



Livin gene plays a role in drug resistance of colon cancer cells

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ABSTRACT

Objective: The objective of this study was to investigate the effect of knockdown of Livin expression on reversing drug resistance phenotype of colon cancer HCT-8/V cells.

Design and methods: Specific short hairpin RNA (shRNA) was chosen and transfected in human colon cancer HCT-8/V cell line. Cell apoptosis and chemosensitivity were evaluated following downregulation of Livin expression.

Results: In the current study, Livin was found to be highly expressed in the HCT-8/V colon cancer cells, which were resistant to several anti-tumor drugs. Knocking down of Livin expression in HCT-8/V cells by specific RNAi facilitated the apoptosis of HCT-8/V cells in response to vincristine (VCR), etoposide (VP-16), and 5-fluorouracil (5-FU). Chemosensitivity assay confirmed the results and demonstrated the reversal of drug resistance phenotype of HCT-8/V cells.

Conclusion: These data suggest that specific silencing of Livin gene expression could be a promising target for further research in clinical chemotherapy of colon cancer.

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Introduction

Inhibition of apoptosis is one of the important mechanisms of tumorigenesis and renders the cancer cells resistant to anti-tumor drugs [1–3]. This process is mediated by a group of proteins, called inhibitor of apoptosis proteins (IAP), which play their antiapoptotic role via inhibiting different caspases activities in the apoptosis pathways. A variety of studies suggest that overexpression of IAP facilitates accumulation and survival of neoplastic cells and improvement of tumor drug resistance to antineoplastic agents [4–6]. So far, eight IAPs have been identified in human cells, including XIAP (ILP-1), c-IAP1 (HIAP-2), c-IAP2 (HIAP-1), NAIP, Survivin, BRUCE (apollon), Livin (ML-IAP, KIAP) and ILP-2 [6]. Livin is an important member of the IAP family, originally identified in melanoma [6]. Since then Livin has emerged as a key member of IAP family and has been shown to be expressed in multiple malignant tumors such as bladder cancer, lung cancer, breast carcinoma, acute lymphoblastic leukemia, gastric cancer and colon cancer [7–13].

Colon cancer is one of the most common malignant tumors in human digestive system. Clinically, failure of chemotherapy resulted from drug resistance remains a challenging problem for treatment of colon cancer. It has already been reported that overexpression of P-glycoprotein (P-gp), a product of multidrug resistant gene 1, and antiapoptotic protein are two common mechanisms of chemoresistance in tumor cells. The role of P-glycoprotein in drug efflux has been well studied [14,15], while the function of antiapoptotic proteins, particularly Livin in reversing chemoresistance has not been fully elucidated. In this study, the colon cancer HCT-8/V cells were found to express high levels of Livin and the role of Livin on the susceptibility of HCT-8/V cells to several drugs was explored by using RNA interference technology.

Materials and methods

Design of shRNA and construction of Livin shRNA vectors

Three segments which are located at 158–178, 625–645, and 1234–1254 in Livin cDNA (NM_139317) were selected as our candidate targets using shRNAs online design tools of Ambion (http://www.ambion.com/techlib/misc/siRNA_tools.html). Another shRNA of nonspecific sequence (mock) was used as a control. These sequences by BLAST search were found no homology with other human genes. Then, these shRNAs were subcloned into pGCSi-H1/Neo/GFP (Genechem, Shanghai, China) plasmid between the BamHI and HindIII enzyme sites and the recombinants generated were

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Table 1
Candidate Livin shRNAs and its target sequences.

Recombinants	Target sequence	Structure of short hairpin RNAs
pSi_158	5'-AAGACAGTGCCAAGTGCCTG-3'	<pre> 5'-AGACAGTGCCAAGTGCCTG^T 3'-TTTCTGTACACGGTTCACGGAC^A T C A A G A </pre>
pSi_625	5'-AAGAGACTTTGTCCACAGTGT-3'	<pre> 5'-GAGACTTTGTCCACAGTGT^T 3'-TTTCTGTAAACAGGTGTACACA^A T C A A G A </pre>
pSi_1234	5'-AACTGTACCTGTTGGATGCT-3'	<pre> 5'-CTGTACCTGTTGGATGCT^T 3'-TTGACATGGACAAAACCTACGA^A T C A A G A </pre>
pSi_NS	Nonspecific control (mock)	<pre> 5'-CTGTACCTGTTGGATGCT^T 3'-TTGACATGGACAAAACCTACGA^A T C A A G A </pre>

named pSi_158, pSi_625, pSi_1234 and pSi_NS (Table 1). The inserts in those recombinants were confirmed by DNA sequencing.

Cell culture and transfection

HCT-8/V cells (a human colon cancer cell line with drug resistance to vincristine from KEYGEN, Nanjing, China) were maintained in RPMI 1640 medium (Hyclone) supplemented with 10% of fetal bovine serum (FBS, Hyclone), 2.05 mM of L-glutamine, 100 U/mL of penicillin and 100 µg/mL of streptomycin at 37 °C with 5% CO₂. In order to ensure maintenance of the drug resistance phenotype, cell culture medium for the HCT-8/V was supplemented with 0.08 µg/mL vincristine. For transfection, cells were seeded in a 6-well plate at a concentration of 1×10^6 cells per well and allowed overnight growth to reach 75–85% confluency. Cells were then transfected with the mixture of 1.6 µg plasmid DNA and 4 µL of Lipofectamine 2000 (Invitrogen, USA) in 96 µL serum-free medium. FBS (0.5 mL/well) was added at 4 h post-transfection. At 48 h, the medium was replaced by selective medium (containing 400 µg/mL G418 (Invitrogen, USA), 10% FBS and antibiotics), and the untransfected cells were eliminated within 10 days after transfection.

RNA preparation and real-time PCR

Total RNA from cultured cells was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacture's protocol. The first strand of cDNA was obtained using RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA). Livin and β-actin (NM_001101) expression was determined by real-time PCR with LightCycler (Roche), using SYBR green PCR Master Mix (Applied Biosystems, USA). The primers for Livin (AF311388) were as follows: forward primer 5'-GTCCCCAGCTGTCAGTTC-3' and reverse primer 5'-CGTCTCCGGT TCTTCCCA-3', for internal control gene β-actin, forward primer 5'-GTCATCACCATTGGCAAT GAG-3', reverse primer 5'-CGTCACTTCATGATGAGTT-3'. The amplification conditions for the Livin cDNA included preincubation for 2 min, 8 min polymerase activation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Each measurement was performed in triplicate. Relative quantification was performed using the standard curve method [16].

Western blotting analysis

Whole cells in each well were collected and lysed in RIPA buffer (Beyotime, Jiangsu, China) at 4 °C for 30 min. Then, the samples were separated by SDS–polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride (PVDF) membranes. Target proteins were detected after incubation with appropriate first antibody (Livin, P-gp and housekeeping gene β-actin monoclonal antibody (ProSCI, CA, USA)) in Tris-Buffered saline plus 0.1% Tween-20 (TBS-T) containing 5% fat-free milk at 25 °C for 2 h, followed by incubation with HRP (horseradish peroxidase)-conjugated secondary antibodies (anti-mouse immunoglobulin antibodies (Jackson, PA, USA)) at 25 °C for 1 h. Bound antibodies were developed using the Super ECL plus detection Regent (Applygen, China). Cells transfected with shRNAs that had the highest down-expression of Livin were selected for the following assays.

Cells apoptosis analysis

Apoptotic cells were evaluated using an Annexin V/FITC kit (Beyotime, Jiangsu, China). Cells were stained according to the manufacturer's protocol and analyzed by flow cytometry (Cytomics FC 500, Beckman Coulter, USA). Moreover, the morphological alterations of apoptosis of HCT-8/V were observed by fluorescence microscopy (DMI IRB4000, LEICA, Germany) using Hoechst 33258 (Beyotime, Jiangsu, China) staining.

Chemosensitivity assay

HCT-8/V cells were treated separately with concentration gradient of anticancer agents before and after Livin gene knockdown. All the anticancer agents were obtained from the Department of Pharmacy of Affiliated Hospital of Nantong University. The anticancer agents tested were vincristine (VCR), etoposide (VP-16), and 5-fluorouracil (5-FU). Drugs were tested in six dilutions corresponding to 200%, 100%, 50%, 25%, 12.5% and 6.25% of each drug standard test drug concentration (TDC, 100% TDC used as follows: VCR 0.4 µg/mL, VP-16 48.0 µg/mL, 5-FU 22.5 µg/mL) as previously described [17,18].

The cells were seeded at the density of 5000 cells per well of 96-well plates in RPMI 1640 medium, each well was repeated in

triplicate. After an overnight incubation, the medium was replaced by fresh one containing the test drugs. All the cells were treated for 72 h. The cell confluency was about 45–55% at the time of exposure. At the end of the treatment, cell viability was determined colorimetrically by using Cell Counting Kit-8 (Dojindo, JAPAN) according to manufacturer's protocol. After incubation for 2 h, the 96-well plate was scanned by an enzyme-linked immunosorbent assay (ELISA) reader at 450 nm for OD values to calculate percentage of cell inhibition and IC50 (inhibitory concentration to achieve 50% cell death). The degree of inhibition of drug-treated cells is expressed as a percentage of the untreated cell control. The growth inhibition formula is as follows: % growth inhibition = $[1 - (\text{test} - \text{MinV}) / (\text{MaxV} - \text{MinV})] \times 100$, where the viability was calculated with regard to the untreated cell control, which was set to 100% viability (maximal viability, MaxV). The dead cell control (hydrogen peroxide treated cells) was set to 0% viability (minimal viability, MinV). The TDCs were plotted against the corresponding inhibition values using SPSS 13.0, resulting in the inhibition curves. The IC50 value of drugs was calculated through different concentrations of the drug and the corresponding % growth inhibition as previously described [17] by ED50Plus v1.0, a tool of pharmacological analysis.

Statistical analysis

The significance was calculated using one-way ANOVA and UNIANOVA. A value of $P < 0.05$ was taken as statistically significant. Data were presented as mean with standard deviation (mean \pm SD).

Results

Transfection and selective culture

HCT-8/V cells were then transfected with different shRNA constructs. At 48 h post-transfection, GFP-positive cells could be observed by fluorescence microscopy. In the following days, the selective medium containing G418 was used to eliminate non-transfected cells and at day 10, most of the survived cells were GFP-positive (Fig. 1), indicating that shRNAs were successfully expressed in these cells. The cells were used in the following assays.

Identification of specific shRNA against Livin

The knockdown efficiencies of three candidate Livin shRNAs in HCT-8/V cells were first evaluated using quantitative RT-PCR, and relative Livin mRNA levels were normalized against mRNA levels of an internal

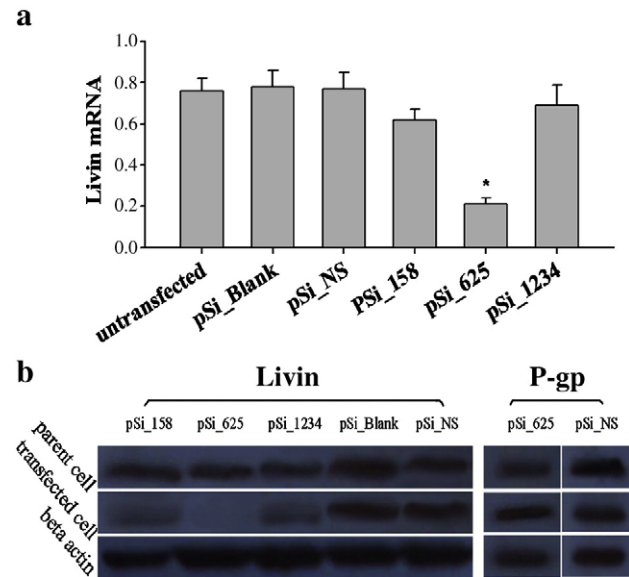


Fig. 2. Detection of Livin mRNA in the transfected and the untransfected HCT-8/V cells (parent cell) by quantitative RT-PCR and evaluation of Livin and P-gp protein by western blotting. (a) Livin mRNA detected by quantitative RT-PCR. Expression of Livin in HCT-8/V cell was shown a ratio of copies of Livin to copies of internal control gene β -actin in the same sample. By comparison, pSi_625 inhibited Livin mRNA most effectively. Values are expressed as means \pm SD ($*P < 0.001$). (b) Western blotting analysis of Livin and P-gp protein in HCT-8/V cells transfected with shRNAs. β -actin expression was used to monitor protein loading between lanes (a) Livin expression in parent cell and (b) Livin expression in pSi_158, pSi_625, pSi_1234, pSi_Blank and mock transfected HCT-8/V cells, P-gp expression in pSi_625 and mock transfected HCT-8/V cells.

control gene β -actin. As shown in Fig. 2a, the cells transfected with pSi_625 (0.21 ± 0.03 , $*P < 0.001$) showed a significant decrease in mRNA level compared with those of vector control pSi_Blank (0.78 ± 0.11) and mock pSi_NS (0.77 ± 0.10). Moreover, the decrease in Livin mRNA level was moderate in pSi_158 (0.58 ± 0.05) and pSi_1234 (0.66 ± 0.06) transfected cells. In addition, the expression of Livin was confirmed by using Western blotting. Fig. 2b shows the level of Livin protein was dramatically decreased in cells with pSi_625 recombinant, compared with the others. There was no change in P-gp protein expression in HCT-8/V cells before and after knockdown of Livin by pSi_625 (Fig. 2b). These results disclosed that pSi_625 recombinant was able to effectively silence Livin expression both in mRNA and protein levels. As a result, the cells transfected with pSi_625 were collected and used for following experiments.

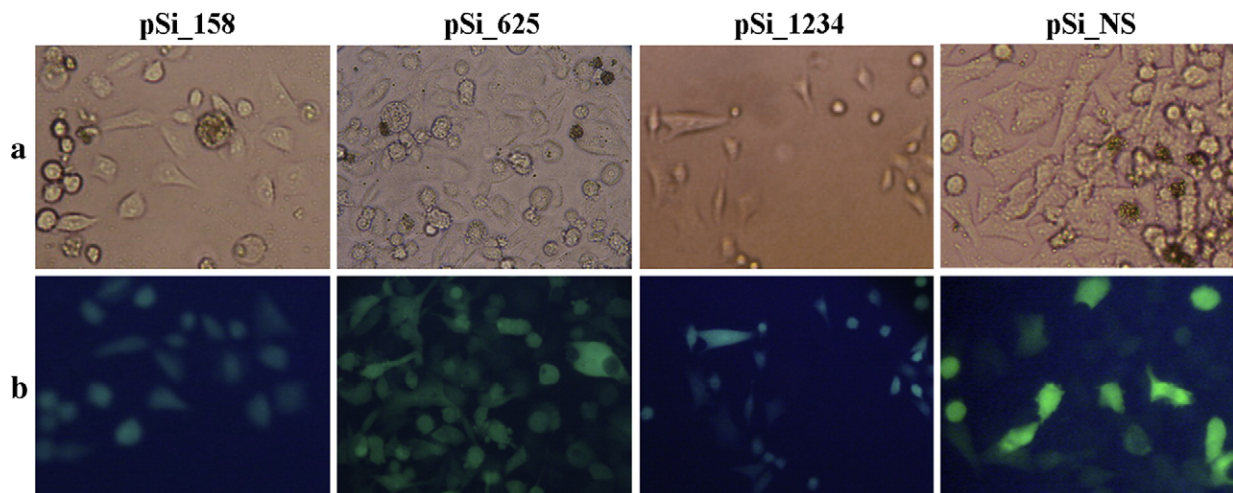


Fig. 1. Three candidate shRNAs recombinants (pSi_158, pSi_625, pSi_1234) and mock shRNA (pSi_NS) were successfully transfected into HCT-8/V cells and expressed shRNAs and GFP. (a: routine field, b: fluorescence field corresponding to row a).

Knockdown of Livin promoted cell apoptosis

In the absence of anticancer drugs, there was negligible apoptosis for HCT-8/V cells and HCT-8/V cells transfected with pSi_625 or pSi_NS. To compare cell apoptosis in medium containing anticancer agents before and after down-expression of Livin, flow cytometry and Hoechst 33258 staining were used to evaluate cell apoptosis. Analysis of flow cytometry indicated that, as shown in Fig. 3, HCT-8/V cells with pSi_625 maintained in medium containing VP-16, VCR and 5-FU for 72 h underwent much more apoptosis compared with the untransfected cells. The apoptotic rates of HCT-8/V cells significantly increased respectively from 0.9%, 0.1% and 3.1% to 18.8%, 22.8% and 23.4% in the medium containing VP-16, VCR and 5-FU. Moreover, Hoechst 33258 apoptosis staining showed typical apoptosis morphology using fluorescence microscopy (Fig. 4). Taken together, these results suggested that down-expression of Livin mediated by pSi_625 could

promote apoptosis of HCT-8/V cells in medium containing VP-16, VCR and 5-FU of 100% TDC.

Effect of knockdown of Livin on reversal of drug resistance

The growth inhibition curves of the 3 anti-tumor drugs at six different TDCs were shown in Figs. 5A–C. It was demonstrated that the Livin expression in HCT-8/V cells was knocked down by pSi_625, but not by the control pSi_NS, resulting in much higher cell growth inhibition at different anti-tumor drug concentrations. The IC50s of VCR, VP-16 and 5-FU for the untransfected HCT-8/V cells were $89.71 \pm 5.45\%$ TDC, $47.65 \pm 5.28\%$ TDC and $56.30 \pm 4.70\%$ TDC respectively, but the IC50s of VCR, VP-16 and 5-FU for the HCT-8/V dropped to $23.79 \pm 5.42\%$ TDC ($P < 0.001$), $21.15 \pm 9.66\%$ TDC ($P < 0.001$), and $22.31 \pm 6.71\%$ TDC ($P < 0.001$) respectively when Livin expression was silenced (Fig. 5D). These results suggest that there was a significant enhancement in chemosensitivity of VCR,

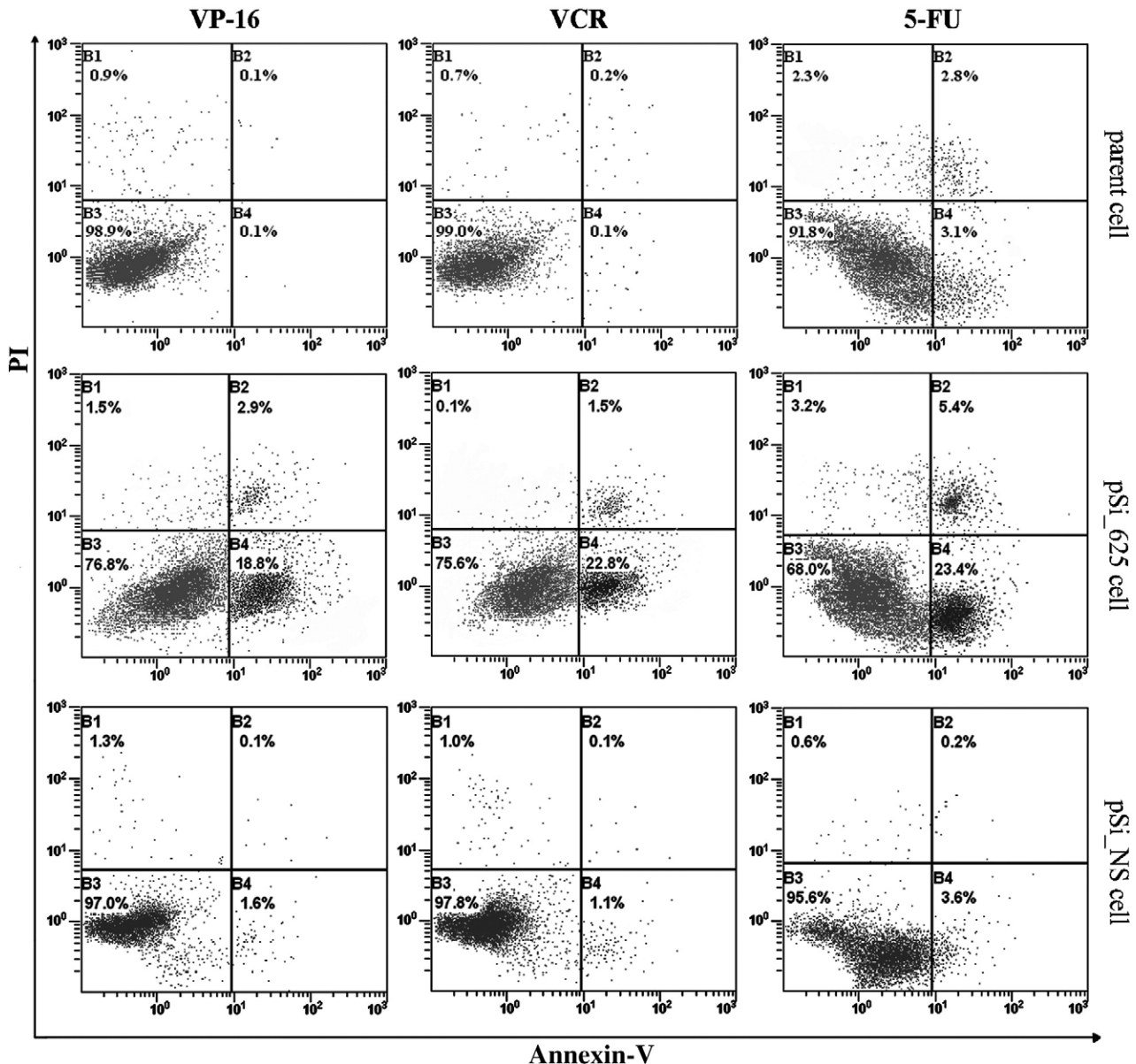


Fig. 3. Evaluation of apoptosis in HCT-8/V cells before and after RNAi with pSi_625 by flow cytometry. FITC-labeled Annexin V and fluorescent dye PI were used to stain cells after 72 h drug treatment. The data were presented as percent apoptotic cells versus total cells.

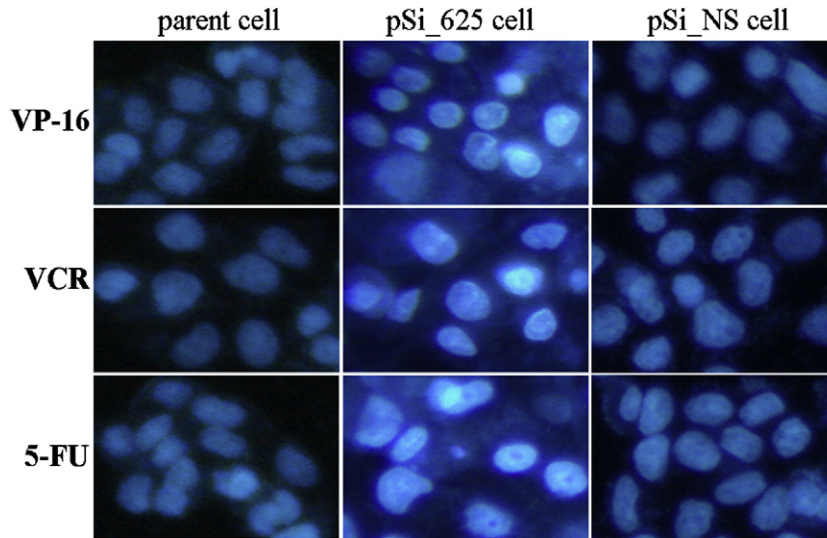


Fig. 4. Observation of Hoechst33258 apoptosis staining by fluorescence microscopy. Nuclei of viable parent cell and pSi_NS cell were normal and nuclei of apoptotic pSi_625 cell were compacted, which maintained in medium containing VP-16, VCR and 5-FU of 100% TDC for 72 h.

VP-16 and 5-FU to Livin knocked down HCT-8/V cells compared with the untransfected cells or control shRNA transfected cells.

Discussion

Failure of chemotherapy results from a variety of complicated factors, including the increase in drug efflux, specific metabolism of antineoplastic agents, mutation of the specific drug targets, the disability of drug transport proteins, activation of DNA repair and apoptosis evasion of neoplastic cells [12,13].

Livin has one baculovirus IAP repeat (BIR) domain and a RING finger motif. In some cases, it showed different antiapoptotic property [19]. The main antiapoptotic mechanism of Livin is that it binds

upstream (e.g., caspase-9) and downstream (e.g., caspase-3 and caspase-7) caspases but not caspase-8 by means of BIR domain [20]. To date, two alternatively spliced transcripts of Livin gene have been identified, which were named Livin- α and Livin- β respectively. Although there is only 54-nt differences in the 6th exon between the longer Livin- α and the shorter Livin- β , antiapoptotic property of the two isoforms has subtle differences. Crnković-Mertens et al. [21] reported that the targeted inhibition of Livin β , but not of Livin α , retarded the growth of HeLa cells in clonogenic survival assays and silencing of Livin β , but not of Livin α , sensitized HeLa cells to different proapoptotic stimuli such as UV irradiation, tumor necrosis factor α , or etoposide. Another study [22] suggested that the specific down-regulation of Livin expression in lung cancer cells significantly

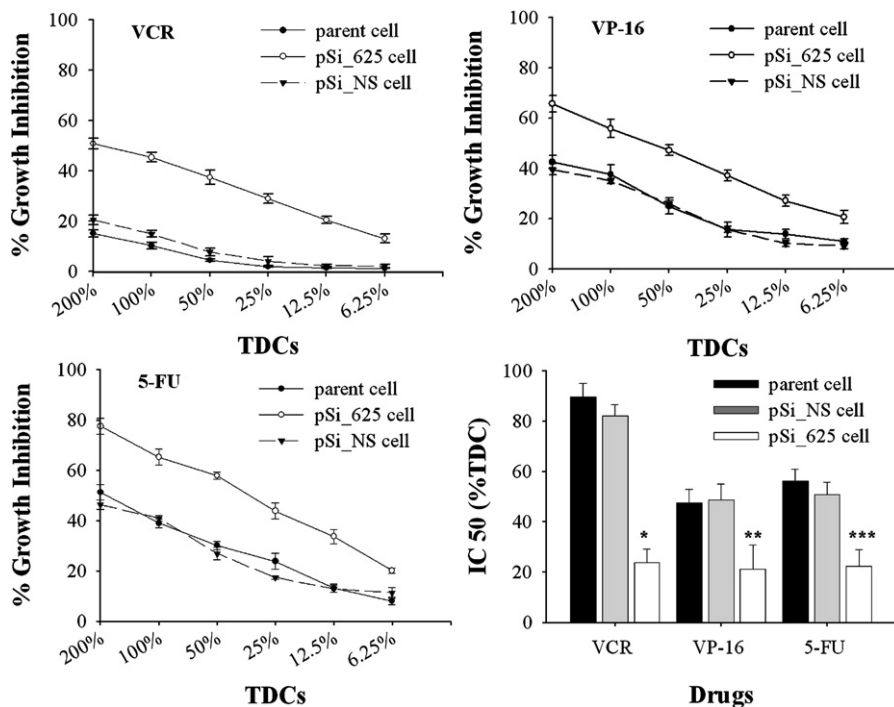


Fig. 5. Inhibition curves of anticancer drugs at six different test drug concentrations. (A) Inhibition curve of VCR for pSi_625 cell, pSi_NS cell and parent cell. (B) Inhibition curve of VP-16 for pSi625 cell, pSi_NS cell and parent cell. (C) Inhibition curve of 5-FU for pSi625 cell, pSi_NS cell and parent cell. (D) IC50s of VCR, VP-16 and 5-FU to pSi625 cell, pSi_NS cell and parent cell before and after silencing Livin (VCR * $p < 0.001$, VP-16 ** $p < 0.001$ and 5-FU *** $p < 0.001$).

inhibited *in vitro* cell proliferation and *in vivo* tumorigenicity and led to cell arrest in the G1/G0 phase of cell cycle, eventual apoptosis and chemosensitivity enhancement in lung cancer cells. These investigations indicate that Livin plays a role which is relevant to drug resistance of chemotherapy.

RNA interference has been used for silencing gene expression in cells and experimental animals. shRNA reagents are used to bind to and promote the degradation of target RNAs by harnessing an endogenous biological pathway. However the effects of shRNA reagents on silencing target RNAs vary wildly, which are related to the secondary structure of target RNAs [25]. The pSi_625 shows more interference efficiency, which may contribute to its low disruption energy as compared with other two target sequences of candidate shRNAs. In this study, we designed three candidate shRNAs targeting both Livin α and Livin β and found that pSi_625 could inhibit expression of Livin more effectively than other candidate recombinants in HCT-8/V cells with drug resistance phenotype. Then, we assessed chemosensitivity and apoptosis of HCT-8/V cells that transfected pSi_625.

Exposure of neoplastic cells to a single cytotoxic agent can give rise to resistance to structurally and functionally unrelated agents, this is so called multidrug resistance which is largely caused by P-glycoprotein (P-gp), the adenosine triphosphate binding cassette-transporter [23,24]. HCT-8/V can be maintained in routine medium supplemented VP16, VCR and 5-FU respectively as a result of overexpression of antiapoptotic protein Livin and efflux pump P-gp, as well as other potential drug resistance factors. After silencing Livin mediated by pSi_625, we found that the capacity to drug resistance of HCT-8/V cells to VP16, VCR and 5-FU reduced significantly. The IC50s of HCT-8/V cells in medium containing VP16, VCR and 5-FU decreased to $23.79 \pm 5.42\%$ TDC, $21.15 \pm 9.66\%$ TDC, and $22.31 \pm 6.71\%$ TDC respectively, compared with the untransfected cells. Accordingly, the apoptosis rates of HCT-8/V in medium containing VP16, VCR and 5-FU have an obvious increase in contrast with parent HCT-8/V cells ($P < 0.05$). However, the level of P-gp protein expression still keeps high level after Livin silencing (Fig. 2b). These results suggested that cancer cells with drug resistance phenotype have higher apoptosis threshold caused by various factors such as overexpression of antiapoptotic proteins and P-gp, and disrupting either factor could result in a decrease in chemoresistance of cells. Thus, Livin might play an accomplice role in tumor resistance to chemotherapeutic drugs along with P-gp, although the drug resistance mechanisms of Livin and P-gp are different and interrelationships between Livin and P-gp need a further study.

In summary, our data suggest that a combination of down-regulation of Livin and anticancer drugs can significantly reverse drug resistance phenotype of colon cancer cells. This study sheds light on specific silencing of Livin gene expression, which could be a promising target for further research in clinical chemotherapy of colon cancer and other related cancers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clinbiochem.2010.02.004.

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