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PROGNOSTIC SIGNIFICANCE OF NUCLEAR FACTOR KAPPA B (p65) AMONG BREAST CANCER PATIENTS IN CAPE COAST TEACHING HOSPITAL

ABRAHAM MENSAH

2024

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PROGNOSTIC SIGNIFICANCE OF NUCLEAR FACTOR KAPPA B (p65) AMONG BREAST CANCER PATIENTS IN CAPE COAST TEACHING HOSPITAL

BY

ABRAHAM MENSAH

Thesis submitted to the Department of Microbiology and Immunology, School of Medical Sciences, College of Health and Allied Sciences, University of Cape Coast, in partial fulfilment of the requirements for the award of Master of Philosophy degree in Infection and Immunity

AUGUST, 2024

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DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this University or elsewhere.

Candidate's Signature: …… Date…….

Name: Abraham Mensah

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

Supervisor's Signature…… Date……

Name: Dr. Roland Osei Saahene

Supervisor's Signature …… Date ……..

Name: Professor Leonard Derkyi-Kwarteng

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ABSTRACT

Breast cancer is known to be aggressive among Africans including Ghanaian women with high mortality. Nuclear factor kappa B (NF-kB), a transcription marker has been linked to malignant tumors including breast cancer. However, the underlying molecular mechanism remains unclear with no known published data on NF-kB prognostic significance among breast cancer patients in Ghana or any other African country. This study aimed to assess the prognostic significance of NF-kB (p65) expression and its association with various clinicopathological features in breast cancer patients at Cape Coast Teaching Hospital (CCTH). A cross-sectional study design and purposive sampling procedure were used to obtain breast cancer tissues from 90 breast cancer patients and normal breast tissues from 15 individuals without breast cancer for NF-kB (p65) examination by immunohistochemical technique and data was analyzed by SPSS version 25. The study revealed that NF-kB (p65) was expressed in 86.7% of breast cancer tissues with a significant relationship to tumor grade, proliferation index (Ki67) and molecular subtype. In terms of high-level expression, tumor grade 3 was about 10 times that of grade 1 (54.2% versus 5.1%), and Ki67 > 20 was 79.7% compared to 20.3% for Ki67 ≤ 20. Triple-negative breast cancer patients had 49.1% overexpression of NF-kB (p65) compared to 17% and 25.4% for luminal A and luminal B respectively. Only 8.5% high expression of NF-kB (p65) was seen in HER 2-enriched cases. There was no strong association between NF-kB (p65) expression and other clinicopathological parameters such as age, hormonal receptors, laterality, lymphovascular invasion, perineural invasion, pathological lymph node stage, pathological tumor stage and HER 2. The AUC predictive value of NF-kB (p65) was 0.919 with a sensitivity of 83% and specificity of 84% ($p < 0.0001$). This first study in Ghana (Africa) shows that NF-kB (p65) is highly expressed among breast cancer patients in CCTH-Ghana and has a link to tumor grade, Ki67 and molecular subtypes. NF-kB (p65) might affect the prognosis of breast cancer patients. To assess the role and linkage of NF-kB (p65) expression as a potential prognostic marker and therapeutic target, additional studies with a bigger sample size and in vitro experiments are required.

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KEYWORDS

Nuclear factor kappa B

Breast cancer

Prognostic significance

Immunohistochemistry

Tumor grade

Ki 67

Molecular subtypes

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DEDICATION

In memory of Madam Beatrice Tawiah

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LIST OF ACRONYMS

BC	Breast Cancer
MBC	Mucinous Breast Cancer
ER	Estrogen Receptor
CSP	Canonical Signaling Pathway
HER 2	Human Epidermal Growth Factor Receptor 2
FISH	Fluorescent In Situ Hybridization
TNBC	Triple-Negative Breast Cancer
LAR	Luminal Androgen Receptor
BL 1	Basal-Like 1
CK	Cytokeratins
SSM	Skin-Sparing Mastectomy
NAC	Nipple-Areola Complex
BCS	Breast Conserving Surgery
RTK	Receptor Tyrosine Kinases
ALND	Axillary Lymph Node Dissection
NF-kB	Nuclear Factor Kappa B
SLN	Sentinel Lymph Node
BRCA	Breast Cancer Gene
RHD	Rel Homology Domain
IKB	Inhibitor of Kappa B
IKK	Iκb Kinase
Eres	Estrogen Response Elements
TNF-A	Tumor Necrosis Factor Alpha
TF	Transcription Factor

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LCC1	Laccase Gene 1
TAM	Tumor Associated Macrophage
FOXA1	Forkhead Box Protein A 1
EMT	Epithelial to Mesenchymal Transition
DAB	3,3'- Diaminobenzidine
EGFR	Epidermal Growth Factor Receptor
HIF	Hypoxia-Inducible Factor
PI3K	Phosphatidylinositol-3-Kinase
IAP	Inhibitors of Apoptosis
NCSP	Non-Canonical Signaling Pathway
NEMO	NF-kB Essential Modulator
MCF7	Michigan Cancer Foundation-7
CXCR4	Chemokine Receptor 4
NIK	NF-kB-Inducing Kinase

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CHAPTER ONE

INTRODUCTION

Breast cancer, a common pathological proliferation of cells in the breast continues to be a global burden among many women. Early screening and detection are key to reducing mortality. However, due to logistic constraints for diagnosis and treatment especially in Africa and some Asian countries, most of the cases are detected at advanced stages resulting in ineffective treatment and mortality (Ginsburg et al., 2020). Current studies suggest that chemotherapy and radiotherapy treatment have some level of effectiveness although it can also increase inflammation and anti-apoptosis by activating Nuclear Factor kappa B (NF-kB); a well-known transcription factor in malignant tumors and chronic inflammation (Kumar Agrawal et al., 2018; Zeng et al., 2020). Therefore, it has become vital for breast cancer research scientists to study the prognostic significance of NF-kB biomarker to improve treatment outcomes. Unfortunately, there is no known published data on NF-kB prognostic significance among breast cancer patients in Africa although the disease is rising on the continent. This research seeks to fill this knowledge gap by providing baseline data for future therapeutic options.

1.0 Background of the Study

Breast carcinoma is the most prevalent cancer among women aged 20 to 50 years worldwide. Additionally, it ranks among leading women's mortality statistics (Iacoviello et al., 2021). Breast cancer (BC) exceeded lung cancer as the world's most common tumor in the Global Cancer statistics for 2020 with annual cases continuing to rise (Britt et al., 2020; Sung et al., 2021).

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A 2018 report by Adeloye shows that there is evidence of growing BC cases in Africa although the generalization of these projections is questionable due to a lack of proper data in several regions (Adeloye et al., 2018). While the Southern African sub-region has the highest incidence rate, the Western African sub-region has the greatest burden when both onset and death rates are taken into account. However, the Central and Eastern African regions have seen the greatest increases in prevalence since 2008 (Azubuike et al., 2018). There is also some disagreement about the nature of the BC characteristic pattern and the precise determining factors. There is an indication that sociodemographic factors are the main drivers that contribute to its incidence and mortality (Azubuike et al., 2018).

In Ghana, just like other developing countries, BC is increasingly becoming common and cases are mostly detected in advanced stages owing to a lack of screening for early detection, inadequate health infrastructure, proper national cancer screening programme, and diagnosis (Bonsu & Ncama, 2019; Olson et al., 2016). Again, it is the commonest cancer to kill women in Ghana and often their tumors are aggressive in nature and associated with young age (Ohene-Yeboah & Adjei, 2012). Additionally, the overall survival rate after five years is still below 50% with no improvement despite molecular advances and non-conventional therapies (Mensah et al., 2016). Furthermore, notwithstanding treatment advances, some patients still experience recurrence and eventually experience metastatic illness, which is typically terminal. Our understanding of the cellular and molecular processes underlying BC development, recurrence and metastasis is not entirely clear; consequently, it is crucial to investigate these mechanisms further in order to identify molecular

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targets that can aid in the development of targeted therapies and/or prognostic prediction.

Recently, the contributions of transcription factors to the development of cancers have been acknowledged. Nuclear factor kappa B (NF-kB), one of the transcription factors with five family members exists as a dimer that binds to specific inhibitors; Inhibitor of kappa B (IKBs), degrade off IKBs under external stimuli via ubiquitin-proteasome pathway and are released, which subsequently translocate to the nucleus and stimulate various target genes (Concetti & Wilson, 2018; Zhu et al., 2017). NF-kB has been linked to chronic inflammation and malignant tumors including BC, through increased expression of target genes (Kumar Agrawal et al., 2018). Furthermore, contrary to expectations, clinical studies have shown that chemotherapy drugs and radiotherapy not only induce cell death in BC but also stimulate this pathway (NF-kB), which stifles caspase cascade signaling by increasing the quantity of anti-apoptotic proteins expressed (Zeng et al., 2020). Inhibiting the NF-kB pathway have been demonstrated in some studies to induce apoptosis, repress proliferation and invasion, and boost chemosensitivity in different kinds of tumor cells (Capece et al., 2020; Kani et al., 2013). Therefore, blocking NF-kB may be a successful cancer therapeutic method. However, there is a general paucity of data on the prognostic worth of NF-kB among BC patients with no known published data in the African context in which genetic makeup and other contributory factors may affect its expression level. This research, therefore, seeks to evaluate the expression levels of NF-kB (p65) among BC patients in Cape Coast Teaching Hospital (CCTH)-Ghana and its association with clinicopathological features.

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1.1 Statement of the Problem

Breast cancer is said to be the second leading terminal disease in women and despite significant advances in BC treatment in the last few decades, BC survival rate of patients leaves much to be desired (DeSantis et al., 2019; Wimmer et al., 2020). Breast cancer (BC), particularly Triple-Negative breast cancer (TNBC), is characterized by no expression levels of progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and estrogen receptor (ER). It typically spread from the tumor site to distant locations, resulting in a poor patient survival rate (Boire et al., 2020; Couch et al., 2015).

Africa is not exempted from the increasing levels of BC (characterized by high fatality rate) due to various socioeconomic factors and late reporting of cases (Adeloye et al., 2018; Azubuike et al., 2018). There is, therefore, the need to understand the pathways involved in BCs better to find alternative therapeutic markers to enhance the prognosis for BC.

NF-kB; a known transcriptional factor that promotes chronic inflammation, tumorigenesis, and anti-apoptosis is gaining attention among BC researchers since most of the chemotherapeutic treatments are interfered with by the NF-kB pathway (Barrios et al., 2021; Zeng et al., 2020).

However, Ghana and the rest of the African countries lacks published data on the prognostic significance of NF-kB (p65) among BC patients even though genetic makeup and other contributory factors may affect its expression level. In order to help close this gap, this research intends to evaluate the prognostic worth of NF-kB (p65) among BC patients in Cape Coast teaching hospital (CCTH).

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1.2 Aim

To determine the prognostic significance of Nuclear Factor kappa B (p65) among breast cancer patients in Cape Coast teaching hospital.

1.3 Specific Objectives

To determine the:

1. Level of NF-kB (p65) expression among the breast cancer tissues.
2. Association between NF-kB (p65) and histological features.
3. Association between NF-kB (p65) and molecular markers; Ki 67, PR, HER 2, ER.
4. Association between molecular subtypes of breast cancer and NF-kB (p65).

1.4 Significance of the Study

Research on the prognostic worth of Nuclear Factor kappa B in BC can provide valuable information for strategic treatment and prevention. Clinical studies have shown that chemotherapy drugs and radiotherapy not only induce cell death in BC but also stimulate the NF-kB pathway, which stifles caspase cascade signaling by increasing the expression levels of anti-apoptotic proteins (Zeng et al., 2020). Therefore, knowing the exact expression levels of NF-kB in BC tissues that have not been subjected to chemotherapy and or radiotherapy treatments can help our understanding of this pathway to improve prognosis in BC patients.

There are rising levels of BC in Africa (Adeloye et al., 2018) including Ghana and early detection and prevention is key to reducing mortality (Bonsu & Ncama, 2019). Notwithstanding this, there is no known published data concerning the prognostic significance of NF-kB (p65) in African BC patients.

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This study may form baseline data for studying NF-kB prognostic worth in Ghanaian BC patients as well as in the African context. Furthermore, this study will provide novel data on NF-kB (p65) expression levels among BC tissues without prior chemotherapeutic or radiotherapeutic treatment in Ghana. Majority of African BC cases are triple-negative which does not respond to current hormonal therapy and/ or chemotherapy drugs. Therefore, this study will provide crucial information on the local and global front on how best to improve targeted treatment and reduce mortality among African BC patients by targeting NF-kB (p65) pathway.

1.5 Delimitations

A cross sectional investigation was conducted and it covers 90 BC tissues and 15 non-BC tissues that served as a control. Tissues with no prior treatment such as chemotherapy and or radiotherapy treatment were included from the CCTH pathology laboratory. Parameters such as tumor size, age, tumor grade, histological type, hormonal receptor status, molecular subtypes and laterality were included in the study. Kumasi Centre for Collaborative Research (KCCR) laboratory was used as a testing center.

1.6 Limitations

The study included only patients tissue samples collected from January 2019 to March 2023 without prior chemotherapy and or radiotherapy treatment. Percentage levels of expression might be different if other previous years were included. NF-kB (p65) was measured by immunohistochemical technique only although polymerase chain reaction testing or Western blot could have been used to further confirm the expression level in the BC tissues. Only p65 was measured and so the expressions of other NF-kB family members were not

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known. Other comorbidities of BC patients that can activates NF-kB were not known.

1.7 Definition of Terms

Nuclear Factor-kappa B: A common quick response transcription factor in immune and inflammatory cells.

Breast Cancer: is the abnormal proliferation of cells that line the breast lobules or ducts.

Immunohistochemistry: a combination of anatomical, immunologic, and metabolic methods to identify cellular components through an antigen-antibody reaction labeled with a distinguishable correspondent molecule**.**

1.8 Organization of the Study

Apart from the abstract, references and appendix, this thesis comprises five chapters. The first chapter discusses the study's background, with emphasis on terms like NF-kB, BC and prognostic significance. In addition, the study's main goal is described, as well as the specific objectives it seeks to achieve. The chapter delves deeper into the concerns that the investigation aims to discuss like the knowledge gap and the purpose of the data generated by the study.

In chapter two, current research from around the world was evaluated and written in accordance with a conceptual model. The main focus was on the gaps in the evaluated literature that needed to be filled, in addition to the methodologies used or modified and why. The chapter provides a global report on the prevalence of BC, diagnostic techniques, NF-kB pathways, and their importance in BC.

The third chapter examines the study's materials and methods. The study setting, and population are all described, along with their clinicopathological

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features. The chapter also describes in detail how samples were obtained, examined, and data analyzed.

In chapter four, the statistically processed data were presented and discussed. The outcome and discussion were centered on how the specific objectives were organized. The final chapter (chapter five) summarizes the thesis's first three chapters before concluding with the research findings and potential interpretations for any inconsistencies in the results. In addition, based on the primary and inconclusive findings, recommendations were given.

1.9 Chapter Summary

The chapter describes the study's background, focusing on keywords like Nuclear Factor Kappa B, breast cancer, and prognostic significance. The primary purpose of the study, as well as specific objectives aimed at addressing the issues are stated. The chapter goes on to discuss the research knowledge gap and the purpose of the study's data.

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CHAPTER TWO

LITERATURE REVIEW

2.0 Introduction

Almost any organ or tissue can develop cancer, which is a broad class of disorders. It starts where there is uncontrolled growth of cells that infect surrounding body parts and or spread to nearby organs. Based on assessment by the World Health Organization (WHO), cancer is one of the major causes of mortality around the globe, amounting to approximately 10 million fatalities in 2020 (WHO, 2020).

Breast cancer (which is a major contributor to total cancer burden globally) does not only affect the rich countries negatively but also the developing nations; putting pressure on healthcare systems, particularly those least prepared to handle this burden in terms of accessibility, diagnosis, and effective treatment. This needs pragmatic steps and a holistic approach to tackle it, especially on the African continent where the disease is known to be more aggressive.

2.1 Breast Cancer Origin

Breast cancer is the abnormal proliferation of cells that line the breast lobules. It is one of the top three cancers worldwide and the main causes of cancer-related deaths in women (Harbeck & Gnant, 2017; Torre et al., 2016). There are no conclusive data on breast cancer origin, despite the believed that breast cancer originates from mutation of mammary bipotent progenitor cells. Some studies also show that germline mutation in breast cancer have a basal-like phenotype while others suggest that pre-neoplastic tissues have higher luminal progenitor cells than normal breast tissues (Zhang et al., 2017).

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2.2 Breast Cancer Epidemiology

Global, regional and country level epidemiological data on breast cancer shows growing trend which requires urgent attention.

2.2.1 Global Analysis of Breast Cancer

As of 2019, 2.09 million cases of BC have been diagnosed with the highest age frequency of 46.3 per 100,000 globally (Mattiuzzi & Lippi, 2019). Australia and New Zealand have the highest rates with an age-standardized rate (ASR) of 94.2, followed closely with 92.6 ASR by Western Europe, North America (84.8), Northern Europe (90.1), and South-Central Asia (25.9) respectively. Western and Eastern Africa has a rate of 27.9 to 37.3 while South-Eastern Asia and Central America have ASR of 38.1 to 38.3 respectively (Huang et al., 2021). According to Azamjah et al., (2019), BC mortality has considerably risen over the last 25 years, with a slope rate of 0.7 per 100,000 from 1990 to 2015. The report shows that BC affects one out of every eight women in the United States, and by 2050, the global incidence of BC is expected to reach nearly 3.2 million new cases annually (Azamjah et al., 2019). These figures reflect the extent of BC incidence, its wider impact, and the necessity for preventive and therapeutic measures to be implemented as soon as possible (Tao et al., 2014).

2.2.2 Breast Cancer Epidemiology in Africa

A review of 41 studies conducted in 22 selected countries in Africa showed an increased rate of incidence of BC from 2000 to 2015 with 24.5 per 100,000 persons. North Africa had the highest rate of 29.3 per 100, 000 compared to Sub-Saharan Africa (SSA) rate of 22.4 per 100 000 (Adeloye et al., 2018). According to limited available data, BC in Cameroon and Central

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African Republic was 5.8% and 15.27% respectively (Cumber et al., 2017). The prevalence of BC cases in sub-Saharan Africa (SSA) is expected to double by 2050 (Cumber et al., 2017). SSA has the highest mortality from BC with a lower than 40% five-year overall survival when compared to wealthy nations such as the United States, which has an 86% chance of survival (Black & Richmond, 2019; Sung et al., 2021b). A recent research conducted in five SSA countries predicted that early detection and appropriate treatment might avert 28% to 37% of breast cancer-related deaths (McCormack et al., 2020). Prevalence of triple-negative BC meta-analysis of 20 out of 52 countries in Africa in 2022 indicated that 27% of all breast cancer in Africa are triple negative out of which 45.7% (highest) were from West Africa compared to 14.9% (lowest) in Central Africa (Hercules et al., 2022). Though the evidence suggests that the rate of BC is increasing in Africa the generalizability of these figures are questionable due to a lack of data in several regions and, as well as discrepancies in data collation and reliability across existing national cancer centers (Adeloye et al, 2018).

2.2.3 Breast Cancer Epidemiology in Ghana

Breast cancer is rapidly becoming a major public health concern in Ghana with expected new BC cases of 4,645 (20.4%), far exceeding the projected 2,062 incidence rates in 2012, with roughly half of them dying (Breast Care International, 2023). Earlier reports suggest that one out of every eight patients die from the disease and was the most common cancer-related death among women in Ghana (Amoako et al., 2019; Ohene-Yeboah & Adjei, 2012). BC is estimated to affect 76 out of every 100,000 Ghanaian women and is expected to rise as the country's population ages and women embrace modern lifestyles (Naku Ghartey Jnr et al., 2016; Obrist et al., 2014). According to

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previous studies, BC in Ghana is increasingly becoming prevalent among younger women, with late-stage stage presentation of the disease (Ohene-Yeboah & Adjei, 2012; Scherber et al., 2014). Lack of screening for early BC detection, inadequate health infrastructure, proper national cancer screening programme and diagnosis serves as a barrier that results in the late presentation (Bonsu & Ncama, 2019). Myths and misconceptions that BC disease is incurable, it is caused by witchcraft or evil spirits, it is a spiritual disease, is the result of a person's failure, that women die from a mastectomy are some reasons why they report at a late stage (Breast Care International, 2023).

2.3 Anatomical Structure, Cells and Functions of the Breast

In general, a normal breast has around 15 to 20 lobes in each breast. They are organized like daisy petals. Each lobe has numerous smaller structures known as lobules. These generate a large number of tiny milk-producing bulbs. There are also ducts directed toward the nipple, which is situated at the center of a dark-skin region named the areola. The lobes, lobules, and bulbs are connected via ducts. The areas between the lobules and ducts are filled with fat. While there are no muscles within the breast, muscles can be found beneath each breast and around the ribs. Again the breast structure has lymph vessels connecting to lymph nodes and are found in groups beneath the arm (https://www.Hopkinsmedicine.org, accessed on 21/4/23). An image of breast anatomy is depicted in Figure 2.1.

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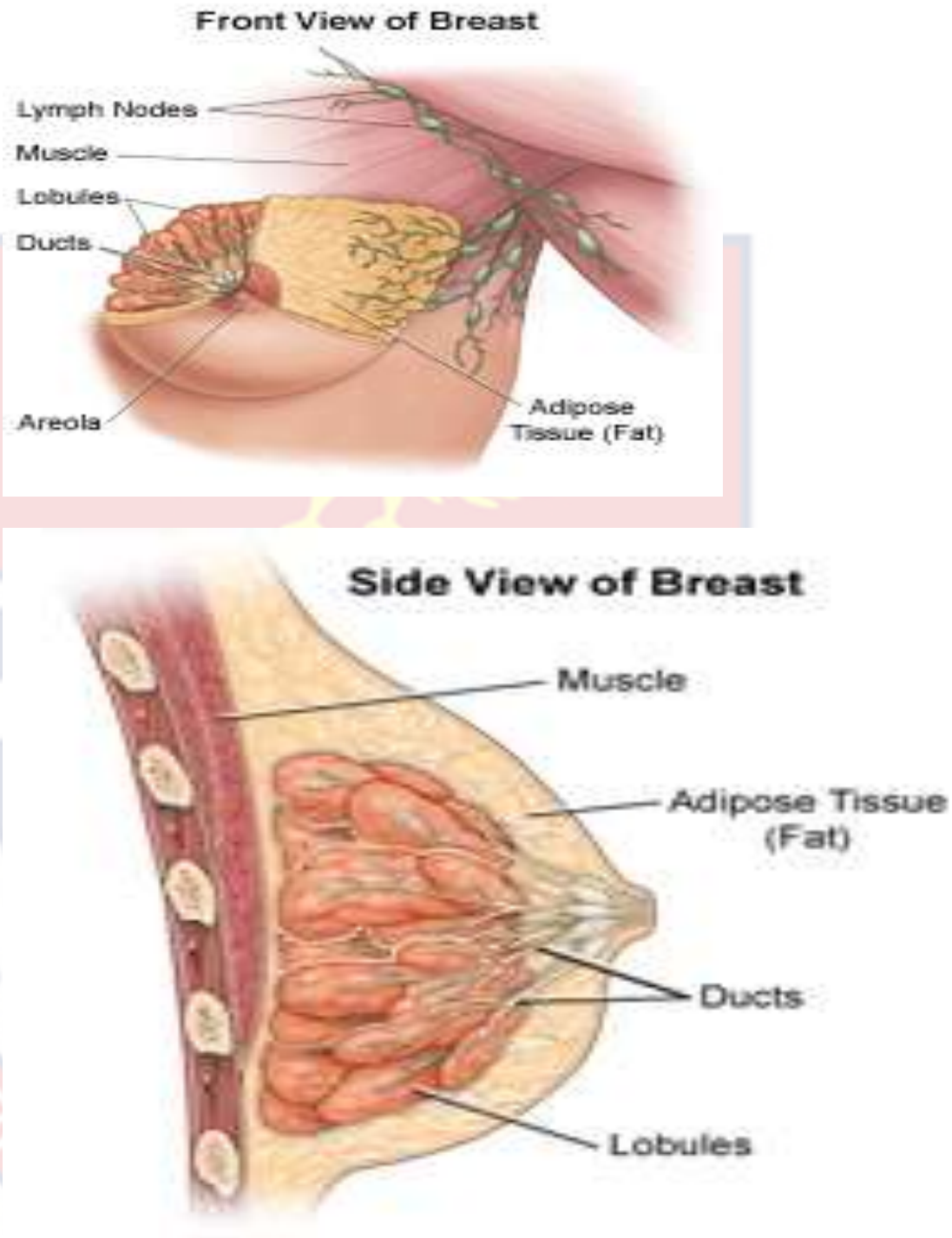

Figure 2.1: Anatomy of the breast. (source: https://www.Hopkinsmedicine. org, accessed on 21/04/23).

Again, the various breast structures, cells and functions are provided in Table 2.1 below.

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Table 2.1: Breast Structures/Cells and Functions

Breast structure/cells	Main function
Nipple/ areola	Expulsion of milk
Interlobular stroma	Function in breast size, shape, and mobility
Intralobular stroma	Support of terminal duct luminal unit
Large duct	Milk distribution channel
Terminal duct luminal unit	Myoepithelial cells contract for milk ejection; luminal cells produce milk.
Luminal cells	Milk production and conduit for milk
Myoepithelial cells	Support for the basement membrane, preservation of luminal cell polarity, and contraction for milk ejection
Fibroblast and myofibroblast	Support of epithelial cells and provide majority of breast volume

Source: *https://www.Hopkinsmedicine.org accessed on 21/4/23*

2.3.1 Normal and Malignant Breast Histology

Normal breast tissues mostly form structured glands as depicted in A, whereas malignant breast tissue B (invasive cancer) loses this structured appearance as seen Figure 2.2

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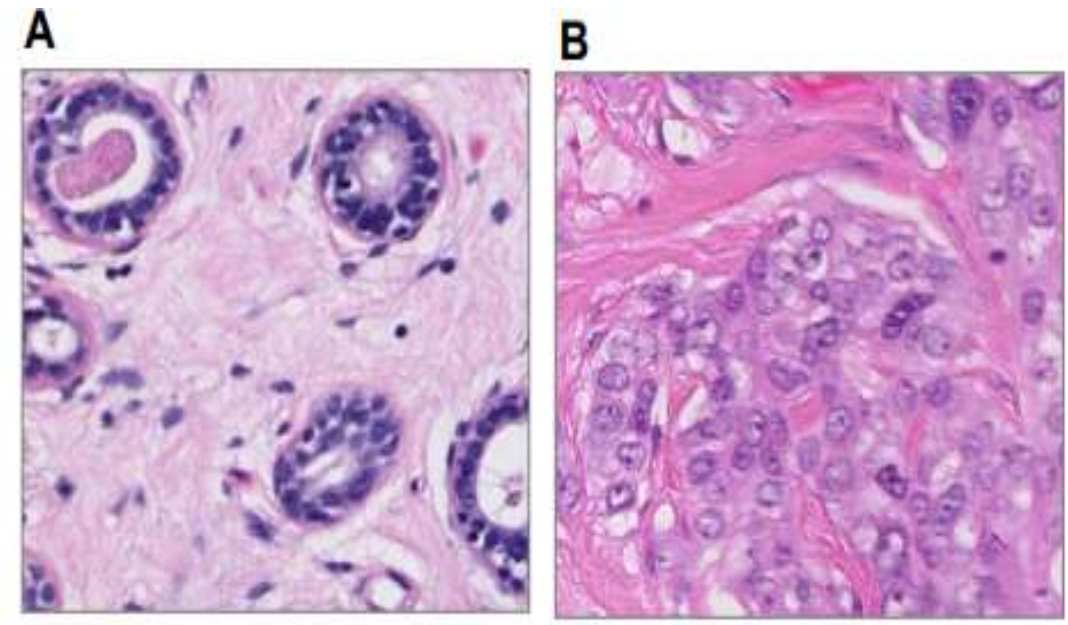

Figure 2.2: Image of normal breast histology (A) and malignant breast histology (B) (adopted from Bennett, 2014).

2. 4 General Risk Factors for Female Breast Cancer

Historically, BC has been associated with numerous risk factors. It includes those that can be altered and those that cannot be altered. Some of the risk factors that cannot be altered are female gender, old age, early menarche, menopause, exposure to early radiation, family history, breast density, race/ethnicity, breastfeeding, genetic mutations, pregnancy and non-cancerous breast diseases. Some of the factors that can be modified include hormonal replacement therapy, diethylstilbestrol, alcoholism, smoking, lack of vitamin supplements, being overweight or obese and consumption of processed foods (Łukasiewicz et al., 2021).

2.4.1 Gender

The major factor that increases the likelihood of BC is the female gender. Women are estimated to have a 100-fold greater likelihood than males to develop BC over their lifetime. The greater frequency of BC in women mainly results from an increase in both progesterone and estrogen stimulation.

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In postmenopausal women, the quantity of androgens and estrogens secreted has been demonstrated to be closely related to the risk of BC. Furthermore, women who are yet to have onset of menopause and those who are in the post-menopausal transition have more sex reproductive hormone fluctuations in their lifetime than men, which puts them at a higher chance of developing breast cancer (Abdelwahab Yousef, 2017; Łukasiewicz et al., 2021; Key et al., 2013).

2.4.2 Age

Various research shows that women over 50 (years) are diagnosed with BC most often, and the likelihood of getting breast cancer rises with age (Feng et al., 2018; McGuire et al., 2015). There is also a link between the diagnosis's age and the overall survival. A woman's chance of survival is lower when BC is discovered before age 50 than when it is diagnosed between 50 and 70 (McGuire et al., 2015). Nevertheless, there are more cases of extremely aggressive TNBC in younger individual patients aged 40 and below (Admoun & Mayrovitz, 2022).

2.4.3 Hereditary

Breast cancer is thought to be hereditary in around 25% of cases. An acquired mutation in either the BRCA1 and/or BRCA2 genes is the most prevalent variant of hereditary BC. These genes facilitate the synthesis of proteins that help healthy cells repair DNA damage. These genes' mutations can cause atypical cell growth, which may result in cancer (Shiovitz & Korde, 2015).

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2.4.4 Ethnic Origin

Differences in ethnic origin and racial background continue to be prevalent among breast cancer patients; the biological processes underlying this observed trend are unknown. Overall, white indigenous women continue to have a very high incidence rate whiles mortality is high among black women with a low survival rate (American Cancer Society, 2016, Yedjou et al., 2019).

2.4.5 Others Miscellaneous Risk Factors

Breast cancer evidence suggests that it is significantly increased in first-degree relatives. Replacement hormonal therapy, obesity, physical inactivity as well as alcohol consumption and smoking have all been linked to an increased risk of BC. The reproductive history of women also influences risk, with nulliparity associated with higher rates than multiparity (Admoun & Mayrovitz, 2022).

2.5 Diagnosing Breast Cancer

A tissue biopsy of the affected area is required to diagnose BC after an abnormal mammogram test. If the existence of BC is established pathologically, the cancer is further evaluated by assessing tumor stage, nodal involvement, and metastases (Cserni et al., 2018). The presence or absence of biomarkers such as hormonal receptors of estrogen and progesterone or HER2 production are also examined. Treatment choices such as surgery, chemotherapy, and radiotherapy are then guided by these assessments (Cserni et al., 2018).

2.6 Histopathological Classification of Breast Cancer

Histology continues to remain as the benchmark for diagnosis and the bedrock for the classification of BC despite molecular and genetic advances. There have been various changes in breast cancer classification throughout the

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years, with the recent WHO classification of breast cancer in 2019 (Cserni, 2020). Some of the histopathological classifications include the following:

2.6.1 *In-situ* Carcinomas

In-situ carcinoma is a type of early-stage carcinoma that is not invasive and thus has no potential to spread. It is presently characterized on the account of myoepithelial cell layer encompassing the neoplastic proliferation. This simply means that normal breast structures such as ducts and acini are either entirely or partially packed with tumor cells but do not continue to spread further. This was formally divided by WHO classification as "ductal carcinoma *in-situ* (DCIS) and lobular carcinoma in-situ" (Tan et al., 2020).

2.6.2 Mucinous Breast Cancer

Mucin production and extracellular mucin presence are the main features of mucinous BC (MBC). About 4% of all BC cases are MBC. It is also known as colloid carcinoma. Based on the degree of cellularity, it has two subtypes (pure type and mixed typed) (Budzik et al., 2021).

A. Over 90% of the tumor mass is made up of the "pure" type (PMBC), which is entirely composed of tumor cells with extracellular and intracellular mucin. PMBC tumors can also be divided into two types: hypocellular tumors (PMBC-A) with cribriform, micropapillary, papillary, tubular, or cordlike growth patterns, and hypercellular tumors (PMBC-B) with solid nests of floating cells (Budzik et al., 2021).
B. The mixed type (MMBC) has less than 90% mucin and infiltrating elements like ductal or lobular breast cancer-like elements (Budzik et al., 2021).

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2.6.3 Invasive Breast Carcinoma of No Specific Type

Invasive ductal carcinoma, not otherwise specified was its previous name. It is an umbrella category of cancers that lacks clear characteristics that set them apart from other histological kinds of breast cancer (Cserni, 2020).

2.6.4 Carcinomas with Apocrine Differentiation

This type of carcinoma has cells with unique cell borders, plenty of fine eosinophilic or vacuolated cytoplasm, large round to oval nuclei, and prominent nucleoli are characteristics of apocrine differentiation, according to light microscopic morphology (Cserni, 2020).

2.6.5 Other types

Carcinomas with a papillary morphology, metaplastic carcinoma, invasive lobular carcinoma, invasive cribriform carcinoma form part of many other breast cancer histology categorizations by different organizations. However, clinical pathologists are more practical in their methods, generally differentiating between ductal and lobular tumors and occasionally a mixed ductal and lobular from other types (Cserni, 2020).

2.7 Histopathological Indicators for Prognosis

There are numerous histopathological indicators of breast cancer that relate to the physical characteristics of the tumor and the stage of the cancer's progress. These variables are used in diagnostics to stage tumors and identify tumors that have distinct prognoses, which allows the best treatment to be chosen.

2.7.1 Tumor Size

The size of breast tumor has traditionally been utilized to stage breast cancer as well as assist suggestions for treatment. It is mostly divided into three

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(< 2 cm, 2-5 cm, >5 cm) categories, although no specific category is well-defined for tumor size. It serves as a predictive factor for both general and cancer-free survival. The higher the size (≥5 cm) the poorer the prognosis (Bennett, 2014; Livingston-Rosanoff et al., 2019).

2.7.2 Tumor Grade

The grade of a tumor is calculated by rating each of its morphological characteristics such as the development of tubules, the number of mitoses (dividing cells), and the size and shape of cellular nuclei, with each score ranging from 1 to 3. This is later combined for a final score of 3 to 9. Grade 1 has aggregates score between 3 and 5 and is most favorable (the cells have a slower growth rate and resemble normal breast tissue), grade 2 has a combined score between 6 and 7 and has intermediate growth between grade 1 and 3, whereas grade 3 has a combined score between 8 and 9 and is less favorable (are likely to multiply and spread more quickly) (www.cancer.org, accessed on 01/06/23). A diagrammatic representation of breast tumor grade is depicted below in Figure 2.3

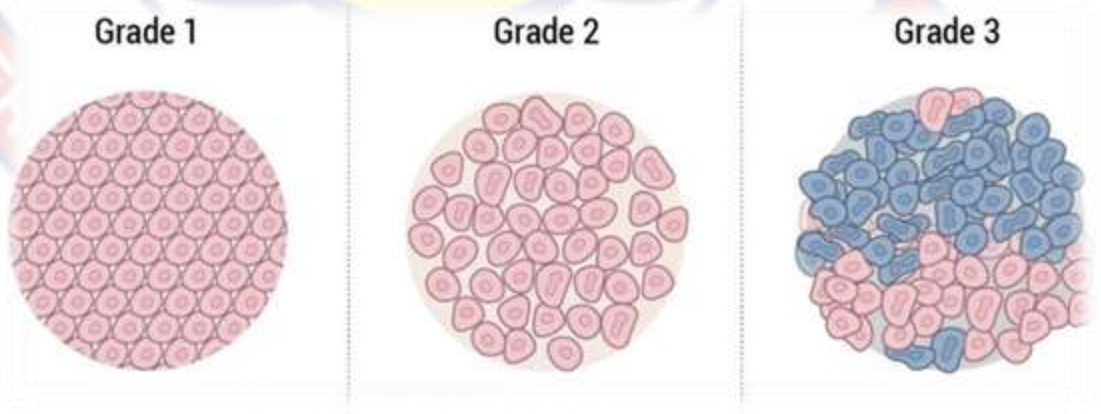

Figure 2.3: A diagram showing various breast tumor grades (source: https://www.Hopkinsmedicine.org, accessed on 21/04/23)

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2.7.3 Nodal Status

One of the best indicators of prognosis for BC in its early stages is the status of the regional lymph nodes. Patients with negative lymph nodes have about 15–30% lower risk of breast cancer recurrence. In contrast, those who have lymph node involvement have about a 70% chance of recurrence. The prognosis is less favourable with increasing node involvement (Bennett, 2014)

2.7.4 Metastasis

Metastasis is simply the spread of a tumor to other organs. 10% or less of newly diagnosed BC show metastases or spread to other organs and about 30% of people with early-stage BC will experience metastasis. Bone is the most frequent distal site, followed by the lung, the brain, and then the liver. Treatment for metastases is supportive and intends to extend life while controlling symptoms and improving quality of life (Bennett, 2014).

2.7.5 Tumor Stage

There are two main staging categories; pathological staging and clinical staging. The pathologic stage examines tissue that was removed during surgery. When prompt or comprehensive surgery is not possible, malignant tumors may occasionally be given a clinical stage based on the findings of a physical examination and imaging tests to aid in treatment planning (www.cancer.org, accessed on 01/06/23)

The BC staging method most frequently employed is the “American Joint Committee on Cancer” (AJCC) tumor-node-metastasis (TNM) system. The AJCC staging system was updated in January 2018 to involves 7 key parameters; the tumor size (T), lymph node (N), metastasis (M), hormonal

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status (ER and PR), HER 2 status and Grade (G) (www.cancer.org, accessed on 01/06/23).

In staging tumors in breast cancer, the letter T, accompanied by numbers from 0 to 4, indicates the size of the primary tumor and whether it has spread into the skin or the chest wall beneath the breast. Higher T values indicate a larger tumor and/or a wider spread to breast-regional tissues. The table below explains each parameter relating to tumor size (T) staging.

Table 2.2 : Various Designation of Tumor Size (T) Staging in Breast Cancer

Tumor size (T) staging	Definition
TX	"Primary tumor cannot be assessed"
T0	"No evidence of a primary tumor"
Tis	"Carcinoma in situ (DCIS, or Paget disease of the breast with no associated tumor mass)"
T1	**"**This includes T1a, T1b, and T1c) and is 2 cm (3/4 of an inch) or less across".
T2	"Tumor is more than 2 cm but not more than 5 cm (2 inches) across".
T3	"Tumor is more than 5 cm across"
T4	"Tumor of any size growing into the chest wall or skin. This includes inflammatory breast cancer"

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Similarly, in lymph node staging, this is done based on how the lymph nodes appear under a microscope. If the cancer has spread to nearby lymph nodes, the letters N and a number between 0 and 3 indicate how many lymph nodes are involved. The table below shows the various categorizations (www.cancer.org, accessed on 01/06/23).

Table 2.3: Lymph Node Staging in Breast Cancer

Lymph node staging	Definition
NX	"It is impossible to evaluate nearby lymph nodes".
N0	"Lymph nodes nearby have not been affected by cancer".
N1	"One to three axillary (underarm) lymph nodes have been affected by the cancer, and/or a sentinel lymph node biopsy revealed cancer in internal mammary lymph nodes".
N2	"Four to nine lymph nodes under the arm have become affected with cancer, or the internal mammary lymph nodes have grown larger".
N3 (N3a, N3b, N3c)	"Cancer has spread to at least four axillary lymph nodes and the internal mammary lymph nodes on sentinel lymph node biopsy, or it has spread to at least ten axillary lymph nodes with at least one area of cancer spread measuring more than two millimeters or it has spread to the supraclavicular nodes, which are the lymph nodes above the collarbone, with at least one area of cancerous spread measuring more than 2 mm."

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Furthermore, metastasis staging involve whether or not the cancer has advanced to distant organs, such as the bones, lungs or liver is indicated by the letters M followed by a 0 or 1 as shown in the table below (www.cancer.org, accessed on 01/06/23).

Table 2.4: Metastasis Staging of Breast Cancer

Metastasis (M) stage	Definition
M0	"X-rays (or other imaging tests) and a physical exam revealed no evidence of a distant spread"
M1	"A biopsy of one of these areas demonstrates that cancer has spread and is larger than 0.2mm and has reached distant organs (most frequently the bones, lungs, brain, or liver) as seen on imaging tests or by physical examination"

2.8 Current Biomarkers for Prognosis

In diagnostic laboratories, there are biochemical markers that can be used. These biomarkers which are premised on the existence of particular protein molecules in the tumor, help to predict the prognosis of the client and, when present, make it possible for the decision on suitable therapeutic applications. The following are the common molecular markers used for prognosis in breast cancer

2.8.1 Estrogen Receptor

Estrogen is a female hormone essential for the formation and development of the breast, and as explained earlier many risk factors related to cancer of the breast are linked to estrogen (Hilton et al., 2018). Estrogen receptors (isoforms ERα and ERβ) are nuclear receptors that control gene expression to mediate estrogen actions. Over 70% of BC cases have estrogen

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receptor alpha (ERα), a key transcription factor that promotes the spread of breast cancer cells with oestradiol serving as the primary growth stimulus (Clusan et al., 2021). ER acts as a ligand-activated transcription factor, controlling the expression of many genes associated with cell division and survival, which in BC results in growth and disease advancement. Estrogen binds to ER, leading to the generation of a homodimer that binds to estrogen response elements (EREs) in target genes and controls their expression. The interaction between co-regulators is critical in specific cell signaling. ER also interacts with several growth factor receptors and cell signaling pathways (Clusan et al., 2021).

Immunohistochemistry (IHC) continues to be the recommended and commonly used ER testing strategy. According to 2020 expect recommendations, positive tumor nuclei of 1% to 100% should be considered as ER-positive whereas 1% to 10% is ER low positive with a comment and 1% or 0% of tumor cell nuclei that are immunoreactive interpreted as negative. Additional experts suggest promoting efficient operation, interpretation, and reporting of patients cases with an initial low to no ER staining result, including creating a policy specific to the lab, outlining further measures taken by the lab to confirm or adjudicate results (Allison & Elizabeth et al., 2020).

2.8.2 Progesterone Receptor

Another steroid hormone receptor is the progesterone receptor (PR), which is used in BC prognosis to indicate the possibility of a tumor responding to endocrine therapy. PR expression is regulated by ER, and it is considered to represent a fully functioning ER route (Li et al., 2022). PR testing largely remained prognostic in ER-positive patients but testing for cancers that are

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invasive using principles similar to those employed in ER testing is still advised (Allison & Hammond et al., 2020).

2.8.3 Human Epidermal Growth Factor Receptor 2 (HER 2)

HER 2 belongs to the receptor tyrosine kinases (RTK) family and regulates cell growth, differentiation and survival. Approximately 15% to 20% of BC express high HER2 levels and are classified as HER2 positive. The tumors typically grow rapidly and have a poor prognosis (Saini et al., 2011). HER2 with a 3+ score on IHC is interpreted as positive, 2+ score is equivocal and a 0 or 1+ score on IHC is diagnosed as negative. Although immunohistochemistry (IHC) is the gold standard for HER2 testing, fluorescent in situ hybridization (FISH) test is also used especially if the IHC score is 2+ (Wolff et al., 2018).

2.8.4 Ki 67

In breast cancer prognosis, Ki67 immunohistochemistry is performed to assess the proliferation status of the tumor. Although it is commonly used, its analytical validity is questionable due to its limited use in treatment plans. The current working group on Ki67 "International Ki67 in Breast Cancer Working Group" propose its clinical utility evident only in anatomically preferable ER-positive and HER2-negative clients to ascertain individuals who do not require adjuvant chemotherapy and also suggest automation scoring to enhance its current limitations (Nielsen et al., 2021).

2.9 Breast Cancer Molecular Subtypes

Keeping with immunohistochemistry and genomic assay testing of molecular markers for prognosis, BC is typically split into four subtypes "Luminal A", "Luminal B", "HER2-enriched" and "triple-negative").

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2.9.1. Luminal A

Luminal A tumors express ER and/or PR but not HER2, as well as a fewer than 20% cell proliferation indicator Ki67. They are low grade, slow growing, and offer a favorable prognosis, with less relapse and a greater likelihood of surviving. Hormone therapy works well for these tumors, but chemotherapy has limited effectiveness (Orrantia-Borunda et al., 2022).

2.9.2 Luminal B

Luminal B tumors are more severe and have a worse prognosis than Luminal A tumors. They are ER-positive. PR can be either positive or negative, and it exhibits a high level of Ki67 expression. They usually have an intermediate/high histologic grade. In addition to chemotherapy, hormone therapy may help these tumors. Because of the increased Ki67, they grow faster than luminal A and have a poor treatment outcome (Inic et al., 2014).

2.9.3 HER2- enriched

The HER2-enriched category makes up for 10-15% of BC and is distinguished by high HER2 levels, negative ER and PR-negative respectively. They are more aggressive, grow more rapidly and have less favorable outcomes than luminal tumors. Their prognosis has improved since the advent of HER2-targeted treatments (Orrantia-Borunda et al., 2022).

2.9.4 Triple-Negative Breast Cancer (TNBC)

TNBC is characterized by the absence of ER, PR, and HER2. They account for approximately 20% of all diagnosed BC. It is particularly prevalent among women under the age of 40, as well as in African-American women. Triple- negative BC is further subdivided into multiple subtypes, including mesenchymal, luminal androgen receptor (LAR), immunomodulatory (IM),

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basal-like (BL1 and BL2) and claudin-low, with the last two accounting for 50-70% and 20-30% of cases, respectively (Kumar & Aggarwal, 2016). Triple-negative BC risk varies depending on the genetic makeup, age, ethnicity, overweight and obesity, and breastfeeding habits (Collignon et al., 2016; Kumar & Aggarwal, 2016). Triple-negative BC is distinguished by its aggressive behavior, early relapse, and proclivity to manifest in advanced stages. It exhibits a high rate of proliferation, changes in DNA repair genes, and increased genome instability. It is a largely undifferentiated, highly proliferative, heterogeneous tumor with variable prognosis subsets, according to histology. By means of IHC, they are divided into basal and non-basal TNBC; the former is distinguished by the presence of cytokeratins (CK)5/6 and human epidermal growth factor type 1, whereas the latter does not have CK 5/6 (Orrantia-Borunda et al., 2022).

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Table 2.5: Summary of Breast Cancer Molecular Subtypes and their Prognosis

Marker/molecular subtype	Luminal A	Luminal B	HER 2 enriched	TNBC
ER	Positive	Positive	Negative	Negative
PR	Positive/negative	Positive/negative	Negative	Negative
HER 2	Negative	Positive	Positive	Negative
Ki67	Low	High	High	High
Prognosis	Good	Intermediate/ poor	Poor	Poor
Cancer growth	Slow	Faster	Faster than luminal types	Fastest
Cancer aggressiveness	Less	More	More than luminal types	Most
Therapy	Hormonal	Hormonal/chemotherapy	chemotherapy/Herceptin	Chemotherapy/experimental
Frequency	50%	15%	20%	15%

Source: (Orrantia-Borunda et al., 2022)

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2.10 Types of Breast Cancer Treatment

Breast cancer treatment differs depending on tumor characteristics such as grade, size, stage as well as other specific biomarkers present on the tumor. A multimodal approach to treating breast cancer includes surgery for operable tumors, neoadjuvant chemotherapy, radiotherapy, adjuvant chemotherapy, and hormonal therapy.

2.10.1 Surgery

Women with invasive BC can choose between Breast Conservation Surgery (BCS) and Mastectomy, depending on a variety of tumor and patient-specific factors. The surgical scope of the operation determines the type of the procedure. (Freeman et al., 2018).

Mastectomy is the total surgical removal of the cancerous breast and is still the standard treatment for invasive BC among individuals with large tumors and in certain people who choose mastectomy after a thorough review of all alternatives (Bennett, 2014).

Breast Conservation Surgery (BCS) also known as lumpectomy removes the cancer while leaving as much normal breast as practicable. “Skin-sparing” (SSM) and “nipple-sparing” (NSM) are two relatively new conservative surgical alternatives for BC patients. Most of the breast skin is preserved in SSM to create a pocket that allows for immediate reconstructive surgery with an implant or autologous transplant to achieve a quality cosmetic outcome. The “nipple-areola complex” (NAC) is also conserved in NSM. According to meta-analyses, the outcomes of SSM and NSM are no different from those of non-conservative mastectomies. Many women can now choose BCS over a total mastectomy (Bennett, 2014; Galimberti et al., 2017).

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Again, axillary surgery can be performed through Axillary Lymph Node Dissection (ALND) or Sentinel Lymph Node (SLN) surgery. ALND is a non-dye procedure whereby the surgeon removes a number (usually fewer than 20) underarm lymph nodes whiles in SLN surgery the surgeon injects a dye and then removes only the lymph nodes under the arm that picked up the dye. These lymph nodes are most likely where the cancer will spread first. Removal of only one or a few lymph nodes reduces the possibility of serious side effects that can occur after an axillary lymph node dissection such as arm swelling (www.cancer.org accessed on 01/06/2023).

Although ALND was an important part of BC surgery, SLN biopsy has emerged as a precise technique for axillary staging. ALND can be avoided not just in patients with negative SLNs, but also for individuals with a few positive SLNs receiving breast and/or axillary radiation. ALND, despite its limitations, continues to be the preferred procedure for patients with clinically positive nodes. It is worthy to note that as a result of a greater application of systemic and radiation therapy, the need for axillary surgery in the treatment of BC has decreased (Noguchi et al., 2021).

2.10.2 Chemotherapy

Chemotherapy for BC patients is usually grouped into two types; neoadjuvant chemotherapy and adjuvant chemotherapy. Neoadjuvant chemotherapy is systemic chemotherapy used before the application of local treatment modalities (such as surgery or radiotherapy). Again, patients with locally advanced BC and those in the middle stages of the disease are advised to use it. It aims to make inoperable BC operable, transform BC that requires breast removal into breast-sparing BC, and provide a drug foundation in the

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periodic review treatment to improve the prognosis of patients (An et al., 2021). Adjuvant chemotherapy (which is given after initial treatment) is also effective for many female patients because it reduces mortality and recurrence in patients under 50 years by 27% and 35% respectively. In women aged 50 to 69 adjuvant chemotherapy reduce cancer recurrence by 20% (Bennett, 2014).

However, because some forms of BC are certainly going to be resistant to chemotherapy, it is crucial to choose the right patients in order to prevent unwarranted risks and adverse effects for patients who are unlikely to benefit from the course of treatment (Bennett, 2014). Patients who have luminal B tumors are frequently chemosensitive. Again, ER-positive tumors have a tendency to be less responsive to chemotherapy when compared to ER-negative tumors. Finding a way to recognize patients that will respond to chemotherapy, optimizing disease-free and life expectancy, and lowering side effects, is crucial to avoid unnecessary treatment (Bennett, 2014).

To avoid this, combination therapy is usually used to enhance the therapeutic effectiveness and efficiency of BC treatment. Moreover, to prevent needlessly increasing the amount of therapeutic agents to be administered, the number of agents must be kept to a minimum unless there is proof that adding more agents to the treatment plan will result in additional benefits (Fisusi & Akala, 2019). Whiles some agents may increase toxicity when added to current regimens without significantly improving therapeutic efficacy, recent advances have provided the option of using nanoparticles in combination therapy to further increase the benefits that can be derived from such a treatment strategy (Fisusi & Akala, 2019).

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2.10.3 Radiotherapy

In most cases of early-stage BC, radiation therapy is advised following breast-conserving surgery because it enables organ preservation. In cases of advanced breast cancer management, post mastectomy radiotherapy is still a widely acceptable level of care (Castaneda & Strasser, 2017). Additionally, recent developments in the usage of hypofractionated radiotherapy are becoming increasingly popular due to their "comparable toxicity profiles" and the ability to treat individuals who need a shorter therapeutic phase, adding both value and expenditure savings to the treatment of BC (Castaneda & Strasser, 2017).

In recent trials, regional lymph node irradiation promises to offer better target coverage and less long-term toxicity. There have also been two significant trials that support the use of partial breast irradiation for patients with low-risk BC (Haussmann et al., 2020). Although neoadjuvant and radiochemotherapy is still regarded as a risk factor for poor wound healing and a cosmetic outcome, a recent publications seem to have changed this perception (Haussmann et al., 2020).

2.10.4 Hormonal Therapy

Hormonal therapy is utilized for the treatment of ER positive tumors because many tumors depend on estrogen for growth. This endocrine therapy may be used as neoadjuvant therapy to shrink tumors before surgery or as adjuvant therapy following surgery for early cancer. Based on their unique mode of action, hormonal therapy has been categorized into three;

- Selective estrogen receptor modulators
- Specified estrogen receptor degraders

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- Aromatase inhibitors (Bennett, 2014).

A diagrammatic representation of the three modes of action is depicted in Figure 2.4.

Selective Estrogen Receptor Modulators **(**SERM) inhibits estrogen from attaching to ER. The optimal endocrine therapy for patients with ER-positive BC for many years has been the anti-estrogen tamoxifen. Tamoxifen binds to the ER and modifies its conformation. As a result, the bond with nuclear transcriptional co-activators is disrupted, changing the downstream consequences (Bennett, 2014; Hernando et al., 2021). SERMS exhibit a combination of agonist as well as antagonist activity, based on the intended organ. It has antagonistic effects on the breast and performs the function of an anti-estrogen. Although tamoxifen is more tolerable than chemotherapy, it still has some drawbacks, such as a higher risk of endometrial cancer. Despite (tamoxifen) being ER-positive sensitive, many patients do not benefit from this therapy, and many carcinomas that do initially respond to it develop resistance (Bennett, 2014; Hernando et al., 2021).

Another group of anti-estrogens known as Specified Estrogen Receptor Degraders (SERD) works by destroying the ER and preventing ER-dependent signaling. The only SERD that has received approval is Fulvestrant. It improves ER ubiquitination, prevents dimerization, and inhibits the transcription of estrogen-dependent genes as a result. Trials have shown that when used as a first-line treatment, it is just as effective as tamoxifen and is frequently utilized for patients following the emergence of tamoxifen resistance. The main advantage of this medication over tamoxifen is that it functions as a pure antagonist and has no additional agonistic effect on the endometrium. New

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SERDs are being designed to overcome the drawbacks (bioavailability) of fulvestrant, some of which are in phase III clinical trials and others in early clinical development. In preliminary and early studies, newer SERDs have shown improved pharmacokinetic and bioavailability when compared with fulvestrant, and they may have a higher likelihood of producing clinical benefits (Bennett, 2014; Hernando et al., 2021).

In Aromatase Inhibitors (AIs), the enzyme aromatase transforms testosterone into estrogen. There are several clinical benefits of inhibiting this enzyme in BC based on research. For patients with post-menopausal BC, AIs are now the first line of treatment. They bind to the aromatase enzyme and prevent the transformation of androgens into estrogens. There are two classes both of which reduce the levels of estrogen in the bloodstream. Because peripheral aromatase produces the majority of the circulating estrogen, postmenopausal women benefit from AIs the most (Chumsri, 2015).

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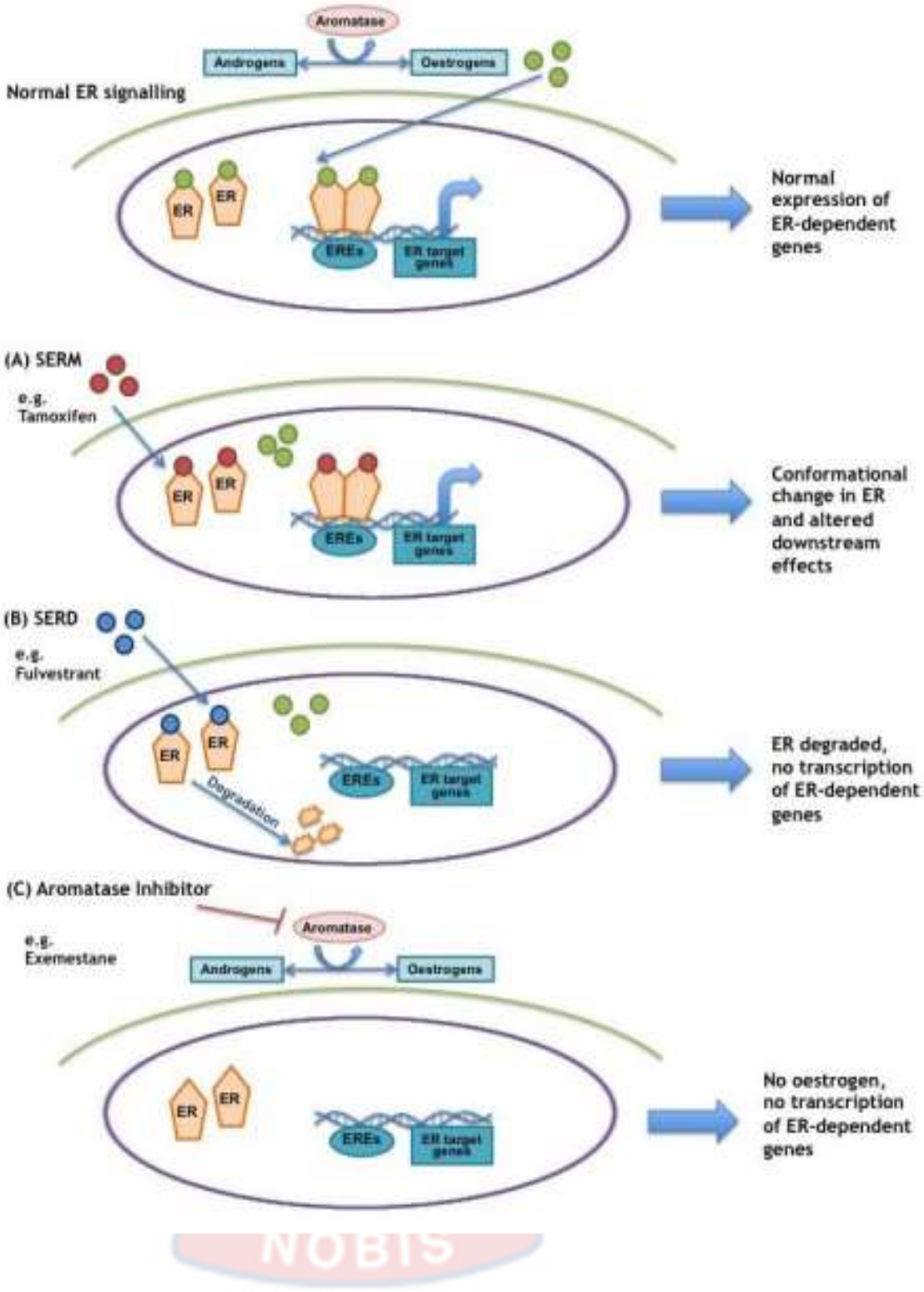

Figure 2.4: A diagrammatic representation of mechanism of various hormonal therapy in breast cancer (Bennett, 2014).

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2.10.5 Targeted Therapy

Patients who have BC cells overexpressing particular distinctive proteins on their surface, permitting an aberrant growth pattern are treated with targeted therapy. Occasionally antibodies are employed as they function similarly to the human immune system (Zanardi et al., 2015).

The progression of BC is significantly influenced by estrogen and estrogen receptors. This is the justification for the traditional practice of blocking the estrogen signaling route in women with estrogen-positive BC by targeting estrogen. Tamoxifen was the first medication approved for estrogen positive metastatic BC, reducing recurrences by roughly 40%–50%. An alternative therapy for treating estrogen-dependent BC is the use of aromatase inhibitors, for example exemestane, letrozole, and anastrozole. These drugs prevent the biosynthesis of androgens by inhibiting the aromatase enzyme, which lowers the estrogen levels in tumor cells (Zanardi et al., 2015).

For BC types that are not hormone-dependent, there are additional treatments available such as targeting of HER2 proteins. The HER2 protein is regarded as a useful biomarker for therapy due to its common overexpressed receptor signature in BC. Trastuzumab, a recombinant antibody (Herceptin) is the approved drug for HER2-positive BCs. Trastuzumab is a microtubule inhibitor and is effectively transported into HER2-positive BC cells by the conjugated monoclonal antibody trastuzumab emtansine, which can be used to treat the disease. Other drugs that came after, including lapatinib and pertuzumab, did not demonstrate immunity to the emergence of resistance mechanisms while also having negative patient side effects (Masoud & Pagès, 2017).

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Unfortunately, TNBC does not currently have any effective targeted therapies. This is because TNBC lack the markers (ER, PR, HER2) currently utilized for targeted therapies (Bennett, 2014). The traditional approach is to administer chemotherapy to the patients, especially anthracycline and taxane. In TNBCs that are resistant to anthracycline and taxane medications, recent findings indicate that the microtubule-stabilizing substance ixabepilone when combined with capecitabine may be effective. In addition to chemotherapy, patients with hereditary gene mutations which account for 20% or less of TNBC may also benefit from the use of pharmacological inhibitors of the enzyme polyadenosine diphosphate ribose polymerase inhibitors. To identify potential therapeutic targets, more research must be done on the numerous pathways that fuel the growth of this particular BC subtype (Zanardi et al., 2015).

2.11 Resistance Mechanisms in Breast Cancer

Endocrine resistance in breast cancer is the commonest mechanism of resistance in breast cancer patients. This comprise intrinsic resistance and acquired resistance (Belachew & Sewasew, 2021). Endocrine therapy, which primarily aims to prevent estrogen from acting on estrogen receptors can be limited by endocrine resistance in cases of ER-positive BC through De novo or acquired resistance. Therefore, more estrogen receptor genomic study is ongoing to identify various therapeutic targets (Belachew & Sewasew, 2021).

On average, 30% of ER-positive tumors exhibited de novo tamoxifen resistance, thus lack of ER expression to hormonal therapy, especially to tamoxifen. Again, recent documented studies have shown that patients with inactive alleles of cytochrome P450/2D6 fail to break down tamoxifen to its

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active metabolite and are less receptive to tamoxifen (Belachew & Sewasew, 2021).

In furtherance, acquired resistance develops through various mechanisms such as loss of ER expression following endocrine therapy. Loss of ER expression is caused by transcriptional repression and population remodeling of the ER gene. This causes a change from an initial ER-positive to an ER-negative phenotype, and endocrine therapy that targets ER specifically does not suppress breast cancer (Chang, 2012). This occurs in an averagely 20% of breast cancer cases. Moreover, a mutation in ER gene and altered expression patterns of co-regulatory proteins are all contributing factors to acquired resistance in BC (Belachew & Sewasew, 2021).

2.12 The Nuclear Factor-kappa B Complex

A diagrammatic representation of the NF-kB complex structure is shown below in Figure 2.5.

2.12.1 NF-kB Family

The NF-kB is a family of five different transcription factors with unique proteins, namely RelA or p65, RelB, c-Rel, p150/NF-kB1 and p100 also known as NF-kB2 (Hoesel & Schmid, 2013). They possess the rel homology domain (RHD) in the N-terminus, which is required to facilitate the binding of DNA, interaction with its inhibitors as well as dimerization. It functions as a stressor mostly in the microenvironment and influences the expression of key regulatory genes like immunity, inflammatory response, apoptosis, and tumor growth (Hoesel & Schmid, 2013; Zinatizadeh et al., 2021). It is found in the cell's cytoplasm and is activated by a variety of cellular triggers. The two common pathways for NF-kB activation are the canonical (classical) and non-canonical

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(non-classical) pathways. NF-kB activation involves complicated biomolecular interactions with adapter protein molecules as well as phosphorylation and ubiquitinase enzymes resulting in translocation into the nucleus to regulate gene expression (Zinatizadeh et al., 2021).

2.12.2 Inhibitor of Kappa B (IKB) Family

IκBα, IκBβ, IκBϵ and BCL-3 are the four proteins that make up the IKB family. Ankyrin (ANK) repeats, which act as a binding mediator of IKBs to the NF-kB family are present in these proteins and serve to identify them. Because their DNA-binding RHD domains are covalently linked to an inhibitory domain that is similar to IKB, p100 and p105 can also be regarded as members of the IKB family based on the presence of ankyrin repeats (Hoesel & Schmid, 2013; Zinatizadeh et al., 2021)

2.12.3 IκB kinase (IKK) Family

The three most crucial IKK complex members are IKKα, IKK β and IKKγ. In the NF-κB pathways, the IKKs serve to induce IκB degradation and release NF-κB dimers, in addition to participating in post-translational modification of the NF-κB subunits (Hoesel & Schmid, 2013; Pavitra et al., 2023).

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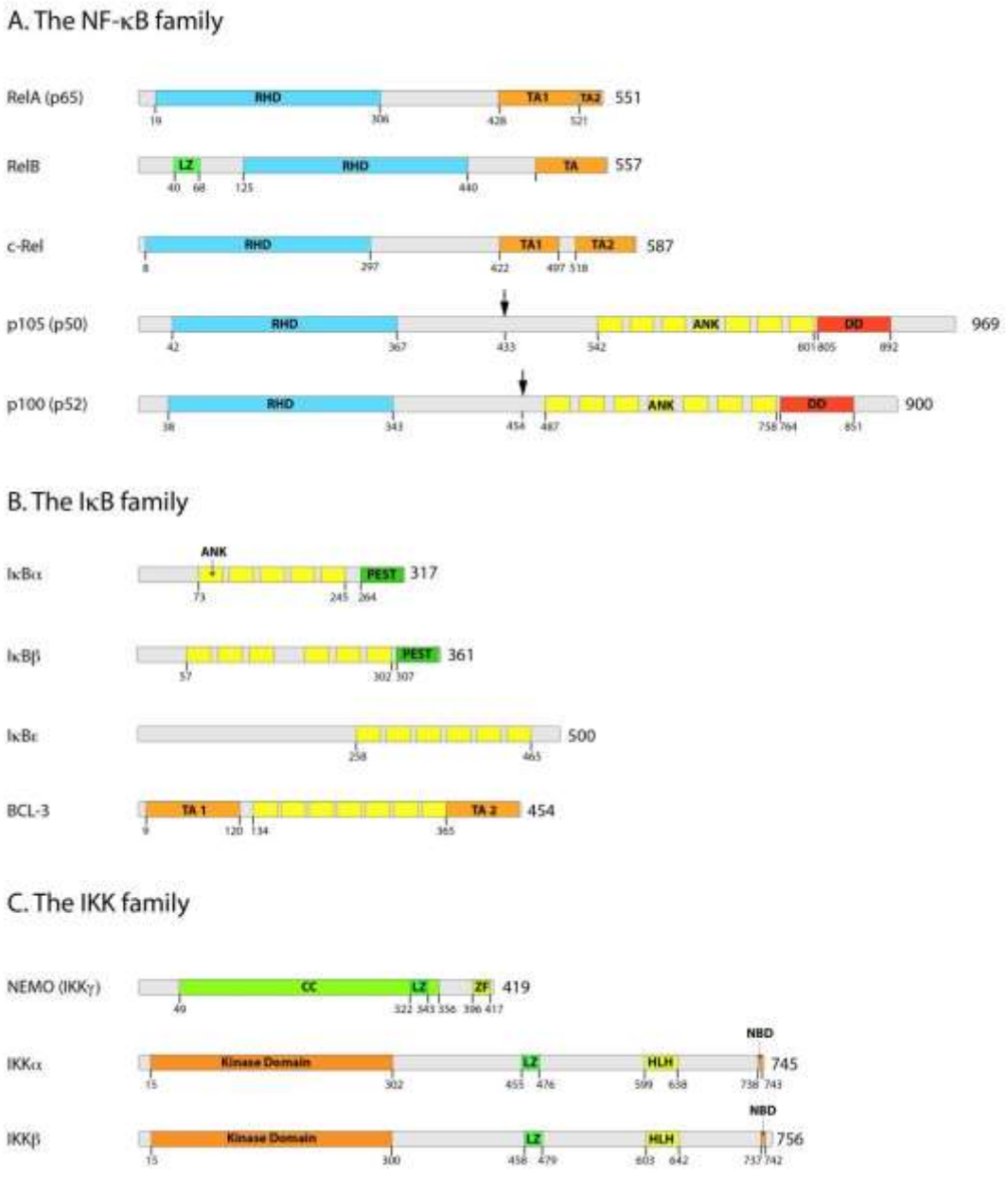

Figure 2.5: Structural representation of NF-kB complex (Hoesel & Schmid, 2013)

2.13 Mechanisms of Signaling NF-kB Pathways

The canonical signaling pathway (CSP) and the non-canonical signaling pathway (NCSP) are the two common ways of signaling in NF-kB.

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2.13.1 The Canonical NF-κB Pathway

The canonical signaling pathway (CSP) starts with IkB proteins and is triggered by tumor necrosis factor-alpha (TNF-α), which responds to ultraviolet (UV) rays, growth factors, cytokines, bacteria, and mitogens (Liu et al., 2010). TNF-α interaction with tumor necrosis factor receptor 1 (TNFR1) is the first step in the pathway. The catalytic subunits IKKβ and IKKα and the regulatory subunit NF-kB essential modulator (NEMO) of the IKK complex are recruited as a result of the ligand's binding to its receptor (Bennett, 2014; Pavitra et al., 2023). The predominant subunit IKK β then phosphorylates IκBα (usually binds to p50/p65 heterodimer) and the resulting ubiquitination releases NF-kB (p50/p65) from the cytoplasm, which then makes it easier for it to move to the nucleus, where it attaches to NF-kB sites in the DNA and triggers the expression of various regulatory genes (Bennett, 2014; Pavitra et al., 2023). A diagrammatic representation of the canonical signaling pathway is depicted in Figure 2.6 below.

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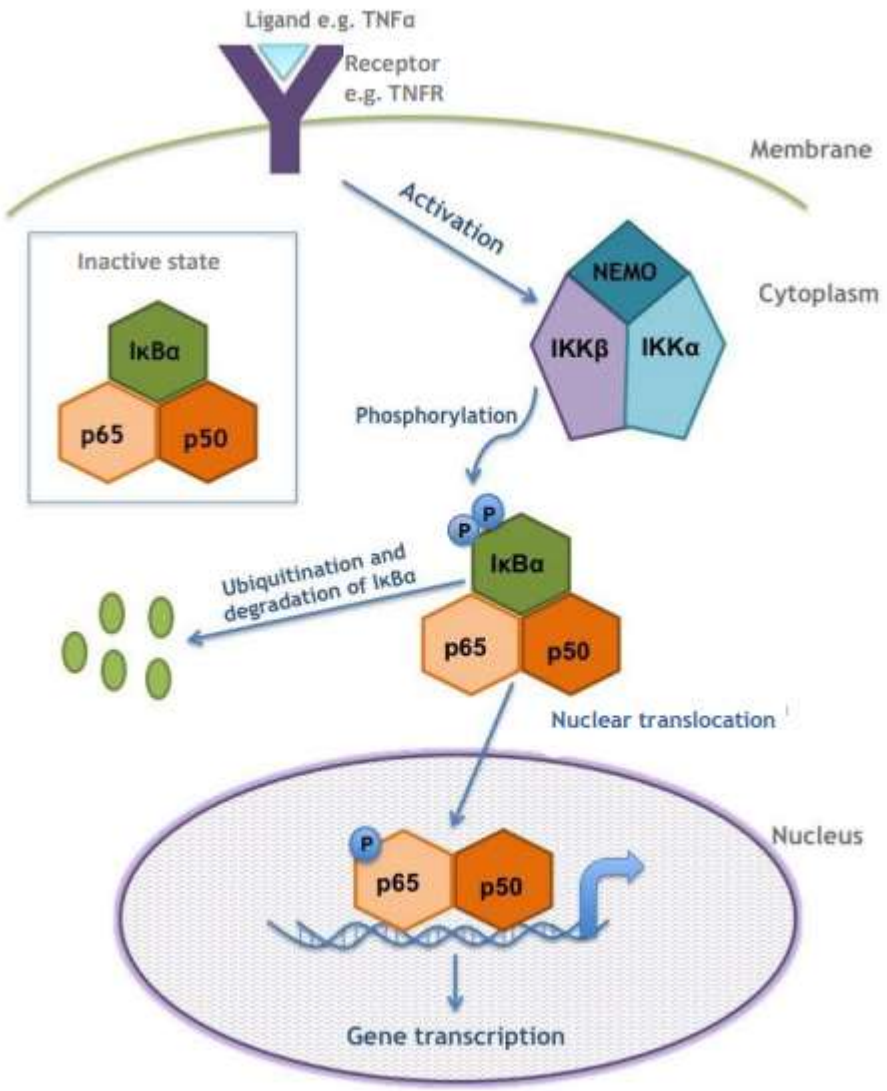

Figure 2.6: A diagram showing the canonical signaling pathway of NF-kB (Bennett, 2014).

2.13.2 The Non-Canonical NF-κB Pathway

Unlike the NF-kB canonical pathway, which depends on IKKβ and NEMO, this requires IKKα kinase activity. Lipopolysaccharides, stress, lymphotoxin B, viruses, and growth factors all can start the non-canonical signaling pathway. TNF receptor, CD40 helps to activate RelB, which in turn activates NF-κB-Inducing Kinase (NIK) and causes its phosphorylation before being degraded. Inducing the expression of chemokine genes, p52 and RelB

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migrate to the nucleus after NIK activates IKK and breaks down p100 (Bennett, 2014; Pavitra et al., 2023). Figure 2.7 below depicts a graphic representation of the non-canonical signaling pathway.

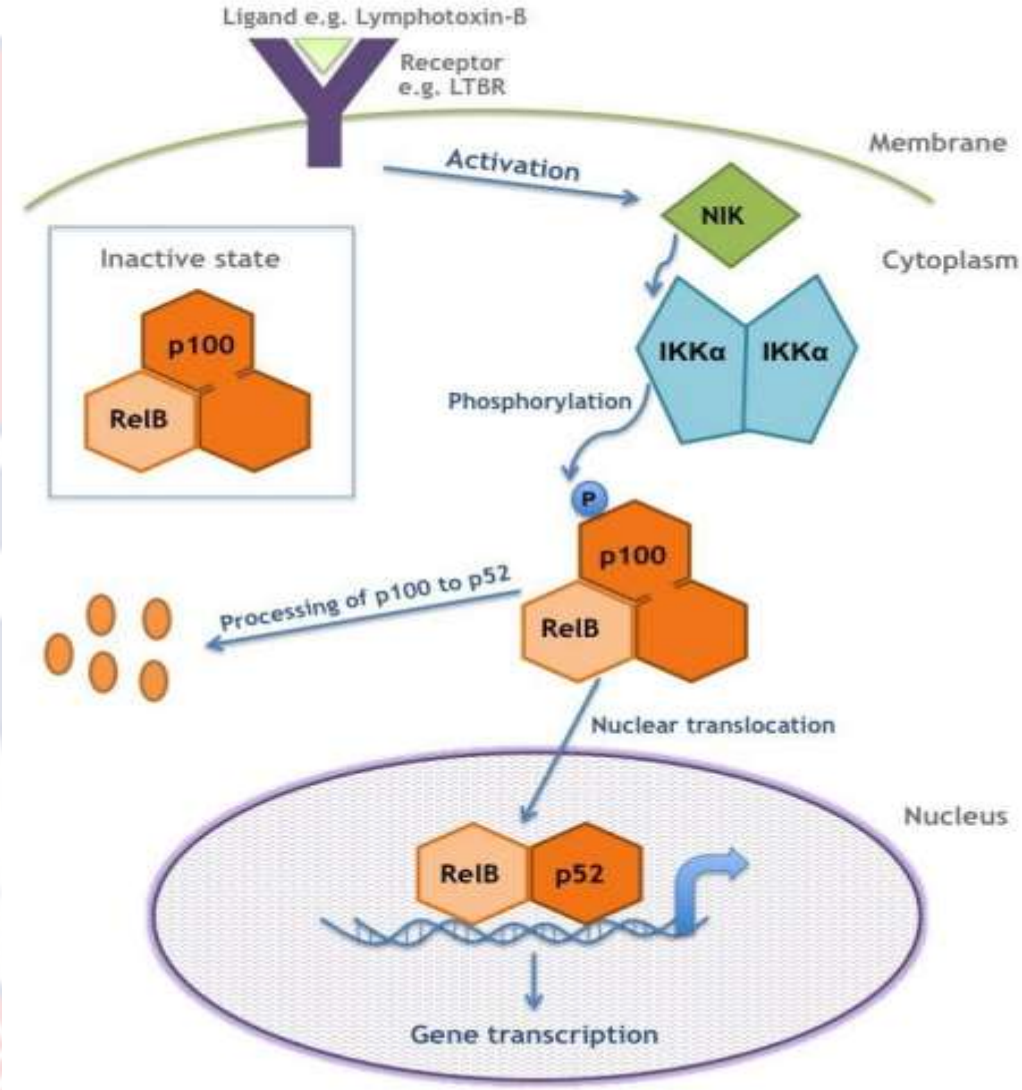

Figure 2.7: A diagrammatic representation of the non-canonical signaling pathway of NF-kB (Bennett, 2014).

2.14 NF-kB and Breast Cancer

NF-kB plays a crucial function in proper mammary gland growth through the action of the receptor activator of NF-kB ligand (RANKL) and the decoy receptor osteoprotegerin (OPG). Again, it has been observed that the

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mammary epithelium's expression and activity of NF-kB change throughout conception, lactation, and involution, and together with IKK plays a vital role in the proliferation of the mammary epithelium during post-natal development (Bennett, 2014).

However, it is emerging that RANKL activation of NF-kB induces cellular proliferation by targeting cyclin D1 in addition to preventing apoptosis and promoting tumor cell renewal (Devanaboyina et al., 2022). In support of NF-kB pathway in BC initiation and progression, different genetic signatures studied showed mounting indications that NF-kB activation occurs in cancerous stem cells (Rani et al., 2019). It enhances the invasiveness and metastatic potential of stem cells in addition to producing a microenvironment favorable for their survival. NF-kB and chemokine receptor 4 (CXCR4) have been shown to maintain cancerous cells' stemness and promote their migration, according to a line of evidence. When compared to nearby normal tissue, breast tumors showed higher expression of NF-kB1, NF-kB2, and c-Rel. RelA/p65 expression was also increased and activated NF-kB activity was found in hormone-resistant BC cells (Rani et al., 2019).

In a different study, it was found that parthenolide, an NF-kB inhibitor, restored tamoxifen sensitivity in resistant MCF7 cell lines by inhibiting NF-kB (Rani et al., 2019). In contrast to MCF-7 estrogen-dependent cells, LCC1 estrogen-independent, tumor-associated macrophage (TAM)-sensitive BC cells showed increased NF-kB1 and NF-kB2 expression and DNA binding, which further supported NF-kB's role in the endocrine resistance pathway. The expression of E2-responsive genes was increased by blocking NF-kB, which binds to the ER on EREs (Rani et al., 2019).

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Furthermore, NF-kB promotes IL-6 and IL-8 production, which is thought to be how BC progresses. According to another study, foxhead box A 1 (FOXA1) inhibits NF-kB's ability to bind to the IL-6 promoter, which results in the suppression of IL-6 expression. The study came to the conclusion that reduced FOXA1 expression causes tamoxifen-resistant cells to exhibit characteristics of cancer stem cells by preferentially binding NF-kB to the IL-6 promoter and upregulate IL-6 (Rani et al., 2019).

Current evidence shows that NF-kB can considerably enhance the expression of 60 related genes and that this upregulation promotes the growth and metastasis of tumors. Following translocation into the nucleus, the subunit p65 exhibits transcriptional activity, triggers the expression of target genes, and forms a network that controls cell cycle, promotes cell invasion, tumorigenesis, inflammation, metastasis, and ultimately results in resistance to radiotherapy and chemotherapy via a crosstalk with different pathways (Pavitra et al., 2023). NF-kB cross-talk with epidermal growth factor receptor (EGFR) pathway, signal transducer and activator of transcription 3 (STAT3) pathway and hypoxia-inducible factor (HIF) to modulation its transcriptional activity in BC (Pavitra et al., 2023).

According to reports, EGFR and members of its family are present in BC in large numbers and are linked to downstream NF-kB activation, primarily in ER-negative BC cells (Sukocheva et al., 2022). HER2 overexpression activates NF-kB by triggering this pathway in BC cells. Furthermore, mutated p53 also help activate NF-kB and EGFR which stimulate the expression of TGF and collectively induce angiogenesis. As a result, inhibiting the EGFR-NF-kB

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pathway may provide a method of treating BC as well as other cancers (Pavitra et al., 2023).

Moreover, a research report indicates that 70% of BC cells, particularly TNBC, have unnaturally elevated STAT3 levels, which contribute to the pathogenesis of the disease. Different genes involved in anti-apoptosis, cell cycle regulation, and the production of chemokines and cytokines are controlled by STAT3 and NF-kB. The acetyltransferase p300 initiates the STAT3-mediated acetylation of NF-kB, which stimulates the modification of RelA and increases the NF-kB nuclear retention end eventually aids in tumorigenesis (Marotta et al., 2011; Yu et al., 2015).

In terms of hypoxia cross-talk with NF-kB in breast cancer, hypoxia inducible factor (HIF) maintains a process whereby hypoxia causes tumor growth to become drug-resistant and phenotypically metastatic. However, NF-kB regulates HIF-1α transcription and bind precisely to the HIF-1α promoter site, which also has p65 binding sites for the direction of oxidative stress and inflammation-induced cancer cell progression (Pavitra et al., 2023).

There is also evidence that the receptor-interacting protein kinase 2 directly activates NF-kB and other pathways and plays an instrumental role in contributing to the development of immunity and inflammation. It stimulates toll-like receptors in BC, which drive tumor cells toward cancer stem cell phenotype. Additionally, triple-negative BC has an overexpression of the TNF receptor, which has been shown to promote metastasis and sensitize BC cells through the activation of NF-kB (Pavitra et al., 2023).

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2.15 Role of NF-kB and Breast Cancer Resistance to Therapy

Overall increase in anti-apoptotic signaling in BC is caused by the activation of NF-kB, which enhances the transcription of a number of pro-survival factors such as cyclin D1, and inhibitors of apoptosis (IAPs). Radio, chemo, and endocrine therapies eventually become less effective as a result of this activation (Pavitra et al., 2023).

2.15.1 Role of NF-kB in Endocrine Resistance

NF-kB activation primarily leads to tumor progression in ER-negative tumors. A reciprocal inhibition is found between ER-positive and NF-kB. ER expression reduces inflammation, prevents NF-kB from migrating into the nucleus, and also breaks down its DNA-binding protein. Beside this, there is close to 50% of ER-positive patients who do not respond to the treatment due to tamoxifen resistance. This resistance mechanism subsequently leads to high levels of NF-kB expression. As a result, the medication that should block NF-kB and lower ER instead causes progression of the disease (Pavitra et al., 2023).

2.15.2 Role of NF-kB in Radiotherapy Resistance

Previous research demonstrated that radiotherapy resistance develops regardless of dose, however, it has been noted that clinical doses stimulate intracellular and extracellular signaling, activate NF-kB and cause the secretion of TNF- α. This mechanism supports long-term favorable effects of NF-kB and TNF-α interaction to augment resistance in BC patients (Pavitra et al., 2023).

2.16 NF-kB Inhibitor for Breast Cancer Therapy

Prior in vitro research has demonstrated the feasibility of inhibiting NF-kB activation in reducing a number of pro-inflammatory effects. This makes NF-kB an appealing target for BC therapy. Few direct NF-kB inhibitors are in

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use, despite the fact that some drugs have been shown to have indirect NF-kB inhibiting abilities. These NF-kB inhibitors (Bortezomib, thalidomide) can lower NF-kB activity, which enhances BC treatment results (Pavitra et al., 2023).

2.17 Chapter Summary

In brief, the chapter reviewed various similar literature on global breast cancer epidemiology, the African and Ghanaian context of BC, various clinicopathological features used in classifying breast cancer, current strength and challenges associated with treatments, complexity of NF-kB interaction in BC and NF-kB as a possible next target for improving treatment outcomes among breast cancer patients.

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CHAPTER THREE

METHODOLOGY

3.0 Introduction

The study used archived BC tissues to assess the prognostic significance of NF-kB among BC patients in CCTH-Ghana. The association between clinicopathological features of archived BC tissues and NF-kB (p65) expression was established. The chapter details the method and materials used in achieving the objectives of the study. Thus it covers the research approach, the study location, and the sampling technique. It further discusses data retrieval, laboratory techniques used and data analysis.

3.1 Study Design and Sampling Technique

A cross-sectional study design was used to recruit patients' tissue samples received by the department from January 2019 to March 2023 and a purposive sampling method was employed to select breast cancer tissues without prior chemotherapy and/ or radiotherapy treatment before sampling.

3.2 Study Area

The study was carried out from Department of Pathology, Cape Coast Teaching Hospital, and current laboratory work was done at Kumasi Center for Collaborative Research (KCCR) laboratory. CCTH has a 400-bed capacity and serves as a teaching and referral facility for School of Medical Sciences, University of Cape Coast (UCC) and the region respectively. It is bordered to the north by Abura township, to the south by Pedu Estate, to the east by Nkanfoa, and to the west by Abura /Pedu estate (www.ccthghana.org accessed on 17/04/2023). The Department of Pathology provides various histopathology,

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cytopathology and autopsy services including breast cancer diagnosis and treatment.

3.3 Inclusion Criteria

Breast cancer tissue collected from patients with no prior treatment such as chemotherapy and or radiotherapy with a completed laboratory request form containing variables like age, hormonal receptor status, tumor size, perineural invasion, lymphovascular invasion, pathological lymph node stage, tumor grade, pathological tumor stage, tumor type and molecular subtypes were included.

3.4 Exclusion Criteria

All tissues collected from patients that have prior treatment with chemotherapy and or radiotherapy were excluded. BC tissues without histological and demographic data were excluded. Tissues from patients with other cancers were excluded. Male breast cancer tissues were also excluded.

3.5 Sample Size Determination

The minimum number (N) of patients' archived tissues included in this study was determined by the Cochran's formula below:

$$N = \frac{Z^2 (P)(1-P)}{(d)^2}$$

Where Z = 1.96 is the standard score (95% confidence interval)

P = 0.102 (10.2%) is the prevalence of BC in Ghana (Globocan, 2020)

d = allowable error (d) of 5%

The calculated sample size = $\{(1.96)^2 \times 0.102 \times (1-0.102)\} \div (0.05)^2 = 140.74$

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However, based on the available data of breast cancer patients from January 2019 to March 2023 at the CCTH pathology department and strict inclusion criteria, 105 (90 pathological tissues and 15 control tissues) samples were obtained for further works.

3.6 Clinical Data Retrieval

Clinicopathological information such as age, pathological lymph node status, Ki67, tumor location and size, type of tumor and grade, perineural invasion, lymphovascular invasion, HER 2, the hormonal status of ER and PR, were obtained from the patient's laboratory request form and aligned with individual tissue block numbers for selection at CCTH pathology unit. All tissues were confirmed with haematoxylin-eosin stain for pathological classification of breast cancer before selection.

3.7 Laboratory Work

All archived BC tissues without prior treatment received within the period of study were assessed for NF-kB (p65). Embedded tissues were sent to Kumasi Centre for Collaborative Research (KCCR) for histological sectioning and immunohistochemical detection of NF-kB (p65) antigens.

3.7.1 Tissue Sectioning

The immunohistochemistry slides were first labeled with various tissue identification numbers and using a microtome (Biobase, China) the tissues were trimmed to smoothen the surface and later sectioned into 4um. This was quickly placed in a lukewarm water bath (Thermo Fisher paraffin section floatation bath). The labeled slides (positively charged) were then used to gently pick the tissues from the water bath and allowed to dry on the slides. The slides were

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then incubated in a drying oven (Digi Heat-J. P selectra) overnight at 55-60 ℃ before deparaffinizing the sections.

3.7.2 Deparaffinization of Tissues

Tissues were then deparaffinized and rehydrated with xylene (two changes for 10 minutes each) and various grades of ethanol (100%, 90%, and 70% each for 3 minutes) respectively. This was followed by washing the specimen twice with distilled water for 3 minutes each.

3.7.3 Antigen Retrieval by Heat-induced Epitome Method

The fixation process in paraffin-embedded tissue frequently masks antigenic sites, hence antigen retrieval is needed to disrupt the protein crosslinks to expose these sites to make it possible for antibody binding. The heat-induced technique was used by placing slides in an epitome retrieval solution from Novocastra with a pH 9.0 in a rubber bowl. This was later placed in Biocare medical decloaking chamber with a temperature adjustment to 95 ℃ and a retrieval time of 30 minutes. The tissues were further allowed to cool for extra 35 minutes in the buffer before washing with reaction buffer solution for 2 minutes each.

3.7.4 Blocking of Endogenous Peroxidase and other proteins

In order to eliminate endogenous peroxidase and other non-specific binding protein activities, tissues were incubated (10 minutes) in H_2O_2 (3%) diluted with distilled water and blocking reagent (Biocare medicals) respectively. Tissues were rinsed in reaction buffer for 3 minutes before and following blocking.

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3.7.5 Addition of Primary and Secondary Antibodies and Antibody optimization trials

Tissues were then incubated for 2 hours in NF-kB (p65) rabbit polyclonal antibody IgG from Proteintech (catalog number 10745-1-AP) diluted in Da Vinci green diluent from Biocare Medical with optimization of 1:400 dilutions (various optimizations were performed within the dilution range prior to the final selection as seen in Appendix B). Following the incubation period, tissues were rinsed twice for 3 minutes each with reaction buffer before incubating with MACH 1 Universal horse radish peroxidase (HRP) polymer from Biocare Medical for 40 minutes.

Various dilution trials were performed on selected tissues (both positive and negative controls) with different incubation periods and the best staining appearance and intensity of the primary antibody was selected for the entire work. The overnight incubation result did not differ much from the 2-hour incubation period result. Hence the 2-hour incubation method was used for the research work.

3.7.6 Addition of Chromogen

Two washes were done with reaction buffer for 3 minutes each before the addition of 3,3 diaminobenzidine (DAB) from Biocare medical chromogen and substrate.

3.7.7 Counterstain

Mayer's hematoxylin was then used for counter staining (1-2 minutes) followed by dehydration with ethanol (70%, 90% and 100% for 3 minutes each) and clearing by xylene (two changes for 5 minutes each) respectively. Coverslips were used to mount the slides with the help of a para-mount.

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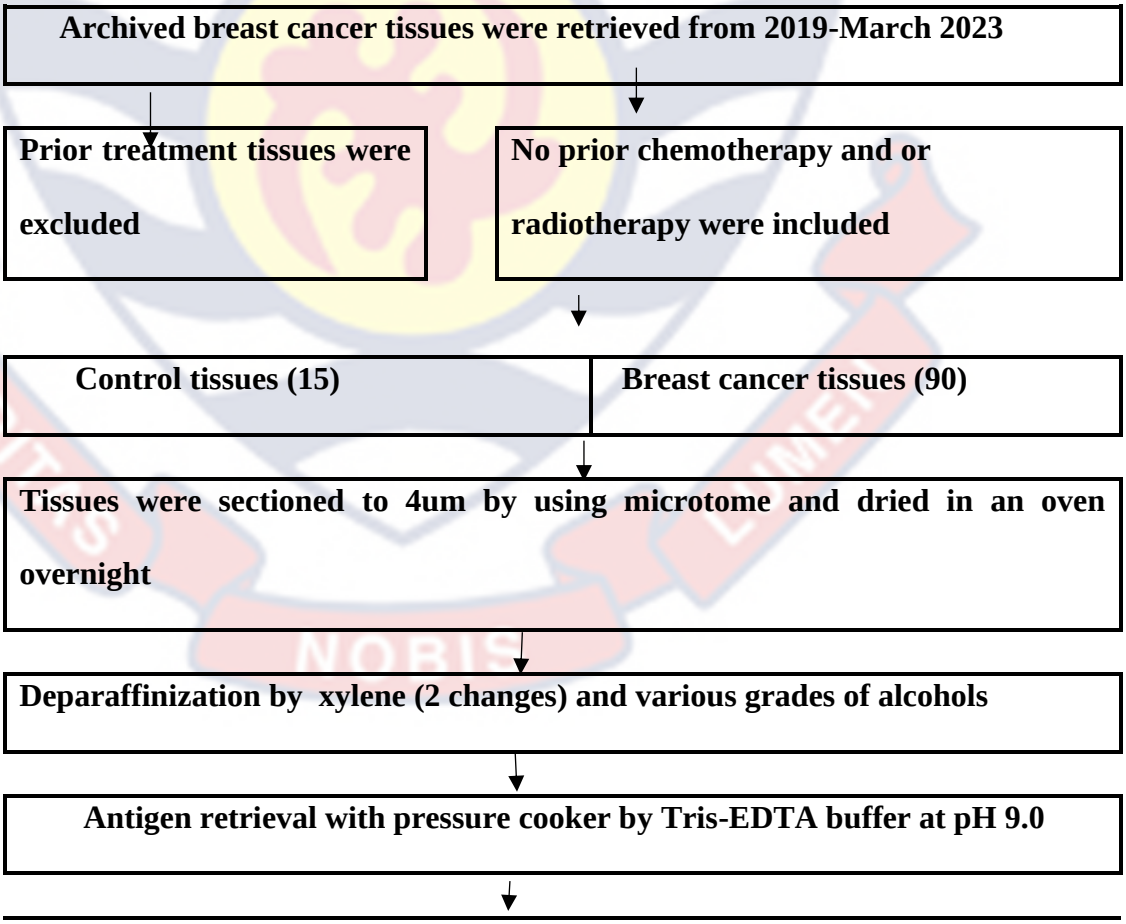
3.7.8 Visualization and Reporting of result

Slides were visualized by two pathologists using a digital microscope (Opto-Edu brand) and BC cells showing NF-kB (p65) positive or negative reactions were recorded. The presence of brownish yellow colour was considered positive either in the cytoplasm or nucleus or both. A combination of the intensity of stain (0= no staining, 1=mild stain, 2=moderate stain, 3=strong stain) and proportion of tumor stained (0= no staining, 1<25%, 2= 25-50%, 3= 51-75%, 4=76-100%) were used to grouped the tissues as either negative (0), low (1-2), moderate (3-4) or high (5-7) stained (Adankwah et al., 2018). Normal breast tissues were used as negative controls as well as an omission of primary antibodies. 3-4 best shots of images were taken x400. The work summary is as illustrated in Figure 3.1



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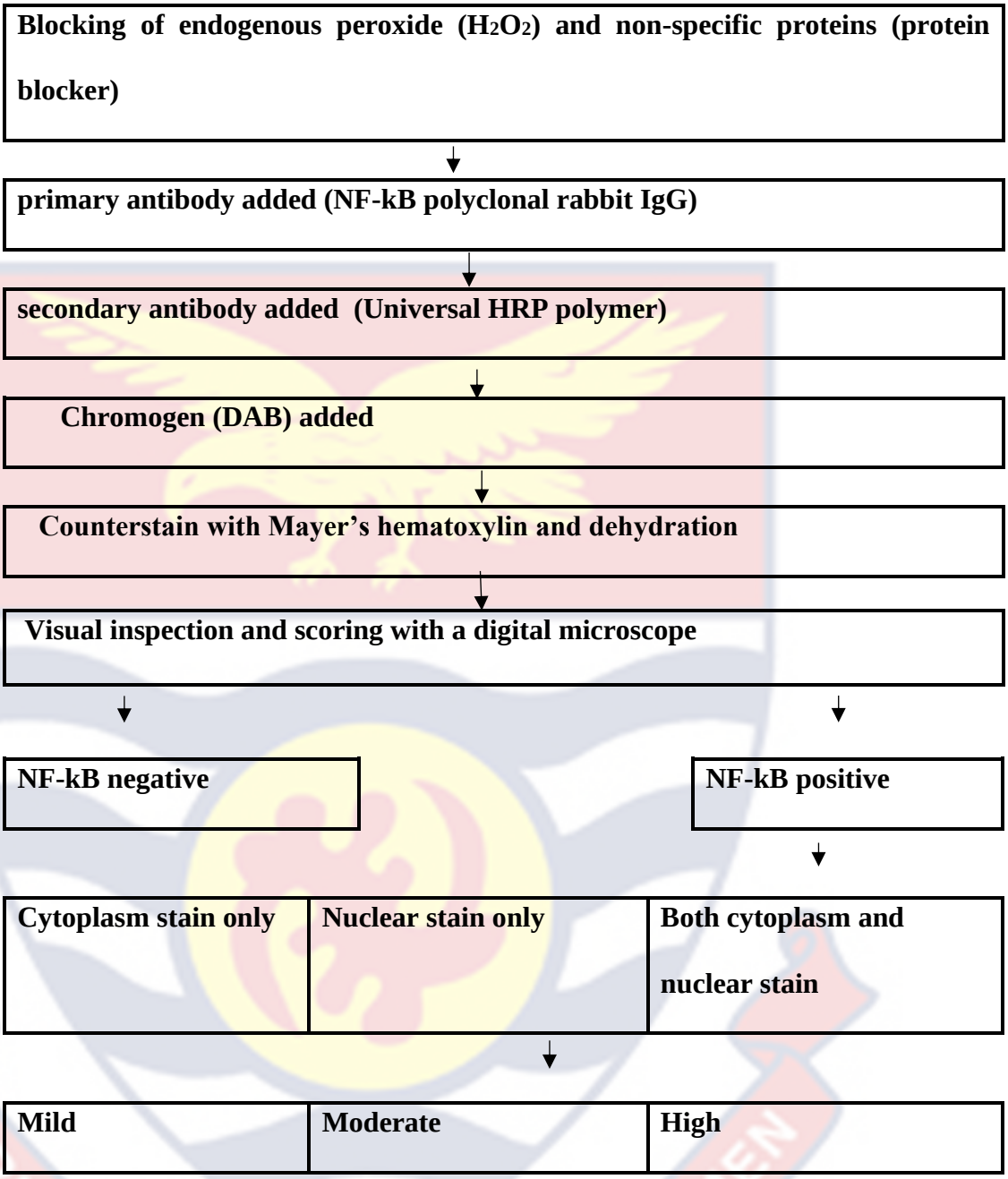

Figure 3.1: Summary workflow for NF-kB (p65) test among breast cancer patients from Cape Coast Teaching Hospital

3.8 Data Analysis

The data generated was entered into Microsoft Excel and later into SPSS version 25 for analysis. Descriptive and Pearson chi-square analysis or Fisher's exact test was done for all demographic data, type of tumor and grade, HER2, perineural invasion, the hormonal status of ER and PR, molecular subtypes, laterality, tumor size, pathological tumor stage, pathological lymph node stage,

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lymphovascular invasion, and presented in tables and figures form with p values < 0.05 considered significant. Logistic regression analysis was also performed to check the association within the significant groups. Sensitivity, specificity, positive predictive value and negative predictive value of the NF-kB (p65) as a prognostic marker was also determined by Receiver Operating Characteristic (ROC) curve analysis using Xlstat.

3.9 Ethical Consideration

The study was carried out in accordance with institutional ethical guidelines. Ethical clearance was obtained from the institutional review board of Cape Coast Teaching Hospital, Central Region (reference number: CCTHERC/EC/2023/060). De-identification of all sample was done before usage for the research.

3.10 Chapter Summary

In this study, CCTH-Ghana and Kumasi Center for Collaborative Research were selected as study centers with 90 archived BC tissues and 15 normal breast tissues from January 2019 - March 2023 as target samples. However, the collection of the blocks did not cover the years before 2019 hence it's possible that the result obtained does not accurately reflect all BC patients seeking care at CCTH-Ghana. Again the study is a cross-sectional study and so cannot determine the dynamics of changes after breast cancer treatment. Moreover, only immunohistochemistry technique was employed in assessing the NF-kB (p65) expression in the BC tissues and the sensitivity may differ when other methods such as polymerase chain reaction (PCR) or Western blot testing were employed.

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CHAPTER FOUR

RESULTS AND DISCUSSION

4.0 Introduction

This research work explored the prognostic significance of NF-kB among BC patients at CCTH-Ghana. NF-kB (p65) expression levels were measured by manual immunohistochemistry technique. This was scored based on the proportion of cytoplasmic and or nuclear stain (0=no staining, 1<25%, 2=25-50%, 3= 51-75%, 4=76-100%) and intensity of the staining (0=no staining, 1 = mild, 2 =moderate, 3 =strong) respectively. The combined proportion and intensity were then grouped as either negative (0), low (1-2), moderate (3-4) or high (5-7). Negative and low staining were considered "low" and moderate and high staining were considered "high" for NF-kB (p65) expression data analysis. The chi-square test was then used to compare the association between the NF-kB (p65) expression (low or high) with various clinicopathological features. Logistic regression was also used to check the odds between statistically significant variables. The diagnostic performance of the NF-kB (p65) marker was determined by using the receiver operating curve (ROC) and area under the curve (AUC) analysis. Data in this research study were mainly depicted in figures and tables.

4.1 Result

4.1.1 Patient's Characteristics from CCTH

The study included 90 female BC patients with an average age of 54.3±13.9. 57 (63.3%) were above 50 years compared to 33 (36.7%) who were below the age of 50 years. Out of the 90 pathological cases, tumor grades 3, 2 and 1 were 43 (47.8%), 37 (41.1%), and 10 (11.1%) respectively. Most of the

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cases had tumor sizes between 2.1 to 5 cm 46 (51.1%) compared to 0-2 cm 15 (16.7%) and >5 cm 29 (32.2%) respectively. In terms of laterality, 59 (65.6%) were from the left side versus 31 (34.4%) from the right side. Regarding hormonal receptors, the majority were negative for both estrogen and progesterone receptors representing 53 (58.9%) and 73 (81.1%) respectively. Positive estrogen receptor was 37 (31.1%) versus 17 (18.9%) for progesterone positive receptor. HER 2 data showed that 70 (77.8%), 13 (14.4%), and 7 (7.8%) of the patients were negative, positive and equivocal respectively. 64 (71.1%) had high Ki67 compared to 26 (28.9%) which were low. Breast cancer molecular subtypes were divided into five; luminal A, luminal B, Triple-negative, HER 2- enriched and others. Triple-negative represented by 38 (42.2%) were the majority. There were equal cases of luminal A and luminal B (n=19; 21.1%) whiles HER 2-enriched was 10 (11.1%) and 4 (4.4%) classified as others.

Regarding the pathological tumor stage, majority of the patients were at T2 (n=41; 45.6%) whereas 6 (6.7%), 13 (14.4%), 19 (21.1%) and 11 (12.2%) were at T0, T1, T3 and T4 respectively. Lymph node stages were N1 (n=23; 25.6%) the highest followed by N0 (n=21; 23.3%), N2 (n=16; 17.8%) and N3 (n=9; 10%) respectively. However, 21 (23.3%) of the pathological lymph node stage (pNX) was unclassified. Forty-seven (52.2%) of the patients had lymphovascular invasion compared to 43 (47.8%) who did not have lymphovascular invasion. Perineural invasion was 12 (13.3%) as compared to the majority 78 (86.7%) who do not have perineural invasion. Sixty-nine (76.7%) of the cases were invasive carcinoma with no specific type (NST) whiles 21 (23.3%) were either mixed carcinoma, mucinous carcinoma,

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cribiform carcinoma, or papillary carcinoma. Complete data on the various clinicopathological features of study participants are depicted in Table 4.1.

Table 4.1: General Clinicopathological Features of Breast Cancer Cases

Variables	Frequency (N)	Percentage (%)
Age group (years)		
0-50	33	36.70
> 50	57	63.3
Tumor grade		
Grade 1	10	11.1
Grade 2	37	41.1
Grade 3	43	47.8
Tumor size (cm)		
0-2	15	16.7
2.1-5.0	46	51.1
>5	29	32.2
Laterality		
Left	59	65.6
Right	31	34.4
Estrogen receptor		
Negative	53	58.9
Positive	37	41.1
Progesterone receptor		
Negative	73	81.1
Positive	17	18.9
HER 2		
Negative	70	77.8
Positive	13	14.4
Equivocal	7	7.8
Ki67		
High	64	71.1
Low	26	28.9

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Molecular subtype		
Triple-Negative	38	42.2
Luminal A	19	21.1
Luminal B	19	21.1
Her 2-Enriched	10	11.1
Others	4	4.4
Pathological tumor stage		
pT0	6	6.7
pT1	13	14.4
pT2	41	45.6
pT3	19	21.1
pT4	11	12.2
Pathological lymph node stage		
pNX	21	23.3
pN0	21	23.3
pN1	23	25.6
pN2	16	17.8
pN3	9	10.0
Lymphovascular invasion		
Absent	43	47.8
Present	47	52.2
Perineural invasion		
Absent	78	86.7
Present	12	13.3
Type of breast cancer		
Invasive carcinoma (NST)	69	76.7.
Others	21	23.3

NST- no specific type, pN-pathological lymph node, pT- pathological tumor, pNX-pathological lymph node stage not assessed, CCTH- Cape Coast Teaching Hospital.

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4.1.2 Immunohistochemical Expression of NF-kB (p65) in Breast Cancer Tissues

Out of the 90 cases, 59 (65.6%) breast cancer tissues expressed high NF-kB (p65) as compared to 31 (34.4%) low expression. The 59 high expression consist of 25 (27.8%) and 34 (37.8%) moderately and strongly stained whereas the 31 low expression were made up of 12 (13.3%) negative and 19 (21.1%) mild respectively. Representative images of immunostaining of BC tissues are depicted in Figure 4.1 NF-kB (p65) immunostaining was seen in either cytoplasm only or both cytoplasm and nucleus of study subjects. 78 (86.7%) of the 90 breast cancer tissues were positive for NF-kB (p65). Cytoplasm stain alone was 68 (87.2%), both cytoplasm and nuclear stain were 10 (12.8%) and no nuclear stain alone was recorded. A few other images of cytoplasmic and nuclear expression of NF-kB are presented in Appendix C.

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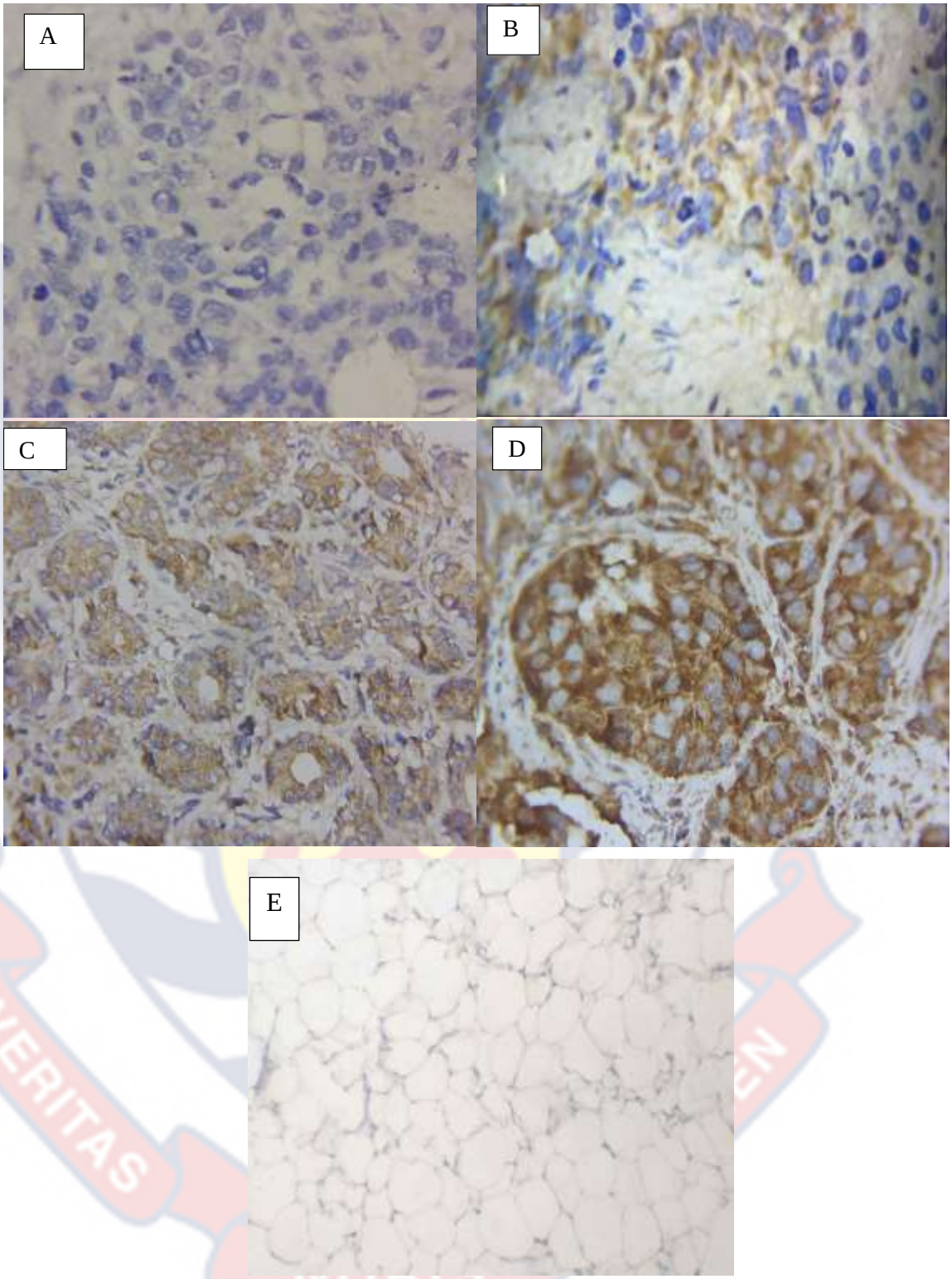

Figure 4.1: Images of NF-kB (p65) expression levels in various BC tissues. A is grade 3 BC tissue with negative NF-kB (p65) expression. B is a grade 3 BC showing a mild expression. C is grade 3 BC with moderate expression whiles D is a grade 3 BC with strong NF-kB (p65) expression respectively. E is normal breast tissue with negative NF-kB (p65) expression. X400 images.

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4.1.3 Association between NF-kB (p65) Status and Histopathological Features

NF-kB (p65) status and its association with histopathological features such as age, primary lymph node stage, type of BC, tumor size, laterality, tumor grade, primary tumor stage and perineural invasion were analyzed by Chi-square (X^2) and p-value of <0.05 was considered significant. NF-kB (p65) was highly expressed among the age group > 50 (62.7%) compared to those below 50 years 22 (37.3%). In terms of low NF-kB (p65) status, 11 (35.5%) versus 20 (64.5%) was observed among age groups 0-50 and those above 50 years respectively. NF-kB (p65) was not statistically significant (p = 1.000) according to age group. NF-kB (p65) marker was significantly associated with tumor grade (p = 0.029) with grade 3 having (n=32; 54.2%) high expression followed by grade 2 (n=24; 40.7%) and grade 1 (n=3; 5.1%) respectively (Table 4.2 and Figure 4.2). There was a moderate odds ratio (Table 4.4) of expressing high NF-kB (p65) in grade 3 tumors (OR = 6.79, 95% CI (1.49-30.92), p-value = 0:013). Although high levels of NF-kB (p65) were seen in tumor sizes 2.1-5.0 cm (n=31; 52.5%), >5 cm (n=21; 35.6%) and 0-2 cm (n=7; 11.9%), this was not significant when compared to its low expression values of 15 (48.4%) for 2.1-5.0 cm, 8 (25.8%) for >5 cm and 8 (25.5%) for 0-2 cm respectively. There was no significant association between NF-kB (p65) and laterality, pathological lymph node stage, pathological tumor stage, lymphovascular invasion, perineural invasion and type of breast carcinoma as depicted in Table 4.2.

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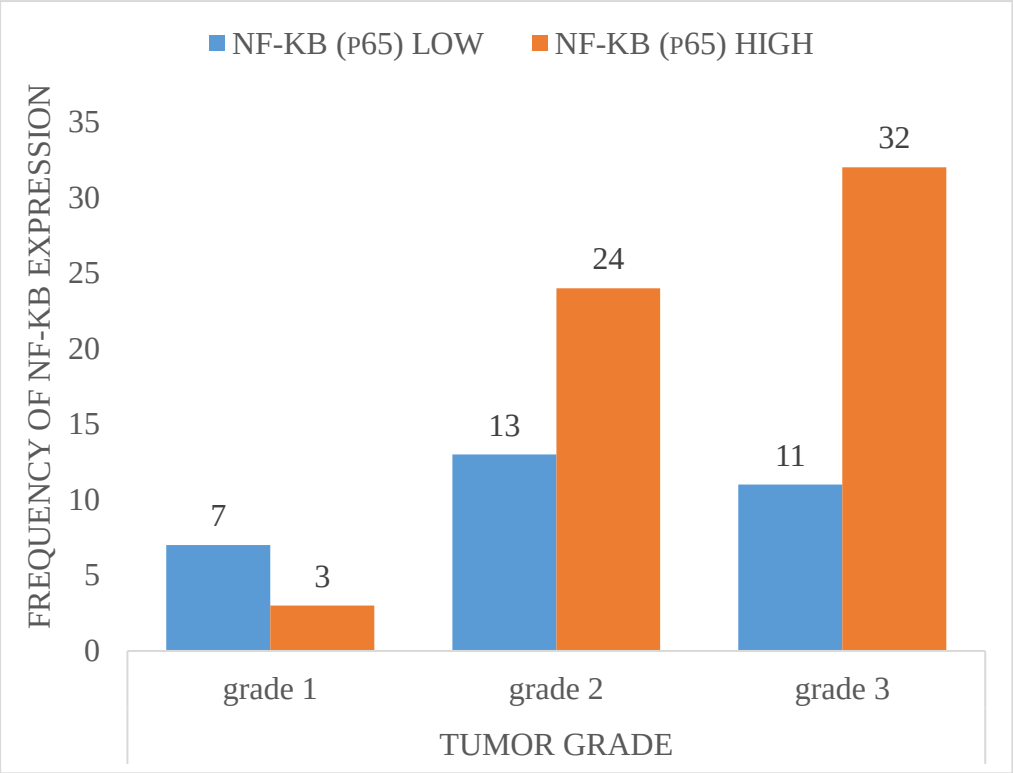

Figure 4.2: Histogram of NF-kB (p65) status among various tumor grades of breast cancer patients

Table 4.2: Association between NF-kB (p65) Status and Histopathological Features

Variables	NF-kB (p65) status			X^2	P value
	Total (90) n (%)	High (59) n (%)	Low (31) n (%)		
Age group (years)					
0-50	33 (36.7)	22 (37.3)	11 (35.5)	0.028	1.000
> 50	57 (63.3)	37 (62.7)	20 (64.5)		
Tumor grade					
Grade 1	10 (11.1)	3 (5.1)	7 (22.6)	7.102	**0.029**
Grade 2	37 (41.1)	24 (40.7)	13 (41.9)		
Grade 3	43 (47.8)	32 (54.2)	11 (35.5)		
Tumor size (cm)					
0-2	15 (16.7)	7 (11.9)	8 (25.8)	3.043	0.218
2.1-5.0	46 (51.1)	31 (52.5)	15 (48.4)		
>5.0	29 (32.2)	21 (35.6)	8 (25.8)		
Laterality					
Left	59 (65.6)	36 (61.0)	23 (74.2)	1.563	0.249
Right	31 (34.4)	23 (39.0)	8 (25.8)		

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Pathological tumor stage					
pT0	6 (6.7)	5 (8.5)	1 (3.2)	7.302	0.121
pT1	13 (14.4)	5 (8.5)	8 (25.8)		
pT2	41 (45.6)	28 (47.5)	13 (42.0)		
pT3	19 (21.1)	15 (25.4)	4 (12.9)		
pT4	11 (12.2)	6 (10.1)	5 (16.1)		
PLNS					
Pnx	21 (23.3)	12(20.3)	9 (29.0)	7.604	0.107
pN0	21 (23.3)	15 (25.4)	6 (19.4)		
pN1	23 (25.6)	12 (20.3)	11 (35.5)		
pN2	16 (17.8)	11 (18.7)	5 (16.1)		
pN3	9 (10.0)	9 (15.3)	0 (0)		
LVI					
Absent	43 (47.8)	25 (42.4)	18 (58.1)	2.006	0.186
Present	47 (52.2)	34 (57.6)	13 (41.9)		
Perineural invasion					
Absent	78 (86.7)	50 (84.7)	28 (90.3)	0.547	0.534
Present	12 (13.3)	9 (15.3)	3 (9.7)		
Type of breast cancer					
Invasive carcinoma (NST)	69 (76.7)	48 (81.4)	21 (67.8)	2.106	0.191
Others	21 (23.3)	11 (18.6)	10 (32.2)		

NST-no specific type, pNX- pathological lymph node stage not assessed, NF-kB- nuclear factor kappa B, LVI- Lymphovascular invasion, PLNS-pathological lymph node stage.

4.1.4 Association between NF-kB (p65) and Prognostic Molecular Markers

Table 4.3 shows the expression levels of NF-kB (p65) among the various molecular markers. There was no significant association between NF-kB (p65) status and estrogen receptors (p = 1.000). There was comparatively similar expression levels of both high and low NF-kB (p65) in estrogen receptor-negative, 35 (59.3%) and 18 (58.1%) as well as in estrogen receptor-positive, 24 (40.7%) and 13 (41.9%) respectively. Progesterone receptor negative had 47 (79.7%) high and 26 (83.9%) low expression levels compared to progesterone positive receptor levels of 12 (20.3%) high and 5 (16.1%) low and this was also not statistically significant (p = 0.779). HER2 had no significant link with NF-kB (p65) status (p = 0.373), however, there was a significant association between NF-kB (p65) status and proliferation index Ki67 (p = 0.026).

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Patients with high Ki67 >20 had a significant odds ratio [OR = 3.27, 95% CI (1.28-8.34), p-value = 0:016] of expressing high NF-kB (p65). Similar significance was also observed in terms of molecular subtypes and NF-kB (p65) expression (p = 0.009) although there was no difference in terms of logistic regression analysis (Table 4.4). A histogram of NF-kB status among various molecular biomarkers analysed (ER,PR, HER2, Ki67) and molecular subtypes are shown in Figures 4.3 and 4.4 respectively.

Table 4.3: Association between NF-kB (p65) and Prognostic Molecular Markers

Variables	NF-kB (p65) status			X^2	p-Value
	Total (90) n (%)	High (59) n (%)	Low (31) n (%)		
Estrogen receptor					
Negative	53 (58.9)	35 (59.3)	18 (58.1)	0.013	1.000
Positive	37 (41.1)	24 (40.7)	13 (41.9)		
Progesterone receptor					
Negative	73 (81.1)	47 (79.7)	26 (83.9)	0.235	0.779
Positive	17 (18.9)	12 (20.3)	5 (16.1)		
HER2					
Negative	70 (77.8)	48 (81.4)	22 (71.0)	1.972	0.373
Positive	13 (14.4)	8 (13.6)	5 (16.1)		
Equivocal	7 (7.8)	3 (5.0)	4 (12.9)		
Ki67					
High	64 (71.1)	47 (79.7)	17 (54.8)	6.095	**0.026**
Low	26 (28.9)	12 (20.3)	14 (45.2)		
Molecular subtype					
Luminal A	19 (21.1)	10 (17.0)	9 (29.0)	13.548	**0.009**
Luminal B	19 (21.1)	15 (25.4)	4 (13.0)		
Triple-negative	38 (42.2)	29 (49.1)	9 (29.0)		
Her2-Enriched	10 (11.1)	5 (8.5)	5 (16.0)		
Others	4 (4.5)	0 (0)	4 (13.0)		

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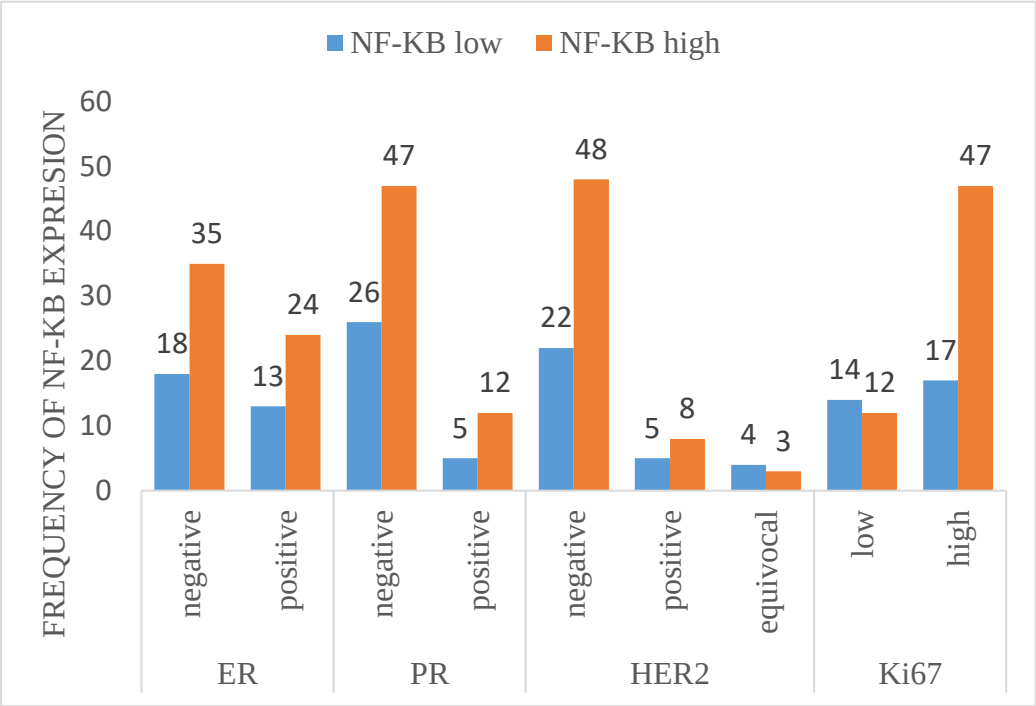

Figure 4.3: Histogram of NF-kB (p65) status among molecular markers of study participants

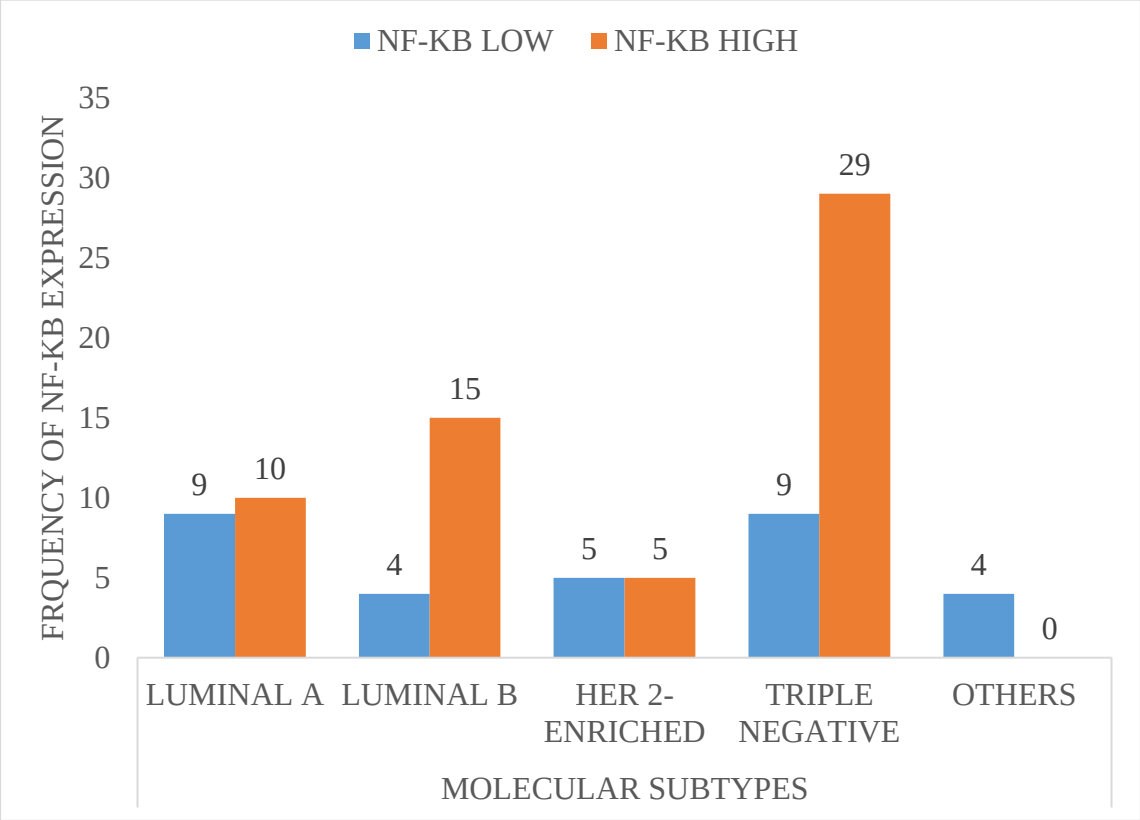

Figure 4.4: Histogram of NF-kB status among breast cancer molecular subtypes

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Table 4.4: Logistic Regression Analysis for NF-kB (p65) Versus Tumor Grade, Molecular Subtypes and Ki67

Variables	Total (90) n (%)	High (59) n (%)	Low (31) n (%)	cOR (95% CI)	p-value
Tumor grade					
Grade 1	10 (11.1)	3 (5.1)	7 (22.6)	1	
Grade 2	37 (41.1)	24 (40.7)	13 (41.9)	4.31 (0.95-19.53)	0.058
Grade 3	43 (47.8)	32 (54.2)	11 (35.5)	6.79 (1.49-30.92)	**0.013**
Molecular subtypes					
Luminal A	19 (21.1)	10 (17.0)	9 (29.0)	1	
Luminal B	19 (21.1)	15 (25.4)	4 (13.0)	3.38 (0.81-14.02)	0.94
Triple-negative	38 (42.2)	29 (49.1)	9 (29.0)	2.90 (0.90-9.35)	0.75
Her2-Enriched	10 (11.1)	5 (8.5)	5 (16.0)	0.90 (0.20-4.17)	0.893
Others	4 (4.5)	0 (0)	4 (13.0)	0.00 (0.0)	0.999
Ki67					
Low	26 (28.9)	12 (20.3)	14 (45.2)	1	
High	64 (71.1)	47 (79.7)	17 (54.8)	3.23 (1.24-8.34)	**0.016**

OR: odds ratio, CI: confidence interval

4.1.5 Diagnostic Performance of NF-kB (p65) Among Study Subjects

To ascertain the diagnostic accuracy of NF-kB among BC patients in Cape Coast, Ghana, a receiver operating characteristic curve was drawn, and the corresponding sensitivity, specificity, positive predictive value, and negative predictive value were determined as shown in Figure 4.5 and Table 4.5 respectively. Overall sensitivity was 83% with a specificity of 84%. The positive predictive value was 90.7% and the negative predictive value was 72.2%. With a cut-off value of ≥0.670, the area under the curve (AUC) value of 0.919 ($p<0.0001$).

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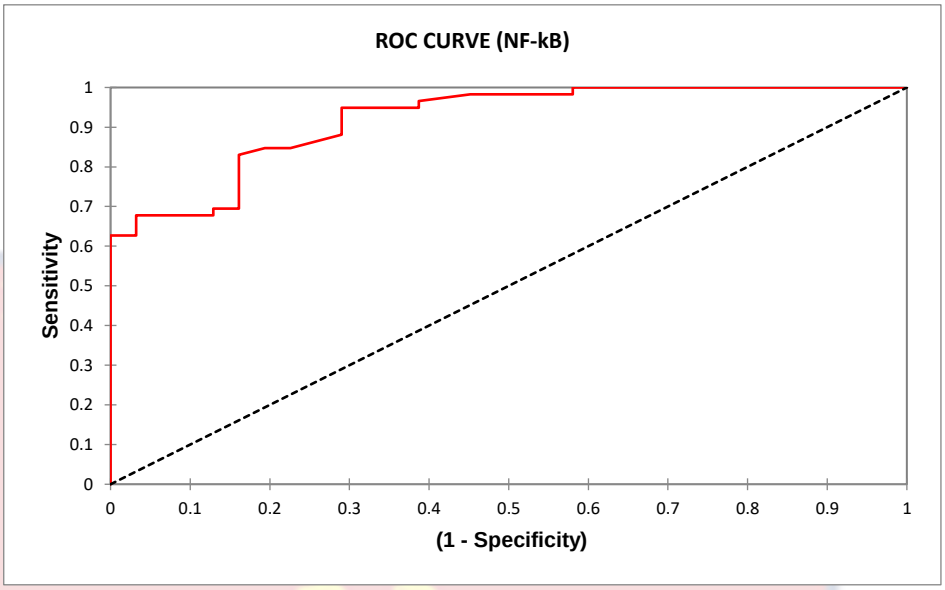

Figure 4.5: Receiver operating characteristic (ROC) curve evaluation of the sensitivity and specificity pattern of NF-kB (p65) marker among breast cancer patients in CCTH

Table 4.5: Diagnostic Performance of NF-kB (p65) among Breast Cancer Patients in CCTH

Marker	Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC	p-value
NF-kB (p65)	≥0.670	83.0	84.0	90.7	72.2	0.919	<0.0001

PPV: positive predictive value, NPV: negative predictive value, AUC: area under the curve

4.2 Discussion

Based on histopathological features of this study, the average age of 54.3±13.9 years follows similar trends of other BC scholarly work done in Ghana (Der et al., 2015; Derkyi-Kwarteng et al., 2020; Ohene-Yeboah & Adjei, 2012). In contrast, BC investigations carried out in Nigeria (Ngwogu et al., 2017) and Uganda (Galukande et al., 2014) had lower than 50 years mean ages respectively. Invasive carcinoma (no specific type) representing 76.7% of this

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current research work was the commonest just like other literature reports (Derkyi-Kwarteng et al., 2020; Ngwogu et al., 2017; Ohene-Yeboah & Adjei, 2012). The left breast was appreciably affected with tumor (65.6%) compared to the right breast (34.4%) in this study which was not observed by Ngwogu et al,. (2017), Derkyi-Kwarteng et al., (2020) and Kumar Agrawal et al., (2018). Tumor grade 3 was the highest followed by grade 2 and 1 respectively. Again, BC stage 2 was seen as the highest in this research. These two observations were not the phenomenon in other studies across Africa (Derkyi-Kwarteng et al., 2020; N' et al., 2012; Ngwogu et al., 2017; Ohene-Yeboah & Adjei, 2012) and maybe largely attributed to sample inclusion criteria. There were no much difference in BC pathological lymph node staging for pNX, pN0, pN1 and pN2 in this study. Pathological lymph node stage 3 was the least observed which is similar to a report by Kumar Agrawal et al., (2018). Breast cancer research has demonstrated that vascular (lymphovascular or perineural) invasion form significant part of the overall outcome of the disease. In this study, 65.5% of BC patients had vascular invasion which is comparable to another study in Ghana with 70.6% (Derkyi-Kwarteng et al., 2020) in contrast to 2013 report of 5.1% (Edmund et al., 2013). This statistic emphasizes the dismal prognosis for BC among women in Ghana.

There were more hormonal receptor negatives in this study as opposed to a 2020 study in Ghana (Derkyi-Kwarteng et al., 2020). TNBC of 42.2% in the current study was higher compared to 35% reported by Galukande et al., (2014) and Derkyi-Kwarteng et al., (2020) respectively. In Soweto, 21% TNBC was recorded in 2013 (Mccormack et al., 2013). Luminal A and Luminal B were similar (21%) in terms of occurrance in this study. However, 38% of Luminal

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A and 5% of Luminal B were reported in Uganda whiles 42.2% Luminal A and 12.3% Luminal B were recorded in a previous study in Ghana respectively (Derkyi-Kwarteng et al., 2020; Galukande et al., 2014).

Currently, there is no known published data on the levels of NF-kB expression in BC patients from the African population, of which Ghana is a part. This study was thus designed to fill a knowledge gap by supplying data on the prognostic significance of NF-kB (p65) among BC patients in CCTH-Ghana. NF-kB (p65) was at first recognized as an important factor in the initiation of immune system responses, but it is now being researched as a potential prognostic marker and a target for cancer treatment. The research on NF-kB (p65) has just began and there is paucity of data on the prognostic relevance of NF-kB (p65). NF-kB (p65) functions as a transcription mediator for target genes associated with carcinogenesis. It also causes resistance to growth inhibitor signals, unregulated proliferation, angiogenesis, metastasis, and a decrease in apoptosis, these being typical cancer hallmarks (Concetti & Wilson, 2018; Saqer Al-Mutairi, 2023; Zhu et al., 2017). Nevertheless, multiple factors influence NF-kB (p65) activation, including the expression of various proteins which regulate its functionality (Concetti & Wilson, 2018; Zhu et al., 2017). This current study in CCTH-Ghana showed that NF-kB (p65) was highly expressed in BC tissues (86.7%) when compared to non-tumor tissues, as determined by immunostaining. Similar expression levels were seen in a study by Kumar Agrawal et al., (2018) where out of the 119 samples, the mean expression levels of NF-kB was 90.1% ±21.8. Another research by Saqer Al-Mutairi, (2023) detected a positive NF-kB (p65) expression of approximatly 86.9% (86 out of 99 BC tissues) with only cytoplamic immunostaining.

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Majority of the BC tissues in this current study also showed cytoplasmic staining of 87.2% whiles few patients had both cytoplasmic and nuclear staining (12.8%) for NF-kB (p65). However, no nuclear stain alone was found.

The available research works describing the prognostic significance of immunohistochemical expression of NF-kB (p65) aspects of BC is contradictory (Saqer Al-Mutairi, 2023) with no single data from Africa. Even though an earlier study outside of the African continent by Montagut et al., (2006) linked nuclear NF-kB (p65) immunostaining to chemoresistance, Baba et al., (2016) found no established association with respect to nuclear expression of NF-kB (p65) and prognosis or response to chemotherapy. He also suggested that tumors with cytoplasmic NF-kB (p65) expression responded successfully and became free of the disease after chemotherapy initiation (Baba et al., 2016). However, a 2021 research reported nuclear NF-kB (p65) status as a prognostic marker for different chemotherapy regimens (Indra et al., 2021). Another study in the same period found that breast cancer cases with low nuclear NF-kB (p65) levels typically respond more effectively to chemotherapy compared to individuals with high expression levels of NF-kB (p65) (Sampepajung et al., 2021). The significance of cytoplasmic NF-kB (p65) is unknown although it is thought that there is a link between increased NF-kB (p65) expression and the molecular modifications that give rise to its activation; deactivation of NF-kB (p65) is believed to keep it in the cytoplasm, which contributes to an improved outcome in especially TNBC patients (Saqer Al-Mutairi, 2023).

Tumor grading has been shown to have predictive and prognostic significance in BC patients in the initial five years after diagnosis. Additionally, a significant link was observed between tumor grading, pathological response

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and survival rate in BC patients with tumor grade 3 and 2 having a poorer outcome compared to tumor grade 1 (Engstrøm et al., 2013; Ogston et al., 2003). This research discovered that NF-kB (p65) expression is significantly associated with tumor grade. This finding buttress earlier reports on the association between NF-kB (p65) expression levels and tumor grade (Jana et al., 2012; Saqer Al-Mutairi, 2023) . On the contrary, neither cytoplasmic nor nuclear NF-kB (p65) expression was found to relate to tumor grade (Kumar Agrawal et al., 2018; Tannahill et al., 2009). This research provides suggestive evidence of NF-kB (p65)'s aggressive role in BC patients in Ghana and its potential impact on disease progression.

There was no statistically significant relationship between NF-kB (p65) expression and histological tumor type, age group, tumor size, lymphovascular invasion, perineural invasion, primary lymph node stage, primary tumor stage, or laterality in this study. A prior investigations also discovered that neither cytoplasmic nor nuclear expression of NF-kB (p65) and clinicopathological features such as primary tumor stage, laterality, tumor type, tumor size, age group, primary lymph node stage are linked (Gershtein et al., 2010; Kumar Agrawal et al., 2018). However, Saqer Al-Mutairi, (2023) and Jana et al., (2012) observed a significant link between cytoplasmic expression of NF-kB (p65) and tumor size.

In support of various studies conducted, this research in Ghana did not also find any association between hormonal estrogen or progesterone receptors and NF-kB (p65) status (Kumar Agrawal et al., 2018; Saqer Al-Mutairi, 2023). In contrast, other studies reported a significant relationship between hormonal

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receptors and NF-kB (p65) expression (Indra et al., 2021; Jana et al., 2012; Tannahill et al., 2009).

HER 2 belongs to the receptor tyrosine kinases (RTK) family and regulates cell growth, differentiation and survival. Approximately 15% to 20% of breast cancer express high levels of HER 2 and are classified as HER 2 positive. These tumors typically grow rapidly and have a poor prognosis (Saini et al., 2011). A similar trend is seen in this research study at CCTH, Ghana, where 14.4% of breast cancer patients were HER 2 positive. NF-kB (p65) was highly expressed in HER 2 negative tissues (81.4%) compared to HER 2 positive (13.6%) in this study. Although not statistically significant, this is consistent with other reports who discovered no link between NF-kB (p65) status and HER 2 expression (Kumar Agrawal et al., 2018; Saqer Al-Mutairi, 2023). Gershtein et al., (2010) observed that HER 2 has a significant link with NF-kB (p65). This inconsistency requires further probe among breast cancer researchers.

Ki67 immunohistochemistry is performed to assess the proliferation status of the tumor although its clinical utility is limited (Nielsen et al., 2021). In this study, Ki67 was grouped as low and high as suggested by Lombardi et al., (2021) and was significantly associated with NF-kB (p65) which is similar to a study carried out in India (Jana et al., 2012) and the United Kingdom (Tannahill et al., 2009). Moreover, studies in Poland (Kumar Agrawal et al., 2018) and Kuwait (Saqer Al-Mutairi, 2023) however did not observe any strong link between NF-kB and Ki67.

In this study, NF-kB (p65) was significantly associated with molecular subtypes of BC patients in Cape Coast-Ghana with triple-negative patients

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having the highest NF-kB (p65) expression levels (49.1%). Triple-negative cases are known to be common among West African women with a poorer prognosis and high mortality (Derkyi-Kwarteng et al., 2020; Hercules et al., 2022). Based on this research, NF-kB (p65) activities could be a potential contributory factor for its aggressiveness.

Reports indicates that activated NF-kB pathway in tumor cells promotes resistance to chemotherapeutics and ionizing radiation, while its inhibition strongly upregulates the responsiveness of cancer cells to these agents (Luo & Zhang, 2017). This study highlights the possibility of NF-kB (p65) contribution toward heightened tumor cell proliferation especially in TNBC cases in Ghana and therefore may worsen the prognosis of patients. Further and larger size research is needed to ascertain the full role of NF-kB (p65) among the Ghanaian population.

4.3 Chapter Summary

The study revealed that NF-kB (p65) was expressed in 78 (86.7%) of breast cancer tissues studied with a significant relationship to tumor grade, proliferation index (Ki67) and molecular subtype. In terms of high-level expression, tumor grade 3 was about 10 times that of grade 1 (54.2% versus 5.1%), Ki67 > 20 was 79.7% compared to 20.3% for Ki67 ≤ 20 and 49.1% for triple-negative molecular subtype. The AUC predictive value of NF-kB (p65) was 0.919 with a sensitivity of 83% and specificity of 84% ($p < 0.0001$) which suggests that NF-kB (p65) can be an excellent additional diagnostic marker in predicting breast cancer in Ghana. There was no strong association between NF-kB (p65) and age, laterality, tumor size, perineural invasion, pathological lymph node stage, lymphovascular invasion, pathological tumor stage, type of invasive

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carcinoma, estrogen and progesterone receptors, and HER2. High levels of NF-kB (p65) expression especially in TNBC cases may negatively affect the prognosis of BC patients in Ghana.

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CHAPTER FIVE

SUMMARY, CONCLUSIONS AND RECOMMENDATION

5.0 Introduction

Breast cancer reached a higher proportion than lung cancer as the global most common tumor in the Global cancer statistics for 2020 with annual cases continuing to rise. Africans of which Ghana is part are not exempted from this global trend of alarming cases of BC and the aggressiveness of the disease is compounded by late-stage reporting, poverty and poor health infrastructure for diagnosis and treatment. Chemotherapy before/after surgery, radiotherapy and endocrine therapy have been at the forefront of treatment of the breast cancer. Although these treatments (chemo and radiotherapy) have some level of effectiveness, current research suggests they can also increase inflammation and anti-apoptosis by activating NF-kB; a transcription factor for many malignant tumors and chronic inflammation. There is also increasing evidence of chemoresistance and endocrine resistance to many of the current regimens attributable to NF-kB pathway activation. Therefore, it has become imperative for breast cancer researchers to study the NF-kB pathways in BC initiation and progression around the world to improve treatment outcomes. Unfortunately, there is no known published data on NF-kB prognostic significance among BC patients in Africa although BC is rising on the continent. In view of this, this research sought to assess the prognostic significance of NF-kB among BC patients in CCTH-Ghana by determining the association between NF-kB (p65) and other clinicopathological features as well as the diagnostic performance of NF-kB (p65). Archived BC tissues with complete clinicopathological data without chemotherapy and or radiotherapy treatments were selected from

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January 2019-March 2023. Using immunohistochemistry technique and microscopy assessment, the expression levels of NF-kB (p65) among the breast cancer tissues were determined. The result obtained from the study were presented in tables, images and figures.

5.1 Summary

The result generated from this cross-sectional study showed that 78 (86.7%) of the 90 BC tissues were positive for NF-kB (p65). Cytoplasm stain alone was 68 (87.2%) and both cytoplasm and nuclear stain were 10 (12.8%). In all 59 (65.6%) breast cancer tissues expressed high NF-kB (p65) as compared to 31 (34.4%) low expression. Tumor grade 3 showed a high level of p65 expression (n=2; 54.2%) when compared to grade 1 (n=3; 5.1%) and grade 2 (n=24; (40.7%) respectively. Ki67 > 20 was 47 (79.7%) compared to 12 (20.3%) for Ki67 ≤ 20 in terms of high-level expression of NF-kB. 29 (49.1%) of the triple-negative molecular subtype also expressed high level NF-kB. Tumor grade, Ki67 and molecular subtypes were significantly associated with NF-kB (p65) expression. There was no strong association between NF-kB (p65) and age, laterality, tumor size, perineural invasion, pathological lymph node stage, lymphovascular invasion, pathological tumor stage, type of invasive carcinoma, estrogen and progesterone receptors, and HER 2. The AUC predictive value of NF-kB (p65) was 0.919 with a sensitivity of 83% and specificity of 84% ($p < 0.0001$) which suggest that NF-kB (p65) can be an excellent additional diagnostic marker in predicting BC in Ghana.

5.2 Conclusion

This first study of the prognostic significance of NF-kB (p65) among breast cancer patients in Cape Coast shows that NF-kB (p65) is highly expressed

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among study participants especially in TNBC patients. This high expression was also linked to tumor grade, proliferation index (Ki67) and molecular subtypes and therefore suggest its involvement in BC progression. NF-kB (p65) might affect the prognosis of BC patients by increasing anti-apoptosis element, enhance cell proliferation and increase chemotherapy resistance especially in TNBC and worsen the prognosis of BC patients in Ghana. Further studies with a larger sample size combined with in vitro experiments may reveal more interesting findings to completely clarify the role and relationship of NF-kB (p65) expression in BC progression to evaluate it as a possible prognostic marker and a potential therapeutic target.

5.3 Recommendation

This research found a link between NF-kB (p65); a transcription factor as a possible contributor to the progression of BC and therefore recommends that NF-kB (p65) test can be included in the molecular test panels for assessing the overall progression of BC patients. Holistic assessment of NF-kB (p65) levels of expression with other biomarkers may help determine BC progression or otherwise since it is linked to tumor grade and Ki67. Again, there is strong relation of BC molecular subtypes and NF-kB (p65) hence knowledge on NF-kB (p65) levels may help clinicians provided targeted treatment for patients with different BC molecular subtypes.

5.4 Suggestions for Further Research

A repeat of the project in another part of Ghana and other African countries with a larger sample size will enhance the robustness of the current findings and also provide more extensive data on the potential geographical variations in NF-kB (p65) expression among BC patient on the continent. The

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current research used only 90 pathological BC tissues which is a small portion of all BC cases in Ghana and so the expression levels of NF-kB (p65) can be generalized. Future studies may also consider longitudinal research where NF-kB (p65) is tested before initiation of chemotherapy and /or radiotherapy treatment and after treatment. This will give the opportunity to assess the dynamic changes in NF-kB (p65) expression in pre, during and after treatment respectively.

Furthermore, genomic profiling of NF-kB (p65) in BC patients among Africans can enhance future targeted therapy for individuals with BC. Future research may consider using both benign and pathological breast tissues in order to compare the expression levels of NF-kB (p65) and its association with other clinicopathological features among the two groups.

The current research used only immunohistochemistry technique to assess the NF-kB (p65) expression in BC patients and suggests that future studies should employ other techniques like Polymerase Chain reaction (PCR) and Western blotting for testing. PCR and Western blotting test are highly sensitive and more accurate in detecting NF-kB (p65) compared to the immunohistochemistry, and would therefore enhance the sensitivity and specificity of the current research.

Lastly, integrating multidisciplinary approach such as combination of clinical data with genomic profiling to gain more holistic understanding of NF-kB (p65) in breast cancer is needed.

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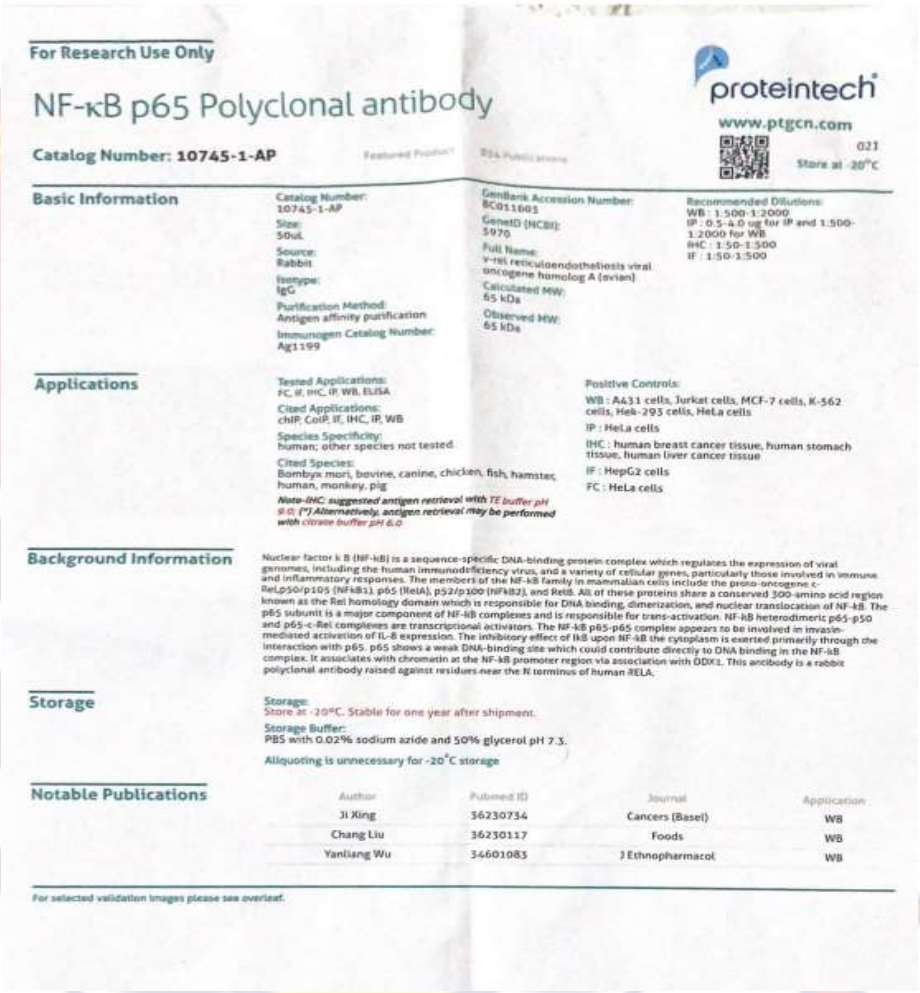
APPENDICES

Appendix A: Product Sheet on NF-kB (p65)



For Research Use Only

NF-κB p65 Polyclonal antibody

proteintech

www.ptgcn.com

Catalog Number: 10745-1-AP

021

Store at -20°C

Basic Information

Catalog Number: 10745-1-AP
Size: 50uL
Source: Rabbit
Isotype: IgG
Purification Method: Antigen affinity purification
Immunogen Catalog Number: Ag1199

GenBank Accession Number: BC011603
GeneID (NCBI): 5970
Full Name: v-rel reticuloendotheliosis viral oncogene homolog A (avian)
Calculated MW: 65 kDa
Observed MW: 65 kDa

Recommended Dilutions:
WB : 1:500-1:2000
IP : 0.5-4.0 ug for IP and 1:500-1:2000 for WB
IHC : 1:50-1:500
IF : 1:50-1:500

Applications

Tested Applications: FC, IF, IHC, IP, WB, ELISA
Cited Applications: ChIP, CoIP, IF, IHC, IP, WB
Species Specificity: human; other species not tested.
Cited Species: Bombyx mori, bovine, canine, chicken, fish, hamster, human, monkey, pig

Note-IHC: suggested antigen retrieval with TE buffer pH 9.0; (*) Alternatively, antigen retrieval may be performed with citrate buffer pH 6.0

Positive Controls:
WB : A431 cells, Jurkat cells, MCF-7 cells, K-562 cells, Hek-293 cells, HeLa cells
IP : HeLa cells
IHC : human breast cancer tissue, human stomach tissue, human liver cancer tissue
IF : HepG2 cells
FC : HeLa cells

Background Information

Nuclear factor k B (NF-kB) is a sequence-specific DNA-binding protein complex which regulates the expression of viral genomes, including the human immunodeficiency virus, and a variety of cellular genes, particularly those involved in immune and inflammatory responses. The members of the NF-kB family in mammalian cells include the proto-oncogene c-Rel,p50/p105 (NFkB1), p65 (RelA), p52/p100 (NFkB2), and RelB. All of these proteins share a conserved 300-amino acid region known as the Rel homology domain which is responsible for DNA binding, dimerization, and nuclear translocation of NF-kB. The p65 subunit is a major component of NF-kB complexes and is responsible for trans-activation. NF-kB heterodimeric p65-p50 and p65-c-Rel complexes are transcriptional activators. The NF-kB p65-p65 complex appears to be involved in invasin-mediated activation of IL-8 expression. The inhibitory effect of IkB upon NF-kB the cytoplasm is exerted primarily through the interaction with p65. p65 shows a weak DNA-binding site which could contribute directly to DNA binding in the NF-kB complex. It associates with chromatin at the NF-kB promoter region via association with DDX1. This antibody is a rabbit polyclonal antibody raised against residues near the N terminus of human RELA.

Storage

Storage:
Store at -20°C. Stable for one year after shipment.
Storage Buffer:
PBS with 0.02% sodium azide and 50% glycerol pH 7.3.
Aliquoting is unnecessary for -20°C storage

Notable Publications

Author	Pubmed ID	Journal	Application
Ji Xing	36230734	Cancers (Basel)	WB
Chang Liu	36230117	Foods	WB
Yanliang Wu	34601083	J Ethnopharmacol	WB

For selected validation images please see overleaf.

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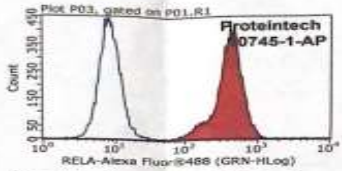
Selected Validation Data

WB result of RELA, p65antibody (10745-1-AP, 1:500) with si-Control and si-RELA,p65 transfected HeLa cells.

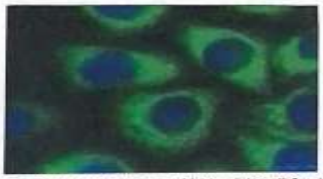
Immunohistochemical analysis of paraffin-embedded human breast cancer using 10745-1-AP (p65 antibody) at dilution of 1:100 (under 40x lens).

IP Result of anti-p65 (IP:10745-1-AP, 3ug; Detection:10745-1-AP 1:1000) with HeLa cells lysate 5000ug.

Various lysates were subjected to SDS PAGE followed by western blot with 10745-1-AP (NF-κB p65 antibody) at dilution of 1:1000 incubated at room temperature for 1.5 hours.



1X10^6 HeLa cells were stained with 0.2ug p65; RELA antibody (10745-1-AP, red) and control antibody (blue). Fixed with 90% MeOH blocked with 3% BSA (30 min). Alexa Fluor 488-conjugated AffiniPure Goat Anti-Rabbit IgG(H+L) with dilution 1:1000.



Immunofluorescent analysis of (-20°C Ethanol) fixed HepG2 cells using 10745-1-AP (p65, RELA antibody) at dilution of 1:100 and Alexa Fluor 488-conjugated AffiniPure Goat Anti-Rabbit IgG(H+L).

For technical support and original validation data for this product please contact:
T: 027-87531629 E: Proteintech-CN@ptglab.com W: ptgcn.com

This product is exclusively available under Proteintech Group brand and is not available to purchase from any other manufacturer.

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Appendix B: Various Dilutions and Incubation Period of NF-kB (p65) Primary Antibody Optimization

Dilution of primary antibody	Incubation period	Action on the tissues	Remarks on trial test
1: 300	2 hours	primary antibody added	positive
1:300	2 hours	Primary antibody omitted	Negative
1:300	Overnight	primary antibody added	Positive
1: 500	2 hours	primary antibody added	positive
1:500	2 hours	Primary antibody omitted	Negative
1:500	Overnight	Primary antibody added	Positive
1:300	Overnight	primary antibody omitted	Negative
1:500	Overnight	primary antibody omitted	Negative
1:400	2 hours	primary antibody omitted	Negative
1: 400	2 hours	primary antibody added	Positive (Selected)

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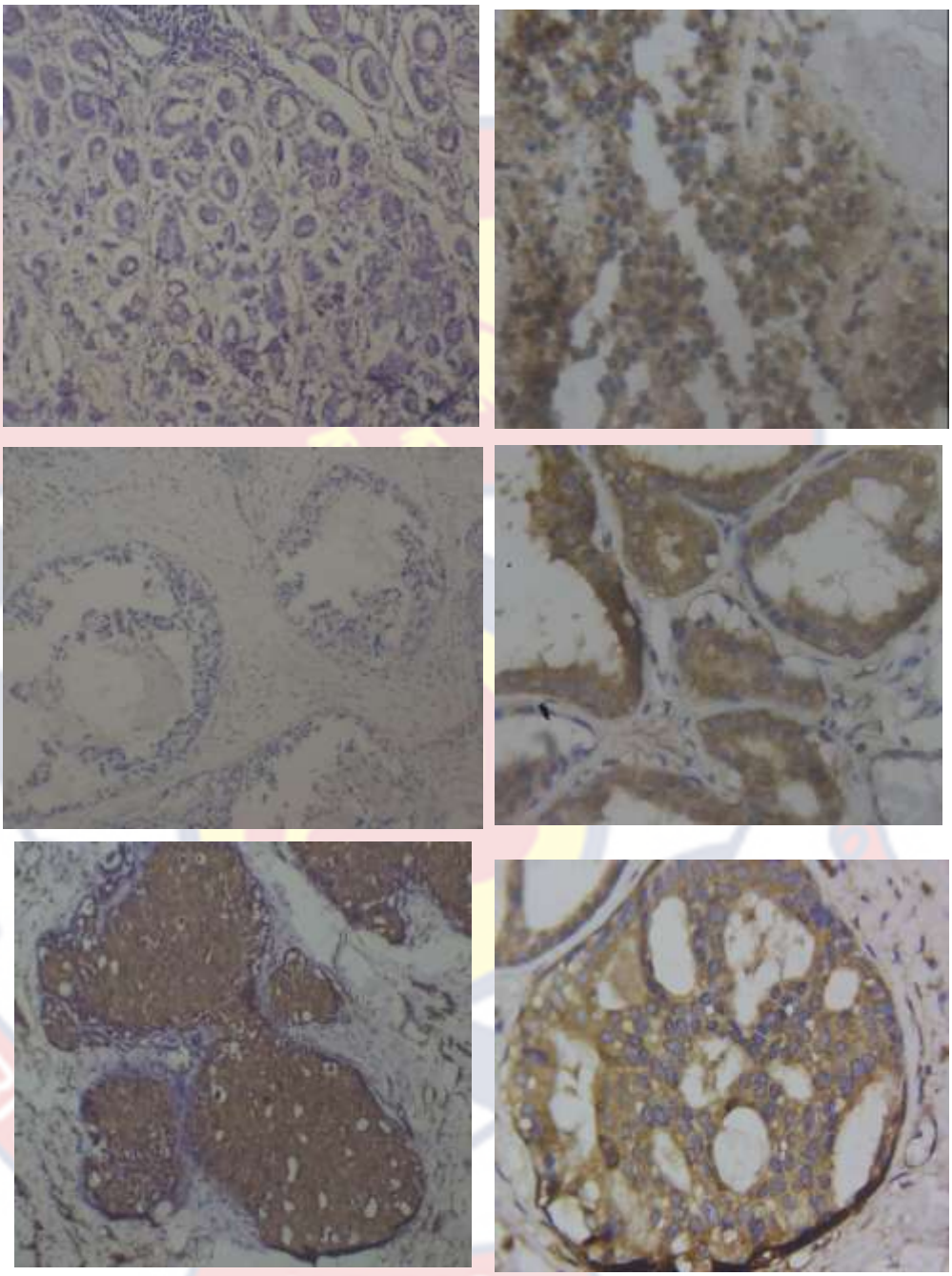
Appendix C: Some Images of Immunohistochemistry Testing of NF-kB (p 65) Expression in Breast Cancer Patients from CCTH



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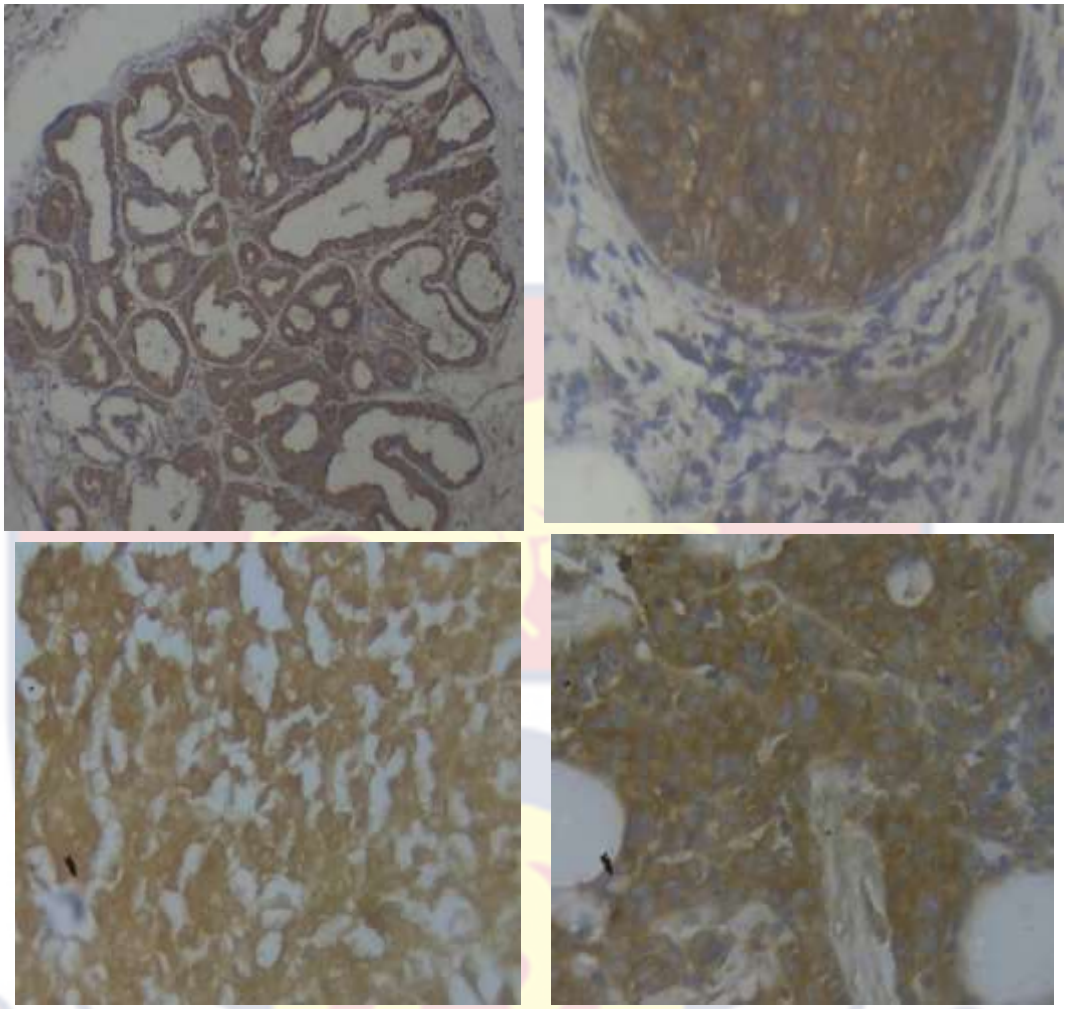

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