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# Amplification of HER-2 gene in breast cancer: immunohistochemical and FISH assessment

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**Abstract**

The invasive mammary cancer is the most frequent malignant tumor of women. Different inherited or acquired molecular genetic alterations have been identified in human breast cancers. A fraction of these cancers, as part of their development, undergoes gene amplification. Among the potential prognostic factors are included the biomarkers which measure or are associated with biological processes involved in tumor progression. Evaluation of HER-2 status is important in the management of patients with breast carcinoma, especially for the identification of those who are eligible for immunotherapy. The aim of our study was to evaluate HER-2 amplification status of human breast cancers by FISH and immunohistochemistry. From the total of 50 tumors included in the study, 17 (34%) presented different degrees of positivity; 33 (66%) did not express the oncoprotein HER-2. HER-2 gene and chromosome 17 status were tested in HER-2 2+ cases using FISH technique. FISH analysis may be useful to better evaluate HER-2 status in breast cancer in uncertain cases, where the immunohistochemistry score is 2+. HER-2 testing results have an important role in the clinical management of breast cancer patients. The identification of HER-2 positive tumors is certainly crucial in order to identify patient candidates for anti-HER-2 therapies.

***Keywords*:** breast cancer, HER-2 protein, HER-2 gene amplification, immunohistochemistry, FISH technique.

## Introduction

The invasive breast cancer represents the most frequent malignant tumor in women. The highest risk areas are North America, Europe and Australia, where 6% of the female population develop invasive breast cancer before the age of 75 years. The risk of developing breast cancer is low in the underdeveloped regions of Southern Africa, the South of the Sahara region and Eastern Asia, including Japan. In these regions, the probability ratio for developing breast cancer under the age of 75 years is 1:3 in comparison with the developed regions mentioned above. The prognosis is very good if diagnosed in the early stages.

Molecular classification of breast cancer based on gene expression profiles of human tumors has been proposed. Luminal, basal-like, normal-like, and erbB2+ subgroups were identified and were shown to have different prognoses [1]. Different inherited or acquired molecular genetic alterations have been identified in human breast cancers. A fraction of breast cancers, as part of their development, undergoes gene amplification. Amplification and overexpression of HER-2 oncogene seems to remain stable over the course of disease and concordance between primary tumor and metastases was noticed [2].

The HER-2 proto-oncogene, also known as neu or c-erbB-2 was initially identified as a transforming gene activated in chemically induced rat neuroblastoma. It is a member of the human epidermal growth factor receptor family, and is located on chromosome 17q21. Its 185-kDa gene product is a transmembrane growth factor receptor with tyrosine kinase activity, and it is known to be involved in the signal transduction of cell growth, cell differentiation, adhesion and motility [3].

The HER (human EGFR-related) family of receptor tyrosine kinases is formed by HER1/EGFR/c-erbB1, HER-2/c-erbB2, HER3/c-erbB3 and HER4/c-erbB4, which share a high degree of structural and functional homology. HER-2/neu, also called ErbB-2 and ERBB2, is a protein that is considered to give higher aggressiveness in breast cancers. HER-2/neu has also been designnated as CD340 (cluster of differentiation 340) and p185. The three additional members of the HER (human EGFR related)–RTK family HER1 (epidermal growth factor receptor (EGFR), c-erbB1), HER3 (c-erbB3) and HER4 (c-erbB4) are of particular interest because of their ability to interact directly with HER-2 [4–6].

Amplification of the HER-2 oncogene or overexpression of HER-2/neu protein has been identified in 10–34% of breast carcinomas [7]. It has been shown that overexpression of this gene is correlated with tumor size, existence of lymph node involvement, absence of hormone receptor expression, aneuploidy [8]. Gene overexpression is currently considered not only as an important possible marker of poor prognosis, but also as a useful determinant of susceptibility to chemotherapy [9].

Recently, a recombinant humanized monoclonal antibody against HER-2 protein, trastuzumab, has been administrated to patients whose breast carcinomas demonstrate HER-2 amplification or overexpression, with clinical effectiveness. Trastuzumab is able to bind to the extracellular domain of the oncoprotein and to block cell proliferation of malignant cells that overexpress the gene. It is also able to induce antibody-dependent cellular toxicity against tumor cells. Thus, it has become very important to accurately evaluate HER-2 status in invasive breast cancers, to establish if the gene is overexpressed or not, especially if anti-HER-2 monoclonal antibody therapy is considered.

Several methods are used in order to detect HER-2 status, but the most widely applied techniques are fluorescence in situ hybridization (FISH) for detecting gene amplification and immunohistochemical studies of paraffin-embedded tissue for detecting HER-2 protein expression [10, 11].

The present study aimed to evaluate HER-2 amplification/overexpression status in breast cancer patients by FISH technique and immunohistochemistry.

## Material and Methods

The study was performed on 50 selected cases, with primary mammary carcinoma, treated at the County Hospital Timişoara. Determining the HER-2 status of breast carcinomas is a prerequisite for the use of the monoclonal antibody therapy. We used immunohistochemistry for the HER-2 oncoprotein and fluorescent *in situ* hybridization (FISH) as a follow up test for ambiguous results.

For each case, 5 μm sections were stained with Hematoxylin–Eosin staining for the establishment of the histopathological type and differentiation stage. Additional sections were prepared for immunohistochemistry and FISH technique evaluation.

In order to determine the HER-2 oncoprotein, we used the polyclonal rabbit anti-human c-erbB2 oncoprotein (code no. A 0485) (Dako). We applied the 5 μm thick paraffin sections on silane covered slides. We prepared three sections of each: for the analysis, for negative control and for the clearly positive tissues.

The main steps for immunohistochemical staining were: deparaffinization in xylene, antibody dilution: 1:200 (6 μL c-erbB2 and 1.5 mL buffer solution), citrate solution treatment for 20 minutes at 700 W, followed by 20 minutes cooling and washing with water and then with buffer solution. Peroxidase was applied for 5 minutes, followed again by washing in buffer solution 2×5 minutes. The primary antibody was applied for 30 minutes, while the link and Streptavidin were applied for 10 minutes each. Each of these three steps was followed by washing in buffer solution 2×5 minutes. The bound antibody was visualized using a DAB-chromogen substrate. The sections were then counterstained with Hematoxylin, and covered with a coverslip and Entelan. Negative control staining was conducted by omission of the primary antibody. Paraffin slides of invasive breast carcinoma were used as a positive control.

Using this staining method, we obtained a strong brown staining located in the cell membrane of malignant cells. DAKO recommends that scoring always be performed within the context of the pathologist's past experience and best judgment in interpreting immunohistochemical stains. Only patients with invasive breast carcinoma should be scored. In cases with carcinoma in situ and invasive carcinoma in the same specimen, only the invasive component should be scored. We graded the stain reaction for c-erbB2 on a scale from 0 to 3+ (Table 1).

**Table 1** – ***HER-2 score (Newcastle system) for immunohistochemical staining***

| Score to report | HER-2 protein overexpression. Assessment | Staining pattern |
|---|---|---|
| 0 | *Negative* | No staining is observed, or membrane staining is observed in <10% of the tumor cells. |
| 1+ | *Negative* | A faint/barely-perceptible membrane staining is detected in >10% of tumor cells. The cells exhibit incomplete membrane staining. |
| 2+ | *Weakly positive* | A weak to moderate complete membrane staining is observed in >10% of tumor cells. |
| 3+ | *Strongly positive* | A strong complete membrane staining is observed in >10% of tumor cells. |

HER-2 protein overexpression was defined as negative (score 0 and 1+) or positive (score 2+ and 3+). Score 2+ was interpreted as equivocal and FISH analysis was required for correct evaluation of the samples.

For FISH analysis, the commercially available dual-color FISH probe PathVysion® from Abbott–Vysis was used. This included fluorescence-labeled DNA-probes for the HER-2 gene locus (Spectrum Orange) and centromere 17 (CEP17, Spectrum Green). FISH technique was performed according to manufacturer's recommended protocol, with small adjustments. For the most representative smears, a hybridisation target area of 22×22 mm was selected. This area was first marked with a waterproof pen. The smears were dehydrated by immersion in 70% ethanol solution for 5 minutes, kept at room temperature, step that were followed by immersion in 80% and 100% ethanol. Then, the slides were denatured for 10 minutes in 70% formamide/ 2×SSC at $73^0$C. Finally, the smears were dehydrated in a series of 70%, 85% and 100% ethanol solutions (2 minutes per each) and dried in an oven at $37^0$C again for 2 minutes. After denaturation at $73^0$C for 5 minutes, the probe hybridization mix was applied. The smears were covered with coverslips, sealed and incubated overnight in a humid chamber at $37^0$C. Next morning, the slides were washed in 0.4×SSC/0.3% Nonidet P40 at $73^0$C for 2 minutes, rinsed twice in 2×SSC/ 0.1% Nonidet P40 for 2 minutes and then air-dried. 4',6-Diamidino-2-phenylindole (DAPI II) was added for counterstaining.

We analyzed the slides with a Zeiss Axio Imager M1 epifluorescence microscope (Zeiss, Jena, Germany) equipped with filter sets for DAPI, Spectrum Orange and Spectrum Green at a magnification of ×1000. Images were captured using MetaSystems digital camera

and analyzed using Isis version 5.2, MetaSystems software for quantitative analysis of samples generated by FISH technique (Altlussheim, Germany). The number of signals and the HER-2/CEP17 ratio were estimated on the tissue specimens. Twenty nuclei were assessed in each area, the chromosome 17 copy number was counted for each cell, and the ratio of Her-2 signals to chromosome 17 signals was calculated. The normal mean Her-2 to chromosome 17 ratio was defined as less than two, and a ratio greater than two was interpreted as gene amplification.

## Results

We investigated HER-2 gene overexpression in 50 female patients with breast cancer. The average age interval was 30–85 years, with a mean of 57.3 years.

Based on interpretation of histopathological examinations, the following histological types of breast cancer have been confirmed:

- 42 (84%) cases of invasive ductal carcinoma;
- three (6%) of invasive lobular carcinoma;
- three (6%) of papillary carcinoma;
- two (4%) of medullary carcinoma;

From the total of 50 tumors, 17 (34%) presented different degrees of positivity with immunohistochemistry; 33 (66%) did not express the oncoprotein c-erbB2. Cases with 0 and 1+ immunostaining scores were considered as HER-2 not overexpressed. Cases with 3+ staining (Figure 1) were classified as showing HER-2 overexpression. The tumors that had an apparently normal ratio had negative immunostaining and those with a strongly positive staining were not further analyzed. Cases with 2+ immunostaining score (Figures 2–5) were interpreted as uncertain and HER-2 status was determined by fluorescence in situ hybridization to reveal genic amplification, if this was present. This cut-off point was predicted on the results of previous breast cancer studies [12]. FISH technique revealed that out of 15 equivocal cases, three HER-2 2+ cases showed HER-2 gene amplification (Figure 6, A–C).

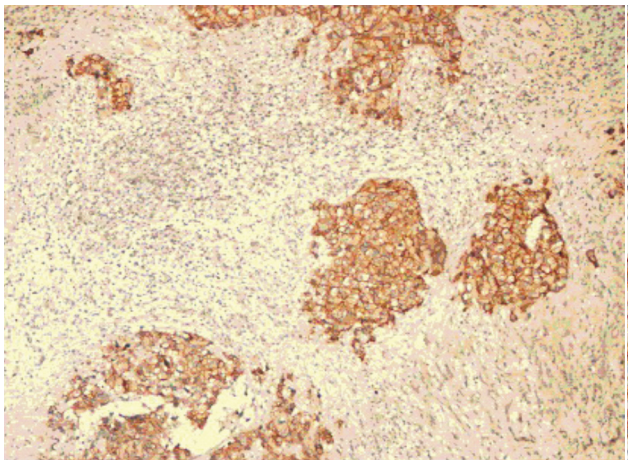

**Figure 1** – ***Intense expression of c-erbB2 oncoprotein in an infiltrative ductal carcinoma (LSAB, ×100).***

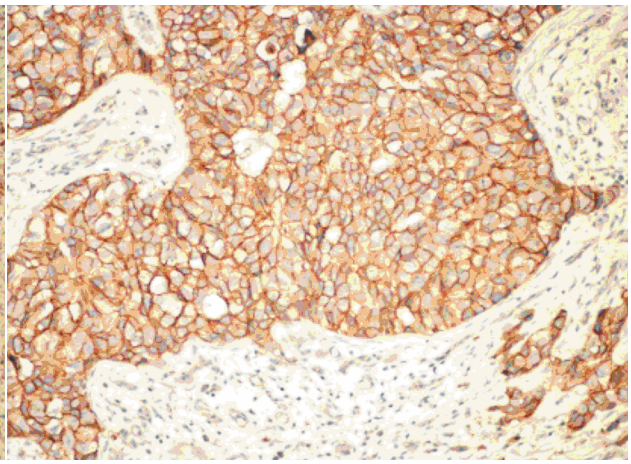

**Figure 2** – ***Moderate intensity of c-erbB2 in an infiltrative ductal carcinoma (LSAB, ×200).***

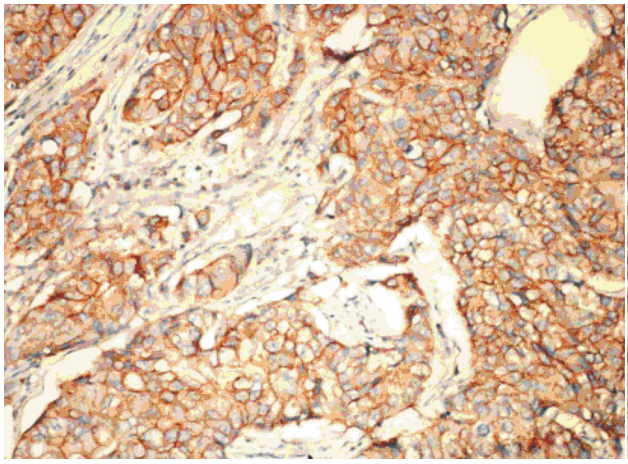

**Figure 3** – ***A weak to moderate complete membrane staining of HER-2 in ductal infiltrative carcinoma (LSAB, ×200).***

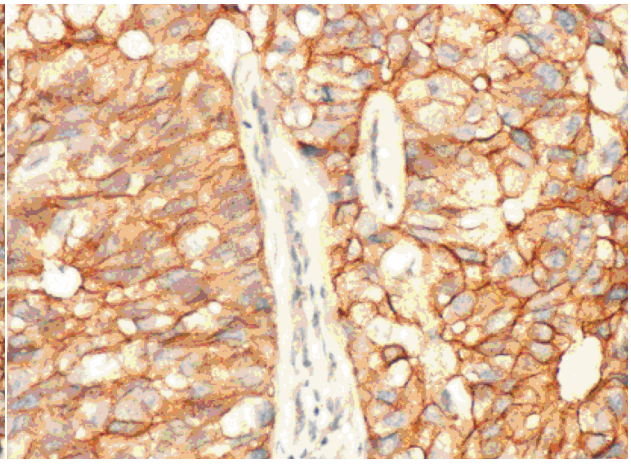

**Figure 4** – ***A weak and complete membrane staining of HER-2 in ductal infiltrative carcinoma (LSAB, ×400).***

### HER-2/CEP17 ratio

HER-2/CEP17 ratio was determined using the rate between HER-2/neu signals and CEP17 signals in 20 nuclei. The total number of HER-2/neu signals was divided by the total number of CEP17 signals. Obtained ratio of ≥2, according to indications given by the Abbott–Vysis Company, revealed gene amplification. According to the guidelines described in this study, we obtained the following results: 12 (80%) of invasive

breast carcinomas were not amplified, and three (20%) cases had HER-2/neu amplification. HER-2/CEP17 rate in cases that were not amplified ranged within 0.7 and 1.9, and in amplified cases, the rate was between 2.3 and 9.3 (one had 2.3, another case had 5.2 and the third one had 9.3).

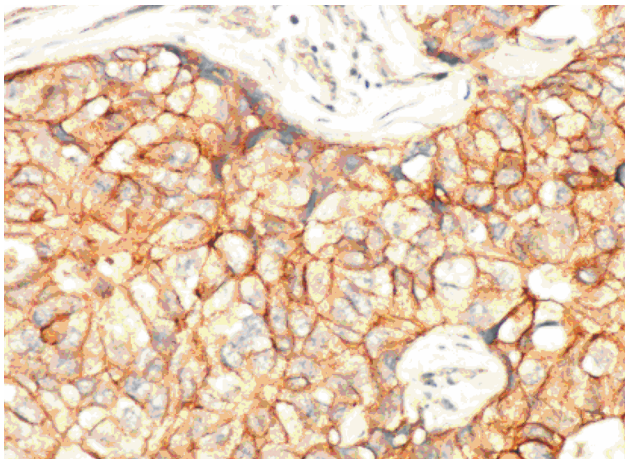

**Figure 5 –** ***A weak and complete membrane staining of HER-2 in ductal infiltrative carcinoma (LSAB, ×400).***

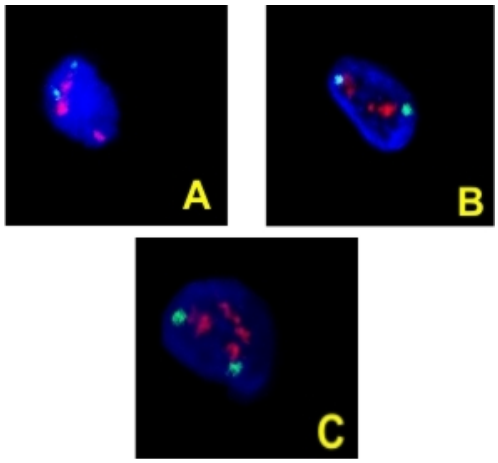


**Figure 6 (A–C) –** ***PathVysion FISH in three cases showing HER-2 gene amplification as red signals. The green signal represents chromosome 17.***

### The mean number of HER-2/neu copies

Other protocols may be used to assess gene amplification, such as InformTM HER-2/neu Gene Detection System, Ventana Medical Systems/Oncor Inc., Tucson, Gaithersburg, USA. This determines only the number of signals/tumor nucleus. In our study, the average value of HER-2/neu copies/tumor nucleus for each breast carcinoma was calculated by dividing the total number of HER-2/neu signals by 20. The result was interpreted in the following way:

- values >4 to ≤10 signals HER-2/neu/cell – "low level" amplification;
- values >10 signals HER-2/neu/cell – "high level" amplification.

Using this rating system, we could appreciate amplification in four cases (23.5%), and of these:

- two cases (4%) had "low level" amplification;
- two cases (4%) had "high level" amplification.

The mean number of HER-2/neu gene copies in cases where:

- amplification did not take place, ranged between 1.1 and 3.9;
- a small rate of amplification was present, ranged between 4.6 and 6.2;
- high amplification was present, was 10.4 and 18.6 respectively.

### Comparison between HER-2/CEP17 rate and the average number of HER-2/neu copies/nucleus

In the 17 studied specimens, amplification could be assessed, in terms of:

- HER-2/CEP17 rate definition, in three cases (representing 20%);
- definition of the average number of HER-2/neu gene copies, in four cases (23.5%).

### Polyploidy and HER-2/neu status

Knowing the level of polyploidy is essential for HER-2/neu status determination. FISH and IHC results are easier to interpret if the number of chromosomes 17/tumor cell is known. Thus, the differential diagnosis of an actual gene amplification and HER-2/neu signal multiplication, which is due to chromosome 17 polyploidy, is more easily established. The group of breast carcinomas with polyploidy have been considered all cases with at least 3.0 signals from chromosome 17. In this study, we identified one case of polyploidy Perhaps in such instances it is appropriate to consider only the absolute number of HER-2/neu signals per tumor nucleus, to determine HER-2/neu status because if only HER-2/CEP17 rate is considered, a minor gene amplification can be ignored.

In our study, we noticed more frequently than we expected overexpression of the protein without gene amplification. Thus, 80% of breast carcinoma without amplification (HER2/CEP17 ratio <2), and 73.33% respectively, of cases without amplification (average number of gene copies per tumor cell ≤4) may have had an overexpression of HER-2/neu protein receptor.

Classification of carcinomas according to the histological type and immunohistochemical HER-2 status is presented in Table 2. There is a significantly higher frequency of HER-2 negative status for each histological type that was analyzed.

**Table 2 –** ***Classification of carcinomas according to the histological type and immunohistochemical HER-2 status***

| Histological type | HER-2- | HER-2+ |
|---|---|---|
| *Invasive ductal carcinoma* | 25 | 14 |
| *Invasive lobular carcinoma* | 4 | 2 |
| *Medullary carcinoma* | 1 | 1 |
| *Papillary carcinoma* | 3 | 0 |

According to the parameters available for evaluation, we performed the molecular classification of breast cancers. This was based on the evaluation of the immunohistochemical status of estrogen receptors, progesterone receptors, as well as cytokeratin CK5 and

EGFR, data taken from the patient's files. Based on the molecular classification, out of 50 cases, 24 were considered to be luminal-A, nine cases luminal-B, 15 cases basal-like and two cases HER-2 positive (3+).

Regarding the evaluation of c-erbB2 oncoprotein in primary mammary cancers, the data from literature differ significantly among various research laboratories. These differences appear due to the application of different research methods, the number of studied cases and the manners of interpreting the results).

## Discussion

The chromosome 17 harbors several important oncogenes and tumor suppressor genes, such as HER-2, TOP2A, DARPP32, p53, and BRCA1 [13]. The HER-2 gene codes for a protein that stimulates normal cell growth, but it also seems to play a significant role in the biology of breast cancer. Overexpression, or an abundance, of HER-2 protein is found in more than 25% of malignant breast tumors and is associated with more aggressive cancer growth and shortened patient survival. It was estimated that in about 90–95% of these cases upregulation is a direct result of gene amplification, but recent studies regarding abnormalities in gene copy number or structure due to chromosomal aneusomy or DNA-aneuploidy revealed that these may lead to altered gene dosage or can directly affect the transcriptional programs for some tumor-associated genes, thus, an increase in DNA copy number alone does not always lead to transcriptional up-regulation [14].

Overexpression of HER-2 is also known to be associated with other carcinoma, including gastric, ovarian, pancreatic or hepatocellular cancers with an adverse outcome [13, 15]. Clinical studies in thousands of patients with breast cancer over the last decade have demonstrated that amplification/overexpression of HER-2 gene is associated with a poor prognosis. In addition to its prognostic use, the HER-2 protein has been targeted in novel immunotherapies initially involving trastuzumab. The first positive clinical results, after introducing the immunotherapy, were reported in patients with metastatic breast cancer, but later primary forms were also studied and the effectiveness of the treatment was demonstrated [16].

Based on this knowledge, researches are now testing different therapies that are targeted to tumors with HER-2 overexpression. HER-2 has attracted considerable attention in breast cancers, where it was noticed that instead of having two gene copies of the gene in one cell, there were multiple copies. Consequently, there is on overexpression of the HER-2 protein on the cell surface, resulting in aberrant cell growth regulation [17]. The effectiveness of these therapies is dependent on accurately evaluating the HER-2 status in the tumors. A variety of methods is available for evaluation of HER-2 status, but for clinical routine and research, the most widely used are immunohistochemistry and FISH [18].

HER-2 amplification/HER-2 overexpression are used as prognostic markers, but also as predictive markers for breast cancers. Thus, in positive cases tumors are faster growing, more aggressive and less responsive to chemotherapy and hormone therapy. HER-2 is used as a prognostic marker in order to predict the course and outcome of the disease. As a predictive marker, HER-2 is used to forecast the patient's response to different therapies, such as chemotherapy or endocrine therapy but also to select patients for anti-HER-2 monoclonal antibody immunotherapy.

Not only cases with metastases, but also a significant proportion of intraductal breast carcinomas demonstrated HER-2 amplification/overexpression, suggesting that the oncogene is activated early in the progression of malignancy [19].

The management of breast cancer has been dramatically changed with the advent of widespread screening programs and the systemic use of adjuvant hormonal therapy and chemotherapy. Recent data have shown that these changes are having a major impact on outcome, and despite increasing incidence, breast cancer mortality is decreasing in most of the Western countries.

There are four major molecular classes of breast cancer: luminal-like, basal-like, normal-like and HER-2 positive [20]. The identification of HER-2 subtype of breast cancer was reassuring to clinicians because it confirmed the clinical impression that tumors with HER-2 overexpression were systematically different from other breast cancers. Only breast cancers with HER-2 amplification respond to the targeted therapy with trastuzumab, which also decreases the risk of recurrence [21]. Other new treatments that specifically target HER-2 are proving to be very effective, such as Lapatinib or certain combinations of anthracycline drugs, which were recently reported to be effective in breast cancers.

It is estimated that about 75% of invasive breast cancers are hormone receptor (estrogen and progesterone) positive [22]. Estrogens play critical roles in development and progression of these tumors. The effects of estrogens are mediated primarily through estrogen receptors (ER) in breast tissue and polymorphisms in the ER genes may alter the functions of these receptors. Progesterone receptors (PR) can also be involved in turning on breast cancer cell growth.

Molecular classification of breast cancer appears to be robust and reproducible and relatively trivial to apply in the clinic [23]. For example, with just three markers (ER, PR, HER-2) it is possible to define four major breast cancer subclasses with major therapeutic implications: ER+/PR+/HER-2-; ER+/HER-2+; ER-/ HER-2+; ER-/PR-/HER-2, subtypes that have distinct differences in prognosis and response to therapy (Table 3) [24].

**Table 3 –** ***Correlation between the immunophenotype of the breast cancer and the elective therapy***

| Immunophenotype | Elective therapy |
|---|---|
| *ER-PR-HER-2-* | Chemotherapy |
| *ER-PR-HER-2+* | Trastuzumab |
| *ER+PR+HER-2-* | Hormonal therapy |
| *ER+PR-HER-2-* | Hormonal therapy |
| *ER-PR-HER-2-* | Hormonal therapy and chemotherapy |
| *ER-PR+HER-2+* | Trastuzumab and hormonal therapy |
| *ER+PR+HER-2+* | Trastuzumab and hormonal therapy +/- chemotherapy |

Breast tissue can now be tested for HER-2 status, routine testing being recommended for most women with breast cancer because the results may affect treatment options. It is recommended that whenever breast cancer recurs or spreads, the cancer cells should be tested for HER-2 [25].

## Conclusions

Her-2 is a well-established prognostic factor for breast cancer cases. The identification of HER-2 positive tumors is certainly crucial in order to identify patients who are candidates for anti-HER-2 therapies. Furthermore, it will be important to evaluate the status of the HER-2, epidermal growth factor receptor (EGFR), and other genes coding for estrogen, progesterone or androgen receptors, prior to treatment decisions. Optimizing treatment strategies according to the molecular classification of breast cancers should be a major priority for future research.

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