Leila SABOUR TAKANLOU

RETROSPECTIVE ANALYSIS OF *HER2 / HSP90 / SIAH* GENE EXPRESSIONS IN INVASIVE DUCTAL CARCINOMA BREAST CANCER PATIENTS

MASTER OF THESIS

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THESIS SUPERVISOR:
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"İnvaziv Duktal Karsinomlu Meme Kanserli Hastalarda *HER2 / HSP90 / SIAH* Gen Ekspresyonlarının Retrospektif Olarak İncelenmesi" adlı çalışmanın, proje safhasından sonuçlanmasına kadar geçen bütün süreçlerde bilimsel etik kurallarına uygun bir şekilde hazırlandığını ve yararlandığım eserlerin kaynaklar bölümünde gösterilenlerden oluştuğunu belirtir ve beyan ederim.

Leila SABOUR TAKANLOU
19.08.2019

KABUL VE ONAY SAYFASI

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Tıbbi Biyoloji Anabilim Dalı Yüksek Lisans öğrencisi Leila SABOUR TAKANLOU tarafından hazırlanan **"İnvaziv Duktal Karsinomlu Meme Kanserli Hastalarda *HER2* / *HSP90* / *SIAH* Gen Ekspresyonlarının Retrospektif Olarak İncelenmesi"** konulu Yüksek Lisans tezi 19/08/2019 günü, ...-..... saatleri arasında yapılan tez savunma sınavında jüri tarafından oy birliği/oy çokluğu ile kabul edilmiştir.

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ÖZELLİKLER	UYGUNDUR	UYGUN DEĞİLDİR	AÇIKLAMA
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Dış Kapak Sayfası	☑	☐	
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DANIŞMAN ONAYI
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İmza:

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Meme kanseri kadınlar arasında en sık görülen kanser türüdür. Sıklığı sürekli artış göstermekte olup, önde gelen ölüm nedeni olmaya devam etmektedir. İnsan epidermal büyüme faktör reseptörü 2 (*HER2*), tirozin kinaz aktivitesine sahip epidermal büyüme faktör reseptör ailesinin bir üyesidir ve meme kanserinin önemli prognostik faktörlerinden biridir. *HER2* için hedefe yönelik tedavilerin başlamasıyla *HER2* pozitif kanserli hastaların klinik seyrinde ciddi iyileşme sağlanmıştır. Günümüzde *HER2* testi değişik yöntemlerle yapılmaktadır ve *HER2* ifade durumunu doğru değerlendirebilmek için bu testlerin standardizasyonu çok önemlidir. Ayrıca *HER2* geni, ısı şok protein ailesinin bir üyesi olan *HSP90*'ı hedefleyerek aktivitesini arttırmaktadır. *SIAH* (yedi homolog homolog-2) gibi *E3* ubiquitin ligazlar ise, *HSP90*'a bağlanarak hedef proteinlerin parçalanmasına yol açan ko-şaperon olarak görev yaparlar. Bu çalışmada, RT-PCR yöntemi ile İHK yöntemi arasındaki korelasyonun değerlendirilmesi amaçlanmıştır. İlaveten, invaziv duktal meme kanserinde *HER2 / HSP90 / SIAH1 / SIAH2* genlerinin ifadeleri ve ifade farklılıklarının hastaların klinikopatolojik özelikleri ile karşılaştırılarak prediktif ve prognostik önemlerinin değerlendirilmesi amaçlanmıştır. Mevcut çalışmada 94 invaziv meme kanserli hastada *HER-2* ifade durumu değerlendirilerek elde edilen bulgular ile *HER2* mRNA ifadelerinin İHK ile 74.35% oranında korele olduğu belirlendi. *HER2*-pozitif meme kanserli hastaların tümör dokularında *HSP90* ve *SIAH2* genlerinin ifadelerinin istatistiksel olarak anlamlı olarak up-regüle edildiği belirlendi (p= 0.047; p= 0.024). İnvaziv duktal meme kanserli hastalarda *HSP90* ve *HER2* mRNA ekspresyonunun arasında korelasyon olduğu, ayrıca *SIAH2* mRNA ekspresyonunun, *HSP90* ve *HER2* ekspresyonları ile korelasyon gösterdiği belirlendi.

Anahtar Kelimeler *HER2, HSP90, SIAH1, SIAH2, IDC*

ENGLISH ABSTRACT

Retrospective Analysis of *HER2 / HSP90 / SIAH* Gene Expressions in Invasive Ductal Carcinoma Breast Cancer Patients

Among females, breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death. Human epidermal growth factor receptor 2 (*HER2*) is a member of the epidermal growth factor receptor family that is an important prognostic factor of breast cancer. The introduction of targeted therapies for *HER2* has dramatically influenced the outcome of patients with *HER2* positive breast cancers. *HER2* testing is realized by different methods and it is crucial to standardize testing techniques to evaluate *HER2* status accurately. *HER2* is a client protein of *HSP90*, a member of the family of heat shock proteins (*HSP*s), which are considered molecular chaperones, as they are responsible for the correct folding of denatured or translated proteins. The *HSP* chaperone activity is tightly regulated by interaction with E3 ubiquitin ligase as seven-in-absentia homolog-2 (*SIAHs*). In this thesis, we aimed to compare RT- PCR and IHC in the detection of *HER2* expression in 94 invasive ductal carcinoma breast cancers, document the level of *HER2/ HSP90/SIAH1/SIAH2* expressions in invasive ductal breast cancer, correlate of 4-gene assay expressions with clinicopathological factors, to assess the predictive and prognostic value of the 4-gene assay with RT-PCR in breast cancer patients. We established that *HER2* mRNA expressions were coordinated 74,35% with IHC. Our study showed that in *HER2*-positive (measured with RT-PCR) breast cancers *HSP90* and *SIAH2* expressions in tumor tissues significantly were up-regulated (p= 0,047; p= 0,024). There was a significant correlation between mRNA expressions of *HSP90* and *HER2*, and also *SIAH2* expression was correlated with *HSP90* and *HER2* expressions.

Keywords *HER2, HSP90, SIAH1, SIAH2, IDC*

1. INTRODUCTION

Among females, breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death, followed by colorectal and lung cancer (for incidence), and vice versa (for mortality); cervical cancer ranks fourth for both incidence and mortality. Invasive ductal carcinomas are breast cancers having malignant ductal proliferation along with stromal invasion in the presence or absence of *DCIS* (ductal carcinoma in situ), apart from their relative proportion. *IDC*s (invasive ductal carcinoma) are a heterogenous group of tumors classified according to cytoarchitectural features, as they have a wide scope of morphological variation. Some of them have enough distinctive features and particular behavior to be classified as special subtypes, while the majority, which constitutes about 75% of *IDC*, fail to exhibit sufficient morphological features to be classified as specific histological types and are generally designated as *IDC* not otherwise specified. Breast cancer is a heterogeneous disease, with treatment decisions and prognosis traditionally guided by immunohistochemistry (*IHC*) markers such as estrogen receptor (*ER*), progesterone receptor (*PR*), human epidermal growth receptor 2 (*HER2*), and *Ki67* (a proliferation index marker), along with tumor size, tumor grade, and nodal status. Breast cancers with *HER2*-overexpressed are found in 15-25% of IDCs and they display a worse prognosis but response good to therapies. *HER2+* breast cancer is often associated with significantly shorter disease-free survival and worse overall survival rates than other subtypes of breast cancer. Four major anti-*HER2* drugs for *HER2+* breast cancer, including monoclonal antibodies, small-molecule tyrosine kinase inhibitors, antibody-drug conjugates, and other emerging anti-*HER2* agents are drugs against the extracellular domain of *HER2*. Crosstalking *HER2* with other pathways, such as epidermal growth factor receptor (*EGFR)/HER1*, insulin-like growth factor1 receptor (*IGF-1R*), estrogen receptor pathway and the *PI3K/Akt/mTOR* pathway has been reported to reduce the effectiveness of trastuzumab. Also, *HER2* is a client protein of *HSP90*, a member of the family of heat shock proteins (*HSP*s), which are considered molecular chaperones, as they are responsible for the correct folding of denatured or translated proteins. It has been suggested that *HSP90* expression may also modulate the effects of oncogenic *HER2*,

representing a potential mechanism of resistance to *HER2* directed drugs. The discrepancy between *HSP90* expression in *IDC* and *ILC* is rather very interesting. High *HSP90* expression in primary breast cancer has been described as a poor prognostic marker in breast cancer. *HSP90* expression was variable in patient tumors compared to cancer cell lines. Whether this expression is also variable between primary and metastatic tumors is unknown at this time. It is important to note that HSP90 inhibitors have been used in breast cancer only in the metastatic and refractory settings. Additionally, studies have not described HSP90 expression as a marker that can predict response to HSP90 inhibitor therapy. On the other hand, *HSP90* inhibitors may potentiate the effects of anti-cancer drugs targeting client proteins of *HSP90.* The *HSP* chaperone activity is tightly regulated by interaction with many co-chaperones; and co-chaperones functions as an *E3* ubiquitin ligase using a modified *RING* finger domain (*U-box*). Co-chaperones is involved in the ubiquitination and degradation of the client *ERBB2* following *HSP90* inhibition. However, studies have shown that *E3* ubiquitin ligase seven-in-absentia homolog-2 (*SIAH2*) with the pharmacological inhibition of *HSP90* suppresses the phosphorylation of *STAT1* and *STAT3. SIAH* proteins are evolutionary conserved RING-type *E3* ubiquitin ligases emerging as critical regulators in both normal development and cancer. *SIAH* proteins exert their primary functions by targeting selected proteins for proteasomal degradation by polyubiquitination. *SIAH* acts as an essential downstream signaling component required for proper *EGFR/HER2* and *RAS* signaling. Activation of the tumor necrosis factor-alpha (*TNF-a*) receptor (*TNFR*) or/and the transforming growth factor-beta (*TGF-b*) receptor (*TGFR*) causes a loss of *SIAH* proteins without influencing their mRNA levels. *SIAH*s are also involved in cytokine signaling modulating the epithelial to mesenchymal transition (*EMT*). As well as, reported which, *SIAH* may represent a useful prognostic biomarker that predicts *DCIS* progression to invasive breast cancer. Two recent genome-wide association studies identified a genetic variant in the *SIAH2* locus associated with *ER*-positive breast cancer, suggesting that the expression of *SIAH2* might be affected by this genetic variant and consequently correlated with *ER*-related tumor progression or hormone therapy response. In addition, studies noted that *SIAH2* significantly predicted first-line tamoxifen treatment failure in breast cancer.

However, the role of *SIAH1 and SIAH2* in breast carcinoma is unclear, we investigated the frequency and expression pattern of *SIAH1 and SIAH2* expression in breast cancer patients.

Aim of this work: (1) document the level of *HER2/ HSP90/ SIAH1/ SIAH2* expression in breast cancer, (2) correlate of *HER2/ HSP90/ SIAH1/ SIAH2* expressions with conventional clinicopathological features, (3) investigate associations of *HER2/ HSP90/ SIAH1/ SIAH2* expressions with intrinsic subtypes of breast cancer, (4) determine the effect of *HER2/ HSP90/ SIAH1/ SIAH2* expressions on relapse-free survival, (5) compare of qRT-PCR and IHC for *HER2* gene status in formalin-fixed paraffin-embedded tissue samples of breast cancer. Here we employed a quantitative PCR method to detect *HER2/ HSP90/ SIAH1/ SIAH2* gene expressions in one hundred formalin-fixed and paraffin-embedded (FFPE) breast cancer tissue samples from the breast cancer patients. Relative expression of *HER2/ HSP90/ SIAH1/ SIAH2* mRNA in the FFPE samples was analyzed using a quantitative RT-PCR (RT-qPCR) method.

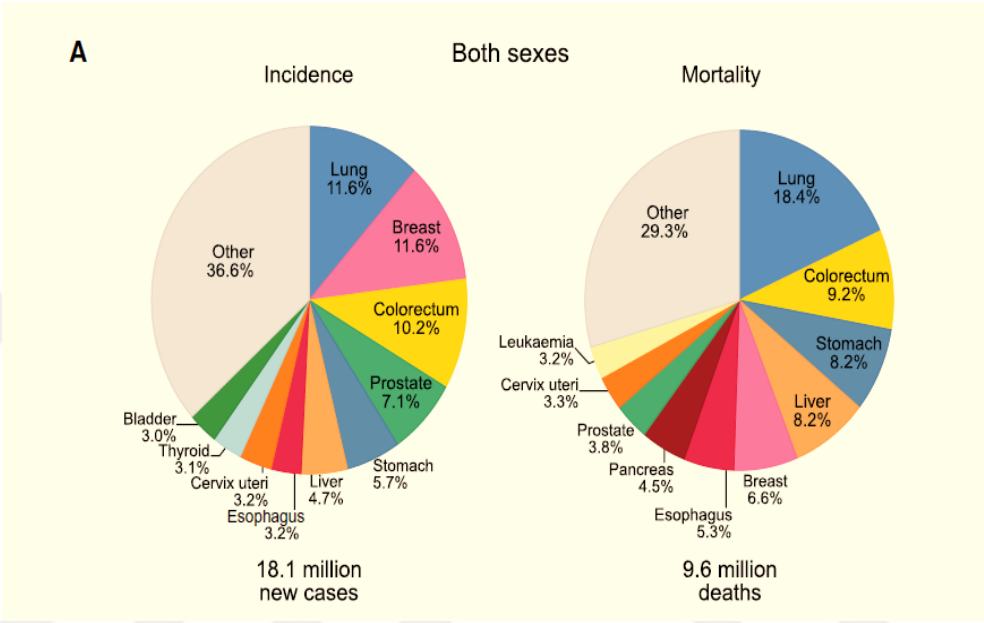
2. BACKGROUND

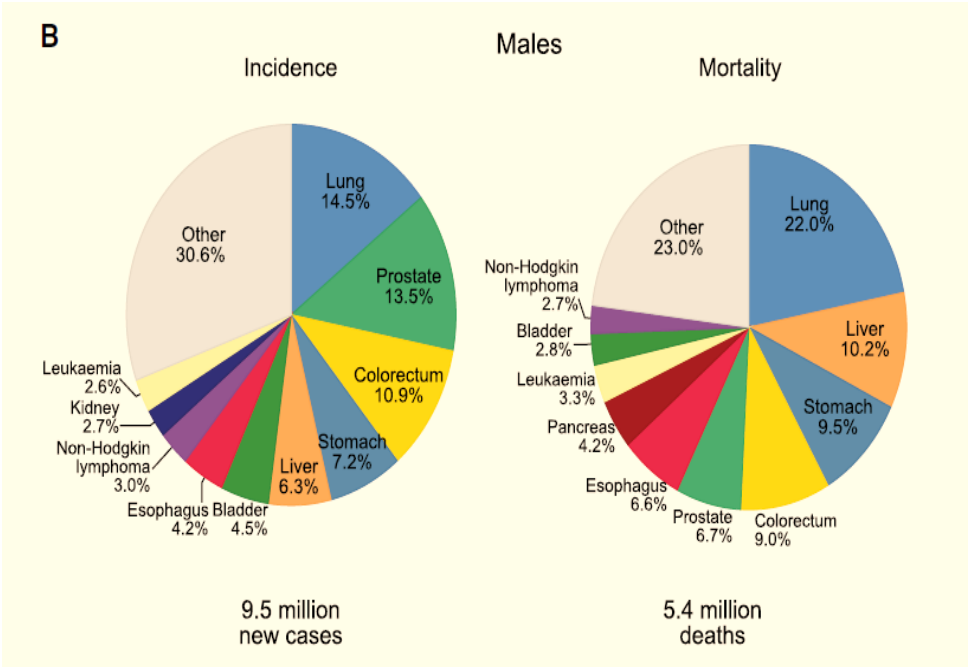
2.1 Breast Cancer Epidemiology

Noncommunicable diseases (*NCDs*) are now responsible for the majority of global deaths, (World Health Organization., 2018) and cancer is expected to rank as the leading cause of death and the single most important barrier to increasing life expectancy in every country of the world in the 21st century. According to estimates from the World Health Organization (WHO) in 2015, cancer is the first or second leading cause of death before age 70 years in 91 of 172 countries. Cancer incidence and mortality are rapidly growing worldwide. The reasons are complex but reflect both aging and growth of the population, as well as changes in the prevalence and distribution of the main risk factors for cancer, several of which are associated with socioeconomic development (Gersten et al., 2002; Omran et al., 1971). Among females, breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death, followed by colorectal and lung cancer (for incidence), and vice versa (for mortality); cervical cancer ranks fourth for both incidence and mortality. The most frequently diagnosed cancer and the leading cause of cancer death, however, substantially vary across countries and within each country depending on the degree of economic development and associated social and lifestyle factors (Bray et al., 2018).

When data of developed and developing countries are reviewed, incidences and profiles of cancers differ between the countries. In developed countries, lung and prostate cancers in men and breast and colorectal cancers in women are more common. On the other hand, lung, stomach and liver cancers in men and breast and cervical cancers in women are more common in developing countries. Bray and et al, provided a status report on the cancer burden worldwide in 2018, based on the GLOBOCAN *2018* estimates of cancer incidence and mortality produced by the International Agency for Research on Cancer (IARC) (Bray et al., 2018). In both sexes combined, lung cancer is the most commonly diagnosed cancer (11.6% of the total cases) and the leading cause of cancer death (18.4% of the total cancer deaths), closely followed by female breast cancer (11.6%), prostate cancer (7.1%), and colorectal cancer (6.1%) for incidence and colorectal cancer (9.2%), stomach cancer

(8.2%), and liver cancer (8.2%) for mortality (Figure 1). Worldwide, it was estimated which there will be about 2.1 million cases of female breast cancer cases in 2018, and accounting for almost 1 in 4 cancer cases among women (Figure 1) (Bray et al., 2018).





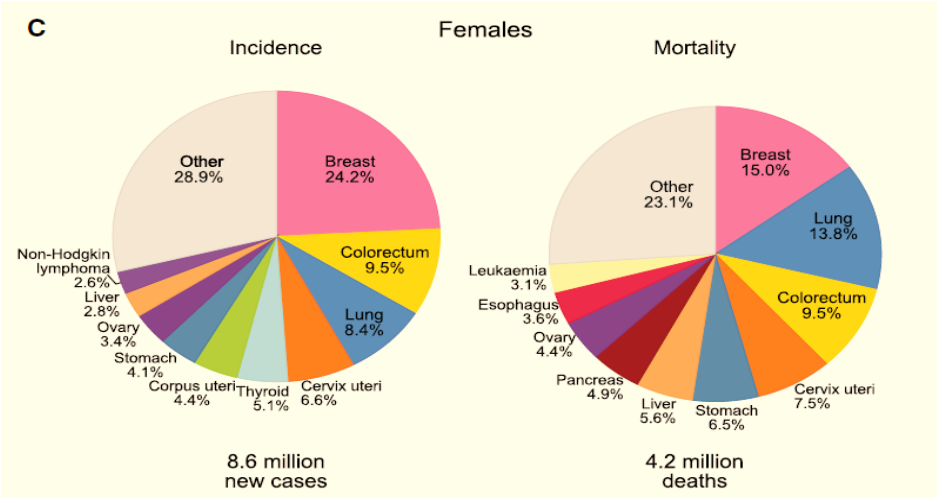


Figure 1. Pie charts present the distribution of cases and deaths for the 10 most common cancers in 2018 for (A) both sexes, (B) males, and (C) females.

The cancer is the most frequently diagnosed in the vast majority of the countries (154 of 185) and is also the leading cause of death in over 100 countries; the main exceptions are Australia/New Zealand, Northern Europe, Northern America (where it is preceded by lung cancer), and many countries in Sub-Saharan Africa (because of elevated cervical cancer rates). Breast cancer incidence rates are highest in Australia/New Zealand, Northern Europe (eg, the United Kingdom, Sweden, Finland, and Denmark), Western Europe (Belgium [with the highest global rates], the Netherlands, and France), Southern Europe (Italy), and Northern America (Figure 2 and Figure 3).

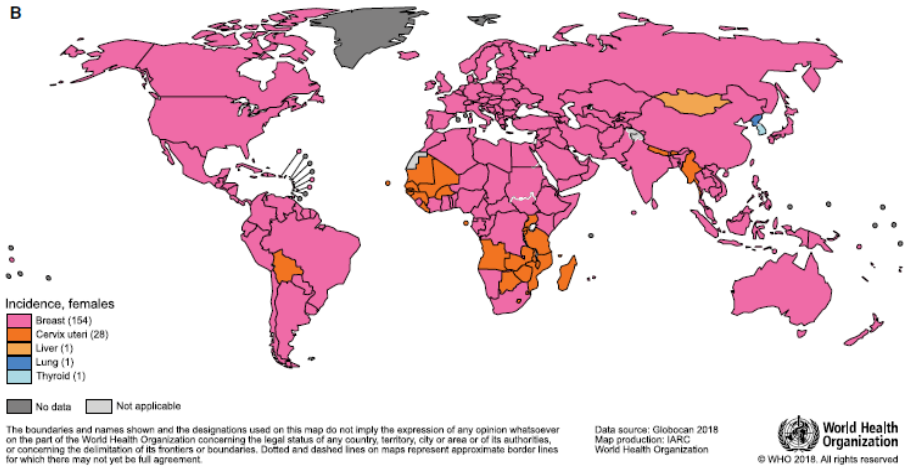


Figure 2. Global maps presenting the most common type of cancer ıncidence in 2018 in each country among (A) women.

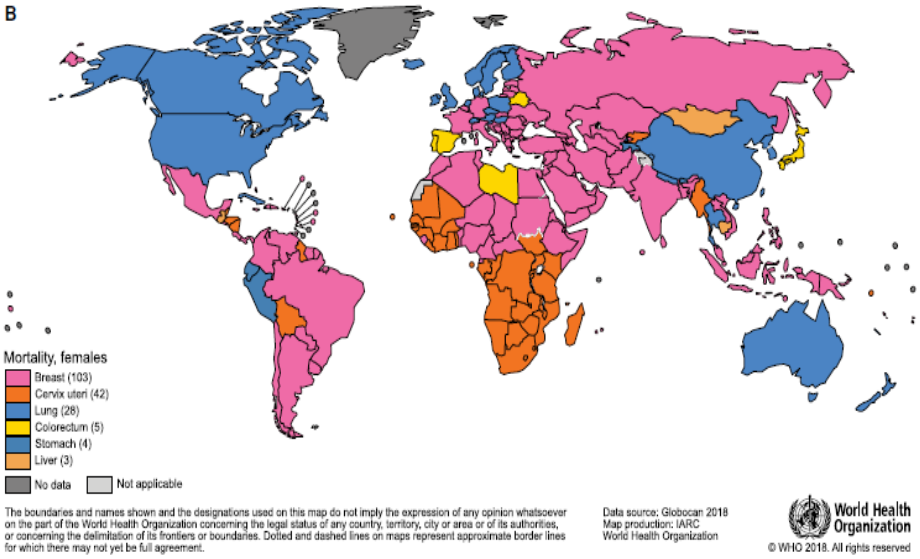


Figure 3. Global maps presenting the most common type of cancer mortality by country in 2018 among women.

Cancer is the most frequently diagnosed disease in Turkey and is also the leading cause of death in Turkey (Figure 4). In Turkey, lung, prostate and urinary bladder cancer are more common in the male population. Breast, thyroid and colorectal cancers appear more in the female population (Figure 5). In Turkey, was estimated which there will be about 22, 345 cases of female breast cancer cases in 2018 (Figure 6); and so far there is no enough statistical data for 2019.

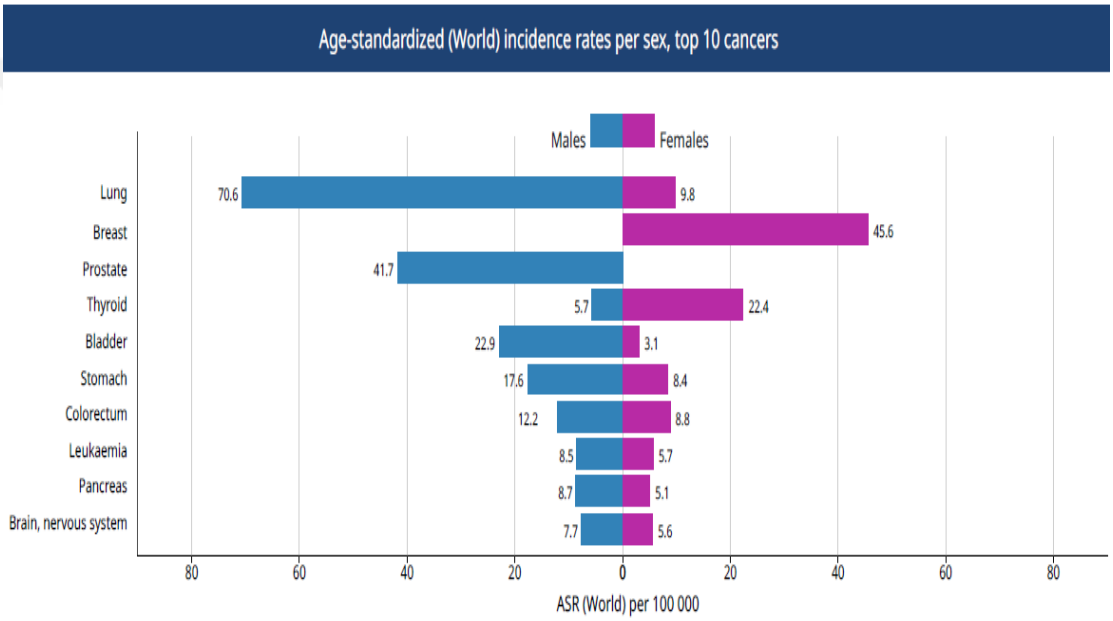


Figure. 4 Incidence rates per sex, the 10 most common cancers in Turkey are shown in descending order of the overall age-standardized rate for both sexes combined. Source: GLOBOCAN 2018.

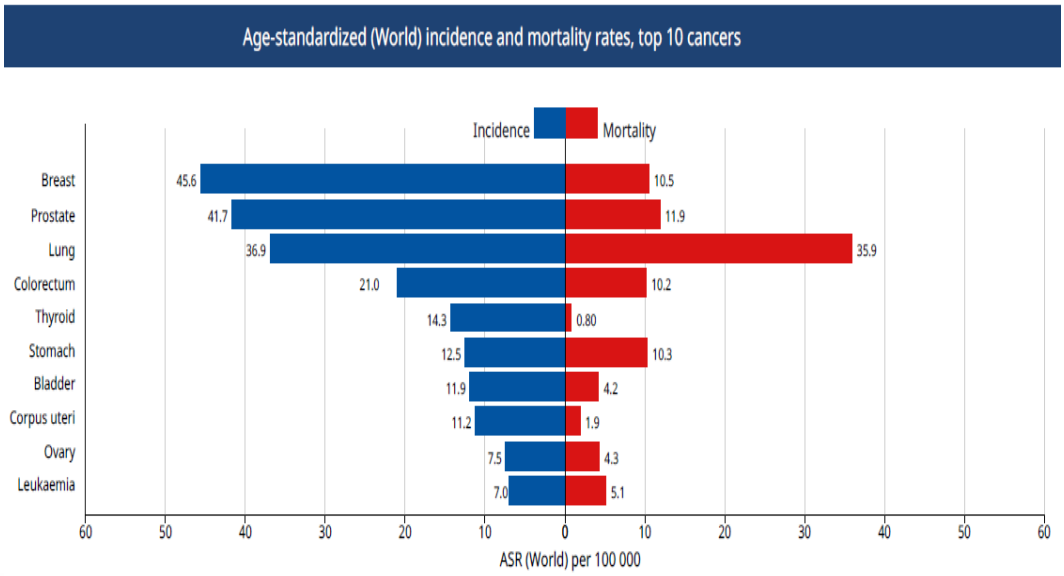


Figure. 5 Incidence and mortality rates, the top 10 cancers in Turkey are shown in descending order of the overall age-standardized rate for both sexes combined. Source: GLOBOCAN 2018.

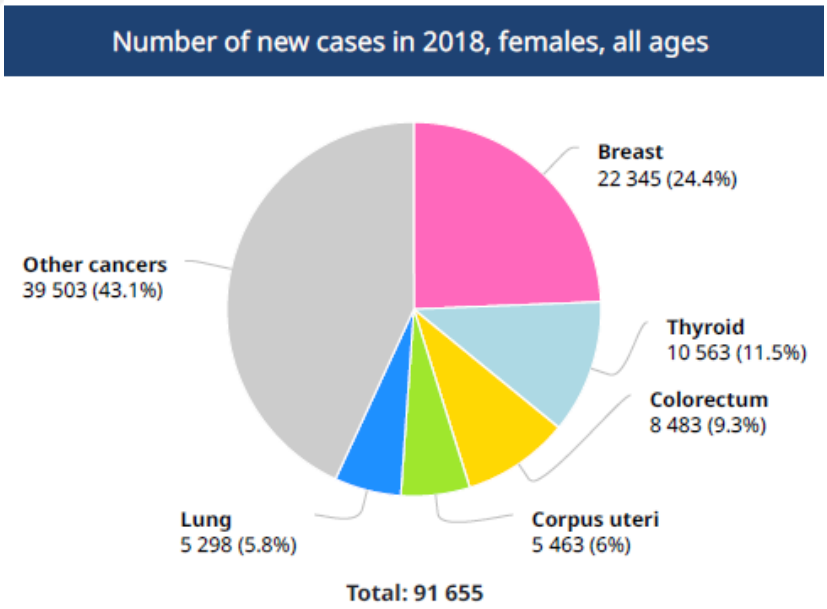


Figure. 6 Number of new cases in 2018, females, all ages. Source: GLOBOCAN 2018.

2.2. The Normal Female Breast

The female breast consists of the mammary gland, a specific type of apocrine gland. It consists of about 20 compartments, alveoli. The alveoli are lined with milk-secreting cells called cuboidal cells, a type of epithelial cells formed as cubes. Myoepithelial cells surround the cuboidal cells and together these cells are called the lobular cells, creating the lobular gland (Sopel et al., 2010), visualized in Figure 7. Myoepithelial cells contracts by stimulation of the hormone oxytocin leading to lactation through milk ducts to the nipples. The milk duct is called lactiferous duct and is lined with epithelial cells surrounded by myoepithelial cells (Sopel et al., 2010). Adipose tissue and connective tissue is surrounding the lobules and the ducts, and also blood vessels and lymphatic vessels.

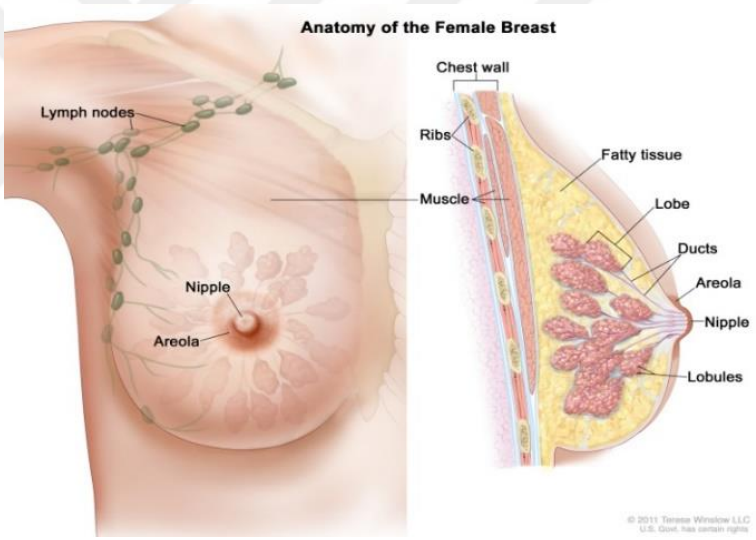


Figure 7. Anatomy of the female breast. The nipple and areola are shown on the outside of the breast. The lymph nodes, lobes, lobules, ducts, and other parts of the inside of the breast are also shown (Sopel et al., 2010).

2.3. Breast Carcinoma and Risk Factors for Breast Cancer

Increasing age is the most important risk factor for most cancers. Other risk factors for breast cancer include the following (Bethesda., 2002):

- Family health history.
- Major inheritance susceptibility.

-Germline mutation of the *BRCA1* and *BRCA2* genes and other breast cancer susceptibility genes.

- Alcohol intake.
- Breast tissue density (mammographic).
- Estrogen (endogenous).

-Menstrual history (early menarche/late menopause).

-Nulliparity.

-Older age at first birth.

- Hormone therapy history.

-Combination estrogen plus progestin hormone replacement therapy.

- Obesity (postmenopausal).
- Personal history of breast cancer.
- Personal history of benign breast disease (BBD) (proliferative forms of BBD).
- Radiation exposure to breast/chest (Bethesda., 2002).

Most of the breast malignancies are adenocarcinomas, which compose more than 95% of breast cancers (Vinay et al., 2010). Invasive ductal carcinoma (*IDC*) is the most common form of invasive breast cancer. It accounts for 55% of breast cancer incidence upon diagnosis (Eheman et al., 2009). Breast carcinomas arise from the same segment of the terminal duct lobular unit (*TDLU*). The typing of invasive breast carcinoma and its histological variants is well established. In general, breast carcinoma is divided into ductal carcinoma in situ (*DCIS*) and *IDC*. However, *DCIS* is a noninvasive potentially malignant intraductal proliferation of epithelial cells that are confined to the ducts and lobules. Invasive or infiltrative carcinoma refers to malignant abnormal proliferation of neoplastic cells in the breast tissue, which has penetrated through the duct wall into the stroma. Invasive carcinoma and carcinoma in situ were classified as ductal and lobular based on the site from which the tumor

originated. Cancers originating from the ducts are known as ductal carcinomas, while those originating from the lobules are known as lobular carcinomas (Vinay et al., 2010).

2.3.1. Microscopic Types of Breast Carcinoma

Breast carcinoma is usually classified primarily by its histological appearance, originating from the inner lining epithelium of the ducts or the lobules that supply the ducts with milk (Rosai et al., 2011).

2.3.1.1. Ductal Carcinoma in Situ and Lobular Carcinoma in Situ

Ductal carcinoma in situ (*DCIS*) is a non-obligate precursor, non-invasive malignancy confined within the basement membrane of the breast ductal system. There is a wide variation in the natural history of *DCIS*. The estimated incidence of progression to invasive ductal carcinoma is at least 13%-50% over a range of 10 or more years after initial diagnosis. Regardless of the treatment strategy, long-term survival is excellent. The controversy surrounding *DCIS* relates to preventing undertreatment, while also avoiding unnecessary treatments. *DCIS*-associated mortality is low, with the expected cumulative breast cancer mortality ten years after *DCIS* estimated to be 2.3% for women < 50 years of age and 1.4% for women > 50 years of age after treatment (Hong et al., 2018). Because of these excellent long-term outcomes, there are various prognostic variables including genetic profiling, grade, necrosis, morphology, and size that are routinely evaluated to guide the management of *DCIS* in efforts to minimize over-treatment. It is especially important as the incidence of *DCIS* has increased in the last 20 years for all races and all ages as a result of the increased utilization of screening mammography (Hong et al., 2018). An accurate histologic diagnosis including grade, histologic type, the presence of calcifications, estrogen, and progesterone as well as understanding the distribution of *DCIS*, is paramount to adequate treatment planning (Hong et al., 2018).

Lobular carcinoma in situ (*LCIS*) is both a risk factor and a non-obligate precursor of breast carcinoma. The relative risk of invasive carcinoma after a diagnosis of classic *LCIS* is approximately 9-10 times that of the general population. *LCIS* occurs predominantly in premenopausal women, with a mean and median age at diagnosis

of 49 and 50 years, respectively (range 20s-80s). *LCIS* is multicentric in 60-80% of patients and bilateral in 20-60% (Wen et al., 2018). *LCIS* and *ILC* share common copy number alterations and somatic mutations. A subset of *LCIS* is clonally related to synchronous or subsequent invasive breast carcinoma. Classic *LCIS* diagnosed on core biopsy with concordant imaging and pathologic findings does not mandate surgical excision. Classic *LCIS* is clinically and mammographically occult. Active surveillance and chemoprevention are management options for classic *LCIS*. The identification of variant *LCIS*, in a needle core biopsy specimen, mandates surgical excision, regardless of radiologic-pathologic concordance. The presence of variant *LCIS* close to the surgical margin of a resection specimen is reported, and re-excision should be considered (Wen et al., 2018).

2.3.1.2. Invasive Ductal Carcinoma and Invasive Lobular Carcinoma

Invasive ductal carcinomas are breast cancers having malignant ductal proliferation along with stromal invasion in the presence or absence of *DCIS*, apart from their relative proportion. The appearance of the invasive component should be determined from the subtypes of *IDC* rather than from the types of *DCIS* or its grade. *IDC* is classified into many histological subtypes according to a wide range of criteria, including cell type (as in apocrine carcinoma), amount, type and location of secretion (as in mucinous carcinoma), architectural features (as in papillary, tubular, and micropapillary carcinoma), and immunohistochemical profile (as in neuroendocrine carcinoma) (Rosai et al., 2011). *IDC*s are a heterogenous group of tumors classified according to cytoarchitectural features, as they have a wide scope of morphological variation. Some of them have enough distinctive features and particular behavior to be classified as special subtypes, while the majority, which constitutes about 75% of *IDC*, fail to exhibit sufficient morphological features to be classified as specific histological types and are generally designated as *IDC* not otherwise specified (Lakhani., 2012).

In comparison with *IDC*, *ILC* is more difficult to detect by standard imaging techniques like mammography (Hogan et al., 2015; Lopez et al., 2009). In general, *ILC* is detected in patients at an older age and at a more advanced stage than *IDC* (Li et al., 2005). Compared to stage/grade- matched *IDC*, patients with *ILC* display

relative late recurrences and worse long-term survival (Li et al., 2005). Endocrine therapy and chemotherapy are frequently used to treat both *ILC* and *IDC*, patients with *ILC* may have lower response rates to neoadjuvant chemotherapy and slightly worse outcomes to tamoxifen compared to patients with *IDC*. The main differences between the two histological subtypes are the lack of E-cadherin (*CDH1*) protein expression in ~90% of *ILC* (Desmedt et al., 2015; Du et al., 2018). *ILC* more often expresses estrogen receptor (*ER*) than *IDC*, with ~90% of *ILC* being *ER*-positive. *ILC* also has high rates (50–70%) of progesterone receptor (*PR*)-positivity, but less than 10% express epidermal growth factor receptor 2 (*HER2/ERBB2*) (Du et al., 2018). While *ILC* generally exhibits lower *Ki67* positivity than *IDC*, it has a higher frequency of *HER2* and *HER3* mutations, *PIK3CA* mutations, *FOXA1* mutations, *ESR1* amplifications, and *PTEN* loss. While there has been a recent characterization of the differences between *ILC* and *IDC* at the genomic level differences in gene expression have not been sufficiently studied (Du et al., 2018).

2.4. Molecular Classification

Breast cancer is a heterogeneous disease, with treatment decisions and prognosis traditionally guided by immunohistochemistry (*IHC*) markers such as estrogen receptor (*ER*), progesterone receptor (*PR*), human epidermal growth receptor 2 (*HER2*), and *Ki67* (a proliferation index marker), along with tumor size, tumor grade, and nodal status. Currently, two methods can determine subtype: gene-based assays and *IHC*-based markers. Three subtypes of breast tumors with different biologic behaviors were discovered using the traditional *IHC* techniques: hormone-receptor-positive, triple-negative, and Human Epidermal Receptor (HER) 2/neu-positive breast cancers (SHerschkowitz et al., 2007; Huang et al., 2003; Sorlie et al., 2001; Sorlie et al., 2003). Genome-wide expression profiling and staggered clustering have now enabled us to identify additional subtypes. Now know that breast cancer comprises different biologic subtypes (Sorlie et al., 2001). They include luminal A, luminal B, HER2-enriched, basal-like, claudin-low, and normal breast-like (Kittaneh., 2011).

2.4.1. Luminal Subtype

Almost 70% of breast cancers are hormone receptor-positive, and they show a more suiteble prognosis than hormone receptor-negative. In breast cancer, more of tumors are luminal A and luminal B subtypes (Prat et al., 2015). The luminal B subtypes tend to display more expression of genes (Sorlie et al., 2001) and distant recurrence-free survival at 5 years and 10 years (Ng et al., 2015; Sorlie et al., 2001). In addition, at 5-year follow-up, basal-like tumors demonstrate worse results than luminal B breast cancers, and at about 10-year follow-up, the survival curves of luminal B breast cancers overlook to transverse those of basal-like tumors (Sorlie et al., 2001). Studies have reported that there are 30-44% discordance values in the classifications based on gene expression predictors and surrogate classifications using *IHC* of *Ki-67* and *PR* rations (Chia et al., 2012). Within *HR*-positive/*HER2*-negative tumors, occurrence values of the non-luminal breast cancer subtypes by gene expression profiling are as follows: the *HER2*-enriched type and the basal-like type breast cancer tumors (Sorlie et al., 2001). The non-luminal subtypes of early breast cancers demonstrated worse results compared to the luminal A breast cancer subtype when they were treated with 5 years of tamoxifen (Prat et al., 2012).

2.4.2. Basal-like Subtype

Basal-like breast cancer tumors have the worst prognosis, while luminal A tumors have the best. As the majority (86%) of triple-negative breast cancers (*TNBC*) those that show as estrogen receptor-negative, progestogen receptor-negative, and HER2-negative communicate to the basal-like breast cancer subtype (Sorlie et al., 2001. However, within the set of *TNBC* tumors, which composed up 10-20% of all tumor breast cancers, all the intrinsic subtypes exist (Figure 8).

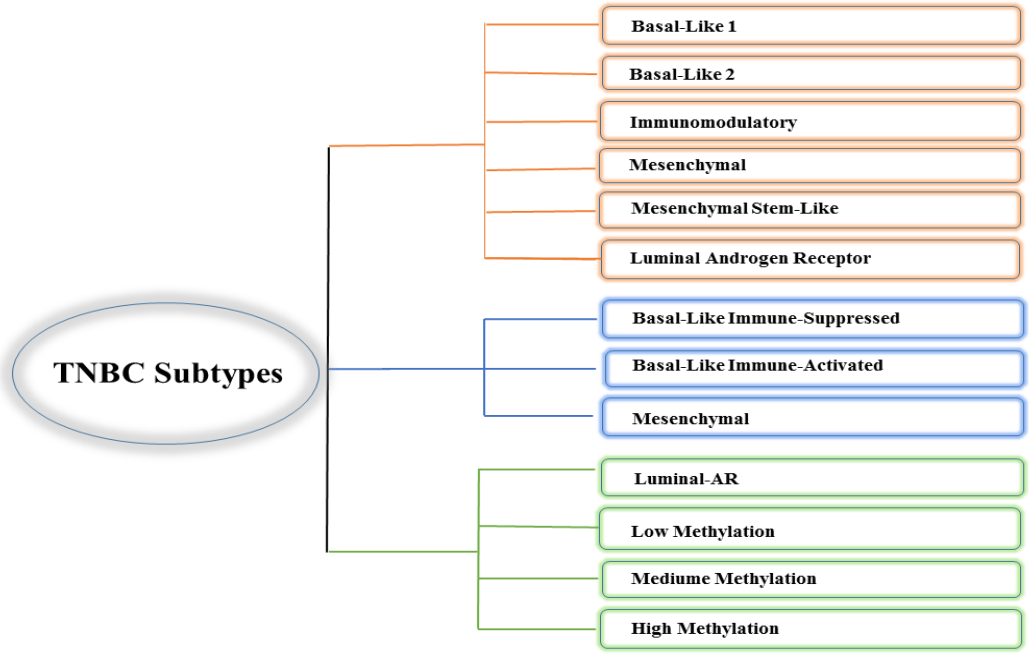


Figure. 8 TNBC classifications. Brian's (Red), Matthew's (Blue), and Clare's methylation subtyping (Green) (Lehmann et al., 2011).

Rates of pathologic complete response following anthracycline or taxane therapy are 25-35%, and patients achieving a pathologic complete response have better results from among those patients with *TNBC* (Liedtke et al., 2008). The distinction between non-basal-like breast cancer subtypes and basal-like breast cancer subtypes within triple-negative breast cancer is main for the choice of chemotherapy, in that carboplatin chemotherapies are as well as docetaxel in basal-like breast cancer subtypes, but less so in other intrinsic subtypes in the metastatic setting (Coates et al., 2015). Tumor-infiltrating lymphocytes are most often found in *TNBC* or *HER2*-positive cancers improved overall survival outcomes (Coates et al., 2015). Also has been suggested that genes involved in immune, inflammatory may be associated to the prognosis of hormone receptor-negative breast cancer tumors, and that proliferation-related gene is associated to the prognosis of hormone receptor-positive breast cancer tumors (Ng et al., 2015).

2.4.3. HER2-Enriched Subtype

Tumors with *HER2* overexpression are found in 15-25% of IDC breast cancers and they demonstrate a worse prognosis but respond well to *HER2*-targeted therapies such as trastuzumab. *HER2*-positive tumors, which displays the potential to give noticing the degree of a patient's response to trastuzumab (Coates et al., 2015). Within the *HER2* subtype of tumors were related with enhanced disease-free survival (DFS) and overall survival (OS) compared to hormone receptor-negative tumors-regardless of clinicopathologic factors-in the 4 years follow-up (Arteaga et al., 2012; Perez et al., 2011). Breast cancer patients with hormone receptor-negative or HER2-positive tumors demonstrated less first relapse in bone and more recurrence in the brain. In addition, women with hormone receptor-negative or *HER2*-positive tumors had a higher pathological complete response rate than those with hormone receptor-positive or *HER2*-positive breast cancer tumors (Gianni et al., 2014; Vaz-Luis et al., 2012). The pathological complete response rate may be enhanced to over 70% using with trastuzumab plus lapatinib or trastuzumab plus pertuzumab in addition to an anthracycline or taxane chemotherapies (Table 1) (Coates et al., 2015).

Table.1 Treatment-oriented classification of subgroups of breast cancer from the St. Gallen Consensus 2015 (Coates et al., 2015).

Clinical grouping		**Note**	**Therapies**
TNBC		*ER-*, *PR-*, and *HER2* negative	Cytotoxic chemotherapy such as anthracycline and taxane
***HER2* positive breast cancers**		ASCO/CAP guidelines[a]	T1[a]:node-negative: not chemotherapy T1[b,c]:node-negative: chemotherapy and trastuzumab
Hormone Receptor-Positive and *HER2* positive		ASCO/CAP guidelines[a])	T1[a]:node-negative: not chemotherapy T1[b,c]:node-negative: chemotherapy and trastuzumab +endocrine therapy
Hormone Receptor-Positive and *HER2* negative		*ER*-positive and/or *PR* positive ≥ 1%[b)]	
Luminal A-like breast cancer	low proliferation	High *ER/PR* and clearly *Ki-67*[c]-low) Low or absent nodal involvement (N 0-3), smaller T size (T1, T2)	Endocrine therapy
Luminal B-like breast cancer	high proliferation	Multiparameter molecular marker 'unfavorable prognosis' if available; lower ER/PR with clearly Ki-67[c]-high); more extensive nodal involvement, histological grade 3	Endocrine therapy and adjuvant

[a)]IHC of HER2 staining 3+ was defined as HER2 positive, and the 0 or 1+ score was HER2 negative. For tumors with 2+ score, HER-2 gene: copies to the CEP 17 ratios of 2.2 or more on FISH were interpreted as amplified. [b)]ER values between 1% and 9% were considered equivocal. [c)]Ki-67 rates should be interpreted in the light of local laboratory values: as an example, if a laboratory has a median Ki-67 score in HR-positive disease of 20%, values of 30% or above could be considered clearly high; those of 10% or less clearly low (Coates et al., 2015).

2.5. Moving Towards An Integrated Classification: METABRIC

2.5.1. Ten Integrative Clusters

Next-generation sequencing has further refined molecular profiles (Ding et al., 2010; Shah et al., 2009). The METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) study identified ten subtypes of breast cancer termed integrative clusters (IntClust). These ten subtypes show characteristic copy number aberrations (*CNA*s) and importantly are associated with distinct patterns of survival and response to neoadjuvant chemotherapy (Curtis et al., 2012). All breast

carcinomas show significant genetic diversity due to both inherited genetic variation and acquired genomic aberrations (Table 2). Inherited variants consist of single nucleotide polymorphisms (*SNP*s) and copy number variants (*CNV*s) and these changes form the background germline genetic landscape of the individual where cancer might develop. Somatic genomic changes, which include single nucleotide variants (mutations) and copy number aberrations (*CNA*s) are acquired and contribute to the initiation and progression of sporadic breast cancers. Genomic aberrations can contribute to carcinogenesis by inducing abnormal gene expression. Through the integrated analysis of both genomic and transcriptomic data across large numbers of breast cancers, the impact of genomic aberrations on the transcriptome can be appreciated (Curtis et al., 2012). Both germline variants (*CNV*s and *SNP*s) and somatic aberrations (*CNA*s) were found to be associated with alterations in gene expression. The 10 integrative clusters were each associated with *CNA*s and gene expression changes (Table 2) These clusters clearly demonstrated the heterogeneity present within tumors classified according to *ER*, *PR* and *HER2* expression, and they divided all of the previously identified intrinsic subtypes into separate groups (Curtis et al., 2012).

Table. 2 Definition of genomic alterations (Curtis et al., 2012).

Genomic alterations	Definition		Description
SNP	Single-nucleotide polymorphism	Germline	Inherited genetic variation in the DNA sequence that occurs when a single nucleotide is altered
CNV	Copy number variant	Germline	Inherited alteration of DNA that results in an abnormal number of copies of one or more segments of DNA (1 kilobase or larger)
SNV	Single-nucleotide variant	Somatic	Acquired genetic variation in the DNA sequence that occurs when a single nucleotide is altered (i.e., point mutation)
CNA	Copy number aberration	Somatic	Acquired alteration of DNA that results in an abnormal number of copies of one or more segments of DNA (1 kilobase or larger)

In METABRIC study, IntClusts 3, 4, 7 and 8 have the best prognosis, IntClusts 1, 6 and 9 have an intermediate prognosis, and IntClusts 2, 5 and 10 a poor prognosis (Dawson et al., 2013). IntClust 4 comprises a mixture of *ER*-positive and *PR*-negative tumors and is characterized by a relative paucity of *CNA*s and a gene expression signature reflecting immune activation. The majority of *ER*-positive and *HER2*-negative tumors are distributed within 8 IntClusts (1, 2, 3, 4, 6, 7, 8 and 9), but have variable degrees of genomic instability and distinct *CNA*s. For example, IntClust 3 has low genomic instability and a high frequency of *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) mutations, IntClust 6 has amplification of 8p12 with upregulation of *ZNF703* (zinc finger protein 703), a common Luminal B breast cancer oncogene (Holland et al., 2011) and IntClust 2 has high genomic instability. IntClust 10, composed of tumors with a high rate of *TP53* mutations and 5q deletion, has a very poor prognosis in the short term, but patients surviving beyond 6 years following treatment have an excellent long-term outcome. IntClust 5, associated with *HER2* amplification. These clusters clearly demonstrated the heterogeneity present within tumors classified according to *ER*, *PR* and *HER2* expression, and they divided all of the previously identified intrinsic subtypes into separate groups (Figure 9) (Holland et al., 2011).

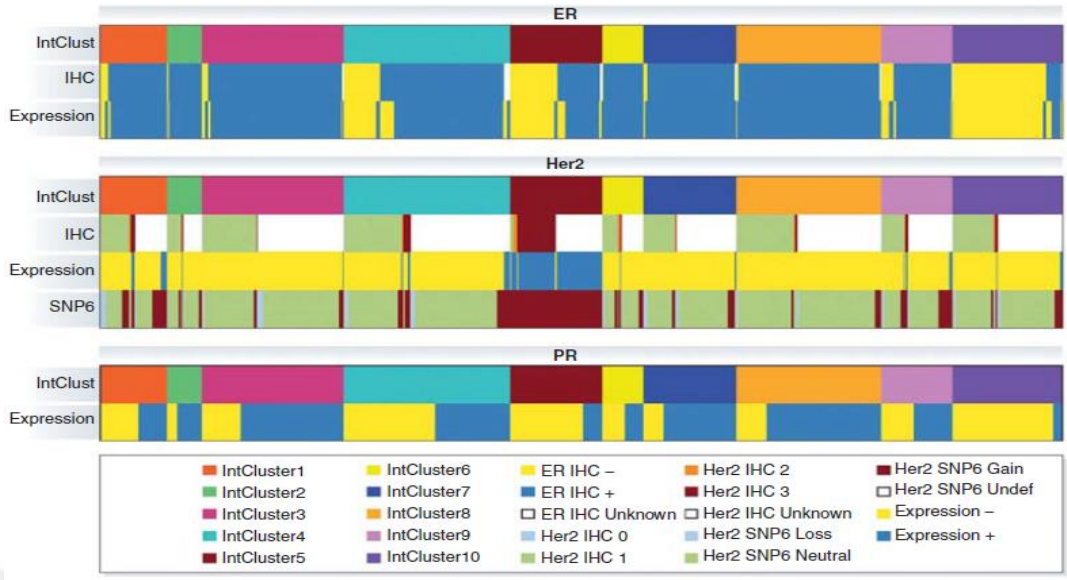


Figure. 9 Relationship between the 10 integrative clusters and *ER* expression, *HER2* expression and *PR* expression (Holland et al., 2011).

2.5.2. Subtype Classification Models (SCMs)

Subtype classification models (*SCM*s) are gene expression–based classifiers used to identify the four primary molecular subtypes of breast cancer (basal-like, *HER2*-enriched, luminal A, and luminal B). *SCM*s have been applied in datasets using different microarray platforms and normalization methods. *SCM*s are based on a mixture of three Gaussian distributions in a two-dimensional space defined by the *ER* and *HER2* gene modules, with a proliferation of aurora kinase A (*AURKA*) module providing discrimination between low and high proliferative tumors (Desmedt et al., 2008; Wirapati et al., 2008). These modules are composed of genes whose expression is specifically correlated with their prototype gene *ER*, *HER2*, or *AURKA* (Desmedt et al., 2008; Wirapati et al., 2008).

2.6. *HER2*-positive Breast Cancer Subtype

Human epidermal growth factor receptor 2 (HER2) positive breast cancer (*HER2+*), which belongs to a subtype of breast cancer with *HER2* gene amplification and/ or *HER2* protein overexpression, accounts for about 25%–30% of all breast cancers (Slamon et al., 1987; Slamon et al., 1990). With aggressive biological behavior and poor clinical outcome, *HER2+* breast cancer is often associated with significantly shorter disease-free survival and worse overall survival rates than other

subtypes of breast cancer. *HER2* is a transmembrane protein with a molecular weight of 185 kDa. It plays a vital role in the regulation of cell growth, survival, and differentiation (Wahler et al., 2015). The overexpression of *HER2* favors cell proliferation by inhibiting cell apoptosis, which therefore leads to malignant tumors (Ménard et al., 2003).

2.6.1. Structure of *HER2*

Human epidermal growth factor receptor 2 (*HER2/neu, ErbB2*) is a 185 kDa transmembrane glycoprotein encoded by the *HER2/neu* oncogene located at chromosome 17q. It belongs to the epidermal grow factor receptor (*EGFR*) family of epithelial tyrosine kinases, which also includes other three distinct receptors: *EGFR* (*ErbB1*), *HER3* (*ErbB3*), and *HER4* (*ErbB4*). Proteins in the *EGFR* family are all transmembrane proteins sharing a common basic molecular structure: an extracellular ligand-binding domain with an amino-terminal, a single transmembrane spanning the region and an intracellular cytoplasmic domain with tyrosine kinase activity (Figure 10) (Lv et al., 2016). The extracellular domain consists of four parts: two repeated ligand-binding domains (*LD1* and *LD2*) responsible for ligand recognition, and cysteine-rich sequences (*CR1* and *CR2*) providing a framework to orientate *LD* regions. The intracellular domain can be divided into two regions: a catalytic tyrosine kinase (*TK*) domain with phosphorylation sites and a carboxyl-terminal tail (*CT*) (Figure 10) (Lv et al., 2016).

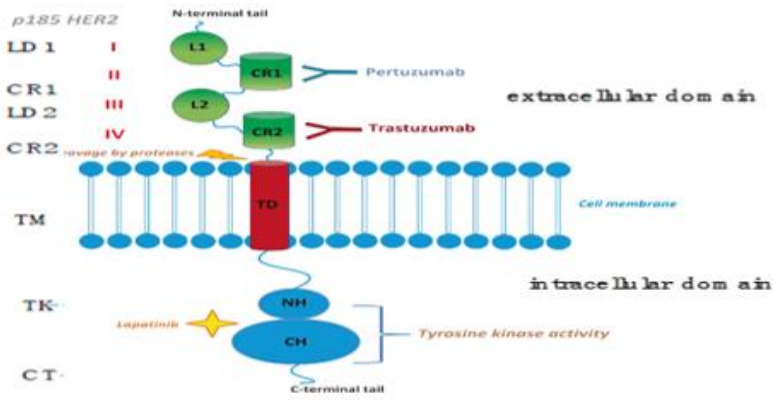


Figure. 10 The basic structure of epidermal growth factor receptor *HER2*, a transmembrane protein (Lv et al., 2016).

2.6.2. *HER2*: Role in Signaling Pathways

Human epidermal growth factor receptors (*EGFR*s, or *HER*s) 1 to 4 constitute a family of tyrosine kinase receptors expressed in normal tissues and in many types of cancer. Human epidermal growth factor receptor-2 (or *HER2*/*neu*, *c-erBb2*) is a member of the *EGFR*s (Feng et al., 2018). Like the others, *HER2* is a receptor tyrosine kinase that consists of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular domain (Feng et al., 2018). The constitutively active form makes *HER2* the preferred component to form dimers with other molecules and grants *HER2* the capability of affecting many cellular functions through various pathways (Feng et al., 2018). Ligand binding and subsequent dimerization stimulate phosphorylation of tyrosine residues in the intracellular domain of *HER2*, leading to the activation of multiple downstream signaling pathways such as the mitogen-activated protein kinase (*MAPK*) and the phosphatidylinositol 4,5-bisphosphate 3-kinase (*PI3K*) pathways (Feng et al., 2018). These signaling pathways are heavily associated with breast tumorigenesis (Figure 11). *HER2* is amplified in various human breast cancer cell lines (Feng et al., 2018). *HER2* signaling amplification results in *HER2* protein overexpression which is linked to tumor cell proliferation and cancer progression. Targeted therapies are developed to bind specific molecules in signaling pathways important for cancer development and progression, providing the most effective therapy in appropriately selected patients. Novel mechanisms underlying the relationship between *HER2* and breast cancer have been uncovered recently. While the interplay of *HER2* and *ER*s has long been recognized, it was recently discovered that a new intermediary factor *MED1* (mediator complex subunit 1) has a significant impact on *HER2*-driven tumorigenesis. The precancerous effect of *HER2* was also found to be linked to inflammation and the expansion of cancer stem-like cells (CSCs) in breast cancer. A newly identified enhancer located at the 30 gene body of *HER2* was reported to be the target locus of known *HER2* regulator, *TFAP2C* (transcription factor AP-2 gamma). Other epigenetic mechanisms, such as DNA methylation and histone modifications, also affect this process (Figure 11) (Feng et al., 2018).

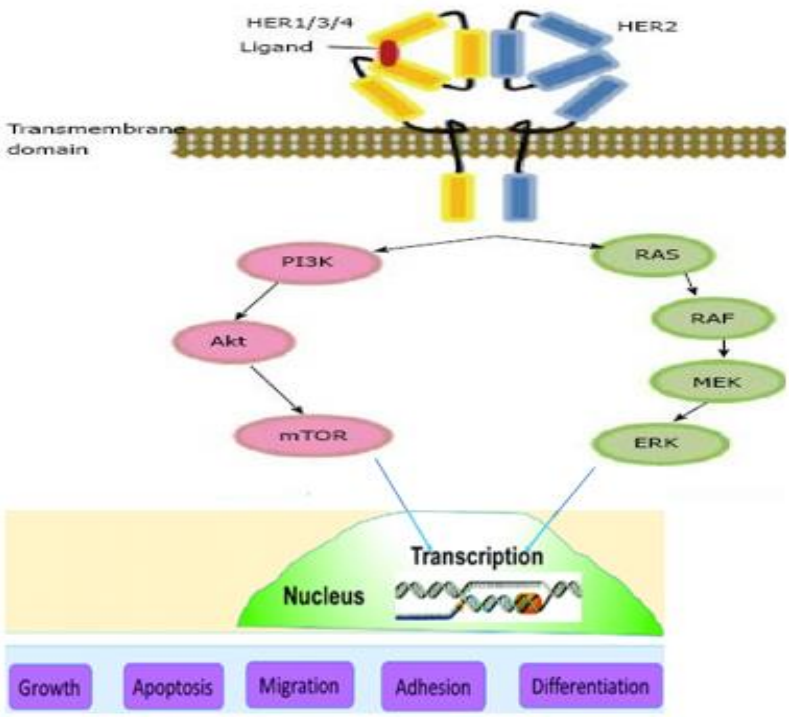


Figure. 11 HER2 signaling pathway (Feng et al., 2018)

2.6.3. Diagnosis of *HER2*-Positive Breast Cancer

IHC and FISH are two tests approved by the food and drug administration for *HER2* testing. Immunohistochemistry is usually utilized as initial testing, followed by florescent in situ hybridization (*FISH)* for subjects with suspicious or discordant outcomes (Xu et al., 2019). The American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) published guidelines on *HER2* testing assay for breast cancer patients, which Wolff et al updated in 2013 (Wolff et al., 2007; Wolff et al., 2013). ASCO/CAP guideline recommendations in 2013, cases with *HER2*/chrom 17(*CEP17*) ratio ≥ 2.0, or cases with *HER2/CEP17* ratio < 2.0 and the average number of *HER2* signals ≥ 6 were scored as positive; and cases with *HER2/CEP17* ratio < 2.0 and the average number of *HER2* signals per cell ≥4 and <6 were scored as suspicious /equivocal when utilizing a dual-probe *FISH* assay. Recently, 2018 ASCO/CAP guidelines clearly defined *HER2 FISH* results of five groups (Wolff et al., 2018). For cases of group 2, 3 and 4, the *HER2* status diagnosis should base on the combined of *FISH* and IHC testing assays. If the *HER2*

Immunohistochemistry result is 2+, the florescent ın situ hybridization result should be recounted by another observer (Figure 12) (Xu et al., 2019).

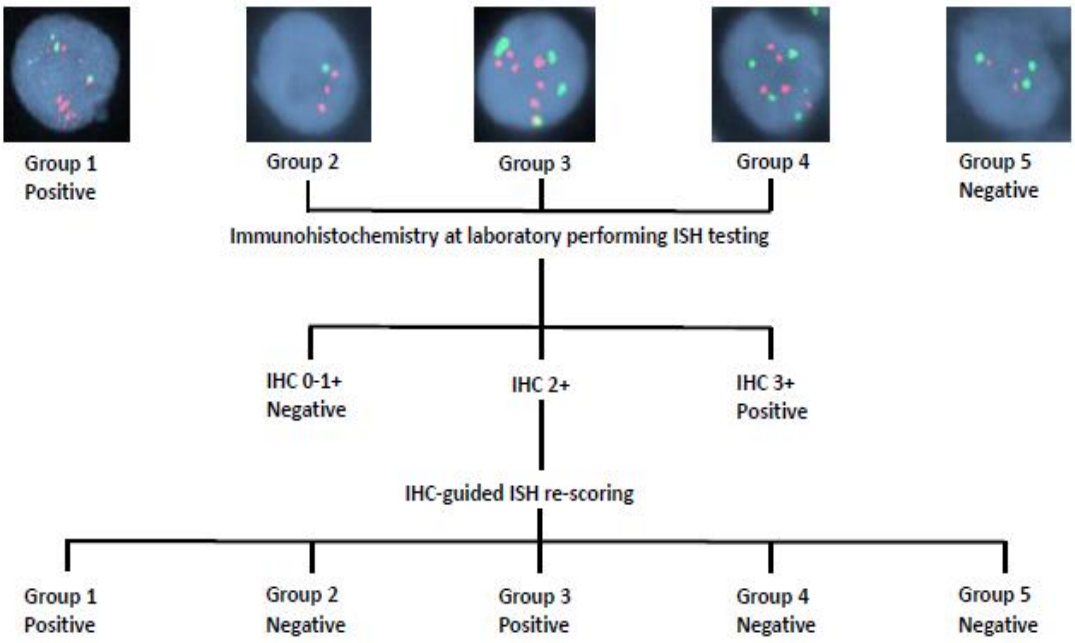


Figure. 12 The 2018 ASCO/CAP (Xu et al., 2019).

2.6.4. Treatment of *HER2*-Positive Breast Cancer

Overexpression of *HER2* is closely related to the development and progression of breast cancer. Therefore, *HER2* becomes a critical target for developing therapeutic drugs against *HER2+* breast cancer. The development of anti-*HER2* therapies has significantly improved the clinical outcome for patients with *HER2+* breast cancer (Incorvati et al., 2013; Lv et al., 2016). Four major anti-*HER2* drugs for *HER2+* breast cancer, including monoclonal antibodies, small-molecule tyrosine kinase inhibitors, antibody-drug conjugate, and other emerging anti-*HER2* agents are drugs against the extracellular domain of *HER2*. (Figure 13) (Lv et al., 2016).

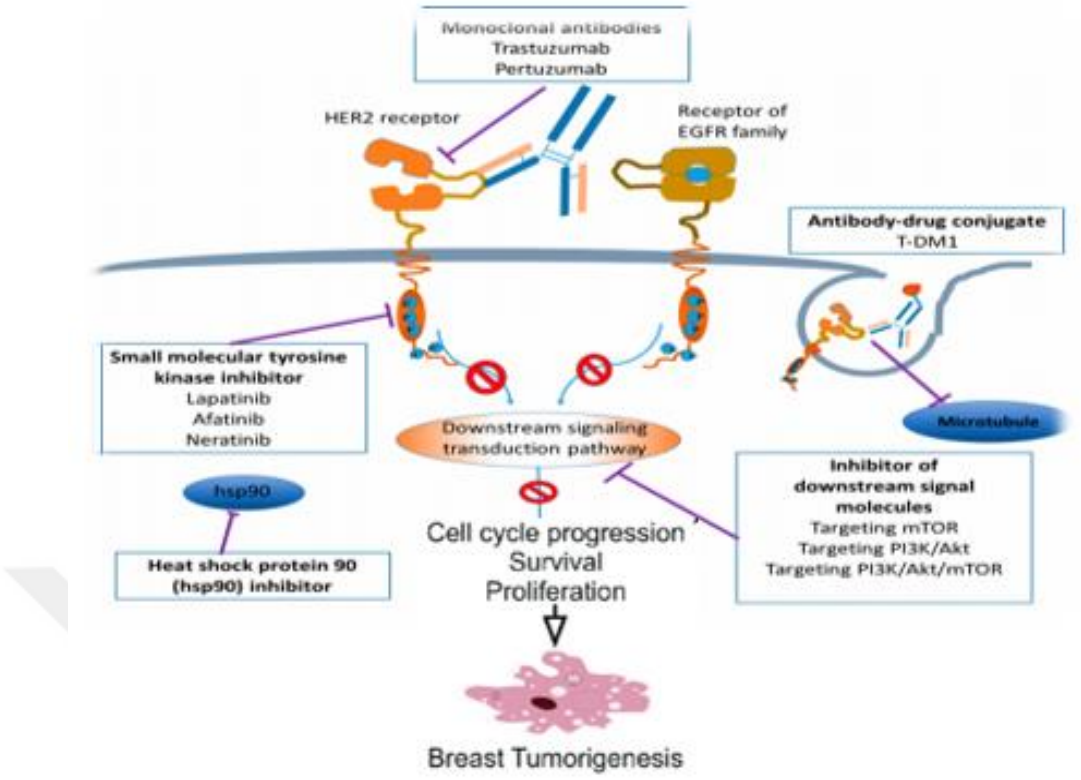


Figure. 13 Molecular approaches for HER2+ breast cancer therapy (Lv et al., 2016).

2.6.4.1. Trastuzumab

Trastuzumab (brand name, Herceptin) is a monoclonal antibody that targets ERBB2 (a tyrosine kinase receptor, also known as *HER2* or *HER-2/neu*). Trastuzumab is only used to treat specific tumors that overexpress *ERBB2*; these tumors are known as "*HER2*-positive" tumors. Trastuzumab is typically used as an adjuvant treatment of early-stage *HER2*-positive breast cancer. Adjuvant therapies are used after primary treatment (such as surgery) to increase the chance of long-term disease-free survival. An example chemotherapy treatment regime is "AC→TH", which stands for adriamycin, cytoxan, then taxol and herceptin. Trastuzumab is also used in the treatment of *HER2*-positive metastatic breast cancer and *HER2*-positive metastatic gastric cancer. Recently, *HER2* targeted therapy has been approved by the FDA for use in the neoadjuvant setting. Neoadjuvant therapy is given before primary therapy, for example, to shrink a tumor to an operable size or to allow for breast-conserving surgery, and to increase the chance of long-term, disease-free survival. In the neoadjuvant setting, pertuzumab, along with trastuzumab

and docetaxel (a chemotherapy agent) can be given pre-operatively (Gianni et al., 2012).

Before treatment with trastuzumab begins, overexpression of the *ERBB2* protein or amplification of the *ERBB2* gene must first be determined. The Food and Drug Administration (FDA) recommends that testing be performed using an FDA-approved test for the specific tumor type (breast or gastric tumor), in a laboratory with demonstrated proficiency with the technology being used. This is because the benefits of trastuzumab have only been proven in patients with tumors that overexpress *ERBB2*. In addition, although trastuzumab is generally well tolerated, the risks of treatment include infusion reactions, pulmonary toxicity, and cardiomyopathy that can result in cardiac failure (Dean., 2015). Trastuzumab targets the *ERBB2* receptor by binding to the juxtamembrane portion of the extracellular domain. This binding limits the receptor's ability to activate its intrinsic tyrosine kinase, which in turn, limits the activation of numerous signaling pathways that can promote the growth of cancerous cells (Lane et al., 2001).

2.6.4.2. Trastuzumab Emtansine (T-DM1)

Trastuzumab emtansine (T-DM1) is an antibody-drug conjugate that is effective and generally well tolerated when administered as a single agent to treat advanced breast cancer. Efficacy has now been demonstrated in randomized trials as the first line, second line, and later than the second-line treatment of advanced breast cancer (Ritchie et al., 2013). T-DM1 is currently being evaluated as adjuvant treatment for early breast cancer. It has several mechanisms of action consisting of the anti-tumor effects of trastuzumab and those of DM1, a cytotoxic anti-microtubule agent released within the target cells upon degradation of the human epidermal growth factor receptor-2 (*HER2*)-T-DM1 complex in lysosomes. At least four molecular mechanisms have been suggested for DM1 anti-tumor activity. First, active DM1 metabolites disrupt the microtubule networks of the target cells, which causes cell cycle arrest at the G2-M phase and apoptotic cell death (Lewis Phillips et al., 2008; Ritchie et al., 2013). The primary resistance of *HER2*-positive metastatic breast cancer to T-DM1 appears to be relatively in frequent, but most patients treated with T-DM1 develop acquired drug resistance. The

mechanisms of resistance are incompletely understood, but mechanisms limiting the binding of trastuzumab to cancer cells may be involved. The cytotoxic effect of T-DM1 may be impaired by inefficient internalization or enhanced recycling of the *HER2*-T-DM1 complex in cancer cells, or impaired lysosomal degradation of trastuzumab or intracellular trafficking of *HER2*. The effect of T-DM1 may also be compromised by multidrug resistance proteins that pump DM1 out of cancer cells (Barok et al., 2014).

2.6.4.3. Pertuzumab

Pertuzumab, a humanized monoclonal antibody and the first in the class of agents called the *HER2* dimerization inhibitors, impairs the ability of *HER2* to bind to other members of the *HER* family. It has a unique and complementary mechanism of action compared with trastuzumab, and the combination has resulted in the enhanced blockade of the HER signaling pathway (Jhaveri et al., 2014). When pertuzumab was used in combination with docetaxel and trastuzumab in the first-line treatment of metastatic *HER2+* breast cancer, it led to an overall survival benefit. Pertuzumab has therefore been approved by the FDA and is currently used as a standard of care for this indication. It is also the first agent in oncology to receive accelerated FDA approval in the neoadjuvant setting. Randomized trials showed that the addition of pertuzumab to trastuzumab-based chemotherapy improves pathologic complete response rates in *HER2+* early-stage breast cancer. A randomized phase III clinical trial with disease-free survival as the primary endpoint is evaluating the safety and efficacy of pertuzumab in the adjuvant setting (Jhaveri et al., 2014).

2.6.5. Mechanisms of Resistance to *HER2* Targeted Therapy

Various factors associated with resistance to *HER2*-targeted therapies have been proposed, which may be generally classified as factors associated with receptor-level effects or those of downstream signaling components. Receptor-level factors affecting drug binding and action might contribute to resistance. *p95HER2* or mutations in the extracellular domain, a *HER2* fragment lacking the extracellular binding domain, is unable to bind trastuzumab but might retain sensitivity to other

HER2-targeted therapies that bind intracellularly. Also, loss of *HER2* amplification has been reported in patients with significant residual disease after neoadjuvant trastuzumab therapy and is associated with poor recurrence-free survival. Increased activation of *HER3* has also been proposed as a contributor to resistance because increased *HER*-family activating ligands have been detected in cells resistant to trastuzumab, which does not inhibit *HER2-HER3* hetero-dimerization, and inhibiting *HER2* has been shown to reactivate *HER3* (Lewis Phillips et al., 2008; Ritchie et al., 2013; Wilks et al., 2015). Factors associated with downstream signaling components have also been investigated as factors related to resistance to *HER2*-targeted therapies. Alterations in regulation of downstream signaling might lead to aberrant activation of signaling pathways downstream of *HER2*. Also, compensatory crosstalk with other pathways, such as between *HER2* and *HER3*, epidermal growth factor receptor (*EGFR)/HER1*, and insulin-like growth factor1 receptor (*IGF-1R*), has been reported to reduce the effectiveness of trastuzumab, and crosstalk between the estrogen receptor pathway and *HER2* and the *PI3K/Akt/mTOR* pathway has been implicated in endocrine resistance (Wilks et al., 2015). Also, *HER2* is a client protein of *HSP90*, a member of the family of heat shock proteins (*HSP*s), which are considered molecular chaperones, as they are responsible for the correct folding of denatured or translated proteins. It has been suggested that *HSP90* expression may also modulate the effects of oncogenic *HER2*, representing a potential mechanism of resistance to *HER2* directed drugs. On the other hand, *HSP90* inhibitors may potentiate the effects of anti-cancer drugs targeting client proteins of *HSP90* (Berezowska et al., 2013).

2.7. HSP90 and Cancer

Most molecular chaperones belong to the class of heat shock proteins (*HSP*s), originally identified as stress-responsive proteins required managing thermal and other proteotoxic stresses. *HSP90* consists of four structural domains an N-terminal Domain (NTD), a charged linker that connects the NTD and Middle Domain (MD), a middle domain and a C-Terminal Domain (CTD). The NTD of *HSP90* is the binding site for ATP and connects to the MD with a charged linker. This domain modulates ATPase activity of *HSP90* by binding to gamma-phosphate of ATP and acts as

recognition and a binding site for co-chaperones and client proteins. Finally, the CTD of *HSP90* helps in the homodimerization of *HSP90* to obtain active conformation (Figure 14) (Li et al., 2013).

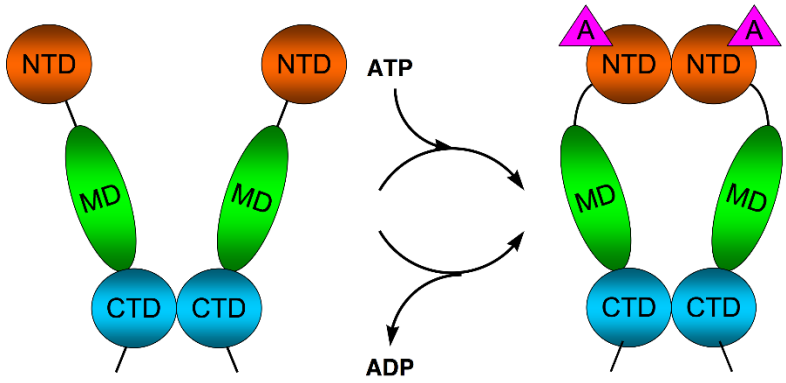


Figure 14. *HSP90* consists of four structural domains (Li et al., 2013).

*HSP*s was designated in accordance to their approximate molecular weight and consequently, the main families of these proteins were distinguished: small *HSP* (s*HPS*), *HSP40*, *HSP70*, *HSP90* and *HSP60* (chaperonins) (Kampinga et al., 2009). Although a number of aberrant signaling pathways in breast cancer have been identified, heat shock protein 90 (*HSP90*), which is one of the most abundant proteins in mammalian cells, plays an important role in folding newly synthesized proteins or stabilizing and refolding denatured proteins after stress, and would influence a large number of signaling pathways. To date, more than 200 *HSP90* clients have been identified, including key regulators in signal transduction and cell cycle control, steroid hormone receptors, and tyrosine and serine/ threonine kinases (Cheng et al., 2012). *HSP90* exists as multiple isoforms that include *HSP90AA1* (an inducible form) and *HSP90AB1* (a constitutive form) in the cytoplasm, *HSP90B1* in the endoplasmic reticulum (Chen et al., 2005). *HSP90* consists of a wide array of client proteins that includes proteins such as Epidermal Growth Factor Receptor (*EGFR), c-Raf, Src, B-Raf, Akt and Met.* Also, *Hsp90* interacts with a variety of proteins that play key roles in breast cancer; including estrogen receptors (*ER*), tumor suppressor *p53* protein, angiogenesis transcription factor *HIF-1*alpha, and a variety of receptor tyrosine kinases, such as *HER2* (Figure 15) (Garg et al., 2016).

The overexpression of *HSP90* has been correlated with high *HER2* and *ER* levels, lymph node status, size of tumors and decreased survival in breast cancer (Pick et al., 2007; Zagouri et al., 2012). *HSP90* mediated deregulation of these critical factors may contribute to the poor prognosis breast cancer (Pick et al., 2007). Importantly, *HSP90* is over expressed in various solid tumors and malignancies. Cancer cells utilize the essential chaperone features of *HSP90* for stabilizing mutated or dysregulated oncoproteins, which promote malignant transformation (Calderwood et al., 2006).

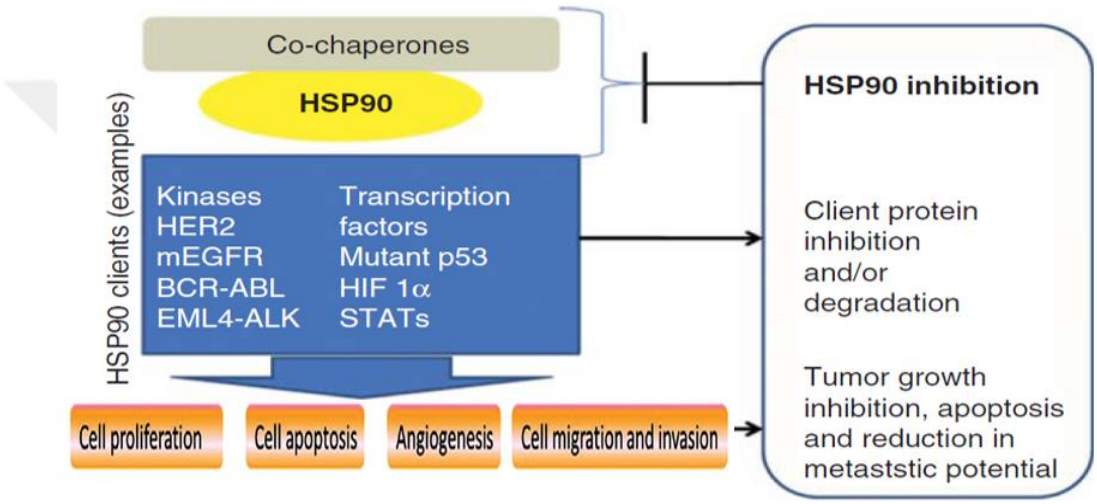


Figure. 15 Molecular signaling pathways modulated by *HSP90* inhibition in cancer (Jhaveri et al., 2014)

2.7.1. *HSP90* and *HER2*- associated Cancer Development

HSP90 is known to regulate Her-2 activity by interacting with a specific loop in its kinase domain. This interaction sequesters *HER2* homodimers and prevents *HER2* dimerization with other *HER* family receptors, thus restricting its catalytic activity. When ligands bind to other *HER* receptors, *HSP90* is dissociated due to steric hindrance and heterodimerization activates *HER2*- mediated signaling pathways, such as *MAPK* and *PI3K* pathways (Citri et al., 2004; Sidera et al., 2008). In addition, surface *HSP90* has been shown to interact with the extracellular domain of *HER2*, activating heregulin-mediated *HER2* pathway, leading to cytoskeletal

rearrangement and contributing to cancer cell motility and invasion (Citri et al., 2004; Sidera et al., 2008). The functional status of *HSP90* is critical for tight regulation of *HER2* and its family members. Alterations of *HSP90* would have a profound impact on *HER2*activity and its oncogenic potential. Indeed, targeting *HSP90* for the treatment of *HER2+* breast cancer is an attractive target for anti-cancer therapy (Jhaveri et al., 2014).

2.7.2. *HSP90* Expression in Breast Carcinogenesis

Elevated *HSP90* expression has been documented in breast ductal carcinomas (Yano et al., 1996; Zagouri et al., 2012), although a significantly decreased *HSP90* expression has been shown in infiltrative lobular carcinomas and lobular neoplasia (Zagouri et al., 2012). The persistent downregulation of *Hsp90* expression throughout the whole lobular series may be contrary to what might have been expected; it is known that *HSP90* overexpression is a feature of invasive ductal carcinomas (Yano et al., 1996; Zagouri et al., 2012). The discrepancy between *HSP90* expression in *IDC* and *ILC* is rather very interesting. High *HSP90* expression in primary breast cancer has been described as a poor prognostic marker in breast cancer (Zagouri et al., 2012). *HSP90* expression was variable in patient tumors compared to cancer cell lines. Whether this expression is also variable between primary and metastatic tumors is unknown at this time. It is important to note that *HSP90* inhibitors have been used in breast cancer only in the metastatic and refractory settings. Additionally, studies have not described *HSP90* expression as a marker that can predict response to *HSP90* inhibitor therapy.

2.7.3. Targeting *HSP90* in *HER2+* Breast Cancer

Breast cancer is an indication where *HSP90* inhibitors should be explored for a variety of reasons. Firstly, inhibition of *HSP90* degrades *HER2*, a client protein, and *HSP90* inhibitors have shown activity in *HER2*-driven xenograft models (Zagouri et al., 2012). Moreover, modulation of estrogen and progesterone receptor signaling has been a long-standing approach to treating breast cancer and both estrogen and progesterone receptors are clients of *HSP90* (Garg et al., 2016; Zagouri et al., 2012). Additionally, the resistance of breast cancer cells to chemotherapy is

known to involve the phosphatidylinositol 3-kinase pathway (Garg et al., 2016), which is modulated by *HSP90* by virtue of one of its key signaling proteins (*AKT*) being a client protein of *HSP90*. Furthermore, inhibition of *HSP90* has also been known to modulate angiogenesis of breast cancer xenografts (Garg et al., 2016; Zagouri et al., 2012). Finally, expression of *HSP90* has been shown to correlate with adverse clinical outcomes, further validating *HSP90* as a target in breast cancer (Zagouri et al., 2012). *Hsp90* inhibitors act mainly in the (N) domain of *Hsp90*, stopping the orchestrated chaperone cycle and leading to ubiquitination and proteasomal degradation of the client proteins and induction of cochaperones such as *HSP70* (Prodromou et al., 2003). The development of *HSP90* inhibitors began with 2 natural products: geldanamycin, a benzoquinone ansamycin, and radicicol, a resorcyclic acid lactone. These are commonly referred to as first-generation *HSP90* inhibitors. As discussed earlier, the first-generation inhibitors (Figure 16) were abandoned in large part because of significant toxicity. The second-generation *HSP90* inhibitors, including AUY922, ganetespib, and AT13387, appear not to have the toxic effects of the first-generation *HSP90* inhibitors. Ganetespib did proceed to a phase 3 trial looking at it in combination with docetaxel, but this trial recently was closed prematurely owing to futility (Jhaveri et al., 2014).

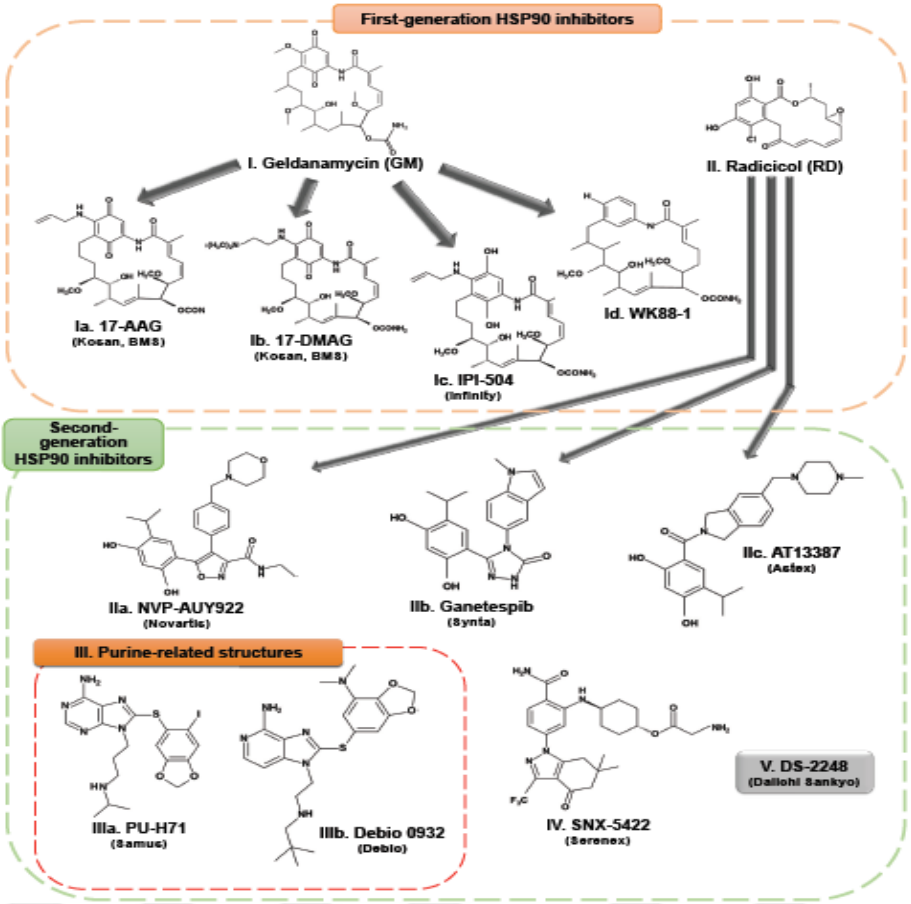


Figure. 16 Structural development of *HSP90* inhibitors (Jhaveri et al., 2014).

2.7.3.1. 17-allylamino-17-demethoxygeldanamycin (17-AAG; tanespimycin)

Tanespimycin is a semi-synthetic derivative of ansamycin antibiotic geldanamycin; a compound with biological activity similar to geldanamycin, but with a more favorable and acceptable safety profile. Tanespimycin has shown considerable preclinical antitumor activity in breast cancer. More specifically, tanespimycin depleted *ER* from *ER*-positive tamoxifen-resistant and *ER*-positive tamoxifen-sensitive breast cancer cells in vitro and inhibited the growth of breast tumor xenografts. Additionally, the combination of tanespimycin and trastuzumab increases ubiquitinylation and decreases the expression of *HER2* in *HER2*-overexpressing breast cancer cell lines. Furthermore, according to preclinical studies, a combination of tanespimycin and chemotherapy (including taxanes, anthracyclines, nucleoside analogs and topoisomerase I inhibitors) is additive and synergistic

(Zagouri et al., 2013). 17-AAG has been tested in more than 30 clinical trials since 1999 either as a single agent or in combination with other approved agents such as bortzemib, imatinib, docetaxel, rituximab, and trastuzumab (Den et al., 2009), and dshowed that the combination of 17-AAG and trastuzumab had activity in patients with *HER2*-positive metastatic breast cancer (Den et al., 2009).

2.7.3.2. Ganetespib (STA-9090)

Ganetespib is a nongeldanamycin- derived *Hsp90* inhibitor developed by Synta Pharmaceuticals that is currently in phase III clinical trial (Proia and Bates 2014). Ganetespib is known to bind to the ATP-binding pocket in the N-terminal domain of *Hsp90a* with a KD value of 110 nM (Jhaveri et al., 2014; Shimamura et al. 2012). Ganetespib, another second-generation *HSP90* inhibitor has shown promising clinical activity (clinical benefit rate of 60%) in combination with paclitaxel and trastuzumab (clinical benefit rate was 60%) in heavily pretreated patients with *HER2*-positive metastatic breast cancer and triple-negative breast cancer (*TNBC*). Ganetespib has shown promising clinical activity (clinical benefit rate of 60%) in combination with paclitaxel and trastuzumab (clinical benefit rate was 60%) in heavily (Jhaveri et al., 2014).

2.7.4. Co-chaperones

The *Hsp* chaperone activity is tightly regulated by interaction with many co-chaperones. Molecular chaperones like *Hsp70* and *Hsp90* fulfill well-defined roles in protein folding and conformational stability via ATP dependent reaction cycles. These folding cycles are controlled by associations with a cohort of non-client protein co-chaperones, such as *Hop*, *p23,* and *Aha1*(Edkins et al., 2015). Pro-folding co-chaperones facilitate the transit of the client protein through the chaperone-mediated folding process. However, chaperones are also involved in ubiquitin-mediated proteasomal degradation of client proteins. Similar to folding complexes, the ability of chaperones to mediate protein degradation is regulated by co-chaperones, such as the C terminal *HSP70* binding protein (*CHIP*). *CHIP* binds to *HSP70* and *HSP90* chaperones through its tetratricopeptide repeat (*TPR*) domain and functions as an *E3* ubiquitin ligase using a modified *RING* finger domain (*U-box*).

This unique combination of domains effectively allows *CHIP* to network chaperone complexes to the ubiquitin-proteasome system (Edkins et al., 2015). Also, *HSP90*-mediated activation and stabilization of client proteins require an ATP-driven chaperone cycle regulated by a number of co-chaperones. Pharmacologic inhibition of *HSP90* disrupts this cycle and leads to the ubiquitin-mediated proteasomal degradation of client proteins. The proposed model is that clients are ubiquitinated and thus targeted to the proteasome by the action of one or more *E3* ubiquitin ligases. Some evidence suggests that the U box containing ligase *CHIP* is involved in the degradation of certain *HSP90* clients (Samant et al., 2014). However, the stability of protein kinase clients such as *ERBB2* is not increased in *CHIP−/−* cells treated with the first in class pharmacologic *HSP90* inhibitor 17-allylamino- 17-demethoxygeldanamycin [17-AAG, tanespimycin], suggesting that other *E3* ubiquitin ligases are also involved. The *Cullin-RING* ligase *Cullin-5* (*CUL5*) is recruited to *HSP90*-containing complexes and is involved in the ubiquitination and degradation of the client *ERBB2* following *HSP90* inhibition (Samant et al., 2014). *Cullin-RING* ligases function as modular, multi-subunit complexes that consist of a Cullin scaffold, a *RING-H2* finger protein, a substrate-recognition subunit, and, in most cases, an adaptor that links the *Cullin* to the substrate recognition subunit. Given the link between *CUL5* and the *HSP90* inhibitor-induced degradation of *ERBB2* (Samant et al., 2014; Xu et al., 2002). Signal transducers and activators of transcription (*STAT*s) are latent, cytoplasmic transcription factors. Janus kinases (*JAK*s) and activated *CDC42*- associated kinase-1 (*ACK1/TNK2*) catalyze the phosphorylation of *STAT1* and the expression of its target genes. *ACK1* and its association with a worse patient outcome, e.g., in pancreatic cancer, hepatocellular carcinoma, stomach cancer, colon cancer, and breast cancer. Catalytically active *ACK1* promotes the phosphorylation and nuclear accumulation of *STAT1* in transformed kidney cells. These processes are associated with *STAT1*-dependent gene expression and interaction between endogenous *STAT1* and ACK1. Moreover, A degradation of *ACK1* by the *E3* ubiquitin ligase seven-in-absentia homolog-2 (*SIAH2*) as well as the pharmacological inhibition of *HSP90* with AT13387 suppresses the phosphorylation of *STAT1* and *STAT3* (Figure 17) (Mahendrarajah et al., 2017).

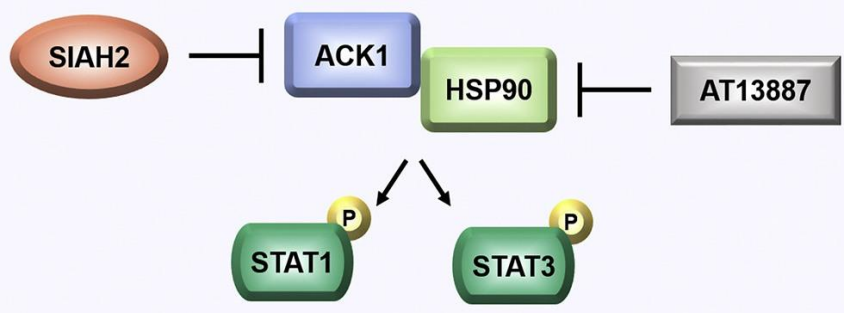


Figure 17. Active *HSP90* is necessary for the activation of *STAT1* and *STAT3* by *ACK1* (Mahendrarajah et al., 2017).

2.8. *SIAH* Gene

SIAH proteins are evolutionary conserved RING-type *E3* ubiquitin ligases emerging as critical regulators in both normal development and cancer. *SIAH* proteins exert their primary functions by targeting selected proteins for proteasomal degradation by polyubiquitination (Hu et al., 1997). *SIAH*s are the human homologs of *Seven-In-Absentia* (*SINA*), an evolutionarily conserved *RING finger E3* ubiquitin ligase and an essential downstream component of the Drosophila *RAS* signaling pathway a critical "gatekeeper" required for proper *RAS* signal transduction. Two homologues, *SIAH1* and *SIAH 2*, exist in humans, and have been shown to play a role in several pathways including those involved in response to DNA damage, the hypoxic response, estrogen signaling, inflammation, and *RAS* signaling (Behling et al., 2011; House et al., 2009). *SIAH* acts as an essential downstream signaling component required for proper *EGFR/HER2* and *RAS* signaling. Additionally, *SIAH* is a downstream "gatekeeper" required for *HER2/RAS*-mediated tumorigenesis and metastasis in human breast cancer (Behling et al., 2011). *SIAH* expression may be dysregulated by several cancer-relevant signaling. Such as the estrogen receptor whose activation via estradiol (*E2*) binding result to receptor dimerization and the induction of *SIAH2* transcription. In the same way, the wingless-type *MMTV* (mouse mammary tumor virus) integration site family member 5A (WNT5a), a negative regulator of *WNT* signaling, is capable to activate *SIAH2* expression. *EGFR* and *HER2*-dependent pathways via mitogen-activated protein kinase (*MEK*) might as

well regulate *SIAH* transcription and thus oncogenic programs in breast cancer cells. *SIAH*s are also involved in regulating the epithelial to mesenchymal transition (*EMT*) (Figure 18) (Knauer et al., 2015).

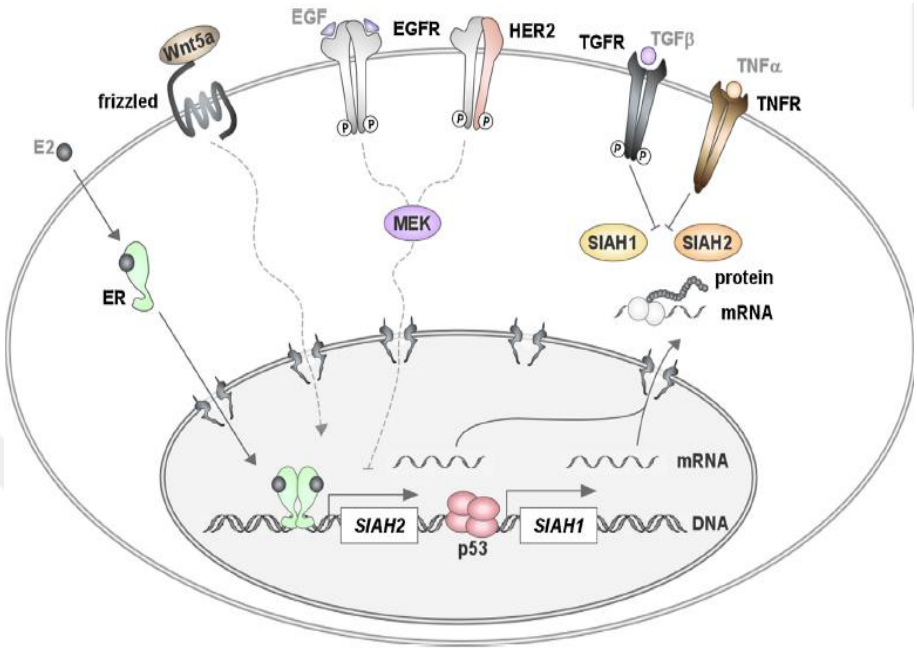


Figure 18. Cancers and the regulation of *SIAH* proteins (Knauer et al., 2015).

EMT has a vital role in tumor cell dissemination and is regulated by a core cassette of transcription factors. Despite recent studies, the molecular pathways that regulate the *EMT* pathway have not yet been fully delineated. One study demonstrated that *SIAH* ubiquitin ligases regulate *Zeb1* protein, a key *EMT* transcription factor (Figure 19) (Chen et al., 2015).

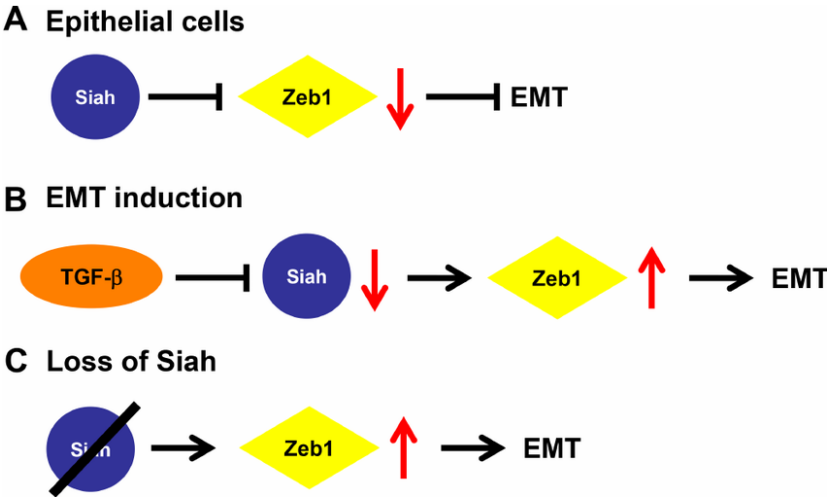


Figure 19. Mechanism of Siah-mediated regulation of EMT.

2.8.1. *SIAH1* Gene and Role of it's in Breast Cancer

The *SIAH1* gene was first identified as a candidate tumor suppressor gene at 16q12.1 in human hepatocellular carcinomas because of frequent loss of heterozygosity and expression down-regulation. Overexpression of *SIAH1* in *MCF7* cells suppresses cell growth by altering the mitotic process. *SIAH1* is a *RING finger E3* targeting h-catenin for ubiquitin-mediated degradation in response to activation of *p53* (Chen et al., 2006). Evidence showed a tumor-suppressive role of *SIAH*s in breast cancers. Enhanced expression of *SIAH1* halts the growth of estrogen receptor-positive MCF-7 breast cancer cells. *SIAH1*-overexpressed locates to the nuclear matrix, changes cellular morphology, and evokes pronounced polyploidy. Also, *SIAH1* controls tubulin spindle formation, cytokinesis, mitotic catastrophe, and cell survival (Figure 20) (Bruzzoni-Giovanelli et al., 1999).

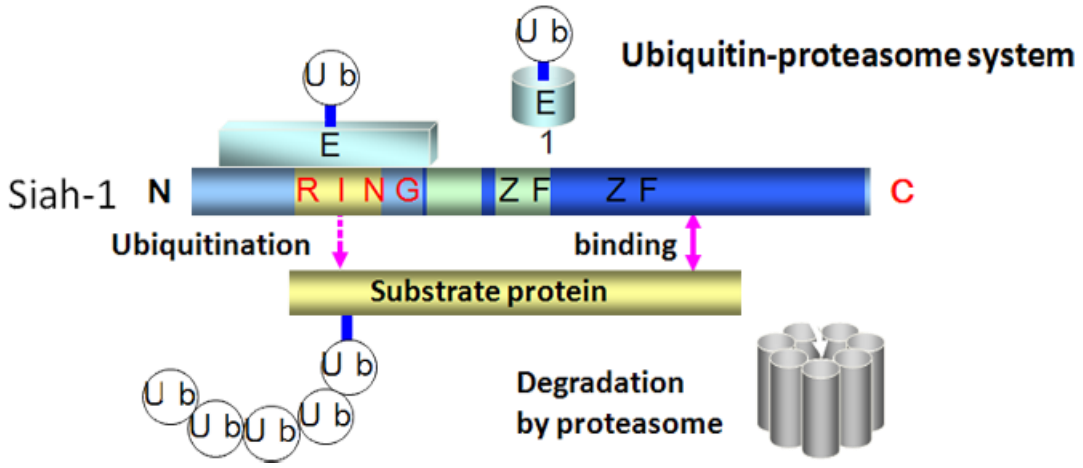


Figure 20. Schema of Siah1 structure (Bruzzoni-Giovanelli et al., 1999).

2.8.2. *SIAH2* Gene and Role of it's in Breast Cancer

Seven In Absentia Homolog 2 (*SIAH2*) protein is an *E3* ubiquitin ligase involved in ubiquitination and proteasome-mediated degradation of other proteins. For example, SIAH2 plays a critical role in controlling the abundance of hypoxia-inducible factor-1α through the prolyl hydroxylases (Interiano et al., 2014; Nakayama et al., 2004). *SIAH2* is also involved in many cancers such as leukemia and prostate cancer. Two recent genome-wide association studies identified a genetic variant in the *SIAH2* locus associated with *ER*-positive breast cancer, suggesting that the expression of *SIAH2* might be affected by this genetic variant and consequently correlated with *ER*-related tumor progression or hormone therapy response (Interiano et al., 2014). In addition, Jansen et al. (Jansen et al., 2009) noted that *SIAH2* significantly predicted first-line tamoxifen treatment failure in breast cancer. Also, the seven in absentia homolog 2 (*SIAH2*) protein plays a significant role in the hypoxic response by regulating the abundance of hypoxia-inducible factor-a. As well as, reported which, *SIAH* may represent a useful prognostic biomarker that predicts *DCIS* progression to invasive breast cancer (Behling et al., 2014).

2.8.3. Regulation of *SIAH* Expression by Hormones and Cytokines

These are critical for the survival of *ER*-positive breast cancer cells, making the *ER* an important target for pharmacological interventions (Zhou et al., 2014). The *SIAH2* gene binds to the group of *ER*-regulated genes (Figure 21). In the absence of estrogen breast cancer cells express activated CDC42 kinase 1 (*ACK1)* and low levels of *SIAH2* (Figure 21; left panel). Induction of *ER* signaling with estrogen induces *SIAH2* to mediate proteasomal degradation of *ACK1*. *SIAH2* interacts with *ACK1* and can regulate the ubiquitinylation of *ACK1* and its subsequent degradation by proteasomes (Figure 21; right panel) (Buchwald et al., 2013). Also, *SIAH2* status is high in *ER*-positive primary breast tumors in MCF-7 breast cancer cells (Interiano et al., 2014). Knock-down of *SIAH2* in MCF-7 breast cancer cells decreases *ER* levels in resting cells. Perhaps, *SIAH2* promotes the proteasomal degradation of a factor decreasing the stability of the *ER*. (Buchwald et al., 2013).

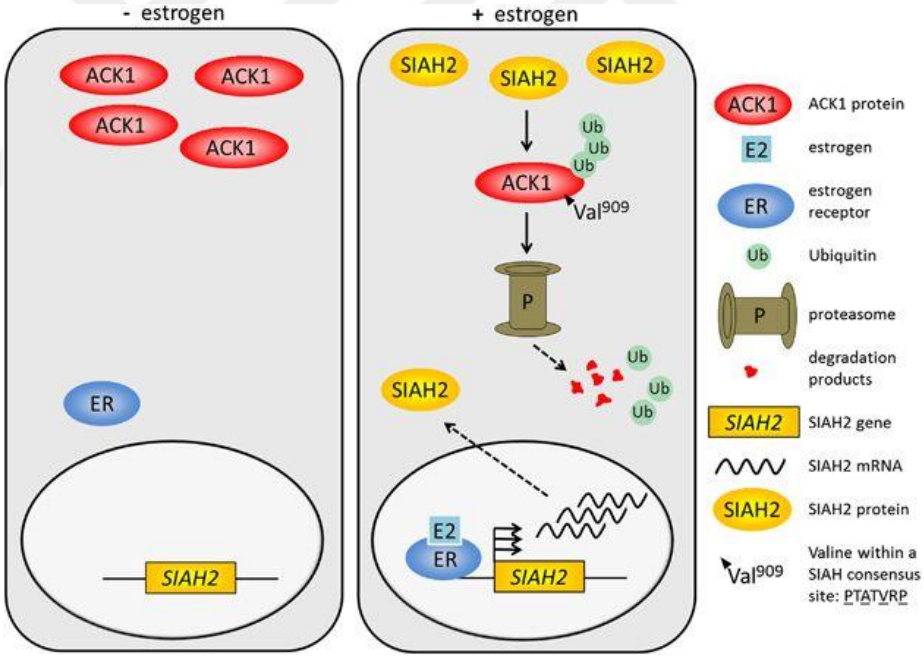


Figure 21. Regulation of SIAH expression by ER (Buchwald et al., 2013).

2.8.4. Cross regulation of *SIAH*s and *p53*

p53 is an important regulator of cellular responses to DNA damage (Wagner et al., 2014). There is an important link between *p53*, *SIAH*s, and the serine/ threonine kinase hypoxia-inducible protein kinase-2 (*HIPK2*). *HIPK2*-activated with the tumor suppressor *p53* interacts and restricts hypoxic gene expressions (Calzado et al., 2009). *SIAH1*/*SIAH2* induces the proteasomal degradation of the *HIPK2* in breast cancer cells and in other tumor-derived cells (Calzado et al., 2009). *HIPK2* phosphorylates *SIAH2* at the residues *T26*, *S28*, and *S68*, and this destabilizes *SIAH2*. Hypoxia promotes the interaction between *SIAH2* and *HIPK2* (Calzado et al., 2009). Consequently, illustrate an autoregulatory control of *p53* signaling by *SIAH*s and *HIPK2*. Also, After DNA damage, activated p53 induces the expression of *SIAH1*, but not *SIAH2*. *SIAH1* is phosphorylated by check point kinases, Ataxia Telangiectasia Mutated (*ATM*) and *Rad3* related protein (*ATR*) in response to DNA-damaged. The phosphorylation of *SIAH1* by *ATM/ATR* exchange the interaction of *HIPK2* and *SIAH1*, thus stabilizing *HIPK2*. Subsequently, *HIPK2* phosphorylates *p53* at Ser 46, leading to the transcription of apoptosis-related genes by *p53* (Figure 22) (D'Orazi et al., 2002).

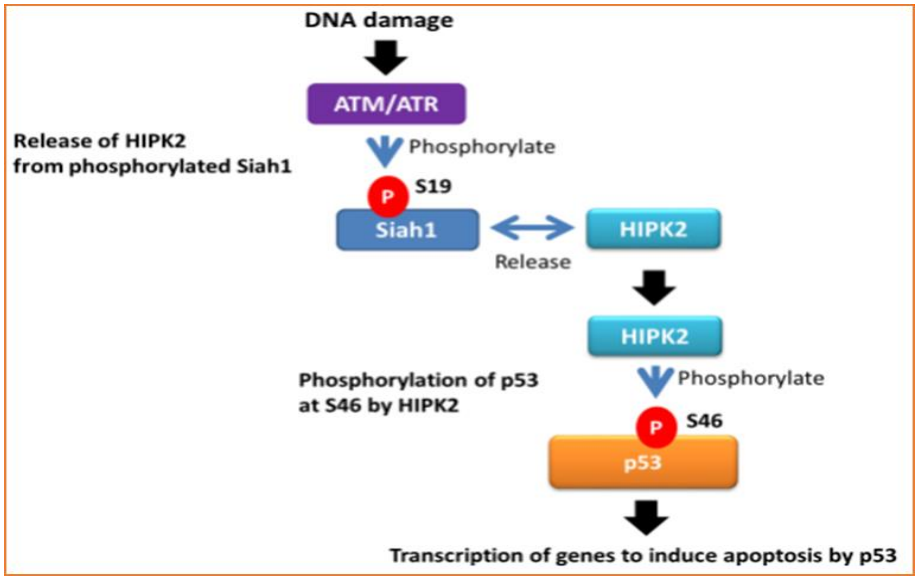


Figure 22. Apoptotic signaling pathway mediated by DNA damage (D'Orazi et al., 2002).

3. MATERIAL AND METHODS

3.1. Materials

3.1.1. Used Equipment

- Refrigerated Centrifuges (Beckman Coulter, ABD)
- Refrigerated Centrifuges (Labogene, Denmark)
- Vortex (VELP Scientifica, İtaly)
- Shaker Incubator (JSR, Korea)
- Thermo-Shaker (Biosan, Latvia)
- -80°C freezer (Panasonic, Japan)
- -80°C freezer (Nuve, Turkey)
- -20°C freezer (Bosch, Turkey)
- -20°C freezer (Ugur, Turkey)
- Ice Systems (Scotsman, USA)
- Ice Systems (Samsung, Turkey)
- UV-Vis Spektrofotometre/Nano Drop (Beckman Coulter, USA)
- Thermal Cycler (Bio-Rad, California, USA)
- Profilex PCR system (Thermo Fisher Scientific, USA)
- UV Imaging Device (Vilber Lourmat, Germany)
- StepOnePlus™ System Real-Time- PCR (Applied Biosystem, USA)

3.1.2. Used Materials

- Agarose (Lonza, Switzerland)
- Ethidium bromide (Amresco, USA)
- Ethanol (Sigma Aldrich, USA)
- FFPE RNA Kit, 50 preps Omega / R6954-01 (Omega, Germany)
- High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA)
- TaqMan® Gene Expression HER2 Assay (Thermo Fisher Scientific, USA)
- TaqMan® Gene Expression SIAH1/2 Assay (Thermo Fisher Scientific, USA)
- TaqMan® Gene Expression HSP90AAAssay (Thermo Fisher Scientific, USA)
- TaqMan® Gene Expression GAPDH Assay (Thermo Fisher Scientific, USA)
- TaqMan® Gene Expression Master Mix (Thermo Fisher Scientific, USA)

3.2. Methods

3.2.1. Clinical Samples and Ethics Statement

We investigated formalin-fixed, paraffin-embedded (FFPE) archival cancer tissue from 94 patients with primary resected invasive ductal breast carcinoma, who underwent surgery during the years 01.01.2010- 01.09.2018 at the Bursa Uludag University (Turkey), and the supervision of sample selection by an experienced pathologist. The usage of human archival tissue for molecular analysis was approved under the number (BUU 2018-22/23) by the local Ethics Committee of the Faculty of Medicine of the Bursa Uludag University.

3.2.2. RNA Extraction

3.2.2.1. Deparaffinization of FFPE Tissue

Archived Formalin-fixed, Paraffin-embedded (FFPE) tissue samples were used for profiling gene expression analysis. Two pieces of 10-µm thick sections FFPE tissue from each paraffin block were collected in sterile 1.5 mL eppendorf tubes and were baked at 56 °C for 15 min to soften the paraffin wax, followed by deparaffinization in xylene and 100% ethanol. In order to remove paraffin from FFPE tissue samples, two pieces of FFPE tissue sections were added with 1 mL of 100% xylene, heated for 5 min at 50 °C to melt the paraffin, and was then centrifuged for 2 min at room temperature at 16,000×g to per µL at the tissue. After centrifugation, xylene was removed and 1 mL of 100% ethanol was added to mix the sample which was again centrifuged at 16,000 ×g for 3 min at room temperature. After centrifugation, EtOH was discarded without disturbing the pellet. The ethanol washing process was repeated twice, during which residual ethanol was removed as much as possible without disturbing the pellet. Finally, the pellet was air-dried for approximately 25 min.

3.2.2.2. Total RNA Extraction

Total RNA was extracted from the tissues using OMEGA reagent (FFPE RNA Kit, Omega, Germany) according to the manufacturer's instructions. The RNA was eluted in 50 µL of preheated Elution Solution and quantified on a UV-Vis Spektrofotometre/Nano Drop (Beckman Coulter, USA) (Table 4). Also, The overall quality of an RNA preparation may be assessed by electrophoresis on an agarose gel electrophoresis. The RNA samples were immediately stored at -80 C, until they were reverse transcribed into cDNA.

Table. 4 The 260/280 ratio and RNA concentration (ng/µl) measured on spektrofotometre for FFPE tumor and normal tissue samples.

NO Sample	Tumor tissue	The ratio of absorbance (260/280)	RNA concentration ng/µl	Normal tissue	The ratio of absorbance (260/280)	RNA concentration ng/µl
1	T	2	752	N	1.93	80
2	T	1.95	594	N	1.85	71
3	T	2	654	N	1.97	327
4	T	1.7	58	N	1.9	133
5	T	1.93	439	N	1.9	359
6	T	1.9	539	N	2	71
7	T	2	591	N	2	50
8	T	1.7	130	N	1.95	154
9	T	1.7	133	N	1.81	235
10	T	1.5	1000	N	1.94	205
11	T	1.3	1031	N	1.94	164
12	T	2	251	N	1.9	130
13	T	2	501	N	2	207
14	T	2	145	N	2.1	149
15	T	2	701	N	1.8	390
16	T	1.9	393	N	1.8	82
17	T	1.9	94	N	1.8	41
18	T	2	449	N	2	510
19	T	2	335	N	1.9	193
20	T	1.9	665	N	2	272
21	T	1.7	1017	N	1.9	137
22	T	1.6	973	N	2	216
23	T	1.6	1021	N	2	830
24	T	2	209	N	2	287
25	T	1.6	1028	N	2	336
26	T	1.9	574	N	2	95
27	T	1.7	970	N	1.8	843
28	T	2	147	N	1.9	465

29	T	1.9	91	N	1.9	147
30	T	2	207	N	1.4	343
31	T	1.54	378	N	2.1	454
32	T	2	77	N	2	767
33	T	1.6	58	N	1.4	29
34	T	2	852	N	2	50
35	T	1.8	977	N	2	541
36	T	2	224	N	2	148
37	T	1.9	805	N	2	544
38	T	1.7	1008	N	2	281
39	T	1.5	1034	N	1.8	52
40	T	1.8	998	N	2	273
41	T	1.8	941	N	2	182
42	T	1.7	1022	N	2	390
43	T	1.8	931	N	2	214
44	T	1.6	926	N	1.9	135
45	T	1.8	85	N	2	87
46	T	1.4	1014	N	2	712
47	T	1.9	588	N	1.9	128
48	T	1.3	1034	N	2	254
49	T	2	591	N	2.1	118
50	T	2	129	N	2	610
51	T	1.1	1002	N	2	156
52	T	1.3	1004	N	2	212
53	T	1.2	1015	N	2	321
54	T	2	399	N	1.9	45
55	T	1.5	991	N	2	136
56	T	1.6	963	N	1.1	1015
57	T	1.2	1041	N	2.1	88
58	T	1.1	1060	N	2	98
59	T	1.3	1060	N	2	444
60	T	2	924	N	2.1	721
61	T	1.9	737	N	1.9	465
62	T	1.3	1011	N	1.6	698
63	T	1.9	291	N	1.7	46
64	T	2	777	N	1.6	396
65	T	1.7	993	N	1.9	126
66	T	1.1	1052	N	2	94
67	T	1.9	882	N	1.7	63
68	T	1.4	1000	N	1.9	371
69	T	1.1	1061	N	2	185
70	T	1.2	1061	N	1.9	65
71	T	2	514	N	1.8	128
72	T	2	514	N	2	123
73	T	1.7	977	N	2.1	71
74	T	1.9	87	N	2	353
75	T	2.1	292	N	2.1	31

76	T	2.1	609	N	2.1	156
77	T	2.1	581	N	2.1	509
78	T	2	107	N	2	70
79	T	2.1	345	N	2.1	87
80	T	1.6	254	N	2.1	126
81	T	2.1	445	N	1.3	98
82	T	2	466	N	2	638
83	T	1.9	898	N	2	394
84	T	2	359	N	2	316
85	T	1.83	894	N	1.9	80
86	T	1.7	236	N	2	95
87	T	2	702	N	1.5	254
88	T	2	200	N	2.1	250
89	T	1.9	360	N	2	420
90	T	2	424	N	2	525
91	T	2	26	N	2	671
92	T	2.1	202	N	1.6	30
93	T	1.64	202	N	1.53	496
94	T	2	415	N	1.9	155
95	T	2	366	N	2	144
96	T	2	453	N	1.9	93
97	T	2.1	634	N	2.1	104
98	T	2.1	5.4	N	2	66
99	T	1.8	351	N	1.9	184
100	T	1.8	541	N	2	541

3.2.3. cDNA Synthesis

Reverse transcription was performed by the TaqMan High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, USA) after RNA extraction. The 20 µL reverse transcription reaction contained dNTPs, MultiScribe Reverse Transcriptase (50 U/ µL), 10x Reverse Transcription Buffer, Random Primer, nuclease-free water, and 10 µL RNA (Table 5).

Table. 5 Preparing the 2x Reverse Transcription Master Mix

Component	Volume/Reaction (µL)
10✕ RT Buffer	2.0
25✕ dNTP Mix (100 mM)	0.8
10✕ RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
Nuclease-free H2O	4.2
Total per Reaction	10.0

Set the reaction volume to 20 µL.

The reaction was carried out at 4 steps (Table 6) on Thermal Cycler (Bio-Rad, California, USA). The cDNA samples were stored at -20 °C until further usage.

Table. 6 Performing Reverse Transcription

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

3.2.4. TaqMan® Gene Expression and Probe Design

Applied Biosystems developed TaqMan® Gene Expression Assays, a genome-wide collection of quantitative, standardized assays for gene expression analysis by real-time quantitative RT-PCR. The TaqMan® Assay utilizes the 5' nuclease activity of Taq DNA polymerase to cleave a fluorescently labeled probe (FAM™-labeled MGB). These assays are a family of more than 700,000

primer/probes sets, which have been pre-designed by using state-of-the-art, extensively validated (~20,000 assays were functionally tested) bioinformatics pipeline. Assays for quantification of gene-encoded transcripts are available for Homo sapiens, Mus musculus, Rattus norvegicus, Drosophila melanogaster, Arabidopsis thaliana, Canis familiaris, Caenorhabditis elegans, and Macaca mulatta (Heid et al., 1996).

Assay design: Each assay includes a single FAM™ dye-labeled TaqMan® probe with a minor groove binder (MGB) moiety and two unlabeled oligonucleotide primers.

These components are designed with a program called Taq Express, which takes into account optimal melting temperature (Tm) requirements, GC-content, buffer/salt conditions, oligonucleotide concentrations, secondary structure, optimal amplicon size, reduction of primer-dimer formation. In case of multi-exon transcripts, probes are placed at each exon-exon boundary to ensure that the primers bind in two distinct exons and that the fluorescent signal is only generated from correctly spliced templates (Heid et al., 1996).

All probes (Applied Biosystems, Foster City, CA, USA) in this study, based on the mRNA sequences of target *HER2, HSP90AA1*, *SIAH1/2*, and reference gene *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) which were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (Table 7).

Table. 7 TaqMan® Gene Expression Assays to evaluate the yield of cDNA conversion.

Gene Target	Kit	Assay ID	Amplicon Length (bp)
GAPDH	TaqMan® Gene Expression Assays, *GAPDH* [Human]	Hs03929097_g1	58
HER2 (ERBB2)	TaqMan® Gene Expression Assays, *HER2* [Human]	Hs01001580_m1	60
HSP90AA1	TaqMan® Gene Expression Assays, *HSP90AA1* [Human]	Hs00743767_sH	133
SIAH1	TaqMan® Gene Expression Assays, *SIAH1* [Human]	Hs 02911337_m1	60
SIAH2	TaqMan® Gene Expression Assays, *SIAH2* [Human]	Hs00192581_m1	107

3.2.5. Real-Time *qRT-PCR*

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) is a major development of PCR technology that enables reliable detection and measurement of products generated during each cycle of the PCR process. This technique became possible after the introduction of an oligonucleotide probe which was designed to hybridize within the target sequence. Cleavage of the probe during PCR because of the 5' nuclease activity of Taq polymerase can be used to detect amplification of the target-specific product (Figure 23).

Techniques to Monitor Degradation of The Probe Including of (1) Intercalation of double-stranded DNA-binding dyes, (2) ^{32}P probe labeling, and (3) Labeling of the probe with fluorescent dyes (Figure 23) (Heid et al., 1996).

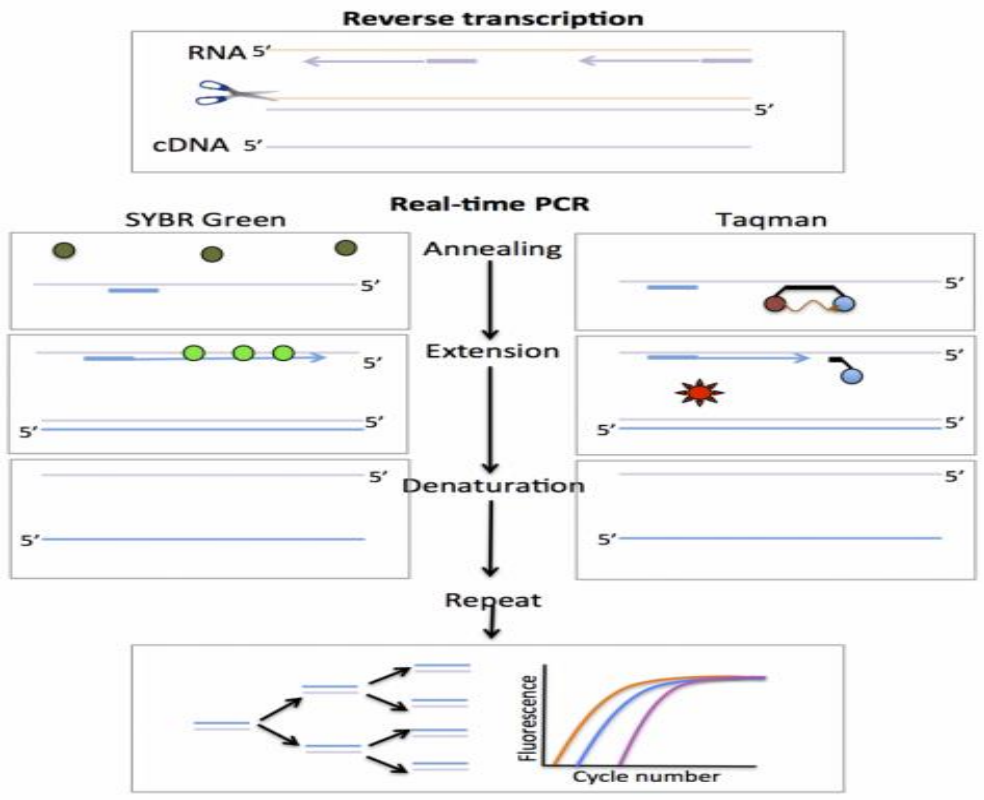


Figure. 23 TaqMan® Gene Expression Assays (Heid et al., 1996)

3.2.6. Standard Curve Construction and Amplification Efficiency Optimization

The baseline is defined as PCR cycles in which a reporter fluorescent signal is accumulating but is beneath the limits of detection of the instrument.

ΔRn is an increment of fluorescent signal at each time point. The ΔRn values are plotted versus the cycle number.

The threshold is an arbitrary level of fluorescence chosen on the basis of the baseline variability. A signal that is detected above the threshold is considered a real signal that can be used to define the threshold cycle (Ct) for a sample. The threshold can be adjusted for each experiment so that it is in the region of exponential amplification across all plots.

Ct is defined as the fractional PCR cycle number at which the reporter fluorescence is greater than the threshold. The Ct is a basic principle of real-time PCR and is an essential component in producing accurate and reproducible data (Figure 24) (Heid et al., 1996).

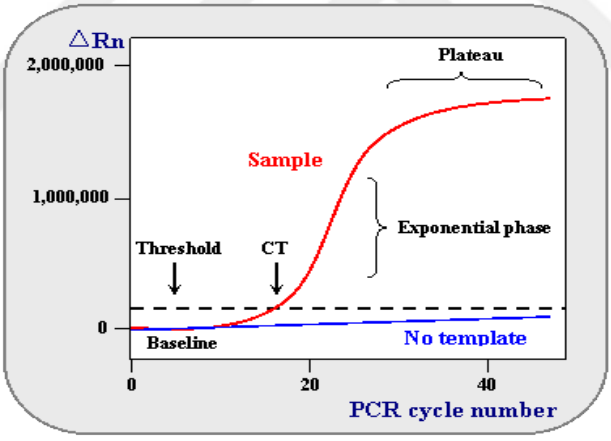


Figure. 24 Standard Curve TaqMan® Gene Expression (Heid et al., 1996)

In the present study, the reaction mix for each sample using the components listed in Table 8. For optimal performance of TaqMan, ® Gene Expression used 100 ng of cDNA. Then the appropriate volume of each reaction mixture transferred to each well of an optical plate, and the plate covered with a MicroAmp® Optical Adhesive Film. For standard 96- well plates and centrifuged the plate briefly to spin down the contents and eliminate air bubbles from the solutions. The plate placed in the Applied Biosystems real-time PCR instrument (Applied Biosystems, Inc.). The assays were started by denaturation for 2 min at 50 °C, 10 min at 95 °C and followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min (Table 9).

Table. 8 Reaction mix for each sample

Component	**Volume (µL) per reaction (20-µL rxns.)**	**Final Conc.**
TaqMan® Universal Master Mix II (2✕)	10.0	1X
TaqMan® Gene Expression Assay (20✕)	1.0	1X
cDNA template + H2O	9.0	100 ng
Total Volume	**20.0**	-

Table. 9 Thermal Cycling Parameters

System	**incubation**	**Polymerase activation**	**PCR**	
	Hold	Hold	Cycle (40 cycles)	
			Denature	Anneal/extend
Temp. (°C)	50	95	95	60
Time (min:sec)	2.0 min	10.00 min	00.15 sec	1.00 min

RT-PCR data analysis: The experimental data were processed by $2^{-\Delta\Delta CT}$ on the premise that the amplification efficiency of the target genes (*HER2*, *HSP90*, and *SIAH1/2*) and reference gene. The average CT value from samples and ΔCt value ($\Delta Ct = Ct_{Target\ gene} - Ct_{GAPDH}$) was calculated, and $2^{-\Delta\Delta CT}$ ($CT = CT_{tumor\ sample} - CT_{control\ sample}$) was computed.

HSP90, *SIAH1/2*, and *HER2* cut-off values were predefined based on previous studies. Since different platforms, normalization strategies and primer/probe lots had been used a constant target-specific shift in Ct values between previous and current assay conditions occurred. The cut-offs from the published previous studies were therefore transformed by the addition of an offset.

3.2.7. Data Analysis and Statistics

Statistical analysis was performed using SPSS version 19.0 (IBM, Armonk, NY, USA). Correlation of gene expression analyses was done using Pearson linear correlation. Receiver operating characteristic (ROC) curve analysis between conventional *HER2 IHC*, and *HER2* mRNA RT-qPCR was performed for all data. Survival analysis was performed using Kaplan-Meier analysis. All tests were 2-sided, and the significance level was set at 0,05. Also graphs made in GraphPad; r and p- values used from SPSS.

4. RESULTS

4.1 Baseline clinical data, consort statement and the central evaluation of HER2

The clinical characteristics are shown in Table 10. A total of 94 invasive ductal carcinoma breast cancer patients were in this study. The mean age was 53,18±11,62 years (median, 52; range, 29 to 82), and the median age at the time of breast cancer diagnosis was 47.

Table. 10 Baseline Clinical and Pathologic Characteristics of the Invasive Ductal Carcinoma Breast Cancer Patients (n =94).

Characteristics	**Number**	**Percent (%)**
Age		
< 50	40	%42.55
≥ 50	54	%57.45
Age at diagnosis of breast cancer		
< 40	21	%22.34
≥ 40	73	%77.65
Family history of cancer		
Positive	27	%52.94
Negative	24	%47.06
Tumor localization		
Right	33	%35.48
Left	41	%44.09
Bilateral	19	%20.43
Grade		
I/ II	50	%54.35
III	42	%45.65
Lymph node		
N0: node-negative	48	%51.07
N1: metastasis involving 1–3 nodes	27	%28.72
N2: at least 4 nodes	19	%20.21
Tumor size (cm)		
< 3 cm	57	%64.04
≥ 3 cm	32	%35.96
Ki-67		
≤15%	29	%32.22
15%–35%	40	%44.45
>35%	21	%23.33
p53 expression		
Positive	23	%35.94
Negative	41	%64.06

Table. 10 continued

In situ component		
No-DCIS (0)	10	%14.70
L-DCIS (<25%)	30	%44.12
H-DCIS (≥25%)	28	%41.18
Lymphatic invasion		
Positive	24	%26.67
Negative	66	%73.33
Vascular invasions		
Positive	10	%11.11
Negative	80	%88.89
Perineural invasion		
Positive	18	%19.78
Negative	73	%80.22
***ER* status**		
Positive	71	%77.17
Negative	21	%22.83
***PR* status**		
Positive	58	%63.04
Negative	34	%36.96
***HER2* status**		
Positive	36	%39.13
Negative	56	%60.87
Subtypes		
Luminal A	46	%52.27
Luminal B	25	%28.41
TNBC	6	%6.82
HER2-enriched	11	%12.50
Recurrence /Metastasis		
With Recurrence	35	%50.72
Without Recurrence	34	%49.28

4.2 Determining criteria to define RT-PCR categories

IHC and RT-PCR comparisons:

To determine *HER2* status, we performed the results of immunohistochemistry and RT-PCR on all 94 cases. In our experience, three major points have to be satisfied for the standardization of molecular methods: (1) the supervision of sample selection by an experienced pathologist, to assure that a suitable amount of infiltrating tumor cells is analyzed and that in situ carcinoma, potentially overexpressing *HER2*, is not included in the specimen; (2) a strict control of the quantity and quality of RNA extracted; (3) the use of extensively validated PCR analysis algorithms to measure the RNA templates in the tumor samples (Zoppoli et al., 2017). Results on the two

different measurements of *HER2* are summarized in Table 13. Immunohistochemical *HER2/neu* were negative in 47 out of 94 cases (50%); 34 cases were score 0 and 13 cases were score 1+, 11 cases (11,70%) were score equivocal (2+) and 36 cases (38,30%) were score 3+ (positive).

According to the ROC analysis, the cut-off value of *HER2* RT-PCR was determined (Table 11, Figure 25). On ROC curve analysis, patients with scores ≤ highest cut-off corresponding to high sensitivity will all be HER 2 negative, and those with scores > lowest cut-off corresponding to high specificity will all be HER 2 positive. The highest likelihood ratio was the relative *HER2* mRNA levels over 7,16 fold change, indicating the highest sensitivity and specificity (p=0,0007).

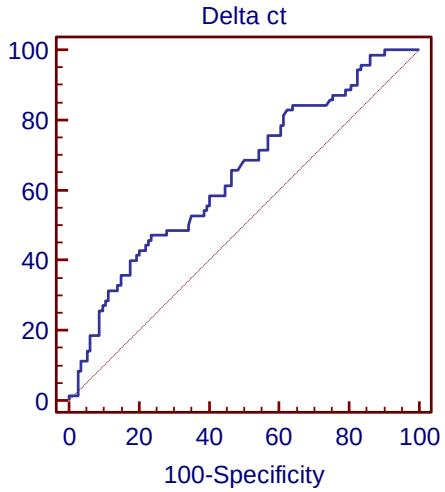


Figure. 25 ROC analysis, the cut-off value of HER2 RT-PCR

Table. 11 ROC curve analysis and clinical cut-off values of *HER2* mRNA RT-PCR (GAPDH Ct value below 25) (n=94).

Cut-off for RT-PCR (Fold change)	Sensitivity %	Normalization gene	Specificity %	95% CI	AUC (Area Under Curve)	*p*-value
7,16	47,14%	GAPDH	76,32%	0,568-0,711	0,641	**0,0007**

ROC, Receiver operating characteristic; CI, Confidence interval.

Based on this result, samples with a relative *HER2* mRNA expression level greater than 7,16 by *HER2* RT-PCR assay were considered positive in this study (Table 12).

Table. 12 Results of between *HER2* IHC (score 0, 1+, 2+, and 3+) versus *HER2* RT-PCR (n=94).

Sample NO	IHC	Real-Time PCR (Fold change)	Sample NO	IHC	Real-Time PCR (Fold change)
1	0	0,15	**5**	2+	4,14
2	0	1,22	**10**	2+	1,3
8	0	0,04	**17**	2+	0,67
9	0	0,85	**46**	2+	2,6
11	0	1,55	**51**	2+	2,57
13	0	1,06	**71**	2+	2,93
15	0	0,08	**75**	2+	0,27
16	0	0,24	**83**	2+	***21,11**
18	0	0,4	**86**	2+	0,21
19	0	0,24	**87**	2+	0,4
22	0	2,16	**93**	2+	1,95
23	0	0,55	**2**	3+	***7,89**
26	0	0,44	**7**	3+	0,16
27	0	4,69	**12**	3+	4,82
28	0	2,68	**14**	3+	***20,82**
30	0	0,74	**20**	3+	0,97
32	0	0,72	**29**	3+	0,95
33	0	1,22	**31**	3+	***8,75**
40	0	0,63	**34**	3+	***27,1**
41	0	3,58	**35**	3+	4,53
42	0	5,82	**36**	3+	2,83
50	0	1,41	**37**	3+	0,5
55	0	1,12	**38**	3+	0,26
61	0	0,08	**39**	3+	***61,39**
62	0	0,19	**43**	3+	0,72
65	0	0,71	**44**	3+	2,33
68	0	1,29	**45**	3+	1,95
69	0	***13,93**	**47**	3+	5,21
70	0	2,17	**48**	3+	***24,93**
74	0	0,57	**49**	3+	0,95
76	0	0,22	**52**	3+	1,05
80	0	***13,18**	**53**	3+	***18,25**
84	0	0,01	**54**	3+	7,11
94	0	1,11	**56**	3+	0,22
1	1+	1,59	**57**	3+	0,81
4	1+	2,17	**58**	3+	3,58
21	1+	1,95	**59**	3+	1,16
24	1+	1,06	**60**	3+	0,97
25	1+	0,53	**64**	3+	5,17
63	1+	***7,16**	**66**	3+	***50,56**
73	1+	1,06	**67**	3+	0,88
77	1+	1,29	**72**	3+	6,19
78	1+	2,12	**81**	3+	***27,86**
79	1+	1,741	**82**	3+	***16,22**
85	1+	0,69	**88**	3+	2,55
89	1+	0,7	**90**	3+	***13,45**
92	1+	1,25	**91**	3+	***23,59**

*** HER2 RT-PCR based-of fold change (cut-off, ≥ 7,16)**

4.3 Concordance between IHC versus RT-PCR

By RT-PCR assay in the cut-off equal 7,16 of the 47 negative patient cases by IHC, 44 cases were classified as negative, of the 11 equivocal patient cases were reported as negative by IHC, 10 cases were classified as negative by RT-PCR assay and 1 equivocal patient cases were reported as positive by RT-PCR. Of 36 positive cases by IHC, only 12 cases were reported as positive by RT-PCR assay with the cut-off equal 7,16. Concordance between *HER2* IHC and *HER2* RT-PCR was 74,35%, that *HER2* IHC 0 (94,12%), and in *HER2* IHC 1+ (92,31%) subgroup. Whereas concordance was not good in *HER2* IHC 3+ cases (33,33%) (Table 13).

Table. 13 HER2 status assessed by immunostaining and real-time RT-PCR (the cut-off equal 7,16).

HER2 status (IHC)		**HER2 status (RT-PCR)**		
Number of patients		**Positive (number of patients)**	**Negative (number of patients)**	**Concordance IHC vs RT-PCR %**
(0)	34	2	32	7,16 vs. 0 (94,12%)
(1+)	13	1	12	7,16 vs. 1+ (92.31%)
(2+)	11	1	10	7,16 vs. 2+ (90.90%)
(3+)	36	12	24	7,16 vs. 3+ (33.33%)

4.4 Expression of *SIAH1, SIAH2, HSP90,* and *HER2* mRNA in IDC breast cancer

Housekeeping genes were widely used to detect the expression of *SIAH1*, *SIAH2*, *HSP90,* and *HER2* genes. In this study, we used *GAPDH* housekeeping gene for the precise detection of the target assays. The Ct value variation of housekeeping genes was small and was less than 25. The comparative Ct method was used to calculate fold changes.

Following values are calculated per sample:

(1) $\Delta Ct_{tumor} = Ct_{target\ gene,\ tumor} - Ct_{reference\ gene,\ treated}$

(2) $\Delta Ct_{normal} = Ct_{target\ gene,\ normal} - Ct_{reference\ gene,\ control}$

(3) $\Delta\Delta Ct = \Delta Ct_{tumor} - \Delta Ct_{normal}$

(4) Fold change= $2^{-\Delta\Delta Ct}$

HSP90, *SIAH1*, and *SIAH2* mRNA gene expressions comparison between tumors and normal tissues calculated by Sabiosciences' data analysis software (https://dataanalysis.qiagen.com), that are made in Table 14.

No significant difference in *HSP90*, *SIAH1*, and *SIAH2* mRNAs expression were found between the normal and tumor tissues. Also, graphs of fold changes for *HSP90*, *SIAH1*, and *SIAH2* mRNA gene expressions comparison between tumors and normal tissues were presented in Figure 26.

Table. 14 *HSP90*, *SIAH1*, and *SIAH2* mRNA gene expressions comparison between tumors and normal tissues in invasive ductal carcinoma patients (n=94) by use of Sabiosciences' data analysis software

PCR Array genes	Normal Tissue	Tumor Tissue				
	2^(- Avg.(Delta(Ct))	2^(- Avg.(Delta(Ct))	95% CI*	Fold Change	Fold Regulation (Up-Down Regulation)	p-value
HSP90	0,009433	0,012584	(0,78;1.89)	1,334	Up- Regulated	0,2604
SIAH1	0,001176	0,001561	(0,68;1,97)	1,3276	Up- Regulated	0,4319
SIAH2	0.004737	0.005125	(0.64, 1.53)	1.082	Up- Regulated	0.3863

The p values are calculated based on a Student's t-test of the replicate 2^(- Delta CT) values for each gene in the control group and treatment groups, according to Sabioscience in Sabiosciences' data analysis software with p-values significant and in up or low fold change2.

*CI: Confidence interval

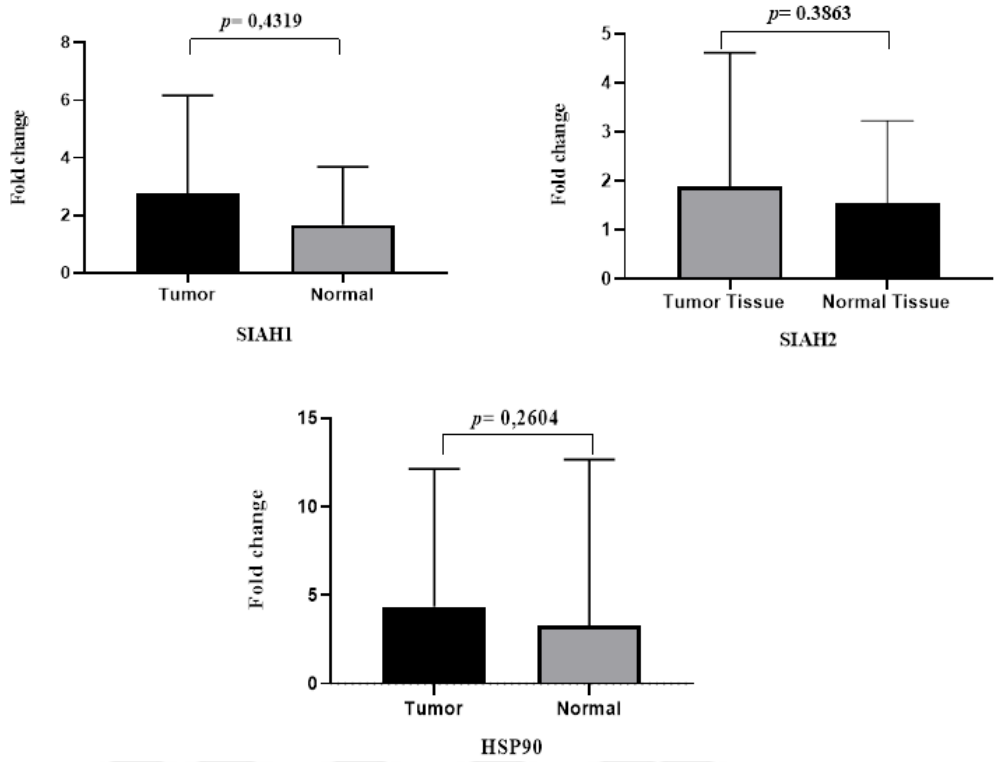


Figure. 26 *HSP90, SIAH1*, and *SIAH2* mRNA gene expressions comparison between tumors and normal tissues in invasive ductal carcinoma (n=94)

4.5 Expression of *SIAH1, SIAH2,* and *HSP90* in *HER2+ RT-PCR* breast cancer

HSP90, SIAH1, and *SIAH2* mRNA gene expressions compared between tumors and normal tissues in *HER2*-positive breast cancers that *HER2* gene significantly up-regulated (≥7,16 fold differences; n=16) by use of Sabiosciences' data analysis software (Table 15). Our study showed that *HSP90* expression in tumor tissues significantly was up-regulated (*p*= 0,047), and also *SIAH2* expression significantly was up-regulated (*p*= 0,024) in *HER2*-positive breast cancers. The *SIAH1* expression in tumor tissues approximately significant was up-regulated (p= 0,376) in *HER2*-positive breast cancers. Also, graphs of fold changes for *HSP90, SIAH1*, and *SIAH2* mRNA gene expressions comparison between tumors and normal tissues in *HER2*-positive breast cancers were presented in Figure 27.

Table. 15 Gene expressions (*HSP90*, *SIAH1*, and *SIAH2*) comparison between tumors and normal tissues in HER2+ breast cancer based of RT-PCR assay; *HER2* gene significantly up-regulated (≥7,16 fold differences) in Sabiosciences' data analysis software (n=16)

PCR Array genes	Normal Tissue	Tumor Tissue			
	2^(-Avg.(Delta(Ct))	2^(-Avg.(Delta(Ct))	Fold change	Fold Regulation (Up-Down Regulation)	p-value
HSP90	0.008689	0.014752	1.697	Up- Regulated	***0.047**
SIAH1	0.000707	0.001601	2.2646	Up- Regulated	0.376
SIAH2	0.002292	0.004333	1.89	Up- Regulated	***0.024**

The p values are calculated based on a Student's t-test of the replicate 2^(- Delta CT) values for each gene in the control group and treatment groups.

*CI: Confidence interval, *: p< 0,05

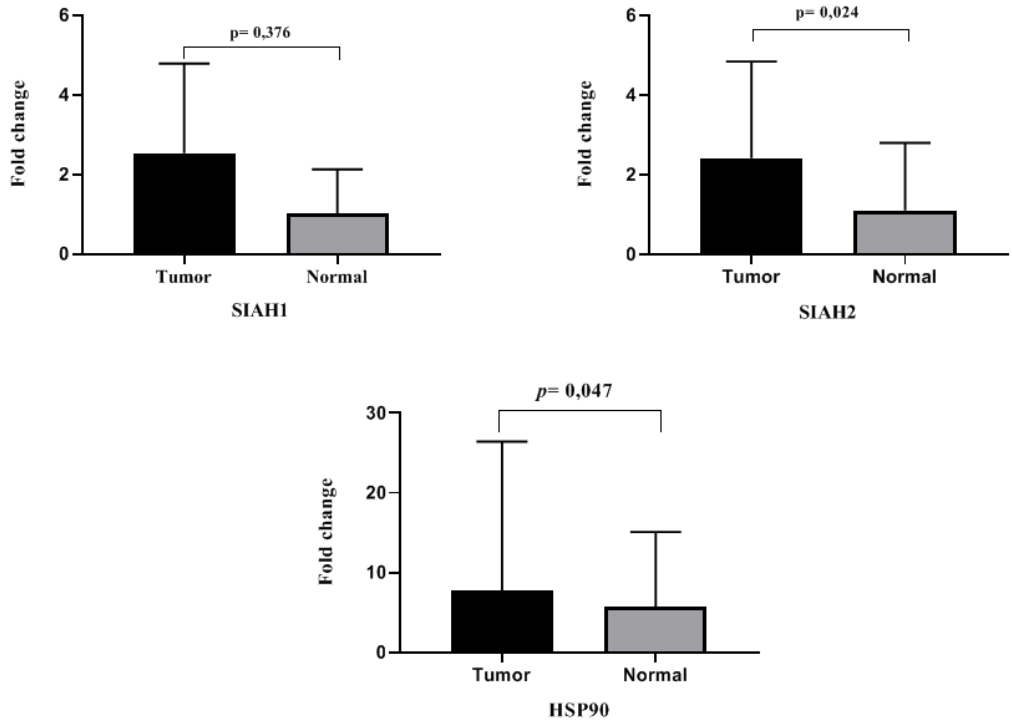


Figure. 27 *HSP90*, *SIAH1*, and *SIAH2* mRNA gene expressions comparison between tumor and normal tissues in *HER2*-positive breast cancers (n=16).

4.6 Correlation of analyzed markers

After normalization with internal reference genes, the median ΔCt value of *HER2*, *HSP90*, *SIAH1* and *SIAH2* mRNA in IDC breast cancer tissue were 4,75; 6,42; 9,40 and 8,13; respectively. In terms of correlation four genes were analyzed in this study.

Correlation analysis showed that the mRNA expression of *HSP90* and *HER2* was linearly correlated (*P*=0,001; r=0,20), and mRNA expression of *HSP90* and *SIAH2* was correlated inversely (*P*=0,000; r=0,45)

In terms of correlation *SIAH2* expression and *HER2*, there were linearly correlation (*P*=0,000; r=0,25) (Figure 28), and between *SIAH1* and *HER2* or *HSP90*, no correlation was seen in our study.

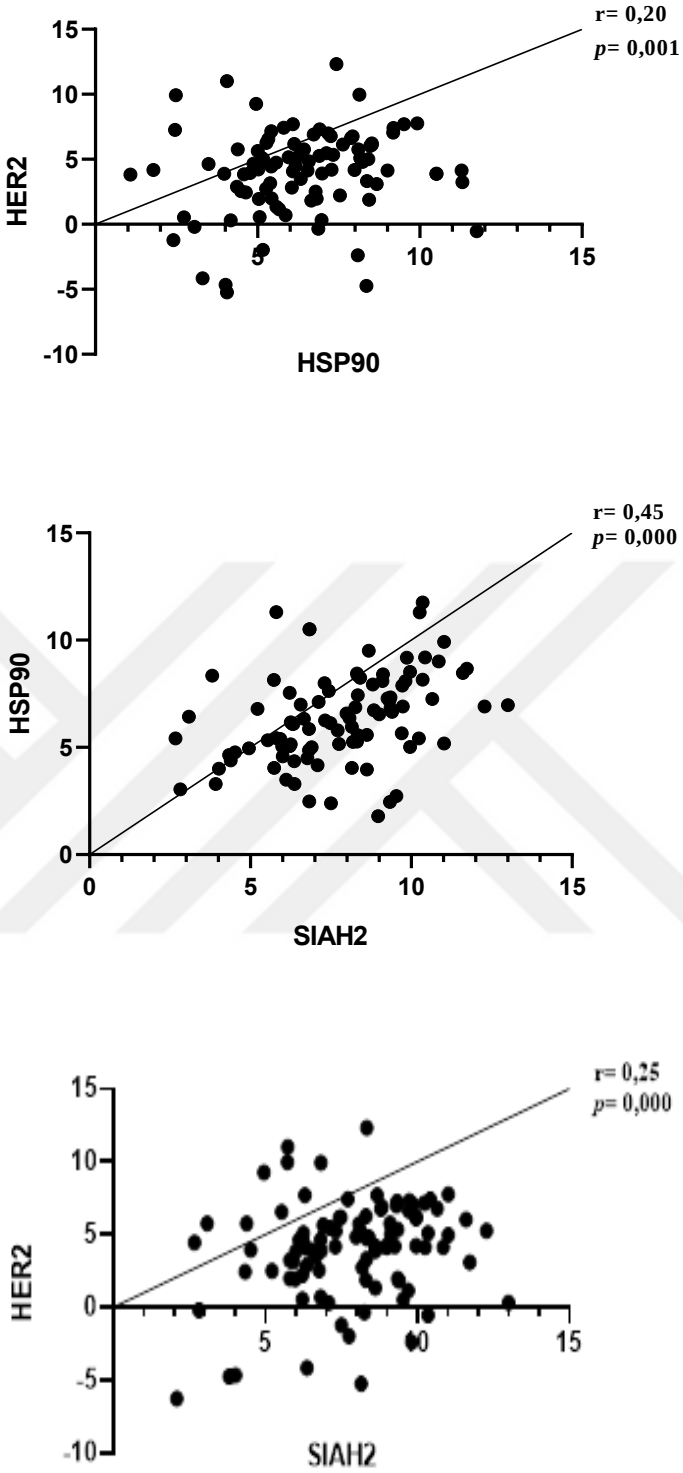


Figure. 28 Correlation of analysis

4.7 Effect of *HER2*-positive RT-PCR on overall survival (OS) in invasive ductal breast cancer

The mean follow-up time range of breast cancer patients was 60 months. The Kaplan–Meier analysis of overall survival performed in 16 *HER2*-positive (RT-PCR) patients with fold change ≥ 7,16. In this study, Overall survival was not significantly different between patients with *HER2*-positive with follow-up of ≥60-month and <60-month (P=0.0543, and 0.1712, respectively) (Table 16).

The test for the interaction between *HER2*-positive RT-PCR (Fold change ≥7,16) group and *HER2* IHC 3+ group were not statistically significant in the cox model (Table 17, Figure 29). Also in the 4 *HER2*-positive RT-PCR because low of number patient, the cox model didn't perform on this group. Therefore with the increase of number *HER2*-positive patients will be could better results.

Table. 16 Overall survival (OS) in patients with *HER2*-positive (RT-PCR) with the follow-up 60-month and lower 60-month (n=16)

Gene	Normal Tissue	Tumor Tissue (Follow-up ≥ 60-month) n=7				Tumor Tissue (Follow-up< 60-month) n=9			
	2^(-Avg.(Delta(Ct))	2^(-Avg.(Delta(Ct))	Fold Regulation	Fold Change	*p*- value	2^(-Avg.(Delta(Ct))	Fold Regulation	Fold Change	*p*- value
HER2-positive (Fold-change ≥7,16)	0.043813	0.726267	16.5765	16.576	0.0543	0.511293	11.6699	11.669	0.1712

Table. 17 Cox Relative Risk Model assessing interaction of *HER2*-positive RT-PCR (Fold change ≥7,16) group and *HER2* IHC 3+

HER2 test assay	Relative Risk Ratio	95% CI	*p*-value
RT-PCR (Fold change ≥7,16) n=16	0.9889	0.3502 - 2.7923	0.9833
IHC (*HER2* 3+ n=24	1.8536	0.6726 - 5.1086	0.2352

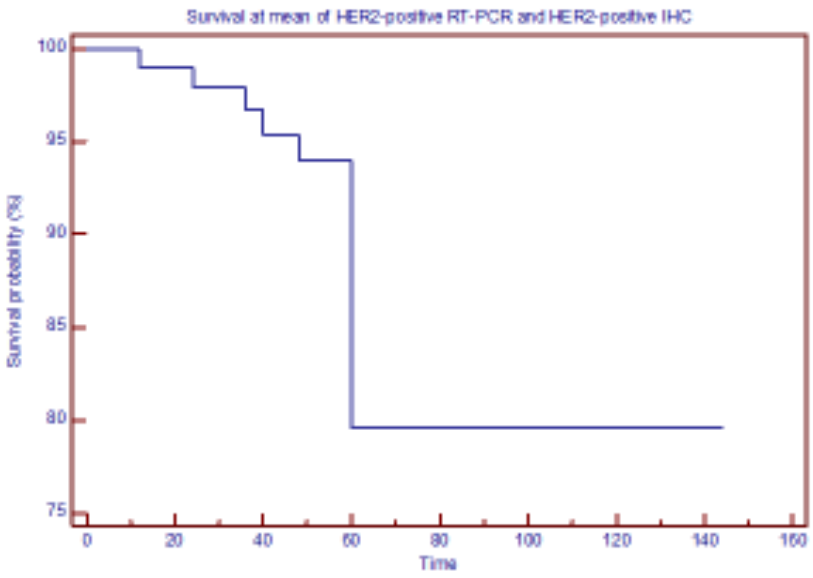


Figure. 29 Cox Model assessing the interaction of *HER2*-positive RT-PCR group and *HER2* IHC 3+

Also Kaplan Meier analysis in two groups, IHC and RT-PCR *HER2*-positive (group1; n=12) and in IHC *HER2*-positive and RT-PCR *HER2*-negative (group 2; n=24), any relation significantly show with proliferation index Ki-67, metastasis status, and tumor grade.

Table. 18 Kaplan Meier analysis in two groups [in both IHC and RT-PCR *HER2*-positive (group1; n=12) and in IHC *HER2*-negative and RT-PCR *HER2*-positive (group 2; n=24)].

Clinicopathologic Features	**Hazard ratio**	**95% CI**	***p*- value**
Ki-67	0.6068	0.1039 to 3.5426	0.5977
Metastasis	1.7386	0.4347 to 6.9541	0.3928
Grade	1.8154	0.4053 to 8.1301	0.3884

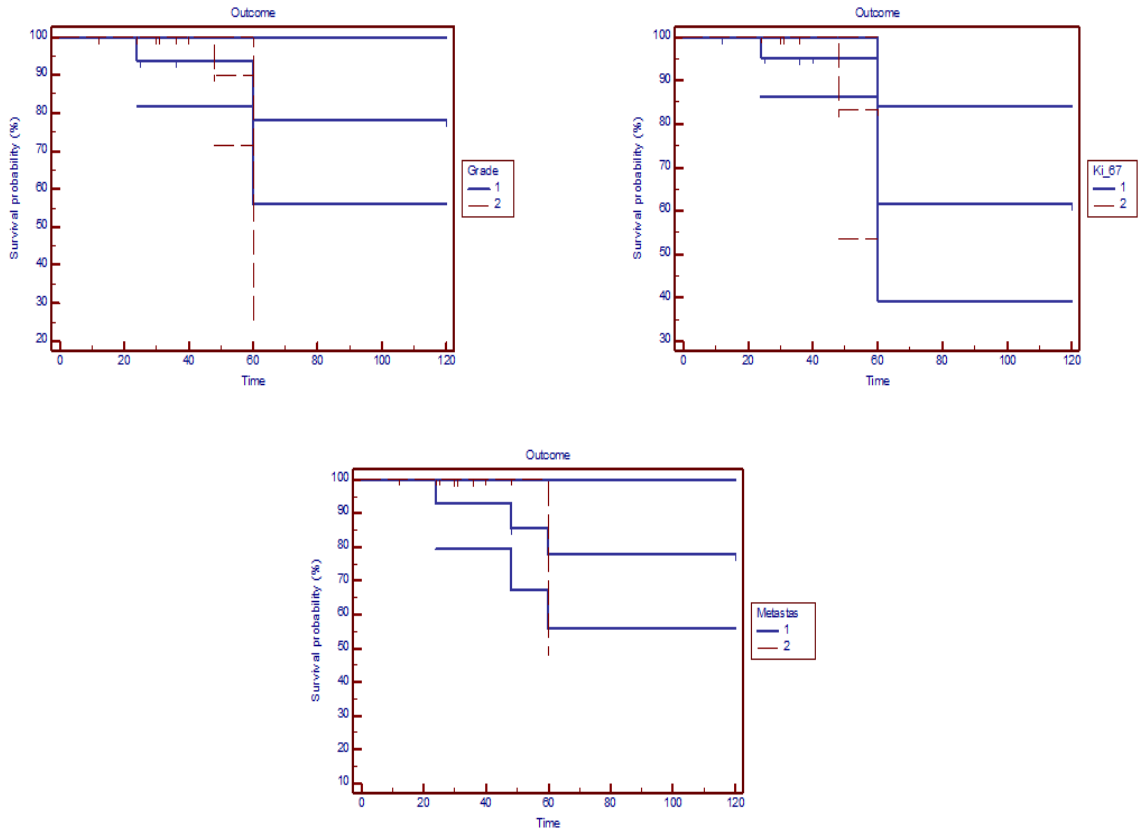


Figure. **30** Relation of clinicopathologic features in IHC and RT-PCR *HER2*-positive breast cancer

5. DISCUSSION AND CONCLUSION

Among females, breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death. IHC4 score is rapid, economical breast cancer prognosis. *ER, PR, Ki-67,* and *HER2* IHC4 biomarkers have been implicated clinically as a prognostic index of disease clinical and distant recurrence outcomes. Grade, age, nodal status, tumor size, and type of drug treatment with IHC4 biomarkers were combined to produce the IHC4 algorithm (Cuzick et al., 2011). *HER2* is an important prognostic factor of breast cancer, and *HER2* overexpression is correlated with aggressive tumors, lower prognosis, and response to chemotherapy (Ciocca et al., 1992; Duffy et al., 2016; Ross et al., 2004). For a clinical decision in neoadjuvant and adjuvant therapies such as trastuzumab, *HER2* testing is validated to predict response to anti-*HER2* therapies (Harris et al., 2016; Wolff et al., 2014). IHC and FISH approved by the FDA for *HER2* testing, that first testing assay is IHC, followed by FISH is applied for samples with equivocal or incompatible results (Hanna et al., 2007; Wolff et al., 2007). However, they are time-consuming, onerous, costly, and difficult to standardize (Nitta et al., 2012; Perez et al., 2010). But, PCR-based methodologies detecting *HER2* positivity at both RNA and DNA levels involve quantitative measurements, does not require observer eyes to interpret also having potential advantages of independence from interobserver variability and can be easily standardized and automated and performed on small samples (Lehmann-Che et al., 2011; Noske et al., 2011; Park et al., 2014). One of the disadvantages of this method is that during RNA extraction for RT-PCR, tumor cells and non-tumor cells may be are mixed, which can the influence on the result and leading to false-negative outcomes (Table 19) (De et al., 2010; Lehmann-Che et al., 2011). Since real-time PCR is the oldest and most obvious, which is also a part of Oncotype DX ®, a 21-gene assay, therefore PCR-based methodologies detecting *HER2* RNA expression can be evaluated by applying various platforms (Paik et al., 2004).

Table. 19 Relative comparisons of IHC and FISH with RT-PCR

	IHC	**FISH**	**RT-PCR**
Detection	Protein expression levels	Gene number alterations, DNA rearrangements	Gene expression levels
Description	Use of antibodies to detect levels of a specific protein	Use of a fluorescently labeled DNA probe to detect specific DNA sequences in chromosome	Conversion of RNA to cDNA by reverse transcriptase then quantification of specific gene sequences using PCR
Sample requirement	Tissue sections, FFPE samples Frozen samples	Tissue sections FFPE samples fresh frozen	FFPE samples fresh frozen specimens
Example assays	IHC4 (ER, PR, HER2, and Ki-67)	HER2 FISH pharmDX Kit	Oncotype DX PAM50, Breast Cancer Index
Determination	Is subjective and Needs experienced eyes	Is semiquantitative and Needs experienced eyes	Is quantitative and Uses numerical values with statistics
Advantages	The simple, inexpensive procedure, Processed slides can be stored for years, and reassessed, Cell morphology can be viewed	High sensitivity and specificity, The resolution is better, Can be applied to both dividing and non-dividing cells	Cost-effective, Rapid results, Sensitive
Disadvantages	The semiquantitative, subjective score, Fixation time can affect results, Results dependent on the quality of antibody used to detect the protein, Usually, only 1-2 proteins can be analyzed per section	costly fluorescence microscope required, results must be captured and stored within a short period (fluorescent signal decays within a few weeks), Only 1-2 DNA regions analyzed per experiment	Requires knowledge of candidate genes
False-negative/positive results	Has a high false-negative/false-positive rate	Is accurate (may give false-negative results in case of polysomy)	Is accurate (following a lot of standardization)

Previously available data on PCR-based platform has compared different analytical methods, both with *HER 2* at DNA as well as RNA levels, and we have chosen to do *HER 2* assessment at the RNA level. Most studies have shown good concordance with conventional platforms. As previously highlighted, concordance is something that we can artificially manipulate by choosing a particular cut-off, and the true value of a test by assessing the clinical benefit of the patient. A comparative analysis of the previous work done at the RNA level has been shown in Table 20. In this present study, we established that *HER2* mRNA expressions were coordinated 74,35% with IHC. In this study, we showed that *HER2* equivocal tumors were closer to *HER2*-negative disease in mRNA RT-PCR that this was similar to the results of Lehmann-Che (Lehmann-Che et al., 2011), also *HER2* IHC 2+ tumors were closer to *HER2*-negative disease in mRNA RT-PCR in our study and this was similarly with results

FISH of Zoppoli as non-amplified (Zoppoli et al., 2017). Twenty four out of 94 cases revealed discordances with IHC method, and only 33,33% with 3+ IHC showed concordance. Vanden et al between *HER2* IHC3+ and PCR- based assessment revealed a concordance 88%, while in our experiment was 33,33%. In survival analysis, in patients, the overexpression of *HER2* with fold change ≥7,16 no had a significant association with overall survival (OS) in groups of follow-up ≥ 60-month and follow-up< 60-month. IHC results may vary substantially as a result of multiple factors, including the time to fixation, duration of fixation, time of processing, antigen retrieval, staining procedure, and staining interpretation (O'Leary et al., 2001; Reiner-Concin et al., 2008). Therefore RT-PCR allows a highly reliable quantitative assessment and could be a useful adjunct to IHC. Discordance between the results of approved assays IHC and RT-PCR-based assays could potentially create confusion in treatment decision-making. Furthermore, the 2013 ASCO/CAP Guidelines clearly indicate that there is insufficient evidence to support the use of non-FDA approved assays (specifically including mRNA assays using RT-PCR) for the clinical determination of *HER2* status due to the lack of published data that demonstrates the clinical validity of these assays based on outcome (Wolff et al., 2013).

Table. 20 Comparison of various studies on RT-PCR

Study	Number of samples(IHC)	Type of tissue	Normalization gene	Cut-off for RT-PCR	Concordance
Our study	94	FFPE	GAPDH	7,16	**74.35%** (94,12% with 0 IHC score, 92,31 with 1+ and 33.33% with 3+ IHC)
Vanden et al., 2005	32	Frozen fresh	GAPDH	3,7	88% (IHC 3+)
Lehmann-Che et al., 2011	466	FFPE	TATA-binding protein	7	97,3% with IHC 2+
Zoppoli et al., 2017	153	FFPE	APP	1,5	90.20% with equivocal FISH (as non-amplified), 88,0% with FISH positive
Cronin et al., 2004	62	FFPE	6 gene reference set	-	Concordance in 3+
Esteva et al., 2005	149	FFPE	5 gene reference set (β Actin, GAPDH)	11,5	84% with IHC
Kostopoulou et al., 2007	195	FFPE	GAPDH	7,1	93%
Cuadros et al.,2010	81	Fresh Frozen	GAPDH	0,99	Suggests RT-PCR may not replace IHC and FISH
Baehner et al.2010	568	FFPE	ACTB, GAPDH, GUSB, RPLP0, and TFRC	11,5	97%
Dabbs et al.,2011	843	FFPE	ACTB, GAPDH, GUSB, RPLP0, and TFRC	11,5	93%

Ductal carcinoma in situ does not exhibit marked *HSP90*-upregulated, while IDC presents with high *HSP90* expression. Findings may imply that the precursor context may not entail the cellular stress present in invasive cancer. Within invasive ductal carcinoma, a higher grade, larger tumor size, higher estrogen receptor expression, and *HER2* positivity associated with higher *HSP90* expression (Zagouri F et al., 2008; Zagouri F et al., 2010). *HER2* is a client protein of *HSP90*, a member of the family of heat shock proteins (*HSP*s), which are considered molecular chaperones, as they are responsible for the correct folding of denatured or translated proteins

(Munster et al., 2001). It has been suggested that *HSP90* expression may also modulate the effects of oncogenic *HER2*, representing a potential mechanism of resistance to *HER2* directed drugs. On the other hand, *HSP90* inhibitors may potentiate the effects of anti-cancer drugs targeting client proteins of *HSP90* (Berezowska et al., 2013). In breast cancer, additional targeting of *HSP90* has been shown to increase trastuzumab efficiency in vivo and in vitro (Modi et al., 2011; Scaltriti et al., 2011). In the present study, *HSP90* expression in *HER2*-positive tumor tissues was up-regulated and significantly mRNA expression of *HSP90* and *HER2* was linearly correlated. Similarly, Jarzab et al was showed that high *HSP90* expression was strongly associate with positive *HER2* staining. Also, they revealed associations of high HSP90 expression with larger tumor size, steroid receptors, and lymph node metastases (Jarzab et al., 2016). Another study found significant differences between high *HSP90* expression and *HER2* overexpression in colon cancer (Drecoll et al., 2014). Seven In Absentia Homolog 2 (*SIAH2*) protein is an *E3* ubiquitin ligase involved in ubiquitination and proteasome-mediated degradation of other proteins. Many *E3*s could be oncogenes or tumor suppressor genes because frequent deregulation of *E3*s has been shown in human carcinogenesis (Chen et al., 2006). Two homologs, *SIAH1* and *SIAH2*, exist in humans, and have been shown to play a role in several pathways including those involved in response to DNA damage, the hypoxic response, estrogen signaling, inflammation, *RAS* signaling, and as an essential downstream signaling component required for proper *EGFR/HER2* (Behling et al., 2011). The role of *SIAH1* in breast cancer remains less well described. In contrast to other cancer types, only a few reports identify *SIAH1* as a pro-tumorigenic protein in breast cancer similar to *SIAH2*, most point to a tumor suppressor role for *SIAH1* in breast cancer (Adam et al., 2015). *SIAH1* is also implicated in *GAPDH* transport to the nucleus in a novel cell death cascade, suggesting that *SIAH* proteins may play additional roles in cell biology (Hara et al., 2006). Kathryn and et al were revealed that *SIAH* may represent a useful prognostic biomarker that predicts DCIS progression to invasive breast cancer (Behling et al., 2011). In the present study, we showed that *SIAH2* expressions in *HER2*-positive tumor tissues were up-regulated. Also, Chan et al were found a significant increase in nuclear *SIAH2* expression from normal breast tissues through to DCIS and

progression to invasive cancers. Seven in Absentia Homolog 2 (*SIAH2*), an *E3* ubiquitin-protein ligase, has been shown to be associated with resistance to antiestrogens (Chan et al., 2011). Chan et al have revealed a significant positive association between SIAH and *HER2* (Chan et al., 2011). Previous several studies suggested that the tumor-suppressive role of *SIAH* proteins, as shown that overexpression of *SIAH1* arrested the growth of hepatoma cells (Yoshibayashi et al., 2007). Further evidence supports a tumor-suppressive role of *SIAH*s in breast cancers. Increased expression of *SIAH1* halts the growth of *ER*-positive *MCF-7* breast cancer cells. Overexpressed SIAH1 locates to the nuclear matrix, alters cellular morphology, and evokes pronounced polyploidy. *SIAH1* controls tubulin spindle formation, cytokinesis, mitotic catastrophe, and cell survival (Bruzzoni-Giovanelli et al., 1999), also report of Adam et al showed that *SIAH1* assumes a tumor-promoting role in breast cancer cells similar to *SIAH2* and that both ubiquitin ligases affect cell migration (Adam et al., 2015). In this study, we show that *SIAH1* expression in IDC tumors was up-regulated, but statistical analysis of *SIAH1* mRNA expressions was no significant. However, it is in conflict with other reports that found *SIAH1* has proapoptotic and antiproliferative properties in breast cancer cells. Notably, in our study correlation analysis showed that the mRNA expression of *HSP90* and *HER2* was linearly correlated, and mRNA expression of *HSP90* and *SIAH2* was correlated. The molecular chaperone heat shock protein 90 (*HSP90*) is required for the activity and stability of its client proteins such as *HER2*. Pharmacologic inhibition of *HSP90* leads to the ubiquitin-mediated degradation of clients, particularly activated or mutant oncogenic protein kinases. Client ubiquitination occurs via the action of one or more *E3* ubiquitin ligases. In the study of Rahul, silencing *CUL5* (Cullin-RING ligase Cullin-5; an *E3* ubiquitin ligase) reduced cellular sensitivity to distinct *HSP90* inhibitors in human cancer cells, and reduced sensitivity to HSP90 inhibition was also observed upon silencing *CUL5* in *ERBB2*-positive breast cancer (Samant., 2014). Also, a clinical study showed a significant decrease in *CUL5* expression in breast tumors versus matched normal tissue (Fay et al., 2003), and this is the first study that we show an increase in *SIAH2* expression correlated with enhance in *HER2* and *HSP90* expressions. Therefore,

SIAH2 seems to plays an important role in multiple aspects of the molecular and cellular response to *HSP90* inhibition and degradation of *HER2*.

Conclusion

Molecular classifications in breast cancer are indicators of genetic tumor heterogeneity, and understanding the molecular and cellular mechanisms of tumor heterogeneity that are relevant to the development of treatment resistance is a major area of research. RT-PCR and IHC are highly concordant methods for *HER2* status assessment, and RT-PCR allows a highly reliable quantitative assessment and could be a useful adjunct to IHC.

Correlation analysis in the present study showed that the mRNA expression of *HSP90* and *HER2* was linearly correlated, and mRNA expression of *HSP90* and *SIAH2* was correlated.

In terms of correlation *SIAH2* expression and *HER2*, there was linearly correlation, and also between *SIAH1* and *HER2* or *HSP90*, no correlation was seen in our study. Therefore, *SIAH2* seems to plays an important role in multiple aspects of the molecular and cellular response to *HSP90* inhibition and degradation of *HER2*. Our findings suggest further investigation of *SIAH2*, with respect to this response in cancer cells.

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7. ABBREVIATIONS

- World Health Organization (WHO)
- Invasive ductal carcinoma (IDC)
- Ductal Carcinoma in situ (DCIS)
- Lobular Carcinoma in situ (LCIS)
- Invasive Lobular Carcinoma (ILC)
- Immunohistochemistry (IHC)
- Estrogen Receptor (ER)
- Progesterone Receptor (PR)
- Human Epidermal Receptor (HER)
- Triple-Negative Breast Cancers (TNBC)
- pathologic Complete Response (pCR)
- METABRIC (Molecular Taxonomy of Breast Cancer International Consortium)
- Single Nucleotide Polymorphisms (SNPs)
- Copy Number Variants (CNVs)
- Copy Number Aberrations (CNAs)
- ZNF703 (zinc finger protein 703)
- PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha)
- Subtype Classification Models (SCMs)
- Aurora Kinase A (AURKA)
- Epidermal Growth Factor Receptors (EGFR)
- Mitogen-Activated Protein Kinase (MAPK)
- American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP)
- Chromosome Enumeration Probe 17(CEP17)
- Food and Drug Administration (FDA)
- Trastuzumab Emtansine (T-DM1)
- Heat Shock Proteins (HSPs)
- N-Terminal Domain (NTD)
- C-Terminal Domain (CTD)
- Middle Domain (MD)
- 17-allylamino-17-demethoxygeldanamycin (17-AAG; tanespimycin; KOS-953)
- Hsp70 binding protein (CHIP)
- Signal Transducers and Activators of Transcription (STATs)
- Janus Kinases (JAKs)
- TFAP2C (transcription factor AP-2 gamma)
- MED1 (mediator complex subunit 1)

- Activated CDC42-Associated Kinase-1 (ACK1/TNK2)
- Seven-In-absentia Homolog-1 (SIAH1)
- Seven-In-absentia Homolog-2 (SIAH2)
- Epithelial to Mesenchymal Transition (EMT)
- Formalin-fixed, Paraffin-embedded (FFPE)
- Cycle threshold (Ct)
- RT- qPCR (Real-Time quantitative reverse transcription PCR)

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9. CURRICULUM VITAE

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