Effect of Genetic Polymorphism of ABCB1 Gene on Anastrozole Efficacy in Iraqi Breast Cancer Women

A Thesis

Submitted to the Council of College of Pharmacy / University of Kerbala as Partial Fulfillment of the Requirements for the Degree of Master of Science in Pharmacology and Toxicology

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(وَفَوْقَ كُلِّ ذِي عِلْمٍ عَلِيمٌ)

صدق الله العلي العظيم

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Dedication

To those who are suffering from cancer, I wish them healthy life.

To my dear parents, who raised me for the love of knowledge when I was child.

To my faithful husband who supported me and made the difficulties easy for me.

To my beloved daughters, who have given a life full of love and smile.

To all persons in my life who touch my heart.

Hiba

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List of Abbreviations

Abbreviation	Meaning
˚C	Centigrade
µl	Microliter
ABC	ATP-binding cassette transporter
ABCB1	ATP- binding cassette transporter family B member 1
AIs	Aromatase inhibitors
ALH	Atypical lobular hyperplasia
ARMS	Amplification refractory mutation system
ATM	Ataxia-Telangiesctasia Mutated
ATAC	Armidex, Tamoxifen Alone or in Combination
ATP	Adenosine triphosphate
BBD	Benign breast disease
BC	Breast cancer
bp	Base pair
BPI	Brief pain inventory
BRCA1	Breast cancer 1 gene
BRCA2	Breast cancer 2 gene
CA 15.3	Cancer antigen 15.3
CEA	Carcinoemberyonic antigen
CLIA	Chemiluminscent immunoassay
CYP 450	Cytochrome p 450
DCIS	Ductal carcinoma in situ
DNA	Deoxyribonucleic acid
E1	Estrone
E1S	Estrone sulfate
E2	Estradiol
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor

ELFA	Enzyme linked fluorescent assay
EMR	Eastern Mediterranean Region
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ESR1	Estrogen receptor 1 gene
FDA	Food and drug administration
FSH	Follicle stimulating hormone
GnRHa	Gonadotropine Releasing Hormone agonist
HER2	Human epidermal growth factor receptor 2
HRT	Hormone replacement therapy
IBC	Invasive breast cancer
IBM, US	International business machine, United state
IDC	Invasive/infiltrating ductal carcinoma
IgG	Immunoglobulin G
ILC	Invasive/infiltrating lobular carcinoma
LCIS	Lobular carcinoma in situ
LH	Leutinizing hormone
LHRH	Leutinizing hormone releasing hormone
MDR1	Multidrug resistance 1
ml	Milliliter
MRI	Magnetic resonance imaging
MUC-1	Mucin-1
NBD	Nucleotide binding domain
PCR	Polymerase chain reaction
PET	Positron emission tomography
Pg/ml	Pecogram per milliliter
P-gp	P-glycoprotein
pm	Round per minute

Pmol/µl	Picomole per microliter
PPT	Protein precipitation buffer
PR	Progesterone receptor
PRα	Progesterone receptor alpha
PRβ	Progesterone receptor beta
RASSF1	Ras association domain family member 1
RBC	Red blood cell
RLUs	Relative light units
rs	reference SNP
SERDs	Selective estrogen receptor degraders
SERMs	Selective estrogen receptor modulators
SLC	Solute carrier transporter
SNPs	Single nucleotide polymorphisms
SPR	Solid phase receptacle
TBE	Tris-borate EDTA buffer
TDLUs	Terminal ductal lobular units
TMD	Transmembrane domain
TNM	Tumor, node and metastasis
u/ml	Unit per milliliter
UGT	UDP- glucuronosyltransferase
UGT1A4	UDP- glucuronosyltransferase family 1 member A4
UGT2B7	UDP- glucuronosyltransferase family 2 member B7
v/cm	Volt per centimeter

Abstract

Background: Breast cancer is the most frequent cancer among the women. It develops as a result of various interactions between genetic and environmental factors.

Anastrozole is one of most the common drugs used in treatment of estrogen receptor (ER) and / or progesterone receptor (PR) positive breast cancer. Anastrozole is widely used in postmenopausal breast cancer women, in addition, it is used in premenopausal women with advanced or metastatic breast cancer. ATP-binding cassette B1 (ABCB1) has been reported to be an efflux transporter of anastrozole. ABCB1 gene is polymorphic and have about 40 single nucleotide polymorphisms, which may affect the pharmacokinetic of drugs and so the clinical outcome. Cytosin 1236 Thymine (C1236T) and Cytosin 3435 Thymine (C3435T) SNPs have been detected in the present study.

Aim of the study: The aim of the present study was to identify the genetic polymorphism of an efflux transporter gene ABCB1 (C1236T and C3435T) in the participated breast cancer women, as well as to investigate the impact of ABCB1 gene polymorphism on anastrozole efficacy, and to investigate its association with the development of anastrozole induced arthralgia.

Patients and methods: This cross-sectional observational study was done at Imam Al-Hussein Medical City / Oncology Center in Kerbala. One hundred women with estrogen receptor and / or progesterone receptor positive breast cancer were selected to participate in this study. All women enroled in this study were taking 1 mg of anastrozole daily before starting the study. Blood samples were taken from eligible patients who had signed informed consent for genetic testing and for the measurement the of estradiol and cancer antigen CA 15.3 levels. This study used Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS PCR) for detection of

C1236T and C3435T. Arthralgia were assessed depending on patient's history in addition to Brief Pain Inventory (BPI) questionnaire.

Results: The results obtained from the present study have been detected various genetic polymorphisms of the tested gene encoding ABCB1 transporter. The findings of this study revealed that there were statistically non-significant association between ABCB1 gene polymorphism (C1236T and C3435T) and the levels of estradiol and tumor marker CA15.3. Also, the current study demonstrated that there was non-significant association between genetic polymorphism of ABCB1 gene and anastrozole induced arthralgia.

Conclusions: This study revealed that ABCB1 gene was highly polymorphic in the participated breast cancer patients with mutant genotypes that had the highest frequency in both C1236T and C3435T. Genetic polymorphism in gene encoding an efflux transporter may not affect the anastrozole efficacy. This study showed that 89% of breast cancer women treated with anastrozole had arthralgia; however, it did not reveal any association between ABCB1 gene polymorphisms with development of arthralgia, as a main side effect of anastrozole.

Chapter One

Introduction

1. Breast cancer

1.1. Preface

Worldwide, breast cancer (BC) is the most common cancer, and is the main cause of death among women (1). It is estimated that about 30% of new cancer diagnosis and about 25% of cancer deaths. Nearly two thirds of young females who have breast cancer have hormone receptor positive and human epidermal growth receptor 2 (HER2) negative tumors (2). There is a large difference in survival rates of breast cancer around the world, with an estimated five years survival of 80% in countries with high income to less than 40% for countries with low income (3). The development of breast cancer occurs as a result of complex interaction between genetic and environmental factors (4).

1.2. Epidemiology of breast cancer

The incidence rates stay highest in countries with high income, and among the women of higher social class, because of range of lifestyle factors, like nutrition and development, childbearing, lactation and physical activity (5). On an average, females with age over 60 years are more likely to have breast cancer while only about 10% to 15% of breast cancer cases occur in females younger than 45 years and this results may be due to different ethnicities or races (6). The rates of incidence are highest in Australia, North Europe, Western Europe, South Europe, and Northern America (7), whereas middle Africa and Eastern Europe had the lowest rates (6).

In 2012, approximately 292,677 of new cases were diagnosed with breast cancer among women population in Eastern Mediterranean Region (EMR) and about 176,139 died from this disease. In Iraq, breast cancer become a major hazard to female health where it has been detected as the maximum-ranked malignancy in Iraqi people since 1986 (8). The annual report of the Iraqi Cancer Registry (2019), demonstrated that breast cancer

had the highest percent and incidence rate among top 10 cancers in Iraq in 2019 (9).

Throughout eight years from 2008 to 2015 examined in the population of Kerbala city, breast cancer showed the highest prevalence in women Figure (1-1) (10).
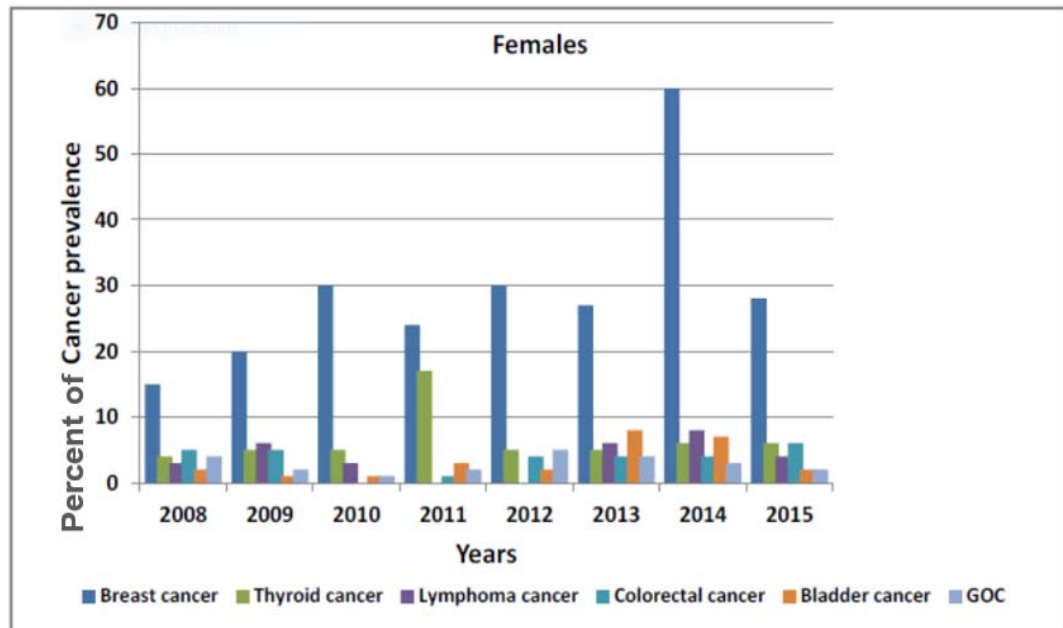
Figure (1-1): Prevalence of breast cancer in females (2008-2015) in population of Kerbala City (10).

The annual report of Iraqi Cancer Registry (2019) showed that breast cancer had the highest incidence rate among other cancers in Kerbala populations (9).

1.3. Pathogenesis

The characteristics of cancer are existent in all cancer cells irrespective of the cause or type, and these involve: uncontrolled growth, angiogenesis (formation of new blood vessel) and apoptosis avoidance, so when the control of apoptosis is lost, the cancer cells survive more longer and offer more time for buildup of mutations which can rise the invasiveness throughout tumor progression, promote angiogenesis, deregulate and interfere with cell proliferation and differentiation (11).

Abnormal methylation of DNA is a hallmark of cancer and may work in different ways to influence transcription, and may cause changes in Ras Association Domain Family Member1 gene (RASSF1) a tumor suppressor gene, and epidermal growth factor receptor gene (EGFR). These changes might be important in influencing preneoplastic alterations in gene expression relevant to development of tumor in breast (12).

One model of usual history of breast cancer assumes that it appear as a result of progression of breast tissue through certain histological forms of benign breast disease (BBD) then followed by carcinoma in situ before eventually emerging into invasive breast cancer (IBC) (13).

Sometimes the disease may develop as a part of hereditary cancer syndrome, produced by mutation in high penetrance susceptibility genes. A significant proportion of hereditary breast cancer (approximately 16%) can be attributed to germline mutations in Breast Cancer 1 and 2 (BRCA1 and 2) early onset genes (14, 15). Regarding to this, identification of BRCA1/2 mutations allows the application of prevention strategies, including magnetic resonance imaging screening or risk minimizing surgeries, which enhance survival (16).

Another gene alteration that has important role in breast cancer is human epidermal growth factor receptor HER2 which is overexpressed and amplified in about 15%-30% of breast cancer (17).

1.4. Etiological factors for breast cancer

The factors that have been associated with an increased hazard for breast cancer progress in women are:

1.4.1. Age

The risk of rising breast cancer increased with age (3). The disease is very unusual before the age of 20 years; however, the occurrence gradually

increases with age, and during 90 years of age, one fifth of females are affected. This reveals that the reproductive hormones made by adrenal glands and ovaries are implicated in the breast cancer pathogenesis (18).

Besides, age at menarche and at menopause plays important role in breast cancer development due to early menarche and delayed menopause will rise the period of exposure to estrogen during a female's reproductive years, however there has to be a cooperation with environmental and genetic factors for progression of breast cancer (19).

1.4.2. Gender

Globally, the number of cases who were newly diagnosed with male breast cancer elevated from about eight thousands (in 1990) to approximately 23 thousands (in 2017) (20). However, it is rare in men and representing only about 1% of cancers in males. Male breast cancer usually exhibits more advanced disease characteristics than females. In addition to expression of estrogen and progesterone receptor, male BC also express androgen receptor (21).

1.4.3. Genetic factors

A recent study showed that there was a significant association between the risk of breast cancer and the protein truncating variants in some genes like BRCA1, BRCA2 and ATM (Ataxia-Telangiesctasia Mutated) (22).

1.4.4. Family history

About 20%-25% of breast cancer cases have a positive family history (3). If the female has a family history of breast cancer, she has an increased risk for developing the disease (23). Females with first degree relatives that had cancer in both breasts showed a risk ratio of 5.4 times when statistics for pre- and postmenopausal breast cancer cases were joined together (24).

1.4.5. Lifestyle factors

Changeable risk factors which represent 21% of all breast cancer deaths throughout the world (18) involve the followings:

1.4.5.1. Diet and alcohol consumption

Foods with high fats content are linked with elevated incidence of breast cancer, because diet rich in fats have high cholesterol which is a precursor for estrogen synthesis and another steroid hormones, so this will expose the breast to larger quantities of estrogen which can trigger the development of cancer (25). It was also noted that diet rich in fibers inhibits the intestinal absorption of estrogen (18). Alcohol drinking has been linked with raised risk of breast cancer (26).

1.4.5.2. Physical activity

It is thought that physical activity may decrease the risk of breast cancer through numerous mechanisms, involving the effect of physical activity on sex hormones, adiposity and insulin resistance (27).

1.4.5.3. Obesity

It's a hazard factor for the progression of BC in postmenopausal females, and this may be due to peripheral conversion of androstenedione to estrone that arises in adipose tissue which lead to increase the estrogen levels in obese postmenopausal females (28, 29).

1.4.6. Endocrine factors

1.4.6.1. Endogenous hormones exposure

Breast cancer is usually seen in infertile females and also more common in females who do not breast-feed their children, while females with first full term pregnancy at an early age has been discovered to be protective particularly if coupled with late menarche and early menopause (so this result in lowering the duration of exposure to estrogen) (18). This is because

the estrogen level is declined in pregnancy and in females that had many babies (30).

1.4.6.2. Exogenous hormones exposure

There is an association between the use of hormone replacement therapy (HRT) and risk of breast cancer. BCs associated to HRT use are frequently hormone receptor positive (31). Females starting hormone therapy nearer to menopause have an elevated risk for breast cancer (32).

Oral contraceptive appeared with modest elevation in risk of breast cancer and this risk is thought to be greater for women who started contraception before age 20 years and the risk is mainly due to estrogen content of the contraceptive (18).

1.5. Classification of breast cancer

Breast cancer can be classified according to its site into: invasive and non-invasive breast cancer (33).

1.5.1. Non-invasive breast cancer

This type can be classified into two distinct classes: ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS) (34). DCIS is the most common type of non-invasive breast cancer, which is restricted to the breast duct (33), whereas in case of lobular carcinoma in situ (LCIS), the lesions looked most often in the terminal duct lobular units (TDLUs). LCIS is thought to rise from atypical lobular hyperplasia (ALH) (35). The term "in situ" illustrates "in place". Although the atypical cells have not spread to other tissues outer the ducts or lobules, they can grow and develop into invasive breast cancer (33).

1.5.2. Invasive breast cancer

Invasive breast cancer appears when atypical cells from within the milk ducts or lobules split out into adjacent breast tissue. Cancerous cells can pass across the breast to various parts of the body through the systemic

circulation or immune system and reach to different organs like bones, lungs, liver and brain, and so forming new cancers. The most common invasive tumor forms involve invasive / infiltrating lobular (ILC) or infiltrating / invasive ductal carcinoma (IDC) (36).

1.5.2.1. Infiltrating lobular carcinoma (ILC)

It originates in the lobules (the milk glands) of the breast, but often spreads to other parts of the body (33). ILC encompasses up to 15% of all cases (37). The morphologic features of ILC is characterized by round, small cells which have insufficient cytoplasm, which penetrate the stroma in single file and enclose benign breast tissues in a targeted mode (38).

1.5.2.2. Infiltrating ductal carcinoma (IDC)

IDC is the major subtype comprising for 70% to 80% of all invasive lesions. It originates in the ducts of milk of the breast and spreads to the wall of the ducts, invading the fatty tissues of the breast and possibly different parts of the body. IDC is further subdivided into tubular, mucinous (colloid), papillary, medullary, and cribriform carcinomas (36).

1.6. Molecular classification of breast cancer

Cancer of breast is a molecularly heterogeneous disease (39). Genomic profiling has assisted classification of breast cancer into molecular subtypes. These subtypes are biologically different entities with special prognosis and therapeutic features (40).

Based on gene expression, breast cancer is classified into four molecular subtypes: luminal A, luminal B, HER2 and basal like (39). Table (1-1) molecular subtypes (41-43).

Table (1-1): Molecular subtypes of breast cancer (41-43).

Molecular Subtypes	Immunohistochemical Surrogates	Pattern of gene expression	Clinical features
Luminal A	ER positive and/or PR positive, HER2 negative	High expression of ER	Sensitive to endocrine therapy, poor response to chemotherapy, overall good prognosis.
Luminal B	ER positive and/or PR positive, HER2 positive	High expression of ER, but it's lower than luminal A	Sensitive to endocrine therapy, better response to chemotherapy than luminal A, poorer prognosis than luminal A.
HER2	ER negative, PR negative, HER2 positive	Low expression of ER, HER2 expression is high	Tend to respond to trastuzumab, better response to anthracycline-based chemotherapy, overall poor prognosis
Basal-like	ER negative, PR negative, HER2 negative (triple negative)	Low expression of ER and HER2, with high expression of basal epithelial genes and basal cytokeratins	Insensitive to endocrine therapy or trastuzumab, respond to platinum-based chemotherapy, overall poor prognosis

1.7. Prognostic factors of breast cancer

Markers for prediction of prognosis and decisions of treatment in breast cancer are estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) (44), Tumor Node and Metastasis (TNM) staging and tumor grade are (45).

1.7.1. Hormonal receptors

Steroid hormone receptor is a superfamily nuclear receptor (46), which regulates cell growth and differentiation and so influencing the normal physiology, behavior and reproduction (47). The steroid hormone receptor family involve glucocorticoid, mineralocorticoid, androgen, progesterone and estrogen receptors. In breast cancer, estrogen and progesterone receptors are regarded as a prognostic factors and the main determinant for treatment decisions (44).

There are two isoforms of estrogen receptor, ERα and ERβ, which are encoded by different genes (48). Each receptor has specific patterns of tissue expression, post-translational modifications and cellular location in normal and disease conditions (44).

Progesterone receptor has two isoforms, PRα and PRβ, which are formed from a single gene by translation at two different start codons (49).

1.7.1.1. Cellular mechanism of estrogen in development of breast cancer

Estrogen plays an important role in growth, differentiation and performance of numerous tissues, involving uterus, breast and urogenital system of males and females. According to this, the progression of cancer in reproductive organs as breast and prostate cancer frequently occurs due to the oestrogen, progesterone and androgens, which exert various biological events in normal and abnormal cells (33).

Estrogen is produced through two different organs. In premenopausal females, the massive quantity of oestrogen (estradiol-17β

and estrone) is generated by the ovaries in response to follicle stimulating and luteinizing hormones, which are derived from the pituitary. However, in postmenopausal females, the amount of estrogen production by ovaries is few or not produced at all. In this case, the oestrogen precursors (testosterone and androstenedione) are generated by adrenal gland and then in peripheral tissues are transformed into estradiol and estrone through aromatization (50).

The role of estrogen in breast carcinogenesis can be demonstrated as following:

a- Receptor dependent

Estrogen acts as promoter (factor that promote the growth of transformed cells or survival of existent transformed cells). During the binding of estrogen to its receptor, it stimulates genes transcription that are involved in cell proliferation, and through the process of mitosis for each cell cycle, a new DNA synthesized and there is point mutation if not repaired this lead to development of breast cancer (51).

b- Receptor independent

Estrogen acts as initiator (factor that stimulate the genetic damage that cause transformation of cells) (52). The reactive estrogen intermediates (quinone / semiquinone) are generated by redox cycling of oestrogen metabolites which are the most expected oestrogen initiators. This reactive intermediates can interact with DNA and producing mutations in critical genes (tumor suppressor genes and oncogenes) which result in transformation and proliferation of abnormal cell (53).

So understanding the role of estrogen in breast carcinogenesis has not only been used to the development of therapeutic interventions but also for prevention of the disease (54).

1.7.2. Human epidermal growth factor receptor 2 (HER2)

It is a tyrosine kinase receptor which is a member of human epidermal growth factor receptor family (55).

In certain types of breast cancer, HER2 gene is amplified which drives protein expression and rise in the number of receptors located on the surface of tumor cells which finally result in excessive cell division and development of tumor. For this reason, patients with HER2 positive breast cancer have substantially shorter survival and disease free survival and poorer prognosis (45).

1.7.3. Tumor Node and Metastasis (TNM) staging

TNM system is used for staging of patients with breast cancer according to size of tumor (T), involvement of regional lymph nodes (N) and metastasis (M) (56). T may be T0 (no evidance of primary tumor), T1 (tumor size <2 cm), T2 (tumor size 2-5 cm), T3 (tumor size > 5 cm), and T4 (tumor extend to skin and chest wall). N may be N0 (no lymph node metastasis), while N1, N2, and N3 (refere different types of lymph nodes involvement). M may be either M0 (refere no distant metastasis) while M1 (refer distant metastasis) (57).

1.7.4. Tumor grade

It is one of the common prognostic factors, which is a combined score dependeds on microscopic assessment of the cytological and morphological features of tumor cells which shows the aggressiveness of a tumor. Breast cancer tumors can be categorized into three grades: grade1 refers to well differentiation and slow growing, grade 2 refers to intermediate differentiation whereas grade 3 refers to poor differentiation and high proliferation (58).

1.8. Tumor markers

Tumor markers are biomarkers which are found in the blood, body tissues or urine that can be raised by the presence of one or more cancer types

(59). For various cancers, tumor markers play significant roles in diagnosis, determining prognosis, expecting response to certain therapies, early finding of recurrence, and also for monitoring of treatment for patients with advanced disease. Cancer antigen 15.3 (CA15.3) and carcinoemberyonic antigen (CEA) are the most commonly used serum tumor markers of breast cancer (60).

The Food and Drug Administration (FDA) have approved to use CA15.3 and CEA as tumor markers for monitoring subjects with breast cancer (61).

1.8.1. Cancer antigen (CA15.3)

It is a member of mucin-1 (MUC-1) family of glycoproteins which is overexpressed in cancers and it is well known as a useful tumor marker (62). The level of CA15.3 in blood can be used for screening of breast cancer as well as other malignancies, involving ovarian, lung, pancreatic, colon and liver cancer. However, CA15.3 is more useful to determine the breast cancer prognosis and for monitoring the efficacy of therapy as it was revealed that the increased level of this tumor marker in serum tend to raise the severity of the breast cancer and/or tumor size (59).

1.8.2. Carcinoembryonic antigen (CEA)

It is a member of cell-surface glycoproteins family and it is useful tumor marker in a variety of adenocarcinoma (61).

Elevated level of CEA is linked with metastatic breast cancer. In patient with breast cancer, the serum concentrations of CEA are depending directly on the size of tumor for both primary and metastatic cases. For breast cancer, CEA is less specific tumor markers than CA15.3 (59).

1.9. Screening and diagnosis of breast cancer

Preceding studies have proposed that early detection of breast cancer with appropriate treatment might reduce the rates of death from breast cancer. Researchers have studied many breast diagnostic methods, involving

mammography, magnetic resonance imaging (MRI), ultrasound breast imaging, positron emission tomography (PET) (63).

1.9.1. Mammography

Mammogram is a radiographic checking of the breast, used for either screening or diagnosis (64). Screening mammograms done for females who are asymptomatic and it is effective in early detection of breast cancer (65), while diagnostic mammograms done for females who have a clinical problems, as a palpable mass or another symptoms of breast disease, and for patients with history of breast cancer within the previous five years (64).

1.9.2. Magnetic Resonance Imaging (MRI)

Magnetic resonance imaging creates image at various cross-sections through application of strong magnetic field with radiofrequency signals. It has been recommended for individuals with high risk of breast cancer. Breast MRI is less specific but more sensitive than mammography in detection of small tumors in those individuals (63).

1.9.3. Ultrasound breast imaging

Ultrasound breast imaging employs high-frequency sound waves to create image (64). It rises the detection rates of cancer for individuals with high risk of breast cancer and it aids to identify solid masses and cysts, but it's less effective compared to mammography (63).

1.9.4. Positron Emission Tomography (PET)

PET utilizes a positron-emitting radionuclide. It's very sensitive and non- invasive molecular imaging technique. PET can be used to visualize the location of tumor in the body, in addition, by using specific radiolabeled tracers, Positron emission tomography is also supposed to imagine the activity and expression of certain molecules and biological processes that affect the behavior of tumors and/or the responsiveness to treatment (66, 67).

1.10. Therapeutic approach of breast cancer

The main objectives of therapy for non-metastatic breast cancer are removing of tumor from the breast and local lymph nodes with prevention of recurrence. Whereas for metastatic breast cancer, therapeutic objectives are symptoms palliation and prolonging life. Presently, metastatic breast cancer stays incurable in almost all affected subjects (68).

The size of tumor and the involvement of lymph nodes remain essential in the management decision making process. Systemic and neoadjuvant endocrine therapies had previously kept for inflammatory and locally advanced breast cancer (69, 70).

1.10.1. Surgery

Surgery plays an important role in breast cancer treatment. It can be used for local control of the cancer. Radical mastectomy which involve excision of the breast with dissection of lymph node in axilla and removal of both pectoralis muscles. While modified radical mastectomy of Patey require removal of the entire breast tissue with a large portion of the skin and the lymph nodes located in the axilla. The pectoralis muscles are conserved (71). Simple mastectomy represents additional modification of the modified radical mastectomy in that it conserves both the axillary lymph nodes and the pectoral muscles (72).

Breast conserving surgery usually includes removing the tumor without removal excess normal breast tissues, which is more aesthetically tolerable to the patient than radical mastectomy. It is more successful in females with early stage of breast cancer, however it is not recommended for females with high risk of local recurrence (73).

1.10.2. Chemotherapy

Chemotherapy involves using of cytotoxic drugs to eradicate cancerous cells. It can be administered either before surgery as neoadjuvant chemotherapy or after surgery as adjuvant chemotherapy (74). In the case of

inflammatory or locally advanced breast cancer, chemotherapy must always be initiated before surgery (69). The advantages from the use of neoadjuvant chemotherapy are the reduction in size of cancerous cells and in some cases eradication of it in addition to accelerates a routine surgical procedure (75). Adjuvant chemotherapy is a systemic therapy administered to patients to treat unnoticed cancer cells in the breast (74).

There are several mechanisms involved in the resistance of cancerous cells for chemotherapy as: genetic factors (gene mutation or amplification) and increased efflux of drugs (76).

1.10.3. Radiation therapy

In breast cancer, radiation may be applied to the whole breast or the chest wall (following mastectomy) or a part of the breast (following lumpectomy) and the local lymph nodes (68). This involve using of high energy rays from radiation that cause killing of cancerous cells and it may be done following the breast cancer surgery to damage the residual cells in the chest (33).

1.10.4. Endocrine therapy

The association between oestrogen and breast cancer has long been studied, and the carcinogenic effects of oestrogen appears to be mostly mediated through its action on their receptors which result in cellular proliferation. Endocrine therapy is an essential treatment for females with estrogen receptor positive tumors (77). It usually includes: selective estrogen receptor modulators or degraders (SERMs, SERDs), aromatase inhibitors (AIs), and ovarian suppression drugs (78).

In early stage breast cancer, the typical duration for endocrine therapy is five years. Today, several investigations support the usage of hormonal therapy for ten years. However, long term hazards and side effects should be balanced against benefits (69).

1.10.4.1 Selective estrogen receptor modulators and selective receptor degraders (SERMs and SERDs)

1.10.4.1.A. Selective estrogen receptor modulators (SERMs)

Selective estrogen receptor modulators are anti-estrogen that are designed to compete with oestrogen and modulate oestrogen receptor activity by altering the cofactors that associate with it. SERMs can be categorized depending on their chemical structure as: triphenylethylenes (tamoxifen), phenylindoles (bazedoxifene), benzothiophenes (raloxifene) and tetrahydronaphthalenes (lasofoxifene) (79).

Tamoxifen is a competitive inhibitor of estrogen receptor which induce conformational changes in these receptor resulting in suppression the transcriptional activity of estrogen receptor. Several another SERMs have been studied, but none of them was obviously superior to tamoxifen in the prevention or treatment of breast cancer (80).

1.10.4.1.B. Selective estrogen receptor degraders (SERDs)

They are anti-estrogen which are designed to bind to estrogen receptor and provoke its degradation and so preventing the dimerization and stopping the estrogen receptor signaling pathway (79). Fulvestrant is an example of selective estrogen receptor degrader, which is effective for females with hormone-sensitive advanced breast cancer (81). It not only blocks the nuclear estrogen receptor, but also the cytoplasmic and the membrane bound receptors (82).

1.10.4.2. Ovarian suppression drugs (gonadotropine-releasing hormone agonist GnRHa)

The chronic non-pulsatile adminsteration of GnRHa, such as gosereline, triptorelin, buserelin, and leuprorelin cause through a period of days to down regulation of gonadotropine-releasing hormone receptors present in pituitary, decreased secretion of leutinizing hormone (LH) and follicle stimulating hormone (FSH) and marked decline of gonadotropin and

estrogen levels (83). So, GnRH agonists administration has mainly substituted every other irreversible procedure as ovarian ablation. It has been revealed that in premenopausal females with breast cancer who did not take chemotherapy, ovarian suppression may decrease both the recurrence and mortality rate (78).

1.10.4.3. Aromatase inhibitors (AIs)

Aromatase inhibitors are anti-estrogen compounds designed to decrease the level of circulating estrogen by inhibiting the activity of aromatase enzyme, which is a microsomal enzyme responsible for conversion of androgen to estrogen (78). The first generation of aromatase inhibitor is aminoglutethimide became accessible about 25 years ago. Although of its effectiveness, aminoglutethimide was incapable to rival tamoxifen because of highest toxicities and lacking of selectivity for aromatase. The second generation of aromatase inhibitor is formestane which is developed in 1993 and it had less side effect than aminoglutethimide. While the third generation of aromatase inhibitors developed in the mid1990s (84).

These generations of AIs include: steroidal compounds, which cause a permanent inhibition of aromatase like exemestane, while the nonsteroidal compounds, which are reversible competitive inhibitors like anastrozole and letrozole (77).

1.10.4.3.A. Anastrozole

The chemical name of anastrozole is 2-[3-(2-Cyanopropan-2-yl)-5-(1,2,4-triazol-1-ylmethyl)phenyl]-2-methylpropanenitrile (85), it is a nonsteroidal compound which blocks the aromatase enzyme reversibly and resulting in lowering the estrogen level. Anastrozole is given in a dose as 1 mg per day for constant suppression of estradiol and estrone (86), but recent investigations have reported that this daily dose may not benefit all females with breast cancer due to interindividual variations which may change the

tolerability and efficacy of the drug and so affecting on its pharmacokinetics and pharmacodynamics (87). Anastrozole and all third generation compounds have become endocrine drugs of choice for postmenopausal breast cancer females because they are associated with a stronger activity and better general tolerability compared with tamoxifen (88). It used as a first line therapy in postmenopausal patients who had estrogen receptor (ER) positive breast cancer, especially in locally advanced or metastatic cases, in addition, anastrozole is indicated in recurrence cases (as alternative option for endocrine therapy) (86). It is approved for treating females with breast cancer following surgery in postmenopausal females (89). Also, anastrozole used in combination with goserelin (GnRH agonist) in premenopausal women with estrogen receptor positive metastatic breast cancer (90). It is usually administered through a period of 2 to 5 years (91) however in some cases, extended use of anastrozole therapy from 5 to 7-8 years may be needed which result in improvement in disease free survival (92).
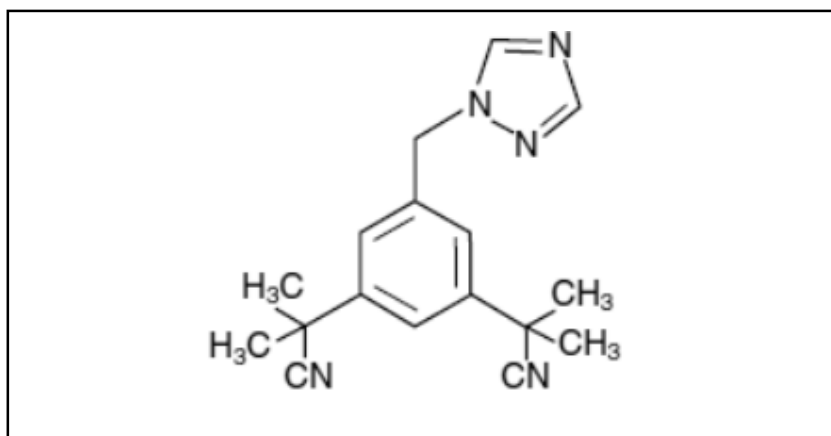
Figure (1-2): Chemical structure of anastrozole (93).

1.10.4.3.B. Mechanism of action

Aromatase (CYP19A1) is the main enzyme that is involved in the biosynthesis of estrogen which catalyzes the rate limiting step of estrogens synthesis from androgens. Anastrozole competes with androgen on the active site of aromatase enzyme (94). This is done by reversible binding of

anastrozole to the hem iron of aromatase enzyme (CYP19A1), and so inhibiting its activity. Due to this reversible interaction between the drug and the enzyme, anastrozole should be continually present to result in inhibition of aromatase (95).
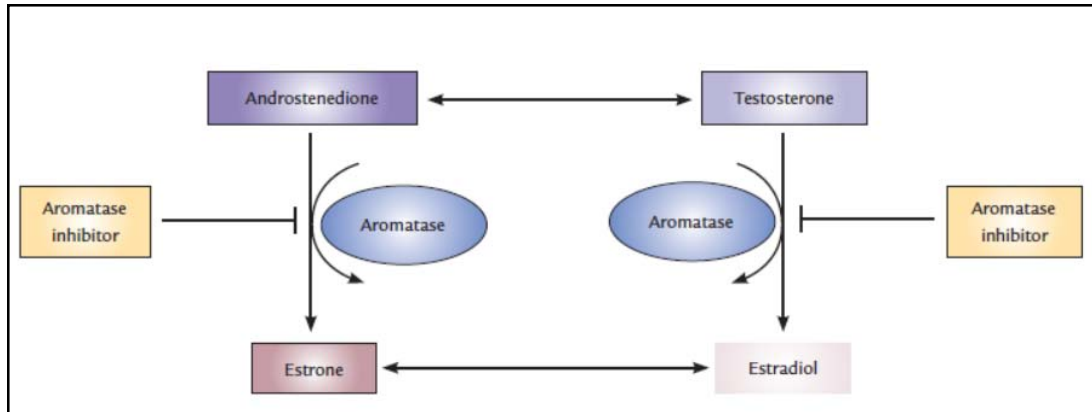
Figure (1-3): Mechanism of action of anastrozole (96).

1.10.4.3.C. Pharmacokinetic of anastrozole

After oral administration of anastrozole, it has been established that it needs 2 to 3 hours to reach maximum concentration (95). Steady state plasma concentrations are reached through 7 to 10 days of therapy with anastrozole (84). Diet intake does not extensively affect steady state concentration. When absorbed, anastrozole is extensively distributed through the body, with approximately 40% of the drug bound to plasma proteins (95).

Anastrozole is mainly metabolized by the liver through cytochrome P450 isoforms (which involve hydroxylation and N-dealkylation reactions) and through UDP-glucuronosyltransferase (UGT) isoforms (which involve glucuronidation reaction) (84). It undergoes hydroxylation reaction catalyzed mainly by CYP3A4 and to a lesser extent through CYP3A5 and CYP2C8 to form hydroxyanastrozole, which may be further glucuronidated through UGT1A4 to form hydroxyanastrozole glucuronide. As well as, anastrozole can be directly conjugated with glucuronide to anastrozole-N-glucuronide. This glucuronidation conjugation

reaction is mainly catalyzed by UGT1A4 and to a lesser extent through UGT1A3 and UGT2B7.

N-dealkylation is another pathway for anastrozole biotransformation that lead to form triazole or 3,5-Bis-(2-methylpropiononitrile)-benzoic acid (97). Figure (1-4) (98).
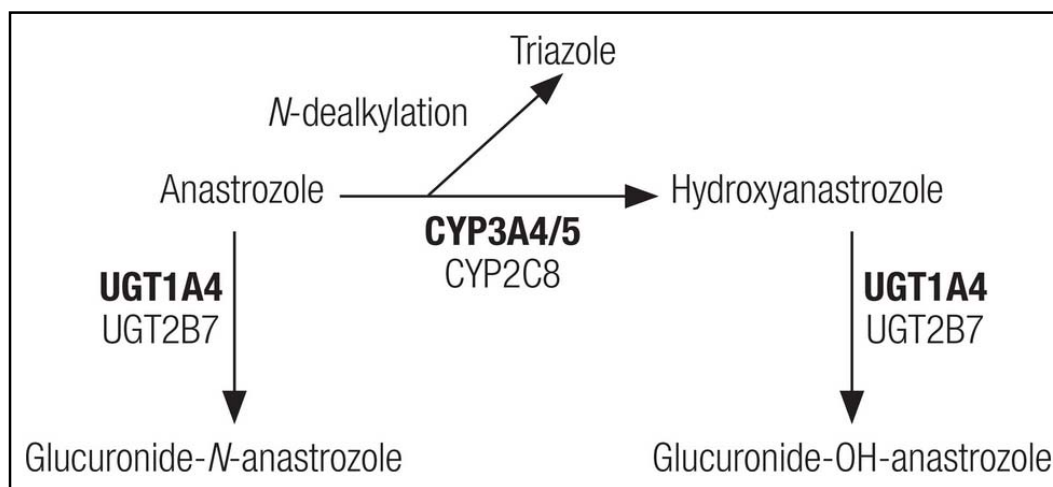
Figure (1-4): Metabolic pathway of anastrozole (98).

The half-life of anastrozole is nearly 50 hours (84). Anastrozole is mainly eliminated as metabolites in the urine (60%), and a portion of it is excreted in the feces. Approximately 10% of the given dose is eliminated unchanged in the urine (95). It can be given for patients with hepatic impairment (mild to moderate) (84).

1.10.4.3.D. Pharmacodynamic of anastrozole

Anastrozole is very selective aromatase inhibitor due to extensive suppression of serum estrogen metabolites (estradiol, estrone, and estrone sulfate) concentrations without considerably influencing the concentrations of adrenal steroids, corticosteroids or gonadotrophins (95). Oestrogen suppression is reached through inhibition of oestrogen biosynthesis by the action of anastrozole (91).

1.10.4.3.E. Medical uses

A- Breast cancer

Anastrozole is indicated for early stage hormone receptor positive breast cancer, chemoprevention of tumor. In postmenopausal females, the drug is used as first line therapy for locally advanced and metastatic breast cancer (86).

B- Infertility

Anastrozole is efficient to treat infertility in anovulatory females by stimulating ovulation (99). It also can be used to treat male infertility due to azoospermia (off-label use) by elevation testosterone level, lowering oestrogen level and suppressing testosterone metabolism in periphery. The goal is to decrease the oestrogenic effect on spermatogenesis (100).

1.10.4.3.F. Adverse effect of anastrozole

A- Osteoporosis: Anastrozole increases bone turnover, lowering bone mineral density and raise the relative risk of fractures. Bone loss caused by anastrozole is resulted from inhibition of aromatiztion of androgens and their conversion to oestrogens in peripheral tissues (101, 102).

B- Other troublesome adverse effects involve: myalgia, arthralgia, hot flashes, dryness of vagina, reduced libido (103), headache, edema as well as nausea (which is a very common gastrointestinal side effect) (86).

1.10.4.3.G. Drug interaction

Because anastrozole is mainly metabolized by CYP450 isoforms, it is very important to observe that many drugs can cause alterations in the activity of CYP3A4 and act as either enzyme inducers or inhibitors.

Antimicrobials (clarithromycin, erythromycin), antifungals (ketoconazole, voriconazole, fluconazole), antivirals (ritonavir, saquinavir), immunosuppresants (cyclosporine) are enzyme inhibitors, which inhibit CYP3A4 resulting in elevation the plasma levels of anastrozole. While

anticonvulsants (carbamazepine, phenytoin, phenobarbital) and antimycobacterials (rifampin) are inducers for CYP3A4 and so leading to decrease the plasma level of anastrozole due to extensive activity of the enzyme (104).

In addition, most of drugs that affect on CYP450 activity also affecting on ABCB1 activity by induction or inhibition (105).

1.11. Drug transporters

Drug transporters are membrane bound proteins with several trans-membrane spanning domains and certain cellular locations and specific membrane orientations which determine their cellular/tissue function (106).

Drug transporters play an important role in pharmacokinetic of drugs and in some cases may lead to drug-drug interaction because these drugs may potentially compete with each other for the binding site on the transporter, thus leading to unpredicted changes in tissue and serum drug levels and potential toxic side effects (107).

The drug transporters that received greatest interest are two major superfamilies of the membrane transport proteins, first, the solute carrier (SLC), second, the ATP-binding cassette (ABC) transporter families, both are involved in translocation of endogenous and exogenous compounds through the cell membranes. The SLC superfamily comprises uptake in addition to efflux transporters while the ABC members are mainly efflux transporters (108). SLC transporters generate concentration gradients through exchange and/or co-transport of ions or act as facilitator transporters while ABC transporters directly cause ATP hydrolysis (106).

Interindividual differences of ABC and SLC transporter expression and function contribute to variations in efficacy of treatment or the incidence of adverse drug reactions (108).

1.11.1. ABC transporter

In human, ATP-binding cassette (ABC) transporters are a large group of membrane protein complexes, which comprise 48 members that are categorized into 7 subfamilies from ABC-A to ABC-G depending on their sequence similarities. ABC transporters are mainly located on the plasma membrane and so decreasing the intracellular concentration of a variety of diverse drugs, drug metabolites and conjugates through exporting (109). In addition, these transporters can transport endogenous compounds like bile acids, uric acid, folate, leukotrienes, and glutathione. Often, there is an overlapping in the substrate specificities of ABC transporters with those of SLC family (107).

Structurally, ABC transporters have two transmembrane domains (TMD) and two nucleotide-binding domains (NBD). The hydrophobic TMDs are structurally diverse, which alternatively recognize and translocate numerous substrates upon conformational changes (110). While NBD consisting of conserved ABC which is responsible for binding and hydrolyzing ATP through ATPase, thus providing energy required for efflux of endogenous and xenobiotic substrates from cytoplasm into extracellular spaces Figure (1-5) (109).
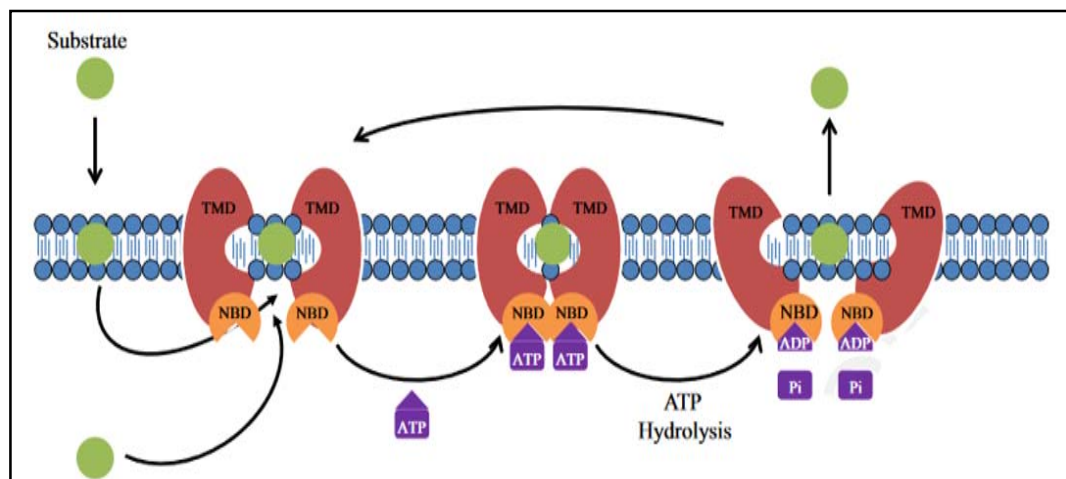
Figure (1-5): ABC transporters and their function (109).

1.11.1.1. ATP-binding cassette B1 (ABCB1) transporter

ATP-binding cassette transporter B1 (ABCB1; multidrug resistance protein 1 MDRI; p-glycoprotein p-gp) is an adenosine triphosphate (ATP)-dependent efflux transporter which is located in the plasma membrane of various cell types. It function as unidirectional efflux transporter for a wide range of endogenous and exogenous compounds, thus providing a protection to the cells from potentially toxic substances (111).

Some of endogenous substances are transported by ABCB1 including phospholipids, ions, sugars and sterols. In addition multiple drugs used in cancer therapy, infection, allergy, hypertension and inflammation are substrates for p-glycoprotien (112). So ABCB1 transporter may have a great effect on the pharmacokinetics of drugs in human. A particular study have revealed that anastrozole is a substrate for ABCB1 transporter (113).

One of the known mechanisms responsible for drug resistance in patients with cancer is the raising ability of cancerous cells to transport drugs out of the cell using ATP-dependent transporters, which may be due to overexpression of these transporters (114), and this result in decreasing the concentration of the drug inside the cells, consequently lowering the efficacy of the drug in damaging the cancerous cells (115, 116).

ABCB1 is located in many organs and tissues, involving the liver, the intestine, proximal renal tubules, endothelial cells in the blood-brain barrier, placenta, breast ductal epithelium, testes, and hematopoietic cells (117, 118).

1.11.1.2. Effect of genetic polymorphisms of ABCB1 gene

Genetic polymorphism is the variation in the sequence of DNA among populations or individuals. These may involve single nucleotide polymorphisms (SNPs), insertion, deletion, sequence repeats. SNPs are the most frequent form of genetic variations among individuals, and present at a specific nucleotide site (119). Some of SNPs are located in the coding

regions while the others present outside the coding regions. Those that falling in the coding regions can be further divided into: synonymous or silent and non-synonymous (120).

ABCB1 gene encode ABCB1 transporter, a polypeptide chain with 1280 amino acids, and it is located on chromosome 7q21 with 28 exons and 28 introns (112, 121), figure (1-6).
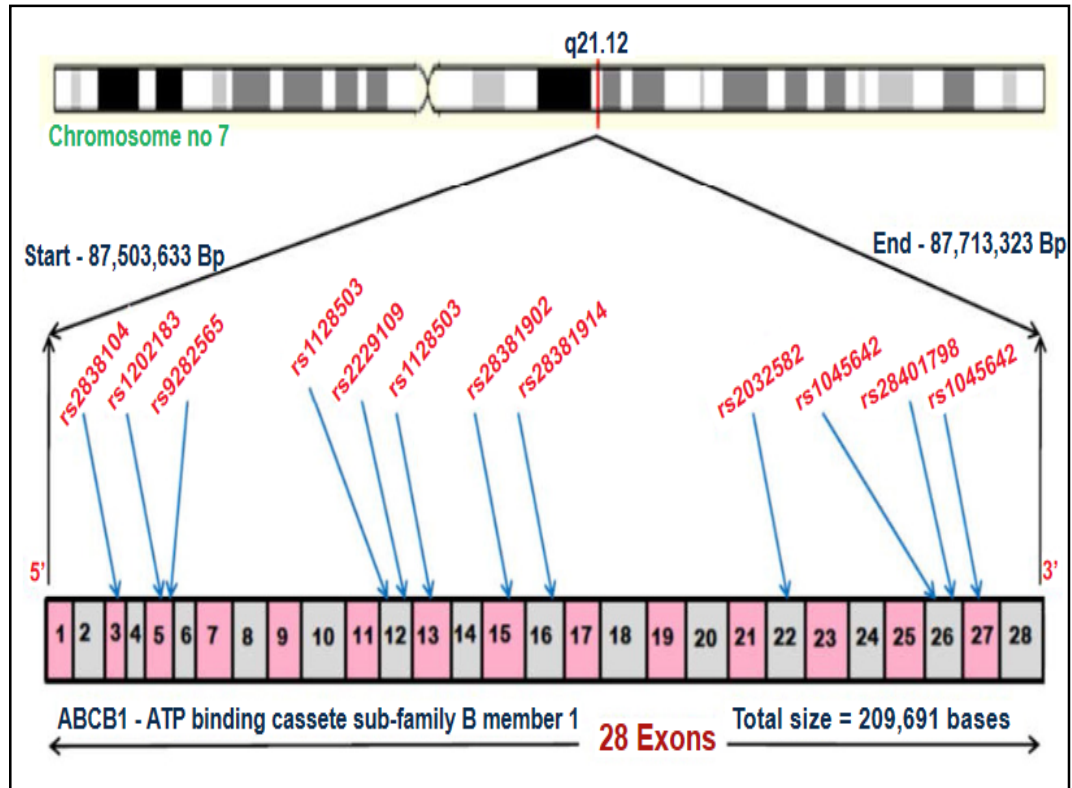
Figure (1-6): The location of ABCB1 gene on chromosome 7, and the exon structur of the gene with loction of SNPs at their corresponding genomic positions (122).

MDR1 gene is polymorphic and have more than 40 single nucleotide polymorphisms (SNPs) (123), and these SNPs may affecting the disposition of drug and clinical outcomes (124, 125).

C1236T (rs1128503) is one of synonymous SNPs of ABCB1 gene, located on exon 12 and it does not involve alteration in amino acid glycine at position 412 (112, 121). Recent study demonstrated that this SNPs is contributed to cancer recurrence (126).

C3435T (rs1045642) is another synonymous SNPs of ABCB1 gene, located on the exon 26, it includes C to T transformation and it does not change the amino acid isoleucine at position 1145 (121).
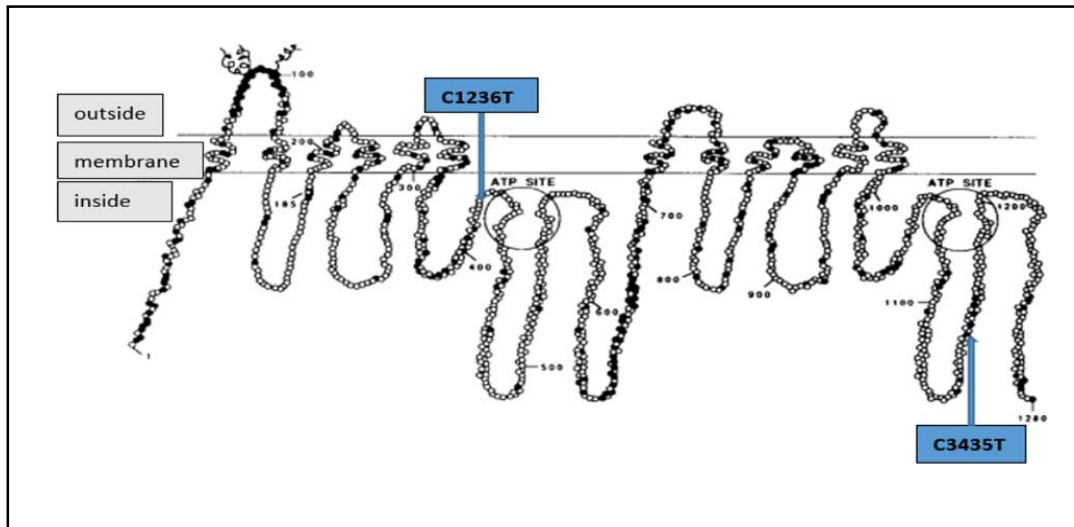
Figure (1-7): Schematic representation of ABCB1 with location of C1236T and C3435T SNPs.

Concerning C3435T, it has been found that patients with breast cancer who carry TT genotype have lower MDR1 expression level than healthy controls, and this may rationalize why the patients that carry TT genotype respond to treatment better than those carrying CC or CT genotype (114).

1.12. Aim of the study

1. To detect the genetic polymorphisms of ABCB1 gene (C1236T and C3435T) in the participated breast cancer women.

2. To investigate the effect of genetic polymorphisms of ABCB1 gene (C1236T and C3435T) on anastrozole efficacy in women with breast cancer.

3. To investigate the association of ABCB1 gene polymorphisms (C1236T and C3435T) with anastrozole induced arthralgia

Chapter Two

Materials, patients & Methods

1. Materials, patients and methods

2.1. Materials

2.1.1. Instruments

The instruments that used in this study with their manufacture and origin are listed in table (2-1).

Table (2-1): Instruments used in this study with their manufacture and origin.

Instrument	Manufacture / Origin
Centrifuge	Hettich / Germany
Digital Camera	Canon / England
Distillator	GFL / Germany
Electrophoresis apparatus	TECHNE ME / England
Freezer (-20 °C)	Concord / Lebanon
High speed centrifuge	Sigma 3-30K / Germany
Hood	LabTech / Korea
Hot plate Stirrer	LabTech / Korea
Mindray CL-series Chemiluminescence Immunoassay Analyzer	Mindray / China
MiniVIDAS	BioMérieux / France
Nanodrop	Bio Drop / England
PCR -thermocycler	TECHNE / England
Refrigerator	Concord / Lebanon
Sensitive balance	DENVER / Germany
UV-transilluminator	Syngene / England
Vortex mixer	Human Twist / Germany
Water bath	LabTech / Korea

2.1.2. Chemicals and kits

Specific chemicals and kits that used in this study are listed in table (2-2).

Table (2-2): Kits and chemicals used in this study with their Manufacture and origin.

Kits and Chemicals	Manufacture / Origin
100 bp DNA ladder	Bioneer / Korea
10X TBE (Tris-Borate-EDTA) Buffer	iNtRON / Korea
Absolute ethanol	BDH / Germany
AccuPower® PCR PreMix	Bioneer / Korea
Agarose D1 low EEO	CONDA / Spain
CA 15.3 kit	Biomérieux / France
Estradiol (CLIA) kit	Mindray / China
Ethidium bromide	iNtRON / Korea
G-DEX^{TM} IIb for blood genomic DNA extraction kit.	iNtRON / Korea
Isopropanole (2-propanol)	SRL / India
Nuclease free water	Promega / USA
Primers	Macrogen / Korea

2.2. Patients

2.2.1. Study population

This study was a cross–sectional observational study that carried out at Imam Al-Hussein Medical City / Oncology Center in Kerbala, during the period from September 2019 till February 2020. The protocol of the study was approved by the Scientific and Ethical Committee of Pharmacy College / Kerbala University, and informed signed consent form was given by each subject after explaining the nature and purpose of study. The study was conducted on 100 females with age (at diagnosis of breast cancer) ranged

from 31 to 75 years, they were taking anastrozole (Armidex®) tablet 1 mg per day orally.

2.2.1.1. Inclusion criteria

The inclusion criteria involved: all females were taking anastrozole at least 6 months before starting the study, and all patients who had estrogen receptor and/or progesterone receptor positive (luminal A only).

2.2.1.2. Exclusion criteria

The exclusion criteria included: women had started anastrozole therapy simultaneously either with adjuvant chemotherapy or adjuvant radiation therapy (or both). Also, women were taking drugs that affect the activity of ABCB1 transporter (inducers or inhibitors) were excluded.

Patients with a previous history of gastrointestinal disorders or surgery were also excluded from the study. In addition, women who had taken known CYP3A4/5 or UGT1A4 inducers or inhibitors were excluded.

2.2.2. Clinical data collection

During the time of blood sample collection, each patient was questioned whether she had used one or more drugs that may interfere with anastrozole metabolism or may affecting the activity of ABCB1 transporter, to make sure that all the potentially interacting drugs were captured in the database.

The data were obtained from the medical records of consenting females and from the patients themselves and these included: age, weight, academic achievement, workplace, marital status, breast feeding, dates of first menarche and last menopause, family history of breast cancer and number, date of first birth –full pregnancy, Date of breast cancer diagnosis, Site (left, right, or both), type of breast cancer, stage and grading, immunohistochemical status (ER, PR, HER2), surgery, chemotherapy,

radiation, presence of arthralgia or other side effects, liver disease or any other diseases, time on anastrozole therapy and duration, and any other drugs used.

2.2.3. Sample collection and analysis

After being approved by Scientific and Ethical Committee of Pharmacy College / Kerbala University, blood samples were taken from eligible females who had signed informed consent. 5 ml of venous blood were withdrawn from each female contributed in this study. 2 ml of blood was placed in EDTA-tube for molecular analysis. 3 ml was placed in gel tube, and serum was obtained after centrifugation of blood at 3000 rpm for 10 minutes, it was used for measurement of estradiol and CA15.3 levels.

2.3. Methods

2.3.1. Molecular analysis

2.3.1.1. DNA Extraction

Genomic DNA was extracted from blood sample as stated by the protocol G-DEX^{TM} IIb for blood genomic DNA extraction kit. The following method is suitable for DNA isolation from blood:

1. 1 ml of blood was centrifuged at 2000 rpm for 1 minute to form the buffy layer.

2. 300 µl of the buffy coat was added into 1.5 ml eppendorf tube, then 900 µl of RBC lysis solution was added. The mixture was mixed thoroughly by vortex and incubated for 5 minutes at room temperature. The tube was inverted again at least once through the time of incubation.

3. The eppendrof tube was centrifuged at 10,000 x g for 1 minute, the supernatant was removed except the white cell pellet and only about 50-100 µl of the remnant was remained.

4. The tube was vortexed strongly to re-suspend the cells.

5. 300 µl of cell lysis solution was added to the re-suspended cells and then pipetting up and down was done to lyse the cells.

6. The mixture was chilled at room temperature. 100 µl of protein precipitation buffer (PPT) was added to cell lysate then vortexed vigorously at high speed for 20 seconds.

7. The eppendorf tube was centrifuged at 13000-16000 x g for 3-5 minutes, the precipitated proteins formed a tight white pellet.

8. 300 µl of the supernatant that containing the DNA was transferred into eppendorf tube, then 300 µl of 100% isopropanol was added and mixed by inverting the tube gently several times.

9. The mixture was centrifuged at 13000-16000 x g for 1 minute, the DNA was noticed as a small white pellet.

10. The supernatant was poured off and the tube was drained briefly on clean adsorbent paper, then 1 ml of 70% ethanol was added and the tube was inverted several times for washing the DNA pellet followed by centrifugation at 13000-16000 x g for 1 minute, carefully the ethanol was poured off.

11. The tube was inverted and drained on clean adsorbent paper and allowed to air dry for 10-15 minutes.

12. 150 µl of DNA rehydration buffer was added.

13. DNA was rehydrated through incubation at 65 ºC for 30-60 minutes.

14. The collected DNA was stored at -20 ºC (127).

2.3.1.1.A Determination of purity and concentration of DNA

The concentration and purity of DNA were measured by using Nano-spectrophotometer (NanoDrop). The DNA purity was measured at A260/A280 ratio. 1 µl of DNA sample was placed on the micro detector of nanodrop. The concentration and purity were documented from the instrument.

2.3.1.2. Primers design

The primers were designed depending on the research introduced by Chen *et al.* (2009) (128). The primers sequences that were utilized for amplification analysis of ABCB1 gene for SNPs identification are shown in table (2-3).

Table (2-3): Primers sequences of SNPs (C1236T and C3435T) with their product sizes (128).

Primers	Primers sequences (5'-> 3')	Product size
1236P1	AAT GTT CAC TTC AGT TAC CCA TCT CG	508 bp
1236P2	AAT GAT TTC CCG TAG AAA CCT TAC	
1236C	TGG TAG ATC TTG AAG CGC	305 bp
1236T	TGC ACC TTC AGG TTC TGA	238 bp
3435P1	TGC TGG TCC TGA AGT TGA TCT GTG AAC	300 bp
3435P2	GGC CAG AGA GGC TGC CAC AT	
3435C	GTG TCA CAG GAA GAG TTC	126 bp
3435T	TCC TTT GCT GCC CTC TCA	209 bp

Lyophilized primers were dissolved with a certain volume of nuclease free water according to instruction of manufacture to give concentration of 100 pmol/µl (represent a stock solution). Table (2-4) represent the volumes of nuclease free water added to each primer to obtain 100 pmol/µl.

Table (2-4): The volumes of nuclease free water added to each primer to obtain 100 pmol/µl cocentration.

Primers	Volume of nuclease free water added (µl)
123P1	290
123P2	300
1236C	320
1236T	320
3435P1	290
3435P2	300
3435C	320
3435T	320

For working solution, 10 µl of stock solution was diluted with 90 µl of nuclease free water to obtain 10 pmol/µl as a final concentration. The stock and working solutions were kept at -20 ºC.

2.3.1.3. Polymerase chain reaction (PCR)

In the present study, ARMS-PCR technique was used for detecting C1236T and C3435T SNPs of ABCB1 gene according to research introduced by Chen *et al* (2009) (128).

2.3.1.3.A. Optimization of the PCR conditions

The optimization of PCR was accomplished after multiple trails to reach the best concentration of the primers and the best annealing temperature.

The best conditions which provided the best results for C1236T and C3435T detection are showed in tables (2-5), (2-6) and (2-7).

2.3.1.3.B. Running the PCR

The PCR mixture was prepared in PCR premix formula as shown in table (2-5).

Table (2-5): Components of PCR working solution.

Component	Volume (µl)
Primer 1 (outer)	0.5
Primer 2 (outer)	0.5
Primer 3 (inner)	1
Primer 4 (inner)	1
DNA sample	5
Nuclease free water	12
Accupower® PCR PreMix (total)	20

In the current study, the thermal program for detecting C1236T is demonstrated in table (2-6).

Table (2-6): PCR program for detecting C1236T.

Steps	Temperature (ºC)	Minute: second	Cycles
Initial denaturation	94	03:00	1
Denaturation	94	00:30	
Annealing	60	00:30	35
Extension	72	00:55	
Final extension	72	05:00	1

The thermal program for detecting C3435T is demonstrated in table (2-7).

Table (2-7): PCR program for detecting C3435T.

Steps	Temperature (ºC)	Minute: second	Cycles
Initial denaturation	94	03:00	1
Denaturation	94	00:30	
Annealing	58	00:30	35
Extension	72	00:55	
Final extension	72	05:00	1

2.3.1.4. Agarose gel electrophoresis

1. Agarose gel 1.5% was prepared by dissolving 0.3 gm of agarose powder in 2 ml of 10X TBE buffer (Tris-Borate-EDTA) and 18 ml of distilled water.

2. The mixture was heated on a hot plate, and left for few seconds when the mixture began to boil.

3. The solution was left to cool and 2 µl of ethidium bromide was added.

4. The comb was fixed on one end of the try to make holes where the samples were loading.

5. After the agarose solution had decanted to try, it has been left to congeal at 25 ˚C.

6. The comb was removed lightly away from the try.

7. The try was stabled into the device chamber, and the chamber was filled with 1X TBE buffer.

8. One of the wells of agarose gel was loaded with 5 µl of DNA ladder while the others were loaded with 5 µl of each PCR products.

9. The voltage of the electrophoresis apparatus was fixed at 45 Volts to ensure an electrical field adjusted with (5) v/cm for 10 cm distance between cathode and anode.

10. At the end of the run, ultraviolet trans-illuminator was used for detection of the bands.

11. The gel was photographed using digital camera (129).

2.3.2. Biochemical parameters

2.3.2.1. Estradiol E2

The quantitative determination of concentration of estradiol level in human serum by Chemiluminescent Immunoassy (CLIA), which is a competitive binding immunoenzymatic assay (130).

<u>Principle</u>

Chemiluminescent (CL) series E2 assay is a competitive binding immunoenzymatic assay to determine the level of estradiol.

In the first step, sample paramagnetic microparticle coated with goat anti-rabbit IgG, sample treatment solution, and polyclonal anti-estradiol antibody (rabbit) were added into a reactive vessel. After incubation, estradiol in the sample will be bound to anti-estradiol antibody.

In the second step, estradiol alkaline phosphatase conjugate was added to the reaction vessel. Estradiol in the serum of sample competes with estradiol alkaline phosphatase conjugate for binding sites on the anti-estradiol antibody. The resulting antigen: antibody complexes were bound to goat anti-rabbit IgG on the microplate, which was magnetically captured while other unbound substances were removed by washing.

In the third step, the substrate solution was added to the reaction vessel. It was catalyzed by estradiol-alkaline phosphatase conjugate in the immunocomplex retained on the microplate.

The resulting chemiluminescent reaction was measured as relative light units (RLUs) by a photomultiplier built into the system. The amount of estradiol present in the sample was inversely proportional to the relative light units generated through the reaction.

The estradiol concentration could be determined through a calibration curve (130).

The expected values of estradiol level:

postmenopausal female <25-84 pg/ml.

follicular phase 20-138 pg/ml.

ovulation phase 100-440 pg/ml.

luteal phase 31-317 pg/ml.

2.3.2.2. Cancer Antigen 15.3 (CA15.3)

The quantitative measurement of CA15.3 levels in human serum was determined by using Enzyme Linked Fluorescent Assay (ELFA) technique by using MiniVidas apparatus (131).

<u>Principle</u>

The assay principle combines two steps enzyme immunoassay sandwich method with a final fluorescent detection (ELFA).

The Solid Phase Receptacle (SPR) served as the solid phase as well as the pipetting device for the assay.

All of the assay steps were performed automatically by the instrument. The reaction medium was cycled in and out of the SPR several times.

The serum of sample was cycled in and out of the SPR several times. This operation enabled the 115D8 which fixed onto the interior wall of the SPR to capture the reactive antigenic determinants of CA15.3 present in the samples. Unbound components were eliminated during the washing steps. Alkaline phosphatase-labeled DF3 antibody is then incubated in the SPR where it bound with the DF3 reactive antigenic determinants. Unbound conjugate was then eliminated during the washing steps.

During the final detection step, the substrate (4-methyl-umbelliferyl phosphate) was cycled in and out of SPR. The conjugate enzyme catalyzed the hydrolysis of this substrate into a fluorescent product (4-methyl-umbelliferyl) the fluorescent of which was measured at 450 nm.

The intensity of the fluorescent was proportional to the concentration of CA 15.3 present in the sample.

At the end of the assay, the results were automatically calculated by the instrument in relation to the calibration curve (131, 132).

The normal value for CA 15.3 is less than 30 u/ml.

2.3.3. Arthralgia assessment

Participants that were classified as having arthralgia depending on the Brief Pain Inventory (BPI). BPI is a multiple items of question that is widely used to assess pain in cancer populations (133). In addition, most of those patients were already diagnosed to have arthralgia and osteoporosis and given by their doctors zoledronic acid (Zometa®) to treat them.

2.3.4. Statistical analysis

For statistical analysis, Statistical Package for Social Sciences (SPSS) version 25, IBM, US was used. The genotype groups were expressed in frequency and percentage. The biochemical parameters were expressed as mean ± SD. Single factor ANOVA was used to examine the differences in the mean of biochemical parameters tested within genotype groups in breast cancer patients. Pearson's correlation coefficient was used to test the relation between the levels of parameters and the detected genotype groups in breast cancer patients. Chi square test (x^2) was used to determine the association between genotype groups and the elevation in tumor marker levels. Odds ratio (OR) and confidence interval 95% (CI-95) were used to examine the association of these genotypes on the elevation of tumor marker CA15.3 and on the development of arthralgia. In all statistical analysis that used in the current study, the probability value (p value) of less than 0.05 is considered as a significant difference.

Chapter Three

Results

3.1. Patient's demographic data

The demographic data of the patients that were participated in this study are shown in table (3-1). The age range at diagnosis of breast cancer was (31-75) years with mean (53.142); the percentage of married women was 94% while the percentage of unmarried women was only 6%; the number of women with pre- or postmenopause were 5 and 95 respectively; about 64% of women who depended on lactation to feed their babies while only 19% and 17% of patients who did not or undertook mixed feeding respectively; the percentages of females who had or did not have family history of breast cancer were 12% and 88% respectively; only 3% of patients with breast cancer in both sides (left and right); 49% and 48% of females in left side and right sides respectively; the percent of patients who had ER and / or PR positive was 100%; the percent of patients that undergo surgery (mastectomy, lumpectomy with or without lymph nodes removal) was 88%; but 12% with no surgery; 84% of women who taken chemotherapy while 16% with no chemotherapy; the percentage of females who taken radiation therapy was 69% and 31% of females with no radiation; arthralgia is one of the most common side effects of anastrozole and so 89% of patients who participated in this study were suffering from arthralgia but only 11% of them without arthralgia.

Table (3-1): Demographic data

Parameters		N (%)
Age at diagnosis of breast cancer		Mean (range) 53.142 (31-75)
Marital status	Married	94 (94%)
	Unmarried	6 (6%)
Pre- or postmenopause	Premenopause	5 (5%)
	Postmenopause	95 (95%)
Breast feeding	Positive	64 (64%)
	Negative	19 (19%)
	Mixed	17 (17%)
Family history	Positive	12 (12%)
	Negative	88 (88%)
Site of breast cancer	Left	49 (49%)
	Right	48 (48%)
	Left + right	3 (3%)
Receptor status	ER and / or PR (positive)	100 (100%)
Surgery	Positive	88 (88%)
	Negative	12 (12%)
Chemotherapy	Positive	84 (84%)
	Negative	16 (16%)
Radiation	Positive	69 (69%)
	Negative	31 (31%)
Arthralgia	Positive	89 (89%)
	Negative	11 (11%)

3.2. Molecular analysis

3.2.1. Results of amplification reaction

The amplification of SNPs of ABCB1 gene: C1236T was shown in 305 bp, 238 bp as in figure (3-1) and C3435T was shown in 126 bp, 209 bp as in figure (3-2).
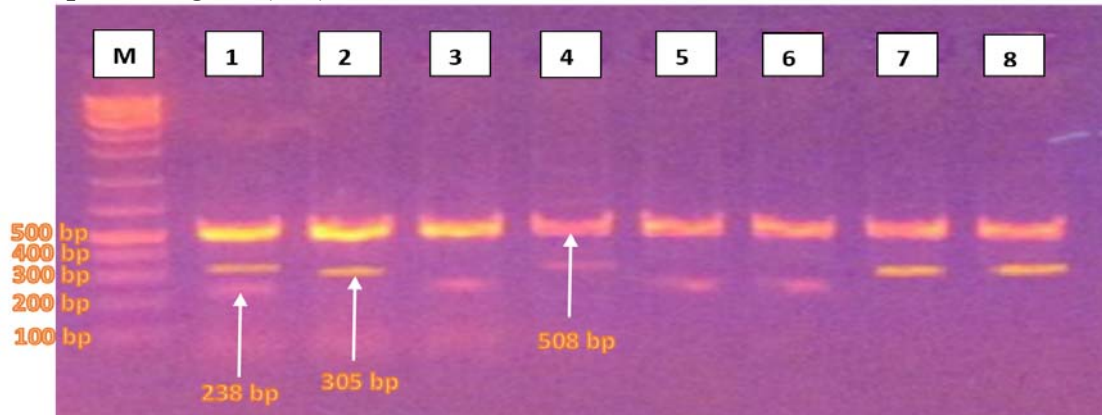
Figure (3-1): DNA samples of PCR amplification of ABCB1 gene (C1236T) showed: Line M: Represented DNA marker (ladder) 100 – 1500 bp, Line 1: Represented CT genotype (heterozygote), Lines 2, 4, 7 and 8: Represented CC genotype (wild) were showed in 305 bp, Lines 3, 5 and 6: Represented TT genotype (mutant) were shown in 238 bp.
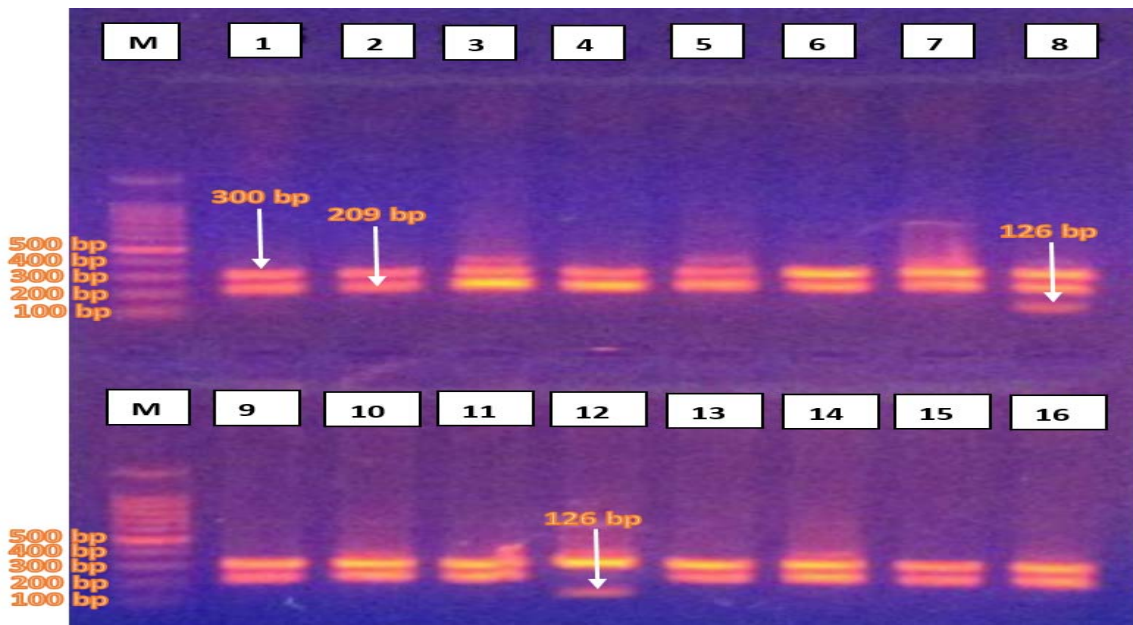
Figure (3-2): DNA samples of PCR amplification of ABCB1 gene (C3435T) showed: Line M: Represented DNA marker (ladder) 100 – 1500 bp, Lines from 1 to 7, from 9 to 11, and from 13 to 16: Represented TT genotype

(mutant) were shown at 209 bp, Line 8: Represented CT genotype (heterozygote), Line 12: Represented CC genotype (wild) were shown at 126 bp.

3.2.2. The genetic basis of ABCB1 gene polymorphisms in patients with breast cancer

3.2.2.1. The genetic distribution of C1236T in breast cancer patients

The frequency and percentage of C1236T genotype that detected in the breast cancer patients are shown in table (3-2).

The most frequent genotype in 100 breast cancer patients recruited in this study was the mutant type (TT) with frequency and percentage 54 and 54% respectively, while the heterozygote type (CT) represent the lowest frequent type with frequency and percentage of 18 and 18% respectively. The wild type of C1236T, which carry CC genotype have been identified in frequency and percentage of 28 and 28% respectively.

Table (3-2): The frequency and percentage of ABCB1 gene (C3435T) genotypes detected in breast cancer patients

SNP	Genotypes	Frequency	Percentage
C1236T	CC (Homozygote, wild type)	28	28%
	CT (Heterozygote)	18	18%
	TT (homozygote, mutant type)	54	54%
	Sum	100	100%

SNP: single nucleotide polymorphism; C: cytosine; T: thymine.

3.2.2.2. The genetic distribution of C3435T in breast cancer patients

The frequency and percentage of C3435T that detected in the breast cancer patients are shown in table (3-3).

In this study, the most frequent genotype of C3435T that detected in 100 breast cancer women was the mutant type (TT) with frequency and

percentage of 76 and 76% respectively, while the frequency and percentage of heterozygote (CT) was 15 and 15% respectively, however the homozygote wild type (CC) represented the lowest frequency and percentage (9, 9%) respectively.

Table (3-3): The frequency and percentage of ABCB1 gene (C3435T) genotypes detected in breast cancer patients.

SNP	Genotypes	Frequency	Percentage
C3435T	CC (Homozygote, wild type)	9	9%
	CT (Heterozygote)	15	15%
	TT (homozygote, mutant type)	76	76%
	Sum	100	100%

3.3. Biochemical parameters

3.3.1. Estradiol levels (E2)

3.3.1.1. Estradiol level in breast cancer patients with ABCB1 gene polymorphism

The (mean ± SD) of estradiol (E2) level in breast cancer women with ABCB1 gene polymorphisms are shown in Table (3-4). The mean of E2 levels in all genotypes was within the normal limit.

For C1236T, the (mean ± SD) of E2 level in BC women in the wild homozygous (CC), heterozygous (CT) and mutant homozygous type (TT) were (19.482 ± 15.060 pg/ml), (22.656 ± 8.02 pg/ml) and (19.716 ± 9.717 pg/ml) respectively. There were non-significant ($p > 0.05$) differences in the means of E2 level between genotype groups.

Concerning C3435T, the (mean ± SD) of of E2 level in BC women in the wild type (CC), heterozygous (CT) and mutant type (TT) were (15.275 ± 6.817 pg/ml), (23.023 ± 18.803 pg/ml) and (20.2 ± 9.450 pg/ml) respectively. There were non-significant ($p > 0.05$) differences in the means of estradiol level between the genotype groups.

Table (3-4): Mean and standard deviation of estradiol levels in breast cancer patients with ABCB1 gene polymorphism.

SNPs	Genotypes	E2 level Mean ± SD pg/ml	P value
C1236T	CC	19.482 ± 15.060 a	0.585
	CT	22.656 ± 8.02 a	
	TT	19.716 ± 9.717 a	
C3435T	CC	15.275 ± 6.817 a	0.260
	CT	23.023 ± 18.803 a	
	TT	20.2 ± 9.450 a	

P value derived from ANOVA test, Significant: p< 0.05, Non-significant: p> 0.05.
a: Similar letters mean non-significant differences.

3.3.1.2. Correlation coefficient between serum estradiol levels in breast cancer patients and ABCB1 gene polymorphism

Concerning the correlation between ABCB1 gene polymorphisms and serum estradiol level, table (3-5) showed no correlation between C1236T and serum estradiol level, in addition there was no correlation between C3435T and serum estradiol level.

Table (3-5): Correlation coefficient between ABCB1 gene polymorphisms and E2 levels.

SNPs	Correlation coefficient	P value (2-tailed)
C1236T	0.005	0.955
C3434T	0.05	0.618

P< 0.05 is significant.

3.3.2. Tumor marker CA15.3 level

3.3.2.1. Tumor marker CA15.3 level in breast cancer patients with ABCB1 gene polymorphism

In the current study, table (3-6) shows mean levels of serum CA15.3 in BC patients with ABCB1 gene polymorphism. The mean level for all genotypes was within the normal value.

For C1236T, the (mean ± SD) of CA15.3 for the heterozygous (CT) was the higher if compared with the mutant or wild type, but it still within the normal value (23.235 ± 14.730 U/ml) while those for wild (CC) and mutant (TT) types were (15.616 ± 6.264 U/ml) and (20.507 ± 16.446 U/ml) respectively. There were only significant differences between the wild type and the heterozygote, but there were non-significant between the wild and the mutant or between the heterozygote and the mutant genotype.

Concerning C3435T, the (mean ± SD) of CA15.3 for the wild (CC), heterozygous (CT) and mutant type (TT) were (14.438 ± 5.43 U/ml), (14.882 ± 6.42 U/ml) and (21.417 ± 15.93 U/ ml) respectively. There were non-significant differences between the serum levels of CA15.3 and the different genotype groups.

Table (3-6): Mean and standard deviation of CA15.3 levels in breast cancer patients with ABCB1 gene polymorphism.

SNPs	Genotypes	CA15.3 level mean ± SD (U/ml)	P value
C1236T	CC	15.616 ± 6.264 a	0.163
	CT	23.235 ± 14.730 b	
	TT	20.507 ± 16.446 ab	
C3435T	CC	14.438 ± 5.43 a	0.09
	CT	14.882 ± 6.42 a	
	TT	21.417 ± 15.93 a	

P value derived from ANOVA test, Significant: p<0.05, Non-significant: p>0.05. Different letters mean significant, similar letters mean non-significant differences.

3.3.2.2. Normal/above values of serum CA15.3 level in breast cancer patients with ABCB1 gene polymorphism

Table (3-7) shows the number and ratio of patients who had normal/above normal values of CA15.3 level in BC patients with ABCB1gene polymorphism. In the present study, 89 patients had normal value of CA15.3 level, while only 11 patients had elevated level of CA15.3.

For C1236T, the number and percentage of women who had CA15.3 levels above the normal limit are shown as follow: no one of patients with (CC) genotype have CA15.3 level above the normal value, 4 (36.4%) of patients with genotype (CT) and 7 (63.6%) patients with (TT) genotype. The number and ratio of patients who had CA15.3 levels within normal value are shown as follow: 28 (31.5%) of patients with (CC) genotype, 14 (15.7%) of patients with (CT) genotype, and 47 (52.8%) of patients with (TT) genotype. The p- value was non-significant, so the two variables (C1236T and CA15.3) are independent on each other.

For C3435T, the frequency and percentage of females who had CA15.3 levels above the normal value are shown as follow: no one of patients with (CC) genotype have above the normal value of CA15.3 level, 2 (18.2%) of patient with (CT) genotype and 9 (81.8 %) patients with (TT) genotype. The number and percentage of patients who had normal readings of CA15.3 level are shown as follow: all of patients 9 (10.11%) with (CC) genotype had normal values of CA15.3, 13 (14.6%) of patients with (CT) genotype and 67 (75.28%) of patients with (TT) genotype. Since the p- value was non-significant, the two variables (C3435T and CA15.3) are independent on each other.

Table (3-7): Number and percentage of patients who had normal or above limits of CA15.3 in breast cancer patients with ABCB1 gene polymorphism.

SNPs	Genotypes	CA15.3 (Above normal) N=11 N (%)	CA15.3 (within normal) N=89 N (%)	
C1236T	CC	0 (0)	28 (31.5)	$^*X^2$=5.98
	CT	4 (36.4)	14 (15.7)	Df= 2
	TT	7 (63.6)	47 (52.8)	p>0.05
C3435T	CC	0 (0)	9 (10.11)	X^2= 1.25
	CT	2 (18.2)	13 (14.6)	Df= 2
	TT	9 (81.8)	67 (75.28)	P> 0.05

* X2: chi--square test, Df: degree of freedom, P< 0.05 regarded significant.

3.3.2.3. Correlation coefficient between ABCB1 gene polymorphisms and CA15.3 levels in breast cancer patients

Concerning the patients of this study, there was no correlation between C1236T and CA15.3, and the p value was non-significant. Besides, there was no correlation between C3435T and CA15.3, and p value was non-significant. Table (3-8).

Table (3-8): Correlation coefficient between ABCB1 gene polymorphisms and CA15.3 levels.

SNPs	Correlation coefficient	P value (2-tailed)
C1236T	-0.14973	0.137
C3435T	-0.0641	0.5261

P< 0.05 is significant.

3.3.2.4. The odds ratios of the detected genotypes of ABCB1 gene polymorphisms in the elevation of serum CA15.3 in the BC patients treated with anastrozole

Table (3-9) shows that C1236T and C3435T have non-significant effect on the elevation of serum CA15.3 level (odds ratio 1.56, p> 0.05) for C1236T and (odds ratio 1.477, p>0.05) for C3435T.

Table (3-9): The odds ratios of ABCB1 gene polymorphisms (C1236T and C3435T) with elevation of CA15.3 levels.

SNPs	Odds ratios (CI-95)		P value
C1236T	1.56	(0.4274 -5.721)	0.499
C3435T	1.477	(0.2965-7.364)	0.633

CI-95: confidence interval 95%, p< 0.05 is significant.

3.4. Arthralgia

3.4.1. The odds ratios of ABCB1 gene polymorphisms and occurrence of arthralgia in BC patients treated with anastrozole

Table (3-10) shows that C1236T and C3435T SNPs in BC women had non-significant effect on the onset of arthralgia (as the main side effect of anastrozole) (odds ratio 0.401, p> 0.05) for C1236T and (odds ratio 1.214, p> 0.05) for C3435T.

Table (3-10): The odds ratios of ABCB1 gene polymorphisms with onset of arthralgia.

SNPs	Odds ratios (CI-95)	P value
C1236T	0.401 (0.099-1.611)	0.1979
C3435T	1.214 (0.295-4.994)	0.787

P< 0.05 is significant.

Chapter Four

Discussion

4. Discussion

Anastrozole is a non-steroidal aromatase inhibitor that suppresses the conversion of androgen to estrogen. Firstly, anastrozole was used alone in postmenopausal breast cancer patients for prevention of disease recurrence, but in premenopausal breast cancer patients, it can be used together with luteinizing hormone releasing hormone agonist for better estrogen suppression than can be reached with tamoxifen and luteinizing hormone releasing hormone agonist (134). In vitro study by Miyajima *et al.* (2013) which showed that anastrozole is a substrate for ABCB1 transporter (113). ABCB1 is highly polymorphic drug transporter and the variation in its gene has been associated with alterations in the disposition, response and toxicity to a wide variety of drugs (135).

Therefore, in order to establish effective therapeutic response and decrease anastrozole side effect, it is important to understand the critical role of ABCB1 gene polymorphisms on the breast cancer treatment response.

To the best of our knowledge, the present study is the first study which focused on the genetic variations of ABCB1 gene in Iraqi BC women and their impact on the breast cancer treatment in the term of therapeutic response to anastrozole and the occurrence of anastrozole side effect.

4.1 Demographic data

Breast cancer is the most frequent type among females and it is a multifactorial disease and its occurrence is associated with different factors such as genetic factors, family history, age and lifestyle factors (136).

Table (3-1) demonstrated the demographic data of the patients that participated in this study. The mean age at diagnosis of breast cancer in the women who contributed in this study was 53.142 years, which is compatible with Thakur *at al.* (2017), they found that the age greater than 50 years is a major risk factor for breast cancer (137). Some of females developed breast cancer at younger age (below 40 years) but this represents a small percentage of the total incidence; however, its combined with more aggressive subtypes and they were expected to exist at an advanced stage and so poorer outcome (138). This finding may be due to early menarche will cause early progression of the breast and so early exposure to estrogen, this result in an increasing the risk for developing breast cancer, while older age at menarche will lower the breast cancer risk in premenopausal females (139).

Some studies reported that the married females are associated with high risk of breast cancer while others showed that the marriage plays a protective role (140, 141), on the other hand, in this study the proportion of married women with breast cancer was higher than the unmarried, in my opinion, these results may be due to genetic factors that contributed in BC development.

In the present study, the ratio of females who fed their babies by breast feeding were higher than the others. Several studies reported that breast feeding has a protective effect against breast cancer which is may be due to during lactation the number of menstrual cycles is decreased and so the cumulative exposure to endogenous hormones also reduced (142). The researcher think that results of the current study can be explained due to small sample size and this study was a cross sectional observational study.

One of the most common risk factors of breast cancer is the family history, which has been revealed by numerous studies. About 15% of all diagnosed breast cancer women had a family history of the disease (143). In the present study, the ratio of women who had family history of BC was 12%. Several studies reported that family history of breast cancer along with genetic susceptibility, remains to predict raised breast cancer hazard in elderly women (144).

4.2. Frequency of the detected genotypes of ABCB1 gene within breast cancer patients

The genotype testing determined the frequencies and percentages of ABCB1gene polymorphisms within breast cancer patients of this study as existing in tables (3-2) and (3-3).

In the present study, for C1236T SNP, the percentage of wild genotype (CC) in 100 breast cancer patients was 28%, the heterozygous type (CT) presented with percentage of 18%, and finally the mutant homozygous type (TT) appeared with percentage of 54%.

In contrast, a study in Saudi Arabia that included 100 patients with breast cancer showed that the percentages of wild genotype (CC), heterozygous (CT), and mutant type (TT) were 73%, 11%, and 16% respectively (145). A study in Indian breast cancer women that enrolled 111 patients showed that the frequencies of C1236T genotype were 23 for CC, 48 for CT and 40 for TT genotype (146). Another study in China that recruited 153 patients revealed that the number of patients who carry CC genotype were 19, while for those that carry CT or TT were 56 and 78 respectively (147). The results of the current study were compatible with results obtained in China in that the mutant (TT) genotype had the higher frequency.

In the current study, for C3435T, the percentage of patients who carrying the wild type (CC) was 9%, the heterozygous (CT) genotype

presented with percentage of 15%, while mutant type (TT) represent the most common genotype observed in 100 patients with percentage 76%.

The frequencies of C3435T were 32.8%, 54.3%, and 13% for wild, heterozygous and mutant types respectively were reported by Jaramillo-Rangle *et al.* (2018) in northeastern Mexico for patients with breast cancer (148); In Morocco Tazzite *et al.* (2016), found that the percentages of CC, CT, and TT genotypes were 50%, 33.3%, and 16.6% respectively were detected in patients with breast cancer (149), these results that were completely different compared to the results of the current study.

In Jordan, Abuhaliema *et al.* (2016), showed that the detected frequency of C3435T genotypes in 150 women with breast cancer were 45.3%, 41.3%, and 13.3% for CC, CT, and TT respectively (150). Taheri *et al.* (2010), that recruited 54 Iranian women (Caucasian) with breast cancer found that the frequency and percentage of C3435T were 10 (18%), 30 (56%), and 14 (26%) for CC, CT, and TT respectively (114).

In the current study, the results have showed that the mutant genotype had the higher frequency for both SNPs (C1236T and C3435T), this may reveal that these two SNPs are associated with risk of breast cancer. This may be due to in vitro study have showed that estrogen hormone is a substrate for ABCB1 transporter (151), and because those individuals who carry mutant genotype had lower expression levels of ABCB1 (152), taken together, this may lead to accumulation of estrogen inside the cells and elevate the risk for developing breast cancer in patients with mutant genotype. Also, the researcher suggests that the cause of high frequency of the mutant genotype may be due to marriage between relatives which may elevate this percent.

4.3. Impact of ABCB1 gene polymorphisms on the estradiol levels in breast cancer patients.

The correlation between the risk of breast cancer and the elevation of blood estrogen levels has been found consistently in different studies (153, 154), these observations support the hypothesis that estrogen is a mammary-gland carcinogen. The mechanisms through which estrogen act as carcinogen are complex and involve initiation and promotion (154). One of the most common mechanisms used for controlling the estrogen synthesis in the body is through inhibition of aromatase enzyme, which catalyze the final steps of estrogen synthesis from androgens through using anastrozole (89).

The findings of this study offer insight into the different concentrations of estradiol within different genotypes of ABCB1 gene table (3-4). Although of using anastrozole, (which inhibit estrogen synthesis) this study found a detectable amount of estradiol in the serum but still within normal values. These results were compatible with study introduced by Abd-Allateef *et al.* (2016) who found a measurable levels of estradiol in breast cancer women treated with anastrozole despite of a close total inhibition of aromatase enzyme (155). The detectable amount of E2 in the current study may be due to polymorphisms in aromatase (CYP19A1) gene. A study found that two SNPs in CYP19A1 that occur in 5′-flanking region (rs6493497 and rs7176005) were associated with elevated baseline activity levels of aromatase also, increased the levels of E2 among women with BC who were treated with anastrozole (156).

The observed results for C1236T and estradiol level showed that the patients who were carrying the mutant genotype (TT) have approximately the same mean levels of estradiol for those carrying wild type (CC). A study conducted by Vaclavikov *et al.* (2008) which demonstrated that patients with breast cancer who carry mutant genotype (TT) have lower levels of ABCB1 expression in their tumors (152), and so the capacity of

these transporter to transport the drug out of the cancer cells may be reduced, depending on this study, the patients who had TT genotype would theoretically respond to treatment better than those with CC or CT genotypes and this may result in decreasing the levels of estradiol in women with mutant genotype. However, results in this study revealed that there were non-significant differences in the levels of estradiol in patients carrying mutant type from those with wild type or heterozygote (and all of these were within the normal value) and there may be other factors affected on these levels of E2 like polymorphisms in other genes.

Concerning C3435T, the women with CC genotype had lower mean estradiol levels, while those with heterozygote had higher levels. Several studies reported that the patients with breast cancer who have TT genotypes have lower expression levels of ABCB1 than patients with CT or CC genotypes (114, 152) , and this may reduce the cellular elimination of drug from tumor cells and may enhance the therapeutic response. According to these studies, women with TT genotype may have well response to treatment, while the results obtained in this study revealed the opposite (patients with CC genotype respond better with mean of estradiol level lower than patients with TT or CT) and not showed any statistical significance. This may be due to several factors affecting on the estradiol levels or genetic polymorphisms in certain genes may influence on the drug response such as CYP19A1. A study found that a genetic polymorphism in CYP19A1 cause variations in estrogen levels among women with breast cancer (157).

The study also researched the correlation between ABCB1 gene polymorphism (C1236T and C3435T) and estradiol levels in patients with breast cancer and it did not reveal any correlation table (3-5). This is may be due to these SNPs are a synonymous SNPs. Both C1236T and C3435T are silent mutations and synonymous SNPs which are unlikely to change protein levels and activity directly (158).

4.4. Impact of ABCB1 gene polymorphisms on the serum tumor marker CA15.3 in breast cancer patients.

Cancer antigen CA15.3 is broadly used as a tumor marker in breast cancer, and generally used for monitoring of cancer treatment and in prognosis of breast cancer (159). Although tumor marker alone is insufficient to assess the response to therapy (160), several studies recommend that tumor marker levels associate with therapy response (161-163). For example, Robertson *et al.* (1999), reported that changes in levels of tumor marker correlate with patients' therapeutic response, as evaluated by imaging methods (161). However, to date, no studies have researched between the response to anastrozole therapy and the CA15.3 levels along with genetic polymorphism in ABCB1 gene table (3-6).

In the present study, concerning C1236T, the patients who had CT genotype had significant highest mean levels of CA15.3 (but still within normal) than those with CC genotype. A study that investigated the effect of SNPs in ABCB1 gene on its expression in patients with breast cancer, it found that individuals with mutant genotype (TT) had significantly decreased ABCB1 expression levels in their tumors (152). Because of this, individuals with TT genotype may have lower capacity for transporting of the drug out of tumor cells and may respond better to treatments. However, this is not compatible with results of this study since the women who carry the wild genotype (CC) had lower mean levels of CA15.3 than those who carrying mutant or heterozygote genotypes (TT or CT respectively).

Concerning C3435T, patients with wild genotype (CC) had the lowest mean levels of serum CA15.3 while those with mutant genotype (TT) had the highest levels but there were non-significant differences. A study that conducted on Iranian breast cancer women investigated the impact of C3435T polymorphisms on ABCB1 gene expression found that the women with TT genotype had significant lower expression level of ABCB1 gene

than those with CT or CC genotypes (114). Another study also showed that there was a significant lower expression levels of ABCB1 in individuals with TT genotype (152). Regarding to these studies, patients who had TT genotype with low ABCB1 expression may had lower capacity for transporting the drug out of the cancerous cells and allow to remain the drug for a longer period and so may improve the response to therapy. However, this is not well-matched with results in this study since patients with TT genotype had the highest mean of serum CA15.3 level but there were non-significant differences in mean levels of CA15.3.

Taken together, for both SNPs these results were within the normal limits and the differences between genotype groups were non-significant (except for CT with CC in C1236T).

In the present study, C1236T and C3435T were not significantly associated with elevation of serum levels of CA15.3 and they were independent variables table (3-7).

There was no correlation between these SNPs and serum CA15.3 levels table (3-8), this is may be due to both SNPs are synonymous. C1236T and C3435T are synonymous SNPs and they are usually not affect the activity of the gene (158).

The present study also studied the linking of polymorphisms in ABCB1 gene and the elevation in serum CA15.3 levels in terms of odds ratio as demonstrated in table (3-9); however, it did not find any significant association. In my opinion, its possible that variations in the age, size of tumor, lifestyle, environmental factors, and other variables between various patients may influence the levels of tumor markers.

4.5. Impact of ABCB1 gene polymorphisms on the development of arthralgia in Iraqi BC women treated with anastrozole.

Arthralgia is one of the most common adverse effect of anastrozole which generally causes joint pain, usually affecting the hands, wrists, knees and hips. Sometimes, the symptoms affecting the quality of life which may cause discontinuation of treatment (164). In the Arimidex, Tamoxifen, Alone or in Combination (ATAC) trail showed that anastrozole was associated with a significant higher occurrence of arthralgia compared with tamoxifen (165).

In the current study, most of the women had arthralgia (89%) (table 3-1), this is possibly due to anastrozole use or may be other factors cause this adverse effect like genetic polymorphism in CYP19A1. In a study that recruited breast cancer women treated with anatrozole found that there was a significant association between the onset of arthralgia and CYP19A1 polymorphism (124). For this reason, this study investigated the association between genetic polymorphisms in ABCB1 gene and the occurrence of arthralgia in the term of odds ratio table (3-10). The results showed that there were non-significant association between C1236T and the development of arthralgia. This is compatible with Gervasini *et al.* (2017) who did not find a significant association between C1236T and the onset of arthralgia (124). Also, for C3435T, the present results revealed that there was non-significant association between this SNP and the occurrence of arthralgia. This is not agreed with Gervasini *et al.* (2017) who found that C3435T was inversely associated with development of arthralgia (124).

Despite that the pathophysiology of arthralgia is not fully explained, estrogen suppression by the action of anastrozole has been assumed to play a key role (166). However, this study found that although all patients had a detectable levels of estradiol, but most of them suffered from arthralgia and this may be due to other factors. A study found that

genetic polymorphism in CYP19A1 (rs4775936) and ESR1 genes were associated with development of arthralgia (167).

Conclusions & Recommendations

Conclusions

Depending on the results that obtained, the followings may be concluded:

1. ABCB1 gene was highly polymorphic and detected with different genotypes and variable frequencies in Iraqi women with BC.

2. For both C1236T and C3435T SNPs, the mutant genotype (TT) was the most predominant than other genotypes which were CC and CT.

3. The highly polymorphic ABCB1 gene that was detected in Iraqi breast cancer women was noted to be non-significantly correlated with variable serum levels of estradiol hormone and cancer antigen CA15.3, indicating there was no impact of ABCB1 genotypes on the levels of these parameters, and so may not influence on anastrozole response.

4. The existing finding showed that there was no association between different genotypes of ABCB1 gene and the onset of arthralgia in Iraqi breast cancer women.

Recommendations

1. Study a large numbers of SNPs for ABCB1 gene along with a large number of BC patients and correlate the effect of these SNPs on anastrozolr response.

2. Study the genetic variations in aromatase gene CYP19A1 which is responsible for estrogen synthesis in patients treated with anastrozole and correlate these results with clinical response.

3. Investigate the genetic variations in enzymes that involved in metabolism of anastrozole might contribute to individual variations in drug response.

4. Research the genetic effects of other genes that may impact on the onset of arthralgia.

5. Further studies are needed to demonstrate the effect of marital status and risk of breast cancer in Iraqi women.

6. Further studies are needed to confirm the association between ABCB1 gene polymorphisms and the development of breast cancer.

Future work

1. Study the ABCB1 gene sequencing for further bio-molecular studies.

2. Study the impact of ABCB1 gene polymorphisms on transporting ratio across the cell which is extremely precise and direct method for explaining the influence of these polymorphisms on the transporting status of ABCB1 transporter. One way to do this by measuring the concentration of anastrozole in plasma and inside the tumor cells.

Limitations of the study

1. Patients: many patients did not agree to contribute in this study, this may be due to their age, fear, and difficulty of drawing blood from their veins.

2. Sample size: this study included small sample size because many patients did not come personally to receive the treatment from the oncology center, in addition to COVID-19 reported by WHO as a global pandemic (168) .

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Appendices

Questionnaire of BC patients

Name: **Age:** **No.:**

Weight: **Height:**

Academic achievement: **Workplace:**

Address: **Phone no.:**

Marital status: **Breast feeding:**

First menarche: **Last menopause:**

Family history of BC: Yes ☐ No ☐

Number ☐

Date of BC daignosis:

Site (left, right, or both): **Stage and Grade:**

ER: **PR:** **HER2:**

Surgery: Yes ☐ No ☐

Chemotherapy: Yes ☐ No ☐

Radiation: Yes ☐ No ☐

Duration of BC:

Date of recurrence: **Site of recurrence:**

Type of BC:

Presence of arthralgia:

Liver diseases: **Other diseases:**

Time on anastrozole therapy:

Other drugs used:

Ministry of Higher Education

and Scientific Research

University of Karbala

College of Pharmacy

Department of Postgraduate Studies

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء

كلية الصيدلة

شعبة الدراسات العليا

Issue No.:

Date:

العدد: د.ع/ 6 / 2651

التاريخ: 17 / 11 / 2019

الى/رئاسة جامعة كربلاء/قسم الدراسات العليا

م/اقرار البحوث العلمية

تحية طيبة..

نود اعلامكم باجتماع اللجنة الاخلاقية السريرية والمشكلة حسب الامر الاداري المرقم (د/8/2317) في (2019/10/1) و المتضمن اقرار صلاحية اجراء وتنفيذ البحوث الخاصة بطلبة الدراسات العليا (الماجستير) في فرع الادوية والسموم.

مع التقدير..

المرفقات:-

-الامر الاداري انفا

-محضر اللجنة الاخلاقية انفا.

أ.م.د. احمد حقي اسماعيل

معاون العميد الشؤون العلمية

2019 / 11 / 17

نسخة منه الى:

- مكتب السيد العميد ، للتفضل بالاطلاع .
- مكتب معاون العميد للشؤون العلمية .
- شعبة الدراسات العليا للحفظ مع الاوليات .
- الصادرة .

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العراق - محافظة كربلاء- مكتب بريد كربلاء- ص ب 1125

الإستنتاجات: كشفت الدراسة ان جين ABCB1 متعدد الأشكال بدرجة عالية في المريضات المصابات بسرطان الثدي، وكانت الأنماط الجينية ذات الطفرة قد مثلت أعلى تكرار في كل من C1236T و C3435T. استنتج أيضا بأن تعدد الأشكال الجينية في الجين المشفر لناقل التدفق ABCB1 قد لا تؤثر في فعالية دواء اناستروزول. أظهرت الدراسة ان معظم النساء المصابات بسرطان الثدي اللاتي عولجن بأناستروزول يعانين من ألم المفاصل، إلا إنها لم تكشف عن أي ارتباط بين تعدد الأشكال الجينية ABCB1 مع تطور آلام المفاصل، كأثر جانبي رئيس للأناستروزول.

الخلاصة

خلفية الدراسة: يعد سرطان الثدي من أكثر أنواع السرطان شيوعا بين النساء. يحدث سرطان الثدي نتيجة التفاعل المتنوع بين العوامل الوراثية والبيئية. يعد عقار الأناستروزول من أكثر الأدوية شيوعا المستخدمة في علاج سرطان الثدي المعتمد على مستقبلات هرمون الأستروجين و / او مستقبلات هرمون البروجستيرون. يستخدم دواء أناستروزول على نطاق واسع في النساء المصابات بسرطان الثدي بعد سن اليأس ٫ بالإضافة الى انه استخدم في النساء اللواتي لديهن سرطان ثدي متقدم او منتشر. أشارت بعض الدراسات الى أن (ABCB1) هو الناقل لدواء أناستروزول خارج الخلية و إن جين ABCB1 هو جين متعدد الاشكال وله تقريبا ٤٠ شكلا تعدديا مختلفا من النوكلوتيدات المفردة ٫ وهذه قد تأثر في الحركية الدوائية للأدوية وبالتالي النتائج السريرية ٫ حيث شخصت طفرتين في هذه الدراسة وهما: C1236T و C3435T.

الهدف: صممت هذه الدراسة لتحديد الشكل الجيني ل ABCB1 عند النساء العراقيات المصابات بسرطان الثدي ٫ بالإضافة الى مدى تأثير تعدد اشكاله الجينية الجيني في فعالية أناستروزول ٫ و كذلك دوره في تطوير ألم المفاصل الناجم عن استخدام الدواء.

المرضى والطرق: أجريت هذه الدراسة العرضية المقطعية في مدينة الإمام الحسين (ع) الطبية / مركز الأورام في كربلاء. اختيرت مائة امرأة مصابة بسرطان الثدي من النوع الإيجابي لمستقبلات هرمون الإستروجين و / او مستقبلات هرمون البروجستيرون للمشاركة في هذه الدراسة. كانت المشاركات في هذه الدراسة يأخذن ١ ملغم من أناستروزول يوميا قبل بدء الدراسة ٫ حيث أخذت عينات الدم من المريضات المؤهلات اللواتي وقَعن على إستمارة الموافقة المسبقة لغرض اجراء الإختبار الجيني ٫ و لقياس مستويات هرمون الاستراديول و دلالة السرطان CA 15.3. إستخدمت هذه الدراسة نظام البوليميرز المتفاعل ذو النوع ARMS للكشف عن C1236T وC3435T. في حين قيمت آلام المفاصل اعتمادا على تاريخ المريض في تناول حمض الزوليدرونيك بلإضافة الى إستبيان BPI.

النتائج: أظهرت هذه الدراسة وجود تعدد الأشكال الجينية المختلفة للجين ABCB1 المشفر للناقل. كما أظهرت النتائج هذه الدراسة وجود علاقة غير معنوية بين تعدد الأشكال الجينية C1236T)ABCB1 و C3435T) ومستويات الاستراديول و كذلك دلالة الورم CA 15.3. في حين أظهرت الدراسة الحالية عدم وجود ارتباط معنوي بين تعدد الأشكال الوراثي للجين ABCB1 وآلام المفاصل التي يسببها دواء اناستروزول.

جمهورية العراق
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جامعة كربلاء
كلية الصيدلة

تأثير تعدد النمط الجيني لجين **ABCB1** في فعالية عقار الاناستروزول عند النساء العراقيات المصابات بسرطان الثدي

رسالة
مقدمة الى كلية الصيدلة ـ جامعة كربلاء
كجزء من متطلبات الحصول على شهادة الماجستير في علم الادوية والسموم

من قبل
هبة صلاح مهدي
بكالوريوس صيدلة (جامعة كربلاء ٢٠١٢)

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