Inhibition of livin expression suppresses cell proliferation and enhances chemosensitivity to cisplatin in human lung adenocarcinoma cells

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Received March 21, 2014; Accepted December 3, 2014

DOI: 10.3892/mmr.2015.3372

Abstract. Livin is a novel member of the inhibitor of apoptosis protein family that has been reported to be overexpressed in various types of human malignancy. Although several studies have demonstrated that livin may be used as an effective target for tumor therapy, few studies have investigated its role in human lung adenocarcinoma. In the present study, two different methods were used in order to investigate the tumor-suppressing effect of livin in human lung adenocarcinoma cells. Firstly, small interfering (si)RNA technology was used to down regulate livin expression; siRNA-mediated knockdown of livin was confirmed using reverse transcription quantitative polymerase chain reaction and western blot analysis, and cell proliferations was assessed using an MTT assay *in vitro*. Secondly, inhibition of livin expression was induced through the synergistic inhibitory effect between flavopiridol and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Experimental results revealed that, following transfection of the livin gene-silencing vector, the gene expression of livin was markedly decreased, SPC-A1 cell proliferation was significantly reduced and the therapeutic effect of the chemotherapy drug cisplatin was markedly improved. This growth inhibitory effect was also observed in the flavopiridol and TRAIL combination treatment group. In the flavopiridol and TRAIL combination treatment group, the protein expression of livin was significantly reduced and the survival rate of SPC-A1 cells was significantly lower than the flavopiridol and TRAIL single operation group. In conclusion, the RNA silencing and the synergistic inhibitory effect between flavopiridol with TRAIL was able to effectively inhibit the expression of livin, significantly decrease SPC-A1 tumor cell proliferation and significantly enhance sensitivity to the chemotherapy drug cisplatin. These findings suggest that livin may be used as a novel target for tumor gene therapy.

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Key words: livin, RNA interference, flavopiridol, tumor necrosis factor-related apoptosis-inducing ligand, cisplatin, SPC-A1 cells

Introduction

Lung cancer is the leading cause of cancer-associated mortality among males and the second leading cause among females worldwide (1). The incidence of lung cancer is increasing in females worldwide and is the main cause of cancer-associated mortality among females in Europe and the USA, exceeding breast and cervical cancer (2-10). Lung adenocarcinoma is currently the most common pathological type of lung cancer (11,12) and is the primary type of lung cancer in females, adolescents and non-smokers.

Livin, a novel member of the inhibitor of apoptosis (IAP) protein family, is not detected in the majority of normal tissues, however, is highly expressed in various types of human malignancy (13-30). Increased activity of this protein may be used as a reliable prognostic factor for initial and late resistance to chemotherapeutic drugs in certain types of human tumor (31-37). Inhibition of livin gene expression may effectively promote tumor cell apoptosis and raise the sensitivity of tumor cells to different treatments *in vitro* (35,36). Although several studies have demonstrated that livin may be used as an effective target for tumor therapy (38-40), few studies have focused on human lung adenocarcinoma. Therefore, the present study aimed to investigate the treatment effect of livin expression inhibition in lung adenocarcinoma.

In the present study, two different methods were used to investigate the tumor-suppressing effect of livin in human lung adenocarcinoma. Firstly, small interfering (si)RNA technology was used to downregulate livin expression, which was confirmed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. In addition, cell proliferation was assessed using an MTT assay *in vitro*. Secondly, inhibition of livin expression was induced through the synergistic inhibitory effect between flavopiridol and tumor necrosis factor-related apoptosis-inducing ligand

(TRAIL). Furthermore, the effect of the inhibition of livin expression on SPC-A1 tumor cell proliferation and sensitivity to the chemotherapy drug cisplatin was investigated. The combination of chemotherapy and downregulation of livin expression may contribute to the treatment of human lung adenocarcinoma drug-resistant tumor cells.

Materials and methods

Materials. The human lung adenocarcinoma SPC-A1 cell line was acquired from Nanjing KeyGen Biotech., Co., Ltd. (Nanjing, China). The primers for livin siRNA and control siRNA were synthesized by Beijing Genomics Institute (Beijing, China). All antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Drugs and reagents. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco-BRL (Carlsbad, CA, USA). TRIzol reagent and Lipofectamine 2000 were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Reverse Transcriptase SYBR Green Master mixture was acquired from Takara Bio, Inc. (Otsu, Japan). The bicinchoninic acid protein assay kit and ECL-Plus kit were purchased from Thermo Scientific (Rockford, IL, USA). The MTT cell proliferation assay kit was purchased from Sangon Biotech (Shanghai, China) and TRAIL was purchased from Merck Millipore (Darmstadt, Germany). In addition, flavopiridol was obtained from Sigma-Aldrich (St. Louis, MO, USA) and Z-VAD-FMK was purchased from R&D Systems (Minneapolis, MN, USA).

Cell culture and transfection. SPC-A1 cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS. All were placed in a humidified incubator, containing 5% CO_2 at 37˚C. SPC-A1 cells were replated at $2x10^5$ cells/well in six-well plates once they had reached exponential phase. When the cell density reached 40-50%, cells were transfected with Lipofectamine 2000 with a pcDNA3.1 expression vector (Invitrogen Life Technologies) and cultured at 37˚C and 5% CO_2 for 24 h. The clone in which the livin-siRNA was transfected was termed the livin-siRNA group, the group transfected with the negative control vector was termed the negative control (NC) group and SPC-A1 cells were termed the control (CON) group. The following RNA silencing sequences were used: siRNA-livin790, forward 5'-GAGAGGUCCAGUCUGAAAG-3' and reverse 5'-CUUUCAGACUGGACCUCUC-3' and siRNA-livin180, forward 5'-CCUAAAGACAGUGCCAAGU-3' and reverse 5'-ACUUGGCACUGUCUUUAGG-3'.

RT-qPCR. Total RNA was isolated with TRIzol reagent and reverse-transcribed to synthesize cDNA. cDNA was subsequently amplified by SYBR-Green based qPCR using the following primers: Livin, forward 5'-GGAGAGAGGTCCAGTCTGAAAGT-3' and reverse 5'-ACCTTGCACGTCCTCTCCTC-3' and Homosapiens histone acetyltransferase (HBOA), forward 5'-ATCAAAGAAATCAGTCAGGAGACG-3' and reverse 5'-CTCTTTGGCTATCCACTCATCAAT-3'. The 25 μl fluorescent PCR reaction mix contained: 8.5 μl ddH_2O, 12.5 μl 2X SYBR Premix Ex Taq II, 2 μl cDNA and 1.0 μl forward and reverse primers (10 μmol/l), respectively. The cycling parameters were as follows: 40 cycles, including denaturation at 95˚C for 5 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec. Melting curve analysis was used to confirm the primer specificity. The comparative cycle threshold (Ct) method was used for calculation of livin mRNA expression. The calculation methods were as follows: ΔCt=Ct (Livin) - Ct (HBOA), ΔΔCt=ΔCt (Treatment) - ΔCt (Control). ΔΔCt mean values were compared between groups. The relative mRNA expression was expressed in $2^{-\Delta\Delta Ct}$.

Western blot analysis. SPC-A1 cells were harvested after 48 h of transfection and extracted using lysis buffer. Cell extracts were separated on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Separated protein bands were electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Finally, 5% skimmed milk powder was used to block the PVDF membranes for 1 h. The membrane was incubated overnight at 4˚C with a 1:100 dilution of mouse monoclonal anti-livin primary antibody (sc-166390; Santa Cruz Biotechnology, Inc.). The following day, goat anti-mouse Immunoglobulin G horesradish peroxidase-conjugated secondary antibodies (sc-2005; Santa Cruz Biotechnology, Inc.) were added at a dilution of 1:2,000 and the mixture was incubated for 2 h at room temperature. PVDF membranes were washed in phosphate-buffered saline four times. The ECL-Plus kit was used to visualize the immunoreactive bands. Relative protein level was normalized by β-actin concentration. Three separate experiments were performed in duplicate for each treatment.

Cell proliferation assay. Cell viability was assessed using an MTT assay. Cells were cultured in 24 well plates at a concentration of $5x10^4$ cells per well and allowed to adhere. SPC-A1 cells were termed the CON group. The group transfected with the negative control vector was termed the NC group. The group transfected with the livin-siRNA vector was termed the livin-siRNA group. The group initially transfected with livin-siRNA and then treated with 1.2 μg/ml cisplatin was termed the livin-siRNA+cisplatin group. The FP group was treated with 100 nmol/l flavopiridol and the T group was treated with 100 ng/ml TRAIL. The cisplatin treatment group was treated with 1.2 μg/ml cisplatin. The F+T treatment group was treated with 100 nmol/l flavopiridol and 100 ng/ml TRAIL. The FP+T+cisplatin treatment group was treated with 100 nmol/l FP, 100 ng/ml TRAIL and 1.2 μg/ml cisplatin. The Z-VAD-FMK group was treated with flavopiridol, 50 μmol/l Z-VAD-FMK and TRAIL. Following treatment for 24 h, 100 μl MTT (0.5 mg/ml) was added to the cells and the mixture was incubated for 4 h at 37˚C. Subsequently, the supernatant was removed, dimethyl sulfoxide was used to dissolve the resultant formazan crystals and the absorbance value was read at 570 nm using a Smartspec 3000 spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) was used for the data analysis. All experiments were repeated three times and data are presented as the mean ± standard deviation. Differences between groups were analyzed using

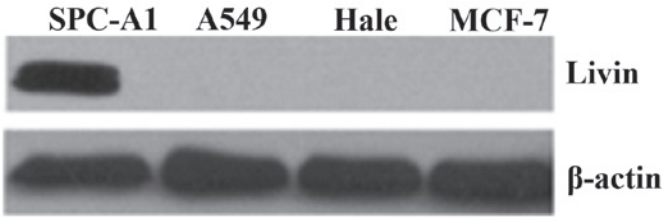

Figure 1. Protein expression of livin in human SPC-A1 cells. The protein expression of livin was investigated by western blot analysis in four cell lines (SPC-A1, A549, Hale and MCF-7). A relatively high protein expression of livin was observed in SPC-A1 cells.

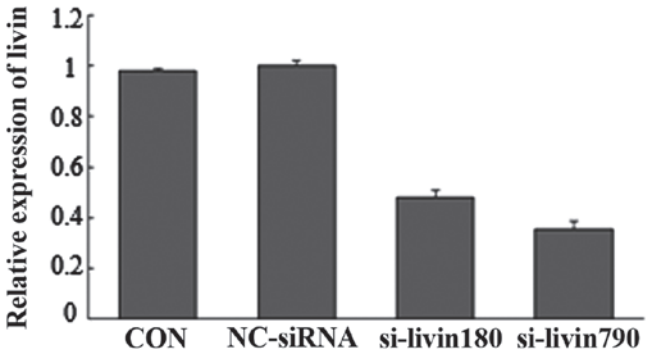

Figure 2. Silencing of livin gene expression by RNAi in SPC-A1 cells. The knockdown efficiency of two candidate siRNAs (si-livin180 and si-livin790) was evaluated using reverse transcription-quantitative polymerase chain reaction. The mRNA expression of livin in cells transfected with si-livin790 and si-livin180 were significantly decreased to 52 and 64.4%, respectively. NC, negative control; CON, control.

one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Protein expression of livin in the human SPC-A1 cell line. The protein expression of livin was investigated using western blot analysis in several cell lines (SPC-A1, A549, Hale and MCF-7). As shown in Fig. 1, livin protein expression was only observed in SPC-A1 cells.

Silencing of livin gene expression by RNAi in SPC-A1 cells. The knockdown efficiency of two candidate siRNA (siRNA-livin180 and siRNA-livin790) was evaluated using RT-qPCR. The results revealed that the livin mRNA level in cells transfected with siRNA-livin790 and siRNA-livin180 were significantly decreased to 52 and 64.4%, respectively (Fig. 2). The most significant silencing effect was observed with siRNA-livin790, therefore, the siRNA-livin790 transfection group was used in the follow-up experiment.

Synergy between flavopiridol and TRAIL affects livin expression. The synergistic inhibitory effect between flavopiridol and TRAIL on livin protein expression was evaluated by western blot analysis. Compared with either flavopiridol or TRAIL alone, combining flavopiridol with TRAIL significantly decreased the protein expression level of livin (Fig. 3). No significant difference was identified between flavopiridol and TRAIL treatment groups.

Effects of inhibitor Z-VAD-FMK on livin protein expression. The result of western blot analysis demonstrated that the

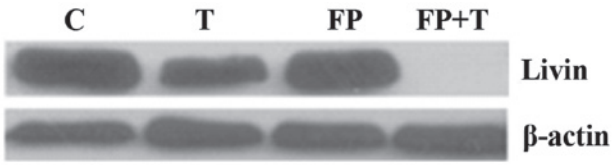

Figure 3. Synergistic inhibitory effect between flavopiridol and TRAIL on the protein expression of livin. The synergistic inhibitory effects between flavopiridol and TRAIL on livin protein expression were evaluated by western blot analysis. Compared with either flavopiridol or TRAIL alone, combining flavopiridol with TRAIL significantly decreased the protein expression level of livin. No significant difference was identified between flavopiridol and TRAIL treatment groups. C, control group; FP, flavopiridol treatment group; T, TRAIL treatment group; FP+T, flavopiridol with TRAIL treatment group; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

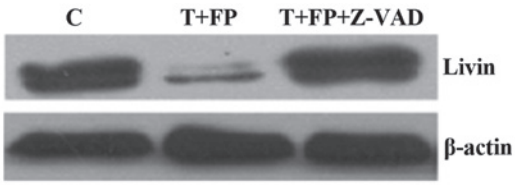

Figure 4. Effect of the caspase inhibitor Z-VAD-FMK on livin protein expression. The effects of caspase inhibitor Z-VAD-FMK on livin protein expression were evaluated by western blot analysis. The synergistic inhibitory effects between flavopiridol and TRAIL were able to effectively inhibit the protein expression of livin, while the caspase inhibitor Z-VAD-FMK reversed this inhibitory effect. C, control group; T+FP, flavopiridol with TRAIL treatment group; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

synergistic inhibitory effect between flavopiridol and TRAIL may effectively inhibit the protein expression of livin, while the caspase inhibitor Z-VAD-FMK reversed this inhibitory

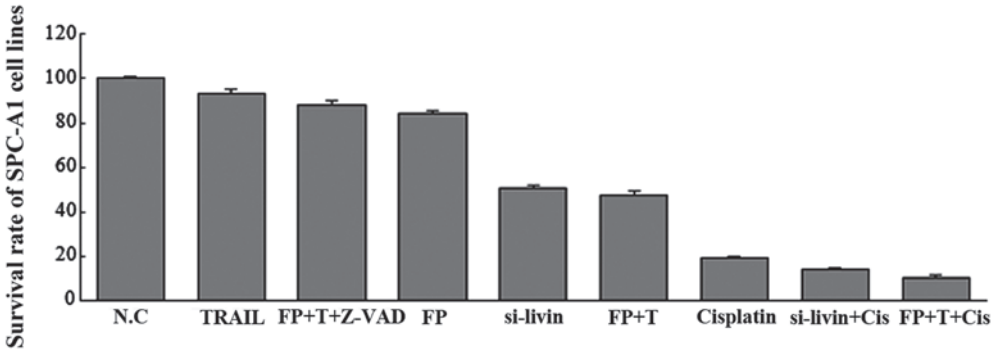

Figure 5. Effect of livin inhibition on cell proliferation. An MTT assay was used to investigate the proliferative activities of SPC-A1 cells. Data was normalized to the control group. Suppression of livin resulted in a significant decrease in the proliferation rate of SPC-A1 cells and enhanced sensitivity to the chemotherapy drug cisplatin. FP, flavopiridol treatment group; T, TRAIL treatment group; FP+T, flavopiridol with TRAIL treatment group; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

effect. As shown in Fig. 4, expression of livin protein in the T+FP+Z-VAD treatment group was significantly higher than the T+FP group and no significant difference was identified between the control group and T+FP+Z-VAD treatment group.

Effect of livin inhibition on cell proliferation. To elucidate the effect of livin inhibition on tumor growth in SPC-A1 cells, an MTT assay was used to evaluate the tumor cell proliferative activities. The survival rate of each group was as follows: Flavopiridol group, 84.30±1.34%; TRAIL group, 93.40±1.56%; F+T combination group, 48.02±1.35%; siRNA-livin group, 50.88±1.14%; cisplatin group, 19.30±0.89%; siRNA-livin+cisplatin group, 14.37±0.81%; FP+T+cisplatin group, 10.86±0.87% and the Z-VAD-FMK group, 88.16±1.64%. The data were normalized to the control group (Fig. 5). As illustrated in Fig. 5, suppression of livin resulted in a significant decrease in the proliferation rate of SPC-A1 cells at 48 h and this suppression effect unexpectedly enhanced the sensitivity of cells to the chemotherapy drug cisplatin.

Discussion

The anti-apoptotic effect of the IAP family is possibly regulated through inhibition of the caspase signaling cascade, inhibiting the cell death receptor tumor necrosis factor receptor-mediated apoptosis signaling pathway and interaction with nuclear factor-κB. The IAP family has several family members, including x-linked inhibitor of apoptosis protein, baculoviral IAP repeat-containing protein 1 and 3, cellular IAP1, Apollon (Bruce), IAP-like protein-1, Survivin and livin. The structure of IAP family members is highly conserved, containing Cys/His baculovirus IAP repeats and a COOH-terminal ring finger, which has E3 ubiquitin ligase activity and is critical in the regulation of proliferation and apoptosis (41).

Livin, a novel member of the IAP family, is important in apoptosis, cell proliferation and cell cycle control (20). It has been demonstrated to be expressed in transformed cells and multiple types of malignant tumor, including neuroblastoma (20) as well as carcinomas of the bladder (21,22), lung (23), nasopharynx (24), kidney (25), liver (26), colon/rectum (27), skin (28), bone (29) and stomach (30). Silencing livin leads to apoptosis induction, cell cycle arrest and proliferation inhibition in malignant tumor cells (42-50).

The present study investigated the clinical significance of livin in SPC-A1 cells and examined the potential of using RNA interference to knock down livin expression, including the subsequent effects on tumor growth in SPC-A1 cells *in vitro*. The present results demonstrated that, following transfection of the livin gene-silencing vector, the expression of the livin gene was significantly decreased, SPC-A1 cell proliferation was significantly reduced and the therapeutic effect of the chemotherapy drug cisplatin was markedly improved. The livin-mediated signaling pathway remains to be elucidated, although a number of studies have demonstrated that silencing livin promotes tumor cell apoptosis by regulating mitomycin, tumor necrosis factor-α, caspase-3 and caspase-9 (45) and mediates gastric tumor cell invasion via MAPK signaling (51). The livin-mediated signaling pathway requires further investigation.

TRAIL, as a member of the tumor necrosis factor gene superfamily, selectively induces apoptosis in numerous transformed cells, excluding normal cells. The synergistic inhibitory effect between TRAIL and chemotherapy drugs has been examined in ovarian carcinoma (52), the combination resulted in increased sensitivity to TRAIL, promoted induction of apoptosis, reduced drug dosage and decreased normal tissue toxicity. Through mediating cell cycle arrest and apoptosis in breast cancer cells, flavopiridol has made important contributions to increasing drug effectiveness and identifying new drug targets (53).

The synergistic inhibitory effect between flavopiridol and TRAIL has been demonstrated in SPC-A1 cells (54). The present study aims to further confirm this synergistic inhibitory effect on apoptosis promotion and examine whether this combination may enhance chemosensitivity. The current results revealed that the expression of livin protein was significantly reduced in the flavopiridol and TRAIL combination treatment group. The survival rate of SPC-A1 cells in the combination treatment group was significantly compared the groups treated with flavopiridol or TRAIL alone. Additionally, the survival rate was lowest in the group treated with a combination of flavopiridol, TRAIL and cisplatin.

In conclusion, the RNA silencing and the synergistic inhibitory effect between flavopiridol with TRAIL was able

to effectively inhibit the expression of livin, significantly decrease SPC-A1 tumor cell proliferation and significantly enhance sensitivity to the chemotherapy drug cisplatin. These findings suggest that livin may be used as a novel target for tumor gene therapy.

Acknowledgements

This study was supported by a grant from the Natural Science Foundation of Yunnan Province (grant no. 2010ZC131).

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