SPANDIDOS PUBLICATIONS

ONCOLOGY REPORTS 45: 18, 2021

Inhibitor of apoptosis protein Livin promotes tumor progression and chemoradioresistance in human anaplastic thyroid cancer

HEE KYUNG KIM[1], SUN-AE KIM[2], EUN KYUNG JUNG[2], KYUNG-HWA LEE[3], JOON KYOO LEE[2], HO-CHEOL KANG[1], YOUNG-EUN JOO[1], SANG CHUL LIM[2] and TAE MI YOON[2]

Departments of [1]Internal Medicine, [2]Otorhinolaryngology-Head and Neck Surgery, and [3]Pathology, Chonnam National University Medical School and Hwasun Hospital, Hwasun, Chonnam 58128, Republic of Korea

Received March 4, 2020; Accepted September 8, 2020

DOI: 10.3892/or.2021.7969

Abstract. Anaplastic thyroid cancer (ATC) is characterized by a rapid and aggressive course of progression. Despite significant advances in surgery, radiotherapy and chemotherapy, the disease-specific mortality due to ATC is approximately 100%. New strategies, such as molecular targeted therapies, are imperative for improving survival. Livin, a member of the human inhibitor of apoptosis protein family, has been found to be associated with tumor progression and poor prognosis in various human cancers. The aim of the present study was to evaluate the role of Livin in cancer progression and chemoradioresistance of ATC and to investigate its potential as a therapeutic target. Endogenous Livin expression in the human BHT101 ATC cell line was silenced by Livin-specific small interfering RNA. To assess the impact of Livin on cancer cell behavior in human ATC cells, various methods such as cell invasion, cell viability and cell apoptosis assays were applied. To assess the expression of Livin and the change of apoptosis-related proteins associated with Livin expression, reverse transcription-quantitative PCR and western blotting were performed. Immunohistochemistry was performed to detect Livin protein expression in human ATC tissues. The association between Livin expression and apoptotic/proliferation index was analyzed in human ATC cells. Livin-knockdown suppressed tumor cell invasion; and conversely, it enhanced cell apoptosis, with elevated expression levels of cleaved caspase-3 and -7 and cleaved PARP. Livin-knockdown enhanced radiation-induced apoptosis, while reducing cell viability following radiotherapy, as well as lenvatinib treatment. In addition, human ATC tissues with high Livin-expression exhibited a high Ki-67 labeling index and low apoptotic index. In summary, these findings indicate the contribution of Livin to tumor progression and chemoradioresistance in ATC.

Correspondence to: Dr Tae Mi Yoon, Department of Otorhinolaryngology-Head and Neck Surgery, Chonnam National University Medical School and Hwasun Hospital, 322 Seoyang-Ro, Hwasun, Chonnam 58128, Republic of Korea
E-mail: yoontm@chonnam.ac.kr

Key words: livin protein, drug resistance, neoplasm, apoptosis, molecular targeted therapy, anaplastic thyroid carcinoma

Introduction

The incidence of thyroid cancer has continued to increase in the USA over the past 30 years (1). A Surveillance, Epidemiology, and End Results Program-based study reported that between 1975 and 2009 there was a three-fold increase in incidence rate, from 4.9 to 14.3 per 100,000 individuals in the USA (1). Although the majority of cases (>90%) are those of differentiated thyroid cancer with a favorable prognosis, ~1% of all cases are anaplastic thyroid cancer (ATC) (2). ATC is one of the most aggressive cancers, with a 1-year survival rate of only 5-20% (3,4). Surgical treatment improves the prognosis of ATC (5); however, early diagnosis remains challenging (4). The progression and metastasis of ATC occur early and rapidly, making it likely that the optimal timing for surgery is missed (3-6). Multimodal therapy consisting of surgery, systemic chemotherapy and external beam radiation therapy is required; however, ATC is known to be highly resistant to any form of therapy (6). Targeted kinase inhibitors based on tumor-derived molecular alterations and emerging immunomediated combination therapies were introduced in 2013, but the prognosis continues to be poor (6).

For a tissue to maintain homeostasis, cell growth and death rates must be balanced (7). The inhibition of a cellular death pathway, apoptosis, is a crucial mechanism underlying the development and progression of cancer, which helps cancer cells escape the immune system (8). Furthermore, the inhibition of apoptosis can lead to resistance to chemotherapy or radiation therapy (9). Inhibitors of apoptosis proteins (IAPs) are regulatory proteins that impede tumor cell apoptosis by inhibiting caspases in the apoptosis signaling pathway (10). Several studies have revealed links between members of the IAP family and cancer (10,11). IAPs play an important role in resistance to chemotherapy and radiotherapy (9,12-15); they are characterized by a domain termed the baculoviral IAP repeat (BIR) (9). Livin, a novel IAP family member, contains of a single BIR domain and a COOH-terminal RING finger domain (9,16). Human Livin manifests as two isoforms, Livin α and β, as a result of two alternatively

spliced transcripts (9). Livin is highly expressed in various tumor cells; however, it has minimal/no expression in most normal adult tissues (17,18). Several studies have shown that Livin expression is associated with aggressive disease course, chemoresistance and poor outcome (17,19-22); moreover, therapeutic sensitivity has been shown to be altered following Livin downregulation (23,24). Therefore, Livin may serve as a potential therapeutic target (25). To the best of our knowledge, there is no study to date on Livin expression in human ATC specimens or cell lines. The aim of the present study was to analyze the role of Livin in the ATC cell line and process the clinical data of patients with ATC, thereby investigating its potential as a therapeutic agent.

Materials and methods

Cell culture and transfection. The BHT101 human ATC cell line was provided by Dr Won Gu Kim (Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea). BHT101 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 20% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37˚C. To knock down the endogenous genetic expression of Livin in ATC cells, small interfering RNAs (siRNAs) were used. Seeded in 6-well plates at a density of 2.0×10^5 cells/well, ATC cells were then transfected with 50 μM Livin-specific siRNA (Bioneer Corporation) or 50 μM negative control siRNA (scrambled siRNA; cat. no. 1027281; Qiagen, Inc.) using Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h at 37˚C. Subsequent experiments were performed after 48 h. Livin-specific siRNA sequences were as follows: Sense, 5'-GGA UGG CUU AAC UCU ACC U-3', and antisense, 5'-AGG UAC AGU UAA GCC AUC C-3'.

Protein isolation and western blot analysis. Cells were lysed using radioimmunoprecipitation assay buffer (Biosesang Inc.), following which the bicinchoninic acid assay was performed to measure protein concentrations. Protein lysates (20-30 μg/lane) after 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis were separated and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were then incubated with 5% bovine serum albumin (Bioshop Canada Inc.) in Tris-buffered saline (TBS)-0.5% Tween-20 at room temperature for 1 h. Subsequently, the membrane was washed four times for 15 min each with TBS-0.5% Tween-20. Specific proteins were detected sequentially using primary antibodies against GAPDH (cat. no. sc-25778; Santa Cruz Biotechnology, Inc.), Livin (α, 36 kDa; β, 34 kDa; cat. no. 5471; Cell Signaling Technology, Inc.), cleaved caspase-3 (cat. no. 9664; Cell Signaling Technology, Inc.), caspase-3 (cat. no. 9662; Cell Signaling Technology, Inc.), cleaved caspase-7 (cat. no. 9491; Cell Signaling Technology, Inc.), caspase-7 (cat. no. 9492; Cell Signaling Technology, Inc.), cleaved poly(ADP-ribose) polymerase (PARP; cat. no. 5625; Cell Signaling Technology, Inc.), and PARP (cat. no. 9542; Cell Signaling Technology, Inc.). After diluting the primary antibodies at 1:1,000 in TBS-0.5% Tween-20, they were incubated with the membranes for 24 h at 4˚C. Anti-rabbit (cat. no. 7074; Cell Signaling Technology, Inc.) or anti-mouse (cat. no. 7076, Cell Signaling Technology, Inc.) horseradish peroxidase (HRP)-conjugated secondary antibodies were diluted at 1:2,000. The membranes were incubated with secondary antibodies at room temperature for 2 h. Using an enhanced chemiluminescence detection system for HRP (EMD Millipore), immunoreactive proteins were visualized and analyzed with an LAS-4000 luminescence image analyzer (FUJIFILM Wako Pure Chemical Corporation). All western blot analysis experiments were run independently and in triplicate.

RNA isolation, reverse transcription (RT) semi-quantitative polymerase chain reaction (qPCR) and RT-qPCR. TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for RNA extraction from cells, according to the manufacturer's protocol. RT was performed using 1 μg total RNA, M-MLV reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.), 1 μl 2 mM dNTP mix (Enzynomics Co., Ltd.), 2 μl 0.1 M dithiothreitol (Invitrogen; Thermo Fisher Scientific, Inc.), 4 μl 5X first-strand buffer (Invitrogen; Thermo Fisher Scientific, Inc,), 1 μl RNase inhibitor (Promega Corporation) and 1 μl oligo(dT) (Bioneer Corporation). The resulting cDNA was amplified using primers specific for Livin and GAPDH (Bioneer Corporation). GoTaq DNA Polymerase and 5X Green GoTaq reaction buffer (Promega Corporation) were used for PCR. qPCR was performed for 5 min at 94˚C for one cycle, 30 sec at 94˚C, 20 sec at 58˚C and 30 sec at 72˚C for 32 cycles, and 7 min at 72˚C for one cycle. The primer sequences were as follows: Livin α and β forward, 5'-CAC ACA GGC CAT CAG GAC AAG-3' and reverse, 5'-ACG GCA CAA AGA CGA TGG AC-3'; and GAPDH forward, 5'-ACC ACA GTC CAT GCC ATC AC-3' and reverse, 5'-TCC ACC CTG TTG CTG TA-3'. The polymerase chain reaction products were separated electrophoretically on a 1% agarose gel containing ethidium bromide. Densitometry was performed using WiseUV (WUV-L20; Daihan Scientific Co., Ltd.) and Multigauge V3.2, (Fuji Co., Ltd.).

RT-qPCR analysis was performed using the QuantiSpeed SYBR-Green Kit (cat. no. 105-02; PhileKorea) with the Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science) and confirmed using melting curve analysis. RT, prior to qPCR, was performed as described above. Amplification plots were used to evaluate the quantification cycle (Cq). The sequences of qPCR primers used were as follows: Livin α and β forward, 5'- CAC ACA GGC CAT CAG GAC AAG-3' and reverse, 5'- ACG GCA CAA AGA CGA TGG AC-3'; and 18S rRNA forward, 5'-GTA ACC CGT TGA ACC CCA TT-3' and reverse, 5'-CCA TCC AAT CGG TAG TAG CG-3'. qPCR was performed at 95˚C for 2 min for one cycle, followed by 95˚C for 10 sec and 60˚C for 25 sec for 42 cycles. Each reaction was repeated independently at least three times. The $2^{-\Delta\Delta Cq}$ method was used to determine the mRNA expression levels (26).

Cell invasion assay. The number of cells that migrated through a 8.0-μm pore Transwell invasion apparatus (cat. no. 3422; Costar, Inc.) was used to determine the extent of cell invasion. A day before the experiment, the upper chamber was coated with 1% gelatin solution for 12 h at 37˚C and then dried for 12 h at room temperature. After transfection for

48 h, the upper chamber cells transfected with 50 μM Livin siRNA or 50 μM negative control siRNA were seeded at $2x10^5$ cells in 120 μl 0.2% bovine serum albumin (BioShop Canada, Inc.) with FBS-free DMEM. As the chemoattractant, 400 μl 0.2% bovine serum albumin with FBS-free DMEM containing fibronectin (cat. no. 361635; EMD Millipore) was loaded into the lower chamber. After a 24 h incubation period, cells at the bottom of the Transwell surface were stained with Diff-Quik solution (Sysmex Corporation). Subsequently, the cells were counted in five random microscopic fields of view at x100 magnification using a light microscope. The results are presented as mean ± standard error of the number of cells per field after three individual experiments.

Apoptosis assay. An Annexin V-fluorescein isothiocyanate (FITC) assay was performed to assess apoptosis. Cells were transfected with either 50 μM Livin siRNA or 50 μM negative control siRNA for 48 h. After 48 h of transfection, the cells were collected following trypsinization, washed twice in phosphate buffered saline, and resuspended in binding buffer (BD Biosciences). After the addition of Annexin V-FITC and 7-amino-actinomycin D (BD Biosciences), the cells were incubated in the dark for 15 min and then resuspended in 400 ml binding buffer. A FACS Calibur flow cytometer (BD Biosciences) and BD Cell Quest version 3.3 software (Becton-Dickinson) were used for cell analysis. The data analysis was executed using WinMDI version 2.9 (The Scripps Research Institute). All apoptosis assay experiments were run in triplicate and independently.

Cell viability assay. After 48 h of transfection, cells seeded in 24-well plates ($1x10^4$ cells/well) were transfected the following day with either 50 μM Livin siRNA or 50 μM negative control siRNA. Cell viability was measured after incubation for 48 h using an EZ-CyTox (tetrazolium salts, WST-1) enhanced cell viability assay kit (cat. no. EZ-3000; Daeil Lab, Inc.) for 1-2 h at 37˚C. The absorbance was read at 460 nm using a microplate reader. All cell viability assay experiments were run in triplicate and independently.

Cell irradiation or lenvatinib treatment. After 48 h of transfection, cells were cultured at 37˚C and treated with γ-irradiation at various doses (5, 10, 20, 30 and 40 Gy) (^{137}Cs, 2.875 Gy/min) using a Gammacell 3000 Elan (Therathronics) at room temperature. A stock solution of lenvatinib (4 mg/ml; Eisai Co., Ltd.) was dissolved in dimethyl sulfoxide (27,28) and diluted at various concentrations (5, 10, 20, 40, 80 and 160 μM) for 24 h at 37˚C for experimental use.

Patients and tumor specimens. Paraffin-embedded tissue sections from 25 patients who underwent surgery for definitive ATC at Chonnam National University Hwasun Hospital (Jeonnam, Korea) between September 2005 and December 2015, were collected to evaluate Livin protein expression. A total of 6 men and 17 women, with a mean age of 73.0±10.7 years (range, 37-85 years), were enrolled in the present study. To ensure the diagnostic accuracy of ATC, two pathologists reviewed the pathological slides of all enrolled patients independently. Two patients were excluded because they were diagnosed with carcinoma showing thymus-like differentiation rather than ATC. All ATC tumor tissues were obtained before either radiotherapy or chemotherapy. All 23 enrolled patients were treated with definitive surgery (total thyroidectomy, 20 patients; subtotal thyroidectomy, 2 patients; hemothyroidectomy, 1 patient), among them, 16 were followed up with adjuvant radiotherapy or concurrent chemoradiotherapy. Hospital records were reviewed thoroughly for clinicopathological characteristics and complete medical history. The date of starting treatment until the date of death or the date of last follow-up was calculated as the survival duration (months).

Immunohistochemistry. Tissue processing and immunohistochemical analysis were performed according to a previously described method (29). The tissue sections were incubated with 1:100-diluted primary antibodies against Livin (cat. no. 5471; Cell Signaling Technology, Inc.) in antibody diluent reagent solution (cat. no. 003118; Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h at 4˚C. The staining results were interpreted by two independent observers without any knowledge of the associated clinical records. Some patients had both ATC and papillary thyroid carcinoma (PTC) in the thyroid gland. Since the Livin staining status may be different in ATC or PTC tissues, in these cases the staining results were interpreted according to the Livin staining status in the ATC tissue. Scores for staining intensity were as follows: 0, no staining of tumor cells; 1+, weak to comparable staining in the cytoplasm and/or nucleus relative to non-tumor cell staining; and 2+, readily appreciable or dark brown staining distinctly marking the tumor cell cytoplasm and/or nucleus. The percentile of stained cells was scored as follows: 0, 0%; 1, 1-25%; 2, 26-50%; and 3, ≥51%. The products of the intensity and percentile scores were the final intensity scores, which were defined as low Livin expression if ≤2 and as high Livin expression if >2.

Assessment of tumor cell proliferation and apoptosis. Tissue processing and immunohistochemical analysis were performed according to a previously described method (29). The tissue sections were incubated with 1:100-diluted primary antibodies against Ki-67 in antibody diluent reagent solution (cat. no. 003118; Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h at 4˚C. Tumor cell proliferation was visualized using Ki-67 (cat. no. ab16667; Abcam) and a light microscope (magnification, x200). The number of Ki-67-positive nuclei per 1,000 tumor cell nuclei was used to determine the Ki-67 labeling index.

To detect and quantify apoptosis, the DeadEnd™ Colorimetric terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) system (cat. no. G7130; Promega Corporation) was used according to the manufacturer's instructions. TUNEL-positive apoptotic cells exhibit darkly stained nuclei or nuclear fragments with a cytoplasmic halo, as observed with a light microscope (magnification, x200). The number of TUNEL-positive nuclei containing apoptotic bodies among 1,000 tumor cell nuclei was defined as the apoptotic index.

Statistical analysis. An unpaired Student's t-test was used to determine the significance of experimental differences. Data are shown as the mean ± standard error. All experimental

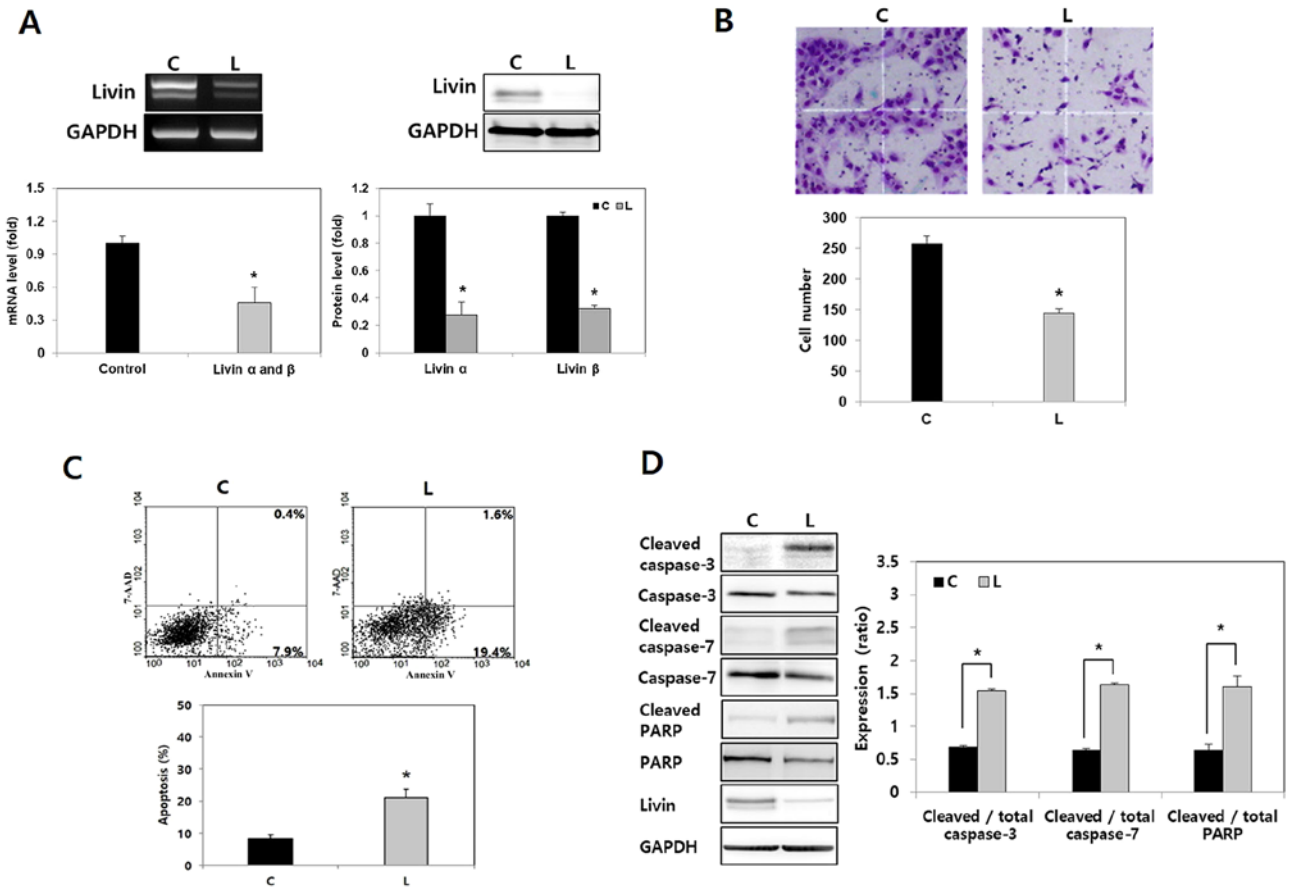

Figure 1. Effect of Livin knockdown on cell invasion and apoptosis in human anaplastic thyroid cancer cells. (A) Compared with negative control siRNA, the mRNA and protein levels of Livin α and Livin β were reduced by Livin siRNA in BHT101 cells, as shown by RT-semi-qPCR, RT-qPCR and western blotting. *P<0.05 vs. C. (B) Compared with negative control cells, significantly fewer Livin knockdown BHT101 cells demonstrated invasion capacity in a cell invasion assay. Magnification, x100. Stained invading cells were counted and presented as the mean ± standard error for three independent experiments. *P<0.05 vs. C. (C) In a cell apoptosis assay, flow cytometry demonstrated that Livin-knockdown BHT101 cells exhibited more apoptosis compared with control cells. *P<0.05 vs. C. (D) Compared with the levels in the control cells, levels of cleaved caspase-3, cleaved caspase-7 and cleaved PARP were higher in Livin-knockdown BHT101 cells. Cleaved proteins were normalized to the corresponding total protein level. *P<0.05. RT, reverse transcription; siRNA, small interfering RNA; C, negative control siRNA-transfected cells; L, Livin-specific siRNA-transfected cells; 7-AAD, 7-amino-actinomycin D; PARP, poly(ADP-ribose)polymerase.

assays were run in triplicate and independently. The survival curves were calculated using the Kaplan-Meier method and assessed with a log-rank test. All analyses were performed using SPSS version 21.0 (IBM, Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

Livin knockdown suppresses tumor cell invasion and enhances tumor cell apoptosis in human ATC cells. In the present study, the role of Livin in tumor progression was investigated using siRNA to inhibit the endogenous expression of Livin in the BHT101 human ATC cells. In the Livin-specific siRNA-treated BHT101 cells, both mRNA and protein levels of Livin α and Livin β were significantly lower than those in the negative control siRNA-treated cells (Fig. 1A). In the cell invasion assay, there were 145.0±6.2 invading Livin-knockdown BHT101 cells compared with 258.2±12.5 invading negative control BHT101 cells (Fig. 1B), which was significantly different (P<0.05).

To evaluate the effect of Livin on apoptosis, an Annexin V apoptosis assay was conducted. The results of the flow cytometric analysis revealed that Livin knockdown significantly increased the proportion of apoptotic cells (P<0.05; Fig. 1C). Next, the expression levels of apoptosis regulatory proteins following transfection with siRNA were evaluated. Levels of cleaved caspase-3, cleaved caspase-7 and cleaved PARP were significantly higher in Livin-knockdown BHT101 cells compared with in the negative control cells (P<0.05; Fig. 1D). These results demonstrated that Livin knockdown leads to tumor cell apoptosis by regulating apoptosis regulatory proteins such as caspase-3, caspase-7 and PARP in human ATC cells.

Livin knockdown enhances radiosensitivity in human ATC cells. It was further examined whether Livin knockdown enhances radiosensitivity by inducing apoptosis in BHT101 cells. Radiation (20 Gy) was applied to cells after transfection with Livin siRNA or negative control siRNA for 48 h. A significantly higher extent or apoptosis was detected with the combination of Livin siRNA and radiation compared with radiation alone (P<0.05; Fig. 2A). Consistently, cleaved caspase-3, cleaved caspase-7 and cleaved PARP levels following radiation treatment were significantly higher in the

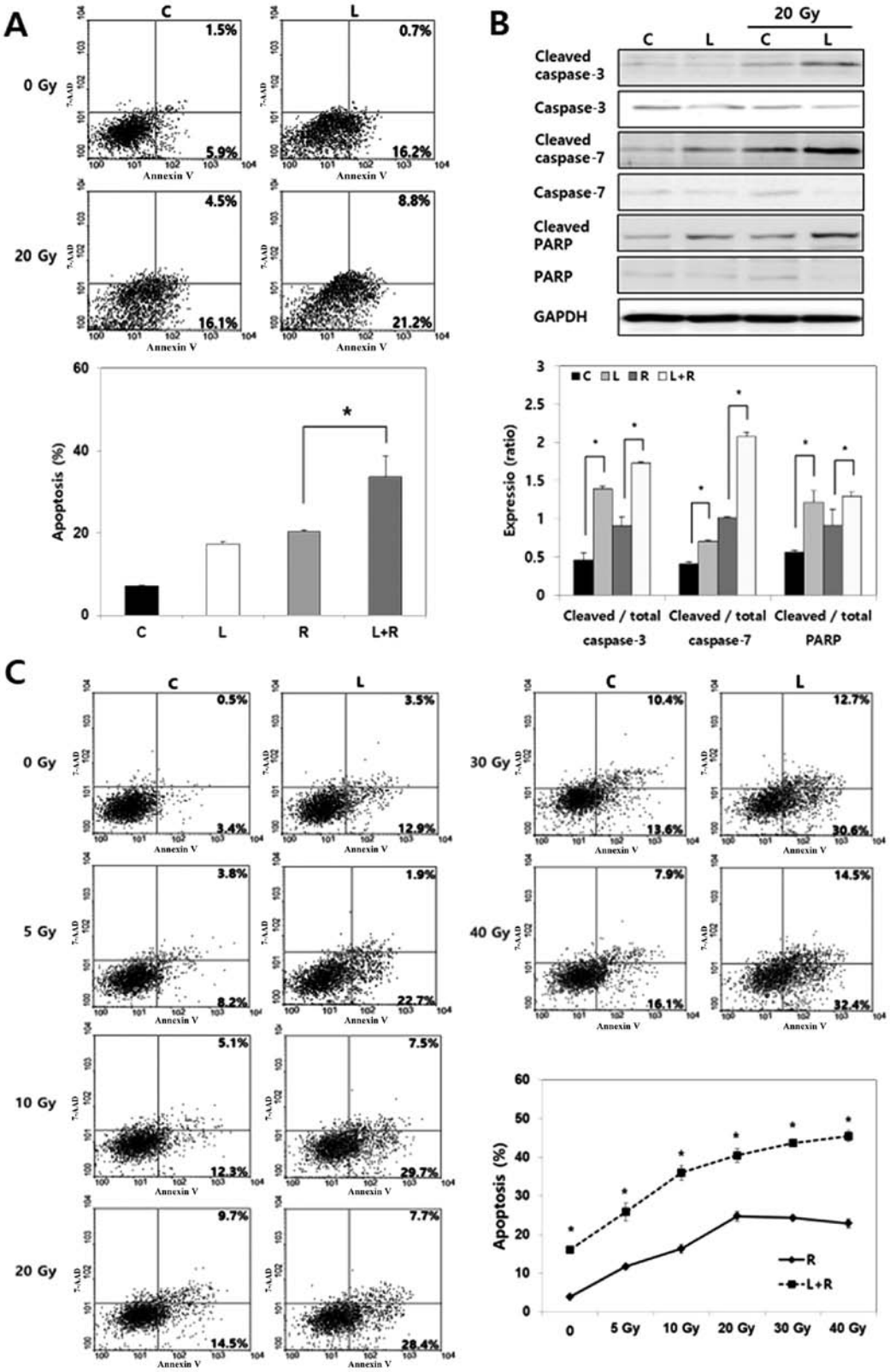

Figure 2. Effect of Livin knockdown on radiosensitivity in human anaplastic thyroid cancer cells. (A) A combination treatment of Livin-knockdown with 20 Gy radiation resulted in significantly more apoptosis of BHT101 cells compared with the control (20 Gy radiation alone). (B) Livin-knockdown cells exhibited greater expression of cleaved caspase-3, cleaved caspase-7 and cleaved PARP compared with control cells (20 Gy radiation alone). Cleaved proteins were normalized to the corresponding total protein level. (C) Under various radiation doses, the combination of Livin knockdown and radiation demonstrated a continuous increase in apoptosis and a larger intergroup difference compared with the control (20 Gy radiation alone). *P<0.05. siRNA, small interfering RNA; C, negative control siRNA transfected cells; L, Livin-specific siRNA-transfected cells; R, radiation, 7-AAD, 7-amino-actinomycin D; PARP, poly(ADP-ribose)polymerase.

Livin-knockdown cells compared with in the control cells (P<0.05; Fig. 2B). The effect of Livin knockdown on apoptosis under various radiation doses was also assessed. Cells treated with radiation alone exhibited radioresistance without any

Table I. Clinicopathological variables of patients with anaplastic thyroid carcinoma (n=23).

Variable	Value
Mean age ± standard deviation (range), years	73.0±10.7 (37.0-85.0)
Sex, n (%)	
Male	6 (26.1)
Female	17 (73.9)
Mean size ± standard deviation (range), cm	5.4±2.9 (0.5-13.0)
Extrathyroidal extension, n (%)	
No	1 (4.3)
Yes	22 (95.7)
PTC component, n (%)	
No	11 (47.8)
Yes	12 (52.2)
Distant metastasis, n (%)	
No	9 (39.1)
Yes	14 (60.9)
Radiotherapy, n (%)	
No	7 (30.4)
Yes	16 (69.6)
Chemotherapy, n (%)	
No	21 (91.3)
Yes	2 (8.7)

PTC, papillary thyroid carcinoma.

further increase in apoptosis under the >20 Gy radiation dose; conversely, the combination of Livin knockdown and radiation resulted in a continuous increase in apoptosis, which was significantly different (P<0.05; Fig. 2C). These findings indicated that a combination of Livin knockdown and radiotherapy enhances radiation-induced apoptosis in human ATC cells.

To determine the effect of Livin knockdown on the cytotoxicity of radiation over time, a cell viability assay was performed with BHT101 cells. Following 20 Gy radiation treatment, the number of viable Livin-knockdown BHT101 cells, calculated by absorbance, was significantly decreased at 48 and 72 h compared with the negative control cells (P<0.05; Fig. 3). As the radiation time increased, the differences in cell viability between the two groups increased. This indicated that Livin knockdown enhances the cytotoxicity of radiotherapy in human ATC cells.

Livin knockdown enhances chemosensitivity of lenvatinib in human ATC cells. It was evaluated whether Livin knockdown enhances chemosensitivity by inducing apoptosis in BHT101 cells. Cells were transfected with Livin siRNA or negative control siRNA for 48 h, following which the cells were treated with lenvatinib (5-80 μM) for 24 h. The combination of Livin siRNA and lenvatinib resulted in a significantly higher level of apoptosis compared with lenvatinib alone at 20 μM

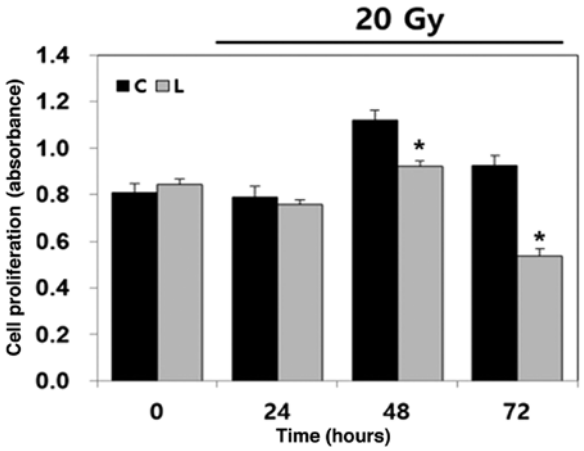

Figure 3. Effect of Livin knockdown on the cytotoxicity of radiation over time in human anaplastic thyroid cancer cells. The number of viable Livin-knockdown BHT101 cells, calculated by absorbance, was significantly lower than that of negative control cells (20 Gy radiation alone) at 48 and 72 h. *P<0.05 vs. C. siRNA, small interfering RNA; C, negative control siRNA-transfected cells; L, Livin-specific siRNA-transfected cells.

(P<0.05; Fig. 4A). Cells treated with lenvatinib alone exhibited chemoresistance with no significant increase in apoptosis was observed even at an increased dose, whereas cells treated with Livin knockdown and lenvatinib showed a continuous increase in apoptosis with increasing doses of lenvatinib. Similarly, the cell viability assay showed that the number of viable Livin-knockdown BHT101 cells, calculated by absorbance, treated with 40 or 80 μM lenvatinib was significantly lower than that of the negative control cells (Fig. 4B). These findings implied that the combination of Livin knockdown and lenvatinib enhances the chemosensitivity of lenvatinib in human ATC cells.

Elevated Livin expression is associated with a high Ki-67 labeling index and low apoptotic index in human ATC tissues. Table I presents the clinicopathological variables of the patients with ATC. The mean size of ATC was 5.4±2.9 cm (range, 0.5-13.0 cm), and 12 patients (52.2%) presented with PTC with ATC. The majority of patients (22/23; 95.7%) had extrathyroidal extension, and 60.9% (14/23) had a distant metastasis at the time of diagnosis. After surgery, 16 patients were treated with adjuvant radiotherapy and 2 patients were treated with adjuvant chemotherapy. Formalin-fixed paraffin-embedded biopsy tissues acquired from 23 patients with ATC were used for immunohistochemical staining to examine Livin protein expression. Livin protein staining demonstrated a heterogenous pattern, with predominantly nuclear and/or cytoplasmic dark brown staining in tumor cells (high expression; Fig. 5A) rather than weak or no staining (low expression; Fig. 5B). According to our grading criteria, 30.4% of patients (7/23) exhibited high Livin expression, while 69.6% of patients (16/23) exhibited low Livin expression. Tumors with high Livin expression had significantly higher Ki-67 labeling and lower apoptotic indexes compared with those with low Livin expression (Table II; P<0.02 and P<0.03, respectively; Fig. 5C and D). These findings indicated that tumors with high Livin expression exhibited higher tumorigenic activity, which potentiated tumor progression in ATC.

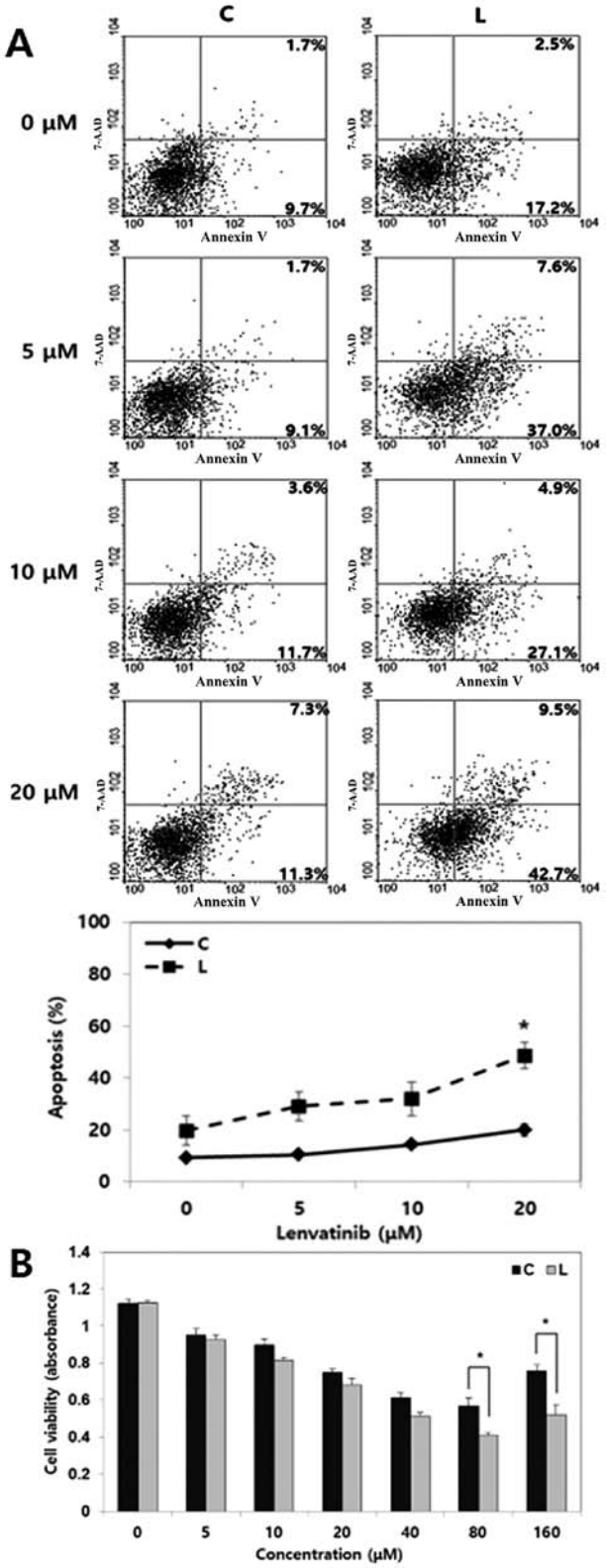

Figure 4. Effect of Livin knockdown on the chemosensitivity of lenvatinib in human anaplastic thyroid cancer cells. (A) In a cell apoptosis assay, a combination treatment of Livin knockdown with lenvatinib resulted in greater apoptosis of BHT101 cells compared with control cells treated with lenvatinib alone. Under various lenvatinib doses, the combination of Livin knockdown and lenvatinib showed a continuous increase in apoptosis than in control cells treated with lenvatinib alone, showing a significant intergroup difference at 20 μM. *P<0.05 vs. C. (B) In a cell viability assay, after applying 40 or 80 μM lenvatinib, the number of viable Livin-knockdown BHT101 cells, calculated by absorbance, was significantly lower than that of negative control cells. *P<0.05. siRNA, small interfering RNA; C, negative control siRNA-transfected cells; L, Livin-specific siRNA-transfected cells.

Table II. Association between Livin expression and proliferation/apoptosis in patients with anaplastic thyroid cancer.

Parameter	Total (n=23)	Livin expression		P-value
		Low (n=16)	High (n=7)	
Ki-67 labeling index[a]	39.3±10.6	35.9±9.6	47.0±8.8	0.02
Apoptotic index[a]	27.4±21.8	32.7±23.5	15.5±10.7	0.03

[a]Data are presented as the mean ± standard deviation.

For the 23 patients with ATC who were enrolled in the present study, the median survival duration was only 2.9 months. Livin expression was not associated with radiotherapy or chemotherapy, and radiotherapy or chemotherapy were not associated with an improved survival (P>0.05; data not shown). Patients with a low Livin expression exhibited a longer median overall survival time compared with patients with high expression (2.9 months in the low-expression group vs. 1.9 months in the high-expression group). However, there was no significant difference in overall survival when analyzed with Kaplan-Meier curves and a log-rank test (P=0.12; Fig. 6).

Discussion

ATC is among the most serious malignant tumors for which no effective treatment currently exists, and multimodal treatment is required (6). Owing to its rarity, most studies on ATC therapy are retrospective (3-5). Therefore, it is difficult to identify the most efficacious treatment regimen for ATC. Several studies have demonstrated that longer survival can be expected in stage IVA and IVB patients, who are eligible for total tumor resection with adjuvant radiotherapy/concurrent chemoradiotherapy (30,31). However, the majority of ATCs exhibit advanced disease progression with regional or systemic metastasis at diagnosis, for which it may be impossible to perform curative surgery. In patients with non-resected ATC, radiation therapy or chemotherapy could be considered. Pezzi *et al* (32) reported improved survival outcome in non-resected ATC patients with radiation therapy with a cumulative dose of >45 Gy. Although conventional chemotherapy involving paclitaxel, docetaxel or doxorubicin is employed, the benefit on survival is only marginal, and the duration of the response is short (33,34). Recently, various tyrosine kinase inhibitors have been approved for cancer treatment. Lenvatinib is a multikinase inhibitor that targets the vascular endothelial growth factor receptor 1-3, fibroblast growth factor receptor 1-4, platelet-derived growth factor receptor-α, and RET and KIT proto-oncogenes (35). Lenvatinib was approved for patients with radioiodine-refractory differentiated thyroid cancer, based on the results of the SELECT trial, which demonstrated marked improvement in progression-free survival and response rate (36). Although the efficacy of lenvatinib in ATC is limited, several studies have shown that it can be employed as a possible treatment for

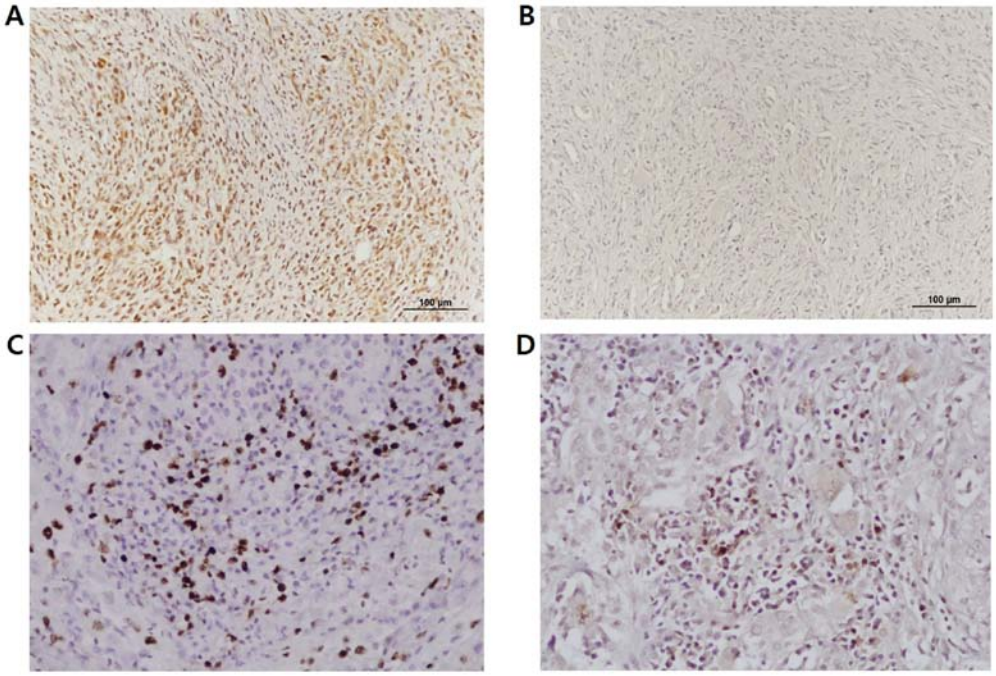

Figure 5. Protein expression of Livin and assessment of tumor cell proliferation/apoptosis in human anaplastic thyroid cancer tissues. Immunostaining of Livin protein revealed (A) dark brown staining distinctly marking the cytoplasm and/or nucleus of the tumor cells (2+ staining intensity) compared with (B) no staining of tumor cells (0 staining intensity). (C) Immunostaining of Ki-67 revealed dark brown staining of nucleus, indicating cell proliferation. (D) The presence of beaded or shrunken chromatin and apoptotic body with a clear halo was examined by TUNEL staining, which indicated apoptotic cells. Magnification, x200.

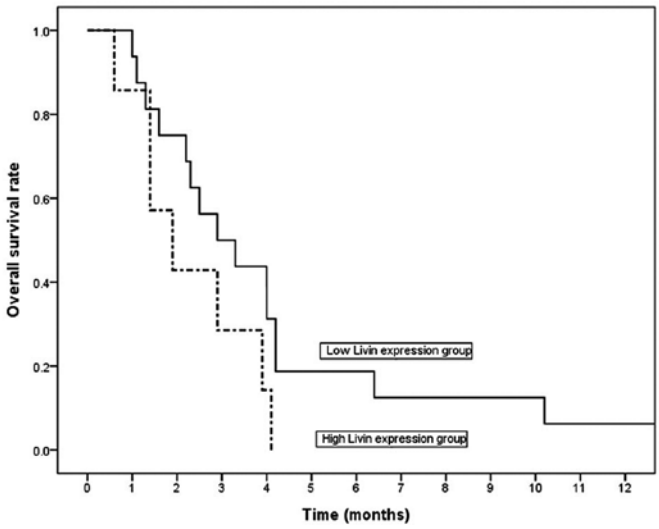

Figure 6. Kaplan-Meier curves of overall survival for patients with anaplastic thyroid cancer according to Livin expression. There was no association between Livin expression and overall survival of patients with anaplastic thyroid cancer (n=23, P=0.12). Solid line, patients with low Livin expression; dotted line, patients with high Livin expression.

ATC. In preclinical human thyroid cancer xenograft models, lenvatinib exhibited significant antitumor activity in five ATC xenografts (35). Several case reports demonstrated its possible efficacy as a therapeutic agent for ATC (37,38). A phase II trial of lenvatinib in 17 ATC patients demonstrated a median progression-free survival of 7.4 months and a median overall survival of 10.6 months with manageable toxicities (39). The objective response rate was 24% and the disease control rate was 94% in the 17 cases. Therefore, the efficacy of lenvatinib in ATC cells was evaluated in the present study.

Apoptosis involves the sensor phase, wherein cell death signals are sensed by monitoring the extra- and intracellular environment, and the effector phase, wherein cell death is caused via mitochondrial apoptosis signaling pathways, which involve the inhibition of downstream caspases (caspase-3, -7 and -9), leading to their inactivation and degradation (7). Resistance to apoptosis can be acquired by cancer cells through a variety of strategies. The IAP group includes structurally related proteins with antiapoptotic potential associated with tumorigenesis and tumor resistance to chemotherapy and radiotherapy (40). IAP family members include NAIP, c-IAP1, c-IAP2, XIAP, survivin, Apollon, ILP-2 and Livin (40). Livin, a newly discovered IAP (41), is not expressed in most normal adult tissues but is expressed in several cancer cell lines (17,18). Similar to other IAP family members, Livin shows an antiapoptotic activity depending on the BIR domain (41). Livin is essential for tumor progression and poor prognosis in several tumors as it regulates the tumor cell sensitivity to chemotherapy and radiotherapy (9,13,14,42-46). Therefore, inactivating Livin may help induce apoptosis and can be considered a treatment modality for certain cancers.

To the best of our knowledge, no previous studies have elucidated the role of Livin in ATC. In the present study, decreased tumor cell invasion and increased apoptosis in Livin-knockdown BHT101 cells were demonstrated. Apoptosis induced by chemotherapy (lenvatinib) and radiotherapy was enhanced in Livin-knockdown BHT101 cells. Furthermore, Livin-knockdown cells demonstrated significantly increased expression of cleaved caspase-3, cleaved caspase-7, and cleaved PARP following radiotherapy. Lenvatinib is a potent angiogenesis inhibitor that targets multiple receptor tyrosine kinases,

including VEGF receptors (35). Targeting tumor angiogenesis can induce nutrient starvation and hypoxia, leading to apoptosis in tumor cells (47). The loss of the effect of suppressing apoptosis during Livin knockdown, appears to strengthen the antitumor effect of lenvatinib and radiotherapy. The obtained results implied that Livin plays a pivotal role in ATC progression and resistance to chemotherapy and radiotherapy.

Several studies have shown that Livin is associated with the aggressive metastasis and prognosis of variable cancers (17,19-22); however, to the best of our knowledge, no studies have been conducted on ATC patients. It is difficult to analyze ATC patients because of the low incidence and short median survival rate. A total of 10,960 patients underwent thyroid surgery between 2005 and 2015 at Chonnam National University Hwasun Hospital (Jeonnam, Korea), of whom only 23 were ATC patients. For those 23 patients, the median survival was only 2.9 months. There was no significant difference in clinical aggressiveness owing to the small number of patients and short survival time; the only differences noted were histological aggressiveness, such as Ki-67 labeling index and apoptotic index, between the high- and low-Livin expression groups. Further studies with a higher number of patients are needed to confirm the differences in clinical characteristics according to Livin expression.

Several studies have identified Livin as a possible diagnostic marker and therapeutic tool for malignant tumors (48-61). El Ali *et al* (48) reported significant differences in the levels of serum anti-Livin antibodies between patients with gastrointestinal cancer and healthy subjects (48). Several studies have also suggested that anti-Livin antibody may be a useful diagnostic marker for a variety of tumors, including those of the gastrointestinal tract, lung and breast (49-51); this also indicates that Livin may be a tumor-associated antigen. Therefore, Livin can serve as a novel immunotherapy target. Zhang *et al* (52) reported that Livin peptide could be used as a novel substitute to trigger cell immunity by loading dendritic cells in combination with chemotherapeutic agents in cases of non-small cell lung cancer. In addition to its potential as a direct immunotherapy agent, prognosis of ATC can be improved by increasing chemosensitivity of ATC cells. The inhibition of apoptosis is one mechanism of chemoresistance because several chemotherapy drugs induce apoptosis (8). As a member of the IAP family, Livin has been shown to cause chemoresistance in various cancers (53-57). Furthermore, several studies have shown that Livin knockdown increases chemosensitivity to multiple anticancer drugs, suggesting its potential as an adjuvant therapeutic agent (58-61).

In conclusion, the present study demonstrated that Livin is positively expressed in ATC and contributes to tumor progression and chemoradioresistance in ATC. However, the current study had some limitations, such as that it was produced using one ATC cell line and that the number of enrolled patients was small. Therefore, further studies including animal studies are needed to support the present results. Although, in summary, the present study suggests the possibility of Livin as a therapeutic target in ATC.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Chonnam National University Hwasun Hospital Institute for Biomedical Science (Hwasun, South Korea; grant no. HCRI 20044) and the National Research Foundation of Korea grant funded by the Korea government (Daejeon, South Korea; grant no. 2020R1F1A1076054).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

HKK and TMY analyzed the data and drafted the manuscript. SAK performed the experimental study. KHL and TMY analyzed the pathological data. TMY and YEJ participated in the design of the study. EKJ, JKL, HCK and SCL contributed to the interpretation of the data. KHL, JKL and SAK revised the manuscript and data including western blotting at the request of reviewers. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Institutional Review Board of Chonnam National University Hwasun Hospital (Hwasun, South Korea; approval no. CNUHH-2020-042). Patients provided written informed consent for the use of resected tissue specimens.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Davies L and Welch HG: Current thyroid cancer trends in the United States. JAMA Otolaryngol Head Neck Surg 140: 317-322, 2014.
2. Sherman SI: Thyroid carcinoma. Lancet 361: 501-511, 2003.
3. Smallridge RC and Copland JA: Anaplastic thyroid carcinoma: Pathogenesis and emerging therapies. Clin Oncol (R Coll Radiol) 22: 486-497, 2010.
4. Kim TY, Kim KW, Jung TS, Kim JM, Kim SW, Chung KW, Kim EY, Gong G, Oh YL, Cho SY, *et al*: Prognostic factors for Korean patients with anaplastic thyroid carcinoma. Head Neck 29: 765-772, 2007.
5. Pierie JP, Muzikansky A, Gaz RD, Faquin WC and Ott MJ: The effect of surgery and radiotherapy on outcome of anaplastic thyroid carcinoma. Ann Surg Oncol 9: 57-64, 2002.
6. Smallridge RC, Ain KB, Asa SL, Bible KC, Brierley JD, Burman KD, Kebebew E, Lee NY, Nikiforov YE, Rosenthal MS, *et al*; American Thyroid Association Anaplastic Thyroid Cancer Guidelines Taskforce: American Thyroid Association guidelines for management of patients with anaplastic thyroid cancer. Thyroid 22: 1104-1139, 2012.
7. Hanahan D and Weinberg RA: The hallmarks of cancer. Cell 100: 57-70, 2000.

8. Igney FH and Krammer PH: Death and anti-death: Tumour resistance to apoptosis. Nat Rev Cancer 2: 277-288, 2002.
9. Liu B, Han M, Wen JK and Wang L: Livin/ML-IAP as a new target for cancer treatment. Cancer Lett 250: 168-176, 2007.
10. Sanna MG, da Silva Correia J, Ducrey O, Lee J, Nomoto K, Schrantz N, Deveraux QL and Ulevitch RJ: IAP suppression of apoptosis involves distinct mechanisms: The TAK1/JNK1 signaling cascade and caspase inhibition. Mol Cell Biol 22: 1754-1766, 2002.
11. Allen SM, Florell SR, Hanks AN, Alexander A, Diedrich MJ, Altieri DC and Grossman D: Survivin expression in mouse skin prevents papilloma regression and promotes chemical-induced tumor progression. Cancer Res 63: 567-572, 2003.
12. Wang L, Zhang Q, Liu B, Han M and Shan B: Challenge and promise: Roles for Livin in progression and therapy of cancer. Mol Cancer Ther 7: 3661-3669, 2008.
13. Chang H and Schimmer AD: Livin/melanoma inhibitor of apoptosis protein as a potential therapeutic target for the treatment of malignancy. Mol Cancer Ther 6: 24-30, 2007.
14. Yan B: Research progress on Livin protein: An inhibitor of apoptosis. Mol Cell Biochem 357: 39-45, 2011.
15. Sun JG, Liao RX, Zhang SX, Duan YZ, Zhuo WL, Wang XX, Wang ZX, Li DZ and Chen ZT: Role of inhibitor of apoptosis protein Livin in radiation resistance in nonsmall cell lung cancer. Cancer Biother Radiopharm 26: 585-592, 2011.
16. Yoon TM, Kim SA, Lee DH, Lee JK, Park YL, Lee KH, Chung IJ, Joo YE and Lim SC: Livin enhances chemoresistance in head and neck squamous cell carcinoma. Oncol Rep 37: 3667-3673, 2017.
17. Tanabe H, Yagihashi A, Tsuji N, Shijubo Y, Abe S and Watanabe N: Expression of survivin mRNA and livin mRNA in non-small-cell lung cancer. Lung Cancer 46: 299-304, 2004.
18. Augello C, Caruso L, Maggioni M, Donadon M, Montorsi M, Santambrogio R, Torzilli G, Vaira V, Pellegrini C, Roncalli M, *et al*: Inhibitors of apoptosis proteins (IAPs) expression and their prognostic significance in hepatocellular carcinoma. BMC Cancer 9: 125, 2009.
19. Gazzaniga P, Gradilone A, Giuliani L, Gandini O, Silvestri I, Nofroni I, Saccani G, Frati L and Aglianò AM: Expression and prognostic significance of LIVIN, SURVIVIN and other apoptosis-related genes in the progression of superficial bladder cancer. Ann Oncol 14: 85-90, 2003.
20. Li CJ, Cong Y, Liu XZ, Zhou X, Shi X, Wu SJ, Zhou GX and Lu M: Research progress on the livin gene and osteosarcomas. Asian Pac J Cancer Prev 15: 8577-8579, 2014.
21. Xiang Y, Yao H, Wang S, Hong M, He J, Cao S, Min H, Song E and Guo X: Prognostic value of Survivin and Livin in nasopharyngeal carcinoma. Laryngoscope 116: 126-130, 2006.
22. Sun K, Liao Q, Chen Z, Chen T and Zhang J: Expression of Livin and PlGF in human osteosarcoma is associated with tumor progression and clinical outcome. Oncol Lett 16: 4953-4960, 2018.
23. Wang R, Lin F, Wang X, Gao P, Dong K, Zou AM, Cheng SY, Wei SH and Zhang HZ: Silencing Livin gene expression to inhibit proliferation and enhance chemosensitivity in tumor cells. Cancer Gene Ther 15: 402-412, 2008.
24. Liu S, Li X, Li Q, Liu H, Shi Y, Zhuo H, Li C and Zhu H: Silencing Livin improved the sensitivity of colon cancer cells to 5-fluorouracil by regulating crosstalk between apoptosis and autophagy. Oncol Lett 15: 7707-7715, 2018.
25. Miura K, Fujibuchi W, Ishida K, Naitoh T, Ogawa H, Ando T, Yazaki N, Watanabe K, Haneda S, Shibata C, *et al*: Inhibitor of apoptosis protein family as diagnostic markers and therapeutic targets of colorectal cancer. Surg Today 41: 175-182, 2011.
26. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.
27. Jing C, Gao Z, Wang R, Yang Z, Shi B and Hou P: Lenvatinib enhances the antitumor effects of paclitaxel in anaplastic thyroid cancer. Am J Cancer Res 7: 903-912, 2017.
28. Ogino H, Hanibuchi M, Kakiuchi S, Trung VT, Goto H, Ikuta K, Yamada T, Uehara H, Tsuruoka A, Uenaka T, *et al*: E7080 suppresses hematogenous multiple organ metastases of lung cancer cells with nonmutated epidermal growth factor receptor. Mol Cancer Ther 10: 1218-1228, 2011.
29. Yoon TM, Kim SA, Park YL, Lee KH, Sung MW, Lee JK, Lim SC, Chung IJ and Joo YE: Expression of the receptor tyrosine kinase recepteur d'origine nantais and its association with tumor progression in hypopharyngeal cancer. Head Neck 35: 1106-1113, 2013.
30. Goffredo P, Thomas SM, Adam MA, Sosa JA and Roman SA: Impact of timeliness of resection and thyroidectomy margin status on survival for patients with anaplastic thyroid cancer: An analysis of 335 cases. Ann Surg Oncol 22: 4166-4174, 2015.
31. Baek SK, Lee MC, Hah JH, Ahn SH, Son YI, Rho YS, Chung PS, Lee YS, Koo BS, Jung KY, *et al*: Role of surgery in the management of anaplastic thyroid carcinoma: Korean nationwide multicenter study of 329 patients with anaplastic thyroid carcinoma, 2000 to 2012. Head Neck 39: 133-139, 2017.
32. Pezzi TA, Mohamed AS, Sheu T, Blanchard P, Sandulache VC, Lai SY, Cabanillas ME, Williams MD, Pezzi CM, Lu C, *et al*: Radiation therapy dose is associated with improved survival for unresected anaplastic thyroid carcinoma: Outcomes from the National Cancer Data Base. Cancer 123: 1653-1661, 2017.
33. Haymart MR, Banerjee M, Yin H, Worden F and Griggs JJ: Marginal treatment benefit in anaplastic thyroid cancer. Cancer 119: 3133-3139, 2013.
34. Tiedje V, Stuschke M, Weber F, Dralle H, Moss L and Führer D: Anaplastic thyroid carcinoma: Review of treatment protocols. Endocr Relat Cancer 25: R153-R161, 2018.
35. Tohyama O, Matsui J, Kodama K, Hata-Sugi N, Kimura T, Okamoto K, Minoshima Y, Iwata M and Funahashi Y: Antitumor activity of lenvatinib (e7080): An angiogenesis inhibitor that targets multiple receptor tyrosine kinases in preclinical human thyroid cancer models. J Thyroid Res 2014: 638747, 2014.
36. Schlumberger M, Tahara M, Wirth LJ, Robinson B, Brose MS, Elisei R, Habra MA, Newbold K, Shah MH, Hoff AO, *et al*: Lenvatinib versus placebo in radioiodine-refractory thyroid cancer. N Engl J Med 372: 621-630, 2015.
37. Oishi K, Takabatake D and Shibuya Y: Efficacy of lenvatinib in a patient with anaplastic thyroid cancer. Endocrinol Diabetes Metab Case Rep 2017: 16-0136, 2017.
38. Koyama S, Miyake N, Fujiwara K, Morisaki T, Fukuhara T, Kitano H and Takeuchi H: Lenvatinib for anaplastic thyroid cancer and lenvatinib-induced thyroid dysfunction. Eur Thyroid J 7: 139-144, 2018.
39. Tahara M, Kiyota N, Yamazaki T, Chayahara N, Nakano K, Inagaki L, Toda K, Enokida T, Minami H, Imamura Y, *et al*: Lenvatinib for Anaplastic Thyroid Cancer. Front Oncol 7: 25, 2017.
40. Deveraux QL and Reed JC: IAP family proteins - suppressors of apoptosis. Genes Dev 13: 239-252, 1999.
41. Kasof GM and Gomes BC: Livin, a novel inhibitor of apoptosis protein family member. J Biol Chem 276: 3238-3246, 2001.
42. Chung CY, Park YL, Kim N, Park HC, Park HB, Myung DS, Kim JS, Cho SB, Lee WS and Joo YE: Expression and prognostic significance of Livin in gastric cancer. Oncol Rep 30: 2520-2528, 2013.
43. Li F, Yin X, Luo X, Li HY, Su X, Wang XY, Chen L, Zheng K and Ren GS: Livin promotes progression of breast cancer through induction of epithelial-mesenchymal transition and activation of AKT signaling. Cell Signal 25: 1413-1422, 2013.
44. Lin X, Li HR, Lin XF, Yu ME, Tu XW, Hua ZD, Lin M, Xu NL, Han LL and Chen YS: Silencing of Livin inhibits tumorigenesis and metastasis via VEGF and MMPs pathway in lung cancer. Int J Oncol 47: 657-667, 2015.
45. Xi RC, Sheng YR, Chen WH, Sheng L, Gang JJ, Tong Z, Shan Z, Ying GH, Dong LC and Chen YS: Expression of survivin and livin predicts early recurrence in non-muscle invasive bladder cancer. J Surg Oncol 107: 550-554, 2013.
46. Chen YS, Li HR, Lin M, Chen G, Xie BS, Xu NL and Lin LF: Livin abrogates apoptosis of SPC-A1 cell by regulating JNKI signaling pathway. Mol Biol Rep 37: 2241-2247, 2010.
47. Hoshi T, Watanabe Miyano S, Watanabe H, Sonobe RM, Seki Y, Ohta E, Nomoto K, Matsui J and Funahashi Y: Lenvatinib induces death of human hepatocellular carcinoma cells harboring an activated FGF signaling pathway through inhibition of FGFR-MAPK cascades. Biochem Biophys Res Commun 513: 1-7, 2019.
48. El Ali Z, Grzymisławski M, Majewski P, Baumann-Antczak A and Kosowicz J: Anti-livin antibodies: Novel markers of malignant gastrointestinal cancers. Pol Arch Med Wewn 120: 26-29, 2010.
49. Yagihashi A, Asanuma K, Tsuji N, Torigoe T, Sato N, Hirata K and Watanabe N: Detection of anti-livin antibody in gastrointestinal cancer patients. Clin Chem 49: 1206-1208, 2003.
50. Yagihashi A, Asanuma K, Kobayashi D, Tsuji N, Shijubo Y, Abe S, Hirohashi Y, Torigoe T, Sato N and Watanabe N: Detection of autoantibodies to livin and survivin in Sera from lung cancer patients. Lung Cancer 48: 217-221, 2005.

51. Yagihashi A, Ohmura T, Asanuma K, Kobayashi D, Tsuji N, Torigoe T, Sato N, Hirata K and Watanabe N: Detection of autoantibodies to survivin and livin in sera from patients with breast cancer. Clin Chim Acta 362: 125-130, 2005.
52. Zhang L, Xu Z, Chen X, Duan Y, Chen Z and Sun J: Clinical benefits of Livin peptide-loaded DCs/CIKs combined with chemotherapy in advanced non-small cell lung cancer. Am J Cancer Res 9: 406-414, 2019.
53. Yin L, Liu S, Li C, Ding S, Bi D, Niu Z, Han L, Li W, Gao D, Liu Z, *et al*: CYLD downregulates Livin and synergistically improves gemcitabine chemosensitivity and decreases migratory/invasive potential in bladder cancer: The effect is autophagy-associated. Tumour Biol 37: 12731-12742, 2016.
54. Wang Z, Liu S, Ding K, Ding S, Li C, Lu J, Gao D, Zhang T and Bi D: Silencing Livin induces apoptotic and autophagic cell death, increasing chemotherapeutic sensitivity to cisplatin of renal carcinoma cells. Tumour Biol 37: 15133-15143, 2016.
55. Liu Y, Guo Q, Zhang H, Li GH, Feng S, Yu XZ, Kong LS, Zhao L and Jin F: Effect of siRNA-Livin on drug resistance to chemotherapy in glioma U251 cells and $CD133^+$ stem cells. Exp Ther Med 10: 1317-1323, 2015.
56. Zhuang L, Shen LD, Li K, Yang RX, Zhang QY, Chen Y, Gao CL, Dong C, Bi Q, Tao JN, *et al*: Inhibition of livin expression suppresses cell proliferation and enhances chemosensitivity to cisplatin in human lung adenocarcinoma cells. Mol Med Rep 12: 547-552, 2015.
57. Zou AM, Wang HF, Zhu WF, Wang FX and Shen JJ: Effect of RNAi-mediated silencing of Livin gene on biological properties of colon cancer cell line LoVo. Genet Mol Res 13: 3832-3841, 2014.
58. Wang TS, Ding QQ, Guo RH, Shen H, Sun J, Lu KH, You SH, Ge HM, Shu YQ and Liu P: Expression of Livin in gastric cancer and induction of apoptosis in SGC-7901 cells by shRNA-mediated silencing of Livin gene. Biomed Pharmacother 64: 333-338, 2010.
59. Wang X, Xu J, Ju S, Ni H, Zhu J and Wang H: Livin gene plays a role in drug resistance of colon cancer cells. Clin Biochem 43: 655-660, 2010.
60. Oh BY, Kim KH, Chung SS and Lee RA: Silencing the livin gene enhances the cytotoxic effects of anticancer drugs on colon cancer cells. Ann Surg Treat Res 91: 273-277, 2016.
61. Yuan D, Liu L, Xu H and Gu D: The effects on cell growth and chemosensitivity by livin RNAi in non-small cell lung cancer. Mol Cell Biochem 320: 133-140, 2009.