

HSPA9 overexpression inhibits apoptin-induced apoptosis in the HepG2 cell line

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Abstract. Apoptin, a small protein derived from chicken anemia virus, possesses the capacity to specifically kill tumor cells while leaving normal cells intact. Previous studies have indicated that the subcellular localization of apoptin appears to be crucial for this tumor-selective activity. Apoptin resides in the cytoplasm of normal cells; however, in cancer cells it translocates into the nucleus. In the present study, purified prokaryotic native His-apoptin served as a bait for capturing apoptin-associated proteins in both a hepatoma carcinoma cell line (HepG2) and a human fetal liver cell line (L-02). The captured proteins obtained from a pull-down assay were separated by two-dimensional gel electrophoresis. Mass spectrometry was employed to detect the effect of HSPA9 overexpression (one of the interacting proteins with apoptin *in vitro*) and downregulation of HSPA9 on HepG2 cells. The data revealed that HSPA9 overexpression resulted in partial distribution of apoptin in the cytoplasm. Notably, HSPA9 overexpression markedly decreased the apoptosis rate of HepG2 cells from 41.2 to 31.7%, while the downregulation of HSPA9 using small interfering RNA significantly enhanced the apoptosis of HepG2 cells. Our results suggest new insights into the localization mechanism of apoptin which is tightly associated with HSPA9 overexpression and its crucial role in cellular

apoptosis both in a tumor cell line (HepG2) and a normal cell line (L-02). These findings shed new light on the elucidation of the underlying mechanism of anticancer action of apoptin.

Introduction

Apoptin, a small viral protein, was originally identified in the chicken anemia virus (1). Research has shown that apoptin specifically induced apoptosis in a broad range of different human cancer cell lines derived from various cancer types but not in their normal cell counterparts (2). Furthermore, apoptosis induced by apoptin is p53-independent (3,4). Yet, the underlying molecular mechanism of the roles of apoptin in inducing apoptosis in cancer cell lines is largely unknown.

Previous studies have demonstrated that apoptin-induced cancer cell apoptosis is tightly associated with its cancer-specific phosphorylation and subcellular localization (5). In the last decade, several apoptin interacting partner proteins have been identified, such as the N-myc-interacting protein (NMI) (6), promyelocytic leukemia protein (PML) (7), anaphase promoting complex/cyclosome subunit 1 (APC/C) (8), death effector domain associated factor (DEDAF) (9), acid ceramidase (10), importin- β 1 (11) and peptidylprolyl isomerase-like 3 (Ppil3) (12). It has been demonstrated that these proteins play crucial roles in apoptin's selective toxicity in different cancer cell lines. Maddika *et al* (13) reported that apoptin interacts with the SH3 domain of the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K) through its proline-rich sequence (amino acid 81-86) and activates PI3K. Furthermore, PI3K downstream effector kinase Akt1 is also activated by apoptin and interacts with it. Subsequently, apoptin and activated Akt1 are translocated to the nucleus (14). Nuclear Akt1 then activates CDK2 by both direct and indirect mechanisms, and the cyclin A-CDK2 complex directly phosphorylates apoptin at Thr-108 and contributes to the regulation of its subcellular location (15). Identification of these apoptin-interacting proteins extends the knowledge of apoptin cancer-specific toxicity. The search for, the identification of, and the characterization of novel apoptin-interacting partner proteins are important to further elucidate the molecular mechanism underlying the cancer-specific toxic effects of apoptin, thereby extensively identifying the detailed mechanisms of its anticancer action.

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Abbreviations: NMI, N-myc interacting protein; PML, promyelocytic leukemia protein; APC/C, anaphase-promoting complex/cyclosome subunit 1; DEDAF, death effector domain-associated factor; Ppil3, peptidylprolyl isomerase-like 3; PI3K, phosphoinositide 3-kinase; HA, haemagglutinin

Key words: HSPA9, apoptin, interacting proteins, apoptosis, hepatoma carcinoma cell line

HSPA9 (mortalin/mthsp70/Grp75/PBP74/mot-2) is a heat uninducible member of the hsp70 protein family (16). It is a 74-kDa protein and resides in mitochondria, endoplasmic reticulum, the plasma membrane and cytoplasmic vesicles (17,18). The function of HSPA9 is related to proliferation, functional maintenance and stress response in cancer cells (16,19-22). Wadhwa *et al.* (23) found that HSPA9 overexpression may enhance cellular growth or proliferation and prolong the cellular lifespan. Expression study of HSPA9 in cancer cells has revealed that it is commonly upregulated in cancers (23), suggesting that this protein is involved in many cellular processes.

In the present study, prokaryotic native His-apoptin fusion protein purified with Ni-NTA affinity chromatography was used as a bait for capturing apoptin-interacting proteins in both a hepatoma carcinoma cell line (HepG2) and a human fetal liver cell line (L-02) by using a pull-down assay. The captured proteins were separated by two-dimensional gel electrophoresis and identified by mass spectrum. Our data indicated that HSPA9, a major protein captured from HepG2 cells by pull-down assay, was overexpressed in the HepG2 cells. This resulted in partial apoptin retention in the cytoplasm which significantly inhibited the apoptosis rate of HepG2 cells from 41.2 to 31.7% induced by apoptin, while the downregulation of HSPA9 by siRNA interference significantly enhanced the apoptosis of HepG2 cells.

Materials and methods

Plasmid construction. The plasmid pcDNA3-apoptin was previously constructed by our laboratory group and preserved (stored at -20°C) (24). In brief, to construct pcDNA3-HA-apoptin, a sequence derived from influenza haemagglutinin (HA) was inserted into pcDNA3-apoptin (25). The apoptin fragment was excised from pcDNA3-apoptin with *EcoRI* and *XhoI* and then inserted into the *EcoRI* and *XhoI* enzyme cutting sites of the pET-28a (+)-vector (Invitrogen, USA). First-strand cDNA was synthesized from 8 µg of total mRNA (purified from the HepG2 cell line) by using a reverse transcriptase-polymerase chain reaction (RT-PCR) kit (Takara, Shiga, Japan) according to the manufacturer's instructions. The HSPA9 fragment was amplified by PCR, and then inserted into the *BamHI* and *XhoI* enzyme cutting sites of the pcDNA4-vector (Invitrogen). The HSPA9 primers (commercially synthesized by Shanghai Biotech Bioscience and Technology Co., Ltd., Shanghai, China) employed in the PCR reaction were as follows: sense, 5'-TGGATCCATGATAAGTGCCAGCCGAGCTGCAGCAG C-3' and antisense, 5'-CTCGAGCTGTTTTTCCTCCTTTTG ATCTTCC-3'.

Expression and purification of native His-apoptin. *Escherichia coli* (*E. coli*) strain BL21(DE3) transformed with pET28a(+)-apoptin was cultured at 37°C overnight, and the *E. coli* solution was diluted at a volume ratio of 1:50 in 1 liter of Luria-Bertani medium containing kanamycin (50 µg/ml), followed by incubation at 37°C. The absorbance (A)₆₀₀ was adjusted to 0.6-0.8, and 1 mM of isopropyl-β-D-thiogalactopyranoside was added to the culture system. The culture temperature was then changed to 24°C and further cultured for 5 h, followed by centrifugation at 5,000 x g at

4°C for 10 min. Resuspension was carried out in equilibrium buffer (containing 50 mM sodium phosphate, 300 mM NaCl, 1 mM PMSF, pH 8.0). Subsequently, the *E. coli* solution was sonicated and centrifugated at 10,000 x g at 4°C for 30 min and filtered through a 0.22-µm pore-size filter. The imidazole concentration of the lysate was adjusted to 150 mM. The lysate was applied to a Ni-NTA resin (Qiagen) affinity column (2 ml) which was equilibrated with 5-fold column volumes of equilibration buffer (containing 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 150 mM imidazole and 1 mM PMSF) and washed with 10-fold column volume equilibration buffer until the A₂₈₀ was stable. The protein was eluted with 5-fold column volumes of elution buffer (containing 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 500 mM imidazole and 1 mM PMSF). Eluting protein-fractions was screened by SDS-PAGE. Protein fractions containing His-apoptin were collected and dialyzed at 4°C for 20 h in solution containing 50 mM sodium phosphate and 300 mM NaCl, pH 7.5.

In vitro pull-down assay. HepG2 or L-02 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mmol/l L-glutamine in a saturated humidity, in an atmosphere of 5% CO₂ at 37°C. Cells were washed twice with ice-cold PBS and lysed by repetitive freeze-thawing in binding buffer (26) (containing 20 mM HEPES, pH 7.4, 20 mM MgCl₂, 150 mM NaCl, and standard inhibitors of proteases and phosphatases) (3). Then the cell lysates were centrifuged (18,000 x g) at 4°C for 20 min. Purified fusion His-apoptin (500 µg/column) was immobilized on 0.5 ml Ni-NTA resin, and incubated with 4 mg total proteins of HepG2 or L-02 cells at 4°C for 1.5 h, and gently shaken on a rotating platform. The mixed liquor was centrifuged and washed 5 times with the binding buffer to avoid nonspecific binding. Apoptin-interacting proteins were eluted with elution buffer (containing 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 500 mM imidazole and 1 mM PMSF), and dialyzed at 4°C overnight. Interacting proteins underwent freeze drying.

Two-dimensional gel electrophoretic analysis of apoptin-interacting proteins. Two-dimensional gel electrophoresis was performed according to the manufacturer's technical guidelines (GE Healthcare). Briefly, the protein samples were solved by rehydration buffer (containing 8 M urea, 2% CHAPS, 1% DTT, 0.5% IPG buffer and 0.002% bromophenol blue). NL IPG strips, 18 cm in length, pH 4.0-7.0 (GE Healthcare), were applied in first-dimensional isoelectrofocusing and then transferred to 12.5% SDS-PAGE for second-dimensional electrophoresis. The gels were visualized by modified silver staining. The maps were analyzed by ImageMaster 2D Platinum 6.0 software (GE Healthcare). The target protein spots were cut from the gel, and then identified by Mass Spectrometry (Thermo Scientific).

Co-immunoprecipitation and immunoblotting. Transfection was carried out using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. HepG2 cells with 70% confluency were transiently co-transfected with pcDNA3-HA-apoptin and pcDNA4-HSPA9, and the pcDNA3-vector and pcDNA4-vector were set up as controls,

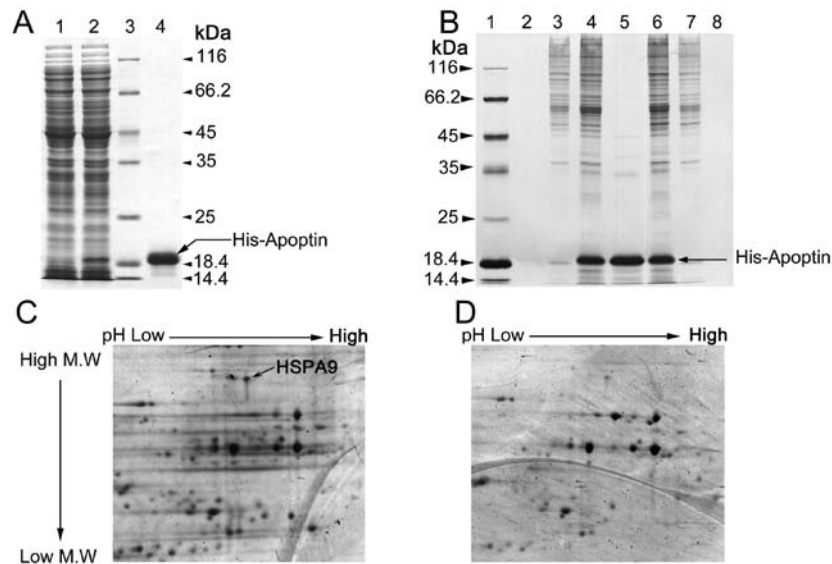


Figure 1. Interaction of HSPA9 and apoptin *in vitro*. (A) Prokaryotic native His-apoptin fusion protein was purified with Ni-NTA affinity chromatography (Coomassie Blue staining). Lane 1, pET-28a(+)-vp3 *E. coli* BL21 (DE3) PlyS total proteins without IPTG induction. Lane 2, pET-28a(+)-vp3 *E. coli* BL21 (DE3) PlyS total proteins with 1 mM IPTG treatment. Lane 3, Protein standard marker. Lane 4, His-apoptin fusion protein purified by Ni-NTA His-Bind[®] resin affinity column. (B) The proteins interacting with apoptin *in vitro* were captured by His pull-down assay using His-apoptin as a bait protein (silver staining). Lane 1, protein standard marker. Lane 2, nonspecific affinity proteins (HepG2) were eluted by elution buffer. Lane 3, HepG2 cellular total proteins and Ni-NTA His-Bind resin nonspecific adhesive protein. Lane 4, interacting proteins with 6His-VP3 in HepG2 cellular total proteins. Lane 5, purified 6His-VP3 proteins. Lane 6, His-apoptin captured interacting proteins in L-02 total proteins. Lane 7, nonspecific adhesive protein with Ni-NTA His-Bind resin in L-02 total proteins. Lane 8, nonspecific proteins eluted by elution buffer using affinity proteins (Ni column solidified 6His-VP3 incubated with L-02 cellular total proteins). (C and D) Two-dimensional electrophoretogram showing the proteins interacting with His-VP3 and 6His-VP3 (modified silver staining). (C) Two-dimensional electrophoretogram-separated apoptin-interacting proteins of His-VP3 captured from HepG2 cells (black arrow indicates HSPA9 in the two-dimensional electrophoretogram). (D) Two-dimensional electrophoretogram-separated apoptin-interacting proteins of 6His-VP3 captured from L-02 cells. M.W., molecular weight.

respectively. HepG2 cells were washed twice with ice-cold PBS 24 h after transfection, and then lysed with ice-cold lysis buffer (containing 50 mM Tris, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM Na₃VO₄, 2 mM EGTA and protease inhibitor cocktail) (14) for 30 min on ice, and then centrifuged (16,000 × g) at 4°C for 20 min. Co-immunoprecipitation was performed using the indicated antibodies, and the immune complex was captured using protein A-agarose beads (Sigma). After washing for 3 times with cell lysis buffer, bead-bound proteins were subjected to immunoblot analysis.

Transient transfection of small interfering RNA (siRNA). siRNA purchased from GenePharma (Shanghai, China) was introduced into HepG2 cells by Lipofectamine 2000. The target sequence was 5'-AAA CTC TAG GAG GTG TCT TTA-3' (27). Briefly, a total of 7 μg siRNA, mixed with 3 μl of Lipofectamine 2000, was added to HepG2 cells in 1.5 ml of DMEM medium, in accordance with the manufacturer's instructions. Transfection medium was replaced with fresh DMEM medium 12 h after incubation. Scrambled siRNA served as the control. Immunoblot assay was conducted to detect the expression levels of HSPA9. The β-actin served as an internal loading control.

Immunofluorescence. Cells cultured on coverslips in 3.5-cm dishes the day before transfection underwent the following preparations prior to immunofluorescence observation. Plasmids or siRNA were used according to Fig. 4. Twenty-four hours after transient transfection, cells were fixed with 4% paraformaldehyde, permeabilized in 0.5% Triton X-100,

blocked in 5% bovine serum albumin (BSA). HA-apoptin was then stained with rat anti-HA monoclonal antibody (1:500) (Tiangen Biotech Co., Ltd., Beijing, China), followed by staining with RBITC-conjugated goat anti-rat IgG secondary antibody (KPL, Gaithersburg, MD, USA). HSPA9 was stained with rabbit anti-HSPA9 monoclonal antibody (1:200) (Cell Signaling Technology, Inc.), followed by staining with FITC-conjugated goat anti-rabbit IgG secondary antibody (KPL). Coverslips were then stained with DAPI, mounted on slides and observed using an Olympus Fv500 confocal microscope (Olympus).

Apoptosis assay. Cells were cultured on coverslips in 3.5-cm dishes the day before transfection. Twenty-four hours after transfection, apoptosis was assessed by cytofluorometric analysis using an Annexin V-fluorescein-5-isothiocyanate (FITC) and propidium iodide (PI) apoptosis detection kit (KeyGen).

Statistical analysis. Experiments were repeated a minimum of three times unless otherwise noted. Experimental data are expressed as the means ± SD. The significance was determined by Student's t-test for comparisons between two groups using SPSS 12.0 software. A level of P<0.05 or 0.01 was considered to indicate a statistically significant result.

Results

***In vitro* pull-down assay identifies HSPA9 as an apoptin-interacting protein.** To identify human cellular proteins that interact with apoptin, we performed a pull-down assay with purified bait protein of the native His-apoptin (Fig. 1A). Apoptin-interacting

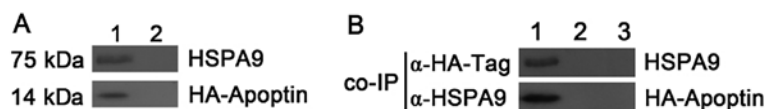


Figure 2. HSPA9 interacts with apoptin in HepG2 cells. HepG2 cells were transiently co-transfected with pcDNA3-HA-apoptin and pcDNA4-HSPA9, and with pcDNA3-vector and pcDNA4-vector as the controls. (A) Immunoblotting showing the co-expression of HSPA9 and HA-apoptin in HepG2 cells. Lane 1, HepG2 cells were co-transfected with pcDNA3-HA-apoptin and pcDNA4-HSPA9. Lane 2, HepG2 cells were co-transfected with the pcDNA3-vector and pcDNA4-vector. (B) Co-immunoprecipitation was carried out to confirm HSPA9 and apoptin interaction. Lane 1, total HepG2 cell extracts immunoprecipitated with the HA-Tag antibody and the immune complexes were analyzed for HSPA9 expression by immunoblotting (the upper strap). Total HepG2 cell extracts immuno-precipitated with the HSPA9 antibody, and the immune complexes were analyzed for HA-apoptin expression by immunoblotting (the lower strap). Lane 2, HepG2 cells were transfected with pcDNA4-HSPA9 or pcDNA3-HA-apoptin respectively. Lane 3, pcDNA3-vector and pcDNA4-vector were transfected into HepG2 cells.

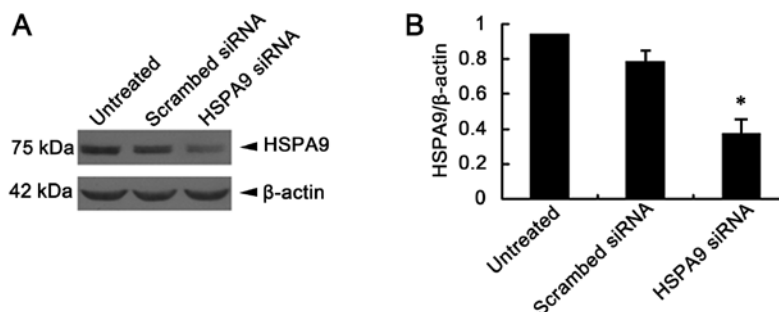


Figure 3. HSPA9 expression is suppressed by siRNA in HepG2 cells. (A) Twenty-four hours after transfection with siRNA, HepG2 cell lysates were immunoblotted to detect the HSPA9 protein level. The expression of HSPA9 in HepG2 cells was markedly downregulated in cells treated with HSPA9 siRNA when compared with that of cells treated with scrambled siRNA or that of the control cells without treatment. (B) The amount of HSPA9 protein was normalized with internal standard β -actin. * $P < 0.05$.

proteins in both hepatoma carcinoma HepG2 cells and human fetal liver L-02 cells were first separated by 12% SDS-PAGE (Fig. 1B). Many protein bands were differentially captured from the HepG2 and L-02 cells. Then two-dimensional gel electrophoresis was conducted for further separation of the captured proteins (Fig. 1C and D). The proteic maps were analyzed by ImageMaster 2D Platinum 6.0. The differential protein spots captured from HepG2 and L-02 cells were excised in gel, and then identified by mass spectrum. HSPA9 was a major interacting protein with apoptin *in vitro* among the identified proteins in the HepG2 cells by mass spectrum, as HSPA9 is much less abundant in the L-02 cells compared with HepG2 cells (23). In our study, we did not detect HSPA9 as an interacting protein with apoptin in L-02 cells *in vitro* (Fig. 1D).

HSPA9 interaction with apoptin in HepG2 cells. To confirm the interaction observed in the pull-down assay, the binding of HSPA9 to apoptin was tested in HepG2 cells by co-immunoprecipitation. HepG2 cells were transiently co-transfected with pcDNA3-HA-VP3 and pcDNA4-HSPA9, respectively. pcDNA3-vector and pcDNA4-vector were transfected into HepG2 cells, respectively, which served as controls in the co-immunoprecipitation test. pcDNA4-HSPA9- or pcDNA3-HA-apoptin-transfected HepG2 cells showed that the overexpression of HSPA9 or HA-apoptin did not adhere to the beads in an unspecific manner. These results suggest that HSPA9 indeed co-precipitated with apoptin (Fig. 2).

siRNA suppresses HSPA9 expression levels in HepG2 cells. Twenty-four hours following transfection with siRNA, HepG2

cell lysates were prepared. The HSPA9 protein expression in HepG2 cells was detected by immunoblotting. Results showed that HSPA9 expression in HepG2 cells was suppressed nearly 60% by siRNA (Fig. 3).

HSPA9 overexpression results in partial cytoplasmic distribution of apoptin. Since HSPA9 and apoptin were found to be interaction partner proteins in HepG2 cells, we performed immunofluorescence to study the effect of HSPA9 overexpression on the subcellular localization of apoptin. Transient co-transfection of plasmid or siRNA is shown in Fig. 4. Results showed that HSPA9 overexpression in HepG2 cells resulted in partial cytoplasmic distribution of apoptin (Fig. 4C).

HSPA9 overexpression in HepG2 cells significantly affects apoptosis induced by apoptin. To investigate the effect of overexpression or suppression of HSPA9 on the apoptosis induced by apoptin in HepG2 cells, HepG2 cells were transiently co-transfected with pcDNA4-VP3 and HSPA9 plasmid or siRNA. Twenty-four hours after transfection, the apoptosis of HepG2 cells was assessed by cytofluorometric analysis. Annexin V and PI-positive cells were considered as apoptotic cells. Our data indicated that overexpression of HSPA9 in HepG2 cells led to a significant reduction in the cell apoptotic rate from 41.2% (control) to 31.7% (co-transfected group) ($P < 0.01$). Notably, the apoptotic rate was increased from 43.6% (co-transfected group) to 52.2% (siRNA group) when HSPA9 was knocked down by siRNA ($P < 0.05$), whereas overexpression or suppression of HSPA9 had no effect on the apoptosis of L-02 cells (Fig. 5C). These results suggest that

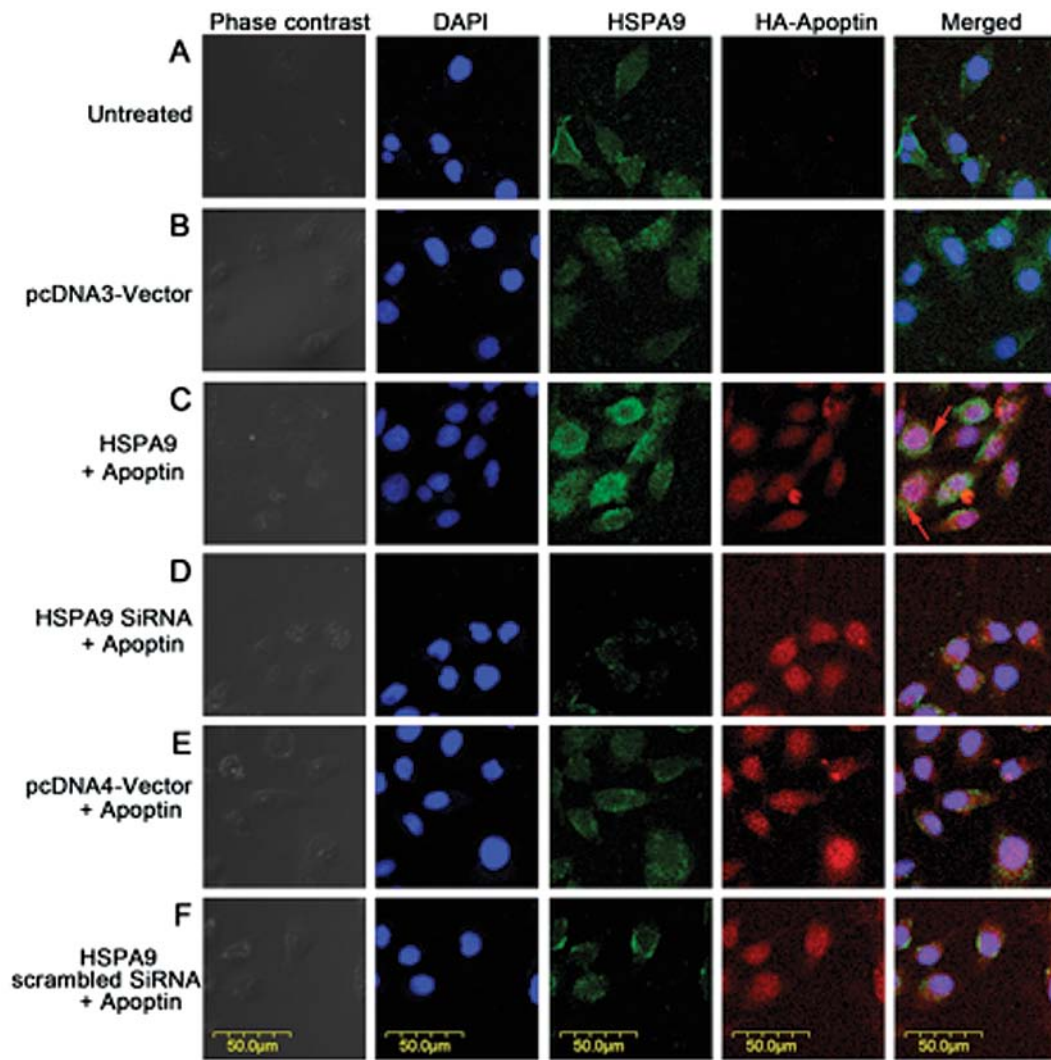


Figure 4. HSPA9 overexpression results in partial cytoplasmic distribution of HA-apoptin. (A) HepG2 cells were untreated as a control. HepG2 cells were transfected with the (B) pcDNA3 vector, (C) pcDNA4-HSPA9 and pcDNA4-VP3 (apoptin) (red arrow indicates that HSPA9 and apoptin were co-localized in the cytoplasm), (D) HSPA9 siRNA and pcDNA4-VP3, (E) pcDNA4 vector and pcDNA4-VP3 (apoptin), or (F) scrambled siRNA and pcDNA4-VP3. DAPI was used for nuclear staining, and HSPA9 was stained with rabbit HSPA9 monoclonal antibody followed by FITC-conjugated goat anti-rabbit IgG secondary antibody. HA-apoptin was stained with rat HA monoclonal antibody, followed by RBITC-conjugated goat anti-rat IgG secondary antibody.

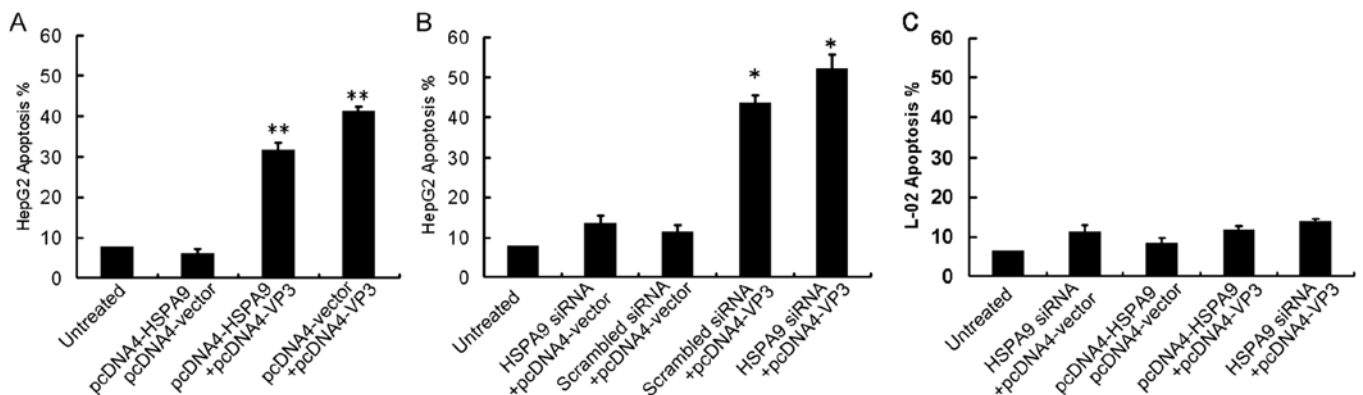


Figure 5. Overexpression or suppression of HSPA9 significantly affects apoptosis induced by apoptin in HepG2 cells, but not in L-02 cells. (A) HepG2 cells were co-transfected with pcDNA4-HSPA9 and pcDNA4 vector, or pcDNA4-HSPA9 and pcDNA4-VP3 or pcDNA4 vector and pcDNA4-VP3, respectively. Apoptosis of control cells and the transfected cells were tested by cytofluorometric analysis. (B) HepG2 cells were co-transfected with HSPA9 siRNA and pcDNA4 vector, or scrambled siRNA and pcDNA4 vector, or scrambled siRNA and pcDNA4-VP3, or HSPA9 siRNA and pcDNA4-VP3, respectively. (C) L-02 cells were transfected with HSPA9 siRNA and pcDNA4 vector, or pcDNA4-HSPA9 and pcDNA4 vector, or pcDNA4-HSPA9 and pcDNA4-VP3 or HSPA9 siRNA and pcDNA4-VP3, respectively. *P<0.05; **P<0.01, compared with the control.

HSPA9 inhibits apoptosis induced by apoptin in tumor cells rather than in normal cells.

Discussion

HSPA9 is a member of the heat shock protein 70 family of chaperones (16) which is localized in mitochondria, in endoplasmic reticulum, on the plasma membrane and in cytoplasmic vesicles (17,18). HSPA9 is a major protein in the mitochondria (28) and it plays a central role in the elaborate protein translocation system responsible for efficient import and export of proteins (29-31). Its role in cell viability and mitochondrial biogenesis was demonstrated by experimental data. A study of the HSPA9 homologue (Ssc1) knocked out from yeast cells revealed that this was lethal to these cells (32), and loss of functional mutations of HSPA9 led to aggregation of yeast mitochondria (33). Previous studies focusing on cancer biology suggest that HSPA9 is a protein which plays a crucial role in anti-apoptosis. It functions by means of counteracting the 'stress' of senescence and apoptosis and as a 'microevolutionary buffer' that neutralizes conformational consequences of mutant proteins. It also aids the acquisition of functions in chaperone-stabilized rogue proteins and promotes cellular invasiveness and motility. Furthermore, HSPA9 plays a pivotal role in the cellular coordination of hyperactivation of proliferation signals and resistance to radiation, heat, hormones and chemotherapeutic agents (34).

To date, the underlying mechanism of the specific toxic effect of apoptin on tumors remains to be elucidated. Maddika *et al* (35) reported that the specific toxic effect of apoptin is independent of death receptors but is related to loss of mitochondrial membrane potential and the release of mitochondrial cell-death mediators via a Nur77-dependent pathway. It has been demonstrated that the nuclear localization of apoptin and the cytoplasmic translocation of Nur77 are the main causes for the toxic effect on cancer cells by apoptin (35). Our results revealed that HSPA9 overexpression led to the partial distribution of apoptin in the cytoplasm. Meanwhile, the apoptin-induced apoptosis rate of HepG2 cells was markedly decreased in response to HSPA9 overexpression. In contrast, when downregulation of HSPA9 expression occurred in HepG2 cells due to siRNA treatment, apoptin's toxicity to HepG2 cells was significantly enhanced in that the apoptosis rate of HepG2 cells was found to be markedly reduced. These findings suggest that HSPA9 may inhibit the cancer-specific toxicity produced by apoptin by means of retaining apoptin's location in the cytoplasm. Thus, we deduced that the overexpression of HSPA9 in HepG2 cells may inhibit the cancer-specific toxicity of apoptin through two ways. On the one hand, the more HSPA9 that was present in the cytoplasm, the more apoptin that was retained in the cytoplasm. At this time, relatively less apoptin was translocated to the nucleus so that less toxicity was produced by apoptin accordingly. On the other hand, HSPA9 overexpression may protect mitochondria from the impairment of pro-apoptosis factor stimulation, thereby contributing to the inhibition of cancer-specific toxicity of apoptin. In contrast, downregulation of HSPA9 levels caused by siRNA treatment attenuated mitochondrial stability (33), which may indirectly enhance the activity of apoptin, therefore leading to the high cancer-

specific toxicity of apoptin in HepG2 cells. Although the overexpression of HSPA9 plays a crucial role in the underlying mechanism in attenuating the anti-apoptosis effect in HepG2 cells, still a high number of HepG2 cells died under the same experimental conditions, suggesting that other factors playing different roles in cancer-specific toxicity also participated in this process and were involved in the relationship between apoptin and HSPA9. However, the precise mechanism still needs extensive investigation.

Taken together, our study showed that HSPA9 is an important apoptin-interacting protein. HSPA9 overexpression prevented apoptin from entering the nucleus to carry out its apoptosis-promoting activity, while downregulation of HSPA9 expression significantly elevated the cancer-specific toxicity of apoptin. Strategies to precisely manipulate the downregulation of HSPA9, so as to strengthen the anticancer effect of apoptin and shed light on novel effective therapeutic strategies for cancer, such as liver cancer are needed and deserve to be intensively studied.

Acknowledgements

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References

1. Noteborn MH, de Boer GF, van Roozelaar DJ, Karreman C, Kranenburg O, Vos JG, Jeurissen SH, Hoebe RC, Zantema A and Koch G: Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle. *J Virol* 65: 3131-3139, 1991.
2. Danen-Van Oorschot AA, Fischer DF, Grimbergen JM, Klein B, Zhuang S, Falkenburg JH, Backendorf C, Quax PH, Van der Eb AJ and Noteborn MH: Apoptin induces apoptosis in human transformed and malignant cells but not in normal cells. *Proc Natl Acad Sci USA* 94: 5843-5847, 1997.
3. Rohn JL, Zhang YH, Aalbers RI, Otto N, Den Hertog J, Henriquez NV, Van De Velde CJ, Kuppen PJ, Mumberg D, Donner P and Noteborn MH: A tumor-specific kinase activity regulates the viral death protein apoptin. *J Biol Chem* 277: 50820-50827, 2004.
4. Teodoro JG, Heilman DW, Parker AE and Green MR: The viral protein apoptin associates with the anaphase-promoting complex to induce G2/M arrest and apoptosis in the absence of p53. *Gene Dev* 18: 1952-1957, 2004.
5. Danen-Van Oorschot AA, Zhang YH, Leliveld SR, Rohn JL, Seelen MC, Bolk MW, Van Zon A, Erkeland SJ, Abrahams JP, Mumberg D and Noteborn MH: Importance of nuclear localization of apoptin for tumor-specific induction of apoptosis. *J Biol Chem* 278: 27729-27736, 2003.
6. Sun GJ, Tong X, Dong Y, Mei ZZ and Sun ZX: Identification of a protein interacting with apoptin from human leucocyte cDNA library by using yeast two-hybrid screening. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao* 34: 369-372, 2002 (In Chinese).
7. Janssen K, Hofmann TG, Jans DA, Hay RT, Schulze-Osthoff K and Fischer U: Apoptin is modified by SUMO conjugation and targeted to promyelocytic leukemia protein nuclear bodies. *Oncogene* 26: 1557-1566, 2007.
8. Teodoro JG, Heilman DW, Parker AE and Green MR: The viral protein apoptin associates with the anaphase-promoting complex to induce G2/M arrest and apoptosis in the absence of p53. *Gene Dev* 18: 1952-1957, 2004.
9. Danen-van Oorschot AA, Voskamp P, Seelen MC, van Miltenburg MH, Bolk MW, Tait SW, Boesen-de Cock JG, Rohn JL, Borst J and Noteborn MH: Human death effector domain-associated factor interacts with the viral apoptosis agonist apoptin and exerts tumor-preferential cell killing. *Cell Death Differ* 11: 564-573, 2004.

10. Liu X, Elojeimy S, El-Zawahry AM, Holman DH, Bielawska A, Bielawski J, Rubinchik S, Guo GW, Dong JY, Keane T, Hannun YA, Tavassoli M and Norris JS: Modulation of ceramide metabolism enhances viral protein apoptin's cytotoxicity in prostate cancer. *Mol Ther* 14: 637-646, 2006.
11. Poon IK, Oro C, Dias MM, Zhang J and Jans DA: Apoptin nuclear accumulation is modulated by a CRM1-recognized nuclear export signal that is active in normal but not in tumor cells. *Cancer Res* 65: 7059-7064, 2005.
12. Huo DH, Yi LN and Yang J: Interaction with Ppil3 leads to the cytoplasmic localization of apoptin in tumor cells. *Biochem Biophys Res Commun* 372: 14-18, 2008.
13. Maddika S, Wiechec E, Ande SR, Poon IK, Fischer U, Wesselborg S, Jans DA, Schulze-Osthoff K and Los M: Interaction with PI3-kinase contributes to the cytotoxic activity of apoptin. *Oncogene* 27: 3060-3065, 2008.
14. Maddika S, Bay GH, Krocak TJ, Ande SR, Maddika S, Wiechec E, Gibson SB and Los M: Akt is transferred to the nucleus of cells treated with apoptin, and it participates in apoptin-induced cell death. *Cell Prolif* 40: 835-848, 2007.
15. Maddika S, Panigrahi S, Wiechec E, Wesselborg S, Fischer U, Schulze-Osthoff K and Los M: Unscheduled Akt-triggered activation of CDK2 as a key effector mechanism of apoptin's anticancer toxicity. *Mol Cell Biol* 29: 1235-1248, 2009.
16. Wadhwa R, Kaul SC, Ikawa Y and Sugimoto Y: Identification of a novel member of mouse hsp70 family. Its association with cellular mortal phenotype. *J Biol Chem* 268: 6615-6621, 1993.
17. Singh B, Soltys BJ, Wu ZC, Patel HV, Freeman KB and Gupta RS: Cloning and some novel characteristics of mitochondrial Hsp70 from Chinese hamster cells. *Exp Cell Res* 234: 205-216, 1997.
18. Ran Q, Wadhwa R, Kawai R, Kaul SC, Sifers RN, Bick RJ, Smith JR and Pereira-Smith OM: Extramitochondrial localization of mortalin/mthsp70/PBP74/GRP75. *Biochem Biophys Res Commun* 275: 174-179, 2000.
19. Craig EA, Kramer J, Shilling J, Werner-Washburne M, Holmes S, Kosc-Smithers J and Nicolet CM: SSC1, an essential member of the yeast HSP70 multigene family, encodes a mitochondrial protein. *Mol Cell Biol* 9: 3000-3008, 1989.
20. Kaul SC, Taira K, Pereira-Smith OM and Wadhwa R: Mortalin: present and prospective. *Exp Gerontol* 37: 1157-1164, 2002.
21. Merrick BA, Walker VR, He C, Patterson RM and Selkirk JK: Induction of novel Grp75 isoforms by 2-deoxyglucose in human and murine fibroblasts. *Cancer Lett* 119: 185-190, 1997.
22. Wadhwa R, Taira K and Kaul SC: Mortalin: a potential candidate for biotechnology and biomedicine. *Histol Histopathol* 17: 1173-1177, 2002.
23. Wadhwa R, Takano S, Kaur K and Deocaris CC, Pereira-Smith OM, Reddel RR and Kaul SC: Upregulation of mortalin/mthsp70/Grp75 contributes to human carcinogenesis. *Int J Cancer* 118: 2973-2980, 2006.
24. Peng DJ, Sun J, Wang YZ, Tian J, Zhang YH, Noteborn MH and Qu S: Inhibition of hepatocarcinoma by systemic delivery of apoptin gene via the hepatic asialoglycoprotein receptor. *Cancer Gene Ther* 14: 66-73, 2006.
25. Drewes G, Ebnet A, Preuss U, Mandelkow EM and Mandelkow E: MARK, a novel family of protein kinases that phosphorylate microtubule-associated proteins and trigger microtubule disruption. *Cell* 89: 297-308, 1997.
26. Rohn JL, Zhang YH, Aalbers RI, Otto N, Den Hertog J, Henriquez NV, Van De Velde CJ, Kuppen PJ, Mumberg D, Donner P and Noteborn MH: A tumor-specific kinase activity regulates the viral death protein apoptin. *J Biol Chem* 277: 50820-50827, 2002.
27. Ohtsuka R, Abe Y, Fujii T, Yamamoto M, Nishimura J, Takayanagi R and Muta K: Mortalin is a novel mediator of erythropoietin signaling. *Eur J Haematol* 79: 114-125, 2007.
28. Bhattacharyya T, Karnezis AN, Murphy SP, Hoang T, Freeman BC, Phillips B and Morimoto RI: Cloning and subcellular localization of human mitochondrial hsp70. *J Biol Chem* 270: 1705-1710, 1995.
29. Koehler CM: New developments in mitochondrial assembly. *Annu Rev Cell Dev Biol* 20: 309-335, 2004.
30. Rehling P, Brandner and Pfanner N: Mitochondrial import and the twin-pore translocase. *Nat Rev Mol Cell Biol* 5: 519-530, 2004.
31. Wiedemann N, Frazier AE and Pfanner N: The protein import machinery of mitochondria. *J Biol Chem* 279: 14473-14476, 2004.
32. Craig EA, Kramer J and Kosc-Smithers J: SSC1, a member of the 70-kDa heat shock protein multigene family of *Saccharomyces cerevisiae*, is essential for growth. *Proc Natl Acad Sci USA* 84: 4156-4160, 1987.
33. Kawai A, Nishikawa S, Hirata A and Endo T: Loss of the mitochondrial Hsp70 functions causes aggregation of mitochondria in yeast cells. *J Cell Sci* 114: 3565-3574, 2001.
34. Kaul SC, Deocaris CC and Wadhwa R: Three faces of mortalin: a housekeeper, guardian and killer. *Exp Gerontol* 42: 263-274, 2007.
35. Maddika S, Booy EP, Johar D, Gibson SB, Ghavami S and Los M: Cancer-specific toxicity of apoptin is independent of death receptors but involves the loss of mitochondrial membrane potential and the release of mitochondrial cell-death mediators by a Nur77-dependent pathway. *J Cell Sci* 118: 4485-4493, 2005.