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Detection of Estrogen Receptor Alpha and Beta Gene Mutations in Iraqi Women with Breast Cancer

A Dissertation

Submitted to the Council of College of Science, Al-Nahrain University, in Partial Fulfillment of the Requirements for the Degree of Doctor in Philosophy of Biotechnology

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وأَنْزَلَ اللهُ عَلَيكَ الكِتابَ و ٱلحِكْمَةَ و عَلَمكَ ما لَمْ

تَكُنْ تَعلَمْ وَ كانَ فَضْلُ اللهِ عليكَ عَظيماً

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Sahar Hadad

Summary

This study was aimed to determine the mutations and single nucleotide polymorphisms (SNPs) in exon 4 and 6 of estrogen receptor alpha (*ESR1*) gene as well as exon 3 and 7 of estrogen receptor beta (*ESR2*) gene in Iraqi women with breast cancer.

Different group of samples (25 of blood with FFPE from the same patient, 15 of fresh tissue with blood and FFPE from the same patient, and formalin fixed paraffin embedded) were collected from women with breast cancer during 1st of April to 1st of September, 2014.

Immunoexpression of estrogen receptor (ER) protein was examined in 50 samples of FFPE by using immunohistochemistry technique. A specific positive immune staining of estrogen receptor was detected in 35 (70%) women with breast cancer. Moreover, a correlation between expression of estrogen protein and risk factors such as age, familiar history, and metastasis to lymph node was studied. A significant difference was noticed between expression of estrogen protein and age, familiar history, and metastasis lymph node while non-significant differences were found between estrogen expression and menopause.

On the other hand, the molecular analysis of exon 4 and 6 in *ESR1* and exon 3 and 7 in *ESR2* has been studied by using PCR. It was found that exon 4 and 6 in *ESR1*were appeared as a single band with size 370 and 300 bp respectively, while exon 3 and 7 in *ESR2* were revealed as a single band with size 151 and 157 bp, respectively.

Moreover, single nucleotide polymorphisms (SNPs) were determined in exon 4 and 6 of *ESR1* and in exon3 and 7 of *ESR2* using DNA sequence. Then, nucleotide sequences of these exons were aligned with the control group (healthy women) and with NCBI. Seven polymorphisms (AAG, AAA, TTT,

AAA, CCG, AAA, and AAC) were detected in exon 4 of *ESR1*, six of them (AAG, AAA, TTT, AAA, AAA, and AAC) were novel SNPs while CCG was notified as a common polymorphism in comparison with variants recorded in different population, all types of polymorphism in exon 4 of *ESR1*were substitution. Whereas no SNPs were detected in exon 6 of *ESR*, nevertheless two polymorphisms, A 375455 C and G 375718 T, in intronic flaking region around this exon were determined.

Regarding *ESR2*, there was no SNP in exon 3 has been identified. While three novel polymorphisms (ACT, AGG and GCA) were detected in exon 7, the type of those polymorphisms was deletion for ACT and AGG while substitution for GCA.

From this study, it can be concluded that some single nucleotide polymorphism in *ESR1* and *ESR2* may effect gene expression.

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List of Abbreviations

Abbreviation	**Meaning**
AJCC	American Joint Committee Cancer
BRCA1,2	Breast and overian cancer
DAP	DAP Dimethyl benzidine

NCBI	National center for biotechnology information
ER	Estrogen receptor
ESR1, ERα	Estrogen receptor alpha
ESR2, ERβ	Estrogen receptor beta
ERE	estrogen response element
HSP	heat-shock proteins
Pmol	Picomole
Primer F	Primer forward
Primer R	Primer reverse
TBE	Tris-Borate EDTA
UV	Ulteraviolet
AI	Alellic impalance
FFPE	Formalin fixed paraffin embedded tissue
LOH	loss of heterozygosity
CGH	comparative genomic hybridization
EGFR	epidermal growth factor receptor

Chapter One
Introduction and Literature Review

1.1. Introduction

Breast cancer is the most common cause of cancer death and the most common form of cancer in women with a 9% incidence of being diagnosed during a lifetime (Lacroix and Leclercq, 2005). In Iraq, breast cancer is the most common type of malignancy in women, it accounts for one third of the registered female cancers according to the latest Iraqi Cancer Registry (Iraqi Cancer Registry, 2009) and this showed a trend for the disease to affect younger age groups (Lawn, 2010). There was an increase in the incidence rates of breast cancer within the last two decades, which became one of the major threats to Iraqi female health.

The estrogen receptor (ER) plays an important role in the pathogenesis and maintenance of breast cancer, it is a ligand-inducible transcription factor which regulates the expression of a variety of genes including some growth factors. The cellular signaling of estrogens mediated through two estrogen receptors, estrogen receptor -alpha (*ESR1*) and estrogen receptor- beta (*ESR*2), both belonging to the nuclear receptor (NR) family of transcription factors (Thomas and Gustafsson, 2011).

The *ESR1* gene is located on chromosome 6q25-27 and it consists of eight exons and spans more than 140 kb, while the *ESR2* gene is located on chromosome 14q22-24, comprises eight exons and spans with 40 kb (Ascenzi *et al*, 2006).

The expression of *ESR1* was studied as a predictive marker of treatment response, it status in breast tumors provided prognostic information and it is the primary target for endocrine therapy (Howell, 2006). While the role of *ESR2* for initiation and development of breast cancer has not yet been clearly established,

it remains controversial (Madeira *et al*., 2013). Several studies suggested that the expression of *ESR2* independently predicted a better disease-free survival in patients treated with endocrine therapy (Hodges-Gallagher *et al*., 2008). However, some data suggested that the positivity of ESR2 protein was associated with low cellular differentiation, which indicated that this receptor might be related to worse overall survival (Qui *et al*., 2009).

Investigation of the molecular mechanisms of carcinogenesis and development of human breast cancer, the regulation of *ESR1* gene expression was an important issue in breast cancer, and the over expression of ESR1 was an initial significant event in its genesis (Hirata *et al*., 2014).

Single nucleotide polymorphisms (SNPs) in specific candidate genes are thought to influence expression and activity of encoding proteins there by predisposing to cancer. Generally, the presence of mutations or SNP in any of the estrogen receptors may have a serious effect on the production of estrogen and it subsequently would effect on the growth of cancer (Siddig *et al*., 2008). SNPs in estrogen receptor α and β have additive effects in increasing risk for developing breast cancer with lymph node metastases (Abbasi *et al*., 2012).

This study was aimed to determine the mutations and single nucleotide polymorphism (SNPs) in the exons of estrogen receptor alpha (*ESR1*) and estrogen receptor beta (*ESR2*) genes in women with breast cancer from Iraq.

1.1. Introduction

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1.2. Literature review

1.2.1. Breast cancer

Cancer is a genetic disease, caused by multiple series of mutations in oncogenes, tumor suppressor genes and stability genes (Vogelstein and Kinzler, 2004). Factors can affect the risk of obtaining these alterations, approximately 5-10% of all breast carcinomas are caused by germ line mutations, in the breast cancer gene mainly 1 and 2 (*BRCA1* and *BRCA2*), the remaining 90-95% is considered sporadic cancers which acquired somatic mutations (Lacroix and Leclercq , 2005).

This disease is the most frequent type of cancer in female that leads to death (Jemal *et al*, 2011). It is a heterogeneous disease that multiple environmental and genetic factors play important roles in causing this disease. Epidemiological studies indicated that age, obesity, family history reproductive factors are associated with the increased risk of breast cancer (McPherson *et al*., 2000; Yuxiang *et al*., 2014).

Tumors are estrogen dependent, meaning that the breast cells are dependent on estrogens to growth and survival (Ali and Coombes, 2002). Approximately 80% breast cancers are classified as estrogen receptor alpha (*ESR1*) positive, defined tumor cells with detectable nuclear estrogen receptor staining in the invasive component of the tumor (DBCG, 2013).

1.2.2. Autonomy of breast

The breast of a female is composed mainly of lobules that are milk-producing glands, ducts which are tiny tubes that carry the milk from the

lobules to the nipple, and stroma contain fatty tissue and connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels (Maxwell and Gabriel, 2009; American Cancer Society, 2010) as shown in Figure (1-1).

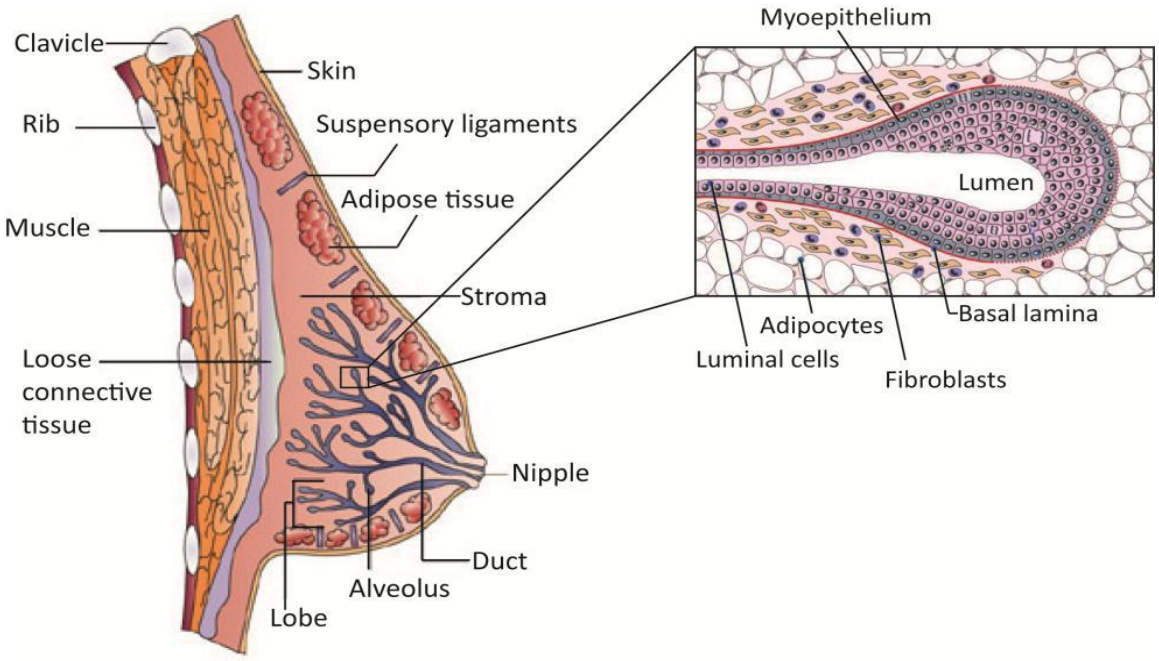


Figure (1-1): Anatomy and structure of female breast (American Cancer Society, 2010).

The structure of female breast from the nipple long ducts terminates in networks of alveoli called lobes. The ducts and alveoli penetrate the stroma and adipose tissue. The illustration includes a magnification of one alveolus visualizing the different cell layers, also shows the rib, while muscle underlying the breast followed by loose connective tissue. Suspensory ligaments and the skin are indicated as well (Ali and Coombes, 2002; Sternlicht, 2006).

Most breast cancers found in the cells that line the ducts (ductal cancers). Some begin in the cells which line the lobules (lobular cancers), while a small

number begin in other tissues. The disease occurs almost entirely in women, but men can get it. Metastasis occurs when localized spreads to the overlying skin and underlying muscles and ribs. Distant metastasis occurs through the lymphatic drainage, vascular system, or neural network which assists to form secondary tumors in preferred organs such as lung, bone, liver, and brain (Abeloff *et al*., 2008; American Joint Committee on Cancer, 2010; American Cancer Society, 2015).

1.2.3. Etiology

Women are at danger of developing breast cancer. Several relatively strong risk factors for breast cancer that affect a large proportion of the general population were known for some time. However, the vast majority of breast cancer cases found in women that have no identifiable risk factors other than their gender and age (Kelsey and Gammon, 1990). The other established risk factors for breast cancer are shown in Table (1-1).

Several factors such as life style and environment can affect developing risk of breast cancer; which includes physical inactivity, obesity after menopause and alcohol consumption (Jemal *et al*., 2011). The predominant risk factor in sporadic breast cancers is hormonal, mainly according to changes in estrogen levels (Brekelmans, 2003).

In breast cancer, estrogens act as tumor promotors, they stimulate proliferation of the breast epithelium and act as an anti-apoptotic effect. Additionally, the oxidative metabolites generated by estrogen metabolism stimulate genotoxic stress (Yager and Davidson, 2006).

Table (1-1): Factors increase the relative risk for breast cancer (Hulka and Moorman, 2001).

Relative Risk	Factors
General factors	• Female Age (65+ vs. <65 years, although risk increases across all ages until age 80) • Certain inherited genetic mutations for breast cancer (BRCA1 and/or BRCA2) • Two or more first-degree relatives with breast cancer diagnosed at an early age • Personal history of breast cancer • High breast tissue density or 75% dense • Biopsy-confirmed atypical hyperplasia • One first-degree relative with breast cancer • High-dose radiation to chest • High bone density (postmenopausal)
Factors that affect circulating hormones	• Late age at first full-term pregnancy (>30 years) • Early menarche (<12 years) • Late menopause (>55 years) • No full-term pregnancies and No breast feeding • Recent oral contraceptive use • Recent and long-term use of HRT • Obesity (postmenopausal) • Personal history of endometrial or ovarian cancer
Other factors	• Alcohol consumption • High socioeconomic status

Prolonged endogenous exposure to estrogens caused by early late menopause, menarche, late age at first full term pregnancy or nulliparity raised the risk of cancer. Also exogenous ovarian hormones, like hormone

replacement therapy or oral contraceptives lead to increase the risk of breast cancer (Rajkumar *et al*., 2001; Anderson and Clarke, 2004).

1.2.4. Diagnostic of breast cancer

There are several methods used for detection of breast cancer

1.2.4-1 Histopathological diagnosis

- **Histology of breast cancer**

Histopathology depends on types, grade and stage. Breast cancer is divided into *in situ* ductal and lobular and invasive disease. There are more than 21 subtypes of invasive breast carcinoma defined in the fourth edition of the WHO classification of tumors of the breast. The most frequent is invasive carcinoma of no special type NST, (also known as invasive ductal carcinoma, NST), and this comprises 40– 75 % of cases. The remaining tumors types are morphologically distinct 'special' types including invasive lobular, tubular, mucinous and metaplastic carcinoma and carcinoma with medullary, neuroendocrine or apocrine features (Rakha and Ellis, 2011; Lakhani *et al*., 2012). Examples of morphological variants of invasive breast cancer are shown in Figure (1-2).

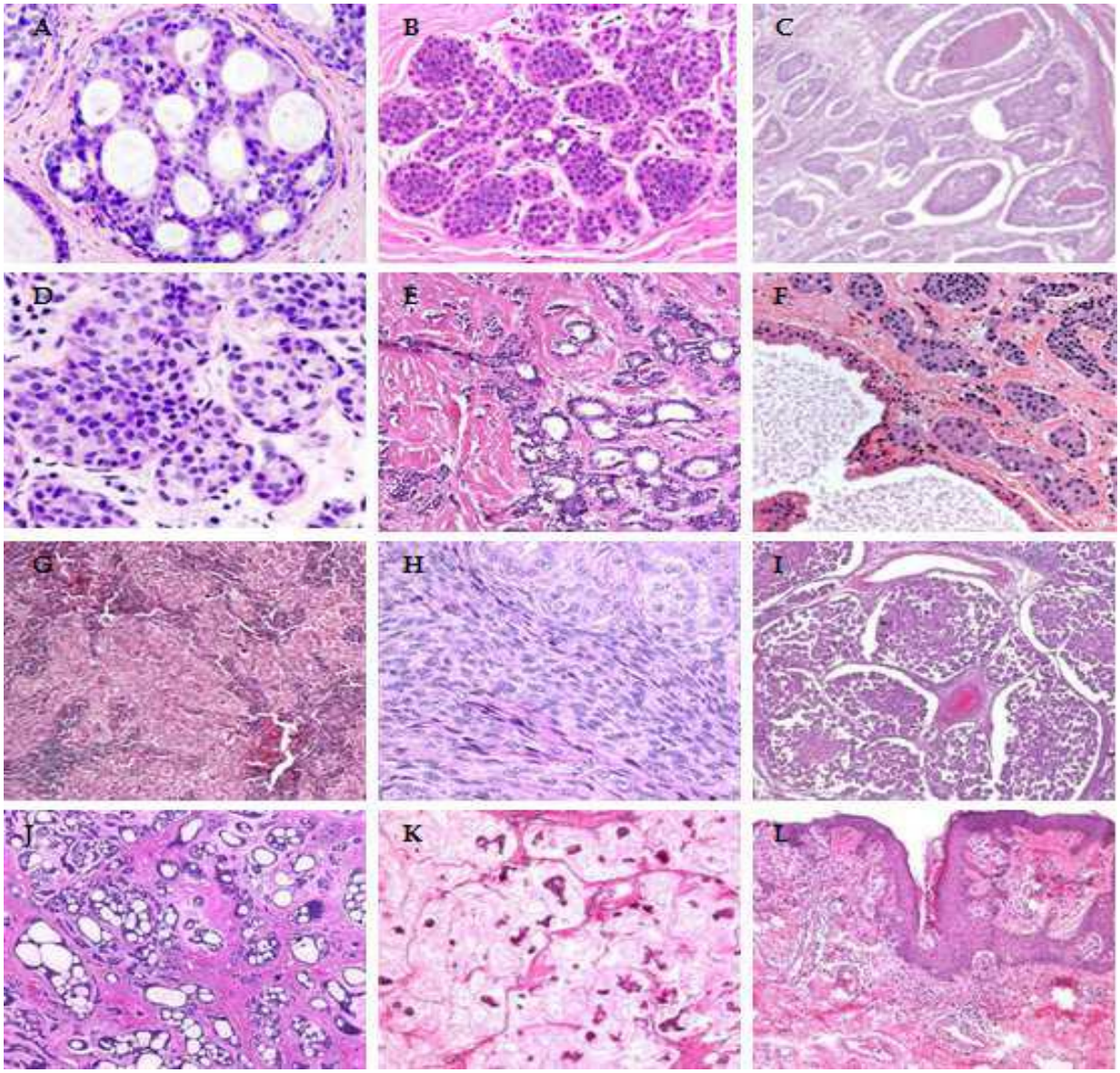


Figure (1-2): Histology of breast carcinoma. Breast carcinoma is classified into Ductal (A), Lobular carcinoma (B) and Inflammatory carcinoma (C). It can be further classified into noninvasive (A-B) and invasive carcinoma (C-L). Invasive cancer includes Inflammatory (C), Invasive lobular (D), tubular (E) apocrine (F), medullary, (G) metaplastic (H), micropapillary, (I) adenoid cystic (J), mucunous carcinoma (K), and paget disease (L) (Reis-Filho *et al*, 2005)

- **Histological grade of breast cancer:**

Histological tumor grade is based on the degree of differentiation of the tumor tissue. In breast cancer, it refers to the semi-quantitative evaluation of morphological characteristics and is a relatively simple and low cost method, requiring only adequately prepared hematoxylin-eosin-stained tumor tissue sections to be assessed by an appropriately trained pathologist using a standard protocol. As shown in Figure (1-3), it is based on the evaluation of three morphological features (Elston and Ellis, 1991; Walker, 2003; Pathology Reporting of Breast Disease, 2005):

(a) Degree of tubule or gland formation

(b) Nuclear pleomorphism

(c) Mitotic count

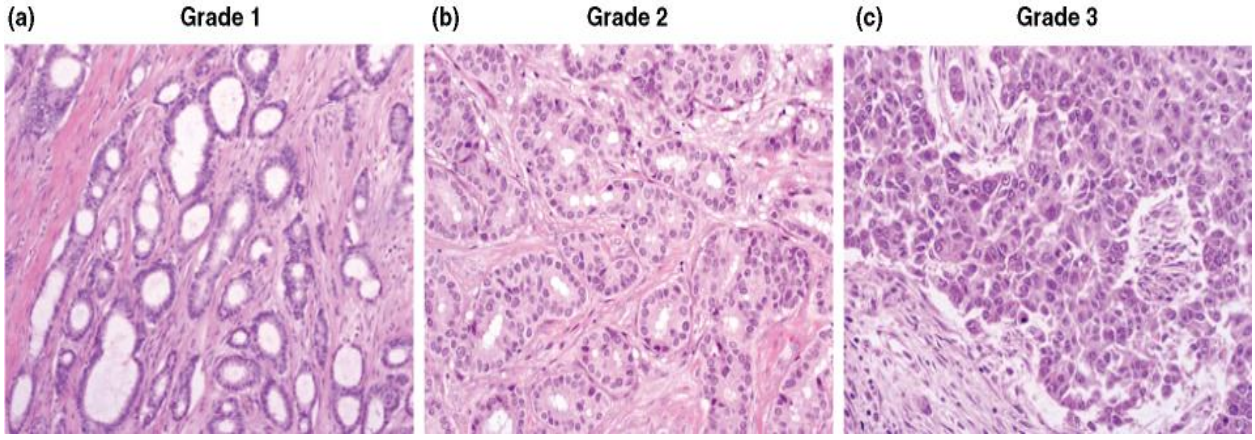


Figure (1-3): Histological grade of breast cancer as assessed by the Nottingham Grading System (Pathology Reporting of Breast Disease, 2005).

(a) A well-diff erentiated tumor (grade 1) that demonstrates high homology to the normal breast terminal duct lobular unit, tubule formation (>75%), a mild degree of nuclear pleomorphism, and low mitotic count.

(b) A moderately diff erentiated tumor (grade 2).

(c) A poorly diff erentiated (grade 3) tumor with a marked degree of cellular pleomorphism and frequent mitoses and no tubule formation (<10%).

- **Classification of breast cancer according to histopathology:**

A-In situ

1. Ductal carcinoma in situ (DCIS) is a spectrum of abnormal breast changes that start in the cells lining the breast ducts. DCIS is considered a noninvasive form of breast cancer because the abnormal cells have not grown beyond the layer of cells where they originated. It is the most common type of in situ breast cancer. Studies suggest that about one-third, and possibly more, of DCIS cases will progress to invasive cancer if left untreated.1 Identifying subtypes of DCIS that are most likely to recur or progress to invasive cancer is an active area of research (Solin *et al*., 2013).

2. Lobular carcinoma *in situ* (LCIS, also known as lobular neoplasia) is not a true cancer or precancer, but an indicator of increased risk for developing invasive cancer.

3. Other *in situ* breast cancers have characteristics of both ductal and lobular carcinomas or have unknown origins.

B-Invasive

Most breast cancers are invasive, or infiltrating. These cancers have broken through the ductal or glandular walls where they originated and grown into surrounding breast tissue.

The TNM classification of tumors uses information on tumor size and how far it has spread within the breast (T), the extent of spread to the nearby lymph nodes (N), and the presence or absence of distant metastases (spread to distant organs) (M) (Edge *et al*., 2010) Once the T, N, and M are determined, a stage of 0, I, II, III, or IV is assigned, with stage 0 being in situ, stage I being early stage invasive cancer, and stage IV being the most advanced disease. The TNM staging system is commonly used in clinical settings shown in Table (1-2) (AJCC, 2010).

Table (1-2): TNM staging systems for esophageal cancer (AJCC, 2010)

Primary tumor (T)			
Tis	Carcinoma in situ	TX	Primary tumor cannot be assessed
T1	Tumor involves ≤ 5 cm of esophageal length, produces no obstruction, and has no circumferential involvement	T0	No evidence of primary tumor
		Tis	High-grade dysplasia[a]
T2	Tumor involves > 5 cm of esophageal length, causes obstruction, or involves the circumference of the esophagus	T1	Tumor invades lamina propria, muscularis mucosae, or submucosa
		T1a	Tumor invades lamina propria or muscularis mucosae
T3	Extraesophageal spread	T1b	Tumor invades submucosa
		T2	Tumor invades muscularis propria
		T3	Tumor invades adventitia
		T4	Tumor invades adjacent structures
		T4a	Resectable tumor invading pleura, pericardium, or diaphragm
		T4b	Unresectable tumor invading other adjacent structures, such as aorta, vertebral body, trachea, etc.
Regional lymph nodes (N)			
NX	Regional nodes cannot be assessed	NX	Regional nodes cannot be assessed
N0	No nodal metastases	N0	No regional nodal metastases
N1	Unilateral, mobile, regional nodal metastases (if clinically evaluable)	N1	Regional lymph node metastases involving 1 to 2 nodes
N2	Bilateral, mobile, regional nodal metastases (if clinically evaluable)	N2	Regional lymph node metastases involving 3 to 6 nodes
N3	Fixed nodes	N3	Regional lymph node metastases involving 7 or more nodes
Distant metastases (M)			
M0	No distant metastases	M0	No distant metastases
M1	Distant metastases	M1	Distant metastases

Pathologic stage grouping, AJCC 2010
Squamous cell carcinoma[b]

GROUP	T	N	M	Grade	Tumor Location[c]
Stage 0	Tis (HGD)	N0	M0	1	Any
Stage IA	T1	N0	M0	1, X	Any
Stage IB	T1	N0	M0	2–3	Any
	T2–3	N0	M0	1, X	Lower, X
Stage IIA	T2–3	N0	M0	1, X	Upper, middle
	T2–3	N0	M0	2–3	Lower, X
Stage IIB	T2–3	N0	M0	2–3	Upper, middle
	T1–2	N1	M0	Any	Any
Stage IIIA	T1–2	N2	M0	Any	Any
	T3	N1	M0	Any	Any
	T4a	N0	M0	Any	Any
Stage IIIB	T3	N2	M0	Any	Any
Stage IIIC	T4a	N1–2	M0	Any	Any
	T4b	Any N	M0	Any	Any
	Any T	N3	M0	Any	Any
Stage IV	Any T	Any N	M1	Any	Any

1.2.4-2. Hormonal diagnosis by using immunohistochemistry, IHC, assay

The published studies used five surrogate IHC markers (ER, PR, HER2, CK5/6, and EGFR) (Carey *et al*., 2006; Al Tamimi *et al*., 2009). At least five main molecular classes of breast cancer are currently recognized:

- **Luminal A:** Luminal A tumor is ER positive, PR positive or negative, HER2 negative, and CK5/6 and EGFR negative. Luminal A was the most frequent subtype and good prognosis and responds well to hormone therapy (Bhargava *et al*., 2009; Al Tamimi *et al*., 2010).
- **Luminal B:** Luminal B tumor identification at the protein level is a point of controversy. The coexpression of HR and HER2 used to define this group, based on the fact that the HER2 associated genes (i.e., ERBB2 and GRB7) are expressed in 30-50% of tumor luminal (Carey *et al*., 2006). As well, this tumor has a poorer prognosis than luminal A tumor and endocrine therapy that require estrogen deprivation in addition to blockage of HER2 pathways (Rastelli and Crispino, 2008).
- **HER2:** the HER2 (+ ve) tumor need to considere separately from pure luminal tumors, which might be further categorized as luminal A and luminal B, with this showing copositivity of HER2 grouped into a separate hybrid category termed (luminal–HER2 hybrids) (Bhargava *et al*., 2009; Al Tamimi *et al*., 2010).
- **Basal:** Basal-like tumor is CK5/6 and/or EGFR positive, ER and PR negative, and HER2 negative (Bhargava *et al*., 2009; Al Tamimi *et al*., 2010). The basal class is named acording to its pattern of expression that is the same to basal epithelial cells and normal myoepithelial cells of mammary tissue (Perou *et al*., 2000).
- **Unclassified:** Unclassified type is considered synonymous with (normal like) breast cancers. This tumor exhibits overexpression of *PIK3R1* and

AKR1C1, in addition to other genomic alterations. Unclassified tumor is ER and PR negative, HER2 negative, and CK5/6 and EGFR negative, it correspond to the triple negative tumors which not exhibit basal markers (Al Tamimi *et al*., 2010).

Cheang and his coworkers (2009) added that a Ki67 proliferation index of more than 13.25% is a hallmark of luminal B tumors. Ki67 is a nuclear marker of cell proliferation. Besides, its expression correlates proportionally to poorer clinical outcomes.

1.2.4-3. Genomic and molecular diagnosis

Molecular profiling of breast cancer phenotypes was firstly investigated using loss of heterozygosity analysis (LOH) and comparative genomic hybridization (CGH), identified in breast cancer as a key genomic alterations (Albertson and Pinkel, 2003; Reis-Filho *et al*., 2005). Although relatively rudimentary, LOH and CGH provided the early framework for a genomic classification system which stratified breast cancers into distinct low- and high-grade arms. This suggested that the development from normal cells through independent pathways subsequent investigations using a CGH and mutational analysis have suggested exceptions (Simpson *et al*., 2005; Lopez-Garcia *et al*., 2010).

Variant of lobular carcinoma starts from a classic low grade lobular carcinoma buttoned up stochastic acquisition of mutations in *TP53*, *HER2* or *MYC* (Simpson *et al*., 2008). Similarly, there was data suggesting that at least some high-grade ER positive breast cancers arise from low grade ER positive cancers. The multistep model will no doubt continue to be refined through the use of newer technology, allowing more information and comprehensive

genomic analysis. Figure (1-4) showed different technologies timeline used in the molecular classification of breast cancer.

The behavior and phenotype of somatic mutations was underpinned, no surprise that the classification of breast tumors has been attempted based on the type and pattern of alterations that occur in the DNA of tumor cells. Research that occurred in 2005 used a high-throughput large-scale genome profiling that includes sequencing analysis to record the genetic mutations underpinning a range of cancers including breast cancer (Cancer Genomic Atlas Network, 2012).

Then, the International Cancer Genome Consortium was launched, with researchers from around the world collaborating for mapping the genomes for at least 50 types of cancer, with different studies underway concerning genetic changes in different types of breast cancer (Stephens *et al*., 2009; Stephens *et al*., 2012). These consortia and other research groups now use 'multi-omic' approaches involving different technology platforms (genomic DNA copy number arrays, DNA methylation arrays, exon sequencing, mRNA arrays, microRNA sequencing and reverse-phase protein arrays) for developing a more global and integrated picture of breast cancer (Shah *et al*., 2012; Ellis *et al*., 2012).

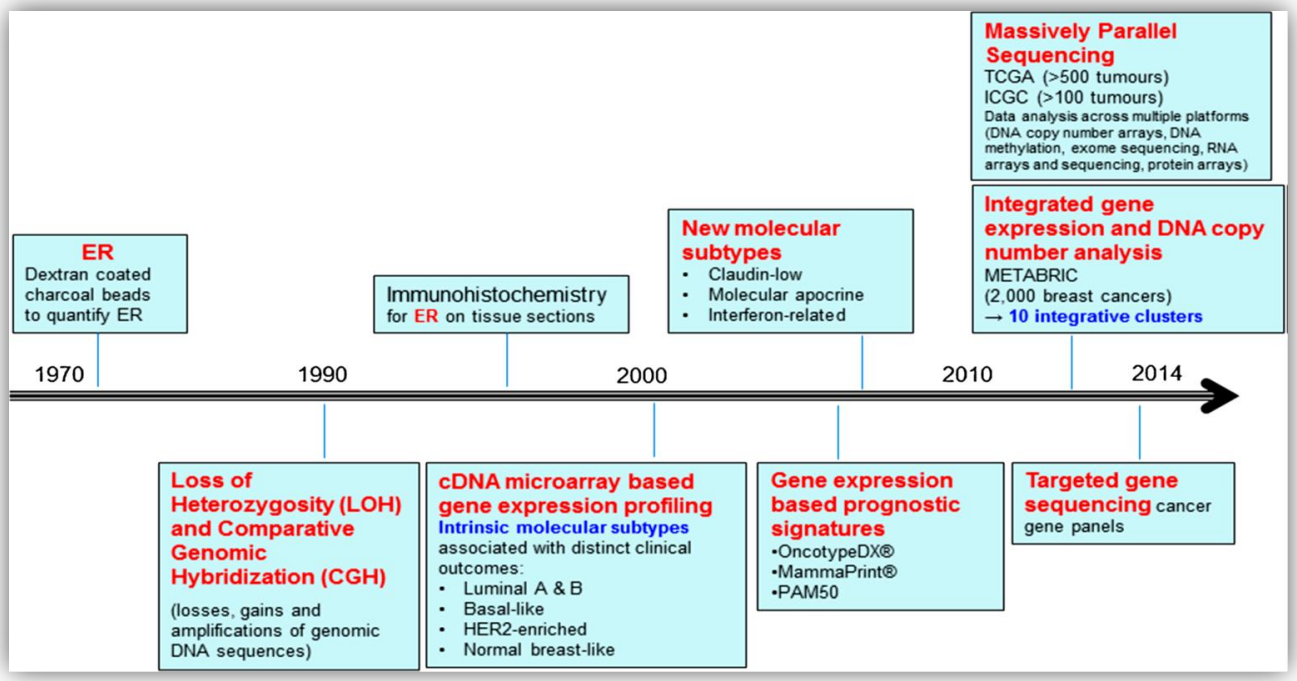


Figure (1-4): Different technologies used in the molecular diagnosis of breast cancer (Vuong *et al*., 2014).

1.2.5. Breast cancer markers

Tumor markers is a useful tool for the clinical management of cancer patients, assisting for diagnostic procedures, staging, therapeutic response evaluation, distant metastasis and recurrence and prognosis detection (Matos *et al*., 2005), helping in the development of new treatment modalities (Pacheco *et al*., 2002).

According to US national institutes of health's working group and biomarkers consortium, a molecular marker is a characteristic measured as an indicator for pathogenic or processes of normal biological, or therapeutic intervention pharmacological response (Mishra and Verma, 2010).

Most of these markers are protein, gene expression patterns and altered DNA identified in tumor tissue as tumor markers (Ventura and Merajver, 2008).

Moreover, there are many markers can be used for the detection of breast cancer:

1. **Hormone Receptors (HR)**

The hormone receptors (HR) are expressed proteins both in the epithelium and in breast stroma which bind to circulating hormones, and mediate their cellular effects (Rosen, 1987; Haslam, 1989).

The HR studied in breast cancer is estrogen receptor (ER). Breast cancers were classified by positive immunehistochemistry (IHC) expression of (Althuis *et al*., 2004). Approximately more than one million women are diagnosed with breast cancer each year and approximately 700.000 of these have positive (+ve) hormone receptors (Piccart-Gebhart, 2011).

The risk factors are closely associated with breast tumors ER (+ve) and may involve mechanisms related to exposure to estrogen, while etiology of breast cancer ER (-ve) should be independent of hormone exposure (Yoo *et al*., 2001; Manjer *et al*., 2001).

2. **Human Epidermal Growth Factor Receptor 2 (*HER-2*)**

Several names have been given for gene, such as *c-erb-2*, *C-rbB-2*, *cerbB-2*, *HER-2*, *HER-2/neu*, *ERBB2*, *erbB2*, *erbB- 2*, *neu/c-erbB-2*/oncogene *neu*, neu protein (Eisenberg and Koifman, 2001). HER-2 is a transmembrane tyrosine kinase receptor belonging to a family of epidermal growth factor receptors structurally related to epidermal growth factor receptor (EGFR) encoded by *ERBB2/HER2* oncogene that is located on chromosome 17q21(Yamamoto *et al*., 1986). Her-2 is express in 20 to 30% of breast cancers and considered a marker of poor prognosis, once it’s over expression it is associated with an

aggressive phenotype of tumor cells, resistance to anti-hormonal, cytotoxic therapies, and low overall survival (Barnes, 1993).

3. **Ki 67 Antigen**

The Ki-67 antigen, first described in 1983, it is non-histone nuclear protein that is tightly linked to the cell cycle and expressed in mid G1, S, G2, and M phases of proliferating cells but not in resting or quiescent cells of the G0 and early G1 phases. Ki-67 score is the most often measured on histological sections by IHC methodology and defined as the percentage of stained invasive carcinoma cells (Gerdes *et al.*, 1984; Reyal, 2012).

4. **Tumor Protein p53**

The p53 is involve in several critical pathways including cell cycle arrest, apoptosis, DNA repair, and cellular senescence, which it is essential for normal cellular homeostasis and genome integrity maintenance. Alteration of *TP53* gene or posttranslational modification in p53 protein leads to alter in response to cellular stress (Hussain and Harris, 2006). In breast cancer, approximately 30% of patients display *TP53* gene mutation, frequency fluctuates from more than 80% in basal-like to less than 15% in luminal-A subtypes (Sorlie *et al*, 2001). According to Allred *et al*. (1993), mutant p53 protein expression is associated with a high tumor proliferation rate, early disease recurrence, and early death in node negative breast cancer.

5. **Carbohydrate 15-3 and carcinoembryonic antigens (CA 15-3 and CEA)**

CA 15-3 combination with CEA is also a relevant tumor marker in breast cancer (Vizcarra *et al*., 1997). The serum marker CA 15-3 has a superior prognostic relevance in relation to CEA (Geraghty *et al*., 1992). Ebeling *et al*., (2002) reported that the prognostic value of CEA is higher than CA 15-3, which demonstrated that these markers have conflicting implications in breast

carcinogenesis. The CEA is a glycoprotein which expressed in vast majority of human colorectal, gastric, and pancreatic cancers, as well as in breast carcinomas and nonsmall cell lung carcinomas (Thompson *et al*., 1991). Determination of CEA in breast cancer is indicative of tumor size and nodal involvement (Mendes *et al*., 2010).

6. **Breast cancer susceptibility genes (BRCA1 and BRCA2)**

Approximately 80% of the cases related to familial breast cancer are associated with one gene of hereditary susceptibility for breast and ovarian cancer, *BRCA1* and *BRCA2*. The *BRCA* genes are classified as tumors-suppressor genes, because the loss of wild-type allele has been observed in tumors of heterozygous carriers. BRCA proteins play important roles in different cellular processes, including transcriptional and activation regulation, repair of DNA damage, cellular proliferation, beyond the control of cell cycle, and differentiation (Welcsh *et al*., 2000; Venkitaraman, 2002). The roles of maternal or paternal inheritance of BRCA mutation affect risk of breast cancer. Shapira *et al.* (2011) showed that lifetime risk was higher in *BRCA* mutation inherited from the father, compared to the mother.

1.2.6. Hormone in breast cancer

Hormone receptor status is a key parameter for molecular classifications of breast cancer (Perou, 2000; Carey *et al*., 2006). They serves as a marker of hormone- dependent growth and responsiveness predictor to hormonal treatments, researchers have hypothesized that factors mediated by hormones may be more strongly associated with breast cancers that express hormone receptors when compared with those that are receptor-negative (Althuis, 2004; Ma *et al*., 2006).

Female hormones that affect mammary gland growing are potential risk factors for breast cancer (Corwin, 2008). Hormones act by binding to specific receptors on the target cells to form a complex to elicit a cellular response. Only the target tissue will express the receptor for a given hormone and then be able to respond to it. Hormone receptors may be located on the surface of the cell or within the cell (Platet *et al*., 2004).

The growth of many breast cancers is produced by female hormone estrogen. Women which have estrogen receptor positive tumors are better prognosis compared to women with ER-negative tumors, with the worse outcome. The hormone type helping in determines how cancer develops and the types of possible treatment available. Estrogen receptor positive means that breast cancer relies on estrogen to help it for grow (American Cancer Society, 2012).

1.2.7. Estrogen

Estrogens are steroid hormones synthesized from cholesterol precursors primarily in the ovaries, also in adipose tissue, vascular endothelium, bone, and some sites of the brain (Simpson *et al*., 1999). The most abundant form of estrogen is 17β-estradiol, which can metabolize into estriol and estrone (Heldring *et al*., 2007).

It is synthesis under regulation of the hypothalamic pituitary-gonadal (HPG)-axis, where a negative feedback loop causes the level of estrogen to cycle. Upon menopause, synthesis of estrogens occurs by the ovaries ceases and eventually terminates (Boron and Boulpaep, 2009).

Like all steroid hormones, estrogens readily diffuse across the cell membrane. Once inside the cell, they bind to and activate estrogen receptors which in turn

modulate the expression of many genes (Whitehead and Nussey, 2001; Prossnitz *et al*., 2007; Micevych and Kelly, 2012; Soltysik and Czekaj, 2013).

The three major naturally occurring estrogens in women are estrone (E1), estradiol (E2), and estriol (E3). Estradiol (E2) is the estrogen predominant during reproductive years both in terms of absolute serum levels as well as in terms of estrogenic activity. During menopause, estrone is the predominant circulating estrogen and during pregnancy estriol is the predominant circulating estrogen in terms of serum levels (Fang *et al*., 2001).

Estradiol (E2) is the most important estrogen in non-pregnant females that are between the menarche and menopause stages of life. However, during pregnancy the role shifts to estriol (E3), and estrone becomes the primary form of estrogen in the body of postmenopausal women. Another type of estrogen is produced only during pregnancy called estetrol (E4) (Nelson and Bulun, 2001).

In the female, estradiol acts as a growth hormone for tissue of the reproductive organs, promote the development of female secondary sexual characteristics, such as breasts, and are also involved in the thickening of the endometrium and other aspects of regulating the menstrual cycle, supporting the lining of the cervical glands, and the lining of the fallopian tubes (Hewitt and Korach, 2003; Baum, 2002).

In breast cancer about 80% of breast cancers, once established, rely on supplies of the hormone estrogen to grow: they are known as hormone-sensitive or hormone-receptor-positive cancers. Suppression of the production of estrogen in the body is a treatment for these cancers. Hormone-receptor-positive breast cancers are treated with drugs which suppress the production of estrogen in the body. Hormonal therapy technique, in the context of treatment of breast cancer, is known variously as hormonal therapy, hormone therapy, or anti-estrogen therapy (Kurzer, 2002)

1.2.8. The Estrogen Receptor

The main estrogen action seems to be mediated through the two receptors; the classic receptor called ER α and the later identified ER β (Ali and Coombes, 2002). The two receptor subtypes both belong to the nuclear receptor (NR) family (Heldring *et al*., 2007), and are very similar in structure and functional organization. The receptors are, however, encoded by unique genes and exhibit different tissue distribution of the breast. The ERα is only present in the nuclei of epithelial cells lining the ducts and lobules, while the ERβ is present in the nuclei of both epithelial and myoepithelial cells (Speirs *et al*, 2002). ERα appears to be the predominant regulator of estrogen regulated genes in breast cancer, while ERβ only seems to exhibit an insignificant impact (Palmieri *et al*, 2002; Fuqua *et al*, 2003).

The ERs belong to the nuclear receptor (NR) super family of ligand-regulated transcription factors. ER α was the first ER to be characterized, and it is used as a marker for the diagnosis and treatment of breast cancer. ER β, discovered in the mid 1990's, remains less characterized including its relation to breast cancer development (Osborne, 1998; Mosselman *et al*., 1996).

Both ERs are co-expressed in a number of tissues including the mammary gland, epididymis, thyroid, adrenal, bone, and certain regions of the brain (Matthews and Gustafsson, 2003). However, within some of these tissues, such as the endometrium and the prostate, they are expressed in different cell types (Taylor and Al-Azzawi, 2000; Latil *et al*., 2001).

ER alpha is a dominant ER in the uterus, liver, kidney, and heart, whereas ER beta is a dominant ER in the ovaries, prostate, lung, gastrointestinal tract,

bladder, and hematopoietic and central nervous system (Dahlman-Wright *et al*., 2006). Estrogen signaling plays a critical role in many physiological processes, including development regulation, growth and function of many organ systems in the body. Therefore, aberrations in estrogen signaling are associated not only with different types of cancer, including breast, endometrial and ovarian cancers, but also with diseases such as osteoporosis, depression and eating disorders (Nilsson *et al*., 2001).

ER alpha is expressed in 70% of breast tumors at levels that are significantly higher than ER beta. ER alpha drives proliferation and promotes the survival of breast cancer cells (Planas-Silva *et al*., 2001). ER alpha is the principal biomarker for directing endocrine therapies and is the primary therapeutic target in breast cancer (Musgrove and Sutherland, 2009). Endocrine therapies include selective ER modulators (SERMs) such as tamoxifen, that acts as an estrogen receptor antagonist in the breast, and fulvestrant, an antagonist that induces proteasomal degradation of ER alpha, as well as aromatase inhibitors such as letrozole and exemestane that block the synthesis of estrogen and deprive the receptor of hormone (Johnston and Dowsett, 2003).

1.2.9. Mechanism of estrogen action

Regulation of gene expression as a consequence of estrogen receptor (ER) activation is obtained through various genomic and non-genomic actions (Fig. 1-3). Classical ER signaling is activated by ligand binding and subsequent conformational changes and dimerization of ER (Ali and Coombes, 2002). The ER dimer, in complex with various co-regulators, directly binds to specific estrogen receptor elements (EREs) on the DNA to either activate or repress expression of ER regulated target genes (Fig. 1-5 A).

A second genomic pathway is termed the ERE-independent or the non-classical, since the ER does not bind directly to the DNA (Musgrove and Sutherland, 2009; Barone *et al*., 2010). In this pathway, the ligand activated ER is dependent on interaction with other transcription factors, particularly members of the family of activating protein 1 (AP-1), specificity protein 1 (Sp1), and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (fig. 1-5 B) (Barone *et al*., 2010).

The third genomic pathway does not require estrogen binding, and is therefore termed ligand-independent. Activation of ER is the result of phosphorylation of AF-1, DNA binding domain (DBD), and the hinge domains caused by second messengers downstream of activated receptor tyrosine kinases (RTKs) (Fig. 1-5 C).

The last known type of ER signaling pathway is nongenomic. Here ER is activated upon binding of the ligand to a cytoplasmic or membrane-associated ER receptor. Ligand binding induces assembly of functional protein complexes, consisting of e.g. the kinase c-Src, which subsequently activates intracellular signaling cascades.

This in turn activates various transcription factors which can affect the gene expression of proteins implicated in growth, proliferation and survival (Fig. 1-5 D). Many factors have been found to regulate ER mediated transcription both in the presence and absence of estrogen, e.g. cyclin D which activates ER mediated transcription in the absence of estrogen and enhances transcription in its presence (Zwijsen *et al*., 1997).

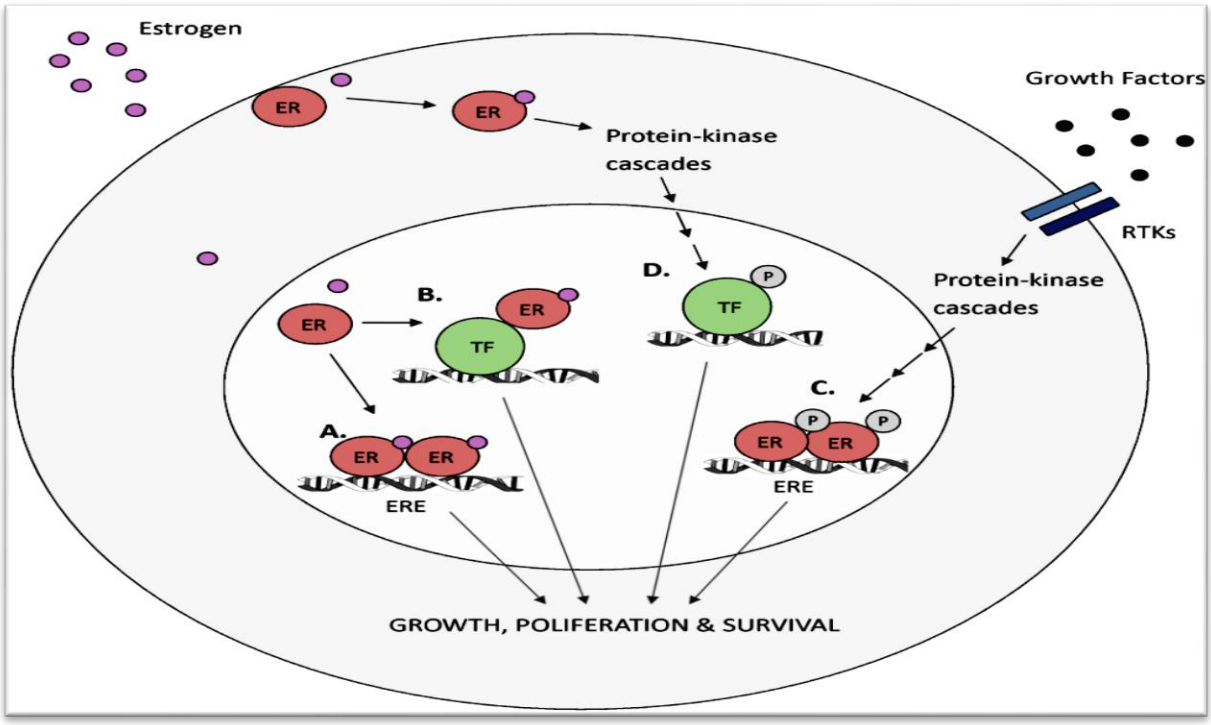


Figure (1-5): Estrogen receptor signaling (Musgrove and Sutherland, 2009).

A. Classical activation of the estrogen receptor (ER) by ligand binding and subsequent dimerization and binding to the estrogen response elements (EREs).

B. The non-classical pathway: ER binds and regulates gene transcription of ER regulated genes via various transcription factors (TFs).

C. The ligandin dependent pathway: ER is activated by phosphorylation, caused by protein-kinase-cascades downstream of the activated receptor tyrosine kinases (RTKs).

D. The nongenomic pathway is activated upon binding of the ligand to a cytoplasmic or membrane-associated ER receptor. This initiates intracellular signaling cascades activating various TFs capable of affecting gene expression (Bjornstrom and Sjoberg, 2005).

1.2.10. Structural and functional organization of estrogen receptor genes

Estrogen receptors (ER) were products of distinct genes localized on different chromosomes; human *ESR1* gene was encoded on chromosome 6q24-q27 (Gosden *et al.*, 1986), while the gene encoding human *ESR2* gene was localized on chromosome 14q22-q24 (Enmark *et al.*, 1997).

Estrogen (E2) binding to the receptor induces the ligand binding domain (LBD) to undergo a conformational change, upon which the receptor dimerizes, binds to DNA, and stimulates gene expression (Cowley *et al.*, 1997; Katzenellenbogen and Katzenellenbogen, 2000).

ER alpha and ER beta share in the DBD 96% amino acid identity, approximately 53% amino acid identity in the LBD and 30% or less in other domains, involved in localization and transactivation (Ogawa *et al*., 1998).

As well, there were some differences in potential transcriptional activation of the two receptors which activated estrogen receptors form dimers. It was assumed in cell types, the two receptor subtypes are co-expressed, the formation of alpha/beta heterodimers played an important role in estrogen signaling, affecting patterns of gene regulation distinct from those gene regulated by the ER homodimers (Matthews and Gustafsson, 2003).

Both ER encoding genes had complex promoter structures. The ER alpha gene was transcribed from at least nine promoters (A, B, C, D, T2, T, E1, F and E), into multiple transcripts which can vary in their 5' untranslated regions (5'UTRs) (Kos *et al*., 2001). The significance of the multiple promoters in the ERalpha gene was still unclear. The ERβ gene was transcribed from at least two promoters, named 0K and 0N, leading to two different messenger RNAs that display a distinct tissue distribution (Swedenborg *et al*., 2009).

ESR1 (*ER* alpha) and *ESR2* (*ER* beta) genes contained eight exons, separated by seven long intronic sequences. ERs contained 6 regions in their structural

protein common for all nuclear receptors, namely: A, B, C, D, E and F which form functionally different but interacting domains, as shown in Figure (1-6).

Exon 1 encoded the A/B region in *ESR1* and *ESR2*; exons 2 and 3 encoded part of the C region. Exon 4 encoded the remaining part of region C, the whole of region D and part of region E. Exons 5 to 8 contained the rest of region E and region F was encoded by part of exon 8 (Ascenzi *et al.*, 2006).

The DBD was responsible for binding to specific DNA sequences (Estrogen Responsive Elements or EREs) in target gene promoter regions. High structure similarity in this region suggests similar target promoter sites for both receptors.

The A/B region located in the N-terminus of the protein encompassed the AF-1 domain responsible for ligand independent transactivation. The AF-1 domain was the least conserved part among the two ERs with only 30% homology and it is functional only in the ER alpha subtype (Hall and McDonnell, 1999).

The C-terminus of the protein contains the ligand dependent transactivation domain AF-2, the ligand binding domain (LBD) and the homo-hetero dimerization site.

Homology between the E/F regions of both proteins was only 53%, and it explains differences in ligand binding affinities between the two receptors. The hinge region localized in the D domain contains the nuclear localization signal of the estrogen receptors as well as post translational modification sites (Sentis *et al.*, 2005).

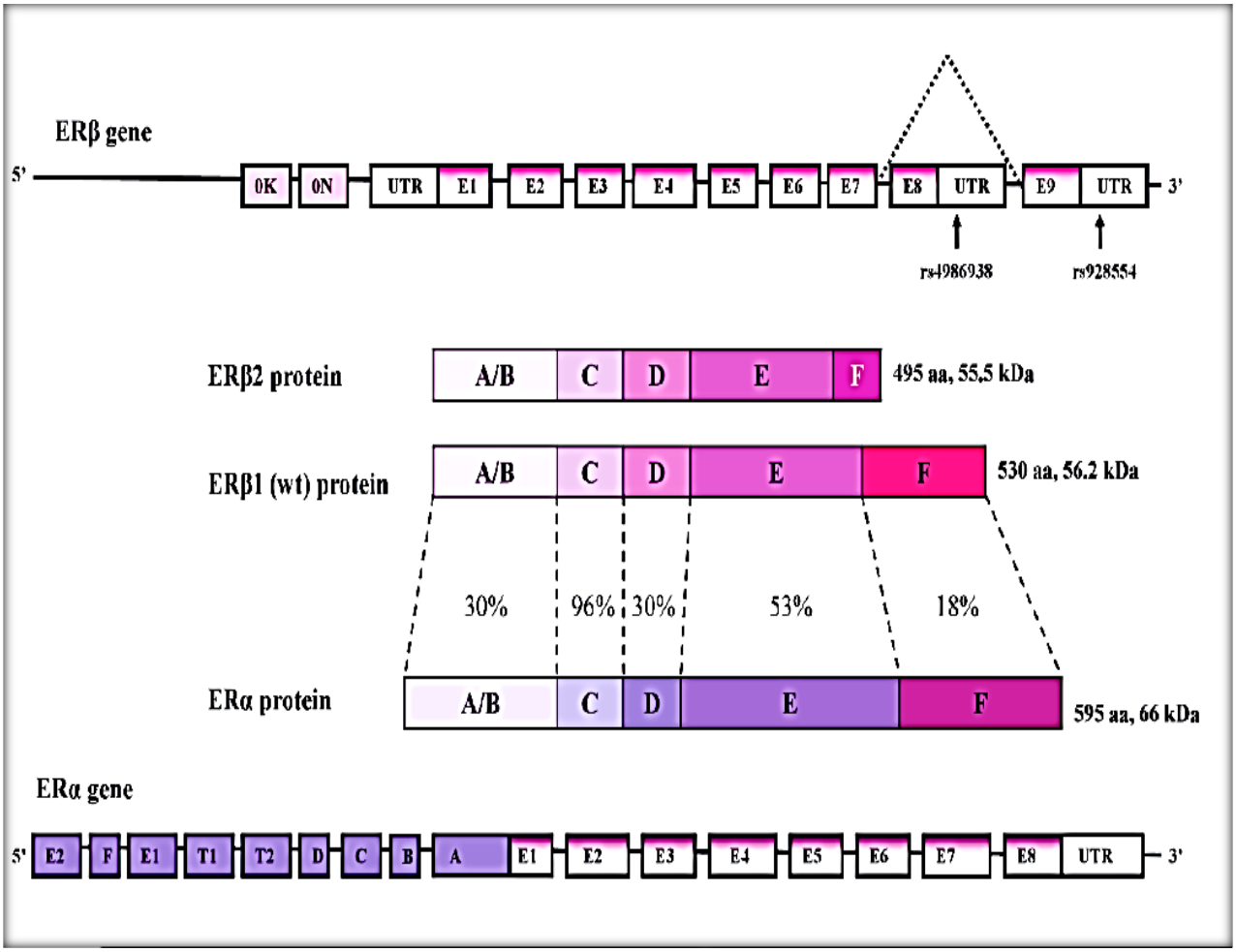


Figure (1-6): Structure of human *ESR*1 and *ESR*2 genes (Ogawa *et al*., 1998; Matthews and Gustafsson, 2003).

1.2.11. Truncated estrogen receptor variant isoforms in breast cancer

Exon-deleted mRNA variants of both estrogen receptor subtypes are found in normal breast and breast cancer tissues. Truncated estrogen receptor proteins possess unique structures that detect their interactions with components of intracellular signaling pathways and as well their subcellular localization and function (Thomas and Gustafsson, 2011).

The truncated estrogen receptor alpha isoforms are:

1- Estrogen receptor alpha-36 (ERα-36) found in both ER alpha (+ve) and (-ve) breast cancer cells (Lee *et al*., 2008). This ER alpha isoform lacks the

AF-1 and a large portion of the LBD, and localized in both cytoplasm and plasma membrane which mediates non-genomic oncogenic signaling in the presence and absence of ligand (Wang *et al*., 2006).

2- Estrogen receptor alpha-46 (ERα 46) expressed in breast cancer cells lacks only the AF-1 domain (Flouriot *et al*., 2000). ERα46 is named according to predict its molecular weight of 46 kDa and corresponded to a deletion of the first coding exon. It inhibited the function of the wild-type ER alpha and formed dimers with ER alpha and beta. ER alpha-46 showed to mediate membrane-associated estrogen signaling and was implicated in estrogen-dependent growth of breast cancer cells (Marquez and Pietras, 2001).

3- Estrogen receptor alpha Δ3 (ERα Δ3) employed another isoform that lacked exon 3 that is part of the DBD. ERα Δ3 was found to decrease proliferation and invasion in breast cancer cells (Erenburg *et al*., 1997).

On the other hand, five estrogen receptor beta isoforms resulted from alternative splicing of the last coding exon. Of all five variants, only three isoforms protein forms are found in breast cancer as following:

1- Wild-type estrogen receptor beta (ERβ1).

2- Estrogen receptor beta 2 (ERβ2) also known as (ERβcx).

3- Estrogen receptor beta 5 (ERβ5) detected in clinical breast cancer (Marotti *et al*., 2010; Huang *et al*., 2014).

Differential splicing found in the ligand binding domain (LBD), ERβ2 and ERβ5 either had disoriented or completely lack helix 12 required for ligand-dependent activation function (AF-2), and thus determined activity of receptor in response to ligand binding (Leung *et al*., 2006). A growing body of evidence points to different roles of ERβ isoforms in biology of breast cancer and

suggests its potential prognostic role for patient management (Dhimolea *et al*., 2015).

ERβ2 found to bind ERα and inhibited ligand-induced ERα transcriptional activity, suggesting that ERβ2 had an important role in the functional neutralizing of ERα, so ERβ2 can be significant for the diagnosis and treatment of breast cancer (Zhao *et al*., 2010).

1.2.12. Estrogen signaling in breast cancer

The normal cell utilizes signal transduction in various essential cellular functions; cell differentiation, homeostasis, cell survival, controlled proliferation and cell death. Carcinogenic cells alter and exploit these signal transduction pathways in order to achieve malignant growth (Hanahan and Weinberg, 2000).

In hormone-dependent cancers, estrogen was taken up from the blood plasma or from local production diffuses into the cancer cell and binds estrogen receptor (ER), thus causing the dissociation of heat shock proteins from the ER molecule (Johnston and Dowsett, 2003). The ligand-bound molecule dimerises, associates with other coactivator or corepressor proteins and subsequently binds to conserved estrogen response element (ERE) sequences within the promoter regions of genes over which it exerts transcriptional control as shown in Figure (1-7) (Dixon, 2014).

The majority of breast cancers are dependent on activation of the ER, and subsequent transcription of target genes, which are important for growth, proliferation and survival (Musgrove and Sutherland, 2009).

Endocrine therapies work by manipulating endocrine signaling by the exogenous administration of hormone antagonists designed to inhibit the biosynthesis and activity of estrogen. Endocrine therapies are considered to be

cytostatic rather than cytotoxic, leading to reduced proliferation and reduction of growth rate (Dowsett *et al*., 2006).

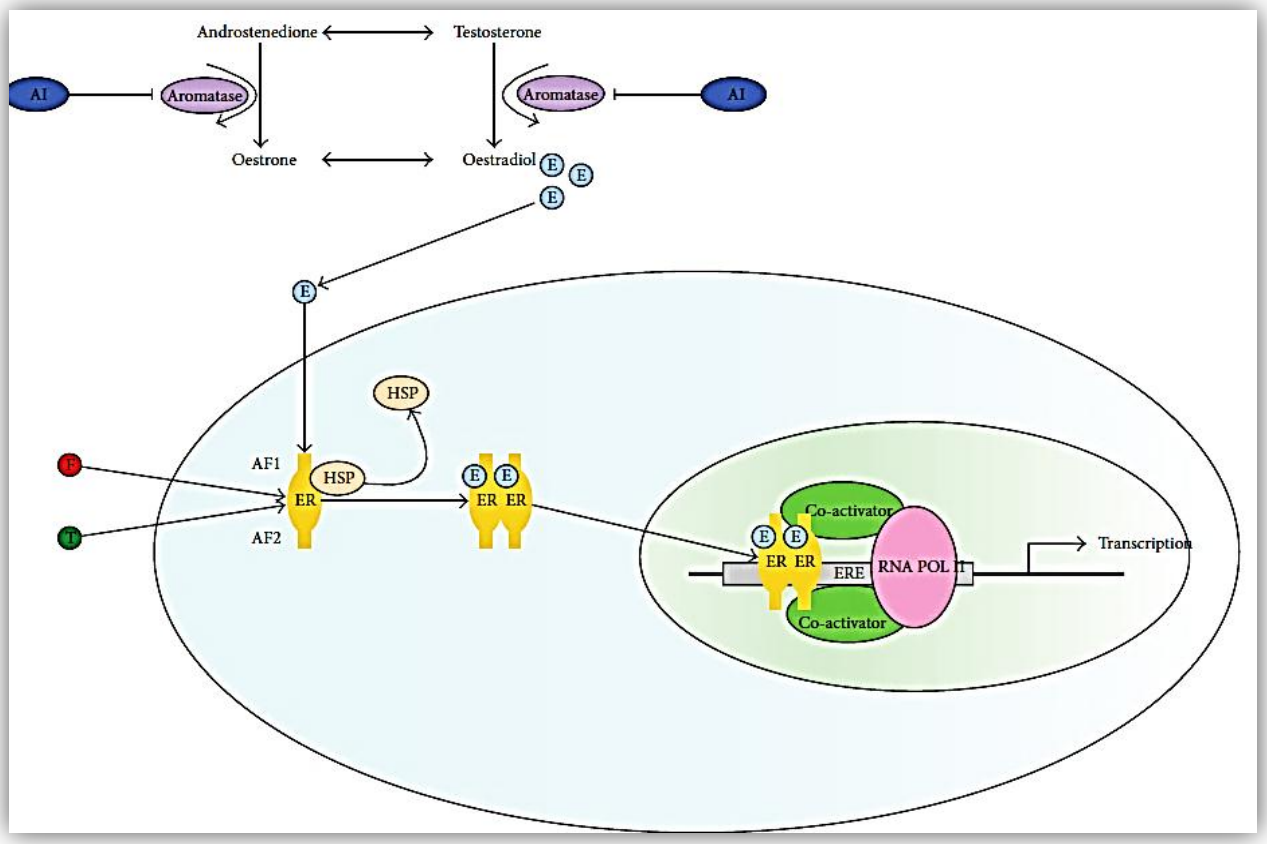


Figure (1-7): Estrogen production via enzymatic conversion of androgens to estrogen by aromatase and estrogen receptor signalling in breast cancer. Estrogen (E), estrogen receptor alpha (ER), estrogen response element (ERE) and heat-shock proteins (HSP). The targets of commonly used endocrine therapies in the pathway are shown: aromatase inhibitors (AI), tamoxifen (T) and fulvestrant (F) (Dixon, 2014).

1.2.13. Mutations

Breast carcinogenesis involves various environmental and genetic factors (Moran *et al*., 2011). Environmental factors are more controlled readily than genetic and racial factors (Bhurgri, 2004). The most common risk factors are excessive estrogen stimuli (Cheung, 2007), and family history of breast and ovarian cancer (Hankinson, 2008). However, because of genetic predisposition, about 5-10% of all breast malignancy is caused by mutation in autosomal dominant genes. Two types of genetic variations are involved in breast cancer, one is the gain of function mutation in proto-oncogene, and the second is loss of function mutation in tumor suppresser gene. This result in the uncontrolled cell division and growth, failure of DNA repair mechanism, and disturbance of cell cycle check point. Women with inherited loss-of-function mutation have 70% risk of developing breast cancer before the age of 70 years (Loman *et al*., 1998).

The major cause of breast cancer was mutation in tumor suppressor genes *BRCA1* and *BRCA2* (Sheikh *et al*., 2015). Germ line mutation in BRCA 1 and 2 was accounted for 16% of all hereditary breast malignancy (Vander *et al*., 2011). Limited data was available on tumor protein (TP53), checkpoint kinase 2 (CHEK2), and estrogen Receptor (ESR) mutations involved in the development of breast cancer (Amir *et al*., 2010).

Breast cancer is combined with *ESR1* and *ESR2* gene polymorphisms, substantial evidence that recorded ER participates in mammary gland tumorigenesis and ER is among the genes that affect breast cancer susceptibility (Oh *et al*., 2006; Sakoda *et al*., 2011). In fact, ESR1 protein over expression was common in breast cancer (Holst *et al*., 2007). Identification of somatic mutation of *ESR1* gene was identified, but estrogen receptor alpha germ-line mutation was rarely occurring in the breast cancer patients. The systematic association study in joint effects of polymorphisms in these two

genes for breast cancer risk, found that single nucleotide polymorphisms in estrogen receptor alpha and beta were correlated with various aspects of breast cancer in Iran and have additive effects in increasing risk for developing breast cancer with lymph node metastases (Abbasi *et al*., 2012).

Over expression of mutant ER was found to enhance proliferation and confer resistance to tamoxifen. Other studies also identified additional ER mutations in the ligand binding domain which also result in constitutive activity and may represent potential mechanisms for acquired endocrine therapy resistance (Merenbakh-Lamin *et al*., 2013; Jeselsohn *et al* ., 2014).

Estrogen receptor alpha (*ESR1*) mutations are significantly enriched in endocrine therapy-resistant, metastatic breast cancer and are rare or nonexistent in treatment-naïve, primary tumors as in Figure (1-8). Mutations appear to be strongly correlated with endocrine therapy resistance, a causal relationship between *ESR1* mutations and endocrine therapy resistance remains. Significant upregulation of ER-responsive genes that harboring *ESR1* mutations, suggests that ER signaling is active in these tumors and may play a role in conferring endocrine therapy resistance (Robinson *et al*., 2013). Detection of *ESR*1 mutations may prompt a clinician to change the treatment regimen from an aromatase inhibitor to an anti-estrogen. It is already known that patients who develop resistance to aromatase inhibitors often respond to anti-estrogen therapy (Ingle *et al*., 2006; Eberwine *et al*., 2014).

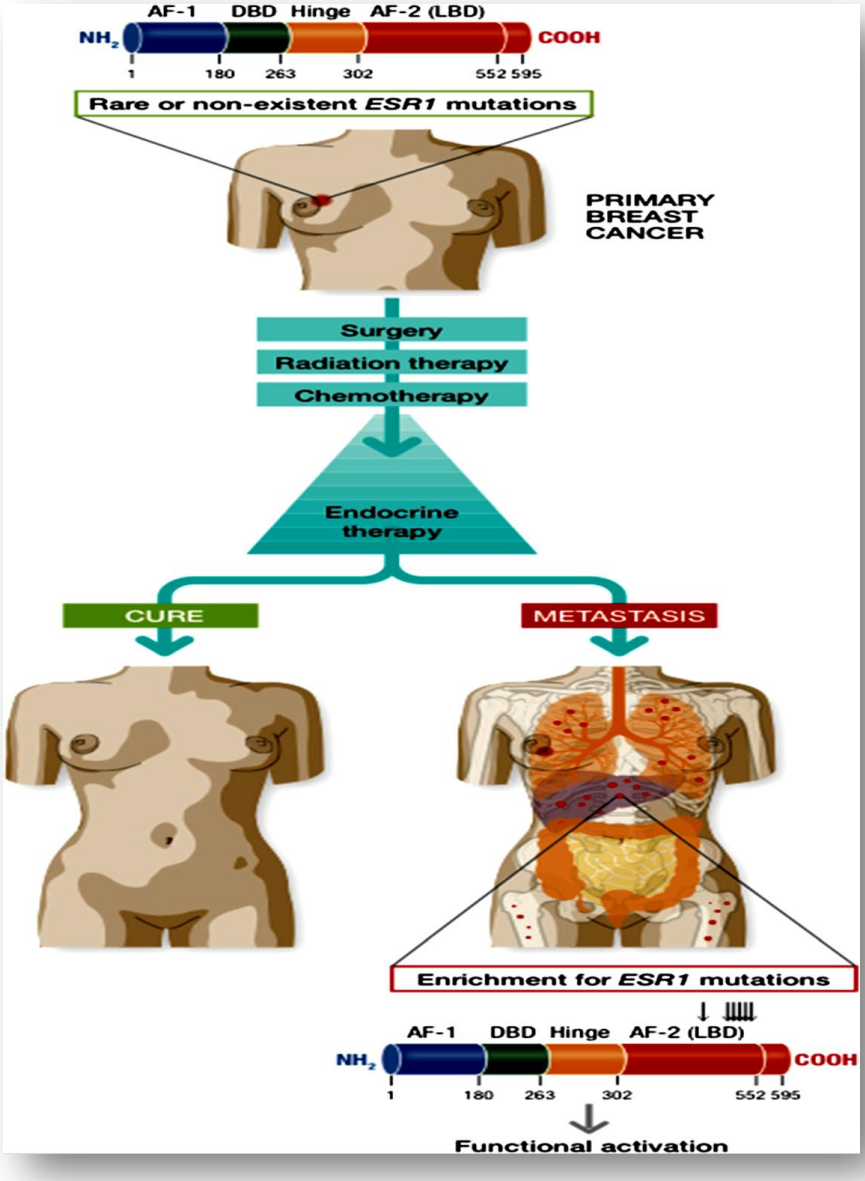


Figure (1-8): Estrogen receptor alpha (*ESR1*) mutations in estrogen receptor positive breast cancer. ESR1 mutations are rare or nonexistent in primary breast tumors and are significantly enriched in metastatic, endocrine therapy-resistant breast cancer. Nearly all *ESR1* mutations localize to the ligand-binding domain (LBD) of the estrogen receptor (ER) and often result in constitutive activation of the ER. DBD, DNA-binding domain (Eberwine *et al*., 2014)

Chapter Two
Materials and Methods

2. Materials and Methods

2.1. Materials

2.1.1. Equipment and apparatus

The apparatus that used in this study were shown in Table (2-1).

Table (2-1): Equipment and apparatus used in this study

Equipment and apparatus	Company (Origin)
Autoclave	Hirayama (Japan)
Electrophoresis equipment	Bio-Red (USA)
Gel –documentation	Bio-Red (USA)
Microcentrifuge	Eppendorf (USA)
Micropipette	Phelps (Germany)
Microtome	Lipshaw (MFG)
Microwave	LG (Korea)
Nanodrop spectrophotometer	Techne (UK)
Oven	Sanyo (Japan)
PCR master cycler gradient	Techne (UK)
PCR thermal cycler	Techne (Japan)
Sensitive balance	Mettlev (Switzerland)
U.V transilliuminator and Camera	Flowgen (UK)
Vortex	Scientific industries (USA)
Water bath	Memmert (Germany)

2.1.2. Chemicals and biological compounds

The chemicals and biological materials used in this study were shown in Table (2-2).

Table (2-2): Chemicals and biological materials used in this study

Chemical compounds	Company (Origin)
Absolute Ethyl Alcohol	Sanyo (Japan)
Agarose	Promega (USA)
Ethidium bromide	Promega (USA)
Green Master Mix	Promega (USA)
Loading dye	Promega (USA)
Mineral Oil	Promega (USA)
Proteinase K	Qiagen (Germany)
Tris borate EDTA (TBE) buffer	Promega (USA)
DNA ladder (100-1000 bp)	Promega (USA)

2.1.3. Kits

The kits used in this study were shown in Table (2-3).

Table (2-3): Kits used in this study

Kits	Company (Origin)
DNA Extraction Mini Prep System	Promega (USA)
QIAamp DNA blood mini cat no 51104	Qiagene (USA)
QIAamp FFPE tissue cat no 56404	Qiagene (USA)
Maxwell®16 tissue DNA purification kit	Promega (USA)
ReliaPrep FFPE gDNA miniprep	Promega (USA)

ER pharmDX^{TM} kits	Dako (Denmark)

2.1.4. Primers used for detection exons in *ESR1* and *ESR2* genes

For detection of exon 4 and 6 in *ESR1* and exon 3 and 7 in *ESR2* genes, several primers were used (Table 2-4).

Table (2-4): Primers for detection of certain exons in *ESR1* and *ESR2* genes and their sequences

Exon	Reference	Oligonucleotides	Sequence (5'→3')	Product size (bp)
***ESR1* gene exon 4**	Abbasi *et al*. (2013)	Forward	ACCTGTGTTTTCAGGGATACGA	370
		reveres	GCTGCGCTTCGCATTCTTAC	
***ESR1* gene exon 6**	Babayan *et al*. (2013)	Forward	CCCTTTCATGTCTTGTGGAAG	300
		Reverse	ATGCCTTTGGAGTGGGTAGA	
***ESR2* gene exon 3**	Abbasi *et al*. (2013)	Forward	TTGCTCCCTAGAGAGACACTGA	151
		Reverse	CTTCACACGACCAGACTCCA	
***ESR2* ene exon 7**	Abbasi *et al*. (2013)	Forward	GATGAGGGGAAATGCGTAGA	156
		Reverse	GGCCCAGCTGTGTGATTACT	

2.2. Methods

2.2.1. Collection of samples

Fifty samples were collected from women with breast cancer during 1st of April to 1st of September 2014, that were divided to three groups as following:

A- Blood and FFPE samples group

Peripheral blood samples were collected from 25 women attended to Alawram Hospital in Baghdad city who were diagnosed and pathologically confirmed with breast cancer in different hospitals in Bagdad with a mean age of 57 years without chemotherapy.

In addition, 10 samples of blood were collected from healthy women, as a control group, at a median age of 45 year, those women had no history of breast cancer and any other neoplastic diseases. Moreover, women with hysterectomy and artificial menopause, or exposed to any kind of radiation and chemotherapy in their lifetime were excluded from this study. Demographical and risk factor data were also recorded using a short structured questionnaire, including information on age, number of children, average lactation term, family history of breast cancer, menopausal status, race, age at onset, they also were asked for blocks of FFPE samples to study the estrogen receptor expression in breast cancer tissue. All blood samples were stored at -4 $^{\circ}$C until use for a further study.

B- Fresh tissue, blood and FFPE samples group

Women, suspected with breast cancer at a mean age of 55 years, attended to Janeen Hospital in Baghdad. Those patients were firstly diagnosed by fine needle aspiration (FNA) or lumpectomy. If the patient had breast cancer, the mastectomy was done. Fifteen samples of fresh tissues (untreated samples) with blood samples from the same women with breast cancer were collected. The fresh tissue sample was divided into two parts; the first one put in normal saline and kept at -20 $^{\circ}$C, while the second part put in formalin then embedded in paraffin and submitted for histopatholgical examination and

imunohistochimstry for estrogen receptor (ER). In addition blood samples from the same women with breast cancer were collected and stored at -4 °C until used for further study. Histopathological paraffin blocks were sectioned into 4 µm thickness on positively charged slides in order to be used for the immunohistochemistry (IHC) assay (Elwood *et al*., 2001).

C- Formalin fixed paraffin embedded samples (FFPE) group

Thirty five samples of formalin fixed paraffin embedded (FFPE) tissues from women with breast cancer attended to Al-Ta`leme Hospital in Baghdad were collected at the mean age of 58 years.

All information about patients was categorized using certain survey data sheets. The data sheets encompassed name, age, sex, number of children, family history of breast cancer, menopausal status, statue final diagnosis, and staging and histopathological records showing the degree of differentiation of breast cancer patients.

The blocks of these samples were sectioned into 4 µm thickness on positively charged slides in order to be used for the immunohistochemistry (IHC) assay (Elwood *et al*., 2001).

2.2.2. Immunohistochemistry (IHC) assay

The expression of estrogen receptor protein was examined by using kit and the estrogen antibody (Monoclonal Mouse Anti-human Estrogen Receptor) from Dako (Danimark).

A- Immunohistochemical staining procedure (Dako, Danimark)

- Paraffin embedded tissue samples were cut in 4 mm thick section and placed on super frost charged slides.

- The sections were back in hot air at 65 ^{o}C for overnight or at 80 ^{o}C for 1 hr.
- The sections were deparaffinized in prewarmed 2X xylene for 5min, rehydration in absolute, 95%, 70% and 30% ethanol for 5 min, then washed for 5 min in distilled water. The excess water tapped off any remaining water around, the specimen was removed carefully by using soft tissue to keep reagent within prescribed area. Endogenouse peroxidase activity was block by covering the section with a peroxidase blocking agent (3% H_2O_2) for 20-30 min.
- The slides rinsed gently with distilled water from a wash bottle (have not direct focus flow on tissue) and placed in fresh PBS buffer for 5 min.
- The excess and remaining buffer was removed as in step 3. Enough primary antibodies are applied to cover the section and incubated at 37 ^{o}C for one hour.
- The slides were rinsed gently with buffer solution from a wash bottle and placed in fresh buffer bath for 5 min. Immediately excess buffer was taped and the slides wiped as before. Enough link antibody (Biotinylated Antibody) was applied to cover the sections, then the slides were placed in humid chamber and incubated at 37 ^{o}C for 1 hr.
- The slides rinsed and wiped as in step 3. Streptavidin reagents was applied to cover the sections, then the slides were placed in the humid champers and incubated at 37 ^{o}C for 30 min.
- The slides were rinsed and wiped as in step 3, 3.3-Diamino benzidine (DAB) was freshly prepared by adding one drop of chromogen to 1 ml

of substrate buffer. The sections were covered with DAB solution and placed in hummed chamber then incubated at 37 ^{o}C for 10 min.

- The slides were rinsed with distilled water from a wash bottle and immersed in bath of hematoxyline for 30 sec., then rinsed with running tap water for 2 min.
- The slides were gradually dehydrated with ethanol (30%, 70%, 95%, 99%) for 1 min and cleared with xylene 2X for 1min then mounted.

B- Staining and scoring interpretations (Dako, Danimark)

The criteria of positive reaction for ER were dark brown intra-nuclear precipitate. The staining was assessed by scoring the proportion and intensity performed at X 40 objective lens. The proportion score interpreperation were (0= none,1 = <1/100, 2= 1/100-1/10, 3= 1/10-1/3, 4= 1/3-2/3, 5= >2/3). Any brown nuclear staining was counted towards the proportion score. Whereas the intensity score was represented the average intensity of the positive cells and interpretation as (0 =none, 1= weak, 2= intermediate, 3= strong).

Estrogen and progesterone receptors scoring was used by assessing the proportion score (PS) and intensity score (IS) in average to high power field (HPF) 400X as below.

A total score (TS) = sum of PS and IS (0 – 8)

A positive result is defined as TS ≥ 3 which was validated and described by Allerd *et al*. (1998), as shown in Figure (2-2).

C- Positive control tissue (Dako, Danimark)

The positive control tissue was examined first to ascertain that all reagents were functioning properly. Positive reactivity was indicated by the presence of brown reaction production at the site of the target antigen. If the positive

control tissues fail to demonstrate the expected staining pattern, all results with the test specimen should be considered invalid.

D- Negative control tissue (Dako, Danimark)

The negative control tissue should be examined after the positive control tissue to verify the labeling of the target antigen by the primary antibody. The absence of specific staining in the negative control tissue confirmed the lack of antibody cross-reactivity to cell/cellular components. If specific staining other than that described in positive control occurs in the negative control tissue, results with the patient specimen should be considered invalid.

E- Photomicrography

Images were acquired at different magnifications (10X, 20X, 400X) using LEICA dissecting microscope 750 (Germany). The files were saved in a joint photographic experts group (JPEG) format.

Negative control Positive control

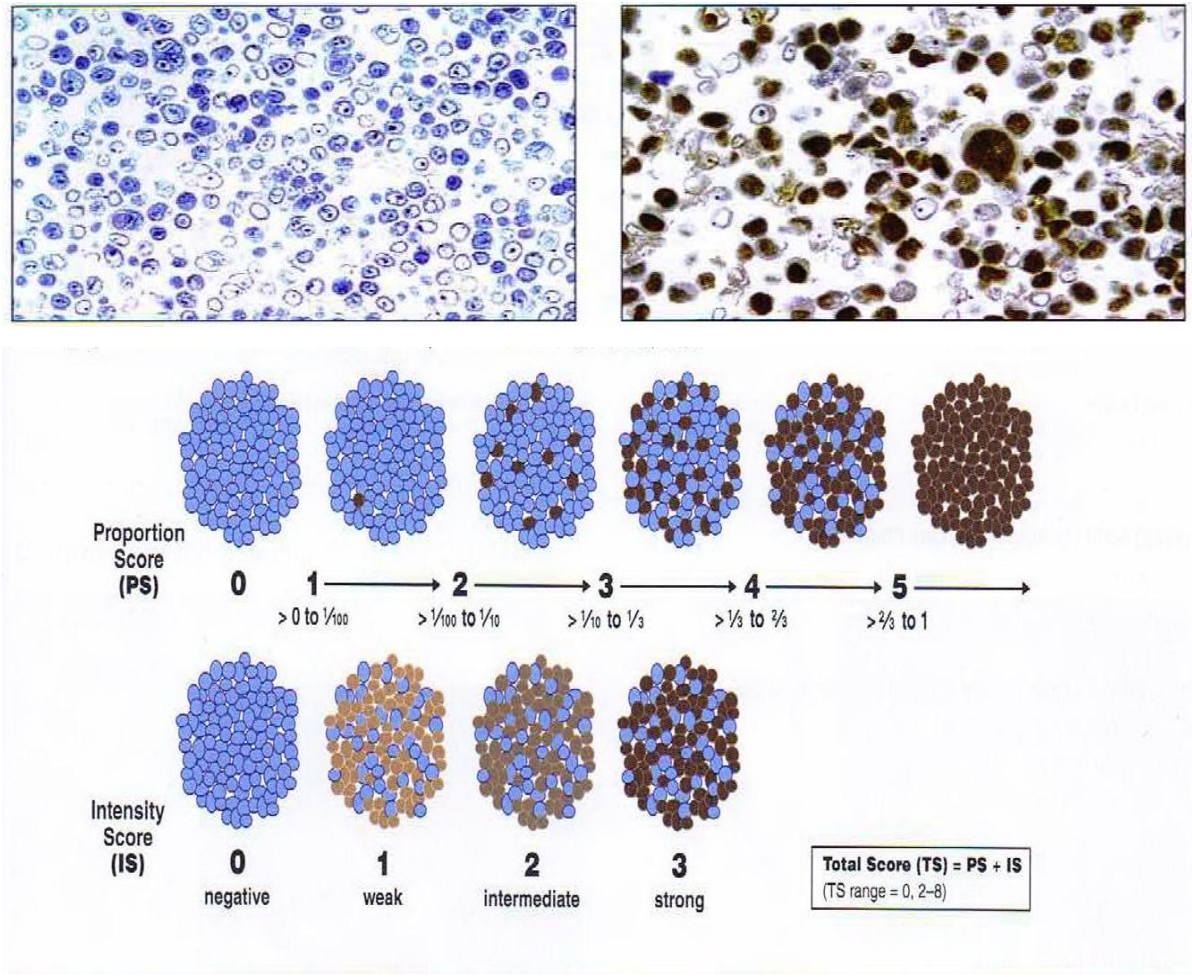


Figure (2-1): Negative control, positive control and scoring of estrogen receptor (Allerd *et al*., 1998)

2.2.3. Molecular study

2.2.3- 1. DNA extraction

2.2.3-1.1. DNA extraction from blood samples

The DNA was extracted from blood samples by using two different kits:

A: Mini Prep System kit (Promega, USA)

The DNA was extracted according to the reliaprep blood genomic DNA MiniPrep system (Promega, USA). This kit contained cell lysis buffer,

proteinase K solution (PK), binding buffer, column wash solution, collection tubes, and nuclease free water.

Procedure

The Extraction of DNA from peripheral blood samples was done as below:

- The blood sample was mixed thoroughly for at least 10 min in a shaker at room temperature.
- Proteinase K solution (20µl) was added into micro centrifuge tube. 200 µl of blood was added to Proteinase K solution and mixed briefly.
- Cell lysis buffer (200 µl) was added to the tube and mixed for at least 10 sec by vortex, then incubated at 56°C for 10 min.
- Binding buffer (250 µl) was added to the tube and mixed for 10 sec by vortex. The mixture of samples were transferred to the filter column set and centrifuged at maximum speed for 1 min. The collection tube containing the supernatant was discarded and replaced by a fresh collection tube.
- Column wash solution (500 µl) was added to the column and centrifuged for 3 min at maximum speed and the supernatant was discarded.
- Nuclease free water (50 µl) was added to the column and centrifuged for 1min at maximum speed. The nuclease free water was discarded and the eluted was stored at -20°C.

B: QIAamp DNA blood mini (Qiagen, USA)

It consisted of collection tubes (2 ml), AL buffer, ATL buffer, concentrated AW1 buffer, concentrated AW2 buffer, AE buffer, one vial of Qiagen protease K, and protease solvent 1.2 ml.

The blood samples were equilibrated at 25°C, the heat blokes were heated to 56°C for use, AE buffer or D.W. was wormed to room temperature for elution. AW1buffer, AW2 buffer, and Qiagen protease was prepared according to the instructions and buffer AL dissolved then incubated at 56°C.

Procedure

- Qiagen protease (20 µl) was pipetted into the bottom of a 1.5 ml microcentrifuge tube, 200 µl of samples was added to the microcentrifuge tube. The whole blood was used up to 200 µl. If the sample volume was less than 200 µl, the appropriate volume of PBS was added.
- Buffer AL (200 µl) was added to the sample, mixed by pulse-vortex for 15 sec, and incubated at 56°C for 10 min, the DNA yield reached a maximum after lysis for 10 min at 56°C.
- Briefly, the mixture was centrifuged in the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid, 200 µl of 96% ethanol was added to the sample, and mixed again by pulse-vortex for 15 sec. After mixing, briefly centrifuged in 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
- Carefully the mixture was applied to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim, the cap closed and centrifuged at 8000 rpm for 1 min. QIAamp Mini spin column placed in a cleaned 2 ml collection tube, and discarded the tube containing the filtrate.
- The spinning column was closed to avoid aerosol formation during centrifugation. Centrifugation is performed at 8000 rpm to reduce noise.

Centrifugation at full speed will not affect the yield or purity of the DNA. If the lysate did not completely pass through the column after centrifugation, the step of centrifugation was done again at higher speed until the QIAamp Mini spin column is empty.

- Carefully to the QIAamp Mini spin column, 500 µl buffer AW1 was added without wetting the rim, centrifuged at 8000 rpm for 1 min, the QIAamp Mini spin column was carefully opened and 500 µl of AW2 buffer added without wetting the rim. Centrifugation at full speed (14,000 rpm) for 3 min was done.
- The QIAamp mini spin column was placed in a new 2 ml collection tube and discards the old collection tube with the filtrate, and centrifuged at full speed for 1 min.
- QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube, and discarded the collection tube containing the filtrate. Carefully 200 µl of AE buffer or distilled water was added to the QIAamp mini spin column, incubated at room temperature (15–25°C) for 1 min, and then centrifuged at 8000 rpm for 1 min.

2.2.3-1.2. DNA extraction from fresh tissues samples (Promega, USA)

The DNA was extracted from fresh tissue samples by using Maxwell® 16 Tissue DNA Purification Kit (Promega, USA); it was used with the Maxwell® 16 instruments for automated purification of genomic DNA from fresh or thawed tissue samples. DNA can be purified from tissue samples of up to 500 mg. The purified DNA is suitable for use in adirect downstream analysis by standard amplification methods.

A- Tissue sample volume and preprocessing requirements

The total yield of genomic DNA from tissue samples depends on the sample size (weight) and tissue type. It is normal for different tissue types of the same mass to give different genomic DNA yields. The Maxwell® 16 Tissue DNA Purification Kit was designed to purify genomic DNA from up to 500 mg of tissue. Exceeding this recommended sample size may adversely affect yield and quality of the purified genomic DNA, and the contents are shown in Figure (2-4).

B- Procedure

The fresh or thawed tissue was placed (up to 500 mg) into well no. 1 of predispensed cartridge which contained lysis buffer as described in Figure (2-5). Optional RNase treatment in some cases was used. To remove copurified total RNA, an RNase treatment can be performed by adding 5µl of RNase A per milliliter of elution buffer.

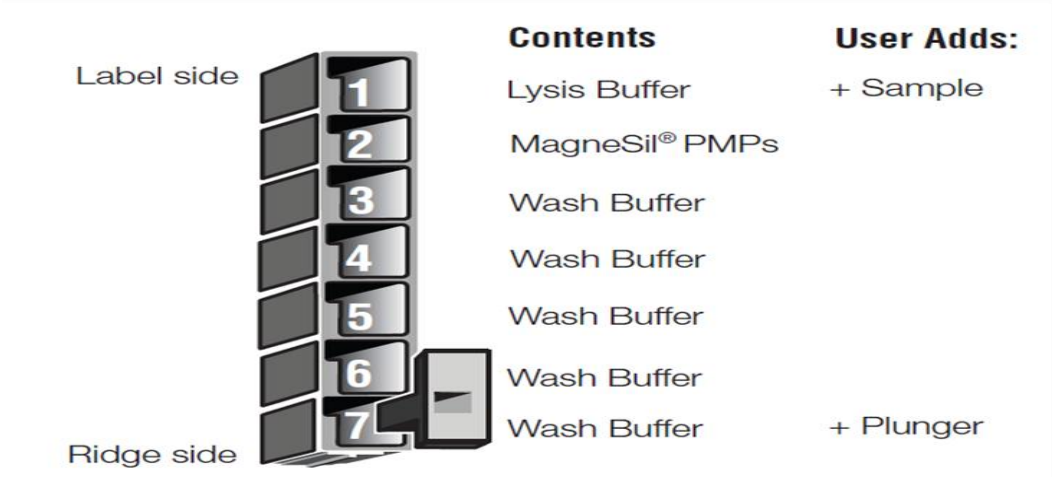


Figure (2-2): Maxwell® 16 Tissue DNA Purification Kit cartridge contents

The following process was done as follows:

- Each cartridge was placed to be used into the holder with the ridged side of the cartridge facing toward the numbered side of the rack. The

seal was removed from each cartridge. One plunger was placed into well no.7 of each cartridge such that the bottom of the plunger at the bottom of the cartridge (well no.7 was the well closest to the ridged side of the cartridge). The plunger will fit loosely in the cartridge.

- The sample was added to well no. 1(Well no. 1 is the well closest to the cartridge label and furthest from the user).
- The program was set up and the run begun.

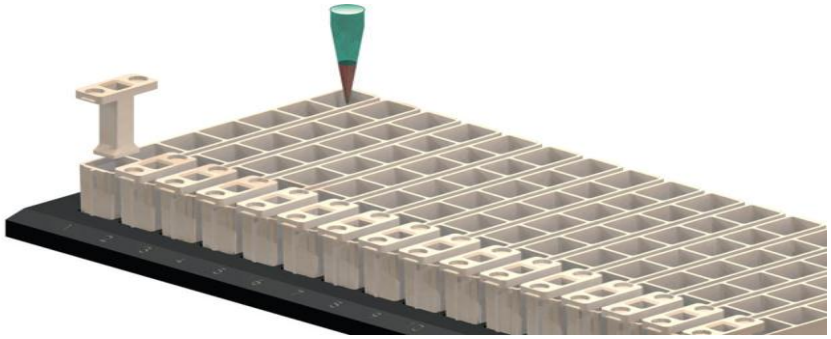


Figure (2-3): Maxwell® 16 DNA Purification cartridge, the contents of a cartridge for the MaxwellR 16 blood, cell and tissue DNA purification kit, in all cases the sample is added to well no.1

2.2.3-1.3. DNA extraction from formalin fixed paraffin embedded (FFPE) samples (promega, USA)

ReliaPrep™ FFPE genomic DNA Miniprep (promega, USA) was used. It contained proteinase K (PK), BL buffer, wash solution, RNase A, elution buffer, packages, collection tubes and packages ReliaPrep™ FFPE binding columns.

1- Preparation of FFPE sections

By using a sterile blade, excess paraffin trimmed off the tissue block, 5-50 μm of sections were cut from FFPE blocks using a microtome, then placed in

a 1.5 or 2ml microcentrifuge tube, equivalent of ≤100µm of tissue slices may be processed per reaction.

2- DNA isolation from FFPE samples

A- Deparaffinization using mineral oil

The mineral oil (300µl) was added to ≤ 50 microns of FFPE sections, while 500µl of mineral oil were added to sections ≥ 50 microns. The mixture was incubated at 80°C for 1 min then vortex had done for mixing.

B- Sample lysis

A 200 µl lysis buffer was added to the sample, spun at 10,000 rpm for 15 sec at room temperature. Two phases were formed, a lower (aqueous) phase and an upper (oil) phase, 20µl proteinase K was added to the lower phase, the lower phase was mixed by pipetting, incubated at 56°C for 24 hr, and then incubated at 80°C for 1 hr, the sample was allowed to cool at room temperature, and centrifuged briefly at room temperature to collect any drops from the inside of the lid. Directly to the lysed sample in the lower phase (10µl) RNase A was added, the lower phase was mixed by pipetting, and incubated at room temperature (20–25°C) for 5 min.

C- Nucleic acid binding

To the lysed sample, 220 µl of BL buffer and 240µl of absolute ethanol were added, vortex briefly to mix, and spun at 10,000 rpm for 15 sec at room temperature. Two phases was formed; a lower (aqueous) phase and an upper (oil) phase. For each sample to be processed, a binding column was placed into one of the collection tubes. The entire lower (aqueous) phase was

transferred, including any precipitate that may have formed, to the binding column/collection tube. The columns were assembled and caped. The assembly was spanned at 10,000 rpm for 30 sec at room temperature; the flow was discarded through, and reinserted the binding column into the collection tube.

D- Column washing and elution

To the binding column, 500µl of wash solution of 1X (with ethanol) was added, and spun at 10,000 × g for 30 sec at room temperature. The flow was discarded; reinserted the binding column into the same collection tube used for the nucleic acid binding. The cap was opened on the binding column and the binding column/collection tube spanned assembly at 16,000 × g for 3 min at room temperature to dry the column. The binding columns transferred to a clean 1.5ml microcentrifuge tube, discarded the collection tube, then 30–50µl of elution buffer was added to the column and caped the column, spanned at 16,000 × g for 1 min at room temperature, removed and discarded the binding column and stored the eluted DNA at –20°C.

2.2.3-2. Estimation of DNA

The DNA concentration of each sample was measured by using Nanodrop at 260nm, the DNA purity was estimated as a ratio of A260/ A280.

2.2.3-3. Amplification of DNA by PCR

- The Go Taq® Master Mix was thawed at room temperature; the master mix was mixed by vortex then spun in a micro centrifuge.
- The reaction mix (25 µl) was prepared by mixing the components: 12.5 µl of 1X Go Taq® Green Master Mix, 0.5 µl of 0.2 µM forward primer, 0.5 µl of 0.2 µM reverse primer, 5 µl of < 250 mg DNA template and

6.5 µl of nuclease free water. The optimal values of various steps for DNA amplification were shown in Table (2-5).

Table (2-5): Optimal values of various steps of DNA amplification

Components	Volume (µl)	Concentration
Go Taq® Green Master Mix	12.5	2x
Forward primer	0.5	0.2 µM
Reverse primer	0.5	0.2 µM
DNA template	5	< 250 mg
Nuclease free water	6.5	-
Final volume	25	

The reaction was placed in a thermal cycler using the protocol shown in Table (2-6).

Table (2-6): Optimal condition for DNA amplification by PCR

Thermal cycler protocol	No. of cycle	Temperature - time
Initial Denaturation	1	94°C - 3 min
Denaturation	30	94°C - 30 sec
Annealing		57°C - 30 sec
Extension		72°C - 30 sec
Final Extension	1	72°C - 10 min

2.2.3-4. Agarose gel electrophoresis (Sambrok and Russell, 2001)

Electrophoresis through agarose gels is a standard method used to separate, identify, and purify DNA fragments of varying sizes ranging from 100 bp to 25 kb (Sambrok and Russell, 2001).

It was done by preparing 1.5% agarose gel as described by Sambrok and Russell (2001), as below:

A- Preparation of solution

1X Tris borate EDTA (TBE) buffer was prepared by diluting 10X TBE buffer with distilled water (one volume of 10X TBE buffer with 9 volume of distal water; 1:10 dilution).

B- Preparation of agarose gel

- Agarose (1.5 g) placed into a glass beaker, and then 100 ml of 1X (TBE) buffer were added.
- The solution was stirred on a hot plate or in oven until the agarose was dissolved and the solution be cleared; the solution was allowed to cool to about 55°C before pouring.
- A concentration of 0.5 µg/ml of ethidium bromide was added to the solution.
- Gel tray ends were sealed with tape or another custom-made dam.
- The comb was placed in the gel tray about 1 inch from one end of the tray.
- The gel solution was poured into the tray and allowed to solidify for about 20 min at room temperature.
- The comb was gently removed, and then the tray was placed in an electrophoresis chamber and covered (just until wells are submerged) with electrophoresis buffer (the same buffer used to prepare the agarose).

- At this step, 7µl of samples were loaded on each well with extreme cautions to avoid damage of the wells and cross contamination of neighboring wells.
- Electrophoresis at 90-100 volts, for 60-120 min or until dye markers have migrated an appropriate distance, depending on the size of DNA to be visualized.
- Sometimes, the gel was not stained with ethidium bromide during the run, so the gel in 0.5 µg/µl ethidium bromide was stained after the electrophoresis was finished for 15 min, and then DNA bands which to be visible under a short wave of UV light (365nm) were examined.

C- Gel electrophoresis conditions

The PCR products were electrophoresed on 1.5% agarose gel. The gel was run at a 5V/ cm for 45 min. A sample positive control, a negative control, and a 100 bp DNA ladder were loaded on the gel.

D- Photo documentation

Agarose gel was visualized by a UV transilluminator, agarose gel was placed above the UV transilluminator device and exposed to UV light and the photos were captured using a digital camera.

2.2.3-4. Assessment of products and DNA sequencing

Before Sequencing, DNA concentration of PCR product was measured using Nano drop (ACTGene, USA). The concentrations of all products were more than 100 ng/ml; they were considered to be the least concentration required for DNA to be sequenced, while less than 100ng/ml of products was discarded and either repeated the amplification or replaced with another one.

DNA capillary sequencing was performed at the National Instrumentation Center for Environmental Management (NICEM), using the ABI Prism 3100xl genetic analyzer (Applied Biosystems®, US). According to manufacturer's recommendations, the amplified products were divided into two sets and transferred into PCR strips; one set used for the forward primer and the second for the reverse primer. Following sequencing, the resultant sequences were tested for length and quality using BioEdid and Mega 6.

The obtained sequences were aligned using BLAST software with normal sequence from National Center for Biotechnology Information (NCBI) and examined for presence of SNPs and mutations.

2.2.4. Statistical analysis

The data were translated into a computerized database structure. The database was examined for errors using range and logical data cleaning methods. The statistical significance of association between all categorical variables was assessed by a linear regression.

The statistical significance of the association between all categorized variables was assessed by a linear regression. The statistic was done using Minitab 15 statistical analysis software; another one was ANOVA which was used to compare groups among each other and the control. In all statistical analyses, a P value < 0.05 was considered to be significant.

Chapter Three
Results and Discussion

3. Results and Discussion

3.1. Characteristics of women with breast cancer in Iraqi

In this study, 50 samples were collected from women with breast cancer that divided to three groups. Table (3-1) revealed that this disease was found in women with mean age 57 year, 22% of women appeared with familial history, 48% of women were menopause and 62% of women suffered from metastasis to lymph node.

Table (3-1): Characteristics of women with breast cancer

Sample group	No.	Mean age (year)	Familiar History %		Menopause %		Metastasis to lymph node	
			+ve	-ve	+ve	-ve	+ve	-ve
Blood and FFPE	25	57	24	76	48	52	64	36
Frozen tissues, blood, and FFPE	15	55	20	80	26	73	60	40
FFBE	10	58	20	80	60	40	60	40
Total	**50**	**57**	**22% +ve**		**48% +ve**		**62% +ve**	

3.2. Immunohistochemistry (IHC) study

3.2.1. Expression of estrogen receptor in women with breast cancer by using IHC

Immunoexpression of estrogen protein was examined in 50 samples of formalin fixed paraffin embedded tissue (FFPE) by using immunohistochemistry technique. The result showed a specific positive immune staining of estrogen receptor in 35 (70%) women with breast cancer (Fig. 3-1).

The estrogen receptor (ER) in both normal and tumor cells is required for growing cells. In normal cell undergoing repeated cycles of proliferation and death while cancer cell multiplied in the presence of estrogen as cancer cell continue to grow and multiply lead to produce a tumor (Ewlwood and Craig Jordan, 2003). Estrogen induces the secretion of growth factors from the stroma which stimulate epithelial cells to proliferate (Koibuchi *et al*., 1999; Elwood *et al*., 2001).

The status of estrogen receptor expression in mammary tumors is considered as a major prognostic factor of breast cancer where the negativity/low expression is associated with poor prognosis (Saghir *et al*., 2006). The expression of ER alpha is associated with breast cancer biology and development of tumors, ER alpha expression in tumor tissues is a favorable predictor of prognosis in endocrine treatment (Hayashi *et al*., 2003). Detection of estrogen receptor expression by immunohistochemistry assay is considered as a routine test used in taking decisions on hormonal therapy for breast cancer (Holst *et al*., 2007). It has been shown that ESR1 protein was confirmed as prognostic factor for detection breast cancer in approximately 70% of all breast cancers (Harvey *et al*., 1999; Pinhel *et al*., 2012; Hirata *et al*., 2014; Dixon, 2014). While in Iraq, it was found that estrogen receptor positive tumors were reported in 65.1% of the cases (Iraqi Cancer Registry, 2009).

Moreover, immunohistochemistry results showed a correlation between ER positivity and SNP formation and risk of breast carcinoma (Herynk and Fuqua, 2004).

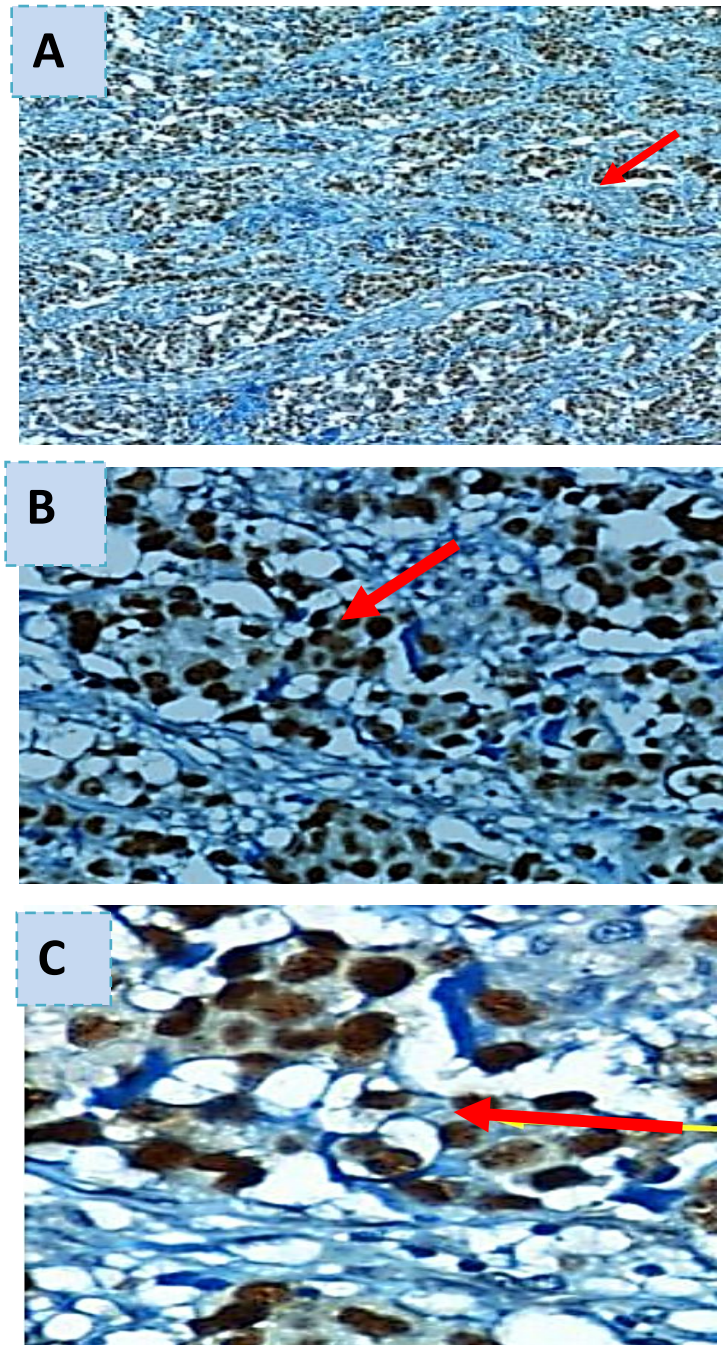


Figure (3-1): A representative case of infiltrative ductal carcinoma of the breast in a 50 years old female tested by immunohistochemistry and specific antibodies to ER. Malignant cells appeared as dark brown stain reflecting positive expression to ER. A, B and C represent different magnifications of the same section at X100, X200, and X400, respectively

3.2.2. Association between immunoexpression of estrogen receptor (ER) protein and risk factors

The association between several risk factors (age, familiar history, menopause, and metastasis to lymph node) and expression of estrogen protein in fifty samples of FFPE collected from women with breast cancer was studied.

The first risk factors was age, Table (3-2) showed a significant expression ($P<0.01$) of estrogen receptor protein in 36 women with breast cancer at an age more than 55 year old in comparison with 14 women at age less than 55 years old; the percentage of expression of estrogen receptor was 78% and 50%, respectively. These results are consistent with the general acceptable concept of breast cancer incidence increases with age (Yoo *et al*., 2001; Hirata *et al*., 2014). The older woman was more likely to get breast cancer than younger, so breast cancer rate was low in women under 40, it increases after age 40 and were highest in women over age 70 (Howlader *et al*., 2015; American Cancer Society, 2015).

In previous study it was found that age was considered as a risk factor for breast cancer, the risk of developing this disease increased when getting older and it was not the same for all women but in combination with other risk factors the chance increased to develop breast cancer (American Cancer Society, 2012).ER is reflected by marked changes in its signaling pathway and/or expression of the two estrogen receptors (ER alpha and ER beta) during breast carcinogenesis and progression (Kang *et al*., 2002; Su *et al*., 2012, Hirata *et al*., 2014). In addition, ER alpha expression in tumor tissues was shown to be a favorable marker of prognosis of breast carcinogenesis (Hayashi *et al*., 2003).

Table (3-2): Expression of estrogen receptor protein in fifty FFPE samples from women with breast cancer in Iraq

Risk factors for 50 patients	**Estrogen Expression**	***P*-value**
	ER +ve (%)	
Age		
>55(36)	28 (78%)	0.00217**
<55(14)	7 (50%)	
Familiar history		
+ve (11)	6 (54%)	0.0001**
-ve (39)	29 (74%)	
Menopause		
+ve (24)	17 (70%)	0.0341**
-ve (26)	18 (69%)	
Metastasis to lymph node		
+ve (31)	25 (80.6%)	0.00394**
-ve (19)	10 (52.6%)	

*** represents a non-significant value of *P*<0.05 where ** a *P*< 0.01 is defined as a significant, +ve: positive status of the risk factor, -ve: negative status of the risk factor.**

The second risk factor was familiar history, it was noticed that 39 women without familiar history achieved high percentage of expression of estrogen receptor (74%) while 11 women with familiar history revealed low percentage of expression of estrogen receptor (54%). In previous studies it confirmed that 10-30% of women with breast cancer had a family history are more likely to have ER positive tumors than others without family history ((Hulka and Moorman, 2001; Molino *et al*., 2004; Reis-Filho and Lakhani, 2008). Indeed, the development of

breast cancer could also attribute to gene mutations that could be either inherited from one parent (familial cancer) or acquired after conception (sporadic cancers). It was important to note that just few of the inherited mutations (high penetrate mutations) were correlated with a breast cancer risk, while mostly were associated with a smaller increase (low penetrate mutations) breast cancer risk and a less family history prominent of breast cancer, these mutations were known as low penetrate mutations (Barbour, 2003).

Menopause was the third risk factor was included in the present study, it was found a significant difference in the expression of estrogen receptor in women at menopause or pre-menopause; the percentage of ER was 70% in menapousal women. Indeed, the production of estrogen was usually seen in the normal tissues of breast, muscle and bone, which processed by aromatase conversion of androgens hormones (androstenedione and testosterone) (Johnston and Dowsett, 2003). Residual levels of estrogen were also seen in the blood by 20-folds higher in post-menopausal compared with pre-menopausal women (Simpson and Dowsett, 2002; Larionov *et al*., 2003). The noticeable increase of estrogen was correlated with the high risk of breast cancer (Key, 2002; Dixon, 2014). Moreover, women at post-menopausal having high levels of endogenous hormones "estrogen and testosterone" had twice breast cancer risk comparing with those at the lowest levels (Hankinson and Eliassen, 2007; Fuhrman *et al*., 2012). High circulating hormone levels were correlated with and can reflect the effects of other factors for breast cancer risk, as in postmenopausal obesity (Endogenous Hormones Breast Cancer Collaborative Group, 2011). On the other hand, this issue gets more complicated particularly with the hormonal levels changes during the menstrual cycle with women at the pre-menopausal stage (Kaaks *et al*., 2005; Zeleniuch-Jacquotte *et al*., 2012).

In addition, this study was correlated between breast cancer and metastasis of lymph node. It noticed that 31 women with metastasized lymph node had high percentage (80.6%) of expression of estrogen protein while 19 women without metastasize showed low percentage of expression of estrogen (52.6%). The role of this risk factor is still controversial (Bartlett *et al*., 2007; Eleftherios, 2009) and inconsistent findings of ER distribution were observed between younger and older patients (Wildiers *et al*., 2009).

The ER-dependent mechanisms was due to genetic and epigenetic changes of the ER alpha gene, such as promoter hypermethylation, truncated isoforms, or post-translational modifications, which influence either its function or lack of protein expression (Herynk and Fuqua, 2004; Musgrove *et al*., 2009). Moreover, the estrogen receptor-independent pathway that altered cell cycle via signaling mechanism and activation of escape pathways. All these could contribute to the failure of systemic therapy leading to outgrowth of metastases in other organs (Osborne and Schiff, 2011).

3.3. Molecular study

3.3.1. Purity and concentration of extracted DNA from women with breast cancer

The DNA was successfully extracted from all samples of blood and frozen tissue, while 10 of 35 (28%) samples of formalin fixed paraffin embedded (FFPE) were success. The purity of DNA extracted from blood samples was ranged from 1.7 to 2 while the concentration of DNA was ranged from 50 to 120 µg/ml. The same value was estimated from frozen tissue samples; it ranged from 1.8 to 2 and DNA concentration ranged from 75 to 150 µg/ml. Whereas less purity of DNA

was determined from FFBE samples, it ranged from 1 to 1.5 and the DNA concentration ranged from 15 to 50 µg/ml (Table 3-2).

Table (3-3): Purity and concentration of DNA extracted from different samples collected from women with breast cancer

Samples	Purity	Concentration (µg/ml
Blood	1.7-2	50-120
Frozen tissue	1.8-2	75-150
FFPE	1-1.5	15-50

Indeed, the extraction of DNA from blood was easy, fast, and efficient with good purity and concentration, so as samples that collected from frozen tissue. Notable, frozen tissue samples needed advanced instrument and expensive material for DNA extraction. While the extraction of DNA from FFPE samples were expensive, complicated, and consumed time.

The results of DNA purity and concentration were oscillated and this may return to the kind of sample collection, preservation, fixation in formalin and concentration of formalin that used, condition and material used in procedure of embedded in paraffin, and conditions of storage the blocks that contain tissue.

These results were similar to that confirmed by Cong and Xiang (2012) who found that DNA extracted from FFPE was poor quality, time consuming with low concentration and purity of DNA, which result in false-negative of false-positive results according to not good preservation of samples, an incomplete tissue fixation and/or tissue over fixation.

Moreover, a comparison between samples (blood, frozen tissue and FFPE) used in this study, it has been found that blood and frozen tissue are more favorable and are gold standards to detect sequencing. Blood samples was more favorable

because its collection is so easy, fast, does not need professional surgery to collect it and easy for extraction of DNA and not required expensive materials or kit. While frozen tissue is collect from patient exposed to surgery, also it need expensive kit, and sometime advance instrument for DNA extraction. In addition, the blood and frozen tissue samples from the same women with breast cancer showed no difference in detection of mutation, if mutation is detected in blood it is also found in the tissue sample for the same patient and visa versa, and this proves in the result of this study.

Regarding, FFBE samples used in this study, the extraction of DNA was difficult, moreover not all samples gave good sequence and figures for detection mutations, some sequences were not suitable for analysis and alignment. As well the mutation rate in FFPE sample of the studied genes was low when compared with blood and frozen tissue samples. Cong and Xiang (2012) reported the same results.

3.3.2. Amplification of exons in estrogen receptor alpha (*ESR1*) and beta (*ESR2*) genes

The samples (blood, frozen tissue, and FFPE) that showed positivity to estrogen receptor protein were selected for molecular study. 10 samples from blood, 7 sample from frozen tissue, and 7 samples from FFPE were selected.

The exon 4 and 6 in estrogen receptor alpha (*ESR1*) gene were detected by using PCR. Figure (3-2) and (3-3) showed that exon 4 and 6 in *ESR1* were appeared as a band size with 370 bp and 300 bp respectively.

In previous study, Hsiao *et al*. (2004) and Abbasi *et al*. (2013) found different results in Iranian women; the exon 4 had a band size 329 bp. This may return to using different PCR program.

On the other hand, exon 3 and 7 in estrogen receptor beta (*ESR2*) appeared as a band with size 151 bp and 157 bp respectively (Fig 3-4 and 3-5).

In Iranian women, the size of exon 3 and 7 in *ESR2* was also detected (Abbasi, 2010). The exon 3 had the same sized that found in this study, while exon 7 showed different size with 329 bp, this may due to the differences in PCR program.

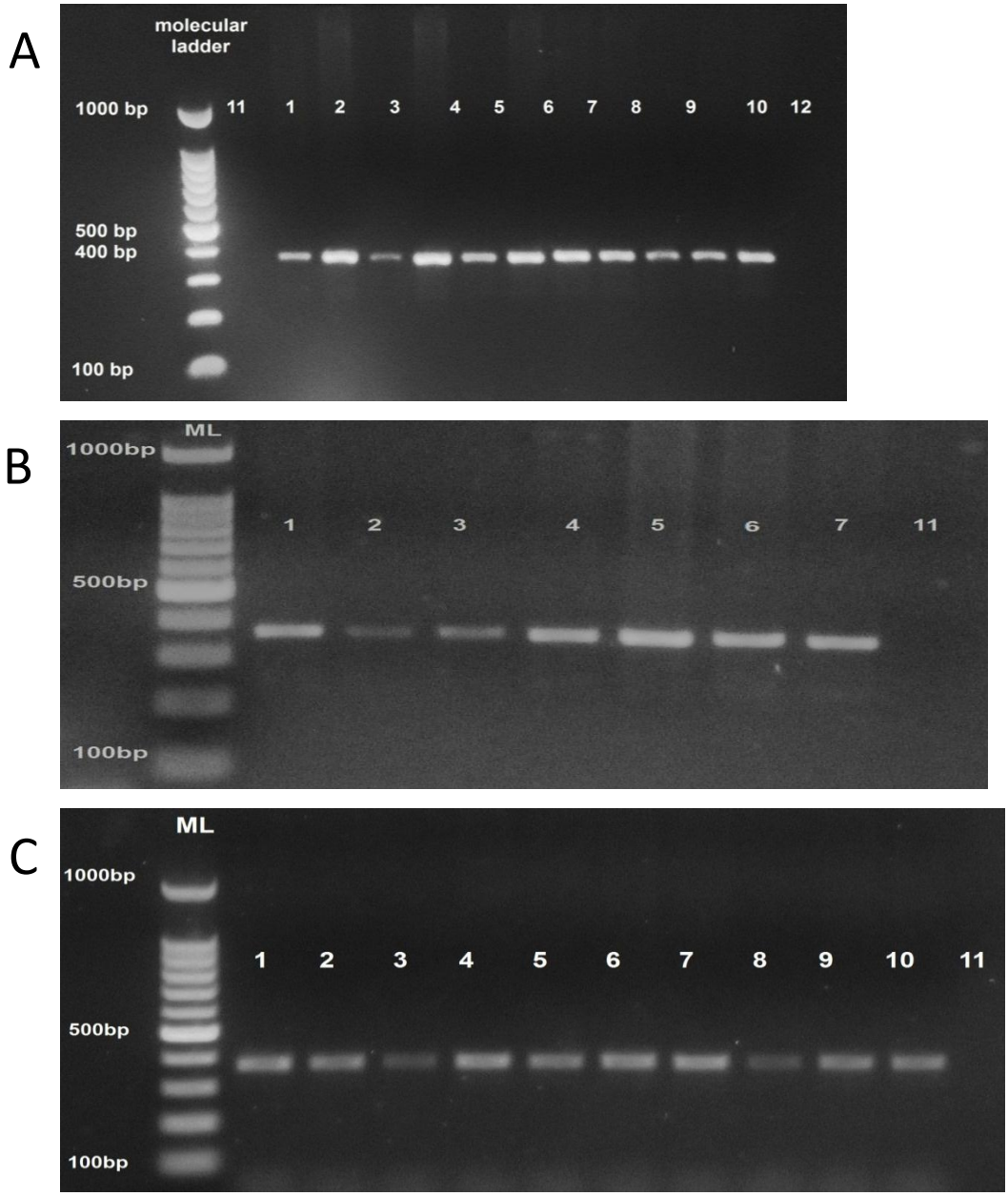


Figure (3-2): Amplification of exon 4 in estrogen receptor alpha set 1 primer with 370 bp. A: blood samples; Lanes 1-10 represent DNA from women with breast cancer, lane 11 represents DNA of negative control, lane 12 represents DNA from healthy subjects. B: frozen tissue samples; Lanes 1-7 represent samples from women with breast cancer, lane 11 represents DNA in negative control. C. FFPE samples; Lanes 1-10 represent DNA from women with breast cancer, lane 11 represents control negative. Agarose 1.5%, 5V/cm for 45 min, ML: molecular ladder (100-1000 bp)

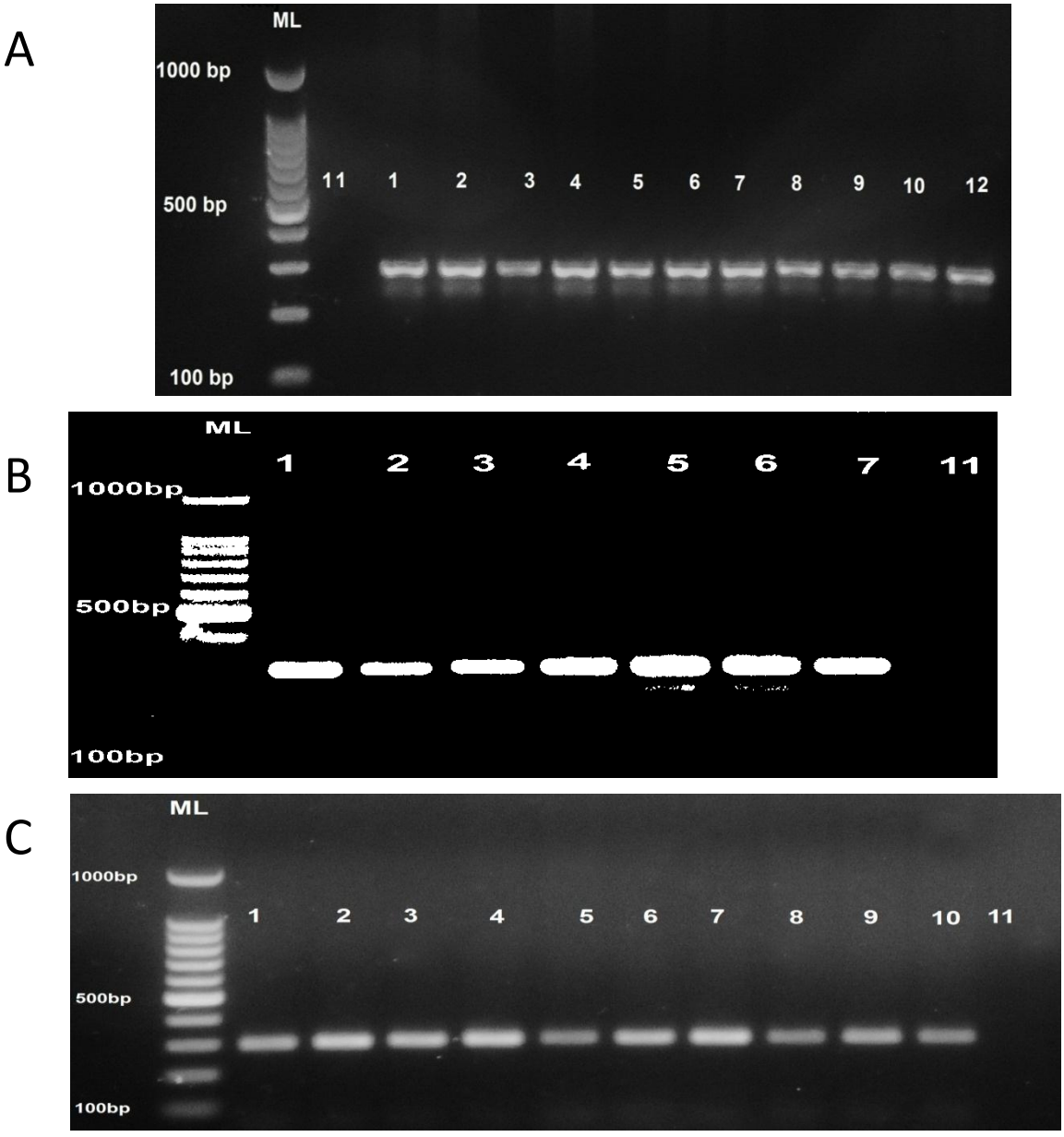
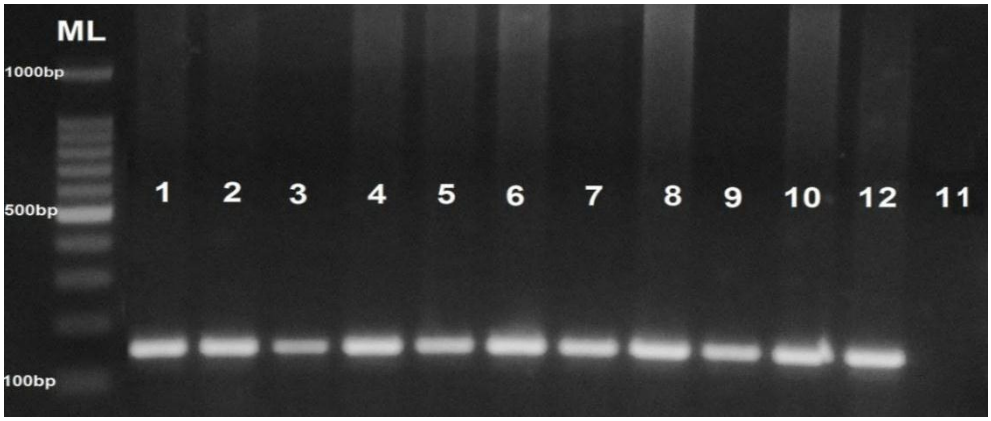
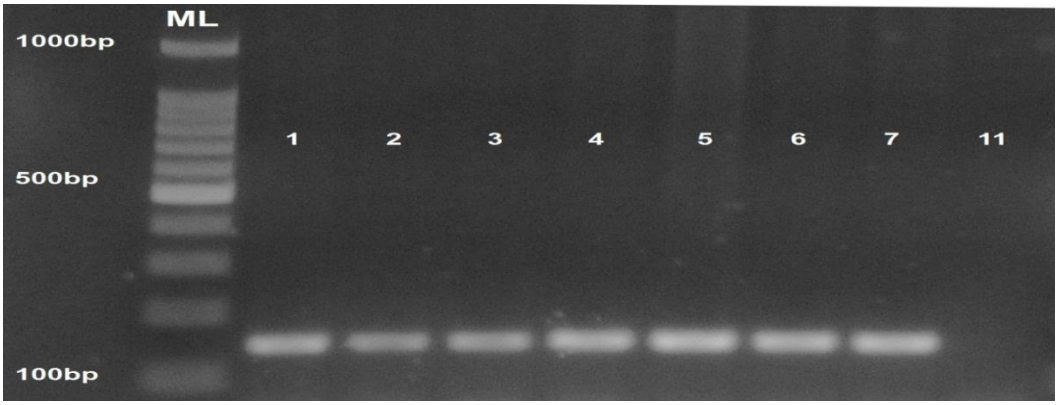


Figure (3-3): Amplification reaction of exon 6 in estrogen receptor alpha set 1 primer with 300 bp. A: blood samples; Lanes 1-10 represent DNA from women with breast cancer, lane 11 represents control negative, lane 12 represents DNA from healthy subjects. B: frozen tissue samples; Lanes 1-7 represent DNA from women with breast cancer, lane 11 represents DNA from control negative. C. FFPE samples; Lanes 1-10 represent samples from women with breast cancer, lane 11 represents DNA from control negative. Agarose 1.5%, 5V/cm for 45 min, ML: molecular ladder (100-1000 bp)

A



B



C

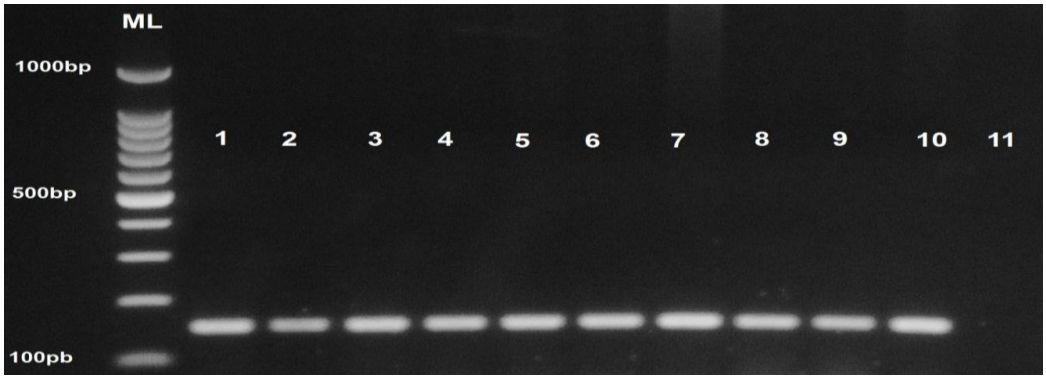


Figure (3-4): Amplification of exon 3 in estrogen receptor beta set 1 primer with 151 bp. A: blood samples; Lanes 1-10 represent DNA from women with breast cancer, lane 11 represents control negative, lane 12 represents DNA from healthy subjects. B: frozen tissue samples; Lanes 1-7 represent DNA from women with breast cancer, lane 11 represents control negative. C. FFPE samples; Lanes 1-10 represent DNA from women with breast cancer, lane 11 represents control negative. Agarose 1.5%, 5V/cm for 45 min, ML: molecular ladder (100-1000 bp)

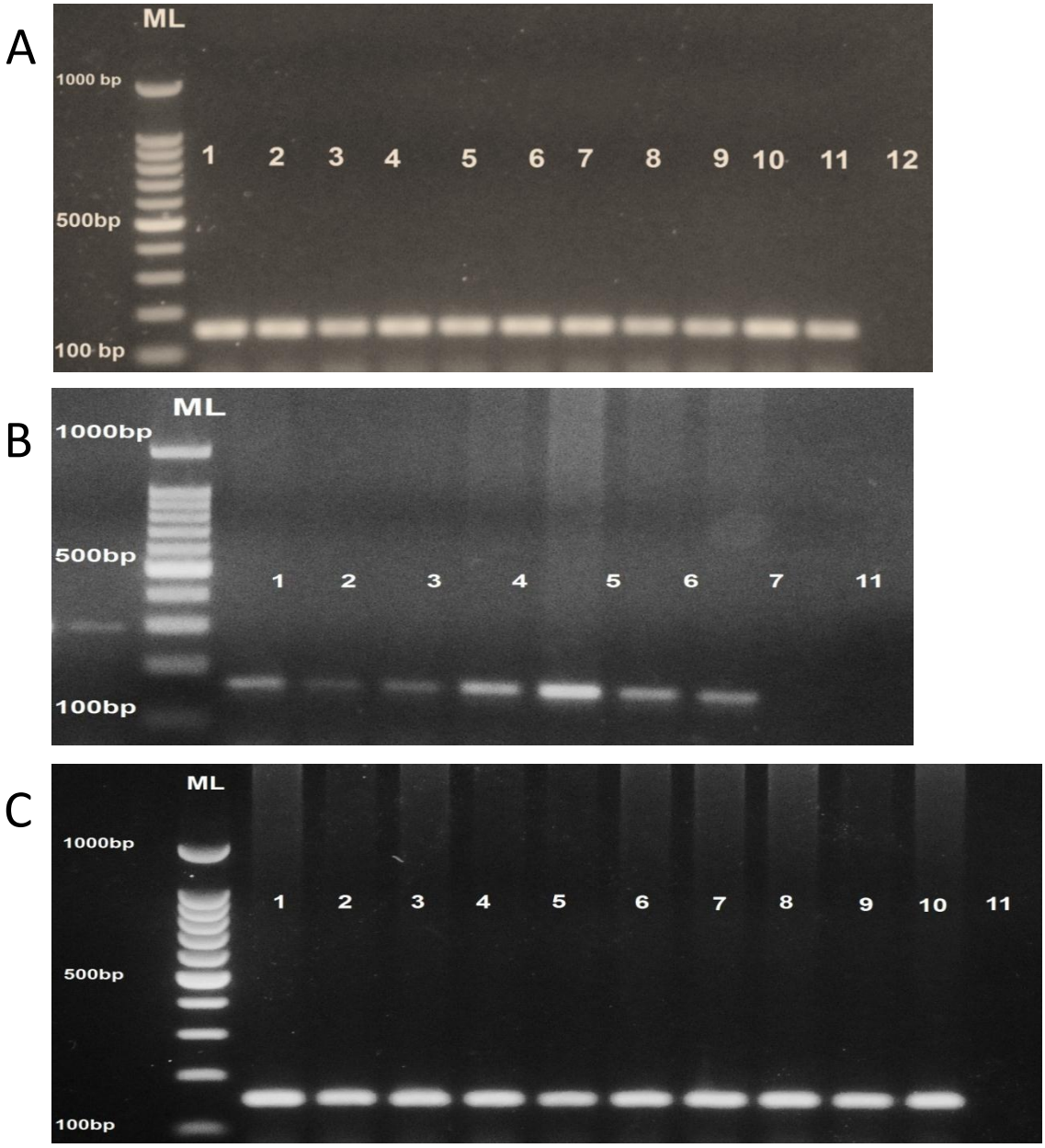


Figure (3-5): Amplification of exon 7 in estrogen receptor beta set 1 primer with 157 bp. A: blood samples; Lanes 1-10 represent DNA from women with breast cancer, lane 11 represents control negative, lane 12 represents DNA from healthy subjects. B: frozen tissue samples; Lanes 1-7 represent DNA from women with breast cancer, lane 11 represents control negative. C. FFPE samples; Lanes 1-10 represent DNA from women with breast cancer, lane 11 represents DNA of control negative. Agarose 1.5%, 5V/cm for 45 min, ML: molecular ladder (100-1000 bp)

3.3.3. Polymorphisms in estrogen receptor alpha (*ESR1*) and estrogen receptor beta (*ESR2*) genes

3.3.3-1. Polymorphisms in estrogen receptor alpha gene (*ESR1*)

A: Polymorphisms of exon 4 in *ESR1*

In exon 4 of *ESR1* gene, seven polymorphisms (AAG, AAA, TTT, AAA, CCG, AAA, and AAC) were detected. The type of polymorphisms, position and their effects on gene expression were described in Table (3-5). All mutations in exon 4 of *ESR1* were substitution polymorphisms that converted one base to another and then caused either no changing in the produced protein and this called silent polymorphism (sense mutation), or caused an exchange in the produced protein and this called missense mutation.

Table (3-5): Polymorphisms in exon 4 of *ESR1* gene in women with breast cancer

No.	Mutation	Type	Position	Wild type codon	Mutated codon	Chang of amino acid*	Effect on translation	Kind of mutation	No. of patie-nts
1	A → G	Substitution	258740	GAG	AAG	G → G	Silent mutation	Point mutation	2
2	G → A	Substitution	258777	AGA	AAA	R → K	Missense mutation	Point mutation	1
3	C → T	Substitution	258762	TCT	TTT	D → N	Missense mutation	Point mutation	3
4	G → A	Substitution	258826	AAG	AAA	K → K	Silent mutation	Point mutation	1
5	C → G	Substitution	258892	CCC	CCG	P → P	Silent mutation	Point mutation	3
6	G → A	Substitution	258921	AGA	AAA	R → K	Missense mutation	Point mutation	6

7	G → A	Substitution	258977	GAC	AAC	D → N	Missense mutation	Point mutation	6

*** G: Glycine, R: Arginine, K: Lysine, D: Aspartic acid, N: Asparagine, P: Proline**

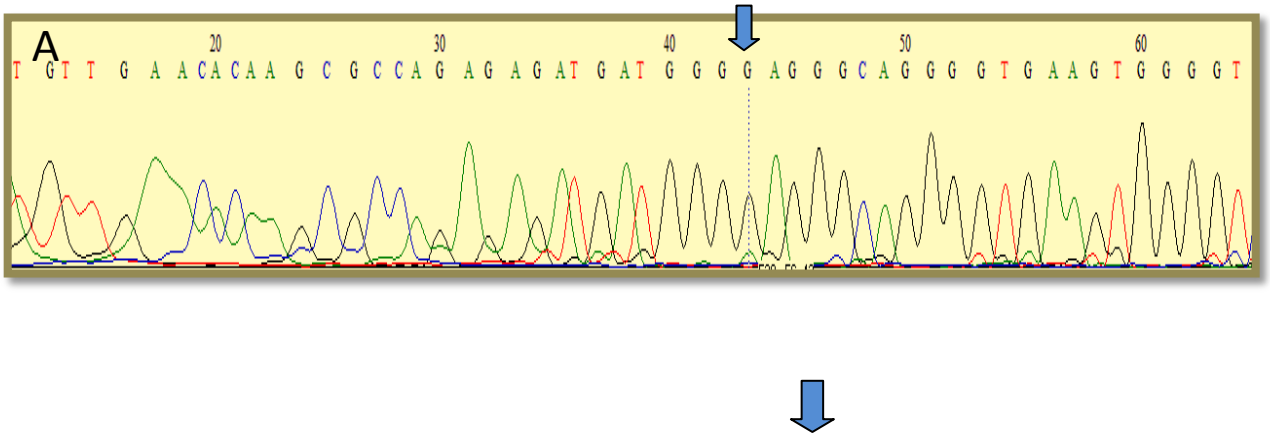
The sequences of exon 4 in *ESR*1 gene were aligned with control group (healthy women) and with NCBI as shown below (Fig. 3-6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19) as well as the nucleotide sequence profile of control group (healthy women) was aligned with NCBI as shown in appendix- 3.

- **Sequences profile and alignment of each polymorphism in exon 4 of *ESR1***

1- AAG

The sequencing result revealed the presence of SNP A →G (Table 3-5). The identified SNP was a silent polymorphism (sense mutation), it was substitution polymorphism. The common codon GAG was converted to AAG. This point mutation had no effect on gene expression in which the changing codon still encoding the same amino acid, Glycine. This polymorphism was found in 2 (8 %) samples; one from blood and the other from FFBE.

Figure (3-6) showed nucleotide sequencing profile for women with breast cancer compared with healthy women.



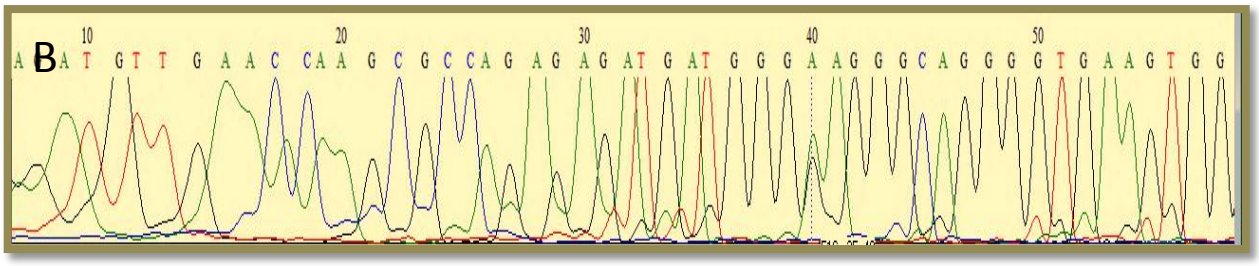


Figure (3-6): Nucleotide sequencing profile of exon 4 in *ESR1*gene polymorphism at position 258740 as edited by Finich TV software. A: healthy women as a control, B: women with breast cancer, G substitution by A

Then an alignment of nucleotides sequencing of exon 4 in *ESR1* for women with breast cancer compared with control in NCBI center using automated sequencer and analyzed by BLAST data. The query number represents the current results while the subject represents the reference sequence, the blue color refers to exon while the black color represents the intron region around exon as shown in Figure (3-7).

Homo sapiens estrogen receptor 1 (*ESR1*), RefSeqGene on chromosome 6
Sequence ID: ref|NG_008493.2|Length: 419779Number of Matches: 1
Related Information
Map Viewer-aligned genomic context
Range 1: 258698 to 259031GenBankGraphics Next Match Previous Match First Match
Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
560 bits(620)	6e-156	331/334(99%)	3/334(0%)	Plus/plus

Features:

```
Query  8       GGAGGGAGAATGTTGAAACACAAGCGCCAGAGAGATGATGGGAAGGGCAGGGGTGAAGTG  67
               ||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  258698  GGAGGGAGAATGTTGAAACACAAGCGCCAGAGAGATGATGGGGAGGGCAGGGGTGAAGTG  258757

Query  68      GGGTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGATCAAACGC  127
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  258758  GGGTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGATCAAACGC  258817

Query  128     TCTAAGAAGAACAGCCTGGCCTTGTCCCTGACGGCCGACCAGATGGTCAGTGCCTTGTTG  187
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  258818  TCTAAGAAGAACAGCCTGGCCTTGTCCCTGACGGCCGACCAGATGGTCAGTGCCTTGTTG  258877

Query  188     GATGCTGAGCCCCCCATACTCTATTCCGAGTATGATCCTACCAAACCCTTCAGTGAAGCT  247
               ||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  258878  GATGCTGAGCCCCCCATACTCTATTCCGAGTATGATCCTACCAGACCCTTCAGTGAAGCT  258937

Query  248     TCGATGATGGGCTTACTGACCAACCTGGCAAACAGGGAGCTGGTTCACATGATCAACTGG  307
               |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  258938  TCGATGATGGGCTTACTGACCAACCTGGCAGACAGGGAGCTGGTTCACATGATCAACTGG  258997

Query  308     GCGAAGAGGGTGCCAGGTAAGAATGCGAAGCGCA  341
               |||||||||||||||||||||||||||||||||
Sbjct  258998  GCGAAGAGGGTGCCAGGTAAGAATGCGAAGCGCA  259031
```

Figure (3-7): Alignment of exon 4 in *ESR1* gene sequence of women with breast cancer polymorphism at position 258740 using automated sequencer was analyzed by BLAST data, query number represents the current results while the subject represents the reference sequence. Blue color represents exon region, black color represented intron region while the single nucleotide polymorphism represents red color, G substitution by A

2- AAA

The sequencing result displayed the presence of SNP G→ A (Table 3-5). The identified SNP was missense mutation, it was substitution polymorphism. The common codon AGA was converted to AAA. This point mutation caused alteration in gene expression because of alteration in amino acid; the Arginine was converted to Lysine. This polymorphism was found in one (4.1%) sample of FFBE.

Nucleotide sequencing profile for women with breast cancer compared with control of healthy women was demonstrated in figure (3-8).

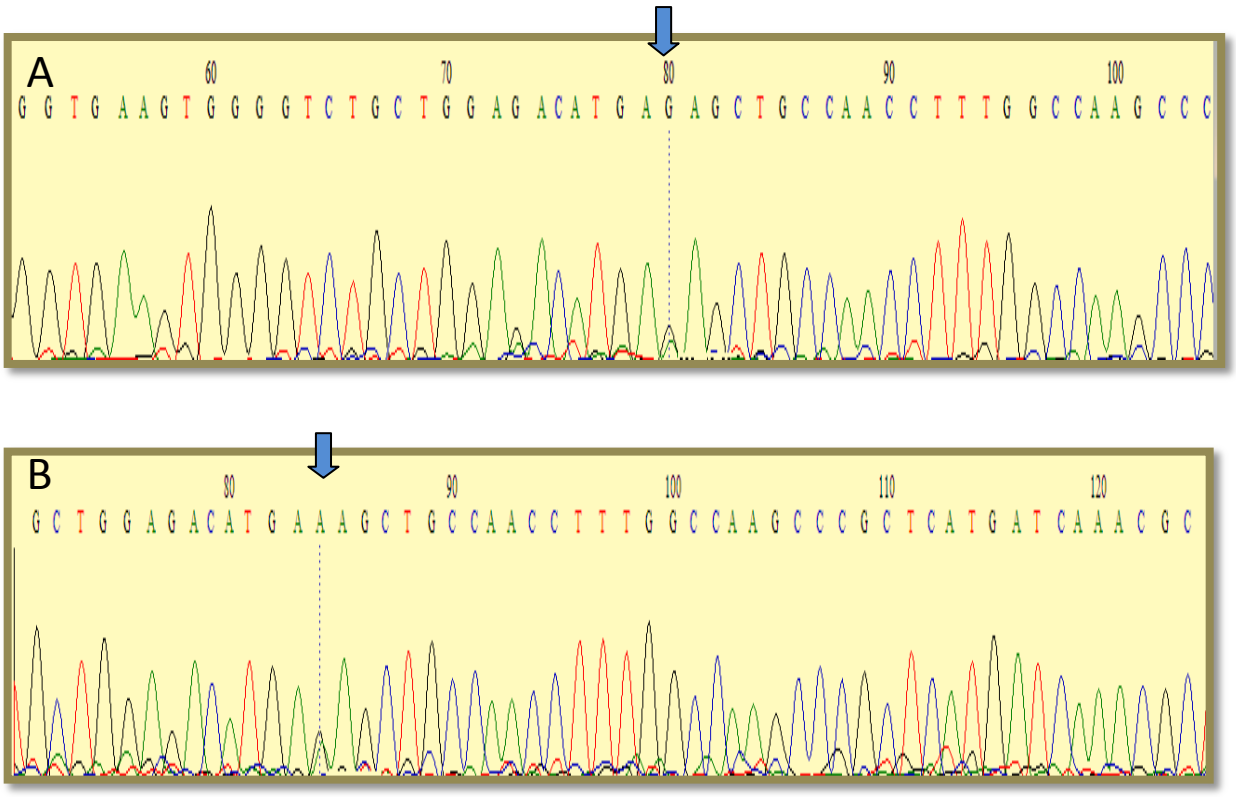


Figure (3-8): Nucleotide sequencing profile of exon 4 in *ESR1* gene polymorphism at position 258777 as edited by Finich TV software. A: healthy women as a control, B: women with breast cancer, G substitution by A

Then an alignment of nucleotides sequencing of exon 4 in *ESR1* for women with breast cancer compared with control in NCBI center using automated sequencer and analyzed by BLAST data. The query number represents the current results while the subject represents the reference sequence, the blue color refers to exon while the black color represents the intron region around exon as shown in Figure (3-9).

ref|NG_008493.2| Homo sapiens estrogen receptor 1 (*ESR1*), RefSeqGene on chromosome6
Length:419779

Score	Expect	Identities	Gaps	Strand
582 bits(315)	**1e-162**	**323/325(99%)**	**2/325(0%)**	**Plus/Plus**

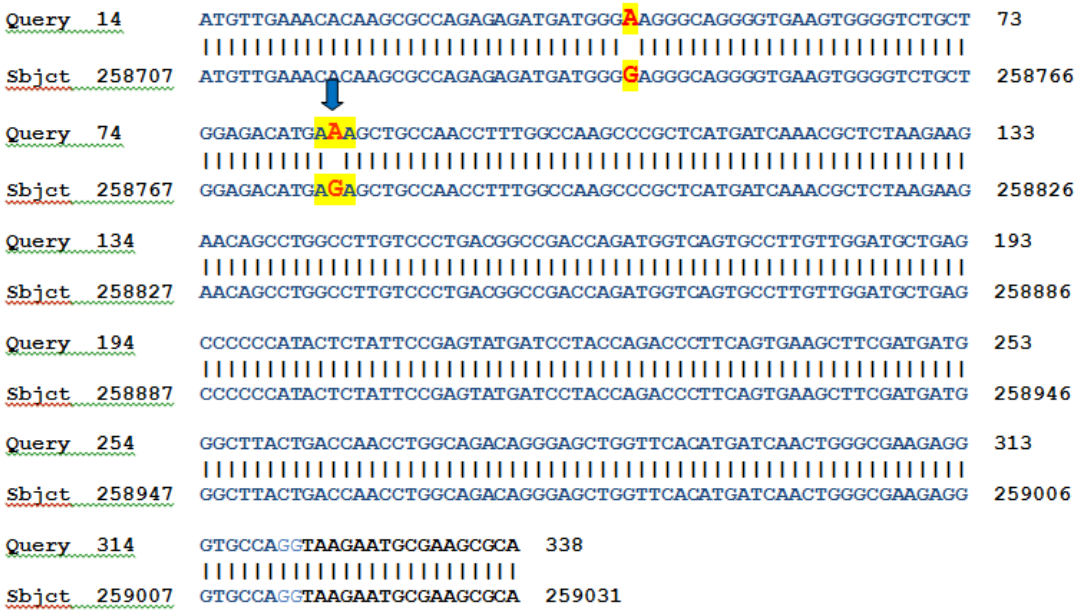


Figure (3-9): Alignment of exon 4 in *ESR1* gene sequence of women with breast cancer polymorphism at position 258777 using automated sequencer was analyzed by BLAST data, query number represents the current results while the subject represents the reference sequence. Blue color represents exon region, black color represented intron region while the single nucleotide polymorphism represents red color, G substitution by A

3- TTT

The sequencing result illustrated the presence of SNP C → T (Table 3-5). The identified SNP was missense mutation, it was substitution polymorphism. The common codon TCT was converted to TTT. This point mutation caused alteration in gene expression because of revision in amino acid; the Aspartic acid was converted to Asparagine. This polymorphism was found in 3 (13%) samples of blood.

Figure (3-10) showed nucleotide sequencing profile for women with breast cancer compared with healthy women.

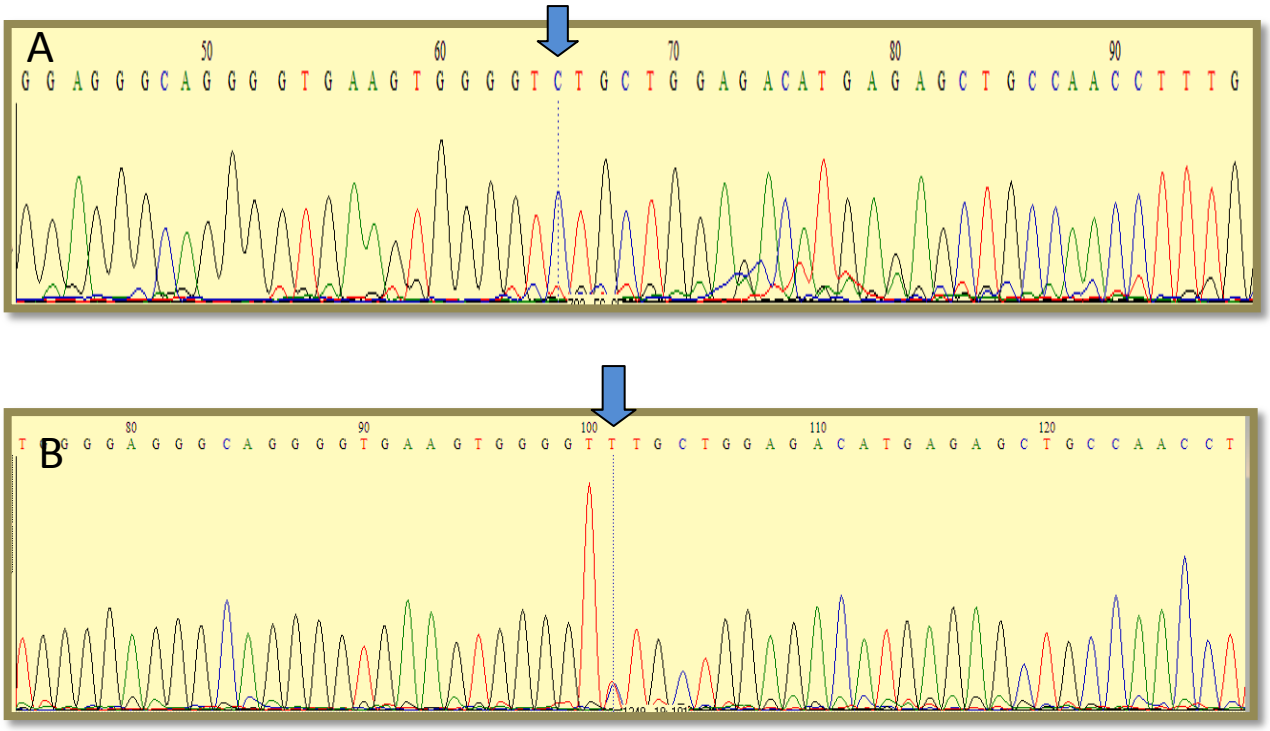


Figure (3-10): Nucleotide sequencing profile of exon 4 in *ESR1* gene polymorphism at position 258762 as edited by Finich TV software. A: healthy women as a control, B: women with breast cancer, C substitution by T

An alignment of nucleotides sequencing of exon 4 in *ESR1* for women with breast cancer was done and compared with control in NCBI center using automated sequencer and analyzed by BLAST data. The query number represents the current results while the subject represents the reference sequence, the blue color refers to exon while the black color represents the intron region around exon as shown in Figure (3-11).

Homo sapiens estrogen receptor 1 (*ESR1*), RefSeqGene on chromosome 6
Sequence ID: ref|008493.2|Length: 419779Number of Matches: 1
Related Information
Map Viewer-aligned genomic context
Range 1: 258665 to 258986GenBankGraphics Next Match Previous Match First Match
Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
582bits(315)	1e-162	321/322(99%)	1/322(0%)	Plus/Plus

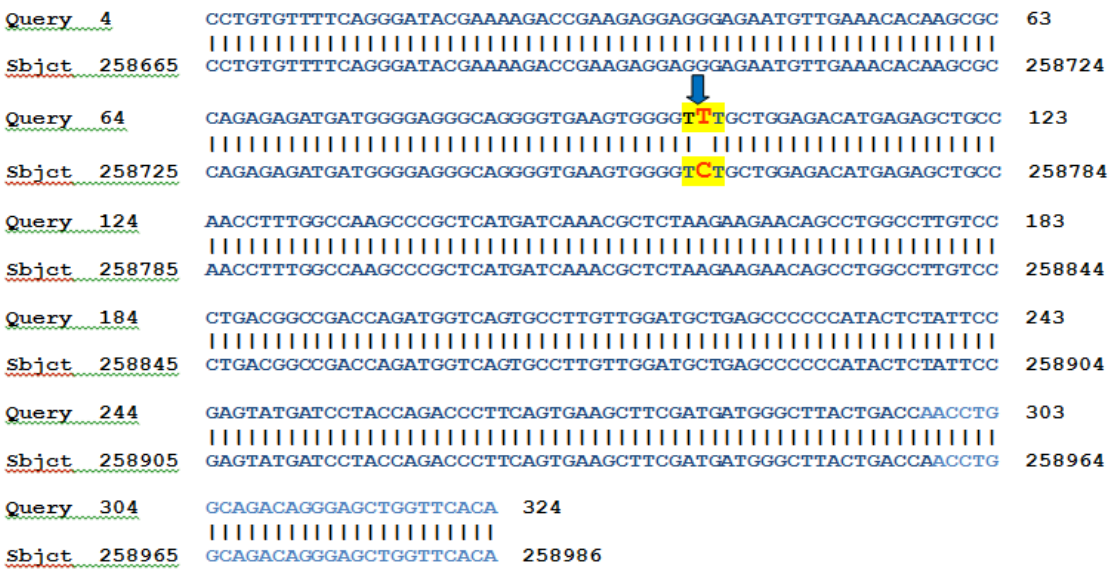


Figure (3-11): Alignment of exon 4 in *ESR1* gene sequence of women with breast cancer polymorphism at position 258762 using automated sequencer was analyzed by BLAST data, query number represents the current results while the subject represents the reference sequence. Blue color represents exon region, the single nucleotide polymorphism represents red color, C substitution by T

4- AAA

The sequencing result exposed the presence of SNP G → A (Table 3-5). The identified SNP was a silent polymorphism (sense mutation), it was substitution polymorphism. The common codon AAG was converted to AAA. This point mutation had no effect on gene expression in which the changing codon still encoded the same amino acid, Lysine. This polymorphism was found in 1 (4.1%) sample from frozen tissue and it appeared in the blood of the same patient.

Figure (3-12) showed nucleotide sequencing profile for women with breast cancer compared with healthy women.

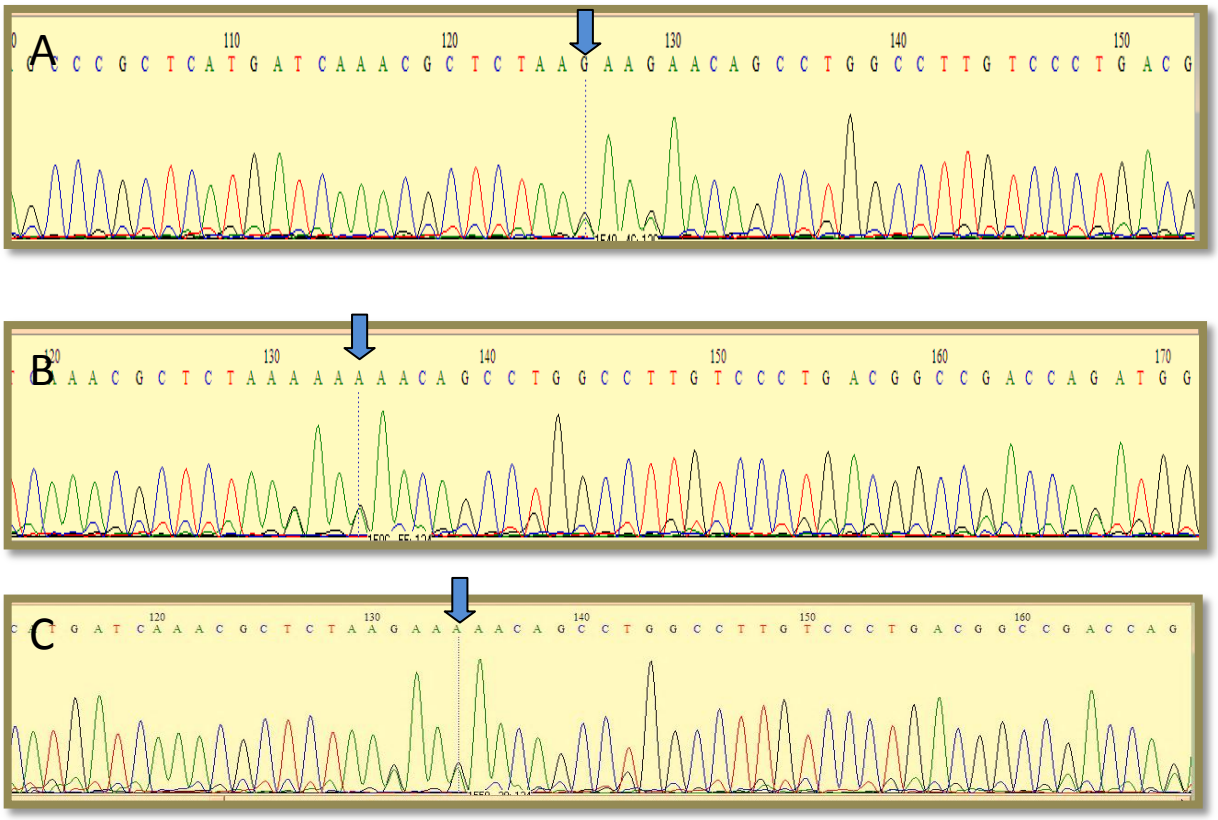


Figure (3-12): Nucleotide sequencing profile of exon 4 in *ESR1* gene polymorphism at position 258826 as edited by Finich TV software. A: healthy women as a control, B: women with breast cancer (blood sample), C: women with breast cancer (tissue sample), G substitution by A

Then nucleotides sequencing of exon 4 in *ESR1* for women with breast cancer were aligned and compared with control in NCBI center using automated sequencer and analyzed by BLAST data. The query number represents the current results while the subject represents the reference sequence, the blue color refers to exon while the black color represents the intron region around exon as shown in Figure (3-13).

A

Homo sapiens estrogen receptor 1 (*ESR1*), RefSeqGene on chromosome 6
Sequence ID: ref|NG_008493.2|Length: 419779Number of Matches: 1
Related Information
Map Viewer-aligned genomic context
Range 1: 258714 to 259026GenBankGraphics Next Match Previous Match First Match
Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
544 bits(294)	8e-151	310/313(99%)	3/313(0%)	Plus/Plus

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Sbjct  258714  AACACAAGCGCCAGAGAGATGATGGGGAGGGCAGGGGTGAAGTGGGGTCTGCTGGAGACA  258773

Query  82      TGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGATCAAACGCTCTAAGAAAAACAGCC  141
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Sbjct  258774  TGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGATCAAACGCTCTAAGAAGAACAGCC  258833

Query  142     TGGCCTTGTCCCTGACGGCCGACCAGATGGTCAGTGCCTTGTTGGATGCTGAGCCCCCCA  201
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Sbjct  258834  TGGCCTTGTCCCTGACGGCCGACCAGATGGTCAGTGCCTTGTTGGATGCTGAGCCCCCCA  258893

Query  202     TACTCTATTCCGAGTATGATCCTACCAAACCCTTCAGTGAAGCTTCGATGATGGGCTTAC  261
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Sbjct  258894  TACTCTATTCCGAGTATGATCCTACCAGACCCTTCAGTGAAGCTTCGATGATGGGCTTAC  258953

Query  262     TGACCAACCTGGCAGACAGGGAGCTGGTTCACATGATCAACTGGGCGAAAAGGGTGCCAG  321
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Sbjct  258954  TGACCAACCTGGCAGACAGGGAGCTGGTTCACATGATCAACTGGGCGAAGAGGGTGCCAG  259013

Query  322     GTAAGAATGGGAA  332
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Sbjct  259014  GTAAGAATGCGAA  259026
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B

Homo sapiens estrogen receptor 1 (*ESR1*), RefSeqGene on chromosome 6
Sequence ID: ref|NG_008493.2||Length: 419779Number of Matches: 1
Related Information
Map Viewer-aligned genomic context
Range 1: 258700 to 259033GenBankGraphics Next Match Previous Match First Match
Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
562 bits(304)	2e-156	332/337(98%)	5/337(1%)	Plus/Plus

Figure (3-13): Alignment of exon 4 in *ESR1* gene sequence of women with breast cancer polymorphism at position 258826 using automated sequencer was analyzed by BLAST data, query number represents the current results while the subject represents the reference sequence. Blue color represents exon region, black color represented intron region while the single nucleotide polymorphism represents red color; A: blood sample, B: Frozen tissue sample, G substitution by A, N represents any one of nucleic acid

5- CCG

The sequencing result illustrated the presence of SNP C →G (Table 3-5). The identified SNP was a silent polymorphism (sense mutation), it was substitution polymorphism. The common codon CCC was converted to CCG. This point mutation had no effect on gene expression because it still coded for the same amino acid, Proline. This polymorphism found in 3 (13%) samples of blood.

Figure (3-14) showed nucleotide sequencing profile for women with breast cancer compared with healthy women.

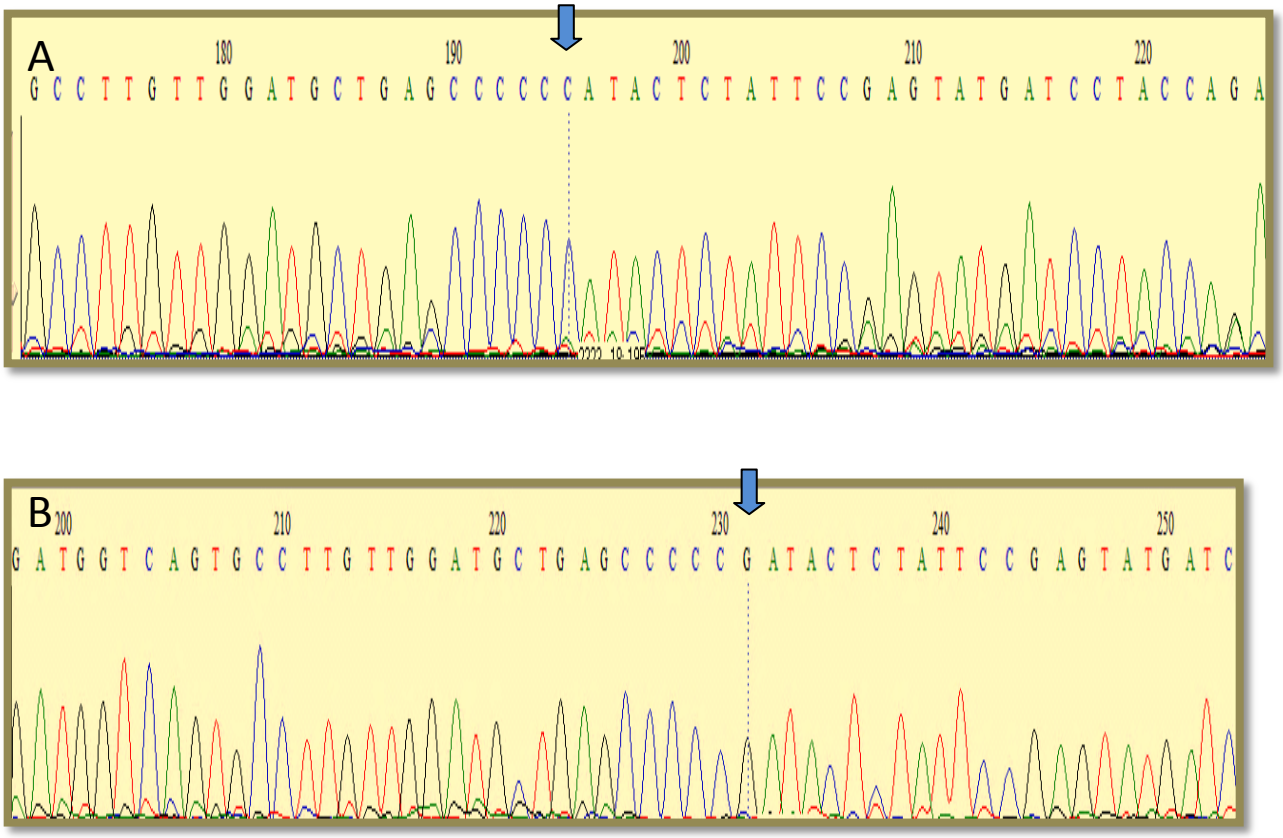

Figure (3-14): Nucleotide sequencing profile of exon 4 in *ESR1* gene polymorphism at position 258892 as edited by Finich TV software. A: healthy women as a control, B: women with breast cancer, C Substitution by G

Then an alignment of nucleotides sequencing of exon 4 in *ESR1* for women with breast cancer compared with control in NCBI center using automated sequencer and analyzed by BLAST data. The query number represents the current results while the subject represents the reference sequence, the blue color refers to exon while the black color represents the intron region around exon as shown in Figure (3-15).

Homo sapiens estrogen receptor 1 (*ESR1*), RefSeqGene on chromosome 6
Sequence ID: ref|NG_008493.2|Length: 419779Number of Matches: 1
Related Information
Map Viewer-aligned genomic context
Range 1: 258697 to 259031GenBankGraphics Next Match Previous Match First Match
Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
580 bits(642)	6e-162	333/335(99%)	2/335(0%)	Plus/Plus

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Sbjct  258697  AGGAGGGAGAATGTTGAAACACAAGCGCCAGAGAGATGATGGGGAGGGCAGGGGTGAAGT  258756

Query  60      GGGGTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGATCAAACG  119
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Sbjct  258757  GGGGTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGATCAAACG  258816

Query  120     CTCTAAGAAGAACAGCCTGGCCTTGTCCCTGACGGCCGACCAGATGGTCAGTGCCTTGTT  179
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Sbjct  258817  CTCTAAGAAGAACAGCCTGGCCTTGTCCCTGACGGCCGACCAGATGGTCAGTGCCTTGTT  258876

Query  180     GGATGCTGAGCCCCCGATACTCTATTCCGAGTATGATCCTACCAAACCCTTCAGTGAAGC  239
               |||||||||||||| |||||||||||||||||||||||||| ||||||||||||||||
Sbjct  258877  GGATGCTGAGCCCCCCATACTCTATTCCGAGTATGATCCTACCAGACCCTTCAGTGAAGC  258936

Query  240     TTCGATGATGGGCTTACTGACCAACCTGGCAGACAGGGAGCTGGTTCACATGATCAACTG  299
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Sbjct  258937  TTCGATGATGGGCTTACTGACCAACCTGGCAGACAGGGAGCTGGTTCACATGATCAACTG  258996

Query  300     GGCGAAGAGGGTGCCAGGTAAGAATGCGAAGCGCA  334
               ||||||||||||||||||||||||||||||||||
Sbjct  258997  GGCGAAGAGGGTGCCAGGTAAGAATGCGAAGCGCA  259031
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Figure (3-15): Alignment of exon 4 in *ESR1* gene sequence of women with breast cancer polymorphism at position 258892 using automated sequencer was analyzed by BLAST data, query number represents the current results while the subject represents the reference sequence. Blue color represents exon region, black color represented intron region while the single nucleotide polymorphism represents red color, C Substitution by G

6- AAA

The sequencing result revealed the presence of SNP G → A (Table 3-5). The identified SNP was a missense mutation, it was substitution polymorphism. The common codon AGA was converted to AAA. This point mutation altered the gene expression because of changing in amino acid; the Arginine was converted to Lysine. This polymorphism was found in 6 (25%) samples; 5 samples from blood and only one samples from frozen tissue and also appeared in the blood sample of the same patient.

Nucleotide sequencing profile for women with breast cancer in comparison with healthy women was shown in Figure (3-16).

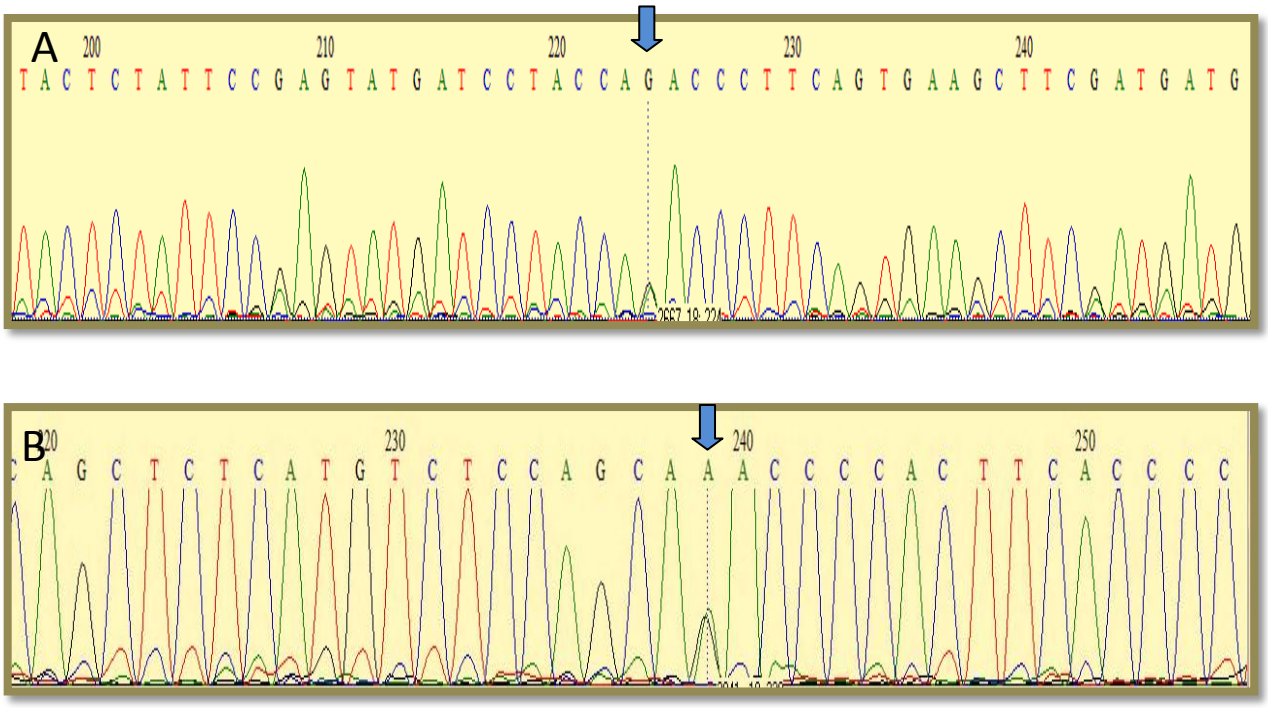

Figure (3.16): Nucleotide sequencing profile of exon 4 in *ESR1* gene polymorphism at position 258921 as edited by Finich TV software. A: healthy women as a control, B: women with breast cancer, G Substitution by A

The alignment of nucleotides sequencing of exon4 in *ESR1* for women with breast cancer was done and compared with control in NCBI center using automated sequencer and analyzed by BLAST data. The query number represents the current results while the subject represents the reference sequence, the blue color refers to exon while the black color represents the intron region around exon as shown in Figure (3-17).

Homo sapiens estrogen receptor 1 (*ESR1*), RefSeqGene on chromosome 6
Sequence ID: ref|NG_008493.2|Length: 419779Number of Matches: 1
Related Information
Map Viewer-aligned genomic context
Range 1: 258700 to 259031GenBankGraphics Next Match Previous Match
Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
574 bits(636)	3e-160	329/332 (99%)	3/332(0%)	Plus/Plus

```
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Sbjct  258698  GGAGGGAGAATGTTGAAACACAAGCGCCAGAGAGATGATGGGGAGGGCAGGGGTGAAGTG  258757

Query  68      GGGTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGATCAAACGC  127
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  258758  GGGTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGATCAAACGC  258817

Query  128     TCTAAGAAGAACAGCCTGGCCTTGTCCCTGACGGCCGACCAGATGGTCAGTGCCTTGTTG  187
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  258818  TCTAAGAAGAACAGCCTGGCCTTGTCCCTGACGGCCGACCAGATGGTCAGTGCCTTGTTG  258877

Query  188     GATGCTGAGCCCCCCATACTCTATTCCGAGTATGATCCTACCAAACCCTTCAGTGAAGCT  247
               ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  258878  GATGCTGAGCCCCCCATACTCTATTCCGAGTATGATCCTACCAGACCCTTCAGTGAAGCT  258937

Query  248     TCGATGATGGGCTTACTGACCAACCTGGCAAACAGGGAGCTGGTTCACATGATCAACTGG  307
               |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  258938  TCGATGATGGGCTTACTGACCAACCTGGCAGACAGGGAGCTGGTTCACATGATCAACTGG  258997

Query  308     GCGAAGAGGGTGCCAGGTAAGAATGCGAAGCGCA  341
               |||||||||||||||||||||||||||||||||
Sbjct  258998  GCGAAGAGGGTGCCAGGTAAGAATGCGAAGCGCA  259031
```

Figure (3-17): Alignment of exon 4 in *ESR1* gene sequence of women with breast cancer polymorphism at position 258921 using automated sequencer was analyzed by BLAST data, query number represents the current results while the subject represents the reference sequence. Blue color represents exon region, black color represented intron region while the single nucleotide polymorphism represents red color, G Substitution by A

7- AAC

The sequencing result revealed the presence of SNP G →A (Table 3-5). The identified SNP was missense mutation, it was substitution polymorphism. The common codon GAC was converted to AAG. This point mutation caused alteration in gene expression because of changing in amino acid; the Aspartic acid was converted Asparagine. This polymorphism was found in 6 (25%) samples, 5 samples from blood and one sample from frozen tissue as well it appeared in the blood sample from the same patient.

Figure (3-18) showed nucleotide sequencing profile for women with breast cancer compared with healthy women.

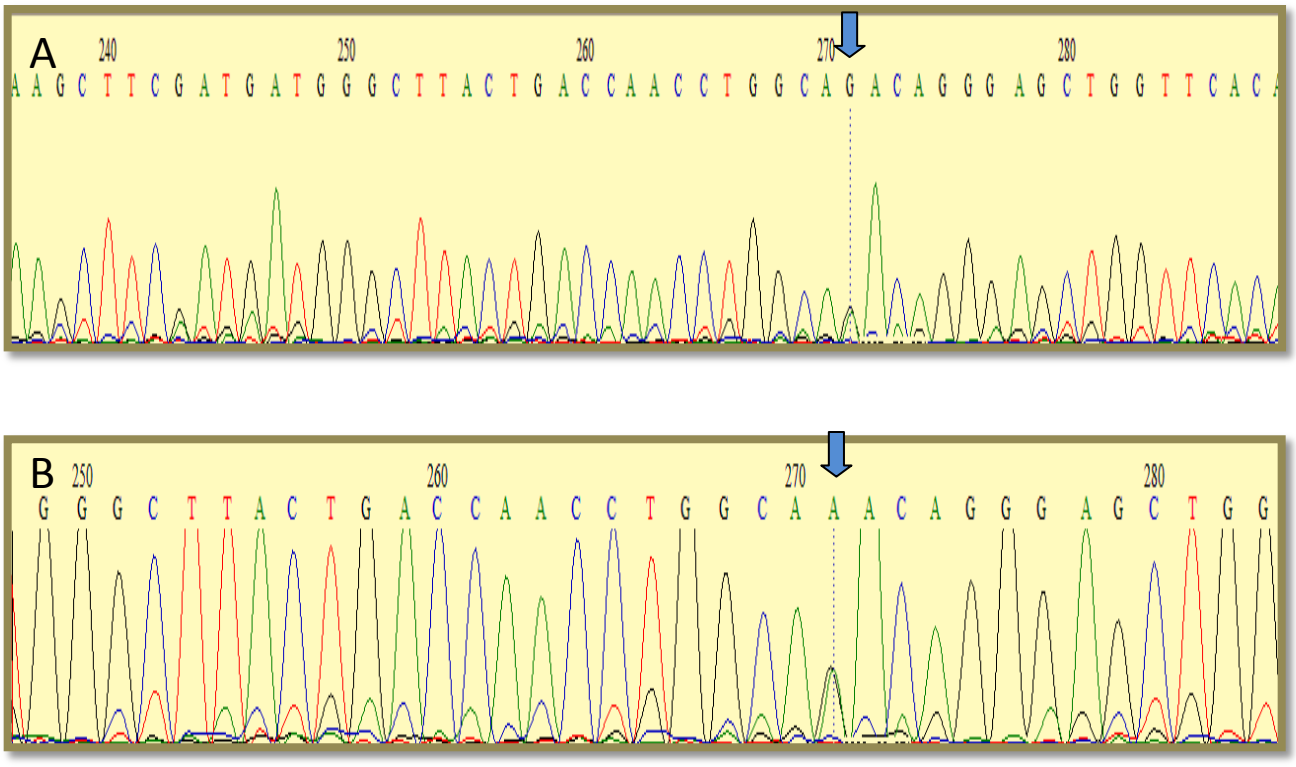

Figure (3-18): Nucleotide sequencing profile of exon 4 in *ESR1* gene polymorphism at position 258977 as edited by Finich TV software. A: healthy women as a control, B: women with breast cancer, G Substitution by A

Then an alignment of nucleotides sequencing of exon 4 in *ESR1* for women with breast cancer compared with control in NCBI center using automated sequencer and analyzed by BLAST data. The query number represents the current results while the subject represents the reference sequence, the blue color refers to exon while the black color represents the intron region around exon as shown in Figure (3-19).

Homo sapiens estrogen receptor 1 (*ESR1*), RefSeqGene on chromosome 6
Sequence ID: ref|NG_008493.2|Length: 419779Number of Matches: 1
Related Information
Map Viewer-aligned genomic context
Range 1: 258700 to 259031GenBankGraphics Next Match Previous Match
Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
574 bits(636)	3e-160	329/332 (99%)	3/332(0%)	Plus/Plus

```
Query  8       GGAGGGAGAATGTTGAAACACAAGCGCCAGAGAGATGATGGGAAGGGCAGGGGTGAAGTG  67
               ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  258698  GGAGGGAGAATGTTGAAACACAAGCGCCAGAGAGATGATGGGGAGGGCAGGGGTGAAGTG  258757

Query  68      GGGTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGATCAAACGC  127
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  258758  GGGTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGATCAAACGC  258817

Query  128     TCTAAGAAGAACAGCCTGGCCTTGTCCCTGACGGCCGACCAGATGGTCAGTGCCTTGTTG  187
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  258818  TCTAAGAAGAACAGCCTGGCCTTGTCCCTGACGGCCGACCAGATGGTCAGTGCCTTGTTG  258877

Query  188     GATGCTGAGCCCCCCATACTCTATTCCGAGTATGATCCTACCAAACCCTTCAGTGAAGCT  247
               ||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  258878  GATGCTGAGCCCCCCATACTCTATTCCGAGTATGATCCTACCAGACCCTTCAGTGAAGCT  258937

Query  248     TCGATGATGGGCTTACTGACCAACCTGGCAAACAGGGAGCTGGTTCACATGATCAACTGG  307
               |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  258938  TCGATGATGGGCTTACTGACCAACCTGGCAGACAGGGAGCTGGTTCACATGATCAACTGG  258997

Query  308     GCGAAGAGGGTGCCAGGTAAGAATGCGAAGCGCA  341
               ||||||||||||||||||||||||||||||||||
Sbjct  258998  GCGAAGAGGGTGCCAGGTAAGAATGCGAAGCGCA  259031
```

Figure (3-19): Alignment of exon 4 in *ESR1* gene sequence of women with breast cancer polymorphism at position 258977 using automated sequencer was analyzed by BLAST data, query number represents the current results while the subject represents the reference sequence. Blue color represents exon region, black color represented intron region while the single nucleotide polymorphism represents red color, G Substitution by A

B: Polymorphisms of exon 6 in *ESR1*

The results of nucleotide sequencing profile of exon 6 in *ESR1* showed no polymorphisms in women with breast cancer when compared with healthy women as shown in Figure (3-20).

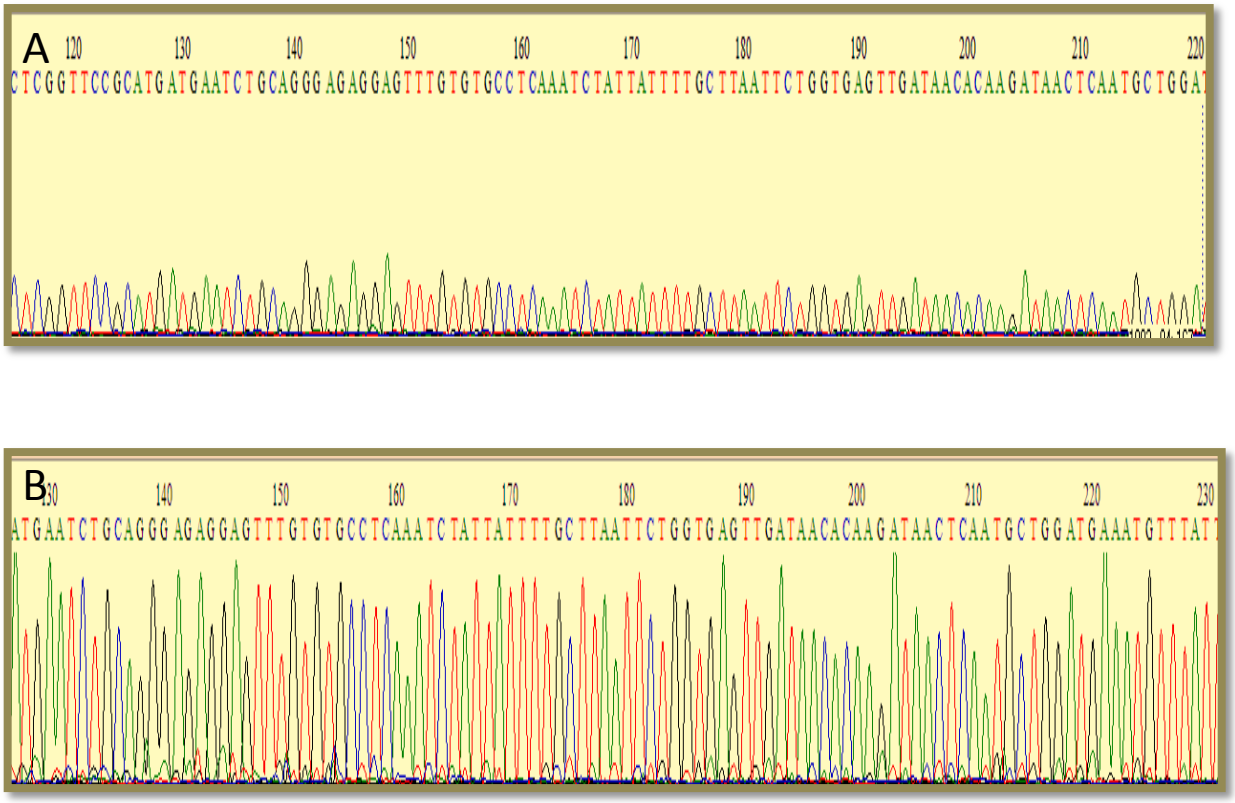

Figure (3-20): Nucleotide sequencing profile of exon 6 in *ESR1* gene as edited by Finich TV software. A: healthy women as a control, B: women with breast cancer

Then nucleotides sequencing of exon 6 in *ESR1* for women with breast cancer were aligned and compared with control in NCBI center using automated sequencer and analyzed by BLAST data. The query number represents the current results while the subject represents the reference sequence, the blue color refers to exon while the black color represents the intron region around exon as shown in Figure (3.21).

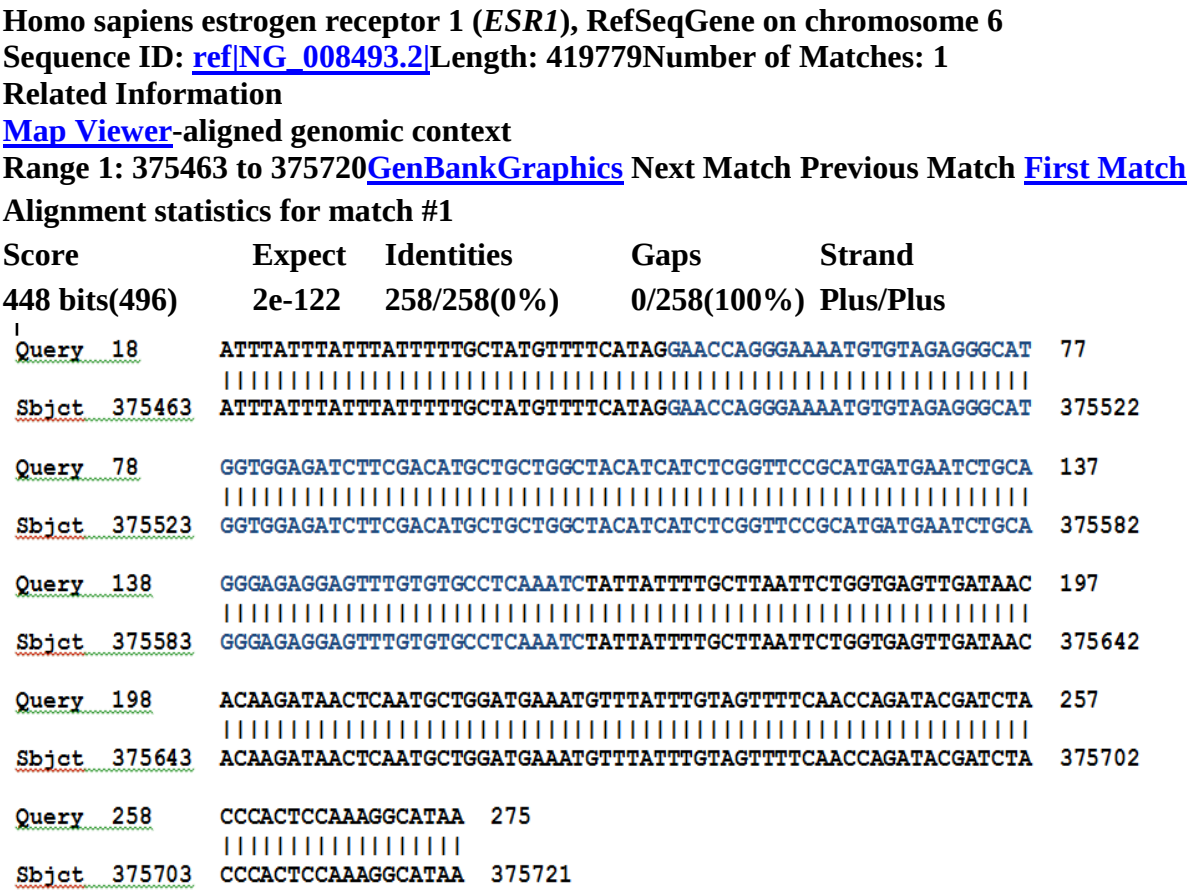

Figure (3-21): Alignment of exon 6 in *ESR1* gene sequence of women with breast cancer using automated sequencer was analyzed by BLAST data, query number represents the current results while the subject represents the reference sequence, blue color represents exon region, black color represented intron region

Notably, two intronic polymorphisms of estrogen receptor alpha were recorded in the flaking region around exon 6 as shown in Table (3-5).

Table (3-6): Polymorphisms in intronic reign flaking exon 6 of estrogen receptor alpha

No.	Polymorphism	Type of polymorphism	Position	No. of patients
1	A ⟶ C	substitution	A 375455 C	1
2	G ⟶ T	substitution	G 375735 T	4

- **Sequences and alignment of each polymorphism in exon6**

1- A 375455 C

The sequencing result displayed the presence of SNP A ⟶C (Table 3-6). This kind of SNP was found at position 375455 on *Homo sapiens* estrogen receptor alpha (*ESR1*) gene, the reference sequence gene on chromosome 6, and polymorphism type was substitution. This polymorphism was found in one (4.1%) sample of FFBE.

The nucleotide sequencing profile for women with breast cancer was compared with healthy women (Fig. 3-22).

An alignment of nucleotides sequencing of exon 6 in *ESR1* for women with breast cancer compared with control in NCBI center using automated sequencer and analyzed by BLAST data. The query number represents the current results while the subject represents the reference sequence, the blue color refers to exon while the black color represents the intron region around exon as shown in Figure (3-23).

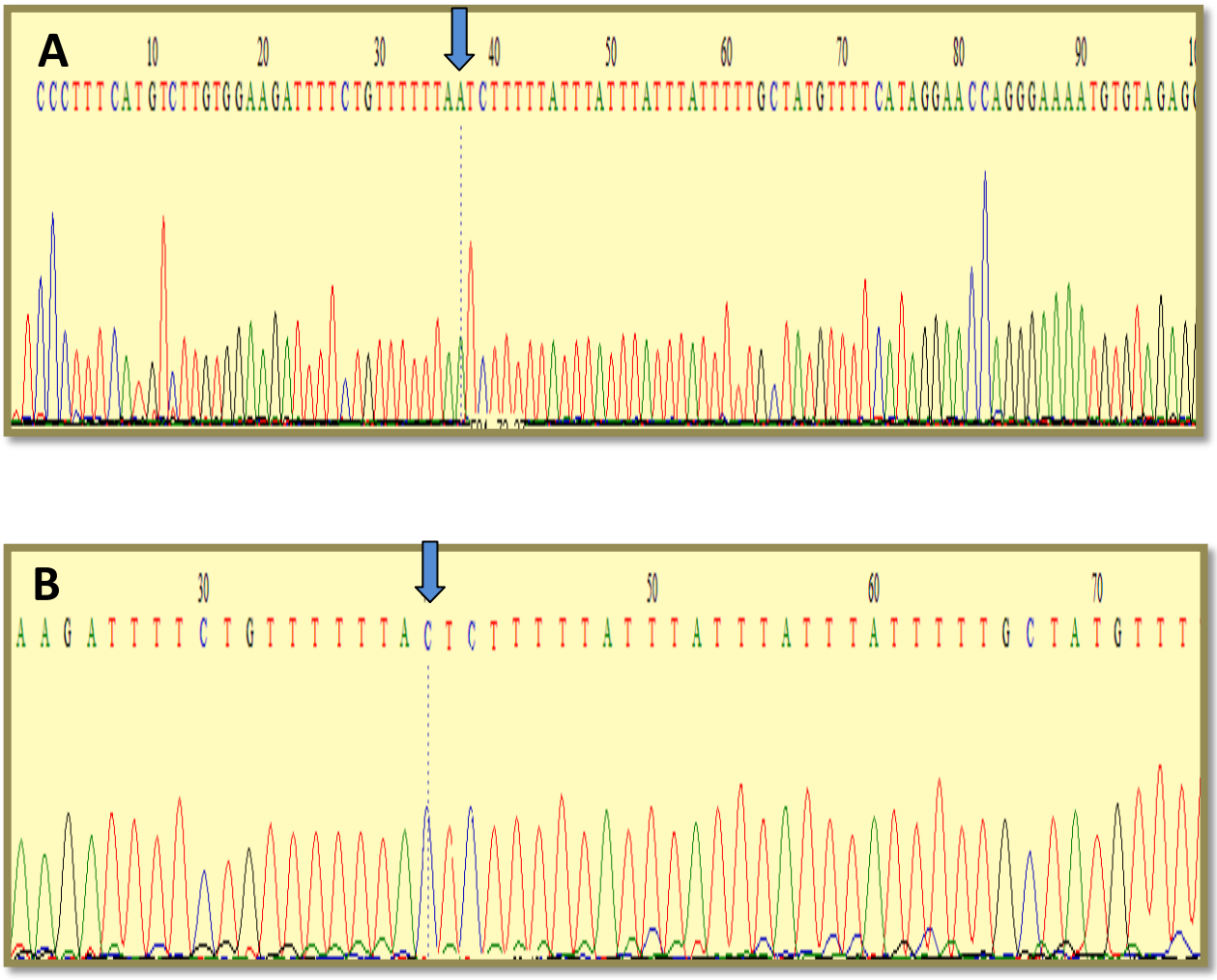

Figure (3-22): Nucleotide sequencing profile of exon 6 in *ESR1* gene polymorphism at position 375455as edited by Finich TV software. A: healthy women as a control, B: women with breast cancer, A substituted by C

Homo sapiens estrogen receptor 1 (*ESR1*), RefSeqGene on chromosome 6
Sequence ID: ref|NG_008493.2|Length: 419779Number of Matches: 1
Related Information
Map Viewer-aligned genomic context
Range 1: 375419 to 375720GenBankGraphics Next Match Previous Match First Match
Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
549 bits(297)	1e-152	301/302(99%)	1/302(0%)	Plus/Plus

Figure (3-23): Alignment of exon 6 in *ESR1* gene sequence of women with breast cancer polymorphism at position 375455 using automated sequencer was analyzed by BLAST data, query number represents the current results while the subject represents the reference sequence. Blue color represents exon region, black color represented intron region while the single nucleotide polymorphism represents red color, A substituted by C

2- G 375735 T

The sequencing result revealed the presence of SNP G →T (Table 3-6). This kind of SNP found at position 375718 on *Homo sapiens* estrogen receptor alpha (*ESR1*), the reference sequence gene on chromosome 6, and the type of this polymorphism was substitution. This polymorphism was found in 4 (16.6%) samples; one sample from blood, two samples from FFBE and one sample from frozen tissue and blood sample from the same patient.

Figure (3-24) showed nucleotide sequencing profile for women with breast cancer compared with healthy women.

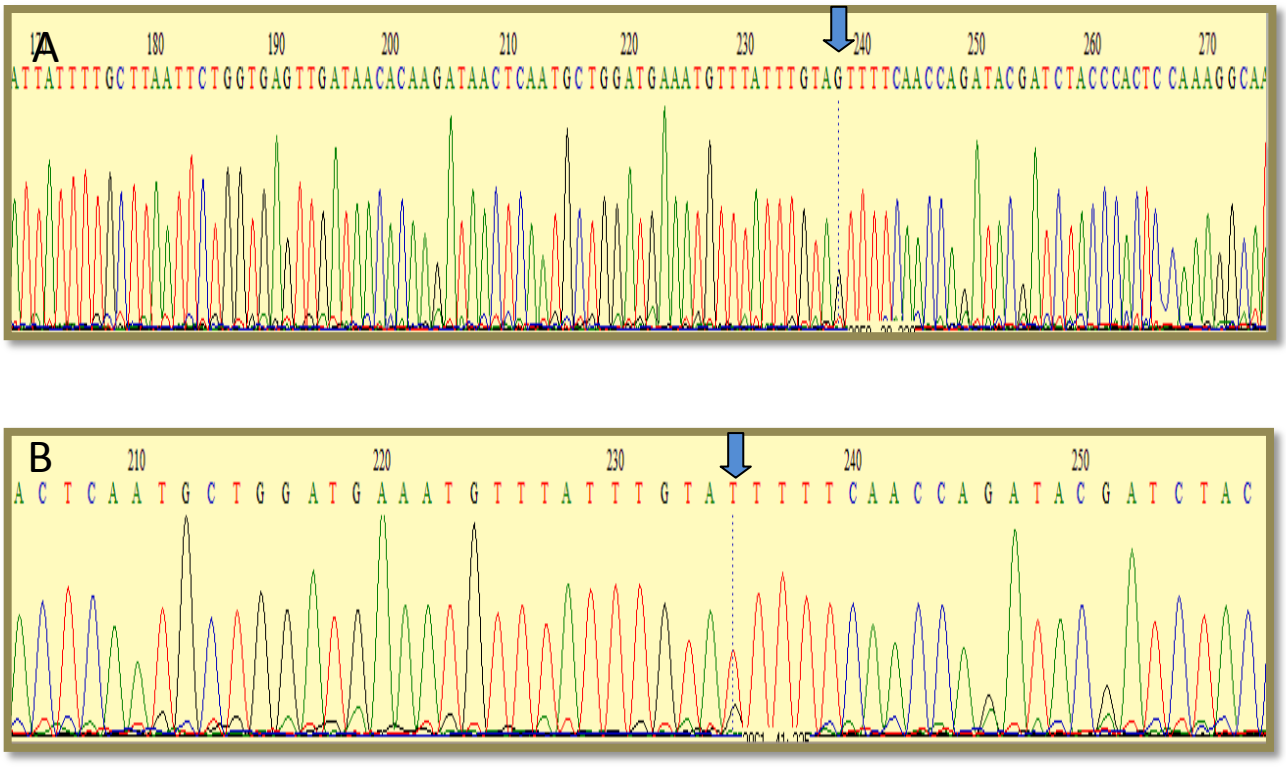

Figure (3-24): Nucleotide sequencing profile of exon 6 in *ESR1* gene polymorphism at position 375735 as edited using Finich TV software. A: control sample (healthy women), B: women with breast cancer, G substituted by T

The alignment of nucleotides sequencing of exon 6 in *ESR1* for women with breast cancer was done and compared with control in NCBI center using automated sequencer and analyzed by BLAST data. The query number represents the current results while the subject represents the reference sequence, the blue color refers to exon while the black color represents the intron region around exon as shown in Figure (3-25).

Homo sapiens estrogen receptor 1 (*ESR1*), RefSeqGene on chromosome 6
Sequence ID: ref|NG_008493.2|Length: 419779Number of Matches: 1
Related Information
Map Viewer-aligned genomic context
Range 1: 375462 to 375718GenBankGraphics Next Match Previous Match First Match
Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
470 bits(254)	7e-129	256/257(99%)	1/257(0%)	Plus/Plus

```
Query  16      TATTTATTTATTTATTTTTGCTATGTTTTCATAGGAACCAGGGAAAATGTGTAGAGGGCA  75
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  375462  TATTTATTTATTTATTTTTGCTATGTTTTCATAGGAACCAGGGAAAATGTGTAGAGGGCA  375521

Query  76      TGGTGGAGATCTTCGACATGCTGCTGGCTACATCATCTCGGTTCCGCATGATGAATCTGC  135
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  375522  TGGTGGAGATCTTCGACATGCTGCTGGCTACATCATCTCGGTTCCGCATGATGAATCTGC  375581

Query  136     AGGGAGAGGAGTTTGTGTGCCTCAAATCTATTATTTTGCTTAATTCTGGTGAGTTGATAA  195
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  375582  AGGGAGAGGAGTTTGTGTGCCTCAAATCTATTATTTTGCTTAATTCTGGTGAGTTGATAA  375641

Query  196     CACAAGATAACTCAATGCTGGATGAAATGTTTATTTGTATTTTTCAACCAGATACGATCT  255
               |||||||||||||||||||||||||||||||||||  ||||||||||||||||||||
Sbjct  375642  CACAAGATAACTCAATGCTGGATGAAATGTTTATTTGTAGTTTTCAACCAGATACGATCT  375701

Query  256     ACCCACTCCAAAGGCAT  272
               |||||||||||||||||
Sbjct  375702  ACCCACTCCAAAGGCAT  375718
```

Figure (3-25): Alignment of exon 6 in *ESR1* gene sequence of women with breast cancer using automated sequencer was analyzed by BLAST data, query number represents the current results while the subject represents the reference sequence. Blue color represents exon region, black color represented intron region while the single nucleotide polymorphism represents red color

The mutation and polymorphism of cancer-associated *ESR1* gene found to predict tumor formation and prognosis (Clarke *et al*., 1997). *ERS1* was representing a surrogate marker for predicting breast cancer developing later in life (Abbasi *et al*., 2013). Several functionally important intronic and exonic loci of *ESR1* gene polymorphisms that are associated with breast cancer have been examined (Hsiao *et al*., 2004).

In this study, 7 polymorphisms (AAG, AAA, TTT, AAA, CCG, AAA, and AAC) were detected in exon 4 which are part of DNA binding domain (DBD) of *ESR1* gene. Six of these polymorphisms (AAG, AAA, TTT, AAA, AAA, and AAC) were novel substitution polymorphism, four SNPs of them (AAA, TTT, AAA, and AAC) were missense polymorphism which causing alteration in amino acid while single nucleotide polymorphism AAG and AAA were silent polymorphisms that not caused any alteration in amino acid.

As known, estrogen receptor (ER) activation participated in development and progression of breast cancer because of alteration in pathways of *ESR1* occurred during development of breast cancer and that associated with breast cancer risk and investigation (Abbasi *et al*., 2009). The function of ER was as a hormone dependent transcriptional regulator that plays significant role in breast cancer development (Kang *et al*., 2002; Vasconcelos *et al*., 2002).

Identification of a novel acquired mutation of *ESR1* gene in women with metastatic breast cancer may lead to develop resistance to endocrine treatment. The mutations cause a conformational change, which mimics the conformation of activated ligand-bound receptor that lead to change the ligand-independent activity then result in resistance to endocrine treatment (Merenbakh-Lamin *et al*., 2013).

The relationship between *ESR1* mutations and resistance to endocrine therapy remains to be investigated, however, there was a significant upregulation of estrogen receptor responsive genes in *ESR1* mutations tumors, suggesting that estrogen receptor signaling was active and may play a role in conferring endocrine therapy resistance (Robinson *et al*., 2013). The mutations in *ESR1* may prompt a clinician to change the treatment regimen from an aromatase inhibitor to an anti-estrogen, so women who developed resistance to aromatase inhibitors often responded to anti-estrogen therapy (Ingle *et al*., 2006; Alluri *et al*., 2014). Moreover, the SNPs that determined in this study may effect copy number of *ESR1* gene and may cause resistant to treatment because amplification was an abnormal status and normal ER protein expression (ER +ve) was requisite for response to treatment (Nielsen *et al*., 2011; Markiewicz *et al*., 2013).

In addition, CCG polymorphism was detected in exon 4 of *ESR1*; it was silent polymorphism and did not alter amino acid. The same result was register in Portugal, Sudan, Iranian women with breast cancer (Vasconcelos *et al*., 2002; Siddig *et al*., 2008; Azimi and Abbasi, 2009). The correlation between silent polymorphisms and phenotypes was not well understood. One of the possibilities is that this silent polymorphism may have an association with another polymorphism that directly influences breast cancer phenotype. Another explanation is that nucleotide structure at the silent polymorphic site may change the level of gene expression for *ESR1*, and this may lead to the association of lymph node metastasis in breast cancer (Azimi and Abbasi, 2009). In contrast no mutation in exon 4 of *ESR1* had seen in German women with breast cancer (Babayan *et al*., 2013).

Whereas a point mutation A908G (Lys303→Arg) was detected in exon 4 of estrogen receptor alpha (*ESR*1) in Iranian and Japanese women (Abbasi *et al*.,

2013; Zheng *et al*., 2003). The somatic mutation may increase sensitivity to estrogen and this may lead to increasing of proliferation at subphysiological level of estrogen and stimulated binding to transcription factor2 at low level of hormone (Zheng *et al*., 2003). While other studies (Southey *et al*., 1998; Kang *et al*., 2002) were shown no association between *ESR1* polymorphism and breast cancer. This may due to the small size of samples or chose only few SNPs.

Regarding polymorphisms in exon 6 of *ESR1*, the current result revealed no polymorphism was seen. This result was similar to that has been done by Babayan *et al*. (2013) on German women with breast cancer. On the other hand, the current results showed two novel polymorphisms, A 375455 C and G 375718 T, in the intronic region flaking exon 6 of *ESR1* gene.

Polymorphisms in non-coding region are potential regulatory polymorphisms, which including promoter/upstream, downstream and intron regions, which may affect transcription. In intron and untranslated regions transcribed as RNA led to affect transcription, stability and translation RNA splicing; or unknown function in intergenic regions (Liao and Lee, 2010; Sadee *et al*., 2011; Albert, 2011).

The spliceosome as a part of the regulatory elements is known to guide the splicing complex and RNA binding proteins to correctly localize the intron/exon sites. Mutations in regulatory elements lead to de-regulation of splicing and alternative splicing within the affected gene, also it may effect transacting factors activities which components of the splicing machinery. Mutations in splicing mechanism may either cause disease directly or contribute to the susceptibility or severity of the disease (Ward and Cooper, 2010).

3.3.3-2. Polymorphisms in estrogen receptor beta (*ESR2*)

A: polymorphisms of exon 3 in *ESR2*

The results of nucleotide sequencing profile of exon 3 in *ESR2* showed no polymorphisms in women with breast cancer when compared with healthy women (Fig. 3-26).

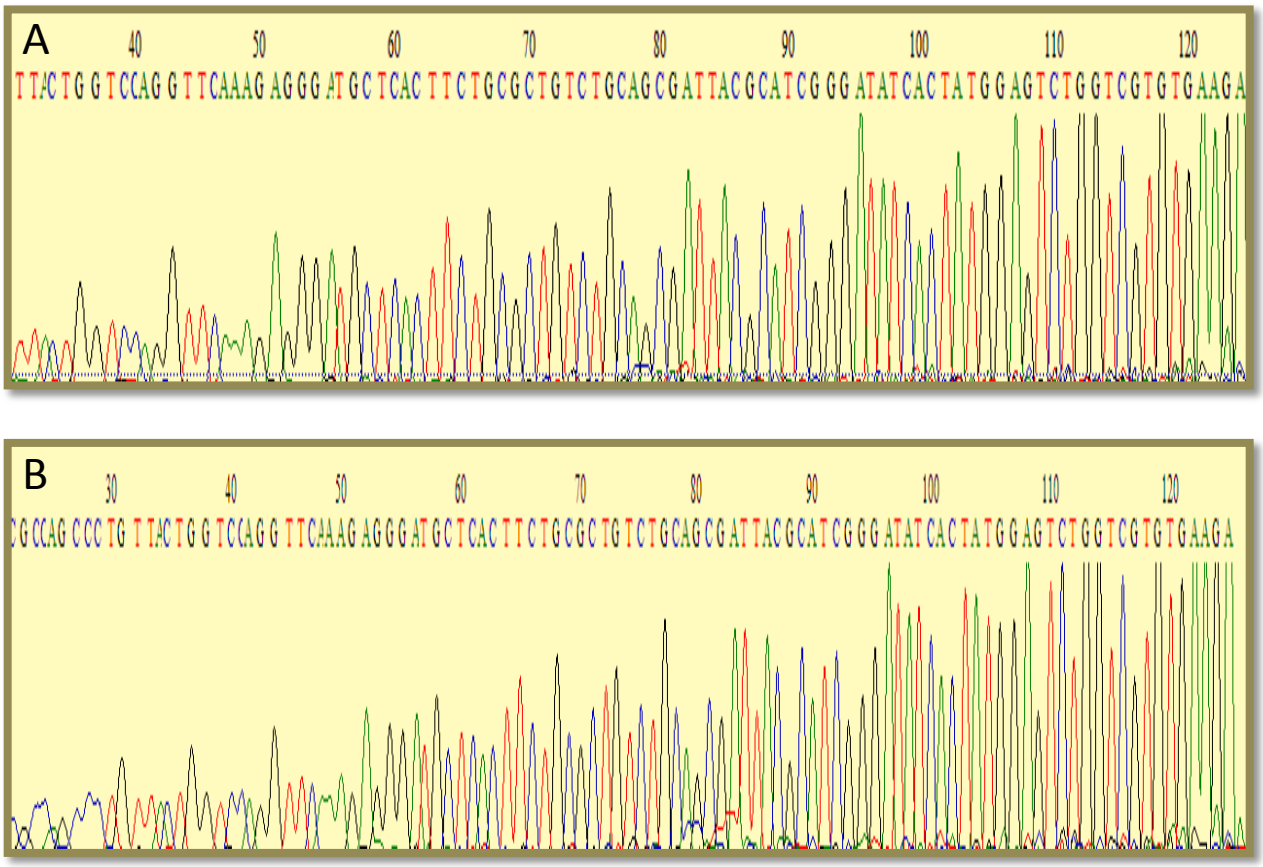

Figure (3-26): Nucleotide sequencing profile of exon 3 in *ESR2* gene as edited by Finich TV software. A: healthy women as a control, B: women with breast cancer

An alignment of nucleotides sequencing of exon 3 in *ESR2* for women with breast cancer was done and compared with control in NCBI center using automated sequencer and analyzed by BLAST data. The query number represented the current results while the subject represented the reference sequence; the blue color refers to exon while the black color represents the intron region around exon as shown in Figure (3-27).

Homo sapiens estrogen receptor 2 (ER beta) (ESR2), RefSeqGene on chromosome 14
Sequence ID: ref|NG_011535.1|Length: 118518Number of Matches: 1
Related Information
Map Viewer-aligned genomic context
Range 1: 63415 to 63537GenBankGraphics Next Match Previous Match First Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
217 bits(117)	4e-53	124/124(0%)	0/124(100%)	Plus/Plus

```
Query  2      AAGGTTAGTGGGAACCGTTGCGCCAGCCCTGTTACTGGTCCAGGTTCAAAGAGGGATGCT  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  63415  AAGGTTAGTGGGAACCGTTGCGCCAGCCCTGTTACTGGTCCAGGTTCAAAGAGGGATGCT  63473

Query  61     CACTTCTGCGCTGTCTGCAGCGATTACGCATCGGGATATCACTATGGAGTCTGGTCGTGT  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  63474  CACTTCTGCGCTGTCTGCAGCGATTACGCATCGGGATATCACTATGGAGTCTGGTCGTGT  63533

Query  121    GAAG  124
              ||||
Sbjct  63534  GAAG  63537
```

Figure (3-27): Alignment of exon 3 in *ESR2* gene sequence of women with breast cancer using automated sequencer was analyzed by BLAST data, query number represents the current results while the subject represents the reference sequence. Blue color represents exon region, black color represented intron region

B: Polymorphisms in exon 7 of *ESR2*

For exon 7 in *ESR2*, three polymorphisms (ACT, AGG and GCA) were detected. The type of polymorphism, position and their effect on gene expression were demonstrated in Table (3-7). The types of polymorphisms in exon 7 of *ESR2* were two deletion mutations and one substitution polymorphism. These mutations either caused reregulation of gene expression and in this case it called missense mutation, or no change in gene expression and this called silent polymorphism (sense mutation).

Table (3-7): Polymorphisms in exon 7 of *ESR*2 gene in women with breast cancer

No.	Polymorphism	Type	Position	Wild type codon	Mutated codon	Chang of amino acid*	Effect on translation	Kind of mutation	No. Of patient
1	Missing A	Deletion	93927	A-C	ACT	N → T	Missense mutation	Point mutation	1
2	Missing A	Deletion	93936	A-G	AGG	K → R	Missense mutation	Point mutation	6
3	T → A	Substit-ution	93919	GCT	GCA	A→ A	Silent mutation	Point mutation	1

*** N: Asparagine, T: Threonine, K: Lysine, R: Arginine, A: Alanine**

The sequences of exon 7 in *ESR2* gene were aligned with control group (healthy women) and compared with NCBI as shown below (Fig. 3-28, 29, 30, 31), also the nucleotide sequence profile of control group (healthy women) were aligned with NCBI was shown in appendix- 3.

- **Sequences and alignment of mutations in exon7 of *ESR2***

1- ACT and AGG

The sequencing result of exon 7 in *ESR2* gene in Table (3-7) showed two deletion mutations (missense mutation). In the first polymorphism, the common codon AAC was converted to ACT, this type of mutation was affected gene expression because of an alteration in amino acid was occurred; Asparagine was converted to Threonine. This kind of mutation found in 1 (4.1%) sample of blood.

While in the second polymorphisms, the common codon AAG was converted to AGG. This polymorphism caused changing in gene expression because of conversion of amino acid; Lysine was changed to Arginine. This kind of mutation appeared in 6 (25%) samples; 3 samples from blood and the other samples from FFPE.

Then the nucleotide sequencing profile for women with breast cancer compared with control group (Fig. 3-28) .

An alignment of nucleotides sequencing of exon 7 in *ESR2* for women with breast cancer was done and compared with control in NCBI center using automated sequencer and analyzed by BLAST data. The query number represented the current results while the subject represented the reference sequence, the blue color refers to exon while the black color represents the intron region around exon as shown in Figure (3-29).

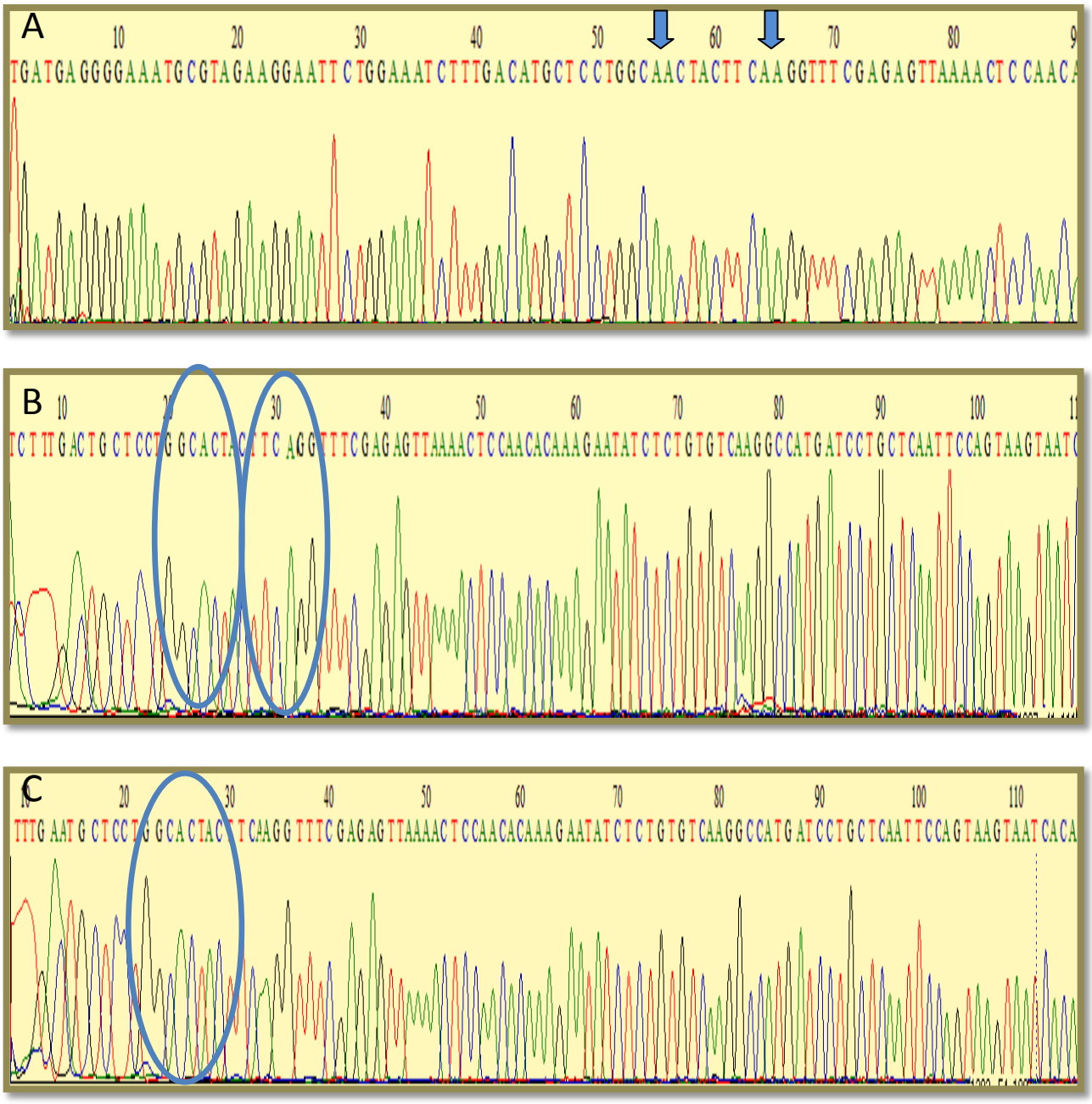

Figure (3-28): Nucleotide sequencing profile of exon 7 in *ESR2* gene there is two deletion mutations the first one at position 93927 and the second at position 93936 as edited by Finich TV software. A: healthy women as a control, B and C: women with breast cancer

Homo sapiens estrogen receptor 2 (ER beta) (*ESR2*), RefSeqGene on chromosome 14
Sequence ID: ref|NG_011535.1|Length: 118518Number of Matches: 1
Related Information
Map Viewer-aligned genomic context
Range 1: 93905 to 94028GenBankGraphics Next Match Previous Match First Match
Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
199 bits(220)	7e-48	122/124 (98%)	2/124(1%)	Plus/Plus

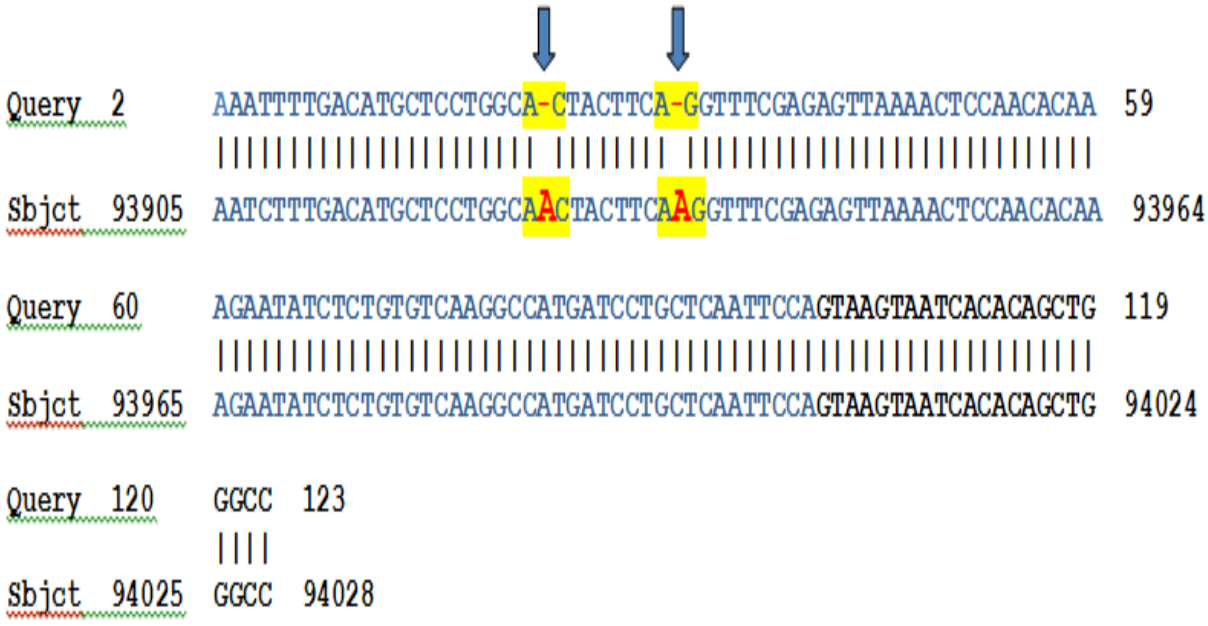

Figure (3-29): Alignment of exon 7 in *ESR2* gene sequence of women with breast cancer, there is two deletion mutations the first one at position 93927 and the second at position 93936 using automated sequencer was analyzed by BLAST data, query number represents the current results while the subject represents the reference sequence. Blue color represents exon region, black color represented intron region while the single nucleotide polymorphism represents red color

2-GCA

The sequencing result revealed the presence of SNP T → G (Table 3-7). The identified SNP was a silent polymorphism (sense mutation), it was substitution mutation. The common codon GCT was converted to GCA. This point mutation had no affect on gene expression in which the altered codons still encode the same amino acid, Alanine. This polymorphism found in one (4.1%) sample of blood.

Figure (3-30) showed nucleotide sequencing profile for women with breast cancer that compared with control of healthy women.

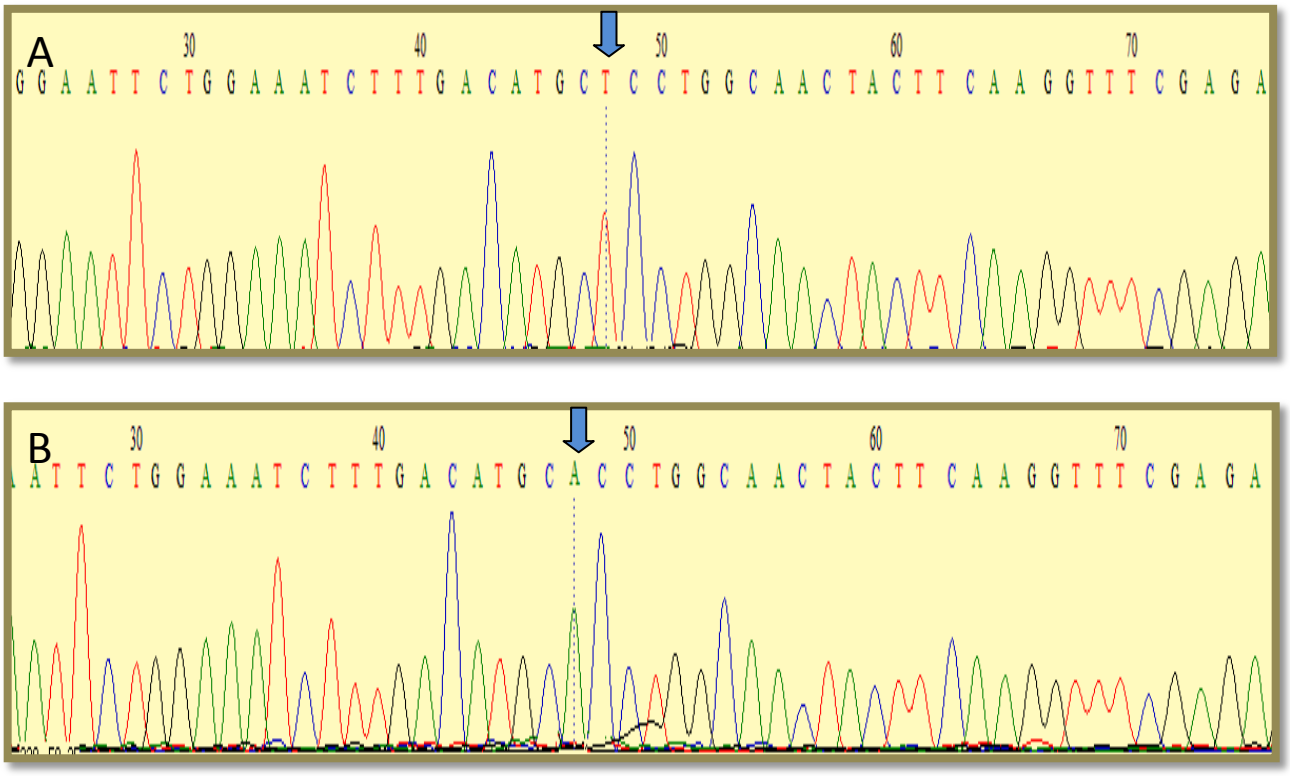

Figure (3-30): Nucleotide sequencing profile of exon 7 in *ESR2* gene polymorphism at position 93919 as edited by Finich TV software. A: healthy women as a control, B: women with breast cancer, which T substituted by A

Then an alignment of nucleotides sequencing of exon 7 in *ESR2* for women with breast cancer was done and compared with control in NCBI center using automated sequencer and analyzed by BLAST data. The query number represents the current results while the subject represents the reference sequence, the blue color refers to exon while the black color represents the intron region around exon as shown in Figure (3-31).

Homo sapiens estrogen receptor 2 (ER beta) (*ESR2*), RefSeqGene on chromosome 14
Sequence ID: ref|NG_011535.1|Length: 118518Number of Matches: 1
Related Information
Map Viewer-aligned genomic context
Range 1: 93873 to 93988GenBankGraphics Next Match Previous Match First Match
Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
202 bits(109)	1e-48	115/116(99%)	1/116(0%)	Plus/Plus

Figure (3-31): Alignment of exon 7 in *ESR2* gene sequence of women with breast cancer polymorphism at position 93919 using automated sequencer was analyzed by BLAST data, query number represents the current results while the subject represents the reference sequence. Blue color represents exon region, black color represented intron region while the single nucleotide polymorphism represents red color, which T substituted by A

From the result above, it was found that Iraqi women had no mutations in exon 3 of *ESR2*. This result was similar to that reported in Iranian population (Abbasi *et al*., 2009).

On the other hand, this study was detected three novel mutations (ACT, AGG and GCA) in exon 7 of *ESR2* gene. Concerning, the two single nucleotide polymorphisms ACT and AGG were deletion mutations that lead to change amino acid produced. This polymorphism caused a frame shift in the translational region. Frame shift changes had a higher effect on the polypeptide than missense or nonsense mutations. In the latter only one amino acid changes, frame shift causes a change in all amino acids of a certain gene. In addition, this type of genetic difference leaded to variation in copy number. Variation in gene copy number might influence the activity of genes and body functions lead to developing many diseases in human body and resist to drugs (Genetics Home Reference, 2016). The changing changes in reading frame and copy number could affect gene expression that associated with breast cancer risk.

On the other hand, the single nucleotide polymorphism GCA was substitution variation. It was silent polymorphism that not changes the gene expression. In chines women, it was recorded another silent polymorphism C (33390) G L 392 L by using direct sequencing of the *ESR2* gene (Zheng *et al*., 2003). The same result was noticed in Iranian women.

Indeed, there was positive association between breast cancer risk and single nucleotide polymorphisms (SNPs) in exon 7. Although it was a silent synonymous change, it may inactivate genes by inducing the splicing machinery to skip the exons (Cartegni *et al*., 2002; Fackenthal *et al*., 2002). In fact, the SNP located in

an exonic splicing enhancer motif and may affect the accuracy and efficiency of *ESR2* pre-mRNA splicing.

Abbasi (2010) suggested that polymorphism was associated with different factors of breast cancer and lymph node metastasis. The link between the silent polymorphism and phenotypes was unclear, but there was a link between silent polymorphism and an extra mutation that contribute to breast cancer, or the nucleotide structure of silent polymorphism site could change the level of gene expression of *ESR*2 that may contribute to the metastasis to lymph node in this disease. The relation between variation/mutation and lymph node metastasis deserves further study clinical indicator prior to surgical evaluation because lymphatic metastasis was linked to disease localization and progression, and lymph node metastasis is considered an important marker for tumor therapy (Fisher *et al*., 1993; Goldhirsch *et al*., 1995).

However, *ESR2* contribution to the risk of breast cancer risk has to be further examined. However, the role of *ESR2* variants in breast cancer may work together with other genes in certain types of breast cancer and in certain populations. Future studies need to examine the sequence of *ESR2* variants (Haldosen *et al*., 2014).

Single nucleotide polymorphisms (SNPs) considered the most common form of genetic variation that affected the way a person responded to the environment factor which may modify disease risk. In this study, non-synonymous SNPs in exon 4 of *ESR1* gene (AAA, AAC, AAA, TTT) and two deletion mutations in exon 7 of *ESR2* ACT and AGG were detected within the regulatory region of the genome coding to different amino acid. Although large number of SNPs were shown to have little or no effect on gene regulation and protein activity, other mutations where base changes may have deleterious effects. The non-synonymous SNPs changing amino acid in proteins studied because of their influence on

protein activity. If SNPs within regulatory regions of the gene, it caused dis-regulation of gene transcription and may change the structure folds of mRNA lead to influence gene expression (Brian *et al*., 2008; Aurekha *et al*., 2009).

As well as, in the present study, it has been found silent polymorphisms (synonymous) in different locations in exon 4 of *ESR1* gene (AAG, CCG and AAA) and in exon 7of *ESR2* gene (GCA). The studied polymorphism was a base-pair exchange in the third codon position central to the *ESR1* gene that does not alter the encoded amino acid (synonymous). This polymorphism could be regarded as a marker, potentiality in linkage disequilibrium with another functional locus (Wedre'n *et al*., 2004). It was reported that this polymorphism may indirectly affect the protein function through alteration of the RNA half-life or protein translation, hence indirectly affecting the level of ESR1 protein (Herynk and Fuqua, 2004).

Additionally study from the National Cancer Institute found that silent a SNP affected protein function because of enforcing the cell for reading a different DNA codon which it already does, while the aforesaid protein sequence eventually was made. Thus, silent change in an altered protein conformation leads to slow folding rhythm resulting, which functions in turn influence. These non-concordances provide an appraisement of the prevalence of allelic imbalance (AI) in normal breast tissue. Although AI may not be a cancer precursor, the frequency of AI in these cancer patients may be a demonstration of an aberrant ongoing malignant transformation process in an individual's breast tissue (Li *et al*., 2002; Braakhuis *et al*., 2003; Larson *et al*., 2005).

Conclusions and Recommendations

Conclusions

From the results of this study, it can be concluded:

1. An investigation of estrogen receptor protein expression in women could be a prognostic factor for developing mammary tumors.
2. ER protein overexpression correlated with the presence of ER mutations and accumulation as determined by DNA sequencing and immunohistochemistry.
3. There were several mutations in *ESR1* and *ESR2* genes, some of them were silent mutations and others were missense mutations which may lead to effect gene expression and cause resistance to endocrine therapy.
4. Entronic polymorphism in *ESR1* gene may effect the regulation of gene expression.
5. Sequencing is a reliable tool to follow up *ESR1* and *ESR2* gene status in breast cancer which is useful for prediction response to chemotherapy.
6. No differences in the type of mutations were detected in blood and frozen samples collected from patients.
7. DNA can be successfully extracted from blood and frozen tissue samples with high purity and quality, while FFPE samples are not favorable for DNA extraction, and may give false positive or negative results because of processing for preparation FFPE samples.

Recommendations

In the light of the conclusions down, it can be recommended:

1. Study the role of methylation in *ESR1* and *ESR2* in women with breast cancer.
2. Using micro RNA for regulation the expression of genes related with breast cancer.
3. Study the effect of novel mutations that detected in exon 4 of *ESR1* and exon 7 of *ESR2* as well as mutation in intronic region flanking exon 6 on developing of breast cancer.

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الخلاصه

هدفت هذه الدراسة إلى تحديد الطفرات والنوكليوتيدات المفردة المتعدة الاشكال في اكسون 4 و 6 لجين مستقبلات الاستروجين ألفا (*ESR1*) بالاضافه الى اكسون 3 و 7 لجين مستقبلات الاستروجين بيتا (*ESR2*) لنساء مصابات بمرض سرطان الثدي في العراق.

جمعت عينات مختلفه (الدم، النسيج مجمد مع الدم من المريض نفسه، والنسيج المطمور في شمع البارافين، FFPE) خلال الفتره الاول من نيسان الى الاول من ايلول 2014.

فحص التعبير المناعي (Immunoexpression) لبروتين مستقبلات الاستروجين (ER) في 50 عينه لنساء مصابات بسرطان الثدي باستخدام تقنية المناعية Immunohistochimstry, و لوحظ وجود صبغة مناعية موجبة لمستقبلات الاستروجين في 35 (70٪) من النساء المصابات بسرطان الثدي. وعلاوة على ذلك، تم دراسه العلاقه بين مستقبلات الاستروجين و عدد من عوامل الخطوره كالعمر,تاريخ العائله, انقطاع الطمث, و سرطان العقد المفاويه. حيث لوحظ وجود فروق معنويه بين التعبير المناعي لمستقبلات الاستروجين في النساء المصابات بسرطان الثدي وبعض عوامل الخطوره مثل (العمر, التاريخ العائلي, و سرطان العقد المفاويه) في حين لم تظهر اي فرق معنوي لتعبير مستقبلات الاستروجين مع انقطاع الطمث.

درس التحليل الجزيئي لاكسونات 4 و6 في جين مستقبلات الاستروجين الفا (*ESR1*) بالاضافه الى اكسون 3 و7 في جين مستقبلات الاستروجين بيتا (*ESR2*) باستخدام تقنيه تفاعل السلسله المتبلمره (PCR). وقد ظهر اكسون 4 و 6 في جين *ESR1* كحزمه بحجم 370 و300 زوج قاعدي على التوالي، في حين تم الكشف عن اكسون 3 و 7 في جين *ESR2* كحزمه بحجم 151 و 157 زوج قاعدي، على التوالي.

كما وشملت الدراسة على تحديد النيوكليوتيدات المفرده متعدده الاشكال (SNP) في اكسون 4 و 6 في جين *ESR1* واكسون 3 و 7 في جين *ESR2* باستخدام تسلسل الحمض النووي (DNA sequencing). تم محاذاة تسلسل النوكليوتيدات لهذه الإكسونات مع مجموعة السيطرة (امرأة سليمة) وNCBI.

شخصت وجود 7 اكسون في *ESR1* من 4 من النيوكليوتيدات المفردة متعددة الاشكال (AAG, AAA ، AAC،حيث كانت ستة منها (AAA, TTT, AAA, CCG, AAA, and AAC) كانت من النيوكليوتيدات CCG) جديده (اصيله)، في حين AAC and، AAA، AAA، TTT المفردة متعددة الاشكال التي سجلت سابقا في عدد من سكان الدول المختلفه. كانت كل انماط من نوع الاستبدال. بينما لم يتم *ESR1* من 4النيوكليوتيدات المفردة متعددة الاشكال في اكسون ، ومع ذلك لوحظ *ESR1* لجين 6ملاحضه اي النيوكليوتيدات المفرده متعدده الاشكال في اكسون Gو C 375455 Aظهوراثنين من النيوكليوتيدات المفرده متعدده الاشكال,
, في منطقة الانترون حول هذا الاكسون.T 375718

، تبين أنه لم يكن هناك اي نيوكليوتيدات المفردة متعددة الاشكال في اكسون *ESR2* وفيما يتعلق بجين حيث لوحظ وجود ثلاثة من *ESR2* لجين 7. لكن تم الكشف عن ثلاث طفرات جديدة في اكسون 3)، وان اثنين من النيوكليوتيدات GCAوAGG, ACT نيوكليوتيدات المفردة متعددة الأشكال جديدة ( ) AGG و ACT من نوع طفرات الحذف (*ESR2* لجين 7المفرده متعدده الاشكال في اكسون استبدال.GCAوالآخيره

نستنتج من هذه الدراسة وجود اكثر من النيوكليوتيدات واحده مفردة متعددة الاشكال في الاكسون الواحد، بالإضافة الى العديد من النيوكليوتيدات المفردة متعددة الاشكال في الإكسونات المختلفة لنفس الجين.

الأهداء

الى الذين بذلوا ارواحهم الطاهره في سبيل الله وسقوا ارض الوطن بدمائهم الزكيه..... شهداء العراق الشرفاء ...

الى من علمني كيف أتعلم...ومضى على نهله دون ان اوفه ما يستحق.... والدي الغالي....

الي منبع الدفء والحنان .... أمي....

الى مصدر الهامي و قوتي و سندي نصفي الثاني ... زوجي الحبيب... اركان

الى هديه الله سبحانه وتعالى ومنّته, نور عيني ابنتي الغاليه جنّات....

الى نبض قلبي.... ابني الغالي محمد رضا ....

الى استاذي الفاضل صاحب الاخلاق الرفيعه .... د. حميد

الى احبتي اخواني واخواتي....

اليهم جميعا اهدي ثمره جهدي المتواضع.....

سحر

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعةالنهرين
كلية العلوم

الكشف عن الطفرات الموجوده في جين مستقبلات الاستروجين الفا و بيتا لنساء عراقيات مصابات بسرطان الثدي

أطروحة

مقدمة الى مجلس كلية العلوم- جامعة النهرين كجزء من متطلبات نيل

درجة دكتوراه فلسفة في العلوم- تقانة الاحيائية

من قبل

سحر مدحت حسين

بكالوريوس علوم/ تقانة احيائية/ جامعة النهرين/ 2004

ماجستير علوم/ تقانة احيائية/ جامعة النهرين/ 2007

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رمضان 2016

حزيران 1437