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Original Article

Correlation between Ubiquitin E3 Ligases (*SIAH*s) 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

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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, p95*HER2* 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.

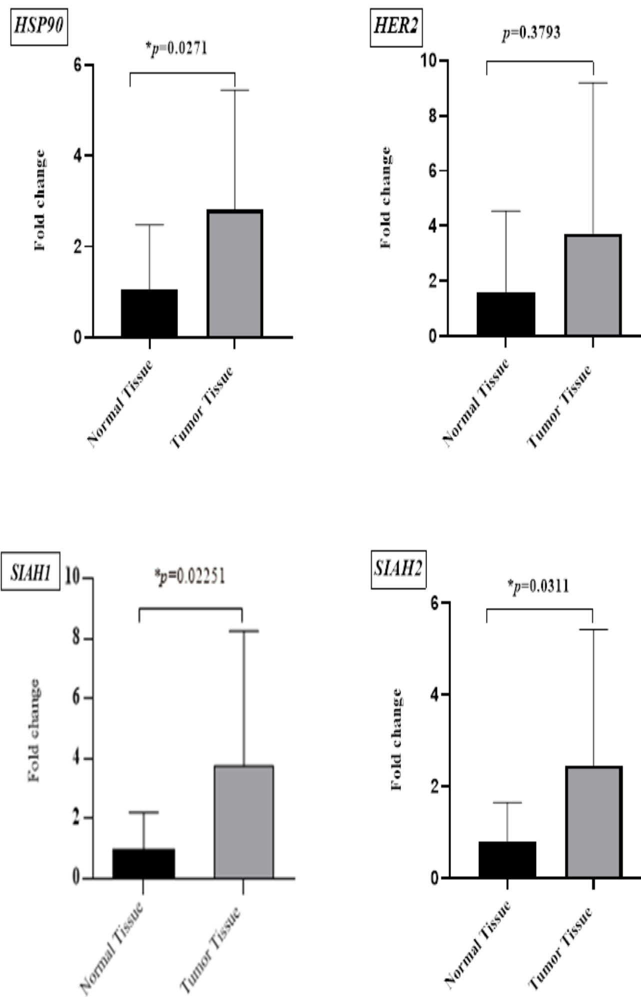

Fig. 1: Comparison of *HSP90*, *SIAH1*, *SIAH2*, and *HER2* mRNA mean expression levels in breast tumors and normal tissues

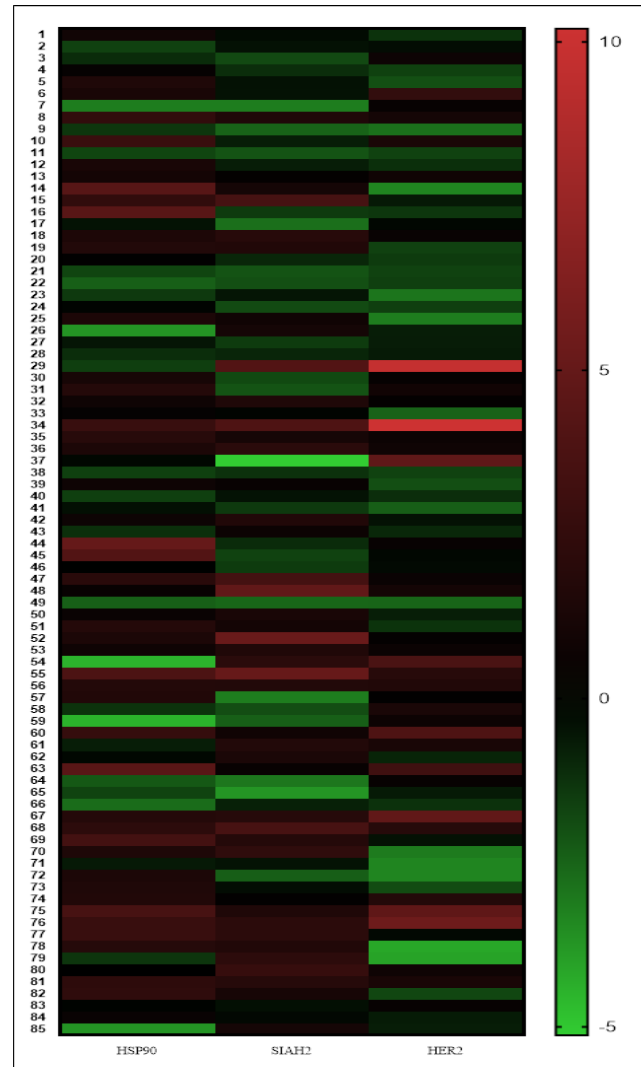

Fig. 2: Heat map of gene expression of *HSP90*, *SIAH2*, and *HER2* mRNA mean expression levels in breast tumors and normal tissues. The red cells (high expression of a gene), black cells (intermediate expression of a gene), and green cells (low expression of a gene)

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).

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.

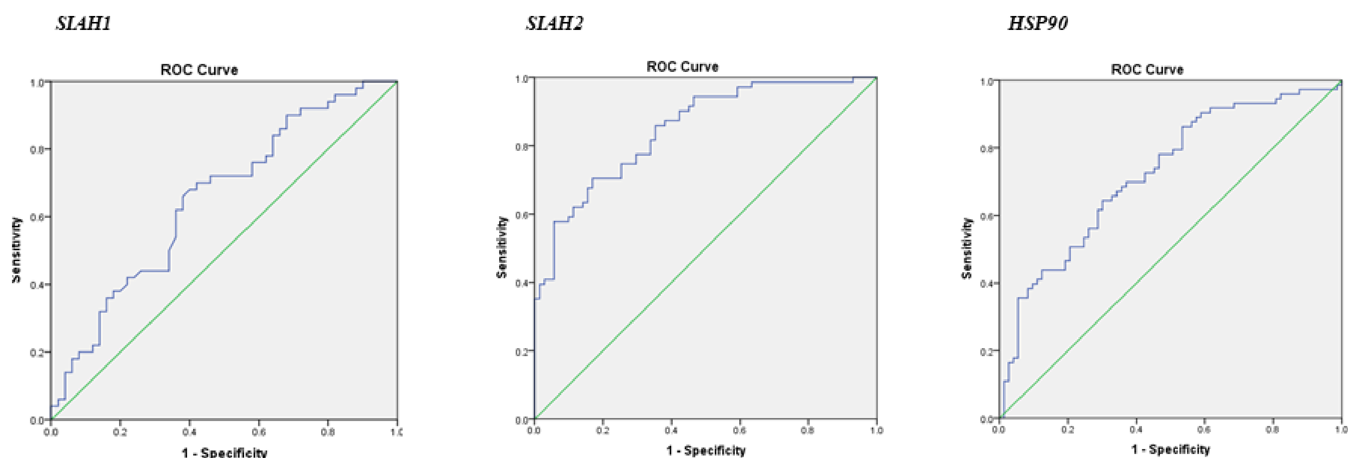

Fig. 3: Based on the AUC value of *SIAH1*, *SIAH2* and *HSP90* fold change

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*** [a]	***Asymptotic Sig.*** [b]	***Asymptotic 95% Confidence Interval***	
				Lower Bound	Upper Bound
SIAH1	.652	.055	.009	.545	.759
SIAH2	.848	.031	.000	.787	.910
HSP90	.724	.042	.000	.642	.806

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*	2.008	0.0375*	1.835	0.050*
PR status (+)	1.847	0.2282	2.492	0.1720	2.029	0.070
HER2 status (IHC 3+)	1.893	0.0312*	2.3912	0.1492	2.148	0.0865
Recurrence/ Metastasis (+)	2.268	0.0447*	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*
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*.

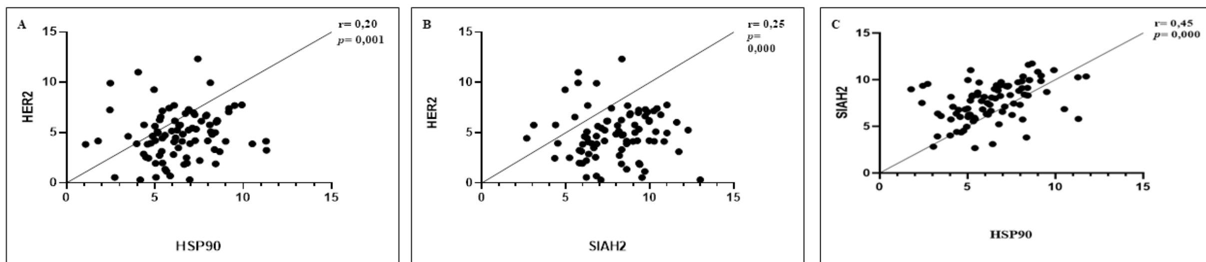

Fig. 4: The correlation analysis of between *HER2* mRNA expression and (A) *HSP90*, (B) *SIAH2*, (C) *HSP90* mRNA expression and *SIAH2*

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,

exclusively those related to aggressive features and poor prognosis, could improve the diagnosis and therapy of breast cancer patients (12).

HSP90 modulates the stabilization of oncogenic and anti-oncogenic proteins such as ER, PR, essential components of *HER2* signaling (*HER2*, *AKT*, *RAF*, and *HIF1a*), 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 studt, 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.

"*SIAH*s 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). *SIAH*s 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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