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Induction of apoptosis by Hax-1 siRNA in melanoma cells

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Abstract

HS1-associated protein X-1 (Hax-1) is a novel intracellular protein and recent studies suggested that it is an anti-apoptotic factor in different tumors. Hax-1 expression was upregulated in various metastatic tumors and cancer cell lines, including melanoma. To understand the role of Hax-1 in melanoma development and progression, we constructed Hax-1 short interfering RNA (siRNA) expression vectors to downregulate Hax-1 expression in a human melanoma A375 cell line. One of the two Hax-1 RNA interference (RNAi) constructs significantly reduced melanoma cell viability, which was due to induction of apoptosis in A375 cells. Molecularly, the induced apoptosis through downregulation of Hax-1 expression was mediated by activation of caspase-3 and poly-ADP-ribose polymerase (PARP) enzymatic activity in A375 cells. The data indicate that Hax-1 plays a role in suppression of apoptosis and promotion of melanoma cell growth, suggesting that this Hax-1 siRNA has a therapeutic indication in control of melanoma.
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Keywords: Hax-1; RNAi; Apoptosis; Melanoma

1. Introduction

Malignant melanoma is a devastating type of cancer because of its high metastatic potential. Its incidence has been increasing on average by 7% a year worldwide. Metastatic disease is the major cause of death among melanoma patients because at the time of diagnosis a large number of them already have metastasis. The long-term survival for patients with metastatic melanoma is only 5% (Brown and Nelson, 1999). Currently, clinical therapy for control of melanoma includes surgery, radiation, and polychemotherapy; however, many metastatic melanomas are typically resistant to radiation and treatments with currently available cytotoxic chemotherapeutic agents have not been effective (Margolin et al., 1998; Tang et al., 1998). Therefore, emphasis is placed on early detection and novel targeted therapeutic regimens are also being investigated. One of them is to induce melanoma cells to undergo apoptosis because some melanomas maintain an anti-apoptotic phenotype. Published studies demonstrated that a number of anti-apoptotic proteins, such as bcl-2, Bcl-xL, and X-linked inhibitor of apoptosis (XIAP), are overexpressed in melanoma and may confer resistance to chemotherapy (Tang et al., 1998; Bush and Li, 2003).

HS1-associated protein X-1 (Hax-1) is a novel intracellular protein, which is localized mainly in mitochondria, but also in endoplasmic reticulum and nuclear envelope in the cells (Suzuki et al., 1997). Several studies have revealed that Hax-1 interacts with viral proteins and plays a role in promoting virus-infected cells survival by inhibition of apoptosis (Dufva et al., 2001; Sharp et al., 2002; Yedavalli et al., 2005). A recent study showed that Hax-1, a substrate of caspase-3, inhibits the apoptotic process by inhibiting caspase-3 activity

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Nomenclature

Hax-1	HS1-associated protein X-1
siRNA	short interfering RNA
RNAi	RNA interference
PAPP	poly-ADP-ribose polymerase
XIAP	X-linked inhibitor of apoptosis
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
PBS	phosphate-buffered saline
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS-T	Tris-buffered saline/Tween
PVDF	polyvinylidene difluoride
MTT	methyl thiazolyl tetrazolium
DMSO	dimethylsulfoxide
ELISA	enzyme linked immunosorbent assay
Q-RT-PCR	Quantitative real-time reverse transcription-polymerase chain reaction
UV	ultraviolet

(Lee et al., 2008). Moreover, in cardiac myocytes and HaCaT cells, Hax-1 could protect them from apoptosis through a caspase-dependent pathway (Mirmohammadsadegh et al., 2003; Han et al., 2006). Other studies have shown that Hax-1 expression was upregulated in different types of tumors, including melanoma (Velculescu et al., 1995; Jiang et al., 2003; Mirmohammadsadegh et al., 2003; Yamaguchi et al., 2005). However, the importance of the Hax-1 gene in melanoma is unknown. Therefore, we have studied the role of Hax-1 in melanoma by using an siRNA technique, which is a promising new tool to evaluate gene functions in cells and has potential applications for therapy (Almeida and Allshire, 2005; Aagaard and Rossi, 2007).

2. Materials and methods

2.1. Cell culture

Human melanoma A375 cells and HaCaT (spontaneously immortalized human epidermal keratinocyte) cells were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Hang Zhou, Sijiqing, China) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The cell culture protocols were in accordance with the use of cell lines of the UKCCC guidelines.

2.2. Western blotting

A375 and HaCaT cells were seeded in 25 cm^2 flask at 1×10^5 cells in DMEM supplemented with 10% FBS and incubated overnight. Western blotting analysis was performed as described previously, with some modifications (Cilenti et al., 2004). Briefly, the cells were washed twice with ice-cold 0.01 M phosphate-buffered saline (PBS, pH 7.4), and lysed using a buffer containing a protease inhibitor cocktail (Roche, Indianapolis, IN, USA). 20 μg of total cellular protein from each of the cell lines were separated on 8–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (BioRad Laboratories, Hercules, CA, USA). The membranes were blocked in 5% non-fat powdered milk/Tris-buffered saline/Tween 20 (TBS-T) overnight at 4 °C. The following day, the membranes were incubated with primary antibody against Hax-1 (BD Biosciences, CA, USA), caspase-3, cleaved poly-ADP-ribose polymerase (PARP) or β-actin (all from Santa Cruz Biotechnologies, Santa Cruz, CA, USA) overnight at 4 °C. The membranes were washed three times with TBS-T and then incubated with secondary antibodies (all from Santa Cruz) for 1 h at 4 °C. The membranes were washed three times with TBS-T for 10 min and then incubated with chemiluminescence solution (Roche) for 1–2 min, and exposed to X-ray film.

2.3. Construction of Hax-1 siRNA expression vectors

The Hax-1 cDNA sequences (NM_006118) were obtained from GeneBank and Hax-1 siRNA sequences were selected by using an siRNA selection program from Whitehead Institute for Biomedical Research (Cambridge, MA, http://jura.wi.mit.edu/bioc/siRNA). The oligonucleotides were synthesized and purified by Shanghai Biological Engineering Company (Shanghai, China). The oligonucleotides were annealed and cloned into the linearized pSUPER/puro vector (oligoengine, Seattle, WA), which contains a polymerase-III H1-RNA gene promoter. The Hax-1 siRNA constructs were amplified and verified by DNA sequencing and were named pSUPER-siH1 and pSUPER-siH2, respectively. The scrambled control plasmid (pSUPER-C) was a circular plasmid encoding an shRNA that did not match any sequence found in the human genome databases. The sequences used to construct the vectors are as follows: Hax-1-siRNA1 (targeting Hax-1 nucleotide positions at 924–942), 5′-GATCCCCTCACCAAGACCTCCAGCCCTTCAAGAGA GGGCTGGAGGTCTTGGTGATTTTTA-3′ and Hax-1-siRNA2 (nucleotide positions at 310–328), 5′-GATC CCCGGTTCCATAGTCCTCAGCATTCAAGAGATGCTGA GGACTATGGAACCTTTTTA-3′. The scrambled sequence used as a control vector was 5′-GATCCCCAGAGCGA-GAGCCTCTATATTTCAAGAGAATATAGAGGCTCTCGCT CTTTTTTA-3′.

2.4. Gene transfection

A375 cells were seeded in 6-well plates at a density of 1×10^5 cells per well in DMEM supplemented with 10% FBS and grown to 80% confluent overnight before gene transfection. pSUPER-siH1, pSUPER-siH2 or pSUPER-C plasmids (2 μg each) were transfected using Lipofectamine 2000 (Invitrogen) in 2 ml serum-free medium per well according to the

manufacturer's instructions. After two days post-transfection, cells were transferred to the selection media, which contained 1 μg/ml puromycin (Sigma Chemicals, St. Louis, MO) in DMEM supplemented with 10% FBS. On day 7, surviving cells were collected to establish the stable cell population.

2.5. Quantitative real-time reverse transcription-polymerase chain reaction (Q-RT-PCR)

RNA isolation and Q-RT-PCR analysis were performed as described previously (Gorreta et al., 2005). Briefly, total RNA from the cells was extracted using Trizol (Invitrogen) and cDNAs were synthesized in 20 μl reaction system (Takara, Dalian, China). PCR was performed in 96-well plates with a BioRad iQ5 and MyiQ™ Real-Time PCR Detection System (BioRad) with an SYBR-GreenMaster Mix (Toyobo, Japan) in 50 μl reaction mixtures. The experiments were repeated 3 times. The PCR cycles were: 95 °C for 5 min; 95 °C for 15 s, 54 °C for 15 s, and 72 °C for 45 s for 40 cycles of PCR reactions. Hax-1 mRNA expression was normalized against β-actin and the expression level was calculated using the ΔΔCT (deltadelta threshold cycle) method. The primer sequences used were 5′-AGACACTTCGGGACTCAATG-3′ and 5′-GCTCCTCCAC-TATCCCATCT-3′ for Hax-1; 5′-CCTGTACGCCAAC ACAGTGC-3′ and 5′-ATACTCCTGCTTGCTGAT-CC-3′ for β-actin.

2.6. Methyl thiazolyl tetrazolium (MTT) assay

Cells were seeded at 3×10^3 per well in 96-well plates and grown in DMEM supplemented with 10% FBS at 37 °C for 96 h. 20 μl MTT (Sigma Chemicals, St. Louis, MO) solution (5 mg/ml) was added to the culture medium at different time points, and incubated at 37 °C in 5% CO_2 in air for an additional 4 h. The reaction was terminated with dimethylsulfoxide (DMSO, 150 μl per well) and the cells were lysed for 10 min. Absorbance values were determined by using the enzyme linked immunosorbent assay (ELISA) reader (DASIT, Milan, Italy) at 490 nm.

2.7. Flow cytometric analysis of cell cycle and apoptosis

Cells were seeded into a 6-well culture plate and transfected with Hax-1 siRNA vectors using Lipofectamine 2000. After two days post-transfection, the cells were washed twice with ice-cold PBS and fixed in 70% ice-cold ethanol for 4 h. Cell pellets were resuspended in RNase A (50 μg/ml) solution for 30 min at room temperature, and stained with propidium iodide solution (20 μg/ml) from Oncogene Company (Merck Chemicals Ltd., Germany) at 4 °C for 1 h in the dark. Cell cycle analysis was performed by using Flow Cytometry (Coulter Biosciences, Hialeah, FL). To detect apoptosis after Hax-1 siRNA transfection, an Annexin V–fluorescein isothiocyanate kit (Oncogene) was used. In brief, identical cell cultures were washed twice with ice-cold PBS and resuspended in a binding buffer. Annexin V–fluorescein isothiocyanate solution and propidium iodide solution were added and incubated in the dark at room temperature for 15 min. The apoptosis rate was analyzed by a Flow Cytometry (Coulter Biosciences).

2.8. Hoechst 33258 staining

A Hoechst 33258 cell apoptosis staining kit (Beyotime, Nanjing, China) was used to confirm morphological changes in the nuclei. The transfected cells were seeded onto sterile glass coverslips placed in 6-well plates and incubated for 48 h. The cells were fixed, washed twice with PBS and stained with Hoechst 33258 staining solution for 5 min at room temperature. The slides were evaluated and images were captured using a Nikon 80i fluorescence microscope equipped with ACT-2U Imaging Software (Nikon, Japan). Apoptotic cells were defined by the condensation of nuclear chromatin or fragmentation to the nuclear membrane.

2.9. Caspase-3 activity assay

Caspase-3 enzymatic activity was determined using a caspase activity kit (Clontech, Mountain View, CA) according to the manufacturer's protocol. Briefly, cell lysates were prepared and caspase-3 substrate (Ac-DVED-pNA) was added to cell lysates. The mixtures were incubated at 37 °C for 1 h and measured with an ELISA reader at an absorbance of 405 nm for caspase-3 activity. For control experiments, the lysates were incubated with the caspase-3 inhibitor DEVD-fmk (Clontech) for 30 min before addition of the substrate. We also evaluated the caspase-3 activity of Hax-1-transfected cells under ultraviolet (UV) stimuli. Cells were exposed to UV radiation (2.4 J/cm^2 for 5 min) as described previously (Li et al., 2004) and incubated for 12 h in fresh DMEM supplemented with 10% FBS. The caspase-3 activity was then detected.

2.10. Statistical analysis

All numeric data were presented as mean ± standard deviation. Statistical analysis was performed by using Student's two-tailed *t*-test with SPSS statistical package, version 10 (SPSS Inc., Chicago, IL). A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Downregulation of Hax-1 expression by Hax-1 siRNA

We first assessed Hax-1 expression in the melanoma cell line A375 by using western blotting analysis. Compared to positive control (Mirmohammadsadegh et al., 2003), A375 cells expressed very high levels of Hax-1 protein (Fig. 1). We constructed two Hax-1 siRNA expression vectors. After transfection of these vectors into A375 cells, Q-RT-PCR and Western blotting analyses showed that one of these Hax-1 siRNA vectors successfully downregulated Hax-1 expression in A375 cells (Fig. 2). However, another Hax-1 siRNA,

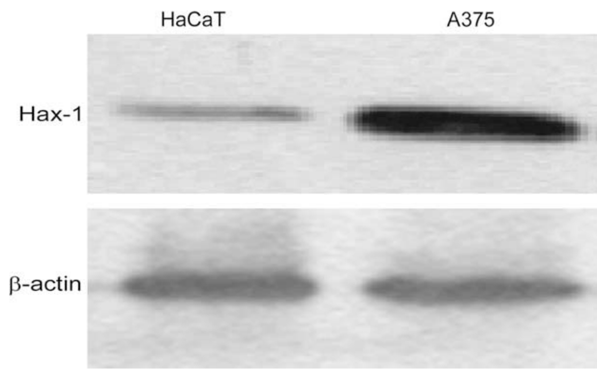

Fig. 1. Western blotting analysis of Hax-1 expression in A375 cells. Both HaCaT (a positive control) and A375 cells were grown in monolayer and total cellular protein was extracted and subjected to Western blotting analysis.

namely pSUPER-siH2 and scrambled control vector had no effects on suppression of Hax-1 expression (Fig. 2).

3.2. Reduced cell viability after downregulation of Hax-1 expression due to induction of apoptosis

To explore the role of Hax-1 in melanoma, we first determined cell viability after Hax-1 siRNA transfection by using MTT assay. The viability of A375 cells after pSUPER-siH1 transfection was significantly reduced (Fig. 3). To determine the causes of the reduced cell viability after downregulation of Hax-1 expression, a flow cytometry analysis of cell cycle distribution was carried out. Downregulation of Hax-1 expression had a minimal effect on cycle distributions, including G1, S and G2/M phases in A375 cells (data not shown). On the other hand, after pSUPER-siH1 transfection the population of sub-G1 phase increased (a hypodiploid DNA peak), suggesting that downregulation of Hax-1 induced apoptosis in A375 cells. Thereafter, we measured the apoptosis levels in the cells. The pSUPER-siH1-transfected A375 cells had an increased apoptosis rate of 83.6%, as compared with the cells transfected with pSUPER-C and the parental A375 cells (Fig. 4). Furthermore, apoptosis induced by the downregulation of Hax-1 in A375 cells following

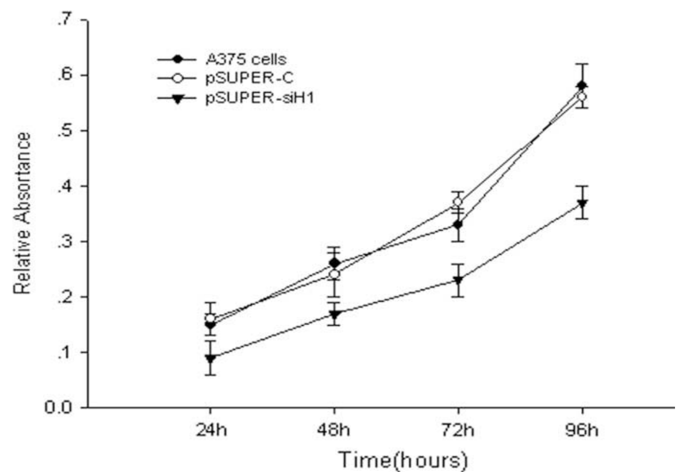

Fig. 3. MTT assay. Cell viability was detected using MTT assay after Hax-1 siRNA transfection (see details in Materials and methods section). Data are shown as mean ± SD from 3 independent experiments.

transfection with pSUPER-siH1 showed apoptotic morphology features (using light microscopy), such as cell shrinkage, chromatin compaction, condensation of cytoplasm, and detachment of the cellular monolayer. Hoechst 33258 staining showed that there were significant morphological changes in nuclear chromatin. In the control cultures, the nuclei were stained a less bright blue and the color was homogeneous. However, condensed chromatin could be observed in cells transfected with pSUPER-siH1 and some formed apoptotic bodies (Fig. 5).

3.3. Induced apoptosis after downregulation of Hax-1 expression via activation of caspase-3 and PARP activity

Both caspase-3 and PARP upregulation occur molecularly at the end stages of apoptosis cascades, which are excellent markers for apoptosis. We detected their activation after Hax-1 siRNA transfection in A375 cells. Western blotting data clearly showed that pSUPER-siH1 transfectants increased the active forms of caspase-3 and PARP expression compared to the

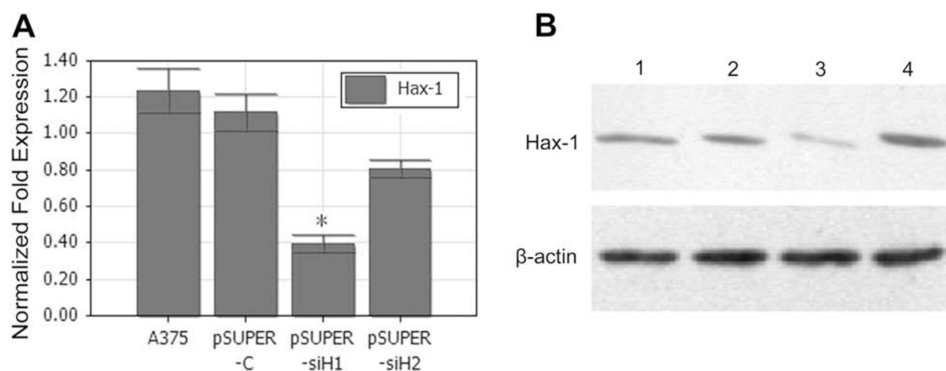

Fig. 2. Downregulation of Hax-1 expression using Hax-1 siRNA. Two Hax-1 siRNAs were constructed and transfected into A375 cells. Quantitative real-time PCR (A) and Western blotting (B) analysis were used to detect Hax-1 mRNA and protein expression, respectively. (A) Each bar represents mean ± SD of 3 independent experiments. *$P < 0.05$. β-Actin was used as a control. (B) Western blotting detection of protein expression. Lane 1, parental A375 cells; lane 2, pSUPER-C; lane 3, pSUPER-siH1; lane 4, pSUPER-siH2.

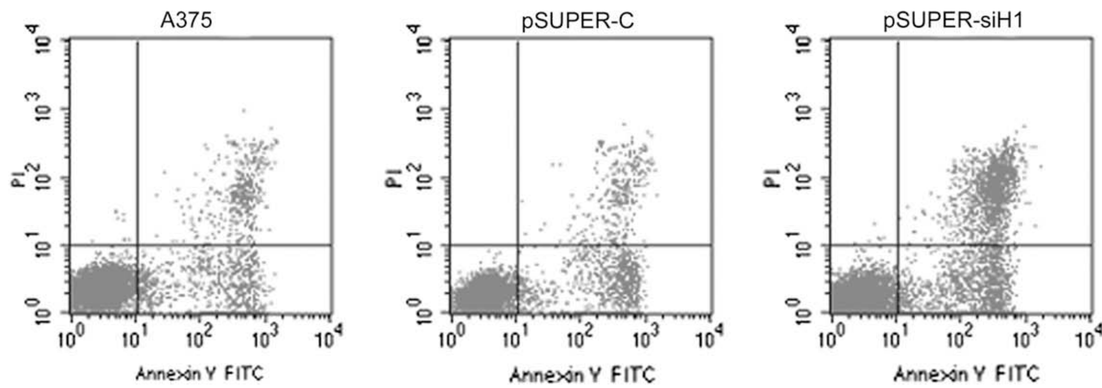

Fig. 4. Flow cytometric assay. Apoptosis rate was detected using Annexin V/FITC and propidium iodide (PI) staining followed by flow cytometric analysis. The cell were grown and transfected with Hax-1 siRNA or control siRNA and the cells were subjected to Annexin V and PI staining (see details in method section) and flow cytometric analysis.

controls (Fig. 6A). The caspase-3 enzymatic activity was also measured (Fig. 6B); caspase-3 enzymatic activity in the pSUPER-siH1-transfected cells increased >2-fold as compared to control cells and the increased caspase-3 activity was blocked by the inhibitor DEVD-fmk. Moreover, UV radiation (2.4 J/cm^2 for 5 min) caused 5.0 and 4.9-fold induction of caspase-3 enzymatic activity in pSUPER-C-transfected cells and parental A375 cells, but UV enhanced Hax-1 downregulation even more (5.9-fold induction, see Fig. 6B).

4. Discussion

Hax-1 siRNA expression vector was used to downregulate Hax-1 expression in melanoma cells. One of the Hax-1 siRNA constructs significantly reduced the melanoma cell viability and induced them to undergo apoptosis. Molecularly, activation of caspase-3 and PARP enzymatic activity were responsible for apoptosis in the A375 cells. The data suggest that Hax-1 plays a role in suppression of apoptosis and promotion of melanoma cell growth, which indicates that the Hax-1 siRNA may have a therapeutic clinical indication, but further study is warranted to determine whether this Hax-1 siRNA has the potential to control melanoma.

Hax-1, a novel 35 kDa protein, was cloned when yeast 2-hybrid screening was used to identify a protein that interacts with HS1. The latter is expressed specifically in hemopoietic and lymphoid cells, and is thought to be involved in B cell signal transduction and the receptor-mediated apoptotic and proliferative responses (Suzuki et al., 1997). Therefore, Hax-1 was identified as a novel protein without significant homology to any other proteins. Immunofluorescence microscopy showed that Hax-1 to be localized in the mitochondria, endoplasmic reticulum, and nuclear envelope (Suzuki et al., 1997). The amino acid sequence of Hax-1 shows homology to the BH1 and BH2 domains of the Bcl-2 family protein (Sharp et al., 2002), which suggests that Hax-1 may play a role in apoptosis. Subsequently, Hax-1 was found to interact with some apoptosis-regulating proteins, such as Epstein-Bar virus nuclear antigen leader protein (EBNA-LP), protein K15 of Kaposis's sarcoma-associated herpes virus, Omi/HtrA2 protease, and the Vpr protein of the human immunodeficiency virus (Kawaguchi et al., 2000; Sharp et al., 2002; Cilenti et al., 2004; Yedavalli et al., 2005). Furthermore, overexpression of Hax-1 protein can protect different types of cells from apoptosis (Suzuki et al., 1997; Lee et al., 2008). In contrast, downregulation of Hax-1 by antisense cDNA can induce apoptosis in HaCaT cells (Mirmohammadsadegh, et al., 2003). The data support the role of Hax-1 as an anti-apoptotic factor.

Apoptosis is an important mechanism regulating cell survival and normal homeostasis in the human body. Altered apoptosis processes will promote carcinogenesis and increase resistance to chemotherapy in cancer patients (Kaufmann and Gores, 2000; Schmitt, 2003). Several studies have shown that Hax-1 is upregulated in some metastatic tumors and tumor cell

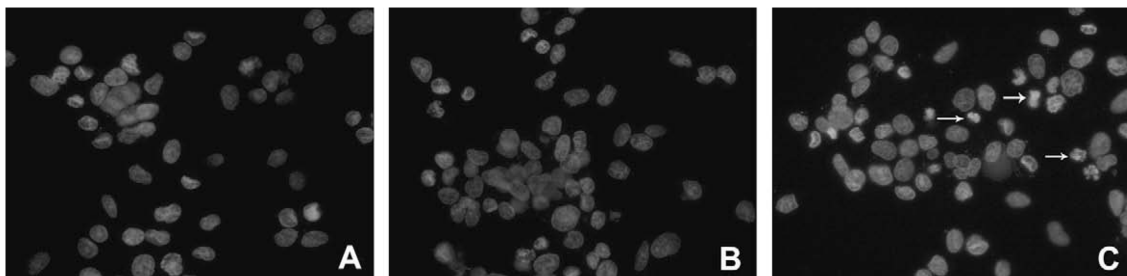

Fig. 5. Hoechst 33258 staining. The cells were grown and transfected with Hax-1 siRNA or control siRNA. After that, the cells were stained with Hoechst 33258 solution and reviewed under a microscope. A, A375 cells without any transfection; B, pSUPER-C-transfected A375 cells; and C, pSUPER-siH1-transfected A375 cells.

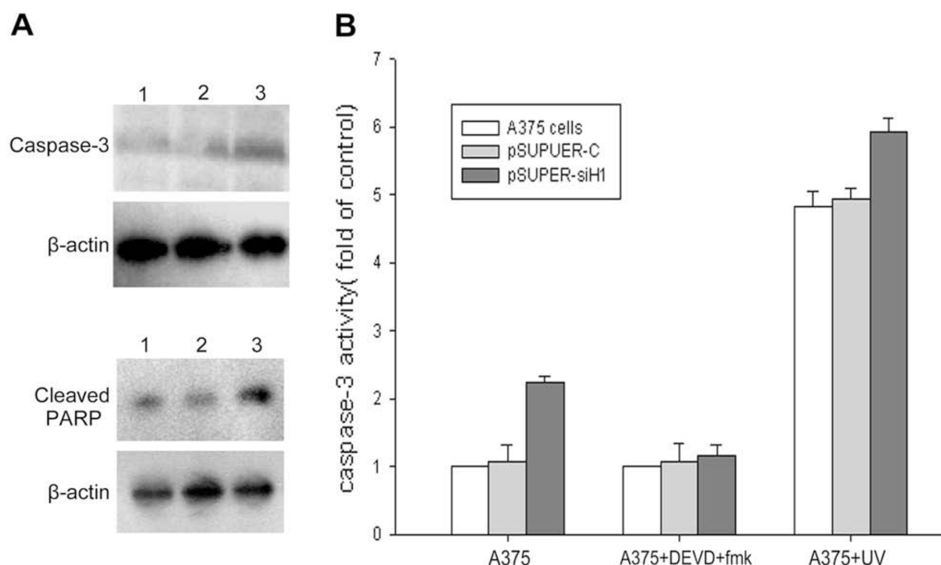

Fig. 6. Detection of caspase-3 and PARP activation. A, Western blotting analysis. The cells were grown and transfected with Hax-1 siRNA or control vector. After that, total cellular protein was extracted and subjected to Western blotting analysis. Lane 1, parental A375 cells; lane 2, pSUPER-C, and lane 3, pSUPER-siH1. B, ELISA assay. The cells were grown and transfected with Hax-1 siRNA or control vector and then exposed to UV for 5 min and incubated for additional 12 h. After that, total cell lysates were used for ELISA to detect caspase-3 activities. Each bar represents mean ± SD of 3 independent experiments. DEVD-fmk is a caspase-3 inhibitor.

lines, including melanoma cell lines (Velculescu et al., 1995; Jiang et al., 2003; Mirmohammadsadegh et al., 2003; Yamaguchi et al., 2005). We have focused on Hax-1 due to its noted anti-apoptosis effect. Hax-1 protein was overexpressed in A375 cells compared to HaCaT cells. We constructed the two Hax-1 siRNA vectors to downregulate Hax-1 expression in A375 cells and showed that transfection of pSUPER-siH1 effectively reduced Hax-1 expression and induced apoptosis in A375 cells. This finding may show that Hax-1 siRNA might help control melanoma because RNAi techniques provide a rapid and versatile method that inactivates expression of the target genes (Brummelkamp et al., 2002; Sun et al., 2007).

Apoptosis is characterized by cell shrinkage and rounding, membrane blebbing, chromatin condensation and breakdown, as well as caspase activation (Ziegler and Groscurth, 2004; Rello et al., 2005). Cysteine aspartases (caspases), a family of proteases, are known to play a key role in signal transduction cascades that culminate in apoptosis. Among all of the caspases, caspase-3 is one of the key executioners of apoptosis and results in DNA fragmentation and cell death (Porter and Janicke, 1999; Slee et al., 2001). Moreover, the activation of caspase-3 results in cleavage of the DNA-repair enzyme PARP and the latter is also a land marker during apoptosis induction (Kuida et al., 1996; Cohen, 1997). In the present study, Hax-1 downregulation led to an increase in the expression of the active form of caspase-3 and PARP proteins. The caspase-3 activity induced by Hax-1 downregulation was inhibited by a caspase-3 inhibitor, indicating that Hax-1-inhibited apoptosis required the suppression of caspase-3 activation. A recent study showed that Hax-1 is a substrate of caspase-3 and therefore inhibits the apoptotic process (Lee et al., 2008). Our results confirmed that Hax-1 inhibits apoptosis through a caspase-dependent pathway.

Hax-1 can also promote survival of virus-infected cells (Kawaguchi et al., 2000; Sharp et al., 2002; Cilenti et al., 2004; Yedavalli et al., 2005) and another study showed that overexpression of Hax-1 increases cell viability under H_2O_2 stimuli in HEK239 cells (Vafiadaki et al., 2007). The data indicate that Hax-1 can regulate tumor cell proliferation and the downregulation of Hax-1 expression promotes apoptosis in melanoma cells. Thus, we can conclude that Hax-1 is involved in regulation of cell growth through inhibition of apoptosis. Therefore, targeting Hax-1 expression may be of use in the therapy of melanoma, which is now under further study.

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References

Aagaard L, Rossi JJ. RNAi therapeutics: principles, prospects and challenges. Adv Drug Deliv Rev 2007;59:75–86.

Almeida R, Allshire RC. RNA silencing and genome regulation. Trends Cell Biol 2005;15:251–8.

Brown TJ, Nelson BR. Malignant melanoma: a clinical review. Cutis 1999;63: 275–8. 281–4.

Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. Science 2002;296:550–3.

Bush JA, Li G. The role of Bcl-2 family members in the progression of cutaneous melanoma. Clin Exp Metastasis 2003;20:531–9.

Cilenti L, Soundarapandian MM, Kyriazis GA, Stratico V, Singh S, Gupta S, et al. Regulation of HAX-1 anti-apoptotic protein by Omi/HtrA2 protease during cell death. J Biol Chem 2004;279:50295–301.

Cohen GM. Caspases: the executioners of apoptosis. Biochem J 1997;326(Pt 1):1−16.

Dufva M, Olsson M, Rymo L. Epstein-Barr virus nuclear antigen 5 interacts with HAX-1, a possible component of the B-cell receptor signalling pathway. J Gen Virol 2001;82:1581−7.

Gorreta F, Runfola TP, VanMeter AJ, Barzaghi D, Chandhoke V, Del Giacco L. Identification of thioredoxin reductase 1-regulated genes using small interference RNA and cDNA microarray. Cancer Biol Ther 2005;4:1079−88.

Han Y, Chen YS, Liu Z, Bodyak N, Rigor D, Bisping E, et al. Overexpression of HAX-1 protects cardiac myocytes from apoptosis through caspase-9 inhibition. Circ Res 2006;99:415−23.

Jiang Y, Zhang W, Kondo K, Klco JM, St Martin TB, Dufault MR, et al. Gene expression profiling in a renal cell carcinoma cell line: dissecting VHL and hypoxia-dependent pathways. Mol Cancer Res 2003;1:453−62.

Kaufmann SH, Gores GJ. Apoptosis in cancer: cause and cure. Bioessays 2000;22:1007−17.

Kawaguchi Y, Nakajima K, Igarashi M, Morita T, Tanaka M, Suzuki M, et al. Interaction of Epstein-Barr virus nuclear antigen leader protein (EBNA-LP) with HS1-associated protein X-1: implication of cytoplasmic function of EBNA-LP. J Virol 2000;74:10104−11.

Kuida K, Zheng TS, Na S, Kuan C, Yang D, Karasuyama H, et al. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 1996;384:368−72.

Lee AY, Lee Y, Park YK, Bae KH, Cho S, Lee do H, et al. HS 1-associated protein X-1 is cleaved by caspase-3 during apoptosis. Mol Cells 2008;25:86−90.

Li LH, Wu LJ, Zhou B, Wu Z, Tashiro S, Onodera S, et al. Silymarin prevents UV irradiation-induced A375-S2 cell apoptosis. Biol Pharm Bull 2004;27:1031−6.

Margolin KA, Liu PY, Flaherty LE, Sosman JA, Walker MJ, Smith JW, et al. Phase II study of carmustine, dacarbazine, cisplatin, and tamoxifen in advanced melanoma: a Southwest Oncology Group study. J Clin Oncol 1998;16:664−9.

Mirmohammadsadegh A, Tartler U, Michel G, Baer A, Walz M, Wolf R, et al. HAX-1, identified by differential display reverse transcription polymerase chain reaction, is overexpressed in lesional psoriasis. J Invest Dermatol 2003;120:1045−51.

Porter AG, Janicke RU. Emerging roles of caspase-3 in apoptosis. Cell Death Differ 1999;6:99−104.

Rello S, Stockert JC, Moreno V, Gamez A, Pacheco M, Juarranz A, et al. Morphological criteria to distinguish cell death induced by apoptotic and necrotic treatments. Apoptosis 2005;10:201−8.

Schmitt CA. Senescence, apoptosis and therapy-cutting the lifelines of cancer. Nat Rev Cancer 2003;3:286−95.

Sharp TV, Wang HW, Koumi A, Hollyman D, Endo Y, Ye H, et al. K15 protein of Kaposi's sarcoma-associated herpesvirus is latently expressed and binds to HAX-1, a protein with antiapoptotic function. J Virol 2002;76:802−16.

Slee EA, Adrain C, Martin SJ. Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. J Biol Chem 2001;276:7320−6.

Sun Y, Yi H, Zhang PF, Li MY, Li C, Li F, et al. Identification of differential proteins in nasopharyngeal carcinoma cells with p53 silence by proteome analysis. FEBS Lett 2007;581:131−9.

Suzuki Y, Demoliere C, Kitamura D, Takeshita H, Deuschle U, Watanabe T. HAX-1, a novel intracellular protein, localized on mitochondria, directly associates with HS1, a substrate of Src family tyrosine kinases. J Immunol 1997;158:2736−44.

Tang L, Tron VA, Reed JC, Mah KJ, Krajewska M, Li G, et al. Expression of apoptosis regulators in cutaneous malignant melanoma. Clin Cancer Res 1998;4:1865−71.

Vafiadaki E, Sanoudou D, Arvanitis DA, Catino DH, Kranias EG, Kontrogianni-Konstantopoulos A. Phospholamban interacts with HAX-1, a mitochondrial protein with anti-apoptotic function. J Mol Biol 2007;367:65−79.

Velculescu VE, Zhang L, Vogelstein B, Kinzler KW. Serial analysis of gene expression. Science 1995;270:484−7.

Yamaguchi H, Wyckoff J, Condeelis J. Cell migration in tumors. Curr Opin Cell Biol 2005;17:559−64.

Yedavalli VS, Shih HM, Chiang YP, Lu CY, Chang LY, Chen MY, et al. Human immunodeficiency virus type 1 Vpr interacts with antiapoptotic mitochondrial protein HAX-1. J Virol 2005;79:13735−46.

Ziegler U, Groscurth P. Morphological features of cell death. News Physiol Sci 2004;19:124−8.