Expression and prognostic significance of Livin in gastric cancer

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Abstract. Livin is one of the most important members of the inhibitor of apoptosis protein family. It is overexpressed in several types of tumors and may have prognostic significance. The present study investigated the biological role of Livin in the oncogenic behavior of gastric cancer cells, the expression of Livin in gastric cancer tissue and the relationship of its expression with various clinicopathological parameters and patient survival. Small interfering RNA blocked Livin gene expression in AGS and SNU638 human gastric cancer cell lines. The expression of Livin was investigated in gastric cancer tissues by RT-PCR, western blotting and immunohistochemistry. The associations with various clinicopathological parameters and survival were analyzed. Livin knockdown inhibited tumor cell migration, invasion and proliferation in AGS and SNU638 cells. Livin knockdown induced apoptosis by activating caspase-3, caspase-7 and PARP. Livin knockdown induced cell cycle arrest by a decrease in cyclin D1, cyclin-dependent kinase 4 and 6 and an increase in expression of p21 and p27. The ERK1/2 and JNK signaling pathways were inhibited by Livin knockdown. Livin expression was upregulated in gastric cancer tissues at the mRNA and protein levels. However, no significant correlation was found between Livin expression and various clinicopathological parameters including survival. In conclusion, Livin expression may be important in the alteration of invasive and oncogenic phenotypes of gastric cancer cells. The prognostic relevance of Livin remains unclear.

Introduction

Gastric cancer (GC) incidence and mortality have decreased. Yet, GC remains one of the major causes of cancer-related mortality worldwide (1). Despite advances in diagnosis and treatment, the 5-year survival rate of GC is only 20%, and progressive behavior including invasion and metastasis remain major contributors to GC-related morbidity and mortality (1). The progression of GC is a complex, multistep process involving multiple genetic and epigenetic alterations of oncogenes, tumor-suppressor genes, DNA repair genes, cell cycle regulators and signaling molecules (2,3).

Apoptosis is an active mechanism of cell death controlling many biologic events, including embryonic development, differentiation and morphogenesis of tissues (4,5). Normal tissue homeostasis requires a regulated balance between cell proliferation and cell death (4,5). Loss of apoptosis regulation can lead to a variety of diseases including cancer. Increased resistance to apoptosis is an important hallmark for the growth of many cancer cell types (6-8).

The inhibitor of apoptosis protein (IAP) family consists of a group of intracellular proteins that are essential for the regulation of apoptosis (9). IAPs bind directly and potentially inhibit a complex array of cysteine aspartyl-specific proteases, caspase-3, -7 and -9, which are responsible for apoptosis and which are induced by diverse pro-apoptotic stimuli (9). Livin is one of the potent members of the IAP family; it is undetectable in most normal tissues but is upregulated in a wide variety of human cancers (10-13). Livin promotes the invasion, growth and apoptotic resistance in a variety of human cancer cells (10-13). Livin overexpression is associated with poor prognosis and resistance to radiotherapy and chemotherapy in several types of human cancers (14-17). These findings have raised interest in Livin as a potential novel therapeutic target for the treatment of human cancers.

While still not completely resolved, several studies have revealed various aspects of the biologic significance of Livin in human GC (18-20). Livin is overexpressed in GC tissues, when compared to its expression in normal gastric tissues adjacent to cancer and benign gastric lesions, and its overexpression has been associated with various prognostic variables (19,20). Silencing of the Livin gene in human GC cells was reported to induce apoptosis and render the cells more susceptible to chemotherapeutic agents (20).

The present study applied small interfering RNA (siRNA) targeting of the Livin gene to investigate the effect of Livin knockdown on biologic behavior of human GC cell lines. In addition, Livin expression was investigated in a well-defined series of GC cases with long-term follow-up, with a focus on patient survival.

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Key words: Livin, apoptosis, prognosis, gastric neoplasm

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Materials and methods

Patients and tissue samples. Twenty GC tissues and paired normal gastric tissues were collected by endoscopic biopsy at Chonnam National University Hwasun Hospital (Jeonnam, Korea) for use in the preparation of mRNA and protein. For immunohistochemistry, tumor specimens were collected from 149 consecutive patients who underwent surgery between January 1998 and December 1999. None of the patients had received preoperative radiotherapy or chemotherapy. All underwent primary tumor resection with regional lymph node dissection. Formalin-fixed and paraffin-embedded tissue blocks were selected by viewing the original pathologic slides and choosing blocks that showed the junction between normal gastric epithelium and the tumor. The histologic grade was classified according to previously established criteria (21,22). Tumor staging used the American Joint Committee on Cancer (AJCC) staging system (23). Patient characteristics including gender, age at time of surgery, tumor size, stage, survival and follow-up information were obtained from hospital records and, when necessary, by contact with attending pathologists and physicians. The observation time was the interval between the time of surgery and last contact (death or last follow-up). The study group comprised 101 males and 48 females, with a mean ± standard deviation (SD) age of 58.0±11.1 years (range, 25-83 years). The mean ± SD size of the tumors was 4.5±2.9 cm (range, 0.2-20.0 cm). The mean follow-up period was 100.1 months (range, 0-176.4 months). All specimens were collected following the informed consent of patients. The study was approved by the Ethics Committee of Chonnam National University Hwasun Hospital.

Cell culture and siRNA transfection. Cell lines derived from AGS and SNU638 human GC cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (both from Hyclone, Logan, UT, USA) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in 5% CO2 at 37°C. Gene knockdown was performed using the specific siRNA. Livin siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and scramble siRNA (Qiagen, Valencia, CA, USA) were transfected with Lipofectamine® RNAiMAX (Invitrogen, Carlsbad, CA, USA) for 48 h according to the manufacturer's recommendations.

Reverse transcription-polymerase chain reaction (RT-PCR). RNA isolation was performed using TRIzol reagent (Invitrogen) following the instructions provided by the manufacturer. Reverse transcription was carried out using 1 µg of RNA and MMLV transcription reagents (invitrogen) according to the manufacturer's recommendations. The amplification of specific DNA was performed with Taq polymerase and specific primers for Livin (5'-CACACAGGCCATCAGGACAAG-3'/5'-ACGGCACAAGAGCATGGGAC-3'), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5'-ACCACAGTCATGACATCAC-3'/5'-TCCACACCCCTGTTGCTGTA-3', as an internal control).

Western blotting. Total cell extracts were prepared using Pierce® RIPA buffer (Thermo Scientific, Rockford, IL, USA) with Halt™ protease and phosphatase inhibitor cocktail (Thermo Scientific). Total cell extracts were separated on polyacrylamide gels and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The specific proteins were blotted with the primary antibody to Livin, Survivin and β-tubulin (Santa Cruz Biotechnology, Inc.); extracellular signal-regulated kinase (ERK), phospho-ERK, p38, phospho-p38, c-Jun NH2-terminal kinase (JNK), phospho-JNK, cleaved caspase-3, -7, -9, cleaved poly(ADP-ribose) polymerase (PARP), cyclin-dependent kinase 4 (CDK4), CDK6, cyclin D1, cyclin D3, cyclin B1, p21, p27, p57, p15 and p16 (Cell Signaling Technology, Danvers, MA, USA). The membranes were developed using the enhanced chemiluminescence detection system, horseradish peroxidase substrate (Millipore) and a model LS-4000 luminescent image analyzer (FujiFilm, Tokyo, Japan).

Cell invasion assay. The invasive ability was calculated as the number of cells passing through the gelatin-coated Transwell filter chambers (Corning, NY, USA). Viable cells (2x105) in 0.2% bovine serum albumin (BSA) were seeded in the upper chambers of Transwell units. Human plasma fibronectin (Calbiochem, La Jolla, CA, USA) as a chemoattractant was added to 0.2% BSA located in the lower chambers of the units. After 24 h of incubation in a 5% CO2, humidified incubator, the cells on the upper surface of each filter were carefully removed with a cotton swab, and cells that had traversed the filter to invade the opposite surface of the filter were stained with Diff-Quik (Sysmex, Kobe, Japan). The number of invaded cells was determined in five random fields using light microscopy, and the mean value was calculated from data obtained from three separate chambers.

Cell migration assay. Cell migration was determined using the Culture-Inserts (2x0.22 cm2; Ibidi, Regensburg, Germany). To create a wound gap, cells were seeded on the Culture-Inserts, which were gently removed using sterile tweezers following a 24-h incubation. The progression of wound closure was photographed using an inverted microscope. The distance between gaps was normalized to 1 cm after capture of three random sites.

Cell viability. Cell viability was determined by the EZ-CyTox (tetrazolium salts, WST-1) cell viability assay kit (Daelil Lab Service Co., Seoul, Korea). After application of WST-1 reagent at 37°C at determined times, cell viability was measured using an Infinite M200 microplate reader with Magellan V6 data analysis software (both from Tecan, Grödig, Austria). All assays were performed three times in sets of three replicate wells.

Flow cytometric analysis. For Annexin V staining, live cells were washed in phosphate-buffered saline (PBS) and then incubated with Annexin V fluorescein isothiocyanate (FITC; R&D Systems, Minneapolis, MN, USA). For cell cycle analysis, cells were incubated in 10 µg/ml ribonuclease A (Sigma-Aldrich) and 50 µg/ml propidium iodide (PI) at room temperature in the dark. BD Cell Quest® v3.3 (Becton-Dickinson, San Jose, CA, USA) and WinMDI v2.9 (The Scripps Research Institute, San Diego, CA, USA) were
used to analyze the population of Annexin V-positive cells and sub-G1 phase.

**Immunohistochemistry.** Paraffin tissue sections from patients were rehydrated through descending ethanol and were retrieved with citrate buffer (pH 6.0). Thereafter, endogenous peroxidase activity was quenched using Peroxidase-Blocking Solution (Dako, Carpinteria, CA, USA) and the tissues were incubated with polyclonal rabbit anti-human Livin in primary Diluent Solution (Invitrogen) overnight at 4°C. After washing, antibody binding was visualized using a Dako Real™ Envision horseradish peroxidase/3,3'-diaminobenzidine detection system (Dako). Stained tissues were photographed using a light microscope.

**Evaluation of Livin expression.** Assessment of immunostained specimens was performed independently by two observers without knowledge of the clinicalopathological data. In the event of a discrepancy, a consensus was reached after further evaluation. The intensity of positive cancer cells was graded on a 4-point scale: 0, no staining of cancer cells; 1, weak staining; 2, moderate staining; and 3, strong staining. The percentage of staining of cancer cells was rated on a 4-grade scale: 0, none; 1, <10%; 2, 10-50%; 3, >50%. The intensity rating was multiplied by the percent staining rating to obtain an overall score. The mean overall score for 149 tumors analyzed was 4.0. This score was chosen as the cut-off point for discrimination of Livin expression (>4, positive expression; ≤4, negative expression).

**Statistical analyses.** For comparison of intergroups, data were derived from at least three independent experiments. The data are presented as means ± SD, and the Student’s t-test was used to determine statistical significance. The χ² test and Fisher’s exact test, where appropriate, were used to compare expression of Livin with various clinicopathological parameters. Actuarial survival rates of patients with positive or negative Livin expression were evaluated according to the Kaplan-Meier method and the differences were tested with a log-rank test. The statistical software program used was Statistical Package for the Social Sciences (SPSS/PC+ 15.0; SPSS, Chicago, IL, USA). A P-value <0.05 indicated a statistically significant difference.

**Results**

**Inhibition of oncogenic behavior of GC cells by Livin siRNA.** We investigated the biologic roles of the Livin gene on oncogenic behavior using siRNA in AGS and SNU638 human GC cell lines. Livin gene expression consistently showed a specific reduction at the mRNA and protein levels in cells transfected with Livin siRNA (Fig. 1). To evaluate whether blocking Livin gene expression affects the oncogenic behavior of GC cells, cell migration, invasion and proliferation were assayed. The artificial wound gap in plates of the scramble siRNA-transfected AGS cells was significantly narrower than that of the Livin siRNA-transfected AGS cells at 12 h (P=0.049) (Fig. 2A).

Transfection of Livin siRNA inhibited AGS and SNU638 cell invasion from 1145.8±562.5 and 799.2±243.3 invaded cells/field (scramble siRNA) to 286.0±175.8 and 119.2±58.7 invaded cells/field (P=0.017 and P=0.002, respectively) (Fig. 2B). Significant decreases in cell proliferation were observed after 72 h in the Livin siRNA-transfected AGS cells (P=0.022) and SNU638 cells (P=0.009), when compared to the scramble siRNA-transfected cells (Fig. 2C).

**Induction of apoptosis in GC cells by Livin siRNA.** Apoptosis induced by transfection of siRNA was assayed using flow cytometry. The cell apoptotic rate induced by transfection of Livin siRNA was significantly increased, compared with that induced by transfection of the scramble siRNA (19.8 vs. 31.4%) in AGS cells, but Livin knockdown had a minimal influence on SNU638 cell apoptosis (15.7 vs. 21.7%) (Fig. 3A). Next, we investigated the activation of caspases, which are critical mediators of apoptosis. Expression of cleaved caspase-3, and -7 and PARP was upregulated in the AGS and SNU638 cells after transfection with Livin siRNA. The protein level of Survivin was reduced by transfection of Livin siRNA in AGS and SNU638 cells (Fig. 3B).

**Induction of cell cycle arrest in GC cells by Livin siRNA.** Flow cytometry was used to detect whether blocking of Livin gene expression alters cell cycle distribution. Transfection of Livin siRNA resulted in cell cycle arrest in the G0/G1 phase of AGS and SNU638 cells (Fig. 4A). Next, the effects of Livin on various CDK inhibitors (CDKIs), cyclins and CDKs, involved in cell cycle progression were assessed. The cyclin D1, CDK4 and CDK6 protein levels were significantly decreased following transfection of Livin siRNA in AGS and SNU638 cells, the p21 and p27 protein levels were significantly increased, and the p57, p15 and p16 protein levels were not altered in response to Livin knockdown (Fig. 4B).

**Impact of Livin knockdown on the mitogen-activated protein kinase (MAPK) signaling pathway involved in the apoptosis and cell cycle arrest of GC cells.** The effect of Livin on stimulation of MAPK signaling pathways leading to apoptosis and cell cycle arrest in AGS and SNU638 cells was investigated. The phosphorylation levels of ERK1/2 and JNK were...
Figure 2. Blocking of Livin gene expression using small interfering RNA (siRNA) inhibits the migration, invasion and proliferation of gastric cancer cells. (A) Effect of Livin knockdown on cell migration. The wound healing assay using siRNA-transfected cells was performed and graphs indicating cell migration are displayed in terms of the relative healing distances (mean ± SE, n=3; *P<0.05). Cell migration was significantly reduced in the Livin siRNA-transfected AGS cells. (B) Effect of Livin knockdown on cell invasion. The number of invaded cells was fewer in the Livin siRNA-transfected AGS and SNU638 cells than the number in the scramble siRNA-transfected cells. Invading cells were stained, counted and the results between groups are presented as a graph (mean ± SE, n=6; *P<0.05). (C) Effect of Livin knockdown on cell proliferation. Cell proliferation was reduced in the Livin siRNA-transfected AGS and SNU638 cells (mean ± SE, n=3; *P<0.05). SS, scramble siRNA; LS, Livin siRNA.
downregulated in Livin siRNA-transfected AGS and SNU638 cells. The phosphorylation level of p38 was not altered by transfection with Livin siRNA (Fig. 5).

Livin mRNA and protein expression in GC tissues. To confirm the results of the GC cell line studies, the expression of Livin at the mRNA and protein levels was evaluated by RT-PCR and Western blotting in human GC tissues, paired normal gastric mucosa, and metastatic or non-metastatic lymph node tissues of the same patients acquired by endoscopic biopsy and from surgical specimens. In endoscopic biopsy specimens, Livin expression was upregulated in cancer tissues when compared to that in the paired normal mucosa at the mRNA and protein levels (P=0.006 and P=0.040, respectively) (Fig. 6). In surgical specimens, immunohistochemical staining of Livin protein was undetected or only weakly stained in the normal gastric mucosa. Immunohistochemical staining of the GC specimens localized Livin expression to the cancer cells, with no expression evident in the stromal compartment of the cancers (Fig. 7A). Immunohistochemical staining of Livin in metastatic lymph node tissues was significantly stronger than that in non-metastatic lymph node tissues (Fig. 7B). The score for immunohistochemical staining of Livin in metastatic lymph node tissues was significantly higher than that in non-metastatic lymph node tissues (P<0.001) (Fig. 7C).

Correlation between Livin expression and clinicopathological parameters in GCs. To study the prognostic role of Livin in GC progression, we investigated the association between expression of the Livin protein immunohistochemically in formalin-fixed, paraffin-embedded tissue blocks obtained from 149 GC patients and the clinicopathological data, including survival. Expression of Livin protein was detected in 56 of the 149 (37.6%) GCs analyzed (Table I). The correlation between Livin expression and clinicopathological parameters is summarized in Table I. No significant correlation was found between Livin expression and various clinicopathological parameters including age, gender, tumor size, Lauren classification, histologic grade, depth of invasion, lymph node metastasis, distant metastasis, or stage. Analysis of the survival for all patients showed that Livin expression did not correlate with survival (P=0.144) (Fig. 8).
Livin is a recently identified member of the IAP family with a single baculovirus IAP repeat (BIR) domain and a COOH-terminal ring domain, which plays an important role in regulating apoptosis (10-13). Many aspects of classical tumor biology research have been investigated. Proposed hallmarks of cancer cells include sustained proliferative signaling, selection of aggressive subtype of cancer cells, replicative immortality, resistance to cell death and deregulation of cellular energetics (6-8). In the present study, Livin knockdown inhibited tumor cell migration, invasion, proliferation, and induced apoptosis and cell cycle arrest in GC cells. These results suggest that Livin may contribute to GC cell invasion and metastasis.

Livin was previously found to inhibit apoptosis by binding to caspase-3, -7 and -9, and its E3 ubiquitin-ligase activity promotes the degradation of IAP antagonist SMAC/DIABLO (10-13). In the present study, the expression of cleaved caspase-3, -7 and PARP was upregulated in GC cells after Livin knockdown. Therefore, Livin inhibits apoptosis by suppressing the activity of caspases in GC cells.

Regulation of cell cycle progression appears to be achieved principally by activity of cyclins, CDKs and CDKIs at the G1/S and G2/M phase transitions (24,25). Cell proliferation is achieved through the transition of cells from G0/G1 arrest into the active cell cycle (24,25). Dysregulation of cell cycle components may lead to tumor formation. The formation of tumors occurs when genes such as cyclin, CDKs and CDKIs mutate, causing cells to multiply uncontrollably (26-28). In the
Livin knockdown induced cell cycle arrest in the G0/G1 phase by decreasing the expression of cyclin D1, CDK4 and CDK6, and by increasing p21 and p27 expression. Therefore, Livin may contribute to GC progression via cell cycle dysregulation.

Livin was found to have anti-apoptotic potential through the activation of JNK1 or MAPK signaling pathways (29-31). Given this knowledge, we evaluated whether Livin knockdown induces apoptosis and cell cycle arrest in GC cells via the regulation of MAPK signaling pathways. We found that the ERK1/2 and JNK signaling pathways were inhibited by Livin knockdown. These results suggest that Livin may regulate GC cell behavior through the MAPK signaling pathways.

Expression of Livin is pronounced in various human cancer types including GC and has been linked with cancer development and progression (14-20). Appropriately, we evaluated the expression of Livin in GC tissues and paired normal gastric mucosa of the same patients obtained by endoscopic biopsy. Livin expression was significantly upregulated in cancer tissues when compared to its expression in paired normal mucosa at the mRNA and protein levels in fresh endoscopic biopsy specimens, confirming previous findings (19,20). These results suggest that Livin may play an important role in the evolution of gastric carcinogenesis.

Livin expression was significantly upregulated in metastatic lymph node tissues when compared to that in the non-metastatic lymph node tissues in fresh surgical specimens. These results suggest that Livin is associated with GC progression.

Finally, we assessed the expression of Livin and its prognostic relevance in a well-defined series of human GCs with complete clinicopathological data including survival. No significant correlation was found between Livin expression and various clinicopathological parameters including age,
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Gender, tumor size, Lauren classification, histologic grade, depth of invasion, lymph node metastasis, distant metastasis, or tumor stage. Furthermore, Livin expression did not correlate with survival. Previously, Livin expression was found to be associated with poor differentiation and lymph node metastasis (19,20). There are several possible explanations for this discrepancy. First, the overall expression of Livin as reported in different studies is difficult to compare due to different scoring systems and different antibodies used. Second, the discrepancy may reflect, in part, the relatively small sample size. Third, dysfunction of genes is caused by a complex process of genetic mutation, epigenetic alteration, and posttranscriptional modification. Therefore, the expression of Livin as detected by immunohistochemistry does not always imply its functional activity in human cancers. Fourth, the steps involved in cancer development and progression are not dependent on Livin-mediated apoptotic regulation alone and are regulated by many biological processes including growth, angiogenesis and invasion. Further studies are warranted to clarify the impact of Livin on the biologic and prognostic significance in GC.

Taken together, the data support the view that Livin expression may play an important role in the evolution of gastric carcinogenesis. The prognostic relevance of Livin in GC remains unclear.

Acknowledgements

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References


Table 1. Correlation between the Livin expression and the clinicopathological parameters of gastric cancer.

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WD, well-differentiated; MD, moderately differentiated, PD, poorly differentiated; NA, not available.

Figure 8. Kaplan-Meier survival curve correlating overall gastric cancer patient survival according to positive (solid line) and negative expression (dotted line) of Livin (P=0.144).


