

# Down-regulation of Hsp90 could change cell cycle distribution and increase drug sensitivity of tumor cells

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**Subject headings** Hsp90; antisense RNA; cell cycle; gene transfection; drug resistance, multiple

## Abstracts

**AIM** To construct Hsp90 antisense RNA eukaryotic expression vector, transfect it into SGC7901 and SGC7901/VCR of MDR-type human gastric cancer cell lines, HCC7402 of human hepatic cancer and Ec109 of human esophageal cancer cell lines, and to study the cell cycle distribution of the gene transected cells and their response to chemotherapeutic drugs.

**METHODS** A 1.03kb cDNA sequence of Hsp90 $\beta$  was obtained from the primary plasmid pHSP90 by EcoR I and BamH I nuclease digestion and was cloned to the EcoR I and BamH I site of the pcDNA by T4DNA ligase and an antisense orientation of Hsp90 $\beta$  expression vector was constructed. The constructs were transfected with lipofectamine and positive clones were selected with G418. The expression of RNA was determined with dot blotting and RNase protection assay, and the expression of Hsp90 protein determined with western blot. Cell cycle distribution of the transfectants was analyzed with flow cytometry, and the drug sensitivity of the transfectants to Adriamycin (ADR), vincristine (VCR), mitomycin (MMC) and cyclophosphamide (CTX) with MTT and intracellular drug concentration of the transfectants was determined with flow

## cytometry.

**RESULTS** In EcoR I and BamH I restriction analysis, the size and the direction of the cloned sequence of Hsp90 $\beta$  remained what had been designed and the gene constructs were named pcDNA-Hsp90. AH-SGC790, AH-SGC7901/VCR, AH-HCC7402 and AH-Ec109 cell clones all expressed Hsp90 anti-sense RNA. The expression of Hsp90 was down-regulated in AH-SGC7901, AH SGC7901/VCR, AH-HCC7402 and AH-Ec109 cell clones. Cell cycle distribution was changed differently. In AH-SGC7901/VCR and AH-Ec109 cells, G1 phase cells were increased; S phase and G2 phase cells were decreased as compared with their parental cell lines. In AH-SGC7901 cell, G1 phase cells were decreased, G2 phase cells increased and S phase cells were not changed, and in AH-HCC7402 cells G1, S and G2 phase cells remained unchanged as compared with their parental cell lines. The sensitivity of AH SGC7901, AH-SGC7901/VCR, AH-HCC7402 and AH-Ec109 to chemotherapeutic drugs, the sensitivity of AH-SGC7901/VCR to ADR, VCR, MMC and CTX the sensitivity of AH-HCC7402 to ADR and VCR, and the sensitivity of Ec109 to ADR, VCR and CTX all increased as compared with their parental cell lines. The mean fluorescence intensity of ADR in AH-SGC7901, AH-SGC7901/VCR, AH-HCC7402 and AH-Ec109 was also significantly elevated ( $P < 0.05$ ).

**CONCLUSION** Down-regulation of Hsp90 could change cell cycle distribution and increase the drug sensitivity of tumor cells.

## INTRODUCTION

Heat shock proteins (HSPs) are a highly conserved group of intracellular proteins whose synthesis is increased in response to a variety of stressful stimuli<sup>[1]</sup>. HSPs are classified by molecular weight into groups of HSP110, HSP90, HSP70, HSP60, small molecular HSP and ubiquitin. HSP90 is a main chaperone protein in the cell plasma<sup>[2]</sup>. Recent studies have suggested that HSP90

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expression is increased in tumor tissues<sup>[3,4]</sup> and is closely related to multi-drug resistance protein P-gp<sup>[5,6]</sup>. To further investigate the biological roles of HSP90 in the processes of carcinogenesis and the possibility of down regulation of HSP90 to reverse the drug resistance of tumor cells, we have constructed the Hsp90 antisense RNA eukaryotic expression vector, transfected it into SGC7901 of human gastric cancer SGC7901/VCR of multi-drug resistant human gastric cancer, HCC7402 of human hepatic cancer and Ec109 of human esophageal cancer cell lines. We also studied the cell cycle distribution of the gene transected cells and their response to chemotherapeutic drugs.

## MATERIAL AND METHODS

### Materials

**Reagents** EcoR I and BamH I nuclease was purchased from Sino-American Biotech Inc T4 DNA ligase from Promega Company. Other reagents included guanidinium isothiocyanate lysis solution (guanidinium isothiocyanate, citric acid sodium, sarkosyl, ( $\gamma$ -<sup>32</sup>P) ATP, T4 phage polynucleotide kinase, 20 × SSC (NaCl, citric acid sodium), 50 × Denhardt (Ficoll, N-Polyvinylpyrrolidone and BSA), pre-hybridization solution (6 × SSC, 0.5% SDS, 5 × Denhardt, 100 mg/L salmon sperm DNA) TBE (89 mmol/L Tris-boric acid, 2 mmol/L EDTA), 5 × hybridization buffer (200mM-PIPES pH 6.4, 2mM-NaCl, 5mM EDTA), formamide hybridization buffer (formamide: 5 × hybridization buffer = 4:1), RNase S<sub>1</sub>, RNase S<sub>1</sub> buffer (10mM Tris-Cl pH 7.5, 300mM NaCl, 5mM EDTA), 50mg/L proteinase K, 0.5% SDS (w/v), phenol, chloroform, RNA loading buffer (80% formamide, 1mM EDTA pH 8.0, 0.1% bromophenol blue, 0.1% Xylene Cyanol), acrylamide, bisacrylamide, 10% saturated ammonium sulfate, urea, lipofactamine, G418, RPMI1640, goat anti-human HSP90  $\beta$  monoclonal antibody, HRP-labeled donkey anti-goat IgG, Lysis buffer (50 mmol/L Tris • Cl, pH 8.0, 150 mmol/L NaCl, 0.02% NaN<sub>3</sub>, 0.1% SDS, 100  $\mu$ L/mL PMSF, 1 mg/L aprotinin, 1% NP-40, 0.5% deoxycholic acid), TEMED, Tris-glycine working solution (25 mmol/L Tris • Cl, 250 mmol/L glycine, 0.1% SDS), 2 × SDS gel-loading buffer (100 mmol/L Tris • Cl, pH 6.8, 200 mmol/L DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol), electroblotting buffer (39 mmol/L glycine, 48 mmol/L Tris • Cl, 0.037% SDS, 20% methanol), tetrachloride naphenol, propidium iodide, adriamycin (ADR), vincristine (VCR), Mitomycin (MMC) and cyclophosphamide (CTX).

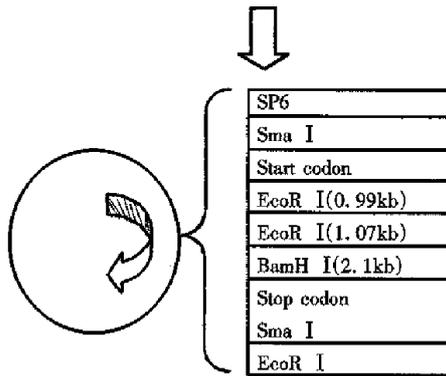
**Cell lines** SGC7901 of human gastric cancer cell line was provided by the Military Medical Scientific Chinese Academy, of Military Medical Sciences, SGC7901/VCR of MDR-type

human gastric cancer cell line was from our institute, HCC7402 of human hepatic cancer cell line from Pathological Department of Fourth Military Medical University (FMMU). Ec109 of human esophageal cancer cell line was from Biochemical Department of FMMU.

### Methods

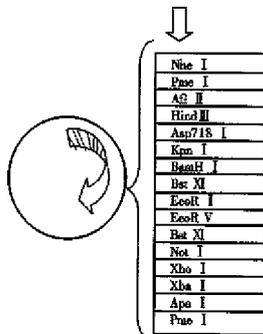
**Construction of HSP90 antisense RNA eukaryotic expression vector** pHSP90 of HSP90 cDNA plasmid was supplied by YAMAMOTO. Eukaryotic expression vector pcDNA 3.1(+) was supplied by Yang Jing-Hua. *E. coli* JM109 was introduced by original source our institute. ① Plasmid map analyses: Analysis of the nuclease site of pHSP90 plasmid map of HSP90 prokaryotic expression vector found that there were two EcoR I nuclease sites and one BamH I nuclease sites in the gene sequence of HSP90 cDNA, of which EcoR I nuclease site is located at 0.99kb and 1.07kb respectively and BamH I nuclease site is located at 2.1kb. There was another EcoR I nuclease site at prime 3 of the pHSP90 plasmid following the stop codon of HSP90 cDNA (Figure 1). There was one EcoR I and one BamH I nuclease site on the multi-clonal nuclease sites of prokaryotic expression vector pcDNA 3.1(+), of which EcoR I is located at prime 3 and BamH I is located at prime 5 (Figure 2). With EcoR I and BamH I nuclease, a 1.03kb fragment of 1.07kb-2.1kb could be digested from HSP90 cDNA (EcoR I was at prime 3, BamH I at prime 5). By inserting the digested fragment (1.03kb) into EcoR I and BamH I nuclease site of pcDNA 3.1(+), the HSP90 antisense RNA eukaryotic expression vector could be constructed.

② The primary plasmid pHSP90 was extracted by alkali lysis. First, pHSP90 was digested by EcoR I and electrophoresed on agarose-gel. Two fragments of 1.43kb and 3.99kb could be seen under ultraviolet from the gel. Of them, the 1.43kb-DNA was excised from the gel and purified by the frozen-melting method. The purified DNA was further digested by BamH I nuclease and electrophoresed, and a 1.3kb fragment (EcoR-I was at prime 3, BamH I was at prime 5) was obtained. The specific fragment (0.5  $\mu$ g) and 0.1  $\mu$ g-pcDNA 3.1(+) linearized by EcoR I and BamH I nuclease was ligated by T4 DNA ligase under 16°C for 18 h (Figure 3). The ligated products were transformed into the sensitized *E. coli* JM109, grew on ampicilline resistant agarose gel. Positive clones were selected and amplified and a small amount of plasmid was extracted. The reconstructed plasmid was digested by either EcoR I or BamH I or by both EcoR I and BamH I nuclease to determine the size and the direction of the fragment.

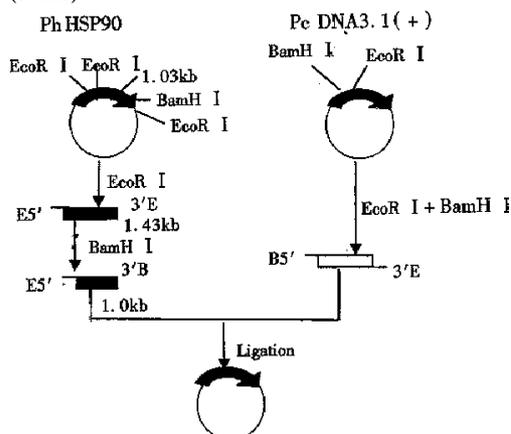


**Figure 1** Plasmid map of phHSP90.

Vector: pSP64(3kb); Inserts: human HSP90 $\beta$  cDNA (2.5kb); Insert site: Sma I of multiclone enzyme restriction site of pSP64; Promoter: SP6; Drug resistance: Amp



**Figure 2** Plasmid map of eukaryotic expression vector pcDNA 3.1 (+). (5.4kb)



**Figure 3** Construction of pcDNA-HSP90 of HSP90 antisense RNA eukaryotic expression vector.

**Gene transfection and clone selection.** The pcDNA-HSP90 of HSP90 anti-sense RNA constructs was extracted by the alkali lysis method, purified by PEG, transfected into SGC7901, SGC7901/VCR, HCC7402 and Ec109 by Lipofectamine and selected by G418. In a six-well culture plate, seed  $1 \times 10^5$  cells per well in 2 mL appropriate complete growth

medium. Incubate the cells at 37°C in a CO<sub>2</sub> incubator until the cells are 80% confluent. After rinsing the cells with serum free and antibiotics-free medium, add pcDNA-HSP90-Lipofectamine mixture (or pcDNA 3.1(+)-Lipofectamine mixture as control). Incubate the cells at 37°C in a CO<sub>2</sub> incubator for 6 hours. Then change the medium with RPMI1640 culture medium containing 10% fetal bovine serum. G418 (400 mg/L) was added to select the resistant clone after 48-72 hours. The medium was changed every 3-4 days and colonies were collected approximately 3 weeks later.

**RNA analyses.** ① Probe designing and synthesizing. Reading 30bp from 1641<sup>st</sup> bp of HSP90 cDNA, the gene sequence is 5' ATT GAC GAG TAC TGT GTG CAG CAG CTC AAG-3'. This gene fragment was matched with the gene bank from the Internet. We found that this sequence could only match with the HSP90 gene, and no homology with any other available genes. So this selected sequence was used as a probe and was synthesized by the Shanghai Biotech Company.  $\beta$ -actin was donated by Dr. Zhao Qing-Chuan and the sequence is  $\beta$ -actin-2095: 5'-ACT ATG TTT GAG ACC TTC AA-3'. ② Oligonucleotide probes labeled by T4 phage polynucleotide kinase. Add pure water to a total volume of 19.5  $\mu$ L in 10pmol/ $\mu$ L Oligonucleotide (1.0  $\mu$ L),  $10 \times$  T4 buffer (2.0  $\mu$ L) and 10pmol [ $\gamma$ -<sup>32</sup>P]ATP (5.0  $\mu$ L). After being mixed, suck 0.5  $\mu$ L-10  $\mu$ L 10 mmol/L Tris-Cl (pH 8.0) to assay the radioactivity ratio. Then 1  $\mu$ L T4 phage polynucleotide kinase was added and mixed and bathed in 37°C for 45 min, then bathed in 68°C for 10min to inactive T4 phage polynucleotide kinase. Afterwards, suck 0.5  $\mu$ L above solution and added to 10  $\mu$ L 10 mmol/L Tris-Cl (pH 8.0) to assay the radioactivity ratio. Precipitate the probes with ethanol and resolubilized to 20  $\mu$ L 1mmol/L EDTA deionized water and stored at -20°C. Radioactivity ratio of the oligonucleotide was assayed by the TCA method<sup>[7]</sup>. ③ RNA isolation. Total RNA was obtained from the cells by the guanidium isothiocyanate-phenol-chloroform extraction method. Cells of  $1 \times 10^6$  were collected and washed with PBS, guanidinium isothiocyanate lysis solution 500  $\mu$ L, 3 mol/L- NaAc 100  $\mu$ L, water saturated phenol and chloroform each 500  $\mu$ L-were added, ice bathed for 20 min and centrifuged at 4°C (12 000r/min) for 15 min. The upper water layer was sucked up to another eppendoff tube, extracted with anequal volume of phenol/chloroform (1/1), precipitated by isopropanol, centrifuged at 4°C (12 000r/min) for 15 min, washed with 70% ethanol once, resuspended to three-evaporated water, treated by

50  $\mu$ L DEPC quantitated and stored at  $-20^{\circ}\text{C}$ . ④ RNA dot blotting. The quantitated RNA was denatured by formaldehyde and was dropped to the nitrocellulose membrane and was dried in  $80^{\circ}\text{C}$  dry oven for 2 h. After pre-hybridization at  $68^{\circ}\text{C}$  water bathing for 2 h, probes were added to the hybridization bag and water bathed for 16 h-24 h at  $68^{\circ}\text{C}$ . The hybridization membrane was picked out and washed with  $2 \times \text{SSC}$  and 0.1% SDS for 20 min at room temperature and with  $0.2 \times \text{SSC}$  and 0.1% SDS at  $68^{\circ}\text{C}$  3 times for 10 min. Then the nitrocellulose membrane was dried by filter paper and was covered by fresh-keeping film. The membrane was exposed to X-ray film in the presence of intensifying screens at  $-20^{\circ}\text{C}$  for 48 h-72 h. The film was then developed to observe the results. ⑤ RNase protection assay<sup>[8]</sup>. RNA (10  $\mu$ L) from the cells were precipitated and resuspended in 30  $\mu$ L formamide hybridization buffer. Radiolabeled Oligonucleotide probes (1  $\mu$ L) and  $\beta$ -actin probes (1  $\mu$ L) diluted to  $1 \times 10^5$  cpm with hybridization buffer were added to the total RNA. The mixture was heated at  $85^{\circ}\text{C}$  for 5 min and the RNAs hybridized at  $45^{\circ}\text{C}$  or  $50^{\circ}\text{C}$  overnight. After hybridization, 2  $\mu$ L RNase S1 buffer, 16  $\mu$ L water and 2  $\mu$ L RNase S1 (2 mg/L) were added and the mixture was incubated at  $37^{\circ}\text{C}$  for 60 min. The solution, which now contained RNase protected fragments, was deproteinized with 50 mg/L proteinase K and 0.5% SDS (w/v) at  $37^{\circ}\text{C}$  for 15 min, extracted with an equal volume of phenol/chloroform (1/1), coprecipitated with 10  $\mu$ g yeast RNA, and resuspended in 8  $\mu$ L loading buffer. The samples were electrophoresed on a 18% acrylamide/6M urea polyacrylamide sequencing gel (200 v, 120 min). Gels were radioautographed at  $-20^{\circ}\text{C}$  for 48 h. Its size was determined according to the standard molecular weight marker.

**Protein analyses** Cells were lysed by lysis buffer and the protein was quantitated. Then SDS-PAGE and immunoblot was performed as usual. Goat anti-HSP90- $\beta$  (1:100) was reacted with the electroblotting for 2 h and HRP-labeled donkey anti-goat IgG (1:500) reacted with the electroblotting for 1 h at room temperature. After washing with PBS (0.01 mol/L pH 7.4), tetrachloride nephrol was used to develop the protein strip and photographed.

**Growth analyses** The pcDNA-HSP90 gene transfected cells and their parental cells were seeded into 24-well plates as pairs at a concentration of  $1 \times 10^4$ /well, 2 mL each well, and cultured in the incubator with a condition of 5%  $\text{CO}_2$ ,  $37^{\circ}\text{C}$ . The cell morphology and growth feature were observed

under microscope. Three wells of each kind of cells were digested by 0.25% trypsin and counted, and its mean value was used to draw the growth curve. Cell doubling time of the exponential phase was calculated by Patterson's formula,  $T_d = T \cdot \lg 2 / \lg (N_1/N_0)$ . Here  $T_d$  is cell doubling time (hour),  $T$  is the time needed for cell growth from  $N_0$  to  $N_1$

**Cell cycle analyses.** Cells growing well were digested by 0.25% trypsin, washed by PBS, fixed by cold ethanol at  $4^{\circ}\text{C}$  and dyed with PI, and then were analyzed by flow cytometry.

**The drug sensitivity analyses.** MTT methods were used. The pcDNA-HSP90 gene transfected cells and their parental cells were seeded into 96well plates as pairs at a concentration of  $5 \times 10^3$ /well, 200  $\mu$ L each well, cultured in the incubator with a condition of 5%  $\text{CO}_2$ ,  $37^{\circ}\text{C}$  overnight. Add adriamycin, vincristine, mitomycin or cyclophosphamide at a concentration of 2  $\mu$ g/ $\mu$ L, 2  $\mu$ g/ $\mu$ L, 2  $\mu$ g/ $\mu$ L and 400  $\mu$ g/ $\mu$ L according to the peak plasma concentration of different antineoplastic drugs, and diluted at a ratio of 1:10 respectively and seed 3 weeks for each concentration. Forty-eight hours later, add tetrazolium blue 20  $\mu$ L to each well at a concentration of 5 mg/L PBS. Culturing was continued for 4 hours and the supernatant was removed. Add 150  $\mu$ L DMSO to react for 15 minutes. ELISA reader was used to measure the OD value of each well at A490 nm and the ratio of living cells. The living cell ratio = A490 of the experimental well/that of control well. Dose-effector curve was drawn and  $\text{IC}_{50}$  and resistant index (RI) were determined.  $\text{RI} = \text{IC}_{50}$  of parental cells/that of the gene transfected cells.

**Intracellular drug concentration analyses** In a 35 mm tissue culture plate, seed  $3 \times 10^5$  cells in to 2 mL complete growth medium. Incubate the cells at  $37^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator until the cells are 70% confluent. Add adriamycin to make its final concentration to 10 mg/L. After culture for 1 hour, cells were digested by 0.25% trypsin, washed by PBS and were analyzed by flow cytometry at 575 nm.

#### Statistical analysis

The growth of pcDNA-HSP90 transfectants and their associated controls were compared using two sample *t* tests. Cell cycle fractions, sensitivity of tumor cells to chemotherapeutic drugs and intracellular drug concentrations of pcDNA-HSP90 transfectants and their associated controls were compared using ANOVA.

## RESULTS

### The constructed HSP90 antisense RNA expression vector

Two gene fragments of 1.43kb and 3.99kb were isolated by agarose-gel electrophoresis after the primary plasmid pHSP90 was digested by EcoR. After the 1.43kb gene fragment was further digested by BamH I, a 1.03kb fragment was obtained, of which BamH I was at its prime 3, EcoR I was at its prime 5. The eukaryotic expression vector pcDNA 3.1(+) was digested by EcoR I and BamH I nuclease and formed linearized plasmid, of which EcoR I was at its prime 3 and BamH I was at its prime 5. With the ligated positive reconstructs digested by EcoR I and BamH I nuclease, we could obtain a 5.4kb vector DNA and 1.03kb inserted fragment. If the ligated positive reconstructs were digested by either EcoR I or BamH I nuclease, we could see a linearized reconstructs (Figure 4). These results indicated that the size and the direction of the inserted HSP90 cDNA fragment was what had been expected. The reconstructed expression vector was named pcDNA-HSP90.

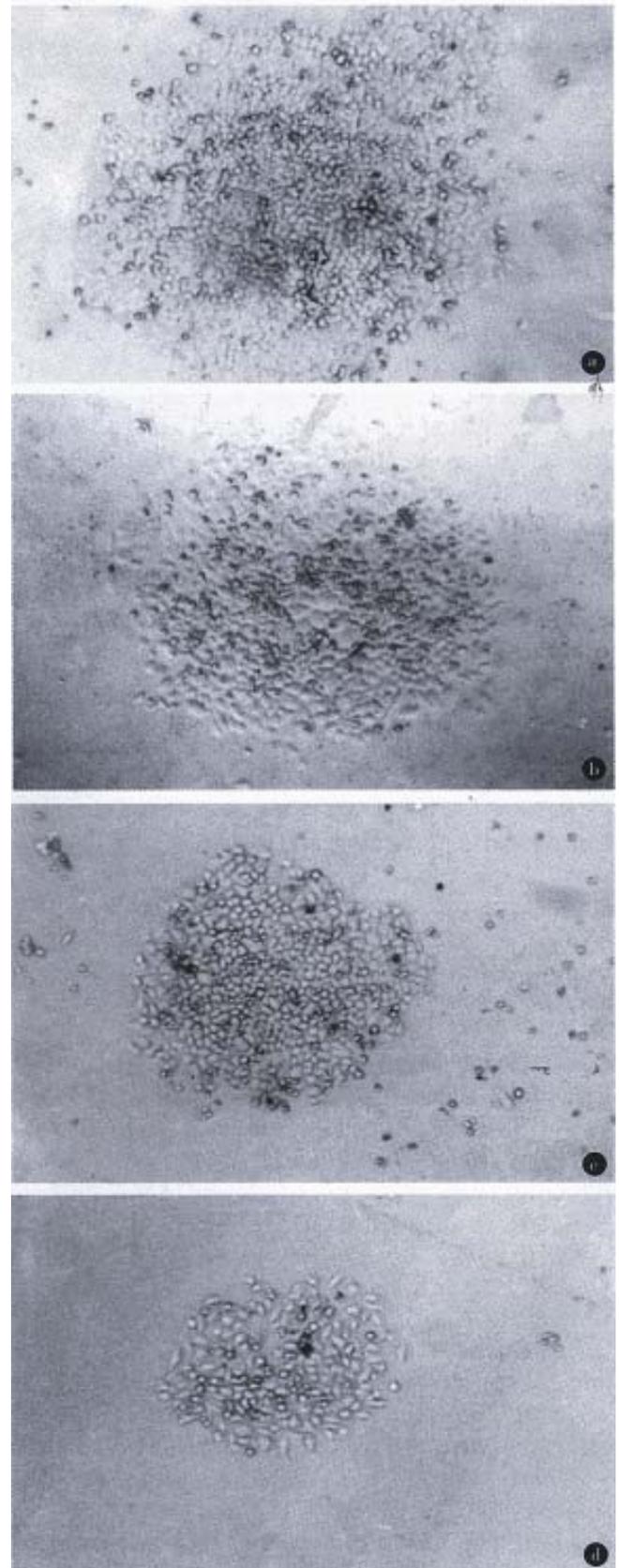


**Figure 4** Nuclease restriction analysis of pcDNA-HSP90 of HSP90 anti-sense RNA eukaryotic expression vector.

1. pHSP90; 2. pHSP90/EcoR I; 3. HSP90/EcoR I-BamH I; 4.  $\lambda$  DNA/EcoR I+Hind III marker (21227, 5148, 4268, 2027, 1375, 125bp); 5. pcDNA 3.1(+); 6. pcDNA 3.1 (+)/EcoR I+BamH I; 7. pcDNA-HSP90; 8. pcDNA-HSP90/EcoR I+BamH; 9. pcDNA-HSP90/EcoR I; 10. pcDNA-HSP90/BamH I

### Gene transfection and clone selection

The positive colonies were selected approximately 3 weeks later. pcDNA-HSP90 of HSP90 anti-sense RNA constructs transfected cell lines were named AH-SGC7901, AH-SGC7901/VCR, AH-HCC7402 and AH-Ec109 respectively (Figure 5). pcDNA3.1 (+) transfected cell lines were named SGC7901-pcDNA, SGC7901/VCR-pcDNA, HCC7402 pcDNA and Ec109-pcDNA.



**Figure 5** SGC7901, SGC7901/VCR, HCC7402 and Ec109 cells transfected with pcDNA-HSP90 of HSP90 antisense RNA constructs, selected by G418, positive clones were obtained and were named a. AH-SGC7901; b. AH-SGC7901/VCR; c. AH-HCC7402; d. AH-Ec109.

### Dot blotting and RNase protection assay

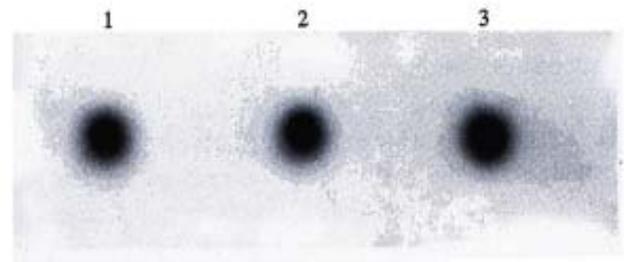
**Dot blotting.** All three groups of pcDNA-HSP90 transfectants, pcDNA3.1(+) transfectants and their parental cells had positive signals of  $\beta$ -actin (Figure 6). Only pcDNA-HSP90 transfected cell group had positive signals of HSP90 anti-sense RNA (Figure 7), indicating that pcDNA-HSP90 transfected cell group had the expression of HSP90 anti-sense RNA.

**RNase protection assay (Figure 8).** HSP90 anti-sense RNA transfectants AH-SGC7901/VCR (1), AH-SGC7901 (4), AH-HCC7402 (7) and AH-Ec109 (10) cells had two positive stripes (Hsp90 anti-sense RNA,  $\beta$ -actin) while pcDNA3.1(+) transfected group and the blank control cell group (parental cell line) had only one positive stripe ( $\beta$ -actin), also indicating that pcDNA-HSP90 transfected cell group had the expression of HSP90 anti-sense RNA and further confirmed that pcDNA-HSP90 had been transfected into the aim cells and had the expression of HSP90 anti-sense RNA.

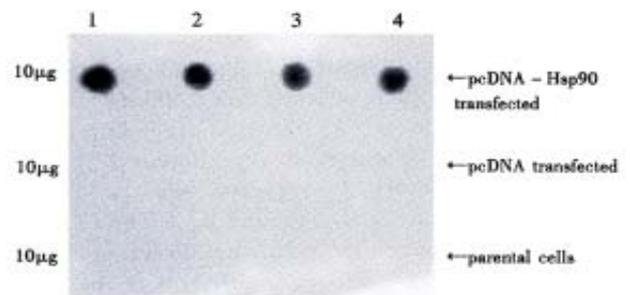
**The expression of Hsp90 protein in the gene transfected cells.** Western blot results showed that the expression of Hsp90 $\beta$  in pcDNA 3.1(+) transfected cells and in their parental cells were almost the same. In Hsp90 antisense RNA transfected cells (AH-SGC7901, AH-SGC7901/VCR, AH-HCC7402 and AH-Ec109), Hsp90 $\beta$  expression was lower than that of their parental cells and pcDNA3.1(+) transfected cells (Figure 9).

**Growth of the gene transfected cell lines.** Compared with their parental cells, the growth of pcDNA-HSP90 gene transfected cells was inhibited in different degrees (Figure 10). The growth inhibiting rate of the 10th day AH-SGC7901 to SGC7901 was 24.28%, of AH-SGC7901/VCR to SGC7901/VCR 27.58%, AH-HCC7402 to HCC7402 10.51% and AH-Ec109 to Ec109 66.91%.

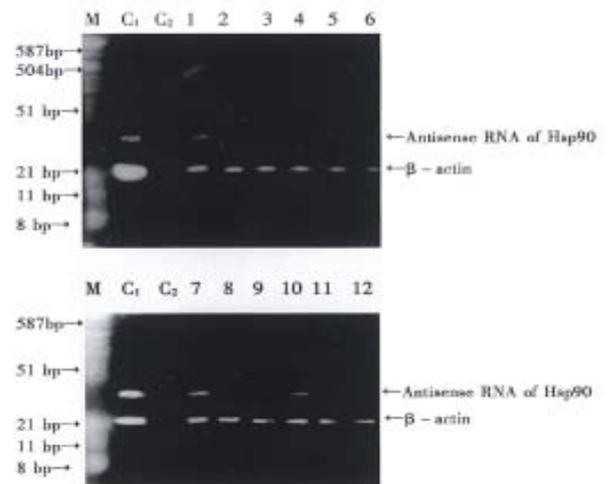
In SGC7901 and AH-SGC7901, the Td calculated from the cell growth curve was 26.6 h and 27.72 h, respectively. The Td of AH-SGC7901 was prolonged 1.12 has compared with its parental cell SGC7901. Td of SGC7901/VCR and AH-SGC7901/VCR was 55.53 h and 72.22 h, respectively. Td of HCC7402 and AH-HCC7402, and Ec109 and AH-Ec109 was 40.66 h and 43.40 h, and 25.03 h and 41.00 h, respectively. These results indicated that the Td of AH-SGC7901/VCR and AH-Ec109 was significantly longer than that of their parental cells ( $P < 0.05$ ), while that of AH-SGC7901 and AH-HCC7402 had no obvious changes as against that of their parental cells ( $P > 0.005$ ).



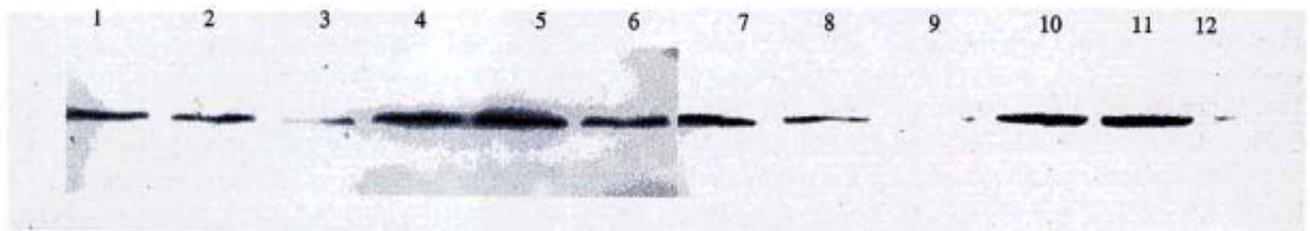
**Figure 6** The expression of  $\beta$ -actin mRNA in AH-SGC7901 of pcDNA-HSP90 transfected cell line, SGC7901-pcDNA of pcDNA 3.1(+) transfected cell line and its parental cell line SGC7901 by Dot blot. 1: SGC7901; 2: SGC7901-pcDNA; 3: AH-SGC7901



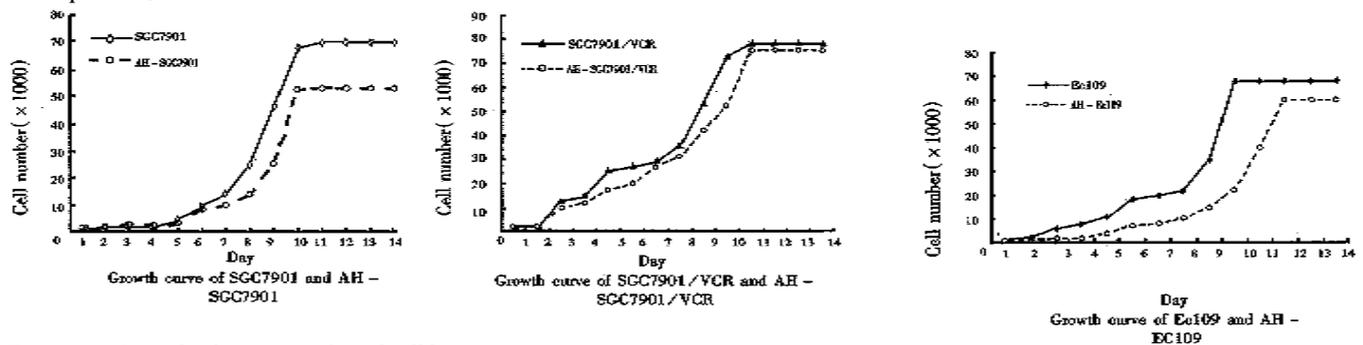
**Figure 7** The expression of HSP90 anti-sense RNA in pcDNA-HSP90 transfected, pcDNA 3.1(+) transfected and the parental cell lines of SGC7901, SGC7901/VCR, HCC7402 and Ec109 by Dot blot. 1: SGC7901; 2: SGC7901/VCR; 3: HCC7402; 4: Ec109



**Figure 8** Expression of hsp90 anti-sense RNA by RNase protection assay. M: PBR322 DNA/Hae III Marker; C1: Positive control,  $\beta$ -actin (20bp) and HSP90 probe (30bp); C2: negative control, probe plus RNase S1, no RNA. 1. AH-SGC7901/VCR; 2. SGC7901-pcDNA/VCR; 3. SGC7901/VCR; 4. AH-SGC7901; 5. SGC7901-pcDNA; 6. SGC7901; 7. AH-HCC7402; 8. HCC7402-pcDNA; 9. HCC7402; 10. AH-Ec109; 11. Ec109-pcDNA; 12. Ec109



**Figure 9** The expression of HSP90 protein in pcDNA-HSP90 transfected cells. 1. SGC7901; 2. SGC7901-pcDNA; 3. AH-SGC7901; 4. SGC7901/VCR; 5. SGC7901/VCR-pcDNA; 6. AH-SGC7901/VCR; 7. HCC7402; 8. HCC7402pcDNA; 9. AH-HCC7402; 10. Ec109; 11. Ec109pcDNA; 12. AHEc109



**Figure 10** Growth of gene transfected cell lines.

**Cell cycle distribution of the transfected cells (Table 1).** We can see from Table 1 that  $G_1$  phase cells of AH-SGC7901 were decreased,  $G_2$  phase cells were increased and S phase had no obvious change as compared with their parental cells, while  $G_1$  phase cells of AH-SGC7901/VCR were increased and S phase and  $G_2$  phase cells were decreased.  $G_1$  phase, S phase and  $G_2$  phase cell of AH-HCC7402 had no obvious changes and  $G_1$  phase cells of AH-Ec109 were increased, S and  $G_2$  phase cells were significantly decreased as compared with their parental cells.

**Table 1** Kinetics of pcDNA-HSP90 transfected cells and their parental cells analyzed with flow cytometry

Cell lines	Cell cycle fraction (%)		
	G0-G1	S	G <sub>2</sub> -M
SGC7901	61.6	28.0	10.4
AH-SGC7901	55.7	28.1	16.2
SGC7901/VCR	62.2	23.5	14.3
AH-SGC7901/VCR	74.7	13.6	11.7
HCC7402	70.7	21.1	8.2
AH-HCC7402	69.5	20.0	10.4
Ec109	56.2	29.5	14.8
AH-Ec109	72.5	19.5	8.0

**Drug sensitivity of the gene transfectants.**  $IC_{50}$  of SGC7901 to ADR, VCR, MMC and CTX was  $1.58 \times 10^{-6}$ ,  $1.99 \times 10^{-3}$ ,  $3.10 \times 10^{-2}$  and 630 mg/L and  $IC_{50}$  of AH-SGC7901 to ADR, VCR, MMC and

CTX was  $1.58 \times 10^{-7}$ ,  $1.99 \times 10^{-7}$ ,  $1.25 \times 10^{-3}$  and 630 mg/L respectively. RI of SGC7901 to ADR, VCR, MMC and CTX was 10, 10 000, 24.8 and 1 compared with AH-SGC7901, i.e., the sensitivity of AH-SGC7901 to ADR, VCR and MMC increased 10, 10 000 and 24.8 times respectively as compared with their parental cell lines, and the sensitivity to CTX had no obvious changes.

$IC_{50}$  of SGC7901/VCR to ADR, VCR, MMC and CTX was 0.398, 1.25, 0.199 and 3981.07 mg/L,  $IC_{50}$  of AH-SGC7901/VCR to ADR, VCR, MMC and CTX was 0.1, 0.199, 0.063 and 794.32 mg/L. RI of SGC7901/VCR to ADR, VCR, MMC and CTX was 3.98, 6.28, 3.15 and 5.01 as compared with AH-SGC7901.

$IC_{50}$  of HCC7402 to ADR, VCR, MMC and CTX was  $1.20 \times 10^{-3}$ ,  $6.33 \times 10^{-4}$ ,  $3.0 \times 10^{-2}$  and 1258.92 mg/L and  $IC_{50}$  of AH-HCC7402 to ADR, VCR, MMC and CTX was  $2.51 \times 10^{-6}$ ,  $6.33 \times 10^{-5}$ ,  $3.00 \times 10^{-2}$  and 1258.92 mg/L. RI of HCC7402 to ADR, VCR, MMC and CTX was 477.7, 10, 1 and 1 or the sensitivity of AH-HCC7402 to ADR and VCR increased 477.7 and 10 times respectively compared with their parental cell lines, and its sensitivity to MMC and CTX had no obvious changes.

$IC_{50}$  of Ec109 to ADR, VCR, MMC and CTX was  $1.0 \times 10^{-1}$ , 44.66, 1.99 and 1258.92 mg/L and  $IC_{50}$  of Ec109 to ADR, VCR, MMC and CTX was  $3.16 \times 10^{-6}$ , 1.99, 1.99 and 398.10 mg/L respectively. RI of Ec109 to ADR, VCR, MMC and CTX was 31 622.77, 22.44, 1.00 and 3.16.

**Intracellular drug concentration of the gene transfectants.** The mean fluorescence intensity of ADR was  $0.286 \pm 0.02$  in SGC7901,  $0.290 \pm 0.026$  in AH-SGC7901 and  $0.279 \pm 0.009$  in SGC 7901 control, which was significantly increased in AH-SGC7901 ( $P < 0.05$ ).

The mean fluorescence intensity of ADR in SGC7901/VCR was  $0.320 \pm 0.050$ ,  $0.323 \pm 0.054$  in AH-SGC7901/VCR which was significantly increased ( $P < 0.05$ ).

The mean fluorescence intensity of ADR was  $0.480 \pm 0.122$  in HCC7402, and significantly increased in AH-HCC7402 ( $0.503 \pm 0.188$ ) ( $P < 0.05$ ).

The mean fluorescence intensity of ADR was  $1.300 \pm 0.361$  in Ec109, and significantly increased in AH-Ec109 ( $1.680 \pm 0.590$ ) ( $P < 0.05$ ).

## DISCUSSION

Antisense RNA is the RNA that is complementary with the mRNA. The antisense RNA could combine with mRNA specifically and inhibit the translation of the RNA. It is one of the gene expression and modulating modes in prokaryotic cells. Antisense RNA also exists in eukaryotic cells but its function is still unknown. Antisense RNA technique is to transfect artificially-composed antisense RNA into the eukaryotic cells, transcript antisense RNA, block the translation of the gene, inhibit the expression of the specific gene and block the function of the gene, and to evaluate the influence of the gene to the cell growth and cell differentiation. In comparison of the ribozyme technique<sup>[8]</sup>, antisense RNA technique has the advantages of simple designing, strong specificity, easy operating, and being economic and time saving. According to the two common nucleases EcoR I and BamH I and their different directions of pHSP90 and pcDNA 3.1(+), the nuclease digested fragment was inserted to pcDNA 3.1(+), and the nuclease restriction analysis showed that the gene clone was successful. This study has laid ground for further understanding the biological roles of HSP90 in tumor cells.

In order to determine whether the HSP90 antisense RNA gene transfected and G418 selected cell clones had the expression of HSP90 antisense RNA, a 30-bp oligonucleotide was synthesized and was labeled with ( $\gamma$ -<sup>32</sup>P) ATP by T4 phage polynucleotide kinase. RNA isolated from the selected clones was first analyzed by dot blotting with the labeled probes. The results showed that the pcDNA-HSP90 transfected cell group had positive signals while the pcDNA 3.1(+) transfected cell group and the blank control cell group had no positive signals, indicating that the pcDNA-HSP90 transfected cell group had the expression of HSP90 antisense RNA.

RNase protection assay<sup>[9,10]</sup> or RNase mapping

is a highly sensitive method for gene expression analysis. This method was primarily used to analyze and quantitate specific RNAs. In the research of gene's characters, it is used to determine the size of the exons. The principle of RNase protection assay is: the labeled oligonucleotide probes were hybridized with cell total RNA or mRNA. The unhybridized single chain RNA was digested by RNase S1. The hybridized double chain was protected and could not be digested by RNase S1. Electrophoresis on acrylamide/urea polyacrylamide sequencing gel, the protected chain could be isolated. Radioautograph could develop the protected chain. Its size could be determined according to the standard-molecular-weight-marker. By this method, 0.1pg globin RNA could be assayed. Its specificity and sensitivity is far greater than Northern blot. In this research, RNase protection assay was also used to determine the expression of HSP90 antisense RNA in the gene transfected and the control cells. The results showed that pcDNA-HSP90 transfected cells had the expression of HSP90 antisense RNA. Analysis of RNA by RNase protection assay is simpler than Northern blot and more specific than Dot blot.

Western blot showed that the expression of HSP90  $\beta$  in AH-SGC7901, AH-SGC7901/VCR, HCC7402 and Ec109 was decreased as compared with their parental cells and their pcDNA transfected groups. This indicated that the antisense RNA of HSP90 has partly blocked the mRNA of HSP90 and inhibited the translation of HSP90 protein.

Both Dot blot and RNase protection assay showed that pcDNA-HSP90 transfected AH-SGC7901, AH-SGC7901/VCR, AH-HCC7402 and AH-Ec109 all had the expression of HSP90 antisense RNA, and with western blot, the expression of HSP90 $\beta$  protein in these cells was decreased when compared with their parental cells and their pcDNA transfected groups. This has established a cell model for further studying the role of HSP90 in these cell lines.

It is known that cell growth is closely related to cell cycle distribution, and cell cycle is regulated by many factors. Oesterreich S<sup>[11]</sup> and Mairesse N<sup>[12]</sup> showed in their studies that hsp27 may be involved in the regulation of cell growth. Fuse *et al*<sup>[13]</sup> had investigated the alternations of cytokinetics and HSP70 expression by hyperthermia in the *in vitro* experimental systems, using two rat glioma cell lines, two human glioblastoma cell lines and rat glioblast cells. They found that HSP70 was increased in all the heat-treated cells. S phase and/or G2/M phase cells were increased in these heat-treated cells. Faassen<sup>[14]</sup> suggested that *in vivo* aging of human T cells resulted in a general defect in the

induction of gene products required for transition from quiescence into the S phase of the cell cycle. This conclusion is supported by observations of diminished inducibility of the lymphokine IL-2 and its receptor during aging. Faassen's study demonstrated that decreased proliferative response to phytohemagglutinin (PHA) was also paralleled by decreased induction during the prereplicative interval of two of the most strongly enhanced proteins in mitogen-activated T cells: HSP90 and P73, which are also members of the heat shock protein family. Diminished induction of HSP90 and p73 was observed in lymphocytes from older subjects (mean age 75 years), regardless of differences in health status of the subject populations.

The above two researches indicated that HSP plays a regulation role in cell growth. Hyperthermic treatment could induce the expression of HSP70, promote the synthesis of DNA and increase the S phase and G<sub>2</sub>/M-phase cell and is beneficial to cell proliferation. In the aged subjects, T cell proliferative response was decreased because of the defects in the induction of HSP90 and P73.

In order to further understand whether decreased expression of HSP90 could influence the tumor growth, we used the cell model we had established and studied their growth. From the cell growth curve it can be seen that the cell growth of the HSP90 antisense RNA transfectants was inhibited in different degrees. Among them, the growth of AH-SGC7901/VCR and AH-Ec109 was significantly decreased as compared with their parental cells, and the cell doubling time prolonged 16.69 h and 15.97 h respectively. The growth of AH-SGC7901 was also inhibited to some degree as compared with its parental cells.

Cell cycle analyses showed that, G<sub>1</sub> phase cells of AH-SGC7901/VCR and AH-Ec109 were increased and S phase cells were decreased as compared with their parental cells, indicating that there was G<sub>1</sub> arrest, causing the growth inhibition of the above two cell groups. G<sub>1</sub> phase cells of AH-SGC7901 were diminished, G<sub>2</sub> phase cells were increased and S phase had no obvious change, indicating that there was G<sub>2</sub>/M arrest, causing slower mitosis, and the decrease of cell mitosis and proliferation. G<sub>1</sub> phase S phase and G<sub>2</sub> phase of AH-HCC7402 all had no obvious changes compared with their parental cells. These results indicate that the expression of HSP90 $\beta$  is related to cell growth. Low expression of HSP90 could cause G<sub>1</sub> arrest and inhibit the synthesis of DNA or cause G<sub>2</sub>/M-arrest and inhibit cell mitosis, therefore inhibiting the cell growth and proliferation. The different inhibition rate in different cell lines may be because of the different heritage background and different blocking rate of HSP90 antisense RNA to HSP90

gene in these gene transfected cells. We concluded that down-regulate the expression of HSP90 $\beta$  could change the cell cycle distribution and inhibit the tumor cell growth.

The development of resistance of tumor cells to anti-cancer drugs is one of the critical issues for successful chemotherapy. Multi-drug resistance is an adaptable reaction of tumor cells to chemotherapeutic drugs. Various kinds of protein expression and/or the changes of enzyme activity could be seen in MDR-type tumor cells. Through the changes of these proteins, tumor cells could avoid being killed by chemical agents. Much evidence suggests that the heat shock proteins (hsp) may be involved in drug resistance<sup>[5,6,11,15-17]</sup>. In our study, the sensitivity of the pcDNA-HSP90 transfected cells to chemotherapeutic drugs increased more significantly than their parental cells. This means that lowered expression of HSP90 could increase sensitivity of the tumor cells to chemotherapeutic drugs. We also found that drug accumulation was increased in the pcDNA-HSP90 transfected cells. HSP90 might be related to the efflux of chemical agents. When HSP90 is decreased, the retention of chemical agents is increased and the intracellular drug concentration is increased, so is the sensitivity of the cells to the anti-cancer drugs. HSP90 is a major cytoplasmic chaperone protein and is related to many protein substrates, e.g. actin, tubulin, protein kinase and steroid receptor<sup>[18,19]</sup>. HSP90 may form hetero-oligomeric complex with some plasmic proteins such as HSP70, p60, Hip/p48, p23, FKBP52, FKBP51 and Cyp-40, involve protein folding, transportation and degradation, and regulate the activity of the protein<sup>[20-25]</sup>.

We thought that HSP90 may regulate the sensitivity of tumor cells to chemotherapeutic drugs through regulating the activity and function of P-gp. Bertram J and co-workers have reported that heat-shock protein hsp90 beta is associated with the P-glycoprotein (P-gp or P170), one of the most prominent components of the drug resistance machinery. They demonstrated that hsp90 beta can be co-precipitated along with P-gp and vice versa. In native agarose gels, both proteins migrated together as one single band as shown by Western blot analysis. This intracellular protein-protein interaction may present a mechanism for the modulation of P-gp function possibly by a stabilization of the protein which seems to be attributed to hsp90 beta. This study supports our research.

Oesterreich S and co-workers<sup>[1]</sup> found that when hsp was induced by elevated temperatures, resistance to doxorubicin (Dox), but not to other commonly used chemotherapeutic agents, was

induced in breast cancer cells. To evaluate the role of hsp27 in this phenomenon, they have transfected MDA-MB-231 breast cancer cells, which normally express low levels of hsp27, with a full-length hsp27 construct. These hsp27-overexpressing cells now display a 3-fold elevated resistance to Dox. They have also derived a MCF-7 breast cancer cell line with amplified endogenous hsp27 which is highly resistant to Dox. When these cells were transfected with an antisense hsp27 construct, they were rendered sensitive to Dox (3-fold). These results suggest that hsp27 specifically confers Dox resistance in human breast cancer cells. Our studies showed that HSP90 was related to MDR, and lowered expression of HSP90 could increase the drug sensitivity of tumor cells by 3-30 000 times indicating that regulation of HSP90 is more effective than hsp27 in this aspect. In different cells with different chemicals, the effect is different, possibly because of the different biological characteristics of the tumor cells and the different mechanism of the anti-cancer drugs.

The mechanisms of HSP90 in sensitizing tumor cells to anti-cancer drugs need to be further studied. This study gives some clues in using HSP inhibitors as a sensitizer to increase the sensitivity of tumor cells to anti-cancer drugs. In conclusion, down-regulation of HSP90 could change the cell cycle distribution and slow the tumor cell growth and increase the sensitivity of tumor cells to anti-cancer drugs.

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