Relationship Between HSP70 and ERBB2 Expression in Breast Cancer Cell Lines Regarding Drug Resistance

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Abstract. *Background: Heat shock protein 70 (HSP70) is known to be downstream of human epidermal growth factor receptor-2 (ERBB2), but little is known regarding the relationship between HSP70 and drug resistance mediated by ERBB2 in breast cancer. Materials and Methods: After infecting breast cancer cells with lentivirus-mediated Lenti-ShHSP70 and Lenti-ShERBB2, we examined the expression of HSP70 and ERBB2 by real-time polymerase chain reaction and western blotting. Results: Compared to the control groups, mRNA expression of HSP70 was decreased in lentivirus-infected, and western blotting indicated a concordant reduction of HSP70 protein. On the other hand, ERBB2 was significantly down-regulated by HSP70 silencing in SK-BR-3 cells at both the mRNA and protein levels. Expression of HSP70 in transfected cells was also reduced by Lenti-ShERBB2. CCK8 viability assay indicated that inhibition of HSP70 increased the sensitivity of SK-BR-3 cells to fluorouracil treatment. Conclusion: HSP70 affects ERBB2 and ERBB2-mediated drug-resistance in breast cancer cells.*

Breast cancer has an overall lifetime risk of 10% of developing metastases (1). There is an upward trend both in morbidity and mortality worldwide (2). Research data show that the clinical therapeutic effect of chemotherapy may be assessed by molecular biomarkers, that constitute targets for the diagnosis of malignant tumours (3, 4).

Heat-shock proteins (HSPs), or stress proteins, are chaperones that are induced by various environmental and pathophysiological stimuli (5), of which the Mr 70,000 heat-shock protein (HSP70) has been shown not only to be required for the maturation of proteins in cell growth under normal conditions, but also involved in the regulation of cell growth and transformation (6, 7). It has been reported that various types of malignant tumours overexpress HSP70, which is closely related to tumourigenesis, malignant phenotype, tumour immunity, resistance to apoptosis and a poor prognosis in the clinical course (8, 9). Many studies have demonstrated that HSP70 is overexpressed in human breast cancer and this correlates with increased cell proliferation, poor differentiation, lymph node metastasis and poor therapeutic outcome in human breast cancer (10, 11).

ERBB2 (also called HER2), a member of the epidermal growth factor receptor family of transmembrane tyrosine kinase type receptors, is one of the most commonly amplified genes in breast cancer. Overexpression of ERBB2 usually results in malignant transformation of cells and is found in 25-30% of all breast cancer cases (12). It is always associated with more aggressive tumour phenotypes, lymph node involvement, distant metastasis, poor prognosis and increased resistance to chemotherapy (such as fluorouracil and paclitaxel) and endocrine therapy (13-15). Overall survival rate and time to relapse for patients with ERBB2-positive breast cancer are significantly shorter than for patients without ERBB2 over-expression (16). Agents targeting ERBB2 have been applied in the clinical treatment of ERBB2-positive breast cancer (16, 17).

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Overall, ERBB2 is a logical target for breast cancer therapy and inhibition of ERBB2 expression leads to the apoptosis of tumour cells (18-20).

Wilson *et al*. evaluated the expression of thousands of genes at the mRNA level and found that a limited number of additional genes were up- or down-regulated by ERBB2 (21). Among them, *HSP70* was found to be up-regulated both in ERBB2-positive breast cancer cell lines and in ERBB2-overexpressing breast cancer. ERBB2 overexpression in breast cancer was associated with differences in the expression pattern of genes of several structural and functional pathways (22).

Therefore, we investigated the relationship between ERBB2 and HSP70 in breast carcinoma. In the present study, we constructed a lentivirus vector-mediating RNAi targeting of *HSP70* gene (pLL2G-shHSP70) and *ERBB2*, and then infected SK-BR-3 cells to knockdown *HSP70* and *ERBB2* expression. After down-regulating the expression of HSP70 and ERBB2, we detected the changes of their expression in terms of mRNA and protein by real-time polymerase chain reaction and western blotting, respectively. We also investigated the effects of silencing of *ERBB2* and *HSP70* genes on the sensitivity of cells to chemotherapeutic drugs.

Materials and Methods

Cell culture techniques. SK-BR-3 (ERBB2$^+$, HSP70$^+$) and MCF-7 (ERBB2$^-$, HSP70$^-$; as control cells) (21, 23) cells were obtained from the Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The cells were grown as a monolayer in RPMI-1640 (Gibco, Carlsbad, CA, USA) containing 10% foetal bovine serum (v/v) and penicillin (100 U/ml)/streptomycin (100 mg/ml. Cells were maintained at 37˚C in a humidified incubator with 5% CO_2.

Lentiviral infection. Construction and production of lentivirus vectors for *ERBB2* are given in our previous study (24). Process for producing SK-BR-3 cells infected with lentivirus carrying human small hairpin RNA (shRNA) for ERBB2 is described elsewhere (25). Lentivirus vectors for shRNA encoding a green fluorescent protein (GFP) sequence was constructed by GENECHEM (Shanghai, China), with the target shRNA sequence of 5'-GGAGGCTGGCAAC ATAACA-3' and the lentivirus vectors containing *HSP70* shRNA were confirmed by sequencing. The transductions of the cells were performed according to the manufacturer's instruction. Briefly, the virus (0.1 ml) was mixed with 0.1 ml complete medium containing polybrene (8 mg/ml) and added to cells, then incubated for 1 h at 37˚C. After that, the cells were incubated in fresh complete medium containing polybrene for 24 h, followed by incubation for 48 h in complete RPMI-1640 medium without polybrene. SK-BR-3 cells infected with lentivirus-mediated Lenti-shHSP70-eGFP were used as study cells; cells infected with negative shRNA (Lenti-shcontrol-eGFP) were used as negative controls, cells without infection served as blank controls, and MCF-7 cells as positive controls. Transduction efficiency was evaluated in cells 48 h after transduction by fluoroscopy. All the cells were harvested after transduction for subsequent studies.

Quantitative real-time reverse transcriptase and quantitative real-time polymerase chain reaction (RT-PCR). Total RNA from breast cancer cell lines was isolated with TRI-Zol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and purity of RNA was determined using a spectrophotometer. Reverse transcriptase was used to create cDNA for further analyses. RT-PCR assays were carried out using SYBR Premix Ex Taq II (Perfect Real-Time; TaKaRa, Yamanashi, Japan) and real-time PCR amplification equipment. Reverse transcriptase and real-time PCR was according to the protocol of the kit instructions. The PCR primers (synthetized by Sangon Biotech, Shanghai, China) used to detect *ERBB2*, *HSP70* and glyceraldehyde phosphate dehydrogenase (*GAPDH*) were as follows: *ERBB2*, sense 5'-CATGTCATCGTCCTCCAGCAG-3', and antisense 5'-TTGACTCTGAATGTCGGCCAA -3', with a product length of 161 bp; *HSP70*, sense 5'-CAGGAAGGCAGAAAG AAAACATA-3', and antisense 5'- GCCACAGTTCACCATTTAC-3', with a product length of 209 bp; *GAPDH*, sense 5'-CTGCACCACC AACTGCTTAG-3', and antisense 5'-TGAAGTCAGAGGAGAC CACC-3', with a product length of 135 bp. The real-time PCR procedure consisted of two steps: one cycle at 95˚C for 10 s, followed by 40 cycles at 95˚C for 5 s and 60˚C for 20 s. The expression of *ERBB2* and *HSP70* was determined by normalization of the threshold cycle of these genes to that of *GAPDH*. Data were analysed using the comparative ΔΔCT method.

Western blotting analysis. Whole-cell proteins were isolated from various breast cancer cell lines. The lysates were centrifuged, and the supernatant was collected and stored at –80˚C according to the manufacturer's instructions. Total protein was loaded (10 μl per well), separated by 10-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes at 60 V for 1 h at 4˚C. The membranes were blocked and incubated with primary antibodies (obtained from Abcam Company, Cambridge, Cambridgeshire, UK; diluted 1:1200 in tris buffered saline (TBS). The membranes were then rinsed thrice with 0.1% Tween-20 phosphate buffered saline (PBS) for 30 min. Secondary antibodies (obtained from Abcam Company, Cambridge, Cambridgeshire, UK; diluted 1:1200 in TBS-A) were used with peroxidase-conjugated affinipure goat anti-mouse IgG (1:8,000) and peroxidase-conjugated affinipure goat anti-rabbit IgG (1:8,000) for 1 h at room temperature. The blotted membranes were then washed three times with 0.1% Tween-20 PBS for 15 min and three times with PBS alone for 15 min. The immunoblots were detected by using an electrochemiluminescence kit (Roche Applied Science, Indianapolis, USA) and exposed in VILBER Fusion FX5 automatic gel imaging analysis system (Vilber Lourmat, Marne La Vallee, Cedex, France).

Cell counting kit-8 (CCK8) assay. The drug sensitivity assay was carried out using the mitochondrial reduction activity assay. According to the protocol of the CCK8 assay kit (Dojindo, Kumamoto, Japan), cells were grown in 96-well culture plates (8,000 cells per well) and treated as required. The experimental group used lentivirus-infected cells and the control group non-infected cells. After 24-h culture, fluorouracil (Aventis Pharma, Essex, Dagenham, UK) was added at the following concentrations: 0.25, 0. 5, 1, 2, 4, 8, 16, 32 μg/ml. Next, cells in each well were incubated with 10 μl of CCK8 at 37˚C for 4 h. Then the optical density (OD) for each well was measured at 450 nm using a microplate reader (Bio-Rad Model 550; Bio-Rad, Hercules, CA,

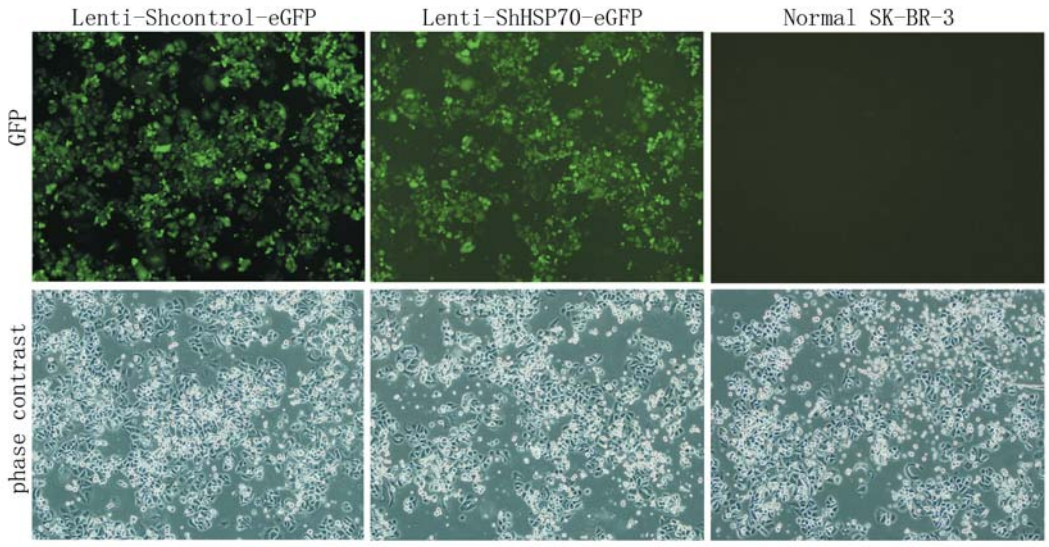

Figure 1. *Phase-contrast imaging and green fluorescent protein (GFP) expression under a fluorescent microscope was taken after 48 h lentivirus infection in SK-BR-3 cells (original magnification, ×100).*

USA). CCK8 experiments were repeated three times on different days. Means and standard deviations of the ODs of the replicates were calculated for each well. The cell-inhibitory rate was calculated according to the following equation: inhibitory rate=[1−($OD_{experiment}$ − OD_{blank})/($OD_{control}$ − OD_{blank})]×100%. The 50% inhibitory concentration (IC_{50}) for the drug was then calculated.

Statistical analysis. The statistical software package SPSS 13.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. For all measurements as needed, the statistical significance between groups was assessed by one-way ANOVA based on homogeneity of variance test. A value of $p<0.05$ was considered to indicate a statistically significant difference.

Results

Infection efficiency of in vitro virus delivery. SK-BR-3 cells were infected with lentiviral vector encoding GFP, resulting in GFP expression in the majority of cultured cells. The infection efficiency was assayed by fluorescence microscopy after cells were infected by lentivirus for 48 h. A robust infection efficiency of 69% was recorded (Figure 1). Fluorescence gradually weakened and the infected cells began to undergo apoptosis on the fifth day, resulting in the gradual decline of the infection rate. SK-BR-3 cells infected with lenti-ShHSP70-eGFP and non-infected cells co-cultured showed no synergistic growth-inhibitory effect when treated with the same protocols.

mRNA expression of HSP70 and ERBB2. Following lentiviral infection of SK-BR-3 cells, the mRNA expression of *HSP70* and *ERBB2* was examined by real-time PCR. PCR analysis showed that the mRNA expression level of *HSP70* was lower in the infected Lenti-ShHSP70-eGFP group than in the Lenti-Shcontrol and normal SK-BR-3 group. There was significant difference in expression between the silenced cells and control cells ($p<0.05$), indicating that the expression of *HSP70* mRNA in SK-BR-3 cells was knocked-down successfully. As shown in Figure 2B, the expression of *ERBB2* mRNA was affected following *HSP70* knock-down, with different expression levels in *HSP70*-silenced cells and control cells. The expression of *ERBB2* in *HSP70*-positive cells (SK-BR-3) was natively at a high level. However, after silencing the expression of *HSP70*, *ERBB2* was down-regulated.

HSP70 and ERBB2 protein expression. The protein expression of HSP70 and ERBB2 was evaluated by western blotting. The grey-scale of the antibody-stained area was measured under identical conditions. The average OD for HSP70 protein expression in the HSP70-shRNA infected group was lower compared to that for the blank control group, and the negative control-shRNA group ($p<0.05$) (Figure 3B). Consistent with the mRNA results, the average OD for ERBB2 protein expression in the SK-BR-3 group, MCF-7, the Lenti-Shcontrol group, and the Lenti-ShHSP70 group significantly differed ($p<0.05$) (Figure 3B). On inhibition by ERBB2-shRNA, the level of HSP70 decreased accordingly ($p<0.05$) (Figure 3B).

Effects of Lenti-ShHSP70-eGFP on cell sensitivity to fluorouracil treatment. After inhibiting *HSP70* expression by Lenti-ShHSP70-eGFP infection, the IC_{50} (index of chemotherapy sensitivity to fluorouracil) was 11.1±0.54

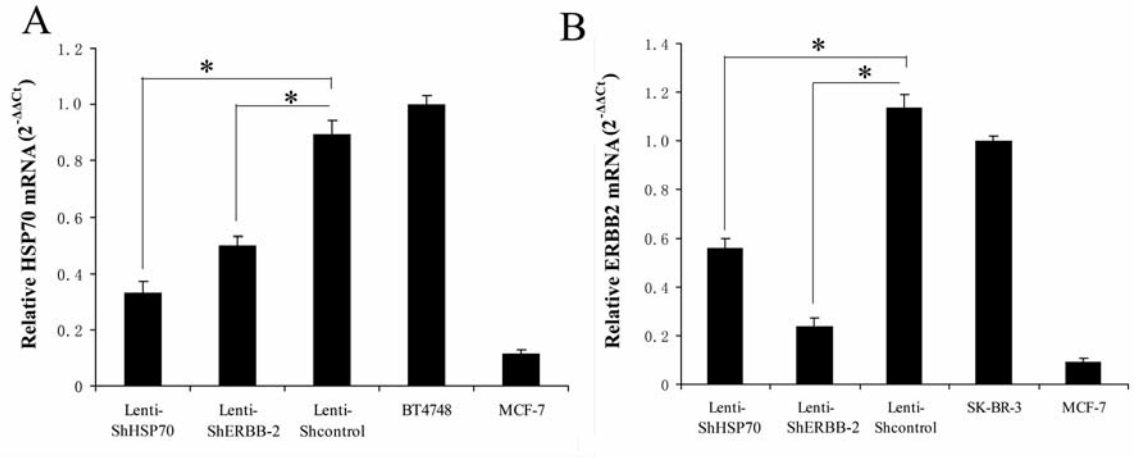

Figure 2. *mRNA levels of heat-shock protein (HSP70) (A) and epidermal growth factor receptor-2 (ERBB2) (B), detected by real-time polymerase chain reaction after SK-BR-3 cells were treated with Lenti-ShHSP70, Lenti-ShHSP70 and Lenti-Shcontrol. HSP70-shRNA infected cells exhibited significantly reduced HSP70 mRNA expression compared to that of Lenti-ShERBB2, the blank control group, and negative control-shRNA group. Similarly ERBB2-shRNA infected cells exhibited significantly reduced ERBB2 mRNA expression. *p<0.05.*

μg/ml for Lenti-ShHSP70 infected cells, 30.01±0.83 μg/ml for SK-BR-3 cells, and 1.15±0.73 μg/ml for MCF-7 cells. After HSP70 was inhibited, the sensitivity to chemotherapy of breast cancer cells improved almost three-fold. This result indicates that the inhibition of HSP70 can increase cell sensitivity to fluorouracil treatment, as indicated in Figure 3C. However, the sensitivity was still lower than that of *ERBB2*-inhibited cells ($p<0.05$).

Discussion

The results of the present study revealed that after down-regulation of HSP70 in SK-BR-3 cells by lentiviral infection, ERBB2 was expressed at a lower level. Concurrently, a range of effects on cell physiology, such as growth inhibition and increased sensitivity to chemotherapy was observed. After SK-BR-3 breast cancer cells were infected with Lenti-ShERBB2, *HSP70* was significantly reduced at both mRNA and protein levels. Although both ERBB2 and HSP70 contribute to the progression of certain types of tumour, our data suggest that HSP70 expression might be strictly associated with *ERBB2* amplification in SK-BR-3 cells.

Research has shown that a DNA vaccine containing HSP70 fused to C-terminal domain and downstream of *ERBB2* could combat an aggressive *ERBB2*-expressing tumor (26). Study of MCF-7 cells found that MP-412, an EGFR and ERBB2 dual inhibitor, depleted not only ERBB2 but also estrogen receptor (ER)-α, and to some extent, affected RAF1 (Raf-1 Proto-Oncogene, Serine/Threonine Kinase), while MP-412 activated HSP70 expression (27). Surprisingly, specific depletion of HSP70 chaperone proteins involved in the control of folding and transport of other proteins resulted in striking changes in gene expression (28). The interaction of lysyl oxidase propeptide with HSP70 and c-RAF inhibits a critical intermediate in RAS (renin-angiotensin system)-induced MEK (mitogen-activated protein/extracellular signal-regulated kinase kinase) signaling and plays an important role in the function of this tumour suppressor. RAF-MEK-MAPK and PAK-JNKK-JNK, two cascades of serine/threonine kinases, regulate the activity of a number of transcription factors, implicated in the signaling network of ERBB (22). HSP70 proteins are implicated in resistance to chemotherapy in cancer (29), and their detection is important for cancer treatment and prognosis. Studies have also demonstrated a correlation between nuclear HSP70 expression and drug resistance in patients with breast cancer treated with induction chemotherapy (30, 31). There is one clinical study showing that high levels of HSP70 in recurrent breast tumours correlates with low response to other kinds of treatment such as radiotherapy and thermotherapy (32, 33). Since the HSP family plays a critical role in cell growth, survival, immunity, and apoptosis (34), our data suggest that the *HSP70* gene maybe a feasible target for gene silencing.

ERBB2 is a cell-surface receptor tyrosine kinase and becomes internalized upon ligand binding, which can trigger a multitude of signaling pathways, such as MAPK and PI3K (22). Consequently, ERBB2 has been shown to trigger signal transduction leading to cell growth and differentiation. Overexpression of *ERBB2* gene has been found in malignancies of the oral cavity (35, 36). Tumors with *ERBB2* amplification/overexpression have been shown to demonstrate increased aggressiveness and metastatic potential and associated with decreased overall survival (34, 37). ERBB2 overexpression in breast cancer was associated

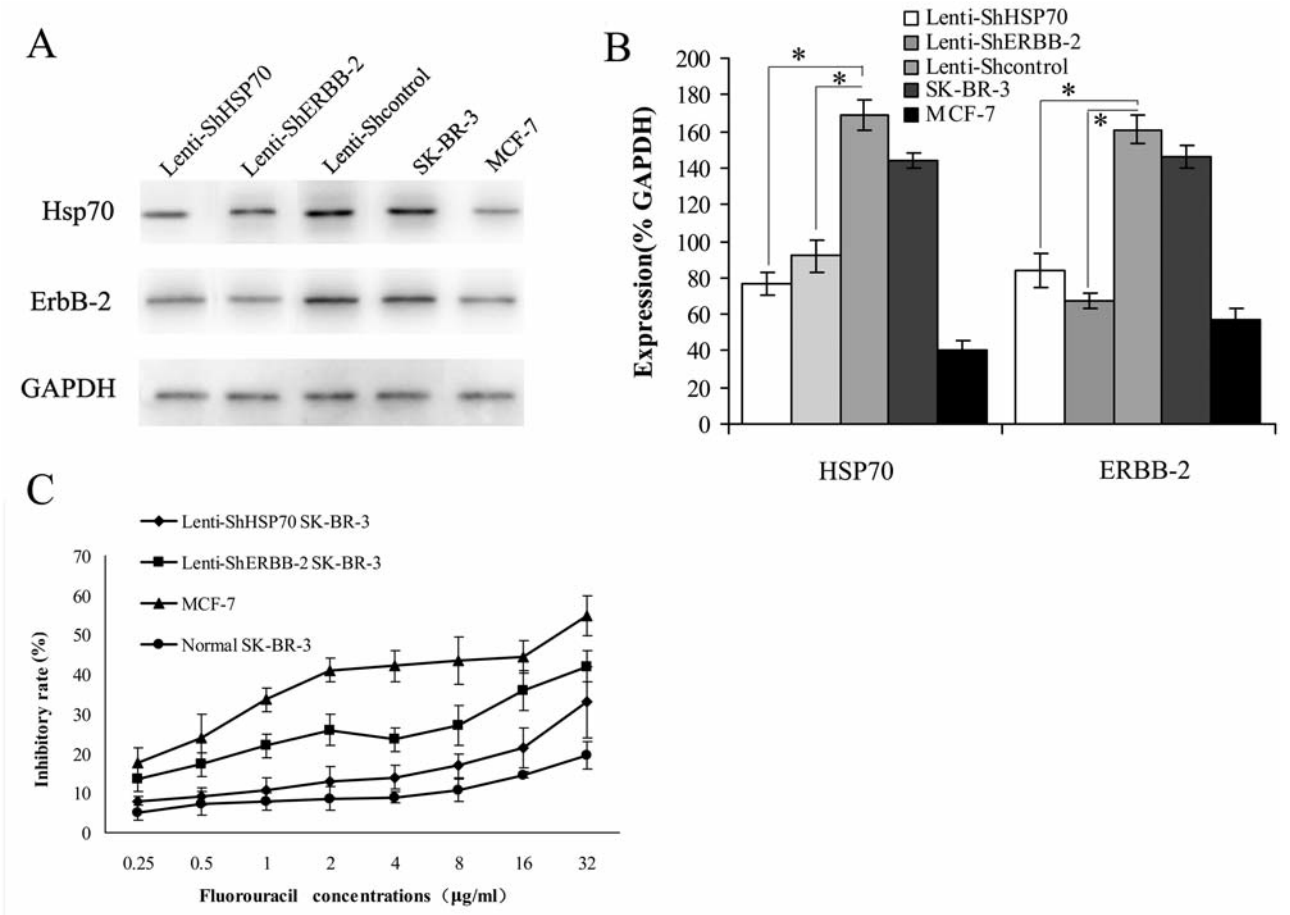

Figure 3. *Levels of heat-shock protein (HSP70) (A) and epidermal growth factor receptor-2 (ERBB2) (B), protein detected by western blotting after SK-BR-3 cells were treated with Lenti-ShHSP70 and Lenti-Shcontrol. Lenti-ShHSP70 significantly reduced HSP70 expression at the protein level compared to Lenti-Shcontrol cells. After interference, the expression of ERBB2 was down-regulated significantly. *p<0.05. C: SK-BR-3 and MCF-7 cell lines were treated with different concentrations of fluorouracil for 24 h and examined under the CCK8 assay. The sensitivity of SK-BR-3 cells to fluorouracil increased after HSP70/ERBB2 interference and the difference was statistically significant (p<0.05).*

with differences in the expression pattern of genes of several structural and functional pathways. Treatment of ERBB2-positive cells with bortezomib, as with ansamycins, seems to shift ERBB2 chaperone association from one that is stabilizing (HSP90) to one that is destabilizing (HSP70). The association of HSP90 with endogenous ERBB2 receptor was found to be lost and replaced by another co-precipitating chaperone protein, HSP70 (38). It has been reported that heat-shock transcription factor 1/HSP70 regulates P-glycoprotein expression at the transcriptional level, since some multidrug-resistant cell lines exhibit constitutively high HSF–DNA binding activity (39). HSP70 and other HSPs may reduce the lethality of antitumor drugs and other cytotoxic insults by preventing apoptosis (40) and hence provide an alternative mechanism for drug resistance (30, 41). There is increasing evidence that HSPs play an important role in the development, maintenance and progression of cancer. HSP90 client proteins include mutated p53, BCR–ABL, RAF1, AKT (protein kinase B), ERBB2 and hypoxia-inducible factor-1-alpha (42, 43). It is known that the carboxyl terminus of HSP70-interacting protein efficiently down-regulates ERBB2 *in vitro* (43). This is somewhat reminiscent of HSP70 and ERBB2 interaction with each other. Whether HSP70 plays a favourable regulatory role in ERBB2-mediated drug resistance of human breast cancer cells, and through which specific signaling pathways still need further investigations. Establishing the role of HSP70 in ERBB2-mediated chemotherapy resistance in breast cancer cells may be urgently necessary.

In summary, our data demonstrate that HSP70 was effectively silenced in SK-BR-3 cells *via* lentiviral infection, providing proof-of-principle for using lentivirus

shRNA in sensitizing fluorouracil-resistant HSP70-overexpressing breast cancer cells to the drug by repressing HSP70 expression. This, in turn, may have important implications for the development of a novel therapy combined with chemotherapy. In addition to new diagnostic, prognostic and therapeutic markers, HSP70 may become a novel target for breast cancer therapy. Future studies are required to examine the exact mechanism of the molecular and biological relationship of HSP70 and ERBB2 in breast cancer cells in detail.

Conflicts of Interests

We declare that we have no competing interests.

Acknowledgements

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