EFFECTS OF 17β-ESTRADIOL AND TAMOXIFEN ON THE INDUCTION OF CHROMOSOMAL ABNORMALITIES AND ON THE EXPRESSION OF *HER2* GENE IN BREAST CANCER CELL LINES

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Turin
2014

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Bogotá
2014

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LIST OF ABBREVIATIONS

aCGH	Arrays Comparative Genomic Hybridization
DNAc	Complementary DNA
ARO	Aromatase
AMPc	Cyclic AMP
IDC	Infiltrating ductal carcinoma
DCIS	Ductal carcinoma in situ
CEA	Carcinoembryonic antigen
CGH	Comparative genomic hybridization
LCIS	Lobular carcinoma in situ
E1	Estrona
E2	17β-estradiol
E3	Estriol
EGFR	Epidermal growth factor receptor
ERα	Estrogen receptor alpha
ERE	Estrogen response elements
FISH	Fluorescence *in situ* hybridization
GPCRs	G protein coupled receptors
GRB7	Growth factor receptor bound protein 7
HER2	Epidermal growth factor receptor
HSP's	Heat shock proteins
AI	Aromatase inhibitors
IARC	International agency for research on cancer
IGF-1R	Growth factor receptor 1 insulin-like
IHC	Immunohistochemistry
ISCN	International system of cytogenetic nomenclature
MAPK	Mitogen activated protein kinase
M-FISH	Multi-color Fluorescence *in situ* hybridization
miRNA	MicroRNAs
NO	Nitric oxide
PI3K	Phosphatidylinositol 3 kinase
PKC	Protein kinase C
PLC	Phospholipase C
PR	Progesterone receptor
ROS	Reactive oxygen species
SERM	Selective estrogen receptor
TAM	Tamoxifen

TGF	Transforming growth factor
TKR	Tyrosine kinase receptors
TNBC	Triple Negative Breast Cancer
uPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
2-OHE1/E2	2-hydroxyestrone / 2-hydroxyestradiol
4-OHE1/E2	4-hydroxyestrone / 4-hydroxyestradiol

SUMMARY

This study covers an area of great importance in the research of breast cancer, related to the study of the effects of both estrogens (E2) and anti-estrogens (Tamoxifen) on chromosomes and of modulation of gene expression. Considering that breast cancer is a very heterogeneous disease and that patients respond differently to treatment, the identification of chromosomal abnormalities as well as genes responsive to 17β-estradiol (E2) and Tamoxifen (TAM) could provide the necessary framework to understand the complex effects of this hormone in target cells and could explain, at least in part, the development of cellular resistance to TAM treatment and the subsequent best therapeutic option. In this order of ideas, we determined the effects of E2 and TAM on the chromosomes and on the modulation of gene expression in four breast cancer cell lines, which represent three of the five subtypes of breast cancer known at present.

The results are presented in six chapters - each one has a group of the results achieved around the cytogenetic characteristics and gene expression profiles of four cell lines and the effects of E2 and TAM incubation on those. The first chapter describes the main features of breast cancer, furthering the use and effects of E2 and TAM treatment. The second chapter presents the cytogenetic and molecular characteristics of four breast cancer cell lines. These results allowed us to supply a comprehensive and specific characterization of complex chromosomal aberrations for MCF7, T47D, BT474 and SKBR3 cell lines, thus providing important information for experimental studies. The third chapter shows a molecular-cytogenetic analysis about chromosome 17 (Chr17), which may be associated with breast cancer development and progression and can potentially be exploited as a therapeutic target. In order to elucidate the structural aberrations affecting Chr17, nine breast cancer cell lines and one triple negative breast cancer (TNBC) case were investigated. The overall high frequency and complexity of Chr17 alterations identified in our study indicate the importance of this chromosome in the pathogenesis of breast cancer. The fourth chapter describes the cytogenetic changes in response to low doses of E2 and TAM in MCF7 and T47D (ER+/HER2-), BT474 (ER+/HER2+) and SKBR3 (ER-/HER2+) human breast cancer cell lines. The results show that all cell lines treated with E2 and TAM at low doses had more karyotype alterations than untreated cells. These aberrations involved numerical changes (endoreduplications, aneuploidy, polyploidy) as well as additional and more complex chromosomal rearrangements. This suggests a karyotypic evolution of certain chromosomal aberrations. Thus, these results could provide insight into the mutagenesis induced by E2 or TAM associated with breast cancer clinical treatments. The fifth chapter shows the results achieved with the effect of the E2 and TAM on HER2 mRNA and protein levels in ER+ and ER- human breast cancer cell lines. They showed that neither E2 or TAM significantly regulate the HER2 mRNA nor protein levels in breast cancer cell lines. Finally, the sixth chapter

includes the results of the effect of E2 and TAM on the modulation of gene expression of 84 key genes commonly involved in the deregulation of signal transduction and other normal biological processes during breast carcinogenesis. The expression pattern of E2 and TAM-regulated genes identified in this study in ER-positive and ER-negative cells could reflect distinctive properties of these cells, which could be exploited to identify the cell response to E2 and know if the genes involved might be used as biomarkers of TAM sensitivity and/or resistance. Indeed, we have already shown that the expression pattern of some of the genes identified in this study clearly discriminates both between ER-positive cells, and between ER-positive and ER-negative cells.

1. GENERAL INTRODUCTION

1.1 JUSTIFICATION

Breast cancer is the type of cancer most frequently present in women from developed and developing countries. This type represents 23% of all female cancers, being highly related to death in women, even though mortality rates are lower than the incidence rates reported for this disease (1). However, this disease has shown a high morbidity and is commonly related to a wide variety of risk factors such as genetic predisposition, exposure to estrogens and the amplification of the *HER2* gene.

Prolonged exposure to estrogen represents a significant risk factor in the development of breast cancer; however, the mechanisms used by this hormone to induce cancer are partially understood. The principal mechanism by which estrogens could induce cancer is through its binding to the specific nuclear receptor known as the estrogen receptor (ER), which generates a potent stimulus for breast gland cell proliferation and increases the risk of DNA mutation during replication (2-4). However, some studies in mice without ovaries that were exposed to dosages of estrogen presented a high incidence of tumor development, indicating that estrogen can cause breast cancer through independent mechanisms of estrogen receptors (ER) (5).

The main estrogen hormone acting in mammary epithelial cells is 17β-estradiol (E2). This hormone is known for its indirect interaction with HER2 and also for increasing cell proliferation and resistance to tamoxifen (TAM) (6). Also, it reduces the *HER2* gene expression by regulating both mRNA and protein levels. In addition, E2 causes genetic lesions such as alterations in the chromosome number (aneuploidies), gene amplification (*c-myc*) and chromosomal structure aberrations in animal models (Syriam hamster, mouse genital tract) (2, 7-10). However, the type and frequency of such chromosomal abnormalities as well as its effect in the amplification and expression of the *HER2* gene are not completely known.

Previous studies in breast tumors have determined that chromosomes 1 and 17 are frequently affected by aneuploidies and structural abnormalities, suggesting that these chromosomes could be useful not only for identifying patients with high risk of developing invasive breast cancer, but as biomarkers of tumor progression. These studies found chromosomal abnormalities in both early and advanced stages of the disease (11). The *HER2* gene is located on chromosome 17 (17q11.2-q12), and is amplified in 10 to 20% of breast cancer cases. This amplification has been correlated to recurrence, metastatic potential, chemo-resistance and poor prognosis (12). The amplification of the *HER2* gene can

display complex patterns of chromosomal aberrations on chromosome 17 (Chr17), involving the long arm in a high proportion. In order to avoid misinterpreting the number of copies of Chr17, some authors have suggested, besides the use of probes for the *HER2* gene and centromere of Chr17, the use of additional probes for other *loci* of this chromosome, including the *SMS* (17p11.2) and *RARA* (17q21.2) genes, which serve as additional control in cases that present multiple copies of the centromere (CEP17). The use of these probes has shown that the polysomy of Chr17 is a rare event in breast cancer and that a copy number greater than 3 in CEP17, observed by FISH, is often associated with the gain or amplification of the centromeric region, showing that Chr17 usually presents complex rearrangements. Nonetheless, the clinical importance of Chr17 abnormalities, including their effect on the *HER2* gene expression, is unknown.

The amplification and over-expression of the *HER2* gene, as well as the time of exposure to the mitogenic hormone E2, are two molecular factors that have been widely associated with mammary tumorigenesis, which has been additionally used in therapy selection between anti-estrogen agents and specific antibodies that interrupt the receptors function (i.e. Tamoxifen). Likewise, a strong association between *HER2* over-expression and E2 was observed. This association leads to ER activation in the absence of ligand and the indirect activation of *HER2* mediated by E2 (9, 13).

Experimental evidence suggests that patients over-expressing the *HER2* protein can develop resistance to TAM; however, the mechanism by which this occurs is unknown. Several studies with breast cancer patients in the metastatic phase, report that more than the 50% of patients who carry ER+ are successful in stopping tumor progression when they are treated with TAM, even though the obvious advantages, the 40% of patients receiving TAM as adjuvant therapy, eventually relapse and die due to their disease. In these cases, the *HER2* expression predicts a poor response to therapy (14, 15).

Paradoxically, it has been reported that TAM possesses a high mutagenic potential. This occurs because it can cause chromosomal ruptures and leads to the generation of translocations and great deletions in animal models (16). However, the type and frequency of chromosomal abnormalities as well as the mechanisms by which TAM induces chromosomal instability are unknown. Additionally, cytogenetic studies about TAM effects at the chromosomal level are very limited.

Taking into account the above, the knowledge of chromosomal abnormalities produced by exposure to E2 and TAM, as its association with the presence or absence of the estrogen receptor, could contribute to a better understanding of the carcinogenic process attributed to E2 and TAM. It might also help to identify candidate genes located in the affected chromosomal regions in order to be used as biomarkers of prognosis and the response to neoplasm treatment.

1.2 PROJECT RELEVANCE

The cell line karyotypes are very important in generating reference guides in order to achieve a better examination and comprehension of the cellular and molecular processes underlying breast neoplasm. The karyotype analysis can provide new ideas regarding the molecular mechanisms leading to cell transformation and allows the clarification of possible cytogenetic aberrations caused by different factors - in the case of the present study, those induced by exposure to E2 and TAM.

The prolonged exposure to estrogen represents a risk factor for the development of breast cancer, but the mechanisms by which these hormones induce cancer are partially understood; likewise, the chromosomal abnormalities produced by E2 and TAM have not been fully described in breast cancer cell lines. Cytogenetic findings in such cell lines could allow the finding of other genes that respond to hormonal actions and possibly involved in tumor development, as well as genes that might be participating in the resistance to anti-estrogen therapy. Similarly, the study of positive and negative cell lines for the presence of the estrogen receptor (ER) and *HER2* could help to understand whether an association exists between these factors and the profile of chromosomal abnormalities.

Currently, the analysis of gene expression profiles (microarrays) applied to breast cancer is considered “the gold standard” in the determination of biomarkers for early detection and therapy response; however, these techniques are very expensive and require a prior knowledge of the affected genes in order to study them. In addition, breast cancer is a heterogeneous disease characterized by the presence of a mosaic of neoplastic cells displaying different genetic aberrations, where several genes can be involved not only in progression but also in the response to therapy. Most of these abnormalities are not observable by the use of techniques such as DNA arrays or aCGH, since these cannot identify low-proportion mosaicisms or balanced structural abnormalities.

Therefore, the applications of cheaper and more accessible techniques, like cytogenetic analysis that allows identifying such alterations, constitute a major need. Even though classic cytogenetic does not possess the resolution of molecular techniques, the application of it might provide a global picture of all the possible chromosomal changes present in individual tumor cells occurring in a highly heterogeneous and polyclonal neoplasm, such as breast cancer.

1.3 OBJECTIVES

1.3.1 General Objective

- To obtain a profile of chromosomal and gene abnormalities induced by E2 and TAM exposure in both ER+ and ER- breast cancer cells.

1.3.2 Specific Objectives

- To identify chromosome abnormalities in the MCF7, T47D, BT474 and SKBR3 cell lines, before and after treatment with E2 and TAM using conventional and molecular cytogenetic techniques (M-FISH).

- To determine the number of copies of *HER2* in the MCF7, T47D, BT474 and SKBR3 cell lines using FISH, before and after treatment with E2 and TAM.

- To identify polysomies and rearrangements in Chr17, assessing the number of copies of *SMS* and *RARA* genes in MCF7, T47D, BT474 and SKBR3 cell lines, before and after treatment with E2 and TAM.

- To establish the profile of chromosome abnormalities induced by the exposure to E2 and TAM and its association with ER and *HER2* gene status.

- To propose a list of genes in the chromosomal regions affected that may be used as potential biomarkers of response or resistance to E2 and TAM exposure.

- To quantify HER2 gene expression at both mRNA and protein level in the MCF7, T47D, BT474 and SKBR3 cell lines, before and after treatment with E2 and TAM.

- To identify the effect of E2 and TAM in the modulation of gene expression of 84 key genes commonly involved in breast carcinogenesis, in MCF7, T47D, BT474 and SKBR3 breast cancer cell lines.

1.4 THEORETICAL FRAMEWORK AND STATE OF ART

1.4.1 Generalities of cancer

Cancer is a group of complex diseases known to make alterations in cellular processes such as proliferation, differentiation and cell death. This generalized loss of control is attributed to the gradual accumulation of mutations on genes involved in the regulation of these processes, which in turn lead to cell transformation marked by uncontrolled cell division, immune system evasion, invasion of tissues different from the one that originated the neoplastic lesion and dissemination throughout the organism (metastasis).

According to the International Agency for Research on Cancer (IARC), particularly the GLOBOCAN program, an estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012, compared with 12.7 million and 7.6 million, respectively, in 2008 (1, 17). The incidence shows an increase compared to previous years that can be explained by the growth and aging of world population, as well as the adoption of lifestyles associated with cancer, including smoking, physical inactivity and bad dietary habits (1).

The factors that cause carcinogenesis are physical (UV radiation), chemical (xenobiotic compounds) and biological agents (virus). Furthermore, hormones, like estrogens, are tumor promoters because they increase the rate of cell proliferation. Some of them are specific to certain types of cancer (i.e. HPV in cervical cancer); however, it is not easy to identify a single cause for this disease, because cancer is a multifactorial disease (18).

Cancer development occurs by steps during which a cell becomes malignant as it accumulates genetic alterations, which confer selective advantages in order to achieve proliferative autonomy and immortality. Thus, the accumulation of genetic alterations may initially increase the rate of cell proliferation, which will give rise to a clonal population of tumor cells. The three steps in the development of cancer include initiation, promotion and progression. In the first step, initiation, a change in the cell's genetic material (mutation in one or more cellular genes controlling key regulatory pathways of the cell) primes the cell to become cancerous. The change in the cell's genetic material may occur spontaneously or be brought on by an agent that causes cancer (a carcinogen). Since initiation is the result of permanent genetic change, any daughter cells produced from the division of the mutated cell will also carry the mutation (19).

The second step is promotion. Agents that cause promotion, or promoters, are defined as chemical compounds capable of causing selective expansion of initiated cells. Agents that cause promotion may be substances in the environment or even some drugs such as sex hormones. Unlike carcinogens, promoters do not cause

cancer by themselves. Promotion has no effect on cells that have not undergone initiation. Thus, several factors, often the combination of a susceptible cell and a carcinogen, are needed to cause cancer.

The last step in carcinogenesis is progression. The term progression refers to the stepwise transformation of a benign tumor to a neoplasm and to malignancy. Progression is associated with a karyotypic change since virtually all tumors that advance are aneuploid (have the wrong number of chromosomes). This karyotypic change is coupled with an increased growth rate, invasiveness, metastasis and an alteration in biochemistry and morphology. Likewise, tumor cells have characteristics that are common to all of them regardless the type of tumor; among them, the following are the most noticeable:

- Minimum requirements of growth factors.
- Not inhibition of proliferation dependent on the density of the cell population.
- Loss of anchorage dependence.
- Changes in the cell morphology and growth habits.
- Inhibition loss by growth contact
- Increase in glucose transport.
- Changes in surface fibronectin expression (may be absent or reduced).
- Release of transforming growth factors and proteases secretion.

These characteristics allow for the differentiation of tumor cell behavior from the behavior of a normal cell; however, the so-called “hallmarks” exist in cancer, being the targets of study for the comprehension of tumor behavior and the design of therapeutic strategies (Figure 1).

1.4.2 Breast Cancer

1.4.2.1 Epidemiology

Breast cancer is the second most common cancer in the world and, by far, the most frequent cancer in women, accounting for 25% (1.67 million) of the total new cancer cases and 15.4% (198,000) of the total cancer deaths in 2012. It is the most common cancer in women both in more and less developed regions with slightly more cases in less developed (883,000) than developed (794,000) regions (1).

Incident rates are high in Western and Northern Europe, Australia/New Zealand and North America, intermediate in South America, the Caribbean and North Africa, and low in Sub-Sahara and Asia (1). The IACR (International Agency for Research on Cancer) estimates that, for Colombia and Italy, breast cancer is the first cause leading of death in women (1). Factors contributing to the

international variation of incidence rates range from reproductive and hormonal factors to those factors related to nutrition and culture, as well as the services available for early detection of cancer in each region (1).

Figure 1. Hallmarks of cancer. The figure shows eight essential changes in the physiology of a transformed cell.

1.4.2.2 Risk Factors

The main risk factors associated to breast cancer development are age, reproductive and familiar factors such as early menarche, first pregnancy at a late age, low parity, late menopause and obesity, as well as the hormonal status and lifestyle (20) (Table 1)

Table 1. Factors that increase the risk of developing breast cancer

RISK FACTOR	RISK GROUP	RELATIVE RISK
Age	Elderly	>10
REPRODUCTIVE FACTORS		
Menarche*	Before 11 years of age	3
First child	Nulliparity	3
Menopause*	After 54 years of age	2
LIFESTYLE		
Diet	High intake of saturated fats	1.5
Body weight (post menopause)	Body mass index >35	2
Alcohol	Excessive intake	1.2
HORMONAL STATUS		
Oral contraceptives	Common use	1.24
Hormone replacement therapy	Use for ≥10 years	1.35
RADIATION	Abnormal exposure after 3 years of age	3
FAMILY HISTORY	Breast cancer in first degree	≥2

*Early menarche and late menopause increase the risk to develop breast cancer due to the increment in the duration of the mammary glands' exposure to ovary hormones (estradiol), most likely because these stimulate the division of breast epithelial cells.

1.4.2.3 Classification

There are several criteria to classify breast cancer, from the histologic, which is the oldest technique, to those that have been proposed with the use of several molecular techniques developed in more recent years. According to the histopathological criteria, breast cancer can be classified as carcinoma *in situ and* invasive carcinoma (infiltrating). Carcinoma *in situ* is in turn classified as ductal (DCIS) or lobular (LCIS), the first one being the most common type. DCIS is in then, classified into 5 subtypes - comedo, cribriform, micropapillary, papillary and solid - while invasive carcinoma is classified into 7 subtypes: infiltrating ductal, lobular invasive, ductal/lobular, mucinous, tubular, medullary, and papillary; among these, the most common type of tumor is the infiltrating ductal carcinoma (IDC)(70%-80%). According to the degree of differentiation, the IDC is sub-classified into well differentiated (grade 1), moderately differentiated (grade 2) and poorly differentiated (grade 3) (21).

Even though it has prognostic value, the previous classification limits the ability to predict the response to therapy. The emergence of molecular techniques (microarrays) has enabled the determination of gene expression profiles, the identification of tumors heterogeneity and the molecular classification of breast cancer. Thus, five subtypes have been proposed based on the expression of estrogen receptors (ER), progesterone receptors (PR), estrogen-associated genes (*ESR1, GATA3* and *FOXA1*), genes associated with the induction of proliferation, the *HER2* gene and other genes located in the region of the *HER2* amplicon on Chr17 (Table 2) (21-23). These subtypes are:

- *Luminal A (RE+, RP+/-, HER2-) and Luminal B (RE+, RP+/-, HER2+)*

These subtypes have a good prognosis and are characterized by expressing genes associated to the estrogen receptor, such as *LIV1* and *cyclin D1* as well as low molecular weight keratins (CK7, CK8, CK18, etc.). ER expression is a condition for treatment with TAM or aromatase inhibitors; on the other hand, it shows a poor response to neoadjuvant chemotherapy. These carcinomas are associated to favorable morphological features such as small tumor size (less than 2 cm), differentiated tumors from low to moderate histological grade, negative axillary lymph nodes and an early stage at the time of diagnosis. Both subtypes express high BCL-2 levels and low proliferation indexes when tested with Ki-67.

- *HER2-positive (RE-, RP-, HER2+)*

Breast carcinoma HER2-positive has increased expression of those genes located in the same region where *HER2* is found, on chromosome 17q, including the growth factor receptor bound to protein 7 (*GRB7*) and is often associated to other markers indicating poor prognosis. Others genes alterated are topoisomerase II-alpha (*TOP2A*), *GATA4* and some genes involved in angiogenesis and proteolysis. This subtype is resistant to endocrine therapy and is usually treated with trastuzumab.

- *Basal (RE-, RP-, HER2- and CK5/6, CK14, p63 and/or EGFR+)*

This subtype is characterized by the overexpression of cytokeratin genes from the basal layer (CK5/6, CK17) and genes related to cell proliferation. Often, these tumors present mutations in the *TP53* gene, overexpress the epidermal growth factor receptor (EGFR) and display an absence of expression of ER, *HER2* and other related genes. This subtype is associated with *BRCA1* gene mutations and also presents the most aggressive behavior despite its high sensitivity to chemotherapy.

- *Similar to normal mammary gland (RE-, RP-, HER2-, CK5/6-, CK14-, p63- and/or EGFR-)*

This carcinoma has similar characteristics with the normal mammary tissue; also, it shows a strong expression of specific genes of adipose tissue and a low expression of the luminal epithelium genes (24, 25). Recently, a new subtype

classified as “Low in Claudine” has been identified (21, 23).

Table 2. Breast cancer molecular subtypes defined from gene expression profiles previously obtained by microarrays. “Adapted from Lancet, 378(9805), Reis-Filho JS, Pusztai L, Gene expression profiling in breast cancer: classification, prognostication, and prediction, 1812-23, Copyright (2011), with permission from Elsevier”

	IHC Markers	Histological Grade	Other Markers	Outcome
Luminal A	RE+: 91 - 100% RP+: 70 - 74% HER2+: 8 - 11% Ki67: Low Basal Markers: -	GI/II: 70-87% GIII: 13-30%	High FOXA1	Good
Luminal B	RE+: 91 - 100% RP+: 41-53% HER2+: 15-24% Ki67: High Basal Markers: -	GI/II: 38-59% GIII: 41-62%	Amplified FGFR1 and ZIC3	Medium/Poor
HER2+	RE+: 29-59% RP+: 25-30% HER2+: 66-71% Ki67: High Basal Markers:-/+	GI/II: 11-45% GIII: 88-93%	High GRB7	Poor
Basal	RE+: 0-19% RP+: 6-13% HER2+:9-13% Ki67: High Basal Markers:-/+	GI/II: 7-12% GIII: 88-93%	RB1:Low CDKN2A: High BRCA1: Low FGFR2: Amp	Poor
Normal	RE+: 44-100% RP+: 22-63% HER2+:0-13% Ki67: Low/Medium Basal Markers:-/+	GI/II: 37-80% GIII: 20-63%		Medium

GI: Tumor grade I; GII: Tumor grade II; GIII: Tumor grade III

1.4.2.4 Prognostic factors

Prognostic factors in breast cancer have been classified in three categories:

Category I: Those of routine use for patient handling. Within this category, the following factors can be found:

- Tumor size: Important prognostic factor, correlated to the survival and lymph node status of the patient. High tumor sizes are correlated with a bad prognosis.
- Lymph node status: Currently, the presence of metastasis in axillary lymph nodes is considered as a bad prognostic indicator in patients with breast cancer in early stages.
- Histological grade: This predicts the survival of patients with breast cancer, where higher histological grades are related to metastasis and short survival.
- Hormone receptors status: Predictive factors for the response to hormone therapy. Estrogen receptor status helps guide treatment for breast cancer. Breast cancers that have a large number of estrogen receptors (estrogen receptor-positive (ER+) tumors) can be treated with hormone therapies like tamoxifen and aromatase inhibitors.

Category II: Biological and clinical factors that require more studies with solid statistical information in order to be validated and that can be optional. Within this category, the following factors can be found:

- HER2: Overexpression of this protein is observed in poorly differentiated breast carcinomas with high histological grade. It predicts resistance to hormone therapy and chemotherapy.
- *TP53:* Mutations in this gene have been associated with high histological grade and aggressiveness.
- Vascular or lymph invasion: Predictor of reduced survival.
- Cellular proliferation markers (Ki-67): A high percentage of this marker has been related to poorly differentiated and large tumors, as well as early recurrence and poor survival.

Category III: Factors that have not been fully studied, for example:

- DNA ploidy analysis: DNA ploidy has not been correlated to clinical results of primary breast cancer patients.
- Angiogenesis: High correlation between number and density of micro-vessels with metastasis percentage and survival with absence of the disease was observed.
- TGF: The Transforming Growth Factor exerts a promoting effect in the growth of some types of breast cancer.
- BCL-2: Good prognostic marker, it also indicates good response to TAM.
- Cathepsin D: The overexpression of this protease has been associated with a high histological risk, great tumor measurement and increased risk of recurrence (26, 27).

Nowadays, *HER2* oncogene is considered as a predictive factor within Category I (according to the level of accumulated evidence) (27) (Table 3).

Table 3. Prognostic factors in breast cancer

CATEGORY I	CATEGORY II	CATEGORY III
Size of the tumor	HER2	DNA ploidy analysis
Lymph node status	TP53	Angiogenesis
Histological Grade	Vascular or lymph invasion	TGF
Histological Type	Cellular proliferation markers (Ki-67)	BCL-2
Hormone Receptors status		Cathepsin D

1.4.2.5 Biomarkers in breast cancer

The identification of those genes responsible for the development and progression of cancer has allowed establishing the definition of breast cancer malignant potential as well as a contribution of a better biological understanding and clinical management of this neoplasia (28).

Some techniques such as Comparative Genomic Hybridization (CGH), CGH arrays and SNP arrays, among others, have allowed the identification of groups of biomarker genes, such as: tumor suppressor genes (*BAP1, PLAGL 1, CDKN2A, PTEN, TSG101, ELF5, KAI1, IGSF, P15, RB1, TP53,* etc.), DNA damage repairing genes (*ATM, BRCA2, CHK2,* etc.), genes involved in apoptosis (*MCL1, BCL2*, etc.) and oncogenes (*KISS1, FGFR1, MYC, CCND1, MTA1, NCOA3, STK15, BCAS1, EMS* and *HER2)* (29).

Many of these genes are prognostic and diagnostic biomarkers, and the determination of their expressions has allowed assessing not only the response to therapy but also the disease progression. Some examples of biomarkers are presented in table 4.

Table 4. Breast cancer biomarkers

BIOMARKER	USE	OBSERVATIONS
STANDARD BIOMARKERS IN CLINICAL PRACTICE		
Estrogens (ER) and Progesterone (PR) Receptors	Prognosis markers of disease progression and predictors of response to anti-estrogen therapy.	Its expression in tumors is also associated with bad prognosis such as amplification of *HER2, c-MYC* and *INT-2* genes and mutation of gene *TP53* (30, 31)
***HER2* (c-erbB-2)**	Prognosis marker of disease progression and predictor of therapy response to trastuzumab	Bad prognosis indicator, it also indicates resistance to therapy. In combination with other factors (ER), *HER2* can be used also to determine prognosis (31)
DNA Ploidy and Phase S	Bad prognostic marker related to increased risk of recurrence and mortality (26)	The lack of tools of standard diagnosis for its determination has hampered its approval as prognosis markers.
Ki-67	Bad prognostic marker	An association between the number of positive cells for Ki-67 and nuclear grade, age, mitotic rate and reduced survival has been reported (31)
CELL CYCLE MARKERS		
Cyclin D1	Progression indicators	Gene amplified in the 20% of breast cancers. It is associated to progression
Cyclin E		High expression levels are correlated to therapy response.
p21 and p27 proteins		Cell cycle regulators, kinase inhibitors dependent of cyclin. Loss or reductions in levels of this protein are associated to poor prognosis.
TUMOR SUPRESSOR GENES		
TP53	Bad prognosis markers in breast cancer.	Mutations in this gene are associated with advanced histological grade, high mitotic index, high rate of cell proliferation, variable association with the amplified state of *HER2, c-MYC* and *RAS* genes, as well as resistance to hormone therapy and chemotherapy.
BRCA1* and *BRCA2		Mutations in this gene are associated with increased risk of developing breast or ovary cancer.
CELLULAR ADHESION MOLECULES		
E-Cadherin	Initiation, promotion, progression and metastasis biomarkers	Absence of this protein is correlated to progression and adversity.
CD44		Presence of this protein is associated to development and progression of breast cancer

Integrins and laminins		Altered expression (low levels) is associated to bad prognosis
PROTEASES ASSOCIATED TO INVASION		
Cathepsin D	Bad prognosis markers	High cathepsin D levels are correlated to reduced survival and metastasis
Urokinase-plasminogen Activator (uPA)		Serum-proteases, which activate plasminogen. High uPA intratumor levels have been associated to reduced survival and metastasis.
ANGIOGENESIS MARKERS		
VEGF (Vascular Endothelial Growth Factor)	Bad prognosis markers	High VEGF levels have been observed in serum, tumor protein extracts and tumor tissues.
CIRCULATING TUMOR BIOMARKERS		
MUC-1 and Carcinoembrionary Antigen (CEA)	Diagnostic markers	Its use in early breast cancer detection is limited due to its low sensitivity and specificity (22)
NEW BIOMARKERS		
ONCOTYPE DX	Even though these techniques are part of methodologies for identifying new markers for early diagnosis, more assays are still required to validate its use in clinical tests.	RT-PCR multigenic multiplex assay, using a set of probes for 21 genes. This set includes 16 genes related to cancer and 5 reference genes (31)
Genomic Micro-arrays and Transcriptional Profiles		Used in the classification of breast cancer and the determination of prognosis (31)
Proteomics: MALDI and SELDI mass spectrometry		Applied in breast cancer to identify new and improved biomarkers in serum and nipple aspirates (32)
MicroRNAs (miARN)		Increased expression (miR-206, 221, 222, 21, 17-5p, 155, 210, etc.); reduced expression (miR-125a/b, let-7, 145, 10b, 372, 34a, 103, 107, 7, 10b, 205, 31); these regulate genes involved in differentiation, proliferation, apoptosis, angiogenesis and metastasis (22, 33, 34)

Although it has been shown that all markers mentioned above are useful for breast cancer, the determination of the presence of ER and *HER2* gene status remains being the "gold standard" for prognosis and therapy, since these are drug targets; furthermore, they are very important to define subtypes, analysis of response and resistance to therapy.

1.4.3 Estrogens and breast cancer

Even though the role of estrogens in the normal development of individuals is known, it has been shown that these also increase the risk of developing breast and uterine cancer (35). Estrogens are comprised of 9 chemically different steroids, highlighting the 17β-estradiol (E2), estrone (E1) and estriol (E3). 17β-estradiol (E2) is the circulating ovarian steroid predominates in women's body, with an increased biological activity in the mammary gland and which is synthesized by the aromatization of testosterone. It plays a regulatory role in a wide variety of biological processes, including reproduction, differentiation, cell proliferation, apoptosis, inflammation, metabolism, homeostasis, brain function and breast development during puberty and sexual maturity (36).

In premenopausal women, the synthesis of the estrogens estradiol (E2) and estrone (E1) is made in the ovaries and peripheral tissues such as adipose tissue, bone and skin, while in postmenopausal women, the synthesis is done only in peripheral tissues, producing a reduction of E2 plasma levels in a 90%.

A prolonged exposure to estrogen as well as high levels of this hormone in the circulation have been associated with an increased risk of developing breast cancer. This exposure may be endogenous or exogenous. Endogenous exposure is related to reproductive factors such as early menarche, late menopause, late first pregnancy and nulliparity. Exogenous exposure results from the use of oral contraceptives (20μg/day) and hormone replacement therapy (2mg/day) and/or food, water and air contamination by plant phytoestrogens, or man-made xeno-estrogens (37).

1.4.3.1 Estrogen receptors (ERs)

Like estrogens, estrogen receptors (ER) have been highly involved in the development and progression of breast cancer. The action of estrogen is mediated by its nuclear receptors ERα and ERβ, which are encoded by different genes (*ESR1* encodes ERα on chromosome 6 and *ESR2* encodes ERβ on chromosome 14), which are expressed in different parts of the body such as the brain, cardiovascular system, urogenital tract, bones, breast and ovarian cells, as well as in neoplasm derived from these tissues. ERs belong to a family of nuclear proteins bound to DNA, which regulate the transcription of a wide variety of genes (which are involved in the development and function of reproductive organs, bone density, regulation of cell cycle, DNA replication, differentiation, apoptosis, angiogenesis, survival and tumor progression) including: *IGFR, CCND1, BCL-2, VEGF* and some growth factors such as heregulins (HER), TGFβ and amphiregulins, which bind and active EGFR (6, 36).

ERα and ERβ receptors contain in their structure six domains: two ligand-independent transcriptional activation domains AF1 (A and B, where MAPKs-mediated phosphorylation is carried out), a DNA-binding domain (C domain), a nuclear localization domain binding and heat shock proteins binding domain (domain D), a ligand-dependent transcriptional activation domain AF2 (E domain) and an F domain, located towards the C-terminal end (38, 39) (Figure 2).

When the ligand is absent, ERs are found predominantly in the nucleus as monomers associated with multiprotein complexes such as heat shock proteins (HSP's) (35, 38). However, recent studies have reported the presence of ERα, ERβ or both on the inner face of the plasmatic membrane, bound either to membrane proteins such as caveolin-1, or associated to other membrane receptors such as IGFR, EGFR or HER2, or to signal adapter molecules such as SHC (6, 38, 40).

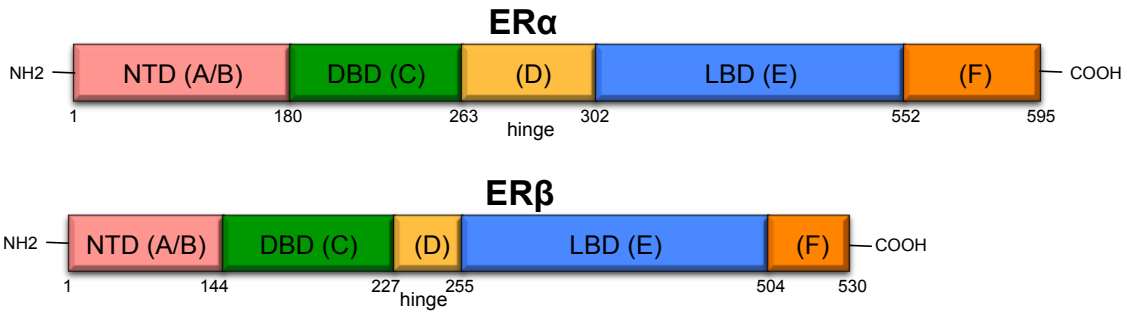

Figure 2. Functional domains of estrogen receptors (ERα and ERβ). Receptors domains structure are indicated in different colors: In pink, the AF1 domain; green, the DNA-binding domain; yellow, the heat shock proteins-binding domain; blue, the ligand-binding domain and orange, the F region.

In normal breast tissue, ERβ plays a role as the dominant receptor, but during carcinogenesis the amount of ERβ decreases whilst the amount of ERα increases. Thus, ERβ was postulated as a tumor suppressor gene in breast cancer (41). Most of the ER present in breast tumors are ERα; however, high levels of this receptor in benign breast epithelium increase the risk to develop breast cancer, and it has been particularly associated with tumor initiation and progression to later stages. Although ERβ function in breast and ovarian cancer is unclear, the interaction between ERβ and ERα is essential for normal development and function of the tissues in which they are expressed (6, 36, 39).

ER detection is widely used in patients with breast cancer as a prognostic marker to predict the risk of progression and as a response predictor to anti-

estrogen therapy. Tumors positive for ER and PR are very well differentiated in histological terms; they show low rates of cell proliferation and diploid content of DNA, among others. These tumors are associated with other poor prognostic markers as the amplification of the *HER2, c-MYC* and *INT-2* genes and *TP53* gene mutation (30, 31).

1.4.3.2 Estrogen regulated genes

The analysis of gene expression profiles allowed the establishment that some hormones such as E2 can modify the expression of genes that regulate the cell cycle, DNA replication and repair, which has been associated with the development of neoplastic lesions. Several authors have described the role of estrogens on regulating growth factor activities, cell cycle regulators, apoptosis regulators and cellular adhesion molecules (Table 5), which have been observed *in vivo* and *in vitro*.

Table 5. Estrogen regulated genes

GENES	REGULATION	FUNCTION	REFERENCES
EGR1 (zf)	Positive or negative	Cell death	(42)
TFAP2A (zf)			
TFAP2B (zf)			
CDK2 (hs)(zf)	Positive	Cell cycle, G1/S and S/G2 transition	(42)
CCNA1 (zf)			
PLK2 (zf)	Positive	Cell cycle, entrance to M, metaphase-anaphase transition, cytokinesis, centrosomes splitting	(42)
CCNB (zf)			
APC (zf)		Cell cycle, metaphase-anaphase transition	
PRC1 (zf)			
JUN (zf)	Positive	Cell cycle progression and DNA replication processes	(34, 42, 43)
E2F4 (zf)(cl)			
PCNA (zf)			
CCND1 (hs)			
Aurora A and B (hs)(zf)			
CDC45L (zf)	Positive	Cell cycle	(42)
EIF4G2 (zf)			
MCM5 (zf)			
TOP1 (zf)			
A1B1 (hs)	Positive	ER Coactivator	(34, 43)

c-MYC (cl)(hs) E2F1 (cl) E2F2 (cl)	Positive	Transcription factors	(34)
IGFBP4 (cl) pS2 (cl) Cyclin A1 (cl)	Positive	Cell proliferation Genes related with cancer Cell proliferation	(44)
MYC- BP1 (cl)	Negative	c-MYC transcriptional repressor	(44)
Replication factor C (cl) IIa Topoisomerase (cl)	Positive	DNA Replication	(45)
FN1 (cl) VIM (cl) CDH2 (cl)	Positive	Cell Adhesion	(45)
CAV1 (cl) CDC42 (cl) HRAS (cl)	Positive	Integrins-mediated signaling pathways	(45)
ITGA6 (cl) ITGB4 and ITGB6 (cl) LAMA3 (cl) LAMB3 (cl) LAMC2 (cl) RHOD (cl) RHOF (cl)	Negative	Integrins-mediated signaling pathways	(45)
KERATINES 5, 7, 8, 16, 17, 18, 6B(lc)	Negative	Cytoskeleton	(45)
CDH1 (cl) MUC1 (cl) DSP (cl)	Negative	Cell adhesion	(45)
NFE2L2 (zf) NQO1 (zf) MAOB (zf)	Negative	Cell protection against damage by oxidative stress	(42)
APP (cl) BNIP3L (cl) CASP14 (cl) CASP6 (cl) TNFSF7 y TNFSF9 (cl)	Negative	Apoptosis	(45)

zf (zebrafish); hs (homosapiens); cl (cell lines)

Likewise, it has been reported that E2 modulates the expression of several microRNAs, most of which act as tumor suppressors since these are involved in the negative regulation of oncogenes. Nonetheless, other microRNAs act as oncogenes by promoting cell proliferation and survival and negatively regulating tumor suppressor genes (34). This estrogen modulation on microRNAs contributes to initiation and progression of breast cancer and could become a therapy target. Some microRNAs modulated by estrogen are listed in table 6.

Table 6. E2 regulated microRNAs

microRNAs	FUNCTION	OBSERVATIONS	REFERENCES
Let-7 family microRNAs (cl)	Limit *c-MYC, Ras* and *E2F2* oncogenes expression	Expressed in luminal type A breast cancer	(34)
miR-98 (cl)		Highly expressed in luminal type A breast cancer	(34)
miR-21 (cl)			(34)
miR-17-5p	Limit the expression of ER coactivator knows as AIB1		(34)
miR- 107, miR-424, miR-570, miR-618 and miR-760 (hs)	Block a significant number of breast cancer transcripts		(46)

cl: MCF-7 y T47D cell lines; hs: homo sapiens

1.4.3.3 Estrogen mediated signaling pathways

The classical action mechanism mediated by ERs (genomic signaling) starts when E2 binds to estrogen receptors (REα and REβ) in the nucleus; this causes a conformational change in the receptor and promotes the release of the inhibitory complex, which consists of many chaperone proteins (Hsp 90 heat shock protein). After E2 is joined to the receptor, they form a dimers (homo-or heterodimere) and bind to DNA in several regulatory regions under the action of estrogens (EREs). However, ERs also regulate the expression of many genes without directly binding to DNA through nonclassical response sites via protein - protein interactions with transcription factors such as Sp-1, AP-1 and GATA1 (39, 42, 47, 48). Genes activated by this route include *IGF-1*, collagenase, *c-MYC*, *cyclin D*, *c-fos* and the low-density lipoprotein receptor (49).

Besides the classic action mechanism mediated by nuclear ERs, a non-genomic effect mediated by ERα and ERβ have been observed (membrane-associated), leading to the activation of the cytoplasmic tyrosine kinase Src

and other signaling molecules such as: (i) insulin-like growth factor receptor 1 (IGF-1R) and the epidermal growth factor receptor (EGFR); (ii) mitogen-activated protein kinases (MAPK), phosphatidyl inositol 3 kinase (PI3K) and Akt; (iii) protein kinase C (PKC) and cyclic AMP (cAMP); (iv) p21 and (v) pathways that promote the release of intracellular calcium (35, 50). These kinases phosphorylate nuclear ERs and their coactivators (AIB1/SRC-3) resulting in the activation of their functions as the transcription regulators of target genes.

These non-genomic activities facilitate cross-communication between signaling pathways mediated by estrogen receptors and signaling pathways mediated by growth factor receptors (i.e. EGFR, IGF-1, HER2), including MAPK, AKT and PKC pathways, which are highly involved in cell proliferation, survival and resistance to endocrine therapy in breast cancer (47, 48, 51).

Additionally, it has been reported that the G protein-coupled receptor (GRP30) is another candidate for non-genomic signaling mediated by estrogen (52). This protein is expressed in ER-positive (MCF7) and ER-negative (SKBR3) breast cancer cell lines, endometrial and ovarian cancer cells and thyroid carcinoma cell lines (38) (Figure 3).

1.4.3.4 Mechanisms of estrogen carcinogenesis

Initially, estrogens (including E2) were classified as non-mutagenic and non-genotoxic molecules because the induction of gene mutations in cells exposed to them had not been determined. However, studies that emerged in 2000, reported the induction of some types of DNA damage, and admitted that estrogen could be potentially mutagenic (2). Estrogen-mediated carcinogenesis is associated with individual characteristics such as age, dose and duration of exposure to E2 and is believed to be a sequential process that promotes tumor initiation and promotion through at least three mechanisms in which it is known that these hormones are involved. Two mechanisms involve estrogen binding to its specific receptors (ERα and ERβ), either to the nuclear or plasma membrane ERs, which, as has already been mentioned, can become a potent stimulus of cell proliferation and increase the risk of genetic injury by causing DNA or chromosomal damage, leading to the appearance of genetic mutations during DNA replication or any other stage of the cell cycle (Figure 4) (2-4).

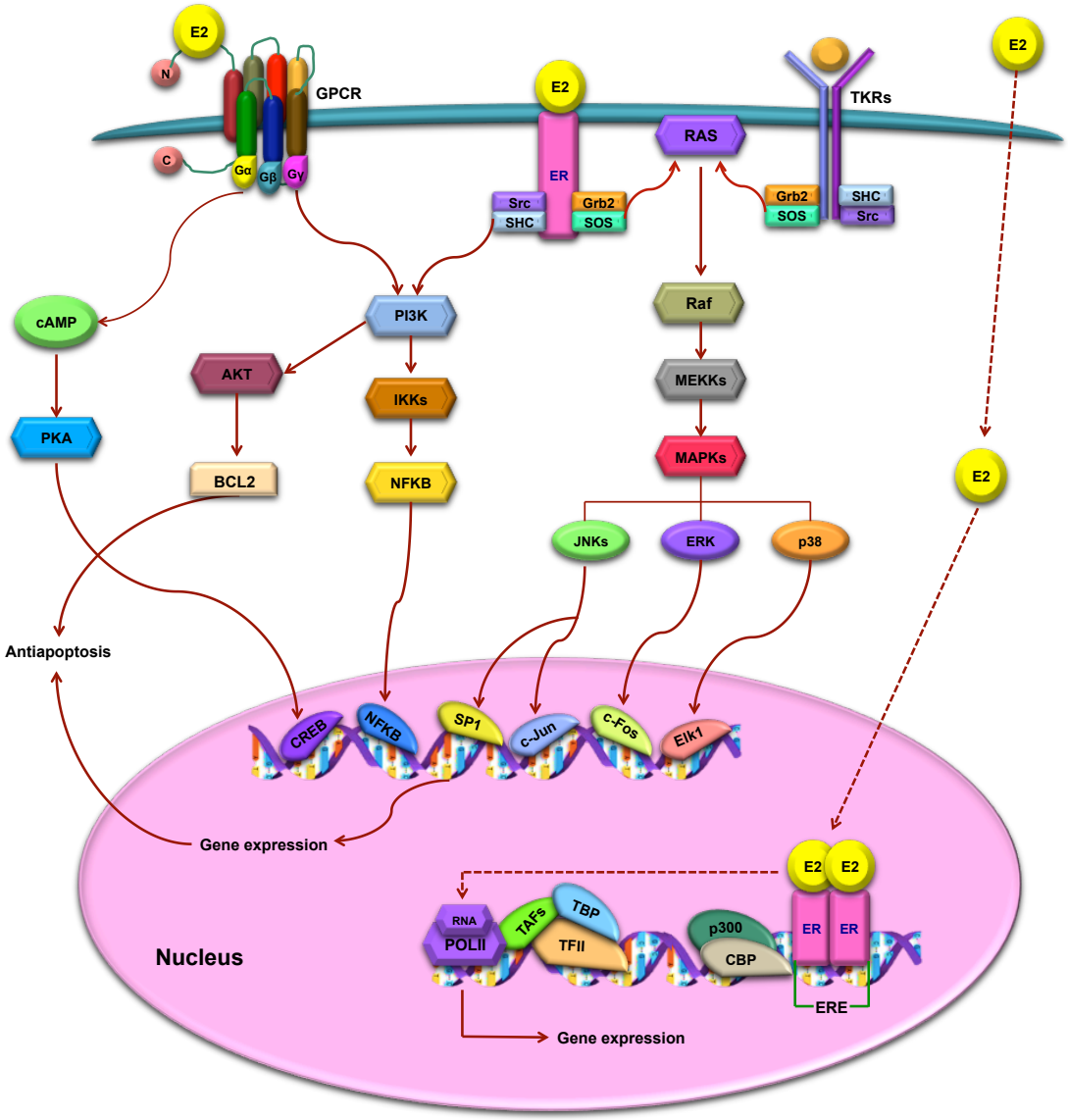

Figure 3. Estrogen mediated signaling pathways. The classical action mechanism of estrogens involves the binding of estrogen to nuclear receptors. The hormone binding to the ER releases the inhibitory complex receptor with heat shock proteins (HSPs), causing conformational changes that allow ERs binding to estrogen response elements (ERE), located in the promoters of target genes and therefore stimulating transcription. ER located in the cell membrane, are also involved in non-genomic signaling pathways. These signaling pathways recruit: tyrosine kinases receptor (TKR), G protein-coupled receptors (GPCRs), Phosphatidyl Inositol-3-kinase (PI3K), serine-threonine kinase (Akt), mitogen - activated protein kinase (MAPK) and members of protein kinases family (PKA and PKC). Modified from http://www.sabiosciences.com/pathway.php?sn=Estrogen_Pathway.

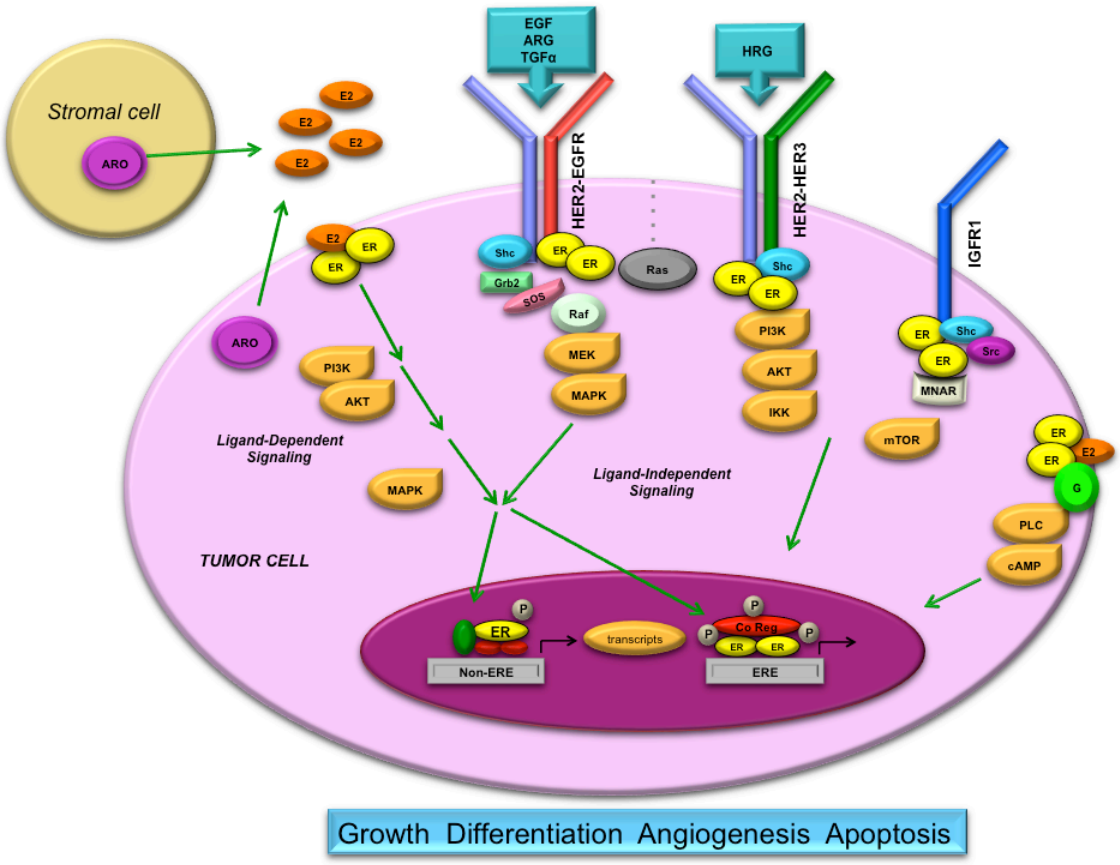

Figure 4. Interactions of estrogens with growth factor receptors in the survival and proliferation of human tumors. It has been reported that estrogens induce cancer by at least three mechanisms, two of which are associated with estrogens binding to their specific receptors (ERα and ERβ), either nuclear or plasma membrane ERs, stimulating cell proliferation and increasing the risk of causing direct damage to DNA during each cell division. Alternative routes for estrogen action include intratumor production of estrogen by the action of aromatase (ARO), the key enzyme in the estrogens biosynthesis, which is being regulated by both nuclear and extranuclear ERs and growth factors that mediate signaling. Similarly, estrogens might regulate angiogenesis associated to tumor through direct interaction with vascular endothelial cells or by the indirect stimulation by the vascular endothelial growth factor (VEGF) secretion from tumors. Adapted from "Clin Cancer Res Copyright 2007, 13(16):4672-6, Pietras RJ, Marquez-Garban DC, Membrane-associated estrogen receptor signaling pathways in human cancers, with permission from AACR".

In addition, alternative pathways may also promote the action of estrogens; within these, the tumor microenvironment plays an important role, where communication between tumor cells and stromal fibroblasts contributes to the intratumoral production of estrogen in breast cancer cells. This production is mediated by the action of aromatase (ARO), a key enzyme in the biosynthesis of estrogens, which is regulated by nuclear and extranuclear ERs as well as growth factors that mediate signaling (Figure 4). Similarly, estrogens may regulate angiogenesis associated to tumors through a direct interaction with vascular endothelial cells or through the indirect stimulation of the vascular endothelial growth factor (VEGF) secretion from tumors (48, 49, 53). This convergence of non-genomic and genomic signaling pathways mediated by ERs provides alternative mechanisms by which estrogens might regulate genetic expressions and, likewise, increase the number of genes regulated by ERs (35).

A third mechanism is associated with the oxidative metabolism of estrogens, including the formation of secondary metabolites that act as reactive oxygen species (ROS) and involve different cytochrome p450 isoforms. The catechol estrogens - particularly the 4-hydroxyestrogen (4-OHE2) - can form semiquinones and 3,4-quinones which bind covalently to guanine or adenine in DNA, destabilizing the molecule and giving rise to unstable adducts (54). The removal of these adducts by the responsible repairing mechanisms, let abasic sites, which can be converted into punctual mutations and serve as initiators of neoplastic transformation (47) (Figure 5). Added to this, the 4-OHE2 and E2 have an affinity for the ER, and the subsequent ligand-receptor binding triggers the classical ER-mediated mechanism; thus, a dual role for this metabolite is established - as a hormonal carcinogen and as a member of signaling pathways dependent ligand (47).

Likewise, during the redox cycling of estrogens, the generation of free radicals occurs from the activity of nuclear or mitochondrial enzymatic systems or non-enzymatic processes, such as iron and copper ions (Fe_2q, Cuq) (which are also involved in the oxygen and electrons transport). These free radicals cause macromolecular damage, modify DNA and produce 8-hydroxidesoxiguanine, lipid adducts and DNA breaks of single or double strands, which are lesions of great importance capable of generating irreparable damage and several types of mutations, including chromosomal aberrations (2, 55-57) (Figure 6). In addition, a similar increase in hydroxyl radicals, which can cause DNA damage, has been identified in human breast tissue from patients with breast cancer (2).

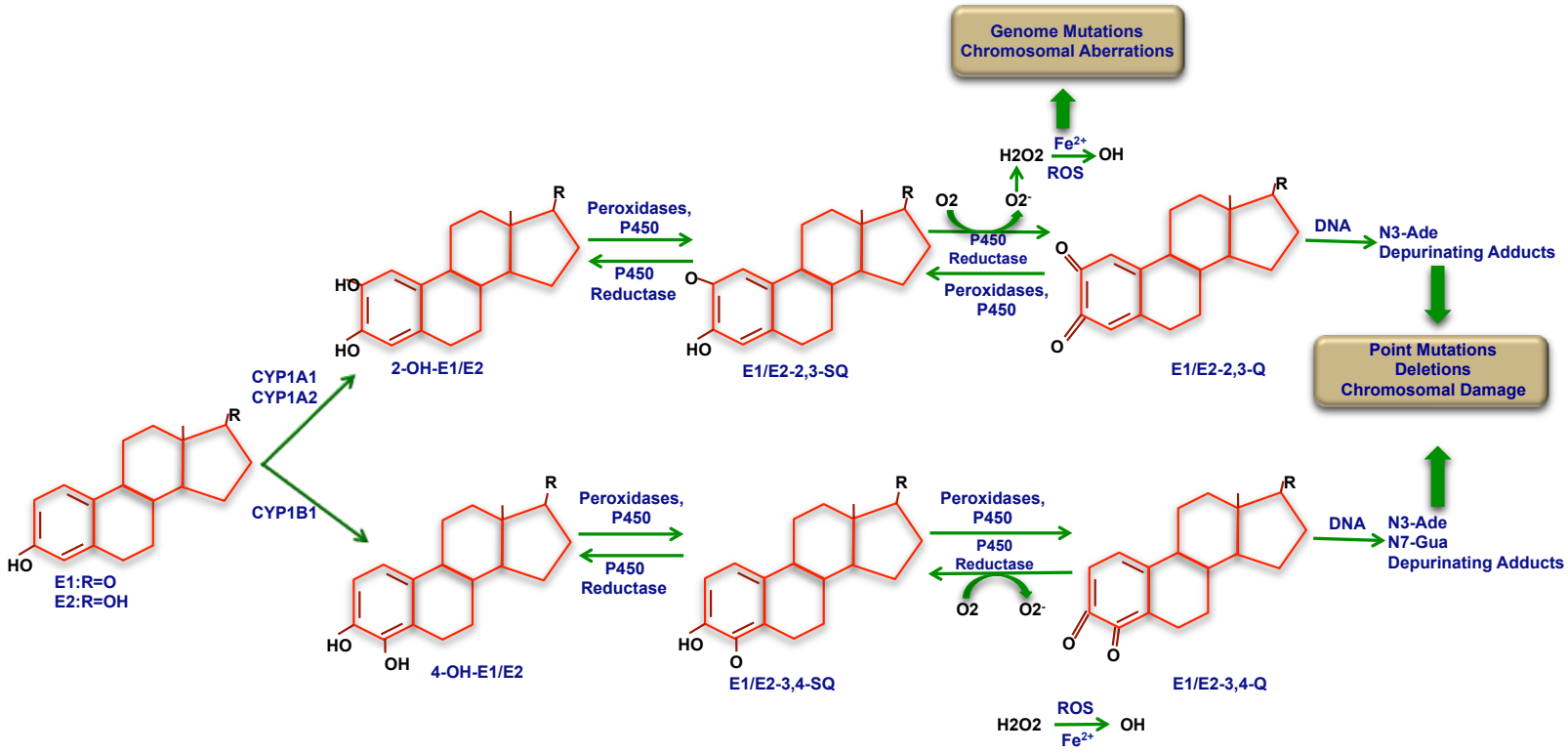

Figure 5. Estrogens metabolism. Oxidative metabolism of estrogens to form catechol estrogens: 2- and 4-hydroxi-estradiol/estrone (2-OH-E1/E2 and 4-OH-E1/E2), mainly through CYP1A1/1A2 and CYP1B1. “Adapted from, Chem Biol Interact,191(1-3), Hevir N, Trost N, Debeljak N, Rizner TL, Expression of estrogen and progesterone receptors and estrogen metabolizing enzymes in different breast cancer cell lines, 206-16, Copyright (2011), with permission from Elsevier”.

The ability of estrogens, particularly E2, to induce mutations in cells was demonstrated in lung cells from a Chinese hamster (V79), where in experiments of exposure at low doses of estrogens (10^{-10} M E2), the appearance of mutations in the *HPRT* gene was observed at low frequencies, while curiously at high doses this effect was not observed. This event has a plausible explanation given that the estrogen metabolism generates catechol estrogen (2 - and 4-hydroxiestradiol), which exhibit pro-oxidant characteristics only at low physiological concentrations, whereas at high concentrations all estrogens (including catechol estrogens) act as antioxidants (2). This would also explain why, in studies using high doses of estrogens, there were no reports of mutagenic effects of this hormone.

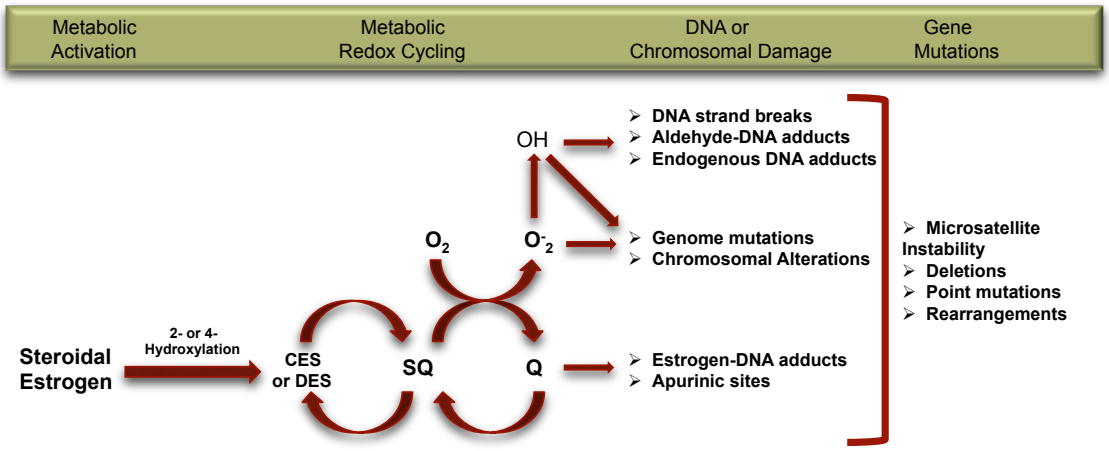

Figure 6. Induction of genetic damage by estrogens. As a result of redox cycling of estrogens are generated free radicals, which cause macromolecular damage, modify DNA, produce lipid adducts and DNA breaks of single or double strands. These lesions are able to generate irreparable damage and several types of alterations, including deletions, point mutations and chromosomal aberrations. OH (hydroxyl group), O^{-}_{2} (superoxide), CES (Conjugated Estrogens), DES (Diethylstilbestrol), SQ (semiquinones), Q (quinones). "Adapted from Mutat Res, 424(1-2), Roy D, Liehr JG, Estrogen, DNA damage and mutations, 107-15, Copyright (1999), with permission from Elsevier"

1.4.4 Importance of *HER2* gene (*ERBB2*) for breast cancer

Human proto-oncogene *HER2* (also called *ERBB2*) is located on the long arm of chromosome 17 (17q11q12) (Figure 7), and codes a 185 - kD protein (p185), which possesses characteristics of tyrosine kinase transmembrane receptors. It also has homology sequences with the epidermal growth factor receptor (EGFR). The HER2 protein belongs to a family of four growth factor receptors

displaying tyrosine kinase activity - including EGFR, HER2, HER3 and HER4 - whose biological activity affects the activation of cellular pathways involved in cell survival and proliferation. The amplification of this gene and the protein overexpression has been associated with a poor prognosis in breast, ovarian, gastric and prostate cancer (58).

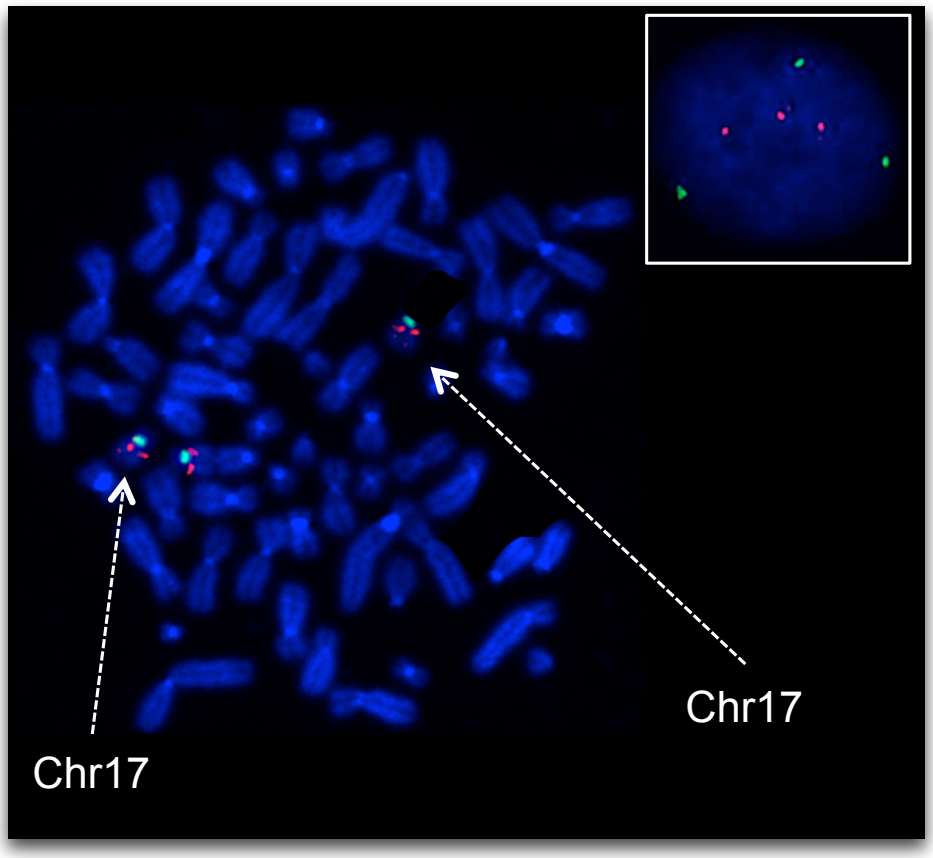

Figure 7. ***HER2* gene location on BT474 cells**. This gene is located in the long arm of chromosome 17 (17q11-q12). The position of the chromosome centromere is shown on a metaphase and a nucleus as the green signal, while *HER2* gene position is shown as the red signal.

1.4.4.1 Structure

HER2 is a type-1 transmembrane glycoprotein consisting of three regions: an N-terminal extracellular domain, a transmembrane α-helix domain and an intracellular tyrosine kinase domain. The N-terminal domain is divided into 4 subdomains (I-IV). Subdomains I and III form a binding site for ligands, while sub-domains II and IV - rich in cysteine - are important for the formation of homo- and heterodimers (Figure 8).

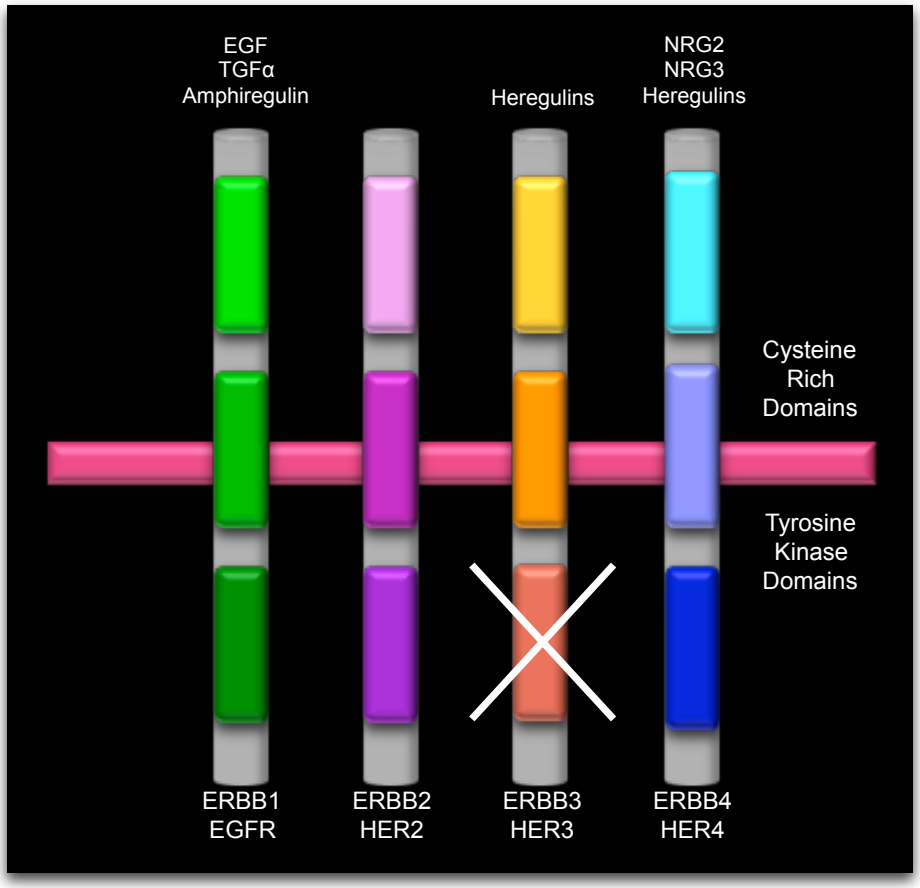

Figure 8. Family of epidermal growth factor receptors. The family of HER receptors is composed by four members: EGFR, HER2, HER3 and HER4. Each receptor is a transmembrane protein comprising three different domains: an extracellular domain, a transmembrane domain and a domain displaying tyrosine-kinase activity. Modified from http://www.biooncology.com/research-education/her/dimer

1.4.4.2 Signaling pathways

Although has not been located a specific binding ligand for HER2, it is usually associated with other family members of the EGFR family (epidermal growth factor receptor) in the formation of functional heterodimers, including HER1 (EGFR), HER3 and HER4. Dimer formation leads to the phosphorylation of the intracellular domain containing binding sites for a variety of molecules, acting as adapters in the activation of cellular signaling pathways - MAPK, PI3K/AKT and phospholipase C - and promoting cell proliferation and survival not only in normal cells, but also in uncontrolled processes in cancer (58, 59) (Figure 9).

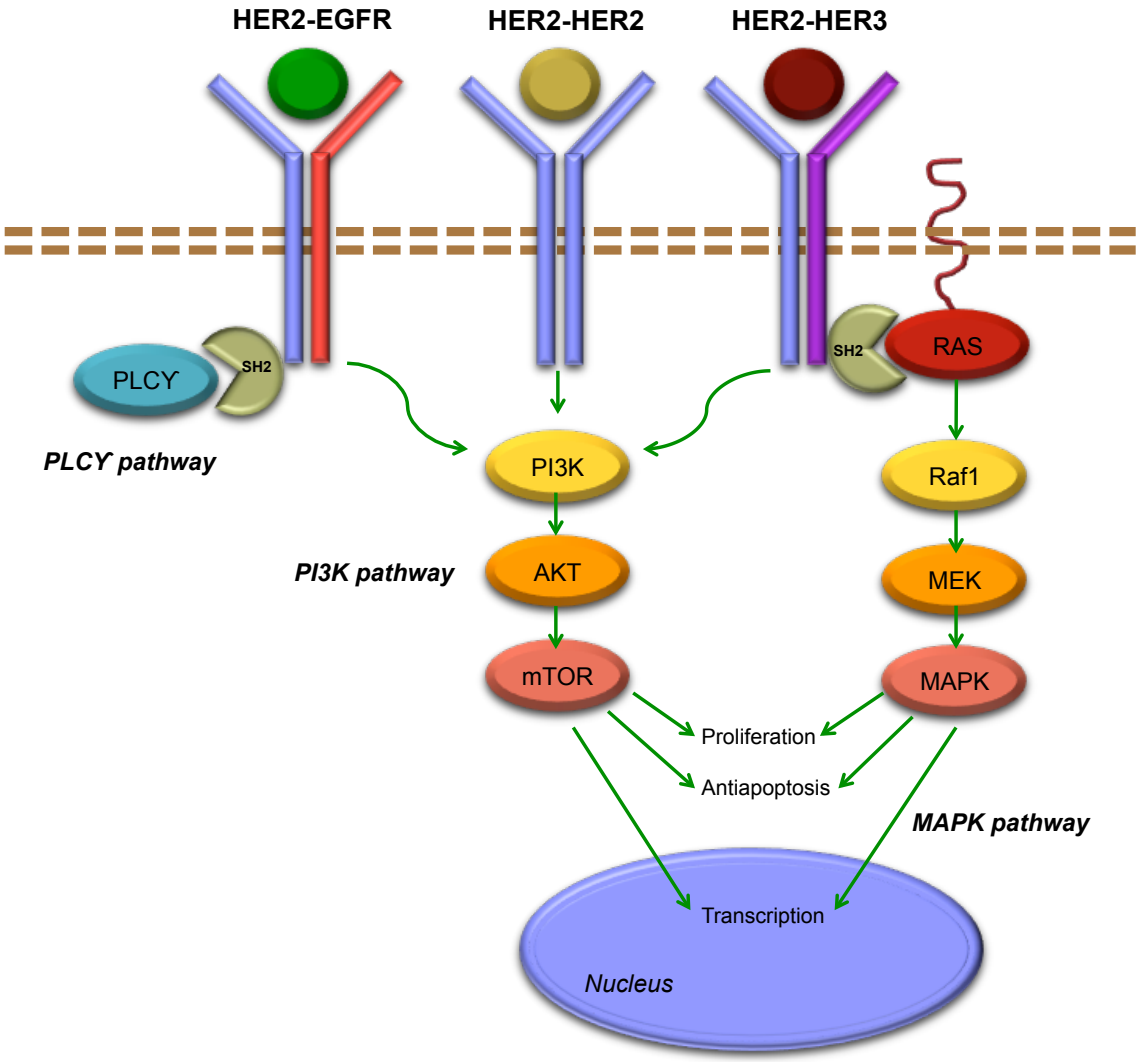

Figure 9. HER2 mediated signaling pathways. PLCγ, PI3K and MAPK are the most common signaling pathways; PI3K and MAPK are highly involved in tumor growth and apoptosis. "Adapted from J Control Release, 146(3), Tai W, Mahato R, Cheng K, The role of HER2 in cancer therapy and targeted drug delivery, 264-75, Copyright (2010), with permission from Elsevier"

1.4.4.3 *HER2* mediated carcinogenesis

HER2 is expressed at low levels in benign lesions and amplified and/or highly overexpressed in 15-20% of all human breast cancers (60-65). Clinical studies in breast cancer showed that *HER2* gene amplification is correlated with recurrence (66), chemoresistance (adriamycin, cytoxan, methotrexate, 5-fluorouracil and TAM), short survival, estrogen and progesterone negative

receptors, high histological grade tumors and positive axillary nodes (67), all of which are considered indicators for poor prognosis. Therefore, *HER2* gene amplification is considered a bad prognostic marker and a predictive factor in response to chemotherapeutics, anti-estrogenics and therapy using specific antibodies to block the receptor function (68).

In addition to aneuploidy and gene amplification, transcriptional deregulation involving enhancer elements located close to the *HER2* promoter, as well as an increased expression of transcriptional factors, have been identified as factors that may cause protein overexpression (62).

1.4.4.4 Relationship between HER2, estrogens and breast cancer

It has been reported in breast cancer that *HER2* oncogene expression is subjected to hormonal regulation, since apparently E2 regulates not only the growth factors expression to HER family (13, 41, 69) but also causes an indirect activation of HER2 protein, thus confirming the existence of a strong association between HER2 overexpression and the activation of signaling pathways mediated by estrogens (13, 41, 69, 70). In HER2-overexpressing tumors, it has been observed an estrogen-independent growth and an increased cross talk between ERs and signaling pathways mediated by EGFR/HER2, which contributes to the development of resistance to endocrine therapy (6, 48).

Several clinical studies have shown that the increased expression of the *HER2* gene is associated more with ER- tumors than with ER+ tumors; the latter is correlated with more aggressive tumor phenotypes and a poor response to TAM treatment (7, 14, 41). Additional evidence suggests that *EGFR* or *HER2* gene overexpression is not a mechanism by which resistance to TAM can be acquired (71); however, the overexpression of both receptors in a tumor, before treatment, predicts poor TAM response. This suggests that the overexpression of these growth factors might contribute to independent hormone growth.

1.4.5 Treatment against breast cancer

Breast cancer is a heterogeneous disease in which each patient has individual characteristics, which has led to the search for new markers in order to improve not only diagnosis but also prognosis and to obtain a better response to therapy. Currently, the strategies for breast cancer treatment attack targets/specific markers that are functionally altered; ERs and the *HER2* gene are examples of these strategies.
The therapeutic management of patients with ER-positive consists in the application of endocrine strategies that seek to block ER with anti-estrogen agent TAM or delete the quantity of ligand (estrogens), either by suppressing gonads in premenopausal women (ovariectomy) or using

aromatase inhibitors in postmenopausal women. These strategies are used for both early and metastatic cases; however, not all patients respond to endocrine therapy, even though patients that initially respond to treatment then acquire resistance to the drug (72).

Although the TAM has been widely used in the treatment of patients with ER+, it has been reported that the use of aromatase inhibitors (AI) such as anastrozole letrazol and exemestan, as well as the use of fulvestrant (a new ER antagonist that binds to the receptor and prevents its dimerization, leading to the degradation and loss of cellular ER) offer better benefits by reducing the estrogens' biosynthesis in target tissues, improving disease-free survival and reducing the risk of recurrence (48, 72, 73). Furthermore, the treatment intended for patients with the *HER2* gene amplification and protein overexpression is to use either monoclonal antibodies that recognize the extracellular *HER2* domain (Trastuzumab) as well as inhibitors of the *HER2* receptor tyrosine-kinase domain (such as lapatinib), or even silencing the gene having RNA interference (58).

1.4.5.1 Tamoxifen

TAM activity is dependent on estradiol circulating levels (E2), which are higher in pre-menopausal women and lower in postmenopausal women. It is a selective estrogen receptors modulator (SERM), because its effect seems to be restricted to ERα positive tumors. The use of this anti-estrogen agent (dose of 20 mg/day) has shown to reduce the occurrence of breast cancer by a 38% of healthy women at high risk of acquiring the disease, decrease the likelihood of recurrence in early breast cancer, prevent the development of cancer in the opposite breast and reduce the risk of developing invasive breast cancer in women who have had ductal carcinoma in situ (DCIS). All the above is explained by TAM's ability to reduce cell proliferation and induce apoptosis in malignant breast cells, among others (74, 75).

In metastatic breast cancer, it has been reported that over 50% of patients with ER-positive tumors get the arrest of tumor progression when they are treated with TAM; however, almost all patients with metastatic disease and the 40% of patients receiving TAM as an adjuvant therapy eventually relapse and die of their disease. Likewise, postmenopausal women with breast cancer at early stages, which have initially responded well to TAM, can eventually become resistant to the drug and develop recurrent tumors (breast tumors and endometrium tumors) (36). Resistance can occur in 30-50% of patients positive for ER (76).

Some explanations for this failure in treatment may be related to:

- Imbalances between estrogens anabolism and catabolism
- Altered bioavailability of TAM

- Alterations in ERα intracellular traffic
- Alterations of signaling pathways involving TAM participation
- Alteration in the regulation of ERα target genes expression at the transcriptional level (36, 77).

Some examples illustrating the latter factors are: resistance to growth inhibitory effects by TGF1, increased AP1 signaling and upregulationof Akt/PI3K, HER2 (78, 79), IGFR-1 (80) and MAP kinases. The activation of these signaling pathways could lead to ligand-independent ER activation by phosphorylation processes (80). Other mechanisms include overexpression of growth factors, increased angiogenesis or heterogeneity of tumor cell population.

TAM is believed to stimulate cellular proliferation by acting on several cellular signaling pathways, including *c-MYC* and MAPKs genes activation. This mitogenic effect might arise as a result of estrogen-altered metabolism (16, 81).

In addition, Aki Mizutani and coworkers (16) reported in 2004 that TAM is carcinogenic and has a high mutagenic potential, blocking replication and causing chromosomal breaking, as well as translocations and deletions that contribute to the initiation and/or tumor progression. Several numerical and structural chromosomal aberrations were observed in rat hepatocytes chromosomes (82) and bone marrow of rats treated with TAM, particularly: chromosomal breaking, deletions, aneuploidy and endoreduplications (83). The induction of chromosomal abnormalities could be due to the formation of DNA adducts favored by TAM, leading to chromosomal instability, which is a very common characteristic associated with carcinogenesis. However, the type and frequency of chromosomal abnormalities and the mechanisms by which TAM induces chromosomal instability are still unknown, and its effect is being studied in order to establish its involvement in disease progression and in the modification of therapeutic response.

1.4.5.2 Trastuzumab and HER2

Trastuzumab is a humanized monoclonal antibody that has been highly effective in treating women with *HER2* positive breast cancer at early or advanced stages and even metastatic stages. This compound binds to the *HER2* extracellular domain, interferes with intracellular signaling pathways mediated by this receptor, and suppresses cell proliferation. A higher survival rate have been reported when this medication is used in combination with chemotherapy (docetaxel, vinorelbine and platinum components) (73).

Currently, other strategies that combine trastuzumab with anti - microtubules agent (trastuzumab- emtansine T-DM1) are being studied, as well as the use of PI3K and mTOR inhibitors or heat shock proteins inhibitors (Tanespimycin)

(73). Likewise, in patients with breast cancer, which are positive for both receptors, HER2 and ER, different therapies are being combined: trastuzumab with letrozole, trastuzumab with anastrazole and lapatinib with letrazole (these drugs block both receptors). In these cases, an increase of 50% in patient survival has been reported (84). Therapeutic strategies combining the use of trastuzumab with endocrine therapies based on inhibitors of relevant points in the intracellular signaling pathways are also assessed, such as Ras/Raf/MAPK and PI3K/Akt/mTOR signaling (31, 48, 84).

Current research in general seeks to obtain genomic and expression profiles in order to identify genes with an important role in generating resistance to endocrine therapy as well as the functioning of cellular signaling pathways involved therein, thus facilitating the creation of customized appropriate therapies, including the selection of doses and chemotherapeutic agents to be used in an individual way (31, 48, 84), in order to achieve the best effect possible.

1.4.6 Cytogenetic' contribution to breast cancer research

Cytogenetic is an invaluable tool for cancer diagnosis and research. The contribution of cytogenetic is conclusive for cancer diagnosis, given that it has contributed to the identification of chromosomal aberrations. In research, it contributes to a better understanding of malignant transformation processes and provides useful information for the development and validation of new cancer treatments.

Although chromosomal banding is widely used to identify chromosomal abnormalities, the resolution is limited because many abnormalities include changes in small segments of chromosomes that cannot be detected by conventional cytogenetic, whereby techniques such as fluorescence *in situ* hybridization (FISH) has been developed. FISH combines cytogenetic techniques with molecular biology techniques; it is based on the use of DNA probes labeled with fluorescence, which recognizes its complementary sequence on the genetic material to be assessed and allows detecting or confirming genetic or chromosomal abnormalities. It is an efficient technique with high reproducibility to pinpoint the location of unique sequences in metaphase chromosomes and facilitate its positioning in banded or unbanded preparations (85).

The FISH technique has numerous technological variants such as spectral karyotyping (SKY, M-FISH), comparative genomic hybridization (CGH) and array comparative genomic hybridization (aCGH), all of which constitute the molecular cytogenetic and provide more accurate detection methods for chromosomal abnormalities in tumor cells. However, these techniques cannot detect balanced chromosomal rearrangements (e.g., translocations or inversions) or low frequency mosaicisms. Together, all are useful tools in determining

the diagnosis, classifying neoplasms in different prognostic subgroups and identifying the most appropriate therapy for each individual.

1.4.6.1 Chromosome markers present in breast cancer

Breast cancer originates from the malignant transformation of normal epithelial cells. Very little is known regarding the appearance order of different alterations in the genome, which include: gene mutations, amplifications, deletions, insertions, translocations, gains and losses of whole chromosomes or parts thereof, among others. Such variety is the result of chromosome polyclonality, which is very characteristic of this neoplasia (86). Some cytogenetic studies suggest that structural abnormalities may occur before numerical abnormalities (87), while other studies have reported the presence of aneuploidies in early breast lesions (88).

It has been shown that monosomies, trisomies, loss of genetic material and over-expression of oncoproteins are significantly correlated with an increased risk of tumor progression, which can be attributed to the gain of oncogenes or loss of tumor suppressor genes located in these chromosomes (61, 89-91). In fact, some authors postulate monosomies as common events playing an important role in the development of breast cancer by demonstrating that invasive intraductal carcinomas have a high degree of monosomies when compared with ductal carcinoma *in situ* (92).

A special interest exists around chromosomes 1 and 17, since several important genes involved in breast cancer, such as the *TP53, HER2* and *BRCA1* (Chr17*)* and *ARHC, P73, MUC1* and *KISS1* (Chr1) genes, are located in these chromosomes (86). All of these genes can be considered as biomarkers for premalignant stages (11, 61, 91).

The most common numerical abnormalities in breast cancer are trisomy of chromosomes 3, 7, 8, 12, 18 and 20 and loss of chromosomes X, 13, 17, 19 and 22 (93-95), while the most common structural abnormalities are those derived from chromosomes 1 and 16, and 1 and 15; deletions of chromosome 1, 3, 6 and 7; inversions of chromosome 7 and isochromosome from chromosome 8 (86, 95, 96). The main chromosomal markers for breast cancer are described in Table 7.

Table 7. Chromosome markers in breast cancer

MARKER	ASSOCIATIONS
der(1;16)(q10;p10) der(1;15)(q10;q10)	Invasive cancer
del(1p)	Poor prognosis
del(1p35-36)	High tumor grade and metastasis
del(1)(q11), del(1)(q12)	Invasive cancer
Gaining in 1q	Primary metastatic disease
del(3p)	Heterozygosity loss (LOH) in 3p11-14, 3p14-23, 3p24-26
Trisomy in 7 (+7)	Low tumor grade
del(7q31)	Poor prognosis and metastasis
Trisomy in 8 (+8)	Metastasis
del(8)(q24)	Low tumor grade and metastasis
del(8p21-22), i(8)(q10)	Metastasis
del(11)(p15)	Familial breast cancer
del(11)(q13)	Metastasis
LOH (13q12-13)	Susceptibility gene for breast cancer (BRCA2) Breast cancer in men
del(16)(q12) and del(16)(q24)	Preinvasive event
del(16)(q21-24)	Invasive disease, distal metastasis
del(17p)	Invasive disease
17q11q12 Amplification	*HER2*, high tumor grade, reduced survival
7q23 Amplification	PPM1D
LOH 17q21	Susceptibility gene for breast cancer (BRCA1) Risk of developing ovary cancer
20q13 Amplification	Aggressive tumors

1.4.6.2 Chromosome abnormalities induced by estrogens

Estrogens can induce chromosomal aberrations, including both numerical (aneuploidy) and structural chromosomal alterations. In both cases, these are induced by secondary metabolites or free radicals generated from oxidative-reduction events (redox) of estrogens (2).

Exposure to natural estrogens or estrogen-like chemicals contributes to the generation of numerical chromosomal changes, both *in vivo* and *in vitro*. It has been reported that estrogens can induce aneuploidies - a key event in the onset of breast cancer correlated with poor clinical outcome and reduced survival (2, 3, 97). In the same way, it has also been reported that estrogens induce or facilitate the acquisition of genetic alterations during neoplastic progression (9, 34, 42-45).

The mechanisms by which E2 exerts its aneugenic activity (induction of aneuploidy) is by direct or indirect pathways. The direct pathway is related to the decrease in the fidelity of mitotic apparatus assembly, since it has been shown that metabolites of these hormones are covalently bound to the C-terminal region of the β-tubulin, and block the polymerization of microtubules.

The indirect pathway is related to the generation of free radicals, which may interfere with chromosomal segregation during anaphase and thus prevents chromosomal disjunction. It has also been reported that E2 induces overexpression of the *Aurora A* and *B* genes, which encodes for centrosome protein kinases associated to mitotic spindle defects, erroneous segregation of chromosomes and genomic instability, as they regulate the mitotic spindle checkpoint between metaphase and anaphase (7-9, 42, 56, 98). In addition, Emma L. Quick and coworkers (97) reported in 2008 that E2 can induce aneuploidy in breast cancer in any chromosome, but with different frequency, and that these occur mainly in chromosomes 1, 7, 8, 11, 12, 17, 18 and 19. Numerical aberrations on chromosome 17 have been correlated with onset, progression and treatment response in breast cancer, as many genes (*HER2, p140, TP53, BRCA1, TOP2A, STARD3)* and changes thereof (*HER2 and STARD3* amplification*, TP53* and *BRCA1* loss, *TOP2A* amplification or deletion) have been identified on this chromosome (99).

HER2 gene amplification has been observed in several breast cancer tumor samples, which also displays chromosomal aneuploidy and other complex chromosomal abnormalities of chromosome 17. To avoid misunderstandings, some authors have suggested the use of additional probes for other loci on chromosome 17, including *SMS* (17p11.2) and *RARA* genes (17q21.2), which serve as additional controls on cases presenting multiple centromere copies (CEP17). The use of these probes have shown that polysomy of chromosome 17 is a rare event in breast

cancer and that a greater copy number than 3 in CEP17, observed by FISH, is often associated with the gain or amplification of the centromere region, showing that chromosome 17 usually presents complex rearrangements (100-105). On the other hand, estrogens are known to cause chromosomal aberrations of the type gene amplifications, translocations and deletions, which confirm their properties as mutagenic and carcinogenic factors (2, 7-10); however, there are few studies that describe the structural chromosomal abnormalities and the frequency in which they occur when cells are exposed to estradiol.