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College of Pharmacy

Evaluation of the Effect of Genetic Polymorphism of SULT1A1 on Therapeutic Response of Tamoxifen in a Sample of Breast Cancer Patients

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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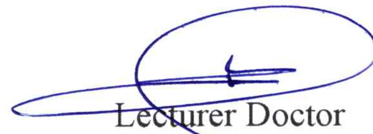

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DEDICATION

To My Father Soul, Allah Almighty bless him.

To My dear mother and their endless love, support and encouragement.

To my beloved brothers for their kindness efforts and for inspiring me to be strong despite many obstacle in life.

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List of Abbreviations	
Abbreviation	Meaning
4-AAP	4-Aminoantipyrine
4-OH TAM	4-hydroxy tamoxifen
ABCB1	ATP- binding cassette transporter family B member 1
AIs	Aromatase inhibitors
ARMS	Amplification refractory mutation system
ATM	Ataxia telangiectasia mutated gene
BAX	Bcl-2 associated X apoptasis regulator gene
BC	Breast cancer
Bcl-2	b-cell lymphoma 2 apoptasis regulator gene
BMI	Body Mass Index
bp	base pair
BRCA1	Breast cancer 1 gene
BRCA2	Breast cancer 2 gene
CA 15-3	Cancer antigen 15-3
CE	Cholesterol esterase
CHOD	Cholesterol oxidase
CLIA	Chemiluminescent immunoassay
CT	Computed tomography
CYP p450	Cytochrome p 450
CYP2D6	Cytochrome P450 Family 2 Subfamily D Member6
CYP3A4	Cytochrome P450 Family 3 Subfamily A Member4
CYP3A5	Cytochrome P450 Family 3 Subfamily A Member5
DCIs	Ductal carcinoma in situ
DNA	Deoxyribonucleic acid
E1	Estrone
E2	Estradiol
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMR	Eastern mediterian region
ER	Estrogen receptor
FSH	Follicle stimulating hormone
G1	Gap 1 phase
HDL	High density lipoprotein
HER2	Human epidermal growth factor receptor 2
HR	Hormone receptor
IDC	Invasive ductal carcinoma
IF	Inner forward
ILC	Invasive lobular carcinoma
IR	Inner reverse

LDL	Low density lipoprotein
LH	Leutinizing hormone
LHRH a	Leutinizing hormone releasing hormone agonist
MAPK	Mitogen activated protein kinase
MUC-1	Mucin-1
NDM TAM	N-desmethyl tamoxifen
OATP1B1	Organic anion transporting polypeptides family member 1B1
OF	Outer forward
OR	Outer reverse
P53	Tumor suppressor gene
PAPS	3′-Phospho adenosine-5′-phospho sulfate
PARP Is	Poly adenosine diphosphate ribose polymerase inhibitors
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
Pmol/µl	Picomole per microliter
POD	peroxidase
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog gene
raf	rapidly accelerated fibrosarcoma
RLUs	Relative light units
rpm	round per minute
rs	reference sequence
SERDs	Selective estrogen receptor degraders
SERMs	Selective estrogen receptor modulators
SNPs	Single nucleotide polymorphisms
SSRIs	Selective serotonin reuptake inhibitors
SULT1A1	Sulfotransferase family1 subfamilyA member 1
TAM	Tamoxifen
TBE	Tris/Borate/EDTA
UGT	Uridine diphosphate glucuronosyl transferase
W1 buffer	Washing buffer
α-OH TAM	Alpha-hydroxy tamoxifen

Abstract

Background Breast cancer is the most frequent malignancy diagnosed in females around the world.

Tamoxifen is the hormonal drug used as selective estrogen receptor modulator in the treatment of hormone receptor positive breast cancer in premenopausal women with early or metastatic stage. Being a prodrug, tamoxifen undergo metabolism by phase I and phase II enzymes into primary and secondary metabolites that show its therapeutic effect. Genetic polymorphism of sulfotransferase *SULT1A1*, one of the phase II drug metabolizing enzymes may affect clinical response to the drug.

Aims of study: The aim of the present study was to identify the genetic polymorphism of *SULT1A1* gene (rs 6839 and rs 9282861) in the participated breast cancer women, and to investigate the impact of *SULT1A1* genetic polymorphism on tamoxifen efficacy.

Patients and methods This is an observational cross-sectional study conducted at Imam Al-Hussein Medical City/Oncology Center in Kerbala. One hundred female patients with breast cancer that was estrogen-receptor and / or progesterone-receptor positive were selected. Patients aged 45-65 years, being on tamoxifen 20mg daily dose for at least 3 months had enrolled in this study. Venous blood samples were drawn from each female after they are signed written informed consent to measure biochemical parameters such as estradiol, CA15-3, vitamin D, calcium, and lipid profile in addition to genetic analysis. The Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS PCR) was used for the detection of *SULT1A1* gene rs 6839 and rs 9282861.

Results: The results of this study revealed that there were various genetic variants of *SULT1A1* gene included rs 6839 (902T>C) and rs 9282861(638C>T). In case of rs 6839, the homozygous wild (TT) is the predominant genotype compared to homozygous mutant (CC) and the heterozygous mutant (TC) genotypes with frequency of 54, 29 and 17 respectively. However, for rs 9282861, the homozygous mutant (TT) is the predominant genotype in comparison to the heterozygous mutant (CT) and homozygous wild (CC) genotypes with frequency of 41, 21 and 38 respectively. The levels of estradiol, tumor marker CA15-3, calcium, vitamin D3, cholesterol, triglycerides, HDL and LDL in the serum showed non-significant association between the studied SNPs of *SULT1A1* gene. Moreover, the current study demonstrated that there is high occurrence of joint pain over 76% with low recurrence rate about 7%.

Conclusion

The current investigation concluded that genetic variation in phase II metabolizing enzyme *SULT1A1* may be contributed to variability in response to tamoxifen therapy in a sample of breast cancer Iraqi women beside variation in the incidence of adverse drug reactions.

Chapter one

Introduction

1. Breast cancer

1.1 Overview

Breast cancer is the most common type of cancer worldwide (1). It causes 25% of all malignancies and 15% of cancer related deaths despite the fact that the mortality rate is decreased in high income countries (2). In Iraq, according to the Iraqi Cancer Registry (ICR), breast cancer is the female malignancy, accounts for 1/3 of all recorded female cancer patients (3). For many years, the role of estrogen in breast carcinogenesis has been recognized. Furthermore, estrogen stimulation, which is mediated by estrogen receptor (ER) is considered the key role in human breast cancer growth and progression (4).

1.2 Epidemiology

Breast cancer is a serious disease that spreads and causes a significant physical, emotional, and financial injury on affected individuals, their families, as well as the community and health systems. In the United States, data collected in 2022 estimated a higher incidence of breast cancer 287,850 new cases (compromising 31% of all cancer types) compared to lung cancer 118,830 case (13%), despite the fact that lung cancer mortality is 21% (of all cancer deaths) overcoming breast cancer mortality which is 15% (5).

Breast cancer incidence trends differ by country, owing to ethnic, cultural and geographic variation (6). Breast cancer rates are rising in middle eastern developing countries such as Egypt, Lebanon, Syria, Jordan, Iran, Iraq, and Saudi Arabia. Despite the fact that the incidence of breast cancer in the middle east is lower than in the western countries, where it ranges from 22 and 71/100,000 women. Women aged 40-49 years accounted for approximately one-third of all breast cancer diagnoses in eastern mediterranean region (EMR). Breast cancer incidence peaks in Asia among

premenopausal women in their 40s, whereas it peaks in western countries among postmenopausal women in their 60s (7). While, western countries having higher incidence of breast cancer with constant and decreasing mortality rates (8).

Several studies have found that Iraqi women are more likely than their western counterparts to be diagnosed with breast cancer at a younger age, in advanced stages and with aggressive behavior. The latest annual report of Iraqi Cancer Registry (ICR) in 2019, illustrated that breast cancer increased in the last two decades and became the first of the top ten malignant neoplasms affecting the community (19.7%), as shown in figure (1-1), followed by bronchus and lungs, while cancer related deaths are due to bronchus and lungs (16%), breast (11.3%), and leukemia (8.6%) (9).

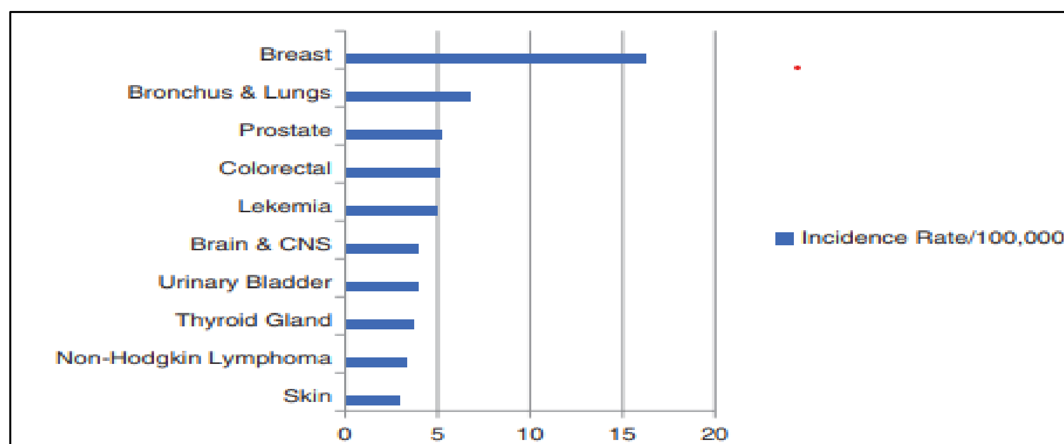


Figure (1-1): Top 10 cancers in Iraq by incidence per 100,000 people in 2019 (9)

1.3 Pathophysiology of breast cancer

Normal breast development is controlled by specific genes and signaling pathways that control cell division, death, differentiation, and motility. Genes dysregulation engaged in multiple signaling pathways leads to the formation of first tumors and metastasis. Metastatic cascade is a multi-step process that includes invasion of the local tumor cell, entry into the vasculature, exit of malignant cells from the circulation, and finally

colonization at distant organs (10). The loss in apoptotic control causes cancer cells to survive longer, gives more time for mutations to accumulate, stimulates angiogenesis, increases invasiveness of tumor, desregulates cell proliferation, and differentiation (11).

Breast carcinogenicity shows itself as cancer of cells in the canals and lobules of the breast, known as invasive ductal carcinoma and invasive lobular carcinoma. There are two divisions of breast tumors: benign and malignant. The most frequent type of benign forms (fibroadenoma) is caused by abnormal growth of cells in the breast canals, resulting in an unusual bump and they do not appear to be spreading to other body parts, which is usually removed with surgery. While in malignant tumors (IDC, ILC) when left untreated, cancer cells can migrate beyond the breast like lymph nodes bone, brain, lung and liver. It is also possible to cure breast cancer if found early enough. If cancer reaches to other parts of the body, it is usually not curable, although it may usually be controlled successfully for a long period (12).

The progress of breast cancer is induced by mutations in the BRCA1 or BRCA2, which are tumor suppressor genes. Cells lacking BRCA1 function are unable to arrest in G2 phase of cell cycle after DNA damage. Deficiency of BRCA2 is responsible for chromosome segregation error, and after several divisions, chromosome abnormalities develop (13).

Human epidermal growth factor receptor-2 (HER2) overexpression is predisposed to poorly differentiated, high-grade tumors, increased the rates of cell proliferation and lymph node involvement, and a relative resistance to certain types of chemotherapy and endocrine therapy. This resulted in a poor prognosis due to increased invasion and metastasis, as well as increased angiogenic activity (14).

1.4 Risk factors for breast cancer

1.4.1 Gender

Breast cancer incidence in females has been extensively documented around the world, in comparison to lower male breast cancer incidence (15).

1.4.2 Age factor

It has been known that the risk of developing breast cancer is highly related to increase with age in general (16). In 2016, women over the ages of 40 and 60 years accounted for 99.3 % and 71.2 % of all breast cancer-related deaths in the United States (17).

1.4.3 Genetic mutations

Gene mutations in BRCA1 and BRCA2 account for 5-10% of breast cancer cases, other genes are less common (p53, PTEN, ATM) which put a person at risk for developing hereditary breast cancer or other cancers (18).

The two well-known antioncogenes (BRCA1 and BRCA2) are found on the long arm of the human chromosome 17 and 13 (19). Patients with positive gene expression have a 90% chance of developing cancer in premenopausal age and should be encouraged to undergo screening and follow up (20).

1.4.4 Endogenous hormone exposure

1.4.4.1 Early menarche and late menopause

The reproductive years of a women can be measured using her age at menarche and her age at menopause. The ovary produces steroid hormones that regulate the growth and function of the breast throughout out this time.

The onset of menarche at a young age and the onset of menopause at a later age have both been linked to an increased risk of breast cancer (21).

1.4.4.2 Age at first birth

It has been proved that women who have their first child before the age of 18 have nearly 1/3 the breast cancer risk as women who have their first child after the age of 35. The findings explain the previously discovered reverse link between overall parity and breast cancer occurance, as women who have their first child earlier likely to have a higher parity later in life (18). Although recent reports indicate that more women in western cultures are remaining delaying childbearing until after 35 years of age may be contributing to the rise in breast cancer incidence (22).

Breast cancer has been associated with delayed childbirth which is due to socioeconomic progress and efficient contraception. Nulliparity and postponed childbirth have been linked to a higher incidence of estrogen receptor positive tumors, but not ER-negative tumors (23).

1.4.4.3 Breast feeding

Breast feeding appears to have a protective effect against the development of breast cancer. Breast feeding may cause a delay in the return of regular ovulatory cycles as well as a decrease in endogenous sex hormone levels. It has been predicted that for every year of breast feeding, there is a 4.3 percent reduction (24).

1.4.5 Exogenous hormone exposure

In postmenopausal women with an intact uterus, combined estrogen plus progestin in hormone replacement therapy, markedly elevated breast cancer risk, delayed breast cancer diagnosis and increased mortality rates. In contrast, postmenopausal women without uterus who used estrogen

alone had a statistically significant lower chance of developing breast cancer and did not prevent breast cancer from being detected (25). Also, the use of oral contraceptives at younger age and long duration increased the risk of breast cancer especially if they are carriers of BRCA1/2 mutations (26).

1.4.6 Life style factors

1.4.6.1 Dietary factors

Diet such as red meat, have limited evidence on their linkage with breast cancer, although it is a possible cause of breast cancer (27). A high dietary fat intake especially the saturated fat may increase the risk of breast cancer in postmenopausal women and associated with mortality and poor prognosis (28).

1.4.6.2 Tobacco smoking

Mutagens from tobacco smoke have been detected in the breast fluid of non-lactating women, despite the fact that the association between smoking and an increased risk of breast cancer is still debated. Until now, evidence has indicated that smoking, at a young age, increases the risk (29).

1.4.6.3 Alcohol consumption

Alcohol consumption can raise estrogen-related hormone levels in the blood and activate estrogen receptor pathways. A meta-analysis of epidemiological studies found that drinking 35-44 grams of alcohol per day raises the risk of breast cancer by 32%, with a 10-grams increase in alcohol consumption per day increased risk by 5% among premenopausal women and 9% among postmenopausal women (30).

1.4.6.4 Obesity

In postmenopausal women, excessive body fat and aromatization in adipose cells, converts androgens from the adrenal glands to estrogen. These higher

levels of circulating estrogen stimulate more estrogen-sensitive breast tissues, predisposed to hyperstimulation and tumor formation. In premenopausal women, obesity has a lower impact as estrogen produced mainly in the ovaries and lower estrogen levels due to significant uptake of estradiol into fat and the higher rate of estrogen clearance by the liver (31).

Hyperinsulinemia and insulin resistance were investigated as risk factors for obesity. Insulin has anabolic effects on cellular metabolism, and insulin receptor overexpression in human cancer cells has been demonstrated (32).

1.4.6.5 Physical activity

It has been shown to reduce the risk of breast cancer in a dose-dependent manner, with modest activity causing a 2% risk reduction and intense activity conferring a 5% risk reduction (33).

1.5 Types of breast cancer

1.5.1 Non-invasive breast cancer

It is malignant tumor that has not spread beyond the ducts or lobules where it is located (34). It is also called in situ, means “in place”. While these abnormal cells not spread outside the lobules or ducts, they can transform into invasive breast cancer (35).

This type of malignancy includes ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS). DCIS occur when atypical cells develop within inner lining of the milk ducts but do not spread to nearby tissues (36). LCIS begins to grow in the breast lobules and has not spread into other breast tissues outside the lobules (37).

1.5.2 Invasive breast cancer

It is also called metastatic breast cancer, when abnormal cells from the milk ducts or lobules spread further into area within breast tissue or extend to other body parts via the lymphatic system or systemic circulation and reach locoregional lymph node, bones, lungs, liver, and brain (38). This type includes invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC).

IDC accounting for 80% of all BC diagnosis (39). It include: Mucinous carcinoma, when unusually large amount of mucous, or mucin, makes up part of the tumor (40). Tubular carcinoma, usually small and consist of tube-shaped structures and has an excellent prognosis (41).

ILC comprises about 10-15% of BC histological types. It is made up of small, uniform, non-cohesive cancer cells that invade the stroma in a single-file pattern (42).

1.5.3 Inflammatory breast cancer

It is rare and very aggressive illness, in which lymph vessels in the skin of the breast blocked by cancer cells. The breast often looks swollen, inflamed and red with (peau d'orange), an orange-peel skin appearance (43).

1.5.4 Paget's disease of the breast

It is rare condition, it appears as erythema, desquamation, and ulceration localized to the nipple with pain or itching (44).

1.6 Molecular subtypes of breast cancer

Four molecular subtypes of invasive BCs are considered based on gene expression profiling by the cancer cells. In clinical practice biomarker assays: ER, PR, HER2 and Ki-67 (proliferation marker reflects rapidly dividing tumor cells) frequently used (45)(46)(47).

Table (1-1): Molecular classification of breast cancer (48)

Molecular Subtypes	Receptor expression	Histological grade/prognosis	Response to therapies
Luminal A 40-50%	ER+ PR+(≥20%) HER2 -	Low grade GI, favorable prognosis, low Ki-67 levels (0-13%)	Respond to endocrine therapy tamoxifen and AIs
Luminal B 20-30%	ER + PR+(<20%) HER2 +/-	Intermediate grade GII, grow faster, worse prognosis than luminal A, intermediate Ki-67 levels (14-19%)	Less response to endocrine therapy, response to chemotherapy, targeted therapy
HER2-enriched 15-20%	ER - PR - HER2 +	High grade GIII, poor prognosis (early locoregional recurrence/ metastasis, poor survival rate), high Ki-67 levels (≥20%)	Respond to trastuzumab (Herceptin), anthracycline based chemotherapy
Triple-negative or basal-like 10-20%	ER- PR- HER2 -	High grade GIII, poor prognosis, high Ki-67 levels, more aggressive than luminal A or B, more frequent in younger patients	No response to endocrine therapy or trastuzumab, sensitive to platinum-based chemotherapy and PARP inhibitors

1.7 The TNM staging of breast cancer (tumor-node and metastasis)

The TNM system can help the physician to determine the outcome of the disease (prognosis) and select an appropriate management plan or evaluate and compared treatment results between different treatment centers (49).

Table (1-2): TNM staging of breast cancer (48)

T=Tumor Size	**T0**	No primary tumor
	Tis	Tumor only in breast ducts or lobules
	T1	≤ 2 cm
	T2	>2- ≤5 cm
	T3	> 5cm
	T4	Tumor of any size with extention to chest wall/skin
N=Lymph nodes	**N0**	No lymph node metastasis
	N1	Metastasis in 1-3 axillary lymph node
	N2	Metastasis in 4-9 axillary lymph node
	N3	Metastasis in ≥10 axillary lymph node, or infra-or supraclavicular lymph nodes
M=Metastasis	**M0**	No evidence of cancer metastasis
	M1	Cancer found in other area of the body

1.8 Stages of breast cancer:

Breast cancer stages determined by the size and type of tumor, as well as how deeply the tumor cells have penetrated the breast tissues (50).

Table (1-3): Breast cancer stage (34)

Stage		**Tumor characteristics**
0 Non-invasive		The tumor begins to grow <2cm, e.g., DCIS
I Invasive	**IA**	Tumor <2cm, lymph node -
	IB	No tumor in the breast or tumor <2 cm, cancer cells of 0.2-2 mm are found in the lymph nodes
II Invasive (Regional)	**IIA**	Tumor <2cm, lymph node +, Tumor 2-5cm, axillary lymph nodes -
	IIB	Tumor >5cm, axillary lymph nodes - , Tumor 2-5cm, axillary lymph nodes +
III Locally advanced	**IIIA**	Tumor >5cm, axillary lymph nodes +, Tumor of any size, axillary lymph nodes +
	IIIB	Tumor of any size, metastasis to skin, chest wall or internal lymph nodes of the mammary gland
	IIIC	Tumor of any size, wide metastasis and more lymph node involved
IV Advanced/Metastatic stage		Tumor spread to other parts of the body (bones, lungs, liver, brain) beyond the breast region

1.9. Estrogen and ER- mediated mammary carcinogenesis

Estrogens are steroid hormones that consists of estrone (E1), 17β-estradiol (E2), and estriol. The most powerful circulating ovarian steroid, 17β-estradiol, is involved in breast development at puberty and sexual maturity. It also contributes to the initiation and progression of target tissue cancers in breast, endometrial, prostate, ovary, lung and colon (51).

Estrogens are synthesized primarily in the ovaries, also in the adipose tissue and adrenal glands. In premenopause, estradiol synthesized in the ovaries, while in postmenopause, estrone is predominantly synthesized in peripheral tissues. Aromatase (encoded by the CYP19A1gene), is the rate-limiting enzyme, catalyzing the conversion of androgens to estrogens (52). Estrone or E1, is produced by aromatization of androstenedione in extra glandular tissues, can be reversibly transformed to estradiol by the enzyme 17β-hydroxysteroid dehydrogenase in peripheral tissues (53).

The women who exposed to estrogen for longer duration, whether through early onset of menstruation, delayed first childbirth, nulliparity, short duration of breast feeding, late menopause, or estrogen replacement medication, have greater risk of developing breast cancer. In the mammary gland, estrogen acts locally increasing synthesis of DNA and stimulating bud development (54).

Two explanations have been proposed to elucidate increased risk: (1) Receptor dependent: binding of 17β-estradiol to its intracellular receptor ERα, resulting in enhanced cell proliferation and mutations, suppression of apoptosis with uncontrollable cell division or growth (2) Receptor independent: metabolism of estradiol to genotoxic metabolites with a resulting increase in DNA mutations (55).

Metabolism of estradiol causing DNA damage by two ways: (a) generation of estradiol-adenine -guanine adducts which are released from the DNA backbone leaving depurinated sites which cause error predisposed to DNA repair with resultant point mutations (b) generation of oxygen free radicals resulting from redox cycling of 4-OH estradiol to the 3-4 estradiol quinone which may also be reduced to semiquinones by cytochrome P450 reductase, this process can produce ROS that cause oxidative DNA damage (56).

1.10 The role of HER2/Neu antigen

This antigene belongs to the human epidermal growth factor receptor family, located on long arm of chromosome 17 (17q21-q22) (57)(53). HER2 overexpressed in 20-30 % of BC that are highly aggressive in nature. It has physiological as well as carcinogenesis and metastasis effect (58).

HER2 having tyrosine kinase activity, thus heterodimerization leads to autophosphorylation of cytoplasmic domain and cell proliferation. Also, it has regulatory role in raf/Ras/MAPK and PI3K/AKT pathways and disruption of these pathways by gene amplification lead to uncontrolled cell growth, division and function (59). Trastuzumab, monoclonal antibody drug inactivate HER2, improving prognosis and help patients live longer (60).

1.11 Diagnosis of breast cancer

1.11.1 Breast Self-Examination (BSE)

Most oncologists instruct females to perform self-examination of the breast to recognize any abnormalities in breast size and shape (61).

1.11.2 Digital mammography

In this system X- ray films replaced with electronics that convert these rays into mammographic photographs of the breast. This usually used in breast cancer screening in healthy women at the age of 40 year before signs and symptoms appear. In females with dense breast tissues, mammography may produce false negative or positive results (62). The implementation of mammography in many countries as the gold standard screening test for breast cancer in early stages increase chance of healing (63).

1.11.3 Breast Ultrasound Imaging

This is rapid and being a standard way to diagnose palpable breast mass (but also, nipple inversion, localised skin retraction bloody nipple discharge) in young women with thick breast where mammography sensitivity is lower. It determines the site and size of the tumor, whether it is solid or filled with fluid, and whether it is biopsied to rule out cancer. BUI complements mammography and clinical examination in all ages (64).

1.11.4 Magnatic Resonance Imaging (MRI)

Is the precise technique to detect breast cancer ≤ 5 mm. It may be used with mammography or ultrasound as a screening tool for detecting breast cancer in high-risk women with strong family history or hereditary breast cancer gene changes (BRCA1, BRCA2) (65). It also used for preoperative evaluation (measure the size and extent of the tumor) in addition to assessment of response to neoadjuvant chemotherapy (66).

1.11.5 Positron EmissionTomography/ComputedTomography (PET/CT)

Important details affecting various body organs can be easily detected by PET and CT. It is highly precise to predict different parts of locoregional lymph node extent and distant metastasis which are not visible by standard imaging. This procedure is used in the management plan to identify the spread of primary tumor. It is also used for restaging after disease relapse and treatment follow up (67).

1.11.6 Tumor markers

Tumor markers are active substances that can show the presence and progression of a tumor cells. They can be monitored at all stages of breast cancer, and assist in prediction of metastasis, treatment, diagnosis and screening (68). Elevated blood levels of CA15-3 are seen in <10 % of patients in the early stages of disease and in ~70% of patients in the advanced stages of breast cancer. The CAl5-3 usually fall after efficient management and may also be elevated in other cancer types and not-cancerous diseases like hepatitis and benign tumors of the breast (69)(70).

1.11.7 Immunohistochemistry

Immunohistochemistry by using Eosin and Hematoxylin staining has provided the valuable information for the optical treatment and aggressive behavior of the BCs (71). Breast cancer tissues are examined for ER/PR, as well as HER2 antigen, in order to identify the disease (72). ER- and/ or PR-positive (hormone sensitive BCs) were undergo treatment with anti-hormone therapy, while HER2-positive breast cancers treated with anti-HER2 drugs (73).

1.11.8 Breast biopsy

Breast biopsy is the best way for breast cancer diagnoses. Breast imaging, clinical examination of the breast and biopsy can all performed for better diagnosis precision and to rule out false positive results as possible (triple test) (74). Axillary-US by using fine needle aspiration (FNA) and /or core needle biopsy (CNB) used to detect axillary lymph nodes (ALNs) metastasis (75).

1.12 Treatments

The goal of breast cancer treatment is to prolong survival, delaying disease progression and enhancing quality of life. Treatment options vary depending on tumor characteristics and clinical stage at diagnosis (76).

Breast cancer is a very traumatic experience for many females around the world. Psychological survey give an image of the emotional and community influences of breast cancer on the lives of females with their impact on better or worse outcome. Most women exposed to breast loss experience asexuality, loss of self-confidence and depression (77) (78).

1.12.1 Surgery

This is the primary management strategy. The amount of tissue removed varies depending on the characteristic of the cancer, whether it has spread, and the patient’s special feelings (79). Lumpectomy (breast conserving surgery), is done by removing the infected part of the breast that contain the malignant tumor with some healthy tissues and surrounding lymph nodes while leaving the intact breast as possible (80).

This procedure is typically performed in women who are in early stage of cancer, although they require additional treatments such as radiotherapy, chemotherapy, or hormonal therapy (81).

Radical mastectomy (complete breast removal) is assumed to be performed in case of advance breast cancer. Reconstructive surgery, involve the use of a prosthetic implant to restore body representation, femininity and sexuality, and positively influence the patient's feelings (82).

1.12.2 Chemotherapy

These cytotoxic drugs causing cell death by apoptosis, either by directly interfering with DNA, or they target key proteins required for cell division. It has a greater effect on rapidly dividing cells, such as cancer cells, than on normal healthy cells (that are able to repopulate) (83). However, rapidly dividing cells such as hair follicles, nails, mouth, digestive tract and bone marrow negatively affected by chemotherapy, side effects such as hair loss, brittle nails, mouth sores, nausea, vomiting, and myelosuppression are usually subside once the treatment is completed (84).

Depending on the patient's condition, neoadjuvant chemotherapy used before surgery to shrink the tumors that are too large to be removed with less extensive surgery, or have many lymph nodes involved with cancer, or are inflammatory breast cancers (85). Adjuvant Chemotherapy regimen such as cyclophosphamide and doxorubicine followed by paclitaxel used following surgery to eliminate any cancer cells that may have been left behind or that have spread to other parts of the body that are so small to be visible on imaging tests and lowers the likelihood of recurrence (86)(87).

1.12.3 Radiation therapy

This option of cancer treatment uses beams of high-energy rays (usually X-rays) to destroy cancer cells or slows their growth by damaging their DNA (88). In case of early breast cancer stages, it can be used before surgery to shrink tumor size and reduce the need for mastectomy so, the lumpectomy and radiation therapy combination is increasingly being used (81). It can be used during surgery (intraoperative radiation) and after surgery to kill any cancer cells remaining in the chest area (89).

1.12.4 Endocrine therapy

The estrogen receptor positive breast cancer account for nearly 3/4 of all breast cancers diagnosed. Hormonal therapy stops the effect of estrogen on breast cancer cells (90). Furthermore, endocrine therapies can be used in sequence to prolong survival and delay the need for chemotherapeutic regimen (91).

Treating breast cancer with endocrine intervention occur by: 1) Direct targeting of ER itself via blocking estrogen's action by selective estrogen receptor modulators (SERMs), e.g., tamoxifen, 2) Lowering estrogen levels, by gonadal suppression via oophorectomy or GnRH agonist in premenopausal women, or by aromatase inhibitors (AIs) in postmenopausal women, 3) Downregulating ER expression by selective estrogen receptor degraders (SERDs) that bind to and breakdown ERs (92).

1.12.4.1 Ovarian function suppression drugs (GnRHa)

There are several strategies for ovarian ablation in premenopausal women with BC, including SERM (tamoxifen), radiotherapy, cytotoxic chemotherapy, surgical removal of the ovaries (oophorectomy), and extended use of luteinizing hormone releasing hormone (LHRH) analogues (goserelin) (93). LHRH is released from the hypothalamus and act on G

protein coupled receptors type1 (GnRHR1) in the pituitary in order to increase secretion of FSH and LH, which in turn stimulate the ovaries to release estrogen (94). LHRH agonist mimic the effect of LHRH, initially produce a surge of ovarian hormones but later reduce production as it downregulate and desensitize LHRH receptors in pituitary gonadotropic cells. This results in reduction of circulating estrogens and slowing the growth of hormone sensitive tumors (95).

1.12.4.2 Aromatase inhibitors (AIs)

These substances are intended to reduce estrogen production by targeting aromatase, this enzyme complex responsible for the final step in the formation of estrogen. Third generation aromatase inhibitors such as letrozole, exemestane and anastrazole are currently used (96). A clinical study found that women treated with aromatase inhibitors decrease the risk of contralateral breast cancer than women treated with tamoxifen (91).

1.12.4.3 Selective estrogen receptor degraders (SERDs)

Fulvestrant is regarded as estrogen antagonist, it shows antineoplastic actions in breast tissues without positive effect on the uterus and bones, this may lead to osteoporosis if used for long duration (97). Fulvestrant reduce the number of ER. These are usually beneficial in postmenopausal women, and those with tamoxifen resistant breast cancer cells (98).

1.12.4.4 Antiestrogen therapy

The most commonly used class of anti-estrogen drugs in breast cancer. They consist of agents called (SERMs) such as (tamoxifen, toremifene and raloxifene) (99).

1.12.4.4.A Tamoxifen

The chemical name of tamoxifen is (Z)2-[4-(1,2-diphenyl-1-butenyl) phenoxy]-N, N-dimethyl ethanamine 2-hydroxy-1,2,3- propane tricarboxylate. Tamoxifen is a nonsteroidal-triphenylethylene analogue, characterized as selective estrogen receptor modulator, that operates as an antagonist in the breast but as an agonist in the uterus and bone, reducing ER function by competing with estrogen for receptor binding (100).

Tamoxifen is the most widely prescribed chemopreventive treatment for high-risk women with ER positive breast cancer. Tamoxifen is recommended as standard adjuvant therapy by the American Society of Clinical Oncology, after surgery usually for 5 years, prevented breast cancer recurrence and contralateral breast cancer by 40-50% in adjuvant breast cancer trials. It is also used to treat metastatic breast cancer (101).

Tamoxifen therapy up to 10 years is more beneficial in lowering mortality and cancer recurrence in high-risk patients (102). The mixed agonist/antagonist actions of tamoxifen are linked not only to its effect in the prevention and treatment of BC and its positive effects on bone mineral density maintenance, but also to some negative side effects, such as an elevated risk of endometrial cancer and thromboembolic events (103)(104).

The quantitative ER and PR analysis may help to predict whether adjuvant tamoxifen therapy is likely to be beneficial to select or reject individuals for hormonal treatment. Cancers with high quantitative ER amount or a positive PR show the best response (105)(106).

De novo or acquired resistance develops in a significant proportion of individuals with localized illness and patients with advanced disease who initially respond to tamoxifen (107).

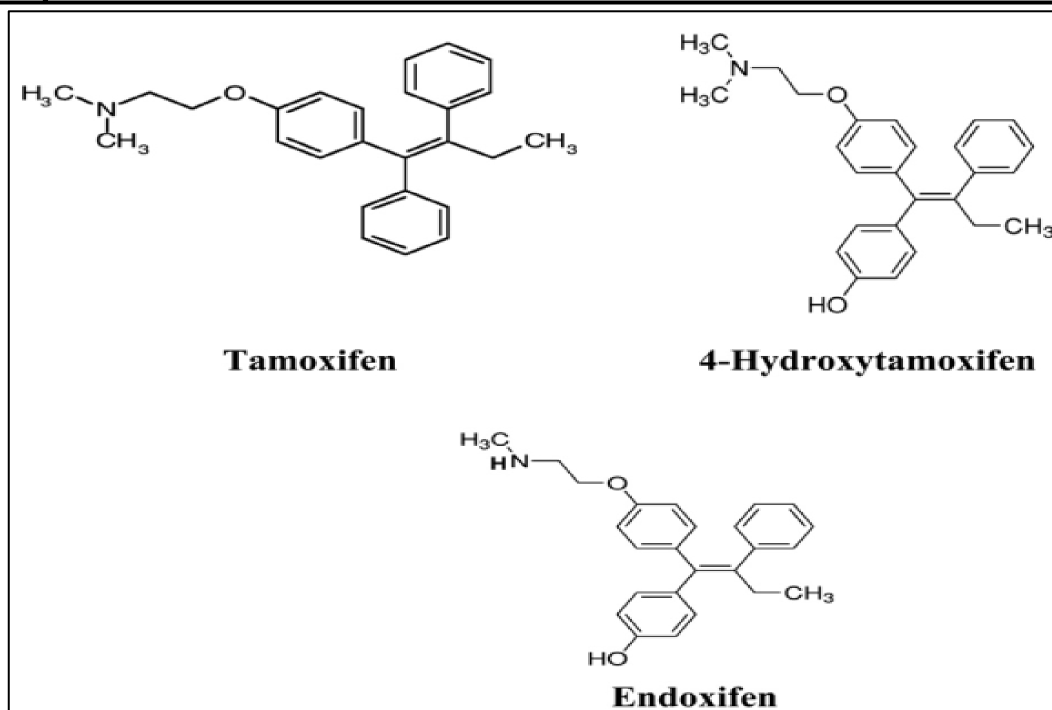


Figure (1-2): Chemical structures of tamoxifen and their active metabolites, 4-hydroxytamoxifen and endoxifen (108)

1.12.4.4.B Mechanism of action of tamoxifen

Estradiol binds to ER in a target cell, this initiates a sequence of events. The estrogen–ER complex homodimerizes and binds to discrete DNA sequences in the regulatory regions of estrogen-sensitive genes known as estrogen response elements (ERE). The two transcriptional activation functions of the estrogen–ER complex, AF1 and AF2, interact with other proteins (transcriptional coactivators) to stimulate the RNA polymerase II activity (RNA Pol II), and thus regulating gene activity (103).

Tamoxifen inhibits binding of estrogen to the ER in a competitive manner. Tamoxifen–ER complex also homodimerizes and binds to the estrogen-responsive element of estrogen-sensitive genes. Moreover, only AFl is still active and inactivation of AF2 reduces transcriptional activity of the estrogen-responsive gene as well as coactivator binding. In a consequence, tamoxifen prevents Gl phase of the cell cycle and limiting cell growth (109). It stimulates the production of the inhibitory growth factor

'transforming growth factor β', which may act on breast carcinoma cells in a negative paracrine manner. Tamoxifen reduces the production of insulin-like growth factor (IGF-1) (133), which acts as a potent mitogen for breast cancer (134).

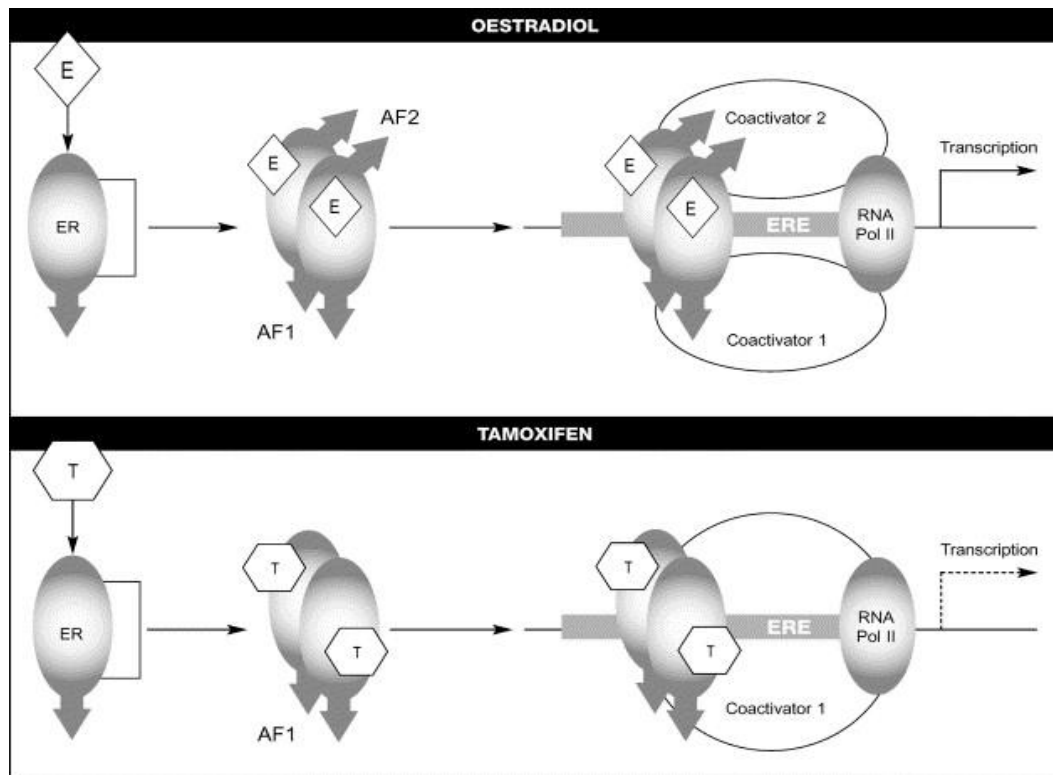


Figure (1-3): Mechanism of action of tamoxifen (109)
*E: Estrogen, ER: Estrogen Receptor, T: Tamoxifen

1.12.4.4.C Tamoxifen pharmacokinetics

Tamoxifen is easily absorbed following oral administration, with peak plasma concentrations observed in 4-7 hours about 40 µg/L and steady-state levels attained in 3-4 weeks of treatment. Tamoxifen is more than 99% plasma protein-bound, mainly to albumin (110). After a single oral dosage of 20 mg tamoxifen, concentration in breast tissue and lymph nodes were high, exceed serum ratio. The volume of distribution is 50-60 L/kg and only trace amount detected in the cerebrospinal fluid (111). The drug elimination is biphasic, with an initial half-life of 7 hours and terminal half-lives of around 7-11 days. Tamoxifen is highly processed in the liver, and the primary route of elimination is biliary excretion. The amount excreted

unchanged in urine is minimal <1% (112). Tamoxifen extensively metabolized in the liver, primarily by the large family of cytochrome P450 (CYP) enzymes CYP2D6, CYP2B6, CYP2C9, CYP2C19 and CYP3A4/5, into several primary and secondary metabolites that exhibit more antiestrogenic action in breast cancer cells than tamoxifen itself. Phase II drug metabolizing enzymes, including sulfotransferases (SULTs) and UDP-glucuronosyltransferases (UGTs), proceed the metabolic conversions to even more hydrophilic forms, facilitating excretion (113), figure (1-4).

Tamoxifen is mainly metabolized into its primary metabolites, N-desmethyl-tamoxifen (NDM-tamoxifen) and 4-OH-tamoxifen by CYP3A4/5 and CYP2D6 enzyme respectively. Thereafter, a second transformation from its primary metabolites, into the active metabolite endoxifen (4-OH-NDM-tamoxifen) occurs (114). N-demethylation and 4-hydroxylation are the two major tamoxifen metabolic routes, accounting for approximately 92% and 7% of tamoxifen biotransformation respectively (115).

Both 4-OHTAM and endoxifen have much 100-fold higher ER affinity and are ~30-100-fold higher antiestrogen activity than tamoxifen. Although endoxifen has an antiestrogenic effect similar to 4-OHTAM, its higher plasma concentration 5-10 times, regarded it the main active metabolite (116). Endoxifen not only inhibits ER but also targets ERα (coded for by the ESR1 gene) for proteasomal degradation (117)(118). Norendoxifen, is the final known active metabolite of tamoxifen formed by demethylation of endoxifen with dual aromatase and ER inhibitory properties (119). Several minor metabolic routes were defined: Flavin monooxygenases FMO1 and FMO3 metabolized tamoxifen to tamoxifen-N-oxide (TNO), which can be reduced back to tamoxifen in vitro by CYP3A4 (113). Moreover, 4´-hydroxytamoxifen, mediated by CYP2B6 and CYP2D6

and α-hydroxytamoxifen, produced mainly by CYP3A4. Whereas, NDM-tamoxifen is also converted to N,N-didesmethyltamoxifen, demethylated by CYP3A4, 4′-hydroxy-N-desmethyltamoxifen, and α-hydroxy-N-desmethyltamoxifen (120). The 4-OHTAM sulfation pathway by the major SULT isoforms SULT1A1, 2A1 and human estrogen sulfotransferase SULT1E1 leads to detoxification of TAM (121). While, only the SULT1A1 enzyme is responsible for the inactivation of endoxifen into endoxifen sulfate (115). Tamoxifen and 4-OHTAM are glucuronidated by UGT1A4 to the corresponding N-glucuronides. Furthermore, UGT1A8, UGT1A10, UGT2B7, UGT2B15 and UGT1A4 are involved in the O-glucuronidation of 4-OHTAM and endoxifen (122)(123).

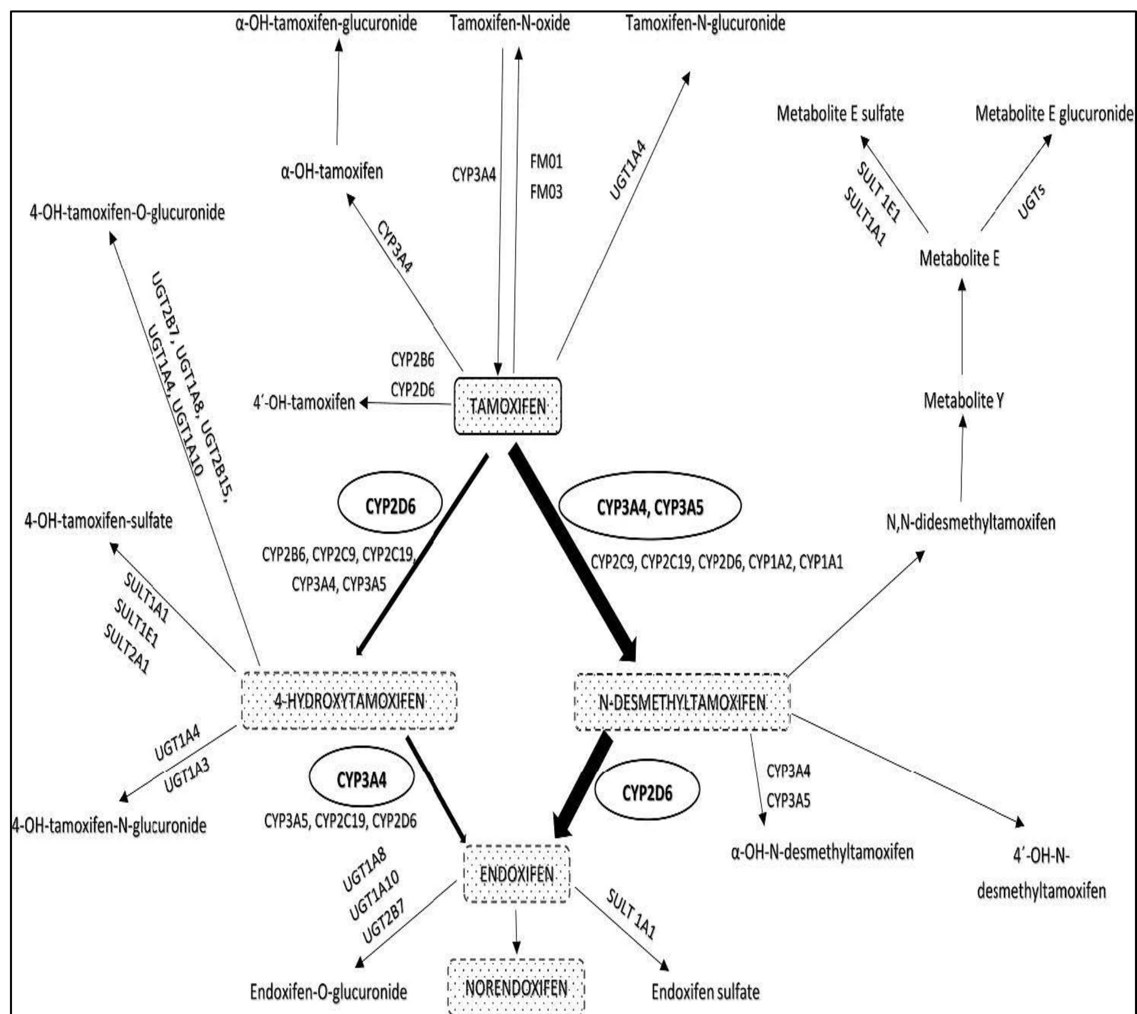


Figure (1-4): Metabolic conversion of tamoxifen (112)

*Major metabolic pathways are highlighted with bold arrows

1.12.4.4.D Tamoxifen pharmacodynamics

Tamoxifen binding antagonizes estrogen binding and produces a nuclear complex that decreases DNA synthesis and inhibits estrogen action in breast cancer cells. Thus, estrogen cannot bind to the cancer cell, and this cell can not receive estrogen's signals to grow and multiply. Numerous genetic variations can cause differences in the response of the organism to the drug (110).

1.12.4.4.E Medical uses of tamoxifen

A- Breast cancer: Tamoxifen is prescribed for the treatment of ER positive early, locally advanced and metastatic BCs in pre- and postmenopausal women, ductal carcinoma in situ, and as primary chemoprevention in high-risk women. It can be used as neo- or adjuvant therapy, with lymph node negative/positive (124).

B- Infertility: Tamoxifen used for ovulation induction in 50%-90% of infertile women with anovulatory disorders, and that 30%–50% of such women become pregnant (125). It is effective in women after failure of clomiphene therapy. Tamoxifen does not cause ovary hyperstimulation or multiple pregnancies. These findings may be linked to a higher cervical mucus score and improved corpus luteum function (126).

C- Gynecomastia: The off-label tamoxifen treatment, is an option for male with breast cancer, infertility, and idiopathic gynecomastia. Recently, it also suggested to prevent gynecomastia in prostate cancer patients who receiving anti-androgen therapy (127).

1.12.4.4 F. Side effects of tamoxifen

A- Endometrial cancer: Tamoxifen raises the risk of endometrial cancer by 3-fold, however not all women are similarly at risk (128). It is thought to be caused by estrogenic stimulation of the endometrium, or by the genotoxic

impact of tamoxifen metabolites such as α-OHT that catalyzes the irreversible binding of tamoxifen to DNA (129)(130).

The presence of CYP3A4 in the human endometrium and that regional metabolism of tamoxifen to α-OHT possibly attributes to its activity. Sulfonation of α-OHT in phase II metabolic pathway, produce a sulfate ester that is much more reactive than α-OHT itself (131).

B- The most frequent side effects of tamoxifen are hot flashes, nausea and fatigue. Joint pain, weight gain, insomnia, mood swings, uterine bleeding, vaginal dryness or discharge and loss of libido are less frequent (132). Deep vein thrombosis, venous thromboembolism, pulmonary embolism, cardiovascular- and ischemic cerebrovascular events are rare (133).

1.12.4.4.G Drug interactions

Tamoxifen is metabolized mainly in the liver by CYP2D6 and CYP3A4/5 to active metabolites such as endoxifen (134). The concurrent use of CYP2D6 inhibitors by selective serotonin reuptake inhibitors (SSRIs) such as paroxetine, fluoxetine (potent CYP2D6 inhibitors), while escitalopram, cimetidine, amiodarone, ticlopidine and haloperidol (moderate/weak inhibitors) would substantially reduce endoxifen plasma concentrations (135)(136). In addition, CYP3A4 inhibition result in decreased TAM efficacy (134). Rifampin reduced tamoxifen bioavailability (due to induced efflux transporter, ABCB1), or increased tamoxifen metabolism via induction of UGTs (phase II metabolism via glucuronide conjugation of endoxifen itself) (137).

1.12.4.4.H Pharmacogenomics of tamoxifen

Pharmacogenomics is an important player in the health field because it predicts possible drug responses and is useful in avoiding side effects and optimizing administered doses. After standard dosing, there is significant interindividual variation in steady-state levels of the two most potent tamoxifen metabolites, 4-hydroxy-tamoxifen and endoxifen. The majority of these differences could be attributed to CYP2D6 genetic polymorphisms (101).

Currently, there are over 100 CYP2D6 allelic variants with varying metabolic activities. The predicted phenotype allowing metabolizers to be classified into four groups: poor, intermediate, extensive, and ultrarapid metabolizers. Patients carrying CYP2D6 alleles with high-predicted enzymatic activity have high serum levels of 4OHTAM and endoxifen. Lower endoxifen and N-desmethyl-tamoxifen concentrations are expected to be associated with the CYP3A4*22, CYP3A5*3 and CYP3A5*6 polymorphisms, which indicates reduced or no metabolic activity (138).

The metabolism of tamoxifen was altered by CYP2C19*2 CYP2C19*17 variant alleles that have either no or increased metabolic activity (139). The enzymes responsible for the elimination and inactivation of tamoxifen and its metabolites via conjugation with either a sulfate or a glucuronide may also be subject to significant genetic variation. Patients with adjuvant tamoxifen-treated breast cancer who had SULT1A1*2/*2 and either UGT2B15*1/*2 or UGT2B15*2/*2 had a significantly lower 5 year survival (140).

Previous studies found that genetic polymorphism of OATP1B1(infflux transporter), resulted in the decreased intake of tamoxifen and endoxifen into cells, which may represent a potential therapeutic strategy for treatment of breast cancer (141). The effectiveness

of breast cancer treatment, including tamoxifen, is also influenced by genetic variants of ABCB1(efflux transporter), which will be crucial in the future for developing personalised anticancer therapies (142).

1.12.4.4.I Effect of genetic polymorphisms of *SULT1A1* gene on tamoxifen

There are 13 members of human cytosolic sulfotransferase *SULT* gene family. The most intensively studied *SULT* genes are *SULT1A* subfamily. *SULT1A1* is in close proximity to the three other members of the *SULT1A* subfamily: *SULT1A2*, *SULT1A3*, and *SULT1A4* that located on the short arm of chromosome 16 (16p12.1-p11.2) and shared more than 93% amino acid sequence identity (143).

Two broad classes of sulfotransferases have been identified: 1) Membrane-bound SULTs are localized in the Golgi apparatus and are responsible for the sulfonation of (large molecules) peptides, proteins, lipids and glycosaminoglycans, and 2) the soluble cytosolic SULTs catalyze sulfonation of xenobiotics (detoxication, carcinogen bioactivation) and small endobiotic molecules such as steroids, bile acids and neurotransmitters (144). Sulfotransferases exhibit wide tissue distribution. The members of *SULT1A* subfamily have been identified in liver, brain, breast, intestine, jejunum, lung, adrenal gland, endometrium, placenta, kidney and blood platelets. SULT1A1 exhibits the highest level of expression of all SULT1 enzymes in the liver (145).

The thermostable phenol sulfotransferase SULT1A1, has been shown to be the primary enzyme responsible for the sulfation of tamoxifen. The variaty in sulfonation capacity may be important in determining an individual's response to xenobiotics, and recent studies have begun to suggest roles for *SULT* polymorphism in disease susceptibility (146).

Since many of the tamoxifen-related enzymes are polymorphic, genetic variances in tamoxifen-related outcomes may contribute for interindividual or interethnic variation. The majority of patients receiving adjuvant tamoxifen may relapse, indicating that the benefit is not equal. Tamoxifen and its metabolites are degraded by phase II drug-metabolizing enzymes. Following normal dosage, their interpatient diversity may thus lead to changes in steady-state levels and drug-related active chemical elimination (107).

In tamoxifen metabolism, SULT1A1 is primarily convert 4-OHTAM into inactive 4-OHTAM sulfate and endaxifen into inactive endoxifen sulfate. Furthermore, SULT1A1 is implicated in the inactivation of NDM-tamoxifen into metabolite e sulfate after several sequential reaction (147).

Recently, rs6839 (902T>C), a *SULT1A1* SNP, was discovered in the 3′-untranslated region of the *SULT1A1* gene. This SNP is linked to lower *SULT1A1* enzymatic activity. Carriers of two variants in *SULT1A1* (rs6839) have been reported to have 10–15% higher levels of endoxifen and 4-OHTAM. However, this SNP had a minor influence on the concentrations and metabolic ratios of tamoxifen and its active metabolite, as well as recurrence free survival in women taking adjuvant tamoxifen (148).

A genetic polymorphism in exon 7 of the *SULT1A1* gene rs9282861 (638C>T) causes an amino acid shift from arginine to histidine at the conserved position 213 of the protein (Arg213His) (149). The rs 9282861 was merged to rs1042028 on October 12, 2018.

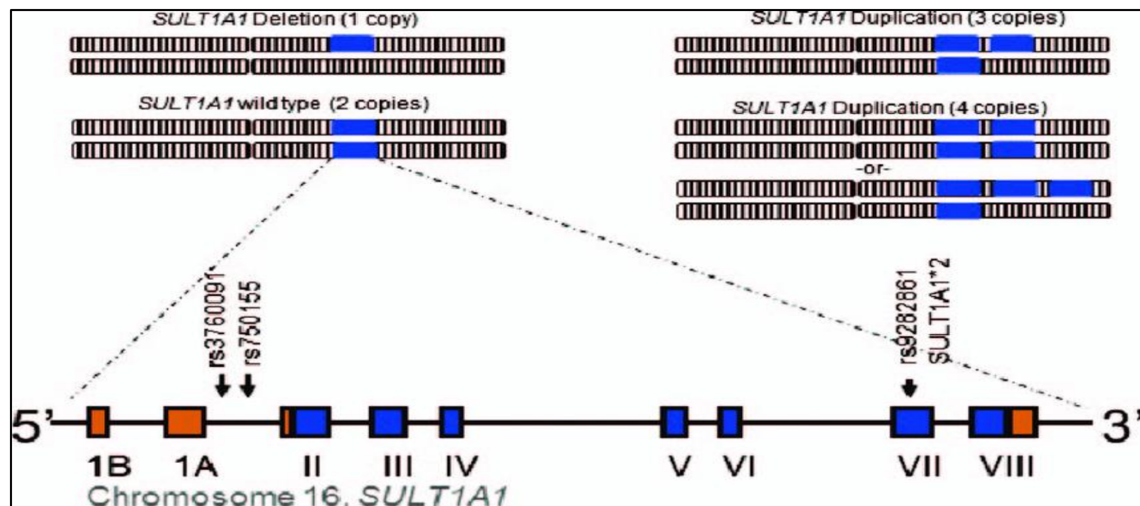


Figure (1-5): SULT1A1 locus with both a copy number variation and multiple single nucleotide polymorphisms, with rs 9282861 SNP depicted by the blue box VII (150)

The variant T allele produces an enzyme with twice reduced catalytic activity and poorer thermostability than the wild C allele. Patients who are homozygous for *SULT1A1* rs9282861 mutation have a higher chance of survival. T allele supports the concept that the decreased enzymatic activity associated with the homozygous TT variant genotype may result in slower clearance of 4-OHTAM, thereby extending its duration of action (151).

1.13 Aims of the study

1. To identify the *SULT1A1* gene polymorphisms (rs 6839 and rs 9282861) in the Iraqi breast cancer women participated in this study.

2. To investigate the impat of genetic polymorphisms of *SULT1A1* gene (rs 6839 and rs 9282861) on tamoxifen effectiveness and incidence of adverse effects in women with breast cancer.

3. To determine wheather any associations exist between gene polymorphisms of *SULT1A1* (rs 6839 and rs 9282861) with serum levels of CA15-3, estradiol, vitamin D, calcium and lipid profile in patients on tamoxifen.

Chapter two

Patients, Materials and Methods

2. Patients, Materials and Methods

2.1 Patients

This is a cross-sectional observational study conducted at Imam Al-Hussein Medical City / Oncology Center in Kerbala during the period from the start of November 2021 till the end of Augest 2022. One hundred female patients aged 45-65 years, being on tamoxifen 20mg daily dose for at least 3 months had enrolled in this study. Patients had attended oncology center for follow-up after they have already been diagnosed with breast cancer. The Scientific and Ethical Committee of College of Pharmacy / University of Kerbala gave its approval to the study′s protocol, and after describing the nature and goal of the study, each patient signed an informed consent form.

2.1.1 Inclusion Criteria

Breast cancer female with hormone receptor positive estrogen receptor and/or progesterone receptor, aged 45years and above being on tamoxifen 20mg/ day for at least 3 months.

2.1.2 Exclusion Criteria

Patients had started taking tamoxifen at the same time as adjuvant chemotherapy or adjuvant radiotherapy (or both). Females who are hypertensive or diabetic and taken cocurrent medications like antihypertensive, antidiabetics, lipid-lowering agents and drugs that inhibit CYP2D6 activity like fluoxetine, were not included. Furthermore, Pregnant and lactating women and females with other malignant diseases or thromboembolic events such as deep vein thrombosis and pulmonary embolism were also excluded from the study.

2.1.3 Clinical data collection

Some clinical information was collected from patients themselves while they were receiving treatment, such as age, weight, height, academic achievement, workplace, marital status, family history, menopausal status and breast feeding. Another information taken from patient's medical records in the center which include date of diagnosis, site and type of breast cancer, stage and grade, dose, duration and time of tamoxifen. Immunohistochemical status (ER, PR, HER2), surgery, chemotherapy, radiation, presence of joint pain or other side effects, recurrence and any other drugs used are also recoded.

2.1.4 Blood sample collection

After an overnight fast, five milliliters of venous blood were drawn from each female. Three milliliters of blood were placed in a gel tube and allowed to clot for 15 minutes before being centrifuged at 3000 rpm for 10 minutes to separate the serum that was used to measure biochemical parameters such as estradiol, CA15-3, vitamin D, calcium, and lipid profile. The remaining two milliliters of blood were kept in an EDTA tube for the genetic assay. Precautions have been taken in clinical settings to prevent Covid 19 infection.

2.2 Materials

2.2.1 Chemicals, Kits, and their Suppliers

The specific kits and chemicals and their manufactures origins are listed in table (2-1).

Table (2-1) Chemicals, kits, and their producing companies

Chemicals and Kits	Manufacture	Origin
Agarose powder	Bio Basic	Canada
CA15-3 kit	Mindray	China
Calcium kit	Mindray	China
DNA Extraction Kit from blood favor prep	Favorgen	Taiwan
DNA ladder marker (100 bp)	Bioneer	Korea
Estradiol kit	Snibe Diagnostic	China
Ethanol	SDI	Iraq
Ethidium Bromide	Intron	Korea
Lipid profile kit (triglyceride, cholesterol, HDL and LDL)	Mindray	China
Nuclease free water	Bioneer	Korea
PCR green master mix Kit	Promega	USA
Primers for detection of SULT1A1gene: rs 6839 T>C and rs 9282861 C>T	Macrogen	Korea
TBE buffer	Bioneer	Korea
Vitamin D kit	Snibe Diagnostic	China

2.2.2 Instruments and their suppliers

The instruments and the manufacturing companies utilized in genetic and biochemical analysis are listed in table (2-2).

Table (2-2) Instruments and the manufacturing companies

Instruments	Manufacture	Origin
Centrifuge	Hettich	Germany
Digital camera	Canon	England
Distillator	GFL	Germany
Gel Electrophoresis apparatus	Cleaver	UK
Hood	LabTech	Korea
Hot plate stirrer	LabTech	Korea
Incubator	Binder	Germany
MAGLUMI 800 Fully-Auto Chemiluminescence Immunoassay Analyzer	Snibe Diagnostic	China

Micropipettes	SLAMMED	Japan
Mindray BS -series (BS-240 pro) analyzer	Mindray	China
Mindray CL-series (CL-900i) Chemiluminescence Immunoassay Analyzer	Mindray	China
PCR machine (Thermocycler)	Veriti	USA
Refrigerator/ freezer (-20 ˚C)	Concord	Lebanon
Sensitive balance	AND	Taiwan
UV- Trans illuminator	Syngene	England
Vortex mixer	HumanTwist	Germany
Water bath	LabTech	Korea

2.3 Methods

2.3.1 Biochemical and Hormonal Assay Methods

2.3.1.1 Estimation of serum estradiol

This is in vitro quantitative determination of estradiol in human serum using the MAGLUMI Fully-auto chemiluminescence immunoassay analyzer "CLIA"(152).

Principle

The estradiol assay is a competitive chemiluminescence immunoassay.

First, the sample is well mixed and incubated to create antigen-antibody complexes with monoclonal antibody labeled ABEI and magnetic microbeads coated with pure E2 antigen. The supernatant is decanted after precipitation in a magnetic field, and then a wash cycle is carried out. A chemiluminescence process is then started by adding the starter 1+2. A photomultiplier measures the light signal as a relative light unit (RLU), which is inversely proportional to the concentration of E2 contained in the sample.

2.3.1.2 Estimation of serum tumor marker CA15-3

The CL-series CA15-3 assay is a chemiluminescence immunoassay (CLIA) for the quantitative determination of CA15-3 in human serum (153).

Principle

The CL-series CA15-3 assay is a two-site sandwich assay to determine the level of cancer antigen CA15-3.

In the first step, sample, paramagnetic microparticle coated with monoclonal CA15-3 antibody (mouse) and monoclonal CA15-3 antibody (mouse)-alkaline phosphatase conjugate are added to the reaction vessel. After incubation, CA15-3 present in the sample binds to both anti-CA15-3 antibody coated microparticle and anti-CA15-3 antibody alkaline phosphatase-labeled conjugate to form a sandwich complex. Microparticle is magnetically captured while other unbound substances are removed by washing. In the second step, the substrate solution is added to the reaction vessel. It is catalyzed by anti-CA15-3 antibody (mouse)-alkaline phosphatase conjugate in the immunocomplex retained on the microparticle. The resulting chemiluminescent reaction is measured as a relative light units (RLUs) by a photomultiplier built into the system. The amount of CA15-3 present in the sample is proportional to the light units (RLUs) generated during the reaction.

2.3.1.3 Estimation of serum vitamin D

This is an in vitro chemiluminescence immunoassay for quantifying total 25-OH Vitamin D in human serum using the MAGLUMI series, Fully-auto chemiluminescence immunoassay analyzer (154).

Principle

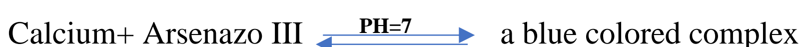
Competitive chemiluminescence immunoassay used to measure 25-OH vitamin D levels. The 25-OH Vitamin D is a two incubation chemiluminescence immunoassay. In the first incubation, the 25-OH vitamin D is dissociated from its binding protein by the displacing reagent, and binds to the 25-OH vitamin D antibody on the magnetic microbeads forming an antigen-antibody complexes. Following a second incubation, the 25- OH vitamin D labeled ABEI are added. The rest unbound material is removed during a wash cycle. Subsequently, the starter 1+2 are added to initiate a flash chemiluminescent reaction. The resulting chemiluminescent reaction is measured as a relative light units (RLUs), which is inversely proportional to the vitamin D content in the sample.

2.3.1.4 Estimation of serum calcium

This is a quantitative determination of serum calcium by using mindray BS series (BS-240Pro) analyzer (155).

Principle

The calcium assay is obtained by a photometric Arsenazo III method.



By utilizing 8-hydroxyquinolone-5-sulfonic acid to block the interference of magnesium, calcium ions combine with Arsenazo III to generate a blue-colored complex at a neutral solution. The amount of calcium present directly correlates with the rise in absorbance.

2.3.1.5 Estimation of serum lipid profile

2.3.1.5.A Estimation of total cholesterol

The total cholesterol in human serum was measured in vitro on BS-240Pro using the enzymatic colorimetric technique (156).

Principle

Cholesterol ester in the sample, under the existence of cholesterol esterase, hydrolyzed into cholesterol and free fatty acid. Total cholesterol oxidized by cholesterol oxidase enzyme to generate cholest -4-en-3-ketone and hydrogen peroxide. The generated hydrogen peroxide, under the existence of peroxidase, react with hydroxybenzoic acid and 4-aminoantipyrin (4-AAP) to produce H_2O and quinone imine pigments. The generated volume of quinone imine pigments is proportional to the TC volume in the sample, by measuring the generated pigment volume at specific wavelength, total cholesterol concentration can be calculated.

Cholesterol esters + H_2O $\xrightarrow{CE}$ Cholesterol + Free fatty acid

Cholesterol + O_2 $\xrightarrow{CHOD}$ Cholest-4-en-3-one + H_2O_2

2 H_2O_2 + 4-AAP + Para hydroxy benzoic acid $\xrightarrow{POD}$ Quinone-imine dye + 4 H_2O

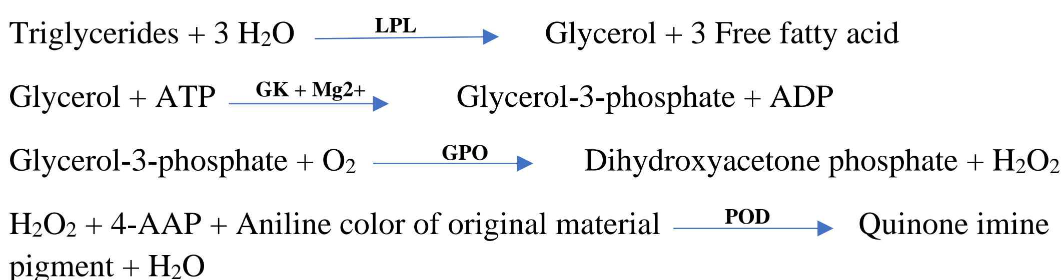
2.3.1.5.B Estimation of serum triglyceride

Triglycerides (TG) in serum were estimated in vitro using the BS240Pro system using the enzymatic quantitative colorimetric technique. (156).

Principle

Triglycerides in the sample hydrolyzed by lipoprotein lipase into glycerol and free fatty acid. The Glycerol is then phosphorelated under the existence of glycerol kinase and adenosine triphosphate (ATP) to glycerol-3-phosphate. Under the exixtance of glycerol phosphate oxidase (GPO), it react with O_2 to produce dihydroxyaceton phosphate and H_2O_2. Catalyzed by

peroxidase (POD), H_2O_2 react with aniline color of the original material and 4-amino-antipyrine to produce H_2O and quinone imine pigment, the volume of quinone imine pigment is proportional to triglyceride content in the sample. By measuring the generated pigment volume at specific wave length, the triglyceride concentration of the sample can be calculated.

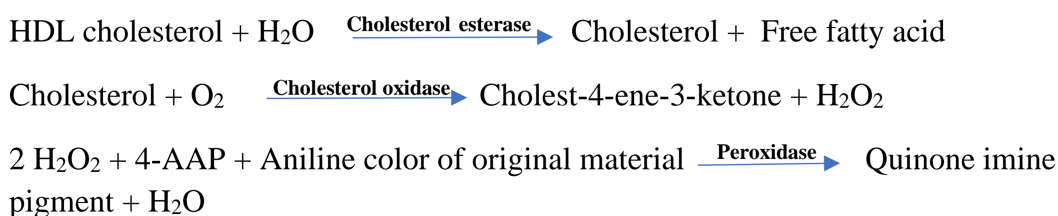


2.3.1.5.C Estimation of serum HDL

On the BS240Pro system, an in vitro quantitative enzymatic colorimetric test was used to estimate high-density lipoprotein (HDL) in serum (157).

Principle

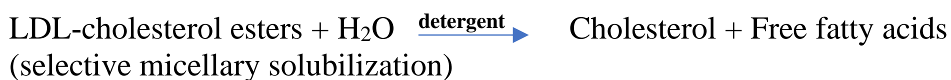
High density lipoprotein cholesterol in the sample, under the existance of surfactant in the reagent, is selectively hydrolyzed by cholesterol esterase into cholesterol and free fatty acid. The generated cholesterol oxidized by cholesterol oxidase to generate cholest -4-en-3-ketone and hydrogen peroxide. Under the exisistance of peroxidase, H_2O_2 react with aniline color of the original material and 4-amino-antipyrine to produce H_2O and quinone imine pigment, the volume of quinone imine pigment is proportional to HDL content in the sample. By measuring the final pigment volume at specific wave length, the HDL concentration of the sample can be calculated.



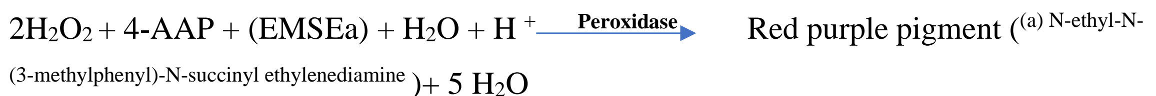
2.3.1.5.D Estimation of serum LDL

Cholesterol esters and free cholesterol in Low-Density Lipoprotein (LDL) are determined using a cholesterol enzymatic method that involves the use of cholesterol esterase and cholesterol oxidase in the presence of surfactants that selectively solubilize the only LDL. Surfactants and a sugar compound inhibit the enzyme reactions to lipoproteins other than LDL. Cholesterol levels in HDL, VLDL, and chylomicron are not recognized.

Cholesterol esterase breaks down cholesterol esters quantitatively into cholesterol and free fatty acids.



Cholesterol oxidase converts cholesterol to Δ4-cholestenone and hydrogen peroxide in the presence of oxygen.



In the presence of peroxidase, the hydrogen peroxide generated combines with 4-aminoantipyrine and EMSE to produce a red-purple color. Using a photometer, the color intensity of this dye is determined in relation to the level of cholesterol (158).

2.3.2 Genetic Analysis

2.3.2.1 Extraction of Genomic DNA from Blood Sample

Favor Prep Genomic DNA Mini kit from Favorgen was used to provide a fast and easy method for purification of total DNA from blood and various biological samples and to yield pure DNA suitable for storage and immediate application.

RBC Lysis

1. Pipet 300 µl of whole blood and added into 1.5 ml microcentrifuge tube.
2. 30µl of proteinase K enzyme was added to the microcentrifuge tube and then mix by pulse vortex.
3. Add 900 µl (3X) of RBC lysis buffer and mix by inversion.
4. The sample mixture incubated at room temperature for 10 min. Make sure that the sample mixture become deep-red and transparent after incubation.
5. Centrifuge at 3000x g for 5 min. and completely remove the supernatant.
6. Add 100µl of RBC lysis buffer to the pellet and resuspend the cells by pipetting.

Cell Lysis

7. 200µl of FABG buffer was added into the sample mixture and mixed well by vortexing.
8. For 10 minutes, the mixture was incubated at room temperature. The tube was turned over every 3 minutes during the incubation.
9. The elution buffer was incubated at 70 ºC water bath.

DNA Binding

10. 200µl of absolute ethanol was added to the sample and mixed well by the vortex for 10 sec.
11. The FABG column was placed to a collection tube. The mixture was applied carefully to the FABG column, close the cap, and centrifuged at 14,000 rpm for 1 min, then the filtrate was discarded and a new collection tube is replaced.

Column Washing

12. 400µl of W1 buffer was added to the FABG column and centrifuged for 30 sec at 14,000 rpm and discarded the filtrate and the FABG column placed back to the collection tube.
13. 600µl of Wash buffer was added to the FABG column and centrifuged for 30 sec at 14,000 rpm and discarded the filtrate then the FABG column placed back to the collection tube for additional centrifuging for 3min to dry the column.

Elution

14. The dry FABG column placed to a new 1.5 ml microcentrifuge tube and 100 µl of preheated Elution buffer or TE was added directly to the membrane center of FABG column
15. The FABG column was incubated for 10 min in an incubator then centrifuged for 1min at 14,000 rpm to elute the DNA. The DNA fragment stored at -20 ˚C

2.3.2.2 Primers design

The primers were created using Primer-BLAST software depending on https://WWW.ncbi.nlm.nih.gov/websites and bought as a lyophilized package from Macrogen, Korea. The primer sequences that were used for the SULT1A1 gene amplification study for SNPs detection are presented in tables (2–3) and (2-4).

Table (2-3): Primers sequences of SULT1A1 rs6839 T > C

Primers	Primers Sequence (5'- 3')	Primer size (bp)	Product size (bp)
I-F	GCACACTCCCTCTGCAGTGCCT	22	T allele: 189 C allele: 136
I-R	AGCTGTGAGAGGGGCTCCTTGG	22	
O-F	CAGCCTCCAAATTGCTGGGATTACA	25	Two outer primers: 283
O-R	GGATGAGACTCCAGCTTTGCTCCC	24	
I-F: Inner Forward, I-R: Inner Reverse, O-F: Outer Forward, O-R: Outer Reverse			

Table (2-4): Primers sequences of SULT1A1 rs9282861 C > T

Primers	Primers Sequence (5'- 3')	Primer size (bp)	Product size (bp)
I-F	GGTCTCCTCTGGCAGGGCGT	20	T allele: 191 C allele: 147
I-R	AAAAGATCCTGGAGTTTGTGGGTCG	25	
O-F	GGGAGATGCTGTGGTCCATGAAC	23	Two outer primers: 293
O-R	AGGAGTTGGCTCTGCAGGGTTTCT	24	
I-F: Inner Forward, I-R: Inner Reverse, O-F: Outer Forward, O-R: Outer Reverse			

Each lyophilized primer was first dissolved in a particular amount of nuclease-free water to create a stock solution with a concentration of 100 pmol/µl. Next, a diluted work solution was created by adding 90µl of nuclease-free water to 10µl of each stock solution. This work solution was stored at -20°C until use.

Table (2-5): The volumes of nuclease free water added to each primer to obtain 100 pmol/µl cocentration

Primers		Volume of nuclease free water added (µl)
rs6839 T > C	**rs9282861 C > T**	
I-F	I-F	250
I-R	I-R	250
O-F	O-F	250
O-R	O-R	250

2.3.2.3 Polymerase chain reaction (PCR)

In the current study, amplification refractory mutation system polymerase chain reaction (ARMS-PCR) technique was used for detecting rs6839 and rs9282861 SNPs of SULT1A1 gene.

2.3.2.3.A Optimization of PCR Conditions

Following a number of trials, the PCR reaction was optimized to determine the ideal annealing temperature and the ideal number of amplification cycles.

2.3.2.3.B Running the PCR

In a microcentrifuge tube, the PCR mixture was created by adding 12.5 µl of green master mix (Promega/USA), 0.5 µl of inner forward and reverse primers, 1.5 µl of outer forward and reverse primers, 4 µl of DNA and the volume was brought to 25 µl with 4.5 µl of nuclease free water. Amplified segments were separated by gel electrophoresis apparatus using agarose gel 1.5% and ethidium bromide stain and observed under ultraviolet (UV) trans-illuminator. Mixing the PCR component with DNA and employing the optimum PCR program are shown below in table (2-6), (2-7) and (2-8).

Table (2-6): PCR mixture for genotyping of SULT1A1 rs 6839 T > C and rs 9282861 C > T

Component	**Volume** (µl)
Inner Forward primer	0.5
Inner Reverse primer	0.5
Outer Forward primer	1.5
Outer Reverse primer	1.5
DNA template	4
Nuclease free water	4.5
Green Master mix	12.5
Total	25

Table (2-7): PCR condition for genotyping of rs6839 T > C

Steps	**Temperatures/ ºC**	**Minute/Second**	**Cycles**
Initial denaturation	94	05:00	1
Denaturation	94	00:35	
Annealing	62	00:30	35
Extension	72	01:00	
Final extension	72	07:00	1

Table (2-8): PCR condition for genotyping of rs9282861 C>T

Steps	Temperatures/ºC	Minute/Second	Cycles
Initial denaturation	94	05:00	1
Denaturation	94	00:35	35
Annealing	60	00:30	
Extension	72	01:00	
Final extension	72	07:00	1

2.3.2.4 Agarose Gel Electrophoresis

1. To make Agarose gel, 1.5g of agarose powder was dissolved in 100ml of 1x TBE buffer (Tris-Borate-EDTA), which is prepared by taken 10ml of 10x TBE buffer and 90ml of distilled water.
2. On a hot plate, boil the solution for a few minutes.
3. After allowing the solution to cool, 4 µl of ethidium bromide was added.
4. The comb was fixed to the end of the tray to create wells for the loading of PCR products.
5. After curefully pouring the agarose gel into the tray, it was left at room temperature for 20 minutes to harden.
6. The comb was gently taken out of the tray.
7. The tray was fixed inside an electrophoresis chamber and filled with a TBE buffer.
8. One of the wells loaded with 5 µl of DNA ladder and the other wells were loaded with 4 µl of each PCR products.
9. The voltage of the electrophoresis equipment was tuned to produce an electrical field of 5 v.cm-1 at a distance of 10 cm between the cathode and anode.
10. The UV trans-illuminator operating at 320-336 nm was employed for band detection at the end of the run, which lasted around 90 minutes.
11. A photo of the gel was taken using a digital camera.

2.4 Statistical analysis

The data of participants in this study were analysed by using the statistical package for social sciences (SPSS) version 28, IBM, US. Scale variables presented in mean ± standard deviation (Mean±SD). Student's t-test for two independent samples was used to compare means between groups. One way ANOVA test was used to compare more than two means. The correlation between categorical variables was assessed using the Chi-Square statistic. When the Chi-Square is not appropriate, Fisher's Exact test is performed instead. P value <0.05 is regarded as a significant difference.

Chapter three

Results

3.1 Demographic characteristic of patients:

The studied sample included 100 female patients with breast cancer. The mean age was 51year ± 4.85 (range 45-65) (table 3-1). The majority of participants were married (93%). The women with known family history of BC were (44%) in corresponding to (56%) had no family history.

Over half of women (55%) have right side breast cancer compared to (45%) who had cancer in left side. Lymph node was involved in (64%)of patient and absent in the others. Patients have both estrogen receptor ER positive/ progesterone receptor PR positive was (98%) and only (2%) were ER positive /PR negetive, with higher percentage of HER2 negetive (67%) compared with HER2 positive (33%).

The percentage of patients who undergo previous surgery, radiotherapy and chemotherapy were (94%), (79%) and (91%) respectively. A high proportion of participants (76%) reported joint pain with hot flashes (13%) and (2%) endometrial hyperplasia. Patients who had recurrence and those who did not (7%) and (93%), respectively.

Table (3-1): Description of demographic and disease characteristics of the studied patients (n=100)

<table>
<tr><th colspan="3">Variables</th><th>Values</th></tr>
<tr><td colspan="3">Age (Years) M±SD</td><td>51.08 ± 4.85</td></tr>
<tr><td colspan="3">BMI (Kg/m^2) M±SD</td><td>28.30 ± 5.57</td></tr>
<tr><td colspan="3">Duration of tamoxifen (Years) M±SD</td><td>3.41 ± 2.36</td></tr>
<tr><td colspan="3">Duration of disease (Years) M±SD</td><td>4.18 ± 2.50</td></tr>
<tr><td rowspan="2">Marital status %</td><td colspan="2">Married</td><td>93</td></tr>
<tr><td colspan="2">Single</td><td>7</td></tr>
<tr><td rowspan="2">Family history%</td><td colspan="2">Yes</td><td>44</td></tr>
<tr><td colspan="2">No</td><td>56</td></tr>
<tr><td rowspan="2">Breast cancer Side %</td><td colspan="2">Left breast</td><td>45</td></tr>
<tr><td colspan="2">Right breast</td><td>55</td></tr>
<tr><td rowspan="2">Lymph node involvement %</td><td colspan="2">Yes</td><td>64</td></tr>
<tr><td colspan="2">No</td><td>36</td></tr>
<tr><td rowspan="2">Surgery %</td><td colspan="2">Yes</td><td>94</td></tr>
<tr><td colspan="2">No</td><td>6</td></tr>
<tr><td rowspan="2">Chemotherapy %</td><td colspan="2">Yes</td><td>91</td></tr>
<tr><td colspan="2">No</td><td>9</td></tr>
<tr><td rowspan="2">Radiotherapy %</td><td colspan="2">Yes</td><td>79</td></tr>
<tr><td colspan="2">No</td><td>21</td></tr>
<tr><td rowspan="4">Immunohistochemical tests %</td><td rowspan="2">HER2</td><td>Negative</td><td>67</td></tr>
<tr><td>Positive</td><td>33</td></tr>
<tr><td colspan="2">Positive for both ER/PR</td><td>98</td></tr>
<tr><td colspan="2">ER positive /PR negative</td><td>2</td></tr>
<tr><td rowspan="4">Side effects %</td><td colspan="2">Hot flashes</td><td>13</td></tr>
<tr><td colspan="2">Joint pain</td><td>76</td></tr>
<tr><td colspan="2">Both (Hot flashes & Joint pain)</td><td>9</td></tr>
<tr><td colspan="2">Endometrial hyperplasia</td><td>2</td></tr>
<tr><td rowspan="2">Recurrence %</td><td colspan="2">Yes</td><td>7</td></tr>
<tr><td colspan="2">No</td><td>93</td></tr>
<tr><td colspan="4">Results presented by mean ± standard deviation and percentage</td></tr>
</table>

The mean and standard deviation of laboratory parameters of participants are listed in table (3-2).

Table (3-2): Description of mean levels of laboratory biomarkers of the studied patients (n=100)

Biomarkers	Mean±SD	Reference range
Estradiol	33.14 ± 11.25	10-66 pg/mL
CA15-3	17.99 ± 6.29	Up to 35 ng/mL
Vitamin D	11.81 ± 5.69	30-100 ng/mL
Calcium	8.87 ± 2.58	8.1-10.4 mg/dL
Cholesterol	176.80 ± 31.61	50-200 mg/dL
Triglyceride	166.53 ± 61.99	35-150 mg/dL
HDL	46.03 ± 11.35	35-65 mg/dL
LDL	94.98 ± 28.82	0-140 mg/dL

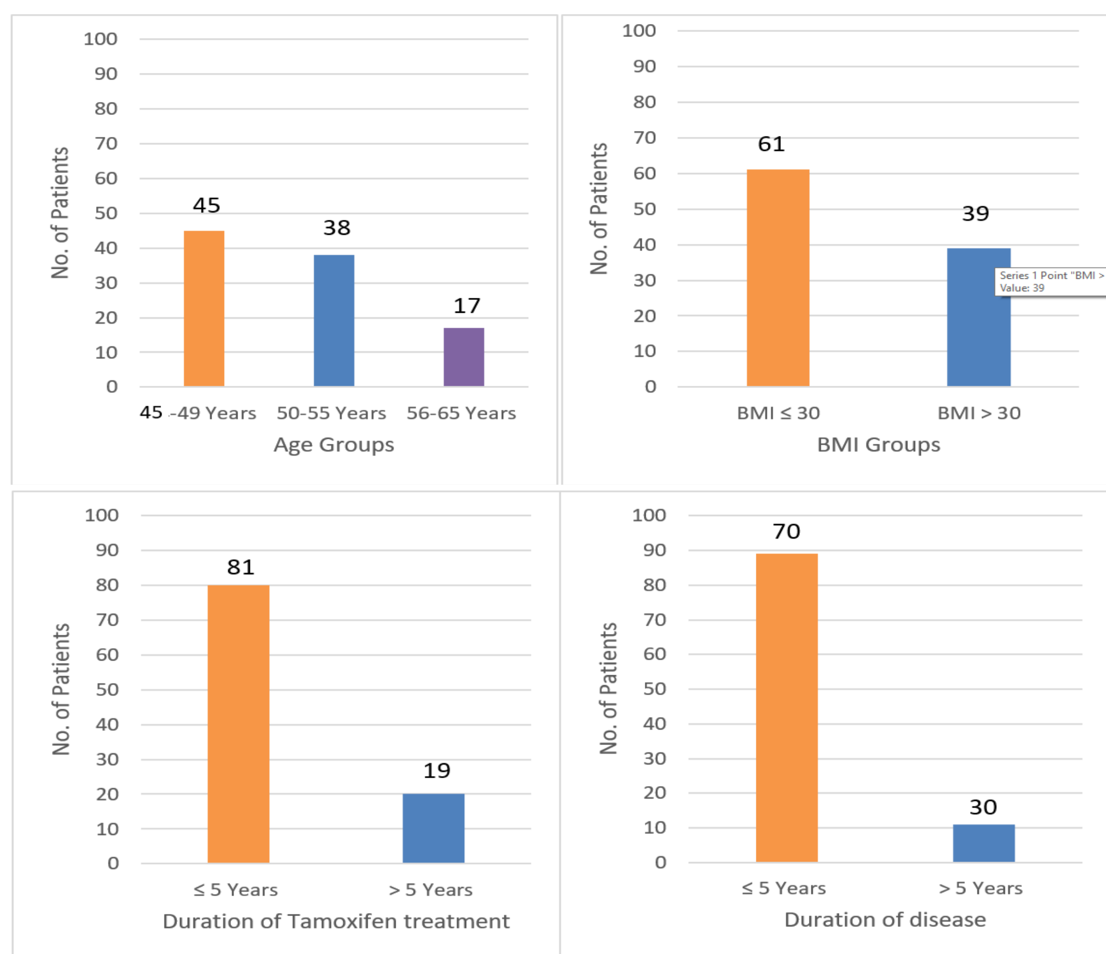


Figure (3-1): Demographic data of studied patients, age, BMI, duration of tamoxifen treatment and disease

To show the association between laboratory biomarkers and age categories of studied patients (table 3-3), ANOVA test was conducted to compare mean serum level of estradiol, CA15-3, vitamin D3, calcium, cholesterol, TG, HDL, LDL among three age groups, which ranged from 44-49 year, 50-55 year and 56-65 year respectively. There were statistically high significant differences (P= 0.001) for estradiol, CA15-3, vitamin D3, calcium, and significant differences (P= 0.039) for LDL.

Table (3-3): Comparison of laboratory biomarkers with age categories of studied patients

Biomarkers	Age Category			P value
	45-49 years n= (45)	**50-55 years** n= (38)	**56-65 years** n= (17)	
Estradiol	34.53 ± 10.51	31.36 ± 11.94	33.48 ± 11.73	0.001**
CA15-3	18.96 ± 8.34	17.99 ± 6.29	17.38 ± 5.57	0.001**
Vit. D3	11.15 ± 5.44	12.06 ± 5.94	13.00 ± 5.92	0.001**
Calcium	8.55 ± 1.14	8.75 ± 1.18	10.00 ± 5.73	0.001**
Cholesterol	176.64 ± 29.55	179.82 ± 30.02	163.18 ± 22.93	0.095
TG	162.05 ± 51.83	149.50 ± 60.91	148.17± 53.82	0.133
HDL	44.94 ± 11.41	47.54 ± 11.78	45.52 ± 10.47	0.615
LDL	97.54 ± 28.75	98.59 ± 30.35	80.15 ± 21.35	0.039*

Results shown as mean ± standard deviation, P value < 0.001 considered high significant**
P value < 0.05 considered significant*

To show the association between laboratory biomarkers and BMI categories of studied patients (table 3-4), Student's t-test was conducted to compare mean serum level of estradiol, CA15-3, vitamin D3, calcium, cholesterol, TG, HDL, LDL between two groups, non-obese and obese. There was statistically high significant difference (p= 0.002) for vitamin D3.

Table (3-4): Comparison of laboratory biomarkers with BMI categories of studied patients

Biomarkers	BMI category		P value
	(Non-obese) n= (61)	(Obese) n= (39)	
Estradiol	32.86 ± 11.22	33.59 ± 11.44	0.520
CA15-3	17.49 ± 6.25	19.62 ± 8.28	0.110
Vit. D3	10.76 ± 5.32	13.45 ± 5.94	0.002**
Calcium	8.55 ± 1.06	9.37 ± 3.89	0.410
Cholesterol	176.07 ± 43.08	177.95 ± 27.66	0.532
TG	167.76 ± 82.49	164.59 ± 64.49	0.989
HDL	45.84 ± 11.66	46.31 ± 10.98	0.740
LDL	94.83 ± 30.30	95.21 ± 26.73	0.969

Results shown as mean ± standard deviation, (Non-obese) BMI≤ 30 Kg/m2, (Obese) > 30 Kg/m2
P < 0.002 considered high significant**

To compare between laboratory biomarkers and duration of tamoxifen therpy of studied patients (table 3-5), Student's t-test was conducted to compare mean serum level of estradiol, CA15-3, vitamin D3, calcium, cholesterol, TG, HDL, LDL between two groups, below and above 5year. There was no statistically significant difference ($p > 0.05$).

Table (3-5): Comparison of laboratory biomarkers with duration of tamoxifen treatment groups

Biomarkers	Duration of tamoxifen treatment groups		P value
	≤5 Years n= (81)	>5 Years n= (19)	
Estradiol	33.60 ± 11.44	31.18 ± 10.46	0.642
CA15-3	18.51 ± 7.39	17.51 ± 6.11	0.651
Vit. D3	11.96 ± 5.69	11.16 ± 5.83	0.480
Calcium	8.96 ± 2.82	8.51 ± 1.06	0.363
Cholesterol	177.04 ± 33.23	175.79 ± 24.12	0.829
TG	165.48 ± 74.16	170.94 ± 83.70	0.799
HDL	46.01 ± 11.01	46.05 ± 13.03	0.819
LDL	95.50 ± 28.37	92.76 ± 31.39	0.906
Results shown as mean ± standard deviation, P value > 0.05 considered non significant			

To compare between laboratory biomarkers and duration of disease of studied patients (table 3-6), Student's t-test was conducted to compare mean serum level of estradiol, CA15-3, vitamin D3, calcium, cholesterol, TG, HDL, LDL between two groups, below and above 5year. There was no statistically significant difference ($p > 0.05$).

Table (3-6): Comparison of laboratory biomarkers with duration of disease groups of studied patients

Biomarkers	Disease duration		P value
	≤5 Years n= (70)	>5 Years n= (30)	
Estradiol	33.70 ± 11.46	31.84 ± 10.85	0.580
CA15-3	18.34 ± 7.14	18.3 ± 7.28	0.400
Vit. D3	12.23 ± 5.71	10.83 ± 5.63	0.200
Calcium	9.03 ± 3.01	8.51 ± 1.03	0.260
Cholesterol	175.91 ± 30.48	178.78 ± 34.51	0.743
TG	166.64 ± 72.24	166.25 ± 84.38	0.979
HDL	46.47 ± 10.67	44.96 ± 12.92	0.306
LDL	95.08 ±27.52	94.74 ± 32.17	0.991
Results shown as mean ± standard deviation, P value > 0.05 considered non significant			

3.2. Genetic analysis

3.2.1. Results of genotyping of *SULT1A1* gene

The results of SULT1A1 genotype rs 6839 was shown a clear band with a molecular size 136 bp and 189 bp (Figure 3-2) and rs 9282861 was shown in 147 and 191 bp (Figure 3-3). The size of amplicon was determined by comparison with DNA ladder 100 - 1000 bp.

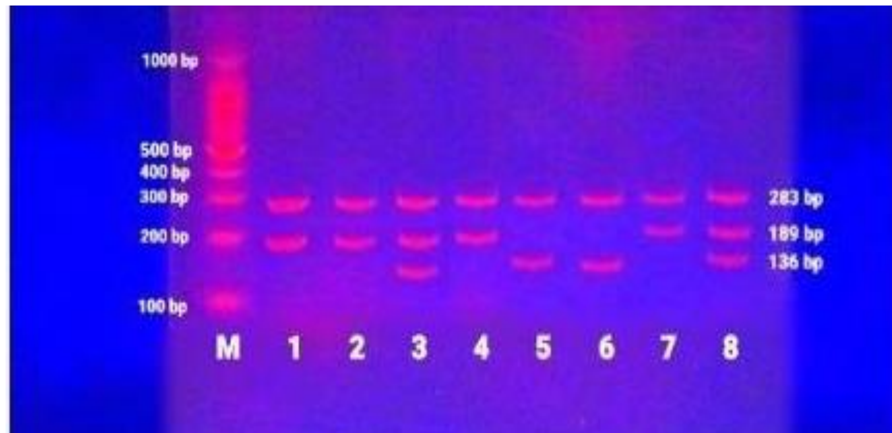


Figure (3-2): Polymerase chain reaction amplification of *SULT1A1* gene rs 6839 showing: line M: represented the DNA ladder (100-1000 bp), where lines 1,2,4, and 7 represented the **TT** (**wild**) genotype shown in 189 bp, lines 3 and 8 represented the **TC** (**heterozygous mutant**) genotype, and lines 5 and 6 represented the **CC** (**homozygous mutant**) genotype shown in 136 bp.

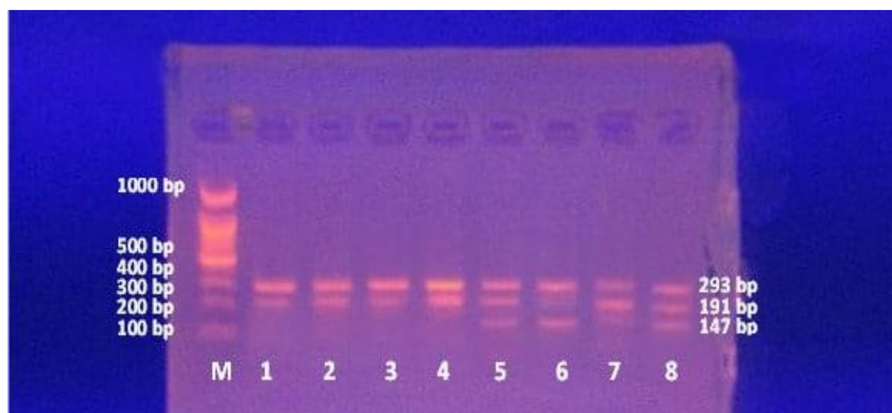


Figure (3-3): Polymerase chain reaction amplification of *SULT1A1* gene rs 9282861 showing: line M: represented the DNA ladder (100- 1000 bp), lines 1, 2, 3, 4 and 7 represented the **TT** (**homozygous mutant**) genotype which was shown in 191 bp, lines 5 and 8 represented the **CT** (**heterozygous mutant**) genotype, and line 6 represented the **CC** (**wild**) genotype, which was shown in 147 bp.

3.2.2 The genetic distribution of *SULT1A1* genotype

3.2.2.1. Genetic distribution of *SULT1A1* rs 6839 in breast cancer patients

The frequency and percentage of rs 6839 genotype detected in breast cancer patients are shown in table (3-7). The wild type (TT), which had a frequency and percentage of 54 and 54%, was the most common genotype among the 100 breast cancer patients recruited in this study, whereas the heterozygous mutant type (TC), which had a frequency and percentage of 17 and 17%, was the least common. The frequency and percentage of the homozygous mutant type (CC), were found to be 29 and 29%, respectively.

Table (3-7): Distribution of SULT1A1 gene rs 6839 in patients with breast cancer

Genotype	Group n=100	Frequency	%
rs 6839	TT (wild)	54	54%
	TC(mutant heterozygous)	17	17%
	CC(mutant homozygous)	29	29%
Data presented by numbers and percentage Wild represent major genotype, Mutant represent minor genotype			

The result of comparison between observed and anticipated values for SNP with rs 6839 in the tested population were shown in figure (3-4), and table (3-8). The distribution and percentage of individuals having rs 6839 differ from those expected under Hardy–Weinberg equilibrium {number of observed vs expected were: TT (54, 39.1); CC (29, 14.1); TC (17, 46.9) (goodness-of-fit $\chi 2$ for rs 6839 40.526 , $P < 0.001$) and therefore it was statistically significant.

Table (3-8): Hardy–Weinberg equilibrium for rs 6839 genotype in studied patients

Genotype n=100	Frequency%	Alleles		Hardy–Weinberg equilibrium χ^2 test
		T	C	
TT (Homozygous wild type)	54	0.625	0.375	40.619 **P < 0.001 [S]**
TC (Heterozygous mutant type)	17			
CC (Homozygous mutant type)	29			

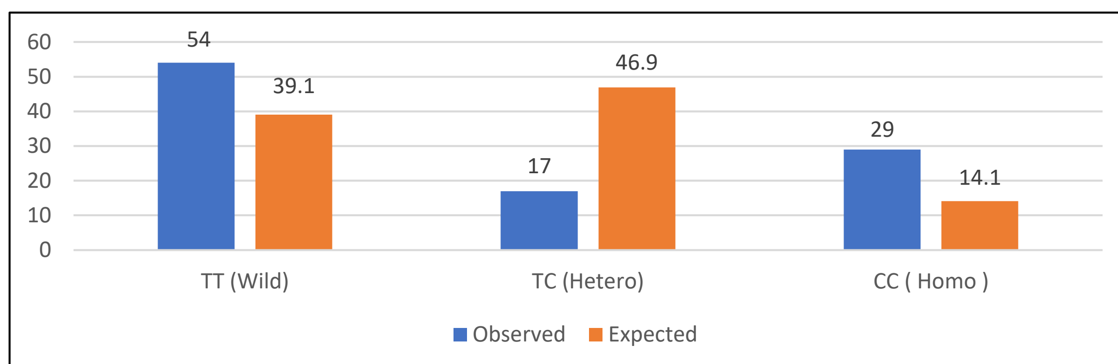


Figure (3-4): Observed vs expected genotype frequencies % of rs 6839 among individuals' sample

3.2.2.2. Genetic distribution of *SULT1A1* rs 9282861 in breast cancer patients

The frequency and percentage of rs 9282861 genotype identified in breast cancer patients are displayed in table (3-9). The homozygous mutant type (TT), was the most frequent genotype among the 100 breast cancer patients recruited in this study with frequency and percentage of 41 and 41%, while the heterozygous mtant type (CT), with a frequency and percentage of 21 and 21%, was the lowest frequent genotype. The frequency and percentage of the wild type (CC), was 38 and 38%, respectively.

Table (3-9): Distribution of *SULT1A1* gene rs 9282861 in patients with breast cancer

Genotype	Group n=100	Frequency	%
rs 9282861	CC (Wild)	38	38%
	CT(mutant heterozygous)	21	21%
	TT(mutant homozygous)	41	41%
Data presented by numbers and percentage Wild represent major genotype, Mutant represent minor genotype			

The result of comparison between observed and anticipated values for SNP with rs 9282861 in the tested population were shown in figure (3-5), and table (3-10). The distribution and percentage of individuals having rs 9282861 differ from those expected under Hardy–Weinberg equilibrium {number of observed vs expected were: CC (38, 23.5); TT (41, 26.5); CT (21, 50) (goodness-of-fit χ^2 for rs 9282861 33.595, P < 0.001) and therefore it was statistically significant.

Table (3-10): Hardy–Weinberg equilibrium for rs 9282861 genotype in studied patients

Genotypes n=100	Frequency%	Alleles		Hardy–Weinberg equilibrium χ^2 test
		T	C	
CC (Homozygous wild type)	38	0.515	0.485	33.595 **P < 0.001 [S]**
CT (Heterozygous mutant type)	21			
TT (Homozygous mutant type)	41			

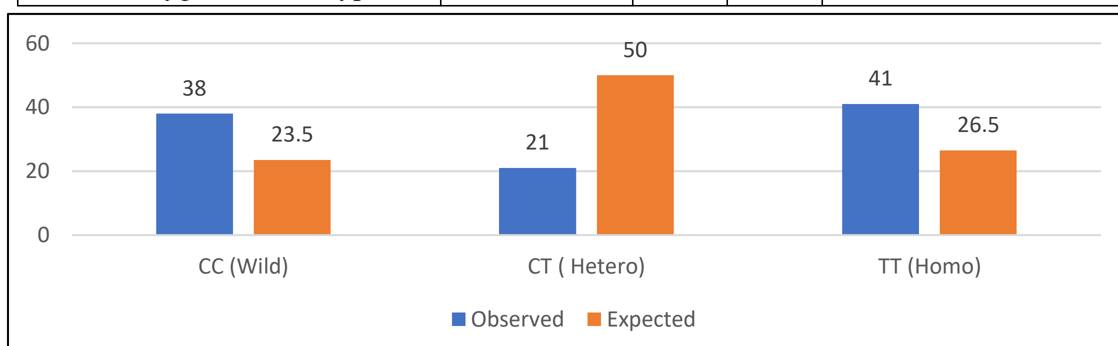


Figure (3-5): Observed vs expected genotype frequencies % of rs 9282861 among individuals' sample

3.3 Relationship between demographic characteristics and *SULT1A1* gene

3.3.1. Relationship between demographic characteristics and *SULT1A1* rs 6839 SNP

To show the difference between demographic characteristics and rs 6839 SNP (table 3-11), a Chi-Square test performed to compare the age, BMI, duration of treatment and disease, marital status, family history, breast cancer side, lymph node involvement, surgery, chemotherpy, radiotherapy, HER2, ER/PR, side effect, recurrence and the genotypes of the rs 6839 SNP. There was no statistically significant difference among them ($p > 0.05$).

Table (3-11) Difference among demographic characteristic in rs 6839 SNP

Demographic parameters		Genotype n=100			P value
		TT n=54	TC n=17	CC n=29	
Age group	44 - 49 Years	23	8	14	0.95
	50 - 55 Years	21	7	10	
	56 - 65 Years	10	2	5	
BMI group	Obese	32	10	19	0.83
	Non obese	22	7	10	
Treatment group	<5 years	46	13	22	0.51
	>5 years	8	4	7	
Diagnosis group	<5 years	38	13	19	0.73
	>5 years	16	4	10	
Marital status	Married	53	15	25	0.08
	Single	1	2	4	
Family history	Yes	25	8	11	0.73
	No	29	9	18	
Breast cancer Side	Left breast	29	8	19	0.42
	Right breast	25	9	10	
Lymph Node involvement	Positive	30	12	22	0.15
	Negative	24	5	7	
Surgery	Positive	51	16	27	0.97
	Negative	3	1	2	
Chemotherpy	Positive	50	16	25	0.55
	Negative	4	1	4	
Radiotherpy	Positive	44	11	24	0.28
	Negative	10	6	5	
HER2	Positive	15	5	13	0.27
	Negative	39	12	16	
ER / PR	ER+/PR+	52	17	29	0.41
	ER+/PR-	2	0	0	
Side effect	Hot flashes	5	1	7	0.21
	Joint pain	44	14	18	
	Both (Hot flashes & Joint pain)	3	2	4	
	Endometrial hyperplasia	2	0	0	
Recurrence	Yes	3	1	3	0.70
	No	51	16	26	
Results are shown as n= number of patients and percentage					

3.3.2. Relationship between demographic characteristic and *SULT1A1* rs 9282861 SNP

To show the difference between demographic characteristics and rs 9282861 SNP (table 3-12), a Chi-Square test was conducted to compare the age, BMI, duration of treatment and disease, marital status, family history, breast cancer side, lymph node involvement, surgery, chemotherpy, radiotherapy, HER2, ER/PR, side effect and recurrence between the genotypes of rs 9282861 SNP, there was no statistically significant difference among them ($p > 0.05$).

Table (3-12): Difference between demographic characteristic in rs 9282861 SNP

Demographic parameters		Genotype n=100			P value
		CC n=38	CT n=21	TT n=41	
Age group	44 - 49 Years	21	9	15	0.13
	50 - 55 Years	15	8	15	
	56 - 65 Years	2	4	11	
BMI group	Obese	22	15	24	0.54
	Non obese	16	6	17	
Treatment group	<5 Years	33	17	31	0.44
	> 5 Years	5	4	10	
Diagnosis group	<5 Years	30	14	26	0.3
	>5 Years	8	7	15	
Marital status	Married	35	20	38	0.89
	Single	3	1	3	
Family history	Yes	17	6	21	0.23
	No	21	15	20	
Breast cancer Side	Left breast	23	11	22	0.77
	Right breast	15	10	19	
Lymph node involvement	Positive	27	12	25	0.49
	Negative	11	9	16	
Surgery	Positive	38	20	36	0.07
	Negative	0	1	5	
Chemotherpy	Positive	34	17	40	0.08
	Negative	4	4	1	
Radiotherpy	Positive	30	16	33	0.92
	Negative	8	5	8	
HER2	Positive	12	8	13	0.85
	Negative	26	13	28	
ER/PR	ER+/PR+	38	21	39	0.23
	ER+/PR-	0	0	2	
Side effect	Hot flashes	2	6	5	0.19
	Joint pain	31	13	32	
	Both (Hot flashes & Joint pain)	4	1	4	
	Endometrial hyperplasia	1	1	0	
Recurrence	Yes	4	2	1	0.32
	No	34	19	40	

Results are shown as n= number of patients and percentage

3.4. Effect of tamoxifen treatment on laboratory parameters according to different single nucleotide polymorphism SNP of *SULT1A1* gene in a studied breast cancer patients

3.4.1. Effect of tamoxifen on laboratory parameters according to the genotypes of rs 6839 T>C SNP

To show the difference between serum levels of biomarkers: E2, CA15-3, vitamin D3, and calcium based on rs 6839 SNP groups (table 3-13), one-way ANOVA test was performed. No significant difference was found between the measured biomarkers and rs 6839 SNP groups ($p > 0.05$).

Table (3-13): Difference between alleles of *SULT1A1* genotype rs 6839 T>C SNP with mean levels of biomarkers in breast cancer patients who receive tamoxifen treatment

Lab. parameters	rs 6839 n= 100			P value
	TT n=54	TC n=17	CC n=29	
S. E2	31.04 ± 10.15	35.14 ± 11.96	35.89 ± 12.34	0.12
S. CA15-3	18.67 ± 6.85	16.27 ± 4.04	17.72 ± 6.24	0.38
S. vitamin D3	12.07 ± 6.08	10.50 ± 6.16	12.09 ± 4.68	0.58
S. calcium	8.78 ± 1.11	8.15 ± 1.26	8.66 ± 0.93	0.11
Results are shown as mean ± SD, P value > 0.05 considered non significant				

To show the difference between mean serum levels of lipid profile markers: Choleterol, TG, HDL, LDL and rs 6839 SNP groups (table 3-14), one-way ANOVA test was also performed to compare the means. Additionally, the results showed that there was no significant difference ($P > 0.05$).

Table (3-14): Difference between alleles of *SULT1A1* genotype rs 6839 T>C SNP with mean levels of lipid profile in breast cancer patients who receive tamoxifen treatment

Lipid Profile	rs 6839 n=100			P value
	TT n=54	TC n=17	CC n=29	
S. Chol	176.18 ± 30.44	167.00 ± 30.82	179.41 ± 25.01	0.37
S. TG	163.07 ± 57.79	139.29 ± 52.47	148.90 ± 52.15	0.24
S. HDL	44.98 ± 11.49	48.41 ± 8.31	46.56 ± 12.66	0.54
S. LDL	93.73 ± 29.79	94.84 ± 29.68	97.39 ± 27.31	0.86
Results are shown as mean ± SD, P value > 0.05 considered non significant				

3.4.2. Effect of tamoxifen on laboratory parameters according to the genotypes of rs 9282861 C>T SNP:

The difference between mean serum levels of estradiol, CA15-3, vitamin D3, calcium and rs 9282861 SNP groups (table 3-15), was also examined as a clinical outcome in breast cancer patients, using a one-way ANOVA test. No significant difference was found between the various genotypes of rs 9282861 SNP ($P > 0.05$).

Table (3-15): Difference between alleles of *SULT1A1* genotype rs 9282861 C>T SNP with mean levels of biomarkers in breast cancer patients who receive tamoxifen treatment

Lab. parameters	rs 9282861 n= 100			P value
	CC n=38	CT n=21	TT n=41	
S. E2	34.72 ± 11.21	31.74 ± 9.86	32.40 ± 12.04	0.53
S. CA15-3	18.17 ± 6.72	18.92 ± 7.11	17.33 ± 5.48	0.63
S. vitamin D3	11.47 ± 5.71	11.13 ± 5.33	12.48 ± 5.93	0.60
S. calcium	8.57 ± 1.26	8.73 ± 1.06	8.66 ± 0.99	0.86
Results are shown as mean ± SD, P value > 0.05 considered non significant				

In addition, the difference between serum levels of cholesterol, triglyceride, HDL and LDL and rs 9282861 SNP groups (table 3-16) by using a one-way ANOVA test, did not reveal any statistical significant differences across genotypes ($p > 0.05$).

Table (3-16): Difference between alleles of *SULT1A1* genotype rs 9282861 C>T SNP with mean levels of lipid profile in breast cancer patients who receive tamoxifen treatment

Lipid Profile	rs 9282861 n=100			P value
	CC n=38	CT n=21	TT n=41	
S. Chol	174.99 ± 30.63	182.91 ± 26.51	172.32 ± 28.81	0.39
S. TG	159.13 ± 49.28	148.90 ± 63.06	154.10 ± 58.13	0.79
S. HDL	46.32 ± 11.32	46.19 ± 13.87	45.67 ± 10.21	0.96
S. LDL	99.47 ± 29.41	92.37 ± 27.15	92.16 ± 29.28	0.48
Results are shown as mean ± SD, P value > 0.05 considered non significant				

3.5. Difference between both *SULT1A1* gene rs 6839 SNP & rs 9282861 SNP are mutant SNP and either rs 6839 SNP or rs 9282861 SNP mutant or no mutation:

The difference between both SULT1A1 gene rs 6839 SNP & rs 9282861 SNP are mutant and either rs 6839 SNP or rs 9282861 SNP mutant or no mutation, was examined by performing a Student′s t-test. Study group was classified into two sub groups based on: Group 1-Patients having mutation in both rs 6839 SNP & rs 9282861 SNP, Group 2-Patients who have mutation in either rs 6839 SNP/ rs 9282861 SNP or patients who not showing any gene mutation. In relation to laboratory parameters (mean), serum levels of estradiol & vitamin D3 were observed to be higher in patient group who had mutations in both rs 6839 & rs 9282861 SNPs , while, CA15-3 & calcium were found to be slightly lower as shown in table (3-17), no significant differences were found since ($P > 0.05$).

Table (3-17): Difference in mean levels of biomarkers between both rs 6839 SNP & rs 9282861 mutant SNP and either rs 6839 SNP or rs 9282861 SNP mutant or no mutation

Lab. Parameters	Patient genotype n=100		P value
	Either rs 6839 or rs 9282861 SNP mutant or no mutation n=90	Both rs 6839 & rs 9282861 SNP mutant n=10	
S. E2	32.84 ± 10.90	35.92 ± 14.45	0.41
S. CA15-3	18.06 ± 6.42	17.31 ± 5.09	0.72
S. vitamin D3	11.73 ± 5.76	12.56 ± 5.35	0.66
S. calcium	8.65 ± 1.11	8.52 ± 1.11	0.73
Results are shown as mean ± SD, P value > 0.05 considered non significant			

Interestingly, lipid profile parameters were decreased markedly when compared the two groups using a Student′s t-test. Patients who had mutations in both rs 6839 & rs 9282861 SNP were showing lower levels of lipid profile, although these differences were not statistically significant (P> 0.05), as shown in table (3-18).

Table (3-18): Difference in mean levels of lipid profile between both rs 6839 SNP & rs 9282861 mutant SNP and either rs 6839 SNP or rs 9282861 SNP mutant or no mutation

Lipid Profile	Patient genotype n=100		P value
	Either rs 6839 SNP or rs 9282861 SNP mutant or no mutation n=90	Both rs 6839 SNP & rs 9282861 SNP mutant n=10	
S. Chol	176.54 ± 30.05	166.70 ± 15.88	0.11
S. TG	157.65 ± 55.66	130.30 ± 51.06	0.14
S. HDL	46.49 ± 11.44	41.82 ± 10.09	0.21
S. LDL	95.12 ± 29.97	93.72 ± 15.96	0.81
Results are shown as mean ± SD, P value > 0.05 considered non significant			

3.6 Association of side effects and recurrence with demographic data

Across tabulation analysis by using a Fisher's Exact test was conducted between demographic data and side effects (table 3-19). There was no statistically significant association between them (p> 0.05).

Table (3-19): Association of side effects with demographic data

Group		Hot flashes	Joint pain	Both	Endometrial hyperplasia	P value
Age group	44 - 49 yr	5	33	7	0	0.252
	50 - 55 yr	6	29	1	2	
	56 - 65 yr	2	14	1	0	
BMI group	Obese	7	48	5	1	0.887
	Non obese	6	28	4	1	
Duration of treatment	<5 yr	10	62	7	2	0.876
	>5 yr	3	14	2	0	
Duration of diagnosis	<5 yr	8	53	7	2	0.668
	>5 yr	5	23	2	0	
Results are shown as number or percentage						

Across tabulation analysis by using the Fisher's Exact test was conducted between demographic data and recurrence of disease (table 3-20). With regard to the recurrence of breast cancer, only age groups were shown to have a statistically significant correlation (P=0.025). Other demographic data and recurrence were not significantly associated (P>0.05).

Table (3-20): Association of recurrence with demographic data

Group		Recurrence		P value
		No	Yes	
Age group	44 - 49 yr	44	1	0.025*
	50 - 55 yr	32	6	
	56 - 65 yr	17	0	
BMI group	Obese	57	4	0.828
	Non obese	36	3	
Duration of treatment	<5 yr	75	6	0.742
	>5 yr	18	1	
Duration of diagnosis	<5 yr	66	4	0.441
	>5 yr	27	3	
Results are shown as number or percentage , *P value <0.05 considered significantly different				

Chapter four

Discussion

4. Discussion

Tamoxifen has a complicated metabolism that involves both oxidative and conjugative processes. Sulfation and glucuronidation are major mechanisms in phase II tamoxifen metabolism. Sulfotransferase SULT1A1 catalyze the formation of sulfates from 4-hydroxytamoxifen and endoxifen (159).

To the best of our knowledge, this is the first study that has mainly focused on the genetic variants of the *SULT1A1* gene in Iraqi women with breast cancer and their influence on breast cancer treatment in terms of therapeutic response to tamoxifen, the occurrence of tamoxifen side effects and recurrence.

4.1 Demographic data

Numerous risk factors may accounted for breast cancer include genetic predisposition, early menarche, late menopause, low parity, oral contraceptives and long-term hormone replacement therapy (HRT), high breast density, a history of atypical hyperplasia and ionising radiation, obesity, and modern life style factors such as diet, cigarette smoking, and alcohol consumption (160).

According to table (3-1), the mean age of breast cancer incidence in Iraqi women participating in the current study was 51 years, which is consistent with the Nada Alwan et al. (2019) study conducted at Medical City Teaching Hospital in Baghdad. In this study, less than half of the patients (44 in total) have a family history of breast cancer (161), which is a higher ratio than in Hasan et al. (2020) study with 20 cases (n=100) and 20 controls (n=200) performed in Merjan and Al Hilla teaching hospitals, which found a statistically significant link between positive family history and breast cancer (162).

The hormones estrogen or progesterone can encourage the development of breast cancer in tumor cells that are hormone receptor positive. HER2 positivity means that tumor cells generate an abundance of the HER2/neu protein, which has been associated with some aggressive forms of breast cancer (163). The higher the ER and PR content in breast cancer, the higher response rate to hormonal therapy about 73%. ER expression is linked with age and menopausal status. It is more frequently detected in postmenopausal than in premenopausal women and in older than younger women with breast cancers (106).

Lymph node involvement in breast cancer has long been recognized as an important prognostic factor. Positive axillary lymph nodes predict an increased risk of local and distant recurrence, that has a direct impact on mortality and worse prognosis. The survival rates are up to 40% lower in node-positive than node-negative patients (164).

4.2 Effect of tamoxifen on studied laboratory biomarkers

The current study shows the variations in serum levels of estradiol, tumor marker CA15-3, vitamin D, calcium and lipid profile in Iraqi breast cancer women on tamoxifen therapy.

In 60–70% of breast cancer patients, estrogen receptor (ER) expression is dysregulated. Estrogen plays a direct role in cellular proliferation by regulating the expression of ER target genes in several tissues, such as the breast (165).

4.2.1 Effect of tamoxifen on hormones (estradiol)

The primary circulating ovarian steroid, 17-β estradiol (E2) is the most physiologically active hormone in breast tissue, stimulates breast growth between puberty and sexual maturity. During menopause E2 plasma levels drop by 90%.

The concentrations of estradiol in breast cancer tissues do not differ between pre- and postmenopausal women, despite significantly different circulating estrogen levels in these two groups of women. This suggests that de novo biosynthesis, or peripheral aromatization of ovarian and adrenal androgens, is much more significant than the uptake of this hormone from the circulation, which may not contribute significantly to the total content of this hormone in breast tumors (166).

Hormones are responsible for around 78 percent of breast cancers. Stopping the function of estrogen in the breast is therefore a well-known treatment method for avoiding recurrence, and it may be accomplished by either inhibiting the hormone's activity on estrogen receptors or stopping its biosynthesis (167).

Because it is an estrogen-dependent tumor, its beginning and development are significantly connected with estrogen levels, despite the fact that people maintain a dynamic equilibrium at various physiological phases. Furthermore, persistent low E2 concentrations are sufficient to raise breast cancer risk in premenopausal women, despite the fact that high E2 levels are linked to breast cancer in postmenopausal women (168).

4.2.2 Effect of tamoxifen on tumor marker CA 15-3

In order to monitor cancer development, determine prognosis, plan treatment by anticipating treatment response, and screen for disease progression following surgery, early chemotherapy, and radiation, tumor markers have been recognized as a noninvasive and cost-effective tool. The most often utilized tumor markers in breast cancer are cancer antigen 15-3 (CA 15-3). Different kinds of epithelial cells exhibit variable expression of CA 15-3, a member of the mucin-1 (MUC-1) glycoprotein family. It is abnormally overexpressed in 90% of breast cancer (70)(169).

The mean results of the tumor marker in this study are within normal range (table 3-2), with statistically significant difference (p value<0.05) among age groups (table 3-3). Little evidence available concerning the effect of tamoxifen and the tumor marker, however a study done at AL- Amal National Hospital in Baghdad (2019), found that among 140 Iraqi breast cancer women who received the drug, tumor marker CA15-3 levels and estradiol levels showed lower levels in non-recurrent corresponding to recurrent breast cancer group (170).

4.2.3 Effect of tamoxifen on serum vitamin D and calcium level

Vitamin D serves as a precursor to the hormone calcitriol (1,25-dihydroxyvitamin D3), which regulates several processes in numerous human organs (l69). By being exposed to the sun, the skin may produce its own vitamin D, and, to a lesser extent, gained through food and supplementation. It is generally known that vitamin D affects bone mineralization and plays a role in maintaining calcium homeostasis (171)(172). Aside from its advantages for bone health, its role in the prevention and treatment of a variety of diseases, including cancer, autoimmune disorders, and cardiovascular disease, has been examined (173)(174)(175).

According to laboratory results, vitamin D may have significant anticancer effects (176) which including anti-proliferative, pro-apoptotic, pro-differentiating, anti-inflammatory, anti-invasion, and anti-angiogenesis (177)(178)(179). Previous research has revealed that the link between vitamin D and breast cancer risk is higher in premenopausal women than in postmenopausal women (180). Earlier studies in postmenopausal women found no connection between vitamin D level and breast cancer risk (181).

Serum calcium concentrations are tightly regulated by physiology because they have a significant impact on key physiological processes including heart rate and nerve conducion. The skeleton is the reservoir of calcium in the blood. The calcium-sensing receptor on the parathyroid glands instructs parathyroid cells to produce and release parathyroid hormone (PTH) into the circulation when levels of ionized calcium in serum fall below their set point. By promoting the conversion of 25-hydroxyvitamin D (25-OHD) to l,25(OH)2D in the kidney, decreasing calcium excretion in the urine, and releasing calcium from the skeleton into the circulation, parathyriod hormone works to preserve calcium. By preventing future PTH production, which has anti-apoptotic effects and may promote invasiveness and tumor development, the ensuing rise in ionized calcium in the blood restores calcium equilibrium and may be linked to tumor protective properties (182)(183).

According to results from a prospective cohort research, blood calcium levels are favorably correlated with breast cancer risk in overweight peri-/postmenopausal women with a BMI of above 25 kg/m^2. Serum calcium levels are also negatively correlated with breast cancer risk in premenopausal women (184).

4.2.4 Effect of age and BMI

The results show that serum vitamin D and calcium were positively increased with age, while serum estradiol levels decreased with age. There was statistically significant change in mean serum level of estradiol, vitamin D and calcium when comparing the mean results among different age groups of postmenopausal women (p value=0.001). A case control study carried by Mallika and Gopinath in 2017 (185) on Indian women with test group (n=100) being on tamoxifen from 1-4 years and control group (n=50) having breast carcinoma and not taking tamoxifen,

these groups further divided into pre- and postmenopausal women. The results showed that there is no significant change in serum calcium between test and control group and so it did not have any unfavorable effect on bone.

According to the body mass index, our study results revealed that increased mean results of serum estradiol, vitamin D and calcium with increasing BMI, which was in contrast to the study carried by Al-biati et al.2017 (186). Their research, which was done at the Baquba Teaching Hospital in Diyala, Iraq, demonstrated that tamoxifen treatment for obese breast cancer patients lowers serum estradiol levels in treated patients, with tamoxifen having a positive effect on serum vitamin D levels as opposed to letrozole, which has a negative effect and lowers serum vitamin D levels. Other studies showed that overweight and obese people with extra adipose tissue, have a negative connection between vitamin D and BMI that may be explained by a volume-distribution effect with reduced bioavailability of fat-soluble vitamin D or it may become sequestered in adipose tissue (187)(188).

The results of multicenter cohort study by James et al.(2003) on 3385 women with breast cancer, showed that obesity was not related with a significant increase in the risk of recurrence or a change in the effectiveness of tamoxifen. However, obesity is linked to less favorable disease features upon diagnosis, including larger tumors and more lymph nodes that are impacted (189). The impact of adipose tissue volume on hormone levels is highlighted in the main premise. The aromatase enzyme in adipose tissue with high fat volume, converts androgen into estrogen, which increases blood estrogen levels. This process is also mediated by a decrease in sex hormone binding globulin (SHBG), which lowers estrogen activity (190).

4.2.5 Effect of treatment and disease duration

Sampling results in our study such as estradiol, vitamin D and calcium were compared according to duration of tamoxifen use and duration of breast cancer (below or above five-year interval), no significant difference was found between the groups (p value>0.05), but it was found that serum levels were decreased with increased treatment or disease duration. The inverse result in Kim et al. study (191) showed that patients who underwent anti-hormonal medication (tamoxifen) had serum 25OHD levels that were considerably higher at 6 and l2 months compared to baseline levels, whereas serum 25OHD levels were reduced with chemotherapy.

Finding of Yetkin et al. (192) study stated that tamoxifen and vitamin D together inhibit the growth of cancer cells and cause cell cycle arrest at the Go/Gl phase. According to the expression results of p53, Bcl-2, and Bax expression, it was discovered that combination was more efficient at driving cells toward apoptosis. Therefore, it's crucial to keep patients' serum 25(OH)D levels within the normal range in order to continue the anticancer benefits (193).

4.2.6 Effect of tamoxifen on lipid profile

The lipid profile appears to influence the development of female breast cancer, particularly in the presence of an elevated BMI. Proteins and lipids combine to form lipoproteins, which are specialized clusters in the blood that transport lipids. These are vital cell membrane components that are crucial for cell development and division. They are also required for normal and malignant tissue cell integrity. The majority of energy in the body is stored as triglycerides (194). Triglycerides and cholesterol are initially packaged into lipoproteins and transported in plasma before being taken up and degraded by cells for cellular functions. As a result, by providing lipids to cancerous cells and tumors, lipoproteins may be crucial in the development of cancer. There have been several findings of increased

plasma lipid levels in breast cancer patients, including TG, total cholesterol, and LDL (195).

The mammary tissue is densely packed with lipids. Recent study by Anber ZN,2020 investigated the role of endogenous and dietary lipids in the etiology and prognosis of cancer (196).

According to this study, mean serum lipid levels such as total cholesterol, HDL, and LDL cholesterol are within normal ranges; however, triglyceride levels showed a slight increase above normal ranges (table 3-2). Although, there is significant difference of low density lipoprotein level among different age groups of breast cancer patients (p value= 0.039) as seen in table (3-3).

Al-biati et al.(2017) also reported that after three months of tamoxifen therapy, serum cholesterol decreased while triglyceride and HDL cholesterol increased (186). While Mallika and Gopinath study (2017) found a statistically significant reduction in total cholesterol and LDL cholesterol in the test group compared to the control group, plasma triglyceride and HDL cholesterol levels did not differ (185).

Che Lin and his colleages (2014) stated that tamoxifen had significant impact on serum lipid profile by lowering total cholesterol, triglyceride, low density lipoprotein and increasing high density lipoprotein cholesterol after initiation of therapy (197).

4.3 Frequency of the detected genotypes of *SULT1A1* gene within breast cancer

Sulfotransferase enzymes SULTs catalyze the transfer of sulfonate SO3- from the universal sulfonate donor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to the hydroxyl or amino group of an acceptor molecule. Generally speaking, sulfonation has been considered a detoxifying

route that produces more water-soluble conjugates and facilitates their excretion through the kidneys or bile (143).

The genetic differences in *SULT1A1* is of great interest because this enzyme is participates in the metabolism of endo- and xenobiotics, procarcinogen activation/detoxification, and the disposition of several therapeutic agents including acetaminophen, minoxidil, diethylstilbestrol and 4-hydroxy tamoxifen (198).

Sulfated tamoxifen metabolites are formed by the Phase II enzyme SULT1A1 (199). Because 4-OH TAM has a substantially greater affinity than the parent compound for binding to the estrogen receptor, sulfation of 4-OH TAM may have a considerable impact on the effectiveness of tamoxifen treatment. As a result, SULT1A1 activity could be an important indicator of tamoxifen therapeutic efficacy, and modification of *SULT1A1* activity by genetic and/or environmental factors may affect treatment outcomes (149).

Among the 200 Egyptians genotyped for *SULT1A1*, the genotype distribution and alleles frequency for rs9282861 were CC 149, CT 48, and TT 3, with 0.865 and 0.135 for the C and T alleles, respectively (200). These findings are not compatible with recent research that indicates the mutant TT genotype predominates over the wild or heterozygote genotypes.

In the 97 Japanese participants with rs9282861, the frequency distribution of high activity allele (CC) was 78 with and low activity alleles were 19, p value >0.037 associated with *SULT1A1* activity did not match our results. While 3-UTR rs 6839 SNP were not associated with enzymatic activity, p value >0.05 (201).

The genotype distribution of 97 liver specimens from the United States was obtained. For rs 6839, the allele frequencies for wild TT 35, mutant TC 43, and CC 19 were supplied, whereas for rs 9282861, the allele frequencies for wild CC 37, mutant CT 40, and TT 20 were given (148). These findings are different from our findings for both SNPs.

Previous research by Yu et al.2010 (202) and Sanchez et al.2018 (148) confirmed that lower enzymatic activity of SULT1A1 in the presence of the rs 6839 SNP would result in higher concentrations of endoxifen and 4-OH TAM due to their lower elimination. According to these findings, the low activity group (CC genotype) had a lower risk of relapse than the medium (TC) and high activity groups (TT). Despite the fact that only a few patients relapsed in our study, they were evenly distributed between (CC genotype) and (TT genotype), as seen in the table (3-11).

The findings of Tengström et al.(2012), a study of 412 Finnish breast cancer patients in which 65 patients received adjuvant tamoxifen, are comparable to our findings regarding the fact that risk of recurrence was more prominent in genotypes CC and CT and less prominent in those with the genotype TT. This supports the idea that the homozygous TT variant genotype of SULT1A1 rs9282861 may have a functional consequence of decreased enzymatic activity and thermostability that may result in slower removal of 4-OH-TAM and enhancing survival (151).

Nowell et al.(2002), and Wegman et al.(2005) found that homozygous (mutant TT genotype) had a worse outcome in tamoxifen-treated breast cancer patients than both homozygous (wild CC genotype) and heterozygous (mutant CT genotype) *SULT1A1* carriers of rs9282861. However, Consequently, the effect of *SULT1A1* and clinical outcome among tamoxifen-treated patients is still unclear (149) (203).

Nowell, hypothesized that hepatic sulfation of 4-OH TAM, followed by kidney reabsorption and further desulfation by steroid sulfatase expressed in breast tumors, would slow the rate of clearance of 4-OH TAM and result in higher levels of circulating metabolite in individuals with higher activity SULT1A1 alleles. Furthermore, the presence of *SULT1A1* in breast tumors, in conjunction with steroid sulfatase, may result in the cycling of 4-

OH TAM between sulfated and non-sulfated forms within the cancerous cell. thus, prolonging the duration of action of active 4-OH TAM.

In a systematic and meta-analysis study based on ethnicity, Forat -Yazdi et al.(2017) discovered that the *SULT1A1* Arg213His (rs9282861) polymorphism increased breast cancer risk in Asians but not in Caucasians. The studies included were published between 2000 and 2013. There were twelve studies of Caucasian descendants and eight studies of Asian descendants among those. These studies were carried out in the United States, Austria, India, Korea, Sweden, Germany, Finland, China, Taiwan, Russia, and Italy (204). Although Iraq is considered to be part of the Asian continent, we found no difference in cancer risk or relapse between polymorphisms in our study.

Wang et al.(2010) performed a meta-analysis of 14 published case–control studies find no significant association of the Arg213His (rs9282861) polymorphism with breast cancer incidence. A more thorough investigation of ethnic populations, showed a significant association between the variant homozygote TT with a 2.27-fold greater risk of breast cancer in the Asian population as compared to people with the CC genotype (205).

In the current study, Genotype distribution for both *SULT1A1* SNPs rs6839 and rs9282861 were not in Hardy-Weinberg equilibrium (χ^2=40.619 and 33.595, respectively, with significant p value < 0.001), as shown in table (3-8) and (3-10), this may be attributable to the small sample size which may not accurately reflect the whole population.

4.4 Association of tamoxifen with side effects

Patients who use tamoxifen or aromatase inhibitors (AIs) for extended periods of time as adjuvant hormonal therapy frequently have adverse effects that negatively affect their quality of life, including joint

discomfort and arthralgia. It is possible that younger patients have a higher frequency of arthralgia than their older counterparts due to the acute reduction of sex hormones caused by hormonal therapy (206).

Synovium, subchondral bone, and joint cartilage all have ER. Estrogen has immunosuppressive and anti-inflammatory effects. Acute estrogen withdrawal activates the nuclear factor κB transcription factor, which then increases proinflammatory cytokines production, and eventually tissue destruction. Estrogen receptors can also be found in the limbic system and the dorsal root ganglion. In the central nervous system, estrogens inhibit pain signaling (207).

Previous research has demonstrated that bone mineral density, bone turnover indicators, and parathyroid hormone levels are significantly influenced by vitamin D levels. As a result, low 25(OH)D leves are strongly associated with higher rate of fractures in bones and decreased physical performance, particularly in postmenopausal women (208)(209).

Seventy six percent of the patient included in the present study experience joint pain in comparision to other side effects such as hot flashes (13%) and endometrial hyperplasia (2%), table (3-1).

Tamoxifen is also linked to other menopausal side effects such as hot flashes, night sweats, and loss of libido because it lowers circulating estrogen levels. These side effects can have a significant impact on quality of life and result in noncompliance (210).

4.5 Limitations of the study

1. Patients: many patients refused to participate in the study, possibly because they were afraid of having blood drawn from the veins.
2. Sample size: small sample size was obtained because many patients did not come to the oncology center personally because they lived far away or, in some cases, in another province; instead, their relatives received treatment from the center, and also because of COVID-19.

Conclusions and Recommendations

Conclusions

Based on the findings, the following conclusions can be reached:

1. *SULT1A1* gene was highly polymorphic and detected with different genotypes and variable frequencies in a sample of Iraqi breast cancer women.
2. For rs 6839, the wild type TT was more frequent than the mutant types TC and CC, whereas for rs9282861, the mutant genotype TT was more frequent than the other genotypes CC and CT.
3. It was noted that the genetic variants of *SULT1A1* gene were not-significantly correlated with serum levels of estradiol hormone, cancer antigen CA15-3, vitamin D, calcium and lipid profile. The levels of these parameters may alter among different genotypes of *SULT1A1* gene and therefore, may influence tamoxifen response.
4. The findings showed the decrease in vitamin D3 level may be associated with joint pain in a studied Iraqi breast cancer women .

Recommenations and future works

1. More large-scale and multicenter research would be required to assess the impact of genetic polymorphism of *SULT1A1* on tamoxifen response in Iraqi breast cancer women.
2. Studying genetic variants in other enzymes involved in metabolism of tamoxifen such as CYP 450 enzymes e.g. CYP2D6, CYP3A4/5, CYP2B6, CYP2C9, CYP2C19, UGTs, and transporters such as ABCB1 and OATP1B1 could contribute to inter-individual variability in tamoxifen response.
3. In the clinical setting, we recommend that genetic tests should be developed to expect an individual′s response to tamoxifen therapy, and that personalized drugs with greater efficacy and safety should be developed.
4. To prove the effect of gene polymorphism of metabolizing enzyme on bioavailability of tamoxifen, the plasma level of tamoxifen metabolites should be determined.

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Appendices

Questionnaire of breast cancer patients

Name:	**Phone no.**	**No.**
Age:	**Weight:**	**Height:**

Workplace: **Academic achievement:**

Address: **Marital status:**

First menarche: **Last menarche:**

Family history of breast cancer:

Date of breast cancer diagnosis:

Site (left, right):

Stage & Grading

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

Surgery:

Radiotherapy:

Chemotherapy:

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

Other diseases:

Dose of tamoxifen:

Time on tamoxifen Therapy:

Presence of osteoporosis & other Side effects:

Other drugs used:

Lab. Data:

الخلاصة

خلفية الدراسة: سرطان الثدي هو الورم الخبيث الأكثر شيوعًا بين الإناث في جميع أنحاء العالم.

تاموكسفين هو العلاج الهرموني الذي يستخدم كمُحَوِّل انتقائي لمستقبلات الاستروجين في علاج سرطان الثدي الإيجابي لمستقبلات الهرمونات لدى النساء قبل انقطاع الطمث في المرحلة المبكرة أو المنتشر. كونه عقارًا أوليًا، يخضع عقار تاموكسفين لعملية التحول الحيوي بواسطة إنزيمات المرحلة 1 والمرحلة 2 إلى مستقلبات أولية وثانوية تظهر تأثيره العلاجي. يؤثر تعدد الأشكال الجيني لـ Sulfotransferase SULT1A1، أحد إنزيمات استقلاب الدواء في المرحلة الثانية، على الاستجابة السريرية للعقار.

الهدف من الدراسة: الهدف من هذه الدراسة هو تحديد تعدد الأشكال الجيني لجين SULT1A1 (rs 6839 و rs 9282861) في النساء المصابات بسرطان الثدي المشاركات، وكذلك للتحقق في تأثير تعدد الأشكال الجيني SULT1A1 على فعالية عقار تاموكسفين.

المرضى وطريقة العمل: هذه الدراسة العرضية المقطعية أجريت في مدينة الإمام الحسين (ع) الطبية / مركز الأورام في كربلاء. تم اختيار مائة مريضة بسرطان الثدي الإيجابي لمستقبلات هرمون الاستروجين و / أو لمستقبلات هرمون البروجسترون. تم تسجيل المرضى الذين تتراوح أعمارهم بين 45-65 عامًا، والذين يتناولون عقار تاموكسفين 20 ملغ يوميًا لمدة 3 أشهر على الأقل في هذه الدراسة. تم سحب عينات الدم الوريدي من كل أنثى مصابه بعد التوقيع على استمارة الموافقة المكتوبة مسبقا لقياس تراكيز المعلمات البيوكيميائية مثل استراديول، CA15-3، فيتامين D3، الكالسيوم، انواع الدهون بالإضافة إلى التحليل الجيني. استخدم في هذه الدراسة تفاعل البوليميراز المتسلسل لنظام التحور الحراري التضخمي (ARMS PCR) للكشف عن rs 6839 و rs 9282861.

النتائج: كشفت نتائج هذه الدراسة أن هناك متغيرات جينية مختلفة من الجين SULT1A1 تشمل rs 6839 (902T> C) و rs 9282861 (638C> T). في حالة rs 6839، يسود النمط الجيني متماثل الزيجة الطبيعي (TT)على النمط الجيني متماثل الزيجة الطافر (CC) والنمط الجيني متغاير الزيجة الطافر(TC) بمعدل تكرار54، 29، 17 على التوالي، اما بالنسبة لـ rs 9282861 ، وجد ان النمط الجيني متماثل الزيجة الطافر (TT) يسود على النمط الجيني متغاير الزيجة الطافر (CT) والنمط الجيني متماثل الزيجة الطبيعي (CC) بمعدل تكرار41، 21، 38 على التوالي. أظهرت مستويات استراديول، ودلالة الورم CA15-3، والكالسيوم، وفيتامين D3، والكوليسترول، والدهون الثلاثية، و HDL و LDL في المصل ارتباطًا غير معنوي بين SNPs المدروسة لجين

SULT1A1. كما أظهرت الدراسة الحالية أن هناك نسبة عالية من آلام المفاصل تزيد عن 76٪ مع ظهور جديد للمرض بنسبة لا تتعدى 7٪ من المرضى.

الخلاصة: خلصت الدراسة الحالية إلى أن الاختلاف الجيني في المرحلة الثانية من إنزيم الاستقلاب SULT1A1 قد يساهم في التباين في الاستجابة لعقار التاموكسفين في عينة من النساء العراقيات المصابات بسرطان الثدي إلى جانب التباين في حدوث التفاعلات الدوائية الضارة.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء

كلية الصيدلة

تقييم تأثير تعدد الاشكال الجيني لجين SULT1A1 على الاستجابة العلاجية لعقار التاموكسفين في عينة من مرضى سرطان الثدي

رسالة

مقدمة الى كلية الصيدلة – جامعة كربلاء

كجزء من متطلبات الحصول على شهادة الماجستير في علم الادوية والسموم

من قبل

أسماء عبد الأمير عبد الرضا

بكالوريوس صيدلة (جامعة كربلاء 2011)

باشراف

الأستاذ الدكتور

احمد صالح صاحب

الدكتور

كرار كاظم محسن

٢٠٢٢ ميلادي

١٤٤٤ هجري