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Silencing livin gene by siRNA leads to apoptosis induction, cell cycle arrest, and proliferation inhibition in malignant melanoma LiBr cells

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Abstract

Aim: The aim of the present study was to investigate the effects of silencing the livin gene by small interfering RNA (siRNA) on the expression of livin and the effects on apoptosis, cell cycle, and proliferation in human malignant melanoma LiBr cells. **Methods:** Three chemically-synthetic siRNA duplexes targeting livin were transiently transfected into the LiBr cells, and the effects on livin expression were detected both at the mRNA level by real-time RT-PCR and at the protein level by Western blotting. Apoptosis was evaluated by terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling assay, flow cytometric analysis, and the expression of procaspase-3 and activated caspase-3 analysis by Western blotting. Cell cycle was analyzed by flow cytometry. Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. **Results:** One of the 3 designed siRNA could effectively knock down the livin expression both at the mRNA and protein levels in dose- and time-dependent manners; 100 nmol/L with maximum downregulation on mRNA at 48 h, and on the protein at 72 h after transfection. Silencing livin could significantly induce apoptosis, arrest cell cycle at the G_0/G_1 phase, and inhibit proliferation in LiBr cells. Meanwhile, caspase-3 was activated. **Conclusion:** The livin gene could serve as a potential molecular target for gene therapy by siRNA for malignant melanoma.

Introduction

Livin, also called melanoma inhibitor of apoptosis proteins (IAP)or kidney IAP, is a recently discovered inhibitor member of the apoptosis protein (IAP) family[1–3]. Similar to other IAP that are able to block apoptosis in a caspase-dependent or -independent manner, livin is selectively expressed in most human neoplasms, but not or to a lesser extent in normal differentiated tissues, and appears to be involved in tumor cell resistance to apoptosis induced by a variety of stimuli[4]. Moreover, livin has a differential expression pattern and higher expression rate in tissue samples and primary cultures derived from malignant melanoma patients, as well as in malignant melanoma cell lines[1,2,5,6]. In addition, high levels of livin expression were correlated with

tumor progression and a lower survival rate, as well as the resistance of the cells to chemotherapy both *in vitro* and in melanoma patients receiving chemotherapy[5]. Therefore, the overexpression of livin renders malignant melanoma cells resistant to apoptotic stimuli and potentially contributes to the pathogenesis of this malignancy.

The discovery that small interfering RNA (siRNA) duplexes can trigger RNA interference (RNAi) for post-transcriptional gene silencing to knock down the expression of target genes in mammalian cells has opened the innovative access to developing the technique into therapeutics[7]. Over the past few years, RNAi-based therapies have been successfully implemented in a variety of cancer models[8]. The overexpression of livin, especially in malignant melanoma cells, implies that livin may be a potential target of RNAi by

knocking down its expression to modulate the apoptosis deficiency for gene therapy in malignant melanoma.

In this study, to investigate the biological effect of silencing the livin gene on human malignant melanoma LiBr[9] cells, three chemically synthesized siRNAs targeting to livin were transiently transfected into LiBr cells and the effects on apoptosis, cell cycle and proliferation were observed *in vitro*.

Materials and methods

Cell line and reagents Malignant melanoma cell line LiBr[9] was a kind gift from Professor Tian-wen GAO (Fourth Military Medical University, Xi'an, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Lipofectamine 2000, and TRIzol were purchased from Invitrogen (Carlsbad, CA, USA). The SYBR ExScript RT-PCR kits were from TaKaRa (Dalian, China). The mouse monoclonal antibody to livin (human; 88C570) was from Alexis Biochemicals (Lausen, Switzerland). The rabbit polyclonal antibody to caspase-3 (human; sc-7148) was from Santa Cruz (Santa Cruz, CA, USA). The rabbit polyclonal antibody to β-actin (human) was from Zhongshan (Beijing, China). Goat anti-mouse immunoglobulin G (IgG)/horseradish peroxidase (HRP) and goat anti-rabbit IgG/HRP were also from Zhongshan (China). The *in situ* cell death assay kit was from Keygen (Nanjing, China). The Annexin V-fluorescein-isothiocyanate (FITC) kit was from Jingmei Biotech (Shenzhen, China).

siRNA design and preparation The siRNA targetting to livin was designed according to the characterization of siRNA by Elbashir *et al*[7] and Reynolds *et al*[10]. As livin has 2 splicing variants, livin α and livin β[11], and the 2 isoforms possess an identical 843 bp sequence, except for an additional 54 bp in livin α, all 3 designed siRNA target livin β (GenBank Accession No NM_022161). The siRNA duplexes were designed and 3 were identified using the siRNA selection web server (http://jura.wi.mit.edu/bioc/siRNA), a online design tool at WHITEHEAD for siRNA. siRNA-1 (sense: 5'-GGC CUG GAC ACC UGC AGA GdTdT-3' and antisense: 5'-CUC UGC AGG UGU CCA GGC CdTdT-3'), siRNA-2 (sense: 5'-GGU GCU UCU UCU GCU AUG GdTdT-3' and antisense: 5'-CCA UAG CAG AAG AAG CAC CdTdT-3'), and siRNA-3 (sense: 5'-GAG AGG UCC AGU CUG AAA GdTdT-3' and antisense: 5'-CUU UCA GAC UGG ACC UCU CdTdT-3') targeted to sites 294–312, 541–559, and 790–808 of human livin mRNA sequences, respectively. The negative control duplexes of siRNA (siRNA-NC), with random sequences, did not target any known mammalian gene. All of the siRNA duplexes were chemically synthesized and 1.0 optical density of siRNA-NC was labeled with fluorophore FAM by Shanghai GenePharma (Shanghai, China).

Cell culture and transfection The LiBr cells were cultured in DMEM supplemented with 10% FBS and without antibiotics at 37 °C in a humidified 5% (*v/v*) CO_2 incubator. Transfection with siRNA was carried out with Lipofectamine 2000 according to the procedure recommended by the manufacturer.

Determination of transfection efficiency Six hours after transfection with FAM-labeled siRNA-NC at various final concentrations, the cells were analyzed by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with Cell Quest Software (Becton Dickinson).

Real-time RT-PCR The total RNA was extracted from the LiBr cells using TRIzol reagent according to the manufacturer's protocol. First-strand cDNA was synthesized using the ExScript RT-PCR reagent kit according to the manufacturer's instructions. The specific primers for livin (forward: 5'-GTC AGT TCC TGC TCC GGT CAA-3', reverse: 5'-GGG CAC TTT CAG ACT GGA CCTC-3', 189 bp) and for GAPDH (forward: 5'-GCA CCG TCA AGG CTG AGAAC-3', reverse: 5'-ATG GTG GTG AAG ACG CCA GT-3', 142 bp) were designed and synthesized by TaKaRa. Real-time PCR was prepared using the SYBR premix Ex *Taq* kit according to the manufacturer's protocol, and amplification was performed on an ABI Prism 7000 detection system (Applied Biosystems, Foster, CA, USA) according to the conditions recommended by the manufacturer, with an initial denaturation step at 95 °C for 10 s, followed by 40 cycles of denaturation at 95 °C for 5 s and 60 °C for 31 s. At the end of the amplification, a melting curve (disassociation curve) was run to ensure that only a single specific product was amplified. The data were analyzed by ABI Prism 7000 SDS software and the cycle threshold (Ct) values were determined. The modification of the $2^{-\Delta\Delta Ct}$ method[12] was used to calculate changes of the relative expression of livin normalized against GAPDH.

Western blotting The total protein was extracted from the LiBr cells using RIPA lysis buffer and 1:100 dilution of a protease inhibitor cocktail (Sigma, St Louis, MO, USA). Western blotting was performed as described in a previous study[13] and modified. Thirty micrograms of the protein lysate was resolved electrophoretically on SDS-PAGE (12% for livin and 15% for caspase-3) and blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). After being blocked for 1 h in blocking buffer (5% non-fat dried milk and 0.5% Tween-20 in TBS) and separately incubated with an antihuman livin mouse monoclonal antibody (1:1000), antihuman caspase-3 rabbit polyclonal antibody (1:500), and antihuman β-actin rabbit polyclonal antibody (1:3000) for 2 h

at room temperature, the blots were washed 3 times with TBST (0.5% Tween in TBS) and incubated for 1.5 h with goat antimouse IgG/HRP (1:3000) or goat antirabbit IgG/HRP (1:3000) at room temperature, followed by washing 3 times with TBST. The signals were visualized with the enhanced chemiluminescence method and developed with X-ray film. The band density was measured by the GEL DOC 2000 system equipped with Quantity One software (Bio-Rad, Hercules, CA, USA) and normalized against the density of the respective housekeeping β-actin.

Terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling assay The cells were seeded at 1.5×10^5 per well in 6-well plates with a coverslip for the seeded cells to grow on. At 72 h post-transfection, apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling (TUNEL) using the *in situ* cell death assay kit according to the instructions of a previous study[14], but with minor modifications: the cells were stained only with 3,3'-diaminobenzidine-tetrachloride and not counterstained. The apoptosis index was calculated as the percentage of cells with definite positive TUNEL staining and was obtained by counting 5 randomly chosen fields in each slide under a light microscope.

Flow cytometric analysis The LiBr cells were seeded at 7.5×10^4 per well in 12-well plates and transfected as described earlier. At 72 h post-transfection, the cells were harvested, stained with FITC-labeled Annexin V and propidium iodide (PI; Sigma, USA) to explore apoptosis on the FACSCalibur flow cytometer using Cell Quest software.

In addition, the cells were harvested, fixed in 70% ethanol for 12 h at 4 °C, and stained with PI in a phosphate-buffered saline solution containing RNase (Roche, Basel, Switzerland) for the cell cycle analysis using Modfit software (Variety Software House, Topsham, ME, USA).

***In vitro* cell proliferation assay** The cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The LiBr cells were seeded at 5×10^3 per well in 96-well flat-bottom plates 1 d before transfection. After transfection for 24, 48, 72, and 96 h, followed by the addition of 20 μL of 5 mg/mL MTT (Sigma, USA) to each well, the cells were incubated for another 4 h, and 150 μL DMSO (Sigma, USA) was added and then lysed for 15 min. The absorbance value was measured on a microplate reader (Bio-Rad, USA) at 490 nm.

Statistical analysis The obtained data were statistically evaluated by ANOVA and presented as mean±SD from 3 independent experiments. Probability values of less than 0.05 were considered significant. All analyses were carried out using SPSS 10.0 statistical software (SPSS, Chicago, IL, USA).

Results

Expression of livin and transfection efficiency with siRNA in LiBr cells To identify whether LiBr cells express livin, Western blotting was performed. Unexceptionally, like other malignant melanoma cell lines, the LiBr cells expressed the livin protein obviously (data not shown).

To evaluate the transfection efficiency of LiBr cells with the siRNA duplexes, flow cytometric analysis was performed 6 h after transfection with FAM-labeled siRNA-NC and showed a significant transfection efficiency of 83.27%± 4.11% at 75 nmol/L in a dose-dependent manner within 25–75 nmol/L ($P<0.05$). Thus, 75 nmol/L siRNA duplexes served as the initial concentration in the following experiments for the selection and validation of optimal siRNA sequences.

Knockdown of livin expression in LiBr cells by RNAi To select and validate effective siRNA target sites and determine the optimal dose- and time-response effect of silencing livin, the expression of livin in the LiBr cells that were separately transfected with siRNA-1, siRNA-2, or siRNA-3 at various concentrations was detected by real-time RT–PCR and Western blotting at 24, 48, 72, and 96 h post-transfection, respectively. Markedly, the silencing effects of siRNA-1, siRNA-2, or siRNA-3 on livin varied greatly (data not shown), and of them, only siRNA-3 achieved the highest silencing efficacy in dose- and time-dependent manners; 100 nmol/L of siRNA-3 with a maximum downregulation of 76.94%± 6.33% on mRNA at 48 h and of 83.39%±5.44% on the protein at 72 h after transfection, respectively (partial data shown in Figure 1).

Therefore, siRNA-3 was selected as the effective siRNA sequence, and 100 nmol/L as the final concentration for use in investigating the effects of silencing livin on LiBr cells at 72 h post-transfection in the following experiments (except for the MTT assay).

Induction of apoptosis in LiBr cells by siRNA The TUNEL assay was performed to investigate whether silencing the livin gene induces apoptosis. The apoptosis index of cells transfected with siRNA-3 was 15.97%±2.56%, significantly higher than that of the cells in the blank (6.23%± 1.12%), mock (6.47%±1.51%), and siRNA-NC (7.00%±1.11%; $P<0.05$) groups. In contrast, the apoptotic cells in the blank, mock, and siRNA-NC group did not differ from each other ($P>0.05$; Figure 2A).

To further confirm the induction of apoptosis and quan-
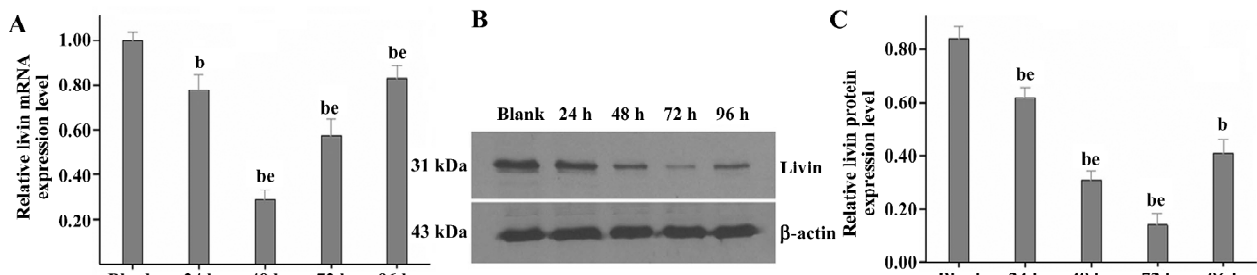
Figure 1. Livin expression in LiBr cells at 24, 48, 72, or 96 h post-transfection with 100 nmol/L siRNA-3. (A) relative livin mRNA expression level by real-time RT-PCR and normalized against GAPDH. (B) representative livin protein expression by Western blotting. (C) relative livin protein expression level by Western blotting and normalized against β-actin. [b]$P<0.05$ *vs* blank group. [e]$P<0.05$ for the linear regression analysis.
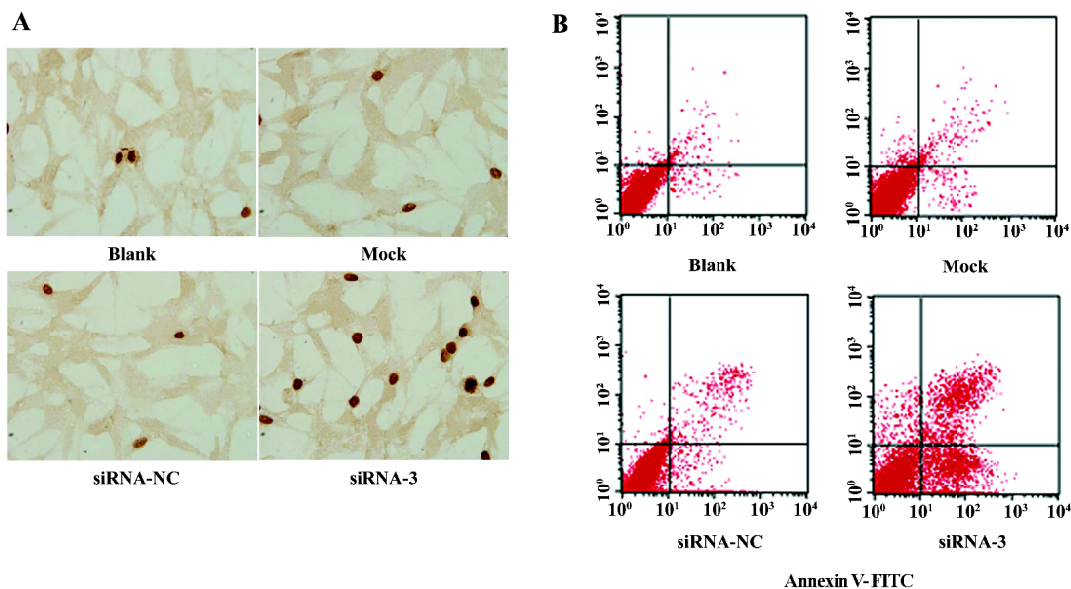

Figure 2. Induction of apoptosis in LiBr cells at 72 h post-transfection with 100 nmol/L siRNA-3. (A) representative photomicrographs showing positive staining (dark brown) in apoptotic cell nucleus by TUNEL assay. (B) representative dot plots showing early apoptosis (LR, Annexin V[+]/PI[−]), late apoptosis (UR, Annexin V[+]/PI[+]), necrotic cells (UL, Annexin V[−]/PI[+]), and normal cells (LL, Annexin V[−]/PI[−]) by flow cytometric analysis.

tify the apoptotic cells by silencing livin, flow cytometric analysis was performed. The early apoptotic rate of cells transfected with siRNA-3 increased to 28.67%±5.55% and the late apoptotic rate increased to 12.91%±3.77%, which were significantly greater than those in of the cells in the blank, mock, and siRNA-NC groups ($P<0.05$); there were no significant differences in the apoptotic cells among the blank, mock, and siRNA-NC groups ($P>0.05$; Figure 2B).

To explore whether silencing livin activates caspase-3 in LiBr cells, the expression of procaspase-3 and activated caspase-3 was analyzed by Western blotting. The protein level of procaspase-3 was obviously downregulated ($P<$

0.05) and the cleaved form of caspase-3 was found in cells transfected with siRNA-3 compared with the blank control. In contrast, the levels of procaspase-3 were unchanged ($P>0.05$) and no cleaved fragment was detected in the cells of the blank, mock, and siRNA-NC groups (Figure 3).

Cell cycle arrest in LiBr cells by siRNA To examine whether silencing livin leads to cell cycle arrest, the phase distribution of the cell cycle was analysed by flow cytometry. Compared with the blank, mock, and siRNA-NC groups, there were great changes of cell cycle distribution in the cells transfected with siRNA-3; the cells blocked in the G_0/G_1 phase increased to 69.41%±4.41% ($P<0.05$), while the cells in the S
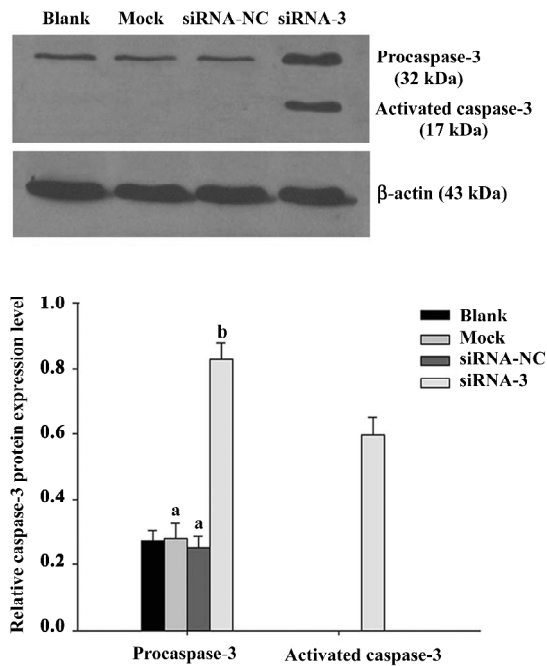
Figure 3. Expression of procaspase-3 and activated caspase-3 in LiBr cells at 72 h post-transfection with 100 nmol/L siRNA-3. (A) representative protein expression of procaspase-3 and activated caspase-3 by Western blotting. (B) relative expression levels of procaspase-3 and activated caspase-3 protein by Western blotting and normalized against β-actin. [a]$P>0.05$, [b]$P<0.05$ *vs* blank group.

Figure 4. Cell cycle arrest and inhibition of cell proliferation in LiBr cells at 72 h post-transfection with 100 nmol/L siRNA-3. (A) representative histograms of cell cycle distribution showing percentage of cell-gated populations in the G_0/G_1, S, and G_2/M phases by flow cytometric analysis. (B) cell growth curve showing the proliferation inhibition in the LiBr cells with siRNA-3 by MTT assay. [b]$P<0.05$ *vs* blank group. [e]$P<0.05$ *vs* 24, 48, and 96 h groups.
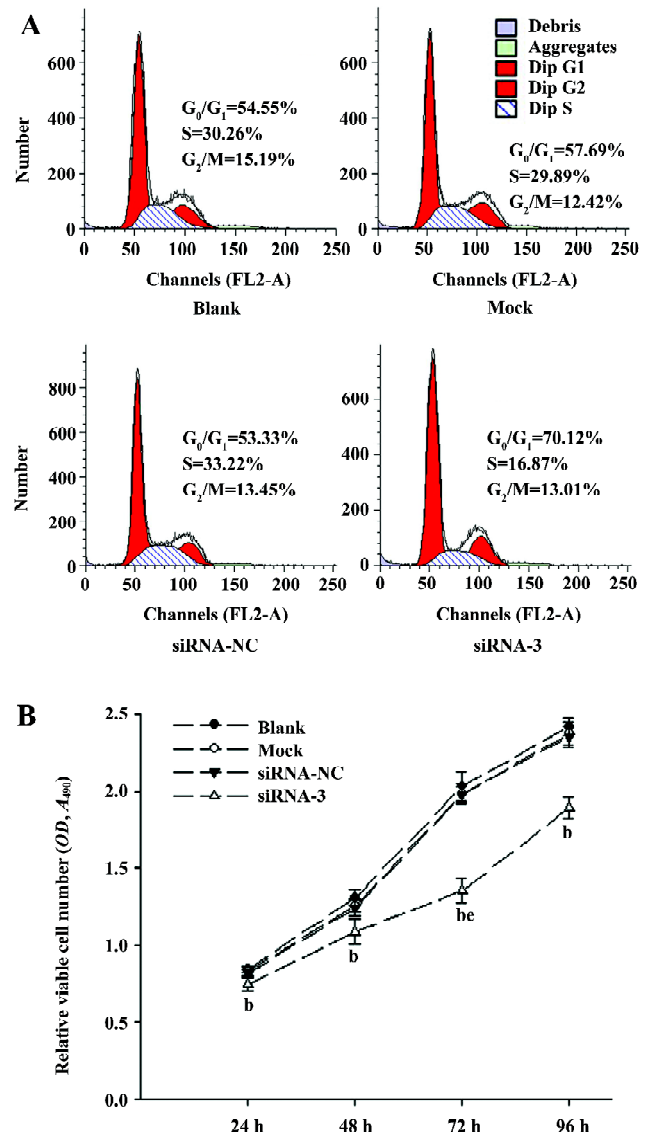
phase decreased to $18.59\%\pm2.65\%$ ($P<0.05$). In contrast, there were no notable differences in the cell cycle distribution among the blank, mock, and siRNA-NC groups ($P>0.05$) (Figure 4A).

Inhibition of the proliferation in LiBr cells by siRNA The MTT assay showed that, compared with the blank group, the proliferation of cells transfected with siRNA-3 was remarkably inhibited from 24 to 96 h ($P<0.05$), with the highest inhibitory rate of $33.35\%\pm3.46\%$ ($P<0.05$ *vs* 24, 48, and 96 h groups) at 72 h post-transfection, but at 96 h, the inhibitory rate decreased to $21.82\%\pm3.20\%$, which indicated that the number of viable cells began to increase. In contrast, there was no obvious difference in cell proliferation among the blank, mock, and siRNA-NC groups ($P>0.05$; Figure 4B).

Discussion

As resistance to apoptosis is a hallmark of various cancers and may be the underlying basis for tumorigenesis and tumor progression, strategies of inducing cancer cells to apoptosis are being designed[15,16]. Caspases are critical for the induction of apoptosis and their decreased expression is correlated with an increased grade of cancer, while the increased expression of caspases renders the cancer cells susceptible to chemotherapy[17,18]. However, the endogenous functions of caspases are inhibited by IAP that bind activated caspases in cancer cells[19]. Thus, removing the negative effects of the IAP represents a promising strategy to sensitize cancer cells to apoptosis[15]. RNAi technique can be applied to increase the apoptotic susceptibility of cancer

cells by knocking down anti-apoptotic genes, such as certain IAP that were overexpressed. IAP seem to fit ideally as a specific molecular target because they are differentially overexpressed in many cases of malignant cells, but not in their healthy counterparts, and act at the effector level of the apoptosis pathways[15]. Up until now, 8 IAP have been identified in human cells, and among them, livin has been recently identified[4]. Previous reports have indicated that livin was overexpressed in a variety of malignancies and especially in malignant melanoma cells[1,2,5,6]. Considering that malignant melanoma, the most dangerous form of skin cancer, is resistant to currently available therapeutics[20], silencing the livin gene may be an encouraging approach for the gene therapy of malignant melanoma.

Our present study demonstrates that the expression of livin in LiBr cells could be knocked down specifically and effectively by siRNA in dose-and time-dependent manners. The silencing efficiency of the 3 designed specific siRNA duplexes that were separately targeted to the sites upstream (294–312), midstream (541–559), and downstream (790–808) of livin mRNA demonstrated striking differences. Only siRNA-3 resulted in a significant downregulation of livin expression. This phenomenon was believed to be associated with the positional effects[21].

In this study, we observed that silencing livin could notably promote apoptosis in LiBr cells. In accordance with the observations of increasing apoptotic cells, the cleaved form of caspase-3 (17 kDa) was found and the expression of procaspase-3 protein was upregulated. IAP are able to inhibit apoptosis by direct binding and the inhibition of certain caspases through 1 or more repeats of a highly-conserved 70 amino acid domain termed the baculovirus IAP repeat (BIR) domains. Livin with only 1 BIR domain is also able to inhibit caspases-3, -7, and -9[15]. Caspases form the core activation cascade of apoptosis with upstream or initiator caspases-8, -9, and -10, and downstream or effector caspases-3, -6, and -7. Caspase-3 is the effector protein of both the intrinsic and extrinsic pathways of apoptosis initiation[15]. The cleaved form of caspase-3 induced by siRNA indicated that silencing livin released caspase-3 from negative regulation by livin to activation and triggered apoptosis in the LiBr cells. This is in agreement with a previously published study on HeLa cells[22].

Our results demonstrated for the first time that silencing livin by siRNA leads to cell cycle arrest at the G_0/G_1 phase. In addition to the suppression of apoptosis, cancer cells are also characterized by deregulated cell proliferation, which is generally associated with accelerated G_1/S and G_2/M cell cycle transitions[23]. Some signals of DNA damage lead to

cell cycle arrest at the G_1 and/or G_2 phases, reducing the rate of DNA synthesis or resulting in apoptosis[24,25]. The checkpoint responses and induction of apoptosis are considered to be major mechanisms for reducing both the initiation and progression of cancer[26,27]. In this study, the accumulation in the G_0/G_1 phase and the reduction in the S phase of LiBr cells treated with siRNA-3 indicated that silencing the livin gene resulted in a decrease of the ability of cells to progress from the G_0/G_1 to the S phase and synthesize DNA, eventually resulting in the inhibition of proliferation, which was confirmed by MTT assay. The study of cell proliferation with time-courses showed that the highest inhibitory rate was accordance with the maximum of the livin protein being downregulated at 72 h post-transfection. In this study, we provide preliminary evidence that silencing livin can change the cell cycle in LiBr cells. The results of a previous study indicated that livin might be regulated by cell cycle proteins similar to survivin based on the observation that livin and survivin have a similar subcellular localization[1]. Whether livin acts as a bifunctional protein associated with the regulation of the cell cycle and the inhibition of apoptosis-like survivin needs to be further explored.

In conclusion, silencing the livin gene by siRNA can significantly knock down the expression of livin, induce apoptosis, arrest the cell cycle at the G_0/G_1 phase, and inhibit proliferation in LiBr cells. Livin can serve as a potential molecular target to malignant melanoma in gene therapy by siRNA. The validated sequence of siRNA-3 can be used to construct vectors for stable expression in future.

Acknowledgements

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