Down-regulation of Heat-shock Protein 70 (HSP-70) Correlated with Responsiveness to Neoadjuvant Aromatase Inhibitor Therapy in Breast Cancer Patients

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Abstract. *Background: Aromatase inhibitor (AI) has been established as an effective endocrine therapy in estrogen receptor (ER)-positive postmenopausal breast cancer patients. Our recent proteomic analysis demonstrated that ten proteins were significantly altered in their expression levels before and after the therapy in the patients receiving neoadjuvant AI. Among these newly identified proteins, heat-shock protein 70 (HSP-70) was the most significantly correlated with both clinical and pathological responses. Therefore, in this study, we further evaluated the significance of this HSP-70 alteration using immunohistochemistry. Materials and Methods: A total of 32 patients treated with neoadjuvant exemestane or letrozole in whom pre- and post-treatment tumor tissues were available were included. Immunohistochemical evaluation of ER, progesterone receptor (PgR), Her-2, Ki-67 and HSP-70 was performed. Results obtained were compared to both clinical and biological responses of the patients. Results: The majority of the patients responded to treatment (16 patients with partial response, 14 with stable disease and 2 with progressive disease). The means of ER, Ki-67 and HSP-70 were significantly different between treatment responders and non-responders. Decrement of HSP-70 and Ki-67 after AI treatment and pretreatment Ki-67 labeling index of >10% tumor cells were significantly associated with clinical responsiveness to AI treatment ($p<0.0001$). There was a significant positive correlation between changes of HSP-70 and Ki-67 before and after the therapy. Conclusion: Decrement of HSP-70 in breast carcinoma cells plays important roles in therapeutic mechanisms of AIs through suppressing tumor cell proliferation in breast cancer patients.*

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Aromatase inhibitor (AI) has become a gold standard of endocrine therapy for estrogen receptor (ER)-positive post-menopausal women with breast cancer (1-5). Breast cancer patients have been in general presenting at earlier clinical stages due to a wide availability of screening programs and increased breast cancer awareness among the general population, but it is also true that there are patients who manifest with advanced clinical stages on their first visit to clinicians (6-7). Neoadjuvant therapy aiming for tumor shrinkage could allow the choice of breast conservative surgery for these advanced breast cancer cases (8). In these neoadjuvant settings, chemotherapy has been frequently used but adverse effects and complications are quite common among the patients. Therefore, the ideas of using endocrine therapy in these neoadjuvant settings have evolved, at least for ER-positive breast cancer patients. However, the determination of objective therapeutic effectiveness in neoadjuvant endocrine therapy has not been well established, with the possible exception of alterations of Ki-67 labeling index (LI) before and after the treatment (9). In addition, the alterations of carcinoma cell biology following the therapy have not been well studied compared to those of chemotherapy, with the exception of recent studies of

microarray analysis reported by Miller *et al*. (10) and Mackay *et al*. (11). We have recently demonstrated that ten proteins had different expression profiles after three months of neoadjuvant AI compared to those before the therapy using proteomic approach in ER-positive postmenopausal breast cancer patients (12). Among these proteins, heat-shock protein 70 (HSP-70) was most significantly correlated with both clinical and biological responses of the patients. Therefore, in this study, we further examined the potential role of HSP-70 in therapeutic effectiveness of neoadjuvant AI therapy using immunohistochemistry and correlated the findings with clinicopathological features and biological responses of these patients.

Materials and Methods

Patients. A neoadjuvant clinical, trial termed Celecoxib Anti-Aromatase Neoadjuvant trial (CAAN trial), was conducted on post-menopausal breast cancer patients and its details were previously reported (13). Briefly, all the patients were postmenopausal women with invasive ductal breast carcinoma and positive ER/PgR status determined by immunohistochemistry. These patients either suffered from local advanced breast cancer, in which the purpose of neoadjuvant treatment was to downstage the cancer for a better chance of subsequent surgical complete resection, or they were anticipated to have high operative risks due to advanced age or co-morbidities that prevented them from upfront surgical treatment. The treatment duration was three months of AI and all the patients were randomized into three different treatment groups: group A patients received combined treatment of exemestane 25 mg daily and celecoxib 400 mg twice daily; group B received exemestane 25 mg daily; and group C received letrozole 2.5 mg daily. As reported previously, there were no significant differences in term of clinical and pathological responses among these three different treatment groups (13). Therefore, the responses toward AI were not influenced by the use of celecoxib.

Institutional Ethical Committee approval of this analysis was obtained from The University of Hong Kong and Queen Mary Hospital, Hong Kong. Informed consent of participation in the trial were obtained from all the patients before enrollment into the trial. During the treatment period, participating patients were monitored serially with both clinical and radiological assessments for the responses to treatments and potential adverse effects. After completion of 3-month treatment, standard surgical treatments were offered to the patients. The responses to AI treatment were measured according to RECIST scales in the outpatient clinics at the Queen Mary Hospital, The University of Hong Kong (14).

Immunohistochemistry. Mouse monoclonal antibodies for ER, PgR and HER2 were purchased from Roche Diagnostics, Switzerland. Mouse monoclonal antibody for Ki-67 was purchased from DAKO Cytomation (Glostrup, Denmark). Mouse monoclonal antibody for HSP-70 (HSPA2) was purchased from ABNOVA (Taipei, Taiwan). The dilutions of primary antibodies were as follows: ER, PgR and HER2 ready for use; Ki-67, 1:100; HSPA2, 1:200. ER, PgR and HER2 were stained by auto-immunohistochemical system BENCHMARK® XT (Roche Diagnostics). Ki-67 and HSPA2 were immunostained by a biotin-streptavidin method using Histofine kit

Table I. *Demographic data (mean+SD) of the studied patients*.

Total no. of patients: 32	
Mean age (years)	71.0+9.3 (51–93)
Pre-treatment mean (range) tumour size (cm)	
Clinical assessment	4.1+1.2 (2.0–8.0)
USG assessment	3.0+0.9 (1.2–5.5)
Treatment arm	
A	12 (37.5%)
B	10 (31.3%)
C	10 (31.3%)
RECIST response	
CR	0 (0.0%)
PR	16 (50.0%)
SD	14 (43.8%)
PD	2 (6.3%)
Objective treatment response	
Responder (size reduction)	28 (87.5%)
Non-responder (size increment)	4 (12.5%)
Biological treatment response	
Group 1 (increase)	8 (25.0%)
Group 2 (no change)	5 (15.6%)
Group 3 (decrease)	19 (59.4%)

CR: Complete response; PR: partial response; SD: stable disease; PD: progressive disease.

Table II. *Data on the biological markers of the studied patients*.

ER (mean Allred's score)	
Pre-treatment	7.0+1.6
Post-treatment	7.4+1.2
PgR (mean Allred's score)	
Pre-treatment	6.7+1.7
Post-treatment	5.2+2.3
Her-2 (IHC score, no. of patients)	
Pre-treatment (0–1+, 2+, 3+)	15, 11, 6
Post-treatment (0–1+, 2+, 3+)	18, 9, 5
Ki-67	
Pre-treatment (mean %)	17.6+14.0
Post-treatment (mean %)	10.0+9.3
High pre-treatment Ki-67 index* (no. of patients)	
Yes	22 (68.8%)
No	10 (31.2%)
HSP-70	
Pre-treatment (mean H score)	85.9+42.6
Post-treatment (mean H score)	56.1+30.4
Change of HSP-70 after AI treatment (no. of patients)	
Down-regulation	24 (75%)
Up-regulation	8 (25%)

*Pretreatment Ki-67>10% is considered as having a high proliferative index. Results expressed in mean+SD. ER: Estrogen receptor; PgR: progesterone receptor; Her-2: human epidermal growth factor receptor type 2; Ki-67: Ki-67 protein; HSP-70: heat-shock protein 70.

(Nichirei Co. Ltd, Tokyo, Japan). Antigen retrieval for Ki-67 analysis was performed by heating the slides in an autoclave at 121˚C for 5 min in citric acid buffer (2 mmol/l citric acid and 9 mmol/l trisodium citrate dehydrate, pH 6.0). These slides were further incubated with the primary antibodies for 12-18 h in a moist chamber at 4˚C. The antigen-antibody complex was then visualized with 3,3'-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer, pH 7.6, and 0.006% H_2O_2), and counterstained with hematoxylin. Immunohistochemical H-score was calculated by adding the sum of 100× 1+ (weak), 2+ (moderate) and 3+ (strong) of staining intensity. All IHC stained slides were evaluated independently by two authors (CY and NP).

Biological response as determined by Ki-67 alterations. There has been no consensus on the absolute value of the pretreatment Ki-67 level at which the definition of high proliferative index is set at this juncture (15-32). However, several investigators reported that setting the cut-off Ki-67 level of >10% as the definition of high proliferative index was associated significantly with poorer disease-free survival (DFS) and overall survival (OS) regardless of the nodal status in breast cancer patients (16, 28-29). Therefore in our study, pretreatment tumor specimens with Ki-67 level of >10% were tentatively defined as the highly proliferative group. In addition to the RECIST criteria, the responses to AI treatment were also graded according to their change in proliferative index as the biological response. The changes of Ki-67 were also tentatively classified into three groups using the criteria reported by Ellis *et al*. (33), Miller *et al*. (34) and Chanplakorn *et al*. (35), in which the significant changes were defined as more than 40% of the original measurement: group 1, the increased group (Ki-67 increment more than 40%); group 2, the unchanged group (increment or reduction of Ki-67 less than 40%); and group 3, the decreased group (Ki-67 level reduction more than 40%).

Statistical analysis. The software SPSS 15.0 (Inc, Chicago, IL, USA) was used. Independent student *t*-test was used to test the correlation of parametric variables while Pearson Chi-square test was used to test the correlation between non-parametric variables.

Results

Clinicopathological features of the patients. Clinical and pathological findings of the patients are summarized in Table I. Patients were evenly distributed into three treatment arms described above. There was no complete response (CR) achieved in the study, and 2 patients were found to have progressive disease (PD). Together with the 2 patients who had stable disease (SD) associated with size increment during the course of treatment, in all, 4 patients had a size increment after this 3-month neoadjuvant treatment. Therefore, the proportion of objective responders was 87.5% (n=28), while that of non-responders was 12.5% (n=4).

Immunohistochemistry. The great majority of the patients (n=22) had high Ki-67 level before treatment (Ki-67>10%; Table II). There were 23 and 24 patients who demonstrated significant decrement in proliferative index and HSP-70 expression respectively after treatment. There were no

Table III. *Comparison of the mean differences of various factors among objective treatment responders (with size reduction) and non-responders (with size increment)*.

	Responders	Non-responders	*p*-Value
Age	71.36 + 9.75	69.00+5.72	0.643
ER	0.185+1.11	2.000+2.71	0.019*
PgR	–1.864+1.83	0.250+4.11	0.097
Her-2	–3.214+0.86	0.500+1.00	0.090
Ki-67	–9.861+12.74	8.875+15.31	0.012*
% Change of Ki-67	–42.20+61.24	272.55+392.35	<0.0001*
HSP-70	–36.961+48.91	20.025+61.82	0.043*

Data shown as mean+SD; independent sample *t*-test used for comparison of means between responders and non-responders. ER: Estrogen receptor; PgR: progesterone receptor; Her-2: human epidermal growth factor receptor type 2; Ki-67: Ki-67 protein; HSP-70: heat-shock protein 70. *p-Value <0.05 is considered as statistically significant.

Table IV. *Correlation of treatment response with different factors*.

	Objective treatment response		
	Responders (no. of pts)	Non-responders (no. of pts)	*p*-Value
Pre-treatment Ki-67 level (high/low)*	21/7	1/3	<0.0001
Change in Ki-67 after treatment (decrease/increase)	23/5	1/3	<0.0001
Change in HSP-70 after treatment (decrease/increase)	23/5	1/3	<0.0001

Pearson Chi-square test used, p-value<0.05 considered as statistically significant. *Pretreatment Ki-67>10% is considered as high. Other factors with non-significant correlations are not shown.

Table V. *Comparison of the mean differences of various factors among biological responders and non-responders in terms of % change of Ki-67*.

	Biological responders (% decrease in Ki-67≥40%)	Biological non-responders (% increase in Ki-67≥40%)	*p*-Value
Age	70.58±9.86	74.60±8.41	0.414
ER	0.111±1.21	1.60±2.51	0.073
PgR	–2.31±2.15	0.40±2.88	0.034*
Her-2	–0.42±0.96	0.20±1.10	0.224
HSP-70	–46.56±44.19	16.22±73.10	0.022*

Data shown as mean+SD; independent sample *t*-test used for comparison of means between responders and non-responders. ER: Estrogen receptor; PgR: progesterone receptor; Her-2: human epidermal growth factor receptor type 2; Ki-67: Ki-67 protein; HSP-70: heat-shock protein 70. *p-Value <0.05 is considered as statistically significant.

significant differences in both clinical and biological responses among these three treatment groups (p=0.202 and 0.057 respectively in Pearson Chi-square test, results not shown in table).

Changes of ER, Ki-67 and HSP-70 expression were statistically significant among objective treatment responders and non-responders (p-value=0.019, 0.012 and 0.043 respectively) (Figure 1, Table III). The clinical treatment response was significantly correlated with the biological response (42.2% mean Ki-67 reduction in the responder group and more than 2-fold Ki-67 increment (272.55%) in the non-responder group, p<0.0001). Results of Allred's score of ER in tumor cells were similar before and after treatment in responders (0.185±1.11), while an increment of Allred's score of 2.000±2.71 was detected in non-responders.

Immunoreactivity of Ki-67 and HSP-70 demonstrated both significant and consistent reductions among treatment responders. Table IV summarized significant factors associated with treatment responses including the pretreatment high proliferative index determined by Ki-67 LI and post-treatment decrement of Ki-67 and HSP-70. The pretreatment Ki-67 was also significantly associated with decrement of HSP-70 (p<0.0001 on Pearson Chi square test, data not shown in table). Representative illustrations of immunoreactivity of HSP-70 before and after the treatment are demonstrated in Figure 2.

Factors associated with biological response of the patients. A total of 19 patients (59.4%) had significant decrement of Ki-67 level following the treatment, *i.e.* they were biological responders, and the biological non-responders were 5 patients (15.6%). The remaining 8 patients (25%) did not have significant alterations of Ki-67 LI after completion of treatments. Table V summarizes the changes of biological markers before and after the treatment among the three subgroups of patients. Using the changes of Ki-67 level as a marker for biological response, the down-regulation of HSP-70 still represented a significant predictor for AI response (p=0.022) (Figure 3). Change in PgR expression was also found to be a significant factor (p=0.034).

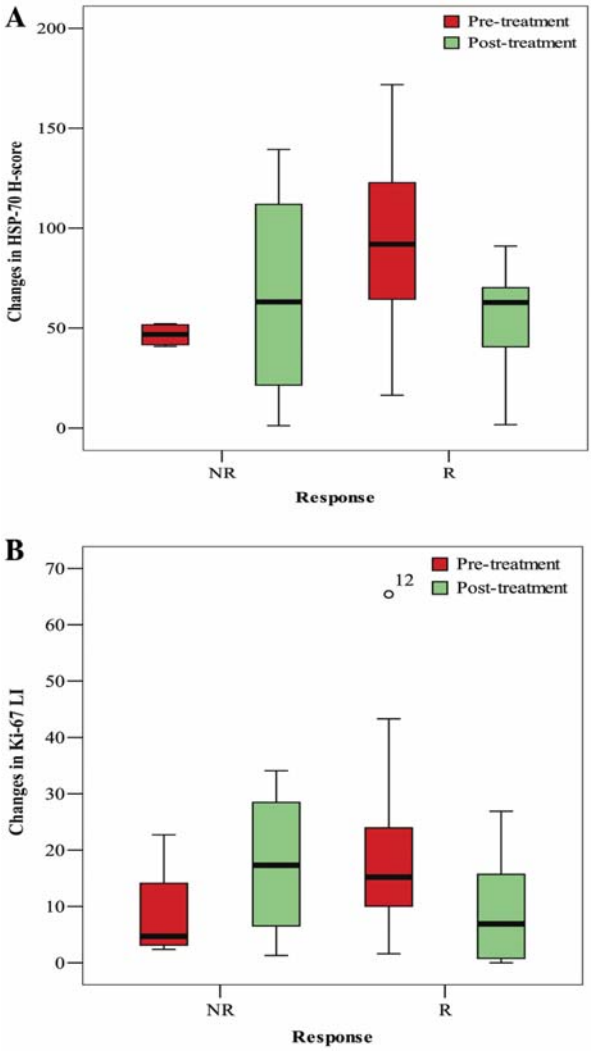

Figure 1. *Changes (mean difference) of HSP-70 (A) and Ki-67 (B) among the clinical objective responders (R) and non-responders (NR).*

Discussion

AI has been established an effective treatment for ER positive postmenopausal breast cancer but the problem of *de novo* resistance has remained the major clinical obstacle. It is also very important to evaluate the changes of carcinoma tissues following AI treatment in order for us to have a better understanding of the mechanisms of AI actions on breast carcinomas. Various alterations of histopathological features following AI treatment have been reported in the literature, which included decreased cellularity, increased interstitial fibrosis, decreased histological grading and others (34, 36) but it is also true that there have been no histological parameters following neoadjuvant AI therapy which are able to predict clinical outcome of the subsequent adjuvant AI treatment at this juncture.

Chen *et al*. reported significant reduction of PgR and Ki-67 levels following the letrozole neoadjuvant trial (37). Decrement in Ki-67 after AI treatment was also reported by Ellis *et al*. (38). Among these factors, an alteration of Ki-67 has been probably the most consistent finding among different studies. Dowsett *et al*. reported the serial changes of Ki-67 level among 330 post-menopausal breast cancer women taking neoadjuvant anastrozole at the 2nd week and 12th week in the IMPACT trial (39). The change of Ki-67 level was more substantial in the anastrozole-treated group than in the tamoxifen-treated or combined groups and the degrees of such changes were also more pronounced at the

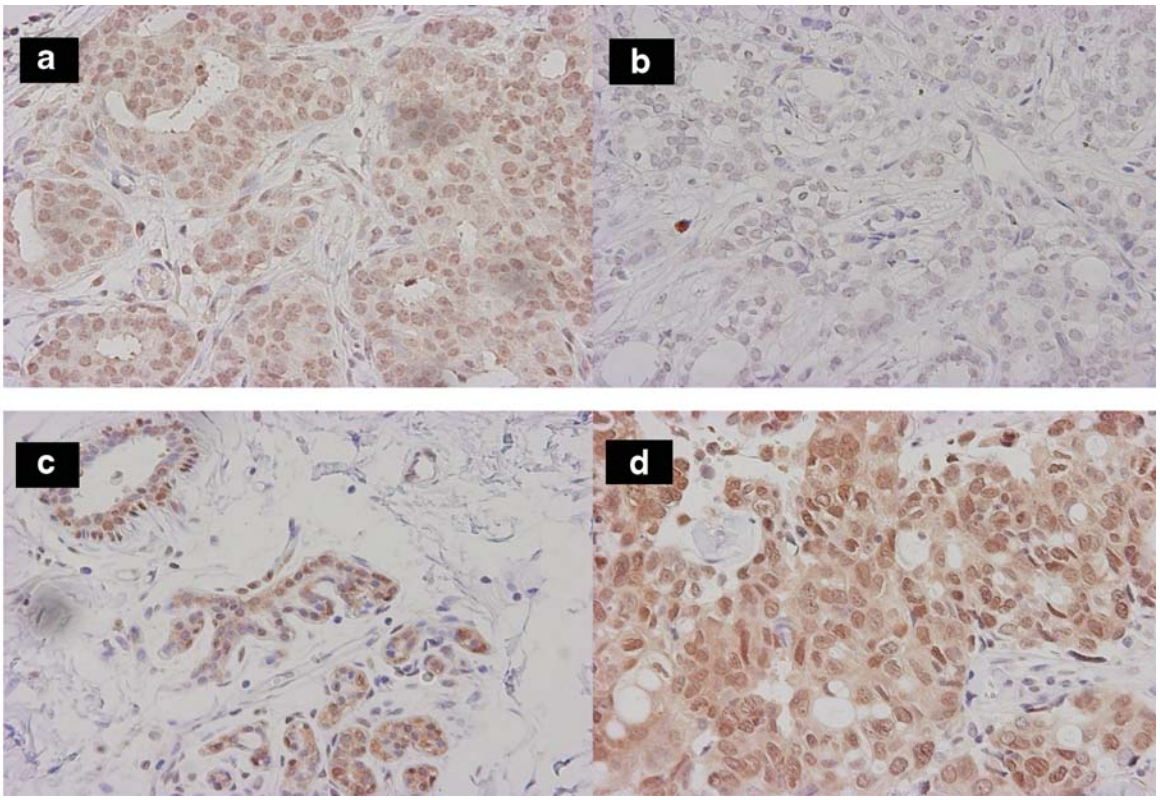

Figure 2. *Representative images of HSP-70 IHC staining (400× magnification). a: pre-treatment and b: post-treatment showing down-regulation of HSP-70 in patient #71 (responder). c: pre-treatment and d: post-treatment showed up-regulation of HSP-70 in patient #100 (non-responder).*

2nd week of treatment (93% of patients showed a certain degree of reduction) than at the 12th week. The suppression of Ki-67 LI with the use of AI (anatrozole) was also reported to be correlated with a better recurrence-free survival (9). Results of our present study also demonstrated that the reduction of Ki-67 predicted the observed response to treatment with neoadjuvant AI, which is consistent with those in the previously reported studies. The pretreatment cut-off values of Ki-67 level have been in dispute but those of 10% appear to be widely accepted. The pretreatment high Ki-67 level of carcinoma, defined as >10%, was associated with decrement of Ki-67 and HSP-70 after completion of treatment, which was also found to be a significant predictor for treatment response of the patients as well. Results of these findings also suggest that endocrine therapy may still be effectively used in ER-positive cases associated with high cell proliferation but this awaits further investigations for clarification.

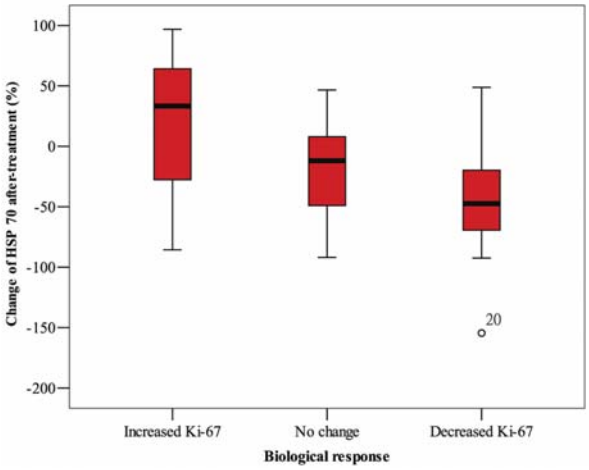

Figure 3. *Mean differences of HSP-70 among different categories of biological responders (p=0.22 between non-responders and responders).*

It is also important to evaluate wide-scale alterations of proteins or genes before and after neoadjuvant AI therapy. The expression patterns of various proteins underwent various changes following AI treatment: Ki-67, aromatase, ER-alpha, ER-beta, PgR, cyclin D1, p53, phosphorylated form of ER-alpha Ser118, ER-alpha Ser167, and p44/42 MAPK Thr202/Tyr204 expression were all decreased, while that of expression of STAT 5 and IFBP5 were increased after 6 months of treatment (9, 40-41). The alterations of gene profiles after 2

weeks of letrozole were also reported (11). In this study, there were extensive changes in both up- and down-regulated gene profiling even after a short period of AI treatment. These data clearly indicated that ER-positive carcinoma cells developed extensive adaptive changes under estrogen depletion caused by AI treatment. Such adaptive changes are also reasonably considered partly to protect carcinoma cells from cellular apoptosis and render them developing into phenotypic-resistant strains. The recent demonstration of significant alterations of the enzymes estrogen sulfatase (STS) and 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1), other than aromatase being involved in intratumoral estrogen production, has also been considered in the spectrum of these adaptive cellular alterations responding to estrogen depletion (35). These adaptive changes may ultimately result in development of *de novo* resistance to AI. Normanno *et al*. also suggested that these adaptive changes are developed in a stepwise manner toward different types of endocrine therapies (42). In addition, these phenotypic changes have been usually considered to be derived from a series of protein interactions. It is therefore important to evaluate the changes of these proteins after exposure to endocrine therapy in the following two aspects. The first is to elucidate the mechanism of *de novo* resistance, which may provide novel therapeutic approaches. Secondly, the results may lead to an availability of potential predictors for subsequent adjuvant AI treatment, which is obviously of enormous help in determining the clinical management strategy.

To our knowledge, this is the first study reporting that the down-regulation of HSP-70 is significantly associated with treatment response of neoadjuvant AI in ER-positive postmenopausal breast cancer patients. HSPs belong to a group of inducible proteins under various cellular stresses such as heat shock, chemotherapy and other anticancer therapies or other lethal conditions (43-44). HSPs are usually classified according to their molecular weights, such as HSP100, HSP90, HSP70, HSP60 and small HSPs. While the main cellular function of HSP is usually considered ATP-dependent protein chaperoning, HSP is also considered important in the process of post-translational protein folding, keeping the proteins in correct configurations for their stability. This generally protects carcinoma cells from apoptosis [45]. Under stressful cellular conditions, elevated HSP-70 levels allow the cells to cope with increased levels of unfolded and denatured proteins. HSP-70 is therefore generally considered important in maintaining several house-keeping functions such as an import of proteins into cellular compartments; folding of proteins in the cytosol, endoplasmic reticulum and mitochondria; degradation of unstable proteins; dissolution of protein complexes; control of regulatory proteins; refolding of misfolded proteins; and translocation of precursor proteins into mitochondria (44). Thanner *et al*. demonstrated the correlations of HSP-70 expression with overall survival and survival after recurrence in node-negative breast cancer patients (46). Koshiyama *et al*. also reported that HSP-70 expression was related to either hormonal regulation of cell proliferation and/or down-regulation of sex steroid receptors in estrogen dependent human endometrium (47). Down-modulation of HSP-70 by anti-sense construct was also reported to have chemosensitizing and even cytotoxic properties *in vitro* (48-50). An inhibitor, ADD70 (AIF-derived decoy for HSP-70), was reported to demonstrate promising results in animal models for colon cancer and melanoma (51). In particular for breast cancer under the state of estrogen deprivation, ERs can be activated by non-ligand binding manner *via* cross-talk mechanisms by various signal transduction pathways, which at least includes AKT, MAPK and PI3K (42). One of the common sites for hyperphosphorylation by these kinases is Ser-118 loci of the ER (52). Since HSP-70 is closely related to Akt, the use of a novel HSP-70 inhibitor was reported to decrease Akt expression in a cell line study (53). This has explained the potential roles of HSP-70 in the cross-talking to ER under the stress of estrogen depletion.

In our present study, decreased levels of of HSP-70 in patients following the neoadjuvant therapy were associated with clinical and biological response to AI. It is unlikely that AI can directly down-regulate the expression of HSP-70 but those carcinoma cells unable to change the expression of HSP-70 to chaperone an increasing load of unfolded proteins and accommodate the need to stabilize the proteins involved in cross-talk mechanisms to ERs would have a greater chance of undergoing apoptosis; but further investigations are required to test this hypothesis.

Disclosure/Conflict of interest

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