Correlation between Ubiquitin E3 Ligases (SIAHs) and Heat Shock Protein 90 in Breast Cancer Patients

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Abstract

Background: Breast cancer is a heterogeneous disease and differences in the expression levels of the ER, PR, and HER2 the triplet of established biomarkers used for clinical decision-making have been reported among breast cancer patients. Furthermore, resistance to anti-estrogen and anti-HER2 therapies emerges in a considerable rate of breast cancer patients, and novel drug therapies are required. Several anomalous signaling pathways have been known in breast cancer have been known; heat shock protein 90 (HSP90) is one of the most plenty proteins in breast cells. The family of ubiquitin ligases such as SIAH1 and SIAH2 is known to specifically target misfolded proteins to the proteasome; also, they have been illustrated to play a role in RAS signaling and as an essential downstream signaling component required for EGFR/HER2 in breast cancer.

Methods: The expression of SIAH2, HSP90, and HER2 was assessed by quantitative Real-Time PCR in 85 invasive ductal carcinoma breast tumor samples at Uludag University Hospital in Turkey during the years 2018–2019, and its association with the clinicopathologic variables of patients was evaluated.

Results: HSP90, SIAH1, and SIAH2 were significantly (P=0.0271, P=0.022, and P=0.0311) upregulated tumor tissue of patients with breast cancer. Moreover, this study observed a significant association between the high expression of SIAH2/ HSP90 with ER status, high expression of HSP90 with Recurrence/ Metastasis, and high expression of SIAH2 with Ki-67 proliferation index.

Conclusion: The HSP90 and SIAH2 expressions play a significant role in breast cancer development by combining the experimental and clinical data obtained from the literature.

Keywords: Breast cancer; Invasive ductal carcinoma; Ubiquitin-protein ligases

Introduction

Breast cancer is a heterogeneous disease with morphologic and genetic alterations varied that pose a challenge to its diagnosis and treatment. Two classifications of breast carcinoma are ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC), in which IDCs with wide morphological variation are a heterogeneous group of breast cancers (1). In recent times, the
detection of cancer biomarkers has become a focal point of cancer research, and biomarkers play a requisite role in the administration of invasive breast cancer patients (2, 3). IHC4 score (ER, PR, HER2, and Ki-67) is a rapid, economical breast cancer prognosis (4).

HER2 (human epidermal growth factor receptor 2) is an important prognostic factor of breast cancer, and HER2 overexpression is correlated with aggressive tumors, lower prognosis, and response to chemotherapy (5-7). Trastuzumab is routinely used at the early stages of adjuvant and neoadjuvant therapy in HER2-positive breast cancer (8). There are various factors related to resistance to anti-HER2 therapies, such as loss of HER2 amplification, p95HER2 or mutations in the extracellular domain, crosstalk of HER2 with the PI3K/Akt/mTOR, and the estrogen receptor pathway (9, 10). Furthermore, high HSP90 expression may regulate the HER2 activation and offering the main mechanism of resistance to HER2 inhibitors (11).

So far, more than 200 HSP90 clients have been recognized, inclusive of key regulators in signal transduction and cell cycle control, steroid hormone receptors, and tyrosine and serine/threonine kinases (12, 13). The HSP90 expression has been correlated with high ER levels, high HER2 levels, lymph node status, size of tumors, and reduced survival in breast cancer; HSP90 overexpression is a feature of IDC breast cancers (14-16). HSP90 inhibitors may enhance the effects of anticancer agents that target client proteins of HSP90 such as HER2 (11). HSP90 inhibitors such as tanespimycin reduced ER in ER-positive tamoxifen-sensitive and ER-positive tamoxifen-resistant breast cancer cells and repressed the growth of breast tumors. Moreover, the combining inhibitors of HSP90 (Tanespimycin and Ganetespib) and trastuzumab expanded ubiquitinylation and reduce the expression of HER2 in HER2-overexpressed breast cancer cell lines (17). Conclusively, the overexpression of HSP90 has been exhibited to be associated with opposite clinical outcomes, further validating HSP90 as a target in breast cancer (15).

The first step in the activation of Ubiquitin occurs through a thioester bond catalyzed by an Ubiquitin-activating (E1) enzymes prior transfer to the Ubiquitin-conjugating (E2) enzymes. The final step in transmission of ubiquitin to the cellular targets, Ubiquitin-conjugating (E2) enzymes react with E3 ubiquitin ligases and become targets for the proteasome (18, 19). The Ubiquitylation–proteasome system is major intracellular misfolded protein degradation pathways, (20, 21) and it works with molecular chaperones in this process (22, 23). Moreover, the ubiquitin ligase functions have been demonstrated in the degradation of ErbB2/HER2 (HSP90 client protein kinases) following inhibition of HSP90 (24). Seven In Absentia Homolog (SIAH) proteins are E3 ubiquitin ligase, and SIAHs are involved in cancers such as prostate cancer, leukemia, and breast cancer. Furthermore, SIAH has been proposed as a beneficial prognostic biomarker that predicts DCIS progression to IDC breast cancer (25, 26). There are two homologs for SIAH in humans; SIAH1 and SIAH2 play roles in different pathways inclusive of the hypoxic response, inflammation, those involved in response to DNA damage, RAS signaling, estrogen signaling, and EGFR/HER2 signaling (26-29). As well as SIAHs are involved in cytokine signaling modulating the epithelial to mesenchymal transition (EMT) (30).

Here we employed a quantitative PCR method to detect HER2, SIAH2, and HSP90 gene expressions in 85 formalin-fixed and paraffin-embedded (FFPE) breast cancer tissue samples from breast cancer patients.

Materials and Methods

Clinical Samples and Ethics Statement
We examined 85 formalin-fixed, paraffin-embedded (FFPE) cancer tissues with invasive ductal breast carcinoma and normal tissues at Uludag University Hospital in Turkey during the years 2018–2019.
The usage of breast cancer tissues for molecular analysis was ratified under the number (BUU 2018-14/26) by the Ethics Committee of the Faculty of Medicine of the Bursa Uludag University.

**Total RNA Extraction**

Total RNA was extracted from the tissue samples including tumor and adjacent normal tissues of the same patient using OMEGA reagent (FFPE RNA Kit, Omega, Germany) according to the manufacturer’s instructions.

**cDNA Synthesis and Real-Time qRT-PCR**

Reverse transcription were performed by the TaqMan High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, USA). 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. The reaction was carried out at 4 steps (Step 1: 25 °C, 10 min; Step 2: 37 °C, 120 min; Step 3: 85 °C, 5 min; Step 4: 4 °C, ∞) on Thermal Cycler (Bio-Rad, California, USA). In the present study, the reaction mix for each sample used of TaqMan®, Gene Expression, in a volume of 20 μL including in 4 μL preamplified of cDNA (50 ng), 1 μL of TaqMan gene expression assay, 5 μL of dH2O, and 10 μL of the Universal Master Mix (2x). The qRT-PCR reactions were accommodated in 96-well plates in the Applied Biosystems RT-PCR instrument. 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 sec and 60 °C for 1 min. In addition, all probes (Applied Biosystems, Foster City, CA, USA) in this study, based on the mRNA sequences of target HER2, HSP90AA1, SIAH2, and reference gene GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) acquired from GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) (Table 1).

**Table 1:** 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        | 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 |

**Data Analysis and Statistics**

Statistical analysis was performed using SPSS version 21.0 (IBM Corp., Armonk, NY, USA). Correlation of gene expression analyses was done using Pearson linear correlation. Survival analysis was performed using Kaplan-Meier analysis. All tests were 2-2-sided, and the significance level was set at 0.05.

**Results**

**Baseline Clinical Data, Consort Statement**

The clinical characteristics are shown in Table 2. Overall, 85 invasive ductal carcinoma breast cancer patients were investigated in this study. The mean age was 53.18±11.62 years (median, 52), and the median age at the time of breast cancer diagnosis was 47.
### Table 2: Baseline Clinical and Pathologic Characteristics of the IDC Patients (n =85)

| Characteristics                  | Number | Percent (%) |
|----------------------------------|--------|-------------|
| Age (yr)                         |        |             |
| < 50                             | 36     | 42.35       |
| ≥ 50                             | 49     | 57.65       |
| Grade                            |        |             |
| I/ II                            | 45     | 52.94       |
| III                              | 40     | 47.06       |
| Lymph node                       |        |             |
| N0: node-negative                 | 40     | 47.06       |
| N1: metastasis involving 1–3 nodes| 26     | 30.59       |
| N2: at least 4 nodes             | 19     | 22.35       |
| Tumor size (cm)                  |        |             |
| < 3 cm                           | 55     | 64.71       |
| ≥ 3 cm                           | 30     | 35.29       |
| Ki-67                            |        |             |
| ≤15%                             | 27     | 31.76       |
| 15%–35%                          | 38     | 44.71       |
| >35%                             | 20     | 23.53       |
| In situ component                |        |             |
| No-DCIS (0)                      | 10     | 11.76       |
| Low-DCIS (<25%)                  | 30     | 35.30       |
| High-DCIS (≥25%)                 | 28     | 32.94       |
| Missing data                     | 17     | 20.00       |
| ER status                        |        |             |
| Positive                         | 64     | 75.30       |
| Negative                         | 21     | 24.70       |
| PR status                        |        |             |
| Positive                         | 51     | 60.00       |
| Negative                         | 34     | 40.00       |
| HER2 status                      |        |             |
| Positive                         | 30     | 35.30       |
| Negative                         | 55     | 64.70       |
| Recurrence/ Metastasis           |        |             |
| With Recurrence                  | 35     | 41.18       |
| Without Recurrence               | 34     | 40.00       |
| Missing data                     | 16     | 18.82       |

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

In this study, HSP90, SIAH2, and HER2 mRNA gene expressions comparison between tumors and normal tissues were calculated by Sabiosciences' data analysis software (https://dataanalysis.qiagen.com).

Comparison of HSP90, SIAH1, and SIAH2 mRNA mean expression levels in breast tumor and normal tissues indicate a significant increase in breast cancer patients with 1.93, 2.09, and 1.82 fold increase in tumor samples compared to the normal tissues ($P= 0.0271$, $P= 0.0225$, and $P= 0.0311$, respectively). Whereas, the analysis of HER2 gene expression in tumoral tissues with a 1.66 fold increase was not significantly higher than normal tissues ($P= 0.3793$) (Fig. 1). A Heatmap of gene expressions is demonstrated in Fig. 2.
Based on the cutoff value of SIAH1, SIAH2 and HSP90 fold changes, tumor breast cancers were identified as upregulated (AUC= 0.848, P<0.001; AUC=0.848, P<0.001; AUC=0.724, P<0.001, respectively) (Table 3, Fig. 3).

**Fig. 1:** Comparison of HSP90, SIAH1, SIAH2, and HER2 mRNA mean expression levels in breast tumors and normal tissues

**Fig. 2:** Heat map of gene expression of HSP90, SIAH2, and HER2 mRNA mean expression levels in breast tumors and normal tissues. The red cells (high expression of a gene), black cells (intermediate expression of a gene), and green cells (low expression of a gene)

**Association between HSP90 and SIAH1/2 expression and clinicopathological characteristics of the Breast Cancer Patients**

The correlation of HSP90 and SIAH1/2 expression with clinicopathological parameters (Ki-67, ER, PR, and HER2 were assessed with immunohistochemistry (IHC)) are shown in Table 4.
Fig. 3: Based on the AUC value of SIAH1, SIAH2 and HSP90 fold change

Table 3: Sensitivity, Specificity, and Area under the ROC Curve for Base Excess at the Optimal Cut-off values of SIAH1/2 and HSP90 mRNA

| Gene   | AUC   | Std. Error<sup>a</sup> | Asymptotic Sig.<sup>b</sup> | Asymptotic 95% Confidence Interval |
|--------|-------|------------------------|----------------------------|-----------------------------------|
|        |       |                        |                            | Lower Bound                       |
|        |       |                        |                            | Upper Bound                       |
| SIAH1  | .652  | .055                   | .009                       | .545                              |
| SIAH2  | .848  | .031                   | .000                       | .787                              |
| HSP90  | .724  | .042                   | .000                       | .642                              |

a. Under the nonparametric assumption  
b. Null hypothesis: true area= 0.5  
AUC. The area under the curve

Table 4: HSP90 and SIAH2 Expression and Clinicopathologic Characteristics in 85 Breast Cancer Patients

| Characteristics                  | HSP90 expression | SIAH1 expression | SIAH2 expression |
|----------------------------------|------------------|------------------|------------------|
|                                  | Fold Change      | P-value          | Fold Change      | P-value          | Fold Change      | P-value          |
| ER status (+)                    | 1.846            | 0.0423<sup>*</sup> | 2.008            | 0.0375<sup>*</sup> | 1.835            | 0.050<sup>*</sup> |
| PR status (+)                    | 1.847            | 0.2282           | 2.492            | 0.1720           | 2.029            | 0.070            |
| HER2 status (IHC 3+)             | 1.893            | 0.0312<sup>*</sup> | 2.3912           | 0.1492           | 2.148            | 0.0865           |
| Recurrence/ Metastasis (+)       | 2.268            | 0.0447<sup>*</sup> | 3.9347           | 0.0657           | 1.669            | 0.2387           |
| Age (< 50)                       | 1.802            | 0.2048           | 2.6326           | 0.0610           | 1.0401           | 0.735            |
| Grade III                        | 1.872            | 0.2078           | 2.0991           | 0.0973           | 1.566            | 0.4900           |
| Lymph node (N2: at least 4 nodes) | 4.295         | 0.0931           | 2.1332           | 0.2632           | 0.628            | 0.2852           |
| Lymph node (N1: 1–3 nodes)       | 1.365            | 0.1386           | 2.1495           | 0.3219           | 1.121            | 0.2585           |
| Tumor size (>3 cm)               | 1.809            | 0.0808           | 1.3896           | 0.6203           | 1.604            | 0.1878           |
| Ki-67 (>35%)                     | 1.899            | 0.1291           | 1.4756           | 0.4766           | 1.501            | 0.5429           |
| Ki-67 (15%–35%)                  | 1.510            | 0.3094           | 3.5840           | 0.0540           | 1.944            | 0.0154<sup>*</sup> |
| In situ component (≥25%)         | 0.896            | 0.5271           | 1.7248           | 0.2210           | 1.573            | 0.8611           |
**HSP90 expression and clinicopathological characteristics**

HSP90 gene expression, significantly associated with ER-positive (*P*=0.0423), HER2-positive (*P*=0.0312), and recurrence/metastasis rates (*P*=0.0447) in histopathological tumoral tissues; but no significantly associated with histopathological tumor staging, in situ component, and ki-67 status of the breast cancer patients. We observed that HSP90 expression increased 4.295 fold change (*P*=0.0931) in breast cancer lymph node (N2: at least 4 nodes) compared with lymph node (N1: 1–3 nodes) patients. Moreover, tumor tissues with size >3 cm (1.89-fold change; *P*=0.0808) presented an increase in HSP90 expression compared with normal tissues.

**SIAH1 expression and clinicopathological characteristics**

SIAH1 overexpression was not related to age, PR, recurrence/metastasis rates, tumor staging, lymph node involvement, Ki-67 status, and in situ component status of the breast cancer patients. The SIAH1 overexpression was associated with the ER-positive (*P*= 0.0375), and SIAH1 expression in tumor tissues approximately significant was up-regulated (*P*= 0.0657) in recurrence/metastasis positive breast cancers.

**SIAH2 expression and clinicopathological characteristics**

SIAH2 overexpression was not related to age, PR, recurrence/metastasis rates, tumor staging, lymph node involvement, and in situ component status of the breast cancer patients. SIAH2 overexpression was associated with the ER-positive (*P*= 0.050) and ki-67 status (Ki-67 (15%–35%), *P* = 0.0154). Besides, the high expression of SIAH2 showed close to being significant in HER2-positive tumors (*P*= 0.070).

**HER2 mRNA expression tends to be correlated with HSP90 and SIAH1/2 high expressions**

Furthermore, the correlation between HER2 and HSP90, SIAH2 were analyzed. Results of correlation were shown that HSP90 scores were higher in high-level HER2 mRNA expression cases (Fig. 4A; *P*=0.001, *r*=0.20). Additionally, analysis of gene expression data demonstrated that SIAH2 expression was significantly correlated with HER2 mRNA level (Fig. 4B; *P*<0.001, *r*=0.25). HSP90 mRNA expression was positively associated with the expression of SIAH2 (Fig. 4C; *P*<0.001, *r*=0.45). There was no relationship between the SIAH1 and HER2, SIAH1 and HSP90, or SIAH1 and SIAH2.

**Kaplan–Meier Analysis**

The Kaplan-Meier survival analysis showed no effect of the SIAH1, SIAH2, and HSP90 high expressions on the overall survival of breast cancer patients (5 years follow-up) (*P* = 0.090, *P*= 0.971, and *P*= 0.582, respectively).

**Discussion**

The resistance to anti-estrogen and anti-HER2 therapies emerges in a considerable rate of breast cancer patients, and novel drug therapies are required. Recognition of other molecular factors,
HSP90 modulates the stabilization of oncogenic and anti-oncogenic proteins such as ER, PR, essential components of HER2 signaling (HER2, AKT, RAF, and HIF1α), and EGFR in breast cancer (11, 31, 32). The prognostic significance of HSP proteins in breast cancer is better reflected in their impact on patient survival, and increased HSP90 expression was associated with increased death rates (33).

DCIS does not exhibit marked HSP90-upregulated, while IDC presents with high HSP90 expression (34, 35). In previous studies, the negative impact of overexpression HSP90 on patient survival was illustrated; also, the overexpression of HSP90 was severely correlated with larger tumor size, histological grade, and lymph node (34, 35).

The studies on HSP90 expression have been published from breast cancer cell lines xenografts and not from tumor biopsy samples, and the association of HSP90 expression with clinical features has not been broadly studied in the context of molecular subtypes of breast cancer. Our findings showed that HSP90 gene expression, significantly associated with ER-positive, HER2-positive, and recurrence/metastasis rates in histopathological tumoral tissues, whereas no significant correlation was observed between histopathological tumor sizes. This is in contrast with the results that demonstrated a significant difference between high-level HSP90 expression with larger tumor size; whereas in our study, tumor tissues with size >3 cm presented an increase in HSP90 expression (1.89-fold change) compared with normal tissues (16).

We observed that HSP90 expression increased 4.295-fold change in breast cancer lymph node with at least 4 nodes. Furthermore, significant difference were showed between high-level HSP90 expression with lymph node metastases. “HSP90 expression was different in patients' tumors in comparison with cancer cell lines; whether overexpression of HSP90 is also different between primary and metastatic tumors is unclear at this time” (15).

In studies of pre-clinical and clinical breast cancer models demonstrated that the potentially increased aggressiveness, related to overexpressing the Hsp90. HER-2 is the most sensitive HSP90 client, and HER2-amplified are potently inhibited by HSP90 inhibitors in breast cancer cells (36). In the present study, HSP90 expression in tumor tissues was up-regulated, and significantly mRNA expression of HSP90 and HER2 was linearly correlated.

“SIAHs are the human homologs of Seven-In-Absentia (SINA), an evolutionarily conserved RING finger E3 ubiquitin ligase, and two SINA homologs have been identified in the human genome, SIAH1 and SIAH2” (37). SIAHs have been shown to play a role in different pathways including estrogen signaling, RAS signaling, and as an essential downstream signaling component required for suitable EGFR/HER2, and also in pathways those involved in response to DNA damage, the hypoxic response (26). Some studies reported pro-tumorigenic roles of SIAH1 and SIAH2, whereas studies often identify SIAH1 as a tumor suppressor in breast cancer (38).

SIAH2 as an E3 ubiquitin ligase involved in proteasome-mediated degradation and ubiquitination of proteins (25, 39). SIAH may represent as a beneficial prognostic biomarker that predicts ductal carcinoma in situ progression to invasive ductal breast cancer (26).

In our study, there was a significant increase in the expression of SIAH2 levels in invasive ductal carcinoma (IDC) tissues. A significant increase was found in SIAH2 expression in DCIS progression to invasive cancers (40). A significant positive association was revealed between SIAH and HER2, which is in line with our study. In the present study, SIAH2 overexpression was associated with the ER-positive, which was similar to that of Chan et al (40), and also our findings showed that SIAH2 gene expression, significantly associated with Ki-67 (15%-35%) proliferation index.

The high expression of SIAH2 showed close to being significant in patients with HER2-positive
breast cancer. Notably, in this study correlation analysis showed that the mRNA expression of HSP90 and HER2 was linearly and mRNA expression of HSP90 and SIAH2 was correlated. The heat shock protein 90 (HSP90) activates/stabilizes its target proteins such as HER2.

The repression of HSP90 causes the deterioration of oncogenic protein kinase activated or mutated; the ubiquitination of client proteins takes place by means of the action of E3 ubiquitin ligases. CUL5 (Cullin-RING ligase Cullin-5) is an E3 ubiquitin ligase, which the overexpression of CUL5 has been shown in breast cancer patients (41). The silencing of CUL5 (Cullin-RING ligase Cullin-5; an E3 ubiquitin ligase) was decreased cellular susceptibility to HSP90 inhibitors in HER2-positive breast cancers (24).

**Conclusion**

The mRNA expression of HSP90 and HER2 was related, and also mRNA expression of HSP90 and SIAH2 was correlated. In terms of the correlation between SIAH2 expression and HER2, there was a linear correlation in our study. Therefore, SIAH2 can contribute as a cellular and molecular response to HSP90 inhibitors in the treatment of HER2-positive breast cancer.

**Journalism Ethics considerations**

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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**Conflict of interest**

The authors declare no conflict of interest.

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