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Comparative, Molecular, Immunohistochemical and Tissue Microarray Study on Iraqi and Italian Breast Cancer Patients

A Dissertation

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FAROOQ

Summary

Breast cancer is the most common cancer of women worldwide. It is a heterogeneous disease. The incidence rate of this disease increased in the last years in Iraq. The current study was conducted to study the immunohistochemical and molecular parameters for the breast tumors by using tissue microarray (TMA) technique on Iraqi and Italian patients with breast carcinoma. Histopathological data for the patients were obtained. Formalin-fixed, paraffin-embedded (FFPE) samples were used in this study. Three TMAs recipient blocks were designed and constructed for a first time on Iraqi samples. Fifty-five Iraqi breast cancer cases used as the first TMA block; thirty Italian breast cancer cases were used to construct the second TMA block and fifteen Iraqi benign breast lesions, which used as a control and to construct the third TMA. For TMAs construction, each donor sample were represented in triplicate, cores were transferred from each donor patient sample to the recipient block. Hematoxylin and Eosin (H and E) stained then immunohistochemically (IHC) studied for estrogen receptor (ER), progesterone receptor (PR), Ki67, p53 and HER-2 for the three TMAs. The mean age of the Iraqi breast cancer patients in this study 49.38 ±8.29 years old; ranging from 36-69 years old. The mean age of the Italian patients 57.00 ±8.52; the range 39-73 years old. The mean age of the Iraqi patients was lower than the Italian breast cancer cases used in this study. ER positive expression was found in 26 (47.3%) out of 55 cases of the Iraqi breast cancer cases, In the Italian TMA, 23 (76%) cases out of 30 sample showed positively expressed ER; ER expression in the Iraqi samples were lower than the Italian samples. PR expression was observed in 35 (63.6%) out of 55 samples of the Iraqi breast cancer. For the Italian breast cancer TMA, 23 out of 30 cases had positively expressed PR (76%). Ki67 showed positive expression in 30 out of

55 of the Iraqi breast cancer (54.5%), while in the Italian breast cancer TMA 23 out of 30 cases were positively expressed (76%).

The positive immunohistochemical expression of p53 in the Iraqi malignant breast TMA were observed in 48 out of 55 samples (87.2%). For the Italian TMA, 23 out of 30 samples were positively expressed of p53 (76%).

Overexpression of HER-2 (score 2+ and 3+) was observed in 30 of the Iraqi breast cancer samples (54.5%) and in 16 out of 30 of the Italian breast cancer patients (53.3%).

Correlation between the five markers were studied statistically and the study revealed that there was a significant relationship between the HER-2 expression and expression of ER and p53 (*P* value <0.01), but there was no significant relationship between HER-2 expression with the expression of PR and Ki67 (*P* value >0.01).

Twelve cases out of 55 Iraqi samples were triple negative (negative for ER, PR and HER-2 overexpression) (21.8%), whereas only one case out of 30 cases of the Italian samples was triple negative (3.3%). The results indicated that the Iraqi breast cancer cases used in this study were phenotypicaly more aggressive than the Italian breast cancer samples.

We utilized TMA sections for the validation of HER-2 molecular testing. This study reviewed the validity and reliability of the two most commonly used tests for HER-2 detection: IHC and FISH. Depending on a statistical analysis of studies comparing IHC and FISH, FISH assay revealed that there was amplification in the *HER-2* gene in (88%) of IHC2+ cases, and were amplified in (98.2%) of the IHC3+. For the Italian samples, positive amplification was observed in (81.80%) of the IHC2+, and amplified in (100%) for IHC3+.

DNA extraction was performed successfully from the formalin fixed, paraffin embedded (FFPE) sections. After DNA quantification, only the DNA

samples extracted from the Italian samples were successfully used for polymerase chain reaction (PCR) amplification of *BRCA1* gene exon 11. Agarose gel electrophoresis of the PCR product showed the presence of a band with size of 185 bp, which represents the correct expected band in 24 out of 30 samples used in this study. PCR products were purified, and their DNA sequencing was performed, and analyzed by National Center for Biotechnology Information (NCBI) and the results revealed 4 samples with deletion mutation and 3 samples with point mutation.

In conclusion, the mean age of the Iraqi of the Iraqi breast cancer patients was lower than that of the Italian breast cancer patients. TMA was successfully used as a rapid, economic and effective method for IHC and FISH studies. There were a differences in the ER, PR, Ki67, p53 and HER-2 expression between the Iraqi and the Italian samples. ER and PR expression in the Italian cases were higher than the Iraqi cases which means that the Iraqi breast cancer was more aggressive than the Italian samples and the negative cases not responding to the hormonal therapy. Triple negative cases were higher in the Iraqi samples than the Italian samples. The Iraqi breast cancer samples were phenotypicaly more aggressive than the Italian breast cancer samples. FISH is the gold standard method to assess the response to the therapy for HER-2 positive cases. DNA was successfully extracted from FFPE sections and used for *BRCA1* exon11 to determine the mutations in the sporadic breast cancer cases, the DNA sequencing results and analysis showed 7 mutations in *BRCA1* exon11.

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List of Abbreviations

Ab	Antibody
AJCC	American Joint Committee for Cancer
ASCO	American Society of Clinical Oncology
Bcl-2	B-cell leukemia/lymphoma 2
bp	Base pair
BRCA	Breast Cancer Susceptibility Gene
CAP	The College of American Pathologist
CDK2	Cyclin-dependent kinase-2
CDK-2	Cyclin-dependent kinase-2
CEP	Chromosome enumeration probe
CISH	Chromogenic *In Situ* Hybridization
DAB	3, 3-Diaminobenzidine
DAPI	4'-6-Diamidino-2-phenylindole
DCIS	Ductal Carcinoma *in situ*
DNA	Deoxy Ribonucleic Acid
dNTP	Deoxyribonucleotide trisphosphate
DPX	Distyrene, plasticizer, xylene
DU	Depleted Uranium
EGFR	Epidermal growth factor receptor
ER	Estrogen Receptor
FFPE	Formalin-Fixed, Paraffin-Embedded
FISH	Fluorescent *In Situ* Hybridization
H and E	Hematoxylin and Eosin
HER-2	Human Epidermal Growth Factor Receptor-2
HRP	Horse Radish Peroxidase
IDC	Invasive Ductal Carcinoma
IHC	Immunohistochemistry
ILC	Invasive Lobular Carcinoma
LCIS	Lobular Carcinoma *In Situ*
MCF-7	breast cancer cell line
Mcm-2	The minichromosome maintenance protein 2
MDM2	Mouse Double Minute2

MIB-1	The monoclonal murine antibody MIB-1
NCBI	National Centre for Biotechnology Informations
NPI	Nottingham Prognostic Index
P	Probability
PAI	Plasminogen Activator Inhibitor
PCR	Polymerase Chain Reaction
PI3K	Phosphoinositide-3 Kinase
PR	Progesterone Receptor
PTEN	Phosphatase and Tensin Marker
RNA	Ribonucleic Acid
SERM	Selective estrogen-receptor modulator
SSC	sodium chloride and sodium citrate
TAE	Tris, Acetic acid, EDTA
TMA	Tissue Microarray
TN	Triple Negative
TNBC	Triple Negative Breast Cancer
TNM	Tumor Node Metastasis
uPA	urokinase-type Plasminogen Activator
VEGF	Vascular Endothelial Growth Factor

Chapter One

Introduction

and

Literature Review

1.1 Introduction

The recorded history of breast cancer traces back thousands of years. It is no surprise that from the dawn of history, scientists and physicians have written about cancer. Incidents of breast cancer have been documented back to the early Egyptians when the popular treatment was cautery of the diseased tissue (Kardinal and Yarbro, 1979). The disease occurs mostly in women, but does occur rarely in men. Breast cancer continues to be one of the most common cancers and a major cause of death among women worldwide; an estimated 1,301,867 new breast cancers were diagnosed and 464,854 people were die from the disease in 2007 (American Cancer Society, 2007). In Iraq, according to the latest Iraqi Cancer Registry reports, cancer of the breast is the commonest type of malignancy in females and there is a general trend towards an increase in the frequency and incidence of breast cancer in younger age group. The most common histo-pathological types were invasive ductal carcinoma (IDC) (77.2%), and invasive lobular carcinoma (ILC) (9.8%). Patients less than 30 years old age formed about 5% of cases, whereas about 75% of the cases occurred in women older than 40 years. The highest number of cases is between 40-50 years old age groups (Iraqi Cancer Board, 2000).

Breast cancer is associated with different types of somatic genetic alterations such as mutations in proto-oncogenes and tumor suppressor genes. The tumor is highly heterogeneous, with a wide range of biological, clinical and pathological characteristics (Ferlay *et al.*, 2001). Breast cancer is the most common malignancy in women, and it is curable if diagnosed at an early stage. Traditional prognostic factors include the axillary lymph node status, tumor size, nuclear grade and histologic grade. Interest in novel prognostic markers is based on the fact that a significant number of patients with early-stage breast cancer harbor microscopic metastasis at the time of diagnosis. It

is now well established that adjuvant systemic therapy improves survival in patients with early-stage breast cancer, the early detection of breast cancer is a major prognostic factor in the management of the disease. In particular, detecting breast cancer in its pre-invasive form, as ductal carcinoma *in situ* (DCIS) improves prognosis greatly compared with invasive tumors (Early Breast Cancer Trialists' Collaborative Group, 1998).

Prognostic and predictive tumors markers are tools allowing for more precise treatment allocation of patients. A prognostic marker (or a factor) is associated with the clinical outcome of the disease, ideally for untreated patients. A predictive marker can be defined as a factor that indicates sensitivity or resistance to a specific treatment. Predictive markers are important in oncology as different cancers vary widely in their response to particular therapies. Molecular oncology is one of the most promising fields that may contribute considerably to the diagnosis of breast cancer, its metastases, and addressing major problems with early detection, accurate staging, and monitoring of breast cancer patients (Hayes *et al.*, 1998; Esteva and Hortobagyi, 2004). The only recommended predictive markers in oncology are ER and PR for selecting endocrine-sensitive breast cancers and HER-2 for identifying breast cancer patients with metastatic disease who may benefit from trastuzumab, HER-2 protein monoclonal antibody (Michael, 2005). A large number of molecular markers have been studied to determine their ability to predict prognosis or response to therapy, or both. Prognostic and predictive molecular markers commonly used in clinical practice include Ki-67, ER, PR, and HER-2. Prognostic indices that integrate clinical, histologic, and molecular parameters need to be developed and validated in conjunction with novel bioinformatic methodologies to aid clinical decision-making. Modern array technologies allow for the simultaneous screening of virtually all human genes on the DNA and RNA level. Studies using such

techniques have lead to the identification of hundreds of genes with a potential role in cancer or other diseases. The validation of all of these candidate genes requires in situ analysis of high numbers of clinical tissues samples. The tissue microarray (TMA) technology greatly facilitates such analysis. In this method, minute tissue samples (0.6 mm in diameter) from up to 1,000 different tissues can be analyzed on one microscope glass slide. All in situ methods suitable for histological studies can be applied to TMAs without major changes of protocols, including immunohistochemistry, fluorescence in situ hybridization, or RNA in situ hybridization. Because all tissues are analyzed simultaneously with the same batch of reagents, TMA studies provide an unprecedented degree of standardization, speed, and cost efficiency (Dancau *et al.*, 2010).

Objectives of this study:

1. To apply Tissue Microarrays (TMAs) technique for the diagnosis of breast cancer.
2. Studying and comparing the immunohistochemical expression of tumor markers (ER, PR, Ki67, p53 and HER-2) in Italian and Iraqi breast carcinoma cases.
3. To study the correlation of these tumor markers with each others.
4. Studying the amplification of the *HER-2* gene by using Fluorescent *in situ* Hybridization (FISH) technique.
5. Determination of the mutations in Breast Cancer susceptibility gene (*BRCA1*) exon 11 in the sporadic breast cancer.

1.2 Literature Review

1.2.1 Breast Cancer Statistics and Epidemiology

The disease occurs mostly in women, but rarely affects men. Breast cancer continues to be one of the most common cancers and a major cause of death among women worldwide. The American Cancer Society estimated that 1,301,867 new breast cancers will be diagnosed and 464,854 people will die from the disease (American Cancer Society, 2007): breast cancer accounts for about 23% of all female cancers in the world. Furthermore, approximately 4.4 million living women around the world were diagnosed with breast cancer in the last years. The breast cancer incidence rate is high in Europe and North America, accounting for more than half of all breast cancer cases around the world, while incidence rates in Africa and Asia are low. The highest rate is in North America (99.4 per 100 000), and Central Africa has the lowest incidence rate (16.5 per 100 000) (Parkin *et al.*, 2005). Studies of racial/ethnic characteristics of breast cancer reveal that non-Hispanic white, Hawaiian, and black women have the highest levels of breast cancer risk. Other Asian/Pacific Islander groups and Hispanic women have lower levels of risk (Pike *et al.*, 1993).

Depleted Uranium (DU) weaponry has been used against Iraq since the First Gulf War in 1991, also hundreds of tons of DU expenditure were also used during the invasion of Iraq in 2003 which increased the radioactive contamination impact. Breast cancer becomes the most common tumor type in women in Iraq; over the last years, there has been a threefold increase in the incidence of breast cancer (Al-Azzawi, 2006). Accordingly, millions of Iraqi's have received higher doses of radioactivity than ordinary background levels. Consequently, thousands of Iraqi children and their families suffered from different radiation related diseases such as leukemia, congenital

malformations, malignancies, congenital heart diseases, chromosomal aberration and multiple malformations. Women in the contaminated areas suffered high rates of miscarriages and sterility (Al Ghurabi, 2002).

1.2.2 Breast Cancer Histology

Breast tumors are composed of three basic cellular components: epithelial cells, stromal cells and vascular components. The epithelial cells become transformed and constitute the malignant portion of the tumor. Stromal cells do not typically become transformed, but they do secrete growth factors and other regulatory elements. Growth factors secreted by the malignant cells promote the growth and proliferation of endothelial cells, thereby increasing the vascular component. This neovascularization serves to supply the tumor with the blood circulation needed for growth and metastases (Cotran, 2002).

The main components of the female breast are lobules (milk-producing glands), ducts (milk passages that connect the lobules and the nipple), and stroma (fatty tissue and ligaments surrounding the ducts and lobules, blood vessels, and lymphatic vessels). Each breast has 15 to 20 lobes, which have many smaller lobules. Thin tubes, called ducts, connect the lobes and lobules (Figure 1-1) (Engel, 1996). The most common type of breast cancer is ductal cancer; it is formed in the cells of the ducts. Cancer that starts in lobes or lobules is called lobular cancer; it is more often found in both breasts than other types of breast cancer. Cancers also are classified as non-invasive (in situ) and invasive (infiltrating). Invasive breast cancer has a tendency to spread to other tissues of the breast and/or metastasize to other regions of the body. The term *in situ* (Latin phrase meaning in place) refers to cancer that has not spread past the area where it initially developed. Ductal Carcinoma *in situ* (DCIS) is a pre-invasive form of breast cancer where ductal cancer cells grow only inside the ducts of the breast. Lobular Carcinoma *in situ* (LCIS) is

usually encountered as an incidental finding in a breast biopsy, manifested by proliferation of the cells in the lobes or the lobules. Invasive Ductal Carcinoma (IDC) is the most common form of breast cancer, comprising about 65-85% of all cases, the cancer cells arise from the lining of milk duct, then break through the ductal wall and invade nearby breast tissue. The cancer cells may remain localized near the site of origin or spread (metastasize) throughout the body, carried by bloodstream or lymphatic system. Invasive Lobular Carcinoma (ILC) comprises 5 to 10 percent of breast cancers, starting in the milk-producing lobules and then breaking into the surrounding breast tissue or other parts of the body (Lester and Cotran, 1999). A less common type of breast cancer is inflammatory breast cancer characterized by general inflammation of the breast. Other rare types of breast cancer are medullary carcinoma (an invasive breast cancer that forms a distinct boundary between tumor tissue and normal tissue), mucinous carcinoma (formed by the mucus-producing cancer cells), and tubular carcinoma (WHO, 1981).

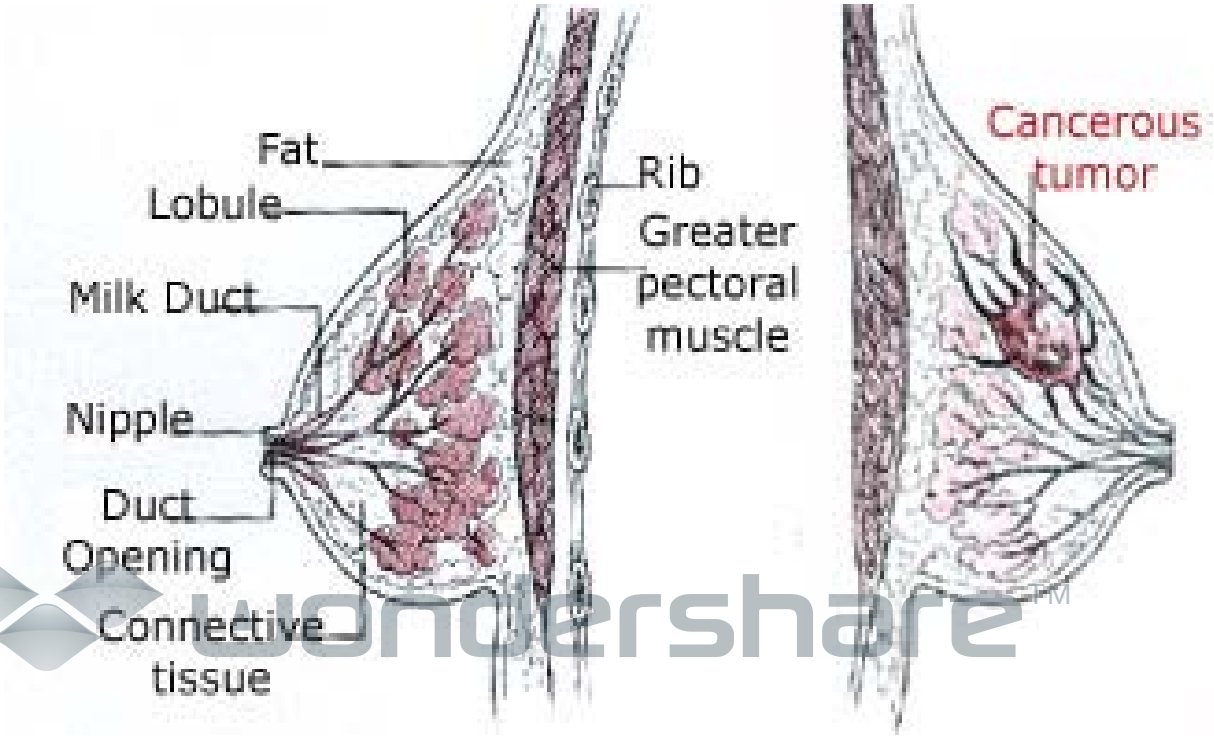


Figure (1-1) Histology of the Female Breast (Engel, 1996)

1.2.3 Staging of Breast Cancer

Tumor staging systems provide information about extent of disease that can be used to guide treatment recommendations and provide estimates of patient prognosis. In addition, the staging system provides a framework for reporting treatment outcomes and thereby permits the efficacy of new treatments to be assessed. Changes in the staging system are periodically required in order to incorporate new diagnostic and therapeutic advances that affect risks of disease recurrence and patient survival (Singletary *et al.*, 2002). The staging systems published by American Joint Committee for Cancer (AJCC) for breast cancer are based on the size and extent of invasion of the primary tumor (T), the clinical absence or presence of palpable axillary lymph nodes and evidence of their local invasion (N), together with the clinical and imaging evidence of distant metastases (M). This is then translated into the TNM classification (Wendy *et al.*, 2003).

Clinical stage according to the TNM (Tumor Node Metastasis) staging system is the most well established prognostic marker in breast cancer. Patients are stratified into different prognostic categories (stage 0 to IV) by the TNM system outlined in table (1-1) (Singletary *et al.*, 2002).

Table (1-1): Definition of TNM Staging in Breast Cancer (Singletary *et al.*, 2002)

Stage	Tumor	Node	Metastasis
Stage 0	**Tis**	**N0**	**M0**
Stage I	**T1**	**N0**	**M0**
Stage IIA	**T0**	**N1**	**M0**
	T1	**N1**	**M0**
	T2	**N0**	**M0**
Stage IIB	**T2**	**N1**	**M0**
	T3	**N0**	**M0**

Stage IIIA	**T0**	**N2**	**M0**
	T1	**N2**	**M0**
	T2	**N2**	**M0**
	T3	**N1**	**M0**
	T3	**N2**	**M0**
Stage IIIB	**T4**	**N0**	**M0**
	T4	**N1**	**M0**
	T4	**N2**	**M0**
Stage IIIC	**Any T**	**N3**	**M0**
Stage IV	**Any T**	**Any N**	**M1**

Definitions of TNM staging in breast cancer by Singletary *et al.* (2002):

Primary Tumor: TX, primary tumor cannot be assessed. T0, no evidence of primary tumor. Tis, carcinoma *in situ*. T1, tumor with <2 cm in greatest dimensions. T2, tumor with >2 cm but not >5 cm. T3, tumor with >5 cm. T4, inflammatory carcinoma or tumors with extension to skin or chest wall.

Regional lymph nodes (N): NX, not assessable. N0, no regional lymph node metastasis. N1, metastasis in ipsilateral axillary lymph node. N2, metastasis in ipsilateral axillary lymph nodes fixed or ipsilateral internal mammary lymph node metastasis. N3, metastasis in infraclavicular lymph node(s) or metastasis in both ipsilateral internal mammary lymph node and ipsilateral axillary lymph node, or in the ipsilateral supraclavicular lymph node alone.

Distant metastasis (M): MX, not assessable. M0, no distant metastasis. M1, distant metastasis present.

1.2.4 Grading of Breast Cancer

Histological grade provides prognostic information in many tumors, including breast cancers. Two main methods have evolved, based either on nuclear factors or a combination of cellular features (nuclear, cytological and architectural). The latter method is the most widely accepted for grading breast cancer and has been refined with the stricter definition of more objective criteria. The histopathological grade according to the Elston and Ellis method is based on the sum of three separate morphological components in the breast cancer: tubule formation, nuclear pleomorphism and mitotic count. Several studies have demonstrated that this method yields independent prognostic information for survival in human breast cancer (Elston and Ellis, 1991). This recognition of histological grade as an important prognostic factor has now led to methods devised for assessing grade on cytological preparations, with the aim of obtaining prognostic information preoperatively (Hunt *et al.*, 1990; Rakha *et al.*, 2008).

1.2.5 Breast Cancer Risk Factors

A risk factor is anything that increases the chance of getting a disease. Every woman is at risk for developing breast cancer. Several relatively strong risk factors for breast cancer that affect large proportions of the general population have been known for some time. However, the vast majority of breast cancer cases occur in women who have no identifiable risk factors other than their gender (Kelsey and Gammon, 1990).

Numerous risk factors for breast cancer have been identified, including a female gender, age, previous breast cancer, benign breast disease, hereditary factors (family history of breast cancer), early age at menarche, late age at menopause, late age at first full-term pregnancy, postmenopausal obesity, low

physical activity, race/ethnicity and high-dose exposure to ionizing radiation early in life (Bernstein, 1998; Wu *et al.*, 2002).

The "speculated" risk factors for breast cancer include never having been pregnant, having only one pregnancy rather than many, not breast feeding after pregnancy, use of postmenopausal estrogen replacement therapy or postmenopausal hormone (estrogen/progestin) replacement therapy, use of oral contraceptives, certain specific dietary practices (high intake of fat and low intakes of fiber, fruits, and vegetables, low intake of phytoestrogens), alcohol consumption, tobacco smoking, and abortion. Although men can and do develop breast cancer, the disease is 100 times more likely to occur in a woman than in a man. The risk of breast cancer is higher in middle-aged and elderly women than in young women, this risk increases as a woman ages, rising sharply after the age of 40 (Wu *et al.*, 2002; Edwards *et al.*, 2002). When breast cancer occurs in adolescents and young women, it tends to be more aggressive and has a worse prognosis than when it occurs in older women. Benign breast disease is an important risk factor for a later breast cancer, which can develop in either breast. It encompasses a spectrum of histologic entities, usually subdivided into nonproliferative lesions, proliferative lesions without atypia, and atypical hyperplasia, with an increased risk of breast cancer associated with proliferative or atypical lesions (Connolly and Schnitt, 1993).

The risk of breast cancer is higher among women who have a close blood relative (mother, sister, or daughter) who have had the disease. The increase in risk is especially high if the relative developed breast cancer before the age of 50 in one or in both breasts (Claus *et al.*, 2003).

Factors that increase lifetime exposure to estrogens and progesterone include early age at menarche (12 years or younger), regular ovulation, and

late age at menopause (55 years or older). Convincing evidence exists for the overall role of age at menarche and the first full term pregnancy in breast cancer risk. A younger age at menarche is associated with an earlier onset of ovulatory cycles and consequently with longer exposure to estrogens, which increases cell proliferation. The earlier the first full term pregnancy, the earlier in life cells undergoing differentiation, thus decreasing the risk of mammary cell transformation and/or proliferation (Pike *et al.*, 1993). Estrogen and progesterone most likely induce breast cancer by increasing cell proliferation as well as through genotoxicity, both hormones act via nuclear receptors (Yager and Davidson, 2006). For about a decade after the pregnancy; risk is increased, probably due to the hormonal stimulation of already initiated breast epithelial cells. The additional contribution of cyclical estrogen exposure is less clear, and much evidence indicates that progestins add to breast cancer risk. High endogenous estrogen levels in postmenopausal women are now well established as an important cause of breast cancer, and many known risk factors appear to operate through this pathway (Lambe *et al.*, 1994).

Most breast cancer cases are sporadic, and 15% to 20% of cases are due to inherited mutations in several breast cancer–predisposing genes. Contributions of particular note include the discovery of several gene mutations in breast cancer susceptibility genes (*BRCA1*, *BRCA2* and *PTEN* genes) and quantification of the risks associated with them. It has been shown that germline mutations in the *BRCA1* and *BRCA2* genes account for a large proportion of cases of hereditary breast cancer (Ford and Easton, 1995). A minority of hereditary breast cancer cases is attributable to germline mutations in high-penetrance breast cancer susceptibility genes *BRCA1* and *BRCA2* (Nathanson *et al.*, 2001).

Epidemiological studies have shown that in women with a family history of breast cancer, the risk of breast cancer is increased two to threefold. Studies have also shown that there are families in which breast cancer risk is inherited in an autosomal-dominant fashion ('hereditary breast cancer'). The denser woman's breast tissue is, the higher her chance for breast cancer. It is speculated that "dense breast tissue may contain extra-abundant growth factors that stimulate cancer formation" (Engel, 1996).

While most biological risk factors are unmodifiable, there are several lifestyle factors that can be modified, including diet, alcohol intake, age when bearing the first child or having no children, use of oral contraceptives, and physical activity levels (Engel, 1996; American Cancer Society, 2002).

Alcohol intake increases endogenous estrogen levels that may contribute to the observed increase in risk among regular drinkers and users of oral contraceptives and are probably due to their estrogenic (and probably progestational) effects (Collaborative Group on Hormonal Factors in Breast Cancer, 1996; Dorgan *et al*., 2001).

1.2.6 Prognostic and Predictive Markers in Breast Cancer

Interest in novel prognostic markers is attributed to the fact that a significant number of patients with early-stage breast cancer harbor microscopic metastasis at the time of diagnosis. It is now well established that adjuvant systemic therapy improves survival in patients with early-stage breast cancer, therefore, the identification of biological markers that might have the ability to predict therapeutic response is crucial. Axillary lymph node status, tumor size and tumor grade are of prognostic value in patients with operable breast cancer, but only the expression of hormonal receptors is a clinically useful marker that predicts response to treatment (Early Breast Cancer Trialists' Collaborative Group, 1998).

Programmed cell death proceeds through at least two pathways, which both can be regulated at multiple levels. The extrinsic apoptotic pathway consists of cell surface receptors (death receptors), their inhibitory counterparts (decoy death receptors) and downstream cytoplasmic proteins such as caspase activators. The intrinsic apoptotic pathway focused on the mitochondria, which contains several apoptogenic factors (Kroemer, 1999).

A variety of prognostic factors are assessed in invasive breast cancer, including tumor size, grade, lymph nude status, ER/PR status, and presence of lympho-vascular invasion. The Nottingham Prognostic Index (NPI) is widely used to predict survival and risk of relapse, and thus help select appropriate adjuvant systemic therapy. NPI= 0.2 x tumor size (cm) + histologic grade + nodal status. Nodal status is scored 1-3: 1= node –ve; 2= 1-3 nodes +ve; 3= >3 nodes for breast cancer. Histological grade is also scored 1-3. If treated with surgery alone 10 years survival rates are: NPI <2.4: 95%; NPI 2.4-3.4; 85%; NPI 3.4-4.4; 70%; NPI 4.4-5.4; 50%; NPI> 5.4; 20% (longmore *et al.*, 2004).

1.2.6.1 Estrogen Receptors (ER) and Progesterone Receptors (PR)

The link between growth of breast cancer and sex hormone activity was suggested as early in 1896 when Dr. Beatson reported drastic clinical response for three breast cancer cases after surgical removal of their ovaries. Although the concept of hormonal regulation was not known at that time, Beatson stated that the ovaries seemed to have control over proliferation of the local epithelium in the breast (Beatson, 1896). The analyses of oestrogen and progesterone receptors in breast cancers quickly provided the evidence that they could aid the identification of those cancers that were more likely to respond to endocrine treatment (McGuire *et al.*, 1974).

Estrogen receptors (ERs) are nuclear receptor proteins that have an estrogen-binding domain and a DNA binding domain (Rayter, 1991). The *ER* and *PR* genes are located at chromosome 6q25.1 and 11q22, respectively (Menasce *et al.*, 1993). The biological actions of estrogens are mediated by estrogen binding to one of two specific estrogen receptors (ERs), ERα and ERβ, which belong to the nuclear receptor superfamily, a family of ligand-regulated transcription factors (Salmon *et al.*, 2001). ERα and ERβ are products of different genes and exhibit tissue- and cell-type specific expression. Normal human breast epithelium in the non-pregnant, non-lactating woman is, unlike the endometrium, not very sensitive to proliferative stimulus from the sex-hormones estrogen and progesterone (Anderson *et al.*, 1998). Estrogens regulate many physiological processes, including normal cell growth, development, and tissue-specific gene regulation in the reproductive tract and in the central nervous and skeletal systems. Estrogens also influence the pathological processes of hormone-dependent diseases, such as breast, endometrial, and ovarian cancers, as well as osteoporosis (Couse and Korach, 1999). The ER pathway plays a critical role in the pathophysiology of human breast cancer; overexpression of ERα is a well-established prognostic and predictive factor in breast cancer patients; while the prognostic significance of ERβ is not well defined (Speirs and Kerin, 2000). The ER and PR status can be measured using immunohistochemistry (IHC). The results of IHC correlate closely with biochemical ligand-binding assays and with clinical response rates to endocrine therapy (Holmes *et al.*, 1990; Nadji *et al.*, 2005). Normal breast epithelial cells express no or very low basal levels of estrogen receptor or progesterone receptor, and immunohistochemical studies show that only 7% of these cells stain positively for ER or PR (Lapidus *et al.*, 1998). In contrast, approximately 70% of all breast cancers overexpress ER and half of these tumors also overexpress PR, as detected by ligand binding or immunohistochemical

analysis (Chebil *et al.*, 2003). The clinical values of ER and PR as tumor markers are well established and recommended to be analyzed in all primary breast cancers according to the American Society of Clinical Oncology (ASCO) guidelines for tumor markers (Bast *et al.*, 2001). Selective estrogen-receptor modulators (SERMs) are synthetic nonsteroidal compounds that switch on and switch off target sites throughout the body. Tamoxifen, the pioneering SERM, blocks estrogen action by binding to the ER in breast cancers. Tamoxifen has been used ubiquitously in clinical practice during the last 30 years for the treatment of breast cancer and is currently available to reduce the risk of breast cancer in high-risk women. Unlike tamoxifen, raloxifene does not increase the incidence of endometrial cancer. Raloxifene maintains bone density (estrogen-like effect) in postmenopausal osteoporotic women, but at the same time reduces the incidence of breast cancer in both high and low-risk (osteoporotic) postmenopausal women. Clearly, the simple ER model of estrogen action can no longer be used to explain SERM action at different sites around the body. Instead, a new model has evolved based on the discovery of protein partners that modulate estrogen action at distinct target sites. Coactivators are the principal players that assemble a complex of functional proteins around the ligand ER complex to initiate transcription of a target gene at its promoter site. A promiscuous SERM ER complex creates a stimulatory signal in growth factor receptor–rich breast or endometrial cancer cells. These events cause drug-resistant, SERM-stimulated growth. The sometimes surprising pharmacology of SERMs has resulted in a growing interest in the development of new selective medicines for other members of the nuclear receptor superfamily. This will allow the precise treatment of diseases that was previously considered impossible (Jordan and O'Malley, 2007).

1.2.6.2 Proliferation Marker *Ki67*

Ki-67 was identified by Gerdes *et al.* in (1991) as a nuclear nonhistone protein, shortly after the corresponding antibody was described by the same group (Gerdes *et al.*, 1983) in the city of Kiel (hence "Ki") after immunization of mice with the Hodgkin's lymphoma cell line L428 (67 refers to the clone number on the 96-well plate in which it was found). The *Ki-67* gene is on the long arm of human chromosome 10 (10q25) (Fonatsch, 1991). Two alternative mRNA species resulting from alternative splicing encode two isoforms of the protein. The "large" Ki-67 protein isoform has a calculated molecular mass of 359 kD, and the "small" isoform, a mass of 320 kD (Schluter *et al.*, 1993). *Ki67* is a large nuclear protein (395 kD) and has been hypothesized to be involved in several different cellular functions such as cell cycle regulation, ribosomal RNA processing, organizing DNA or to have a structural role in the nucleus (Scholzen and Gerdes, 2000). The Ki67 protein is a proliferation marker expressed in cycling (non-G0) cells in the G1, S, G2 and M-phases of the cell cycle, while quiescent or resting cells in the G0 phase did not express the Ki67 antigen (Gerdes *et al.*, 1984). It is rapidly catabolized at the end of the M phase, and is undetectable in resting (G0 and early G1) cells (Fitzgibbons, 2000). The expression of Ki67 varies in intensity throughout the cell cycle, and this has raised concern that it could lead to a misclassification of cycling cells as resting ones. Evidence indicates that levels of Ki67 are low during G1 and early S-phase and progressively increase to reach a maximum during mitosis. A rapid decrease in expression starts during anaphase and telophase (Lopez *et al.*, 1991). The original monoclonal Ki67 antibody presented by (Gerdes *et al.*, 1983) only works on frozen tumor sections while several additional monoclonal antibodies against the Ki67 antigen have been produced for use on paraffin embedded tumor specimens. The well documented of these antibodies is the monoclonal

murine antibody MIB-1, which has higher sensitivity for detecting Ki67 than other comparable antibodies (Rose *et al.*, 1994). Breast cancers expressing high levels of Ki67, are associated with worse outcomes (Trihia *et al.*, 2003). Ki67 is not included in routine clinical decision-making because of a lack of clarity regarding how Ki67 measurements should influence clinical decisions. Recent studies (Dowsett *et al.*, 2007) indicate that changes in Ki67 expression after neoadjuvant endocrine treatment may predict long-term outcome. High Ki67 is a sign of poor prognosis associated with a good chance of clinical response to chemotherapy, but its independent significance is modest and does not merit measurements in most routine clinical scenarios. However, its application as a pharmacodynamic intermediate marker of the effectiveness of medical therapy holds great promise for rapid evaluation of new drugs (Ander *et al.*, 2005). Overall, the total evidence value of the reports addressing the prognostic and predictive value of Ki67 is not yet considered sufficient to warrant recommendation for clinical use of Ki67 (Bast *et al.*, 2001).

Many studies have investigated the correlation between Ki-67 and other well-known markers of proliferation like S-phase fraction as measured by flow cytometry (Brown *et al.*, 1996), mitotic index, tyrosine kinase **(**Spyratos *et al.*, 2002), and *in vivo* uptake of bromodeoxyuridine (a laborious but highly reliable procedure considered by many to be the gold standard of cell proliferation measurements). Each been regularly reported to correlate well with Ki67 (Moriki *et al.*, 1996; Thor *et al.*, 1999). The correlation of Ki67 with proliferating cell nuclear antigen has been reported as positive, but weak (Moriki *et al.*, 1996),and the multiplicity of functions developed by proliferating cell nuclear antigen may account for this discrepancy (Prosperi, 1997). Studies of the correlation with DNA ploidy are also conflicting **(**Spyratos *et al.*, 2002) and no correlation has been found with thymidilate synthase (Trihia *et al.*, 2003). A recently described marker of cell

proliferation, the minichromosome maintenance protein 2 (Mcm-2), showed a strong correlation with Ki-67 although it seemed to be more sensitive in detecting cycling cells (Gonzalez *et al.*, 2003).

1.2.6.3 Human Epidermal Growth Factor Receptor-2 (HER-2)

The *neu* gene (also known as c-erbB2 or *ERBB2*) was originally identified in rat neuroectodermal tumors and later its close human relative was isolated (Rubin and Yarden, 2001). The HER-2 protein is one of the HER family of human epidermal growth factor receptors consisting of four members: HER-1, HER-2, HER-3 and HER-4 which have its homologue to the epidermal growth factor receptor family of transmembrane receptors, all of which are involved in the regulation of cell proliferation. In HER-1, HER-2, and HER-4, tyrosine kinase activity prompts increased cell proliferation. To be activated, these receptors must dimerase with themselves or with another receptor within the family. Therefore, cancer treatment agents that target more than one receptor may offer some advantage over those, which target a single receptor (Ross *et al.*, 2003). The transmembrane HER receptors have important roles in the network of cell signals controlling cell growth and differentiation. In a normal cell, the activity of the receptors is strictly controlled, most significantly through the HER-2 receptor (Rubin and Yarden, 2001).

HER-2 is a proto-oncogene located on chromosome 17, which encodes for p185 kD; a transmembrane glycoprotein with tyrosine kinase activity and structural homology to the human epidermal growth factor receptor (EGFR; HER-2), expressed in many cell systems, triggers a rich network of signaling pathways, and plays an important role in normal growth and development. Overexpression and/or amplification of HER-2 are detected in approximately 20% to 30% of invasive ductal carcinomas of the breast (Pauletti *et al.*, 2000).

HER-2 expression in cancer is a direct result of gene amplification in approximately 90-95% of cases (Caterina and Jorge, 2008). Although the activity of the HER receptor is strictly controlled in normal cells, HER-2 receptor overexpression plays a pivotal role in transformation and tumorigenesis. *HER-2* gene amplification and/or overexpression of the receptor have been detected in subsets of a wide range of human cancers including breast cancer, and is an indicator of poor prognosis. It is proposed that overexpressed HER-2 in combination with HER-3 causes high activity of cell-signaling networks, thereby resulting in tumor cell proliferation. Thus, the HER-2 receptor is an attractive target for new anti-cancer treatments (Neve *et al.*, 2001). HER-2 positivity can be represented as overexpression or amplification. HER-2 overexpression is defined as an abnormal increase in the number of HER-2 protein receptors on the cell surface. Normal breast epithelium has approximately 20,000 receptor molecules, whereas a HER-2 positive tumor can have as many as 2 million receptor molecules. *HER-2* gene amplification is characterized as an abnormal increase in the number of *HER-2* gene copies located within the cell nucleus. HER-2 overexpression and *HER-2* gene amplification are commonly, but not always, correlated (Harris *et al.*, 2007).

The primary interest in HER-2, at present, is focused on its potential predictive capabilities. Overexpression of HER-2 is associated with a worse outcome using tamoxifen therapy, while the aromatase inhibitors letrozole and anastrozole have been demonstrated in small randomized studies to result in higher response rates compared with tamoxifen in patients with receptor positive breast cancer (Dowsett, 2003; Ellis *et al.*, 2003).

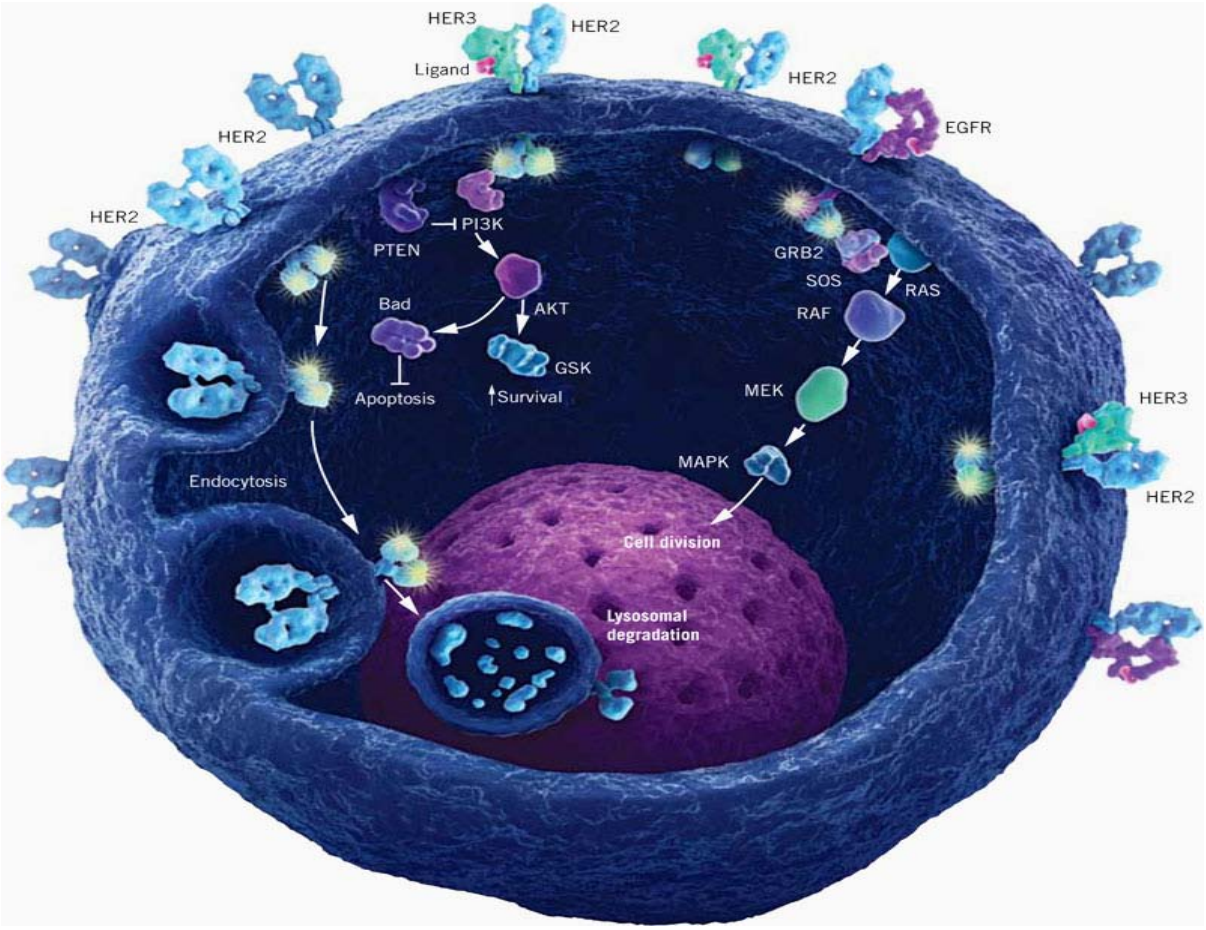


Figure (1-2) HER Family (Sundaresan *et al.*, 1999)

There are several possible uses of HER-2 status. HER-2 positivity is associated with worse prognosis (higher rate of recurrence and mortality) in patients with newly diagnosed breast cancer who do not receive any adjuvant systemic therapy. Thus, HER-2 status might be incorporated into a clinical decision, along with other prognostic factors, regarding whether or not to give any adjuvant systemic therapy. HER-2 status is also predictive for several systemic therapies (Yamauchi *et al.*, 2001). Methods for tumor HER-2 assessment include fluorescence in situ hybridization (FISH), which assesses gene amplification, and immunohistochemistry (IHC), which assesses protein expression. These most widely used technologies are ideally suited for routine and archival paraffin embedded tissue and are evaluated by direct

visualization of tumor cells. Published gene-protein correlation studies, in general, show good-to-excellent concordance (Jacobs *et al.*, 1999). Based on the numerous studies assessing the validity of IHC and FISH testing for *HER-2* gene, the American Society of Clinical Oncology (ASCO) and the College of American Pathologist (CAP) have released new guidelines for the testing of HER-2 status in breast cancer (Gown, 2008). Overall method concordance rates of 82% to 92% have been reported and ASCO/CAP expressed no preference for one method over the other. The FISH method uses fluorescent DNA probes for *HER-2* and chromosome enumeration probe (CEP17). The ratio of *HER-2* to CEP 17 is positive for gene amplification when =2.2 and is negative (no amplification) when =1.8. Ratios of 1.8 to 2.2 are reported as equivocal. IHC test results for HER-2 are based on cell membrane stain intensity and percent of tumor cells stained. IHC results that are negative (0 and 1+) or strongly positive (3+) are associated with negative and positive protein overexpression, respectively (Wolff *et al.*, 2007). In this regard, HER-2 positivity appears to be associated with relative, but not absolute, resistance to endocrine therapies (Konecny *et al.*, 2003). Although controversial, preclinical and clinical studies have suggested that this effect may be specific to SERM therapy, such as tamoxifen (ER blocker), and perhaps not to estrogen depletion therapies, such as with aromatase inhibitors (e.g. anastrozole) (Ellis *et al.*, 2001). HER-2 status also appears to be predictive for either resistance or sensitivity to different types of chemotherapeutic agents. HER-2 may be associated with relative, but not absolute, lower benefit from nonanthracycline, nontaxane-containing chemotherapy regimens (Menard *et al.*, 2001). In contrast, retrospectively obtained results from prospectively conducted randomized clinical trials appear more definitive in suggesting that HER-2 positivity is associated with response to anthracycline therapy. This effect may be secondary to co-amplification of *HER-2* with topoisomerase II, which is the direct target of

these agents (Pritchard *et al.*, 2006). Preliminary data also suggest that HER-2 may predict for response and benefit from paclitaxel in either the metastatic or the adjuvant settings (Hayes *et al.*, 2006). Perhaps most importantly, several studies have now shown that agents which, target HER-2 are remarkably effective in both the metastatic and adjuvant settings. Trastuzumab against HER-2 protein, a humanized monoclonal antibody, improves response rates, time to progression, and even survival when used alone or added to chemotherapy in metastatic breast cancer (Slamon *et al.*, 2001; Fleming *et al.*, 2001). Trastuzumab is also active as a single agent (Cobleigh *et al.*, 1999).

1.2.6.4 Tumor Suppressor Gene *p53*

First described in 1979, *p53* was the first tumor suppressor gene to be identified. *p53* functions to eliminate and inhibit proliferation of abnormal cells, thereby preventing neoplastic development. The *p53* signalling pathway is in a standby mode under normal cellular conditions (Hollstein *et al.*, 1991). Activation occurs in response to cellular stresses, and several independent pathways of *p53* activation have been identified to be dependent on distinct upstream regulatory kinases (Vogelstein *et al.*, 2001). The human *p53* gene is located at the short arm of chromosome 17 (17p13.1) (McBride *et al.*, 1986). The *p53* has 11 exons (the first is not translated) and encodes a 53-kD nuclear transcription factor that functions as a multifunctional transcription factor involved in the control of cell cycle, in repair after DNA damage, and in apoptosis. A mutation in the *p53* gene is the most common mutation found in malignant cells, and somatic mutations are present in 20-30% of all breast cancers (Soussi and Beroud, 2001).

Overexpression of p53 by immunohistochemistry (IHC) has been identified in 11–55% of invasive breast carcinomas. A large number of studies have assessed the prognostic value of *p53* alterations, yielding some

conflicting findings (Levin *et al.*, 1991). Expression of *p53* is induced by diverse forms of cellular stresses such as hypoxia or DNA damage caused by carcinogens, ionizing radiation and UV light (Graeber, 1994; Harris, 1996). *p53* plays a key role in mediating cell response to various stresses, mainly by inducing or repressing a number of genes involved in cell cycle arrest, senscence, apoptosis, DNA repair, and angiogenesis (Lacroix *et al.*, 2006). The *p53* gene altered in breast carcinomas in approximately 20–40% of all cases depending on tumor size and stage of the disease. It seems to be an early event in breast tumorigenesis (Fridman and Lowe, 2003).

All data available on *p53* mutation analyses of human breast carcinomas, as well data from transgenic animal studies and experimental cell studies, support an important role for *p53* in mammary carcinogenesis. Although only a fraction of breast tumors harbor a *p53* mutation, accumulating data over the past years point to an inactivation of the *p53* activity by alterations of either upstream or downstream targets in the *p53* pathway in a large proportion of breast tumors. Several other mechanisms for inactivation of *p53* itself have been described including amplification of one of the many *p53* binding proteins such as MDM2 (Mouse Double Minute2), alterations in genes coding for proteins responsible for the phosphorylation, acetylation and ribosylation of the p53 protein, and in genes coding for transcription factors of the *p53* gene itself (Sax *et al.*, 2002).

These discoveries will provide a foundation, when performing high-throughput genome analyses allowing the analyses of all genes in the *p53* pathway simultaneously, and will certainly provide new insight into its role in breast tumorigenesis. Molecular pathological analyses of specific components in the *p53* pathway are likely to have an impact on the diagnosis, prognosis, and selection of the right treatment for individual breast cancer patients. The majority of genetic alterations in the *p53* gene found in breast cancer tumors

are point mutations leading to translation of a stable, mal-functional protein with extended half-life which accumulates in the cell, and is therefore detectable by immunohistochemistry (IHC) (Borresen-Dale, 2003). Although the biological properties of *p53* suggest a potential clinical usefulness, and many study results are promising, but the documentation of the predictive and prognostic value for *p53* is not yet solid enough, for the recommendation to include *p53* determination in routine clinical management of breast cancer patients (Bast *et al.*, 2001).

Tumor suppressor *p53* can induce apoptosis through both pathways intrinsic and extrinsic by activating transcription of pro-apoptotic genes, although the intrinsic pathway contribution to *p53*-mediated cell death is not clearly defined. The best described link between *p53* and apoptosis is the *p53* mediated regulation of transcription of pro-apoptotic members of Bcl2 family (Sax *et al.*, 2002; Fridman and Lowe, 2003). Among the biological markers investigated, *p53* and *Bcl-2* genes have received considerable attention as promising prognostic and predictive markers (Coradini and Daidone, 2004). These genes are involved in growth control and apoptosis pathways, which appear to play an important role in tumor progression and in response to anticancer agents.

1.2.6.5 Breast Cancer Susceptibility Genes 1/2 (*BRCA1* and *BRCA2*)

BRCA1/2 genes are tumor suppressor genes, the mutations of which could result in hereditary breast cancers. Several measures have been tried to reduce the risk of hereditary breast cancers. The initial results after prophylactic oophorectomy, bilateral prophylactic mastectomy and oral administration of tamoxifen show their effects on risk reduction. *BRCA1/2* associated breast cancers have their own pathologic characteristics (Teng *et al.*, 2008).

BRCA1 is classified as a tumor suppressor gene and plays an important role in surveillance of cell cycle and repair of DNA damage. About 5% of breast cancer cases are attributable to the inheritance of a mutant *BRCA1* allele. A mutant *BRCA1* allele is present among about 50% of families with a predisposition to breast cancer and among about 80% of families with a predisposition to both breast and ovarian cancer (Szabo and King, 1995). *BRCA1* is mapped to chromosome 17q21 via a genetic linkage analysis in 23 early-onset breast cancer families (Hall *et al*., 1990), and encodes a protein of 220 kD, composed of 1863 amino acids (Miki *et al*., 1994). *BRCA1* has 24 exons, including 2 non-translating exons. *BRCA1* contains only two recognizable protein motifs, a RING finger domain near the N-terminus and a BRCT domain at the C-terminus. RING fingers are cysteine-rich sequences that coordinate the binding of two zinc ions and are found in a number of diverse proteins. This type of domain may facilitate both protein–protein and protein–DNA interactions. The BRCT domain is a phylogenetically conserved sequence found in proteins involved in DNA repair and cell cycle regulation (Callebaut and Mornon, 1997).

In 1995, a second gene termed *BRCA2* was found related to hereditary breast cancer (Wooster *et al*., 1995). It covers about 70 kb of genomic sequence in 13q12, encoding a protein of 3418 amino acids. The coding region of *BRCA2* is composed of 27 exons with a non-translating exon. However, the gene sequence of *BRCA2* bears no obvious homology to any known gene including *BRCA1*, and the protein contains no defined functional domains (Tavtigian *et al*., 1996; Wooster *et al*., 1995). *BRCA2* can bind with *BRCA1*, participating in DNA damage repair pathway associated with the activation of homologous recombination and double-strand break repair (Chen *et al*., 1999). For their key role in maintaining genomic integrity and supervising cell cycle, mutations in *BRCA1* and *BRCA2* are found strongly

related to hereditary breast cancers. However, the types of mutation differ in distribution by ethnicity and geographic location (Syrjakoski *et al*., 2000). Biochemical, genetic, and cytological studies have implicated multiple roles for *BRCA1* gene product in the regulation of gene transcription, DNA damage repair, proliferation, and apoptosis.

Accumulating data reveal an important role of *BRCA1* protein in cell cycle checkpoints following DNA damage (Xu *et al.*, 2001). The pathological BRCA allelic variants may cause altered function of the proteins, altered transcriptional activity and/or altered DNA repair; the accumulation of defects results in diffuse chromosome instability, which could be directly responsible for cancer formation. In fact, mutations in *BRCA1* and *BRCA2*, conferring a highly increased susceptibility to breast and ovarian cancer, do not lead to cancer by themselves. The current consensus is that these are 'caretaker' genes which, when removed, allow other genetic defects to accumulate. The nature of these other molecular events may define the pathways through which *BRCA1* and *BRCA2* function. A caretaker's role is to maintain the integrity of the genome (Kinzler and Vogelstein, 1997).

High estrogen levels may increase normal *BRCA1* expression in an attempt to ensure genomic stability in the face of a potential estrogen-induced increase in genomic damage. Mutated *BRCA1* in inherited breast cancer or down-regulated *BRCA1* in sporadic breast cancer is unable to repair genomic damage induced by high levels of estrogens, increasing the likelihood that other mutations will occur and that a normal cell will ultimately become transformed. This interaction between estrogens and *BRCA1* probably explains, at least in part, why *BRCA1* mutation carriers exhibit a significantly increased risk of breast cancer and a moderately increased cancer risk in other estrogen-regulated sites (ovaries, prostate, and possibly the colon), but not in non-estrogen-regulated tissues (Clarke, 2000). *BRCA1* mRNA expression and

ER mRNA expression are closely linked to each other, suggesting a functional relationship between the two genes (Seery *et al.*, 1999). Furthermore, methylation of the *BRCA1* promoter appears to be strongly correlated with a lack of ER or progesterone receptor expression (Catteau *et al.*, 1999). In accordance with these observations, *BRCA1* was recently shown to have the ability to regulate the cellular response to estrogens (Fan *et al.*, 1999).

1.2.6.6 Other Markers

The *Bcl-2* (B-cell leukemia/lymphoma 2) gene was first identified at the breakpoint of a chromosomal translocation t(14:18) in B-follicular lymphoma (Tsuijimoto *et al.* 1985). *Bcl-2* is a cytoplasmic protein belonging to the *Bcl-2* family, and is expressed in normal glandular epithelium, but it is overexpressed in 25%–50% of breast cancers (Van Slooten *et al.*, 1996). It encodes a 26 kD protein that protects cells against apoptosis. Overexpression of *Bcl-2* has been shown to suppress the initiation of apoptosis in response to a number of stimuli, including anticancer drugs in a number of systems (Teixeira *et al.* 1995). *Bcl-2* is the first example of an oncogene that inhibits cell death rather than promoting proliferation. Accordingly, it was shown that the pathway toward tumorigenesis depends not only on the ability to escape growth control but also depends on the ability to prevent apoptosis (Vaux *et al.*, 1988).

Deregulation of cell cycle control is a hallmark of cancer. The primary cyclins (A, B1, D1, D3 and E) are crucial for cell cycle progression. Secondary cyclins (C and H) have putative indirect effects on cell cycle propulsion. Dysregulation of the cyclin-dependent kinase-2 (CDK2)–bound cyclins plays an important role in the pathogenesis of cancer (Pamies and Crawford, 1996). High levels of cyclin E expression are found in many types

of cancer, and elevated levels of the E1 cell cycle protein have been associated with a poor prognosis in primary breast cancer patients. The cell cycle regulatory protein cyclin E has been described as a strong prognostic factor for breast cancer (Ferrara, 2004).

Angiogenesis, the development of new blood vessels, is required for the growth of microscopic cancers into larger, clinically relevant tumors. Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen *in vitro* and an angiogenic inducer in a variety of *in vivo* models. It is believed to play a central role in angiogenesis through a variety of mechanisms, including effects on endothelial cell proliferation, survival, and migration (Ferrara, 2004). VEGF is overexpressed even in early stage breast cancers. VEGF expression is associated with both increased tumor microvessel density and increased risk for breast cancer recurrence (Gasparini *et al.*, 1997).

The urokinase-type plasminogen activator (uPA) is a serine protease that plays an important role in the invasion and metastasis process through degradation of the extracellular matrix. High levels of tissue uPA and its inhibitors (plasminogen activator inhibitor PAI-1, PAI-2) measured using ELISAs have been correlated with poor outcome in node-negative breast cancer patients (de Witte *et al.,* 1999). uPA was found to be an independent prognostic marker for outcome, although less powerful than axillary node status (Janicke *et al.,* 2001). Serine protease uPA and its inhibitor PAI-1 may be the markers that improve the selection of candidates for adjuvant systemic therapy and may also be the markers that could facilitate treatment decision in each individual patient, which is of a most importance (Čufer, 2004).

In 1997 *PTEN* gene was identified as a tumor suppressor gene encoding a cytoplasmic protein that controls cellular processes. *PTEN* is a tumor

suppressor gene, phosphatase and tensin homologue deleted from chromosome 10. *PTEN* is located on chromosome 10q23 and encodes protein regulating various signal transduction pathways and modulating cell growth processes, cell migration and apoptosis (Di Cristofano and Pandolfi, 2000).

In primary breast carcinomas, both somatic and germ-line *PTEN* mutations occur. The *PTEN* gene is frequently mutated or inactivated in a high proportion of human cancers, including up to 30% of breast cancers, resulting in hyper-activation of the PI3K/Akt signaling pathway. It is loss is associated with an intrinsic activation of the AKT pathway and is known to confer resistance to inhibitors of the HER-family (Pandolfi, 2004). In addition, germline mutations in the *PTEN* gene are associated with multi-neoplastic, autosomally dominant syndromes in humans, which feature a predisposition to formation of several different malignancies, including breast cancer (20–50%) of affected females (Liaw *et al.*, 1997; Marsh, *et al.*, 1997).

The protein product, PTEN, is a lipid phosphatase, the enzymatic activity of which primarily serves to remove phosphate groups from key intracellular phosphoinositide signaling molecules. This activity normally serves to restrict growth and survival signals by limiting activity of the phosphoinositide-3 kinase (PI3K) pathway. Multiple lines of evidence support the notion that this function is critical to the ability of *PTEN* to maintain cell homeostasis. Indeed, the absence of functional *PTEN* in cancer cells leads to constitutive activation of downstream components of the PI3K pathway, including the Akt and mTOR kinases. In model organisms, inactivation of these kinases can reverse the effects of *PTEN* loss. These data raise the possibility that drugs targeting these kinases, or PI3K itself, might have significant therapeutic activity in *PTEN*-null cancers. Akt kinase inhibitors are still in development (Sansal and seller, 2004).

1.2.7 Tissue Microarrays (TMAs)

Advances in genomics and proteomics dramatically increased the need to evaluate large numbers of molecular targets for their diagnostic, predictive or prognostic value in clinical oncology. Conventional molecular pathology techniques are often tedious, time-consuming, and require a lot of tissue, thereby limiting both the number of tissues and the number of targets that can be evaluated. The TMA technology allows the simultaneous analysis of up to 1,000 tissue samples at a time, either at the DNA, RNA or protein level on a single microscope glass slide. Hematoxylin-Eosin stained slides with adequate representative tissues are required before starting, as eventually the quantity of TMA will be represented by the quality of each tissue selected. Minute tissue cylinders (typically between 0.6 mm to 2 mm in diameter) are taken from different primary tumor blocks (the 'donor' blocks) and subsequently assembled in an array-like format into one empty 'recipient' block. Regular Microtome can be used to cut sections from these TMA blocks. The recipient block provides a template block that can be utilized for detection and localization studies in tissue-based assays, e.g. immunohistochemistry (IHC) and *in situ* hybridization (ISH) (Kononen *et al.,* 1998; Kallioniemi *et al.,* 2001). Most of the applications of the TMA technology are associated with the field of cancer research, including analysis of the frequency of molecular alterations in large tumor materials, exploration of tumor progression, identification of predictive or prognostic factors and validation of newly discovered genes as diagnostic and therapeutic targets (Kallioniemi *et al.,* 2001). Tissue microarray (TMA) technology allows a massive acceleration of studies correlating molecular *in situ* findings with clinico-pathological information. In this technique, cylindrical tissue samples are taken from up to 1000 different archival tissue blocks and subsequently placed into one empty 'recipient' paraffin block. Sections from TMA blocks

can be used for all different types of *in situ* tissue analyses including immunohistochemistry and *in situ* hybridization. Multiple studies have demonstrated that findings obtained on TMAs are highly representative of their donor tissues, despite the small size of the individual specimens (diameter 0.6 mm). It is anticipated that TMAs will soon become a widely used tool for all types of tissue-based research. The availability of TMAs containing highly characterized tissues will enable every researcher to perform studies involving thousands of tumors rapidly. Therefore, TMAs will lead to a significant acceleration of the transition of basic research findings into clinical applications (Brown and Huntsman, 2007).

1.2.8 Immunohistochemistry (IHC) and Fluorescent *in situ* Hybridization (FISH)

Immunohistochemistry (IHC) is the localization of antigens in tissue sections by the use of labeled antibody as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, radioactive element or colloidal gold. IHC is a molecular technique that combines principles from anatomical, immunological and biochemical techniques for the identification of specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label (Warford, 1994; Wolfe and Herrington, 1997). IHC makes it possible to visualize the distribution and localization of specific cellular components within a cell or tissue. The term immunohistochemistry is often used interchangeably with immunocytochemistry and immunostaining. Immunohistochemistry is widely used in surgical pathology and serves as a diagnostic, prognostic, and predictive tool (Goldstein, 2007).

Fluorescent *in situ* hybridization (FISH) is a molecular cytogenetic technique used for the detection of specific chromosomal rearrangements, and

is applicable to many different specimen types. In FISH, a synthetic DNA molecule, or probe, specifically targeted against the target gene is tagged and then bound to the DNA in the tissue section and detected either directly, or following amplification of signal by secondary antibodies. Unlike most other techniques used to study chromosomes, FISH does not have to be performed on cells that are actively dividing. This makes it a very versatile procedure (Oliveira and French, 2005). FISH is now widely applied to the detection of specific normal and aberrant DNA sequences in both intact cells (either in interphase or in metaphase) and isolated chromosomes. FISH is perceived to be safer and has the added advantage that multiple fluorochromes can be used to discriminate different targets simultaneously (Bartlett, 2004). FISH is now applied in an increasing number of molecular diagnostic areas, including karyotype analysis, gene mapping, disease diagnosis, and therapeutic targeting. Moreover, it has many applications in research: identification of non-random chromosome rearrangements, identification of translocation molecular breakpoint, identification of commonly deleted regions, characterization of somatic cells hybrids, identification of amplified genes, and the study of mechanism of rearrangements. The particular advantage of multicolor (2-4+) FISH, is that gene amplification (*HER-2* in breast cancer), gene rearrangements, microdeletions, chromosomal duplication and viral infections may now be rapidly diagnosed (Bartlett *et al.*, 2001).

1.2.9 Polymerase Chain Reaction (PCR)

A technique, invented in 1987 by Mullis, for amplifying DNA sequences *in vitro* by separating the DNA into two strands and incubating it with oligonucleotide primers and DNA polymerase (Mullis, 1990). It can amplify a specific sequence of DNA by as many as one billion times and is important in biotechnology, forensics, medicine, and genetic research. PCR amplification can turn a few molecules of a specific target nucleic acid (too little to be

analyzed directly or used in biochemical reactions) into as much as a microgram of DNA. Two oligonucleotide primers flank and define the target sequence to be amplified. These primers hybridize to opposite strands of the DNA to serve as initiation points for the synthesis of new DNA strands. A thermostable DNA polymerase, such as *Taq* DNA polymerase, catalyzes this synthesis (Mullis and Faloona, 1987).

Chapter Two

Subjects, Materials

and

Methods

2.1 Samples collection

2.1.1 Iraqi Breast Cancer Patients

Fifty-five Iraqi breast cancer paraffin blocks were collected from patients attended to Jenin Private laboratory, Jenin Private Hospital, Baghdad, Iraq between 2006-2008. The personal informations for each patient were obtained, which included: name, age, and the pathological data, including histologic tumor grade and stage were obtained from the clinical records of the patients and validated by an experienced histopathologist. All cases included invasive ductal and invasive lobular carcinoma and in situ ductal carcinoma type as confirmed by the histopathological records and examinations. Hematoxylin and eosin (H and E) stained slides were prepared from the paraffin embedded blocks and examined by a histopathologist for histopathological diagnosis and determining the degree of differentiation of the tumor.

2.1.2 Italian Breast Cancer Patients

Thirty paraffin blocks were taken from patients attending the department of Biomorphological and Functional Science, Policlinico, University of Naples Federico II, Naples, Italy, clinical and pathological data were also recorded.

2.1.3 Iraqi Benign Breast Lesions

Fifteen samples from Iraqi patients with benign breast lesions were used in this study as control cases, which included (2 fiberoadenomas and thirteen other benign breast lesions).

2.2 Tissue Microarrays (TMAs)

2.2.1 Hematoxylin and Eosin (H and E)

Hematoxylin and Eosin (H and E) stained slides were reviewed by pathologist and areas containing the most representative tumor tissues were marked on both the slides and corresponding paraffin of the donor blocks for tissue microarray construction. The samples were sectioned into 5µm sections then used directly for H and E staining.

2.2.1.1 Materials and Instruments for H and E

1. Ethanol (Carlo Erba, Italy)
2. Xylene (Carlo Erba, Italy)
3. Gill`s Hematoxylin Solution (Bio-Optica, Italy)
4. Deionized Distilled Water
5. Eosin 1% (Carlo Erba, Italy)
6. Superfrost® plus glass slides (Thermo Scientific, GmbH and Co KG, Germany).

2.2.1.2 Method of H and E staining (Kiernan, 1999)

The staining was prepared by using H and E staining system (LEICA ST5020, LEICA, Germany). The instrument programmed as follows for optimum staining:

1. The slides were incubated in oven at 65°C for 15 min for drying
2. The slides then washed in xylene for 10 min (step 2 repeated with fresh xylene) to dissolve the paraffin
3. The slides were transferred to 100% ethanol for 3 min (step 3 were repeated with fresh 100% ethanol)
4. The slides then immersed with 95% ethanol for 3 min, 70% ethanol for 1 min and deionized water for 5 min
5. Stained with Gill's Hematoxylin for 10 min

6. Washed in water for 10 min then deionized water for 1 min.
7. Stained with Eosin 1% for 4 min
8. The slides were washed in water for 5 min, then 95% ethanol for 2 min.
9. The slides were washed with 100% ethanol for 1 min (repeat step 9 with fresh 100% ethanol).
10. The slides were washed with 100% ethanol for 2 min
11. The slides were immersed with xylene for 1 min (step 11 repeated with fresh xylene).
12. Then with fresh xylene for 2 min
13. The slides were mounted and covered slipped by (LEICA CV5020, LEICA; Germany) the slides were left for air dried for at least 2 hr and examined under the double headed light microscope by a histopathologist to choose the most representative area for TMAs construction.

2.2.2 Design and Construction of the Tissue Microarrays (TMAs)

TMAs were constructed from formalin-fixed, paraffin embedded tissues. H and E stained slides with adequate representative tissue were prepared before starting the TMA construction; the goal of the TMA is to present the representative tissue on the array. The optimal method is to mark the area of interest on the H and E slides, cylindrical cores of paraffin embedded tissue were removed from the donor block and transferred to a blank recipient block; the recipient block were prepared to host the donor cores.

The block should not contain bubbles that could generate breakages in the block during punching. Also it was very important to ensure the inside of the cassette filled with paraffin. After building of the TMA, it is very important to choose the recipient stop kit thicker than the donor stop kit. After the construction of the TMA, the recipient block was removed from the machine

and incubated in paraffin at 60°C for 5 min to allow the paraffin penetrating the cores and to avoid the core loss during the sectioning. Then on cold plate -4°C for 1 hr, 5µm sections from the TMA recipient block were made, transferred to a superfrost plus glass slides.

It is very important to be in the right order to facilitate exploitation of the slides under the microscope. The slides were incubated overnight at 37°C to dry, and then used in IHC and FISH studies. It is recommended to perform all the sections at once thus eliminating the losses due to block facing each time.

The distribution and position of the cores were determined in advance with the TMA-designer Software (Alphelys-TMA Designer®2, Plaisir, France).

Three TMA blocks were assembled in this study; two different TMA blocks using the instrument (Minicore, Alyphelys, Plaisir, France as shown in figure (2-1)) inserting all the samples taken from Iraq, a benign breast lesions TMA and a malignant breast TMA were performed. The first included 15 samples from fibroadenomas and mastopathy; the second included 55 malignant samples and the third TMA constructed by using 30 Italian malignant breast samples taken from the Department of Biomorphological and Functional Sciences, Policlinico, University of Naples “Federico II”, Naples, Italy. Each TMA had specimens in triplicate, 45 cores for the benign breast lesions TMA in one recipient block, 168 cores for the 55 Iraqi malignant breast TMA in one recipient block (three of them reference cores in the last three cores were a thyroid tissue). 90 cores for the 30 Italian samples in one recipient block; each core measured 600 µm in diameter and the spacing between the cores 1500 µm. This allows the distribution and positioning of the cores to be easily examined by the histopathologist.

Figure (2-1) Minicore Tissue Microarrayer (Alphelys-TMA, Plaisir, France).

2.3 Immunohistochemistry (IHC)

Immunohistochemical staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumors, specific molecular markers are characteristic of particular cellular events such as proliferation or cell death (apoptosis) (Polak and Van Noorden, 2003).

2.3.1 Materials and Equipments for the IHC

1. Ethanol (Carlo Erba, Italy)
2. Xylene (Carlo Erba, Italy)
3. Distilled Water
4. EnVision™ *FLEX*, Target Retrieval Solution, Citrate Buffer (Low and High pH, 10X) (Dako, Denmark)

5. EnVision™ *FLEX*, Peroxidase-Blocking Reagent (Dako, Denmark)
6. EnVision™ *FLEX*, Antibody Diluent (Dako, Denmark)
7. EnVision™ *FLEX* / HRP (Dako, Denmark)
8. EnVision™ *FLEX*, Substrate Buffer (Dako, Denmark)
9. EnVision™ *FLEX* DAB+ Chromogen (3, 3-Diaminobenzidine, 3 x 3 mL (one drop of DAB for each 1ml of the substrate buffer)
10. Gill`s Hematoxylin Solution counterstain (Bio-Optica, Italy)
11. EnVision™ *FLEX*, 10X Wash Buffer (Phosphate Buffered Saline + 1% Tween 20) diluted in distilled water (Dako, Denmark)
12. DAKO Autostainer (Universal staining system, USA) as shown in figure (2-2)

Figure (2-2) DAKO Autostainer, Universal staining system.

13. Dako Water Bath (DakoCytomation, USA)
14. LEICA CV5020 Mounting System (LEICA, Germany)
15. Coplin Jars (Bio-Optica, Italy)
16. Slides Rack (25 Slides each)
17. Fume Hood (Bio-Optica, Italy)
18. Double Headed Microscopy (LEICA DME, LEICA, Germany)
19. Liquid Blocker, Super pap pen (Daido Sangyo, Japan)
20. DPX mounting medium, which is a mixture of Distyrene (a polystyrene), plasticizer (tricresyl phosphate), and xylene (Carlo Erba, Italy)
21. Micropipette different volumes (pipetman, France)
22. Oven (Heraeus, Germany)
23. Lab Marker
24. Glass cover slips.
25. Primary Antibodies the details of the antibodies shown in table (2-1)

Table (2-1) Primary antibodies used in this study

No.	Antibody	Company	Code	Clone	Dilution
1	Monoclonal Mouse Anti Human ER	DakoCytomation	M7047	1D5	1/150
2	Monoclonal Mouse Anti Human PR	DakoCytomation	M3569	PgR636	1/150
3	Monoclonal Mouse Anti Human Ki67	DakoCytomation	M7240	MIB-1	1/25
4	Monoclonal Mouse Anti Human p53	DakoCytomation	M7001	DO-7	1/50
5	Polyclonal Rabbit Antihuman c-erb-2 oncoprotein (HER-2)	Dako	A0485		1/250

2.3.2 Method of IHC (Ramos-Vara, 2005)

Immunohistochemistry studies were performed on the three TMAs under the same conditions using DAKO Autostainer, Universal staining system, USA. The staining performed as the instruction of the EnVision™ *FLEX* detection system from Dako, the program and protocol used as follows:

1. The design of the program, the number of the slides, the position of the slides, the amount of the solutions, the amount of the solution to be added, the incubation time for the solutions and the washing steps were programmed previously. The program for each run was designed depending on the number of the slides used during the run (maximum 48 slides) and the position of the dropping of the solutions (three sites on the slide for each site 100 µl maximum 300µl for a slide).

2. Deparaffinizition of the TMAs slides were performed by passing of the slides in xylene (twice) and graduated dilutions of alcohol (100% twice, 95%, 70%, and 50%) and incubated in distilled water for 10 min.

3. Demasking (Antigen Retrieval) for the TMAs slides were performed by incubating the slides in (EnVision™ *FLEX*, Target Retrieval Solution, Citrate Buffer; pH9 for ER, PR and p53, pH6 for HER-2 and Ki67) for 60 min at 97°C in water bath. The 60 minute optimal incubation time was determined following several trials using different incubation times (20, 30, 45, 60 and 90 min) for Ag retrieval of Iraqi TMA samples. The slides were left to cool for 20 min at room temperature then the edges surrounding the TMA samples were marked by the liquid blocker pap pen to avoid the distribution of the materials out of the samples during the run of the IHC.

4. The TMAs slides were transferred quickly to the autostainer racks (DAKO Autostainer, Universal staining system) and drying of the samples was avoided by adding the cooled antigen retrieval solution.

5. All the TMAs slides were washed once with washing buffer, EnVision™ *FLEX*.

6. Peroxidase-Blocking Reagent was added for all the TMAs slides and incubated for 10 min. It contains H_2O_2, which is the substrate for peroxidase, because some cells or tissues contain endogenous peroxidase. Using HRP conjugated antibody may result in high, non-specific background staining; this non-specific background can be significantly reduced by pre-treatment of cells/tissues with hydrogen peroxide prior to incubation with HRP conjugated antibody.

7. TMAs slides were washed with the washing buffer, blocking reagent (Ab diluent) was added for 30 min to block the excess site of another nonspecific proteins and reducing the background formation.

8. TMAs slides washed with washing buffer.

9. The optimum dilution of each primary antibody (the dilution of each Ab and the volume were determined and optimized previously as shown in table 2-1) was added to the TMA slides, incubated for 60 min then washed vigorously with washing buffer. Incubations without the specific antibodies were used as negative controls. MCF-7 breast cancer cell line slides were used as a positive control.

10. EnVision™ *FLEX* / HRP added and incubated for 40 min then washed with washing buffer.

11. EnVision™ *FLEX* DAB+ Chromogen 3, 3-Diaminobenzidine was added and incubated for 10 min (the addition and incubation time of DAB is very important and previously optimized to avoid the strong

background formation in the stained slides that will interfere with the results) and washed vigorously two times.

12. Gill`s Hematoxylin Solution added as a counterstain for 4 min, the slides were washed vigorously with water.
13. Dehydration was done for the slides, then mounting by a DPX mounting medium was done using LEICA CV5020 mounting system. The stained slides were left to dry at room temperature in the fume hood for at least one hr. The stained TMAs slides were scored together with a qualified pathologist using a double headed light microscope.

Cut off values for all the antibodies used in the study were done with the help of a pathologist. According to DAKO protocol cut off values of HER2 receptor were done according to DAKO scoring system, using the following categories: 0, negative result or membrane staining in <10% of the tumor cells; 1+; weak and incomplete membrane staining in >10% of the tumor cells; 2+; weak or moderate, complete membrane staining in >10% of the tumor cells; 3+; strong complete membrane staining in >10% of the tumor cells). Negative and 1+ indicated normal HER-2 expression; 2+ was reserved to intermediate cases, whereas 3+ identified cases showing deregulated HER-2 expression.

The scoring for ER, PR was done as described by Allred *et al.* (1998). Staining signal was scored; two parameters evaluated in immunohistochemical preparations of hormone receptors are the number of tumor cell nuclei stained and the intensity of the reaction. For estrogen receptor and progesterone receptor, every tumor was given a score which represents the outcome of the multiplication of the intensity of the staining (no staining = 0; low level = 1; medium staining = 2; strong staining = 3) by the percentage of stained cells (0% = 0; under 10% = 1; 10-50% = 2; 51-80%

= 3; over 80% = 4). Using this system, the maximum score is 12 (with over 80% of the cells showing strong staining). Many laboratories set a threshold for ER positivity at a minimum of 10% nuclear staining. The common scoring system includes "-"(<10%), "+" (10%-30%), "++" (30%-80%) and "+++" (>80%). Other laboratories may use 5% of nuclear staining as the threshold for ER positivity. However, National institute of health recommends that any positive ER staining is considered as a positive result. The intensity of marker expression was quantified using the following scores: 0=negative, 1=weakly positive, 2=moderately positive, 3=strongly positive, the same were applied for PR.

Scoring of Ki67 was employed according to Tan *et al.* (2002): only distinct nuclear staining of invasive carcinoma cells was used for scoring via the light microscope, which was determined semiquantitatively as nil (no immunostaining), low (10% or less immunopositivity) or high (>10% immunoreactive cells).

Scoring of p53 was used according to Sophia *et al.* (1999). Positive nuclei staining of p53 in at least 10 % of the cell nuclei were considered p53 over-expression, while those with less than 10 % positive cell nuclei were considered normal expression. (Negative, score 0; weak or mild staining (5 to < 10 % score 1); moderate staining (10 to < 25 % score 2); strong staining (25 to < 50 % score 3) and highly strong staining (over 50 % score 4).

2.4 Fluorescent *in situ* Hybridization (FISH)

The sample DNA (metaphase chromosomes or interphase nuclei) is first denatured, a fluorescently labeled probe of interest is then added to the denatured sample mixture and hybridizes with the sample DNA at the target

site as it re-anneals back into a double helix. The probe signal can then be seen through a fluorescent microscope and the sample DNA can be scored for the presence or absence of the signal. The FISH procedure essentially requires 2 types of DNA, target and probe. Target DNA is a heterogeneous population of DNA molecules immobilized on a solid surface (e.g., glass slides) as either chromosome spreads (e.g., metaphase spreads) or interphase cells (e.g., tissue sections or cytological preparations). Probe DNA, by contrast, is a population of labeled DNA molecules specific to certain chromosomes, chromosome regions or genes. Commonly used fluorochromes include fluorescein (green signal), rhodamine (red signal) and Texas Red (red signal) (Wilkinson, 1998). Vysis DNA FISH Probes were hybridized to cells obtained from FFPE samples, samples deparaffinized and pretreated to maximize tissue permeability and hybridization. After denaturation of the sample DNA, the probe is applied on the sample slide and hybridization occurs. After hybridization, unbound probe is removed via rapid wash procedure followed by application of the counterstain to detect the cell nucleus.

2.4.1 Materials and Equipments for FISH

1. 4-6-Diamidino-2-phenylindole (DAPI) II (Vysis 32-804831, USA)
2. Formamide (Fisher, USA)
3. NP-40 (Vysis #32-804818, USA)
4. 20x SSC (Vysis #32-804850, USA) 20X SSC is a powder containing sodium chloride and sodium citrate. It is to make 20X SSC solution and subsequent dilutions (2X SSC and 0.4X SSC) for denaturization and wash solutions.
5. Probes (minimize light exposure)
6. Denaturant solution 49 ml Formamide, 7 ml 20xSSC, 14 ml dH_2O, pH to 7.0-8.0, store at 4˚C
7. Ethanol washing solutions 70%, 85% and 100%.

8. Hybrite instrument for hybridization (Vysis, USA)
9. Olympus Fluorescent Microscope BX61 (Japan).

2.4.2 Method of FISH

The method used in this experiment as recommended by the Vysis and supplied with the Kit.

2.4.2.1 Pre-Preparation

1. 5 µm thick slides incubated for 30 min in 2X SSC (pH 7.0) prewarmed to 37˚C in a coplin jar.
2. Slides dehydrated sequentially in 70%, 85% and 100 % ethanol series, 2 min each. Air dried.

2.4.2.2 Probe Preparation

1. 7 µl buffer for the probes
2. 1 µl distilled water
3. 1 µl of each probe, CEP6 SpectrumGreen probe for cytomer and CEP17 SpectrumOrange probe for *HER-2* gene
4. Centrifuged 1-3 seconds
5. Vortex and recentrifuged
6. Heated for 5 min. at 73˚C in a water bath to denature (Always water bath set 2˚C higher and measure temperature inside coplin jar)
7. Used immediately (or kept for a short while longer at 73˚C if required)

2.4.2.3 Slide preparation

1. Denaturant solution (70% formamide/ 2x SSC) placed in 73˚C water bath inside coplin jar
2. Slides denatured for 5 min.
3. Dehydrated in 70%, 85% and 100% ethanol for 2 min. each. Air dried.

4. 10 µl denatured probe applied and covered with a cover glass. Hybridizing area marked on the slides using a diamond scribe. Sealed carefully with rubber cement.
5. Slides placed in a prewarmed humidified box (Hybrite), wrapped in metal foil to protect against light and incubated overnight at 42˚C.

2.4.2.4 Rapid wash procedure

1. 0.4x SSC/ 0.3% NP-40 placed in a 73˚C water bath
2. Cover glass removed and immediately placed into wash tank with 0.4x SSC/ 0.3% NP-40
3. All the slides left in coplin jar for 2 min
4. Slides placed in 2x SSC/ 0.1% NP-40 at room temperature 1 min
5. Air dried in darkness
6. 20 µl of DAPI solution applied to the target area and put on cover glass
7. Slides examined on a fluorescence microscope to score the results. Photos of the amplified and non amplified *HER-2* gene were taken from the samples.

Scoring of FISH for *HER-2* gene was performed; the *HER*-2 gene and centromere chromosome 17 (CEP17) signals were manually counted using a fluorescent microscope. A *HER-2*/CEP17 ratio of equal or more than two was considered amplified, and no amplification if less than two scoring were performed for tumor cells between 20 to 60 tumor cells, The PathVysion (Vysis Inc., Downers Grove, IL, USA) test requires a ratio (*HER-2* gene to CEP 17) of 2.0 or greater for the sample to be considered amplified. All the detailed informations about the IHC and the FISH assays for *HER-2* gene were shown in figure (2-3)

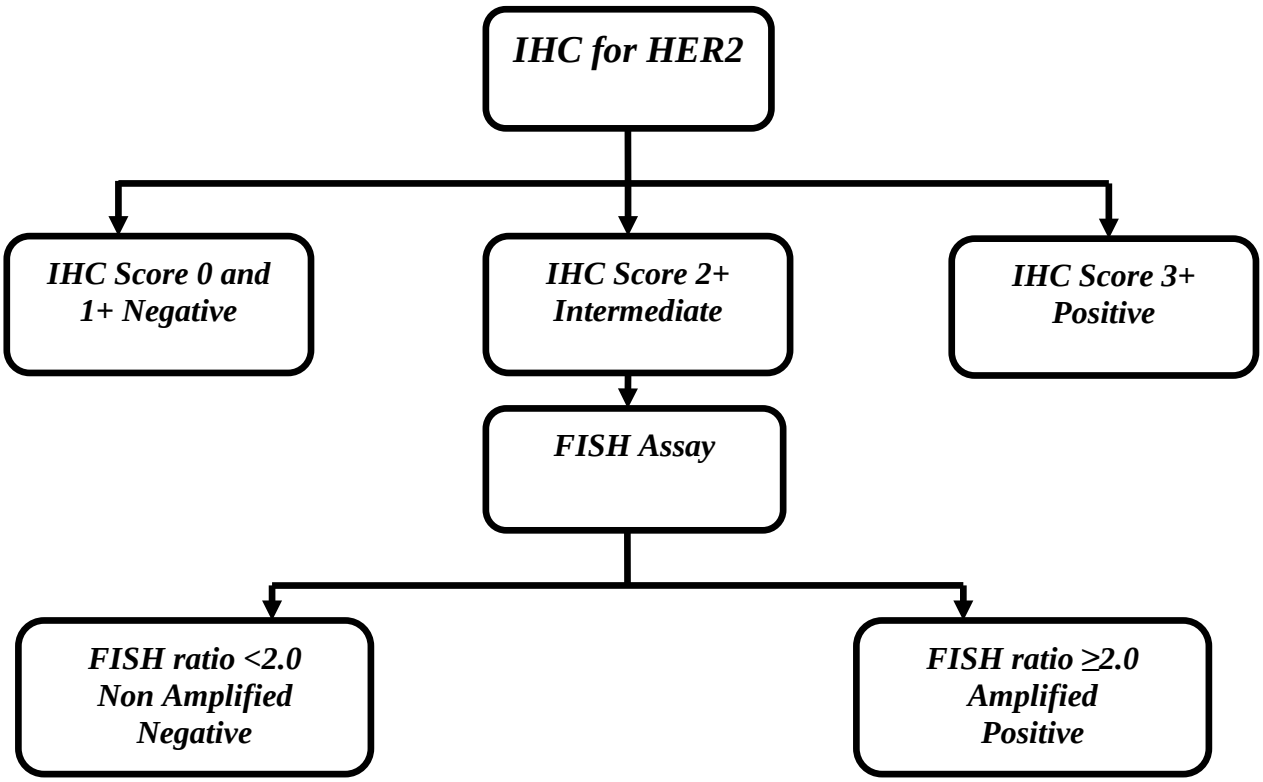


Figure (2-3) Algorithm of Immunohistochemistry (IHC) and Fluorescent *in situ* Hybridization (FISH) for *HER-2* gene

2.5 Isolation of DNA from Formalin-Fixed, Paraffin-Embedded Tissues (FFPE)

The standard practice in pathology laboratories, however, is to collect and store clinical tissue as formalin-fixed, paraffin-embedded (FFPE) samples. The protocol describes the removal of paraffin by extraction with xylene and then with absolute ethanol; the tissue samples then processed according to the QIAamp DNA extraction from FFPE Tissue Kit 50 protocol.

2.5.1 Materials and Equipments for Isolation of DNA from FFPE Tissues

1. (QIAGEN, Catalog Number 56404, Germany)
2. Xylene (Carlo Erba, Italy)
3. Absolute Ethanol (Carlo Erba, Italy)

4. Microcentrifuge Tube (2 ml)
5. Collection Tubes (2ml x150 pieces)
6. MiniElute Columns (50 Spin Column)
7. ATL Buffer (10 ml); before starting the procedure, the ATL Buffer checked whether the precipitate has formed. If necessary, dissolve by heating to 70°C with gentle agitation
8. Proteinase K 20 mg\ml (6 ml)
9. AL Buffer (12ml); before starting the procedure, the AL Buffer was checked whether containing a precipitate or not. The precipitate dissolved by heating to 70°C with gentle agitation
10. AW1 Buffer concentrated (19 ml). Preparing Buffer AW1 was done by adding 25 ml ethanol (96–100%) to the bottle containing 19 ml Buffer AW1 concentrate. Reconstituted Buffer AW1 can be stored at room temperature (15–25°C) for up to1 year.
11. AW2 Buffer concentrated (13 ml). Preparing Buffer AW2 was done by adding 30 ml ethanol (96–100%) to the bottle containing 13 ml Buffer AW2 concentrate. Reconstituted Buffer AW2 can be stored at room temperature (15–25°C) for up to 1 year.
12. Distilled Water
13. Vortex (Vibromix)
14. Eppendorf Centrifuge 5415D (Eppendorf, Germany)
15. Thermomixer Comfort (Eppendorf, Germany).

2.5.2 Method of DNA Isolation from FFPE Tissues

The method followed as described by QIAamp DNA extraction from FFPE Tissue Kit 50 protocol.

1. Cut 2-3 sections of 10 µm of FFPE and added into 2 ml microcentrifuge tube.

2. 1200 µl of fresh xylene were added mixed well, vortex vigorously for 15 seconds.
3. Centrifuged at full speed for 5 min at room temperature
4. Supernatant removed by pipeting, (care must be taken to avoid losing any of the pellets).
5. Steps (2, 3 and 4) repeated.
6. 1200 µl ethanol (96-100%) were added to the pellet to remove residual xylene and mixed gently by vortexing.
7. Centrifuged at full speed for 5 min at room temperature
8. The ethanol removed carefully by pipeting, (care must be taken to avoid losing any of the pellets).
9. Steps (5-7) repeated once.
10. The opened microcentrifuge tubes were incubated at 37°C for at least 10-15 min until the ethanol has completely evaporated (make sure that the ethanol completely evaporated).
11. The tissue pellet resuspended in 180 µl of ATL.
12. 20 µl of Proteinase K were added to the samples, mixed by vortexing and incubated at 56 °C in the Thermomixer Comfort until the tissues were completely lysed (it is preferred to be overnight). Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on rocking platform.
13. The microcentrifuge tubes briefly centrifuged to remove the drops from the inside of the lid, 200 µl of AL buffer added to the samples, mixed by pulse-vortexing for 15 seconds, and incubated at 70°C for 10 min.
14. 200µl of ethanol (96-100%) added to the samples, and mixed by pulse-vortexing for 15 seconds.
15. The mixture including the precipitate was carefully applied to the QIAamp spin column (in a 2 ml collection tube) without wetting the

rim. The cap closed tightly, and centrifuged at 8000 rpm for 1 min. The QIAamp spin column placed into a clean 2 ml collection tube and the tubes containing the filtrate were discarded.

16. The QIAamp spin column opened carefully and 500 µl of AW1 buffer added without wetting the rim. The cap closed tightly and centrifuged at 8000 rpm for 1 min. The QIAamp spin column placed in a clean 2 ml collection tube and the collection tube containing the filtrate discarded.
17. The QIAamp spin column opened carefully and 500 µl of AW2 buffer added without wetting the rim. The cap closed tightly and centrifuged at full speed at 14000 rpm for 3 min. The QIAamp spin column placed in a clean 2 ml collection tube, and the collection tube containing the filtrate discarded.
18. The QIAamp spin tube opened carefully and 200 µl distilled water added and incubated at room temperature for 3 min, and then centrifuged at 8000 rpm for 1 min.

2.6 Quantification of the DNA Concentration

Quantification is performed based on the spectrum measurement at the defined pathway. The concentration of extracted DNA was measured by using spectrophotometric method (Sambrook *et al.*, 1989). The principle of spectrophotometric measurement depends upon the amount of UV irradiation absorbed by the nitrogen bases composing the DNA. One µl of the sample can be measured without using a cuvette or capillaries by using NanoDrop (ND-1000 UV-Vis Spectrophotometer, (NanoDrop Technologies, USA). The NanoDrop ND-1000 is a full-spectrum spectrophotometer (UV and visible spectrum, 220-750 nm) for measuring the absorbance of DNA, RNA, proteins and dyes.

2.6.1 Materials and Equipments for DNA Quantification

1. NanoDrop Spectrophotometer (ND-1000 UV-Vis Spectrophotometer, From NanoDrop Technologies, USA)
2. DNA samples in Distilled Water
3. Distilled Water used as blank
4. Micropipette (pipetman , France)

2.6.2 Method of DNA Quantification

The newly developed sample retention system allows for measurement of small samples volumes: the sample droplet is held in place by surface tension when it is slightly compressed between the pedestal and the sample arm; this generates the defined pathway of 1 mm. The spectrum measurement is then performed with two optical fibers installed in the pedestal (emitting light of a Xenon lamp) and the sample arm (spectrometer with linear CCD array). Quantification is performed based on the spectrum measurement at the defined pathway of 1 mm. DNA, RNA, protein or dye in 1 µl can be measured without using a cuvette or capillaries.

One µl of the distilled water was measured as a blank, and then 1 µl of the samples applied sequentially between each two samples. Clean the sample arm with a fresh dry Kleenex tissue and then apply the next sample to the sample arm and measure the DNA concentration. Measurement only takes 10 sec. Sample measurements are possible over a wide range of concentrations. Multiple types of readings can be performed; for example, DNA and RNA samples absorbance are measured at 230 nm, 260 nm and 280 nm. The absorbance values, concentrations and the ratios at 230/260 nm and 260/280 nm (to evaluate purity of DNA) are calculated by the NanoDrop ND-1000 software and displayed on the attached computer (PC with Microsoft Windows 2000). All measurements were automatically stored in a table and archived in the computer.

To determine the degree of contamination of the DNA with protein, a measurement at 280nm and the ratio A260/A280 were calculated. Pure DNA will give an A260/A280 ratio of 1.8 or higher. Values for A260/A280 of less than 1.8 indicate contamination of the DNA samples. For an A260/A280 value of 1.5, the percentage of protein in the DNA preparation is about 50%. For good PCR results, DNA is required with an A260/A280 ratio of 1.6 or higher. The purity and concentration of the DNA is of a crucial importance for optimal test results.

2.7 PCR for Exon 11 of the BRCA1 Gene

2.7.1 Primers design

The primers used in this study were oligonucleotides complementary to the sequence flanking the exon 11 of BRCA1 gene. The oligonucleotide primers sequence were designed depending on the website www.primer3.com. The primers were synthesized and quality controlled in the synthesis unit at the CEINGE Biotecnologie Avanzate. The features of the primers shown in table (2-2).

2.7.2 Materials and Equipments for the PCR

1. PCR Primers

0.75 µl is used from each primer (forward and reverse) for each 25 µl final volume of the reaction components in a concentration about 2 mM. Before this, many pairs of primers (6 pairs) designed for amplification of BRCA1 genes were tried in Italy, together with optimization for the DNA concentration, primer concentration, buffer components and other factors were implemented to give a good amplification for these genes. These modifications, then, were applied in this study.

Table (2-2) Oligonucleotide primer sequences for BRCA1 gene exon11

Primer for BRCA1 Exon11	Length	Tm	Sequence 5´-3´	GC%	Product Size
Forward	**18b**	**56**	**GCCAGTTGGTTGATTTCC**	**50**	**185 bp**
Reverse	**19b**	**57**	**CACGCTTCTCAGTGGTGTT**	**52**	**185 bp**

2. Master Mix Buffers

HotMasterMix were supplied from 5 PRIME HotMasterMix. 5 PRIME HotMasterMix is a 2.5x concentrated, ready-to-use reagent mix for performing methods classified as "hot start" PCR with minimal pipettings steps. The kit contains all reagents necessary to perform the polymerase chain reaction except primer and template DNA. The 5 PRIME HotMasterMix contains HotMaster *Taq* DNA Polymerase (50 U/ml), 2.5x HotMaster *Taq* Buffer pH 8.5 with 6.25 mM $Mg(OAc)_2$, 500 µM of each dNTP and stabilizers. This corresponds to final concentrations in the PCR reaction of 1.0 U/50 µl *Taq* DNA polymerase, 45 mM KCl, 2.5 mM Mg^{2+}, and 200 µM of each dNTP.

3. Molecular Biology Grade Water, nuclease-free
4. DNA Template (Samples positive and negative controls).
5. MyCycler (Thermal cycler, BIO-RAD, USA).
6. Vortex (Vibromix)
7. Eppendorf centrifuge 5415D (Eppendorf, Germany)
8. Set of Micropipettes Eppendorf (Eppendorf, Germany)
9. 2 ml eppendorf tubes
10. 200µl PCR tubes

2.7.3 PCR Protocol

Before starting the preparations, it is very important to mix the 5 PRIME HotMasterMix thoroughly to avoid localized differences in salt concentrations and all the other components of the PCR reaction.

1. Molecular Biology Grade Water was added in a 2 ml eppendorf tube (12 µl of the Molecular Biology Grade Water used for each 25µl final reaction volume).

2. 5 PRIME HotMasterMix were added into a tube containing the water in a volume (10 µl for the 25 µl final reaction volume).

3. Primers (forward and reverse) were added in a volume 0.75 µl for each 25 µl final PCR reaction volume. The tubes were closed and mixed well by vortexing, then centrifuged briefly to collect the liquid.

4. 24 µl of the reaction mix were dispensed into a 200 µl PCR tubes and the genomic DNA was added (depending on the DNA concentration one µl from the 100ng/µl for the DNA isolated from FFPE). The tube closed and mixed well by vortexing. The tubes centrifuged briefly to collect liquid at bottom of tubes. A positive control, a sample well amplified by these primers, was extracted from FFPE. The negative control contains all the PCR reaction mix plus molecular grade distilled water instead of the target DNA or genomic DNA.

5. PCR program designed using the thermal cycler. The thermal cycler was preheated (>90°C) before placing the PCR tube(s) on the cycler block.

6. The PCR protocol used in this experiment is called touchdown PCR, a type of modified method of PCR used to amplify samples that are very

difficult to amplify by normal PCR. This method allows to add an intermediate steps that amplify degraded and difficult samples.

24 samples out of 30 samples used in this study were amplified. All the samples used in this study were Italian. Extraction of DNA and RNA from the Iraqi samples produced a good quantity of the DNA and RNA, but the quality of the extracted DNA and RNA were not good enough to use these samples in the PCR studies like Normal PCR, Real Time PCR and Oncotype test.

The PCR protocol used in this study was:

94°C for 5 min initial denaturation

(94°C for 30 seconds intermediate denaturation, 45°C for 25 seconds annealing step, 72°C for 30 seconds intermediate extension)

94°C for 30 seconds final denaturation,

57°C for 25 seconds primer annealing,

72°C for 30 seconds then 72°C for 7 min elongation and 4°C on hold.

These steps were repeated for 40 times to produce the final product of the PCR.

2.7.4 Agarose Gel Electrophoresis for PCR Product

PCR products were detected by agarose gel electrophoresis and ethidium bromide staining at a final concentration of 0.5µg/ml for 45 minutes, the DNA bands visualized under UV transilluminator at 356nm. Results were documented using gel documentation system and printed directly using thermal printer.

2.7.4.1 Materials and Equipments of Agarose Gel Electrophoresis

1. Agarose 1.5 % (Sigma, USA) was prepared by adding 1.5 g of agarose to 100 ml of 1x Tris, Acetic acid, EDTA (TAE) buffer mixed well and dissolved by heating (avoid evaporation) then cooled for 10 min at

room temperature. Then poured into the gel tray and left for 20 min for solidification, then transferred to the electrophoresis system to start the run after loading the samples

2. 50% TAE (1x 40ml Tris, 20 mM Acetic Acid, and 1 mM EDTA set the pH 8.3, BIO-RAD, Catalog No. 161-0773, USA)
3. Ethidium Bromide. 1µl added to the gel when it was at 70°C, mixed well to visualize the band by the UV transilluminator
4. Loading Dye Bromophenol blue
5. 100 bp DNA Ladder (New England Biolabs, USA)
6. Microwave (SEVERIN)
7. Vortex (Vibromix)
8. Power Supply (Consort, Belgium)
9. Gel Documentation and UV transilluminator system (BIO-RAD, USA)
10. Distilled Water

2.7.4.2 Method of Agarose Gel Electrophoresis

When the PCR run reach holding, the gel transferred to the tank and filled with the TAE buffer to a limit covering the gel. The samples were taken for agarose gel electrophoresis. 5 µl from each sample were mixed well with the 1 µl loading dye. The 100 bp DNA ladder (marker) loaded in a volume of 2.5µl in the first well then the samples were loaded into the wells of the gel in volume of 5 µl in an ordered form. Positive control and negative control are then added in the last two wells. The voltage used for the electrophoresis 5 volts/cm of the gel. 45 min later the distance of bands were checked by UV transilluminator system. Video printed photos were made by gel documentation.

2.7.5 DNA Sequencing

Sequencing of the PCR product for the 24 samples and the positive control was done in the sequencing unit at the CEINGE Biotecnologie

Avanzate, Naples, Italy. Sequencing of the DNA products was performed for the 48 samples. In the beginning purified and then sequenced at the sequencing unit in CEINGE Biotecnologie Avanzate. After sequencing, the results were analyzed by the Codon Code Aligner program. Detection of the mutations in the exon11 of BRCA1 gene was performed with the comparison of international database in National Center for Biotechnology Information (NCBI).

2.9 Statistical Analyses

Chi-square test and mean ± S.D. were used for the clinicopathological studies. ANOVA test and *P* value were used for IHC and FISH studies, 2-tailed significance for correlation between the five tumor markers (ER, PR, Ki67, p53 and HER-2) all the statistical analyses were carried out in SPSS version 13.0 (SPSS, Inc., Chicago, USA) and Microsoft Excel.

Chapter Three

Results

and

Discussion

3.1 Clinicopathological Characteristics of the Patients

Fifty five Iraqi breast cancer samples were included in this study to design and construct a TMA. All samples were from females. The mean age of the patients (49.38±8.29) years old; ranging from (36-69) years old; eight cases were under (40) years old (14.5%); twenty cases between (40-49) years old about (36.36%); ninteen cases were between (50-59) years old about (34.54%) and eight cases above (60) years old about (14.5%). Fortyeight cases (87.3%) had invasive ductal carcinoma (moderately differentiated); while five cases had invasive ductal carcinoma (poorly differentiated); one case was suffering from invasive lobular carcinoma and one case was suffering from invasive ductal carcinoma papillary type (Table 3-1).

Thirty Italian breast carcinoma samples were included in the present study to design and construct a TMA taken from the archive of the department of Biomorphological and Functional Science, Policlinico; University of Naples Federico II, Italy. The mean age of the Italian patients (57.00 ±8.52); the range (39-73) years old. One case was under forty years old (3.3%); five cases were between (40-49) (16.6%); twelve cases between (50-59) (40%); ten cases between (60-69) (33.3%); and two cases above (70) years old (6.6%). The diagnostic report for the patients revealed that all the samples were invasive ductal carcinoma (moderately differentiated). The clinical and diagnostic data showed that the avearage age of the Iraqi patients with breast cancer were lower than the Italian patients, so the risk factor of the age for the Iraqi population was lower than the Italian population (Table 3-1).

Fifteen samples were used as control cases; thirteen cases were having benign breast lesions (simple cystic mastopathy) and two were fibroadenomas cases. These samples taken from Iraqi population were included in the present study as control cases. The diagnostic data were shown in table (3-2).

Table (3-1) Clinicopathological characteristics of the breast cancer patients

Parameter	Iraqi Samples (55)		Italian Samples(30)	
Age (Years)	No.	%	No.	%
<40	8	14.5	1	3.3
40-49	20	36.4	5	16.2
50-59	19	34.5	12	40
≥60	8	14.5	12	40
Tumor Type				
Invasive ductal carcinoma				
Moderately Differentiated	48	87.3	30	100
Poorly Differentiated	5	9		
Papillary Type	1	1.8		
Invasive Lobular carcinoma	1	1.8		
Total	55	100%	30	100%
Tumor Size (cm)				
<2	7	12.7	11	36.7
2-5	39	70.9	13	43.3
≥5	9	16.4	6	20
Histological Grade				
I	4	7.2	7	23.3
II	36	65.4	16	53.3
III	15	27.3	7	23.3
Axillary Lymph Node				
0	8	14.5	14	46.7
1-3	34	61.8	11	36.7
>3	13	23.6	5	16.2

Table (3-2) Clinicopathological data of the control cases

Parameter	Iraqi Benign Lesion Samples (15)	
Age (Years)	**No.**	**%**
<40	**5**	**33.3**
40-49	**7**	**46.7**
50-59	**3**	**20**
Tumor Type		
Fiberoadenoma	**2**	**13.3**
Benign Breast Lesion	**13**	**86.7**

The present results on Iraqi patients revealed that a high age frequency of cancer occurred between (40-49) years old (36.36%). The Italian breast cancer group showed the highest frequency at the age of (50-59) (40%). The comparison between the two populations revealed that the average age between the two populations was lower and the appearance of breast cancer was earlier in Iraqi women. This may be attributed to many factors, such as environmental factors, the nutrition, low exercise, poor health education. The exposure to a high dose of depleted uranium may be one of the reasons for the increased breast cancer risk in the Iraqi community. Furthermore, there are no national screening programs for the breast cancer patients in all the provinces of the country.

The present results agreed with many studies in Iraq performed on breast cancer and revealed that the peak of age frequency in the Iraqi breast cancer patients was 44.5 years, and that 76.8% were under 50 years (Al-Sanati, 2009). Al-Anbari (2009), revealed a mean age of the 48.7 years and that 32.6% of the cancer patients were in the peak age frequency of 40-49 years.

The risk of breast cancer is higher in middle-aged and elderly women than in young women. This risk increases as a woman ages, rising sharply after the

age of 40. In the United States, more than three-fourths of all breast cancers occur in women aged 50 or older (Wu *et al.*, 2002).

Breast cancer affects up to one in eight women in developed countries with a median age of 61 years at diagnosis. Approximately 2% of breast cancers occur in young women between 20 and 34 years of age and 11% between 35 and 44 years of age (Ries *et al.*, 2008). In USA during 2002-2006, 50% of women who developed breast cancer were at the age 61 or younger at the time of diagnosis (American Cancer Society, 2010).

3.2 Design and construction of the three TMAs

The results of the present study demonstrated that TMAs successfully used to test IHC and FISH for the Iraqi and Italian samples. Furthermore, this technique was applied and validated, for the first time, on Iraqi samples. Samples of the TMA paraffin blocks are shown in figure (3-1).

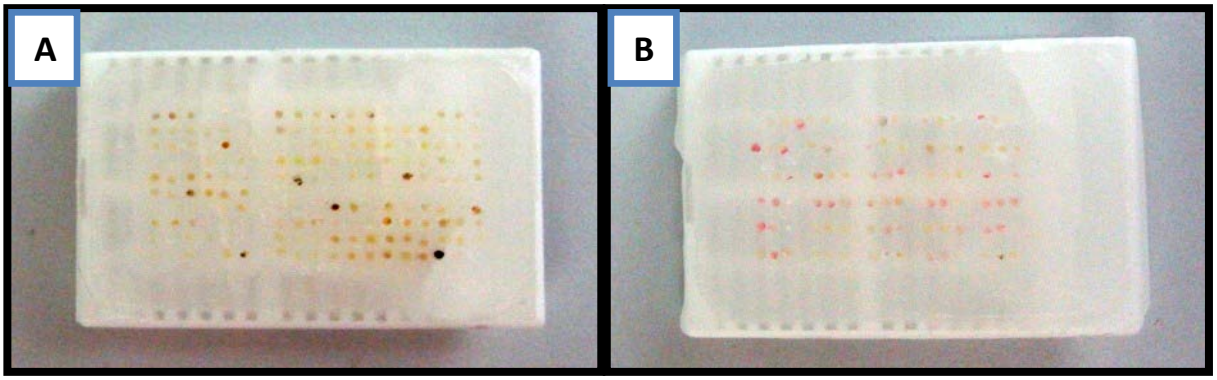


Figure (3-1) Samples of the TMA blocks (A) TMA contains the 55 Iraqi Breast cancer cases in a triplicate core 168 cores, (B) TMA block contanis the 30 Italian Breast cancer samples triplicate 90 cores.

The design of the TMAs program and the distribution of the samples on the recipient block were performed by Minicore TMA designer program. In the same time, the recipient block must be prepared in advance for the TMAs

construction to avoid the breakage of the block. Figure (3-2) shows the samples of the TMA slide and samples of the cores stained by IHC.

The results of TMAs demonstrated that the minute tissue samples in any array format can be sufficiently representative of their donor tumors to establish association between the molecular alterations and the clinical data.

High-throughput tissue microarray technology facilitates the assessment of the clinical relevance of molecular markers by enabling the simultaneous analysis of hundreds of tissue specimens. The widespread adoption of TMAs in many laboratories replaces the conventional one-slide–one-section approach, in which individual archival specimens were placed on separate microscope slides, with the ability to assess RNA, DNA, or protein expression in hundreds of individual patient specimens in a single experiment (Henshall, 2003).

The high speed of arraying, the lack of a significant damage to donor blocks, and the regular arrangement of arrayed specimens, which greatly facilitate automated analysis, are the most significant advantages of the TMA technology over previous concepts of analyzing multiple different tissues in one paraffin block (Kononen *et al.*, 1998).

The use of TMA sections for the measurement of breast cancer prognostic and predictive markers using both IHC and *in situ* hybridization has been validated (Zhang *et al.*, 2003). Similarly, TMA would be useful for evaluating chromogenic *in situ* hybridization (CISH), another technique for HER-2 analysis. Recent data have suggested that CISH is comparable to FISH for HER-2 analysis (Gupta *et al*., 2003).

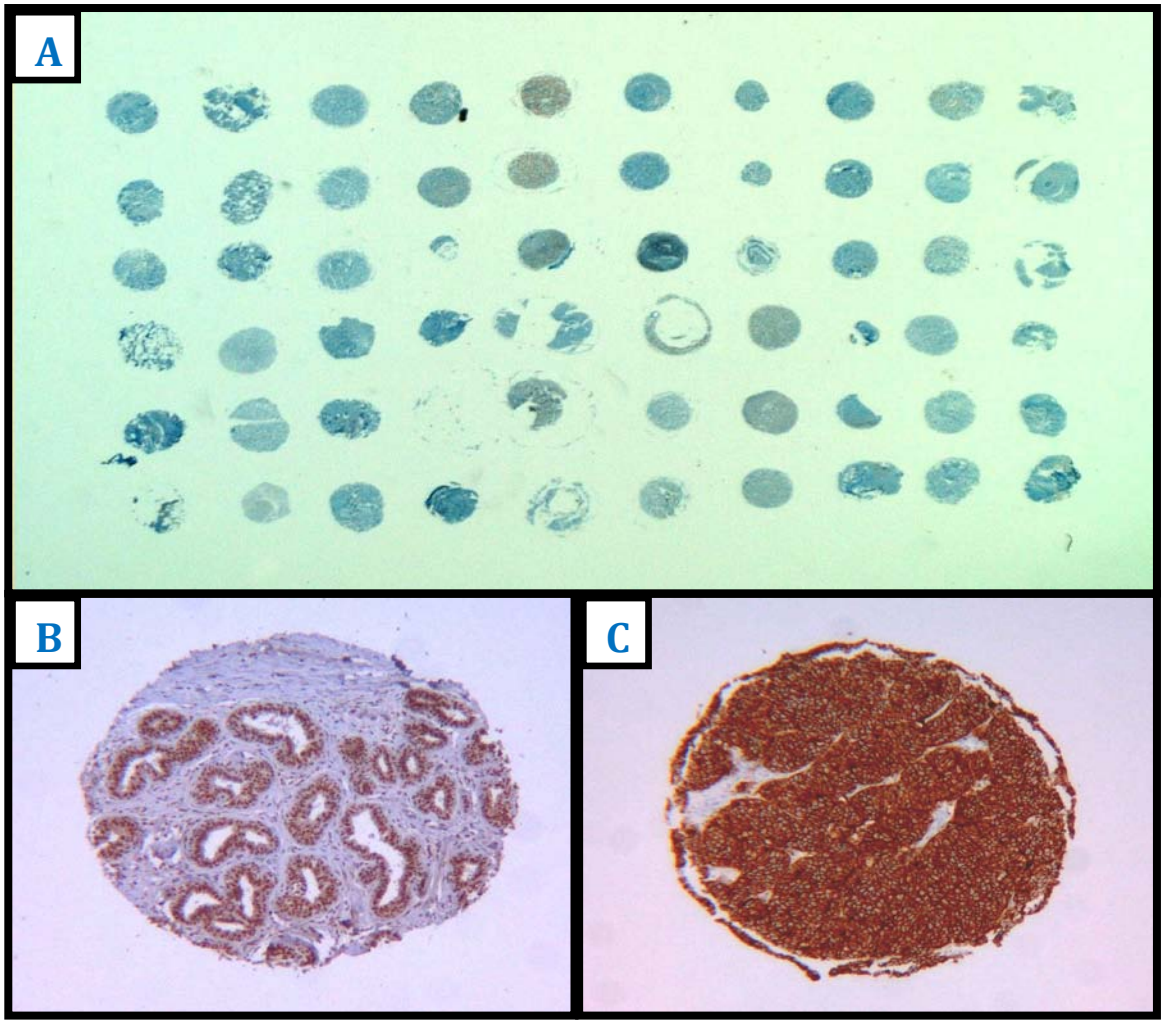


Figure (3-2) Samples of the TMA slides, (A) TMA slide, (B) TMA core stained by IHC with p53 (40X), (C) TMA core stained by IHC with HER-2 (40X).

3.3 IHC Results of the Three TMAs

3.3.1 IHC expression of ER in the three TMAs

Expression of ER in the three TMAs were shown in the nuclei of the cells and detected by IHC technique. Depending on the scoring system used for the ER, two parameters were dependent; the intesity of the staining of the nuclei and the percentage of the tumor cells giving positive expression. The intensity of the nuclei of the stained cells were negative if there is no expression.

The IHC study for the ER expression of the revealed that ER, positive expression was found in 26 (47.3%) out of 55 cases, while 29 cases were not

expressed or negative cases. In the Italian TMA 23 (76%) cases out of 30 samples were positively expressed, while 7 cases were negatively expressed. In the 15 benign cases, 3 (20%) were positively expressed, but the positivity was a weak, and in a low percentage. The results revealed that the percentage of the ER expression in the Italian cases were higher than the Iraqi cases (*P* value 0.01). So there was a significant difference in the immunoexpression of ER between the malignant and the benign cases used as a control cases. These results are shown in figure (3-3), figure (3-4) shows photos of ductal carcinoma stained by IHC, brown stained nuclei indicated positive ER expression and blue stained nuclei indicated no expression for ER in these cells.

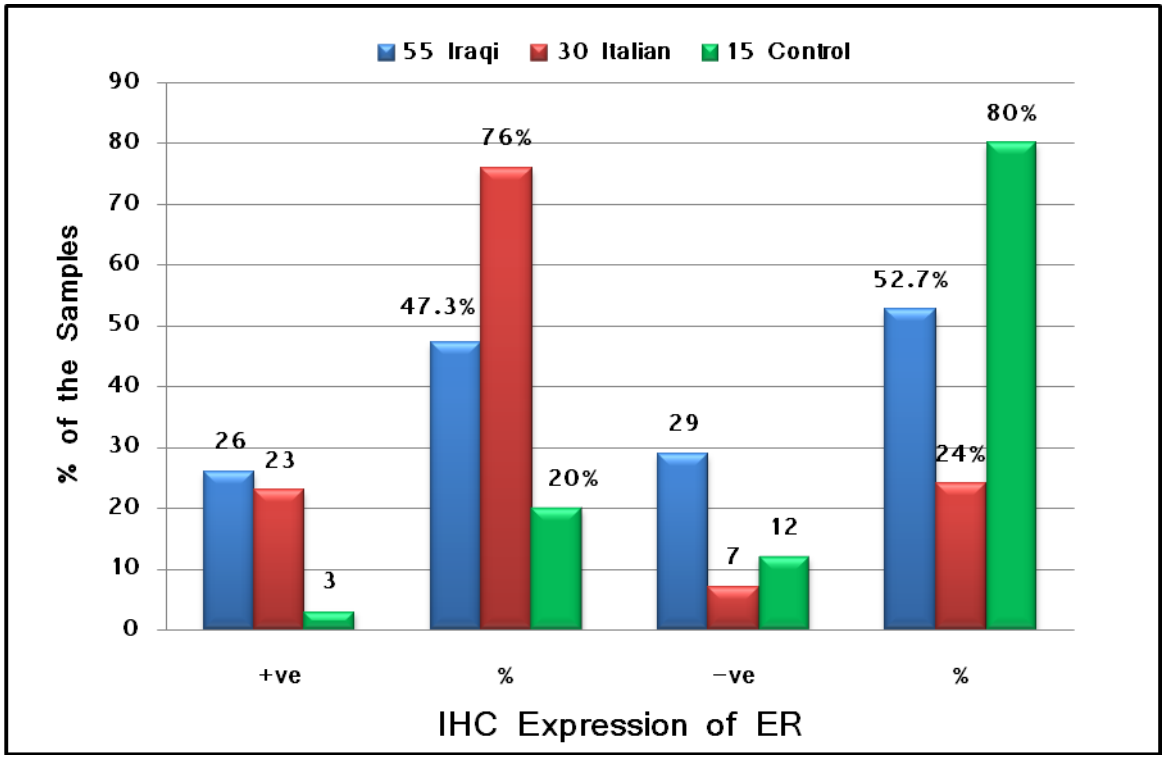


Figure (3-3) Immunohistochemical staining of ER for the three TMAs of the breast samples (Iraqi, Italian and Control).

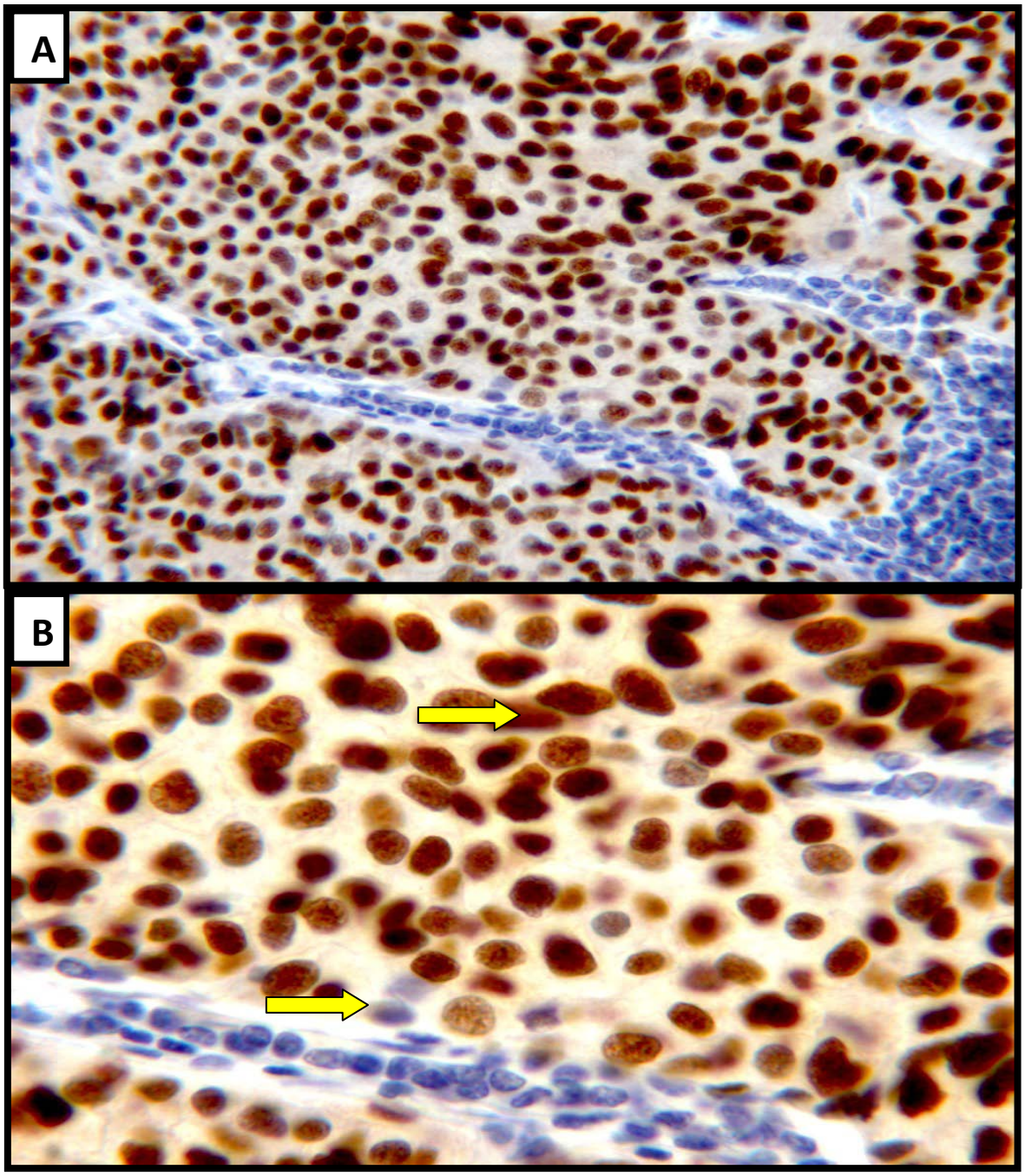


Figure (3-4) Immunohistochemical Expression of ER, ductal carcinoma, strong nuclear staining score 3+ (brown stained nuclei), blue stained nuclei negative (no expression) for ER; (A) X200, (B) X400

3.3.2 IHC of PR in the Three Breast TMAs

The same scoring and cut off values systems of the ER were used for the PR expression. Analysis of the Iraqi breast cancer TMA revealed that 35 out of 55 samples were positively expressed PR (63.60%). For the Italian breast cancer TMA, 23 out of 30 cases positively expressed PR (76%). For the Iraqi benign breast lesion TMA, 3 out of 15 samples positively expressed PR but in a weak intensity and low percentage (20%), (P <0.01). So there was a significant difference in the immunoexpression of the PR between the malignant and benign lesion samples (which were used as a normal control of PR expression) as shown in figure (3-5), figure (3-6) shows photos of ductal carcinoma stained by IHC, brown stained nuclei indicated positive PR expression and blue stained nuclei indicated no expression for PR in these cells.

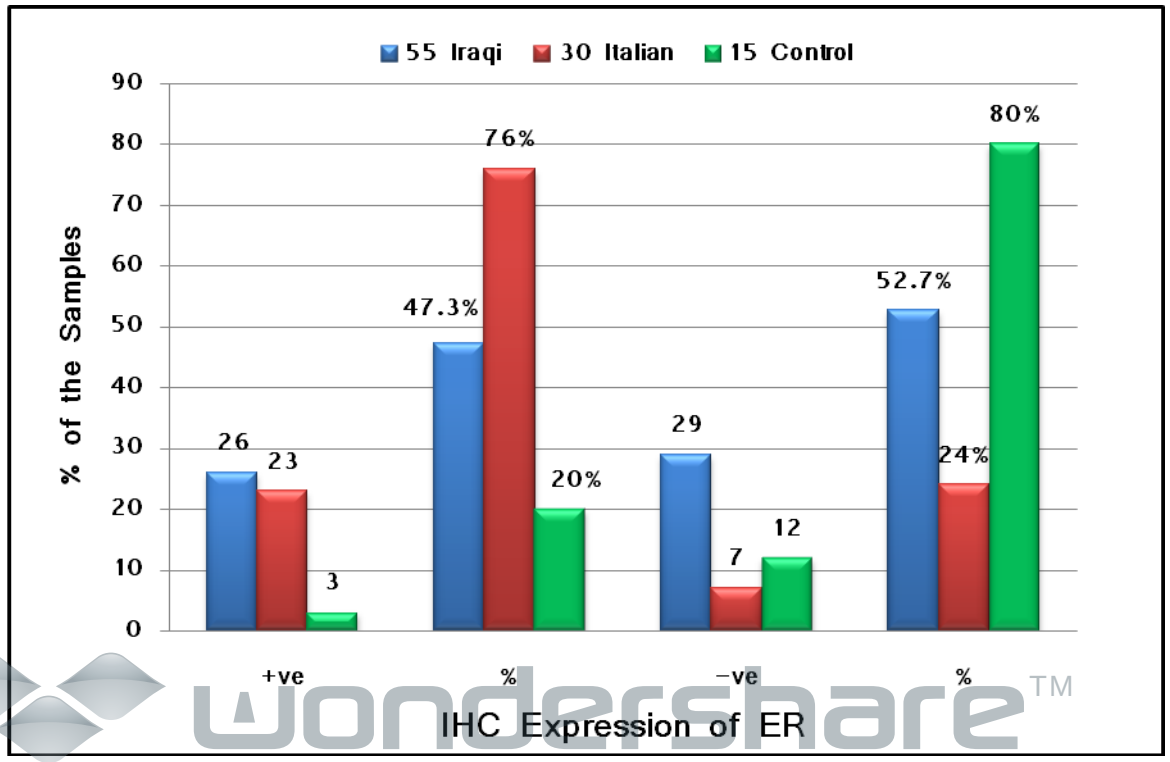


Figure (3-5) IHC staining of PR for the three TMAs (Iraqi, Italian and control).

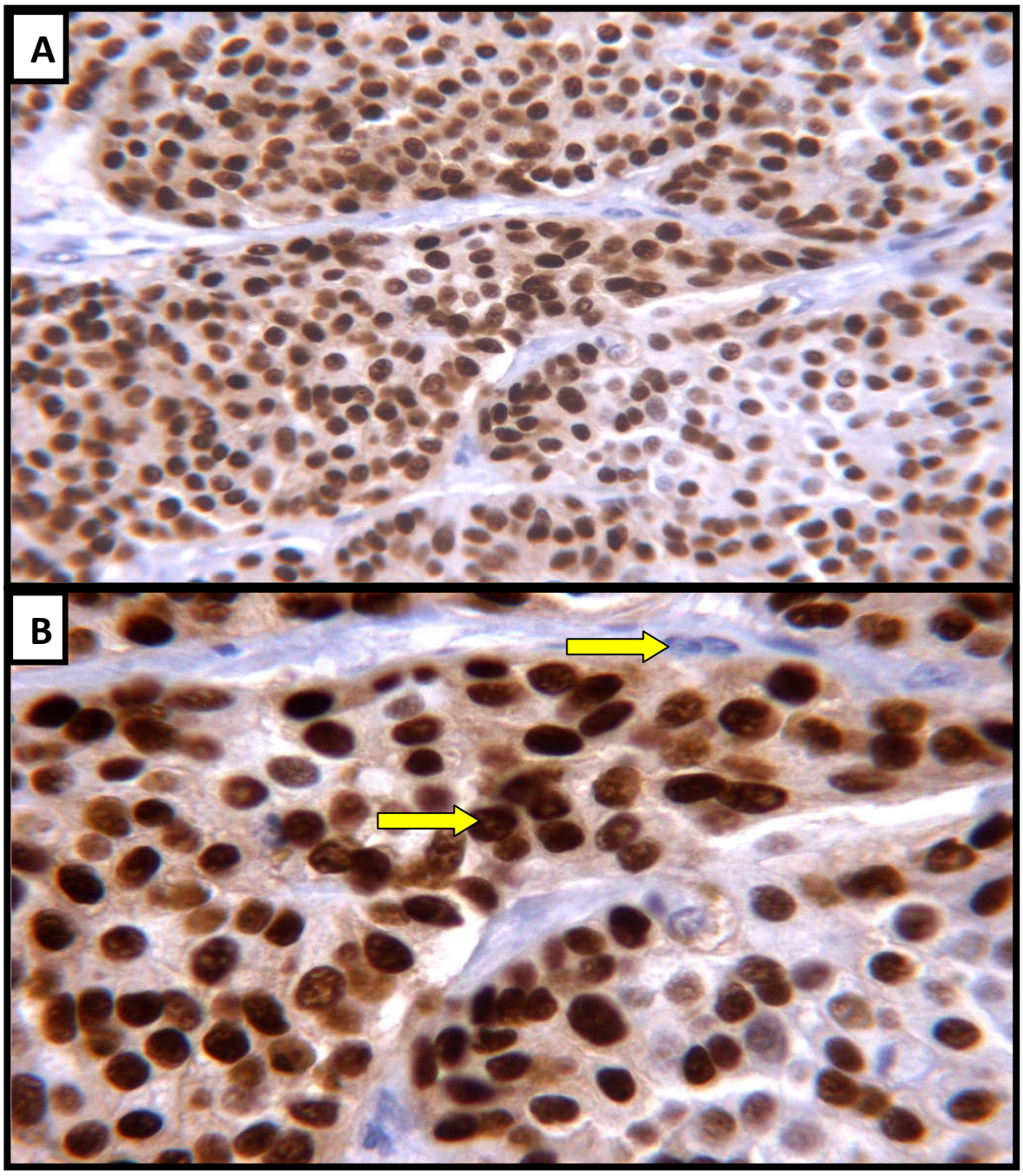


Figure (3-6) Immunohistochemical Expression of PR, ductal carcinoma, strong nuclear staining score 3+ (brown stained nuclei), blue stained nuclei negative (no expression) for ER; (A) X200, (B) X400

Chu *et al.* (2001) reported that 63.9% of the White American women with breast cancer had ER+/PR+. Many studies have demonstrated the differences of the hormone receptor status between the different populations and races. Li *et al.* (2002) demonstrated that the relatives to non-Hispanic whites, various African, south American and east Asian nationalities living in United States had 1.4 to 3.1-fold-elevated risks, presenting with negative ER/ PR.

Our results about ER status of the Iraqi samples revealed that they were decreased in comparison with the international data, but agreed with the results of Huang *et al.,* (2000) who showed that women reporting radiation exposure to the chest from medical procedures, (although few in number), had an increased risk of ER-PR- but a decreased risk of ER+PR+ breast cancer before reaching menopause.

In a study done on 88 Iraqi women with metastatic breast cancer, 34.2% of cases were ER+PR+ and 43.8% ER-PR- (Al-Alwan, 2000). Sughayer *et al.* (2006) revealed that 57.5% of the Jordanian women showed positively expressed PR and this results relatively agreed with the results obtained in this study concerning the Iraqi breast samples.

In Austria, Stierer and his colleagues (1993), reported that ER was positive in 80.6% and PR was positive in 61.3% of primary breast cancer patients.

3.3.3 IHC expression of Ki67 in the Three Breast TMAs

Ki67 is a nuclear antigen that could be detected by IHC techinque. For the Iraqi breast cancer TMA, 30 out of 55 showed positively expressed Ki67 (54.54%); for the Italian breast cancer TMA, 23 out of 30 cases were positively expressed (76%); for the Iraqi benign breast TMA, 2 out of 15 samples exhibited positively expressed Ki67 protein (13%) (P <0.01), so there was a significant difference between the expression of the malignant and the benign cases (Figure 3-7), figure (3-8) shows photos of ductal carcinoma

stained by IHC, brown stained nuclei indicated positive Ki67 expression and blue stained nuclei indicated no expression for Ki67 in these cells.

Our results agreed with the result obtained by Tan *et al.* (2005) which revealed that (43%) of the studied breast cancer cases showed high expression of Ki67.

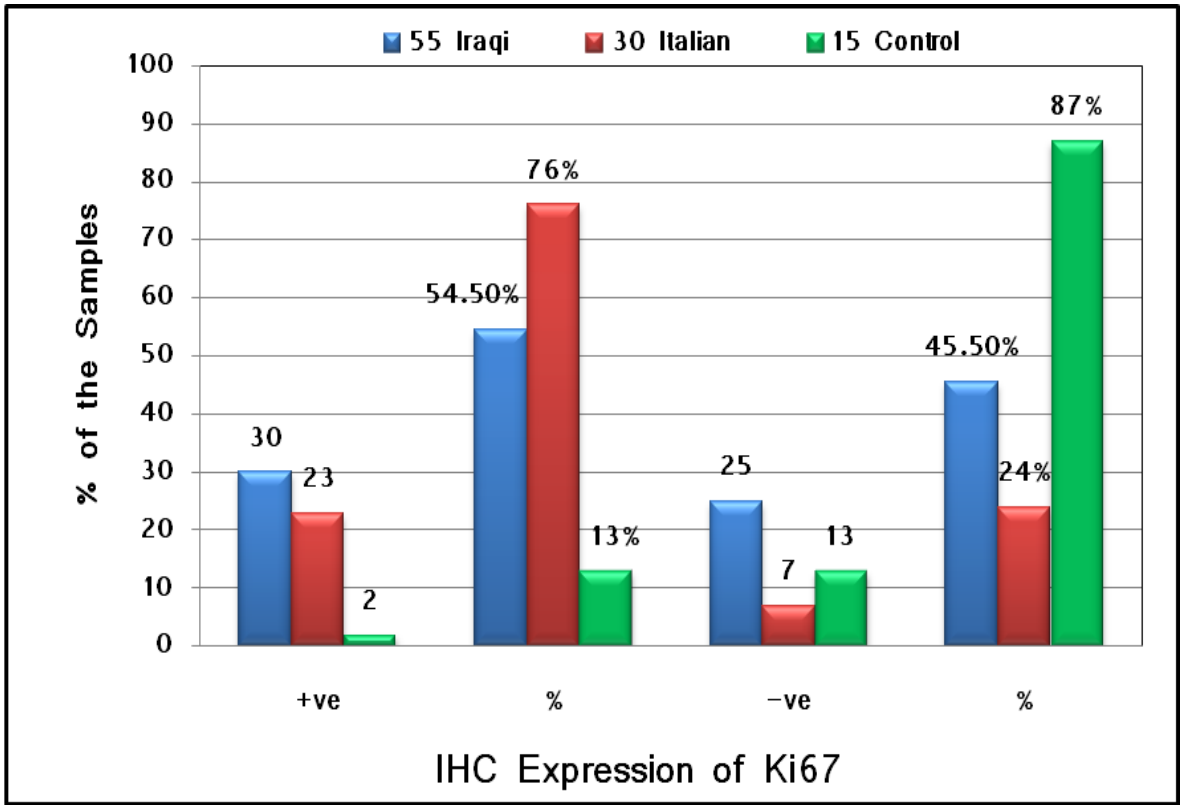


Figure (3-7) Immunohistochemical expression of Ki67 in the three TMAs of breast samples.

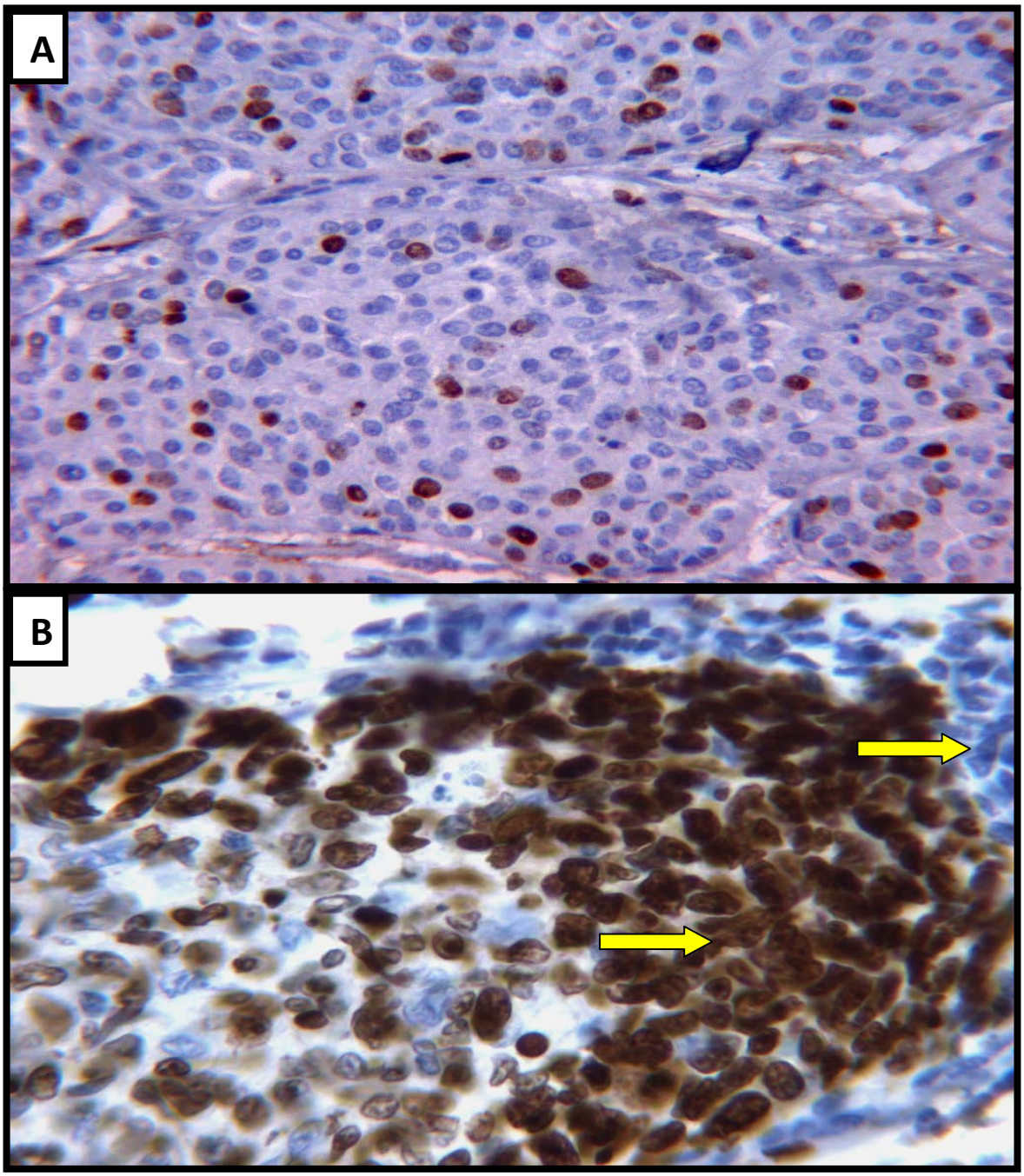


Figure (3-8) Immunohistochemical Expression of Ki67, ductal carcinoma, strong nuclear staining score 3+ (brown stained nuclei), blue stained nuclei negative (no expression) for Ki67; (A) X200, (B) X400

3.3.4 IHC expression of p53 in the three breast TMAs

The immunoexpression of p53 were observed in the nuclei of the tumor cells and measured by IHC technique dpending on the intensity of the positive expression (-ve, 1+, 2+ and 3+) and the percentage of the tumor cells positively expressed p53 (0-100%). For the Iraqi malignant breast TMA, the positive expression of the p53 were observed in 48 out of 55 samples (87.2%), a fact that represents an over expression of p53. For the Italian TMA 23 out of 30 samples showed positively expressed p53 (76%) which was lower than the Iraqi samples. In the benign samples 2 out of 15 samples had positively expressed p53 (13%) (P <0.01), indicating that there was a significant difference between the malignant breast samples expression and the control samples figure (3-9).

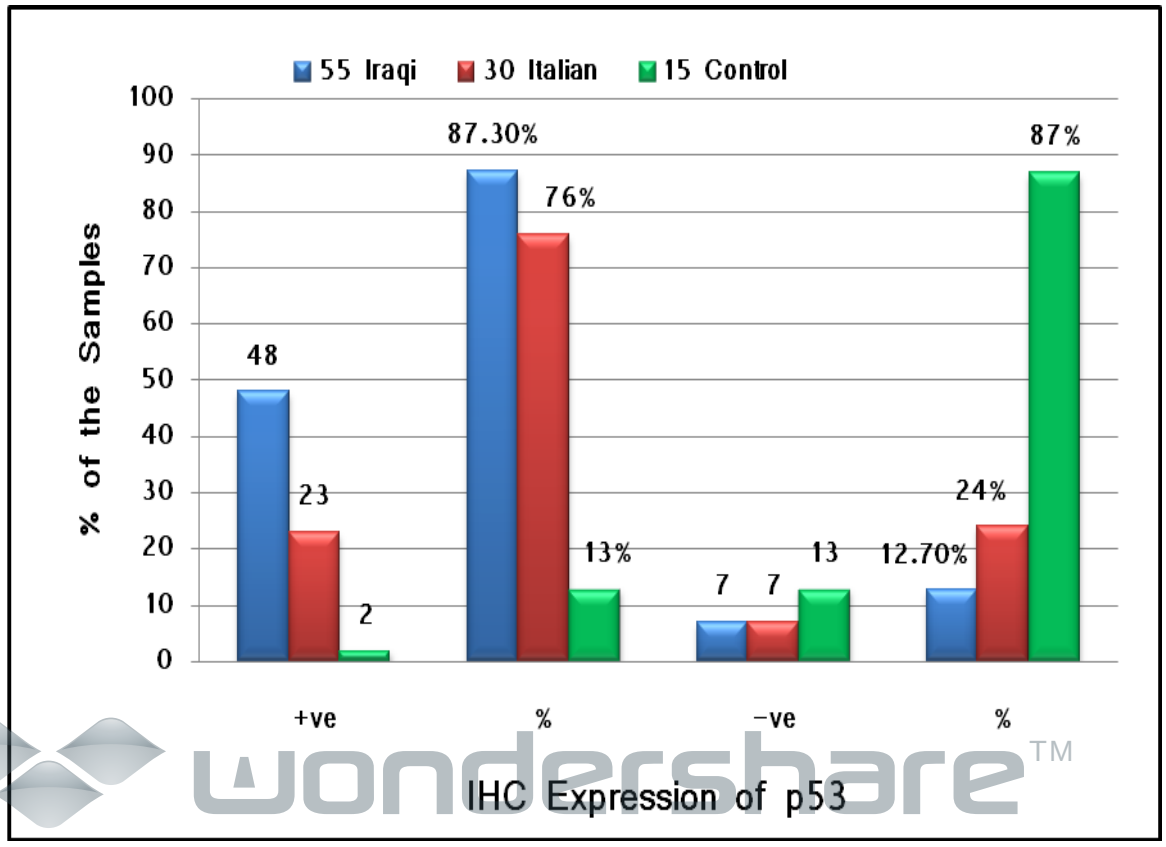


Figure (3-9) Immunostaining of p53 in the three TMAs of the breast samples.

In regard to the p53 overexpression in the Iraqi breast cancer patients, which revealed a more aggressive disease and worse overall survival compared to the already reported above-mentioned studies, this finding may be attributed to the long time exposure to ionic radiations, poor health education and inadequate breast cancer screening programs in our country. Many patients were probably admitted to hospitals when their breast tumors had already reached a very advanced stage. The results showed in figure (3-9), photos of ductal carcinoma stained for p53 by IHC are shown in figure (3-10) brown stained nuclei indicated postive expression and blue stained nuclei indcated negtive expression or no expression for p53.

Al-Sanati, (2009) reported that 57% of the Iraqi breast cancer patients showed p53 overexpression, while Al-Janabi, (2003) revealed that only 44% of breast cancer patients had overexpressed p53.

Abnormal p53 expression were detected in only 29% of tumors of the breast (Rolland *et al.*, 2007). This result disagrees with the present results about p53 expression in the Iraqi and Italian breast cancer samples. Yamashita *et al.*, (2003) reported that p53 overexpressed in only 29% of the breast cancer, and accumulation of p53 protein was significantly associated with poor prognosis in his study. However, they revealed that an overexpression of HER-2 and p53 accumulation is a strong prognostic molecular marker in breast cancer. p53 mutation remains the most common genetic change identified in human neoplasia. Gasco *et al.*, (2002) reported that p53 mutation is associated with a more aggressive disease and a worse overall survival. The frequency of mutation in p53 is, however, lower in breast cancer than in other solid tumors. Changes, both genetic and epigenetic, have been identified in regulators of p53 activity and in some downstream transcriptional targets of p53 in breast cancers that express wild-type p53. Molecular pathological analysis of the structure and expression of constituents of the p53 pathway is

likely to have a value in diagnosis, prognostic assessment and, ultimately in treatment of breast cancer.

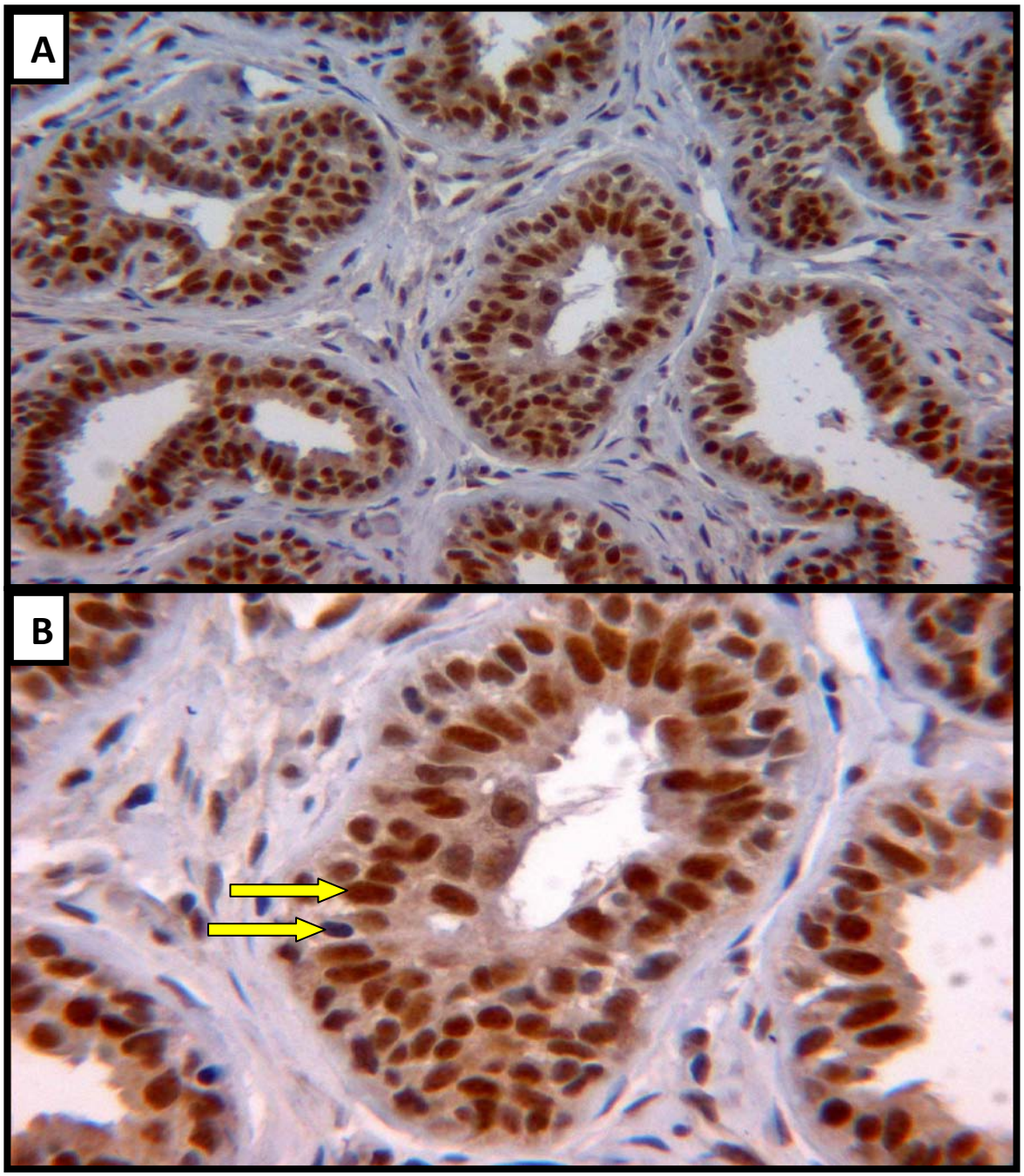


Figure (3-10) Immunohistochemical Expression of p53, strong nuclear staining score 3+ (brown stained nuclei), blue stained nuclei negative (no expression); (A) X200, (B) X400.

3.3.5 Expression of HER-2 in the three breast TMAs samples

HER-2 is expressed in the membrane of the cells, so it differs from the previously studied markers which were nuclei expressed markers.

Negative or no membrane expression were observed in 11 out of 55 samples (20%), i.e., it scored 0. Weak and incomplete membrane staining was found in 14 out of 55 samples (25.45%) and scored 1+; the cut off value for the two scores 0 and 1+ was 0 according to the Dako system for HER-2 scoring. Negative cases and 1+ cases were considered as a normal HER-2 expression. Moderate HER-2 expression was found in 18 out of 55 samples (32.7%), and were considered weak or moderate membrane staining in >10% of the tumor cells, i.e., scored 2+ and the cut off value 2. Twelve out of 55 samples (21.8%) showed complete membrane staining in >10% of the tumor cells, with scoring 3+ and the cut off value 3+. Accordingly, moderate and strong expression were detected in 30 out of the 55 samples (54.50%) (Figure 3-11).

In the Italian TMA, 4 samples showed no expression and were negative samples (13.3%), 10 samples showed weak and incomplete membrane staining (33.33%) and scored 1+; the cut off value was 0. These cases were considered as a normal HER-2 expression. Eleven samples showed overexpressed HER-2, and scored 2+ (36.7%); while five samples showed strong positive (3+) (16.7%), indicating a deregulation of HER-2 expression. Negative and 1+ scored samples were observed in 14 samples (46.6%), whereas moderate and strong expressions were found in 16 samples (53.3%) (Figure 3-12).

For the benign lesions TMA, no expression or negative was observed in 12 samples (80%); 3 samples expressed a weak or incomplete membrane staining, and scored 1+ (20%). The negative and score 1+ indicated normally expressed HER-2 and used as a control cases (Figure3-13).

The comparison between HER-2 expression in the three TMA samples were shown in figure (3-14)

Sections from MCF-7 breast carcinoma cell line were used as a positive control and showed strong positive expression. Negative control were also used; sections of breast carcinoma stained just without using the primary Ab for HER-2 in order to check the sensitivity and the specificity of the IHC staining (data not shown).

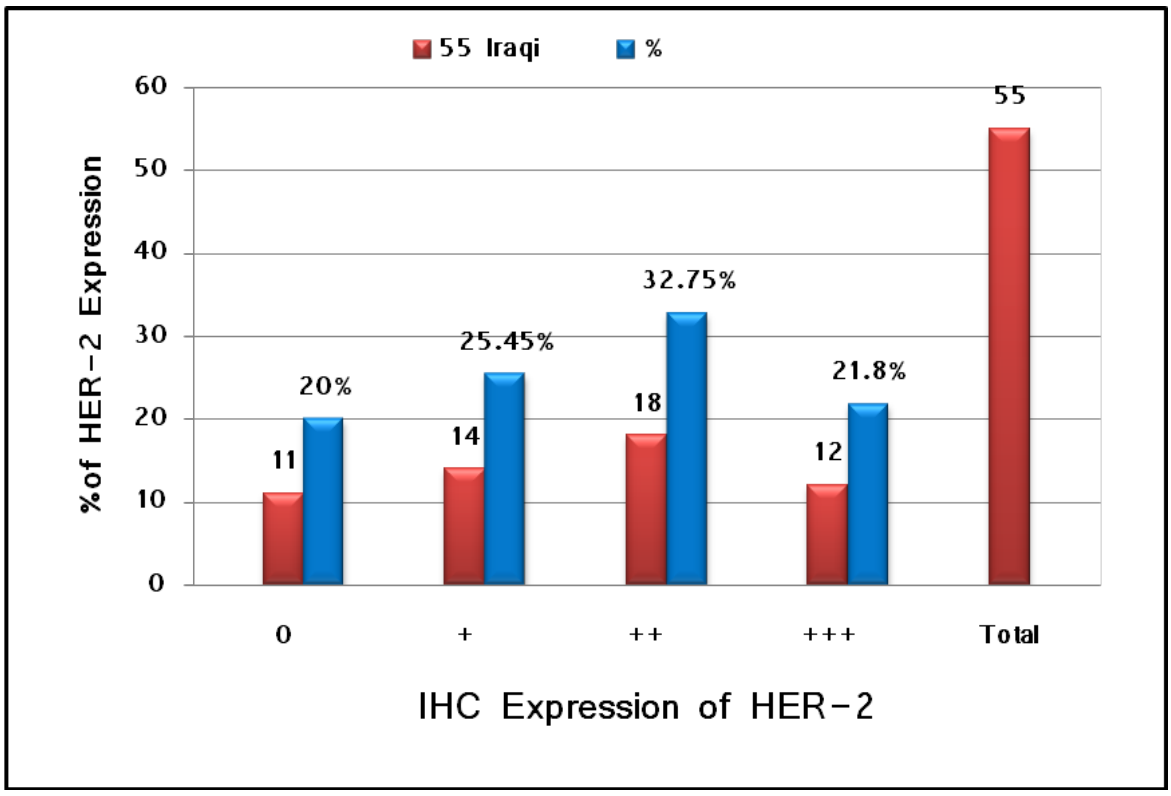


Figure (3-11) HER-2 expression in the TMA contains 55 Iraqi malignant breast samples.

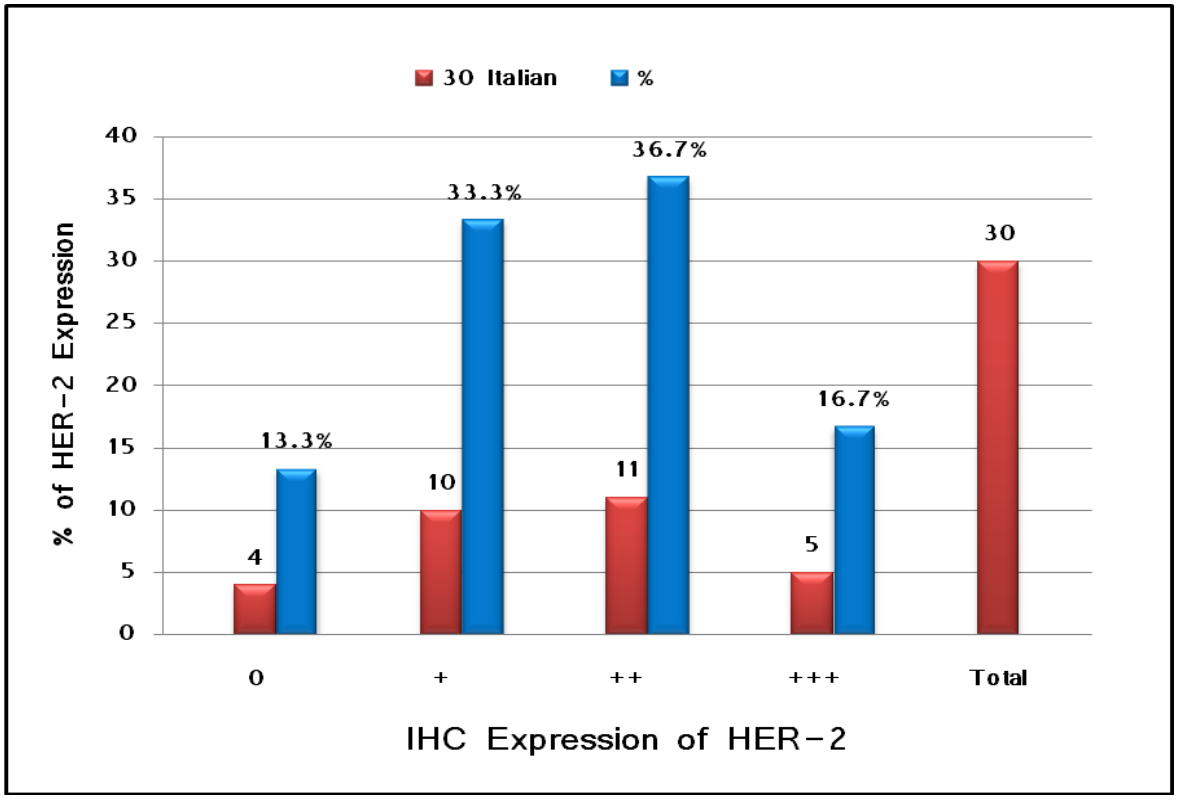


Figure (3-12) HER-2 expression in the Italian TMA contains 30 breast cancer samples.

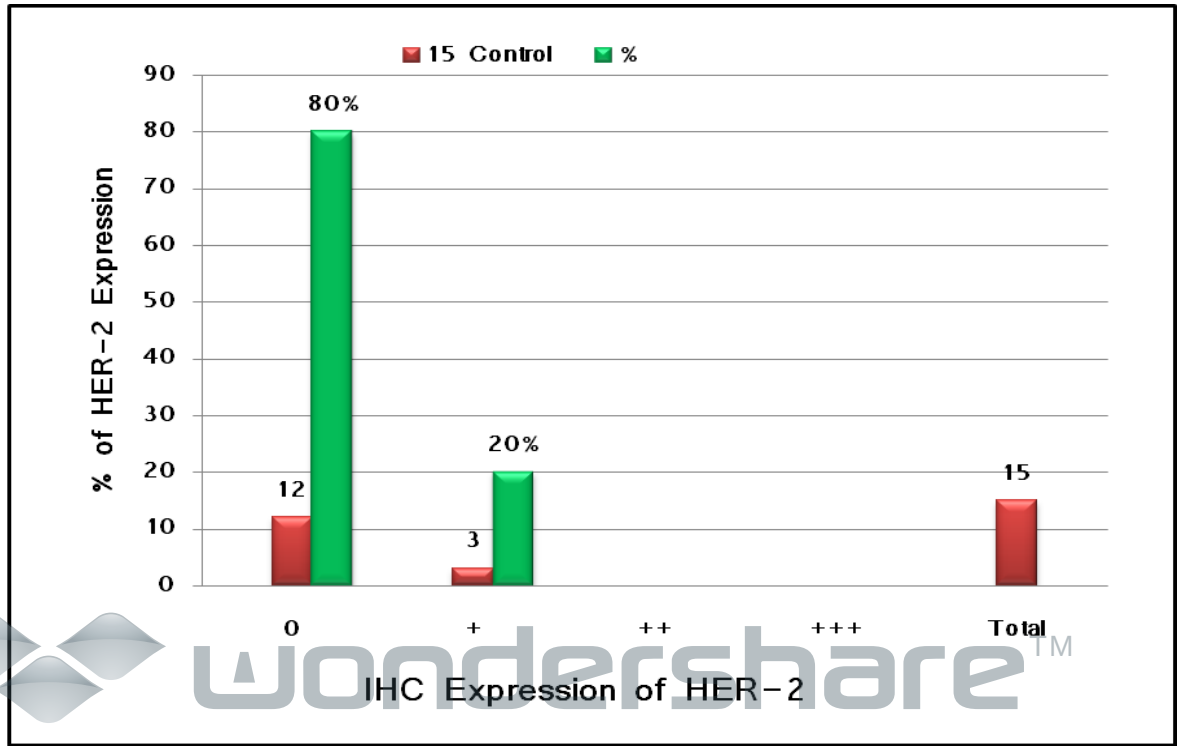
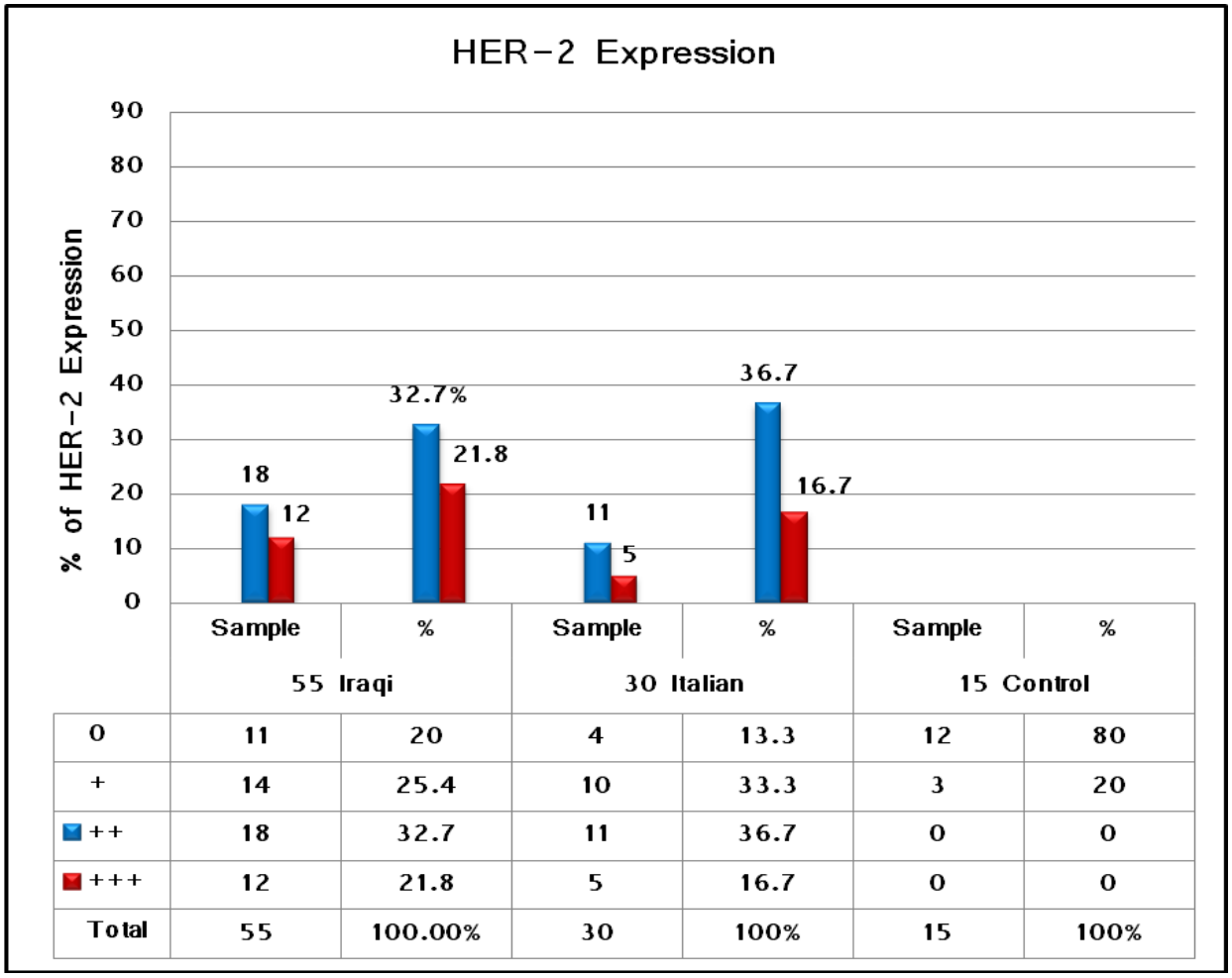


Figure (3-13) HER-2 expression in the control TMA contains 15 Iraqi benign breast lesions.



	55 Iraqi		30 Italian		15 Control	
	Sample	%	Sample	%	Sample	%
0	11	20	4	13.3	12	80
+	14	25.4	10	33.3	3	20
++	18	32.7	11	36.7	0	0
+++	12	21.8	5	16.7	0	0
Total	55	100.00%	30	100%	15	100%

Figure (3-14) Immunohistochemical of HER-2 Expression in the Three TMAs of the Breast Samples.

HER-2 expression were successfully determined by IHC in this study for the three TMA samples; only score 2+ and 3+ were considered positive for HER-2 expression. Iraqi breast cancer overexpression were observed in (54.5%), Italian breast cancer overexpression was detected in (53.3%), while the benign breast group, weak positive expression was detected in 20% of this group, which was used as a control group in this study (Figure 3-14), sample photos of HER-2 overexpression are shown in figure (3-15), brown stained membrane which indicates the positive expression of HER-2 and the negative expression showed no membrane staining.

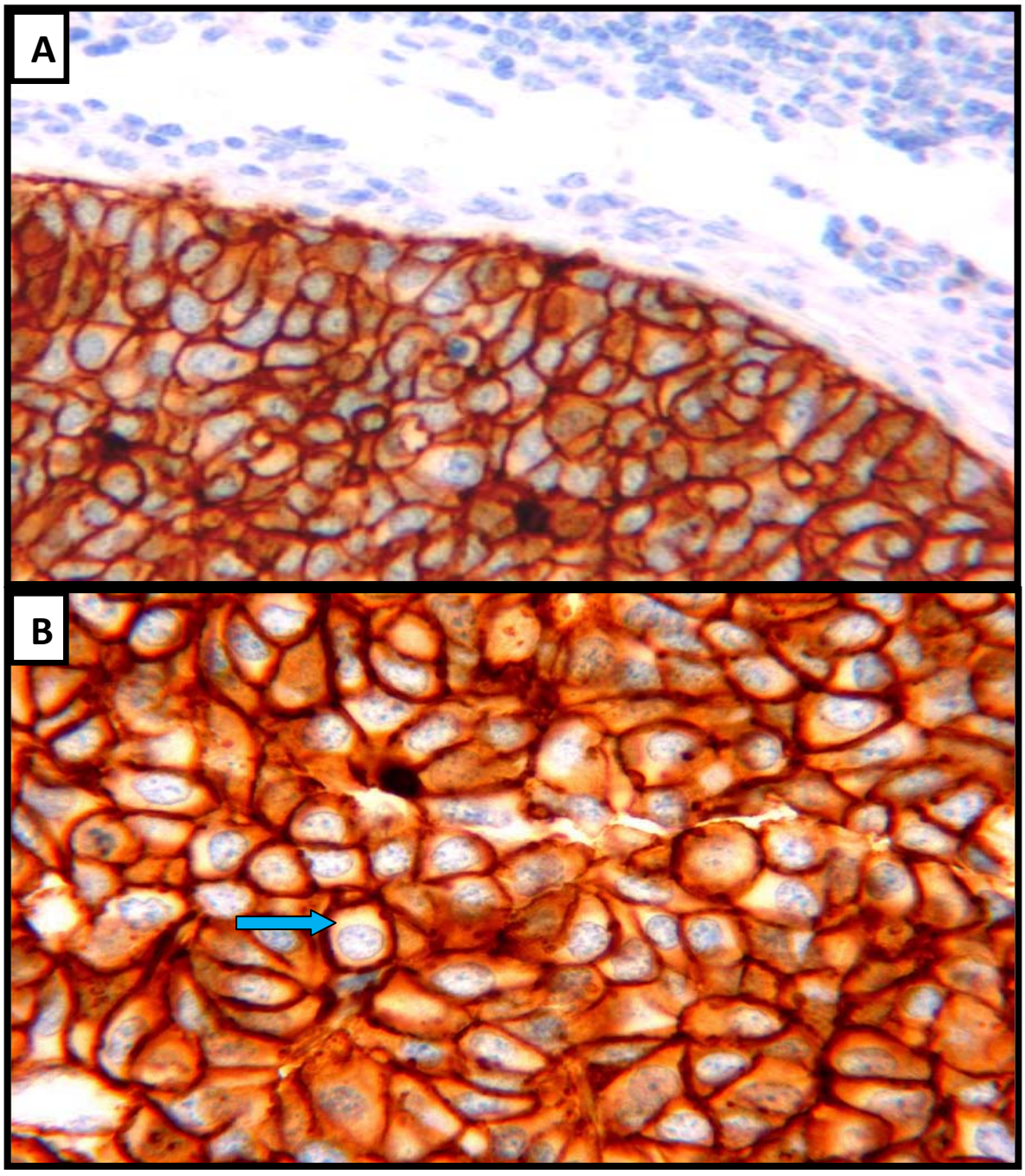


Figure (3-15) Immunohistochemical Expression of HER-2 strong positive expression (complete membrane staining in >10% of the tumor cells) score 3+, breast carcinoma, (A) X200, (B) X400.

AL-Dujaily *et al.* (2008) found that the HER-2 immunoexpression was positive in 67.8% of breast cancer patients group from the midlle of Iraq (Al Kufa district), while it was negative in all benign breast lesions (fibroadenoma). HER-2 immunostaining was significantly associated with histological type and recurrence of breast cancer. It was positively, yet non significantly, correlated with tumor grade, suggesting that HER-2 overexpression plays an important role in the pathogenesis of breast cancer and is associated with a worse prognosis. Furthermore, Al-dujaly *et al.* (2008) findings indicated that in regions exposed to high levels of depleted uranium, HER-2 overexpression was high, but its correlation with age, grade, stage, tumor size, and lymph node involvement are similar to studies that have been conducted on populations not exposed to depleted uranium. Exposure to depelted uranium may be one of the factors responsible for overexpression of HER-2 observed in Iraqi patients of the present study.

The results obtained in this study agreed with that of Hanna (2009) who reported an overexpression of HER-2 protein in (40%) of patients with breast carcinoma, suggesting that the high level of HER-2 positivity might be attributed to the small sample size (only 30 cases), which was subsequently reflected on the percentage of HER-2 positive cases.

Dendukuri *et al.* (2007) reported HER-2 overexpression in about (28.2%) of the studied cases, a finding which is lower than the results obtained in our study. Lebeau *et al.*, 2001 observed that HER-2 overexpression was detected in 42% (35/84) of patients with invasive breast cancer. Carlsson *et al.* (2004) reported that HER-2 overexpression (HER-2-scores 2 or 3) was found in 55% of both the primary tumors and of the lymph node metastases.

HER-2 proto-oncogene is amplified and/or overexpressed in approximately 25–30% of invasive breast cancers. It has been well

documented that positive HER-2 breast cancer patients have predictive role as they respond well to the blocking effect of these receptors by anti-HER-2 receptor antibody using monoclonal (Herceptin) drug that plays a role in the regression of the tumor size and prevention of recurrence (Slamon *et al.*, 1989). On the other hand, Ross and Fletcher (1998), reported that HER-2 positivity occurs in 20-30% of breast cancers, and has been associated with more rapid tumor growth, increased risk of recurrence following surgery, shortened survival and poor response to conventional chemotherapy.

Clearly there are differences in the HER-2 overexpression between the above-mentioned studies. This may be attributed to several factors, including the variable number of the samples, time and type of the fixative used, accuracy of the test, antigen retrieval, and non specific staining.

3.3.6 Correlation between the five IHC markers

Correlation between the five markers used in the present study was studied statistically. Analysis of the Iraqi patients TMAs revealed that there was a significance between the HER-2 expression and (ER and p53) expressions (*P* value <0.01), but there was no significant relationship between HER-2 expression with (PR and Ki67) expressions (*P* value >0.01) (Table 3-3).

For the Italian samples, HER-2 showed no significant relationship with the other markers, but there was a significant relationship between p53 expression with ER and PR expressions (Table 3-4). HER-2 and p53 expressions correlated with recurrence. In the current study, there was no significant association between ER, PR, Ki67 and P53 expression. These results are in accordance with other studies that examined these markers in DCIS treated exclusively by local excision (Tan *et al.*, 2002; Cornfield *et al.*, 2004). Ruiz *et al.* (2006) applied TMA to compare molecular features with proliferative activity in the breast cancer and reported that Ki67 increased

significantly with high expression levels of p53, as well as with amplifications of HER-2. They also revealed that Ki67 was significantly decreased in cancers with high expression levels of ER and PR.

Table (3-3) The correlations between the five IHC markers in the Iraqi Breast cancer TMA

		ER	PR	Ki67	p53	HER-2
ER	Pearson Correlation		.416(**)	.211	.351(**)	.382(**)
	Sig. (2-tailed)		.002	.122	.009	.004
	N	55	55	55	55	55
PR	Pearson Correlation	.416(**)		.031	.215	.169
	Sig. (2-tailed)	.002		.822	.115	.216
	N	55	55	55	55	55
Ki67	Pearson Correlation	.211	.031		.218	.219
	Sig. (2-tailed)	.122	.822		.109	.108
	N	55	55	55	55	55
p53	Pearson Correlation	.351(**)	.215	.218		.492(**)
	Sig. (2-tailed)	.009	.115	.109		.000
	N	55	55	55	55	55
HER-2	Pearson Correlation	.382(**)	.169	.219	.492(**)	
	Sig. (2-tailed)	.004	.216	.108	.000	
	N	55	55	55	55	55

** Correlation is significant at the 0.01 level (2-tailed).

Table (3-4) The correlations between the five IHC markers in the Italian Breast cancer TMA

		ER	PR	Ki67	p53	HER-2
ER	Pearson Correlation		.353	.094	.664(**)	.184
	Sig. (2-tailed)		.056	.621	.000	.331
	N	30	30	30	30	30
PR	Pearson Correlation	.353		-.214	.617(**)	.240
	Sig. (2-tailed)	.056		.256	.000	.202
	N	30	30	30	30	30
Ki67	Pearson Correlation	.094	-.214		-.091	-.021
	Sig. (2-tailed)	.621	.256		.633	.913
	N	30	30	30	30	30
p53	Pearson Correlation	.664(**)	.617(**)	-.091		.262
	Sig. (2-tailed)	.000	.000	.633		.162
	N	30	30	30	30	30
HER-2	Pearson Correlation	.184	.240	-.021	.262	
	Sig. (2-tailed)	.331	.202	.913	.162	
	N	30	30	30	30	30

** Correlation is significant at the 0.01 level (2-tailed).

3.4 Triple Negative (ER, PR and HER-2 Negative) Samples

Triple negative cases mean that the case is negative for ER, PR and HER-2. Twelve cases out of 55 were triple negatives from the Iraqi samples (21.80%), whereas only one case out of 30 cases was triple negatives from the Italian samples (3.3%). This finding indicates that the Iraqi breast cancer cases were phenotypicaly more aggressive than the Italian breast cancer samples.

DNA extractions from these samples were performed to study the mutations in BRCA1/2 genes and their relationship with the aggressive phenotype of the Iraqi breast samples. Good quantities of DNA were obtained from these samples using DNA quantification by NanoDrop spectrophotometer. After DNA quantification, the samples were amplified by PCR but no results were obtained during the migration on agarose gel electrophoresis of the PCR products. Furthermore, optimization was performed for most of the reaction parameters but was unsuccessful too, only the positive control band appeared. One of the probable reasons could be the fact that the samples were exposed to the fixative for a long time, which then cross linked with the DNA and degraded, so that it was not useful for PCR study of these samples (Table 3-5).

Triple-negative (ER-negative, PR-negative, HER-2 not overexpressed) breast cancer has distinct clinical and pathologic features, and represents a clinical problem because of its relatively poor prognosis, aggressive behaviour and lack of targeted therapies, leaving chemotherapy as the mainstay of treatment. Most triple-negative tumors fall into the basal-like molecular subtype of breast cancer (Reis-Filho and Tutt, 2008).

Table (3-5) The triple negative samples among Iraqi and Italian patients

Iraqi Samples Individual Number	ER	PR	HER-2
4	-ve	-ve	-ve
7	-ve	-ve	-ve
13	-ve	-ve	-ve
15	-ve	-ve	-ve
28	-ve	-ve	+
30	-ve	-ve	+
31	-ve	-ve	+
36	-ve	-ve	-ve
39	-ve	-ve	+
41	-ve	-ve	+
45	-ve	-ve	-ve
50	-ve	-ve	-ve
Total=12			
Italian Sample	ER	PR	HER-2
25	-ve	-ve	-ve

Triple-negative (TN) breast cancer remains a major challenge to physicians and patients, and a source of great interest to laboratory investigators. Although TN accounts for a relatively small minority of breast cancer cases, it is responsible for a disproportionate number of breast cancer deaths. Moreover, there have been fewer advances in the treatment of TN than

has been seen with other subtypes. For these reasons, new research initiatives for TN are critical. The investigation of TN is one facet of an emerging effort that regards breast cancer as a collection of separate diseases rather than a single heterogeneous entity, a fact that represents an important step towards the individualization of therapy (Nanda and Olopade, 2008).

Triple negative cases were observed in (21.80%) of the Iraqi breast cancer cases as compared to the one case (3.3%) of the Italian samples, indicating that the Iraqi breast samples were more aggressive with poor prognosis than the Italian group. The present Iraqi results showed higher percentage than those obtained by Cleator *et al.*, (2007) who reported that TN cases accounts for 15% of all types of breast cancer and is characterized by an aggressive clinical course and poor outcome.

3.5 FISH assay for *HER-2* gene

FISH technique is used to detect the gene amplification of *HER-2* gene. FISH assay is considered positive for *HER-2* gene amplification if the average of *HER-2* signals (Orange) per nucleus is ≥ 4, or if the average ratio of *HER-2* signals (Orange) to chromosome 17 centromere signals (Green) is ≥ 2 . This scoring protocol was recommended by the kit manufacturer.

3.5.1 IHC and FISH assays of *HER-2* gene for the Iraqi breast cancer TMA

Twenty-three cases (41.8 %) showed negative scores for both IHC and FISH assays, (scores 0 and 1+ were considered negative for IHC; for FISH score 1 was considered as non amplified or negative. The results showed that the percentage of the *HER-2* gene signals to the centromere of chromosome 17 signals was one, which means that the same copies of the *HER-2* gene and the centromere are located within the same nuclei of the tumor cells.

Five cases (9.1%) were found negative for FISH assay and scored (1.5). These five cases distributed among different IHC scores as follows: one case

belongs to IHC score 0, one case to IHC score 1+, two cases to IHC score 2+ and one case to IHC score 3+. Thirty cases showed positive results for IHC, distributed between IHC score 2+ and 3+; eighteen cases of IHC belong to score 2+ and twelve cases of IHC belong to score 3+. Twenty seven FISH positive cases were arranged as follows; nine cases (16.4 %) scored 2 which means that the number of the *HER-2* gene signals was double the number of the centromere of the chromosome 17 signals; five cases (9.1 %) were scored 2.5; nine cases (16.4 %) scored 3; three cases (5.5 %) scored 3.5, whereas only one case (1.8 %) scored 4.

Two cases of the IHC positive score 2+ and one case of score 3+ were found negative for FISH assay and scored 1.5, and considered non amplified or negative for *HER-2* gene amplification. The statistic analysis revealed that the *P* value for the IHC and the FISH assays was <0.001, indicating the presence of a significant relation between the IHC and FISH assays for the *HER-2* gene, (Table 3-6).

Table (3-6) HER-2 scoring by IHC and FISH of the 55 Iraqi breast cancer samples

IHC Score	FISH Score							Total IHC	% IHC	P value IHC
	1.00	1.50	2.00	2.50	3.00	3.50	4.00			
.00	10	1	0	0	0	0	0	11	20.0	
1.00	13	1	0	0	0	0	0	14	25.5	
2.00	0	2	7	3	5	1	0	18	32.7	
3.00	0	1	2	2	4	2	1	12	21.8	
Total (FISH)	23	5	9	5	9	3	1	55	100.0	0.001
% FISH	41.8	9.1	16.4	9.1	16.4	5.5	1.8	100.0		
P value FISH	0.001									

3.5.2 IHC and FISH assays of *HER-2* gene for the Italian patients

IHC results for the HER-2 receptor of the 30 Italian breast cancer samples revealed that 4 cases (13.3%) scored zero, 10 cases (33.3%) scored 1+; eleven cases (36.7%) scored 2+ and 5 cases (16.7%) scored 3+. On the other hand, FISH assay showed that 12 cases (40.0%) scored 1; 4 cases (13.3%) scored 1.5; 8 cases (26.7%) were scored 2, 5 cases (16.7%) scored 2.5 and only 1 case (3.3%) scored 3.

According to the manufacturer manual scores 1 and 1.5 were considered negative or non amplified and scores equal or greater than 2 were considered positive. The statistical analysis for the *P* value was <0.001, indicating that a significant relationship results between the IHC and FISH assays for *HER-2* gene (Table 3-7).

Table (3-7) HER-2 scoring by IHC and FISH of the 30 Italian breast cancer samples

IHC Score	FISH Score					Total IHC	% IHC	*P* value (IHC)
	1.00	1.50	2.00	2.50	3.00			
.00	4	0	0	0	0	4	13.3	0.001
1.00	8	2	0	0	0	10	33.3	
2.00	0	2	7	2	0	11	36.7	
3.00	0	0	1	3	1	5	16.7	
Total FISH	12	4	8	5	1	30	100.0	
% FISH	40.0	13.3	26.7	16.7	3.3	100.0		
P value (FISH)	0.001							

3.5.3 FISH score for the *HER-2* gene IHC score 2+

Analysis of Iraqi samples revealed that there were a total of 18 cases with HER-2 scores 2+ by IHC. Sixteen cases out of 18 cases were found positive for *HER-2* gene by FISH assay and 2 cases were found negative for FISH assay. The *P* value (<0.001), suggesting that IHC and FISH were significantly related. On the other hand, analysis of Italian samples showed that 11 cases were IHC2+; 2 samples were negative for FISH assay which showed no amplification for *HER-2* gene, while 9 cases were positive for FISH assay and scored ≥ 2 and revealed amplification of *HER-2* gene. The calculated *P* value was (<0.001) which means that there was a significant relationship between the IHC 2+ cases and FISH assay (Table 3-8).

Table (3-8) IHC2+ and FISH scores for *HER-2* gene

Iraqi IHC2+	**FISH score for Iraqi IHC2+**						***P* value**
	1.50	**2.00**	**2.50**	**3.00**	**3.50**	**Total**	**0.001**
	2	**7**	**3**	**5**	**1**	**18**	
Italian IHC2+	**FISH score for Italian IHC2+**						***P* value**
	1.50	**2.00**	**2.50**			**Total**	**0.001**
	2	**7**	**2**			**11**	

3.5.4 IHC and FISH assay for the Iraqi Benign breast lesions TMA

The results obtained from IHC and FISH assays revealed that all the cases were negative for HER-2 expression and *HER-2* gene amplification. The *P* value was <0.001, indicating the presence of a significant relationship between the two tests. The benign cases were used as control cases for no amplification (Table 3-9). The samples photos of no-amplification and amplification of *HER-2* gene were shown in figure (3-16).

Table (3-9) FISH and IHC of the Iraqi benign breast lesions for HER-2

	N	Minimum	Maximum	Mean	Standard Deviation	*P* value
FISH	**15**	**1.00**	**1.50**	**1.17**	**.2**	**0.001**
N	**15**					
IHC	**0**	**1+**	**2+**	**3+**	**Total**	
	12	**3**	**0**	**0**	**15**	

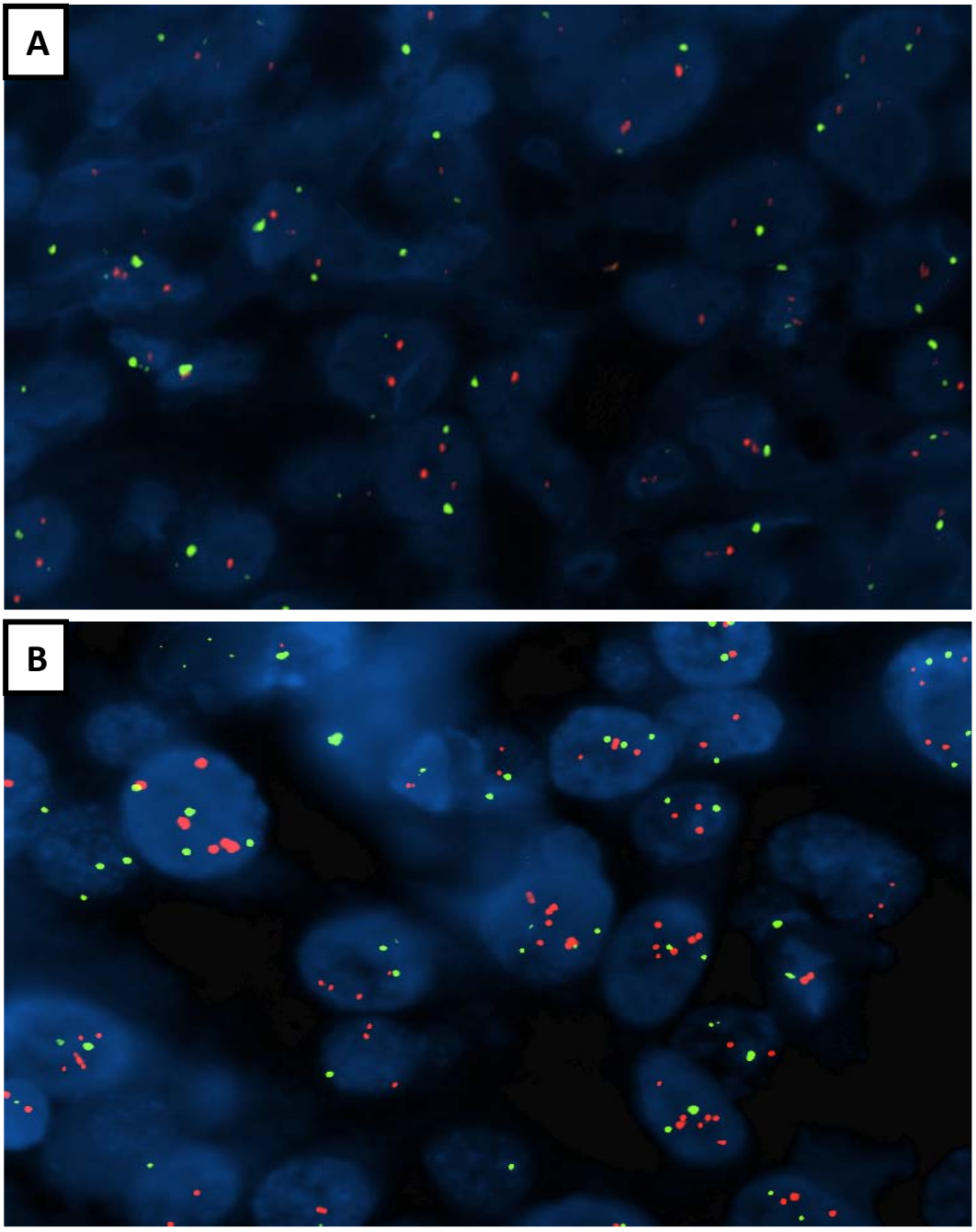


Figure (3-16) FISH for *HER-2* gene signals (Orange) and the centromere of chromosome 17 signals (Green) of breast carcinoma. A; no amplification, B; amplification. X600

HER-2 positive breast cancer is characterized by amplification of the *HER-2* oncogene and/or overexpression of its protein (Hicks and Tubbs, 2005). In a normal cell producing HER-2, there are two copies of the gene and about 50,000 copies of the protein at the cell surface. In comparison, in HER-2 producing cancers, there are more than 2 copies of the gene and more than 1,000,000 copies of the protein at the cell surface. HER-2 positivity occurs in 20-30% of breast cancers. It was associated with more rapid tumor growth, increased risk of recurrence following surgery, shortened survival and poor response to conventional chemotherapy (Ross and Fletcher, 1998).

However, wolf *et al.*, (2007) reported that a positive HER-2 result is defined as IHC staining of 3+ (uniform membrane staining) in more than 30% of invasive tumor cells. A FISH result of more than six *HER-2* gene copies per nucleus or a FISH ratio of *HER-2* gene signals to chromosome 17 signals of more than 2 also indicate HER-2 positivity. A negative result is an IHC staining of 0 or 1+, or a FISH result of fewer than four *HER-2* gene copies per nucleus, or FISH ratio of less than 1.8. Equivocal results require additional studies to arrive at a final determination (Wolff *et al.*, 2007).

This study reviewed the validity and reliability of the two most commonly used tests for HER-2 detection: IHC and FISH (this technique was successfully used for a first time on the Iraqi breast cancer samples. The present result is in agreement with a study of Romond *et al.*, (2005), who reported that the percentage of patients alive and disease-free at the end of 3 years was 75.4% in the no-treated control group and 87.1% in the trastuzumab group.

Elkin *et al.* (2004) reported the analysis of FISH was only among women in the metastatic phase of cancer and their analyses adjusted for the efficacy of trastuzumab.

TMA was validated and successfully used in this study for IHC and FISH. The IHC results (scored as 0, 1+, 2+, and 3+) were compared to the results

obtained by FISH. We utilized TMA sections for the validation of HER-2 molecular testing. Nevertheless, these sections enabled us to rapidly achieve technical validation for *HER-2* gene FISH testing and to reduce the time and cost of the test.

In summary, using FISH assay for the Iraqi patients revealed that there was amplification in the *HER-2* gene (49.2%) of all the cases positive for *HER-2* gene amplification, and that (88%) were amplified in the IHC2+, and (98.2%) were amplified in the IHC3+. For the Italian samples, about (46.7%) of all the cases exhibited positive amplification and (81.80%) amplified in the IHC2+, and (100%) were amplified for IHC3+.

However, Wolff *et al.*, (2007), provided guidelines for improving the accuracy of HER-2 testing in invasive breast cancer; and summarized the utility of HER-2 as a predictive marker. The authors concluded that when carefully validated tissue testing is performed, the available data do not clearly demonstrate the superiority of either IHC or *in situ* hybridization (ISH) as a predictor of benefit from anti-HER-2 therapy. Overall, the panel recommended that HER-2 status should be determined for all invasive breast cancer patients and that a testing algorithm that relies on accurate, reproducible test performance, including newly available tests such as chromogenic *in situ* hybridization (CISH), should be considered.

Though FISH has been demonstrated to have less than 100% sensitivity and specificity, it is widely regarded as the more valid test. Retrospective analyses of data from clinical trials have demonstrated that the benefit of trastuzumab is limited to patients who are FISH positive. Therefore, it was a reasonable assumption to consider FISH as the gold standard test to assess the response to therapy (Dowsett *et al.*, 2003).

3.6 DNA Extraction from FFPE Sections

DNA extraction was performed successfully from the FFPE sections of the Iraqi and Italian breast cancer samples, as well as benign breast lesions samples. From the DNA quantification data obtained by spectrophotometric method, the concentrations and the purity of the Iraqi breast cancer and benign samples were not good enough, but the quality of the extracted DNA was not good enough for amplification by PCR, and that all the attempts for getting successful PCR amplification failed. For the Italian samples, the DNA was extracted, and both the concentrations (46-244 ng/µl) and the purity (1.70-2.50) of the thirty samples were quantified (Table 3-10).

Most of the pathological specimens were collected and archived as formalin-fixed, paraffin-embedded (FFPE) blocks. These samples represent an invaluable source of research material providing both molecular and clinical information. Unfortunately DNA and RNA extraction from FFPE tissues is challenging, as fixation causes modifications and consequent cross-linkage of biomolecules. Furthermore, nucleic acids are often degraded into smaller fragments. Despite these challenges, damaged DNA can be extracted and analyzed with variety of standard molecular biology techniques (Metz *et al.*, 2004).

The results obtained from the Italian samples agreed with finding of (Gilbert *et al.*, 2007) who reported that the DNA degradation is typical to FFPE tissues, and only rather short DNA fragments are easily amplified from these samples. With the FFPE tissue samples, the maximum length of the successfully amplified PCR product was approximately 300 bp. But the Iraqi samples were not efficient for PCR amplification, and the nucleic acid degraded because of the over fixation, so that many tests were cancelled like BRCA1/2 mutations, real time PCR and short interfering RNA effect.

Table (3-10) The quantification of DNA of the 30 Italian breast cancer samples isolated from FFPE sections

Sample Individual Number	DNA Conc. ng/µl	Purity A260/A280
1	47	2.00
2	213	1.99
3	146	1.80
4	154	2.50
5	106	1.86
6	46	2.00
7	244	1.88
8	201	2.00
9	243	1.92
10	65	1.70
11	153	2.00
12	146	2.00
13	176	1.70
14	70	2.00
15	90	1.90
16	124	2.30
17	67	1.80
18	66	2.00
19	56	1.90
20	82	2.00
21	241	2.30
22	195	2.30
23	122	2.00
24	154	2.00
25	134	1.70
26	84	2.30

27	**135**	**2.40**
28	**190**	**2.20**
29	**84**	**1.85**
30	**96**	**2.30**

3.7 PCR for BRCA1 Exon11

3.7.1 Optimization of PCR reaction

After DNA quantification, the DNA samples were used for PCR amplification, repeated runs of PCR were done to amplify the DNA extracted from Iraqi breast cancer and benign breast lesions samples, but there were no amplification. Optimization (Primers concentration, dNTPs concentration, salt concentration and DNA concentration) were performed for these samples but no results were obtained when migrated on agarose gel electrophoresis. Several possibilities could explain why these DNA and RNA were degraded. One possibility is that the fixation time of the FFPE was probably too long so that the fixative interfered with the genetic materials and degraded it. The other possibility may be the type of the fixative used. Further studies are required to consider these problems when doing research on such samples. It would be better to use fresh samples or blood or frozen samples for DNA and RNA studies.

3.7.2 Primers used for BRCA1 gene exon11

Six pairs of primers were used to amplify six regions within the BRCA1 gene. Optimization of the PCR amplification parameters have been done to get a successful amplification but most of it were not functioning. Another pair of primers designed in this study (composed of 18 bases for the forward and 19 bases for reverse) successfully amplified exon11, and the size of the PCR product was 185 bases. Many optimization reactions were performed on this primer set, and repeated optimization runs of PCR were done. Attempts

succeeded to amplify 24 samples out of 30 Italian breast cancer samples. The optimum concentration of the primers was 0,2 µM which worked well in amplifying the samples. All the positive samples were used together in the same PCR run plus the positive and the negative controls (Figure 3-17).

3.7.3 PCR amplification program

Touchdown PCR program was successfully used in this study, which designed to amplify samples that were hard to amplify, specifically the DNA samples extracted from FFPE sections. It was found that 25µl was the optimum reaction volume for this study.

3.7.4 Agarose gel electrophoresis

PCR products were run on 1.5% agarose gel electrophoresis in 1x TAE buffer and stained with ethidium bromide. Results showed the presence of a band with size of 185 bp, which represents the correct expected band in all the samples and positive control (Figure 3-17).

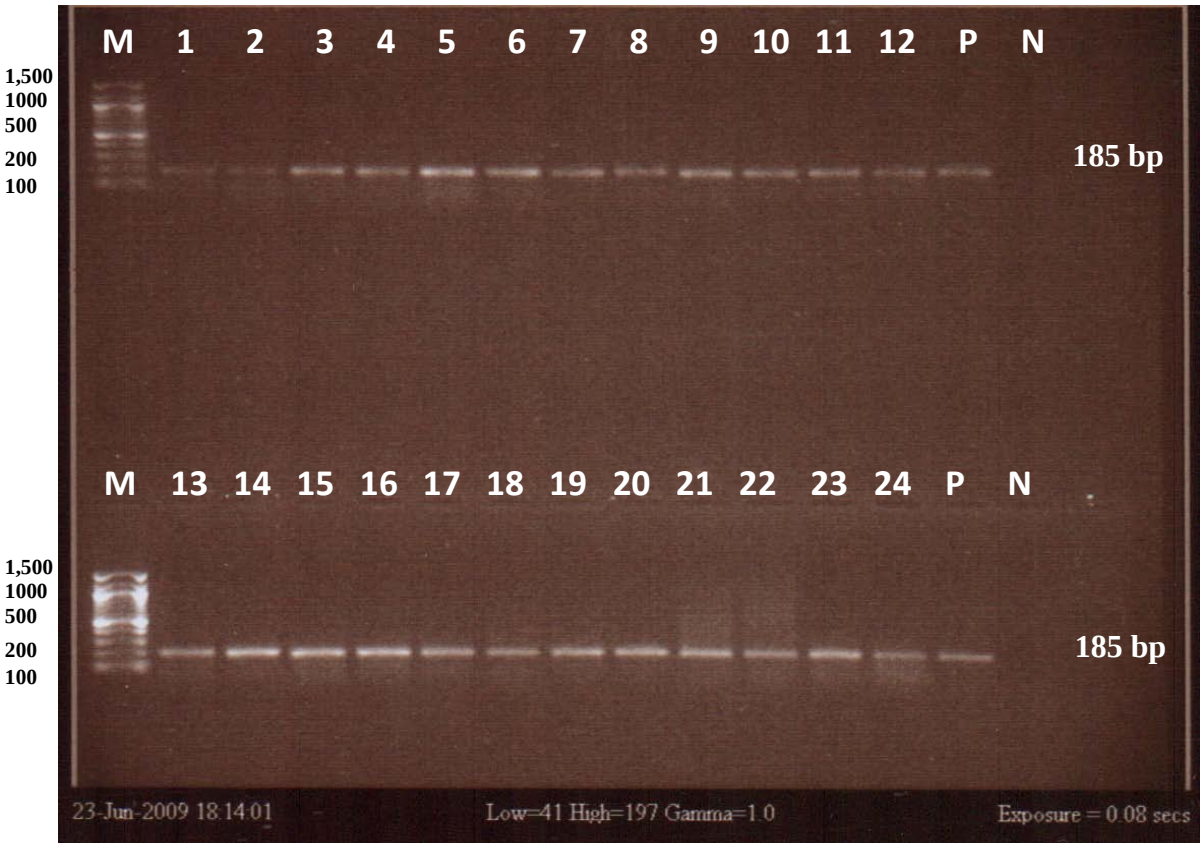


Figure (3-17) Gel electrophoresis of the PCR products of BRCA1 gene exon 11, lanes (1-24), breast cancer samples with the products size 185 bp, M, 100 bp DNA ladder (1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp respectively), P, positive control and N, negative control, 1.5% agarose gel in TAE buffer using 5V/cm for 45 min.

3.8 Mutation detection by the DNA sequencing technique

PCR products were purified in preparation for sequencing. The DNA sequencing was performed at the sequencing unit of the CEINGE Biotecnologie Avanzate, University of Naples Federico II, Italy. The sequences were analyzed to detect the presence of mutations in the PCR product and compared with the international database on the National Center For Biotechnology Information (NCBI), then the mutations were scored and

the expected mutations were shown in table (3-11). The sequences of the normal and patients were shown in the figure (3-18).

Table (3-11) The expected mutations in BRCA1 exon11

Expected Mutations	Number of Samples	Subject sequence of the NCBI
Deletion	**4**	**6521077, 6521071**
Point Mutation	**3**	**150599**
Total	**7**	

The expected mutations were found after the analysis of the sequences in the NCBI. The mutations were found in the base number 6521077 and 6521071 of the gene from the NCBI. It was repeated in 4 samples out of 24 samples at the same base. There was another mutation found in three samples and expected to be a point mutation, repeated in 3 out of 24 samples (48 sequences) (Table 3-11). The sequence of the normal and the patients were shown in figure (3-18).

Nucleotide sequence of exon 11

ACCTCCAAGGTGTATGAAGTATGTATTTTTTTAATGACAATTCAG
TTTTTGAGTACCTTGTTATTTTTGTATATTTTCAGCTGCTTGTGAATT
TTCTGAGACGGATGTAACAAATACTGAACATCATCAACCCAGTAAT
AATGATTTG

Sequence alignment of exon 11 using BLAST from NCBI

ref|NW_001838436.2| Homo sapiens chromosome 17 genomic contig, alternate assembly
(based on HuRef), whole genome shotgun sequence Length=1610766

Features in this part of subject sequence:
breast cancer 1, early onset isoform BRCA1-delta9-11
breast cancer 1, early onset isoform BRCA1-delta9-10-11b
Score = 274 bits (148), Expect = 2e-71
Identities = 148/148 (100%), Gaps = 0/148 (0%)
Strand=Plus/Plus

```
Query  1       ACCTCCAAGGTGTATGAAGTATGTATTTTTTTAATGACAATTCAGTTTTTGAGTACCTTG  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  150452  ACCTCCAAGGTGTATGAAGTATGTATTTTTTTAATGACAATTCAGTTTTTGAGTACCTTG  150511

Query  61      TTATTTTTGTATATTTTCAGCTGCTTGTGAATTTTCTGAGACGGATGTAACAAATACTGA  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  150512  TTATTTTTGTATATTTTCAGCTGCTTGTGAATTTTCTGAGACGGATGTAACAAATACTGA  150571

Query  121     ACATCATCAACCCAGTAATAATGATTTG  148
               ||||||||||||||||||||||||||||
Sbjct  150572  ACATCATCAACCCAGTAATAATGATTTG  150599
```

Sample 4

ref|NT_010783.15| Homo sapiens chromosome 17 genomic contig, GRCh37 reference primary assembly

Length=44983201

Features in this part of subject sequence:

breast cancer 1, early onset isoform BRCA1-delta9-11

breast cancer 1, early onset isoform BRCA1-delta9-10

Score = 241 bits (130), Expect = 2e-61

Identities = 133/134 (99%), Gaps = 1/134 (0%)

Strand=Plus/Minus

```
Query  1       ATTTTTTT-ATGACAATTCAGTTTTTGAGTACCTTGTTATTTTTGTATATTTTCAGCTGC  59
               |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6521085 ATTTTTTTAATGACAATTCAGTTTTTGAGTACCTTGTTATTTTTGTATATTTTCAGCTGC  6521026

Query  60      TTGTGAATTTTCTGAGACGGATGTAACAAATACTGAACATCATCAACCCAGTAATAATGA  119
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6521025 TTGTGAATTTTCTGAGACGGATGTAACAAATACTGAACATCATCAACCCAGTAATAATGA  6520966


Query  120     TTTGAACACCACTG  133
               ||||||||||||||
Sbjct  6520965 TTTGAACACCACTG  6520952
```

Sample 6

> ref|NT_010783.15| Homo sapiens chromosome 17 genomic contig, GRCh37 reference primary assembly

Length=44983201

Features in this part of subject sequence:

breast cancer 1, early onset isoform BRCA1-delta9-11

breast cancer 1, early onset isoform BRCA1-delta9-10

Score = 237 bits (128), Expect = 2e-60

Identities = 136/141 (96%), Gaps = 2/141 (1%)

Strand=Plus/Minus

```
Query  1       TTTTTT-ANGAC-ATTCNGTTTTTGAGTACNTTGTTATTTTTGTATATTTTCAGCTGCTT  58
               |||||| | ||| |||| |||||||||||| ||||||||||||||||||||||||||||
Sbjct  6521083 TTTTTTAATGACAATTCAGTTTTTGAGTACCTTGTTATTTTTGTATATTTTCAGCTGCTT  6521024

Query  59      GTGAATTTTCTGAGACGGATGTAACAAATACTGAACATCATCAACCCAGTAATAATGATT  118
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6521023 GTGAATTTTCTGAGACGGATGTAACAAATACTGAACATCATCAACCCAGTAATAATGATT  6520964


Query  119     TGAACACCACTGAGAAGCGTG  139
               ||||||||||||||||||||
Sbjct  6520963 TGAACACCACTGAGAAGCGTG  6520943
```

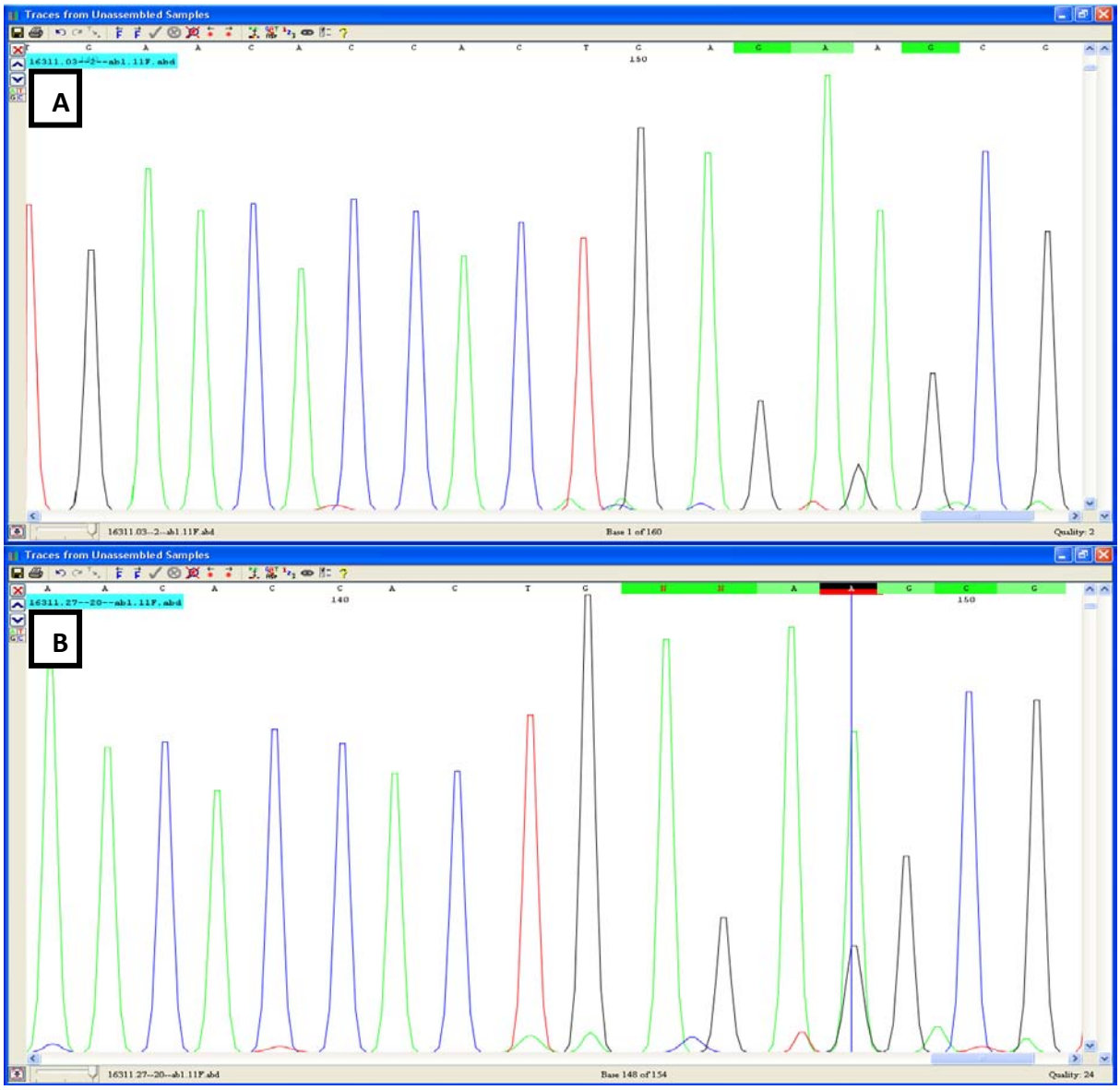


Figure (3-18) The sequences of the normal (A) and Italian breast cancer patients (mutation was shown as a double peak (B)) for BRCA1 gene exon 11.

AL-Sanati (2009) reported that the Iraqi breast cancer BRCA1 mutations have been detected in five patients (6.2%). Three patients had one mutation in **5382insC**, and the other two had two mutations both in **185delAG** and **5382insC.**

Capalbo *et al.* (2006) identified 25 and 52 variants in the BRCA1 and BRCA2 genes, respectively. Seventeen of them represent novel variants,

including four deleterious truncating mutations in the BRCA2 gene (472insA, E33X, C1630X and IVS6+1G>C). Twenty-seven of the 99 probands harbored BRCA1 and BRCA2 pathogenic germline mutations, indicating an overall detection rate of 27.3% and increasing by more than 15% the spectrum of mutations in the Italian population.

Cortesi *et al.* (2003) found the 3358T-A BRCA1 nonsense mutation in three patients with breast cancer, two patients with ovarian cancer and one patient with uterine adenocarcinoma. Furthermore, 13 healthy individuals were carriers of the same BRCA1 mutation.

Claes *et al.* (2004) reported that 49 (18 distinct) BRCA1 mutations and 26 (10 distinct) BRCA2 mutations were identified in Belgian population. The vast majority of the mutations were predicted to lead to a premature stop codon (39 frameshifts, 15 nonsense mutations and 24 splice site disruptions).

In only 7.5% of the sporadic patients diagnosed with breast cancer at young age, BRCA1/2 mutations were identified. A possible role for genetic variants in DNA double strand break repair genes has been hypothesized in this patient group, since a significant proportion of these patients showed elevated chromosomal radiosensitivity by in vitro assays (Baeyens *et al.*, 2002).

Chapter Four

Conclusions

and

Recommendations

4.1 Conclusions

1. The mean age of the Iraqi breast cancer patients was lower than that of the Italian breast cancer patients, and the breast cancer appears earlier in Iraqi women.
2. TMA is a rapid and effective method for IHC and FISH studies.
3. The percentage of the ER and PR expression in the Italian cases were higher than the Iraqi cases, which means that the Iraqi breast cancer was more aggressive than the Italian counterpart.
4. The percentage of Ki67 expression in the Italian cases was higher than the Iraqi breast cancer cases.
5. Positive immunoexpression of p53 was found in (87.2%) of the Iraqi breast cancer cases and was overexpressed in (76%) of the Italian breast cancer cases.
6. HER-2 overexpression was observed in (54.5%) of the Iraqi breast cancer, while overexpressed in (53.3%) of the Italian breast cancer.
7. There was a significant relationship between the HER-2 expression and (ER and p53) expressions, but there was no significant relationship between HER-2 expression with (PR and Ki67) expressions.
8. Triple negative cases were found higher in the Iraqi samples than the Italian samples. Suggesting that the Iraqi samples were phenotypicaly more aggressive than the Italian breast cancer samples and not easily responding to the hormonal and anti HER-2 receptor therapy.
9. FISH is the gold standard method to assess HER-2 positive cases.
10. DNA was successfully extracted from FFPE sections and used for BRCA1 exon 11 studies.
11. Four deletion mutations and three point mutations were found in BRCA1 exon11using DNA sequencing technique.

4.2 Recommendations

1. It is highly recommended to search for new prognostic and predictive markers for breast cancer.
2. Searching the molecular markers in precancerous lesions will be a strong effective factor in breast cancer diagnosis.
3. Using a new molecular technique for breast cancer diagnosis like DNA Microarrays to study the genes profile expression, Oncotype testing, Micro-RNA and quantitative Real Time PCR.
4. Getting fresh samples is better for molecular studies like PCR.
5. Using TMA technique for studying the Iraqi cancers on large number of patients.

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الخلاصة

يعد سرطان الثدي من اكثر انواع السرطانات شيوعا بين النساء في العالم، وهو مرض متباين الاصل. لقد ازداد معدل انتشار هذا المرض في العراق في السنوات الاخيرة. اجريت الدراسة الحالية لدراسة كيم بإئية مناعية نسيج بةٍ(IHC) والتهجين الموقعي الومضاني (FISH) و تفاعل التضاعف التسلسلي(PCR) للدنا لجين ال -(BRCA1 exon11) لمرضى سرطان الثدي وذلك بأستخدام تقنية المصفوفة الدقيقة للنسيج (Tissue Microarray). استخدمت هذه التقنية وبنجاح لأول مرة في القطر على نماذج مرضى سرطان الثدي. والبيانات التشريحية المرضية للمرضى اشارت الى ان متوسط العمر لمرضى سرطان الثدي العراقيين (49.38 ±8.3) سنة تتراوح ما بين (36-69) سنة. بينما متوسط عمر المرضى الايطاليين (57±8.52) سنة تتراوح ما بين (39-73) سنة . وأن مؤشر عامل العمر في المرضى العراقيين كان اقل من المرضى الايطاليين مما يدل على ان ظهور هذا المرض في المجتمع العراقي اسرع ويظهر بوقت مبكر مقارنة بالمرضى الايطاليين. لقد تم استخدام عينات من انسجة مثبتة بالفورمالين، مغمورة في شمع البارافين. ثلاثة نماذج من مصفوفة النسيج الدقيق تم تصميمها وتركيبها؛ النموذج الاول ويحتوي خمسة وخمسون نموذج من مرضى سرطان الثدي العراقيين؛ ثلاثون نموذج من سرطان الثدي لمرضى ايطاليين تم استخدامها لتشكيل النموذج الثاني؛ خمسة عشرة نموذج من الورم الحميد للثدي تم استخدامها لتشكيل النموذج الثالث والذي يمثل مجموعة السيطرة. كل نموذج تم تمثيله وذلك بنقل ثلاث مكررات من النموذج الواهب تم تحويلها الى النموذج المس تقبل. مقاطع من هذه النماذج الثلاث تم تقطيعها ووضعها على شرائح موجبة الشحنة وتم تصبيغ هذه النماذج بصبغة ال-(H&E) ومن ثم تم دراسة الكيمياء المناعية النسيجية لمستقبلات الاستروجين(ER) والبروجسترون(PR) و Ki67 وp53 وHER-2 لجميع العينات الثلاثة العراقية والايطالية والسيطرة.

أظهرت النتائج أن (47.3%)26 نموذج من اصل 55 قد وجدت موجبة التعبير لمستقبلات الاستروجين في المجموعة العراقية، وأن (76%)23 نموذج من اصل 30 نموذج كانت موجبة التعبير لمستقبلات الاستروجين في المجموعة الايطالية. وأن تعبير الاستروجين كان اقل في النماذج العراقية مقارنة بالايطالية. وقد ظهر وجود تعبير مستقبلات البروجسترون في (63.6%)35 من اصل 55 نموذجا عراقيا، وبالنسبة للنماذج الايطالية فقد لوحظ أن (76%)23 من اصل 30 نموذجا كانت موجبة التعبير لمستقبلات البروجسترون. من ناحية اخرى فأن 30 نموذجا (54.5%) من اصل 55 كانت موجبة التعبير لـKi67 في النماذج العراقية، وأن 23 نموذجا (76%) من اصل 30 كانت موجبة التعبير بالنسبة للنماذج الايطالية. وكذلك فأن 48 نموذجا (87.2%) من اصل 55 قد وجدت موجبة التعبير لل-p53 في

النماذج العراقية. أما بالنسبة للنماذج الايطالية فأن 23 نموذجا (76%) من اصل 30 وجدت موجبة التعبير لل-p53.

وكانت زيادة تعبير ال-HER-2 قد وجدت في 30 مريضا (54.5%) بالنسبة للنماذج العراقية، بينما كانت موجودة في 16 مريض (53.3%) بالنسبة للنماذج الايطالية. وأظهرت النتائج ايضا أن 12 نموذج كانت سالبة التعبير لمستقبلات الاستروجين والبروجسترون وال -HER-2 (السلبي الثلاثي) (21.8%) بالنسبة للنماذج العراقية. فيما كانت حالة واحدة فقط (3.3%) سالبة للثلاث مستقبلات في النماذج الايطالية. واظهرت النتائج انه النماذج العراقية كانت ظاهريا اكثر حدة مقارنة بالنماذج الايطالية التي استخدمت في هذه الدراسة.

تم دراسة الترابط بين خمس مؤشرات سرطان وكشفت الدراسة عن أن هناك علاقة معنوية بين تعبير الـHER-2 وتعبير الـ(ER وp53) (P <0.01) ، ولكن لم تكن هناك علاقة معنوية بين تعبير ا لـ HER-2 مع (PR وKi67) (P >0.01).

لقد قمنا باستخدام تقنية ال -TMA من أجل التحقق من الاختبارات الجزيئية HER-2. تستعرض هذه الدراسة مدى صحة ومصداقية الاختبارين الأكثر شيوعا للكشف عن HER-2 : IHC وال-FISH (هذا الاختباراستخدم بنجاح لأول مرة على عينات سرطان الثدي العراقية). اعتمادا على التحليل الإحصائي من الدراسات التي تقارن IHC و FISH. فأن اختبار ال -FISH اظهر أنه كان هناك تضاعف في جين الـHER-2 في (88%) من حالات الـ +IHC2، و (98.2 ٪) في +IHC3. اما في العينات الايطالية، فأن التضاعف قد لوحظ في (81.81 ٪) +IHC2، و (100 ٪) في +IHC3.

تم استخلاص الحامض النووي بنجاح من مقاطع -FFPE(Formalin-Fixed, Paraffin Embedded). بعد تقييم تراكيز الحامض النووي، فقط عينات الحامض النووي المستخلص من العينات الإيطالية قد استخدمت بنجاح لتفاعل التضاعف المتسلسل لجين BRCA1, exon11. الترحيل الكهربائي لنماذج الدنا على هلام الأغاروس أظهر وجود حزمة بحجم 185 زوج قاعدي، والذي يمثل الحجم الصحيح المتوقع في ال 24 نموذج من أصل 30 عينة المستخدمة في هذه الدراسة. تم تنقية نواتج التفاعل التسلسلي المتضاعف ودراسة تسلسل الحامض النووي وتحليلها بواسطة NCBI والنتائج اظهرت 4 عينات وجدت فيها طفرة الحذف و 3 عينات تحمل طفرة نقطية.

تستنتج هذه الدراسة بان متوسط العمر للمرضى للعراقيين كان أقل منه في المرضى الايطاليين ، وسرطان الثدي يظهر مبكرا في النساء العراقيات. وأن المصفوفة الدقيقة للنسيج استخدمت وبنجاح كطريقة سريعة واقتصادية وفعالة في دراسة الIHC والFISH.

لوحظت فروقات في تعبير مستقبلات الاستروجين والبروجسترون وال Ki67- وال p53- والHER-2 بين النماذج العراقية والايطالية. ولقد كان تعبير مستقبلات الاستروجين والبروجسترون اعلى في النماذج الايطالية مما يدل على ان طبيعة السرطان في المرضى العراقيين اكثر عدوانية بالمقارنة مع طبيعة السرطان في المرضى الايطاليين، الحالات السالبة لم تستجب للعلاج الهرموني. ولقد وجدت علاقة معنوية بين تعبير جينات الHER-2 وتعبير الER والp53، لكن لم توجد علاقة معنوية بين تعبير الHER-2 وتعبير الPR والKi67. حالات الثلاثي السالب كانت في النماذج العراقية اكثر مما هو عليه في الايطالية ويمثل هذا احدى الدلائل على كون مرض سرطان الثدي في النماذج العراقية اكثر عدوانية واقل استجابة للعلاج الهرموني والعلاج بالضد احادي النسيلة (Monoclonal Antibody). يعتبر اختبار الFISH الاختبار الذهبي بالنسبة لتوضيح حالة ال HER-2- واستجابته للعلاج بالضد احادي النسيلة. لقد تم استخلاص الدنا وبنجاح من مقاطع ال FFPE- واستخدم لدراسة التفاعل التسلسلي المتضاعف لجين ال BRCA1 Exon11-. تعتبر دراسة تسلسل القواعد الناتروجينية للدنا (DNA Sequencing) طريقة فعالة لكشف الطفرات في نماذج الدنا.

بِسْمِ اللّهِ الرَّحْمَنِ الرَّحِيمِ

(اقْرَأْ بِاسْمِ رَبِّكَ الَّذِي خَلَقَ {1} خَلَقَ
الْإِنسَانَ مِنْ عَلَقٍ {2} اقْرَأْ وَرَبُّكَ الْأَكْرَمُ
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مَا لَمْ يَعْلَمْ {5})

صدق الله العظيم

{سورة العلق1-5}

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وزارة التعليم العالي والبحث العلمي
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دراسة مقارنة جزيئية وكيميائية نسيجية مناعية والمصفوفة المجهرية للنسيج لمرضى عراقيين وايطاليين مصابين بسرطان الثدي

اطروحة

مقدمة الى مجلس كلية العلوم / جامعة النهرين وهي جزء من متطلبات نيل درجة

دكتوراه فلسفه في التقانة الاحيائية

من قبل

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