Immunohistochemical and molecular study in women with breast cancer

A thesis

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BY

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2014
بسم الله الرحمن الرحيم
وَقَلَ رَبِّ تَزِدِنِي عِلْمًا
صِدَاق اللَّهِ الْعَلِيِّ الْعَظِيمِ
وطِه (۱۱۴)

الإهداء
إلى منار النفوس 

النبي محمد ﷺ 

إلى يعسوب الدين............ علي 

ابن أبي طالب ﷺ 

إلى منذ البشرية 

المهدي عج 

إلى مصدر قوتي 

أبي 

إلى واحة راحتتي 

أمي 

إلى شمعتي الغالية 

حسن 

إلى سندي .......... أخي و أخواتي 

إلى كل أصدقاءي ........ وأولهم 

جنان 

إيمان
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<td>Avidin-biotin complex</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer gene 1</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast cancer gene 2</td>
</tr>
<tr>
<td>C-erb B2</td>
<td>Erythroid leukemia oncogene homologue 2</td>
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<tr>
<td>CISH</td>
<td>Chromomeric in situ hybridization</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzedin</td>
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<td>DCIS</td>
<td>Ductal carcinoma in situ</td>
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<tr>
<td>DNA</td>
<td>Deoxyribose Nucleic Acid</td>
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<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
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<tr>
<td>ERBB</td>
<td>Avian Erythroblastosis Oncogene B</td>
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<tr>
<td>FFPE</td>
<td>Formalin fixed paraffin embedded</td>
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<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>FNA</td>
<td>Fine Needle Aspiration</td>
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<td>H&amp;E</td>
<td>Hematoxyline and Eosin</td>
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<td>HER2</td>
<td>Human Epidermal Growth Factor (EGF) Receptor-2</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone Replacement Therapy</td>
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<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
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<tr>
<td>IDC</td>
<td>Invasive Ductal Carcinoma</td>
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<td>IHC</td>
<td>Immunohistochemical</td>
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<td>ILC</td>
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<td>Metaplastic breast carcinoma</td>
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<tr>
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<td>Terminal Duct–Lobular Unit</td>
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<td>Tyrosine Kinase</td>
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<td>TGFα</td>
<td>Transforming growth factor</td>
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Summary

This study was done in the immunohistchimistry laboratory of AL-SADER hospital/ AL-NAJAF AL ASHRAF and International Institute of early detection of cancer /Medical City /Baghdad. Confirmed 50 cases of breast carcinoma (50 female cases with breast cancer were (43 infiltrating ductal carcinoma IDC), 2 infiltrating lobular carcinoma (ILC), and 5 other types carcinoma), included in this study were reviewed in the period between January 2010 and April 2013 against a group of 20 cases with benign breast lesions was included for comparison. Immunohistochemistry (IHC) were used to evaluate the expression of Her2/neu, Estrogen(ER), and Progesterone(PR) receptor test and to correlate immunohistochemical findings with prognostic parameters for breast carcinoma such as patients' age, tumor size, histological type, histological grade.

All samples from patients under study were included paraffin embedded formalin fixed blocks tissue. Results observed that estrogen positive receptors were in 48% (24/50) of the cases and progesterone positive receptors in 46 % (23/50) of the cases, and these data revealed that presence of hormone receptor expression in the majority and cancer was considered hormone receptor positive. The present results on Iraqi women patients revealed that a high age frequency of cancer occurred between 41-50 years of age (46%), and more than 24% of the patients have family history either it is the first or second degree. The histopathology diagnosis showed that a high percentage in Iraqi cases with infiltrated ductal carcinoma represented (86%), while the invasive lobular carcinoma represented (4%) and the mixed carcinoma was represented (10%). Results
revealed that 12 % of patients were in grade 1, 20 % were in grade 2 and 68 % were in grade 3, so most of our patients were in grade 2 and 3. Clinicopathological assessments for ER and PR revealed that there was no statistically significant association between expression and the age, family history, tumor types or stages (P>0.05). On the other hand the expression of Her-2/neu did not show any statistical significant difference with the age, tumor grade or type except there was a significant correlation between her2/neu expression and family history (P<0.01). About 56% of positive ER had a strong positive stain while 42% of PR had strong positive stain.

The results of immunohistochemical analysis for all breast cancer subtypes according to molecular subtype classification showed that luminal A was 54%, Luminal B 4%, Her2/neu subtype 10% and Basel like 32%. Her2/neu over-expression was observed in 14% of Iraqi breast carcinoma affecting female patients. This group presents apparently an aggressive form of breast carcinoma with high histological grade and negative ER.

Four different DNA extraction protocols (Phenol/chloroform, Genaid, Qiagen, and Promega extractions kits) were carried out to evaluate the yield and quality of DNA from formalin fixed paraffin embedded tissue samples(FFPE). The results demonstrated the successful use and optimization of a rapid, reliable and effective protocol for DNA amplification directly from paraffin-embedded tissue eliminating the deparaffinization and DNA isolation steps with maximum yield in the range of was 160 - 293µg obtained with Promega DNA extraction kit, while all of the other kits produce smear DNA in most FFPE cases.
For analysis of HER2/neu gene amplification in FFPE breast cancer cases a standard PCR was used by using forward and reverse primers were performed and PCR amplified products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining against benign tissue. HER2/neu amplified fragment band displayed a strong band in one case and estimated to have a size of 126 bp. A HER2/neu DNA band was extracted from lane and purified using a gel extraction kit and sequenced using the ABI DNA sequencing kit. Samples were loaded into the ABI 310 Genetic Analyzer and the results are shown. BLAST analysis of the DNA sequence was carried out in the National Center for Biotechnology Information’s (NCBI) Genbank and found that the sequence corresponds to the HER2/neu gene fragment on exon 3.
Introduction

Breast cancer represents the most common malignancy affecting women in developed countries, with more than 200,000 new cases diagnosed yearly in the US. Moreover, the incidence rates have increased rapidly in previously low-incidence areas, such as China, partly due to changes in lifestyle and professional habits, as well as the progression of urbanization (Jemal et al., 2011).

The last decade has witnessed significant achievements in the management of advanced breast cancer, including the introduction of novel chemotherapeutic agents (Thomas et al., 2007), the use of aromatase inhibitors in post-menopausal women (Bonneterre et al., 2001) and the benefits derived from molecular-targeted agents, e.g., trastuzumab (Marty et al., 2005) and lapatinib (Geyer et al., 2006), in patients with epidermal growth factor receptor2 (HER2)-overexpression tumors. Despite aggressive multidisciplinary treatment approaches, the prognosis of metastatic breast cancer remains poor, with a median survival of 20 months (Dawood et al., 2008).

HER2, a member of the epidermal growth factor receptor family, is a major target for molecular-targeted therapy in breast cancer. HER2 locates at human chromosome 17q11.2-12, encoding a transmembrane tyrosine kinase that is composed of three distinct regions: an extracellular region containing a ligand-binding domain, a transmembrane domain and an intracellular region harboring a tyrosine kinase domain. Ligand binding leads to receptor dimerization, autophosphorylation and subsequent activation of intrinsic tyrosine kinase activity. Activation of HER2 receptors initiates a series of downstream signaling pathways that regulate various cellular functions, including cell proliferation, apoptosis, angiogenesis and motility.
Although HER2 is not expressed on the cell surface of many normal tissues (Press et al., 1994), HER2 gene amplification and protein overexpression are present in 20-30% of breast cancers. HER2 receptor has become an important target for targeted cancer therapy with trastuzumab (Herceptin®). Trastuzumab, a humanized monoclonal antibody has revolutionized therapy for patients with metastatic breast cancers. Studies have indicated that trastuzumab is particularly effective in the treatment of HER2-positive metastatic breast cancer (Vogel and Franco, 2003).

Overexpression of HER2 has been identified in human breast cancers (Rexer et al., 2013). Although similar HER2 receptor expression between primary breast cancers and metastatic lymph nodes has also been reported (Cho et al., 2008), there are only a few reports regarding the comparison of the HER2 status between the primary breast cancer and the distant metastatic lesions. To date, the literature regarding the concordance of HER2 receptor expression between primary and local-regional recurrences is sparse. Uncertain whether recurrences have the identical or similar HER2 receptor expression pattern as the primary breast cancer (Gancberg, 2002).

Receptor overexpression, together with a similar expression in both primary tumors and disseminated lesions, is considered necessary for the success of targeted therapy, particularly targeted nuclide radiotherapy. In receptor-mediated tumor targeting nuclide radiotherapy, tumor cells are killed with delivered radiation and therapeutic efficiency is mainly dependent on the receptor expression (Carlsson et al., 2003). However, in most studies, samples for analysis are usually obtained from the primary lesion, and the status of the targeted molecules is determined based only on the primary tumor. The expression of HER2 was investigated immunohis-
tochemically in a series of Fixed formalin paraffin embedded primary breast cancer samples and corresponding local-regional recurrent lesions.

The aim of the study is to estimate the clinical-pathological parameters which characterize the study group and focus on comparative investigation of hormonal receptors (estrogen receptor ER and progesterone receptor PR) and Her2/neu oncoprotein expression, according to which we rank the cases into molecular classification subtypes, determining certain correlations between them and morphoclinical prognostic factors in addition to screening mutation in her2/neu in Iraqi breast cancer women; So to achieve these goals the following steps were done:

1- Assessment of the estrogen, progesterone and Her2/neu receptors by IHC methods.
2- Use different DNA extraction protocol from FFPE samples and purification of DNA.
3- Assessment the overexpression of her2/neu protein in human breast cancer in comparison to normal breast tissue and benign breast lesion using specific primer for her2/neu.
4- Screening the mutation in her2/neu receptors using specific primers.
5. Sequencing of the HER2positive mutation sample.
Chapter One

1. Cancer

Throughout people’s lives, the cells in their bodies are growing, dividing, and replacing themselves. Many genes produce proteins that are involved in controlling the processes of cell growth and division. A change (mutation) occurring in the DNA molecules can disrupt the genes and produce defective proteins and cells. Abnormal cells can start dividing uncontrollably, eventually forming a new growth known as a “tumor” or “neoplasm” (Blachford, 2002). Cancer arises as a result of a number of genetic alterations in the dividing cell (Bågeman, 2008).

Cancer is a class of diseases or disorders that is characterized by uncontrolled division of cells and the ability of these abnormal cells to spread, either by direct growth into adjacent tissues through invasion, or by implantation into distant sites by metastasis (where cancer cells are transported through the bloodstream or lymphatic system) (Ghanim, 2009 and AL Kashwan, 2009). Cancer may affect people at all ages, but risk tends to increase with age. It is one of the principal causes of death in developed and developing countries (Blachford, 2002).

1.1 Breast cancer

As general consideration breast cancer is a heterogeneous disease with substantial genotypic and phenotypic diversity (Bågeman, 2008 and Luis et al., 2012), and it can be subdivided into clinical, histopathological and molecular subtypes (Penault-Llorca and Viale, 2012). It is the second most common malignant tumor that occurs in women most commonly from the inner lining of milk ducts, or lobules that supply the ducts with milk (Sariego, 2010). Although this tumor generally appears in sporadic form, ranging from 5% and 10% of all cases, it is considered as hereditary disease.
due to inherited autosomal dominant mutations in several susceptibility genes (Al Ghurabi, 2002). Indeed About 40% to 50% of hereditary breast cancers and most hereditary breast and ovarian cancers are thought to be caused by mutations in breast cancer susceptibility gene 1 (BRCA1) (Jumaah, 2013). Worldwide, about 1.1 million cases are diagnosed annually (Kumor, 2009), and the fifth most common cause of cancer death after lung cancer, stomach cancer, liver cancer, and colon cancer in Iraq. (Iraqi Cancer Registry Center, 2009 and Al Bederi, 2011).

1.1.1 Histology of breast

The breast or mammary gland is covered by skin and subcutaneous tissue and rests on the pectorals muscle, from which it is separated by a fascia. The morphofunctional unit of the organ is the single gland, a complex branching structure that is topographically arranged into lobes (Chang et al., 1982) and which is made up of two major components: the terminal duct–lobular unit (TDLU) and the large duct system. The TDLU is formed by the lobule and terminal ductule and represents the secretary portion of the gland. It connects with the subsegmental duct, which in turn leads to a segmental duct, and this to a collecting (lactiferous or galactophorous) duct, which empties into the nipple. A fusiform dilation located beneath the nipple. Inside a woman’s breast are 15 to 20 sections (lobes). Each lobe is made of many smaller sections (lobules). Lobules have groups of tiny glands that can make milk. After a baby is born, breast milk flows from the lobules through thin tubes (ducts) to the nipple. Fibrous tissue and fat fill the spaces between the lobules and ducts (Ribate, 2007).
1.1.2 Etiology of Breast Cancer

Etiological factors responsible for breast cancer development are still not completely known, but epidemiological evidences significantly suggest on three possible groups of genetic, endocrine and exogenous factors. Genetic mutations responsible for breast carcinogenesis are:

1. activation of proto-oncogene (HER2/neu, located at 17q).
2. inactivation (loss or mutation) of tumor suppressor genes: like p53, BRCA1 and NF1.
3. inactivation of genes responsible for DNA repair (Diallo et al., 2001 and Sting et al., 2006).

Breast cancer family history is important for the first generation of female family members i.e. mother, daughter and sister. Women whose mothers had bilateral breast cancer before menopause carry the highest risk. They have nine times higher risk than others, i.e. 50% of them may develop breast malignancy. Endocrine factors are connected to the endogenous hyperestrogenism, and exogenous intake is connected to the intake of oral contraceptives (OC) and to hormone replacement therapy (HRT). The most important risk factors are: long period of generative time (earlier menstruation and later menopause), infertility, late age at first full-term pregnancy and obesity (Weidner and Semple, 1992).

Influence of physical exercise on the age of the first menstruation is very significant, e.g. girls who exercise regularly whether they practice ballet, swimming or running, start their period later than others. It was demonstrated that girls who did ballet started their menstruation at the average age of 15.4 years in comparison to the control group who started menstruation at the average age of 12.5 years (Di Costanzo et al., 1997).

There are evidences that hyperestrogenism is connected to fibrocystic epithelial hyperplasia. Moderately increased (although disputed) risk is determined by exogenous estrogen (long usage of OC or HRT in
menopause). Many studies have been published about the influence of OC and HRT on breast cancer, with controversial results and the only clear conclusion is that they have no protective effect against breast cancer (Gamallo et al., 1993). Breast cancer cells in women produce different growth factors TGFα (Transforming growth factor) and PDGF (Platelet derived growth factor). Estrogens stimulate the production of these growth factors and it is possible that interactions of circulating hormones, hormonal receptors of cancer cells and autocrine growth factors have a role in the progression of breast cancer.

Measurement of the quantity of hormonal receptors in breast tissue is used to predict response of breast cancer cells to hormonal therapy. In the postmenopausal period larger source of estrogen is fat tissue, where conversion of adrenal androstendion into estrogen occurs (Maier et al., 1977). Women younger than 50 have little or no increased risk connected to the body mass (BM), while women over 60 with 10 kg overweight have an elevated risk (80%) of breast cancer development (Jensen et al., 1997). Visceral obesity is common in over-weighted patients with breast cancer (Rapin et al., 1988 and Reinfuss et al., 1995).

1.1.3 Histopathology of breast cancer

The vast majority of breast cancers arise in the epithelial cells of the TDLU, and are therefore classified as carcinoma. Breast carcinomas are classified pathologically on the basis of their morphology and growth pattern. The majority of the breast carcinomas (about 60%), however, cannot be classified satisfactorily according to specialized pathological subtypes and are designated as invasive ductal carcinoma (IDC) Not Otherwise Specified (NOS). Another common name for these tumors is IDC of No Special Type (NST). The “special type” pathological subtypes of breast carcinoma include invasive lobular carcinoma (ILC), medullary
breast carcinoma (MC), mucinous breast carcinoma, tubular breast carcinoma, and metaplastic breast carcinoma (MBC) (Rosai, 2011).

- **Invasive ductal carcinoma (IDC):**

  Invasive ductal carcinoma not otherwise specified (IDC NOS) is a very heterogeneous group of tumors which includes all breast carcinomas that cannot be classified as a special pathological subtype. The tumors are classified as IDC mixed-type tumors when a special type component of more than 50% is present in addition to the IDC NOS component. In 80% of the IDC cases a precursor lesion of ductal carcinoma *in situ* (DCIS) is present, often of high grade comedo type. Although IDC NOS is generally considered to be a diverse group of breast carcinomas that cannot be assigned to one of the currently-known specialized pathological subtypes, many breast pathologists would agree that there may be one or more specialized subtypes still to be defined in this subgroup of carcinomas (American Cancer Society, 2011).

- **Invasive lobular carcinoma (ILC):**

  ILC is the most common of the special types, accounting for 10-15% of the breast cancers, thus is consider the most second common pathological subtype after IDC NOS. The remainder of the special types of breast carcinoma each do not account for more than 5% of breast tumors, with metaplastic breast cancer being the rarest (less than 1% of breast carcinomas) (Rosai, 2011).

  The classical pattern of invasive lobular carcinoma (ILC) is characterized by small rounded cells with scant cytoplasm, that diffusely grow through the stroma, often in strings of cells called “Indian files”. In most ILC cases, a lobular carcinoma *in situ* (LCIS) component is present, although DCIS has also been observed (Villadsen *et al.*, 2007). In addition
to classical ILC, other variants of ILC have been described including pleomorphic, alveolar, and solid lobular carcinoma (Weidner and Semple, 1992). Classical ILC tumors are frequently low grade tumors because of the morphologically uniform cells and a low mitotic index. Therefore, they have a more favorable prognosis than the ILC variants that have more marked nuclear pleomorphism and thus are of higher grade (Di Costanzo et al., 1997).

Complete loss of expression of the cell adhesion molecule E-cadherin has been observed for the majority (about 80%) of the ILCs and has been associated with truncating mutations of the E-cadherin gene in 50% of ILC breast cancers (Gamallo et al., 1993).

● Medullary carcinoma (MC)

Medullary carcinomas (MC) are poorly differentiated carcinomas with a syncitial growth pattern, absence of glandular structures, moderate to marked nuclear pleomorphism, complete histological circumscription of the tumor, and diffuse lymphocytic infiltrate. MCs are typically high grade tumors and are mostly ER-negative. However, the prognosis of MC is remarkably favorable and better than common IDC NOS, with 10 year survival rates of 50-90% depending on the criteria used. This probably is because less than 10% of the patients present with lymph node metastases (Jensen et al., 1997). Notably, 11% of MCs carry BRCA1 germ line mutations, which is about seven times more frequent than among breast cancers as a whole (Eisinger et al., 1998). Reciprocally, 13-20-% of BRCA1 mutant tumors are medullary carcinomas or carcinomas with medullary features (Marcus et al., 1997).

● Mucinous carcinoma
Mucinous or colloid carcinoma of the breast is characterized by clusters of small and uniform cells floating in a sea of extracellular mucin. DCIS is found to be present in 60-75% of mucinous carcinomas and may have any of the conventional patterns of DCIS (cribriform, comedo, papillary or micropapillary). Mucinous carcinomas are typically ER-positive and mostly also PR-positive (Shousha et al., 1986 and Diab et al., 1999).

Mucinous carcinoma has a very good prognosis with ten-year survival rates of 80-100%, although patients with mixed variants of mucinous carcinoma tend to do worse. The favorable prognosis probably is because pure mucinous carcinomas infrequently metastasize (Diab et al., 1999).

- **Tubular carcinoma**

  Tubular carcinomas are very well differentiated tumors that are characterized by single-layered open tubules, absence of necrosis and/or very few mitoses and minor nuclear pleomorphism. Almost two thirds of these tumors have a low-grade DCIS component (Rosai, 2001).

### 1.1.4 Classification of Breast Cancer

Fifty percent of women diagnosed with breast cancer will survive the disease without recurrence, whereas 15% of the patients will survive the disease despite a recurrence within 15 years. However, one third of breast cancer patients will die of metastases of the primary cancer within 15 years from diagnosis. It is therefore important to distinguish patients with a good prognosis that do not need additional therapy from patients with a poorer prognosis that will benefit from additional therapy. Reliable prognostic and predictive factors that classify breast tumors accurately are thus imperative for the clinician and have been a major focus in breast cancer research (Strachan and Read, 2011).

#### 1.1.4.1 Traditional prognostic and predictive factors
TNM stage as yet, the most powerful predictor of breast cancer recurrence is tumor stage. The TNM method for tumor staging is based on three tumor characteristics at the time of diagnosis: tumor size (T), axillary lymph node involvement (N) and the presence of metastases (M). Together these three factors define tumor stages I through IV (Table 1-1). Almost 90% of all patients with stage I cancers survive at least 5 years after diagnosis, whereas 5 year survival rates for stage II and III cancers are 60-80% and 40-50%, respectively. Patients who have a stage IV cancer have a very poor 10-year survival of less than 5%. The TNM stage of the tumor is thus a very strong indicator of the 5-year survival of the patient (Rosai, 2011).

● Axillary lymph node status

The extent of axillary lymph node involvement at the time of diagnosis is one of the most reliable independent prognostic factors for breast cancer. Patients with tumor free lymph nodes have a far better prognosis than patients with positive lymph nodes, with about 15-45% of node-negative patients having a disease recurrence compared to 50-70% of node-positive patients. Additionally, the risk of disease recurrence as well as mortality increases with an increasing number of lymph nodes involved (Nemoto et al., 1980; Fitzgibbons et al., 2000 and Weiss et al., 2003). The lymph node status is determined by the sentinel node procedure for staging purposes, followed by an axillary node dissection when metastases are present in sentinel nodes (Giuliano et al., 1995 and Allred and Elledge, 1999).

● Tumor size

The size of the tumor is a very strong prognostic factor, even after 20 years of follow-up. Although some pathologists measure the macroscopic size or the microscopic size of the tumor including both the invasive part and the
in situ components, only the microscopic size of the invasive part of the tumor is clinically significant.

Table 1-1 TNM stage classification (adapted by Tavassoli and Devilee, 2003).

<table>
<thead>
<tr>
<th>T</th>
<th>Primary tumor size</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>No evidence for primary tumor</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>T1</td>
<td>Tumor of 2 cm or less in greatest dimension</td>
</tr>
<tr>
<td>T2</td>
<td>Tumor larger than 2 cm, but not more than 5 cm in greatest dimension</td>
</tr>
<tr>
<td>T3</td>
<td>Tumor larger than 5 cm in greatest dimension</td>
</tr>
<tr>
<td>T4</td>
<td>Tumor of any size with direct extension to chest wall of skin</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N</th>
<th>Regional lymph node involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0</td>
<td>No regional lymph node metastases</td>
</tr>
<tr>
<td>N1</td>
<td>Metastases in movable ipsilateral axillary lymph node(s)</td>
</tr>
<tr>
<td>N2</td>
<td>Metastases in fixed ipsilateral axillary lymph node(s) or in clinically apparent ipsilateral internal mammary lymph node(s) in the absence</td>
</tr>
<tr>
<td></td>
<td>Of clinically evident axillary lymph node involvement</td>
</tr>
</tbody>
</table>
N3  Metastases in ipsilateral infraclavicular lymph node(s) with or without axillary lymph node involvement; or in clinically apparent ipsilateral internal mammary lymph node(s) in the presence of clinically evident axillary lymph node metastases

**M**  **Presence of distant metastases**

M0  No distant metastases

M1  Distant métastases

<table>
<thead>
<tr>
<th>Stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>I</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IIA</td>
<td>T0</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IIB</td>
<td>T2</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IIIA</td>
<td>T0</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N1, N2</td>
<td>M0</td>
</tr>
<tr>
<td>IIIB</td>
<td>T4</td>
<td>N0, N1, N2</td>
<td>M0</td>
</tr>
<tr>
<td>IIIC</td>
<td>Any T</td>
<td>N3</td>
<td>M0</td>
</tr>
</tbody>
</table>
The size of the primary tumor (T), involvement of regional lymph nodes (N) and the presence of distant metastases (M) together define the stage of the breast tumor at the time of diagnosis.

Tumor size is directly correlated to axillary lymph node involvement, as larger tumors frequently have more positive lymph nodes. However, larger tumor size is correlated with a worse prognosis independent of lymph node status (Carter et al., 1989; Rosen et al., 1993 and Quiet et al., 1995). This is mainly because lymph node-negative patients with a tumor smaller than 1cm have a far better prognosis than patients with a tumor larger than 2cm (80% versus 65%, respectively) (Chia et al., 2004).

**Histological tumor grade**

Although not as strong as TNM stage, lymph node status or tumor size, the histological grade of a tumor is a good prognostic marker for breast cancer patients. Tumor grade is determined by the Scarf-Bloom-Richardson Grading system, modified by Elston and Ellis (1991). According to this grading system 1, 2 or 3 points are given for each of the following tumor characteristics: tubule formation, nuclear pleomorphism, and mitotic count. The sum of these points forms a score of 1 to 9 that determines the grade or differentiation status of the tumor (Table 1-2).

**Table 1-2 Histological grade (adapted from Tavassoli and Devilee, 2003)**

<table>
<thead>
<tr>
<th>Score</th>
<th>Tubule formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>More than 75% of the tumor has tubule formation</td>
</tr>
<tr>
<td>2</td>
<td>10% to 75% of the tumor has tubule formation</td>
</tr>
<tr>
<td>3</td>
<td>Less than 10% of the tumor has tubule formation</td>
</tr>
<tr>
<td>Score</td>
<td>Nuclear pleomorphism</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------</td>
</tr>
<tr>
<td>1</td>
<td>Nuclei are small and uniform in size and shape</td>
</tr>
<tr>
<td>2</td>
<td>Nuclei are moderate in nuclear size and variation</td>
</tr>
<tr>
<td>3</td>
<td>Nuclei have marked variation, are relatively large, and have prominent or multiple nucleoli</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Score</th>
<th>Mitotic count (per 10 high power fields with field area of 0.274 mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0-9 mitoses</td>
</tr>
<tr>
<td>2</td>
<td>10-19 mitoses</td>
</tr>
<tr>
<td>3</td>
<td>More than 20 mitoses</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grade</th>
<th>Differentiation status</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Well differentiated</td>
<td>3-5 points</td>
</tr>
<tr>
<td>II</td>
<td>Moderately differentiated</td>
<td>6-7 points</td>
</tr>
<tr>
<td>III</td>
<td>Poorly differentiated</td>
<td>8-9 points</td>
</tr>
</tbody>
</table>

Tumors with a low grade are well differentiated and predict a more favorable prognosis for the patient than poorly differentiated tumors with a high grade. The ten-year survival of patients with the lowest grade is 90-95% as opposed to 30-80% for patients with the highest grade (Frkovic-Grazio and Bracko, 2002). Additionally, higher grade is associated with negative hormone receptor status and low grade with positive hormone receptor status. Therefore, histological grade is correlated with response to either endocrine therapy (low grade) or chemotherapy (high grade).
1.1.5 Breast cancer classification by Gene expression profiling

The tumors were divided into surrogates of the genetically defined subgroups of breast cancers and the subtype definitions were observed in table (1-4) as follows: luminal A (ER or PR+HER2-), luminal B (ER or PR+HER2+), HER2 overexpressing (ER-PR-HER2+), triple-negative (ER-PR-HER2-), basal-like (ER-PR-HER2-CK5+), non-classified (ER-PR-HER2-CK5-) and luminobasal (ER or PR+CK5+). In multivariate analysis, tumor size and HER2 positivity were a significant risk of early cancer relapse (Strachan and Read, 2011).

1.1.6 Epidemiology of breast cancer

Over the past 25 years, breast cancer incidence rates have risen globally, with the highest rates in industrial countries. Reasons for this trend include change in reproductive patterns, increased screening, dietary changes and decreased activity (Israyelyan, 2003). Breast cancer is the most common type of cancer among women in the United States (other than skin cancer). In 2012, about 227,000 American women diagnosed with breast cancer (National Cancer Institute, 2012). It is the second most common cause of cancer death in Thai women (Thongsuksai et al., 2000) and in the United States (Wonghongkul et al., 2006). In Bangkok, Thailand, every 20.5 in 100,000 women diagnosed with breast cancer (Jordan et al., 2009). In the United States, every one in eight women diagnosed with breast cancer before the age of 85 (Schag et al., 1993 and Mols et al., 2005). Due to advances in breast cancer detection and treatments, women’s chances of surviving are increasing (Lopez et al., 2005). In the United States breast cancer survivors represent 22% of the estimated 10.1 million cancer
survivors and 40% of all female cancer survivors (Knobf, 2007 and Padunchewit, 2010).

The first of the commonest ten cancers by Primary site and gender in Iraq in 2009 was breast cancer as observed in table (1-3). A total of (2987) new cases of Breast cancer were recorded among Iraqis in year 2009, of these (81) cases were males and (2906) cases were females with incidence rate about 19.59/10000 and registered as 9.43 Cases /10⁵ POP (Iraqi Cancer Registry Center Publications. Ministry of Health, Iraqi Cancer Board, 2009).

Table 1-3

Distribution the number of cancer cases in Iraqi population

(Iraqi Cancer Registry Center Publications, 2009)

<table>
<thead>
<tr>
<th>PRIMARY SITE</th>
<th>NO. OF CASES</th>
<th>MALE</th>
<th>FEMALE</th>
<th>% OF TOTAL</th>
<th>REGISTERED CASES /10⁵ POP.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- Breast</td>
<td>2987</td>
<td>81</td>
<td>2906</td>
<td>19.59</td>
<td>9.43</td>
</tr>
<tr>
<td>2- Bronchus and Lung</td>
<td>1570</td>
<td>1151</td>
<td>419</td>
<td>10.29</td>
<td>4.96</td>
</tr>
<tr>
<td>3- Brain and Other CNS**</td>
<td>962</td>
<td>531</td>
<td>431</td>
<td>6.31</td>
<td>3.04</td>
</tr>
<tr>
<td>4- Urinary Bladder</td>
<td>957</td>
<td>743</td>
<td>214</td>
<td>6.27</td>
<td>3.02</td>
</tr>
<tr>
<td>5- Leukemia*</td>
<td>908</td>
<td>506</td>
<td>402</td>
<td>5.95</td>
<td>2.87</td>
</tr>
<tr>
<td>6- Non–Hodgkin Lymphomas***</td>
<td>737</td>
<td>458</td>
<td>279</td>
<td>4.83</td>
<td>2.33</td>
</tr>
<tr>
<td>7- Colorectal</td>
<td>701</td>
<td>387</td>
<td>314</td>
<td>4.60</td>
<td>2.21</td>
</tr>
<tr>
<td>8- Skin</td>
<td>609</td>
<td>352</td>
<td>257</td>
<td>3.99</td>
<td>1.92</td>
</tr>
<tr>
<td>9- Stomach</td>
<td>538</td>
<td>307</td>
<td>231</td>
<td>3.53</td>
<td>1.70</td>
</tr>
<tr>
<td>10- Larynx</td>
<td>377</td>
<td>279</td>
<td>98</td>
<td>2.47</td>
<td>1.19</td>
</tr>
<tr>
<td>Total Ten</td>
<td>10346</td>
<td>4795</td>
<td>5551</td>
<td>67.84</td>
<td>32.67</td>
</tr>
<tr>
<td>All Sites</td>
<td>15251</td>
<td>7201</td>
<td>8050</td>
<td>100.00</td>
<td>48.16</td>
</tr>
</tbody>
</table>

1.1.7 Risk factors for breast cancer

The fact that a woman’s breast cancer risk increases within one or two generations when moving from a low risk to a high-risk area (Ziegler et al., 1993) indicates that not only genetic factors but also the environment affects breast cancer risk (Lichtenstein et al., 2000). Genetic variants and
Several breast cancer risk factors are also associated with prognosis. Paradoxically, a factor that confers an increased breast cancer risk may be associated with an improved prognosis if the woman develops breast cancer (Al-Bediri, 2011).

1- **Age**: It is well established that the incidence of breast cancer increases with age and will be double every 10 years until the menopause, when the ratio of increase is slow (Baum, 2002).

2- **Age at first-term pregnancy**: Nulliparous women have an increased risk of developing breast cancer (Stewart and Kleihnes, 2003). However, among parous women protection is related to early age for the first full-term pregnancy. If the first birth is delayed to the mid or late thirties, the women are at a greater risk of developing breast cancer than is a nulliparous woman (Bilimoria and Morrow, 1995).

3- **Weight**: Being overweight is associated with a doubling the risk of breast cancer in postmenopausal women. (Curry et al., 2003).

4- **Geographical variation**: there is an overall difference in incidence and death rate of breast cancer between different countries. The biggest difference is between Eastern and Western countries. The lowest rates are in South East Asia and Africa, while the highest rates are in North America, North-West Europe, Australia and New Zealand. However women from Japan who emigrate to the United States, show that their rates of breast cancer rise to become similar to United States rates within one or two generations indicating that factors relating to everyday activities are important than inherited factors in breast cancer (Baum, 2002).

5- **Family history**: Women who have a first-degree relative with breast carcinoma have a risk two or three times that of the general population, a risk further increased if the relative was affected at an early age and/or had
bilateral disease. The aspects related to the discovery of the genes responsible for predisposition to breast carcinoma (Skolnick and Cannon-Albright, 1992).

6-Menstrual and reproductive history: Increased risk is correlated with early menarche, nulliparity, late age at first birth, and late menopause (Kelsey et al., 1993 and Pathak et al., 2000). Breast carcinoma is rare in women who have been castrated; oophorectomy before 35 years of age reduces the risk to one-third. Women who have their first child before the age of 18 years have only one-third the risk of those whose first child is delayed until age 30 (Wang et al., 1985). A reduction in the risk of breast carcinoma among premenopausal women who have lactated has been documented, but no such effect was detected among postmenopausal women (Newcomb et al., 1994). Breast carcinoma risk is increased in postmenopausal women with a hyperandrogenic plasma hormone profile (Rosai, 2011).

7-Contraceptive agents: The various epidemiologic studies that have been done in this field have shown no increased risk, or at most a very low increase among young long-term users (Ross et al., 2000 and Padunchewit, 2010). The tumors that have developed in this population have not differed qualitatively from those seen in control cases (Fechner, 1970 and Fechner, 1971).

8- Ionizing radiation: An increased risk of breast carcinoma has been documented with exposure to ionizing radiation, particularly if this exposure occurred at the time of breast development (Goss and Sierra, 1998).

9-Others: A peculiar association between breast carcinoma and meningioma has been repeatedly noted (Bonito et al., 1993). Even more peculiar is that fact that sometimes the breast carcinoma is found to metastasize within the meningioma. Patients with ataxia–telangiectasia syndrome and with Cowden syndrome have an excess risk of breast cancer (Schrager et al., 1998).

10-Genetics: Hereditary breast cancer accounts for only 5-10% of all breast cancers and germ line mutations with the two major breast cancer susceptibility genes BRCA1 and BRCA2 (Bågeman, 2008) being responsible for a small fraction (~2-3%) of all breast cancers (Loman et al., 2001 and Narod and Foulkes, 2004). In addition to BRCA1 and BRCA2, P53 and
Phosphatase and tensin homolog (PTEN) are considered to be high-penetrance breast cancer susceptibility genes, whereas Ataxia telangiectasia mutated (ATM), BRCA1 interacting protein C-terminal helicase 1 (BRIPI), Checkpoint Homolog 2 (CHEK2), and Partner and Localizer of BRCA2 (PALB2) are considered to be moderate-penetrance susceptibility genes (Liaw et al., 1997 and Stratton and Rahman, 2008). A large proportion of familial aggregation of breast cancer, and possibly non-familial disease, is considered to be due to the effect of low-risk alleles, some being very common and possibly acting via polygenic (Bågeman, 2008).

11-Gene-Environment Interactions: Gene-environment studies may yield new insights with respect to breast cancer. A given exposure, e.g. lifestyle, may have different or even opposite effects on breast cancer risk or prognosis in women, depending on their genetic variants. When taking both genetic and environmental factors into account the potential effect on breast cancer may not be detected, since both women with an increased breast cancer risk and the other with a decreased risk are combined in one group of cases (Bågeman, 2008). In the event of a gene-environment interaction, the combined effect is often greater than that of the genetic variant or lifestyle factor itself (Le Marchand and Wilchand, 2008).

If these genetic variants are also associated with tumor characteristics, they may be even more relevant. Bågeman, 2008 demonstrated that the genetic polymorphisms have been selected based on a minor allele frequency (MAF) in European populations of >5%, thus increasing the chance of the results being of general interest and clinically useful.

1.1.8 Genetic alterations in breast cancer

Molecular oncology is one of the most promising fields that may contribute considerably to diagnosis of breast cancer and its metastases addressing major problems with early detection, accurate staging, and monitoring of breast cancer patients (Israyelyan, 2003 and Jwad, 2012). Genetic testing for mutations in breast cancer susceptibility genes offers some women and their families the opportunity for risk-reducing intervention (Lewis, 2007 and Teo et al., 2013) medical risk reduction gene-targeted therapeutics (Ashworth, 2008).
Breast cancer development can be triggered by mutations of the signals in the network that controls cell division, and can be associated with genetic predisposition (e.g., mutations in BRCA1 and BRCA2 genes), exposure to some environmental factor (e.g., radiation exposure of the chest), or both (Kennedy et al., 2005). So it is an interplay between genetic changes and environmental factors (Dickson and Russo, 2000). The predisposition for breast cancer is inherited but not everyone will develop cancer (Al Bederi, 2011).

1.2 The monoclonal HER2/neu gene:

The human epidermal growth factor (EGF) receptor-2 (HER2; also referred to as HER2/neu and as ERBB2) gene, is encoded by *ERBB2*, a known proto-oncogene located at the long arm of human chromosome 17 (17q12). HER2 is named because it has a similar structure to human epidermal growth factor receptor, or HER1. Neu is so named because it was derived from a rodent glioblastoma cell line, a type of neural tumor. ErbB-2 was named for its similarity to *ERBB* (avian erythroblastosis oncogene B), the oncogene later found to code for EGFR. Gene cloning showed that HER2, Neu, and ErbB-2 are all encoded by the same gene (Coussens et al., 1985). Gene located at position 17q12 on chromosome 17, is a 185 kDa protein (p185), is amplified (i.e., gene copy number greater than 2) and/or the HER2 protein is overexpressed (i.e., cell membrane has excess of HER2 protein molecules compared to normal cells) in approximately 18 to 20 percent of breast cancer cases (Slamon et al., 1987; Yaziji et al., 2004; Hanna and Kwok, 2006 and Wolff et al., 2007).
Figure (1-1) : Ideogram Chromosome 17
Partial map that indicated HER2 gene (Strachan and Read, 2011).

Amplification and/or overexpression of HER2 have been associated with increased tumor aggressiveness and poor prognosis. Amplification or over-expression of this gene has been shown to play an important role in the pathogenesis and progression of certain aggressive types of breast cancer and in recent years it has evolved to become an important biomarker and target of therapy for the disease. The HER2 gene is one of four (HER1 through HER4) in the EGF receptor gene family; All of the ErbB family proteins are typical tyrosine kinases in molecular structure, consisting of a ligand binding extracellular region, a single membrane spanning region, and a domain containing cytoplasmic tyrosine kinase (Leahy, 2004). Most of the ErbB family proteins are ligand-activated, meaning that when ligands bind to their extracellular regions. It induces receptor dimerization of the cytoplasmic
kinase, which causes autophosphorylation, triggering binding of specific signaling molecules that initiate downstream signaling events (Crutcher and Amber, 2007).

HER2 is the only ErbB receptor that does not exhibit specific Ligand binding, and possesses several unique characteristics. Because HER2 does not have a direct Ligand by which it is activated, it is thought that it might act as a coreceptor for the other ErbB receptors. HER2 is a transmembrane tyrosine kinase receptor and a member of the ErbB protein family, more commonly known as the epidermal growth factor receptor (EGFR) family (Leahy, 2004).

1.2.1 Structure of HER2

Structures of the entire extracellular region (Cho et al., 2003) and the first three domains of HER2 show it to adopt a very different conformation than inactivated forms of sEGFR or sHER3 (Garrett et al., 2003). The “snap-like” hairpin loop from domain II is present in HER2, but it is exposed to solvent and does not mediate a contact with domain IV. Instead, an extensive (1200 Å) and highly complementary contact is made between domains I and III, which appears to fix the orientation of the domain I/II pair relative to the domain III/IV pair. The interface between domains I and III is conserved in all three crystal forms of HER2 and appears to be a fixed feature of HER2 homologs. Two key hydrophobic residues buried at this interface, Leu 443 and Leu 472, are conserved in all HER2 homologs but not in other ErbB receptors. The HER2 domain I/II and III/IV pairs align well with the corresponding pairs from all other ErbB receptors, suggesting that the interdomain orientations within these pairs are relatively rigid. By fixing the orientation of the domain I/II and III/IV pairs relative to one another, the HER2 specific interaction between domains I and III thus fixes the
conformation of the entire HER2 extracellular region (Leahy, 2004 and Crutcher and Amber, 2007).

1.2.2 Function of HER2

Oncogene encoded an activated form of a growth factor receptor provided exciting insight into the origins of cancer and presaged discovery of the involvement of EGFR and related receptors in the genesis and severity of many human cancers (Tang and Lippman, 1998; Blume-Jensen and Hunter, 2001 and Holbro et al., 2003). The nature of the ErbB oncogene also indicated that the extracellular region not only mediates ligand dependent activation but also contributes to maintaining the kinase in an inactive state in the absence of ligand.

1.3 HER2 and Breast cancer

The tumors of approximately 25%–30% of the patients with breast cancer over express HER2 protein (Joensuu et al., 2013), and this overexpression is correlated with a poor clinical outcome (van de Vijver et al., 1988; Ross and Fletcher, 1999). It is strongly associated with increased disease recurrence and a worse prognosis (Tan and Yu, 2007). Aberrant expression or activity of two members of the human epidermal growth factor family of receptors (HER), HER1 and HER2, have been connected to 20–30% of breast cancer cases (Yarden, 2001 and Moghimi et al., 2013).

HER2 is a signaling tyrosine kinase receptor that causes increased cell proliferation, tumor invasiveness, accelerated angiogenesis and reduced apoptosis, ultimately translating into an aggressive disease, resistant to
traditional systemic therapy, increased probability for recurrent disease and decreased survival (Kruser and Wheeler, 2010 and Emde et al., 2011).

1.3.1 HER2 with other cancers

The human epidermal growth factor receptor-2 (HER2) gene is amplified and the HER2 protein overexpressed in approximately 18–20 percent of breast cancer cases. Amplification or overexpression of HER2 is associated with poor prognosis (Seidenfeld et al., 2008). Evidence from randomized trials demonstrates that adding trastuzumab, a therapeutic monoclonal antibody that targets HER2, to adjuvant chemotherapy regimens for HER2-positive breast cancer improves survival. HER2 is highly expressed in a significant proportion of breast cancer, ovarian cancer, and gastric cancer (Tai et al., 2010) and is overexpressed in other epithelial malignancies such as thyroid, lung, salivary gland/head and neck and prostate cancers (Seidenfeld et al., 2008) also it is aggressive forms of uterine cancer, such as uterine serous endometrial carcinoma (Santin et al., 2008).

1.4 Diagnosis of breast cancer

There are many essential methods that are used in breast cancer diagnosis:

1-Diagnostic Mammogram: The widespread use of mammography has radically changed the diagnostic approach to breast cancer. Extremely small tumors (1 to 2mm) can be detected with this technique, which relies primarily on the presence of calcification (McLelland, 1991).

2-Blood tests: Samples from the blood of a patient will be taken to check her general health, the number of cells in her blood and to see how well her kidneys and liver are working. Her blood may also be tested to see whether
it contains particular chemicals (markers), which are sometimes produced by cancer cells (Jamkit, 2006).

3-Biopsy: Fluid or tissue is taken from breast to help find out if there is cancer. Some suspicious areas can be seen on a mammogram but cannot be felt during a clinical breast exam. Doctors can use imaging procedures to help see the area and remove tissue. Such procedures include ultrasound guided, needle-localized, or stereotactic biopsy (National Cancer Institute, 2007).

1.5 Prognostic Tumor Markers:

Among the most important tumor markers that were used to assess the prognosis are the following:

1- HER2/neu. This identifies a subset of patients with poor prognosis, particularly those with lymph node metastasis (Press et al., 1994).

2- Bcl-2 expression, a relationship between Bcl-2 protein expression and long term survival in breast carcinoma has been shown (Hurlimann et al., 1995).

3- B-Catenin activity when increased in breast cancer cells is independently associated with poor prognosis.

4- Ki-67: It is a cell proliferation marker and its high expression is a marker of poor prognosis (Li et al., 2004).

1.6 Tumor Markers

Tumor markers are substances that can be found in abnormal amounts in the blood, urine and tissues of some patients with cancer (Lemoine, 1994). Markers are needed to predict cancer progression and the risk of late recurrence. A small number of single biomarkers, including estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER2), and proliferation marker Ki-67 have been used for
several years to predict the prognosis of breast cancer and to guide its therapy (Joensuu et al., 2013).

The College of American Pathology (CAP) and the American Society of Clinical Oncology (ASCO) on April 19, 2010; the two organizations issued joint guideline recommendations with the primary goal of improving the accuracy of HER2 testing and of estrogen receptor (ER) and progesterone receptor (PR) immunohistochemical (IHC) testing in clinical practice and their utility as prognostic and predictive markers in breast cancer (Hammond et al., 2011).

1.6.1 Hormone receptors

A crucial development in the treatment of breast carcinoma has been the realization that the presence of hormone (estrogen and progesterone) receptors in the tumor tissue correlates well with response to hormone therapy and chemotherapy (Hawkins et al., 1980 and Barnes and Hanby, 2001). As a matter of fact, estrogen receptor status is regarded at present as the most powerful predictive marker in breast cancer management (Payne et al., 2008). Estrogen and progesterone receptors are codependent variables, progesterone receptor (PR) being a weaker predictor of response to endocrine therapy than estrogen receptor (ER) (Mohsin et al., 2004). Traditionally, these hormone receptors were measured by the dextran-coated charcoal and sucrose gradient assay, but this has been replaced by the immunohistochemical method, on the grounds that it offers several important advantages (it does not require fresh tissue, it can be done with minute amounts of tumor, etc.), and that the correlation between the two methods is very good (Taylor, 1996; Harvey et al., 1999 and Zafrani et al., 2001).

Several attempts have been made to semiquantitative the immunohistochemical method by standardizing the technical procedure and
reporting, and by using the appropriate controls (Wells et al., 2004; Phillips et al., 2007 and Yaziji et al., 2008). Delay in fixation significantly affects the results, whereas fixation time within reasonable boundaries (between 1 and 9 hours) does not (Ibarra et al., 2010). Regarding the issue of a proper control, Battifora's team has proposed a very innovative procedure, which they refer to as the Quicgel method (Riera et al., 1999). Although the idea is very ingenious (as we have been accustomed to expect from this group), it may be a little too complex to be widely adopted. At this point, which is believed that satisfactory standardization of the immunohistochemical method has not yet been achieved. As a matter of fact, an experienced worker in the field has made the provocative statement that this goal is actually beyond the power of the technique (Nadji, 2008).

The two parameters evaluated in immunohistochemical preparations of hormone receptors are the number of tumor cell nuclei stained and the intensity of the reaction (Rosai, 2011). The first is expressed as a percentage of the entire tumor cell nuclei population. The two parameters are sometimes combined into a scoring system, of which three major versions exist (including the popular Allred scoring system) (Harvey et al., 1999 and Regitnig et al., 2002). Although several sophisticated image analysis programs have been devised for this purpose (Baddoura et al., 1991), in most laboratories these estimations are done visually. Hormone receptors can also be evaluated in paraffin-embedded breast tissue by the in situ hybridization technique and by PCR (Graham et al., 1991 and Carmeci et al., 1997).

About 80% of breast cancers are ER positive, so that an ER-negative rate of 30% or higher suggests that some problems exist with the assay. Not much correlation exists between the cytoarchitectural type of breast carcinoma and the presence of hormone receptor protein; (Scawn and
specifically, no statistically significant difference has been found between ductal-type and lobular-type tumors. However, as a group, ER-negative breast carcinomas tend to have a grade 3 histology, pushing margins, lymphoid stroma, comedo-type necrosis, and central fibrosis/necrosis (Putti et al., 2005). Most medullary, metaplastic, and apocrine carcinomas are negative, whereas mucinous, tubular and lobular carcinomas have a high rate of positivity (Nadji et al., 2005). In ductal carcinoma in situ (DCIS), a predominance of large cells is the best morphologic predictor of ER-negative status (Bur et al.,1992). The positivity in lobular carcinoma in situ is particularly strong, and present in both the glands and the surrounding stroma (Middleton et al., 2007). It is very unusual for an ER-negative cancer to turn ER-positive, whereas the reverse is more common, especially if there has been an intervening tamoxifen therapy (Rosai, 2011). Generally, estrogen receptor concentrations are lower in tumors of premenopausal women than in those of postmenopausal women (Honma et al., 2003). Fisher et al., 1980 found the presence of estrogen receptors to be significantly associated with high nuclear and low histological grades, absence of tumor necrosis, presence of marked tumor elastosis, and older patient age groups. Hormone receptor positivity also correlates with BCL2 immunoreactivity (Bhargava et al.,1994) and absence of P53 mutations,( Caleffi et al.,1994) and correlates inversely with the presence of epidermal growth factor receptors (van Agthoven et al.,1994). It should be pointed out that most breast carcinoma cells also have receptors for androgens, and that these may be found in the absence of estrogen and progesterone receptors (Bayer-Garner and Smoller,2000). As a matter of fact, they seem to be more common in ER-negative tumors. Tumor types said to have frequent expression of androgen receptors are lobular carcinoma, apocrine carcinoma, and Paget disease (Liegl et al., 2005; Riva et al., 2005 and Niemeier et al.,2010).
1.6.1.1 Estrogen receptor

The sex steroid hormone estrogen is important in both men and women for a variety of physiologic processes. Estrogen affects growth, differentiation, and function of tissues of the reproductive system, including the mammary glands, uterus, vagina, and ovaries in females, and the testis, epididymis and prostate in males. All of the effects of estrogens are mediated through their binding to nuclear proteins called estrogen receptors (ER), transcription factors that regulate expression of estrogen-responsive genes (Osborne et al., 2000). In the 1960s, the triphenylethylene tamoxifen was synthesized, and it demonstrated antiproliferative effects in the breast. Shortly thereafter, in trials in patients with metastatic breast cancer, it was shown to be an effective therapy in those patients whose tumors expressed ER (Saez and Osborne, 1989). Tamoxifen thus became widely known as an antiestrogen, a misnomer that persists today (Osborne, 1998 and Padunchewit, 2010).

1.6.1.2 Progesterone receptor

Progesterone (PR) is a sex steroid essential for pregnancy and lactation produced almost entirely by the ovarian corpus luteum (CL) and the placenta. Normal endometrial function requires both estrogen (ER) [which mediates cell growth and induction of progesterone receptors (PR)] and PR, which counteracts ER stimulation and down regulates the receptors for ER and PR. The normal balance achieved by sequential actions of ER and PR is essential to the normal cyclic functions of human endometrium (Young and Lessey, 2010). It is important main marker to diagnosed breast cancer according to molecular subtype (table 1-4).

Table 1-4: Use of Immunohistochemistry as surrogate marker for the molecular subtypes of breast cancer (Rosai, 2011)
**Molecular subtype**

<table>
<thead>
<tr>
<th>Immunoprofile</th>
<th>Luminal A</th>
<th>Luminal B</th>
<th>HER2/neu</th>
<th>Basal-like</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER, PR</td>
<td>ER and/or PR+</td>
<td>ER and/or PR+</td>
<td>ER–, PR–</td>
<td>ER–, PR–</td>
</tr>
<tr>
<td>HER2 and others</td>
<td>HER2– Low Ki-67 (&lt;14%)</td>
<td>HER2+ or HER2– Ki-67 =14%</td>
<td>HER2+</td>
<td>HER2– CK5/6 and/or EGFR+</td>
</tr>
</tbody>
</table>

Modified from (Cheang *et al.*, 2008; Cheang *et al.*, 2009 and Schnitt, 2010)

**1.7 HER/ErbB Receptors**

**1.7.1 HER/ErbB Receptor Family**

The HER/ErbB family of receptor tyrosine kinase (RTKs) is important in the transduction of extracellular cues into intracellular signals that allow a cell to adjust to its environment. They are expressed in various tissues of epithelial, mesenchymal, and neuronal origin (Slamon *et al.*, 1987).

Therefore, the RTKs are essential for the embryonic development of the nervous system, cardiovascular system, gastrointestinal system, and other organ systems, as well as biological processes such proliferation, differentiation, migration and apoptosis (Slamon *et al.*, 1987 and Wolff *et al.*, 2007).

The epidermal growth factor receptor (EGFR) or HER/ErbB family of RTKs, includes four members, EGFR/HER1/ErbB1, HER2/ErbB2/Neu, HER3/ErbB3 and HER4/ErbB4. All the HER/ErbB receptors are transmembrane proteins, sharing an extracellular epidermal growth factor (EGF)-like ligand-binding domain composed of two cysteine-rich domains (II and IV) interspersed with unique domains (I and III), a single α-helix transmembrane domain, an intracellular region comprised of a well conserved tyrosine kinase domain, and a divergent number of regulatory carboxyl-terminal tyrosine residues (Wolff *et al.*, 2007).
Domains I and III can form a binding site for the receptor’s potential ligands (Konecny et al., 2001), while domains II and IV are involved in dimerization between two identical HER/ErbB receptors (homodimerization) and between two different HER/ErbB receptors (heterodimerization). Domain II contains a dimerization arm, which is generally believed to be the main contributor for dimerization. It consists of a protruding short hairpin loop that can contact the dimerization arm of its partner (Olayioye, 2001).

Ligand binding activates the HER/ErbB receptor, then induces homodimerization or heterodimerization, which enables the kinase domains to cross-phosphorylate the carboxyl terminal tyrosine residues on the dimerization partner. Once these residues are phosphorylated, they serve as recognition sites for phosphopeptide binding adaptors and signaling proteins, whose recruitment leads to the activation of intracellular pathways, including the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3-kinase (PI3K) pathways (Franklin et al., 2004).

1.7.2 HER/ErbB Receptor Ligand

Activation of the family of HER/ErbB receptors is controlled by spatial and temporal expression of its ligand, members of the EGF-related ligand growth factor family (Burgess et al., 2003 and Cho et al., 2003). There are a number of HER/ErbB-specific ligands, each of which contains an EGF-like domain that confers binding specificity (Negro et al., 2004). These ligands can be divided into three groups. The first group includes EGF, amphiregulin (AR), and transforming growth factor-α (TGF-α), which binds specifically to EGFR/HER1/ErbB1. The second group includes betacellulin (BTC), heparin binding EGF (HB-EGF), and epiptegulin (EPR), which binds both EGFR/HER1/ErbB1 and HER4/ErbB4. The third group is composed of the neuregulins (NRGs), which are also known as
heregulins (HRGs), and contains two subgroups based on their ability to bind HER3/ErbB3 and HER4/ErbB4 (NRG-1 and NRG-2) or only HER4/ErbB4 (NRG-3 and NRG-4) (Cho et al., 2003 and Negro et al., 2004).

HER2/ErbB2 and HER3/ErbB3 are unique because none of the EGF family of ligands binds to HER2/ErbB2 directly, while HER3/ErbB3 does not have intrinsic kinase activity. However, HER2/ErbB2 constitutively adopts an extended or active state, exposing the dimerization arm in a ligand-independent manner, making it constantly poised for interaction and the preferred heterodimerization partner for all other HER/ErbB receptors (Zhang et al., 2009). These HER2/ErbB2 containing heterodimers are responsible for strong and prolonged activation of downstream signaling pathways (Campbell, 2010).

1.7.3 HER/ErbB Receptor Signaling Adaptors

Activation of the intracellular kinase domain, through the phosphorylation of carboxyl terminal tyrosines on HER/ErbB receptors, triggers the association of specific signaling molecules, whose binding initiates downstream signaling events (Citri et al., 2003). Each of the four HER/ErbB receptors has a different set of phosphorylation sites and recruits different combinations of signaling molecules, such as Src homology2 (SH2) and phosphotyrosine binding (PTB) domain-containing molecules (Slamon et al., 1987; Franklin et al., 2004). Known SH2 and PTB domain containing proteins that interact with HER/ErbB receptors include adaptor proteins (Crk, Gab1, Grb2, Grb7, and Shc), protein and lipid kinases (Src and PI3K), protein phosphatases (SHP1 and SHP2), and phospholipase Cγ (Slamon et al., 1987). Only HER3/ErbB3 efficiently activates PI3K due to multiple coupling sites on HER3/ErbB3 for the PI3K regulatory subunit, p85, but this depends on heterodimerization of
HER3/ErbB3 with the constitutively active HER2/ErbB2 (Garrett et al., 2003).

1.7.4 HER/ErbB Receptor Signaling Pathways in Cancer

Cancer cells evade contact inhibition, cell cycle checkpoints, and apoptosis (Bennis et al., 2012). The transforming capacity of HER2/ErbB2 is associated with its overexpression, generally due to gene amplification (Negro et al., 2004). Overexpression of HER2/ErbB2 disrupts the regulators of the G1/S transition and other cell cycle checkpoints to increase cell proliferation of tumor cells through HER2/ErbB2-dependent pathways, such as Ras/Erk, p38 MAPK, and PI3K (Cooper, 1992). Overexpression of HER2/ErbB2 has been shown to occur in a significant portion of breast (Browne, 2009), ovarian (Pronk, 1993), bladder (Rozakis-Adcock, 1992), gastric (Buday and Downward, 1993) and other human malignancies.
The signalling of HER2 is activated through homodimerization and heterodimerization, which transmits growth factor activation signals to multiple downstream signaling pathways. The HER/ErbB receptors are well known mediators of cell proliferation, migration, differentiation, apoptosis, and metastasis. HER2/ErbB2 is often overexpressed, amplified, or mutated in cancers, making it an important therapeutic target. (Adapted from Yarden and Shilo, 2007).

Avoiding cell death is an essential trait acquired during the malignant process (Schulze et al., 2005). A main effector of HER/ErbB signalling, the PI3K/Akt/protein kinase B (PKB) signaling pathway is particularly important in mediating cell survival since several Akt/PKB substrates directly control various apoptotic processes (Citri et al., 2003). For growth beyond a certain size, the primary tumor must improve its oxygen and nutrient supply through the formation of new blood vessels in a process known as angiogenesis. HER/ErbB receptors have been associated with tumor cell production of proangiogenic factors, the most significant factor being vascular endothelial growth factor (VEGF) (Fruman et al., 1998). Breast and lung cancer cell lines with constitutive HER2/ErbB2 activation show elevated VEGF mRNA levels, and the addition of heregulin to these cultures further increases VEGF transcription in a MAPK-dependent manner (Sarbassov, 2005 and Dong and Liu, 2005). Furthermore, treatment of HER2/ErbB2-overexpressing tumor cells with a monoclonal antibody that interferes with the HER2/ErbB2 receptor decreases expression of proangiogenic factors, including VEGF and TGF-α, resulting in a dramatic change in the tumor vasculature and a decrease in tumor size (Hresko and Mueckler, 2005).

The final step of cancer progression involves tumor cells leaving the site of primary growth and forming metastases. For tumors to metastasize, the cells must possess certain characteristics, including the ability to
migrate and invade the surrounding basement membrane and distant
tissues. The functional inactivation of HER2/ErbB2 blocks EGF-, BTC-,
and HRG induced breast cancer cell migration. HER2/ErbB2-containing
heterodimers promote strong activation of the MAPK and PI3K pathways
known to have important roles in promoting cell migration (Zhang, 2012).

Motile cancer cells must also have the ability to invade the surrounding
basal membrane to acquire full metastatic potential, a process requiring
proteolytic activity. HER2/ErbB2 cooperates with hepatocytes growth
factor (Glidden, 2012) and TGF-β (Osaki et al., 2004 and Calvo et al., 2009)
to promote an invasive phenotype. HRG treatment of breast cancer cells
demonstrated an increase expression of matrix metalloproteinase (MMP)-
9 (Luo et al., 2003). As well as serine protease uPA and its receptor
(Goncharenko-Khaider, 2010) leading to increased invasion. HER2/ErbB2
can also contribute to several distinct capabilities required to complete
tumorigenesis (Schulze et al., 2005). Interestingly, EGFR/HER1/ErbB1 is a
target for mutations and deletions in the extracellular or intracellular
domain, some of which promote constitutive kinase activity in the absence
of ligands to drive tumor cell proliferation (Cross, 1995). TGF-α is
frequently co-expressed with EGFR/HER1/ErbB1 in non-small cell lung
cancer (Plas and Thompson, 2003), prostate cancer (Huang et al., 2009),
gastrointestinal stromal tumors (Huan, 2005) and significantly correlates to
worse patient prognosis in invasive breast carcinomas (Inoki and Guan,
2006).
Chapter Two
Subjects, Materials and Methods

2-1 : Subjects:

The samples of this study were formalin-fixed, paraffin-embedded archival tissues collected from the laboratory of histopathology in AL Sadder Medical City in AL-Najaf AL-ASHRAF and from some private laboratories in the same city. It had done on a total number of 70 subjects including 50 patients with malignant breast cancer (Invasive Ductal Carcinoma and other types) with a proved diagnosis of on histopathological examination presented in AL SADER medical city, these patients were from different age groups and different geographic residencies, while the control samples included 20 cases with benign breast lesions were enrolled in this study.

For molecular study, the nominated blocks from the paraffin embedded tissue samples which were chosen to be used for DNA extraction were sliced 4-5 slices each of them is 10 µm in thickness by a special device (Microtome) and were used in DNA extraction. While for immunohistochemistry were used 4 µm sections of multi-block with 10 % Neutral Buffed Formalin fixed and paraffin embedded human tissue. Mounted on Silanized slides (S3003). The sections were dried for 16 hours at 37 ºC followed by 1 hour at 60ºC (Alcides et al., 2012).
2.2 Chemical and Biological materials

The general material used in this study are listed in table (2-1):

<table>
<thead>
<tr>
<th>NO.</th>
<th>Material</th>
<th>Company</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Absolute ethanol</td>
<td>Fluka</td>
<td>Germany</td>
</tr>
<tr>
<td>2</td>
<td>Agarose 100g</td>
<td>BIORON</td>
<td>USA</td>
</tr>
<tr>
<td>3</td>
<td>Blue/Orange 6x Load Dye</td>
<td>Promega</td>
<td>USA</td>
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<td>5</td>
<td>Chloroform</td>
<td>Flow laboratories</td>
<td>UK</td>
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<td>4</td>
<td>DNA Ladder 100 bp</td>
<td>Promega</td>
<td>USA</td>
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<td>EDTA</td>
<td>Flow laboratories</td>
<td>UK</td>
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<td>Flow laboratories</td>
<td>UK</td>
</tr>
<tr>
<td>8</td>
<td>Ethidium Bromide</td>
<td>SIGMA-Aldrich</td>
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<tr>
<td>9</td>
<td>GoTaq® Green Maser Mix2x</td>
<td>Promega</td>
<td>USA</td>
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<td>10</td>
<td>Hematoxylin</td>
<td>Flow laboratories</td>
<td>UK</td>
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<td>11</td>
<td>Isoamyl alcohol</td>
<td>Flow laboratories</td>
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<td>13</td>
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<td>14</td>
<td>Nuclease Free Water</td>
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<td>15</td>
<td>Phenol</td>
<td>Flow laboratories</td>
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<td>16</td>
<td>Primers</td>
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<td>Proteinase K</td>
<td>Promega</td>
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<td>22</td>
<td>SDS</td>
<td>Promega</td>
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<tr>
<td>20</td>
<td>TBE Buffer 10x</td>
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</table>
2-3: Equipment

The general equipment used in this study are listed in table (2-2).

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Company</th>
<th>Origin</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Clock</td>
<td>CUSABIO</td>
<td>China</td>
</tr>
<tr>
<td>2</td>
<td>Digital camera</td>
<td>Sony</td>
<td>Japan</td>
</tr>
<tr>
<td>3</td>
<td>Electric balance</td>
<td>Huma Scale huma</td>
<td>Germany</td>
</tr>
<tr>
<td>4</td>
<td>Eppendorf tubes</td>
<td>Eppendorf</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Incubator</td>
<td>BINDER</td>
<td>Germany</td>
</tr>
<tr>
<td>6</td>
<td>Incubator</td>
<td>SAKURA</td>
<td>Japan</td>
</tr>
<tr>
<td>7</td>
<td>Gel electrophoresis</td>
<td>Major science</td>
<td>USA</td>
</tr>
<tr>
<td>8</td>
<td>Micropipette</td>
<td>SLAMED 200 µL,1000µL</td>
<td>England</td>
</tr>
<tr>
<td>9</td>
<td>Micropipette</td>
<td>Huma Pette Human(0.5-10,5-50, 20-200) µL</td>
<td>Germany</td>
</tr>
<tr>
<td>10</td>
<td>Microfuge ® 18 Centrifuge</td>
<td>BECKMAN CouLTER</td>
<td>Germany</td>
</tr>
<tr>
<td>11</td>
<td>Microtome</td>
<td>LEICA RM2235</td>
<td>Germany</td>
</tr>
<tr>
<td>12</td>
<td>Magnetic Stirrer Hotplate</td>
<td>STUART Scientific</td>
<td>UK</td>
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<tr>
<td>13</td>
<td>Microwave oven</td>
<td>SAMSUNG</td>
<td>Korea</td>
</tr>
<tr>
<td>14</td>
<td>Tank MAX FILL</td>
<td>Hoefer</td>
<td>USA</td>
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Table (2-2) Equipment
### 2-4 Kits

<table>
<thead>
<tr>
<th>No.</th>
<th>Kit Name</th>
<th>Company</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>g SYNC™ DNA Mini Kit</td>
<td>Geneaid</td>
<td>Korea</td>
</tr>
<tr>
<td>2</td>
<td>LSAB+(labeledStreptavidin-Biotin)</td>
<td>Dako</td>
<td>Denmark</td>
</tr>
<tr>
<td>3</td>
<td>QIAamp® DNA Mini Kit</td>
<td>QIAGEN</td>
<td>Germany</td>
</tr>
<tr>
<td>4</td>
<td>ReliaPrep™ FFPE gDNA Miniprep System</td>
<td>Promega</td>
<td>USA</td>
</tr>
</tbody>
</table>

### 2.5: Immunohistochemical Study

The immunostaining method used in the current study was Labeled Strept-Avidin Biotin (LSAB⁺) technique which was applied for HER2, ER and PR staining (Climent et al., 2001). This part of the study was done in the Histopathology laboratories of AL-SADER Medical city in AL-NAJAF AL-ASHRAF.

**Tissues:** 4 μm sections of multi-block with 10% Neutral Buffed Formalin fixed and paraffin embedded human tissue. Mounted on Silanized slides
(S3003). The sections were dried for 16 hours at 37 ºC followed by 1 hour at 60ºC.

**Deparaffinization:** This has been performed previously by immersion in the following:

1. Xylene 5 minutes (twice).
2. 99 % ethanol 5 minutes (three time).
3. 95 % ethanol 5 minutes.
4. 70 % ethanol 5 minutes.
5. 50 % ethanol 5 minutes.
6. 35 % ethanol 5 minutes.
7. Distilled Water.

### 2.5.1 Standard Immunohistochemistry Staining Method Labeled Streptavidin Biotin (LSAB) Method

1. Paraffin section or frozen section to water and rinse in PBS-Tween 20 for 2x2 min.
2. **Antigen Retrieval:** perform antigen retrieval using Epitope Retrieval Solution.
3. **Serum Blocking:** incubate sections in normal serum – species same as secondary antibody.
   - Note: since this protocol uses avidin-biotin detection system, avidin/avidin-biotin block may be needed based on tissue type.
4. **Primary Antibody:** incubate sections with primary antibody (ER, PR and HER2) at appropriate dilution in IHC-Tek™ Antibody Diluent for 1 hour at room temperature or overnight.
5. Rinse in PBS-Tween 20 for 3x2 min.
6. **Peroxidase Blocking:** incubate sections in peroxidase blocking solution for 10 minutes at room temperature.
7. Rinse in PBS-Tween 20 for 3x2 min.

8. **Secondary Antibody**: incubate sections in Biotinylated secondary antibody in PBS for 30 minutes at room temperature.

9. Rinse in PBS-Tween 20 for 3x2 min.

10. **Detection**: incubate sections in HRP-Streptavidin solution for 30 minutes at room temperature.

11. Rinse in PBS-Tween 20 for 3x2 min.

12. **Chromagen/Substrate**: incubate sections in peroxidase substrate solution.

13. Rinse in PBS-Tween 20 for 3x2 min.

14. **Counterstain** with counterstain solution if desired.

15. Rinse in running tap water for 2-5 minutes.

16. Dehydrate through 95% ethanol for 1 minute, 100% ethanol for 2x3min.

17. Clear in xylene for 2x5min.

18. Coverslip with mounting medium.

### 2.5.2 A: Scoring of the immunohistochemical staining for HER2/neu overexpression (Hammond *et al.*, 2011):

**Score 0**: Negative, No staining is observed or membrane staining is observed in less than 10% of the tumor cells.

**Score +1**: Negative, A faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells. The cells are only stained in part of their membrane.
Score +2: Weakly positive, A weak to moderate complete membrane staining is observed in more than 10% of the tumor cells.

Score +3: Strongly positive, A strong complete membrane staining is observed in more than 30% (formerly 10%) of the tumor cells.

B: Scoring of immunohistochemical staining for ER and PR

The manufacture recommended to use Allred’s scoring system (Allred et al., 1998). It is based on two score evlutions (proportion and intensity score), and summarized as the following:

● A Proportion score (P) is assigned representing the proportion of tumor cells with positive nuclear staining, and has a range of 0-5.

● An Intensity score (I) is assigned representing the average staining intensity of all positive tumor cells, and has a range of 0-3.

● A total score (T) is the sum of P plus I, and has range of 0-8.

A result for both ER and PR reactions is defined as:

1. T : 0-2  Negative.
2. T : 3-4  Weak positive.
3. T : 5-6  Intermediate positive.
4. T : 7-8  Strong positive.
Table (2-4)

Kits contents of LSAB + System –HRP kit

<table>
<thead>
<tr>
<th>Edition 06/07</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotinylated Link</td>
</tr>
<tr>
<td>DAB+ Substrate Buffer</td>
</tr>
<tr>
<td>DAB+CHROMOGEN</td>
</tr>
<tr>
<td>Peroxidase BLOCK</td>
</tr>
<tr>
<td>Streptavidin - HRP</td>
</tr>
</tbody>
</table>

Figure (2-1)

Staining Kit (LSAB+ System-HRP) Immunohistochemistry kit (Dako)

The detection system used was Dako Envision Detection kit (K5007). For the purposes of this study, ER and PR staining was considered positive when at least 10% of tumor cells displayed a minimum of 2+ nuclear staining while HER2 was considered positive if at least 30% of tumor cells showed positive at score 3+ cell membrane staining. A borderline/equivocal result was given for HER2 when at least 10% of tumor cells demonstrated 2+ cytoplasmic membrane staining. Tumors that failed to fulfill any of the above criteria were considered triple negative (Teng et al., 2011).
2.6  Molecular Study of Tissue Samples:

2.6.1  Solutions used in DNA Extraction

2.6.1.1  Xylene:

It’s ring aromatic organic solvent that is used to dissolve paraffin in the FFPE samples. It is ready to be used (FLUKA, Germany). Every time, 800µl is added to 25µg of tissue in 1.5µl eppendorf tube to remove the wax.

2.6.1.2  Chloroform: Isoamyl alcohol (24:1)

To prepare 100ml, 96ml of chloroform was added to 4ml of isoamyl alcohol (Maniatis et al., 1982).

2.6.1.3  Lysis Buffer

1. Ten mM Tris-HCl pH 8.0

2. One hindered mM EDTA pH 8.0

3. Fifty mM NaCl

4. 0.5% SDS

5. Two hindered µg/ml Proteinase K (add just before use).

2.6.1.4  Washing Buffer

This was prepared by dissolving 0.140 gm of ammonium acetate in 76ml ethanol, volume completed with D.W to 100ml (Sambrook et al., 1989).
2.6.1.5 Tris-EDTA ( TE ) Buffer

( 10mM Tris-Base ,PH 8.0 ; 1mM Na₂EDTA, PH 8.0 )

This was prepared by dissolving 0.1211gm of Tris- Base , 0.0372 gm of Na₂EDTA in D.W , PH was adjusted to 8.0, volume completed with D.W to 100ml , sterilized by autoclaving and stored at 4ºC (Sambrook et al. ,1989).

2.6.2 DNA Extraction from Formalin-Fixed Paraffin Embedded Tissue (FFPE).

Formalin fixation and paraffin embedding ( FFPE ) is a commonly used method for archiving tissue specimens .The ability to extract DNA from these samples provides the potential for correlating disease state and tissue morphology with genotype (Chaux et al., 2012) .

Extraction of DNA from FFPE tissue historically has been a challenge because the formalin fixation process results in cross-linking between protein and DNA, as well as between different strands of DNA .

The non optimal preservation of genomic DNA in FFPE complicates its use in many standard downstream analysis applications( Lin, 2009).

In this study , Several methods were used for the extraction process from the paraffin embedded tissue samples . Some of them were using manual methods and the others were using the kits. Methods that have been used for DNA extraction are: a special common manual protocol, g SYNC™ DNA Mini Kit (Geneaid), QIAamp® DNA Mini Kit and ReliaPrep™ FFPE DNA Miniprep System, (Promega ,USA).
2.6.2.1 DNA Extraction by manual Protocol (Sambrook et al., 1989)

1. Paraffin removal

   1. In a fume hood, 800 μl of xylene was added to the labelled tube containing the samples and placed on a rocker with gentle shaking for 5 to 15 minutes to dissolve the paraffin.

   2. The sample is pelleted by spinning at max speed (14,000 rpm or 16 000 x g) in a microcentrifuge for 3 minutes. The xylene supernatant is carefully withdrawn and dispose in a polypropylene tube for discard in the xylene waste. The researcher should be careful not to disturb the pellet.

   3. Xylene wash steps 1 and 2 are repeated until paraffin is fully dissolved. This usually requires two to three washes, depending on the size of the tissue sample. A fully dissolved sample will appear soft, and will sometimes lose its structural integrity. The integrity of the sample can be gently assessed with a pipette tip.

2. Ethanol rehydration

   1. Eight hundred μl of 100% ethanol (v/v) molecular biology grade ethanol was added, vortex then spun for 3 minutes at 14,000 rpm in microcentrifuge. The ethanol supernatant is removed, being careful not to disturb the pellet.

   2. Eight hundred μl of 70% ethanol (100% (v/v) ethanol diluted in distilled water dH₂O) was added, vortex and spun for 3 minutes at 14,000 rpm. The ethanol supernatant is carefully removed.

   3. Eight hundred of 50% ethanol (100% (v/v) ethanol diluted in distilled water dH₂O) was added, vortex and spun for 5 minutes at
14,000 rpm. Ethanol is removed as much as possible by pipetting. The research should be extremely careful of disturbing the pellet at this point as fully rehydrated tissue will not pellet as well as dehydrated tissue.

4. After removing the ethanol supernatant, air-dry the pellet for 5 minutes, being careful not to over dry.

3. Tissue digestion

1. 200-500 μl of Lysis Buffer (formula below) was added. The pellet was re-suspended using a pipette tip or a vortex. Extra effort at this point to fully re-suspend the tissue will result in a more complete digestion of the sample and a better yield of DNA.

2. Samples were incubated at 56°C in a water bath or heating block.

3. For tissue cores, 20 μl of Proteinase K (20 mg/ml stock solution, Invitrogen, AM2548) were spiked morning and evening.

4. The digestion steps are repeated for 2 to 5 days until the tissue was fully dissolved.

4. DNA clean

1. An equal volume of buffer saturated phenol was added and mixed by inversion, then Spun for at least 5 minutes at 14,000 rpm in microcentrifuge.

2. The aqueous layer was transferred to a new tube. Note the interphase: is there a lot of white material.

3. Steps 1 and 2 were repeated on the aqueous fraction until the interphase is clear (typically 3 or more times). Back extractions are performed when the interphase was fuzzy to increase final yield.
4. The aqueous layer was transferred to a new tube and treated with RNase A at 100 μg/ml for 1 hour at 37°C.
5. Steps 1 to 4 were repeated to remove any remaining RNase A and collect the aqueous fraction. Only 1 or 2 buffer saturated phenol steps are needed as there should be much less protein to remove than in the initial lysate.

*To perform back extractions, 50-100 μl of dH_2O is needed to the sample tube containing the interphase and organic portion. The tube is inverted to mix and the sample is spun at 14,000 rpm in a microcentrifuge for 5 minutes. The aqueous phase is collected and added to the previously acquired aqueous extraction. Back extractions are continued until the interphase is clear.

5. DNA precipitation

1. The volume of the collected aqueous layer was estimated.
2. 1/10 the volume of sodium acetate at conc. 3M, pH 5.2 and 1 volume of 100% isopropanol (v/v) [molecular biology grade](or 2.5 volumes of 100% ethanol) were added.
3. Put the above mixture on ice or in a -20°C freezer for 30 minutes or overnight after mixing it well.
4. The mixture was then spun at maximum speed (14,000 rpm) at 4°C in microcentrifuge for 10 minutes
5. The supernatant was discarded.
6. The pellet was washed with 70% ice cold ethanol to remove unwanted salts.
7. The pellet was re-suspended in the buffer of choice (usually dH_2O).
2.6.2.2 Extraction DNA by using gSYNC™ DNA Mini Kit
Tissue Protocol (Geneaid)

Table (2-5)

Kits contents of gSYNC™ DNA Mini Kit (Geneaid)

| ● GST Buffer   |
| ● GSB Buffer  |
| ● W1 Buffer   |
| ● Wash Buffer |
| ● Elution Buffer |
| ● Proteinase K |
| ● GD Column   |
| ● 2ml Collection Tube |

Figure (2-2): gSYNC™ DNA Mini Kit (Geneaid)

1. Samples Preparation

The samples are prepared by slicing sections (up to 25 mg) from blocks of FFPE tissue and transferred to 1.5 ml microcentrifuge tubes.
To prepare Proteinase K, 1.1 ml ddH₂O was added to 11 mg Proteinase K (vortex to dissolve and spin down) and was stored at 4°C.

Absolute ethanol was added to the Wash Buffer prior to initial use.

According to the steps outlined from the manufacturer the researcher has done as following:

1. One ml of xylene was added to the tube and mixed by shaking vigorously.

2. The mixture was incubated at a room temperature for approximately 10 minutes (shaking occasionally during incubation).

3. Centrifuge at 14-16,000 x g for 3 minutes. The supernatant was removed.

4. One ml of absolute ethanol was added to wash the sample pellet and mixed by inverting.

5. Centrifuge at 14-16,000 x g for 3 minutes. The supernatant was removed.

6. Steps 4 and 5 were repeated twice.

7. The tube was opened and incubated at 37°C for 15 minutes to evaporate any ethanol residue.

2. Tissue Dissociation and Lysis

1. Two hundred µl of GST Buffer was added to the tube.

2. Twenty µl of Proteinase K was added to the sample mixture and mixed by vortex.
3. The mixture was incubated at 60°C overnight or until the sample lysate becomes clear.

- The sample was grinded prior to incubation. This was increased the yield.

- The sample is inverted occasionally during incubation to disperse the sample or place in a thermomixer, shaking water bath, or on a rocking platform.

4. Two hundred µl of GSB Buffer was added and vigorously shaken for 10 seconds (it is essential that the sample and GSB Buffer were mixed thoroughly to yield a homogeneous solution).

**3. RNA Degradation**

Following GSB Buffer addition, 5µl of RNaseA (50mg/ml) was added to clear lysate and mixed by shaking vigorously. Then it was incubated at room temperature for 5 minutes.

**4. DNA Binding**

1. Two hundred µl of absolute ethanol was added to the sample lysate and immediately mixed by shaking vigorously for 10 seconds.

   - If precipitate appears, it was broken up as much as possible with pipette.

2. GD Column was placed in a 2 ml Collection Tube.

3. All the mixture was transferred (including any precipitate) to the GD Column.
4. The mixture was centrifuged at 14-16,000 x g for 1 minute.

5. Two ml collection tube containing the flow-through was discarded and the GD column was transferred to a new 2 ml collection tube.

5. The Washing

1. Four hundred µl of W1 Buffer was added to the GD Column.

2. The sample was centrifuged at 14-16,000 x g for 30 seconds and discarded the flow-through.

3. The GD column was placed back in the 2 ml column tube.

4. Six hundred µl of Wash Buffer was added to the GD column.

5. The sample was centrifuged at 14-16,000 x g for 30 seconds and the flow-through was discarded.

6. The GD column was placed back in the 2 ml column tube.

7. The sample was centrifuged again for 3 minutes at 14-16,000 x g to dry the column matrix.

6. DNA Elution

1. The dried GD column was transferred to a clean 1.5 ml microcentrifuge tube.

2. One hundred µl of pre-heated Elution Buffer or TE Buffer was added to the center of the column.

3. The sample was let to stand for at least 3 minutes to ensure the Elution Buffer or TE was absorbed.
4. The sample was centrifuged at 14-16,000 x g for 30 seconds to elute the purified DNA.

2.6.2.3 Extraction of DNA by using QIAamp® DNA Mini Kit (QIAGEN)

Figure (2-3) : QIAamp® DNA Mini Kit (QIAGEN)

Table (2-6)

<table>
<thead>
<tr>
<th>Kits contents of QIAamp® DNA Mini Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash Buffer1 (AW1 Buffer)</td>
</tr>
<tr>
<td>Wash Buffer2 (AW2 Buffer)</td>
</tr>
<tr>
<td>Elution Buffer (AE Buffer )</td>
</tr>
<tr>
<td>Tissue lysis Buffer (ATL Buffer )</td>
</tr>
<tr>
<td>Lysis Buffer (AL Buffer )</td>
</tr>
<tr>
<td>Potienase K</td>
</tr>
<tr>
<td>QIAamp Mini spin columns</td>
</tr>
<tr>
<td>Collection Tubes</td>
</tr>
</tbody>
</table>
According to the steps outlined from the manufacturer the following has been done:

**A. Deparaffinisation of the section**

1. Nine hundred µl Xylene was added to each labeled 1.5 ml microcentrifuge tube containing the curls and vortexed thoroughly.
2. In a bench top centrifuge was spun at top speed for 3mins at room temperature.
3. Supernatant was removed carefully, by using a separate filter tip for each sample.
4. Steps 1-3 were repeated.
5. An amount of 0.9ml of 100% Ethanol was added to each sample, and vortexed thoroughly.
6. The sample was spun for 3mins in bench top centrifuge at top speed.
7. Supernatant was removed carefully.
8. Steps 5-6 were repeated.
9. The supernatant was carefully removed as much as possible.
10. The sample was dried (lids opened) in a heat block at 56ºC for 5-10mins.

**B. Genomic DNA extraction (Qiagen)**

11. The pellet was suspended in 180µl ATL lysis buffer.
12. Fifty five µl of proteinase K was added and vortexed thoroughly.
13. Samples were incubated at 56ºC for 60 mins.
14. Tubes were vortexed.
15. Samples were incubated at 90ºC for 60mins (a weight may be placed on top of samples to stop the tubes popping open), the tubes were spun briefly
to remove the droplets from the lid.

16. Tubes were allowed to cool to room temp.

17. Buffer AL 200μl was added (DNA binding buffer) and mixed.

18. The sample was incubated at 70ºC for 10mins.

19. One hundred μl of Isopropanol was added and mixed.

20. The lysates was transferred (~550μl) into labeled QIAamp Mini Spin Columns.

21. The sample was spun at 8000xg (rcf) for 1min.

22. The flow through was discarded. The column was placed in a fresh collection tube.

23. Buffer AW1 (Wash buffer 1) 500μl was added to the QIAamp mini spin column.

24. The sample was spun at 8000xg for 1min. The flow through was discarded.

25. Five hundred μl of buffer AW2 (Wash buffer 2) was added to the QIAamp Mini Spin Column and spun at 8000xg (rcf) for 1min. The flow through was discarded.

26. Each QIAamp Mini Spin Column was placed in a new collection tube and spun at top speed for 1min to dry the membrane completely. The collection tube was discarded.

27. The QIAamp Mini Spin Column was placed in a fresh labeled 1.5ml microcentrifuge tube.

28. DNA elution buffer 75μl was added to the center of the membrane and incubated at room temp for 5mins.

29. The sample was spun at 8000xg (rcf) for 1min to collect the eluted DNA.
30. The DNA was stored at 4°C for short term and -20°C (Freezer) in the appropriate sample box for longer term storage.

2.6.2.4 DNA Extraction by using ReliaPrep™ FFPE gDNA Miniprep System (Promega)

**Table (2-7)**

Kits contents of ReliaPrep™ FFPE gDNA Miniprep System

<table>
<thead>
<tr>
<th>Mineral oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis Buffer (LBA)</td>
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<tr>
<td>Proteinase K (PK)</td>
</tr>
<tr>
<td>BL Buffer</td>
</tr>
<tr>
<td>Wash Solution</td>
</tr>
<tr>
<td>RNase A</td>
</tr>
<tr>
<td>Elution Buffer</td>
</tr>
<tr>
<td>Collection Tubes</td>
</tr>
<tr>
<td>ReliaPrep™ FFPE Binding Columns</td>
</tr>
</tbody>
</table>
Genomic DNA was extracted using ReliaPrep™ FFPE, DNA Miniprep System, Promega ,USA( table 2-7). DNA extracted according to the manufacturer’s protocol but with slight modifications of its protocol as following ( Lin, 2009 ) :

● **Preparation of 1X Wash Solution**

Twelve ml of 95-100% ethanol were added to the bottle containing 3ml of concentrated Wash Solution.

**A. Preparation of FFPE Sections**

1. using a sterile blade, excess paraffin was trimmed off the tissue block.

2. 5-50 µm sections were cut from FFPE block using a microtome.

3. The sections were placed in a 1.5 or 2ml microcentrifuge tube .the equivalent of ≤ 100 µm of tissue slices may be processed per reaction .

**B. Deparaffinization using Mineral Oil**

1. Mineral oil was added to the sample:

   - For sections ≤ 50 microns, 300 µm of mineral oil was added .

   - For sections ≥ 50 microns, 500 µm of mineral oil was added .

2. The sample was incubated at 80°C for 1 minute .

3. The sample was vortexed to mix.

**1. Sample Lysis**

1. Two hundred µm of Lysis Buffer was added to the sample.
2. The sample was spun at $10,000 \times g$ for 15 seconds at room temperature. Two phases were formed, a lower (aqueous) phase and an upper (oil phase).

3. Twenty µm of Proteinase K was added directly to the lower phase, the lower phase was mixed by pipetting.

4. The sample was incubated at 56º C for 1 hour.

5. The sample was incubated at 80º C for 1 hour.

6. The sample was allowed to cool to room temperature. The sample was centrifuged briefly at room temperature to collect any drops from the inside of the lid.

2. RNase Treatment

1. Ten µm of RNase was added directly to the lysed sample in the lower phase. The lower phase was mixed by pipetting.

2. The sample was incubated at room temperature (20-25 ºC) for 5 minutes.

3. Nucleic Acid Binding

1. Two hundred twenty of BL Buffer was added to the lysed sample.

2. Two hundred forty µl of ethanol (95-100%) was added.

3. The sample was vortexed briefly to mix.

4. The sample was spun at $10,000 \times g$ for 15 seconds at room temperature. Two phases were formed, lower (aqueous) phase and upper phase (oil phase).

5. For each sample to be processed a Binding Column was placed into one of the Collection Tubes provided.
6. The entire lower (aqueous) phase of the sample was transferred, including any precipitate that may have been formed, to the Binding Column / Collection Tubes assembly, and the column was capped.

- The remaining mineral oil was discarded.
- The mineral oil is inert and will not interfere with the extraction procedure if some of the oil phase is carried over to the Binding Column.

7. The assembly was spun at 10,000 × g for 30 seconds at room temperature.

8. The flow was discarded through, and the binding column was reinserted into the collection tube.

4. Column Washing and Elution

1. Five hundred µm of 1X Wash Solution (with ethanol added) was added to the Binding Column. The Column was capped.

2. The sample was spun at 10,000 × g for 30 seconds at room temperature.

3. The flow was discarded through, and the Binding Column was reinserted into same collection tube used for the Nucleic Acid Binding.

4. Five hundred µm of 1X Wash Solution (with ethanol added) was added to the binding column. The column was capped.

5. The sample was spun at 10,000 × g for 30 second at room temperature.

6. The flow was discarded through, and the binding column was reinserted into the collection tube used for the nucleic acid binding.
7. The cap on the binding column was opened, and the binding / collection
tube assembly was spun at 16000 x g for 3 minutes at room temperature to
dry the column.

8. The binding column was transferred to a clean 1.5 ml microcentrifuge
tube and the collection tube was discarded.

9. Thirty to fifty µl of Elution Buffer was added to the column, and the
column was capped.

10. The sample was spun at 16000 x g for 1 minute at room temperature.
The binding column was removed and discarded.

11. The microcentrifuge tube was capped, and the eluted DNA was stored
at -20 ºC (Freezer) in the appropriate sample box for longer term storage.

2.6.3 Solutions used in Agarose Gel Electrophoresis

2.6.3.1 Tris-borate (TBE) Buffer

(0.89M Tris-Base; 0.88M Boric acid; 20mM EDTA, PH 8.0)

To prepare 10X TBE solutions, the component used as following:

108gm of Tris-Base, 55gm of Boric acid, 40ml of 0.5M EDTA (pH
= 8.0) in an appropriate amount of D.W, pH was adjusted to 7.8 and
volume completed to 1 liter with D.W. The solution was sterilized by
autoclave and stored at room temperature (Sambrook et al., 1989).
2.6.3.2 Ethidium Bromide Dye (10 mg/ml)

This was prepared by dissolving 1 gm of Ethidium Bromide in 100 ml of a sterile D.W, and the bottle was kept in the dark (Maniatis et al., 1982). Ethidium is a powerful mutagen; so the gloves and the mask should be worn in all the steps during the work.

2.6.4: Agarose gel electrophoresis

2.6.4.1 Reagents used in agarose gel electrophoresis (Vogelsein and Gillespie, 1979)

- Agarose
- 10 X TBE Buffer.
- Bromophenol blue (Loading dye).
- DNA marker.
- Ethidium bromide (10 mg/ml).

2.6.4.2 Preparation of agarose gel electrophoresis

1. Preparation of 1 Liter of TBE Buffer (1X) (Younan, 2010):

   100 mL of 10X TBE + 900 mL of D.W

2. Agarose gel was prepared according to Maniatis et al., 1982.

   ● A 100 ml of 10 X TBE was placed in a beaker.
   ● Then a 0.8 g, 2 g agarose was added to the buffer (as required) to prepare 0.8% and 2% agarose gel respectively.
(the same concentrations was used in case of low melting agarose gel).

The solution was heated to boiling (using heating stirrer)
Then the solution was allowed to cool down. Then a 10 µL of Ethidium Bromide solution was added.

3. **Casting of the horizontal agarose gel:**

- The gel was assembled to casting tray and the comb was positioned at one end of the tray.
- The agarose solution was dropped into the gel tray after both edges were sealed with tapes and the agarose was allowed to cool at room temperature for 30 minutes (in case of low melting agarose gel was left to cool in refrigerator for more than 30 minutes).
- The comb was carefully removed and the gel replaced in electrophoresis chamber.
- The chamber was filled with an appropriate amount of TBE-electrophoresis buffer so that it covers 1-2 mm over the surface of the gel.

  - To each PCR sample 2µl Blue/Orange 6x Load Dye was added.
  - Ten µl of the 100 bp DNA Ladder with 2µl of Blue/Orange 6x Load Dye was added to the first and last lane of the gel.
Ten μl of the sample was applied to each lane of the gel. The lid was placed on electrophoresis tank.

- The cathode was connected to one side of the unit and the anode to the other side.
- It was run for 1 hour at 120v.
- The DNA was observed by UV transiluminator (λ=305nm).

2.6.5 Molecular analysis of genomic DNA of HER2/neu by using PCR techniques

2.6.5.1 Reagent used in PCR Technique: PCR reaction were carried performed using the following:

1. Primers: In this study, two set of primers has been used, the first primer was used in quantitative PCR (first run), which its sequence was Forward 5′- ATATCCAGGAGGTGCAGGG - 3′ and Reverse 5′- CTTCGAAGCTGCAGCTCCC - 3′, for an expected size of 205 bp. While the primer that used in the nested PCR as the following sequencing Forward 5′- CTCACAACCAAGTGAGGCAG - 3′ and Reverse 5′- CAGGGGTGGTATTCTTCA - 3′, where expected to produce a size of 126 bp table (2-8). These primers was provided by BIONEER CO. (South Korea) that is specific primer to HER2/neu gene.
Table (2-8)

**HER2/neu gene Oligonucleotide primers sequence**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer sequence</th>
<th>Length</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>5' → 3'</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>ATATCCAGGAGGTGCAGGG</td>
<td>19</td>
<td>205 bp</td>
</tr>
<tr>
<td>Reverse</td>
<td>CTTCGAAGCTGCAGCTCCC</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CTCACAACCAAGTGAGGCAG</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>CAGGGGTGGTTATTCTTCA</td>
<td>18</td>
<td>126 bp</td>
</tr>
</tbody>
</table>

2. **Go Taq ®Green Master Mix (2X):**

Go Taq ®Green Master Mix is ready to use mixture that contains Taq DNA polymerase, MgCl₂, pure deoxynucleotides (dNTPs), reaction buffer and blue dye that allow monitoring of progress during electrophoresis, with concentration (2X). Go Taq ®Green Master Mix was supplied by (Promega, USA).

In order to achieve homogeneity of reagents and reduce the risk of contamination a master mix for all samples was prepared containing all the components of the reaction except of genomic DNA (template DNA), mixed gently with sterile distilled water to achieve the appropriate volume.

Amplification was performed on ice in aseptic conditions in laminar air flow using 0.2 ml tight cap eppendorf tubes.

3. **DNA Samples**: Samples already have been used which were prepared by using ReliaPrep™ FFPE gDNA Miniprep System kit (Promega) for extraction DNA.
2.6.5.2 Preparation of polymerase Chain Reaction (PCR) for HER2/neu for first primer:

The amplification was carried out by using Green®Master Mix Kit (Promega, USA). Each PCR reaction was carried out in a total volume of 25µl containing 12.5µl of PCR master mix (Promega, USA), 7.5 µl of nuclease free water, 1 µl (5-10 pmol/ µl) of each primer and 3µl of DNA and the amplification protocol was observed in table 2-9.

Table ( 2-9 )
Reagents were used in PCR (25µL) at final concentration

<table>
<thead>
<tr>
<th>Reagent</th>
<th>size</th>
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<tr>
<td>Primer Forward</td>
<td>1µl</td>
</tr>
<tr>
<td>Primer Reverse</td>
<td>1µl</td>
</tr>
<tr>
<td>DNA Sample</td>
<td>3µl</td>
</tr>
<tr>
<td>Master Mixed</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>D .W</td>
<td>7.5 µl</td>
</tr>
<tr>
<td>Total</td>
<td>25 µl</td>
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</tbody>
</table>

2.6.5.3 PCR Conditions

The optimizing condition were performed on the different degrees of initial steps of PCR amplification were ( 56°C, 58°C and 60°C) which were closed to the essential initial degree 57.5°C that mentioned with primer manufacturing information.
One of the samples were taken, which were characterized by intact DNA and perfect, then the sample was subjected to the previously mentioned protocol of PCR mixture table (2-9) in 200µl tube, where they were prepared for introduction to PCR System. The same sample was subjected to the same conditions, except temperature, which must be identified in this step. The first run was using 56 °C in Annealing degree of second step of PCR system, the second was 58 °C, and the third run was 60 °C for annealing step. This work has been to choose the optimal thermal degree for annealing. In the end of PCR run, the product was taken and examined in 2% agarose gel electrophoresis to detect perfect band after one hour and half at 120 voltage. Then the three samples were taken for testing under UV transiluminator to find out the best one. All these condition were applied on both primers, whether the first or second primer in nested PCR.

Table (2-10): PCR conditions for HER2/neu for first primer

<table>
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<th>NO.</th>
<th>STEPS</th>
<th>Temperature</th>
<th>Time</th>
<th>NO. OF Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>Denaturation 1</td>
<td>95°C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>I</td>
<td>Denaturation 2</td>
<td>95 °C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Annealing</td>
<td>60 °C</td>
<td>30 sec</td>
<td>40</td>
</tr>
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<td>III</td>
<td>Extension 1</td>
<td>72 °C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>Final</td>
<td>Extension 2</td>
<td>72 °C</td>
<td>10 min</td>
<td>1</td>
</tr>
</tbody>
</table>
### Table (2-11): PCR conditions for HER2/neu for second primer (nested PCR)

<table>
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<th>STEPS</th>
<th>Temperature</th>
<th>Time</th>
<th>NO. OF Cycles</th>
</tr>
</thead>
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<td>95°C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>I</td>
<td>Denaturation 2</td>
<td>95 °C</td>
<td>30 sec</td>
<td></td>
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<tr>
<td>II</td>
<td>Annealing</td>
<td>58 °C</td>
<td>30 sec</td>
<td>35</td>
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<td>III</td>
<td>Extension 1</td>
<td>72 °C</td>
<td>30 sec</td>
<td></td>
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<tr>
<td>Final</td>
<td>Extension 2</td>
<td>72 °C</td>
<td>10 min</td>
<td>1</td>
</tr>
</tbody>
</table>

#### Figure (2-5)

Screen of Veriti 96 well Thermal Cycle showing PCR Stages Curve
3.7: Sequencing:

Automated DNA sequencing was performed by using the ABI PRISM 310 Genetic Analyzer (Applied Biosystem, USA) that sequencing done in USA. All the data obtained from automated sequence was edited with a computer based program sequencer. These sequences were compared with the reference sequence (NCBI database) to determine the nature of mutations.

3.8: Statistical analyses

Statistical analyses of all results were analyzed using SPSS Version 10 statistical package using Chi square test at level of significance alpha \( \leq 0.05 \), and correlation-regression test according (r at a significant level of 0.3).
Chapter Three

Results and Discussion

3 -1: Clinicopathological analysis in breast cancer patients

The peak age frequency in the total group studied was in the age category of (41-50 yr) accounting of 50 patients as shown in table(3-1). In this study more than 24% of the patients have family history either it is the first or second degree. The histopathology diagnosis showed that a high percentage in Iraqi cases with infiltrated ductal carcinoma represented (86%), while the invasive lobular carcinoma represented (4%) and the mixed carcinoma represented (10%) which that agreement approximately with Groheux et al., 2013. In current study 12 % of patients were in grade 1, 20% were in grade 2 and 68% were in grade 3, so most of our patients were in grade 2 and 3. The stage of the breast cancer as in the other types of cancer is the most important prognostic parameter.

Table (3-1) Main tumor characteristics in the 50 patients with breast cancer

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<tr>
<th>Age group</th>
<th>Number of patients</th>
<th>%</th>
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<tr>
<td>&lt;40</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>41-50</td>
<td>23</td>
<td>46</td>
</tr>
<tr>
<td>51-60</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>61-70</td>
<td>9</td>
<td>18</td>
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<tr>
<td>&gt;71</td>
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<table>
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<tr>
<th>Family history for breast cancer</th>
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<tr>
<td>Positive</td>
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</table>

<table>
<thead>
<tr>
<th>Type breast cancer of</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infiltrated ductal carcinoma</td>
</tr>
<tr>
<td>Infiltrated lobular carcinoma</td>
</tr>
</tbody>
</table>
Mixed(Ductal & Lobular) carcinoma 5 10

<table>
<thead>
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<th>4-Grade</th>
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</thead>
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<tr>
<td>Grade 1 well differentiation</td>
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<td>12</td>
</tr>
<tr>
<td>Grade 2 Moderate differentiation</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Grade 3 Poor differentiation</td>
<td>34</td>
<td>68</td>
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<table>
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<th>Histological feature type</th>
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<tbody>
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<td>Stage 1</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Stage 2</td>
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<td>Stage 3</td>
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<tr>
<td>Stage 4</td>
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<td>14</td>
</tr>
</tbody>
</table>

The present results on Iraqi women patients revealed that a high age frequency of cancer occurred between 41-50 years of age (46%), that corresponding with other demographic Iraqi studies revealed that the age range accounting for 67 out of 216 breast cancer patients (31%) was most frequent (Alwan, 2010) in Iraq and in agreement with the results of the present study, a group of researchers both in USA and Australia have found that the incidence of breast cancer increasing sharply after the age of 40 (Wu et al., 2002 and Edwards et al., 2002), while some studies differ in their results which show high age frequency between 51-60(38.89%) as reported by Jumaah (2013).

3.2. Immunohistochemical results for Estrogen , Progesterone and Her2/neu Receptors

3.2.1 HER2, Estrogen and Progesterone Receptors

Many sex hormones including estrogen, progesterone and others including Prolactin have been accused to take part in breast cancer
development, in addition, the tumor suppressor genes are also an essential players in the breast carcinogenesis. So a study searching for relations between both is highly indicated as reported by Ghanim, 2009. The hormonal results of the 50 cases with breast cancer were studied as shown in table (3-2) as below:

I : Intensity of staining colour.

P: Proportion of staining tumour cell.

T : Total of intensity and proportion.

<table>
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<tr>
<th>No. of samples</th>
<th>Age Years/old</th>
<th>HER2</th>
<th>I</th>
<th>P</th>
<th>T</th>
<th>ER</th>
<th>I</th>
<th>P</th>
<th>T</th>
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<tr>
<td>32</td>
<td>50</td>
<td>Negative</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>Positive</td>
<td></td>
<td></td>
<td>Negative</td>
<td></td>
</tr>
</tbody>
</table>
The results observed that all samples from patients under study included embedded formalin fixed blocks tissue. Estrogen positive receptors were in 48% (24/50) of the cases and progesterone positive receptors in 46% (23/50) of the cases, and these data revealed that presence of hormone receptor expression in the majority and cancer was considered
hormone receptor positive and they were likely responded to hormonal therapy. These results exactly agree with that mentioned by Joensuu et al., 2013. About 56% of positive ER cases had strong positive stain and 42% of positive PR cases had strong positive stain as observed in table 3-3 and figures 3-1 and 3-2.

**Table (3-3): Distribution of Estrogen, progesterone and her-2/neu receptors status in Iraqi breast cancer patients**

<table>
<thead>
<tr>
<th>The Marker</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen -</td>
<td>26</td>
<td>52</td>
</tr>
<tr>
<td>Estrogen +</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Progesterone -</td>
<td>27</td>
<td>54</td>
</tr>
<tr>
<td>Progesterone +</td>
<td>23</td>
<td>46</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>ER+/PR+</td>
<td>18</td>
<td>36</td>
</tr>
<tr>
<td>ER+/PR -</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>ER-/PR+</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>ER-/PR-</td>
<td>21</td>
<td>42</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

**Her2/neu receptor**

<table>
<thead>
<tr>
<th>Score</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score 0 (Negative)</td>
<td>33</td>
<td>66</td>
</tr>
<tr>
<td>Score +1 (Negative)</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Score +2 (weak)</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Score +3 (positive)</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure (3-1): Representative staining results from H&E and IHC

(A) : Negative Staining for ER in malignant breast cell magnification 10X (score 0).

(B) : ER staining in a metastatic breast cancer, magnification 10X (score 2).
(C): ER staining in a metastatic breast cancer, magnification 40 X (score 3).

Figure (3-2): Representative staining results from H&E and IHC

(A): Negative Staining for Progesterone receptor in malignant breast cell. magnification 10 X (score 0).
(B): PR Positive staining in a metastatic breast cancer, magnification 10X.

(C): PR Positive staining in a metastatic breast cancer, magnification 40X.
3.2.2. HER2 receptors

HER2 were scored based on the intensity and percentage of positive cells on a scale of 0 to 3+. This study demonstrated that (33/50) 66% were score 0 and (4/50) 8% were score+1 which represented as HER2/neu negative (score 0 and score+1) 74%(figure 3-4A,B), (6/50)12% for score+2 which considered equivocal (figure 3-4C) and 7 out of 50 malignant cases (14%) were score+3 that consider as strong positive for HER2/neu expression (figure 3-4D,E) [the percentage appear in figure 3-3], which mean that HER2 genes are over producing the her-2 protein and that those cell growing rapidly and causing cancer. These breast cancer cases tend to be much more aggressive and fast growing while 66% (33/50) had score 0 which mean that HER2/neu protein is normal producing and not causing cancer. That’s where these results of HER2 positive level near consistent that was mentioned by Penault-Llorca and Viale, 2012. When they review estimated that the rate of this discordance is around 7%–26% for HER2 status as these shown in table 3-3.

As well as previous markers which was used in this study, a third marker, HER2 had a great importance in the diagnosis of breast cancer. The clinical activity of anti-HER2 agents has been limited to patients with HER2 tumors as defined by intense membrane staining with HER2 antibodies in the majority of tumor cells(3+ by IHC). In general, HER2 IHC and FISH correlate with each other to IHC to reproducibly assess tumors for HER2 overexpression at outside and/or local laboratories for entry into clinical trials. Intrinsic limitations of IHC are the variability in fixation methods and the impact of fixation of antigenicity of the HER2 protein. Conversely, the more stable DNA, of which the loci are measured by FISH, is less susceptible to tissue fixation. For these reasons, excess copies of the HER2 gene (so-called HER2 positivity) defined by FISH have gained
ground as the standard to define odds of tumor dependence on HER2 and, therefore, response to HER2 antagonists (Abramson and Arteaga, 2011).

**Figure (3-3):** Number of Negative, Equivocal and Positive HER2 Marker with its percentage in breast cancer cases.

**Figure (3-4):** Immunohistochemical (IHC) assessment of the level of Her-2 protein expression in the tumor cell membrane using the approved DAKO Hercep Test kit according to the manufacturer’s instructions.

(A) Cases with no membrane staining are scored as IHC 0 (low power 10x).
**Figure (3-4B):** Cases scored as IHC 1+ demonstrate partial weak membrane staining in more than 10% of tumor cells with no complete circumferential staining (low power 10x).

**Figure (3-4C):** Cases scored as IHC 2+ demonstrate circumferential membrane staining in more than 10% to <30% of tumor cells, but the staining ring is thin (low power 10x).
**Figure (3-4D):** Cases scored as IHC 3+ demonstrate circumferential membrane staining in more than 30% of tumor cells, but the staining ring is thick and has a retractile quality at low power 10x.

**Figure (3-4E):** Cases scored as IHC 3+ demonstrate circumferential membrane staining in more than 30% of tumor cells, but the staining ring is thick and has a retractile quality at high power 40x.
3-3: Correlation between clinicopathological assessment and IHC tumor markers for ER, PR and Her2/neu.

The association between the levels of expression of ER, PR and Her2/neu and the age, family history, tumor grade, tumor type and stage was summarized in Table (3-1). For ER there is no statistically significant association between expression of ER and the age, family history, tumor types or stages (P>0.05). The relationships between the PR of expression and the age, family history, tumor grade, tumor type and stage was also not significant (P>0.05). On the other hand the expression of Her2/neu didn’t show any statistical significant difference with the age, tumor grade or type except there was a significant correlation between her2/neu expression and family history (P<0.01).

The tumor that showing positive receptors has better prognosis and better response to hormonal therapy than those with no receptors (Rosia, 2011). This study demonstrated a 50 malignancy breast carcinoma samples were included wax blocks embedded tissue. ER receptors were in 48%(24/50) of the cases and PR positive receptors in 46% (23/50) of the cases so we concluded that there was hormone receptor expression in the majority of breast cancer in Iraqi patients under study. About 56% of positive ER had a strong positive stain further more a 42% of PR had strong positive stain.

The results were compatible with the Iraqi cancer therapy registry (Iraqi center Board 2007) findings. They found that ER positive tumors were noted in 65% of the cases and PR positive tumors were noted in 45% of the cases. Another study on hormone receptor contents of breast carcinoma specimens belong to Iraqi patients reported higher frequencies for ER and PR equivalent to 61% and 52% respectively (Al-Alwan, 2010). On the other
hand (Sughayer et al., 2006) from Jordanian study found that 50% and 57% of breast cancer samples were positive for ER and PR respectively.

### 3.4 Molecular subtype

According to the classification of molecular subtype of breast cancer, the results show that a group with (ER+, PR+ and HER2-) were 34% (17/50) cases, also when (ER or PR is negative and HER2-) were (10/50) which represented as Luminal A group 54% (27/50), then a group with triple negative (ER-, PR- and HER2-) is 32% (16/50) cases that represent Basal like group, while the other groups appeared least frequency as following: (HER+, ER- and PR-) = 5 Cases which were referred to HER2 subtype group, then (HER+, ER+ and PR+) = 1 Case only and (HER+, ER or PR+) = 1 case also that refer to Luminal B subtype. So the classification of the subtypes of breast cancer for this study illustrated in table (3-4) as following:

<table>
<thead>
<tr>
<th>subtype</th>
<th>ER and/or PR</th>
<th>HER2</th>
<th>No. of Cases</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>overexpression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal A</td>
<td>+</td>
<td>-</td>
<td>27</td>
<td>54</td>
</tr>
<tr>
<td>Luminal B</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>HER2 subtype</td>
<td>-</td>
<td>+</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>
In regard to her2/neu the current results appear to be within the completely reported rates of 20 % to 30 % (Al-masri and Hamad, 2005 ; Azizun-Nisa et al., 2008 and Mudduwa,2009), while less than 20% were reported by other workers (Adebamowo,2008 and Cho et al., 2008). The present study demonstrated that 7 cases of 50 malignant cases revealed 14% were positive for her2/neu expression, while 37 cases out of 50 were with score 0 and score1 and 6 cases with score 2 considered (equivocal ) as her2 negative.

The study was focused on The relationship between hormonal receptors and Her-2/neu status and subsequently correlated the results with the studied clinical and morphoclinical parameters. The study determined that

<table>
<thead>
<tr>
<th>Molecular subtype</th>
<th>-</th>
<th>-</th>
<th>16</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

**Figure (3-5): Distribution of breast cancer cases according to Molecular subtype.**
28.5% (2/7) of cases with positive Her-2/neu score 3+ were also positive for both hormonal receptors. On the contrary, 71.4% of the cases with positive Her-2/neu were characterized by the absence of nuclear stain for both estrogen and progesterone receptors. Analysis of the relationship between the response to hormonal receptors and Her-2/neu status allowed the distribution of 50 breast cancer cases into molecular classification, using “surrogate immuno-histochemical criteria”. 6 cases (12%) with equivocal immunohistochemical stain (Her-2/neu score 2+) were excluded. Thus, we obtained the following incidences of molecular subtypes: as luminal A subtype, was the greatest incidence 54% of cases, followed by basal subtype with 32%. Her-2/neu subtype represented 10% of cases and Luminal B had the lowest incidence, respectively 4% of cases (table 3-4).

The analysis of the obtained correlations between molecular subtypes and morphoclinical parameters were done and illustrated, that luminal A subtype characterized the groups aged over 50 (p<0,05). The majority of cases belonging to luminal B were aged under 50 (p<0,05). Regarding Her-2/neu cases, 40% were found at patients aged under 50 and 60% at the group aged more than 51. The basal group was diagnosis in 90% of the cases aged over 50(p<0,05). This study investigated the relationship between molecular subtypes and tumor size and we observed that tumors of small sizes (T1-T2) prevailed (92% of the cases) in luminal A. Regarding luminal B subtype, 83,4% of cases had dimensions classified in T2-T3. Tumors in the subtypes with negative hormonal receptors had large sizes specific to T2-T3 (60% of Her-2/neu tumors, respectively 91% of basal subtype. As concerns tumor differentiation degree, luminal A subtype was associated in percent of 58% of cases with well and moderately differentiated tumors. Luminal B subtype presented low differentiated tumors in 66,7% of the cases. Her-2/neu subtype was associated in 80% of
cases with low-differentiated tumors G3. Basal subtype presented a moderate and low differentiation degree in 91% of cases. The correct treatment of breast cancer is a multidisciplinary treatment, the sequence of therapeutic methods and their aggressiveness being conditioned by the histopathological type, tumor sizes, adenopathies, the patients’ age and their menopause status. The specific markers ER, PR and Her2/neu are used in treatment response prognosis and in guiding the therapeutic plan.

The majority of patients in the study group were aged over(40) years 88% (44/50). Most tumors had large sizes, 48.9% of the cases were categorized in T2 and T3. 68% of cases were low-differentiated, classified in G3 category, comparatively with only 12% of cases of well differentiated tumors G1. The histologic grade and the nuclear grade are prognostic factors and useful parameter for the stratification by stage, especially for cases without lymph node metastases (Rosen et al., 1993). Evaluation of hormonal status determined that 48% of cases in this study groups were ER+ and 52% ER-, respectively 46% were PR+ and 54% PR-, results which can be compared with those obtained by large studied groups and published in the specialty literature (Rakha et al., 2007).

The cases were categorized into molecular classification. Most of the cases in this study were ranked in luminal A (54%), followed by basal (32%) and Her2 had an incidence of 10 % and luminal B had the lowest incidence 4% of the cases. Specific literature showed that tumors in the Her-2/neu and basal subtypes were more frequently high grade and larger, and they occurred in younger patients compared with those in the luminal A subtype. Tumors over expressing Her-2/neu were statistically significantly more likely to be multicentric / multifocal, have a high nuclear grade and have lymph vascular invasion than luminal A tumors. The luminalB subtype present characteristics that full between those of luminal A and Her-2/neu tumors subtypes (Wiechmann et al., 2009).
3.5 Molecular Study

3.5.1 Optimization of DNA extraction from formalin fixed paraffin embedded tissue.

From 50 samples FFPE were extracted by four DNA extraction protocols (Phenol/Chloroform Extraction, gSYNC™ DNA Mini Kit (Genaid), QIAamp® DNA Mini Kit (Qiagen) and ReliaPrep™FFPE gDNA Miniprep System (Promega). The distribution of collected samples according to preservation years were show in table (3-5). All the samples of FFPE that preservation in 2010 (10/50) collected that represented fifth samples (20%) were failed in obtaining DNA by four protocols used in this study of DNA extraction. While other samples of FFPE collected from period 2011-2013 were differ in their result DNA extraction. Maximum yield of DNA (160-293µg) was obtained with Promega DNA extraction (table 3-6). However, the manual method using phenol/chloroform method had minimum yield in the range of 13.3-17.5 µg/ml was obtained, the kit of Genaid DNA Extraction, its yield was 20.5-39.2µg/ml while the Qiagen DNA Extraction kit was 40-75µg/ml. DNA obtained by using Promega DNA Extraction kit have the ratio of OD\textsubscript{260}/OD\textsubscript{280} of not more than 1.6. Furthermore the quantity of DNA obtained using Promega DNA Extraction kit protocol (169-293µg) was revealed as good result and enough to carry out optimization of PCR and further to analyze multiple cancer markers.
Table 3-5: The distribution of the FFPE samples according to its preservation

<table>
<thead>
<tr>
<th>Years</th>
<th>No. Samples</th>
<th>No. of samples succeed in DNA extraction</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>2010</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2011</td>
<td>12</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td>2012</td>
<td>19</td>
<td>12</td>
<td>63.1</td>
</tr>
<tr>
<td>2013</td>
<td>9</td>
<td>8</td>
<td>88.8</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>

Table 3-6: Spectrophotometric Analysis of FFPE DNA samples from different DNA Extraction methods

<table>
<thead>
<tr>
<th>DNA Extraction Protocol</th>
<th>260/280</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol/ Chloroform Extraction µg</td>
<td>1.1 - 1.2</td>
<td>13.3-17.5</td>
</tr>
<tr>
<td>Genaid DNA Extraction µg</td>
<td>1.0 -1.3</td>
<td>20.5-39.2</td>
</tr>
<tr>
<td>Qiagen DNA Extraction µg</td>
<td>1.3 – 1.5</td>
<td>40-75 µg</td>
</tr>
<tr>
<td>Promega DNA Extraction µg</td>
<td>1.1 – 1.6</td>
<td>160-293 µg</td>
</tr>
</tbody>
</table>

There was a rapidly increasing importance to evaluate a range of susceptibility and tumor markers in formalin-fixed paraffin embedded tissue specimens in molecular epidemiological studies. Polymerase chain reaction (PCP) was one of the easiest molecular biological techniques that can be performed on FFPE tissues. However, the efficiency of PCR was
influenced by a number of factors including the type of fixative and the fixation time, the DNA extraction procedure, PCR amplifiers size, the concentration of template DNA and optimization of PCR (Mahaisavariya \textit{et al.}, 2005). Amongst the four DNA extraction protocols Promega DNA Extraction protocol was found the most reliable method for clinical diagnosis in terms of quantity of DNA obtained, cost effectiveness and short extraction time of 2-3 hours. While phenol/chloroform (manual extraction) was quite laborious, involves a number of steps and was prone to cross contamination.

Formalin was the most widely used for fixative in histopathology diagnosis due to several advantages, but it damages tissue nucleic acids by cross linking it to tissue proteins, which subsequently results in extensive DNA and RNA fragmentation (Bonin \textit{et al.}, 2003 and Lin, 2009). It was very well known that PCR was very difficult with DNA extracted from fixed tissues and fixation intervals were associated with decreased PCR yields and inability to amplify longer DNA targets (Quach \textit{et al.}, 2004). It had been reported previously that DNA amplifications only up to 300 bp were obtained from FFPE postmortem tissues and very often amplifiers only up to 100 bp were obtained (Bonin \textit{et al.}, 2003).

Results of extraction DNA from FFPE in this study showed that it was able to extract good quantity and quality of DNA in a cost-effective manner from FFPE breast cancer tissues and to standardize PCR on paraffin extracted DNA using Promega kit that representad the best and fast method to extraction DNA from FFPE samples, as shown in figure (3-9) while the other three methods used for extraction showed the presence of smear DNA in most cases as shown in figures (3-6, 3-7 and 3-8).
**Figure (3-6):** Gel electrophoresis of DNA extracted from FFPE breast cancer cases by manual method (100V/120 min.) in 0.8% agarose, showing the presence of smear DNA in most cases, visualized under UV after staining with EB.

**Figure (3-7):** Gel electrophoresis of DNA extracted from FFPE breast cancer cases by Genaid kit method (100V/120 min.) in 0.8% agarose, showing the presence of smear DNA in most cases, visualized under UV after staining with EB.
Figure (3-8): Gel electrophoresis of DNA extracted from FFPE breast cancer cases by Qiagen kit method (100V/90 min.) in 0.8% agarose, showing the presence of smear DNA in most cases, visualized under UV after staining with EB.

Figure (3-9): Gel electrophoresis of DNA extracted from FFPE breast cancer cases by Promega kit method (90V/60 min.) in 0.8% agarose, showing the presence of DNA, visualized under UV after staining with EB.
All four tested paraffin for purification and extraction methods resulted in different yield of gDNA from the paraffin-embedded samples tested depending on which protocol used. All methods should be confirmed and tested with more samples to get stronger arguments to use a particular combination. The Promega kit might give better result if more samples are tested. BioMarke, (2006) has presented supporting results for Promega DNA kit, as they showed that 200 bp fragments could be amplified and DNA of higher molecular weight were present in all tested samples from paraffin embedded tissue.

Akalu and others (1999) reported that samples from paraffin-embedded tissue were able to amplify fragments up to 959 bp. In the reported study, gDNA was extracted with an extraction buffer (10 mM Tris–HCl, 1% Tween, 0.1 mg/ml proteinase K, 1 mM EDTA, pH 8.0) and purified with Promega kit or phenol/chloroform (with ethanol precipitation).

The amount of high molecular weight DNA after purification with Promega kit was significantly higher when the total gDNA was analyzed on agarose gel. The gDNA was tested in PCR and some samples purified with Promega amplified fragments of 959bp. These tests indicated clear improvements when kits were used, supporting the notion that the Promega kit in this study should have been tested with more samples. A report by Isola and others (1994) should also be considered. They reported that prolonged digestion with proteinaseK improves the yield of gDNA. While new tests with Promega samples should be digested with proteinase K only 2-3 hours, as in the preparation protocol, to get optimal yield in this short time.

Tests of other paraffin purification and extraction methods were reported by Coombs and others(1999). A combination of digestion with proteinase K, paraffin purification with thermal cycler and gDNA extraction with Chelex-100(media for DNA extraction) gave best results.
They concluded that removal of paraffin and purification are main steps required to obtain good results. Techniques involving melting with oil to remove paraffin showed to be more effective compared to methods using organic solvents to dissolve the paraffin, and melting with oil is also more safer, this technique was done in this study for the paraffin samples (Lin, 2009). Shi and others (2002) reported an extraction method involving heat-treatment and concluded that temperature and pH affect the outcome. High temperature, 120°C, and pH 6-12 showed satisfactory results, considering yield and amplification in PCR. Thus, it is apparent that multiple different methodologies are available for preparing gDNA from paraffin-embedded tissue. Different methods can be preferable in different tests and the optimal method in each given case has to be empirically determined.

The amount of extracted gDNA was not correlated with the amount of tissue, this is probably due to which paraffin block was used for extraction. Perhaps which part of the paraffin block also matters, since no correlation between samples from the same paraffin block could be seen. The quality of extracted gDNA also differs between different paraffin blocks. Fifty two percentage (52%) (26 of 50) of the tested samples were able to amplify a fragment of around 100 bp or 200 bp but only 18% (8 of 50) were able to amplify a fragment around 600 bp. No one of tested samples were able to amplify a fragment around 850 bp. These results indicate that the gDNA is much degraded and this is a probable reason why additional purification steps do not improve the PCR.

Degraded gDNA might influence the yield, that might be referred to the obtained gDNA from a certain amount of tissue, since it is possible that the precipitation efficiency differs between long and short DNA fragments when gDNA is concentrated with precipitation. This hypothesis could be
confirm according to samples with high yield contained more gDNA of high molecular weight and should be able to PCR amplify a long fragment. To evaluate these possibilities, the gDNA samples should be separated on an agarose gel to estimate the distribution of gDNA of different molecular weight.

The age of the paraffin block can be one reason that cause of the poorer quality of the extracted gDNA. The sample from the oldest paraffin block that could not amplify a 600 bp fragment was embedded 2010 (0/10), and group that collected in this year was chosen as a border for the proportion calculation. 50% (6/12) of the samples from paraffin blocks embedded of 2011 were able to amplify PCR fragments. None could however amplify a fragment of 300 bp or longer. 63.1% (12/19) of the samples from paraffin blocks embedded of 2012 were able to amplify PCR fragments of 100 bp to 600 bp. New paraffin block collected in 2013 were determined to have better quality than older samples at 88.8%(8/9). Moreover the age of the paraffin block cannot be the only cause, as some samples from new paraffin blocks did not amplify any fragments and some old blocks were able to amplify at least shorter fragments. Other causes that may be effect than age alone in the influence the quality and here follow some suggestions of possible explanations. The age and/or other conditions, might influence the quality of the tissue before fixation in paraffin. Another important aspect is the time from tissue removal and paraffin fixation. Perhaps, the storage of the paraffin blocks could also influence the quality (Lin, 2009).

The quality of gDNA prepared from paraffin-embedded tissue differs between blocks and gDNA from some blocks seems to be highly degraded. This study clarified that paraffin blocks can be used as source for gDNA in genetic studies. The paraffin purification and DNA extraction method used in this study was considered as a potential preparation method, as some
samples were able to PCR-amplify a 600 bp fragment. Most samples were able to amplify samples around 100-200bp, which make them useful for microsatellite analyses with fragments around that size.

3.5.2. Analysis of HER2/neu Gene Amplification in FFPE Breast Cancer Tumor Samples.

Detection of gene amplification of HER2 can be done by fluorescence in situ hybridization (FISH) or polymerase chain reaction (PCR) methodologies associated with IHC (Menard et al., 2001) and others researchers recommend to more methods to detection this mutation as Real time and sequencing. (Moore, 2012 and Bose et al., 2012).

HER2 mutations detected separately from HER2 gene amplification and DNA sequencing is required to detect them. An analysis of data from a series of studies documenting breast cancer genome sequencing has confirmed that HER2 mutations may be ideal targets for breast cancer treatment. Further, the majority of these mutations are activating mutations that drive breast cancer cell growth in tissue culture (Moore, 2012).

For genomic DNA Analysis the first pair of HER2/neu primers had the following sequence: forward- ATATCCAGGAGGTGAGGG, and reverse- CTTGAAGAGCAGCTGAGGC. The annealing temperatures were calculated based on the lowest melting temperature for the pair of primers. The purified genomic DNA was amplified by PCR with the following conditions: initial denaturing at 95°C for 5 minutes, 40 cycles of denaturing at 95°C, annealing at 60°C, and elongation at 72°C for 10 minutes. The PCR product was then analyzed by gel electrophoresis. The first pair of primers amplified were expected to produce a size of 205 bp figure (3-10).
Figure 3-10: Analysis of FFPE DNA by standard PCR. 2% agarose gel electrophoresis of DNA product. (120V/90 min.), showing the presence of DNA, visualized under UV after staining with EB.

The aim was to analyze gene amplification in FFPE from various stages of breast cancer. The choice of HER2/neu gene because previous reports indicate that it is amplified in >20% of breast cancer. DNA was isolated from FFPE normal tissue, and DC breast carcinomas (materials and methods). To investigate the lower limit of DNA that could be observed by PCR protocol as described in the materials and methods, the HER2/neu gene was amplified using serially diluted FFPE DNA started with 36ng and PCR amplification was examined by agarose gel electrophoresis. It is evident that the intensity of the bands decreases, as less DNA is present. Since the band from the seventh well is fairly strong, we hypothesize that less than 2.25ng of DNA can be detected by the performed method of DNA purification.
The HER2/neu gene was amplified by standard PCR using forward and reverse primers (materials and methods). The HER2/neu primer was used to test the lower limit of DNA that could be detected by DNA purification method which was mentioned. In the first lane represented a 100bp ladder was used as a marker to measure the size of the PCR products. The second to the fourth lanes display the normal product of PCR for HER2/neu gene amplification. Lane 5 displays a negative control, which ensures there is no primer contamination. Results from figure 3-11 shows normal HER2/neu amplification bands in lanes (1, 2, 3, 4). Furthermore another's bands, were detected by a second round of PCR was performed using nested primers to
reamplify the HER2/neu fragment when the bands were appeared in a size 126 bp.

![Image](3-12.png)

**Figure 3-12:** Detection of HER2/neu amplification by nested PCR, 2% agarose gel electrophoresis of DNA product (120V/90 min.), showing the presence of DNA product, visualized under UV after staining with EB.

This figure demonstrates the positive amplification of HER2/neu in one case vs. the normal amplification of HER2/neu in other cases. This data is the result of a nested PCR using the HER2/neu gene from exon3. PCR amplified products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining. The first lane is contains the 100bp ladder, and shows markers at 100 and 200 bp. The next four lanes (1,2,3,4) contains the PCR product of ductal breast cancer genomic DNA cases, which is normal amplification, while the fifth lane show the positive amplification case and Lane 6 shows the negative control and verifies no primer contamination. By performing two rounds of PCR we were
successful in producing single HER2/neu specific bands when HER2/neu gene was positive amplified (figure 3-12).

This study illustrates that Promega kit can be successfully used in providing FFPE sample DNA for gene analysis by PCR. The ability could show the amplification of HER2/neu gene with DNA isolated from FFPE tissue. This results indicated that (1/26) 3.8% of the cases studied were showed positive result for HER2/neu amplification, which corresponds to the literature regarding HER2/neu amplification/ overexpression. In addition, HER2/neu amplification could be detected not only in the invasive stage, but as early as the DCIS stage. This results of mutation in her2/neu agree with that reported by Bose et al., 2012 whom reviewed their data from eight genome-sequencing studies that included nearly1,500 patients. Twenty-five patients with breast cancer had HER2 mutations, nearly all mutations occurred in them who did not have HER2 gene amplification, the hallmark of HER2-positive breast cancer. Bose estimated that HER2 mutations occur in 1 percent to 2 percent of breast cancer cases, but there may be subgroups in which the frequency of this mutation is higher.

3.6 The sequencing

The present study showed there was no amplification detection in 26 FFPE samples except in one case that showed clear amplification band in lane 5 in figure (3-14) and as negative (score 0) in IHC. According to these result the sequencing analysis was suggested to detection mutation by ABI 310 Genetic analyzer used a gel extraction kit and ABI DNA sequencing kit. Samples were loaded into the ABI 310 Genetic Analyzer showed the Panel A, a blast analysis of DNA sequence in the National Center for Biotechnology Information’s (NCBI) Genbank was carried out and found
that the sequence corresponds to the HER2/neu gene fragment on exon3 (figure 3-13). In the panel B, that detected the fragment on exon3 for HER2/neu gene which recognized point mutation at the beginning after GTGC was T and G in the same place were a wild type sequence should be just G. in the sequence just after CCCTGGC the C and T were should normally be C. So there were point mutations were the normal bases of G changed to T in the First raw and C changed to T in the second raw figure (3-14).

Figure 3-13: Electropherogram obtained from ABI 310 Genetic Analyzer.

Figure 3-14: The wild type and mutant template HER2/neu gene, exon 3 sequences.

TGTGCTANGCACCCANCTCTTTGAGGACAACACTATGCCCCTGGCNGTGTAGA
The amplification detection in this case may be attributed to these point mutation but did not affected the expression because not all the mutations that occurs HER2/neu were active mutations and lead to overexpression but some of them silent mutations. The same result that mentioned by Bose et al., (2013). They were found that a few mutations appeared to be “silent events” occurring in in the HER2 gene and was an activating mutation then these patients may benefit from existing HER2-targeted drugs (Bose et al.,2013).

While the majority of HER2 somatic mutations in breast cancer patients are activating mutations that likely drive tumorigenesis. Several patients had mutations that are resistant to the reversible HER2 inhibitor lapatinib. However, not all HER2 mutations were activating mutations.

This result of point mutation in HER2/neu gene was agree with other researchers were mentioned that the mutations consider as clustering at amino acid (aa) residues 755–781, which is in the tyrosine kinase domain of HER2. These mutations either flank or are within the αC helix of the kinase domain. Insertion mutation has been previously described in HER2 at 780–781 non–small cell lung cancer (NSCLC), but the rest of the breast cancer HER2 somatic mutations were distinct (Moore, 2012). The HER2 in-frame deletion of aa 755–759 (del.755–759) matches the EGFR exon 19 deletions found in NSCLC, which are known activating mutations that confer sensitivity to the EGFR tyrosine kinase inhibitor, gefitinib (Shah et al., 2009; Ellis et al., 2012; Shah et al., 2012; Stephens et al., 2012).
The other researchers identified 4 out of 8 somatic variants in HER2 within lobular cancers, three of which were within the tyrosine kinase domain. (The Cancer Genome Atlas Network, 2012).

Compilation of data from seven sequencing studies documented 22 patients with somatic HER2 mutations. These mutations clustered in three regions. The first cluster was at amino acid (aa) 309-310 (exon 8), located in the extracellular domain. These aa residues form part of the HER2 dimerization interface. The second cluster was at aa 755-781, located in the kinase domain (exons 19-20). This was the most common location for HER2 mutations, found in 17 out of 22 patients having somatic mutations here. The third region was at aa 835-896, also in the kinase domain (exons 21-22). Using multiple experimental approaches (cell line experiments, in vitro kinase assays, protein structure modeling and xenograft experiments)(Bose et al., 2012).

So the conclusion of this results could summarized as the following: some mutations occurred in HER2/neu gene, in different types: insertion, deletion, inversion and frame shift. This mutation could detected with sequencing analyses and identify its expression by Real time PCR and IHC.
Conclusions:

According to the results of the present study, the following conclusions could be elucidated:

1. The mean age of the Iraqi breast cancer patients was less than the other countries, and the breast cancer appears earlier and more aggressive in Iraqi women.

2. The risk factors for Iraqi population may differ from other populations and are not subjected to the same criteria because of ethnic background.

3. Patients with benign breast tumors tend to be under the risk of breast cancer.

4. It was found that estrogen, progesterone and HER2 receptors could significantly increase the risk of breast cancer. Estrogen positive receptors were in 48% (24/50) of the cases and progesterone positive receptors in 46% (23/50) of the cases. Also 7 out of 50 malignant cases (14%) were score+3 that consider as strong positive for her-2/neu expression.

5. DNA was successfully extracted from FFPE sections and used for HER2/neu gene detection.

6. The age of FFPE blocks affect in the DNA extraction.

7. As a general rule, DNA extracted from FFPE tissue samples usually suffers a lot of breaks and therefore, it is highly fragmented DNA and need special conditions and skills to be extracted.

8. These findings biologically validate HER2 mutations as good targets for breast cancer treatment, but the appropriate choice of targeted drug is dependent on the precise mutation present.
This study is among the first to functionally characterize mutations identified by breast cancer genome sequencing. A prospective, multi-institutional clinical trial has been launched to screen for HER2 mutation positive patients and determine the clinical outcome of treatment with HER2 targeted drugs.
**Recommendations**

1. Studying other exons in the same gene for the detection of possible novel or already known mutations in Iraqi patients.

2. Using a new molecular technique for breast cancer diagnosis such as real time.

3. Further investigation of larger number of patients with breast cancer for coming study with a longer duration of follow-up and studying the survival rates will provide a better insight and validate our findings.

4. It was suggested that the highly disbelieving hormonal status was that with elevated estrogen and progesterone in Iraqi women, So that more hormonal study is required and its relationship with breast cancer by expanding the studies about the role of steroid hormones as well as prolactin hormone and Ki\textsubscript{67} in breast cancer risk and, hence, determining the risky hormonal status and targeting the therapy.

5. Given the difficulties that we faced in extraction of DNA from FFPE blocks that were prepared in laboratories of Health Ministry of Iraq, we suggested that the protocol of fixation and processing of FFPE samples should be changed to Compatible and fits with molecular study.
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الخلاصة

أجريت هذه الدراسة في مختبرات المناعة النسيجية في مدينة الصدر الطبية /النجف الجنوب وإختبرات الفحص الجيني في المركز الوطني للكشف المبكر عن سرطان / مدينة الطبل / بغداد على 50 عينة من نساء مصابات بسرطان الثدي وكانت عبارة عن نسخة مثبتة بالفورمالين ومطورة بالإبراقين على هيئة قوالب، توزعت أعدادهم نسبة إلى أنواعهم المرضية النسيجية إلى 3 سرطان قنوي متسرّب، 2 حالة سرطان قنوي متشرّب و5 حالات سرطان مختلط، حيث تم جمعها من السنوات مابين 2010 وغاية نيسان 2013، بالإضافة إلى مجموعة أخرى شملت 20 من عينات الورم الحميد للمقارنة بينما كانت عينات السيطرة أنسجة الثدي الطبيعية.

استخدمت فحوصات المناعة النسيجية لتقييم تعبيير المستقبلات لكل من HER2 و البروجيسترون لإيجاد العلاقة بين نتائج المناعة النسيجية ومؤشرات التقدم لسرطان الثدي مع الأخذ في الاعتبار عمر المريض، حجم الورم، النوع النسيجي وكذلك درجة تقدم المرض. بينما النتائج أن نسبة مستلمات الاستروجين الموجودة كانت 44% (5/0) من الحالات ومستلمات البروجيسترون الموجودة 46% (3/2) وهذه البيانات عكست عن وجود تعبيير هرموني موجب في أغلفة الحالات البالغة تكون مستجيبًا للعلاج الهرموني وكذلك بين النتائج في المريضات العصابات السرطانية التي عرف وجود تكرار للمرض يحدث للأعمار مابين 41-01 م(46%) وكان من بين المريضات أكثر من 24% لديهم تاريخ عائلي بالإصابة أما من الدرجة الأولى أو من الدرجة الثانية.

لقد بين التشخيص النسيجي المرضي وجود السرطان الأنبوبي المتسرّب بنسبة عالية في المصابات العصابات تمثلت بـ88% بينما كان السرطان الفصي المتشرّب يمثل 4% وكان المختلط 10% . وأشارت النتائج إلى أن 12% من المريضات كانوا في المرحلة الأولى من المرض و20% في المرحلة الثانية بينما 68% كانوا في المرحلة الثالثة من المرض، وبذلك تكون أغلب الحالات في المرحلة الثانية والثالثة.

أشار التقدير السريري المرضي لمستلمات الاستروجين والبروجيسترون أنها لا توجد فروقات معنوية متربطة بين مستويات التعبيير في المستلمات الهرمونية من جهة ومن العمر التاريخ العائلي، حجم الورم ومرحله المرض من جهة أخرى (p>0.05) وبالمقابل التعبيير
لـ HER2 لا يعرض أي أهمية إحصائية تختلف مع العمر ودرجة الورم أو النوع ماعدا هناك علاقة مهمة بين تعبير الـ HER وتاريخ العائلة (P < 0.01).

كانت مستلمات الاستروجين الموجبة 48% (24/50) بينما كانت نتائج مستلمات البروجسترون الموجبة 26% (13/50) حيث استنتج من ذلك أن التعبير الهرموني للمستلمات تمثل الأغلبية بسرطان الثدي في المريضات العراقيات والتي لها استجابة فعالة للعلاج الهرموني وحوالي 26% من حالات الاستروجين الموجبة كانت نسبة اصطباعها قوية جدا بينما 42% من البروجسترون كانت قوية الاصطباغ.

أظهرت نتائج التحليل المناعي النسيجي لكل أنواع سرطان الثدي والمعتمدة على التصنيف الجزيئي النوعي إن النوع Luminal A كان الأكثر تواجدا بنسبة 54% إما Basal like بنسبة 10% بينما HER2/neu بنسبة 4% وLuminal B بنسبة 22%.

لاحظ أن التعبير المفرط لـ HER2/neu بـ 14% (7/50) من حالات الإصابة بسرطان الثدي في المريضات العراقية وهذه المجموعة كانت بدرجة عالية من الشراسة وبمرحلة نسيجية متقدمة من الإصابة بالسرطان.

استخدمت أربع طرق مختلفة لاستخلاص الحمض النووي (الطريقة الديوية ، عدة استخلاص Promega ، عدة استخلاص Qiagen ، عدة استخلاص Gineaid) حيث استخدمت لتقدير نتاج ونوعية الحمض النووي المستخلص من العينات النسيجية المثبتة بالفورمالين والمطمورة بالبرافين. أثبتت النتائج نجاح استخدام استخلاص الحمض النووي من الأنسجة المثبتة بالفورمالين والمطمورة بالبرافين وبصورة سريعة، موثقة وفعالة في إزالة البرافين وخطوات عزل الحمض النووي مع نتاج عالي ومعدل 247-160 ميكروغرام والذين تم الحصول عليه بواسطة عدة استخلاص Promega مع وجود أسباب تعرقل جودة الحمض النووي المستخلص. بينما بقية طرق الاستخلاص أظهرت حزم غير مثالية للحمض النووي المستخلص. تم استخدام تفاعل البلمرة المتسلسل المتكرر القبئي بمواد مستقبلة وعكسية كانت مجهزة مسبقا واستخدام جل اكاروس 2% المضاد أيشبه صبغي بروميد الإثيديد لتحليل تضاعف في عينات الأنسجة المثبتة بالفورمالين والمطمورة بالبرافين لمرضى سرطان الثدي. تم الحصول على حزم تضاعف جين الـ HER2/neu بصورة واضحة في أحد الحالات والتي كان قياسها 126 زوج قاعدي.
أخذت العينة المذكورة وأُخضعت لنظام معرفة النتائج النوويتيدي باستخدام عدة مسلسل للحمض النووي ABI. العينات حُملت إلى المحل الجيني 310 وتحليلت النتائج بنظام المحال AB 310. المحال الجيني محلل BY BLAST المحال النووي بالمركز العالمي لمعلومات التقنيات الإحيائية (NCBI) ويتطلب إن التسلسل يتطابق مع جين HER2/neu في قطعة الأكسون 3.
دراسة مناعية نسيجية وجزئية للنساء العراقيات المصابات بسرطان الثدي

رسالة مقدمة إلى كلية العلوم / جامعة بغداد
وهي جزء من متطلبات نيل درجة الماجستير في التقنيات الإحيائية
من قبل

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