The effects of Livin shRNA on the response to cisplatin in HepG2 cells

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Abstract. Hepatocellular carcinoma is a lethal malignancy with poor prognosis, partially due to tumor metastasis, recurrence and resistance to chemo- or radio-therapy. Cisplatin can inhibit cancer cell DNA replication, and is widely used in the clinical treatment of tumors. The present study aimed to generate eukaryotic expression vectors for Livin shRNA and to examine the effects of Livin shRNA on the chemosensitivity of HepG2 hepatocellular carcinoma cells. Eukaryotic expression vectors for Livin shRNA (pSD11-U6/Neo/GFP/Livin) were designed and constructed. The HepG2 hepatocellular carcinoma cell line was transfected with this vector using the liposome method. The expression levels of Livin mRNA and protein were measured by quantitative polymerase chain reaction and western blot analysis. The rate of cell growth inhibition was measured using MTT assay following treatment of the cells with cisplatin (2.0 mg/l). DNA sequencing confirmed that the construction of the eukaryotic expression vector for Livin shRNA had been successful. Transfection of these vectors into HepG2 cells led to a significant reduction in the expression levels of Livin mRNA and protein (P<0.05). Cisplatin treatment was associated with significantly higher rates of cell growth inhibition in HepG2 cells transfected with Livin shRNA compared with those that were not transfected (P<0.05). The vectors constructed in the present study produced effective inhibition of the Livin gene in HepG2 cells and increased the chemosensitivity of hepatocellular carcinoma cells.

Introduction

Hepatocellular carcinoma is a common malignant tumor with poor prognosis. RNA interference (RNAi) can reduce oncogene expression and alter the biology of tumor cells. Therefore, application of RNAi to enhance the chemosensitivity of hepatocellular carcinoma to cisplatin, a commonly used anticancer drug that inhibits cancer cell DNA replication, is a potentially important therapeutic strategy (1).

Livin is a member of the inhibitor of the apoptosis (IAP) family and the baculoviral IAP repeat-containing 7 (*BIRC7*) gene that encodes Livin is located on human chromosome 20q13.3, is 46 kb in length and comprises 7 exons and 6 introns (2). Overexpression of Livin has been observed in certain types of cancer tissues and cell lines (3). This may inhibit the cellular apoptosis induced by several anticancer agents. It is also associated with drug resistance in cancer cells (2,4,5). RNA interference (RNAi) is the phenomenon by which double-stranded RNA molecules homologous to a target gene are processed by the enzyme Dicer to produce active small interfering RNA (siRNA) molecules. siRNAs form a complex called the RNA-induced silencing complex (RISC) that includes endonucleases termed argonaut proteins. The RISC, guided by the siRNA, binds to and specifically cleaves mRNA from the target gene, leading to its degradation. This is one form of post-translational gene silencing (2).

In the present study, a eukaryotic expression vector for Livin was constructed. The use of short hairpin (sh)RNA vectors is a popular approach for delivering siRNA. Using the RNAi approach, Livin expression in the HepG2 hepatocellular carcinoma cells was inhibited and the effects of this on the chemosensitivity of the cells were investigated.

Materials and methods

Primary reagents. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), Opti-MEM, and a Lipofectamine 2000 transfection kit were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). RNAiso Plus, a PrimeScript™ Reverse Transcription (RT) Reagent Kit with gDNA Eraser, and an LA PCR Amplification Kit Version 2.1 were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Radioimmunoprecipitation assay (RIPA) buffer, phenylmethylsulfonyl fluoride (PMSF) protease inhibitor and a BCA-100 protein assay kit were purchased from Genechem Co., Ltd. (Shanghai, China). Monoclonal mouse anti-human antibodies against Livin (catalog no. ab118026) and GAPDH (catalog no. ab8245) were purchased from Abcam (Shanghai, China). Horseradish peroxidase (HRP)-labeled goat anti-mouse IgG antibody

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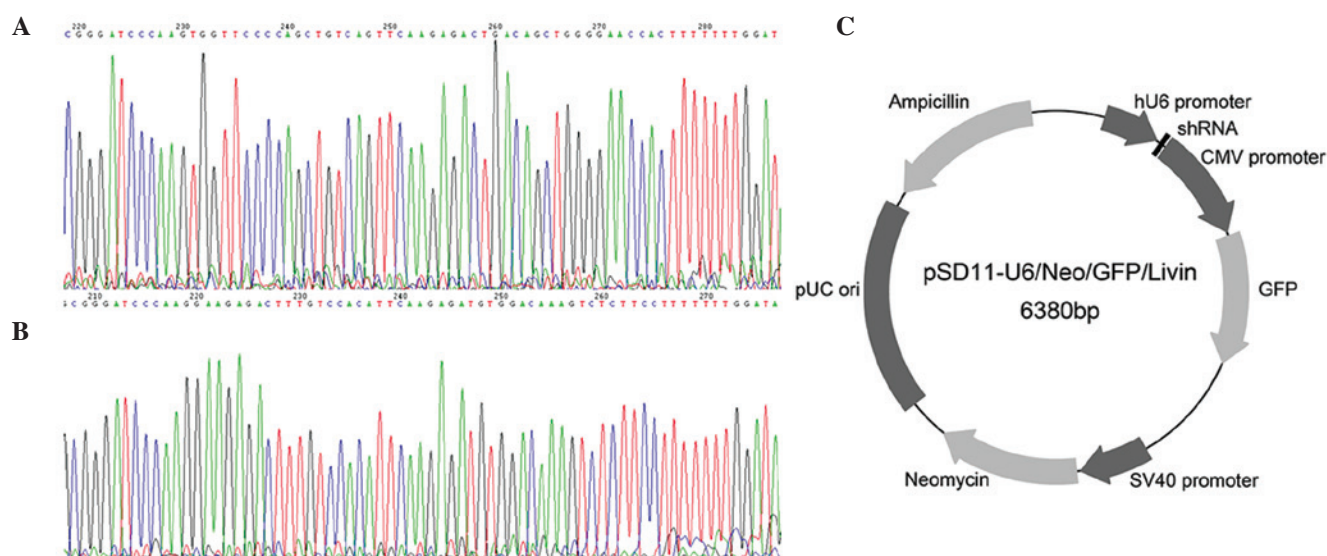E-mail: hchang0721@163.com

Figure 1. Sequencing of recombinant plasmids. (A) Livin 1 and (B) Livin 2. (C) pSD11-U6/Neo/GFP/Livin vector structure. Structure of recombinant plasmid pSD11 (6380 bp). Vector information (in bp): hU6 promoter, 289-616; Polylinker, 617-640; CMV promoter, 671-1259; GFP, 1283-2002; SV40 promoter, 2672-3049; Neomycin, 3077-3871; pUC ori, 4108-5276; Ampicillin, 6233-5373. bp, base pairs.

(catalog no. IH-0041) was purchased from Beijing Dingguo Changsheng Co., Ltd. (Beijing, China). MTT was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. The human hepatocellular carcinoma cell line HepG2 was obtained from the central laboratory of Provincial Hospital Affiliated to Shandong University (Shandong, China). HepG2 cells were seeded at a density of $4x10^5$ cells/well in 6-well plates with DMEM containing 10% FBS in an incubator at 37°C and 5% CO_2. These cells became adherent and 0.25% Trysin-EDTA (Gibco Life Technologies, Carlsbad, CA, USA) was used for passage.

Construction of the Livin shRNA vector. The full-length mRNA sequence of the Livin gene (*BIRC7*; NCBI gene ID, 79444) was identified in the NCBI gene database. Based on shRNA design principles and relevant literature (6), two 19-nucleotide (nt) sequences were selected for use as target sequences for the siRNA vector construction as follows: Livin 1, 5'-GTGGTTCCCCAGCTGTCAG-3' (611-629) and Livin 2, 5'-GGAAGAGACTTTGTCCACA-3' (648-666). BLAST analysis confirmed that the selected sequences were not homologous to other human gene sequences. The sequences were flanked by *Bam*HI and *Hind*III restriction sites at each end. Sense and antisense strands were connected by a 9-nt loop sequence (TTCAAGAGA). Clone construction involved direct annealing ligation; the shRNA coding fragments flanked by restriction sites were annealed and then directly ligated (T4 DNA Ligase; Invitrogen Life Technologies) with the vector prepared by restriction digestion. The structure of the recombinant plasmid pSD11-U6/Neo/ GFP/Livin is presented in Fig. 1. The 2 expression vectors produced in the present study are designated pSD11-Livin1 and pSD11-Livin2. A negative control vector, termed pSD11-NC, was also constructed. It was constructed by re-ligation following the restriction endonuclease digestion. The construction of the Livin shRNA vector was performed

at Shanghai Jikai Biotechnology Co., Ltd. (Shanghai, China) and sequencing was performed at Sangon Biotech Co., Ltd. (Shanghai, China).

Cell transfection. At 24 h prior to transfection, the HepG2 cells were trypsinized (0.25% trypsin in DMEM) and seeded in 6-well plates at a density of $2x10^5$ cells/well. The cells were cultured in DMEM for 24 h, reaching 80% confluence, which was confirmed by inverted microscopy (CKX31 Inverted Microscope, Olympus Corporation, Tokyo, Japan). Cell transfection was performed in accordance with the manufacturer's instructions using the Lipfectamine 2000 kit. The experiments were performed in 4 groups as follows: The Livin 1 group (cells transfected with pSD11-Livin1); the Livin 2 group (cells transfected with pSD11-Livin2); the NC group (cells transfected with pSD11-NC); and the control group (untransfected cells).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). At 48 h following transfection, the total RNA was extracted from cells in each group. RT was performed using the RNAiso Plus kit according to the manufacturer's instructions. The reaction conditions were as follows: 37°C for 15 min and 85°C for 5 sec and then the mix was stored at 4°C. Primer Premier software (version 5.0; http://www.premierbiosoft.com/ primerdesign/index.html) was used to design primers for the fluorescent qPCR. The sequences of the primers used were as follows: Livin, F 5'-CCATCAGCCCCCATTTCT-3' and R 5'-CCATCAGCCCCCATTTCT-3' (the amplicon was 79 bp in length); GAPDH, F 5'-TGCACCACCAACTGCTTAGCA-3' and R 5'-TGCACCACCAACTGCTTAGCA-3' (the amplicon was 87 bp in length). The primers were synthesized by Shanghai Sangon Biotech Co., Ltd. The qPCR was performed on a LightCycler 480 Instrument II using SYBR Green I (Roche Diagnostics, Shanghai, China). The thermal cycling conditions were as follows: Pre-denaturation at 95°C for 30 sec followed by 40 cycles of amplification at 95°C for 5 sec and 60°C for 20 sec. A melting

curve analysis was performed following PCR. To exclude false positive results, PCR for the Livin gene and GAPDH internal reference gene were conducted using blank controls to which no cDNA had been added.

Western blot analysis. At 72 h following transfection, cells were collected from each group. RIPA lysis buffer and PMSF (at a ratio of 100:1) were added to the cell pellets and incubated in an ice bath for 30 min. The samples were centrifuged at 2,000 x g for 30 min at 4˚C. The supernatant was collected and mixed with protein loading buffer (Shenergy Biocolor Bioscience & Technology Company, Shanghai, China) and incubated at 100˚C for 10 min. Protein samples were resolved by 10% polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane (Merck Millipore, Darmstadt, Germany). The membranes were then blocked with Tris-buffered saline and 0.05% Tween-20 (TBS-T) containing 5% skimmed milk powder at 37˚C for 60 min. The mouse anti-human Livin (dilution, 1:1,000) and GAPDH (dilution, 1:3,000) antibodies were added and incubated at 4˚C overnight. The membranes were then washed with TBS-T and incubated with the HRP-labeled goat anti-mouse IgG antibody (dilution, 1:3,000) with shaking at room temperature for 1 h. Following thorough washing with TBS-T, the membranes were immersed in a solution of enhanced chemiluminescence liquid (Immobilon Western Chemiluminescent HRP Substrate, Merck Millipore) and incubated for 5 min at room temperature. The images were visualized using gel imaging equipment; bands from the electrophoresis were analyzed with an AlphaImager® 2200 image analyzer (ProteinSimple Bioscience & Technology Co., Ltd., Shanghai, China).

MTT assay. One day prior to transfection, HepG2 cells were seeded in 96-well plates at a density of 5×10^5 cells/well. Grouping was as described above and each group consisted of 5 replicates. In addition, a blank group was set up containing HepG2 cells only and no added reagents. The transfection procedure was performed using the Lipofectamine 2000 kit, according to the manufacturer's instructions. At 48 h following transfection, DMEM containing 10% FBS and 2.0 mg/l cisplatin (central laboratory of Provincial Hospital Affiliated to Shandong University) was added to the cells. The cells were cultured for an additional 24, 36 or 48 h. The culture medium was removed and replaced with 20 μl MTT (5 mg/ml) solution at the time points indicated. The cells were incubated at 37˚C with 5% CO_2 for 4 h and then 150 μl dimethyl sulfoxide was added to each well and oscillated for 10 min. The optical density (OD) was measured using a Microplate Reader (DNM-9602; Nanjing Perlove Eadial-Video Equipment Co., Ltd., Nanjing, China) at 490 nm. The rate of cell growth inhibition (IR) was calculated using the following formula: IR = (1 - $OD_{Experimental}$/OD_{Blank}) x 100.

Statistical analysis. SPSS statistical software, version 15.0 (SPSS, Inc., Chicago, IL, USA) was used for analysis. Data are expressed as the mean ± standard error. The statistical significance was evaluated by a one-way analysis of variance with Tukey's test. P<0.05 was considered to indicate a statistically significant difference.
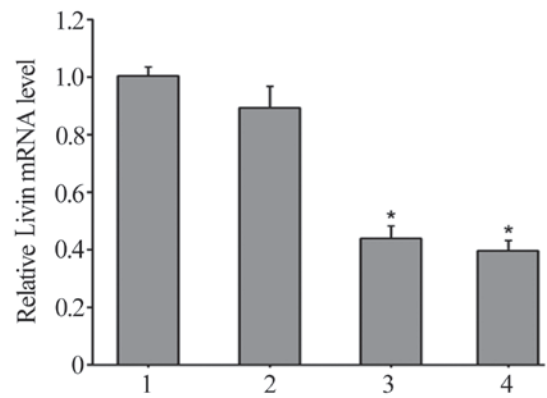

Figure 2. The relative Livin mRNA expression in HepG2 cells following transfection with Livin shRNA expression vectors, was quantified by RT-qPCR. 1, control group (untransfected cells); 2, NC group (cells transfected with pSD11-NC); 3, Livin 1 group (cells transfected with pSD11-Livin1); 4, Livin 2 group (cells transfected with pSD11-Livin2). *P<0.05 vs. the NC and control groups.

Results

Verification of construction of recombinant plasmid. DNA sequencing confirmed that the shRNA coding sequences were correctly inserted into the plasmid vector pSD11-U6/Neo/GFP and that recombinant plasmids pSD-11-Livin1 and pSD11-Livin2 were as designed. Thus, the recombinant plasmids were successfully prepared.

HepG2 cellular mRNA expression changes following transfection. RT-qPCR was performed to quantify the changes in Livin mRNA expression levels. The melting curve for the Livin PCR product peaked at 89.9˚C, and the melting curve for the GAPDH PCR product peaked at 88.1˚C. The PCR products produced a single peak, excluding the possibility of non-specific amplification. The negative control group without cDNA did not produce a melting curve, excluding the possibility of false positive results. GAPDH served as an internal reference gene and relative quantification (Livak method) (7) was used to calculate the relative expression level, presented as ΔΔCt. The results demonstrated that 48 h following transfection, the relative levels of Livin mRNA expression in HepG2 cells in the Livin 1, Livin 2, NC and control groups were 0.44±0.04, 0.40±0.03, 0.90±0.07 and 1.00±0.03, respectively (Fig. 2). The expression levels in the Livin 1 and Livin 2 groups were significantly lower than those of the NC and control groups (P<0.05). These results indicated that pSD11-Livin1 and pSD-11-Livin2 transfection in HepG2 cells specifically reduced the expression levels of Livin mRNA (Fig. 2).

Changes in Livin protein expression in HepG2 cells following transfection. Bands from the western blots were analyzed using an Alpha Imager 2200. The results of this analysis demonstrated that 72 h following transfection, the relative expression levels of Livin protein in the HepG2 cells in the Livin 1, Livin 2 and NC groups were 0.53±0.03, 0.46±0.07, and 0.90±0.06, respectively relative to the control group, which was set to 1. There were significantly lower protein levels in the Livin 1 and Livin 2 groups compared

Table I. Rate of cell growth inhibition (%) by cisplatin in HepG2 cells following transfection with Livin shRNA expression vectors at different time points.

Group	Percentage cell growth inhibition at different time points		
	24 h	36 h	48 h
Control	6.12±0.88	14.75±1.10	23.03±1.07
NC	6.51±1.03	15.34±1.11	23.59±1.32
Livin 1	15.87±0.84[a]	28.00±0.85[a]	36.76±1.20[a]
Livin 2	16.57±1.03[a]	28.10±0.96[a]	37.10±1.48[a]

[a]$P<0.05$ vs. the NC and control groups. Control, untransfected cells; NC, cells transfected with pSD11-NC; Livin 1, cells transfected with pSD11-Livin1; Livin 2, cells transfected with pSD11-Livin2.
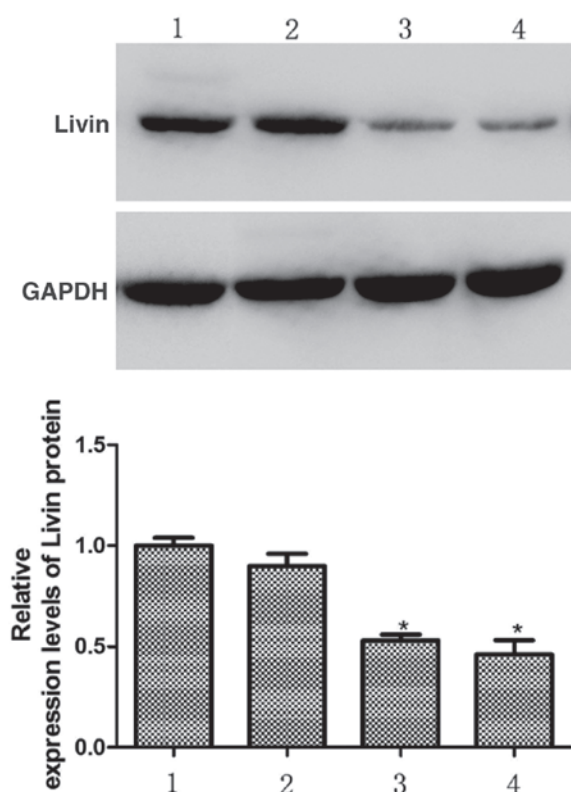

Figure 3. The relative Livin protein expression in HepG2 cells following transfection with Livin shRNA expression vectors was examined by western blot analysis. 1, control group (untransfected cells); 2, NC group (cells transfected with pSD11-NC); 3, Livin 1 group (cells transfected with pSD11-Livin1); 4, Livin 2 group (cells transfected with pSD11-Livin2). *$P<0.05$ vs. NC and control groups.

with the NC and control groups ($P<0.05$). These results indicate that pSD-11-Livin1 and pSD11-Livin2 transfection in HepG2 cells specifically downregulates the expression of Livin protein (Fig. 3).

Inhibition of HepG2 cell growth by cisplatin. The results of the MTT assay demonstrated that at a cisplatin concentration of 2 mg/l, each group exhibited various degrees of growth inhibition. This was recorded at 24, 36 and 48 h, and the rate of inhibition gradually increased over time. The rates of cell growth inhibition in the Livin 1 and Livin 2

groups were significantly higher than in the NC and control groups ($P<0.05$; Table I). No statistically significant differences were identified between the Livin 1 and Livin 2 groups, or between the NC and control groups (Table I).

Discussion

Hepatocellular carcinoma is a common type of malignant tumor in gastroenterological clinical practice. The efficacy of traditional therapeutics for hepatocellular carcinoma, including surgical resection and chemotherapy, is often limited and the disease is difficult to cure; this may be related to the presence of chronic liver conditions, such as hepatitis and cirrhosis. The relationship between changes in gene expression in cancer cells and their chemosensitivity has become an important part of anticancer research (8,9). RNAi provides considerable potential in this line of research. Ashhab *et al* (10) described 6 approaches for introducing siRNAs into mammalian cells. Of these approaches, the design and introduction of shRNA expression vectors into mammalian cells that produce siRNA against a target gene allows relatively stable and specific RNAi without any of the effects of directly introducing siRNA into cells. This strategy also permits inhibition of multiple variants of a single gene or a sequence shared among multiple genes (11).

The post-transcriptional processing of the Livin gene forms 2 types of mature Livin mRNA, Livin α and Livin β, which contain 1,351 and 1,297 nts, respectively. The difference between these 2 types of mRNA is a fragment of 54 nts; the rest of the sequence is the same. Livin α and β encode a protein with the required structure for anti-apoptotic function, a baculovirus IAP repeat (BIR) domain. Although the 2 proteins differ in 18 amino acid residues in this domain, their functions are essentially similar; to bind caspase via the BIR domain, inhibiting its activity and thus inhibiting cancer cell mitochondrial apoptosis (12,13). A number of studies have demonstrated that the overexpression of Livin in certain types of cancer tissues and cell lines can inhibit the apoptosis induced by multiple anticancer agents (14,15). Other previous studies have demonstrated Livin overexpression to be associated with drug resistance in cancer cells (16-18). Previous studies have also produced shRNA expression vectors and used them to stably silence the Livin gene in human bladder cancer T24 cells (19), HeLa cervical cancer (20) and Lovo colon cancer (3) cell lines. The impact of shRNA expression vectors on cancer cell

growth and apoptosis has also been studied. Liang *et al* (21) demonstrated that Livin expression in HepG2 cells increased significantly following the addition of a chemotherapeutic agent, contributing to resistance to chemotherapy. To the best of our knowledge, there have not yet been any studies on the use of RNAi to reduce Livin expression in HepG2 hepatocellular carcinoma cells, or the effects on chemosensitivity.

In the present study, 2 target sequences were selected from the sequence shared by Livin α and β mRNAs, and were used to construct shRNA eukaryotic vectors. Following transfection of the hepatocellular carcinoma cell line HepG2 with these vectors, the expression levels of Livin mRNA and protein were evaluated with RT-qPCR and western blot analysis. The results demonstrated that Livin expression levels were downregulated in HepG2 cells transfected with pSD11-Livin1 and pSD-11-Livin2. The differences from cells transfected with pSD11-NC and control cells were statistically significant, indicating the effectiveness of the selected target sequences. Further experiments were performed on HepG2 cells 48 h following Livin transfection. These cells were treated with 2.0 mg/l cisplatin for various lengths of time and then an MTT assay was performed to determine the absorbance (OD values) and to calculate the rates of cell growth inhibition. The results demonstrated that the cell growth inhibition rate attributable to cisplatin in HepG2 cells transfected with pSD11-Livin1 and pSD11-Livin2 were significantly higher than those in cells transfected with pSD11-NC and untransfected control cells. The present study also indicated that transfection with an shRNA eukaryotic expression vector against Livin mRNA may inhibit Livin gene expression and effectively increase the chemosensitivity of hepatocellular carcinoma cells. The present study provides novel experimental evidence that manipulating the expression of the Livin gene in hepatocellular carcinoma cells can affect their chemosensitivity.

It has been previously demonstrated that chemotherapeutic agents affect the changes made by Livin gene silencing on apoptotic pathways in cancer cells (21,22). Crnkovic-Mertens *et al* (6) demonstrated that in HeLa cells treated with Livin shRNA and a reduced dose of chemotherapeutic agent, Livin gene expression reduced and caspase-3 expression increased significantly. Quintieri *et al* (23) stated that the activation of caspases is key to the cancer cell apoptosis that is induced by chemotherapeutic agents, and that the reduction in caspase activity caused by the factors in cancer cells is a notable cause of the reduction in their chemosensitivity. In the present study, Livin gene expression was increased in hepatocellular carcinoma cells, which may alter the apoptotic pathways, leading to the inhibition of apoptosis induced by chemotherapeutic agents. The combined use of chemotherapeutic agents and RNAi technology to investigate the changes in apoptotic pathways in cancer cells warrants further study. The potential results may aid in the improvement of canonical chemotherapeutic agents and the development of novel adjuvant anticancer drugs.

Acknowledgements

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