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Genetic Mutation in Metastatic Breast Cancer in Luminal A Tumors, which may Cause Resistant to Hormonal Therapy in Palestine

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Thesis Approval

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Declaration

I certify that this thesis submitted for the degree of master's is the result of our research; the content of the thesis is the result of work that has been carried out since the date of approval of the research program. All ethics procedures and guidelines have been appropriately followed while preparing the thesis.

Signed: .....

Dyala Issa Salem Hammad

Date: 6/8/2023

Dedication

Alhamdulillah, Thanks to Allah for the blessing of completing this thesis.

I dedicate my thesis to my family, specifically my father and mother. They have always believed in and supported me

Also, I dedicate my thesis to my brother and best sister, Taleen.

To my friends who have supported and motivated me.

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Abstract

Background:

Molecular classification for breast cancer has many important diagnostic therapeutic and prognostic implications for breast cancer patients, which depends on molecular types presented or missing in cancerous cells. The Luminal A subtype, contain Estrogen receptors (E.R.), Progesterone receptors (P.R.) while missing Human epidermal growth factor-2 (HER2). Those patients are treated by hormonal therapy that targets these receptors to reduce or stop cancer cell growth and survival. In general, these patients have the best prognosis and the disease is more treatable than other subgroups. Some patients may develop hormonal therapy resistance for many reasons. In this research, we want to study the most common genetic mutation that causes hormonal therapy resistance.

Material and method:

This research contains two parts. First, we review a result tested in a tissue sample by Next generation sequencing (NGS) in sixteen patients diagnosed with metastatic breast cancer, Luminal A, treated with hormonal therapy, and developed a disease progression through the treatment management to know the most common mutation in the population. Second, tested three patients that were positive in the first part and targeted these mutations by Polymerase chain reaction (PCR) and sanger sequencing in a blood sample.

Results

The most common mutation that causes hormonal therapy resistance are ESR1 and PIK3CA mutations, and these mutations cannot be detected by PCR method.

Conclusion

These mutations need a specific method that covers all mutations that cause hormonal therapy resistance and disease progression in breast cancer patients. We need a specific method such as ddPCR to detect the mutation in ctDNA liquid biopsy.

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List of Abbreviation

Acronym	Full Form
°C	**Celsius degrees are the unit of temperature**
LCIS	**Lobular carcinoma in situ**
DCIS	**Ductal carcinoma in situ**
IDC	**Invasive ductal carcinoma**
ILC	**Invasive Lobular Carcinoma**
HER2	**Human epidermal growth factor receptor 2**
PIK3CA	**Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha**
TP53	**Tumor protein p53**
IHC	**Immunohistochemistry**
MRI	**Magnetic Resonance Imaging**
BRCA1	**Breast Cancer Gene 1**
BRCA2	**Breast Cancer Gene 2**
DNA	**Deoxyribonucleic Acid**
RNA	**Ribonucleic Acid**

Acronym	Full Form
IV	**Intravenous**
U.V.	**Ultraviolet**
PCR	**Polymerase Chain Reaction**
ddPCR	**Droplet Digital Polymerase Chain Reaction**
Pitt	**Patient**
Bp	**Base pair**
CDK4/6	**Cyclin-Dependent Kinase 4/6**
Sec	**Second**
Min	**Minute**
NF-KB	**Nuclear Factor Kappa B**
ER	**Estrogen Receptor**
RTKs	**Receptor Tyrosine Kinases**
MAPK	**Mitogen-Activated Protein Kinase**
ESR1	**Estrogen Receptor 1**
ESR2	**Estrogen Receptor 2**
PR	**Progesterone Receptor**
DBD	**DNA-Binding Domain**
LBD	**Ligand-Binding Domain**

Acronym	Full Form
AI	**Aromatase inhibitor**
SERMs	**Selective Estrogen Receptor Modulators**
SERDs	**Selective Estrogen Receptor Downregulation**
MBC	**Metastatic Breast Cancer**
ET	**Endocrine Therapy**
JAK	**Janus Kinase**
STAT	**Signal Transducer and Activator of Transcription**
NGS	**Next-Generation Sequencing**
ctDNA	**Circulating Tumor DNA**
CfDNA	**Cell-Free DNA**
FFPE	**Formalin-Fixed Paraffin-Embedded**
PARP	**Poly (ADP-ribose) polymerase**
MEK	**Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase**
MYC	**MYC Proto-Oncogene**
SNVs	**Single-nucleotide variants**
CNVs	**Copy number variants**
AKT1	**AKT Serine/Threonine Kinase 1**

Acronym	Full Form
mTOR	**Mammalian target of rapamycin**
HR	**Hormonal receptor**
EVs	**Extracellular vesicles**
RTKs	**Receptor tyrosine kinases**

Chapter 1

(Introduction)

1.1 The Breast

Anatomically, breasts comprise glandular tissues, lobules, ducts, nipples, fatty tissues, lymph systems, blood vessels, connective tissues, and nerves. The breast contains fibrofatty tissues and glandular tissues, which are divided into lobes, divided into lobules, to finally end in terminal lobular ductal units, where milk is produced. The lobes and lobules are connected by thin tubes called ducts that carry milk from the lobules to the nipples (Rivard et al., 2023)**.** Male breasts share the same anatomy as female breasts but less glandular tissues and lobules than females. Breast tissues depend on hormones to develop and produce milk in lactation, mainly estrogens, progesterone, and prolactin, secreted by the endocrine system. Lymph systems, a part of the immune system, carry white blood cells, and lymph nodes secrete antibodies. This capsule-like structure fights infections and filters interstitial fluid collected from soft tissues and eventually returned to the vascular system (Bujoreanu & Gupta, 2023), (Buettner & Bode, 2012).

1.2 Breast cancer

Breast cancer is one of the most common solid tumors in women and rarely occurs in men. (Rivard et al., 2023). It may start with an inflammatory cancer and then can develop into a malignant tumor. Cancer cells can metastasize to other body parts through the bloodstream or lymph vessels. These cancer cells might start in any site of the breast.

Breast cancer is highly heterogeneous with variable clinical outcomes. Heterogeneity means a difference in tumor cells (intra- or inter-heterogeneity) in cancer cells and a difference between patients; these differences may be due to genetic, protein expression, or environmental factors. Tumor heterogeneity can play an important role in diagnosis, treatments, and patient's responses

to these treatments. Breast heterogeneity has many types; the first type is intra-tumor heterogeneity, defined as the existence of more than one cancer cell clone within a defined tumor mass. The differences between patients could be histological and molecular subtypes which cause differences in the treatment responses and clinical outcomes. The second type is inter-tumor heterogeneity, which occurs in various tumor types and is characterized by genetic alterations in different metastatic tumors. These differences can take place in a single individual (one patient). (Fumagalli & Barberis, 2021).

1.3 Causes and risk factors

Many causes and risk factors may cause breast cancer, including. Gender; females have a higher risk than males of developing breast cancer. Age; old age 50 years or above, they have a higher risk than young ages. Family history or personal history of breast cancer, females who had breast cancer may get cancer in the same or the other breast. Early menstruation or late menopause, radiation therapy, alcohol consumption, hormonal replacement therapy, Late age at first childbirth > 30 years old. (Łukasiewicz et al., 2021).

1.4 Histopathological Classification

Histologically, breast cancer is divided into invasive and noninvasive tumors. Ductal carcinoma in situ (DCIS) and Lobular carcinoma in situ (LCIS) cancerous cells do not invade the basement membrane and rarely metastasize to other organs but have a significant risk of breast cancer development. Invasive breast cancers are numerous and usually develop from the ductal system it's called Invasive ductal carcinoma (IDC) and, to a lesser extent, from the lobular system called invasive lobular carcinoma (ILC), they can spread to other organs by the lymphatic system or

bloodstream. Other rare Subtypes include tubular, mucinous, medullary, and papillary. (Nascimento & Otoni, 2020).

1.5 Breast cancer Staging

The stage of breast cancer measures the breast cancer cell's development. Stages refer to numbers ranging from zero to four. These stages are used as a prognostic factor. Stages from 0 to 2 are considered low stages with better outcomes than the 3 and 4 stages, while stages 3 and 4 are classified as high. The determination of the patient stage is used to choose the best treatment decision. (Zhu & Doğan, 2021).

The most common staging system for breast cancer is TNM staging, which explains the size, amount, and spread of cancer cells. T represents the tumor size and cancer cells spread to any tissue, N represents cancer cells spread to lymph nodes, and M represents metastasis to other organs. Staging attributes as T 0-4, N 0-3, and M 0-1. (Zhu & Doğan, 2021).

1.6 Breast Cancer Grading

Grading illustrates the histological features of tumor cells into numbers (1-3), as grade 1 represents mildly differentiated and the least aggressive tumor cells, whereas grade 3 represents poorly differentiated and the most aggressive tumor cells, with a higher risk for metastasis. (Katsura et al., 2022).

1.7 Molecular classification

Classification of breast cancer divides into groups depending on the expression of specific receptors on cancer cells. This classification is used for treatment, management, and prognosis. (Nascimento & Otoni, 2020).

1.7.1 Hormone Receptor Status

About 70-80% of breast cancers are diagnosed with hormone receptor-positive (Lumachi, 2015). The presence or absence of estrogen and progesterone receptors predict response to estrogen hormones, where hormone-positive receptor tumors are more likely to grow in the presence of estrogens and regress with antiestrogens. At the same time, estrogen receptor-positive tumors have a better prognosis than cancer cells with negative receptors. These receptors are targeted by hormonal treatment and have lower sensitivity to chemotherapy. Estrogen receptors are more common than progesterone receptors, so these receptors are considered biomarkers, and the status of those receptors predicts the efficacy of hormonal treatment. (Orrantia-Borunda et al., 2022).

1.7.2 HER2 Status

Human epidermal growth factor receptor 2 (HER2) is one of the human epidermal growth factor receptors family (HER family); the epidermal growth factor family consists of four members: HER(1-4). (Wieduwilt & Moasser, 2008). Normally, these receptors play a role in cell differentiation, proliferation, and survival for cancer cells. (Iqbal & Iqbal, 2014). HER2 receptors are expressed by the HER2 gene located on chromosome 17. Normally, it exists on the cell surface and is considered an oncogene. Overexpression or amplification of breast cancer cells attributed to HER2 gene. HER2-positive considered to be more aggressive than hormonal receptor-positive tumors. Also, they have a lower prognosis. However, there are many types of treatment for it. (Wieduwilt & Moasser, 2008).

1.7.3 Proliferation Rate Ki67

The Ki67 protein is used to measure the cell division, growth, and proliferation rate. Ki67 is usually a nuclear protein in all cell division phases except the resting phase of G0. The Ki67 protein is measured by the Immunohistochemical method (IHC). The high proliferation of Ki67 in cancer cells has ominous prognostic significance but is also an independent prognostic biomarker for treatment response. (Smolarz et al., 2022). (**Note**; we don't use the proliferation Ki67 in our study).

1.7.4 Molecular subtypes

Depending on the molecular classification, there are four molecular subtypes: Luminal A and B, HER2-enriched, and Basal-like. (Orrantia-Borunda et al., 2022).

The luminal type A is a group of patients with estrogen receptor-positive, progesterone receptor-positive, and HER2 negative, with low expression of Ki67 protein. These patients have a better prognosis than other groups (Orrantia-Borunda et al., 2022).

Luminal type B is a group of patients with estrogen receptor-positive and/or progesterone receptor-positive and HER2 negative or positive, with high levels of protein Ki67. These patients had a slightly worse prognosis. (Orrantia-Borunda et al., 2022).

HER2 enriched is estrogen receptor-negative and progesterone receptor-negative, and HER2 positive. Cancer cells grow faster than luminal cancers, which have a worse prognosis but are usually treated with targeted therapies for HER2 receptors. (Orrantia-Borunda et al., 2022).

Triple-negative cancer or called basal, like triple negative. Estrogen, progesterone, and HER2 receptors are negative. These tumors are more aggressive than luminal A and luminal B breast cancer. (Orrantia-Borunda et al., 2022).

1.8 Diagnosis

Diagnosis of breast cancer uses many tests and procedures, such as; Mammogram, which is an X-ray of the breast and is the most common procedure used to diagnose cancer. Breast ultrasound uses sound waves to produce images of structures deeply in the breast and lymph nodes used, especially when the patient has a solid mass. Breast magnetic resonance imaging (MRI) MRI machines use a magnet and radio waves to produce pictures of the breast. In addition, whole-body C.T. scans, bone scans, and sometimes pet scans can be used. The cornerstone of diagnosis is a biopsy of the mass and sometimes from lymph nodes. (McDonald et al., 2016).

After clinical diagnosis, genetics diagnosis is preferred to confirm breast cancer. There are many comprehensive. Oncomine test Focus Assay lab test is a targeted next-generation sequencing (NGS), multi-biomarker assay that enables the Simultaneous detection of hundreds of variants across 53 genes relevant to solid tumors. Genes detection of hotspots, Single-nucleotide variants SNVs, Insertion–deletion mutations (indels), Copy number variation CNVs, and gene fusions from DNA and RNA in a single workflow these genes lead to tumor development and used as diagnostic, prognostic, and predictive markers of cancer. were shown in appendix1. The assay provides reagents for library construction for DNA and RNA of the multiplex. The assay enables analysis DNA targted genes, and fusions driver genes in a single sequencing run in tumor biopsy.

1.9 Genetic and mutation

Genetic mutations play a significant role in cancer development. In contrast, there are two types of mutation that may occurs. First, acquired mutations (The mutations are acquired during a lifetime and influenced by lifestyle or environmental factors) represent the majority of breast cancer cases. Second, the inherited mutations (The mutations are passed from parents to children) still represent the most challenging cases). PIK3CA and TP53 represent the most common of acquired mutations, while BRCA 1 and BRCA2 subsequently represent the most common inherited mutations on chromosomes 17 and 13. (Shiovitz & Korde, 2015).

1.10 Cell cycle and cancer

Duplication of genetic material during the cell cycle is an essential step for cell division and growth, which is highly regulated by multiple proteins, each responsible for specific functions. When an error occurs during DNA replication, repairing proteins will fix it and repair the new DNA. Unfixed errors during DNA replication cause mutation/s that result in abnormal cells called cancer cells. Therefore, these mutations are used to define tumor aggressiveness and to choose the best treatment choice for each type of cancer, depending on the genetic mutation that occurred. (Matthews et al., 2022).

1.11 Breast cancer management

There are many types of treatment, which can use many types or combinations of therapies together to get the best response. Each patient has a main treatment plan with adjuvant and/or neoadjuvant therapy. The adjuvant and neoadjuvant help the primary treatment to increase the treatment response more effectively and decrease the risk of recurrence. Neoadjuvant therapies

are given to patients before the primary treatment to help reduce the tumor size or kill cancer cells that have spread. After the primary treatment, adjuvant therapies are given to patients to destroy the remaining cancer cells in the whole body (McDonald et al., 2016). The treatment options are based on the type of breast cancer, stage, grade, size, and molecular classification. Each patient has own treatment line depends on the cancer cell characteristics. The most common treatments are used for management:

Surgery is the first type of treatment chosen for breast cancer patients. The surgery may remove the tumor in breast tissue, a partial mastectomy, or a total mastectomy. The type of surgery depends on the type of cancer.

Chemotherapy is an anti-cancer medication that targets cancer cells and destroys them and can also affect healthy cells.

Radiation is the radiation waves that kill and destroy cancer cells and can also affect healthy cells.

Targeted therapy the therapy that are directly target proteins in cancer cells, these protein leads to cancer grows, divides and spreads in the body.

Hormonal Therapy (Endocrine therapy)

This type of therapy prevents the hormone's function by targeting their receptors or hormone production to prevent the fueling of breast cancer cells. So, leads to stopping the growth and death of the cancer cells. There are different therapeutic approaches to treating hormone-dependent tumors. It will be discussed in detail in Chapter Two. (Musheyev & Alayev, 2022).

Objectives of the study:

The study's main objective is to review and study Oncomine panel test results for patients with hormone receptor-positive metastatic breast cancer who have developed a disease progression and developing Resistance to hormonal treatment during the management period.

Another objective is to compare the results obtained from biopsies (Oncomine tests) with blood samples evaluated by polymerase chain reaction (PCR).

Study significance:

Current therapeutic strategies made to overcome endocrine resistance metastatic breast cancer. Identification of genetic mutations responsible for endocrine resistance might help redirect management plans for each patient, help clinicians to select the best modalities for the next Line of treatment, ensure cost-effectiveness in treating those patients, and avoid the expense and toxic effects of chemotherapy.

Moreover, this study will help us determine if we can use blood samples rather than biopsy samples to detect the mutation/s. Because blood samples are noninvasive, more accessible than a biopsy, and less expensive than an Oncomine test, depending on the results, we may create a primes panel for the most common mutation/s that cause resistance.

Chapter 2
(Literature Review)

This chapter provides a literature review and previous studies about endocrine therapy, its Resistance, the genetic association in Resistance, and disease progression.

Hormone receptors

The ovaries and some tissues like skin and fats produce steroid hormones such as Estrogen and progesterone. These hormones affect the central nervous system, cardiovascular maintenance, reproductive system, and fertility (Belachew & Sewasew, 2021). In women, Estrogen promotes the development and maintenance of female sexual characteristics. At the same time, progesterone has a role in the growth of long bones and hormonal fluctuation during the menstrual cycle and pregnancy.

There are two types of estrogen receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ), that share the homology protein level. They are different in cellular actions and are expressed in different tissue types (Musheyev & Alayev, 2022a). ERα, encoded by ESR1, controls cell survival and proliferation genes(Asghari et al., 2022). While ERβ, encoded by ESR2, plays a signaling pathway in the nervous system and reproductive structure development (Dalal et al., 2022). The estrogen receptor is a transcription factor that has six domains. Each one has a specific function for the receptor. These domains are shown in Figure 2.1.

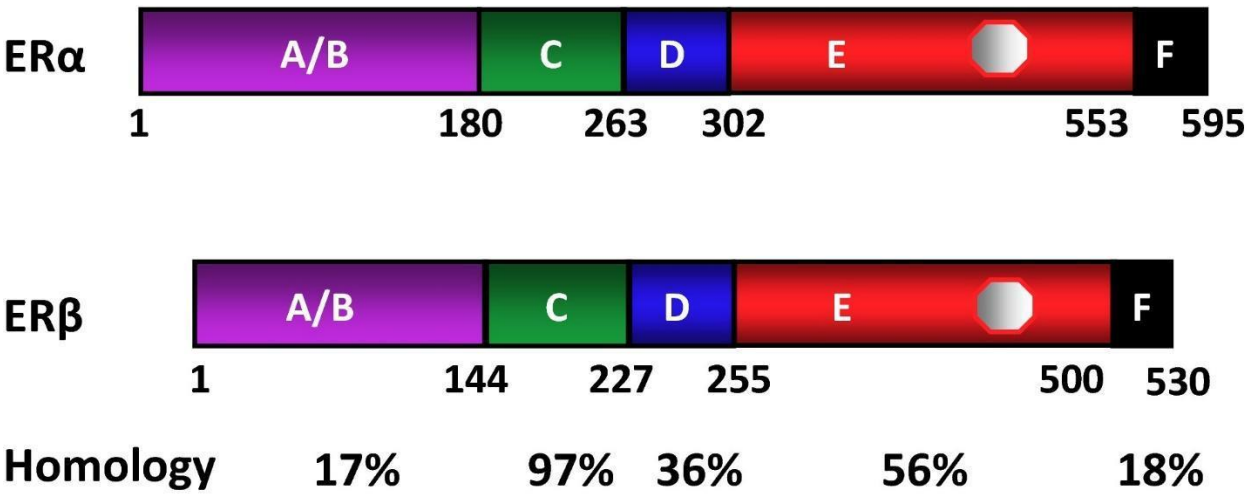

Figure 2.1. structure of the estrogen receptor ERα and ERβ. Estrogen receptor α contains 595 amino acids, while ERβ is 530 amino acids. They structurally contain terminal (A/B) domains. Region (C) is the DNA-binding domain. Domain (D) contains a nuclear localization signal and links with domain (C) to the multifunctional carboxyl-terminal (E) domain. And terminal (F) domain. ERα and Erβ are different, and the percent has shown domain homology (identity) between the two structures. (Yaşar et al., 2017).

Hormonal therapy (Endocrine therapy)

Overexpression of Estrogen is considered an indicator for increased risk of cancer cancer, such as breast cancer. Some of these cancer cells are sensitive to hormones which means these cells need hormones to grow. When estrogen or progesterone receptors are overexpressed in cancer cells surface this will stimulate cancer cells' growth. Referring to breast cancer this type of cancer is called hormone-dependent cancer. 70% of breast cancer tumors express hormonal

receptors such as estrogen receptors in their cells (Z. Li et al., 2022). These receptors are targeted in hormonal therapies (Endocrine therapies) during breast cancer treatment. One of the most common targeted receptors is ERα (Musheyev & Alayev, 2022).

These therapies slow or stop the growth of these tumors by stopping hormone production by eliminating or suppressing the ovaries hormonal production, leading to reduced hormone production in the body (Belachew & Sewasew, 2021). Such as Goserelin Acetate (zoladex)® is a type of hormone therapy which is suppression ovarian hormonal production or by ovarian ablation., also called oophorectomy, which is the surgical removal of ovaries.

Some of therapies are called Selective estrogen receptor modulators (SERMs) bind to estrogen receptors, block estrogen binding. Most common SERMs therapies are approved by the FDA for treatment of breast cancer are tamoxifen (Nolvadex)® and toremifene (Fareston)®.

Other therapies blocking the estrogen production, these medications is called aromatase inhibitors (AI) which block the activity of an enzyme called aromatase. This enzyme converts the androgens to estrogen synthesis in the body. (Belachew & Sewasew, 2021). AI medications such as anastrozole (Arimidex)® and letrozole (Femara)® deactivate aromatase, and exemestane (Aromasin)®, which permanently inactivates aromatase. (Musheyev & Alayev, 2022).

Other drugs are used for luminal A tumors. Fulvestrant (Faslodex)® also blocks Estrogen. It works the same as SERMs, called A selective estrogen receptor degrader or downregulator (SERD) is a type of drug which binds to the estrogen receptor (ER) such as fulvestrant (Faslodex)®. It binds to the estrogen receptor and functions as an estrogen blocker. Studies have shown that fulvestrant is more effective than SERMs (J. Li et al., 2019).

Others therapies are used as hormonal therapy such as, Ribociclib (Kisqali)® is in a of medications class called kinase inhibitors It is a targeted therapy called a CDK4/6 inhibitor. Everolimus (Afinitor)® it also targeted therapy called an mTOR inhibitor. mTOR is a type of protein called a kinase protein. In cancer, mTOR is switched on, which makes the cancer cells grow and produce new blood vessels. mTOR inhibitors therapy can stop cancer cell growing.

Hormonal Therapy Resistant

Many tumors become resistant to these therapies. It may happen during treatment that causes disease progression, or after treatment that causes recurrence. Resistance is divided into two types; primary hormonal Resistance (de novo), a recurrence in the first two years of adjuvant endocrine therapy, or progression of disease within the first six months of first-line hormonal therapy for metastatic breast cancer. And secondary or called acquired Resistance which is a recurrence that occurs on adjuvant endocrine therapy but after the first two years, or recurrence within one year of completing adjuvant endocrine therapy, or disease progression six months after initiating endocrine therapy for metastatic breast cancer (MBC) (Maurer et al., 2017) **in Figure 2.2.**

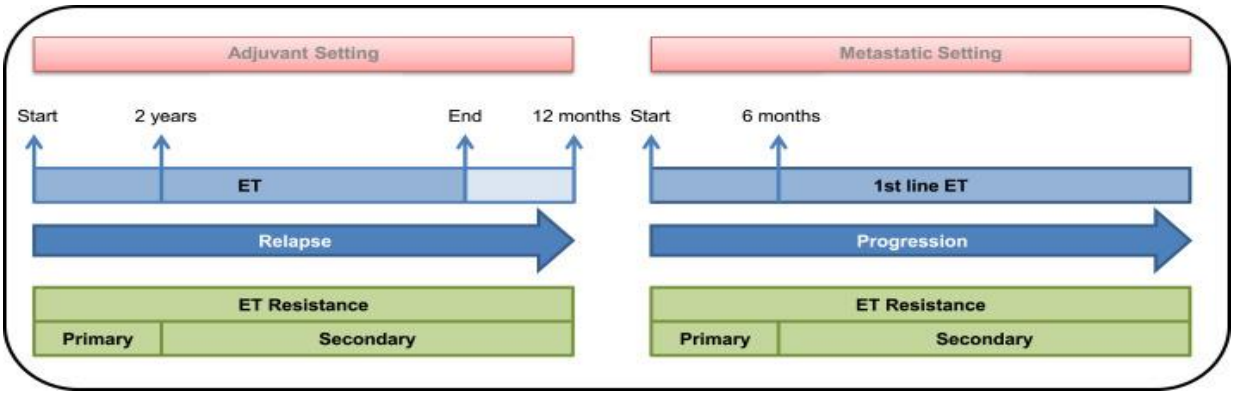

Figure 2.2. Differences between primary and secondary Resistance. In the adjuvant setting. Endocrine therapy (E.T.). (Maurer et al., 2017).

Many mechanisms cause hormonal therapy resistance, such as a loss of E.R. expression, mutations in the E.R. gene, cell cycle regulation, metabolic Resistance, receptor tyrosine kinases, and extracellular vehicles(Belachew & Sewasew, 2021). We want to point out some of them.

Approximately 30% of luminal A tumors developed primary Resistance to tamoxifen therapy (Jordan, 2008). Depending on previous studies, there is an intrinsic mechanism has been in patients who carry inactive cytochrome P450/2D6 (CYP2D6) alleles that cause block convert tamoxifen to the active metabolite, which decreases tamoxifen response (Musgrove & Sutherland, 2009).

Acquired resistance factors

Acquired Resistance occurs by several factors. We mentioned some of them.

E.R. expression loss

Approximately 20% of patients treated with endocrine therapy lose estrogen receptor ER function over time (Early Breast Cancer Trialists' Collaborative Group (Ebctcg), 2011). with loss of ER expression.

Mutation of the ESR1 Gene

Recent data showed that one of the most common causes of endocrine therapy resistance is Estrogen Receptor 1 (ESR1) mutation (Hartmaier et al., 2018). It has been found in the ligand binding domain (Brett et al., 2021).ESR1 mutations are acquired in patients treated with A.I. and other hormonal therapies in the metastatic setting (Brett et al., 2021). The most commonly identified variants are D538G, Y537S, Y537N, and Y537C (Jeselsohn et al., 2015). Mutation in

the E.R. gene causes the loss of its function when the Estrogen bound estrogen receptors. This leads to suppressing Estrogen receptor1 gene expression in a negative feedback loop. In cancer, overexpression of ESR1 causes resistant hormonal therapy(Pejerrey et al., 2018). Approximately 20–40% of patients who are treated by aromatase inhibition (A.I.) for MBC have ESR1 mutations (Nagaraj & Ma, 2021).

Extracellular Vehicles (E.V.s)

Extracellular vehicles are vesicles secreted from the membrane; it varies in size, function, and components. It might carry DNA, RNA, and proteins, and it has cellular information's can activate or affect a receiving cell. Such as activating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factors, which cause hormone therapy resistance in breast cancer.(Wang et al., 2014). Or by secret a, microRNA such as miR-155 (Szczepanek et al., 2022) and miR-222 were shown in studies (Yu et al., 2016).

Receptor Tyrosine Kinases

Receptor Tyrosine Kinases (RTKs) play a role in the cancer cell to activate a cascade pathway such as MAPK, PI3K/Akt, and JAK/STAT these play a role in metastasis and angiogenesis. When a mutation occurs in this gene that affects a treatment response leads to cause disease progression. (Butti et al., 2018)**.**

In breast cancer, RTKs are viewed in different cell types, including Insulin-like growth factor-1 receptor (IGF-1R). The IGF-1R is linked to the growth, development, and metastasis. by effect and work in singling pathways such as PI3K/AKT/mTOR pathway (Bailes & Soloviev, 2021). When the IGF-1R reacts with the estrogen receptor, that causes a re-position of E.R. from the

nucleus to outside the nucleus, which increases activation of E.R. that turn on further activates the pathways and results in acquired endocrine Resistance (Belachew & Sewasew, 2021). Also include, Fibroblast Growth Factor Receptor (FGFR) they are playing a role in breast cancer growth and progression studies have shown the over-expression of FGFR3 that causes tamoxifen resistance by activating the RAS pathway and PI3K pathway (Tomlinson et al., 2012) and FGFR1 promotes cyclin D1 expression in ER-positive breast cancer, resulting in Resistance to antiestrogen (Zhou et al., 2020).

Metabolic Resistance

There are many types of cytochromes converting tamoxifen into pharmacologically active form. The essential one is cytochrome P2D6 (CYP2D6). Any null or reduced enzyme activity for this cytochrome may cause tamoxifen resistance (Hoskins et al., 2009).

PIK3CA Mutation

A class of kinases called PI3Ks is required for healthy cell development and proliferation. class A of PI3Ks consist of a regulatory subunit (p85α) and a catalytic subunit (p110) in which three isoforms exist: p110α (encoded by PIK3CA). The PI3K/AKT/mTOR pathway is vital in cell growth and survival, protein synthesis, and glucose metabolism. This pathway is possibly the most commonly altered pathway in human cancers to increase their proliferation and survival. (Schwartzberg & Vidal, 2020). Such as Mutations in the alpha catalytic subunit of PI3K (*PIK3CA*) are some of the most frequent genomic alterations noted in luminal A breast cancer patients. The inhibition of the PI3K/AKT/mTOR pathway, used as a therapy that reduces cell proliferation and survival, reduces signaling pathways. (Schwartzberg & Vidal, 2020). PIK3CA

mutational analysis in the SOLAR-1 study showed greater reductions in risk for disease progression in mutated PIK3CA patients. It was detected in plasma ctDNA in patients treated with alpelisib plus fulvestrant (André et al., 2021).

Chapter 3

(Material and Methods)

Ethical approval (ethical consideration)

Our thesis research was approved by the research ethics subcommittee of the Faculty of Medicine at Quds University with REC reference number: Ref#: R1-19-22. The consent form was obtained from all patients before the study.

Study Sample

We have selected patients in this study according to inclusion and exclusion criteria: We included specific patients with luminal A metastatic breast cancer who have disease progression or recurrence after hormonal treatment and they are tested with oncomine test. Other types and subtypes of breast cancer patients were excluded from our study.

Data from 16 patients were collected, the majority were females (15 samples), and only one male, data were collected all the available specific patients in our inclusion criteria during 2022 from the medical oncologist doctors in the Palestine hospital (Directorate of Military Medical Services, Hrmalla). The sample patients' numbers are small because our inclusion criteria were particular, and it was difficult to find more patients. The other reason was that the genetic oncomine test is already new in the West Bank, so previous data was small.

Those patients were tested by the oncomine test, we choose this test because it the only test are used in West Bank and have available result data, which is a test of a panel of 53 genes (shown

in the appendix). Those genes were tested from a tumor sample (biopsy) obtained by specialist surgical doctors. Then the results were obtained to see if they had any mutation that caused treatment resistance. This is the first part of our research.

The second part depends on the first part's results. Only three positive oncomine test patients have been attained because the other patients refused to continue the second part of our research.

After that, blood samples were taken to test the positive genes by PCR to study if the tumor tissue (biopsy) sample and blood sample gave us the same results.

All patients signed a consent form according to research protocols, and we what we are studying and why we need a sample. Then, we collected 2 EDTA (Greiner bio-one K3E, 2ml tubes) blood sample tubes from the three patients.

DNA extraction

After we collected the blood sample, we extracted DNA using the Genomic DNA Mini Kit (Blood/Cultured Cell). The procedure is in the appendix(2).

DNA concentration measurement

DNA concentration and purity for each sample are measured by a NanoDrop spectrophotometer device (NanoDrop 2000 Spectrophotometer, Thermo Fisher Scientific).

Primer design

The primers are designed for each patient depending on the type of mutation/s detected in oncomine test using Prime 3 software (https://primer3.ut.ee/). These primers are listed in Table 3.1 below.

No.	Gene	Primers	References	Patient code
1	PIK3CA Exon 21	F. TATTCGACAGCATGCCAATC R. TGTGTGGAAGATCCAATCCA	NM_006218.4	DBC1 DBC3
2	PIK3CA Exon 10	F. AGGGAAAATGACAAAGAACAGCT R. TCTCCATTTTAGCACTTACCTGT	NM_006218.4	DBC2
3	ESR1	F. TGAAGTGCAAGAACGTGGTG R. GAATGCGATGAAGTAGAGCCC	NM_000125.4	DBC2

Table 3.1: The primers are designed by Prime 3 for our patients.

Polymerase Chain Reaction (PCR)

PCR was used to amplify the mutations which are detected in oncomine. The total volume is 25 µl for each PCR reaction, containing a 0.25 µl of primers forward and 0.25 µl of reverse, four µl of extracted DNA, eight µl of H2O, and 12.5 µl of ready mix. All of the samples have the exact total of the PCR product reaction.

Each patient has different primers, so each has a different PCR program.

DBC1 and DBC3 for PIK3CA primer have the same PCR program.

It starts with initial denaturation for 5 min at 95°C with initial denaturation for 5 min at 95°C, after initial denaturation, 34 cycles in the following order, 30 sec at 95°C for denaturation, 30 sec at 51 °C for annealing and 1 min at 72 °C for extension, and final extension for 5 min at 72 °C. for the sample and negative controls without DNA were included.

DBC2 program for PIK3CA and ESR1 primers in the same PCR program

PCR reaction started with initial denaturation for 5 min at 95°C, after initial denaturation, 34 cycles in the following order, 30 sec at 95°C for denaturation, 30 sec at 55 °C for annealing, and 1 min at 72 °C for extension, and final extension for 5 min at 72 °C. for the sample and negative controls without DNA were included.

Gel Electrophoresis

Gel was prepared by weights 2 grams of agarose with 100 ml of TAE (Tris-acetate-EDTA), placed in the microwave for one minute, cooled, and added 8 ml of ethidium bromide. Poured into an agarose gel casting system and turned on the (Consort EV245 electrophoresis) power supply for 60 min in 120 volts. PCR sample products and the DNA ladder (BIO-HELIX - 100bp DNA Ladder RTU (100-1,500 bps)) were loaded in the wells to see sample migration. The ladder has migrated, and the appropriate distance for the movement depends on the DNA size. After the time ended, we saw the results with U.V. light taken in a photo by (Bio-Imaging et al.).

Chapter 4
(Results)

This research has two parts of work. First, the data collection for oncomine test results. Second, the collection of blood samples for PCR test results.

Part 1. Data Reviewed

Sixteen patient data were collected from the medical oncologist doctors from Palestine Hospital (Harmala Hospital) for specifically targeted breast cancer patients diagnosed as Luminal A and metastatic patients and treated with hormonal therapy. During the treatment, they developed disease progression and Resistance to treatment. The collected data have been arranged in Tables.

First, the results in Table 4.1 are the general information about those patients. It contains the hormonal receptors and HER2 status for Luminal A. The receptor estrogen and progesterone are positive, while HER2 is negative. The patient samples are 16 patients. There are 15 females and one male, with variations in age. The coding system (DBC-n-) maintained the patient's privacy.

NO.	CODE	Gender	Age	ER	PR	HER2
pitt1	DBC1	F	67	Positive	Positive	Negative
pitt2	DBC2	F	51	Positive	Positive	Negative
pitt3	DBC3	F	33	Positive	Positive	Negative
pitt4	DBC4	M	43	Positive	Positive	Negative

pitt5	DBC5	F	42	Positive	Positive	Negative
pitt6	DBC6	F	56	Positive	Positive	Negative
pitt7	DBC7	F	39	Positive	Positive	Negative
pitt8	DBC8	F	58	Positive	Positive	Negative
pitt9	DBC9	F	68	Positive	Positive	Negative
pitt10	DBC10	F	45	Positive	Positive	Negative
pitt11	DBC11	F	45	Positive	Positive	Negative
pitt12	DBC12	F	57	Positive	Positive	Negative
pitt13	DBC13	F	47	Positive	Positive	Negative
pitt14	DBC14	F	24	Positive	Positive	Negative
pitt15	DBC15	F	43	Positive	Positive	Negative
pitt16	DBC16	F	31	Positive	Positive	Negative

Table 4.1 The general information was obtained from medical oncologists for targeted patients in this research. Patients' general Data. Patient (Pitt), Estrogen receptor (ER), progesterone receptor (P.R.), Human epidermal growth factor 2 (HER2), and DBC are the codes we are given. Female (F), Male (M).

Second, table 4.2 contains each patient's hormonal treatment line and treatment duration time. Each patient has a specific line of treatment depending on the cancer cells' stage, grade, size, and molecular status. Those patients are Luminal A, and they are sensitive to hormonal therapy, such as Everolimus (Afinitor)®, its mTOR inhibitor. Letrozole (Femara)® is an aromatase inhibitor,

and Tamoxifen, Fulvestrant (Faslodex)® there are a SERD. Ribociclib is a CDK 4 and CDK 6, and exemestane (Aromasin)® is one of the aromatase inhibitor drugs. Moreover, Goserelin (Zoladex)® is ovarian suppression that stops hormonal production.The type of treatment correlates with the mutation type it may occur to develop a disease progression. Also, Table 4.2 contains the metastatic site for each patient. The metastasis in some patients occurs in many organs. 14 of 16 (87.5%) patients have bone metastasis, 5 of 16 (31%) with liver metastasis, also 5 of 16 (31%) with lung metastasis, 3 of 16 (18%) have brain metastasis, and 2 of 16 (12%) with lymph nodes metastasis.

NO.	CODE	Hormonal Therapy Name	Duration of time before disease progression	Metastasis location
Pitt1	DBC1	Ribociclib+Femara	17 Months	Brain and Bone
Pitt2	DBC2	Femara+Faslodex	18 Months	Bone
Pitt3	DBC3	Tamoxifen	2 Months	Bone
Pitt4	DBC4	Tamoxifen	4 Months	Bone
Pitt5	DBC5	Tamoxifen, Everolimus	13 Months	Bone and Brain
Pitt6	DBC6	Ribociclib\Femara,	3 Months	Liver, Bone, and Brain
Pitt7	DBC7	Tamoxifen	2 Months	Lung and Liver
Pitt8	DBC8	Afinitor/Aromasin, Faslodex/Ribo	3 Months	Bone and Liver
Pitt9	DBC9	Femara	2 Years	Lung and Bone
Pitt10	DBC10	Tamoxifen	6 Months	Lung, Liver, and Bone
Pitt11	DBC11	Zoladex/Ribo/Femara	6 Months	Bone, Liver, and Lung
Pitt12	DBC12	Ribo/Femara	1st Line	Lymph Nodes
Pitt13	DBC13	Zoladex/Ribo/Femara	1st Line	Bone
Pitt14	DBC14	Zoladex/Ribo/Femara	3 Months	Lung and Bone
Pitt15	DBC15	Tamoxifen	6 Months	Bone
pitt16	DBC16	Tamoxifen	4 Months	Bone

Table 4.2 Treatment type and metastatic site for each patient.

Third, Table 4.3 shows the oncomine test results for those 16 patients. 8 (50%) of them have an acquired mutation/s that causes Resistance to hormonal treatment during management, the most common mutation here in our data PIK3CA we have 6 of 16 (37.5) patients with this mutation and 2 of 16 (12.5%) with ESR1 mutation, also one (6%) of the patient has an AKT1 mutation. We cannot generalize the result in the population for many reasons: first, we have a small sample size of patients with luminal A, metastatic who developed the progression of a disease and Resistance already few, and second, the oncomine test for those patients are new in the West Bank so the existing data for the test result already few. While 8 (50%) have negative results, the Resistance occurs by other pathways or in other mutated genes not targeted by the 53 genes panel. The type of mutation occurring is connected with the treatment line.

NO.	CODE	Type of mutation Result
pitt1	DBC1	PIK3CA
pitt2	DBC2	PIK3CA and ESR1
pitt3	DBC3	PIK3CA
pitt4	DBC4	NEGATIVE
pitt5	DBC5	NEGATIVE
pitt6	DBC6	NEGATIVE
pitt7	DBC7	NEGATIVE
pitt8	DBC8	ESR1
pitt9	DBC9	PIK3CA
pitt10	DBC10	AKT1

pitt11	DBC11	PIK3CA
pitt12	DBC12	PIK3CA
pitt13	DBC13	NEGATIVE
pitt14	DBC14	NEGATIVE
pitt15	DBC15	NEGATIVE
pitt16	DBC16	NEGATIVE

Table 4.3 oncomine test results.

Part2. Targeted positive patients with PCR and sequencing.

Depending on the oncomine test results, we did part two, selected three patients who were positive for mutations and withdrew blood samples for PCR and sequencing. After collecting the blood sample, we extracted DNA and measured the DNA concentration. To test the quality and quantity of DNA for our extraction.

DNA concentration results (NanoDrop results)

DBC1 DNA concentration is 65.1 ng/ul. The 260/280 results are 1.86. and 260/230 are 2.14.

DBC2 DNA concentration is 16.1 ng/ul. The 260/280 result is 1.42, and the 260/230 is 0.68.

DBC3 DNA concentration is 35.3 ng/ul. The 260/280 result is 0.63, and the 260/230 are 0.50.

Gel electrophoresis results

We used a PCR test to amplify the different mutations for each patient. DBC1 for PIK3CA mutation in Exon 21, DBC2 for ESR1 mutation and PIK3CA mutation in Exon 10, and DBC3 for PIK3CA mutation in Exon 21. The results are shown in the Figures below **Figure 4.1, Figure 4.2, and Figure 4.3**. that shown there are a bands was detected for each primer.

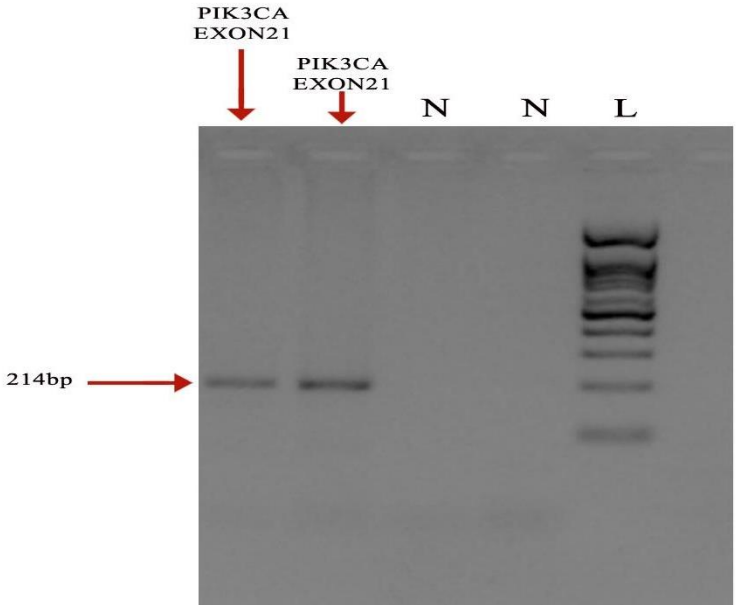

Figure 4.1 Agarose gel photograph to DBC 1 for PIK3CA mutation in Exon21, loaded in four wells with two negative controls. The band's size is 214bp: n (Negative control), L (Ladder), and bp(base pair).

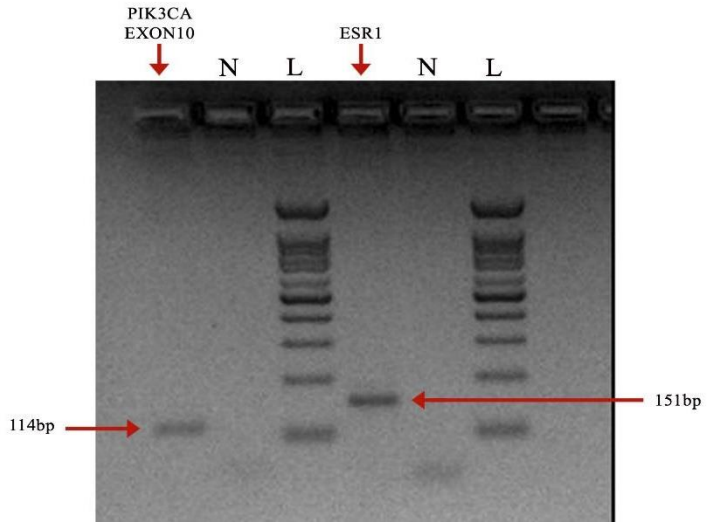

Figure 4.2 Agarose gel photograph to DBC 2 for ESR1 primer and PIK3CA primer. With sizes 151 and 114 bp, respectively, N (Negative control), L (Ladder), and bp(base pair).

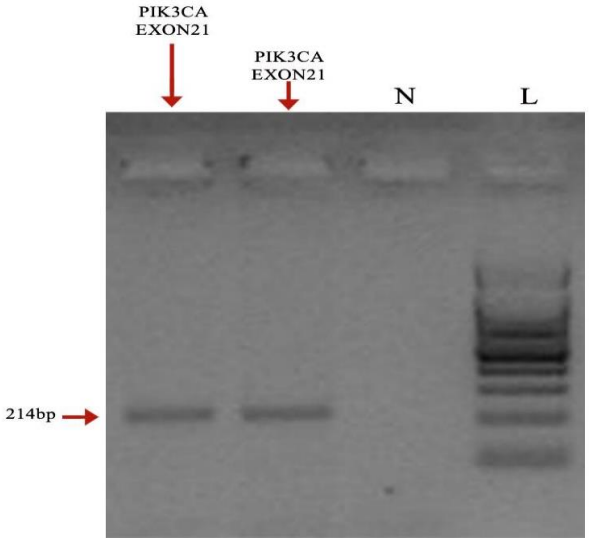

Figure 4.3 Agarose gel photograph to DBC 3 for PIK3CA In Exon 21 with negative control. With a band, the size is 214bp: n (Negative control), L (Ladder), and bp(base pair).

Sequencing result

After the PCR amplification and running in the gel, we sent the samples for sequencing (Hylab, Israel). The results of sequencing are shown in Table 4.4. DBC1, DBC2, and DBC3 samples in PIK3CA mutation and DBC2 for ESR1 mutation for the three patients are not confirmed mutation detection shown in table 4.4. There are no mutations detected by sequencing. This means we cannot use the blood sample by PCR test to detect these mutations.

Table 4.4 Sequencing results for the three patients' mutation/s. DBC1, DBC2, and DBC3.

Code	Gene	Sequencing results
DBC1	PIK3CA (Exon 21)	>DBC1-exon21 TTATTCGACAGCATGCCAATCTCTTCATAAATCTTTTCTCAATGATGCTT GGCTCTGGATGCCAGAACTACAATCTTTTGATGACATTGCATACATTCGA AAGACCCTAGCCTTAGATAAAACTGAGCAAGAGGCTTTGGAGTATTTCA TGAAACAAATGAATGATGCACATCATGGTGGCTGGACAACAAAAATGGA TTGGATCTTCCACACAA
DBC2	PIK3CA (Exon 10)	>DBC2-Exon10 AGAGAATCTCCATTTTAGCACTTACCTGTGACTCCATAGAAAATCTTTCT CCTGCTCAGTGATTTCAGAGAGAGGATCTCGTGTAGAAATTGCTTTGAG CTGTTCTTTGTCATTTTCCCT
DBC2	ESR1	>DBC2-ESR1 TGAAGTGCAAGAACGTGGTGCCCCTCTATGACCTGCTGCTGGAGATGCTG GACGCCCACCGCCTACATGCGCCCACTAGCCGTGGAGGGGCATCCGTGGA GGAGACGGACCAAAGCCACTTGGCCACTGCGGGCTCTACTTCATCGCATT CA
DBC3	PIK3CA (Exon 21)	>DBC3-Exon21 TTATTCGACAGCATGCCAATCTCTTCATAAATCTTTTCTCAATGATGCTT GGCTCTGGATGCCAGAACTACAATCTTTTGATGACATTGCATACATTCGA AAGACCCTA GCCTTAGATAAAACTGAGCAAGAGGCTTTGGAGTATTTCATGAAACAAA TGAATGATGCACATCATGGTGGCTGGACAACAAAAATGGATTGGATCTT CCACACAA

Chapter 5
(Discussion)

5.1. Review Oncomine Test Results

The heterogeneity of breast cancer causes many differences in cells leading to changes in the biological characteristics of cancer cells, such as genetic changes. It depends on whether these changes have been developed to improve tumor classification and implement more targeted treatments or whether the changes cause a mutation through the treatment, affecting the treatment response that causes resistance and disease progression. Luminal A breast cancer has a better prognosis and response for treatment than other subtypes. However, those patients' primary cause of death is driven by hormonal therapy resistance and metastasis (Lei et al., 2019). This research has targeted specific patients who are diagnosed as Luminal A subtype, those with Estrogen and Progesterone receptors are positive and HER2 negative, which are shown in Table 4.1.who developed a disease progression during treatment management to detect the mutations that cause the Resistance and to give them the best Line of treatment. We designed this research in two parts; First, we review and study these mutations that may occur in targeted patients, which are detected by oncomine test for 53 genes panel.

Our sample is 16 patients. Fifteen are females, and one is male with different ages and metastatic sites. Some of them are with metastasis to numerous organs. That showed the most common metastatic site in those breast cancer patients in high percent 14 of 16 (87.5%). That confirmed previous studies. The most common sites in breast cancer patients are bone, liver, lung, and brain (Kennecke et al., 2010).Which is bone metastasis is a universal metastatic site of breast cancer. (Wu et al., 2017). Specifically in Luminal subtypes A and B.(Wu et al., 2017).

The results shown in Table 4.3 are that 8 of 16 (50%) patients are negative. They developed a disease progression with Resistance to hormonal treatment. Our explanation for these results may be other pathways and genes are mutated and not detected in the 53 genes panel, such as metabolic pathways, extracellular vesicles, Forkhead box protein A1 (FOXA1) expression (Fu et al., 2016) microenvironment of tumor and epigenetic factor, for example, microRNA (miRNA) and long non-coding RNA (lncRNA). (Brett et al., 2021).

While 8 of 16 (50%) are positive results. The most common mutations detected are PIK3CA and ESR1 mutations that caused the Resistance to the treatment. Also, one in sixteen patients has an AKT1 mutation. These results cannot generalize for all the population, but studies worldwide confirm that these mutations were detected as the most common mutations that cause hormonal therapy resistance (Lei et al., 2019). The most common mechanism of hormonal therapy resistance is mutations in estrogen receptor alpha (Erα), the *ESR1* gene within the ligand-binding domain that decreases sensitivity to antiestrogen. Most commonly acquired ESR1 mutations occur at D538G and/or Y537S (Brett et al., 2021). in our results, we detect the Y537S in the ESR1 gene in DBC2 patients.

Studies have also identified a prominent role for PI3K pathway dysregulation in metastatic breast cancer progression and endocrine Resistance. PIK3CA is mutated in 40% of Luminal A patients, with the most common variants occurring in E542K or E545K of exon nine and H1047R in exon 21 (Hartkopf et al., 2020). The PIK3CA H1047R variant in exon 21 confirms our data mutation results exist in DBC1 and DBC3 patients. Also, PIK3CA (E545K variant) exists in DBC2 patients.

Knowing mutations that cause Resistance now helps oncologists select the best treatment choice and develop new treatment types that target other pathways to kill cancer cells. Clinical studies

showed a correlation between a treatment type and the mutation type, so many studies showed that patients treated with AI (Augusto et al., 2018) might develop Resistance during the treatment (Augusto et al., 2018). Depending on our data, 5 of 8 mutated patients are treated with Femara therapy (letrozole) as one of the A.I. treatments. Also, several studies have shown that *ESR1* mutations correlate with tamoxifen and fulvestrant treatment. (Dustin et al., 2019).

The new studies showed that cancer cells are susceptible to CDK4/6 inhibitors such as ribociclib, so it should be combined with other hormonal therapy to give a highly effective response to treating patients' Resistance to treatment. (J. Li et al., 2019). Nevertheless, patients DBC1 and DBC12 are treated were ribociclib a CDK4/6 inhibitor families, yet they developed resistance. Clinical trials now work to overcome hormonal therapy resistance by using another targeted therapy or by choosing the best combination therapies (Hanker et al., 2020) such as CDK4/6 inhibitors with AI or fulvestrant combination, is the best choice for first-line treatment for Luminal A metastatic tumors (Hanker et al., 2020). Moreover, developing other new therapies by targeting other pathways, such as inhibitors to the PIK3CA pathway, Signal transducer, and activator of transcription 3 (STAT3) as cancer immunotherapy; MYC is a Protein Coding gene, MEK gene encodes signaling pathway protein in the RAS/MAPK pathway, and Poly (ADP-ribose) polymerase (PARP) pathways (Sunada et al., 2018).

5.2 Detection of mutation by PCR in the blood sample

The second part of this research, DBC1, DBC2, and DBC3 samples in PIK3CA mutation and DBC2 for ESR1 mutation are detected as a band for the primers in gel electrophoresis shown in Figure 4.1, Figure 4.2, and Figure 4.3. But the sequencing results for the three patients are not confirmed mutation detection. The PCR and sequencing cannot uses a test to detect these mutation in blood sample.

There are many comprehensive techniques used to test gene mutations in cancer cells. (Qu, X., et al (2020)) These tests are used to detect the mutation for cancer diagnosis, are inherited cancer by testing the BRCA genes, or if acquired cancer by testing other common genes. Genetic tests to detect mutations lead to treatment resistance. The most common tests are used: Next generation sequencing (NGS) and Polymerase chain reaction (PCR). In this research, the patients are tested first by an oncomine test of one of the NGS for a 53 genes panel in the tumor biopsy sample, followed by a PCR test to detect this mutation in the blood sample for specific primers for mutation detected by NGS, to test the sensitivity of PCR test if can use it as an essential test for breast cancer patients in the blood sample because the blood sample easier than biopsy sample, and the PCR lower cost than NGS. But then, our PCR and sequencing results showed the NGS in tissue samples were sensitive and detectable for these mutations, then PCR in blood samples that confirmed a previous study the NGS was the best method to detect the cancer mutation than other methods (Fusco et al., 2021). Other study shown the NGC test 82% are sensitive than sanger sequencing 57% for PIK3CA mutation detection. It may use a tissue sample or liquid biopsy. (Vollbrecht et al., 2023).

Recent studies have confirmed the development of droplet digital PCR (ddPCR) technology for mutation detection that causes treatment resistance in cancer cells to be more sensitive and reliable for Detection, specifically ESR1 and PIK3CA mutations. This technology is used in liquid biopsy samples to detect the dead tumor cells in the circulation, called circulating tumor DNA (ctDNA). All cells in the body release cell-free DNA (cfDNA) into the bloodstream. But the tumor cell, when it dies, releases the circulating tumor DNA. It was more sensitive to test this ctDNA. It is now the most common successful method to detect PIK3CA and ESR1 (Fusco et al., 2021). All cells in the body release cell-free DNA (cfDNA) into the bloodstream. But the

tumor cell, when it dies, releases the circulating tumor DNA. It was more sensitive to test this ctDNA.

5.3 Conclusion and Limitations

In conclusion, the most common mutations were detected in this research by oncomine test in tissue samples; they are ESR1, PIK3CA, and AKT1. The results confirmed a common mutation that causes hormonal therapy to be resistant worldwide. When testing an ESR1 mutation and PIK3CA in blood samples for three patients by PCR and sequencing, the mutation was not detected, so we cannot use this method to detect these mutations. Recent studies have shown that testing ctDNA in blood samples by ddPCR is the most sensitive method for breast cancer patients.

The limitations of the study are the small sample size that was collected for many reasons, the patients with luminal A, metastatic who developed a diseases progression and Resistance in the population already few, the oncomine test for those patients are new in West Bank, so the existing data for the test result also few not all positive eight patients are tested by PCR. We cannot generalize the study results to the population. And the ddPCR test is unavailable. In the future may collect larger data for oncomine tests in tissue samples and re-test by ddPCR after availability in the blood sample.

5.4 Recommendations

Based on our findings, we recommend to do NGS as liquid biopsy for all breast cancer patients with hormone positive, HER2 negative who develops resistance on hormonal therapy , as its

noninvasive procedure that may replace new solid biopsy from breast cancer patients especially when new biopsy is difficult .

Consequently, new mutation’s will guide oncologists for new targeting therapy that improve survival and prognosis to our patients.

Also, we recommend in future to test these mutations in larger panel that is available in oncomine test such as 81 genes panel .

الطفرات الوراثية في مرضى سرطان الثدي النقيلي المتقدم من نوع luminal A التي تسبب مقاومة للعلاج الهرموني في فلسطين

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الملخص باللغة العربية

التصنيف الجزيئي لسرطان الثدي له أهمية كبيرة في تشخيص مرضى سرطان الثدي وعلاجهم تعتمد على العديد من أنواع فرعية الجزيئية من النوع افرعي للفئة المستهدفة في هذه الدراسة يسمى Luminal A حيث تحتوي الخلية السرطانية على مستقبلات هرمونية تحتوي على مستقبلات الأستروجين(ER) ، مستقبلات البروجسترون (PR) مع فقدان عامل نمو البشرة البشري 2 (HER2) وهم أكثر قابلية للعلاجات من المجموعات الفرعية الأخرى تحديدا للعلاج الهرموني. هؤلاء المرضى يعالجون بالعلاج الهرموني الذي يستهدف هذه المستقبلات مما يؤدي الى تقليل أو وقف نمو الخلايا السرطانية وبالتالي موتها. قد يصاب بعض المرضى بمقاومة العلاج الهرموني لأسباب عديدة. في هذا البحث، نريد دراسة الطفرات الجينية الأكثر شيوعًا تسبب مقاومة العلاج الهرموني.

يحتوي هذا البحث على جزئين. أولاً، نقوم بدراسة وجمع النتائج التي تم اختبارها في عينة الأنسجة بواسطة فحص oncomine على عينات خزعة من الورم لستة عشر مريضًا تم تشخيص إصابتهم بسرطان الثدي و متنقل (METASTSIC)، وتم تشخيصهم انهم من نوع Luminal A، ثانيا ، اختبار ثلاثة من المرضى إيجابيين لطفرة معنية في الجزء الأول وتم استهداف هذه الطفرات بواسطة سلسلة البوليميراز تفاعل (PCR) على عينات دم مأخوذة من المرضى.

النتائج أوضحت ان الطفرات الأكثر شيوعًا التي تسبب مقاومة العلاج الهرموني هي الطفرات ESR1 وPIK3CA ، وهذه الطفرات لا يمكن اكتشافها او فحصها عن طريق فحصPCR .

بالنهاية تحتاج هذه الطفرات إلى طريقة محددة تغطي جميع الطفرات التي تسبب الهرمونات مقاومة العلاج وتطور المرض لدى مرضى سرطان الثدي. مؤخرا تم اكتشاف ان ddPCR الرقمي يستخدم لاكتشاف الطفرة في ctDNA الدم.

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Appendix

DNA target genes									
AKT1	EGFR	FGFR4	JAK3	MYCN	CCND1	ESR1	IDH1	MAP2K2	RAF1
ALK	ERBB2	GNA11	KIT	NRAS	CDK4	FGFR1	IDH2	MET	RET
AR	ERBB3	GNAQ	KRAS	PDGFRA	CDK6	FGFR2	JAK1	MTOR	ROS1
BRAF	ERBB4	HRAS	MAP2K1	PIK3CA	CTNNB1	FGFR3	JAK2	MYC	SMO
DDR2									
Fusion driver genes									
ABL1	AXL	ERBB2	ETV4	FGFR2	NTRK1	PDGFRA	RET	AKT3	ETV1
ALK	BRAF	ERG	ETV5	FGFR3	NTRK2	PPARG	ROS1	EGFR	FGFR1
MET	NTRK3	RAF1							

Appendix1. Oncomine 53 genes panel for cancer mutation

Genomic DNA Mini Kit (Blood/Cultured Cell) Fresh Blood Protocol

IMPORTANT BEFORE USE

- Add absolute ethanol (see the bottle label for volume) to the Wash Buffer prior to initial use
- Collect blood in EDTA-NA_2 treated tubes (or other anticoagulant mixtures)
- Additional requirements: microcentrifuge tubes, centrifuge tube, absolute ethanol, (optional) RNase A (10 mg/ml)

Sample Preparation	• Transfer **up to 300 µl of blood** to a 1.5 ml microcentrifuge tube. NOTE: If the blood sample is more than 300 µl (up to 1 ml), add to a sterile 15 ml centrifuge tube. • Add **3X the sample volume of RBC Lysis Buffer** then mix by inversion. Do not vortex. • Incubate the tube for 10 minutes at room temperature. • Centrifuge for 5 minutes at 3,000 x g then remove the supernatant completely. • Add **100 µl of RBC Lysis Buffer** to resuspend the leukocyte pellet then proceed with Cell Lysis.
Step 1 Cell Lysis	• Add **200 µl of GB Buffer** then shake the 1.5 ml microcentrifuge tube vigorously. • Incubate at 60°C for at least 10 minutes to ensure the sample lysate is clear. • During incubation, invert the tube every 3 minutes. At this time, preheat the required **Elution Buffer** (200 µl per sample) to 60°C (for Step 4 DNA Elution). **Optional Step: RNA Degradation** (If RNA-free gDNA is required, perform this optional step) • Following 60°C incubation, add 5 µl of RNase A (10 mg/ml) to the clear lysate then mix by shaking vigorously. • Incubate at room temperature for 5 minutes.
Step 2 DNA Binding	• Add **200 µl of absolute ethanol** to the lysate then immediately mix by shaking vigorously for 10 seconds. NOTE: If precipitate appears, break it up as much as possible with a pipette. • Place a **GD Column** in a **2 ml Collection Tube**. • Transfer the mixture (including any precipitate) to the **GD Column** then centrifuge at 14-16,000 x g for 5 minutes. • Discard the **2 ml Collection Tube** then place the **GD Column** in a new **2 ml Collection Tube**.
Step 3 Wash	• Add **400 µl of W1 Buffer** to the **GD Column** then centrifuge at 14-16,000 x g for 30-60 seconds. • Discard the flow-through then place the **GD Column** back in the **2 ml Collection Tube**. • Add **600 µl of Wash Buffer (make sure ethanol was added)** to the **GD Column**. • Centrifuge at 14-16,000 x g for 30-60 seconds then discard the flow-through. • Place the **GD Column** back in the **2 ml Collection Tube**. • Centrifuge again for 3 minutes at 14-16,000 x g to dry the column matrix.
Step 4 DNA Elution	Standard elution volume is 100 µl. If less sample is to be used, reduce the elution volume (30-50 µl) to increase DNA concentration. If higher DNA yield is required, repeat DNA Elution step to increase DNA recovery and the total elution volume to approximately 200 µl. • Transfer the dried **GD Column** to a clean 1.5 ml microcentrifuge tube. • Add ***100 µl of pre-heated Elution Buffer***, TE or water to the **CENTER** of the column matrix. • Let stand for at least 3 minutes to ensure the **Elution Buffer**, TE or water is completely absorbed. • Centrifuge at 14-16,000 x g for 30 seconds to elute the purified DNA.

Appendix2. The kit procedure are used for DNA extraction.

التاريخ:_____

إقرار موافقة مريض

المعلومات الشخصية

الاسم: ----------

رقم الهوية: ----------

العمر: ---------

الجنس/الجندر: انثى/ذكر

رقم الهاتف: ----------

العنوان: ----------

هذا البحث لمعرفة سبب مقاومة العلاج الهرموني للمريض من خلال معرفة الطفرات الجينية.

ومساعدة الطبيب في اختيار العلاج المناسب له دون المخاطرة في إعطاء أي علاج يمكن أن يسبب مقاومة للعلاج في المستقبل

- أقر انا (المريض/ة \ ولي أمر عن المريض/ة) ----------بالموافقة على أن المشاركة في بحث علمي بعنوان:

Genetic mutation in Metastatic Breast Cancer in Luminal A Tumor, which may Cause Resistant to Hormonal Therapy in Palestine

الرجاء وضع اشارة (√) في حال الموافقة

- ☐ أقر بأنني قد أخبرت بأن هذا البحث بحث علمي وبأسبابه والفترة الزمنية لهذا البحث.
- ☐ أُخبرت بالجهة التي يجب علي التواصل معها للاستعلام عن أي تساؤل.
- ☐ أُخبرت أنه سيتم استخدام ملفي الطبي وستكون المعلومات في سرية تامة وأنه فقط لغرض استكمال البحث.
- ☐ أوافق على استخدام عينة الخزعة التي تم أخذها لعمل الفحوصات الجينية (في حال تطلب الامر او توفرت)
- ☐ أوافق على عمل فحوصات جينية من عينة الدم ومن الخزعة (في حال تطلب ذلك).
- ☐ أوافق على استخدام نتائج فحوصات جينية سابقة (في حال توفر ذلك).
- ☐ أوافق على أن يتم استخدام نتائج فحوصاتي واستخدامها في البحث العلمي وأيضا في حالة نشر البحث.

توقيع المريض	توقيع الطبيب	توقيع الطالبة
	د. هاني حور	ديالا عيسى حماد

Appendix3. The consent form are signed by the patients.

Al-Quds University
Faculty of Medicine
Abu-Dies, Jerusalem

جامعة القدس
كلية الطب
أبوديس – القدس

Research Ethics subcommittee of Faculty of medicine

Letter of Ethical approval

Date:19/11/2022
Ref#: R1-19-22

Dear Applicants: Dr. Hani Howar and Diala Hammad
Biochemistry and Molecular Biology master program

The Research Ethics subcommittee of faculty of medicine has recently reviewed your proposal entitled (Genetic mutation in Metastatic Breast Cancer in Hormonal Receptor Positive and HER2-Negative Tumor, which may Cause Resistant to Hormonal Therapy. Your proposal is deemed to meet the requirements of research ethics subcommittee at Al-Quds University.
Note: This letter can be used to apply for the central Al-Quds University research ethics committee if needed.

Best of luck,

Dr. Suheir Ereqat
Head of research ethics subcommittee
Faculty of Medicine-Al-Quds university

P.O Box 20002
Tel 02-2799203, Fax 02-2796110

ص. ب 20002
هاتف 022799203 فاكس 022796110

Appendix4. The ethical approval from the research ethics subcommittee in AL-Quds university.