

Hsp27 Expression Does Not Affect Anti-Cancer Drug Sensitivity but Promote Cell Proliferation of Lung Squamous Cell Carcinoma *In Vitro*

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Abstract

Lung cancer is one of the most common malignancies worldwide. Non-Small Cell Lung Cancer (NSCLC) constitutes 85-90% of all lung cancer cases with Squamous Cell Carcinoma (SCC) being one of the subtypes of NSCLC (35%). Unlike adenocarcinoma subtype, treatment options for SCCs are limited as there are currently no molecular targeted therapies specifically designed for the treatment of SCC. There is thus an urgent need to identify novel therapeutic targets to circumvent therapeutic barriers for effective treatment of lung SCCs. Heat Shock Proteins (Hsps) modulate a wide range of cellular responses and is especially known for its ability to help cells survive under stressed conditions. Numerous reports have associated Hsp27 with both tumour-promoting as well as tumour-inhibiting functions, but no studies have been conducted to investigate the role of Hsp27 in lung SCC cells and their cellular responses to commonly used anti-cancer drugs. We reported, for the first time, that Hsp27 does not involve in modulating anti-cancer drug-induced cytotoxicity and affecting the cell survival of Calu-1 and H226 cells. This demonstrates clinical implication as the overexpression of Hsp27 will not compromise the efficacy of standard chemotherapy treatment given to the patients with lung SCC. Crucially, a novel finding from our study is that Hsp27 plays a significant role in promoting cell growth of H226 and Calu-1 cells as silencing of Hsp27 using siRNA transfection diminishes their colony-forming ability. In addition, we also showed the minimal role of Hsp27 in regulating migration and invasion of H226 and Calu-1 cells as demonstrated by wound scratch migration and matrigel invasion assays. Overall, the findings that Hsp27 promotes the growth of lung SCC cells may indicate its potential application as a molecular target for Hsp27 inhibitor, which can be used in molecular-based monotherapy in patients with lung SCC.

Keywords: Hsp27; Lung squamous cell carcinoma; Anti-cancer drug; Cell proliferation

Introduction

As the most common cancer around the globe, lung cancer is currently the leading cause of cancer-related deaths among man in the world[1,2]. Among the different subtypes of lung cancer, Non-Small Cell Lung Cancer (NSCLC) accounts for approximately 85-90% of all lung cancers, which can be further divided into adenocarcinoma, large cell carcinoma and Squamous Cell Carcinoma (SCC) based on the histologic classification[1,3]. Currently, treatment development have

mainly been confined to adenocarcinoma subtype, which is ranked as the most frequent subtype of NSCLC, such as the widely known Epidermal Growth Factor Receptor (EGFR)-targeting inhibitors, gefitinib and cetuximab[4]. However, there are currently no molecular targeted therapies specifically designed for lung SCC which accounts for only 30% of NSCLC[5], thus limiting the treatment options for lung SCC patients[6]. There is thus an urgent need to identify novel therapeutics targets to circumvent therapeutic barriers for effective treatment of lung SCC.

In this search for new therapeutic targets, small heat shock protein of 27kDa (Hsp27) may be a potential target for novel molecular targeted treatments of lung cancer due to its diverse

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and complex roles in wide array of cancers. Hsp27 is a highly conserved mammalian phosphoprotein encoded by the HSPB1 gene, belonging to the family of ATP-independent Small Heat Shock Proteins (sHsps)[7]. Involving in a wide range of cellular processes, Hsp27 is specially known for their ability to help cells to survive under stress conditions. With the role as a molecular chaperone, Hsp27 confer cytoprotective effects by sequestering damaged proteins and inhibiting protein synthesis in stressed cells to prevent accumulation of incorrectly folded protein aggregates. In addition to its role in stressed conditions, it plays a vital part in cells under unstressed conditions through providing cytoskeletal structural ability and facilitating the transportation of newly synthesized proteins to target organelles. However, the function of Hsp27 in cancers reported thus far has been rather controversial, probably due to the intricate networks and multiple activities involved in cancers. Positive correlation between overexpression of Hsp27 and promotion of tumourigenesis has been suggested. For instance, studies have shown that overexpression of Hsp27 increases growth of UMUC-3 bladder cancer cells[8], enhances cell migration and invasion capability as well as promotes resistance to anti-cancer drug, doxorubicin in breast cancer cells[9,10]. On the contrary, other findings oppose the positive correlation that has been mentioned above. For example, an overexpression of Hsp27 was showed to inhibit the growth of Hep-2 human laryngeal cancer cells[11]. Instead of facilitating invasion and migration, Aldrian et al. [12] presented that an overexpression of Hsp27 decreases invasion and migration of human melanoma cells *in vitro*[12]. High Hsp27 expression was also revealed to abolish the multidrug resistance in Adriamycin resistant MCF-7 breast cancer cells[13]. Collectively, these contradictory findings suggest the profound role of Hsp27 in tumourigenesis and malignancy, which is likely to be cancer-type specific. Though the function of Hsp27 has been elucidated in several cancer cell types, no studies have been conducted to investigate the role of Hsp27 in lung SCC cells. This thus demands further research to unravel the actual role of Hsp27 in maintaining the cellular responses in lung SCC cells. In this study, we aim to investigate the functional role of Hsp27 in drug response, tumour growth, invasion and migration of lung SCC. The understanding of Hsp27 functions could eventually help us to identify Hsp27 as a potential therapeutic target and may reveal novel treatment strategies through the use of Hsp27 inhibitors such as OGX-427, to improve current chemotherapeutic regimens in lung SCC.

Materials and Methods

Materials and reagents

Roswell Park Memorial Institute (RPMI)-1640 medium and trypan blue were purchased from Nacalai Tesque (Kyoto, Japan); Fetal Bovine Serum (FBS) was purchased from JR Scientific (North America, USA); L-glutamine and penicillium-streptomycin was purchased from Invitrogen Life Technologies (Brazil); 0.5M Tris-HCL buffer, 1.5M Tris-HCL buffer, Polyvinylidene Difluoride (PVDF) membrane, blotting paper, 30% acrylamide and non-fat dry milk were purchased from Bio-Rad Laboratories (Hercules, CA, USA); 10X Tris glycine, SDS buffer and phosphate-buffered saline (PBS) were purchased

from First BASE (Singapore); Tween-20 was purchased from Sigma-Aldrich (Lyon, France); tetramethylethylenediamine (TEMED) and Dimethyl Sulfoxide (DMSO) were purchased from MP Biomedicals (Illkirch, France); Ammonium Persulfate (APS) was purchased from AppliChem (Darmstadt, Germany); β -mercaptoethanol and isopropanol were purchased from Merck (Darmstadt, Germany); 0.5% trypsin-EDTA was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA); Hsp27 (SC-1048) goat polyclonal IgG, Hsp27 siRNA (SC-29350) and donkey anti-goat IgG-HRP conjugated were purchased from Santa Cruz Biotechnology (CA, USA); anti-rabbit HRP-linked antibody was purchased from Cell Signaling Technology (Beverly, MA, USA); ON-TARGET plus non-targeting pool siRNA (D-001810-10-50) (scrambled siRNA) was purchased from Dharmacon (Thermo Fisher Scientific, USA); Restore TM PLUS western blot stripping buffer and pierce Bicinchoninic Acid (BCA) assay kit were purchased from Thermo Scientific (Rockford, IL, USA); Enhanced Chemiluminescent (ECL) prime western blotting detection reagent was purchased from GE Healthcare (Piscataway, NJ, USA); spectra multicolor broad range protein ladder was purchased from Thermo Fisher Scientific (Vilnius, Lithuania); cis-Diammine (1,1-cyclobutanedicarboxylato)platinum(II) (carboplatin) were purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada); jetPRIMETM transfection reagent, jet-PRIMETM buffer were purchased from Polyplus-transfection (Illkirch, France); methylcellulose was purchased from Tokyo Chemical Industries (Tokyo, Japan); 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega (Madison, WI, USA); cis-diamminedichloroplatinum(II) (cisplatin) was purchased from Pfizer (Perth, WA, Australia); 5-Fluorouracil (5-FU) was purchased from Sigma Aldrich (Steinheim, Germany) and Docetaxel (Taxotere) was purchased from Aventis Pharmaceuticals (Bridgewater, NJ).

Cell lines

All the cell lines (H2066, SW900, H2170, Calu-1, H520, SK-MES-1, H1869, ChaG0-k-1 and H226) used in this study were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The cell lines were cultured in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% FBS, 1% L-glutamine and 1% Penicillin-Streptomycin at 37°C in a humidified atmosphere containing 5% CO₂ and sub-cultured only at 80% to 90% confluency.

Western blot analysis

Cells were seeded in six-well plates at $1-3 \times 10^5$ cells per well (varies on different cell lines) and followed by overnight incubation before harvest by trypsinization. Cell pellets were resuspended in complete lysis buffer (containing miliQ water, phosphatase inhibitor and 10% protease inhibitor) and lysed by sonication for 10 seconds. Lysates were then incubated on ice for 30 min before centrifugation at 14,000 rpm for 20 min at 4°C. Protein concentration in the supernatants was determined by the BCA protein assay kit according to the manufacturer's instructions. 120 μ l of the protein measured were mixed with 40 μ l of 4x loading buffer (containing

4% β -mercaptoethanol, 50 mM Tris-HCl pH6.8, 40% glycerol, 4% SDS-bromophenol blue) and denatured at 95°C for 5 min. 15 μ g of protein lysates were resolved by electrophoresis on 10% SDS polyacrylamide gels at 80 volts for 2 hours at room temperature and subsequently transferred to a PVDF membrane. The PVDF membrane was then blocked with 5% non-fat milk in Phosphate Buffered Saline With Tween-20 (PBST) for 1 hour and probed with primary antibodies (1:3000 for Hsp27 goat polyclonal IgG) overnight at 4°C. After overnight incubation, the membrane was washed with PBST for 2 \times , 30 min each with intermittent shaking, and incubated with HRP-conjugated secondary antibody (anti-goat or β -actin rabbit monoclonal antibodies) for 1 hour at room temperature. The membrane was washed again with PBST before detection using the ECL Prime Western Blotting Detection Reagent and exposure to X-ray film. β -actin was used in each blot as a loading control to ensure equal loading of proteins for each sample. Protein quantification was performed using Image J software (Java-based image processing program developed by National Institutes of Health). Whenever required, blots were washed in PBST and stripped with stripping buffer with intermittent shaking before blocking with 5% non-fat milk in PBST and re-probed with desired antibodies.

RNA interference

1.5×10^5 cells per well (Calu-1 cell line) and 1.2×10^5 cells per well (H226 cell line) were seeded onto 6-well plate (Greiner, Germany) and cultured for 24 h prior to transfection. For optimal siRNA transfection, only cells which are 50% confluent at the time of transfection were transfected. Growth medium in the wells was replaced with 800 μ l of growth medium. To prepare the transfection mix, for each well, 5 μ l (for a final concentration of 50 nM per well) of Hsp27 siRNA from the stock (10 μ M) or 2.5 μ l (for a final concentration of 50 nM per well) of scrambled siRNA (negative control) from the stock (20 μ M) was diluted into 200 μ l of jetPRIME[®] transfection buffer in separate tubes. 4 μ l of jetPRIME[®] transfection reagent was then added and vortex for 10 seconds and spin down briefly. The transfection mix was incubated for 15 min at room temperature to allow complex formation. 200 μ l of transfection mix was added drop wise to each well. The plate was rock gently back and forth and incubated at 37°C for 24 hours.

Cell proliferation analysis

Cell proliferation assay was carried out using MTS. H520 (4×10^3 cells per well), H226 (3×10^3 cells per well) and Calu-1 (5×10^3 cells per well) were seeded onto 96-well plate (Greiner, Germany) and incubated for 24 hours prior to treatment with 100 μ l of increasing concentrations of cisplatin (0.001 to 100 μ M), carboplatin (0.1 to 100 μ M), 5-FU (0.1 to 1000 μ M), docetaxel (0.0001 to 2 μ M) or vehicle (RPMI for cisplatin, 1% DMSO for carboplatin, 1% DMSO for 5-FU, 0.05% DMSO for docetaxel) for 72 hours. 20 μ l of MTS was added to each well and incubated for 3 hours at 37°C. Absorbance was taken at 495 nm using a plate reader (TECAN Infinite M200, Mannedorf, Switzerland). Experiments were performed in triplicates.

Anchorage-dependent clonogenic assay

The anchorage-dependent clonogenic growth assay was carried out to assess the ability of a single cell to grow into a colony. Calu-1 and H226 cells (control, scrambled-treated and Hsp27 siRNA-treated) were seeded onto 6-well plate at a density of 500 cells per well and incubated at 37°C for 14 days. The medium was changed every 7 days. After 14 days, the medium was discarded and cells were washed once with PBS carefully. The cells were then fixed with 4% paraformaldehyde for 10 min, stained with 0.1% crystal violet (in 20% methanol) for 30 min and rinsed in distilled water to remove excess stain. The plates with colonies were left to dry in normal air at room temperature (25°C). Colonies with a diameter of 1 mm and larger were counted as one colony. The number of colonies was expressed as a percentage of control cells (100%). Experiments were performed in triplicates.

Anchorage-independent clonogenic assay

The methylcellulose colony forming assay was carried out to assess the ability of cancer cells to form anchorage-independent colonies *in vitro*. The assay was performed using a methylcellulose semisolid medium consisting of RPMI-1640 medium with 1.5% methylcellulose (supplemented with 10% FBS, 1% L-glutamine and 1% penicillin-streptomycin). Calu-1 and H226 were plated onto 6-well ultra-low attachment plate (Corning Inc., NY, USA) at a density of 2×10^5 cells per well in 4 ml of methylcellulose medium and incubated at 37°C for 21 days. 1 ml of fresh RPMI was added into each well every 7 days. After 21 days, colonies were counted with the light microscope (4x magnification) with the use of an ocular micrometer to evaluate the diameter. Colonies with a diameter of 1 mm or larger were counted as one colony. The number of colonies was expressed as a percentage of control cells (100%). Experiments were performed in triplicates.

Wound scratch migration assay

The wound scratch assay was used to study cell migration *in vitro*. Cells were seeded onto 6-well plate (Greiner, Germany) at a density of 4×10^5 cells per well for Calu-1 and H226 and incubated at 37°C for 24 hours. After 24 hours, a scratch was made by scratching the cell monolayer in a straight line with a p200 pipette tip. The debris was removed and the edge of the scratch was smoothed by washing the cells once with 1ml of RPMI medium and then replaced with 2 ml of RPMI medium. The plates were then incubated at 37°C for 48 hours. The images were taken using a light microscope (4x magnification) fitted with a microscope camera (Olympus) at time intervals of 0, 12, 24, 36 and 48 hours.

To attain the same field during image acquisition, reference point was made by making a gentle scratch on the outer bottom of the plates. The area of wound was analyzed using Java's Image J software. Cell migration towards the wound was expressed as percentage of wound closure:

Percentage of wound closure = $[(A_{t=0h} - A_{t=\Delta h}) / A_{t=0h}] \times 100\%$
 where, $A_{t=0h}$ is the area of wound measured immediately after

scratch was made, and $A_{t=\Delta h}$ is the area of wound measured 12, 24, 36 or 48 hours after the scratch was made.

Matrigel invasion assay

In vitro invasion assay was performed using BD BioCoat Matrigel Invasion Chambers (BD Bioscience, Bedford, MA) and 24-well companion plate (BD Bioscience, Bedford, MA). The falcon cell culture inserts contain an 8 micron pore size PET membrane with a thin layer of MATRIGEL Basement Membrane Matrix. Culture inserts from -20°C were left to thaw to room temperature for 30 min and 500 μl of serum-free RPMI was then added to the bottom of the well and to the inserts. The inserts were incubated at 37°C for 2 hours to allow for rehydration. The media was then removed from the well and inserts without disturbing the layer on the membrane. Using a sterile forceps, the inserts were transferred to empty wells of 24-well plate. In the meantime, Calu-1 and H226 were harvested after trypsinization, washed once with PBS and resuspended with serum-free RPMI containing 1×10^5 cells/ml (Calu-1) or 2×10^5 cells/ml). 750 μl of RPMI (supplemented with 10 % FBS) was added to the bottom of the wells and inserts were transferred to the wells. 500 μl of cell suspension containing 5×10^4 cells (Calu-1) or 1×10^5 cells (H226) was immediately added to the inserts. The plate and inserts were then incubated at 37°C for 48 hours. After 48 hours, non-invading cells were removed by inserting a cotton-tipped swab into the insert and a gentle but firm pressure was applied while moving the tip over the membrane surface. This was repeated with a second swab moistened with medium and the inserts were then rinsed twice with PBS. Invaded cells were fixed with 4 % paraformaldehyde for 10 min, stained with 0.1 % crystal violet (in 20% methanol) for 30 min. Inserts were rinsed in distilled water to remove excess stain and left to dry in normal air at room temperature. Images were taken using a light microscope (4x magnification) fitted with a microscope camera (Olympus). Using predetermined field coordinates, the number of invaded cells was then counted in five microscopic fields. The number of invaded cells was expressed as a percentage of control cells (100%). Experiments were performed in triplicates.

Statistical analysis

Data were expressed as the mean \pm standard deviation (s.d.) of three independent experiments. Student's *t*-test was used in two-group comparisons. *p* values < 0.05 was considered to be statistically significant.

Results

Differential expression of Hsp27 on a panel of lung SCC cell lines

As a basis for further characterization of the roles of Hsp27 on cellular responses in lung SCC cell lines, the basal expression levels of Hsp27 on nine SCC cell lines (H2066, SW900, H2170, Calu-1, H520, SK-MES-1, H1869, ChaG0-k-1 and H226) were first examined using western blot analysis. As shown in Figure 1A, the nine SCC cell lines exhibited varying levels of Hsp27, with the highest expression in H226 cells while the lowest ex-

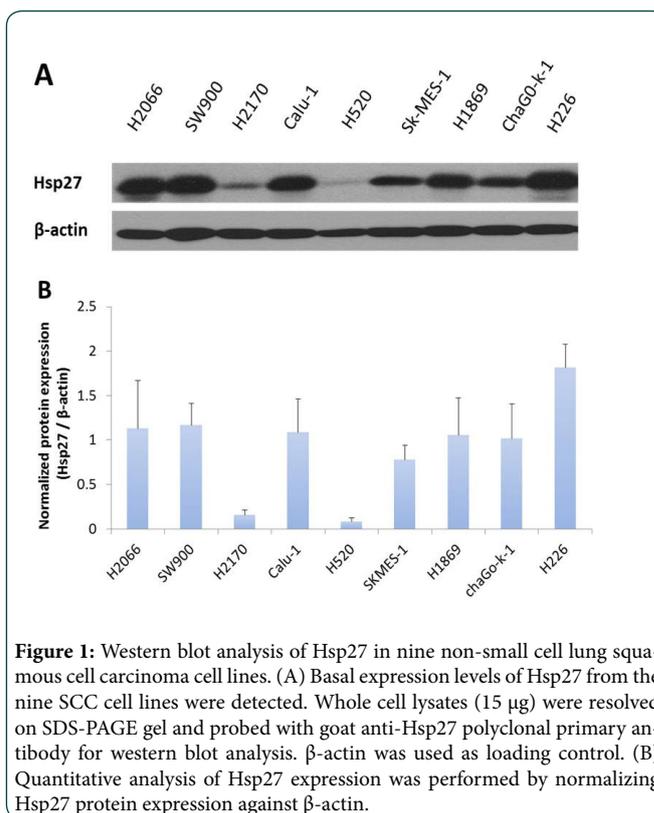


Figure 1: Western blot analysis of Hsp27 in nine non-small cell lung squamous cell carcinoma cell lines. (A) Basal expression levels of Hsp27 from the nine SCC cell lines were detected. Whole cell lysates (15 μg) were resolved on SDS-PAGE gel and probed with goat anti-Hsp27 polyclonal primary antibody for western blot analysis. β -actin was used as loading control. (B) Quantitative analysis of Hsp27 expression was performed by normalizing Hsp27 protein expression against β -actin.

pression in H520 cells, suggesting the differential regulatory mechanisms involved within one particular cancer type. It was noticed that H2066, SW900, Calu-1, SK-MES-1, H1869 and ChaG0-k-1 have a moderately high expression of Hsp27, which is similar in levels across the six cell lines, as shown in Figure 1B, after quantitation by normalizing Hsp27 expression against β -actin.

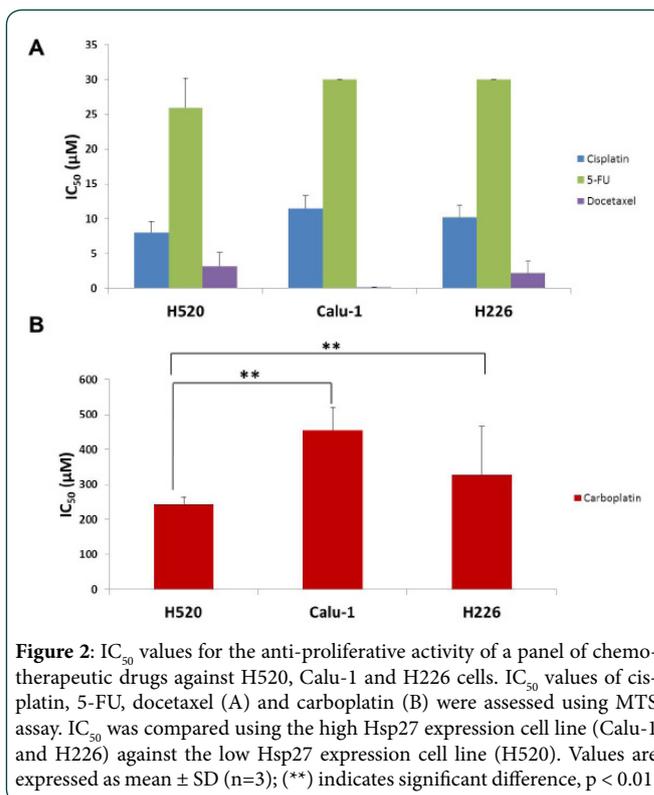


Figure 2: IC_{50} values for the anti-proliferative activity of a panel of chemotherapeutic drugs against H520, Calu-1 and H226 cells. IC_{50} values of cisplatin, 5-FU, docetaxel (A) and carboplatin (B) were assessed using MTS assay. IC_{50} was compared using the high Hsp27 expression cell line (Calu-1 and H226) against the low Hsp27 expression cell line (H520). Values are expressed as mean \pm SD ($n=3$); (**) indicates significant difference, $p < 0.01$.

Hsp27 expression does not affect anti-cancer drug sensitivity in lung SCC cells

To investigate the effects of differential Hsp27 expression on drug sensitivity, Calu-1 and H226 (high Hsp27 expression) and H520 (low Hsp27 expression) were selected to be treated with a panel of four chemotherapeutic drugs, which were cisplatin, 5-fluorouracil (5-FU), docetaxel and carboplatin, and

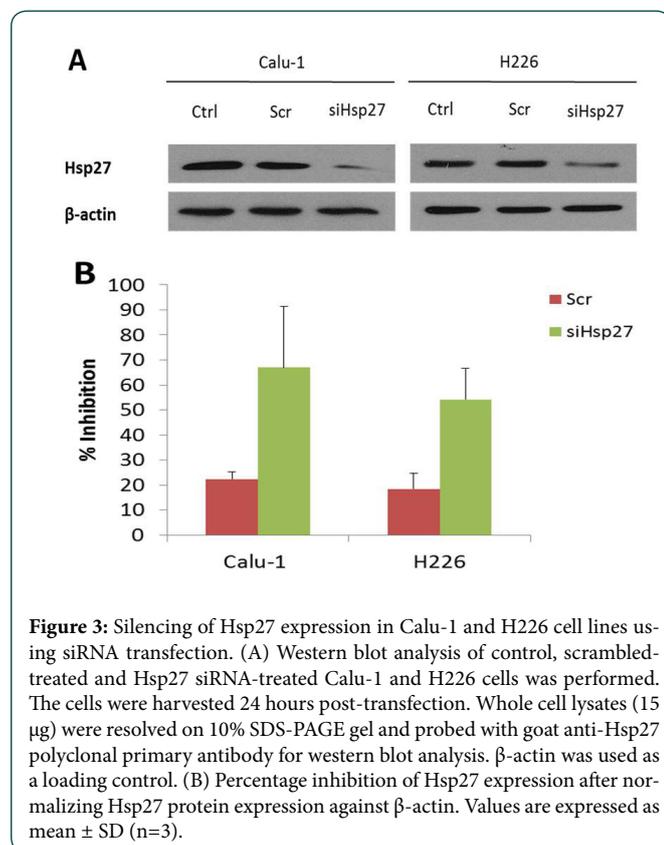


Figure 3: Silencing of Hsp27 expression in Calu-1 and H226 cell lines using siRNA transfection. (A) Western blot analysis of control, scrambled-treated and Hsp27 siRNA-treated Calu-1 and H226 cells was performed. The cells were harvested 24 hours post-transfection. Whole cell lysates (15 μ g) were resolved on 10% SDS-PAGE gel and probed with goat anti-Hsp27 polyclonal primary antibody for western blot analysis. β -actin was used as a loading control. (B) Percentage inhibition of Hsp27 expression after normalizing Hsp27 protein expression against β -actin. Values are expressed as mean \pm SD (n=3).

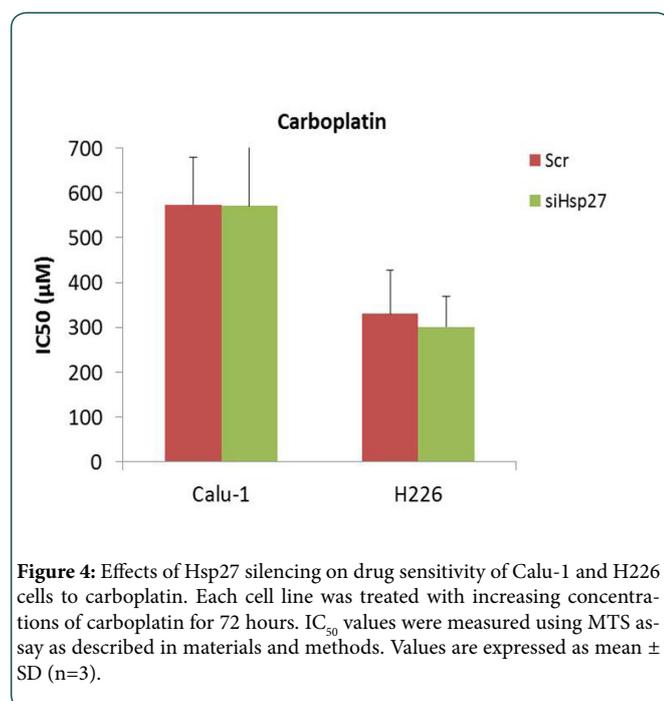


Figure 4: Effects of Hsp27 silencing on drug sensitivity of Calu-1 and H226 cells to carboplatin. Each cell line was treated with increasing concentrations of carboplatin for 72 hours. IC₅₀ values were measured using MTS assay as described in materials and methods. Values are expressed as mean \pm SD (n=3).

assessed by MTS assay. Half-maximal inhibitory concentration (IC₅₀) values are commonly used to evaluate the potency of a compound, in which the lower the IC₅₀ value, the more potent the compound is. According to the results obtained from MTS assay (Figure 2A), it was observed that no significant changes in the IC₅₀ values for Calu-1 and H226 cells as compared to H520 cells following treatment with cisplatin, 5-FU and docetaxel, except for carboplatin. This suggests that the level of Hsp27 expression does not affect the drugs-induced cytotoxicity and cell survival on Calu-1 and H226 cells. However, Calu-1 and H226 cells were more responsive to carboplatin exposure, displaying a significantly higher IC₅₀ value compared to H520 cells receiving the same treatment (Figure 2B). This result thus indicates that the ability of carboplatin to inhibit the proliferation of lung SCC cells may be associated with Hsp27 expression.

No restoration of sensitivity to carboplatin in lung SCC cells after Hsp27 silencing

Considering the fact that Hsp27 may be associated with the ability of carboplatin on inhibition of cell proliferation, silencing of Hsp27 by siRNA transfection on Calu-1 and H226 cells was performed to confirm its role in carboplatin-induced cytotoxicity. Western blot results showed that the expression of Hsp27 decreased significantly for Hsp27 siRNA-treated cells as compared to non-treated (control) and scrambled-treated (negative control) cells, demonstrating the efficacy of the siRNA transfection (Figure 3A). After quantification of the western blot results, there was a 67.0% inhibition and 54.1% inhibition of Hsp27 expression in Hsp27 siRNA-treated Calu-1 and H226 respectively, as compared to the control cells (Figure 3B). Thereafter, scrambled-treated and Hsp27 siRNA-treated Calu-1 and H226 cells were received carboplatin treatment for 72 hours and IC₅₀ values were measured. Quantitative analysis showed that silencing of Hsp27 did not sensitize Calu-1 and H226 cells to carboplatin (Figure 4). Following 72-h carboplatin treatment, the Hsp27 siRNA-treated Calu-1 cells managed to maintain similar drug sensitivity as compared to scrambled-treated Calu-1 cells, with IC₅₀ values of 570 μ M \pm 131 and 573 μ M \pm 105, respectively. Likewise, Hsp27 siRNA-treated and scrambled-treated H226 cells did not show significant difference when treated with carboplatin, with IC₅₀ values of 301 μ M \pm 68 and 330 μ M \pm 97, respectively (Figure 4). Therefore, these results imply that Hsp27 may be partially independent of carboplatin-induced cytotoxicity on lung SCC cells. Taken together, this study demonstrates that the role of Hsp27 expression in modulating chemotherapeutic drug sensitivity is rather minimal.

Suppression of anchorage-dependent and -independent growth of H226 cells by Hsp27 silencing

To examine the effects of Hsp27 silencing on the tumour cell proliferation of Calu-1 and H226 cells, anchorage-dependent and -independent clonogenic assays were conducted. For anchorage-dependent assay, scrambled-treated Calu-1 cells showed rapid proliferation and formed sizable colonies from a single cell (75.1 colonies \pm 5.1). The number of colonies

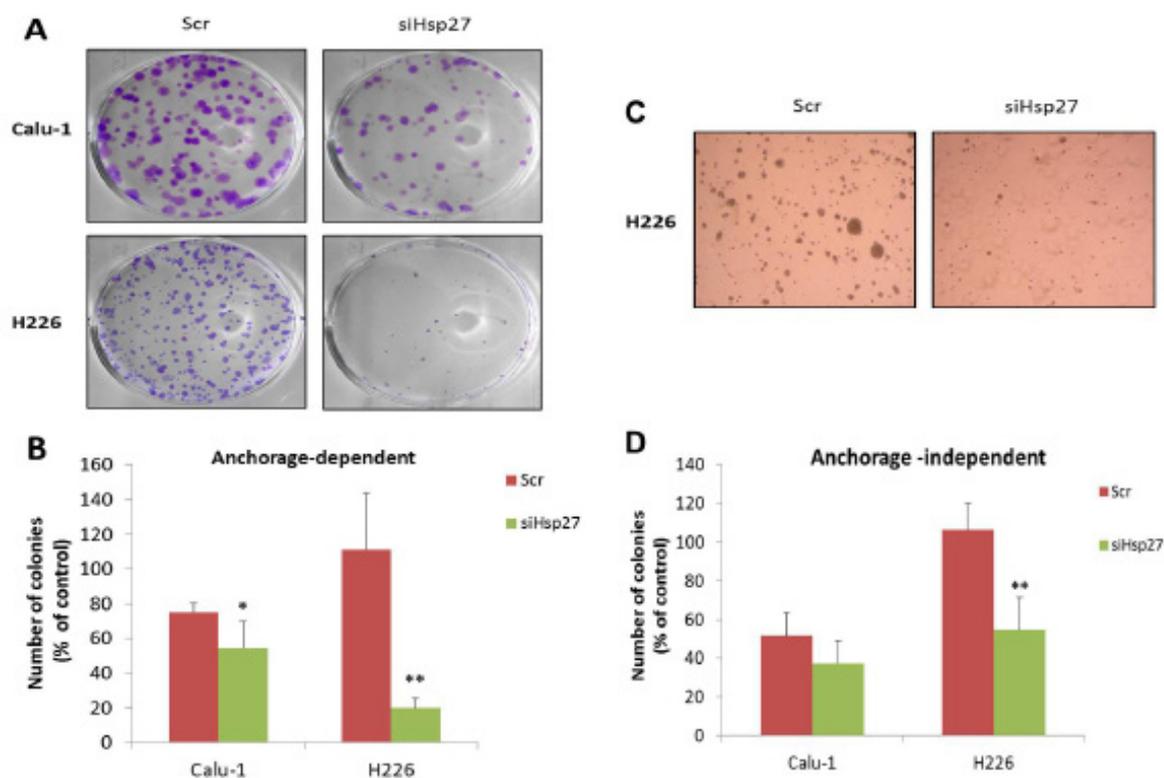


Figure 5: Effects of Hsp27 silencing on anchorage-dependent and -independent growth of Calu-1 and H226 cell lines. Cells were seeded on six-well plate at a density of 500 cells per well and cell colony size formation was determined after 14 days and 21 days for anchorage-dependent and -independent growth assays, respectively. (A) and (C) Representative photographs of crystal violet-stained colonies of scrambled-treated and Hsp27 siRNA-treated Calu-1 and H226 under the light microscope, 4x magnification. (B) and (D) Quantification of colonies formation for scrambled-treated and Hsp27 siRNA-treated Calu-1 and H226 was expressed as percentage of control cells (100%). Values are expressed as mean \pm SD (n=3); (*) indicates significant difference, $p < 0.05$; (**) indicates significant difference, $p < 0.01$.

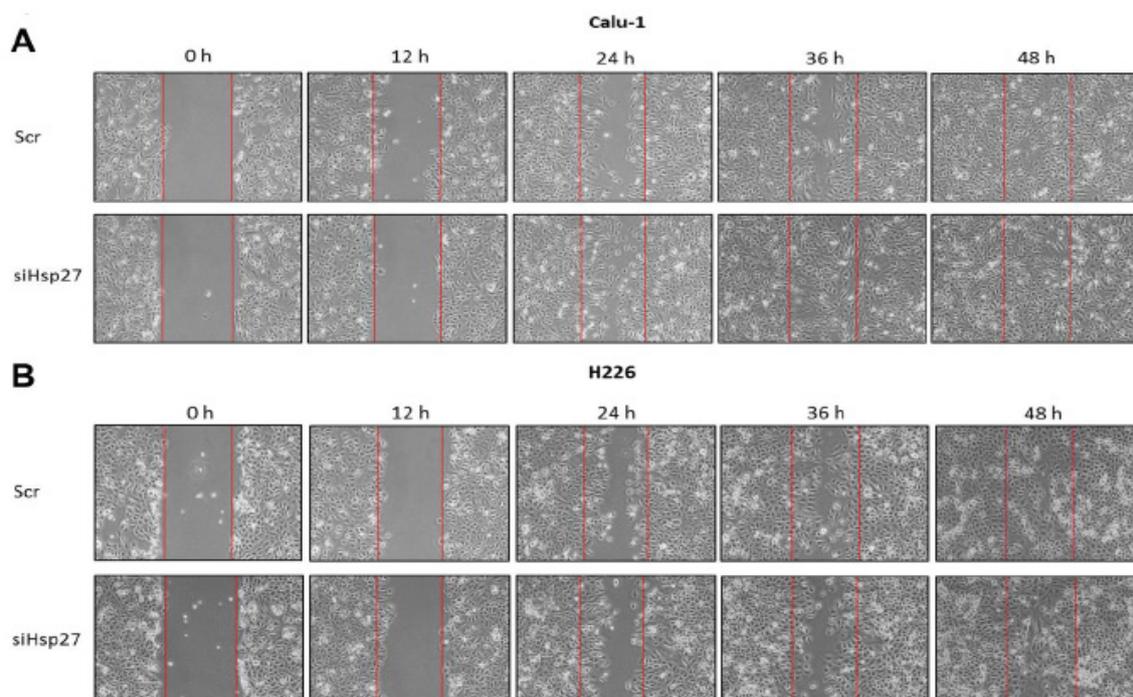


Figure 6: Effects of Hsp27 silencing on migration capability of Calu-1 and H226 cell lines. Representative photographs of wound closure of scrambled-treated and Hsp27 siRNA-treated Calu-1 (A) and H226 cells (B), respectively, at 0 h, 12 h, 24 h, 36 h and 48 h after wound scratch.

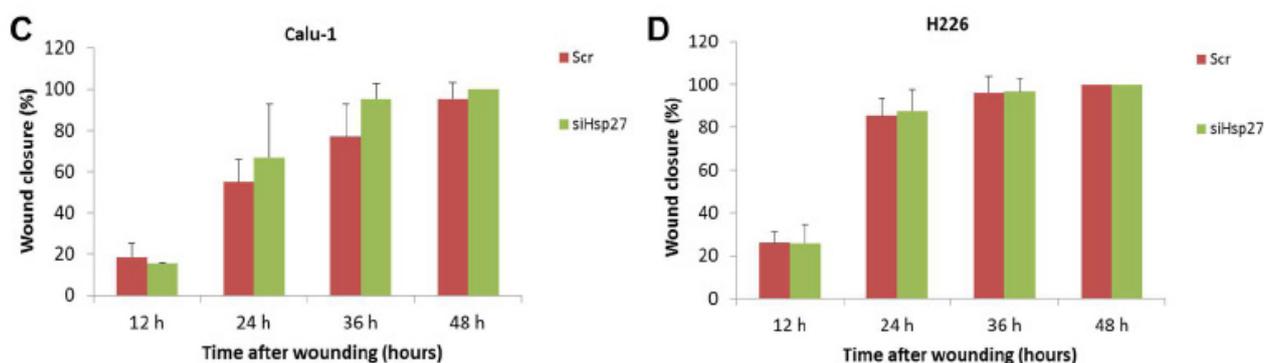


Figure 6: Percentage wound closure of scrambled-treated and Hsp27 siRNA-treated Calu-1 (C) and H226 cells (D) respectively, measured at each time interval with respect to 0 h (0%). Values are expressed as mean \pm SD (n=3).

formed was significantly reduced ($p = 0.02$) by 20.6% in Hsp27 siRNA-treated Calu-1 cells (54.6 colonies \pm 15.2). A greater extent of suppression of colony-forming activity was observed in H226 cells after Hsp27 silencing, with an extremely high reduction rate of 91.5% and smaller colony sizes (19.8 ± 5.4 colonies in Hsp27 siRNA-treated H226 cells vs. 111 ± 32.3 colonies in scrambled-treated cells) (Figure 5A and 5B). These results thus exhibit the ability of Hsp27 to enhance the proliferation of Calu-1 and H226 cells through anchorage-dependent manner, though to varying degrees.

Similarly, analysis of Hsp27 expression on anchorage-independent growth (the ability of cells to form colonies in semi-solid medium) of H226 cells also showed that silencing of Hsp27 remarkably decreased the number of colonies formed in Hsp27-siRNA treated cells (54.8 ± 16.6 colonies) by 51.6% compared to scrambled-treated cells (106.4 ± 13.8 colonies). Interestingly, the effect of Hsp27 silencing on anchorage-independent growth appeared to be less evident in Calu-1 cells than H226 cells. After Hsp27 silencing, the reduction of colonies formation achieved only 14.3%, with 37.6 ± 11.4 colonies in Hsp27 siRNA-treated Calu-1 cells compared to 51.8 ± 11.8 colonies in scrambled-treated cells. Although the decrease of colonies formation did not reach statistical significance, a trend of inhibition of cell proliferation was still observed in Hsp27 siRNA-treated Calu-1 cells. These results indicate that the expression of Hsp27 in Calu-1 and H226 cells play a vital role in promoting anchorage-independent growth on both cell lines.

In addition, cell proliferation MTS assay was also performed to investigate the effects of Hsp27 silencing on cell doubling time. It was detected that cell doubling time for Hsp27 siRNA-treated Calu-1 cells was 8.7 hours longer than scrambled-treated cells, 57.8 ± 21.5 hours and 49.1 ± 2.7 hours, respectively. Similar effects were observed in H226 cells, where cell doubling time for Hsp27 siRNA-treated cells (36.3 ± 7.3) was 4.5 hours longer as compared to negative control siRNA-treated cells (31.7 ± 2.1). Together, these results suggest that silencing of Hsp27 may at least partially affect the proliferation rate of Calu-1 and H226 cells.

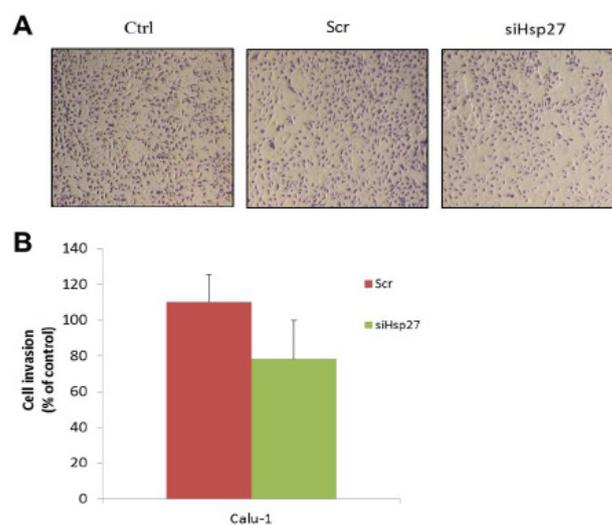


Figure 7: Effects of Hsp27 silencing on invasion capability of Calu-1 cells. The number of invaded cells was counted after 48 hours incubation at 37°C. Representative photographs of invaded cells across Matrigel membrane for (A) scrambled-treated and Hsp27 siRNA-treated Calu-1 under the light microscope, 4x magnification; (B) Number of invaded cells for scrambled-treated and Hsp27 siRNA-treated Calu-1 was expressed as percentage of control cells (100%). Values are expressed as mean \pm SD (n=3).

Hsp27 silencing does not impair migration and invasion capabilities of Calu-1 and H226 cells

Since Hsp27 was found to be involved in affecting the growth of Calu-1 and H226 cells, wound scratch assay was performed to assess the role of Hsp27 in migratory capability of Calu-1 and H226 *in vitro*. As illustrated in Figure 6A and 6C, wound closure for both Hsp27 siRNA-treated and negative control scrambled-treated Calu-1 cells increased in a time-dependent manner after the wound scratch with no significant difference, indicating that silencing of Hsp27 did not effectively inhibit the cell migration in Calu-1 cells even after 48 hours treatment. For instance, the percentage of wound closure at 24 hours, 36 hours and 48 hours after wound scratch was only 12%, 18.2% and 4.5% larger in Hsp27 siRNA-treated cells (24 h: 67.1 ± 25.8 , 36 h: 95.4 ± 7.2 , 48 h: 100) compared to scrambled-treated cells (24 h: 55.1 ± 10.9 , 36 h: 77.2 ± 15.6 , 48 h: 95.5 ± 7.5)

(Figure 6A and 6C). Likewise, no remarkable suppression of wound migration in a time-dependent pattern could be clearly detected in Hsp27 siRNA-treated H226 cells. The percentage of wound closure for Hsp27 siRNA-treated cells (24 h: 87.6 ± 10.2 , 36 h: 96.8 ± 6.3 , 48 h: 100) was similar to scrambled-treated cells (24 h: 85.6 ± 8.2 , 36 h: 96.3 ± 7.6 , 48 h: 100) at each of the time intervals (Figure 6B and 6D).

As invasion is a major step of the metastasis process, the Matrigel invasion assay was performed to assess the role of Hsp27 in invasion capability of Calu-1 and H226 cells. After checking the invasion capability, H226 cells had a relatively weaker invasive capability compared to Calu-1 cells (data not shown). Calu-1 cells were thus used as a model for further invasion experiments. After transfection, the results revealed that there was an insignificant 1.4-fold reduction in the number of invaded cells for Hsp27 siRNA-treated Calu-1 cells (78.3 ± 21.6) compared to scrambled-treated cells (110 ± 15.6) (Figure 7A and 7B), suggesting the minimal role of Hsp27 in promoting invasion of Calu-1 cells.

Discussion

The aims of this study were to determine the roles of Hsp27 in modulating drug sensitivity, cell proliferation, migration and invasion capabilities of lung SCC cells. The present report thus shows that the differential expression of Hsp27 on lung SCC cell lines did not affect the sensitivity to a wide range of chemotherapeutic drugs. Furthermore, Hsp27 silencing caused significant suppression of cell proliferation of Calu-1 and H226 cells in an anchorage-dependent manner. Additionally, its silencing markedly inhibited anchorage-independent growth of H226 cells and to a lesser extent in Calu-1 cells. Finally, it was also identified that the silencing of Hsp27 failed to block the migratory and invasion capabilities of Calu-1 and H226 cells.

The results suggest that differential expression of Hsp27 resulted in no significant difference in the chemotherapeutic drug-induced cytotoxic effects on Calu-1 and H226 cells (both with high expression of Hsp27) compared to H520 cells with low Hsp27 expression following treatment of cisplatin, 5-FU and docetaxel. Although Calu-1 and H226 cells treated with carboplatin demonstrated a marked difference in the IC_{50} values compared to H520 cells, with IC_{50} values of about $243 \mu\text{M}$ and $328 \mu\text{M}$ respectively, the partial suppression of Hsp27 failed to result in sensitization of H226 cells to carboplatin as shown in figure 4, suggesting that the drug resistance of carboplatin in H226 cells may be independent of the overexpression of Hsp27. Altogether, this study presents the evidence that Hsp27 does not involve in altering drug sensitivity to various chemotherapeutic drugs on lung SCC cells. The four drugs (cisplatin, 5-FU, docetaxel and carboplatin) were selected in this study as they are among a list of most common drugs used in the treatment regimen of NSCLC and are considered as standards of care when used in combinational treatment[14]. Briefly, both cisplatin and carboplatin are platinum analogues which cause double stranded and single stranded breaks as well as cross links in DNA. Hence, carboplatin-induced cytotoxicity observed in our study is a result of DNA damage. 5-FU is a pyrimidine antimetabolite, not only inhibiting the enzyme thy-

midine synthase which is essential for DNA synthesis, but also incorporating into RNA to disrupt RNA synthesis. As a plant alkaloid under the class of taxane, docetaxel is also known as microtubule disruptor which can inhibit microtubule disassembly and cause mitotic arrest. These chemotherapeutic drugs function most effectively in proliferating cells, regardless of normal or cancer cells[15]. Previous studies have used these chemotherapeutic drugs to determine the correlation between high Hsp27 expression and drug resistance in different cancer cells. For instance, inhibition of Hsp27 potentiates the pro-apoptotic action of carboplatin in hepatoma HepG2 cells[16]. Furthermore, human ovarian cancer cells treated with Hsp27 antisense gene demonstrated higher sensitivity to cisplatin. This could be due to the ability of Hsp27 to stabilize cytoskeleton[17] since cisplatin is known to disrupt cytoskeleton arrangement[18]. Similarly, murine fibroblast L929 cells with overexpression of Hsp27 displayed a significantly higher resistance to cisplatin, due to the ability of Hsp27 to promote cisplatin-induced Akt activation, thereby enhancing cell survival[19]. These reports show contradictory findings as compared to our results. As various cancers were examined in the different studies, it is likely that the contradictory findings are due to cancer cell-type specificity, in which certain cancers may have higher sensitivity to chemotherapeutic drugs in the presence of Hsp27 expression. Unlike other studies, the present study showed, for the first time, that Hsp27 plays a minimal role in modulating the sensitivity of chemotherapeutic drugs in lung SCC cells. Since our results indicated no correlation between the sensitivity of these chemotherapeutic drugs and the Hsp27 expression, it thus suggests clinical implication to a certain extent as the presence of Hsp27 expression does not affect the response of the standard chemotherapy to patients with lung SCC, thereby maintaining the efficacy of the drugs. In short, the chemotherapy treatment will not be compromised by the expression of Hsp27, thus broadening the treatment options for lung SCC patients.

Although overexpression of Hsp27 may not be responsible for drug resistance, its increased expression is believed to be associated with promotion of cell proliferation of Calu-1 and H226 cells as detected in this study. This postulation can be supported by several studies which proposed the mechanisms of how Hsp27 regulates cell proliferation. For example, Hsp27 can interact with and regulate actin microfilaments which play a significant role during cell division, growth and most importantly, in the process of cytokinesis[20,21]. In addition, it can also promote the translocation of extracellular signal-regulated kinase (ERK) into nucleus, facilitating cell proliferation via MAPK/ERK pathway. Moreover, the ability of Hsp27 to interact with and stabilize Akt can in turn regulate the function of modulatory proteins such as FOXO, GSK3 and eIF4E, which involve in the control of cell proliferation[22]. These studies suggest that Hsp27 activity and its interaction with specific intracellular pathways and growth regulatory proteins could be disrupted by Hsp27 silencing, which can be accounted for the suppression of lung SCC cell growth observed in our study. To further support our results, other studies have demonstrated a positive association between Hsp27 and cancer cell growth. For instance, estrogen-positive breast cancer cells, MCF-7 and

triple-negative breast cancer cells, MDA-MB-231 with over expressed Hsp27 showed a 2-4 fold increase in both anchorage-dependent and -independent growth[23]. It was noticed that the reduction in the number of colony formation in anchorage-independent condition for Calu-1 cells after silencing of Hsp27 did not reach statistical significance, with only 14.3%. To justify this phenomenon, two reasons are proposed. Firstly, our data showed that the siRNA transfection reduced the Hsp27 expression in Calu-1 cells to 37%, which means the transfection did not completely diminish Hsp27 activity. This would indicate that the remaining Hsp27 activity might have supported the growth of Calu-1 cells. Hence, the suppression of Calu-1 anchorage-independent growth may still be dependent on Hsp27 to a certain extent. Secondly, the basal Hsp27 expression detected in Calu-1 cells is relatively lower than H226 cells (see Figure 1), which would mean that the activity of Hsp27 in Calu-1 cells may not as high as that of H226 cells. This may explain the less evident effect of Calu-1 cells to be suppressed in anchorage-independent manner as compared to H226 cells. This therefore implies that higher expression of Hsp27 may be required for the promotion of cell proliferation in lung SCC cells. Nevertheless, there is still a possibility that other players may be responsible for driving the proliferation of lung SCC cells. Further research is required to elucidate the underlying mechanisms of the cell proliferation promotion by Hsp27 in lung SCC cells.

Besides examining the relationship between Hsp27 expression and cell proliferation, our study went further to investigate its role in migration and invasion capabilities of Calu-1 and H226 cells. This is because controlling metastasis remains a major clinical challenge due to the low five-year survival rate for patients with metastatic lung cancer[24]. Moreover, a large proportion of about 70% of patients with lung cancer are present with locally advanced or metastatic disease at the time of diagnosis[25], further emphasizing the need to control metastasis. Our results indicate that Hsp27 silencing did not significantly alter both the migration and invasion capabilities of Calu-1 and H226 cells (Figure 6-7). The observations seen in our study are in contrast with various reports which suggested an involvement of Hsp27 in regulating migration capability of cancer cells. For instance, high Hsp27 expression was demonstrated to result in a delay of cell migration of murine fibroblast NIH-3T3 cells, because of the ability of Hsp27 to modulate regulatory molecules in focal adhesion complexes and thus, increasing cell adhesion by constitutively activating focal adhesion kinase (FAK)[26]. In addition, inhibition of Hsp27 expression via shRNA transfection in MDA-MB-231 cells was showed to significantly reduce cell migration by 70% compared to the control cells, probably due to its ability to stabilize actin filaments during cell migration[27]. Indeed, other study has shown that Hsp27 is able to regulate actin cytoskeletal dynamics and its overexpression enhances actin filament dynamics by promoting F-actin accumulation at the cell cortex with membrane ruffling, pinocytosis, cell motility and buildup of stress fibers[28]. As for invasion capability, decreased cell invasiveness and MMP-9 secretion was revealed in human melanoma cell line, A375 which overexpresses Hsp27[12]. It was also suggested that MMPs can also be regulated by a path-

way upstream of Hsp27, which is p38 MAPK pathway[12]. A competition between Hsp27 and p38-MMPs pathway could result in a decrease of MMP secretion and ultimately cell invasion occurs[12,29]. Besides, Hsp27 could interact with STAT3, a transcription factor of MMP-2, to negatively regulate MMP-2 levels[26]. Despite being shown in other studies extensively, our results present the evidence, for the first time, that Hsp27 may not be the key player in regulating migration and invasion in Calu-1 and H226 cells. This seems likely that lung SCC cells behave differently from other cancer cells in the aspects of migration and invasion due to the different regulation or activation of intracellular transduction signaling pathways. Although Hsp27 is not playing a pivotal role to modulate migration and invasion in lung SCC cells, there is still a possibility that Hsp27 may interact with other unknown proteins to facilitate the process of cell migration and invasion indirectly.

Conclusion

In conclusion, a novel finding from this study was that Hsp27 shows no correlation with the sensitivity of lung SCC cells to various standard chemotherapeutic drugs. This demonstrates clinical benefit to patients with lung SCC who receive the standard chemotherapy as the presence of Hsp27 in lung SCC does not compromise the efficacy of the drugs. More importantly, Hsp27 silencing in H226 cells markedly suppresses their growth by inhibiting their colony forming ability in anchorage-dependent and -independent conditions and thus the cell proliferation, indicating the significant role of Hsp27 in promoting H226 cell growth. In contrast, Hsp27 is likely to play a minor role in the regulation of migration and invasion of Calu-1 and H226 cells. By detailing the different functional roles involved in lung SCC cells, this study suggests the potential applications of Hsp27 as a drug target to suppress the growth of lung SCC cells, paving the way for further research on Hsp27 in the field of anti-cancer drug discovery.

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