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Effect of Genetic Polymorphisms of *CYP3A4* and *UGT1A4* on Anastrozole Efficacy in Iraqi Breast Cancer Women

A Thesis

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

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بِسْمِ اللَّة الرَّحْمَنِ الرَّحِيمِ

﴿وَعَلَّمَكَ مَا لَمْ تَكُنْ تَعْلَمُ وَكَانَ فَضْلُ اللَّة عَلَيْكَ عَظِيمًا﴾

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Dedication

To ……. all the patients who are be ill with cancer …I wish them healthy life

To …… my parents, who drenched the empty cup to give me a drop of love, reaped thorns from my path to pave the way for me to learn.

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List of Abbreviations	
Abbreviations	**Key**
AI	Aromatase Inhibitor
AJCC	American Joint Committee for Cancer
ARMS-PCR	Amplification Refractory Mutation System - Polymerase Chain Reaction
AS-PCR	Allele Specific - Polymerase Chain Reaction
BC	Breast Cancer
BCS	Breast-Conserving Surgery
BPI	Brief Pain Inventory
BRCA1	Breast Cancer gene 1
BRCA2	Breast Cancer gene 2
CA 15.3	Cancer Antigen 15.3
CA 27.29	Cancer Antigen 27.29
CEA	Carcinoembryonic Antigen
CL	Chemiluminescent
CLIA	Chemiluminescent Immunoassy
C_{max}	Concentration Maximum
CT	Computerized Tomography
CYP 1A1	Cytochrome P450 Family 1subfamily A Member 1
CYP3A4	Cytochrome P450 Family 3 Subfamily A Member 4
CYP3A43	Cytochrome P450 Family 3 Subfamily A Member 43
CYP3A5	Cytochrome P450 Family 3 Subfamily A Member 5
CYP3A7	Cytochrome P450 Family 3 Subfamily A Member 7
DCIS	Ductal Carcinoma in situ
DNA	Deoxyribonucleic acid

E1	Oesteron
E2	Oestriol
E3	Estradiol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELFA	Enzyme linked fluorescent assay
ER	Estrogen receptor
ERα	Estrogen receptor alph
ERβ	Estrogen receptor beta
F-A	Forward A
F-C	Forward C
F-T	Forward T
FDA	Food and drug administration
FDG	Fluorodeoxyglucose
FFTP	First Full-Term Pregnancy
FIGO	Federation of International of Gynecologists and Obstetricians
FSH	Follicle Stimulating Hormone
GnRH agonist	Gonadotropin releasing hormone agonist
HER-2	human epidermal growth factor receptor 2
HR	Hormonal receptor
IBM	International Business Machines
I-F	Inter forward
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IMA	Immune medullary agent
I-R	Inter reward

µL	Microliter
MDR	Multidrug resistance
MRI	Magnetic resonance imaging
MUC-1	Mucin -1
O-F	Outer forward
O-R	Outer reward
PCR	Polymerase chain reaction
Pg /ml	Picogram per milliliter
PI3K/AKT	Phosphtidylinosito-3 Kinase/Protein Kinase B
PM	Poor metabolizer
Pmol/ul	Picomole per milliliter
PPT	protein precipitation buffer
RBC	Red blood cell
RLUS	Relative light units
ROS	Reactive oxygen species
Rpm	Rotation per minute
SERDs	Selective Estrogen Receptor Degrader
SERM	Selective Estrogen Receptor Modulator
SNPs	Single nucleotide polymorphisms
SPR	solid phase receptacle
TAM	Tamoxifen
TBE	Tris-borate-EDTA
T_{max}	Time maximum
TNM	Tumor (T),Nodes (N),Metastasis (M)
TP53	Tumor protein -53
u/ml	Uint per milliliter
UGT 2	UDP-Glucuronosyltransferases 2

UGT 3	UDP-Glucuronosyltransferases 3
UGT 8	UDP-Glucuronosyltransferases 8
UGT1A4	UDP-Glucuronosyltransferases 1A4
UV	Ultraviolet
UICC	Union of International Cancer Control
US	United State
v/cm	Volt per centimeter

Abstract

Background: Breast cancer is considered as global problem of health that affects parenchyma of the breast, with a global incidence of 1.7 million women diagnosed with breast cancer per year, around the world breast cancer constituting 25% of all cancers and it is being the second causes of death in women worldwide following lung cancer. In Iraq, generally and in Kerbala women specifically, breast cancer is the top of ten among cancer types, as well as the first cause of death among women.

Aromatase is responsible for final step of estrogen synthesis which plays an important role in breast cancer pathogenesis, anastrozole as aromatase enzyme inhibitor drug that is used in therapy of breast cancer women metabolize mainly through hepatic *CYP3A4* and *UGT1A4*, polymorphism of anastrozole-metabolizing enzymes have a role in interindividual variation in anastrozole response and side effect.

Aims of study: The aims of this study were to detect the genetic polymorphism of anastrozole metabolizing enzyme that encoded by *CYP3A4* gene especially *CYP3A4***22 (G>A) (rs35599367)* and *UGT1A4* gene particularly UGT*1A4***2 (C>A, T)* (rs6755571) in Iraqi breast cancer women, as well as to investigate the impact of *CYP3A4***22 (G>A) (rs35599367)* and UGT*1A4***2 (C>A, T)* (rs6755571) polymorphism on Anastrozole efficacy, in addition to investigate their association with the development of anastrozole induce arthralgia in Iraqi breast cancer women .

Patients and methods: This cross-sectional observational study was done at Imam Al-Hussein Medical City / Oncology Center in Kerbala. One hundred women with (ER and /or PR) positive breast cancer were selected to participate in this study. All women included in this study with age ranged (31-75) were treated with anastrozole tablet (1mg) once daily as standard adjuvant therapy. Blood samples were obtained from eligible women patients who had signed informed consent for genetic testing and

measurement of estradiol (E2) and tumor marker (CA15.3) serum level. This study used Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS PCR) and Allele Specific Polymerase Chain Reaction (AS-PCR) for detection of CYP3A422 (G>A) (rs35599367) and UGT1A4*2 (C>A, T) (rs6755571) respectively. Arthralgia were assessed depending on patient's history in addition to Brief Pain Inventory (BPI) questionnaire.

Results: The obtained results from this study have detected multiple genotypes of *CYP3A4*22 (G>A) (rs35599367)* and *UGT1A4*2 (C>A, T)* (rs6755571) that include the homozygous wild genotype (GG), homozygous mutant (AA) and heterozygous (GA) genotype of *CYP3A4*22* (G>A) (rs35599367) while for *UGT1A4*2* (C>A,T) (rs6755571) was two mutant genotypes detected, homozygous (TT) which was most abundant and homozygous (AA) was founded in very few patients and also homozygous wild (CC) and heterozygous (CT,CA.TA) genotypes also detected in breast cancer patients participated in this study. Regarding to the level of estradiol (E2) and tumor marker (CA 15.3) in the serum, the present study showed that non-significant association ($p>0.05$) between the studied SNPs of both genes and serum estradiol level and tumor marker, with the exception of the homozygous mutant genotype (AA), which found at very low rate (4%) in the breast cancer women included in the study. High number of included breast cancer women in study suffering from arthralgia but the result showed there were no association between *CYP3A4*22* (G>A) (rs35599367) and *UGT1A4*2* (C>A, T) (rs6755571) and arthralgia development.

Conclusions: This study revealed that *CYP3A4*and *UGT1A4* genes were detected different genotypes. Additionally, this study showed that *CYP3A4*22* (G>A) (rs35599367) that mutations were detected using ARMS-PCR technique and *UGT1A4*2* (C>A, T) (rs6755571) where mutations were detected by AS-PCR technique had no effect on drug response (non- significant differences in serum E2 and CA15.3 level among different genotypes of both genes) and on development of arthralgia.

Chapter one

Introduction

1.Breast cancer

tumor is used as a synonym for neoplasm which is a type of abnormal and excessive growth of tissue. The growth is uncoordinated with that of the normal surrounding tissue, and persists in growing abnormally, even if the original trigger is removed, the International Statistical Classification of Diseases and Related Health Problems (ICD) classifies tumor into four main groups : benign tumor, in situ tumor and malignant tumor that simply known as cancers and are the focus of oncology (1).

Breast cancer (BC) is common type of cancers diagnosed among women worldwide, in 2020 (BC) is surpassed lung cancer as the leading cause of cancer incidence; that accounts among women each 4 cancer cases 1 diagnosed with breast cancer and each 6 cancer death 1 is due to BC. Although there is an enhancement in breast cancer survival but it is still the second major cancer related death after lung cancer among women. (2, 3)

Along – time survival is generally defined as a person who still alive 5 years after cancer diagnosis, for BC the survival at 5years is 88% - 90% and for 10 years is 77% after diagnosis, Enhancements in survival can be predictable from early detection and adjuvant (4).

1.2. Epidemiology:

Breast cancer (BC) is the second most popular type of cancer (1.7 million cases), although ranks fifth in relation to cause of death (522,000 about 6.4%) due to the relative positive prognosis; incidence, mortality and survival rates of disease vary largely among different world parts ,this may be due to several factors such as population structure, genetic factors, environment and lifestyle (5, 6) .In 2018, It's accounting for about 29.2% of all cancers type among female in European Union Countries (7). In united states about 316,700 new breast cancer cases was diagnosed in 2019 (8). In

Lebanon, BC incidence rate about (106/100,000), in Syria (21/100,000) and (57/100,000) in Algeria, according to WHO, in Arab countries expected that the breast cancer rate increase to double between (2012-2030) (9).

In Iraq, breast cancer has been considered a major problem of public health, its incidence rising with the increasing size of population.(10). The Iraqi Center Registry annual report in 2019 revealed that the total cancer cases (35,864),breast cancer ranks first in term of percentage and incidence among the top ten cancer types in Iraq ,in general and among women in kerbala in particular (11-13)

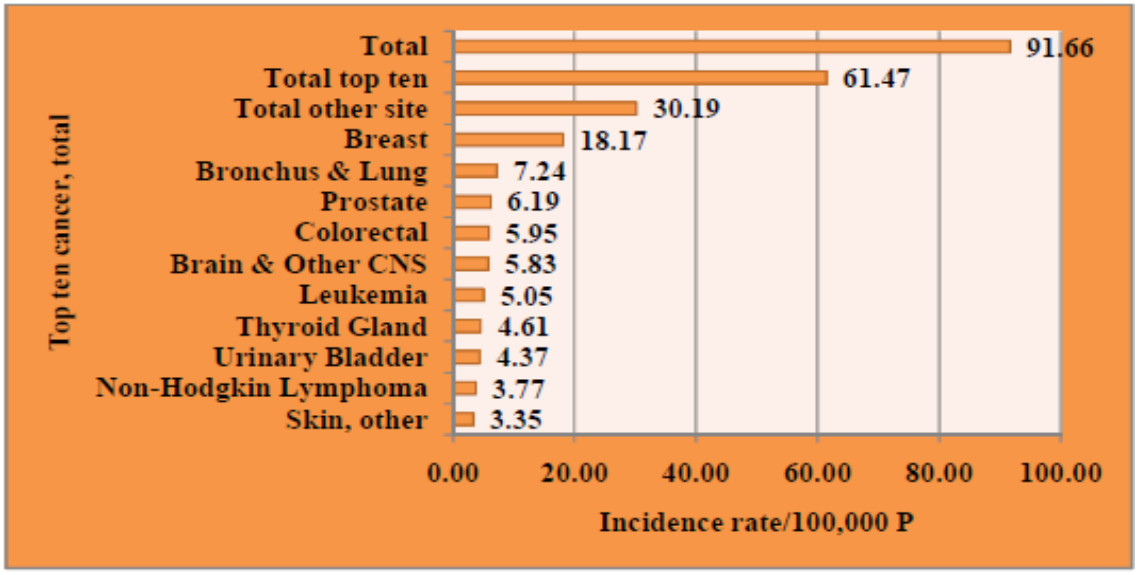

Figure (1.1): Incidence of top-ten cancer in Iraqi population (12)

Figure (1.2): Incidence of breast cancer in kerbala (12)

1.3.Pathogenisis:

The normal development of human is tightly controlled by complex signaling pathways, that allow cells communication with each other and the environment that surround them. Not surprisingly, many of these some signaling pathways are dysregulated or captured by cancer cell (14). Normally when the cells are no longer needed, they under go programed cell death (cell suicide) ;cancer cell charectarized by uncontrolled growth and appotosis avoidenace and this lead to longer survive of cancer cell (16).

Breast cancer is a malignant tumor that starts in the cells of the breast. Like other cancers, there are several factors that can raise the risk of getting breast cancer. Damage to the DNA and genetic mutations can lead to breast cancer, as well as, role of endogenously and exogenously estrogen exposure in breast cancer pathogenesis (15) .

Abnormal methylation of DNA as example for DNA damage is (hypo-or hypermethylation) considered as hallmarke of cancer types; methylation of DNA occurs on cytosine residues of the CpG dinucleotides in DNA. This epigenetic alteration in DNA is heritable but does not alter nucleotide sequence, in contrast to genetic changes. Thus, unlike genetic changes, epigenetic modifications are potentially reversible. About 3–6% of cytosine are methylated in mammals. Approximately 70–80% of CpG sites in the human genome are methylated breast cancer cell is mainly characterized by 50% or more reduction in methylation of CPG isoland of DNA (17).

Another genitic mutation include mutation *PETN* gene incuded PETN protien which is turen off PIT3/AKT pathway which protected cells from suicidal death;so the cell of breast is ready for programed cell death . In som breast cancer ,the gene for the PETN protien is mutated; so the PIT3/AKTpathway is turn on and the cancer cell dose not commit suicide(18). As well as ,Mutations in p53, Breast Cancer gene (*BRCA1*and

BRCA2) are associated with breast cancer in mechansim to correct DNA,these mutations are either inherited or acquired (19).

1.4.Risk factors:

Risk factor can be defined as everything that increase the probability of happening breast cancer ,many of these factors are beyond individuals control (non-modifiable) include :race, pregnancy, age , sex, exposur to chest X-ray, family and personal history, and other factor consider as modifiable factor such as breastfeeding, wight,diet, smoking, physical activity, stress and use of oral contraceptive (20, 21).

1.4.1.Age :

Breast cancer is most commonly diagnosed in femals with age (55-64 years) and the median age of diagnosis is 60 years old ;less than 5% of women with breast cancer < 40 years and as most malignancies breast cancer risk increases with age (22). This indicates that the reproductive hormones produced by the adrenal gland and ovaries are play important role in the pathogenesis of breast cancer (23).

In addition, age of early menarche and late menopause increase BC risk due to early menarche and delayed menopause will escalate the period of estrogen exposure during a female's reproductive years (24).

1.4.2. Reproductive factors:

The reproductive factors that are associated to endogenous estrogen and progesterone exposure, are strongly related with the risk of hormone receptor positive breast cancer (22).

The risk of breast cancer in females having their first birth – full term pregnancy earlier than 25 years old account only one third the risk of breast

cancer in females whose first birth-full term pregnancy later than 35 years old (25) .

The cause of this is change in exposure to estrogen that accompany pregnancy, earlier alteration in breast tissue sensitivity to carcinogenic causing agents or persistent change in hormonal environment have been explain the association between the first birth and risk of breast cancer(26) .

1.4.3. Gender

Breast cancer in men is rare and accounting about ≤ 1% of all diagnosed BC worldwide. The causes of low incidence rate of BC in men are the amount of breast tissue which is relativity low in addition to differences in the hormonal state between men and women. Men breast cancer characterized by low grade, frequently hormonal receptor (HR)-positive and occur with higher stage may be due to delay detection of disease (27, 28).

1.4.4. Family history

The tendency of breast cancer to cluster in families is widely recognized, and most of this familial aggregation is thought to reflect inherited susceptibility (29). However, it is expected that only 20% - 25% of the breast cancer in the first-degree relatives of females affected by the breast cancer may be recognized to known genes mutations which include the high-penetrance susceptibility genes BRCA1 and BRCA2 (30).

1.4.5. Breast feeding:

The reduction in breast cancer risk by 26% have been found in breastfeeding mother >12 month (25). so the breastfeeding having protective effect , prolong breast feeding cause ovulatory suppression ,so

reduce the exposure of breast cell to reproductive hormone and direct breast physically changes that accompany milk (31).

1.4.6. Life style:

1.4.6.1. Obesity:

Obesity has been related to different health disorders one of them was breast cancer. The breast cancer risk was extensively more among women who were obese or overweight similarly for premenopausal and postmenopausal (32).

Numerous theories have explained the association of obesity with postmenopausal breast cancer which include : (1) obesity increase total body aromatization of androgen so this lead to increase level of estrogen in obese women compared with age matched women with normal weight(33). (2) the level of insulin and insulin growth factor(IGF)are higher in obese women and which are able to stimulate the growth of breast epithelial cell (34) .

1.4.6.2. Diet:

There is a strong indication that dietary factors influence the breast cancer risk, so, level of lipid and lipoprotein in blood are also influence by environmental factors and are associated to breast cancer risk (35) .

One of the proposing protective mechanism against breast cancer is decreasing animal protein and increasing intake of vegetable (32).

1.4.6.3. Physical activity:

On average, 20% to 40% reduction in breast cancer risk in most physically active women as compared with the least active women (32)

1.4.6.4. Alcohols:

The association of alcohol drinking with increase of breast cancer risk has been found in many epidemiologic studies through the previous decades (36, 37). since alcohol ingestion results in an elevation of serum estrogen concentrations, it is speculated that the carcinogenic effect of ethanol is mediated, at least in part, by estrogens. In addition, some research has concentrated on acetaldehyde (AA), the first and most toxic metabolite of ethanol oxidation which is by itself carcinogenic and on oxidative stress. Alcohol-related carcinogenesis may also interact with other factors such as smoking, diets, comorbidities and depends on genetic susceptibility (38).

1.4.6.5. Smoking:

The active and passive smoking association with increased risk of breast cancer (37).

Tobacco smoke holds at least 20 type of chemical compound that may induce mammary cancer, several of these compound are lipophilic, and are accumulated in breast tissue and then metabolized and activated by mammary epithelial cells; women who smoke have detectable smoking metabolites in their breast fluid (39),with a higher occurrence of smoking specific DNA adducts and mutation of p53 smoking marks in breast tissue than do nonsmokers (40).

1.4.6.6 Long term use of oral contraceptive pills:

The women that use oral contraceptive for 10 years or more have an increased risk of breast cancer compared with women who don’t or fewer used oral contraceptive ; The risk of breast cancer among oral contraceptive user according to many studies have shown higher in women

with a family history and who carry a BRCA1 or BRCA2 mutation and women who use them before a first full term pregnancy (FFTP) (41).

1.4.7. Hereditary factors:

1.4.7.1. Breast Cancer gene mutation:

One of the risk factor for breast cancer is the inheritance of a mutation in one of the breast cancer susceptibility genes, (BRCA1) that located at chromosome (17q-21) and BRCA2 located at chromosome (13q-12-13) that are tumor suppressor gene (42) , BRCA1 function as checkpoint and DNA repair while BRCA2 act as mediator of homologous recombination mechanism (43).

Breast cancer in BRCA1 carrier women is mainly invasive ductal carcinoma, HR- triple-negative, more aggressive, and hard to detected by mammography; in contrast BRCA2 carrier women is often present with DCIS and more likely to be detected in manograph (44).

1.4.7.2. P53 mutation:

p53 is one of the most important tumor suppressor protein which plays important role in eliminate and inhibit the proliferation of abnormal cells there by preventing neoplastic development(45). Mutation in the TP53 gene that encoding P53 protien often resulting in loss of wild type p53 expression which is found at high incidence in many cancer types including breast cancer (46).

1.4.8. Radiation:

Females who are exposed to chest radiation therapy between (10-30 years of age) had high chance to breast cancer ,because breast tissue more susceptible to carcinogens before it is fully differentiated, conversely

,radiation therapy does not increased BC risk in women with age more than 40 years (25) .

1.5. Histological classification of breast cancer:

From a pathological point of view BC is a heterogeneous neoplasm beginning from the epithelial cell of the milk ducts with variable clinical and histological characteristics (47).Depending on histological appearance, breast cancer is initially divided according to its originated lesion from either the ductal epithelium or lobular epithelium and according to (WHO) that classified BC in to 21 histological types on the basis of cell growth ,morphology and architecture arrangements (21, 48) .

1.5.1. Noninvasive breast cancer:

It is harmful cells that stay in a particular region of the breast tissue, not including spreading to surrounding lobules, ducts or tissue (49).

1.5.1.1 Ductal carcinoma in situ:

It is the most common type and an initial pathologic stage of BC characterized by the growth of tumor cells within the breast duct not spreading across the basement membrane (49) .

1.5.1.2. Lobular carcinoma in situ:

It is characterized by not penetration of basement membrane, only focused in the lobules of breast tissue (50) .

1.5.2. Invasive breast cancer:

It is the harmful cells get through ordinary breast tissue and spread to surrounding tissue or other part of the body such as bone, lung, brain, and liver through the blood and lymph vessels (51, 52). This type

comprise approximately 70% of all cases of breast cancer (53, 54). Metastasis breast cancer consider as an invasive breast cancer that spread to different organs of the body (55, 56) .

1.5.2.1 Invasive ductal carcinoma:

it is invades the ducts and exists in stroma consider as a most prevalent type of invasive breast cancer account for about up to 75% (57) .

1.5.2.2 Invasive lobular carcinoma:

Is the second type that account for about 5% - 15% , which is described as a dangerous tissue infiltration pattern ,providing routine clinical and radiological diagnosis (57).

1.5.3. Inflammatory breast cancer:

It is the generally rare and aggressive type of breast carcinoma that developed rapidly, occurs when cancer cells block the lymphatic vessels in skin covering the breast causing erythema and lumpiness of the skin, and considered a locally advanced cancer that spread from original tumor to nearby lymph nodes (58, 59).

1.6. Molecular classification of breast tissue:

Breast cancer is considered as a complex, multifaceted disease encompassing a great variety of entities that show considerable variation in morphological molecular and clinical attributes. The genome-based frameworks now used for the breast cancer molecular classification (60).

Several breast cancer subtypes classified according to proliferating gene expression profiling and receptor status that include traditional hormone receptor-positive (luminal types) and hormone receptor-negative types (HER-2 and basal-like) (61). As show in table (1-1)

Table (1.1): Molecular classification of breast cancer (62, 63).

Molecular subtype	Gene expression	Receptor status	Histological features	Treatment response
Luminal A	Proliferation gene low expression	ER: positive PR: positive HER2: negative	Low grade	Sensitive to endocrine therapy, variable response to chemotherapy, overall good prognosis
Luminal B	Proliferation gene high expression	ER: positive PR and HER2: positive or negative	Higher grade	Sensitive to endocrine therapy, variable response to chemotherapy, prognosis poorer than of luminal A
HER2	HER2 gene high expression	ER: negative PR: negative HER2: positive	Often high grade	Response to biological therapy such as trastuzumabe, variable response to chemotherapy, over all poor prognosis
Basal - like	Proliferation gene low expression	ER: negative PR: negative HER2: negative	Often high grade	Insensitive to endocrine therapy ,chemotherapy response variable ,poor prognosis

1.7. Prognostic factors in breast cancer:

Prognostic factors are those that measures at the time of diagnosis, which are relate with disease free or overall survival and can frequently be used to expect the nature of tumor (64) .

1.7.1. Hormonal receptors:

In breast cancer, steroid hormone receptors include estrogen receptor and progesterone receptor consider as important prognostic factors and predictive markers for response to endocrine therapy in breast cancer treatment (65).

About 70% of BC are hormone receptor positive tumors, breast cancer HR+ generally has a favorable prognosis, but breast cancer HR- has poor prognosis (66).

Estrogen receptor (ER) is one of nuclear receptor family which has been activated by 17-β-estradiol and there are two isoform of ER i.e. ERα and ERβ that encoded by genes located in different chromosome (67). About 65% of breast cancer women less than 50 years old and 75% of older women with BC are estrogen receptor positive (68).

Progesterone receptor also plays an important role in breast cancer development, it is one of nuclear/steroid receptor family of ligand – dependent transcription factors that regulate gene expression which control target tissue development ,proliferation and differentiation; in addition to endocrine –based cancer pathological processes (69).

1.7.1.1. Cellular mechanism of estrogen in development of breast cancer:

Estrogen is essential for the normal growth and growth of breast tissue and it also considered a major risk factor for breast cancer when exposed to high level of estrogen (70).

Estrogen is phenolic steroid hormone produced by ovaries and secreted into blood and is also produced peripherally in cells expressing the aromatase enzyme, it is lipophilic nature allows it to pass through cell membranes ; estradiol(E2) , oestrone(E1) and oestriol(E3) are the main physiological estrogen form (71).

Estrogen signals through its two receptors (ERα and ERβ),but only ERα is essential for the development of breast and activates proliferative signaling in the normal and breast cancer ,whereas ERβ generally antagonize ERα in the breast(67).

There are two mechanisms involved in carcinogenesis of estrogen:

A- Receptor dependent:

estrogen receptor (ER) mediated stimulation of breast cell proliferation through stimulates transcription of genes involved in proliferation of cell with a concomitant enhanced rate of mutations (during mitosis for each cell cycle a new DNA synthesized and there is point of mutation if not repaired as process continues can lead to development of breast cancer) (72).

B- Receptor independent:

Because estrogen contains aromatic A-ring, it under go oxidative metabolism by CYP1B1 which present in breast tissue result in formation of unstable genotoxic metabolite 3,4-quinones which form adducts with amino acid bases of DNA (adenine and guanine) can result in point of mutation (73).

Also the unstable metabolite of estrogen (2,3- and 3,4-quinones) can undergo redox cycle that is reduced by cytochrome P450 reductase to semiquinones and produced ROS that cause DNA oxidative damage (72).

1.7.2. Human epidermal growth receptor 2:

Human epidermal growth receptor2 (HER2) is a member of tyrosine kinases receptors which is member of epidermal growth factor (EGF) that forming homodimers or heterodimers with other HER receptor cause activation of the several intracellular signaling pathways which involved in enhanced cell differentiation, growth and cell survival (74) .

About 20%-30% of breast cancer women have over expression of HER2, and more spread between younger female; breast cancer progresses rapidly in HER2-positive women; survival rate in HER2-positive female is 20% less than women with HER2 negative (75).

1.7.3. Tumor nodes and metastasis classification of breast cancer:

In the first half of 20th century cancer staging system was recognized and led to the publication of the first Union of International Cancer Control (UICC) staging system in the 1950s and then followed by the Federation of International of Gynecologists and Obstetricians (FIGO) and American Joint Committee for Cancer (AJCC) (76).

This classification is based on clinical and pathological data and it is necessary to known the stage of cancer that indicate the extent of disease and provides indications for treatment and prognosis(77).

The UICC Tumor nodes and metastasis (TNM) classification considers the size of tumor (T) describe the location and size of tumor : T0—no evidence of tumor; T1—the invasive part of the tumor has size ≤20 mm and is carcinoma *in situ*, confined within the ducts or lobules of breast tissue; T2—the invasive part of the tumor is 20–50 mm; T3—the invasive part >50 mm; and T4—the tumor has grown into the chest wall and skin with signs of inflammation, lymph node status (N) : N0—no cancer cells are found in the lymph nodes; N1—the cancer has spread to three nodes; N2—the cancer

has spread to four to nine nodes; and N3—the cancer has spread to ≥10 node and distant metastasis presence (M) : M0—the cancer has not metastasized; and M1—there is evidence of metastasis to another part of body (78, 79).

1.7.4. Stages of breast cancer:

Breast cancer staging plays an important role in local and systemic treatment determination and provides prognostic information, and it is defined pathologically by pathological investigation of the primary tumor and regional lymph nodes after surgical treatment (80).

There are four stages of breast cancer depended on TNM system ,that included stage 0 which indicates apre-cancerous either ductal carcinoma in situ or lobular carcinoma in situ ,stage 1-3 are within the breast or original lymph node and stage 4 which consider a metastatic cancer that has the worst prognosis (81).

1.7.5. Histological grade:

Histological grade means a measure of the differentiation of the breast cancer can be considered a central evaluation for each breast cancer that newly diagnosed and well-established factor in prediction of breast cancer and correlate with response to chemotherapy, grading can be describe the breast cancer cell as low grade that mean well differentiate, intermediate grade that mean moderately differentiated and high grade which mean poorly differentiated (82).

1.7.6 Tumor markers:

Tumor markers can be defined as a substance produced by the tumor itself or by the host in response to a tumor and can be found in blood (serum), urine or body tissues. It is mostly used for determination the

presence of tumor and for evaluating the disease progression status after initial chemotherapy and radiotherapy (83).

There are many categories of breast cancer markers and the most common tumor marker used are CA 15.3, CA 27.29 and CEA. Using of these tumor marker for prediction of BC risk has been usually incorporated in clinical routine but its association with prognosis of breast cancer has not been demonstrated (84, 85).

1.7.6.1 Cancer antigen 15.3:

Cancer antigen 15.3 (CA 15.3) is soluble member of mucin glycoprotein (MUC-1),mucins are over expressed in many adenocarcinomas in glycosylated form that shed into the circulation, CA 15.3 most widely used serum marker in female with breast cancer (83, 86).

Elevation level of CA 15.3 can be detected in early stage of breast cancer but its value is controversial due to lose of tumor and organ specificity and sensitivity but it was mostly reported in patient with advanced or metastases breast cancer, the main purpose from measuring CA 15.3 is for therapy response monitoring along with other clinical feature and radiological imaging (87, 88).

1.7.6.2. Carcinoembryonic antigen:

Carcinoembryonic antigen **(**CEA) is a member of family related cell surface glycoproteins as a kind of cell adhesion molecules and most widely measured and considered as tumor marker for colorectal, lung, gastrointestinal and breast cancer. Elevation the level of CEA in blood associated with metastasis of tumor (83, 86).

1.8. Screening and diagnosis of breast cancer:

Breast cancer is the very common cause of women cancer death in the world and the early cancer diagnosis being essential for an improve prediction of disease and plan for beast treatment and increase survival (89).

The first step in breast cancer diagnosis is clinical examination that include breast examination ,chest region ,and regional lymph nodes in axilla and then imaging breast cancer and finally fine needle biopsy to distinguish the benign from malignant tumor (81).

1.8.1. Mammography:

Mammography is two-dimensional image , depends on the identification of morphological result that are suspicious for breast cancer ,it is the basis for screening and diagnosis of breast cancer (89).

Despite mammography still remains the most widely used method for breast cancer detection but in many studies have revealed a limitation of mammography (90). The mammographic diagnostic accuracy depended on many factors such as breast structure and breast tissue density that mean woman with higher breast density is associated with decrease sensitivity for mammography (91).

1.8.2. Magnetic resonance imaging:

Magnetic resonance imaging (MRI) is well-known technique in breast imaging with several clinical indications for example preoperative staging, neoadjuvant therapy response assessment, scar vs. recurrence and checking of high-risk patient (89).

Magnetic resonance imaging (MRI) is a technology based on the use of radio wave and magnetic field, it can differentiate lesion and breast

abnormalities, so intravenous administration of a gadolinium-containing contrast material is needed to exclude a cancer (92).

If woman has tattoos or permanent make up, she should tell the medical staff and woman who has implantation devices such as intravascular stents or metal screws can have a breast MRI 6 weeks after implantation while pregnant woman is contraindication unless risk vs. benefit (93).

1.8.3. Ultrasound breast imaging:

Ultrasound (US) is extensively used for diagnosis of cancer, screening for additional disease in the breast and localization, assessment of the axilla (89).

Ultrasound (US) consider as an important alternative diagnostic tools to mammography and MRI due to no radiation, faster imaging, higher sensitivity and accuracy and lower cost ,so it become a routine procedure for diagnosing and detecting lump (94).

1.8.4. Positron emission tomography:

Positron emission tomography (PET) is a molecular imaging of breast cancer that depends on using a positron – emitting radionuclide such as Floro-deoxyglucose (FDG), it is a highly sensitive technique and valuable for specific clinical application such as systemic staging of newly diagnosed locally advanced disease, monitoring of treatment response in patient with metastatic disease and detection of suspected disease recurrence (80, 95).

Floro-deoxyglucose (FDG) is a glucose analog that is transported via glucose transporters into cells, phosphorylated by hexokinase and metabolically trapped in tumor cells so the PET reflect to the metabolic activity of the malignant tissue (96).

1.9. Therapeutic approach of breast cancer:

The treatment of breast cancer includes the treatment of local disease with surgery, radiation therapy or both in order to reduce the chance of local recurrence and the risk of metastatic spread, in addition to systemic treatment with chemotherapy, endocrine or hormonal therapy, biological therapy, or compensation of these (97).

The selection of local or systemic treatment depends on several prognostic and predictive factors such as histology of tumor ,clinical and pathological characteristics of the tumor, status of axillary lymph node and tumor hormone receptors (98).

1.9.1. Local treatment of breast cancer:

Local therapy mean removes the cancer from a limited area, such as the breast, chest wall and lymph nodes in the underarm area, it is also helps ensure the cancer does not come back in that area, it is combination of surgery and radiation that applied in early stage of breast cancer before metastasis (99).

1.9.1.1 Surgery:

The main goals of breast cancer surgery are to remove the cancer and determine its stage. Surgical treatment involve breast conserving surgery and mastectomy(100).

In the breast conserving surgery (BCS) which known as partial mastectomy or lumpectomy only the cancerous tissue with a rim of normal tissue (tumor margin) is removed, and this process should be followed by radiation to the breast (99).

While the mastectomy involves total removal of breast ,radical mastectomy involve the remove the breast with dissection of axillary lymph node ,and underlying chest muscles (pectoralis muscles) (101).

Today the modified radical mastectomy involves removal of part of breast and the axillary lymph node without removal of muscle, so this procedure is more famous. The dissection of axillary node is to identify the number of positive nodes and consider an important prognostic factor and staging of breast cancer (102).

1.9.1.2. Radiation:

Radiation therapy is considered as essential component of the breast local treatment, which most frequently starts after a lumpectomy or mastectomy, about 4 to 6 weeks after surgery or chemotherapy, radiation begins (99).

1.9.2. Systemic therapy of breast cancer:

Systemic therapy represents one of the bases in the breast cancer treatment that aims to get rid of cancer cells that may have spread from the breast to other parts of the body and includes drugs therapies that travel through the body, some of these drugs are given by vine and some are given as pills. Because systemic therapy mostly used in addition to breast surgery are often called adjuvant therapy(103, 104). Systemic therapy includes:

1.9.2.1. Chemotherapy:

It is type of systemic breast cancer therapy that travels through bloodstream to affect and treat all parts of the body that involved using cytotoxic drug for stopping the growth and proliferation of malignant cells and consider as adjuvant as well as neoadjuvant therapy (104).

Chemotherapy has non-selective action for this reason it can affect normal cells and lead to sever side effect such as nausea ,vomiting ,diarrhea , anemia and hair loss and can also damage the bone marrow, heart and kidney so in some cases the patient may be dead (105).

In some cases, use combination of drug that have different cytotoxic mechanism, or increase the dosage of drug or using chemotherapy with other therapies such as immunotherapy as combination to overcome the multidrug resistance (MDR) that develop by the cancer cell such as reduce the up take ability of cancer cell or reduce expression of some protein that guide the agent to cell (106, 107).

1.9.2.2. Immunotherapy:

Cancer immunotherapy is classified either to active immunization which is specific stimulation of the immune system or passive immunization which is passive transfer of humoral or cellular materials such as tumor-specific antibody or adoptive T cell that inhibit or kill cancer cell (108).

Immunotherapy can be enhanced by using immune modulatory agent (IMA) which is either stimulate the immune system nonspecifically, or prevents the immune system inactivation by immunosuppressive down modulate mechanisms (109).

1.9.2.3. Hormonal (endocrine) therapy:

Estrogen and progesterone have important role in the regulation of breast tissue growth and differentiation, both steroid hormones are mainly generated in the ovaries and exert their cellular consequences by binding to and activating specific nuclear receptors (ERs and PRs) (110).

About 60%of premenopausal and 75% of postmenopausal female estimated with breast cancer hormone (estrogen and /or progesterone) receptor – positive tumors (111) ;Hormonal therapy is an effective and non-toxic therapy play a critical role in reducing the risk of recurrence of the primary cancer and it is classified according to mechanism of action (103, 112).

1.9.2.3.1. Hormonal therapy that directly target the estrogen receptors:

These types of anti-estrogenic therapy act by interfere with estrogen receptor and include:

A. Selective estrogen receptor modulator:

Tamoxifen is a SERM with mixed antagonistic properties in breast cancer and brain and agnostic properties in bone, liver and uterus. After surgery the use of tamoxifen for (5-10 years) in ER-positive early breast cancer can reduce the annual recurrence rate by 41% and annual mortality rate by 34% (111).

Tamoxifen may be used in pre- and postmenopausal females; the main side effect includes hot flushes, night sweats, vaginal discharge, increased risk of thrombotic event and vaginal bleeding should be evaluated due to increased risk of endometrial cancer (112).

B. Selective estrogen receptor degrader:

The only available agent of this class is Fulvestrant which is highly insoluble compound with poor oral bioavailability and intravenous short half-life , therefore must be given intramuscularly (113).

1.9.2.3.2. Hormonal (endocrine) therapy that deplete estrogen:

In premenopausal women, estrogen synthesized in the perifollicular ovaries via the hypothalamic-pituitary-gonadal axis while in postmenopausal women, adipose tissue is the main site of estrogen synthesis so therapy that depleted estrogen classified according to site of estrogen synthesis (113).

A. Gonadotropin releasing hormone agonist:

Suppression of estrogen in premenopausal females is achieved by radiation to the ovaries or surgical oophorectomy and by using GnRHa such as goserelin (zoladex) that used monthly as depot injection, act by decreasing the gonadotropins and sex hormones production by the gonads so they lower endogenous estrogen production (112, 114).

B. Aromatase inhibitors:

In postmenopausal women, estrogen is no longer released by ovary and is mostly synthesized from nonglandular sources by enzyme called aromatase that can be found in many tissue such as fat ,liver, muscle and in breast cancer cell (110);so aromatase inhibiter will reduce both endogenous estrogen levels and breast cancer incidence ,also reduce the endometrial cancer risk (115).

The first 2 generations of aromatase inhibitors (AIs) were effective in treating breast cancer but caused significant side effects due to their inhibit other steroid hormones such as cortisol and aldosterone (116);while third-generation AIs has increased specificity for aromatase and are either categorized as steroidal (type I) such as exemestane which lead to irreversible inhibition of enzymatic activity or as nonsteroidal (type II) include anastrozole and letrozole that are reversible competitive inhibitors (117).

1.10 Anastrozole:

Anastrozole is type II potent and selective non-steroidal aromatase inhibitor that was patented in 1987 and was approved for medical use in 1995 and approved by the U.S (FDA) as endocrine drugs of choice for treatment and prevention of breast cancer metastasis (118)

The use of anastrozole (1 mg) once daily dose for about five years resulting in impressive reduction in the recurrence and mortality rate of breast cancer (119);but because of interindividual variability that may change the tolerability and efficacy of anastrozole , recent studies have reported that 1 mg anastrozole daily dose may not benefit all patient with breast cancer (120) .

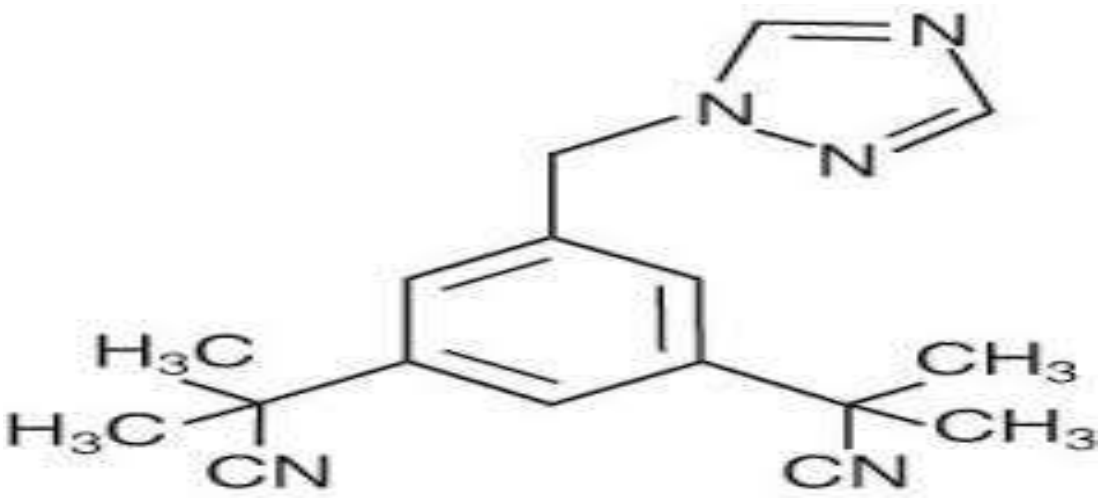

Figure (1.3) :structural formula of anastrozole (121)

1.10.1. Pharmacokinetic of Anastrozole:

Oral administered anastrozole is rapidly absorbed and (T_{max}) is typically reached within 2 hours of dosing under fasting condition while coadministration with food reduces the absorption rate (120); the steady state concentration of anastrozole at 7 days after once daily dosing; The anastrozole protein binding in plasma is 40% and apparent volume of distribution is (98.4L), the elimination half –life of anastrozole is approximately 50 hours and about 85% of anastrozole eliminated by hepatic metabolism so 10% of the administered dosage is eliminated in urine unchanged (122).

1.10.2. Pharmacodynamics of Anastrozole:

Anastrozole is a derivative of benzotriazole that has inhibitory effect on aromatase that suppresses serum oestrone, oestradiol and oestrone sulphate which is the metabolite of estrogen without significantly affecting the concentration of corticosteroid, gonadotropins and adrenal steroid (123).

Aromatase inhibition is primarily achieved by anastrozole that decrease concentration of E1 by 81-87%,E2 by 84-85% and E1S by 94% (124).

1.10.3. Mechanism of action of Anastrozole:

The anti-estrogenic effect of anastrozole is exerted via selective and competitive inhibition of the aromatase enzyme found mainly in the adrenal gland, liver, and fatty tissue (120).

Aromatase is the main enzyme that converts produced androgens into estrogen (110);So anastrozole competitively inhibit of estrogen biosynthesis and which effectively suppress the circulating level of estrogen and subsequently the growth of hormone receptor-positive cancer (124).

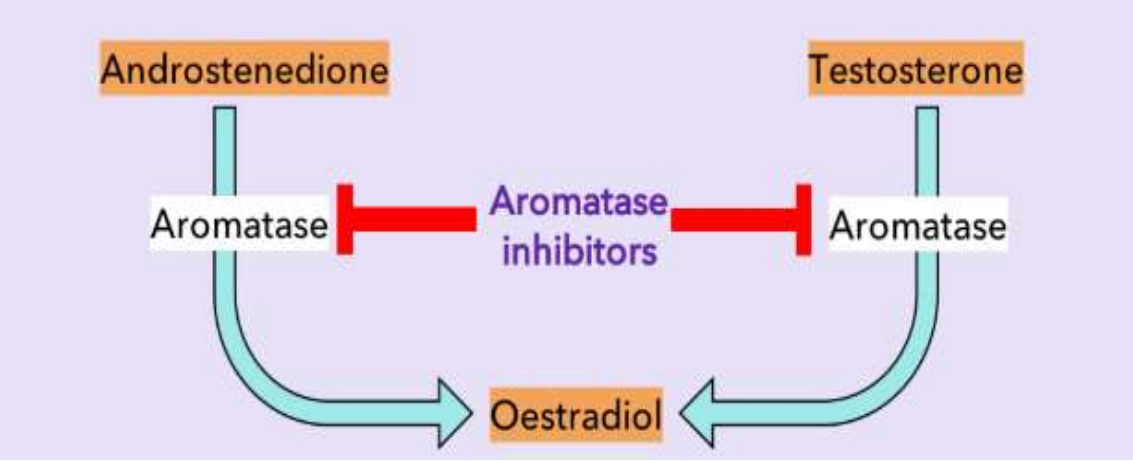

Figure (1.4): Mechanism of action of aromatase inhibitor (anastrazole).

1.10.4. Medical uses of Anastrozole:

- **Breast cancer:**

Anastrozole used in treating postmenopausal women diagnosed with hormone-receptor-positive early breast cancer, after surgery or possibly chemotherapy and radiation to reduce the risk of the cancer recurrence (123); for postmenopausal women diagnosed with advanced-stage or metastatic hormone-receptor-positive breast cancer which the first choice drug because it is associated with a stronger activity and better general tolerability compared with TAM(122);and premenopausal females in which synthesis of estrogen by the ovary is not adequately suppress by anastrozole, so either use LHRH agonist (goserelin) or make surgical ovarian ablation along with anastrozole that cause pituitary gland stimulation lead to transient increase gonadotropin releasing followed by sustained reduction in release of gonadotropin lead to ovarian estrogen suppression (125).

- **Early puberty:**

Anastrozole is used in combination with the antiandrogen bicalutamide in the treatment of peripheral precocious puberty due to familial limited precocious puberty (testotoxicosis) (126).

- **Infertility:**

In male, anastrozole is used to treat infertility due to azoospermia through increase testosterone, decrease level of estrogen and inhibit the testosterone metabolism in periphery in order to reduce the estrogenic effect on spermatogenesis(127).

In female, anastrozole induce ovulation via block estradiol synthesis, reduce estradiol feedback on the hypothalamus-pituitary and elevate FSH production (128).

- **Endometriosis:**

Administration of anastrozole has a significant role in pelvic pain reduction and lesion size so that aromatase inhibitor may be consider as successful treatment of endometriosis(129).

1.10.5 Adverse effect of Anastrozole:

Anastrozole is particularly well tolerated by most patients with breast cancer. But, it may also have associated with adverse effect(130). The most common (more than 10% incidence) adverse effect of anastrozole include hot flash, asthenia, nausea and vomiting, osteoporosis, arthralgia, bone fractures, back pain and peripheral edema (131).

Physiologically, postmenopausal females at high risk of osteoporosis due to physiological decrease of estrogen concentration and the risk is increased in breast cancer postmenopausal women who use anastrozole which decrease estrogen level by inhibited aromatase enzyme that convert androgens to estrogen which play an important role in regulation of bone (132).

Anastrozole induce postmenopausal osteoporosis can be treated and prevented by the use of bisphosphonate such as zoledronic acid which inhibited osteoclastic activity which is use every 4 to every 12 week (133).

1.10.6 Anastrozole drug – interaction:

Anastrozole is mainly metabolized by CYP3A4,so it is very important to observe the drugs that can cause alteration in CYP3A4 activity which act as either enzyme inhibitors or inducers (134).

Table (1.2): Anastrozole drugs interaction (134, 135)

Drugs	**Consequence of co-administration**
Antimicrobials(clarithromycin, erythromycin),antifungals (ketoconazole,voriconazole, fluconazole),antivirals (ritonavir,saquinavir), immunosuppresants (cyclosporine)	Increase Anastrozole plasma level Due to inhibit CYP3A4 activity
anticonvulsants (carbamazepine, phenytoin, phenobarbital) and antimycobacterials (rifampin)	Decrease Anastrozole plasma level Because of induce CYP3A4 activity

1.10.7. Metabolism of Anastrozole:

Liver is the major anastrozole metabolism site through hydroxylation ,N-dealkalytion by CYP3A4 and glucuronidation by UGT1A4 to create three major metabolites (124, 136); anastrozole also can undergo glucuronidation directly into anastrazole N-glucuronide (137).

Triazole is the major circulating anastrozole metabolite which is inactive in addition to a glucuronide conjugate of hydroxyanastrozole and glucuronide of anastrozole are two other anastrozole metabolites that found in plasma and urine (138).

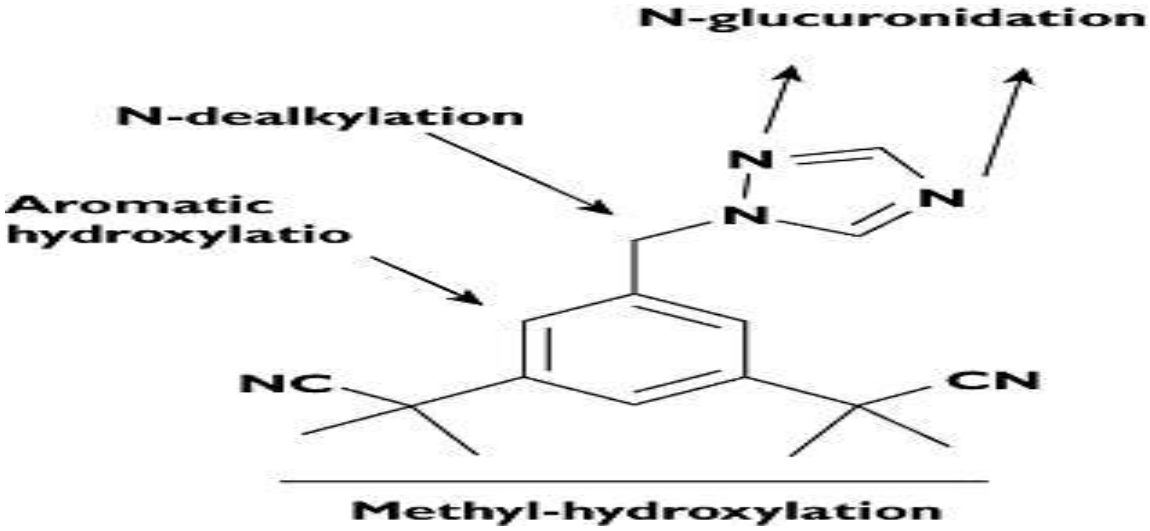

Figure (1.5): Potential site of anastrozole metabolism(139)

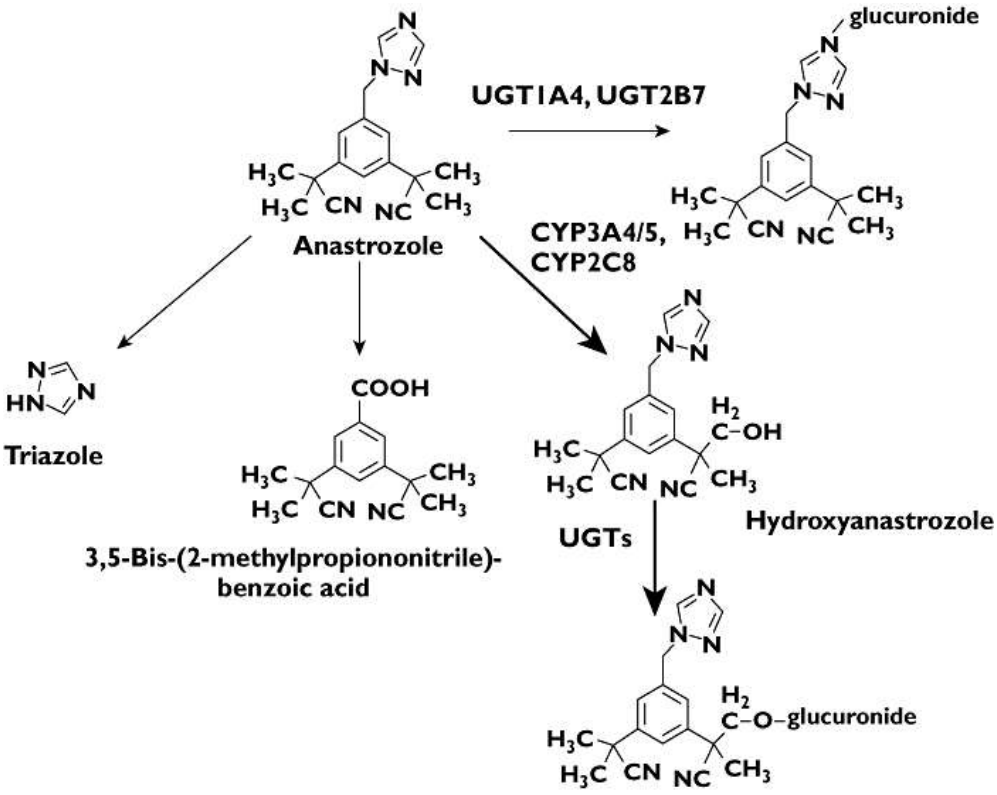

Figure (1.6): Metabolic pathways of anastrozole (138)

1.10.8 Genetic polymorphism of Anastrozole metabolizing enzymes

Single nucleotide polymorphism (SNPs) are the most common type of human genetic variation which are differences in sequence of DNA between individuals or populations, the changes of single base occurring at frequency of 1% within population (140).SNPs either passed from parents to children as DNA sequences in this case called genetic mutation which is most likely associated with diseases or caused by external factors such as radiation or viruses that called mutation (141). SNPs can be either synonymous (silent SNPs) which do not change amino acid or non-synonymous (missenses SNPs) that cause amino acid change and have harmful effect. these two types of SNPs happened in coding region of genes (exon) , single nucleotide polymorphism can be also occurring noncoding (intron) (142).

Genetic Polymorphisms are an important factor in the human susceptibility to disease such as cancer for example mutation of breast cancer susceptible gene (*BRAC1* and *BRAC2*) (30); and in the response and adverse effect of drugs such as the interindividual variation in response to anastrozole due to metabolism-related genes polymorphisms (*CYP3A4* and *UGT1A4*) that may cause drug toxicity or drug efficacy and its metabolites decrease according to whether there is an active or inactive metabolite (143).

CYP3 human family consist of one subfamily only, *CYP3A*, which is located on chromosome 7q22.1. that comprises the four genes CYP3A4,3A5,3A7 and 3A43. CYP3A4 is predominantly found in the liver and also present in many organs and tissue in body where it play important role in metabolism (144).

Anastrozole undergoes phase I N-dealkalytion to create triazole and hydroxylation to produce hydroxyanastrozole thought CYP3A4 (124, 136); so the polymorphisms of *CYP3A4* can effect on concentration and activity of Anastrozole and its metabolites according to type of SNPs,

*CYP3A4*22* (G>A) rs35599367 is the reference SNP of *CYP3A4* gene which it is allele name 3A4*22 and the changing of SNP is from Guanine (wild) to Adenine (mutant) that found in studies decrease enzymatic activity and expression in patients with cancer, anastrazole metabolized to inactive metabolite ,so when activity and expression of (CYP3A4) enzyme decreased as a result of mutation ,this lead to increase duration of action and response to anastrozole (145, 146).

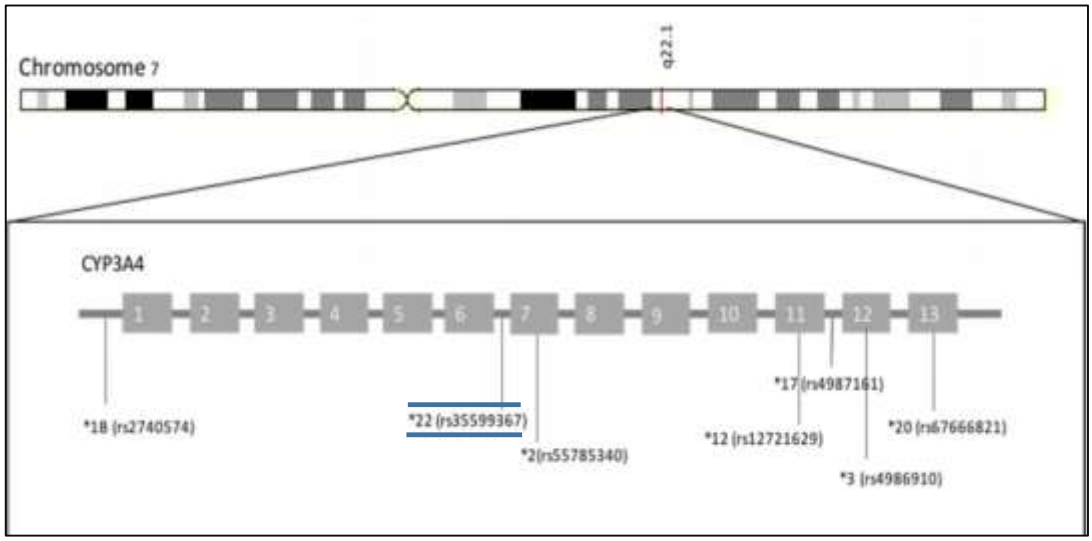

Figure (1.7): Chromosome 7 with single nucleotide polymorphism of *CYP3A4* gene (147)

CYP3A5 and CYP2C8 also play a role in anastrozole metabolism but to lesser extent, the polymorphisms of genes encode these enzymes expressed as inactive form have a small effect on anastrozole metabolism in vivo and vitro (136).

UDP-glucuronosyltransferases are a family of drug metabolizing enzymes contributing to hepatic drug metabolism, UGT involve four families have been identified in humans involve UGT1, UGT2, UGT3 and UGT8 each one involves many subfamilies which distributed in liver and other extrahepatic organs (148).

Anastrozole undergoes glucuronidation by UGT1A4 (which is located on chromosome (2q37.1 kb.) after phase I hydroxylation into hydroxyl anastrozole and also undergo glucuronidation directly to produce anastrozole N-glucuronide (137).

The UGT1A family conjugative activity can be affected by the presence of single- nucleotide polymorphism (SNPs) which have been identified in coding and noncoding region. UGT1A4*2 (C>A,T) Rs6755571 is the reference SNP of UGT1A4 gene which it is allele name 1A4*2 and the changing of SNP is from Cytosine (wild) to Adenine or thiamin (mutant) that found in studies decrease the glucuronidation activity for many drugs (138, 145).

1.11 Aims of study:

1- To investigate the distribution of genetic polymorphism in phase I metabolizing enzyme CYP3A4 by using Amplification refractory mutation system – polymerase chain reaction (ARMS-PCR) of CYP3A4*22 (G>A) rs35599367 and phase II metabolizing enzyme UGT1A4 via Allele Specific Polymerase Chain Reaction (AS-PCR) for UGT1A4*2 (C<A, T) rs6755571 in Iraqi breast cancer women on anastrozole.

2. To study the effect of genetic polymorphism in anastrozole metabolizing enzyme (CYP3A4 and UGT1A4) on drug response through measuring serum level of estradiol (E2) and tumor marker (cancer antigen CA15.3) in addition to evaluate development of anastrozole side effect (arthralgia) according to Brief Pain Inventory (BPI).

Chapter two

Patients and Methods

2. Patients and Methods

2.1 Materials

2.1.1 Chemicals and kits

The specific chemicals and kits used in this study with their manufacture and origin are listed in Table (2.1)

Table (2.1): Chemicals and kits used in this study with their manufacture and origin.

Chemicals and kits	Manufacture/origin
Absolute Ethanol	Bdh/German
Accupower PCR PreMix	Bioneer/korea
Agarose DI low EEO	CONDA pronadisa/spania
CA15.3 Kit	Biomerieux/France
DNA ladder size 1000-1500 bp	Bineer/korea
Estradiol (CLIA)Kit	Mindray/China
Ethidium bromide	Intron/Korea
G-DEX™ IIb for blood genomic DNA extraction kit.	Intron/Korea
Isopropanol (2-propanol)	SRL/India
Nuclease free water	Promega /USA
Primers	Macrogen/Korea
Trise- Borate –EDTA (TBE) Buffer 10X	Intron/Korea

2.1.2 Instruments

Many instruments used in the genetic and biochemical analysis of samples in this study with their manufacture and origin are listed in Table (2.2)

Table (2.2): Instruments used in this study with their manufacture and origin.

Instruments	Manufacture/origin
Autoclave	LabTech / Korea
Centrifuge	Hettic/Germany
Digital camera	Canon/ England
Distillator	GFL/Germany
Electrophoresis apparatus	Techne me/England
Freezer (-20 ˚C)	Concord /Lebanon
High speed Centrifuge	Hettic/Germany
Hood	LabTech /Korea
Hot plate stirrer	LabTech /Korea
Micropipettes	SLAMED/UK
Mindray CL-series ChemiluminescenceImmunoassay Analyze	Mindray/China
MiniVIDAS	BioMérieux/France
Nanodrop	Bio Drop/England
Refrigerator	Concord /Lebanon
Sensitive balance	DENVER/Germany
Thermos cycler (PCR apparatus)	TECHNE / UK
UV-trans illuminator	Syngene /England
Vortex	HumanTwist / Germany
Water bath	LabTech /Korea

2.2 patients

2.2.1 Study samples

This study was a cross-sectional observational study carried out at Imam AL-Hussein Medical city/oncology center in kerbala and laboratories of College of Pharmacy / University of Kerbela, during the period from September 2019 till February 2020. The protocol of the study was approved by the ethical committee of College of Pharmacy / University of Kerbela, and informed signed consent form was given to each subject after explaining the nature and purpose of study. The study was conducted on 100 females with (ER and /or PR) positive breast cancer.

All females included in this study with age ranged from (31 to 75) years mean (53.14) were taking anastrozole tablet 1 mg per day orally at least 6 months after using anastrozole (149).

They were excluded from study if they had started anastrozole therapy simultaneously with either adjuvant chemotherapy or adjuvant radiation therapy (or both) or women who taking drugs that affect the activity of CYP3A4 and/or UGT1A4 enzyme (inducer or inhibitors) were excluded.

In addition, patients with previous history of hepatic disorder or liver surgery were excluded from the study.

2.2.2 Clinical data collection

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

The data were obtained from the medical records of consenting females and from the patients themselves and these includes: age, weight, academic achievement, workplace, marital status, breast feeding, date of first menarche and last menopause, family history of breast cancer and number,

date of breast cancer diagnosis, site (left,right,bilateral), type of breast cancer ,stage and grading, immunohistochemically status (ER,PR,HER2),surgery, chemotherapy, radiation, presence of arthralgia, liver disease or any other diseases, dose of anastrozole ,time on anastrozole therapy and duration, other drugs used.

2.2.3 Sample collection

Blood samples were taken from eligible females who had signed informed consent.

About 6 ml of venous blood were withdrawn from all females contributed in this study, (2 ml) of blood was placed in EDTA- tube for genetic testing, (3ml) was placed in gel tube, and serum was obtained after centrifugation of blood at 5000 rpm for 10 minutes, were it used for measuring estradiol (E2) and tumor marker (CA15.3) level.

2.3 Methods

2.3.1 Molecular analysis

2.3.1.1 DNA Extraction

The DNA extraction was conducted at College of Pharmacy / University of Kerbela / Laboratory of Molecular Biology. DNA genome was extracted from blood sample as started by protocol (G-DEX^{TM} IIb / Intron, Korea) for blood genomic DNA extraction kit. The following method is suitable for DNA extraction from whole blood

1. 2 ml blood was centrifuged at 2000 rpm for 1 minute to produce buffy coat.
2. 300 µl of the formed buffy coat were added to 1.5 ml epindroff tube, then 900 µl of RBC lysis solution was added. The mixture was mixed thoroughly by vortex and incubated for 5 minutes at room

temperature. The tube was inverted again at least once through the time of incubation.

3. The epindroff tube was centrifuged at 10,000 rpm for 1minute, the supernatant was removed except the white cell pellet and only about 50-100 µl of the remnant was remained.
4. The tube was vigorously vortexed to resuspend the cells.
5. 300 µl of cell lysis solution was added to the resuspended cells and then pipetting up and down to lyse the cells.
6. The mixture was chilled to room temperature .100 µl of protein precipitation buffer (ppt) was added to the cell lysate and then vortexed vigorously at high speed for 20 second.
7. The mixture in epindroff tube was centrifuged at 13,000-16.000 rpm for 3-5 minutes. The precipitated proteins were formed a tight white pellet
8. 300 µl of supernatant containing DNA was transferred into a 1.5 ml epindroff tube. 300 µl of 100% Isopropanol(2-propanol) was added and mixed by inverting gently several time.
9. Mixture was centrifuged at 13,000 -16,000 rpm for 1 minutes, the DNA was observed as a small white pellet.
10. The supernatant was poured off and the tube was drained briefly on clean absorbent paper.1 ml of 70% Ethanol was added to sample and the tube was inverted several time to wash DNA, then the tube centrifuged at 13,000-16,000 rpm for 1 minutes. Ethanol was poured off carefully.
11. The tube was inverted and drained briefly on clean absorbent paper and allow to air dry for 10-15 minutes.
12. 150 µl DNA rehydration buffer was added.
13. DNA was rehydrated by incubating at 65 °C for 30-60 minutes.
14. DNA was collected and long – term stored at -20 °C (deep freezing) (150).

2.3.1.2 Quantitation of DNA by spectrophotometric method

Purity and concentration of extracted DNA were measured by using Nano-spectrophotometric method (Nanodrop). The micro detector was cleaned up from blank, then 1μL of samples was applied on the Nanodrop micro detector; the concentration and A260/A280 ratio of DNA were documented from the instrument.

2.3.1.3 Polymerase Chain Reaction

Polymerase chain reaction is the process that involve DNA regions replication through using an enzyme DNA polymerase which permits amplification of desired DNA fragments from one molecules to several million copies; many requirements need to proceed PCR reaction such as two primers which are complementary to the target DNA one primer bind to each side of DNA and between them the desired DNA sequences will be amplified. There are three main steps for any PCR that are cycled about 25-45 times, which include:

A. Denaturation: this step occurs at 94-95°C and requires decoding of double strand DNA into single strands.
B. Annealing: this step occurs at 55-65°C, the reaction begins by stiffen the pair of short oligonucleotide sequences to the ends of the strands of DNA template.
C. Extension: this step occur at 72-74°C, it needs extension of the primers to form new strand complementary to the template table and this happen in the presence of DNA Taq polymerase.(151, 152)

There are several polymerase chain reactions (PCR) techniques differ in the principle, in this study use two techniques:

A. Amplification refractory mutation system (ARMS-PCR): refers to mutation detection method based on specific PCR primers and also called amplification of specific alleles, in which specific set of

primer; two forward primers (forward wild and forward mutant type) and two reveres primers which are complementary to the DNA template which include the region to be amplified (153).

B. Allele specific polymerase chain reaction (AS-PCR): an interaction based on design a specific primers that fuse with the DNA templet (sample the should be amplified) and the 3' end of primer contain the SNP (154).

In addition to DNA templet and primers there are other component which are essential for both techniques include DNA polymerase (*Taq* polymerase), deoxy nucleotide tri-phosphates and buffer solution (151, 152).

2.3.1.4 Primers design

Polymerase chain reaction was performed using a specific primer pairs designed for *CYP3A4*22* (G>A) (rs3559967) and *UGT1A4*2* (C>A, T) (rs6755571) by **Asst. Prof. Dr. Hassan Mahmood Mousa Abo Almaali** using primer blast software and depending on https://www.ncbi.nlm.nih.gov/ websites.

The primer sequences that were utilized for amplification analysis of CYP3A4 and UGT1A4 genes for SNPs identification are shown in Tables (2.3) and (2.4) respectively.

Table (2.3): Primers sequences of *CYP3A4*22* (G>A) (rs35599365) genetic polymorphism.

SNPs	Primer sequence(5 '->3 ')	Product size	Reference
O-F	AGGGGTCTTGTGGATTGTTGA	474 bp	Current study
O-R	CACCTGTCTTGAGCCCCTTAG		Current study

I-F allele G	GATGCAGCTGGCCCTACG	215 bp	Current study
I-R allele A	AGTGTCTCCATCACACCCAGT	297 bp	Current study

Table (2.4): Primers sequences of *UGT1A4*2* (C>A, T) (rs6755571) genetic polymorphism.

SNPs	Primer sequence (5 '->3 ')	Product size	Reference
F-C	CTCCTCCTCAGTGTCCAGC	242 bp	Current study
F-A	GCTCCTCCTCAGTGTCCAGA	243 bp	Current study
F-T	GCTCCTCCTCAGTGTCCAGT	243 bp	Current study
Reverse primer	TGAGTGTAGCCCAGCGTAAC		Current study

lyophilized primers were dissolved with a specific volume of nuclease free water to obtained a primary concentration of (100 Pmol/µL) (as stock solution). Table (2.5) and (2.6) represent the volume of nuclease free water to each primer to obtain 100Pmol/µL.

Table (2.5): The volume of nuclease free water added to each primer of *CYP3A4*22* (G>A) (rs35599367) to obtain 100 Pmol/µL.

Primers of *CYP3A4*22* (rs35599367)	Volume of nuclease water added (µL)
O-F	300
O-R	300
I-F allele G	320
I-R allele A	300

Table (2.6): The volume of nuclease free water added to each primer of *UGT1A4*2* (C>A, T) (rs6755571) to obtain 100 Pmol/µL

Primers of *UGT1A4*2* (rs6755571)	Volume of nuclease water added (µL)
F-C	320
F-A	300
F-T	300
Reverse primer	300

For working solution, 10µL of stock solution was diluted with 90 µl of nuclease free water to get (10 pmol/ µL) as a final concentration (working solution), the primer kept in -20 °C until farther use.

2.3.1.5. Polymerase chain reaction optimization conditions

Optimization of polymerase chain reaction was attained after several trails.

2.3.1.5.1 Optimization of *CYP3A4*22* (G>A) (rs35599367) genetic polymorphism conditions.

*CYP3A4*22* (G>A) (rs35599367) optimization of PCR conditions was prepared by using:

- Different volumes of primers (1µL, o.5 µL)
- Different annealing temperatures (55.7 °C ,60 °C)
- Different volumes of DNA sample (3 µL,5 µL)

The best results of this SNP was obtained in the following conditions:

A. 0.5 µl outer forward primer

B. 0.5 µl outer reverse primer

C. 1 µl inner reverse primer

D. 1 µl inner forward primer

E. 5 µl DNA sample

F. 12 µl nuclease free water

2.4.1.5.2 Optimization of *UGT1A4*2* (C>A, T) (rs6755571) genetic polymorphism conditions.

*UGT1A4*2* (C>A, T) (rs6755571) optimization of PCR conditions was prepared by using:

- Different volumes of primers (1 µL.1.3 µL)
- Different volumes of DNA sample (3 µL,5 µL)
- Different annealing temperatures (55.7 °C ,60 °C)

The best results of this SNP was obtained in the following conditions:

A. 1.3 µL reverse primer

B. 1.3 µL forward (C) primer

C. 1.3 µL forward (A) primer

D. 1.3 µL forward (T) primer

E. 3 µL DNA sample

F. 15 µL nuclease free water

A total volume of reaction was (20 µL) that added to the (500 µL) premix PCR tube that contains premix (5 µL) which centrifuged at 2000 rpm for 10 seconds in a micro centrifuge for mixing the sample tubes and then placed in thermocycler.

2.3.1.6 Running and working solution of PCR

2.3.1.6.1 ARMS-PCR running and working solution

*CYP3A4*22* (G>A) (rs35599367) ARMS-PCR working solution was prepared as follow:

- 0.5µl of 10 poml/µL from each outer forward and outer revers primer, 1 µL of 10 pmol/ µL from each inner forward and inner reverse primer, and 5µL of extracted DNA in PCR premix tube
- The volume was completed to 20 µL with 12 µL nuclease free water.
- The thermal program for *CYP3A4*22* (G>A) (rs35599367) demonstrated in table (2.7).

Table (2.7): PCR thermocycler program for *CYP3A4*22* (G>A) (rs35599367) polymorphism

Steps	Temperature (°C)	Minute: second	Cycles
Initial denaturation	95	03:00	
Denaturation	95	00:30	35
Annealing	60	00:30	
Extension	72	00:55	
Final extension	72	5:00	

2.3.1.6.2 Allele specific –Polymerase chain reaction running

*UGT1A4 *2* (C>A, T) (rs6755571) polymorphism

The AS-PCR mixture for *UGT1A4*2* (C>A, T) (RS6755571) was prepared in PCR premix formula by adding:

1.3µL of revers primer in each tube of PCR premix, 1.3µL of F-C in tube 1 of PCR premix, 1.3µL of F-A in tube 2 of PCR premix, 1.3µL of F-T in tube 3 of PCR premix, 3µL of extracted DNA and the volume was completed to 20 µL with 15 µL of nuclease free water for each tube.

The thermal program for *UGT1A4*2* (C>A, T) (RS6755571) is demonstrated in Table (2.8)

Table (2.8): Polymerase chain reaction thermocycler program for *UGT1A4 *2* (C>A, T) (rs6755571) polymorphism

Steps	Temperature (°C)	Minute: second	Cycles
Initial denaturation	95	03:00	
Denaturation	95	00:30	35
Annealing	60	00:30	
Extension	72	00:55	
Final extension	72	5:00	

2.3.1.7 Agarose Gel Electrophoresis

1. Agarose gel 1.5% was prepared by dissolving 0.3 gm of agarose powder was put in beaker, and then 2mL of 10X TBE buffer (tris-borate-EDTA) and 18 mL of distilled water was added.
2. The mixture was warmed on hot plate, and left for few seconds when the mixture began to boil.
3. The solution was left to cool and 2 µL of ethidium bromide was added.
4. The comb was fixed on one end of the try to make holes where the samples were loading.
5. After the agarose solution had poured to try, it has been left to congeal at 25 ˚C.
6. The comb was removed lightly away from the try.
7. The try was stabled into the device chamber, and the chamber was filled with 1X TBE buffer.
8. One of the wells of agarose gel was loaded with 5 µl of DNA ladder while the others were loaded with 5 µl of each PCR products.
9. The voltage of the electrophoresis apparatus was adjusted at 45 volts to ensure an electrical field adjusted with (5) v/cm for 10 cm distance between cathode and anode.

10. At the end of the run, ultraviolet treans-illuminator was used for detection of the bands.
11. The gel was photographed using digital camera (canon EOS 80 D).(155, 156)

2.4 Biochemical analysis

2.4.1 Estradiol E2

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

- **Principle**

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

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

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

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

The resulting chemiluminescent reaction was measured as relative light units (RLUs) by a photomultiplier built into the system. the

amount of estradiol present in the sample was inversely proportional during the reaction, the estradiol concentration could be determined via a calibration curve (157).

The expected values of estradiol level:

(Postmenopausal female < 25-84 pg/ml, Follicular phase 20-138 pg/ml, Ovulation phase 100-400 pg/ml, luteal phase 31-317 pg/ml).

2.4.2 Tumor marker CA 15-3

The quantitative measurement of CA 15.3 levels in human serum using the Enzyme Linked Fluorescent Assay (ELFA).

➢ Principle

The assay principle was combined a 2-step enzyme immunoassay sandwich method with a final fluorescent detection (ELAF)

The solid phase receptacle (SPR) was served as the solid phase as well as the pipetting device for the assay.

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

The sample was cycled in and out of the SPR several times. This operation enables the 155D8 fixed onto interior wall of the SPR to capture the reactive antigenic determinants present in the sample. Unbound component was eliminated during the washing steps.

Then Alkaline phosphatase-labeled DF3 antibody was incubated in the SPR where it was bonded with the DF3 antibody reactive antigenic determinants. Unbound conjugate was eliminated during the washing steps.

During the final detection step, the substrate (4-Methyl-umbelliferly phosphate) was cycled in and out of the SPR. the conjugate

enzyme was catalyzed the hydrolysis of this substrate into a fluorescent product (4-Methyi-umbelliferone) the fluorescence of which is measured at 450nm. The intensity of fluorescence is proportional to the concentration of CA 15.3 present in the sample.

At the end of assay, the results are automatically calculated by the instrument in relation to calibration curve stored in memory, and then printed out (158, 159).

The normal value of CA 15.3 is 0-30 u/ml

2.5 Arthralgia assessment

Arthralgia was assessed according to Brief Pain Inventory (BPI) (131, 160), in addition to the prescription of zolidronic acide by the physician for the breast cancer patients included in this study.

2.6 Statistical analysis

1. The collected data were established into computer system using a data base program and were recorded in a Microsoft Excel spreadsheet. Statistical analysis was accomplished in SPSS software version 25.IMB, US.
2. The genotyping results expressed as frequency and percentage.
3. The biochemical results were expressed as mean ± SD.
4. ANOVA, a single factor was used to examine the differences in mean of biochemical parameters among detected genotypes.
5. A chi-square test was used to establish any significant differences among serum levels of CA 15.3 among different detected genotypes.
6. Odds ratio and confidence interval 95% (CI 95%) of genotypes detected among breast cancer women were used to examine the association of studied genes polymorphism with elevation serum levels of CA 15-3 and arthralgia development.
7. Pearson's correlation coefficient was used to test the relation between detected genotypes and serum levels of E2 and CA 15-3.
8. In this study, probability value (p-value) in all statistical analysis expressed as the significant values were (<0.05) and non-significant was (>0.05).

Chapter Three

Results

3.1. Demographic data:

Table (3.1) shows the demographic data of breast cancer patients of this study.

The mean and range of patient's age was 53.14 (31-75),number and percentage of married women was 94 (94%) higher than unmarried women 6 (6%) ,number and percentage of pre- and postmenopausal were 5 (5%) and 95 (95%) separately,64 (64%) of women feeding their baby from their breast while 19 (19%) and 17 (17%) are women non- and mixing breast feeding respectively, higher number and percentage of women in study had no family history of breast cancer 88 (88%) corresponding to 12 (12%) of women had family history, 49 (49%) of women had cancer in left breast ,48 (48%) in right breast and 3 (3%) in both side , estrogen and progesterone receptors had an important role in pathogenesis of breast cancer, so 94 (94%) were PR and ER positive and 6 (6%) are PR or ER positive, the first step in breast cancer treatment is surgery 88 (88%) of women made surgery and 12 (12%) didn't made surgery ,84 (84%) and 69 (69%) of women received chemotherapy and radiation respectively, 16 (16%) and 31(31%) didn't received , arthralgia is the main side effect of anastrozole so 89 (89%) of patients in study suffered from arthralgia and 11 (11%) had not.

Table (3.1): Demographic data of breast cancer patients

Parameters		**N %**
Age at diagnosis of breast cancer		mean (range) 53.14(31-75)
Marital status	Married	94(94%)
	unmarried	6 (6%)
Menopausal state	Premenopausal	5 (5%)
	Postmenopausal	95 (95%)

Breast feeding	Yes	64 (64%)
	No	19 (19%)
	Mix	17 (17%)
Family history	Yes	12 (12%)
	No	88 (88%)
Site of breast cancer	Left	49 (49%)
	Right	48(48%)
	Lift + Right	3(3%)
Receptor status	PR and ER (positive)	94(94%)
	PR or ER (positive)	6 (6%)
Surgery	Yes	88 (88%)
	No	12 (12%)
Chemotherapy	Yes	84 (84%)
	No	16 (16%)
Radiation	Yes	69 (69%)
	No	31 (31%)
Arthralgia	Yes	89 (89%)
	No	11 (11%)

3.2 Molecular analysis

3.2.1 Amplification reaction results of *CYP3A4*22* (G>A) (rs35599367) and *UGT1A4*2* (C>T, A) (rs6755571) in the breast cancer patients

The amplification of SNP of *CYP3A4* gene: *CYP3A4*22* (G>A) (rs35599367) was shown in 215 bp, 297bp as in figure (3.1) and amplification of SNP of *UGT1A4* gene: *UGT1A4*2* (C>A, T) (rs6755571) was shown in 243 bp as in figure (3.2 A-C)

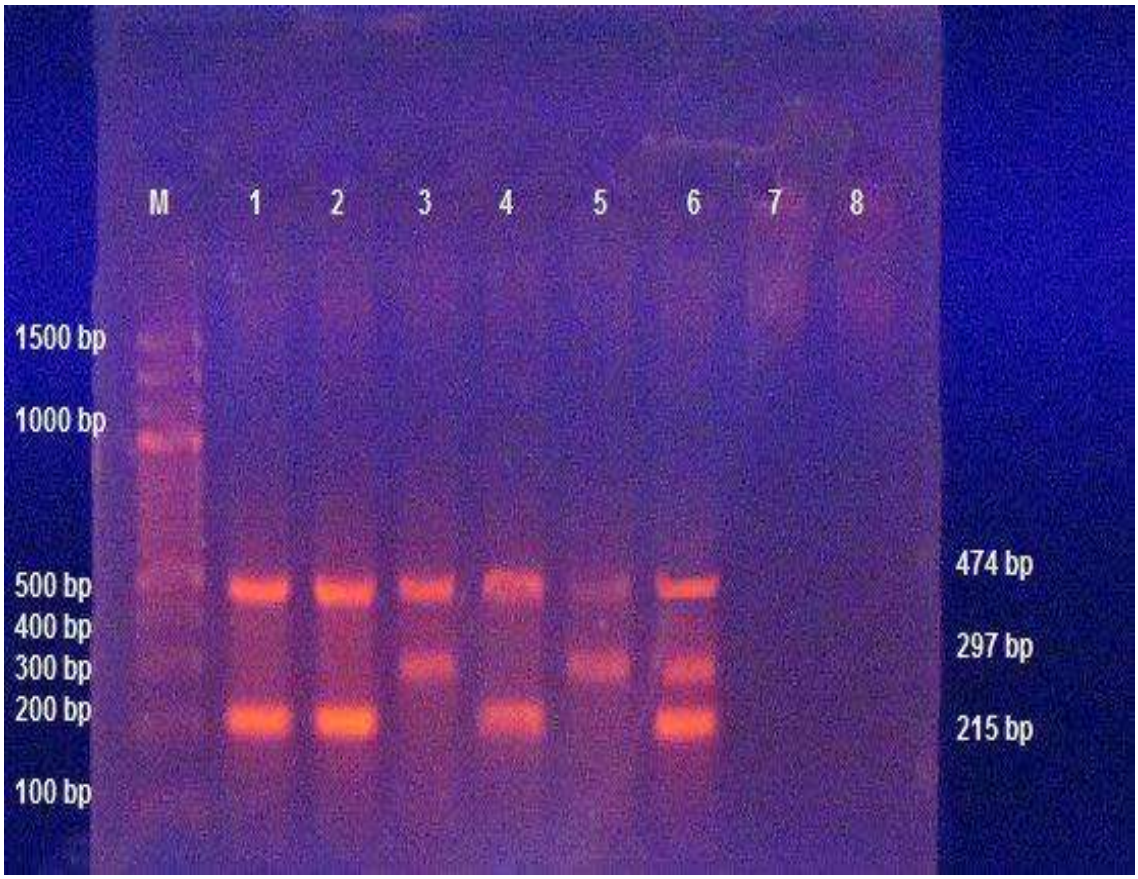

Figure (3.1): The ARMS-PCR of *CYP3A4*22* (G>A) (rs35599367) genetic polymorphism showed: lane M: represented DNA ladder (100-1500 bp), lane 1,2and 4: represented GG genotype (wild)homozygous were shown in (215bp), lane 3and 5: represented AA genotype (mutant) homozygous were showed in (297bp) and lane 6: represented GA genotype (heterozygous),the voltage used in gel electrophoresis is (45 volt)

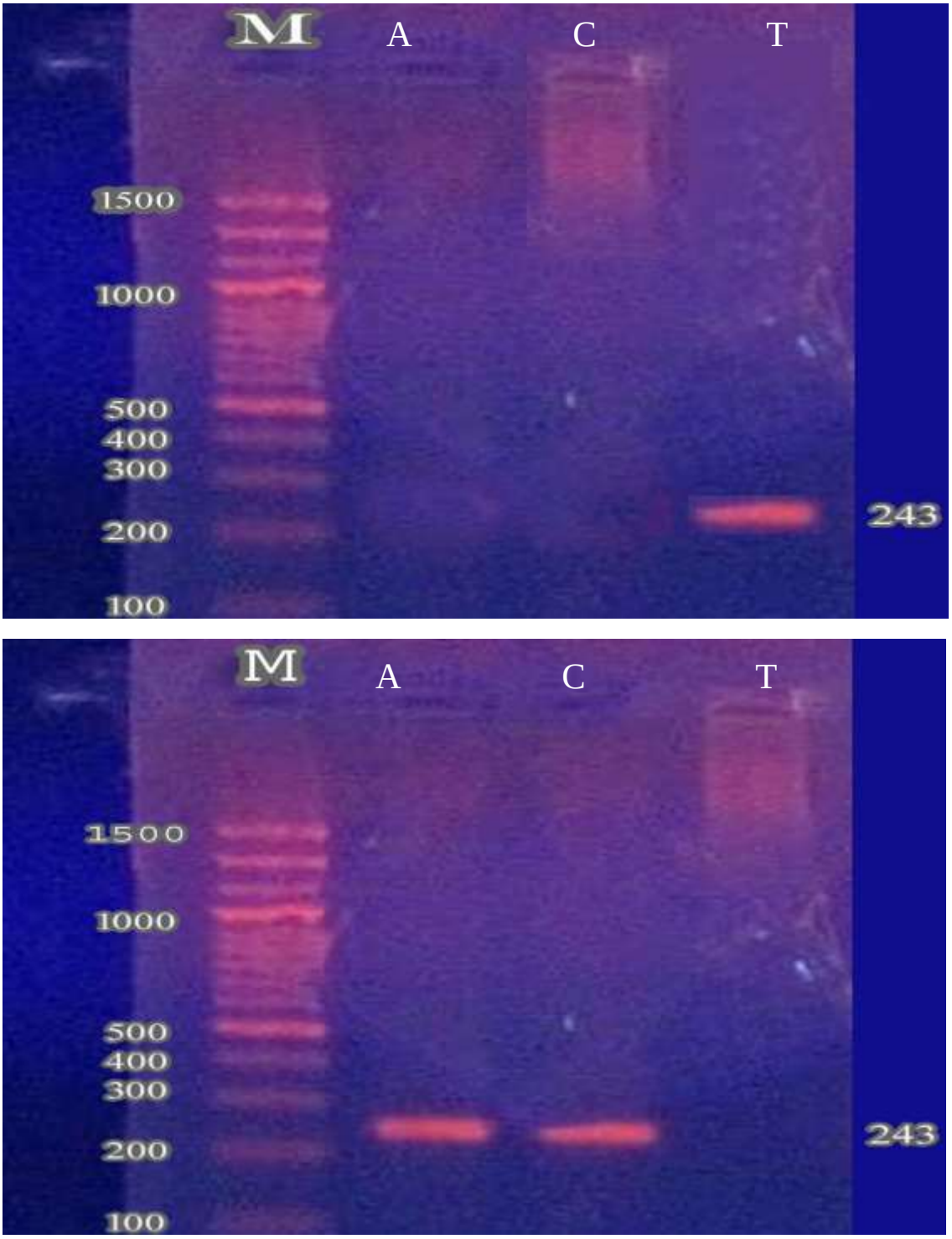

Figure (3.2.A): The (AS-PCR) of *UGT1A4* gene: *UGT1A4*2* (C>T, A) (rs6755571) genetic polymorphism showed: each picture represented one sample, lane M in each sample: represented DNA ladder (100-1500 bp), 1st picture: represented homozygous mutant (TT) genotype was shown in (243bp), 2nd picture: represented heterozygous (AC) genotype was shown in (243bp), the voltage used in gel electrophoresis is (45 volt)

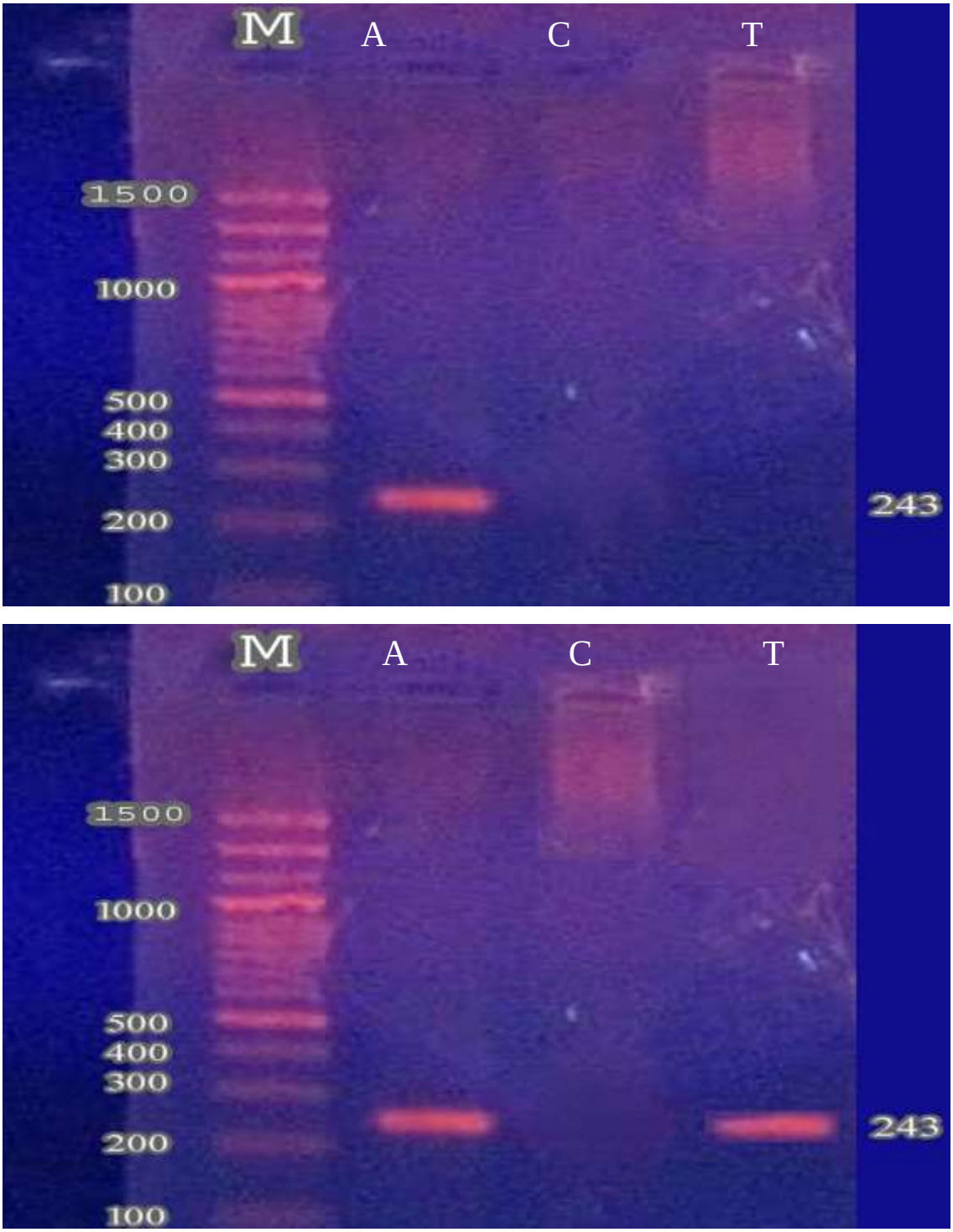

Figure (3.2.B): AS-PCR of *UGT1A4* gene: *UGT1A4*2* (C>T, A) (rs6755571) genetic polymorphism showed: each picture represented one sample, lane M in each sample: represented DNA ladder (100-1500 bp), 1st picture: represented homozygous mutant (AA) genotype was shown in (243bp), 2nd picture: represented heterozygous (AT) genotype was shown in (243bp), the voltage used in gel electrophoresis is (45 volt)

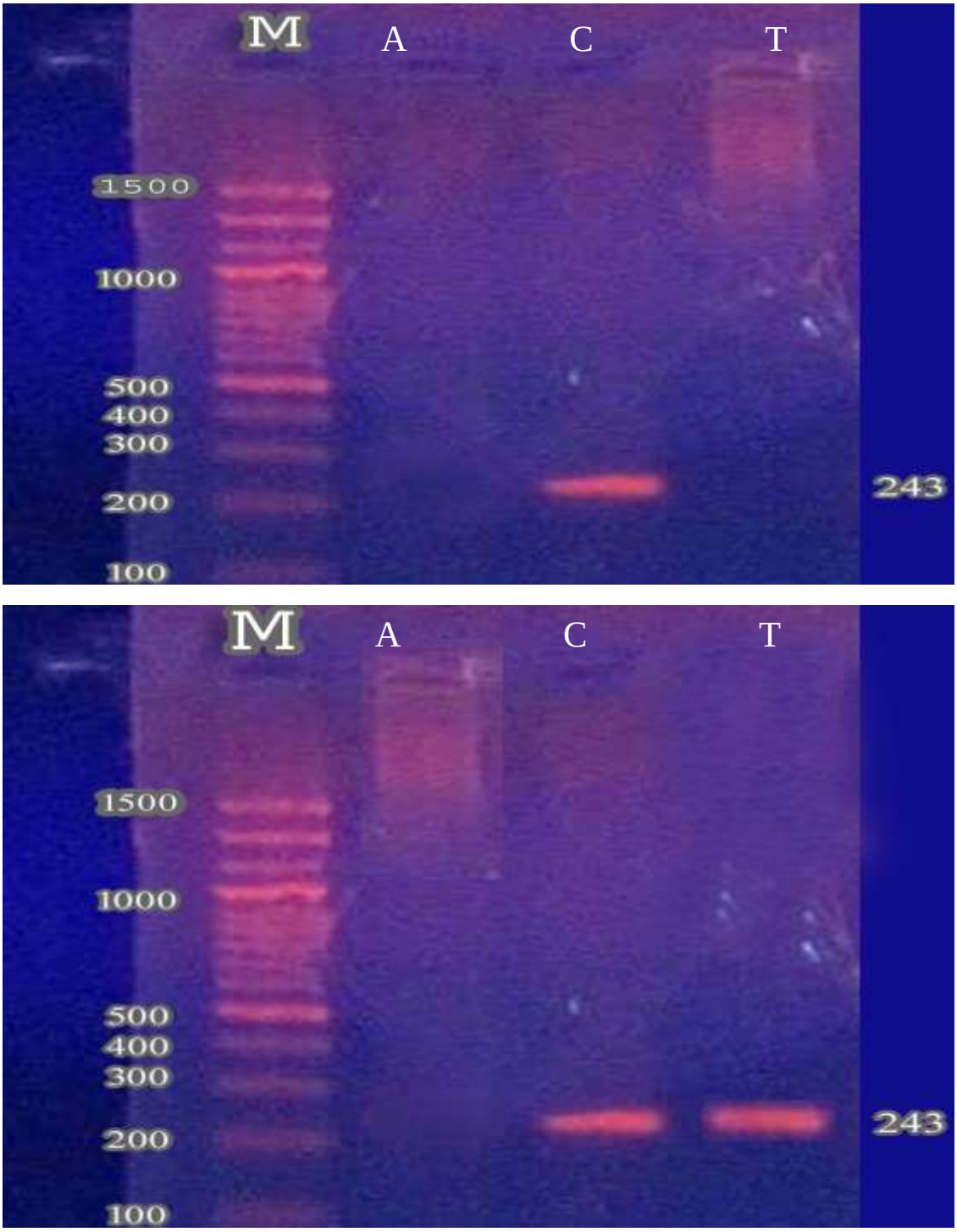

Figure (3.2.C): AS-PCR of UGT1A4 gene: UGT1A4*2 (C>T, A) (rs6755571) genetic polymorphism showed: each picture represented one sample, lane M in each sample: represented DNA ladder (100-1500 bp), 1st picture: represented homozygous wild (CC) genotype was shown in (243bp), 2nd picture: represented heterozygous (CT) genotype was shown in (243bp), the voltage used in gel electrophoresis is (45 volt).

3.2.2 The frequency and percentage of detected genotypes of *CYP3A4*22* (G>A) (rs35599367) and *UGT1A4*2* (C>A, T) (rs6755571) in the breast cancer patients.

For *CYP3A4*22* (G>A) (rs35599367) wild homozygous (GG) genotype was detected with percentage (38%) respectively, while the percentage of homozygous mutant type (AA) was (37%) and heterozygous type (GA) was (25%). as in table (3.2)

Table (3.2): The distribution of detected genotypes of *CYP3A4*22* (G>A) (rs35599367) in the Breast Cancer Patients

SNP	Genotypes	Percentage
rs35599367 (G>A)	GG	38%
	AA	37%
	GA	25%
	Total	100%

For of *UGT1A4*2* (C>A, T) (rs6755571), was detected two mutant genotype (TT) in higher percentage (36%) while (AA) was detected in very little percentage (4%), wild type (CC) was detected in low percentage (8%), anther genotype also detected (CA) (17%), (TA) (6%), (CT) (29%) which were heterozygous, as in table (3.3).

Table (3.3): The distribution of detected genotypes of *UGT1A4 *2* (C>A, T) (rs6755571) in the breast cancer patients

SNP	Genotypes	Percentage
rs6755571 (C>T, A)	CC	8%
	AA	4%
	CA	17%
	CT	29%

	TA	6%
	TT	36%
	Total	100%

3.3 Biochemical analysis

3.3.1 Serum estradiol (E2) level among detected genotyping of *CYP3A4*22* (G>A) (rs35599367) and *UGT1A4*2* (C>A, T) (rs6755571) in breast cancer patients

values of serum estradiol level showed non-significant differences among different detected genotype of *CYP3A4*22* in the (100) breast cancer patients of the current study (p>0.05) as shown in table (3.4).

In the present study, it had been observed that the breast cancer patients harboring mutant genotype (AA) had non-significant higher serum estrogen level (21.072 pg/ml±12.904) while patients with genotype wiled (GG) had the low serum estrogen level (19.631 pg/ml ± 10.509)

Similarly, patients with heterozygous genotype (GA) showed low serum estrogen level (19.696 pg/ml± 9.637)

Table (3.4): Serum estradiol (E2) level among detected genotyping of *CYP3A4*22* (G>A) (rs35599367) in breast cancer patients

SNP	Genotypes	Mean (E2) pg/ml ± SD	P value
rs 35599367 (G>A)	GG	19.631 ± 10.509 a	0.832 N.S.
	GA	19.696 ± 9.637 a	
	AA	21.072 ± 12.904 a	

p value derived from ANOVA test, Significant: p<0.05, non-significant: p>0.05., values expressed as mean± standard deviation (SD), same litters mean no significant differences.

Table (3.5)shows that serum estradiol level was non-significant difference among different *UGT1A4*2* (C>A, T) (rs6755571) genotypes detected in the (100) breast cancer females (p>0.05).

In the present study, it is observed that the breast cancer patients harboring homozygous mutant genotypes (AA, TT) have highest serum estradiol level (26.513 pg/ml±10.327) (21.294 pg/ml±9.680) respectively while patients with wild genotype (CC) have the lowest serum estradiol level (17.068 pg/ml±9.652). Similarly, breast cancer patient with heterozygous genotype (CA, CT, TA) have different low serum estradiol level.

Table (3.5): Serum estradiol (E2) level among detected genotyping of *UGT1A4*2* (C>A, T) (rs6755571) in breast cancer patients

SNP	Genotypes	Mean (E2) pg/ml ± SD	P value
rs 6755571 (C>A,T)	CC	17.068 ± 9.652 a	0.776 N.S.
	CA	19.454 ± 16.96 a	
	AA	26.513 ± 10.327 a	
	CT	19.197 ± 9.849 a	
	TT	21.294 ± 9.680 a	
	TA	20.242 ± 9.691 a	

p value derived from ANOVA test, Significant: p<0.05, non-significant: p>0.05., values expressed as mean± standard deviation (SD), same litters mean no significant differences

3.3.2 Person's correlation of the serum estradiol (E2) with genotypes of *CYP3A4*22* (G>A) (rs35599367) and *UGT1A4*2* (C>A, T) (rs6755571) Gene in breast cancer patients

Table (3.6) shows that there was non- significant correlation between the serum estradiol (E2) level and both *CYP3A4*22* (G>A)

(rs35599367) (correlation's coefficient = -0.055) and *UGT1A4*2* (C>A, T) (rs6755571) (correlation's coefficient = 0.030) (p>0.05)

Table (3.6): Person's correlation coefficient and P values of significance among the serum estradiol (E2) with genotypes of *CYP3A4*22*(G>A) (rs35599367) and *UGT1A4*2*(C>A, T) (rs6755571) in breast cancer patients

SNPs	person's Correlation Coefficient	p-value (2-tial)
(rs 35599367) G>A	-0.055	0.580 N.S
(rs 6755571) C>A,T	0.030	0.763 N.S

P<0.05

3.3.3 Serum tumor marker (CA 15.3) levels among detected genotypes of *CYP3A4*22* (G>A) (rs35599367) and *UGT1A4*2* (C>A, T) (rs6755571) in breast cancer patients.

In the current study, it had been noted that patients with lowest values of serum CA15.3 level (17.016 U/ML±9.516) holding homozygous mutant genotype (AA) of *CYP3A4*22* while patients who have wild genotype (GG) the values (21.453 U/ML±17.843) of serum CA15.3 level was high, also found patients with heterozygous genotype (GA) have low values of serum CA15.3 level (20.283 U/ML± 9.561).

As shows in table (3.7), there were non-significant differences in the values of serum CA 15.3 level among detected genotype of CYP3A4 *22 in breast cancer females (ANOVA p>0.05).

Table (3.7): Serum CA15.3 level among detected genotyping of *CYP3A4*22* (G>A) (rs35599367) in breast cancer patients

SNP	Genotypes	Mean (CA15.3 U/ML) ± SD	P value
rs 35599367 (G>A)	GG	21.453 ± 17.843 a	0.339 N.S.
	GA	20.283 ± 9.561 a	
	AA	17.016 ± 9.516 a	

p value derived from ANOVA test, Significant: p<0.05, non- significant: p>0.05., values expressed as mean± standard deviation (SD), same litters mean no significant differences

Table (3.8) show that values of serum CA15.3 levels in breast cancer patients were significantly different among different *UGT1A4*2* genotypes detected (p<0.05).

Women harboring homozygous mutant genotypes (AA) of *UGT1A4* gene significantly high value (40.673 U/ML±18.055) compared with females with other homozygous mutant (TT) genotype (21.544 U/ML±16.708) while women holding homozygous wild genotype (CC) have low value of serum CA15.3 level (18.221 U/ML±10.536). Additionally, value of serum CA15.3 (17.166 U/ML ± 10.69), (16.64 U/ML±7.838) and (16.541 U/ML ± 12.405) were low in females having heterozygous genotype (CT, TA and CA) separately.

Table 3.8: Serum tumor markerCA15.3 level among detected genotyping of *UGT1A4 *2* (C>A, T) (rs6755571) in breast cancer patients

SNP	Genotypes	Mean (CA15.3) U/ML ± SD	P value
rs 6755571 (C>A,T)	AA	40.673 ± 18.055 a	0.037 S.
	CA	16.541 ± 12.405 b	
	CC	18.221 ± 10.536 b	
	CT	17.166 ± 10.697 b	
	TT	21.544 ± 16.708 b	

	TA	16.64 ± 7.838 b	

p value derived from ANOVA test, Significant: p<0.05, non-significant: p>0.05., values expressed as mean± standard deviation (SD), different litters mean significant differences

3.3.4 Pearson's correlation of the serum tumor marker (CA15.3) with genotypes of *CYP3A4*22* (G>A) (rs35599367) and *UGT1A4*2* (C>A, T) (rs6755571) in breast cancer patients

According to person's Correlation, there were no significant correlation among serum CA15.3 level and both *CYP3A4*22* (person's Correlation Coefficient =0.171) and *UGT1A4*2* (person's Correlation Coefficient=-0.007) (p>0.05) in breast cancer patient as show in table (3.9)

Table (3.9): Person's correlation coefficient among the serum tumor marker (CA15.3) with genotypes of *CYP3A4*22* (G>A) (rs35599367) and *UGT1A4*2* (C>A, T(rs6755571) in breast cancer patients

SNPs	Pearson's Correlation Coefficient	p-value (2-tial)
(rs 35599367) G>A	0.171	0.865 N.S.
(rs 6755571) C>A,T	-0.007	0.942 N.S.

P<0.05

3.3.5 Above or normal serum CA15.3 levels among different detected genotypes of *CYP3A4*22* (G>A) (rs35599367) and *UGT1A4*2* (C>A, T) (rs6755571) in breast cancer patients

Table (3.10) shows the frequency and percentage of patients who had normal or above normal value of CA15.3 in different detected genotypes of *CYP3A4*22* in breast cancer patients. In this study 89 of patients had normal serum CA15.3 level, while 11 patients only had elevated level of CA15.3

The number and percentage of women who had serum CA15.3 levels above the normal limit were shown as follow: 2 (18.18%) patients for each (AA and GA) genotype separately and 7 (63.64%) of patients with (GG) genotype. The number and percentage of patients within normal values were shown as follow: 35 (39.33%) of patients with (AA) mutant genotype ,23(25.84%) of patients with (GA) genotype and 31 (34.83%) with (GG) genotype. The p-value is non-significant, so the two variables (Rs35599367) and CA15.3 are independent on each other.

Table (3.10): The frequency and percentage of patients who had above or normal limit of CA15.3 in different detected genotype of *CYP3A4*22* (G>A) (rs35599367) in breast cancer patients.

SNP	genotypes	CA15.3 level above normal N (%)	CA15.3 level within normal N (%)	
(rs35599367) G>A	GG	7 (63.64%)	31 (34.83%)	X^2= 3.55 Do= 2 P> 0.05
	GA	2 (18.18)	23 (25.84%)	
	AA	2 (18.18%)	35 (39.33%)	
Total (n)		11	89	

X^2=Chi-sq., No significant: p>0.05., Df=degree of freedom

Table (3.11) shows the frequency and percentage of patients who had normal or above normal value of CA 15.3 in different detected genotypes of UGT1A4*2 in breast cancer patients. In this study 89 of patients had

normal serum CA 15.3 level, while 11 patients only had elevated level of CA15.3

The number and percentage of women who had serum CA 15.3 levels above the normal limit were shown as follow: 3 (27.27%) and 2 (18.18%) patients for each mutant (AA and TT) genotype separately, only 3 (27.27%) and 1(9.09%) of patients with (CT and CC) genotype respectively and no one detected with (TA) genotype. The number and percentage of patients within normal values were shown as follow: 27(30.34%) of patients with (TT) mutant genotype, 33(37.08%) of patients with (CT) genotype, 15(16.85%) of patients with (CA) genotype, 7(7.86%) of patients with (CC) wiled genotype, 6(6.74%) of patients with (TA) genotype and only one (1.12%) with mutant (AA) genotype. The p-value is non-significant, so the two variables (rs6755571) and CA15.3are independent on each other.

Table (3.11): The frequency and percentage of patients who had above or normal limit of serum CA15.3 level in different detected genotype of *UGT1A4 *2* (C>A, T) (rs6755571) in breast cancer patients.

SNP	Genotypes	CA15.3 level above normal N (%)	CA15.3 level within normal N (%)	
(rs6755571) C>A,T	CC	1 (9.09%)	7 (7.86%)	X^2= 1.47 Df= 5 P> 0.05
	CA	2 (18.18%)	15 (16.85%)	
	AA	3 (27.27%)	1 (1.12%)	
	CT	3 (27.27%)	33 (37.08%)	
	TA	0 (0%)	6 (6.74%)	
	TT	2 (18.18%)	27(30.34%)	
Total		11	89	

X^2=Chi-sq., No significant: p>0.05., Df=degree of freedom

3.3.6 The odds ratio of the different detected genotypes of *CYP3A4*22* (G>A) (rs35599367) and *UGT1A4*2* (C>A, T) (rs6755571) in elevation of serum CA 15.3 level in breast cancer patients

Table (3.12) shows that the SNP (rs 35599367) of *CYP3A4* gene had non-significant effect in the elevation of serum tumor marker CA 15.3 level in breast cancer women (odds ratio 0.34, p> 0.05). similarly, the SNP (rs 6755571) of UGT1A4 gene has non-significant effect in elevation of serum tumor marker CA15.3 level in breast cancer women (odds ratio 1.29, p> 0.05).

Table (3.12): The odds ratio of the different detected genotypes of *CYP3A4*22* (G>A) (rs35599367) and *UGT1A4*2* (C>A, T) (rs6755571) genes in elevation of serum CA15.3 level in breast cancer patients

SNPs	Odds ratio (Cl -95%)	p-value
rs (35599367) G>A	0.34 (0.0699-1.682)	0.187
rs (6755571) C>A,T	1.29 (0.364-4.536)	0.696

CI-95%: confidence interval, p<0.05

3.4 Arthralgia

3.4.1 The odds ratio of the different detected genotypes of *CYP3A4*22* (G>A) (rs35599367) and *UGT1A4*2* (C>A, T) (rs6755571) in the development of arthralgia in breast cancer patients

Table (3.13) shows that the SNP (rs35599367) of *CYP3A4* gene has non-significant effect in the development of arthralgia in breast cancer women (odds ratio 1.03, p> 0.05), likewise, the SNP (rs6755571) of *UGT1A4* gene has non-significant effect in the development of arthralgia in breast cancer women (odds ratio 1.148, p> 0.05).

Table (3.13): The odds ratio of the different detected genotypes of *CYP3A4*22* (G>A) (rs35599367) and *UGT1A4*2* (C>A, T) (rs6755571) in the development of arthralgia in breast cancer patients

SNPs	**Odds ratio (Cl -95%)**	**p-value**
Rs (35599367) G>A	1.03 (0.280-3.79)	0.963
Rs (6755571) C>A,T	1.148 (0.324-4.359)	0.794

CI-95%: confidence interval, p<0.05

Chapter Four

Discussion

4.Discussion

Anastrozole is a selective third –generation aromatase inhibitor (AI) that has been recognized as the drug of choice in the adjuvant treatment of pre- and postmenopausal breast cancer women, as well as advanced-stage malignancy (161). It has also been investigated in studies for prevention of breast cancer in women who are at high risk of developing disease. A Panel of American society of clinical oncology recommended that optimal adjuvant endocrine therapy for postmenopausal women with receptor –positive breast cancer should include an AI as initial therapy or after treatment with tamoxifen(113).

Anastrozole undergoes extensive hepatic metabolism, the most important enzymes involved in the metabolism of anastrozole are CYP3A4 and UGT1A4 which are highly polymorphic; polymorphism in the genes encoding anastrozole metabolizing enzymes may influence the response and adverse effect of anastrozole and clinical outcome of breast cancer (162).

To the best of our knowledge, this is the first study that investigated Iraqi breast cancer women for interindividual variability of CYP3A4 and UGT1A4 genes involved in metabolism of anastrozole and it influence on response and clinical outcome.

4.1 Demographic data

Breast cancer is a multifactorial disease and several factors contribute to its occurrence, away from genetic predisposition, clinical reproductive, environmental feature and demographic characteristics are other factors could have an impact on developing breast cancer among women (11) .

After female gender, age consider the strongest risk factor for breast cancer, with increased age the risk of disease increased (163) and this matching with this study whereas the age range of women with breast

cancer at diagnosis was (31-75) years with mean (53.14) years ,as showed in table (3-1).

In young women (<35), the breast tumor is more aggressive , larger size, advanced stages, positive lymph nodes and weaker survival than elderly women ;this is may be due to family history, age of menarche and use of contraceptive pills (164).

Some studies have reported that no significant correlation between marriage and breast cancer risk while other studies have shown that marriage is a protective factor for disease outcomes, also there are studies showed that married women have increased breast CA risk compared with the unmarried (20), as in this study ,the explanation of these result may be due to genetic factors that contributed in breast cancer development.

Numerous studies had reported that breastfeeding reduced breast cancer risk, breastfeeding is hypothesized reducing lifetime menstrual cycles number, making women less cumulative exposure to endogenous hormones and the differentiation of ductal cell of breast increased; so less susceptibility to carcinogen (165), in other hand ,there are studies has not been proven the protective effect of breast feeding (2),in this study most of breast cancer women which included in study feeding her baby from breast ,this may be due to small sample size and the study is cross-sectional study.

Several studies showed that breast CA risk increased by 67% among women with a first – degree relative diagnosed with same disease and twofold in female with more than one relative affected, this association may be due to that both patients share inheritable genetic susceptibility (BRCA mutation) (166). While in this study most of females had no family history of breast cancer.

4.2 Molecular analysis

4.2.1 The frequency of detected genotypes of *CYP3A4*22* (G>A) (rs35599367) within breast cancer patients

CYP3A4 is a polymorphic enzyme, as much as 40 times expression of CYP3A4 enzyme varied among individuals and this effect on drug efficacy and safety (167).

The *CYP3A4*22* allele is defined by the intronic rs35599367 variant, which reduces the expression of CYP3A4 mRNA in liver (168). genotyping testing determined the frequency and percentage of *CYP3A4*22* genotypes within breast cancer patients of this study as presented in table (3-2).

In this study, the homozygous wild genotype (GG) of *CYP3A4*22* was found in high number of breast cancer Iraqi women corresponding with other genotypes homozygous mutant and hertozygous (AA, GA) genotype respectively in study. In Gordian population the minor homozygous genotype (AA) of (rs35599367) had very low frequency (2%) (169) ;while in Caucasian breast cancer females the frequency of mutant homozygous (AA) genotype (5%-7%) (170, 171) ;and in African and Asian below(1%) (168).

4.2.2 The frequency of detected genotypes of *UGT1A4*2* (C>A, T) (rs6755571) within breast cancer patients

The key enzymes in human detoxification of endogenous substance and xenobiotic are UGTs which are encoded by a multigene family in human. A relatively small number of UGTs enzymes catalyze the glucuronidation of thousands compounds. Genetic variation within the UGT genes are remarkably common which may cause different genotypes by affecting expression or activity of UGTs enzymes (172).

Glucuronidation is one of major metabolic pathway of anastrozole and its metabolites, so polymorphisms play an important role in individual pharmacological response to anastrozole therapy (172), genotyping testing determined the frequency and percentage of UGT1A4*2 genotypes within breast cancer patients of this study as presented in table (3-3).

The most frequent genotype in breast cancer women recruited in the study was (TT) which contains point mutation and there are no other studies detected this genotype until the time of this study, while (AA) is other mutant genotype, (CC) which is wiled type and (TA) is heterozygous type of (rs6755571) was detected in very little frequency and percentage (4,4%), (8,8%), (6,6%) respectively, other heterozygous genotype (CT, CA) also detected in prevalence (29,29% ,17,17%) separately.

This study disagrees with study of Bojanic K, etal. that found (96%) of breast cancer Croatian patients' population harboring homozygous wiled (CC) genotype and (3.9%) for the heterozygous genotype (CA) while the homozygous mutant genotype (AA) was not detected (86);while the study agree with study of Sutiman N,etal. That shown the Caucasian papulation had (8%) for homozygous mutant (AA) genotype and low frequency (<1%) in Indian but its absence in Malays and Chinese (173). In White, African American the most abounded genotype was homozygous wild genotype (CC) with frequency and percentage (48,52.7%) while frequency of (17,18.7 %)were detected for mutant genotype (AA) and (26,28.6%) for heterozygous (AC) (137).My opinion ,that the higher percentage of homozygous mutant genotype (TT) , *UGT1A4*2* may be related to breast cancer or due to marriage between relatives which may elevate this percent.

4.3 Biochemical analysis

4.3.1 Impact of *CYP3A4*22* (G>A) (rs35599367) on serum estradiol level in breast cancer patients

Estrogen is the major female sex hormone and has an important role in development and progression of breast cancer and other cancer (70),that act as factor that accelerate the growth and /or survival of existing transformed cell (promoters) or factors that provoke genetic damage that lead to cellular transformation (initiators) (73),

The end –products of sequence of transformation of steroid is estrogens (estrone E1, estradiol E2), blocked any steps in conversion pathway of androgens to estrogen especially the final step (aromatase step) which is unique to biosynthesis of estrogen lead to decreased estrogen production and subsequent serum estradiol level (174).

Anastrozole inhibits total body aromatization by 98% , most patients get undetectable systemic estrogen (E2) concentration, undergo successful treatment and experience acceptable side effects, on other hand, some patients experience breast cancer recurrence or metastasis which may be because of the polymorphic effect of *CYP3A4* and/or *UGT1A4* genes (145).

Anastrozole undergo hepatic transformation by CYP3A4 and UGT1A4 to inactive metabolite, so variable activity of CYP3A4 and UGT1A4 enzymes possibly as a result of genetic polymorphism of genes encoding these enzymes may affect drug metabolism (136) .

The noncoding (6-intron) *CYP3A4*22* (rs35599367) affect mRNA accumulation either through affecting splicing, production of stability of transcription and subsequent decrease CYP3A4 enzyme activity (175, 176), many studies conducting with breast cancer patients and with different breast cancer drugs such as docetaxel, tamoxifen and exemestane showed that the breast cancer patients carried (rs35599367) have low enzyme activity which

categorized as PM and high steady –state concentration of drugs(170, 171, 177).

So theoretically and according to the studies mentioned above, breast cancer women who used anastrozole and carried (rs35599367) may be had high concentration of anastrozole and low serum estradiol level, but in this study noted females with mutant genotype (AA) had high serum estradiol level (but still within normal) while wild genotype (GG) had low serum estradiol level and non-significant association of genotype with serum E2 level $p > 0.05$ as in table (3-4). This may be due to these patients may be had another SNP of *CYP3A4* or polymorphic effect of other gene that contribute in anastrozole response or *CYP3A4*22* may be had no effect or increased metabolism of anastrozole.

4.3.2 Impact of *UGT1A4*2* (rs6755571) on serum estradiol level in breast cancer patients

The replacement of cytosine with adenine or thiamin (C >A, T) in exon 1 of UGT1A4 gene was identified and showed that reduced glucuronidation activity for lamotrigine and transandrosterone, while had no effect on tamoxifen and dihydrotesterone glucuronidation (178), similarly Ehmer ,ital. research association of (rs6755571) with reduced glucuronidation activity against mutagenic amines and endogenous steroid (179) .

In this study patients with homozygous wild genotype (CC) had lowest serum estradiol level whereas homozygous mutant genotype (AA,TT) had highest but all of these are still within normal value and non-significant serum estradiol level as shew in table (3-5),however, these result can be explained that (rs6755571) either may be had no effect or increased UGT1A4 activity, but according to impact of these SNP on glucuronidation of other drugs metabolized by UGT1A4 in the studies mentioned above the polymorphism of *UGT1A4* gene (rs 6755571) may be decreased metabolism

of anastrozole and the causes of high estradiol level in the patients with mutant genotype may be another SNP of *UGT1A4* or polymorphic effect of other gene that contribute in Anastrozole response.

Although using anastrozole (which inhibit estrogen synthesis) this study found a detectable amount of estrogen in serum but still within normal values, This result was compatible with study introduced by Mays ;ital. who found a measurable level of estrogen in breast cancer women treated with anastrozole in spite of a close total inhibition of aromatase enzyme (174). However, these finding does not give clear explanation for the variant serum estradiol levels within variant *CYP3A4* genotypes that observed in this study, that's may be due to polymorphism of aromatase gene (*CYP19A1*) that encoding aromatase enzyme (estrogen synthase).

However, the observed association between *CYP3A4*22* genotypes and different estradiol serum levels within Iraqi women with breast cancer who had been treated with anastrozole. |

4.3.3 Impact of *CYP3A4*22* (G>A) (rs35599367) on serum tumor marker (CA 15.3) level in breast cancer patients

Cancer antigen (CA15.3) is considered as tumor marker in breast CA patients while its value is still controversial in early breast cancer stage due to lack of tumor and organ specificity and sensitivity, however, in metastatic breast cancer its used for monitoring therapy response along with other clinical features and radiological imaging (87).

CA15.3 level in healthy individual was within the usual normal range (0-30U/ml) while its level was significantly increased in breast cancer patients and gradually decreased in patients used hormonal therapy such as anastrozole (180). however, to date, no studies have correlate between the response of anastrozole therapy and CA15.3 level along with genetic polymorphism in *CYP3A4*22*, table (3-7).

In this study, the women with wild genotype (GG) had highest serum CA15.3 level compared with women harboring homozygous mutant and heterozygous genotype (AA, GA) which had low serum CA15.3 level but still all the results within normal range and non-significant association with *CYP3A4* polymorphism, so this may be indicate the *CYP3A4*22* increase the activity of enzyme in those patients or may have no effect or may there is other factors contributed in high level of tumor marker (CA15.3).

4.3.4 Impact of *UGT1A4*2* (rs6755571) (C>A, T) on serum tumor marker (CA 15.3) level in breast cancer patients

For *UGT1A4*2* (rs6755571) ,significant highest serum level found in patient with homozygous mutant genotypes (AA, TT) compared with patients harboring homozygous wild and heterozygous genotype (CC, CT, CA, TA) however still with normal values, table (3-8) but these finding is not compatible with studies showed that (rs6755571) decrease the activity of UGT1A4enzyme (178, 179) that's mean the patients with (AA or TT) genotype have low serum level of CA15.3 while in this study patients with (CC, CT, CA, TA) had low serum level ,this may indicate these SNP had low or no effect on CA15.3.

For both SNPs these results were within the normal limits and the differences between genotype groups were non-significant and this may indicate that these SNPs had low or no effect on CA15.3 and so may not impact on drug response.

In the current study, *CYP3A4*22* and *UGT1A4*2* were not significantly associated with elevation of serum levels of CA15.3 and they were independent variables, tables (3-10) and (3-11) respectively.

This study demonstrated that there was no any significant association between *CYP3A4*22* and *UGT1A4*2* polymorphism and elevation serum level of CA 15-3 in term of odds ratio, table (3-12).

In the present study, Both *CYP3A4*22* and *UGT1A4*2* were not significantly correlated with serum levels estradiol and CA15.3 and they were independent variables, tables (3-6) and (3-9), respectively.

4.4. Association of polymorphism of *CYP3A4*22* (G>A) (rs35599367) and *UGT1A4*2* (C>A, T) (rs6755571) with development of arthralgia in breast cancer patients

The majority of research anastrozole associated adverse effect focused on arthralgia. Although pathophysiology of anastrozole induces arthralgia and joint pain was yet to be fully elucidated, the suppression of estrogen through inhibition of aromatase by anastrozole had been postulated to play role (181). Pharmacogentics of anastrozole may be also play a role in development of arthralgia, anastrozole metabolized by (CYP3A4 and UGT1A4) that convert anastrozole to inactive metabolites (136, 138). however, many studies indicated (rs35599367and rs6755571) decrease the expression and activity of CYP3A4 and UGT1A4 enzymes respectively (175, 178), that's mean the drug may be (anastrozole) remain active and lead to development of side effect (arthralgia).

The present study noted that most but not all patients (89%) had arthralgia and in term of odds ratio both SNP had non-significant associated with development of arthralgia, $P<0.05$) table (3-13) so the development of arthralgia in patients of this study may be polymorphism of other gene such as *CYP19A1* (aromatase gene). Many studies observed association of *CYP19A1 w*ith development of arthralgia .(149, 182)

4.5 Conclusions

On the basis of obtained results, the following may be concluded:

1. *CYP3A4*22* and *UGT1A4*2* of Iraqi breast cancer patients were detected with variable frequencies of different genotype that may be leading to different predicted phenotypes and different enzymatic activity.
2. In Iraqi breast cancer women treated with anastrozole, the homozygous wild (GG), homozygous mutant genotype (AA) and heterozygous genotype(GA) of *CYP3A4*22* (rs35599367) were detected in different frequencies by using ARMS-PCR.
3. Two mutant genotypes of *UGT1A4*2* (rs6755571) are detected, (TT) genotype is predominant and (AA) genotype found in low percentage, other genotypes (CC) wild-type and (CA, TA, CT) heterozygous genotypes also observed in different frequencies in Iraqi breast cancer women treated with anastrozole by using AS-PCR.
4. The CYP3A4 and UGT1A4 enzymes that were detected among Iraqi breast cancer patients were observed to be non-significant correlated with variable serum level of estradiol hormone (E2) and tumor marker (CA 15.3) suggesting that *CYP3A4* and *UGT1A4* genotype status had no impact on the level of these parameters.
5. The existing finding suggest that no association between polymorphism of *CYP3A4* and *UGT1A4* genes with development of arthralgia in Iraqi breast cancer women with Anastrozole treatment.

4.6 Recommendations and further work

1. Further large scale and multi center studies are needed in Iraqi breast cancer women receiving Anastrozole to fully define the impact of *CYP3A4* and *UGT1A4* genetic polymorphism on Anastrozole response, increase sample size and use of case-control study instead of cross-sectional observational study
2. Study other polymorphisms of *CYP3A4* and *UGT1A4* in local subject to create agentic map and determine the cause of interindividual variation in Anastrozole response
3. Study the combined effect of *CYP3A4* and *UGT1A4* genotype with predicted phenotype by determining the metabolic ratio of CYP3A4 and UGT1A4 enzymes, which are highly precise and direct method for determination of CYP3A4 and UGT1A4 enzymatic activity .one way to do this by measuring plasma level of Anastrozole and its metabolites.
4. Study genetic variant in other enzymes that involved in metabolism of Anastrozole could contribute to individual variability in Anastrozole response and may warrant further investigation.
5. Study the genotyping of the gene encoding estrogen synthesis (*CYP19A1)* in breast cancer patients who received Anastrozole as adjuvant therapy and correlate these results with clinical outcome.
6. Study the effect of other genes or factors that may the development of arthralgia in breast cancer patients who received Anastrozole as adjuvant therapy.
7. Further studies are needed to demonstrate the effect of marital status and risk of breast cancer.

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Appendices

Questionnaires of breast cancer patients

1. Name
2. Age
3. Wight
4. Academic achievement
5. Marital status
6. Breast feeding
7. Dates of first menarche and last menopause
8. Breast cancer family history
9. Diagnosis date of breast cancer
10. Site of breast cancer (right, left or bilateral)
11. Type of breast cancer
12. Stage and grade of breast cancer
13. Immunohistochemical status
14. Presence of arthralgia or other side effects
15. Date and site of recurrence
16. Time on anastrozole
17. Co-medications

الخلاصة

خلفية البحث: يعتبر سـرطان الثدي واحد من المشـاكل الصـحية العالمية،ويؤثر على انسـجة الثدي،حيث يبلغ معدل الاصـابة في العالم بحوالي (١,٧) مليون امرأة مصـابة بسـرطان الثدي سـنوياً، ويشـكل حوالي (٢٥٪) من جميع انواع السـرطانات حول العالم، ويعتبر السـبب الثاني للوفيات بين النسـاء في العالم بعد سرطان الرئة . في العراق عموماًوفي كربلاء خصـوصـاً ، يحتل سرطان الثدي المرتبة الاولى بين العشر انواع الاولى من السرطان وكذلك السبب الاول للوفاة بين النساء العراقيات.

الاروماتيز ةو الانزيم الرئيسـي في الخطوة النةائية لتصـنيع الاسـتروجين الذي لة دوراً مةماً في حدوث سـرطان الثدي ، الاناسـترازول ةو علاج مثبط لانزيم الاروماتيز حيث يسـتخدم لعلاج سرطان الثدي ،وبفعل انزيمات الكبد CYP3A4 and UGT1A4 يتحول ايضياً الى شكل غير فعال،لذلك الاشـكال المتعددة للنيوكليتدات المفردة (SNPs) للجينات السـؤولة عن ةذة الانزيمات قد تلعب دور في الاختلافات الفردية للاسـتجابة وتطور الاثار الجانبية للعلاج الاناسترازول.

اةداف الدراسة: تهدف هذة الدراسة الكشف عن تعدد الأشكال الجيني للإنزيمات الهاضمة لعقار الأناستروزول المشفرة بواسطة جين *CYP3A4* وخاصة *CYP3A4 * 22* (G>A) (rs35599367) وجين *UGT1A4* خاصة *UGT1A4*2* (C>A,T) (rs675557) في النساء العراقيات المصابات بمرض سرطان الثدي ، وكذلك للتحقيق في تأثير *CYP3A4 * 22* (G>A) (rs35599367)و *UGT1A4 * 2*(C>A,T)(ra6755571) على فعالية عقار الاناسترازول ، بالإضافة إلى التحقق من ارتباطهما مع تطور ألم المفاصل (كتأثير جانبي) لدى النساء العراقيات المصابات بسرطان الثدي المستخدمات لعقار الاناسترازول.

المرضى وطرق العمل:أجريت هذه الدراسة العرضية المقطعية في مدينة الإمام الحسين الطبية / مركز الأورام في كربلاء. تم اختيار مائة امرأة مصابة بسرطان الثدي الإيجابي (ER و / أو PR) للمشاركة في هذة الدراسة. تم علاج جميع النساء المشمولات في ةذة الدراسة اللواتي تتراوح أعمارةن بين (31-75) باستخدام قرص أناستروزول (1 مجم) مرة واحدة يوميًا كعلاج مساعد قياسي. تم الحصول على عينات الدم من النساء المؤهلات اللائي وقعن على الموافقة المسبقة للاختبار الجيني وقياس مستوى الاستراديول (E2) وعلامة الورم (CA15.3) في مصل الدم. استخدمت هذة الدراسة نظام تفاعل البوليميراز المتسلسل (ARMS PCR)وتفاعل البوليميراز المتسلسل المحدد

لأليل (AS-PCR) للكشف عن *CYP3A4*22* (G>A) (rs35599367) و *UGT1A4 * 2* (C>A,T) (rs6755571)على التوالي.كما و تم تقييم آلام المفاصل اعتمادًا على تاريخ المريض من حيث استعمال عقار الزولدرونيك اسد بالإضافة إلى استبيان(BPI).

النتائج: تم الكشف في هذة الدراسة عن عدة انماط وراثية ل *CYP3A4*22* (G>A) (rs35599367) و *UGT1A4*2* (C>A,T) (rs6755571) والتي تشمل النمط الوراثي المتماثل الزيجة (GG) والنمط الوراثي المتماثل الزيجة الطافر (AA) والنمط متغايرة الزيجة (GA) بالنسبة للطفرة *CYP3A4*22* (G>A) (rs35599367) اما بالنسبة للطفرة *UGT1A4*2* (C>A,T) (rs6755571) تم الكشف عن نمطين وراثيين متماثلين الزيحة طافرين وهما (TT) الذي وجدة بكثرة و (AA) الذي وجدة بنسبة قليلة جداً (٤٪) بالاضافة الى النمط الجيني المتماثل الزيجة (CC) وكذلك الانماط غير متماثلة الزيجة (CT,CA,TA)لدى النساء المشمولات في الدراسة.كما واظهرت الدراسة الحالية انة هنالك ارتباط معنوي ضعيف بين الطفرات الجينة المدروسة واختلاف مستوى الاستراديول وعلامة الورم في مصل الدم باستثناء النمط الجيني المتحول (AA) الذي وجدة بنسبة قليلة جداً .عدد كبير من النساء المشمولات بالدراسة يعانين من الام المفاصل المشخص من قبل الطبيب ولكن اظهرت النتائج عدم وجود ارتباط معنوي بين الطفرات الجينية المدروسة وتطور الالم المفصلي لدى النساء المستعملات لعلاج الاناسترازول في هذة الدراسة.

الاستنتاجات: كشفت هذة الدراسة عن وجود انماط جينية مختلفة لجينات (*CYP3A4* and *UGT1A4*, بالاضافة الى ذلك، اوضحت الدراسة ان *CYP3A4*22* (G>A) (rs35599367) الذي تم الكشف عن الطفرات باستخدام تقنية ARMS-PCR و *UGT1A4*2* (C>A,T) (rs6755571) حيث تم الكشف عن الطفرات بتقنية AS-PCR ليس لهما تأثيرعلى الاستجابة الدوائية للاناسترازول (عدم وجوداختلافات معنوية في مستوى الاستراديول E2 وعلامة الورم CA 15.3في مصل الدم بين الانماط الجينية المختلة لكلا الطفرتين) وكذالك الطفرات الجينة التي وجدت للجينات المدروسة ليس لها علاقة بتطور الم المفاصل لدى النساء المشمولات في هذة الدراسة و المستعملات لعقار الاناسترازول كعلاج لسرطان الثدي .

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تأثير تعدد الشكل الجيني (لجينات*UGT1A4* و *CYP3A4*) على الاستجابة لعقار الاناسترازول في النساء المصابات بمرض سرطان الثدي في العراق

رسالة

مقدمة الى كلية الصيدلة / جامعة كربلاء كجزء من متطلبات درجة الماجستير في علم الادوية والسموم

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