast cancers can be categorized into three forms namely; invasive breast cancer, non-invasive
breast cancers do not spread beyond ducts or lobules where it originates. Breast cancers such as
Ductal Carcinoma Insitu (DICS) which grows inside of the milk ducts do not spread to nearby
tissues invasive cancers have the propensity to develop into invasive breast cancer. Invasive
breast cancers occur when cancerous cells spread from milk duct to other tissues within the
breast (Casey et al., 2008). Metastatic breast cancers are invasive cancers that spreads from
origin (breast) to other tissues with in the body resulting in hepatocellular carcinoma in liver,
bronchogenic carcinomas in lung, Osteosarcoma in bones and Glioblastoma multiforme in brain
(Veljkovic & Veljkovic, 2010).

Being female, corpulence, lack of physical exercise, alcohol consumption, and hormonal
treatment during menopause, ionizing radiation, early menstruation and late child bearing are
risk factors in developing breast malignancy (Yager & Davidson, 2006; Goldhirsch et al., 2013).
Certain genetic predisposition plays a minor part in most breast cancer cases. In all breast cancer
cases, genetics is known to be the primary cause of 5-10% of all cases (Yi et al., 2010; Gage,
Research has proven that females whose mother was diagnosed before 50 years of age have an elevated risk level of 1.7 whiles those who mothers were diagnosed after 50 years have an increased risk of 1.4 (Colditz, 2012). In females with zero, one, or two affected relatives, risk of breast cancer is 7.8%, 13.3% and 21.1% with a subsequent mortality from the disease of 2.3%, 4.2%, and 7.6% respectively (Nelson et al., 2012). Those with close degree relatives affected with the disease, the threat of breast cancer amongst the early ages of 40 and 50 is twice that of the broad populace. Genetics plays a major role by causing a hereditary breast cancer syndrome in less than 5% (Pasche, 2010). This comprises of those who carry the BRCA 1 and BRCA 2 gene mutation (Gage, 2012). Mutations in breast cancer accounts for up to 90% of the total genetic influence with a risk factor of 60-80%.

2.1.2 BREAST CANCER CLASSIFICATION

Breast cancers are classified by several scoring systems, one of which is the American Joint Committee on Cancer (AJCC) systems. These grading systems review prognosis and can affect therapy response. Description of breast cancer mainly includes many factors such as female gender, family history on breast cancer, late menopause, prolonged hormonal replacement and genetic mutations (Carlson, 2009). Breast cancer is usually classified primarily by its histological appearance and description. Histological appearances as well as description are usually used to classify breast cancer. The epithelium lining the ducts is mostly where breast cancer is derived from. Breast cancers are classified as either ductal or lobular carcinoma (Sotiriou, 2009). Carcinoma in situ is the proliferation of precancerous cells within a specific tissue section such as the mammary duct without invasion of the immediate tissues. Differently, invasive carcinoma does spread from initial tissue compartment (Carson, 2009; Saslow et al., 2012).
Grade; Grading examines the macroscopic nature of breast cancer cells to the appearance of normal breast tissue. Normal cells in an organ like the breast become differentiated, implying that they take on detailed shapes and forms that reveal their function as part of that organ (Ludwig and Weinstein, 2005). Cancerous cells lack that differentiation ability hence they have poor grades. In cancer, the cells that would generally line up in an orderly way to make up the milk ducts become poorly arranged (Kumar et al., 2010). Cells nuclei become poorly ordered. Pathologist describes cells as well differentiated as low grade, moderately differentiated and poorly differentiated as the cells. Poorly differentiated cancers have a poorer prognosis.

Stage; Breast cancer staging using the TNM system is pivoted on the size of the tumour (T), whether or not tumour has spread far to the lymph nodes (N) in the armpits and whether the tumour has metastasized (M) (Sotiriou et al., 2003; Dunsky et al., 2013). Larger size, nodal spread, and metastasis have a large stage number and a worse prognosis. The main stages are; Stage zero (0) is a pre-cancerous or marker condition, either ductal carcinoma Insitu (DCIS) or lobular carcinoma in situ (LCIS). Stages One (1) to three (3) are in the interior of regional lymph nodes of the breast. Stage four (4) is metastatic cancer that has less favorable prognosis since it has spread beyond the breast and regional lymph nodes (Weigelt et al., 2008). Where possible, imaging studies may be used in staging processes to look for signs of metastasis. However, in cases of breast cancer with low risk for metastasis, the risk associated with PET scan or CT scan outweighs the possible benefits, as these procedures expose the patient to a substantial amount of potentially dangerous ionizing radiation (Vuong et al., 2014).

Receptor Status; Receptors flood the surface, cytoplasm and nucleus of breast cancer cells. Chemical messenger such as hormones bind to receptors, and this leads to signaling pathways which apparently causes changes in the cell. Breast cancer cells may or may not have three
important receptors; Estrogen receptor (ER), Progesterone receptor (PR), and Human epidermal growth factor receptor 2 (HER2) - breast cancers. HER2 breast cancers respond to drugs such as the monoclonal antibody trastuzumab and this has improved prognosis significantly (Dubsky et al., 2013; Vuong et al., 2014). Cells that lack any of the three receptors types are called triple-negative, although they normally do express receptors for other hormones, such as prolactin receptor and androgen receptor.

2.2 CANCER STATISTICS

Cancer is a worldwide disease of concern. Accurate statistics are important both for research and cancer control evaluation. Accurate data on cancer and cancer patterns have been the worry for most developing countries but that is different for the developed country as there are well-instituted cancer registries. Sources for data for cancer include incidence of mortality and population data. Worldwide burden of cancer increases largely because of aging and living cancer prone lifestyles (Elena et al., 2012). There are various cancers distributed worldwide. 14.1 million Cancer cases and 8.2 million cancer deaths were recorded in 2012 GLOBACAN with 27 cancers in men, women, and both sexes.

Lung cancer is the most common cancer (1.82 million cases) and deaths (1.67 million deaths), breast cancer is the second most common cancer (1.67 million new cases, 25% of all cancers ranks 5th as causes of death (522,000 deaths) whiles is the most frequent cause of death in women in less developed regions (324,000 deaths, 14.3% of total) and is also the second cause of death in developed countries (198,000 deaths, 15.4%) after lung cancer. It is followed in by colorectal cancer (1.2 million cases, 608,000), stomach cancer (990,000 cases, 261000 deaths) and liver cancer (649,000 deaths and representing 8.5%). Lung, stomach, and liver predominate among males in developing countries but lung cancer is the most common cancer worldwide.
among men. It runs second to prostate cancer in developed countries (Ferlay et al., 2012). In women, cervical cancer is the second most common cancer in developing countries to breast cancer. The situation in the world is not much different from Ghana. A study conducted by (Wiredu and Armah, 2008) in Ghana revealed 3659 cancer deaths with male to female ratio 1.2:1. The common causes of death in female was malignancies of the breast (17.24%), hematopoietic organs (14.69%), liver (10.97%) and cervix (8.47%) whilst in male, the highest mortality was recorded from liver (21.15%), prostate (17.35%), hematopoietic organs (15.57%) mortality and stomach (7.26%).

### 2.2.1 BREAST CANCER EPIDEMIOLOGY

All over the world, breast cancer is the most common incursive cancer in women (McGuire, 2015). Breast cancer affects 12% of women all over the world. Non-invasive non-melanoma skin cancer is the most common cancer among non-invasive cancers. Less is known of them because they can easily be cured and causes few losses of lives (Riley et al., 2017).

Breast cancer consists of 22.9% of incursive cancers in women and 16% of all female cancers (World Cancer Report, 2014). As at 2012, 25.2% of cancers had been diagnosed in women, ranking it as the leading cancer in women (World Health Organization, 2015). Breast cancer claimed as many as 458,503 which is 13.7% deaths in women as at 2008 whiles 6.0% of cancer deaths are were recorded in both men and women (World Cancer Report, 2012).

There are clear variations in incidents of breast cancer around the world. It is lowest in developing countries and greatest in developed countries. Twelve (12) regions in the world, the standardized age for breast cancer incidence rates per 10,000 women are; East Africa,18; South Central Asia, 22; Sub-Sahara Africa,22; South-Eastern Asia,26; North Africa and Western
Asia, 28; South and Central America, 42; Eastern Europe, 49; Southern Europe, 56; Northern Europe, 73; Oceania, 74; Western Europe, 78; and in North America, 90 (Laurance, 2006; Olson, 2002). There has been a quantum increase in breast cancer cases worldwide since 1970 because of modernized lifestyles. A research conducted strongly related breast cancer to genes and age with 5% of breast cancers occurring to women under 40 years (US Preventive services task force, 2002).

2.3 CANCER BIOMARKERS

The National Cancer Institute (NCI) in the United Kingdom defines biomarkers as a biological molecule found in blood, other body fluids or tissues that are signs of a normal or abnormal process or of a condition or disease like cancer. In areas of research such as proteomics, immunology, gene-expression and microarrays are current developments that present better ways to tackle cancer diagnosis. The necessity to develop cancer biomarkers informed the establishment of the Early Detection Research Network (EDRN) (Srivastava & Kramar, 2000). The motive of cancer screening program is to identify neoplasia at early stages enough that treatment can impact positively on disease (International Conference on Harmonization E9 Expert Working Group, 1999).

Tools for screening breast tumour should not be invasive neither should they be expensive. This is to prevent further harm and also allow many people to have access to it. Substances secreted by malignant tumour tissues mostly contain traces of elements or proteins which is identifiable to such tumours (Greenwald, 1982). Biomarkers however can be indirect in informing screening. Hormonal changes and immune response impacts the growth and profile of a tumour. Biomarkers need to be highly specific for the kind of cancer example, prostate specific antigen (PSA) are highly sensitive to prostate cancer (Margaret et al., 2001). The search for biomarkers
need to begin with preclinical studies comparing tumour tissues with non-tumour tissues. Immunohistochemistry has been extensively used for biomarker studies (Schummer et al., 1999; Swets et al., 1982). Biomarkers are molecular proteins and their presence in blood, or tumor can be indicative of a particular type of tumor, severity of the disease and the site released from.

Studies evidently suggest unbound nucleic acids, which are DNA markers in supernatants of urine are better used in the detection and more sensitive than those found in sediments for the diagnosis of urothelial carcinomas (Thakor & Gambhir, 2013) and differentiating tumor grades (Zhao et al., 2015).

Detectable antigens such as Alpha-feto protein is released into the blood and tissues by germ tumour cell and hepatocellular carcinomas. Calretinin a calcium binding protein is released from tumour types such as mesothelioma, stromal tumours, adrenal cortex and synovial sarcoma. Sarcomas, renal cell, endometrial and lung cancers release the protein vimentin which helps in their diagnosis. Tumor M2 Pyruvate kinase (m2-pk) which is an enzyme for tumor metabolism is released by colorectal cancers, breast cancer, esophageal cancer, stomach cancer, cervical cancer and ovarian cancer (Shastri et al., 2006; Kumar et al., 2007).

Biomarkers potentially have many applications and clinical significance in oncology. These include screening, diagnosis, risk assessment, determination of response to treatment and monitoring of disease progression (Henry & Hayes, 2012). Other use of biomarkers includes guiding antibiotic therapy, evaluating the response to therapy, evaluating the response to therapy and recovery from diseases (Zambon et al., 2008). Biomarkers are used in staging cancer example is measurement of carcinoma embryogenic antigen-125 or classification of the extent of disease example prostate specific antigen levels in blood used to reflect extent of tumour growth
and metastasis. Separate molecular marker and patterns of markers have been used successfully
to classify known tumour into categories that behave contrarily from each other. Biological and
chemotherapy agents are more effective and more widely used than when Tumour, Lymph Node
and Metastasis (TNM) staging was introduced particularly in the adjuvant setting. A biomarker
intended for grading or staging necessarily does not have to be specific as it must be for
screening, early detection or risk assessment. Biomarkers can be categorized into DNA, RNA
and Protein biomarkers. DNA biomarkers; the first markers evaluated for cancer staging are
circulating DNA and tumour cells. Elevated serum DNA levels are associated with metastatic
cancers and with other conditions such as sepsis and autoimmune diseases (Sidransky, 2002; Leo
et al., 1977). Some studies suggest circulating cancer cells in the blood (Kahn et al., 2004; Hoon
et al., 2000) or bone marrow (Nishizuka et al., 2003) as markers for systemic metastases, the
clinical sample sizes for DNA markers have mostly been small (Meng et al., 2004).

RNA biomarkers; a lot of high technologies have been developed to assess the expression of
mRNA in detail whiles most DNA markers are individually evaluated (Paweletz et al., 2001).
Many of the RNA-based biomarkers undergoing clinical testing is made up of multi-gene
molecular framework (Weigelt et al., 2008). The accuracy patterns of RNA markers are better
compared to single-molecule markers, considering which genes to include in the patterns,
additional layers of statistical complexity, prompting new developments in biostatistics,
bioinformatics and data visualization. Molecular markers and their frameworks have been
analyzed by various SUPERVISED ALGORITHMS (Dudsky et al., 2013). Protein biomarkers;
protein cancer biomarkers are single proteins and most are derived from serum. Alpha-Feto
protein (AFP), Beta-Human Chorionic Gonadotropin (B-HCG) and Lactate Dehydrogenase
(LDH) are used in the American Joint Committee on Cancer (AJCC) system to testicular cancer.
Proteins which are not originally used for staging are essential for prognosis and selection of treatment. Protein function is instead often dependent on phosphorylation, glycosylation, post-translational modifications, location in the cell as well as in the tissue (Dudsky et al., 2013; Carol & Caggiano, 2014). All discovered biomarkers have not sufficiently been of great help to health providers in daily practice as each biomarker has limited specificity and sensitivity (Marshall & Reihart, 2009). To get the most from biomarkers, one must combine more than one biomarker.

2.4 DEATH ASSOCIATED PROTEIN KINASES (DAPKs)

The death associated protein kinases comprises a derivation of calcium/calmodulin (Ca^{2+}/Ca^M) dependent serine/threonine kinases whose relatives include DAPk1, DAPk-1 related protein (DRP-1; DAPK-2), Zipper interacting protein kinases (ZIPk; DAPk-3), DAPk1, DRP-1 and ZIP kinases are usually grouped into one superfamily since they share 83% and 80% identity at the amino acid level, respectively with DAPk1. Although DRP1 and ZIPk are not directly regulated in response to cerebral injury, DAPk1 mRNA levels are increased in response to neonatal hypoxia (Schumacher et al., 2002).

DAPk1, ZIPk1 and DRP1 are projected to form a peculiar order to activate apoptic functions (Shani et al., 2004). The upstream protein of all the DAPks is DRp1 by report and is involved in the activation of DAPk1 and ZIPk (Bialik et al., 2006). The binding of ZIP and DAPK 1 to each other via their various catalytic areas phosphorylates ZIPk at six different specific sites in the extra-catalytic C-terminal domain hence leading to amplification of death enhancing signals (Shani et al., 2004).

The translation, modulation and amplification of different signals of stress lead to cell death by kinases forming a death-associated multi-protein complex. DRP1 may be sufficient to ease the
cell death pathway induced by DAP kinases due to the activation from their linear interactions which may be seem to inhibit the upstream protein. However due to the presence of multiple activating sources for these proteins, treatment strategies pointed towards multiple targets have been suggested to yield better outcome (Nair et al., 2013).

2.4.1 ARRANGEMENT OF DAPK DOMAINS

DAPK1 is a 160-kD protein consisting of 1430 amino acids. It consist of a protein kinase domain, a calcium-calmodulin binding region of 62 amino acids, 10 ankyrin repeats each about 30 amino acids in length, two putative P loops, cytoskeletal binding region and a death domain. More than 22 crystal structure of DAPk1, crystalized with their respective inhibitors at resolution higher than 2.5 Angstroms, are reported in the protein Data Bank (PDB) (Zhao et al., 2015).

The crystal structure of DAPk1 is a perfect example of a small-molecule fragment bound to the kinase region, which can be used as the commencement of bioavailable protein kinase inhibitor design allowing in vitro and in vitro target validation evidence with bioavailable kinase inhibitors supports DAPk 1 as a drug discovery target for neurological defects, no clinically promising small-molecule DAPk1 inhibitors is still under research (Henshall et al., 2003).

It is important to gain insight into how their individual domains interrelate with one another, how their dysfunction causes diseases and how the several domain proteins operate in cellular context. This can be understood by the DAPK family of complex (Chen et al., 2005; Stevens et al., 2009).
2.4.2 DAPK 1 LOCALIZATION

The intracellular localization of DAPK 1 is a central part for appreciating its function. It had been published in 1997 that DAPK 1 localized to the cytoskeleton via its cytoskeleton binding domain (Cohen et al., 1997). It was later learnt that DAPK 1 localized to actin stress fibers of the cytoskeleton and that the localization requires its ankyrin repeats region in addition to the cytoskeletal binding domain (Kuo et al., 2003). It has been shown that proteins containing ankyrin repeats are able to check microtubules dependent on the vascular transport (Pan et al., 2008). It has been noted however that although DAPK 1 demonstrates a strong colonization with the cytoskeleton, a diffused DAPK 1 staining in the cytoplasm is also observed in all published work together with the cytoskeletal co staining (Bialik et al., 2004; Kuo et al., 2003).

2.4.3 BINDING SECTION OF CALCIUM-CALMODULIN

Double-locking mechanism is the way for regulating DAPKs since they are Ca2+/CaM-dependent kinases. DAPK1 activity is regulated through numerous phosphorylation sites that are identified within the CaM auto regulatory domain, two of which are Ser 289 and Ser 308 (Bialik et al., 2006). Total activation involves both the dephosphorylation of Ser 308 and CaM-binding segment pulling this domain out from the catalytic activity at low CaM levels. It has been shown that the deletion of the CaM-binding domain from DAPK or the Ser 308 to
Ala generates a constitutively active kinase thereby exhibiting greater Ca\(^{2+}\) independent catalytic activity and killing capability (Cohen et al., 1999).

The overall DAPK-CaM interface is almost 2000Å\(^2\) in area (De Diego et al., 2010). In order to further analyze CaM binding section, Lys 304, Ser 308, Lys 298 and Arg 310 are the dominant interacting residues in the domain of the CaM-binding.

### 2.4.4 THE DEATH DOMAIN AND ANKYRIN REPEATS

The death domain of DAPK 1 is dominantly associated with protein to protein interactions kinase activity and programmed cell death functions. Deletion of the death domain is reported to halt the apoptotic functions of the kinase including TNF-\(\alpha\)-Fas-induced cell death (Cohen et al., 1999).

The use of binding studies, deletion analysis and death domain mini-protein in vivo and in vitro determined that the domain of DAPK1 is the major site for the interaction with tumour suppressor protein tuberin (TSC2). Thus, there is a positive alliance between growth factor stimulation of DAPK and mammalian target of rapamycin complex one (mTORC1) signaling, which eventually may be affected by autophagy, cell survival or apoptosis (Stevens et al., 2009).

Additionally, death domain-containing proteins have links to innate immunity, communicating with Toll-like receptors through bipartite adapter protein such as myeloid differentiation primary response gene 88 (MyD88) (O’Neill et al., 2003).

The domain is the least deliberated domain of DAPK1 and no crystal arrangement has been reported so far. The ankyrin repeats facilitate protein-protein communication and are connected in DAPKs breakdown. S-DAPK-1, an alternative spliced product lacking the DAPK1 kinase
domain regulates the study state levels of the full length DAPK1 and is concerned in the proteasome-independent degradation pathway for DAPK1 (Lin et al., 2009).

The DAPK1 degradation via the ubiquitin-proteasome pathway is propelled by the ankyrin repeats (Connell et al., 2001). The E3 ubiquitin ligase carboxyl terminus of HSC 70-interacting protein (CHIP) which enhances the ubiquitination of HSP 90-interaction proteins will also prompt DAPK1 degradation (Zhang et al., 2007).

### 2.4.5 DEATH-ASSOCIATED PROTEIN KINASE ONE (DAPK1)

DAPK1 is a calcium/calmodulin-dependent serine/threonine kinase complexed in multiple cellular signaling pathways that triggers death, autophagy, and cell survival. Other names of DAPK1 include DKFZp781I035 and EC2.7.11.1 with locus ID 1612. Programmed cell death mediated by DAPK1 is induced by interferon-gamma. DAPK1 encodes structurally unique 160kD and it is made up 8 ankyrin repeats and 2 putative P-loop consensus sites. It is a tumor suppressor or contender (Cohen & Kimchi, 2001).

DAPK1 locus type gene is with the product; 9 and is located on the long arm of chromosome 9, region 3, sub-region 4.1. (9q34.1). DAPK1 is restricted to the first helix of the N-terminal CaM lobe, two residues (Arg 53, Arg 54) of DAPK1 family-specific basic loop, and two additional residues (Arg 23, Lys 222) from other catalytic domain surface loops. The CaM-binding domains are Lys 304, ser 308, Lys 298 and Arg 310 (Inbal et al., 2002).

One of the most up-regulated members of the s6 kinase family is the death associated protein kinase one (DAPK1). DAPK1 is part of a group of kinases like DAPK2, DAPK3, DAP kinase-related apoptosis-inducing protein kinase 1 (DRAK1) and DRAK2 (Zhao et al., 2015). DAPK1
is protein modulated by calcium through the p53 dependent pathways for the activation of death signals (Raval et al., 2007; Raveh et al., 2001).

Substrates of DAPK1 include p21 and p53 (Dryden et al., 2014). Physical, chemical and biological carcinogens which are stimuli to DAPK1, increases expression of the protein which activates dephosphorylation of ser 308 and p53 through the p14/p19ARF pathway, which results in programmed cell death. Not only is DAPK1 activated for apoptosis, studies have shown that DAPK1 is also involved in autophagy, immune response and proliferation (Cohen et al., 1997; Bradley, 2008).

The roles of DAPK1 may change depending on the cell setting. DAPK1 can regulate cell growth by modulating TSC1/TSC2 complex formation in the mammalian target of rapamycin (mTOR) pathway. Phosphorylation of tuberous sclerosis one (TSC1) and tuberous two (TSC2) leads to increased cell growth and protein synthesis and in that way sustaining a homeostatic stability between signals of death and survival. In contrast, DAPK1 is overly expressed in p53 mutant cells. The failure of p53 duty to the cell prevents DAPK1 from prompting apoptosis, thereby ensuring the shift in function from apoptosis towards activation of growth pathway.

In p53 wild type, DAPK1 undergo apoptosis but immediately changes role and proliferate in in a p53 mutant setting, hence the elevation of its expression in different cancers. Studies done on DAPK1 suggest the sensitivity of cancers like ovarian and pancreatic cancers to DAPK1 inhibitors (Zhao et al., 2015; Smith & Pell, 2003) but less is known about its multiple aggression and progression to cancer growth.
2.4.6 INTERACTING PARTNERS OF DAPK1

DAPK1 is defined by relatively large protein with manifold domains and docking motifs that motivates its function and regulation. Understanding protein interactions at the domain level give a universal view of the protein interactions at the domain level gives a global view of the protein interaction complex and perchance of protein functions. However up until now, only certain domains of the DAPK1 protein have been refined by crystallographic studies and the substrates of DAPK1 and the molecular pathway underlying DAPK-mediated signal transduction still remain unclear to a greater degree (Velentza et al., 2001).

Biochemical studies determine that DAPK1 binds syntaxin-1 by their C-terminal domains and phosphorylates it at Ser 188. This phosphorylation result occurs both in vivo and in vitro in Ca^{2+}-dependent manner (Tian et al., 2003). Pyruvate dehydrogenase complex deficiency (PDCD6), a well-identified apoptotic mediator, has been discovered as a binding partner of DAPK1. Co-transfection of DAPK1 cDNA and PDCD6 into a tumour cell line hastened apoptosis via the caspase-3 dependent pathway (Sidransky et al., 2002).

DAPK1 has been found to interact with extracellular-signal regulated kinase one (ERK1) and Extracellular-signal regulated kinase two (ERK2) through a specific docking sequence within its death domain. At Ser735, the phosphorylation of DAPK1 by ERK2 enhances the catalytic activity, in vivo and in vitro of DAPK1. DAPK1 promotes the apoptotic activity of DAPK1 (Chen et al., 2005). More unlike, DAPK1 plays a key role in oxidative stress-induced c-Jun N-terminal Kinases (JNK) signaling via protein kinase D (PDK), which has been acknowledged as a unique substrate of DAPK1 (Eisenberg-Lerner & Kumchi, 2007). It was similarly discovered
that small linear peptide interactions domains or docking motif play a significant role in DAPK1 activities. Immunobinding assays proved that DAPK1 can bind to the full length human Mitotic spindle assembly checkpoint protein one B (MAD1B), thereby stimulating autophagy and blebs in cell membrane (Harrison et al., 2008). DANGER, a novel protein recognized on the reason of its cleaving to inositol 1, 4, 5-trisphosphate receptor (1P3R) protein, contains partial monoclonal antibody 21 (MAB-21) domains. Binding of DANGER directly to DAPK1 catalytic activity and DANGER knock-out mice parade augment neuronal and non-neuronal cell death both in vitro and in vivo (Harrison et al., 2008; Kang et al., 2010).

The regulation of programmed cell death physiologically through its inhibition of DAPK1 signaling is via DANGER. DAPK 1 besides is responsible for the phosphorylation of Prostatic intraepithelial neoplasia one (Pin1) on serine 71 (Ser 71) in its catalytic active site. Such phosphor fully in actives Prostatic intraepithelial neoplasia one (Pin 1) catalytic activity and inhibits is nuclear site and cellular activities (Lee et al., 2011).

2.5 DIAGNOSIS OF CANCER

Currently, there are many diagnosis tests done to detect cancer. Whether from a general practitioner to a specialist, the choice for cancer detection considers; the age and medical conditions of the person, the type of cancer assumed, the cruelty of the symptoms and preceding results of test (Hamilton & Sharp, 2004).

Diagnostic methods are a physical examination, laboratory test on urine, blood etc., and imagine test such as X-ray, PET/CT, MRI, and Ultrasound, nuclear medicine scans such as bone scans, etc., Endoscopy, Genetic tests. Examples are Barium Enema for diagnosing colorectal cancer. Biopsy examination is the most accurate analysis of all cancer tissues currently. Magnetic
Resonance Imaging (MRI) for breast cancer detection, Computed Tomography (CT) scan detect tumour and help determine the stage of cancer. Mammography is used to take an X-ray picture of the breast, this helps locate lumps in the breast for breast cancer detection. Pap smear/test helps diagnosis of cervical cancer by the changes in the cells of the woman cervix. Tumour marker tests are conducted to identify higher levels of biomarkers in blood, urine or body tissues of suspected cancer patients. miRNA is also useful in the diagnosis of cancer (Tie et al., 2009).

Many people die as a result of late diagnosis. A cancer diagnosis has many challenges. Almost all these diagnostic tools listed above are useful after tumourigenesis which affects better treatment. The ability of the person to make meaning of the diagnosis and to adjust to the changes in lifestyle is also a challenge (Hamilton & Sharp, 2004). The future of cancer diagnosis is better as scientists are developing new and extensive methods and machines in the diagnosis of cancer.

Examples are peptidomics which analysis peptides in biological fluids by mass spectrometry and Nanoparticles which has unique biological properties given their small size and large surface area to volume ratio, permitting them to absorb, carry and bind compounds such as small molecular drugs, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins and some probes with high coherence (Thakor & Gambhir, 2013). A better approach of diagnosis is still anticipated as early detection or diagnosis will impact much positively on treatment.
2.5.1 BREAST CANCER DIAGNOSIS

Many of the different breast cancers can be diagnosed by microscopic analysis of a biopsy or tumour sample of the affected breast areas. There are other breast cancers which require specialized laboratory examinations. The commonest methods of investigating breast for cancer include; physical examination by a health care provider and mammography. These two methods can describe an approximate likelihood that a lump is present and may also detect some other lesions such as a simple cyst (Saslow, 2004).

After screening, further test is conducted on sample to reach conclusive diagnosis. Health care provider draws fluid from the lump for microscopic analysis. A fine needle aspiration and cytology-FNAC is used to help conclude the diagnosis. Cancerous cell from the fluid drawn from the lump are screened for under the microscope. Most fluids with cancerous cells are bloody whiles clear fluids drawn from lump are mostly not cancerous. Other options for biopsy include a core biopsy or vacuum- assisted breast biopsy (Yu, 2010), which are protocols in which a section of the breast lump is excised or biopsy in which the entire lump is removed.

There are new ways of diagnosing breast cancer which includes; imaging by ultra sound or MRI. This gives an additional method that helps in diagnostic and basic treatment of breast cancers.

2.6 CANCER TREATMENT

Cancer survivors increase in number by the day due to the aging and growth of population and improvements in treatments. Many cancer survivors must subsist with prolonged effects of treatment as well as physiological apprehensions such as horror of re-occurrence (Krilaviciute et
(Hanahan & Weinberg, 2011).

There are many options to methods of cancer treatment. The methods are grouped into two namely; known cancer treatments and future cancer treatments. Examples of the known cancer treatments include surgery and use of modern technology. It involves removal of tumors and sometimes the lymph nodes by surgery. Modern technology that is used with surgery for treatment to prevent spread and reoccurrence of tumors include Magnetic Resonance Imaging (MRI) scan, Computed Tomography Scan (CT), Ultrasound scan etc. Chemotherapy, Hormonal therapy, Radiation therapy, Adjuvant therapy, Immunotherapy are all used after surgery to help in treatment and prevention of spread (DeSantis et al., 2014).

Targeted cancer treatments like growth signal inhibitors, drugs that induce apoptosis and endogenous agio inhibitors help to treat cancer. Growth in the knowledge of cancer biology has led to remarkable progress in cancer early treatment. Cancer research is currently advancing in so many ways such as Antiangiogenic chemotherapy, Nanotechnology, RNA expression profiling and Proteomics (Singh et al., 2010; Murawa et al., 2013).

2.7 IMMUNOHISTOCHEMISTRY

The diagnoses of infectious and neoplastic procedures of animal species are recurrently done by Immunohistochemical technique. Early detection, diagnosis and prognosis of diseases can now be better detected by biomarker using Immunohistochemical technique. Lately, detection of diseases using immunohistochemistry has been stretched to the analysis of biomarkers that are applied as surrogate endpoints in assessing gene therapy as well as in forecasting clinical responses to other treatments (Seidu et al., 2013). From the inception of immunohistochemistry
(IHC), several reports on applications and methodological changes have been known to be published (Ramos-Vara & Beissenherz, 2000). The emphasis on diagnostic immunohistochemistry has progressed from determination of the cell derivation of malignant tissues towards detecting prognostic markers of possible value in miscellany of therapy (Shi et al., 1993).

### 2.7.1 TECHNIQUE OF IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) is considered for detection of cellular or tissue elements (antigens) by way of antigen-antibody complexes that can be envisaged beneath microscopes by using labelled antibodies. From the inception of fluorescence labelled antibody method by Coons, many labels, enzymes, ferritin, as well as colloidal gold, has been conjugated to antibodies for immunohistochemistry both at the light and electron microscopic levels (Coons et al., 1955). IHC methods involve numerous procedures which have been polished to attain quality staining effects in terms of background staining and sensitivity. Methods such as antibody production, antibody purification, tagging, detection and tissue fixation and processing have progressed remarkably quickly. An exception is the method which involves formalin fixation which progressed quite slowly until there was an improvement by Shi who introduced heat induced antigen retrieval (HIAR) (Shi et al., 1991).

### 2.7.2 ENZYME LABELED-ANTIBODY TECHNIQUE

In this technique, an enzyme is attached to a dye and both are attached to the antibody and are detected by the imaging tool. Horseradish peroxidase (HRP) and calf intestinal alkaline phosphatase (AP) are standard enzymes for labelling. HRP is the most broadly used in amalgamation with chromogen, 3, 3-diaminobenzidine tetra hydrochloride (DAB) at both the light and electron-microscopic status (Yamashita, 2007), as a result the dark brown DAB
reaction deposits that give high-contrast end-products, which are insoluble with organic solvents such as ethanol and xylene, and stable during long-term storage.

2.7.3 HORSERADISH PEROXIDASE-LABELED ANTIBODY TECHNIQUE

The HRP-labelling technique was devised by Nakane and associates (Nakane & Pierce, 1967). In this technique, Antibodies are conjugated to a dextran or amino acid backbone which contains a number of enzymes for detection. Currently there is the production of antibodies with very large number of enzyme molecule for highly sensitive immunoreaction as well as that is bound to antibody to increase dispersion into tissues over conjugates with elevated polymers.

2.7.4 PEROXIDASE-ANTI-PEROXIDASE (PAP) TECHNIQUE

This technique utilizes a peroxidase anti-peroxidase complex. It involves two anti-peroxidase antibodies and three peroxidase molecules and the complex is used as a third coating in the staining method to hold on to the unconjugated primary antibody by a second antibody, which is applied so that one of its two identical binding sites bind to the primary antibody and the other binds to the PAP complex. Alkaline phosphatase has also been used in this method and it follows the same principle (Mason & Sammons, 1978; Cordell et al., 1984). The indirect technique making use of either fluorescence or Horse raddish peroxidase (HRP) conjugate is much less sensitive comparative to peroxidase-anti-peroxidase technique as reported by Sternberger et al. 1970, reported that the peroxidase-anti-peroxidase (PAP) method is much more sensitive than the indirect method using either fluorescein- or HRP-conjugated antibodies (Sternberger et al., 1970). They thought so because of the absence of chemical crosslinking in the preparation of the PAP immune complex which does not require chemical crosslinking and special separation technique of unlabelled enzyme and antibody. With the use of the PAP, there has been a definite quality and provides repeated immunostaining.
2.8 ANTIGEN RETRIEVAL (AR)

Antigen Retrieval (AR) is an uncomplicated, economical, and effective process that leads to suitable IHC staining results in FFPE tissue for a larger numeral of antibodies verified (Shi et al., 2011). This involves the unmasking of the obscured antigenic epitopes that have been masked as a result of formalin fixation (Al-Sanabani, 2013).

At the end of antigen retrieval immunoreactivity is restored and the antigens present in tissues are made accessible to be bound by antibodies which can then be demonstrated by Immunohistochemical stains. There has been the proposal of several hypotheses pertaining to the mechanism of antigen retrieval (Shi et al., 1993), and most of the recent studies in immunohistochemistry has focused on explaining the mechanism of antigen retrieval (Sompuram et al., 2006, Rait et al., 2004).

Until the advent of antigen retrieval, immunohistochemistry was not universally executed on formalin-fixed sections. Back dating to 1975, Huang’s research in IHC was conducted using a protease induced antigen retrieval approach was used to expose antigens in formalin-fixed tissues, but there was little accomplishment due to non-particular digestion of antigens (Huang, 1975). Shi et al., in 1991 then introduced a heat-based antigen retrieval technique.

This method centered on the theory by Fraenkel-Conrat and Olcott that chemical reactions between proteins and formalin can be reversed by heat or alkaline hydrolysis (Fraenkel-Conrat & Olcott, 1948).
2.8.1 HEAT INDUCED ANTIGEN RETRIEVAL METHOD

This method of antigen retrieval utilizes heat/ increased temperature for the retrieval of concealed antigenic epitopes as a result of the aldehyde fixation. It has gained a lot of popularity as and has become the preferred method of antigen retrieval after it was first introduced by Shi et al., in 2011.

Sources of heat in this method include microwave, pressure cooker, hot water bath, tissue floatation bath, domestic electric kettle, autoclave among others. The main effect of heat is probably the transfer of energy that disrupts the protein–protein crosslinks (Ronci et al., 2008).

In addition, the chelation of bound tissues calcium ions and other divalent cations might be also convoluted in the antigen retrieval mechanism making use of buffer and heat (Ramos-Vara & Beissenherz, 2000; Seidu et al., 2013). Several investigators have researched into which retrieval method is best and can be used as standard in immunohistochemistry but have still not come to a definite conclusion.

2.9 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA is a plate oriented assay system planned for identifying and computing peptides, proteins antibodies and hormones. In ELISA, an antigen is rendered immobile to a solid surface and then complex with an antibody that is connected to an enzyme. Recognition is achieved by assessing the conjugate enzyme activity via incubation with a substrate to produce a measurable product (Lui et al., 2016).

The most crucial element of the detection strategy is a highly specific antibody-antigen interaction to form a complex. ELISA can mostly be performed in a 96-well or 384 well
polystyrene coated plates, which will passively bind antibodies and proteins. It is this binding and immobilization of reagents that makes ELISAs not cumbersome to design and execute. Having the reactants of ELISA stagnated to plate surface makes it stress-free to design and execute the test. Having the reactants of the ELISA stabilized to the microplate surface makes it easy to detached non-bound from bound material during the assay (Bishop et al., 2015; Cao et al., 2013).

The ability to wash away nonspecifically bound materials makes the ELISA a potent instrument for measuring specific analytes within a rudimentary preparation (Liu et al., 2017).

2. 9.1 CATEGORIES OF ELISA

ELISA can be accomplished with a number of alterations to the elementary procedure: direct, indirect, competitive or sandwich procedure. The prime phase, immobilization of the antigen of attention, can be completed by direct adsorption by the assay plate or incidentally via a captured antibody that has been attached to the plate. Direct enzyme labeled primary antibody or indirectly enzyme-labeled secondary antibody are ways of identifying antigens in a sample (Ding et al., 2017).

Alkaline phosphatase (AP) or horseradish peroxidases (HRP) are the usual labels for the detection of antibodies. Large selections of substrates are accessible for executing the ELISA with a Horseradish peroxidase (HRP) or Alkaline Phosphatase (AP) conjugate (Bishop et al., 2015). The choice of substrate is dependent on the desired assay reactivity and the equipment obtainable for signal-detection such as spectrophotometer, fluorometer or illuminometer. Among the standard assay formats, the concern was differences in both capture and recognition. It is relevant to distinguish between the precise strategies that exist specifically for the detection
step. However, the capture of an antibody to the plate by direct adsorption to the surface or through a pre-coated detention antibody is as in a sandwich ELISA (Cao et al., 2013).

### 2.9.2 COMPETITIVE ELISA

The significant event of competitive ELISA which is the process of competitive reaction between the sample antigen and antigen bound to the wells of a microtiter plate with the primary antibody. Another name for competitive ELISA is inhibition ELISA. Paramount, the primary antibody is incubated with the sample antigen. After an incubation period, any unbound antibody is washed off (Liu et al., 2017).

The more antigens in the sample, the more primary antibody will be bound to the sample antigen. Therefore, there will be smaller amount of primary antibody available to bind to the antigen coated on the well, resulting in a signal reduction (Han et al., 2014). Key merit of this type of ELISA arises from its high reactivity to compositional alterations in complex antigen mixtures, even when the specific detecting antibody is current in moderately small amounts (Zhang et al., 2007).
CHAPTER THREE

3.0 METHODOLOGY

3.1 EXPERIMENTAL DESIGN

The experimental design was case-control study design.

3.2 STUDY SITE

The study was conducted in Korle-Bu Teaching Hospital. This hospital is the leading national referral center in Ghana and it is presently the third largest hospital in Africa. Pathology and Surgical departments were the sites of study. The Department of Surgery which is one of the early departments of the hospital was where samples were collected from. A total number of 15,100 cases are averagely attended to every year at General Surgical Specialist clinic alone; 10600 cases averagely at the Genito-Urinary clinic; and 4600 cases averagely at the Neurology clinic. It is by far the largest department of the hospital with a bed capacity of 612 and a staff of 860. The Department of Pathology is made up of two units; the Histology and Cytology unit. The department started in the early 1980’s with about four Pathologists and six (6) medical laboratory scientist. Currently, the department has nine (9) Pathologists and Twelve (12) Medical Laboratory Scientists. A total number of 5000 cases averagely are diagnosed yearly. Cases commonly diagnosed are breast biopsies, prostate biopsies, and fibroid biopsies. Recently, cases of gastric cancers are on the rise.

3.3 ETHICAL CLEARANCE

Two ethical clearances were sort. The first was sort from School of Biomedical and Allied Health Sciences of the college of Health Sciences and ethical clearance was granted with the number SBAHS: MD./10550649/AA/5A/2016-2017 (Appendix I). The second clearance was
3.4 STUDY POPULATION

Participants were patients diagnosed with breast cancer at Korle-bu Teaching Hospital. Controls were patients with breast lumps declared not positive for breast cancer. Control blood samples were apparently healthy individuals.

3.5 SAMPLING METHOD

Sampling method was by convenient sampling.

3.5.1 RECRUITMENT OF PARTICIPANTS

Permission was granted by the nursing officer in-charge of the chemotherapy when copy of the ethical clearance and introduction from the hospital was presented to her. The study and what it seeks to achieve was explained to the patients. Upon agreement to be part of the study, patients signed a consent form (Appendix IV) to be recruited as participants. Participants were further grouped into pretreatment and post treatment. The post treatments were participants who were receiving treatment being administered with chemotherapy drugs after being diagnosed with breast cancer and pretreatment participants were the ones who have not received treatment yet after being diagnosed and were first time visitors to the chemotherapy department.

3.5.2 SAMPLE COLLECTION PROCEDURE

Samples used for this study were blood and solid breast tumour samples. Blood (five millilitres) were taken from participants who have consented to be part of the study at chemotherapy suit at Surgery Department in Korle Bu Teaching Hospital. Five (5) milliliters of blood were drawn
into five milliliters (5 mls) serum separator or gel tubes. The blood samples were drawn at the chemotherapy suit when the veins of participants were punctured using cannula in preparation for their treatment. The preparation of the participant for the treatment was done by the nursing officers in the surgical department. These were done to prevent double puncturing of the veins of the participant. The nursing officers administered the chemotherapy treatment through a vein. Each sample was first taken before the nursing officer administers treatment, thus not to subject the patient to the needle twice which would have exposed the patient to pain twice. That would not be best practice especially when sample can be taken from the same cannula which was inserted before treatment. The pathology identification numbers were taken from their pathology reports and their tumour tissue blocks and slides were retrieved from the the Pathology department. All tissue samples were rescreened and grades confirmed by pathologist. Retrieved blocks of participants were prepared for sectioning to obtain four (4) microns think of tissue section which were placed of poly lysine coated glass slides.

3.6 SAMPLE PROCESSING/PREPARATION

3.6.1 IMMUNOHISTOCHEMICAL STAINING

Immunohistochemical staining uses horse raddish peroxidase-streptavidin technique for protein localization. The Immunohistochemical staining was divided into three phases. The phases were; pre-analytical phase, Analytical phase and Post analytical phase. The Pre-analytical phase included; sample procurement followed by, trimming, embedding and ending with tissue microtomy. In this study, re-embedding and microtomy were done as pre analytical stage. This was because the samples were archival. Analytical phase included; dewaxing of tissue sections include pre-incubation steps (example bring section down to water, antigen retrieval, blocking of nonspecific activities), incubation with the primary antibody, and labeling of the antigen-
antibody reaction and ends with slide counter stain and coversliping. Post-analytical; includes interpretation of results and generation of IHC report and its evaluation.

3.6.2 MATERIALS AND REAGENTS.

Breast tumour tissues were 32 malignant breast tumours and 32 non-malignant breast tumours, Phosphate-buffered saline (PBS), at 4°C. Blocking buffer (PBS; containing 2% fraction V bovine serum albumin), at 4°C. Primary DAPK 1 antibody, (Boster Biological Technology company limited, Republic of China), Biotinylated secondary (Reagent B, 6mls, Boster Biological Technology company limited, Republic of China). Mayer’s hematoxylin (Sigma-Aldrich, United Kingdom), Horse raddish peroxidase-streptavidin detection system (Reagent 6mls, Boster Biological Technology Company limited, Republic of China). Sheep-Goat Serum working fluid (Reagent A, 6 mls, Boster Biological Technology Company limited, Republic of China). X10 concentrated citrate buffer, Pasteur pipets (number used 10). Calibrated pipets (10-100 ul and 100-1000 ul), DPX Mountant, (500 mls, Gain Chemical company, Lot: 1002), poly lysine Coated slides, Pressure cooker, (5 Litres, Boffo Wrought Aluminum) and electric hot plate, (Binatone, Son number MPR/EE/0037), Hydrophobic pen (PAP pen), to prevent the spread of regents to other parts. Cotton, Microtome (Leica RM 2125RT, China), microtome blade (stainless seal), Floatation bath (cat. No, 14792; Boekel scientific, Taufkir, Germany), Constructed humidified chamber.

3.6.3 PRE-ANALYTICAL PHASE.

200 archival samples of clients diagnosed with malignant breast tumours and benign breast tumours were retrieved after they signed consent forms and their blood samples were taken at the chemotherapy suit of the Surgical Department. The tissue blocks had already been taken
through fixation using 4% formaldehyde, cut-up, processing (using increasing gradients of ethanol, one troths 50%, one 70%, two troths 90%, two troths of absolute ethanol and xylene for clearing) and finally into the wax on the carousel processing machine (fully automated), processing over-night. Tissues were oriented in the metallic mold and embedded at the embedding chamber at hot plate temperature of 65°C and a cold plate temperature of -5°C.

The microtome used was designed for stainless steel blades which were placed in a blade holder. The block (embedded tissue) were fixed in a chuck and adjusted to four (4) microns. Three (3) thin sections size were cut from each block and an extra section was cut for negative control. Another three (3) sections were cut from a breast tissue block which tested positive during antibody trials and was used as a positive control. Using camel brush, thin sections of tissues were placed on a floatation bath containing water at a temperature of 45°C (over heating the water would destroy the tissue). Tissue sections were picked with poly lysine coated slides slanted at an angle of 60°C. The sections were ready for the analytical phase. Sections were kept away from sunlight to prevent the loss of antigenicity.

3.6.4 ANALYTICAL PHASE.

Dewaxing was done on a hot plate a temperature of 50°C for 5 mins after which sections were brought down to water: this was done by first immersing the tissue sections in xylene for five (5) minutes, then in four (4) changes of ethanol with decreasing concentrations (absolute ethanol, 90% ethanol, 70% ethanol and 50% ethanol) for 5 mins each with agitation. The tissues were then immersed in water.

Fixation and tissue processing modified the three (3) – dimensional structure of proteins, which could render antigens undetectable by specific antibodies because the immunological reactions
depended on the conformation of the antigen. The masking of the antigens were broken using a pressure cooker as shown in figure 1 and 2. Citrate buffer was half diluted 1:10 and put in a coplin jar and placed in the pressure cooker. Distilled water was filled to the level of the diluted citrate buffer in the coplin jar and made to boil to boiling point on a hot plate. When the water reached boiling point, the pressure cooker was opened and slides were placed in the coplin jar and covered and made to boil for 5mins. Pressure cooker was put off and made to cool then the slides were finally brought out and made to cool.

Figure 1 Opened pressure cooker on a hot plate with a coplin jar for antigen retrieval
Figure 2 Closed pressure cooker on a hot plate with a coplin jar for antigen retrieval

Slides were arranged on constructed humidified chamber as shown in figure 3 below. The slides were flooded with 3% hydrogen peroxide (H₂O₂) in deionized water for ten (10) minutes to block endogenous peroxidase activity. Slides were rinsed with Phosphate Buffer Saline three times for three minutes each. Reagent A (Bovine Serum Albumin) was flooded on the sections for 10 minutes at room temperature. The DAPK 1 primary antibody was diluted (1:50 µl) with phosphate buffered saline and was dispensed on the sections except the negative control and kept for five (5) hours at room temperature.

The sections were rinsed with PBS three (3) X for three (3) minutes. Biotinylated secondary antibody was flooded on the sections at room temperature and incubated for fifteen (15) minutes. Slides were rinsed with PBS three (3) times for three (3) minutes. Horse raddish peroxidase (reagent c) was dispensed on the sections at room temperature and incubated for ten (10) minutes. Slides were rinsed with PBS three (3) minutes for three (3) times. Detection of the
immune reaction was done with developing reagents using DAB and H₂O₂. Preparation of working reagent of DAB was prepared by the addition of one (1 ml) of reagent 2 (DAB substrate solution) to one drop (50 µl) of reagent 1 (H₂O₂).

![Coated slides](image1)

![Constructed humidified rack](image2)

**Figure 3 shows constructed humidified staining rack with labeled poly lysine coated slides**

Slides were rinsed thoroughly with tap water. Counterstaining was done using haematoxylin. Staining was done by first bringing the sections down to water; this was done by first immersing the tissue sections in xylene for 5 minutes, then in 4 changes of ethanol with decreasing concentrations (absolute ethanol, 90% ethanol, 70% ethanol and 50% ethanol) for 5 minutes each with agitation.

The samples were immersed in Mayer’s Haematoxylin (Sigma-Aldrich) for five (5) minutes; they were then moved to tap water to blue. The sections were dehydrated in absolute ethanol, cleared in xylene and mounted in distyrene plasticizer xylene (DPX).
3.6.5 POST-ANALYSIS PHASE

In horse raddish peroxidase methods, all positive staining under light microscope appear in shades of brown. Scoring of the staining reaction was done as follows; sections that did not show any 3, 3-dimethylaminobenzidine tetra hydrochloride (DAB) reaction were considered negative and scored as zero.

Those with mild reaction were scored +1, those with moderate staining reaction were scored +2 and those with strong reaction were scored +3. Two biomedical scientists examined the tissue sections independently, using an Olympus light microscope (Olympus CX31, model CX31RBSF). Micrographs were captured using an Olympus digital camera (model DP20-50) mounted on an Olympus microscope (model BX51 TF).

3.7 ENZYME-LINKED IMMUNOSORBENT ASSAY

3.7.1 MATERIALS AND REAGENTS.

Sealing film (two (2) pieces 96) (GenWay ELISA kit; Republic of China); Enzyme coated plate /ml LX 96, Human DAPK1 Standard 48ng/ml (0.5ml X 1 bottle) (GenWay ELISA kit, Republic of China), DAPK 1 Standard/Sample diluent (6ml X 1 bottle)(GenWay ELISA kit; Republic of China), ELISA reagents (6ml X 1bottle) (GenWay ELISA kit, Republic of China), Human DAPK 1 Chromogen solution Reagent A liquid (6ml X 1bottle) (GenWay ELISA kit, Republic of China), Human DAPK 1 Reagent B solution (6ml X 1bottle) (GenWay ELISA kit, Republic of China), Termination solution (6ml X 1bottle) (GenWay ELISA kit Republic of China), Concentrate the washing solution (20mls X 25 times) x 1 bottle (GenWay ELISA kit, Republic of China), Human DAPK 1 Str-HRP conjugate reagent (6mls X 1bottle) (GenWay ELISA kit). ELISA machine; VarioskanLux Thermo scientific. Spectrophotometer, SN:3020 RFF:3020 Vacc
50/60Hz 200VA Thermo Fisher scientific Oy. Rastastie 2, Finland. Pipettes; 1. Finnpippette multichannel (5-50ul, 12tips) Minder Asset Management 0008599, 2. Gilson Precision pipette (10-200); Inc 1(800) 656-2770, Tech JSIGLER M 11980N

Thermo scientific incubator,SHKA4000-7 MaxQ 4000; Disposal tips (200 pieces), Double distilled water, clean tube or Eppendorf tube (50 pieces), Absorbent paper, 500ml beaker.

Kit composition: The sample dilution is 0.05M PBS. The stop solution is 2M H₂SO₄; the washing solution was PBST containing 0.15% Tween-20, if not enough, self-prepare.

3.7.2 EXPERIMENTAL PRINCIPLE

The kit used the competition or inhibition method to detect the content of the death-associated protein kinase one (DAPK1). Wells were pre coated and labeled with DAPK 1 antibody in advance. Samples were added to horse radish peroxidase-labeled recognition antigens in the wells and incubated at 37°C for one (1) hour. Samples compete with solid-phase antigen to form immune complexes. After PBST was washed, the combined HRP catalyzed and converted TMB (Tetramethylbenidine) which was blue in colour to yellow under the action of the acid at 450 nm wavelength suction.

3.7.3 SAMPLE PRE TREATMENT

Blood sample (5 mls) were collected into serum separator and allowed to clot at room temperature and centrifuged at 2500 rpm, for 20 minutes. The serum was collected into other serum separators. Sample was stored at -20°C and was allowed to thaw for thirty (30) minute when ready to run test. Quantity of serum (50µl) used for the test was pipeted into eppendorf tubes as shown in figure 4:
3.7.4 DETAILED OPERATING PROCEDURES

An ELISA 96 well was hand washed to clean and condition the wells for antigen antibody interactions. Horse raddish peroxidase (HRP) holes were first washed with PBS and dried on wash paper. 100 µl diluted PBS solution was gently dispersed in the wells and shaken gently for thirty (30) seconds, then poured off. Further drying was done using dry paper or pad. It was repeated five (5) times.

Design for sample arrangement in well was drawn as shown in figure 5 below. A1 and G1 wells were considered as blank and no samples were added. Reagent A, B and Termination solution were added for zero readings. Wells 2A and 5A were labeled as standard. 50ul of diluted standard was added to each well, and 50 µl enzyme reagent was added to the same wells. Six (6) wells A4-B9 were labeled as controls. The wells contained sera of non-malignant breast tumour sera (as control samples). 50 µl of enzyme reagent and standard reagents were dispensed into the same well. Wells B10-H1 was labeled as sample wells. Sera from malignant breast tumour were put in the wells and DAPK1 reagent was added to it as shown below in figure 5:
Figure 5 shows DAPK 1 pre coated 96 well plate with samples in the wells

It was gently shaken for 20 seconds, after it was covered with the sealing film and incubated at 37°C for sixty (60) minutes. PBS was diluted X10 with distilled water and allowed to stand for a 10 minutes. The film seal is pulled off and the solutions in the wells were discarded, dried with tissue paper. Each well was filled with the PBS working solution and allowed to stand for 30 seconds. The washing was repeated for five (5) times and dried on the tissue (Thermo Scientific, 2010; Engvall & Perlmann, 1971) as shown in figure 6 and arrangement of sample in plate was keyed in the analyzer as shown in figure 7
Figure 6 shows pre coated DAPK 1 96 wells and a multi channeled pipette after results have been read.

Figure 7 shows the investigator labeling the wells on the computer of the ELISA machine: VarioskanLux Thermo Scientific as it appeared in the wells.
CHAPTER FOUR

4.1 RESULTS

4.1.1 DEMOGRAPHICS OF PARTICIPANTS

Individuals diagnosed with breast cancer and receiving treatment at chemotherapy department were the participants. They were sixty-four participants in number. Thirty-two (32) were cases and thirty-two (32) were controls. All participants were females living within Greater Accra and Central regions of Ghana. Ten (10) of the cases were petty traders, fifteen (15) were unemployed and seven (7) of the cases were government workers. The mean age of the breast cancer participants was forty-five (45). The mean age for non-breast cancer participants was forty (40). The highest breast cancer diagnosis made was invasive ductal carcinoma with the commonest grade being grade two.

4.2 RESULTS FOR DAPK 1 IN SERA MEASURED WITH ELISA

The results for DAPK 1 protein in sera were summarized into malignant, pretreatment, post treatment and non-malignant. The DAPK 1 protein was measured in concentration (ng/ml). The table 1 blow shows the results of the total number of participants, mean and standard deviation.
Table 1: The Table below shows the Sample types, their Means and Standard Deviations

<table>
<thead>
<tr>
<th>SAMPLES</th>
<th>NUMBER</th>
<th>MEAN± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre treatment</td>
<td>32</td>
<td>4.2457±1.9201</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>4.6756±1.9041</td>
</tr>
<tr>
<td>Post treatment</td>
<td>17</td>
<td>5.1918±1.9591</td>
</tr>
<tr>
<td>Non-malignant</td>
<td>32</td>
<td>4.9498±1.5302</td>
</tr>
</tbody>
</table>

Result for t-test for sera of malignant and non-malignant breast tumour was 0.110. Result for t-Test for sera of pre-treatment and post treatment malignant breast tumour was 1.000.

Table 2 below shows the significant values of all the sample types.

Table 2: The Table below shows Sample types and their Significant Values

<table>
<thead>
<tr>
<th>SAMPLE TYPE</th>
<th>SAMPLE TYPE</th>
<th>SIGNIFICANT VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-malignant</td>
<td>Malignant</td>
<td>0.11</td>
</tr>
<tr>
<td>Non-malignant</td>
<td>Pretreatment</td>
<td>1.00</td>
</tr>
<tr>
<td>Non-malignant</td>
<td>Post treatment</td>
<td>0.23</td>
</tr>
<tr>
<td>Pre treatment</td>
<td>Post treatment</td>
<td>1.00</td>
</tr>
</tbody>
</table>
4.3 IMMUNOHISTOCHEMISTRY

Results for immunohistochemistry sections were scored by two qualified biomedical scientists and there were no inter observable differences.

Results scored for immunohistochemistry sections were grouped into malignant and non-malignant. Table 3 below includes the total of scores for expression of DAPK 1 and the P-value.

Table 3: The Table below Shows Immunohistochemistry results for DAPK1

<table>
<thead>
<tr>
<th>SAMPLE SIZE</th>
<th>DAPK 1 RESULTS</th>
<th>TOTAL</th>
<th>P. VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>MALIGNANT</td>
<td>8</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>NON-MALIGNANT</td>
<td>28</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>36</td>
<td>19</td>
<td>6</td>
</tr>
</tbody>
</table>

*Shows statistically significant between malignant and non-malignant breast tumour. P-value=0.000008.

4.3.1 ANTIBODY RESULTS

The negative control showed no brown coloration in the cytoplasm of the cells as shown in Figure 8 whiles the positive control showed brown colouration within the cytoplasm of the cells as shown in Figure 10. Included is a test slide Figure 9 showing brown colouration
Figure 8: A micrograph of negative control slide of a breast tissue after Immunohistochemical staining at X 20 magnification showing no brown colouration

Figure 9: A micrograph of test slide of a breast tissue with brown display of colour in the cytoplasm after performing Immunohistochemical staining at X 20
Figure 10: A micrograph of positive control slide of a breast tissue with brown display of colour in the cytoplasm after performing Immunohistochemical staining at X 20 magnification
4.3.2 SAMPLES OF THE RESULTS FOR IMMUNOHISTOCHEMICAL STAINING.

The micrograph below shows a no staining slide which was scored 0 by both biomedical scientists. Stained slide was viewed at magnification of X 20. This is seen as Figure 11 below:

![Micrograph of tissue with a score of zero representing no staining at X 20 magnification](image)

**Figure 11:** A micrograph of tissue with a score of zero representing no staining at X 20 magnification
The micrograph below was scored +1 showing low staining intensity and few stained cells. The micrograph was taken with a magnification of X 20. This micrograph is Figure 12.

Figure 12 A micrograph of a tissue with a +1 score showing low staining intensity at X 20 magnification
Figure 13 A micrograph of stained tissue with a score of +2 representing moderate staining intensity at X 20 magnification

The micrograph for +3 with high intensity and high number of stained cells were scored by both biomedical scientists and is shown below at a magnification of X 20. The micrograph is labeled Figure 14:
**4.4 Tumour Grades and DAPK1 expression**

One (1) grade one tumours, representing 3.13%, 25 samples for grade two representing 78.1% and 4 samples for grade three representing 12.5% and grade x was 2 samples representing 6.25%. The variable differentiation of the tumours I had did not have any impact on the expression of the DAPK 1 protein as variable grades showed different staining intensity across the various tissues with grade two could show a staining intensity of +3 which was the highest of our score and grade three tissues showing a score of +2. All grades were confirmed by a pathologist.
CHAPTER FIVE

DISCUSSION AND CONCLUSION.

5.1 DISCUSSION

Death Associated Protein Kinase One (DAPK 1) is a protein that mediates a range of activities from autophagy, immune responds and DNA fragmentation resulting in programmed cell death. This range of activities of death associated protein kinase one (DAPK1) is considered as the normal functions of the protein in a wild type cell (Zhao et al., 2015).

No work had been done on the protein DAPK1 in tissue and serum considering its use as a diagnostic marker for breast cancer. Due to the increased number of breast cancer cases all over the world and the mortality rate on the increase, the issue of late diagnosis played a major role hence the issue of early diagnosis will contribute to saving many lives. This study, sought to test for the protein in African women, using Immunohistochemistry and ELISA. Further work was done to compare the DAPK 1 protein expression in breast cancer tissues and non-malignant breast tissues.

There were variable expressions of DAPK 1 protein from low to high expressions of the protein in the breast cancer tissues but there were no traces of expression of the protein in the non-malignant breast tissues. The levels of the DAPK 1 proteins expressed in the non-malignant tissues were as a result of some of the cells undergoing apoptosis which is one of the ways through which wild type cells expresses the DAPK1 protein. This was because the nucleus of the cells of the tissues showed pyknosis, karyohexis and traces of karyolysis (Zhao et al., 2015). There was significance difference statistically of the expression of the DAPK 1 between breast cancer tissues and non-malignant breast cancer tissues as the DAPK 1 was over expressed in the
cytoplasm of the cells. The significance value was 0.000008 which is lesser than our P-value \( \leq 0.05 \). The results concerning the expression of the protein in breast cancer tissues are not different from Zhao et al., 2015 as both expressed the DAPK 1 protein in the cytoplasm of breast cancer tissues.

There was presence of the protein DAPK 1 in the sera of the breast cancer clients but no presence in the sera of non-cancer clients. This was not surprising as the participants were not having any form of tumour or mass that could lead to the leakage of the protein from the tissue as a result of necrosis. The malignant breast cancer tumour had the presence of the DAPK 1 in their serum due the effect of the chemotherapy drugs. The activities of drugs have the capacity to cause the lysis and shrinking of the tumour cells there by releasing DAPK 1 protein into the serum (Prioetti et al., 2017).

It could also be the metastatic nature of the breast cancer cells and the growth of the breast cancer tumour as it can lead to pain and destruction of nearby cells as well as tumour cells leading to cell membrane disruption and release of serum in the blood. There was no statistical difference between DAPK 1 protein levels in the sera of breast cancer patients and apparently health people. The statistical P-value was 0.110 and our P-value was P\( \leq 0.05 \). The analysis was done using T test. The results of significance were different from work done by Arko-Boham et al., (2017) on higher serum concentrations of Vimentin and DAPK 1 are associated with aggressive breast tumour phenotypes in Ghanaian. This difference might be due to different sample size as they used larger sample. Lui & Chu (2016), also did some work on promising non-invasive biomarkers in serum for early detection of gastric cancer and they reported no statistical significance with the reason low sample size.
There were participants that had been administered with the chemotherapy drugs such as Doxorubicin, Cyclophosphamide, Paclitaxel, Trastuzumab, 5-fluorouracil. The activity of these drugs is to shrink the tumour cells and also destroy the metastatic cancerous cells in the blood. The knowledge of how these drugs work informed me to have our participants in to Pretreatment and Post treatment.

Fifteen (15) participants were gotten for pretreatment representing 46.9% and 17 for post treatment representing 53.1%. The statistical value was 1.00 which was higher than my P-value \( \leq 0.05 \) hence was not significant. The results suggested that the chemotherapy drugs did not have any effect on the DAPK1 protein per the time the samples were analyzed. This could be due to the quick degradation of the chemotherapy drugs after being administered and the long duration they are booked for their next clinic. The statistical value was 1.000 which was higher than our P-value \( \leq 0.05 \).

What contributed to insignificance might be low number of participants. In 2016, Lui & Chu (2016) did a study on promising non-invasive biomarkers in serum for early detection of gastric cancer reported no statistical significance with the reason of low sample size. This may not be different from mine as low participant numbers may have impacted on the statistical significance of that part of serum portion of this study.

The expression of DAPK1 was compared between post treatment and apparently healthy individuals and statistical value of 0.22 was gotten. This was not significant comparable to P-value \( \leq 0.05 \). This might be due to low sample size.

Further, the expression of the DAPK1 protein was compared between pretreatment and apparently healthy individuals and there was no statistical significance as the value was 0.22.
This could be due to low participants. There was no data to do comparison as no work has been done comparing the protein in serum taking into consideration the effect of chemotherapy drugs or anticancer drugs.

In this study, the main aim of investigating DAPK1 as a non-invasive marker for breast tumour aggression and progression was achieved by the over expression of the protein in tissue and serum of breast cancer clients. All breast cancer tissues stained positive and intense colourations whiles the non-malignant showed no staining to trace of stains. The protein was also expressed in the serum of the breast cancer clients but not in the apparently healthy individuals.

5.2 CONCLUSION

DAPK 1 protein expression levels were elevated in malignant breast tumours whiles there was no expression levels in non-malignant breast tissue participants. The levels of DAPK 1 in sera of both breast cancer and non-breast cancer showed no elevation. The grades of breast cancer tumour had no influence on the expression levels of the DAPK 1 protein in tissues. In conclusion it can be confidently said that Death Associated Protein Kinase One (DAPK 1) is over expressed in breast cancer tumours and its potential use as a biomarker for breast cancer is relevant.

5.3 LIMITATIONS

The conclusion that has been made can only be attributed to DAPK 1 in serum and tissues of breast cancer patients based on the procedures and protocols that were followed from literature to arrive at findings, hence findings from this report cannot be generalized.
5.4 RECOMMENDATIONS

1. I recommend that studies should be done on the expression of the protein DAPK 1 in other cancers such as prostate and gastric cancers in Ghana.

2. I recommend that further studies should be conducted on the expression of the protein DAPK 1 in the serum and tissues of pregnant women diagnosed with breast cancer.

3. I recommend that studies be done on the effect of chemotherapy drugs on the levels and expression of DAPK 1 protein in diagnosed breast cancer patient receiving chemotherapy treatment.

4. I recommend that further studies be done to determine the levels of DAPK 1 protein in post treatment and pretreatment clients using a larger sample size.
REFERENCES


Fraenkel-Conrat, H. & Olcott, H. S. 1948. The reaction of formaldehyde with proteins. V. Cross-linking between amino and primary amide or guanidyl groups. *Journal of the American Chemical Society*, 70, 2673-2684.


Lee, T H., Chen, C H., Suizu, F., Huang, P., Schiene-Fischer, C., Daum, S., Zhang, Y J., Goate,


Lui, X., & Chu, K.M. (2016). Circulating Cell-free DNAs and miRNAs as promising non-


O'Neil, S., Ji, L., Buranahirun, C., Azoff, J., Dhall, G., Khatua, S., Patel, S., Panigrahy, A.,


the catalytic domain of human protein kinase associated with apoptosis and tumor suppression. *Nature Structural & Molecular Biology*, 8, 899-907


World Cancer Report International Agency for Research on Cancer. (2008) Retrieved 26 February 2011. (Cancer stastics often exclude non-melanom skin cancer such as basal-cell carcinoma, which are common but rarely fatal).


125(7), 2707–2720.
APPENDIX I: ETHICAL CLEARANCE FROM SCHOOL OF
BIOMEDICAL AND ALLIED HEALTH SCIENCE

UNIVERSITY OF GHANA
SCHOOL OF BIOMEDICAL AND ALLIED HEALTH SCIENCES

Ref. No.: ………………………………………….

Mr. Bright Afriyie Owusu,
Dept. of Medical Laboratory Sciences,
SBAHS,
Korle Bu.

Dear Mr. Owusu,

ETHICS CLEARANCE


Following a meeting of the Ethics and Protocol Review Committee of the School of Biomedical and Allied Health Sciences held on Tuesday, 14th, March, 2017, I write on behalf of the Committee to approve your research proposal as follows:

TITLE OF RESEARCH PROPOSAL: INVESTIGATION INTO DEATH-ASSOCIATED PROTEIN KINASE ONE (DARK 1) AS NON-INVASIVE MARKER FOR BREAST TUMOR

This approval requires that you submit three-monthly review reports of the protocol to the Committee and a final full review to the Committee on completion of the research. The Committee may observe the procedures and records of the research during and after implementation.

Please note that any significant modification of the research must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this research to the Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee’s duty to review the ethical aspects of any manuscript that may be produced from this research. You will therefore, be required to furnish the Committee with any manuscript for publication.

This reviewed report is valid till 31st, August, 2017

Please always quote the ethical identification number in all future correspondence in relation to this protocol.

Thank you.

Yours sincerely,

Dr. S. D. Amanquah
(Chairman: Ethics and Protocol Review Committee)

Cc: Dean
Head, Dept. of Medical Laboratory Sciences
School Administrator

COLLEGE OF HEALTH SCIENCES

P. O. Box KB 143, Korle Bu, Accra, Ghana.

* Telephone: +233 (0) 302 687 974-5 * Email: sbohs@ug.edu.gh * Website: www.sbohs.ug.edu.gh
APPENDIX II: ETHICAL CLEARANCE FROM KORLE-BU TEACHING HOSPITAL

BRIGHT OWUSU-AFRIYIE
SCHOOL OF BIOMEDICAL AND ALLIED HEALTH SCIENCES
UNIVERSITY OF GHANA, KORLE BU

“INVESTIGATION INTO DEATH-ASSOCIATED PROTEIN KINASE ONE (DAPK1) AS A NON-INVASIVE MARKER FOR TUMOR AGGRESSION AND PROGRESSION”

KBTH – IRB /000100/2016

Investigator: Bright Owusu-Afriyie

27th June, 2017, the Korle-Bu Teaching Hospital Institutional Review Board (KBTH IRB) reviewed and granted approval to the study entitled “Investigation into Death-Associated Protein Kinase One (DAPK1) as a Non-Invasive Marker for Tumor Aggression and Progression”

Please note that the Board requires you to submit a final review report on completion of this study to the KBTH-IRB.

Kindly, note that, any modification/amendment to the approved study protocol without approval from KBTH-IRB renders this certificate invalid.

Please report all serious adverse events related to this study to KBTH-IRB within seven days verbally and fourteen days in writing.

This IRB approval is valid till 30th May, 2018. You are to submit annual report for continuing review.

Sincere regards,

DR. DANIEL ANKRAH
DEPUTY CHAIR (KBTH-IRB)
FOR: CHAIR (KBTH-IRB)

Cc: The Chief Executive Officer
Korle Bu Teaching Hospital

The Director of Medical Affairs
Korle Bu Teaching Hospital
APPENDIX III: LETTER OF PERMISSION FROM DEPARTMENT OF PATHOLOGY, KORLE-BU

SCHOOL OF BIOMEDICAL AND ALLIED HEALTH SCIENCES
COLLEGE OF HEALTH SCIENCES
DEPARTMENT OF PATHOLOGY

Phone: +233-21-661302, 669605
Fax: +233-21-668286
E-mail: Pathology@chs.ug.edu.gh

My Ref. No.
Your Ref. No.

The Chairman
Ethical and Protocol Committee
College of Health Sciences
Korle-Bu

Dear Sir,

LETTER OF PERMISSION - BRIGHT AFRIYIE OWUSU

Mr. Bright Afriyie Owusu is an MSc Professional Medical Laboratory Student in the Department of Pathology, School of Biomedical and Allied Health Sciences, College of Health Sciences. He has sought permission to work with samples (blocks) in the Department and has been granted. He would be conducting his research in the Department on the topic “Investigation into Death Associated Protein Kinase One (DAPK1) as Non-invasive Marker for Breast Tumour”.

Thank you.

Prof R. K. Gyasi
HEAD OF DEPARTMENT

P O Box 4236
Accra.

24TH March, 2017

INTEGRIPROCEDAEAMUS
APPENDIX IV: CONSENT OF PARTICIPATION IN THE
STUDY.

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<th>PERSONAL DETAILS.</th>
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<td>PERSON TO NOTIFY IN CASE OF EMERGENCY.</td>
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APPLICANT INFORMATION

TITLE: INVESTIGATION INTO DEATH ASSOCIATED PROTEIN KINASE ONE AS A NON-INVASIVE MARKER FOR TUMOUR AGGRESSION AND PROGRESSION.

PRINCIPAL INVESTIGATOR: MR. AFRIYIE OWUSU BRIGHT

ADDRESS: UNIVERSITY OF GHANA. GRADUATE SCHOOL.

GENERAL INFORMATION ABOUT WORK.

This study is about a protein called DAPK1 which plays a useful role in normal cell environment but in mutated cells is believed to contribute to the growth and severity of cancer cells. The study seeks to measure levels of this protein in blood and tumour tissue of same participants and to access the impact of the protein in the growth of cancer. This will help inform treatment and contribute to early detection.

POSSIBLE RISK AND DISCOMFORT.

The pain from the pinch of the needle when blood is been drawn from the vein.

POSSIBLE BENEFITS

The study will help in early diagnosis of breast cancer and other cancers, helping to reduce the lives lost and the amount of money spent on treatment because of late diagnosis.

CONFIDENTIALITY

Clients will be assured confidentiality. The information acquired will be kept from public access. Identification numbers will used to code the information as well as samples that will be taken from participants. After research, information gathered will be kept under lock and key.
VOLUNTARY PARTICIPATION

Patient has the right to accept, refuse or opt out of the research at any time of the process and will not be abused verbally or physically.

CONTACTS FOR ADDITIONAL INFORMATION

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<tr>
<th>1. DR. ADU-ARYEE</th>
<th>2. DR. ARKOH-BOHAM BENJAMIN</th>
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<td>HEAD OF SURGERY.</td>
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<td>KORLE BU TEACHING HOSPITAL</td>
<td>LECTURER</td>
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YOUR RIGHT AS A PARTICIPANT.

This research has been reviewed and approved by the Institutional Review Board of Korle Bu Teaching Hospital for Medical Research (KBTH-IRB). If you have any questions about your right as a research participant you contact the IRB office between the hours of 8am-5pm through the landline 0302660766 or email address: rdo@kbth.goo.gh
VOLUNTARY AGREEMENT

The above document describing the benefits, risks, and procedures for the research title INVESTIGATION INTO DEATH ASSOCIATED PROTEIN KINASE ONE AS A NON-INVASIVE MARKER FOR TUMOUR AGGRESSION AND PROGRESSION has been read and explained to me. I have been given an opportunity to have given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

…………………………………………………                           ………………………………
Name and signature or Mark of parent or guardian                             Date

If volunteer cannot read the form themselves, a witness must sign here. I was present while the benefits, risks and procedures were read to take part in the research.

…………………………………………………                           …………………………………
Name and signature of witness                                                           Date

I certify that the nature and purpose, the potential benefits and possible risks associated with participating in this research have been explained to the above individual.

…………………………………………………                           ………………………………
Name and Signature of person who obtained consent.                                   Date
## APPENDIX V: INFORMATION SHEET

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