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Expression of Livin and the Inhibition of Tumor Progression by Livin Silencing in Laryngohypopharyngeal Cancer

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Abstract. *Aim: To evaluate the expression of Livin in human laryngohypopharyngeal squamous cell carcinoma (LHSCC) and investigate whether Livin-knockdown using small interfering-RNA (siRNA) affects tumor aggressiveness in LHSCC cells. Materials and Methods: Immunohistochemistry, reverse transcription-polymerase chain reaction, western blotting, cell invasion assay, cell migration assay, and cell apoptosis assays were performed to assess the impact of Livin on cancer cell behavior in human LHSCC. Results: High immunoreactivity of Livin was observed in 22 (36.7%) of the 60 LHSCC tissues relative to adjacent normal mucosa. In the positive-Livin expression group, distant metastasis tended to occur frequently, but the difference was not statistically significant (p=0.06). Livin-knockdown by siRNA induced cell apoptosis through activation of caspase 3, caspase 7, and poly ADP ribose polymerase in LHSCC cells. Livin-knockdown also resulted in significantly reduced cell invasion and migration in LHSCC cells. Conclusion: siRNA-mediated silencing of Livin may be associated with the reversal of invasive capacity in LHSCC.*

Head and neck squamous cell carcinomas represented approximately 2.4% of all cancers in the United States in 2009 (1). Advanced cancer stage at diagnosis and aggressive tumor biology contribute to poor prognosis, whose 5-year survival rates range from 10% to 40% (2). In the past, treatment of advanced laryngohypopharyngeal squamous cell carcinoma (LHSCC) focused predominantly on cure by comprehensive surgery, and involved total laryngectomy or laryngopharyngectomy with consideration for adjuvant radiotherapy (3, 4). Although this approach is effective and produces high rates of local tumor control, it affects laryngeal function, results in a permanent tracheostomy, and has a negative effect on the patient's quality of life, with consequences such as depression and social isolation (5). Recent efforts to preserve voice and swallowing functions have resulted in surgical organ-preserving procedures with improved reconstructive efforts and/or chemoradiation. Advances in surgical methods, chemotherapy, and radiation therapy have improved locoregional control, while maintaining, but not improving, survival in LHSCC over the last 30 years (3, 6). To improve the survival rate and preserve laryngeal function in patients with advanced LHSCC, new treatment modalities as the molecular target therapy are clearly needed.

Apoptosis is a major barrier to cancer that must be circumvented (7). The imbalance between apoptosis and cell-cycle progression is closely-related to cancer development and advancement. The basal level of apoptosis is tightly controlled by the endogenous inhibitors of apoptosis protein (IAP) gene family, which encodes negative regulatory proteins that prevent cell apoptosis in mammalian cells (8). Livin is a member of the IAP gene family and is overexpressed in tumors including melanoma, breast, colon, prostate, and hepatoma (8-10). High Livin expression in neoplasms correlates with more aggressive behavior, such as shorter disease-free survival or overall survival, and chemoresistance (8, 9). These findings have raised interest in Livin as a target for molecular therapy. However, the significance of Livin in the progression and prognosis of LHSCC is unknown.

In the present study, we examined Livin expression and evaluated the relationship between its expression and clinicopathological factors in a series of advanced LHSCCs. Additionally, to explore the effect of Livin expression and to validate its potential as a novel therapeutic target, we also investigated whether Livin affects tumor cell invasion,

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migration, and apoptosis in LHSCC cells. This study is the first report to demonstrate the impact of Livin expression and its association with tumor progression in LHSCC.

Materials and Methods

Patients and tumor specimens. For evaluation of the protein expression of Livin, paraffin-embedded tissue sections were collected from 60 patients who underwent diagnostic biopsy for advanced LHSCC (stage III and IV) at the Chonnam National University Hwasun Hospital (Jeonnam, Korea) between July 2004 and February 2009. All 60 patients were treated with three cycles of cisplatin-, 5-fluorouracil-, and docetaxel (DCF)-based induction chemotherapy (IC), followed by cisplatin-based concurrent chemoradiation therapy (CCRT) in 56 patients with complete response (CR) or partial response (PR) after IC and two patients who refused operation despite of stable disease (SD) after IC. Another two patients with SD after IC underwent salvage total laryngectomy. CR was defined as no visible or palpable disease in the assessment by physical examination, laryngoscopy, and computed tomography. PR was defined as a >50% decrease in tumor size, compared with the initial measurement. SD was defined as stationary or progressive disease. The patients' clinicopathological characteristics were reviewed in hospital records. Tumors were staged according to the seventh edition of the American Joint Committee on Cancer staging system (11). Survival was measured from the start of chemotherapy to the date of death or date last seen. This study was approved by the Institutional Review Board of the Chonnam National University Hwasun Hospital.

Immunohistochemistry. Paraffin tissue sections were de-paraffinized, rehydrated, and treated with peroxidase-blocking solution (Dako, Carpinteria, CA, USA). The tissues were incubated with polyclonal rabbit anti-human Livin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing in Tris-buffered saline-Tween 20 buffer (TBST), tissues were stained using Dako RealTM Envision horseradish peroxidase (HRP)/3,3'-diaminobenzidine (DAB) detection system (Dako). Tissues were counter-stained with hematoxylin and mounted with coverslips. Two independent observers interpreted staining intensity of the specimens without any knowledge of the patients' clinical information. Assessment for staining intensity was performed as follows: 0, no staning of tumor cells; 1+, weak to comparable staining in cytoplasm and/or the nucleus compared to that of non-tumoral cells; 2+, readily appreciable or dark brown staining distinctly marking the tumor cell cytoplasm and/or nucleus. Specimens with 0 or 1+ staining were regarded as negative expression and those with 2+ staining were regarded as positive expression.

Cell culture and transfection. Cells of SNU 1041 and PCI 1 human LHSCC cell lines were provided from Dr. Sung MW (Seoul National University, Seoul, South Korea). Cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) in a humidified atmosphere of 5% CO_2 at 37˚C. Small-interfering RNA (siRNA) was used to knockdown endogenous Livin gene expression in LHSCC cells. Cells were transfected with Livin-specific siRNA (Bioneer, Daejeon, Korea) and negative control siRNA (Qiagen, Valencia, CA, USA) using Lipofectamine™ 2000 (Invitrogen) for 48 h.

Cell invasion assay. Cell invasion ability was measured by the number of cells that invaded through a transwell invasion apparatus with 8.0 μm pore size (Costar, Cambridge, UK). Alive cells transfected with Livin siRNA or negative control siRNA were seeded at 3×10^5 cells in 120 μl of a 0.2% bovine serum albumin (BSA) suspension in the upper chamber. Subsequently, 400 μl of 0.2% BSA containing 7 μg/ml fibronectin (Calbiochem, La Jolla, CA, USA) as the chemoattractant was loaded into the lower chamber. After incubation for 24 h, cells that had moved to the bottom surface of the Transwell were stained with Diff Quik solution (Sysmex, Kobe, Japan) and calculated in five random squares in the microscopic field of view. Results were expressed as the mean±standard error (SE) of the number of cells/field in three individual experiments.

Cell migration assay (wound healing assay). Cells transfected with Livin siRNA or negative control siRNA were seeded in each well of Culture-Inserts (Ibidi, Bonn, Germany) at 1.5×10^5 cells/well. After incubation for 24 h, each insert was detached and the progression of cell migration was ascertained by photography at 0, 8, 12, and 24 h using an inverted microscope. The distance between gaps was normalized to 1 cm after capture of three random sites.

Cell apoptosis assay. Apoptosis was determined by an Annexin V-fluorescein isothiocyanate (FITC) assay. Forty eight hours after transfection, cells transfected with Livin siRNA or negative control siRNA were collected using trypsin, washed twice in phosphate buffered saline (PBS), and re-suspended in binding buffer (BD Biosciences, San Diego, CA, USA). Annexin V-FITC and 7-amino-actinomycin D (7-AAD; BD Biosciences) were added to the cells, which were then incubated in the dark for 15 min, then re-suspended in 400 ml of binding buffer. Cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Data analysis was performed using standard Cell Quest software (Becton Dickinson).

Protein isolation and western blot analysis. Cells were lysed in RIPA buffer (1 M Tris-HCl, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA) with 1 mM phenylmethanesulfonyl fluoride (PMSF), Halt™ phosphatase inhibitor and Halt™ protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA). Resolved proteins (10-20 μg) were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Specific proteins were sequentially blotted with primary antibodies: Livin, cleaved caspase-3, cleaved caspase-7, cleaved poly ADP ribose polymerase (PARP), and β-actin (Cell Signaling Technology, Danvers, MA, USA) and polyclonal anti-human glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology). Immunoreactive proteins were visualized on the enhanced chemiluminescence (ECL) detection system HRP substrate (Millipore) and the LAS-4000 luminescent image analyzer (Fujifilm, Tokyo, Japan).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from cells was extracted using Trizol reagent (Invitrogen), reverse transcribed, and amplified using specific primers for LIVIN and GAPDH. The primer sequences were: Livin α and Livin β 5'-CAC ACA GGC CAT CAG GAC AAG-3'/ 5'-ACG GCA CAA AGA CGA TGG AC-3' and GAPDH 5'-ACC ACA GTC CAT GCC ATC AC-3'/ 5'-TCC ACC ACC CTG TTG CTG TA-3'. The

sizes of the amplified products were 456 bp for Livin α and 403 bp for Livin β. For cDNA synthesis, 1 μg mRNA was mixed with 50 ng/μl oligo-dT (Promega, Madison, WI, USA), Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen), and RNAsin (Takara Bio, Shiga, Japan). PCR amplification of cDNA was performed using specific primers and GoTaq® DNA polymerase (Promega). PCR products were separated by electrophoresis on a 1% agarose gel containing ethidium bromide.

Statistical analyses. The relationship between Livin expression and various clinicopathologic parameters was compared using the χ^2 test and Fisher's exact test. Survival curves were calculated using the Kaplan-Meier method, and comparison of curves was performed using the log-rank test. Experimental differences between the Livin knockdown group and control group were tested with the Student's t-test. The Statistical Package for the Social Sciences (SPSS) version 21.0 (Microcal Software Inc, Chicago, IL, USA) was used for all analyses. A *p*-value <0.05 was considered statistically significant.

Ethical considerations. Local research ethics committee approval was obtained.

Results

Expression of Livin in human LHSCC tissues. The patients included in this study group were 59 men and one woman. The mean age was 64.4±7.9 years (mean±standard deviation), with a range from 47 to 83 years. The mean follow-up period of patient alive was 50.7±31.1 months with a range from 8.1 to 111.4 months. To decrease the influence according to variable treatment strategies, patients who were treated with the same chemoradiation regimen were included.

Livin protein expression was investigated immunohistochemically in formalin-fixed, paraffin-embedded tissue blocks obtained from 60 LHSCC patients. Immunohistochemical analysis revealed that 22 (36.7%) of 60 LHSCC tissues overexpressed Livin relative to adjacent normal mucosa. A pattern of strong nuclear staining was evident (Figure 1A). In contrast, there was no staining in another LHSCC tissue that serves as negative control (Figure 1B). Twenty two tissues (36.7%) with dark brown staining distinctly marking the tumor cell showed 2+ staining and were regarded as positive expression. Thirty tissues (50.0%) with weak to comparable staining compared to that of non-tumoral cells showed 1+ staining. Eight tissues (13.3%) had no staining. Thirty eight tissues (63.3%) with 1+ or no staining were regarded as negative expression.

Livin expression and clinicopathologic factors in human LHSCC. Patients' data and the correlations between Livin expression and clinicopathological factors in LHSCC are shown in Table I. In the positive Livin expression group, distant metastasis tended to occur frequently, but the difference was not statistically significant (p=0.06). Livin expression in human LHSCC was not associated with age, sex, location, T stage (tumor invasion), N stage (lymph node metastasis), chemotherapy sensitivity, tumor response after chemoradiation therapy, and recurrence (p>0.05) (Table I).

Table I. Correlation between Livin expression and clinicopathological parameters in patients with laryngohyopharyngeal squamous cell carcinoma.

Parameters		Livin expression		
	Total (n=60)	Negative (n=38)	Positive (n=22)	*p*-Value
Age (years)				0.59
<65	29	17	12	
≥65	31	21	10	
Gender				1.00
Male	59	37	22	
Female	1	1	0	
Location				0.18
Larynx	32	23	9	
Hypopharynx	28	15	13	
Stage				1.00
III	23	15	8	
IV	37	23	14	
T stage				0.60
T1, T2	27	16	11	
T3, T4	33	22	11	
N stage				0.54
N0	14	10	4	
N1, N2	46	28	18	
Distant metastasis				0.06
M0	51	35	16	
M1	9	3	6	
Tumor response after IC				0.62
CR, PR	56	36	20	
SD	4	2	2	
Overall tumor response after CRT				0.51
CR	45	28	17	
PR, SD	13	10	3	
Recurrence				0.54
Negative	27	18	9	
Positive	18	10	8	

IC, Induction chemotherapy; CRT, chemoradiation; CR, complete response; PR, partial response; SD, stable disease.

Livin expression and prognostic implication in human LHSCC. For 60 patients with advanced LHSCC enrolled in this study, the 3-year and 5-year overall survival rate was 50% and 42%, respectively. A median duration of overall survival was 69.7 months *versus* 43.2 months for the negative Livin expression group and the positive Livin expression group, respectively. However, no significant statistical difference in overall survival was found between the two groups (p=0.46, Figure 2).

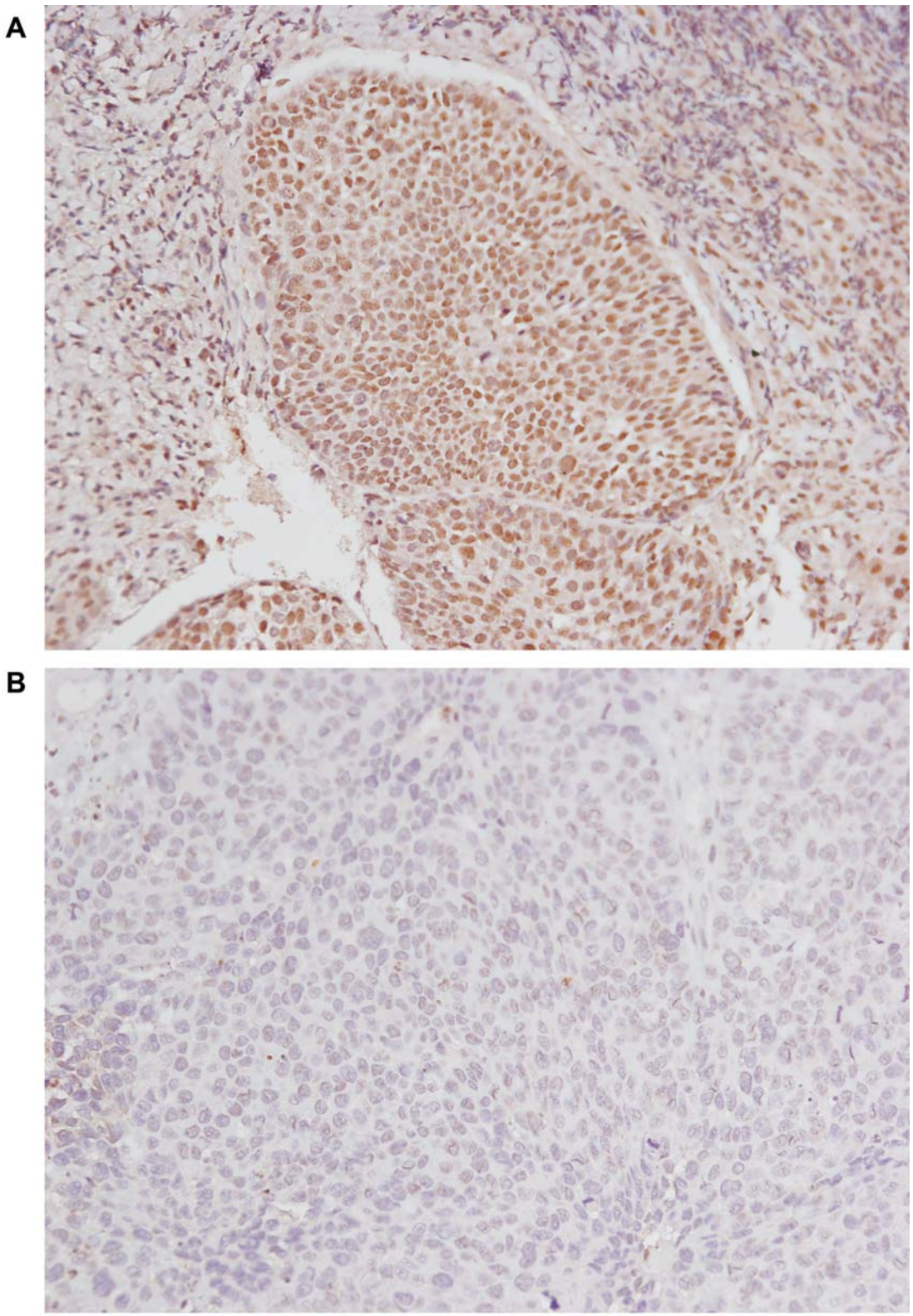

Figure 1. *Immunohistochemical staining of Livin in human laryngohypopharyngeal squamous cell carcinoma (LHSCC) tissue. (A) Human LHSCC cells showed 2+ strong nuclear Livin immunolabelling. ×200. (B) Another setting of human LHSCC cells showing no staining for Livin immunoreactivity served as negative control. ×200.*

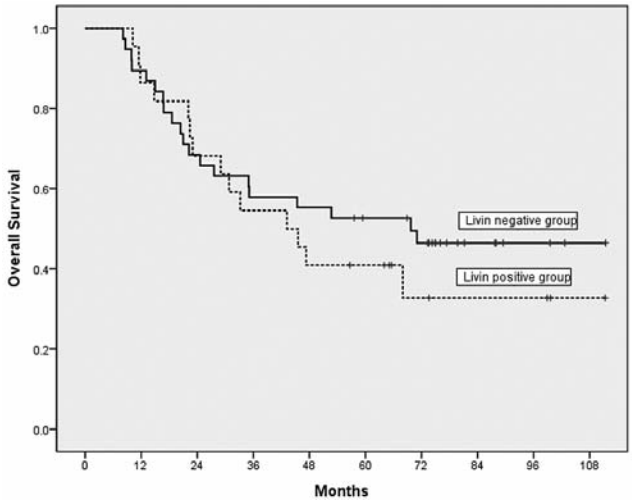

Figure 2. *Overall survival curve according to Livin expression (negative expression, solid line and positive expression, dotted line) in human laryngohypopharyngeal squamous cell carcinoma (p=0.46).*

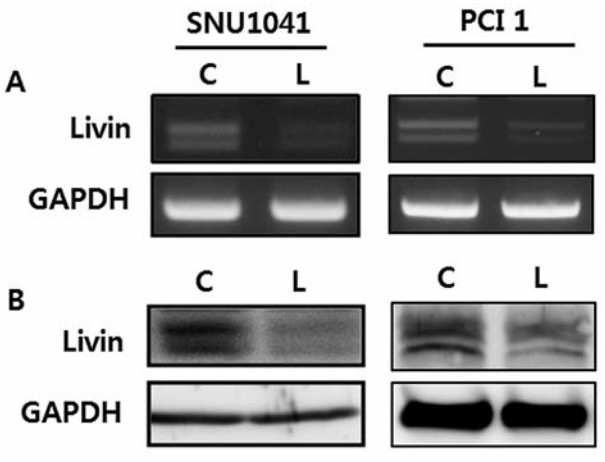

Figure 3. *siRNA-mediated Livin-knockdown in human laryngo-hypopharyngeal squamous cell carcinoma cell lines. The mRNA (A) and protein (B) expression of Livin α and Livin β is decreased by Livin knockdown in SNU 1041 and PCI 1 cells.*

Knockdown of Livin and tumor cell invasion in human LHSCC cell lines. Livin-knockdown was identified by RT-PCR and Western blotting. Livin α and Livin β mRNA and protein expression were reduced by Livin-specific siRNA in SNU 1041 and PCI 1 cells as compared to the negative control siRNA (Figure 3).

In the cell invasion assay, the number of invading Livin siRNA-transfected SNU 1041 and PCI 1 cells was 196.3±3.5 and 38.1±3.4, whereas that for negative control siRNA-transfected cells was 594.6±16.1 and 582.4±15.2, as measured in the five random squares of 0.5×0.5 mm^2 microscope fields. The difference between the two groups was statistically significant ($p<0.001$) (Figure 4). Livin-knockdown resulted in significantly reduced cell invasiveness in human LHSCC cells.

Knockdown of Livin and tumor cell migration in human LHSCC cell lines. In the cell migration assay, the artificial wound gap became significantly narrower in plates of negative control siRNA-transfected cells compared to Livin siRNA-transfected cells at 4 h, 8 h, 12 h, and 24 h in SNU 1041 cells and at 12 h, 24 h, and 36 h in PCI 1 cells ($p<0.01$, Figure 5). Wound gaps were filled in plates of negative control siRNA-transfected cells after 24 h in SNU 1041 cells and after 36 h in PCI 1 cells. On the other hand, wound gaps were wide open in plates of Livin siRNA-transfected cell after 24 h in SNU 1041 cells and after 36 h in PCI 1 cells. The results clearly showed that Livin knockdown resulted in significantly diminished cell migration in human LHSCC cells.

Knockdown of Livin and cell apoptosis in human LHSCC cells lines. To determine the impact of Livin on cell apoptosis, responses of Livin siRNA-transfected cells and negative control siRNA-transfected cells in SNU 1041 and PCI 1 cells was compared by the Annexin V apoptosis assay. Livin-knockdown SNU 1041 and PCI 1 cells displayed increased apoptosis, compared to control cells (Figure 6A and B). The proportion of early apoptotic cells induced by transfection of Livin siRNA was greater than that induced by transfection of negative control siRNA (33.9% *versus* 7.8% and 28.1% *versus* 6.5%, respectively) in SNU 1041 and PCI 1 cells. Additionally, an increase in cleaved caspase 3, cleaved caspase 7, and cleaved PARP, which are all key enzymes of apoptosis, was detected in Livin-knockdown SNU 1041 and PCI 1 cells (Figure 6C). These results suggest that Livin knockdown-induced apoptosis is associated with the modulation of apoptosis regulatory proteins such as caspase 3, caspase 7, and PARP.

Discussion

IAPs are a family of highly conserved cell apoptosis inhibitors found in a wide variety of living organisms from yeast to mammals (9). The IAPs suppress apoptosis induced by a diverse range of stimuli including DNA damage, hypoxia, viral infection, chemotherapeutic drugs, growth factor withdrawal, and nutrient-deprivation, mainly by inhibiting the activity of caspases (7, 8). The dysregulation of the expression of IAPs results in tumorigenesis and chemoresistance (8). Thus, silencing their expression can inhibit tumor progression and prevent cancer treatment failure, which has made them

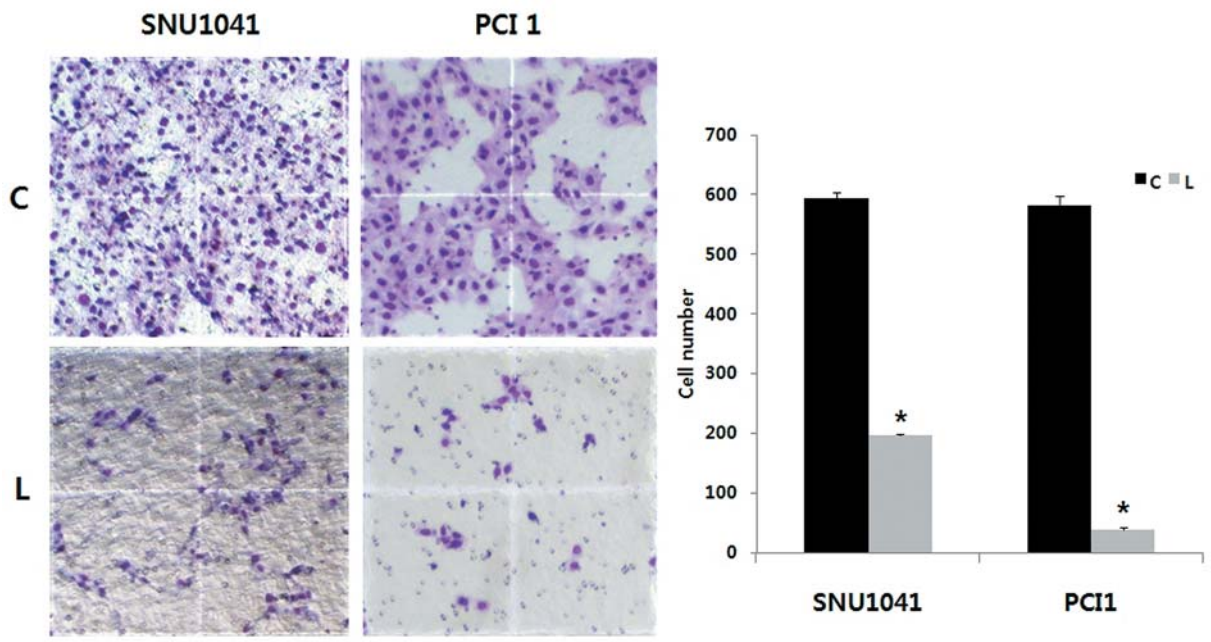

Figure 4. *Effect of Livin knockdown on cell invasion in human laryngohypopharyngeal squamous cell carcinoma cell lines. Invading cells were significantly smaller in Livin siRNA-transfected SNU 1041 and PCI 1 cells (L) than in negative control siRNA-transfected cells (C). Stained invading cells were counted and are represented as a bar graph between groups (mean±SEM, n=3; *p<0.001).*

attractive targets for molecular therapy. Livin is a recently discovered IAP (9). Livin is undetectable in most normal differentiated tissues, but is expressed in various malignancies (8-10). Livin is suggested to be involved in the progression of tumors, and has poorly prognostic significance (12-14). It had been reported that Livin might be involved in the progression of superficial bladder cancer and used as a marker of early recurrence (12). The study of neuroblastoma showed that patients with combined high Livin expression and amplified *Myc* oncogene had significantly shorter median survival (13). All these findings have classified Livin as a new target of molecular therapy (8, 9, 15, 16), but the relationship between Livin and LHSCC has seldom been reported.

The expression and role of Livin in head and neck cancer was only reported that the high Livin expression rate was 28.8% without the significant prognostic implication in nasopharyngeal carcinoma (17). In the presently-studied LHSCC tissues, immunohistochemical staining of Livin protein was predominantly identified in the nucleus of cancer cells and 22 (36.7%) of 60 tissues showed high Livin expression relative to adjacent tissue mucosa. Livin was also overexpressed in LHSCC. This is in agreement with previous reports of Livin expression in melanoma, breast cancer, gastric cancer, colorectal cancer, prostate cancer, bladder cancer, neuroblastoma, and hepatoma (8-10, 12-15). In addition, patients with positive Livin expression tended to suffer distant metastasis. Although this finding had no statistical significance (p=0.06), it is important considering the small number of LHSCC patients. Distant metastasis is directly related to regional metastasis and poor prognosis in LHSCC (18). We need to analyze more cases to confirm the predictive role of Livin in distant metastasis of LHSCC.

Livin overexpression may be correlated with unfavorable survival in LHSCC. In our study, the 5-year overall survival rate of patients with advanced LHSCC treated with DCF-based chemoradiation strategy was 42%. A median duration of overall survival for the negative-Livin expression group was quite longer than for the positive Livin expression group (69.7 months *vs*. 43.2 months). However, this had no statistical significance. It may be because the survival is affected by variable factors and oncogenes, and our cases were not sufficient to provide the statistical significance in sample size and follow-up duration.

Cancer cells are often characterized by increased resistance to apoptosis, which mediates their increased resistance to various stimuli of cell apoptosis (19). Overcoming apoptosis resistance is important to improve treatment outcome for tumors in clinical practice, especially chemoradiotherapy. A number of studies have addressed the importance of Livin in the regulation of tumor cell apoptosis in gastric cancer, lung cancer, melanoma, hepatoma, and glioma (10, 15, 16, 20, 21). In support of the previous

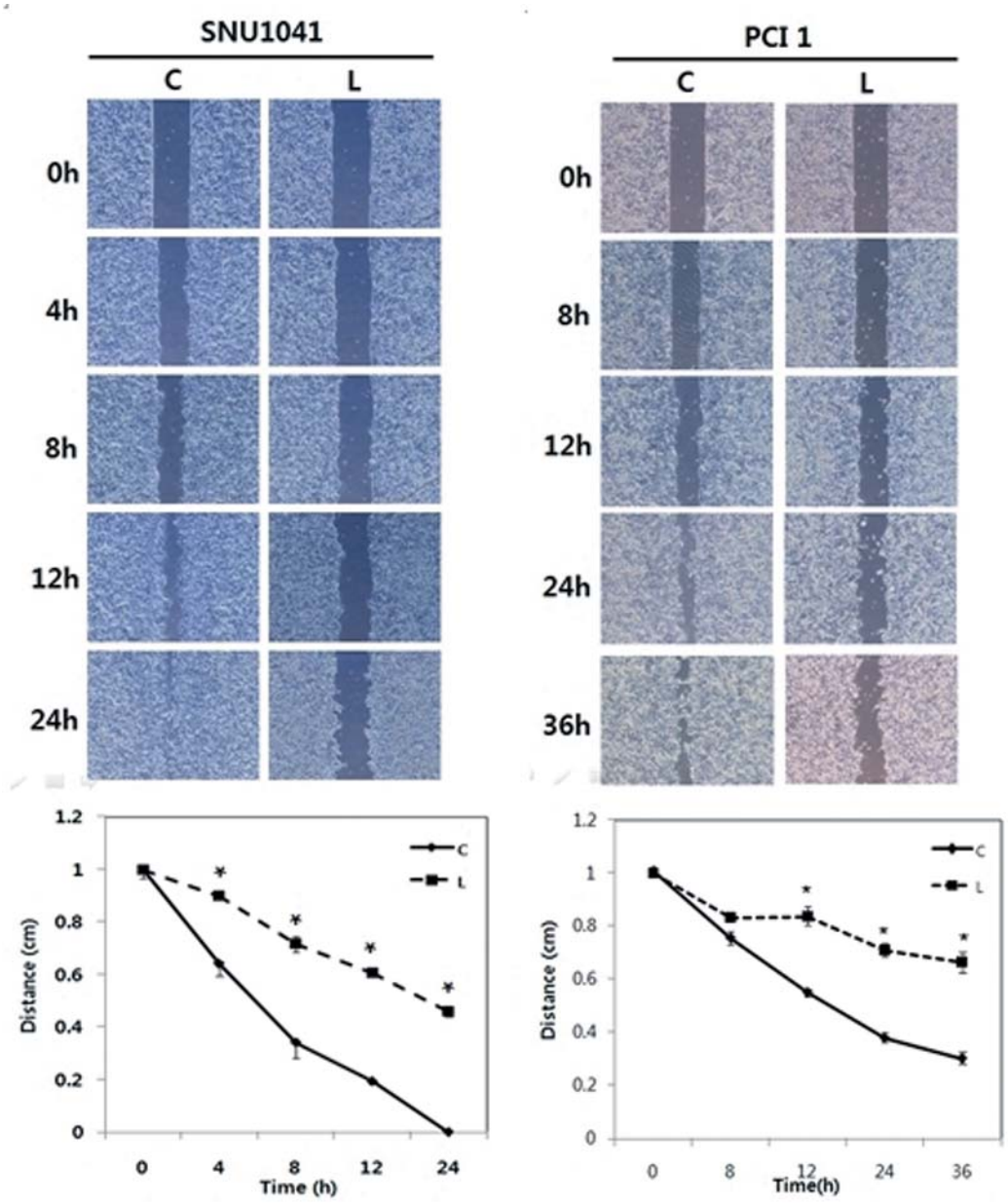

Figure 5. *Effect of Livin knockdown on cell migration in human laryngohypopharyngeal squamous cell carcinoma cell lines. Cell migration showed a marked decrease in Livin siRNA-transfected SNU 1041 and PCI 1 cells (L) rather than in negative control siRNA-transfected cells (C). Cell migration is displayed as relative healing distances measured in three random sites. Values indicate mean±SEM for three independent experiments (*p<0.01).*

studies, presently Livin-knockdown also induced cell apoptosis in LHSCC cells. Induction of apoptosis by Livin-knockdown was mediated through activation of caspase 3, caspase 7, and PARP. These results indicate that Livin induces tumorigenic activities such as apoptosis resistance *via* the caspase pathway in LHSCC. However, in our case series of LHSCC, Livin expression was unexpectedly not associated with chemosensitivity and tumor response after chemoradiation. Further studies on apoptosis-related oncogenes interacting with Livin are required in LHSCC.

In addition to its widely known role in cell apoptosis, Livin may be involved in tumor invasion and metastasis in LHSCC. Tumor invasion and metastasis are key prognostic factors (16). Induction of these activities results in a more invasive phenotype in cancer cells. Livin induced cell invasion in prostate cancer and hepatocellular carcinoma (16, 22). In the

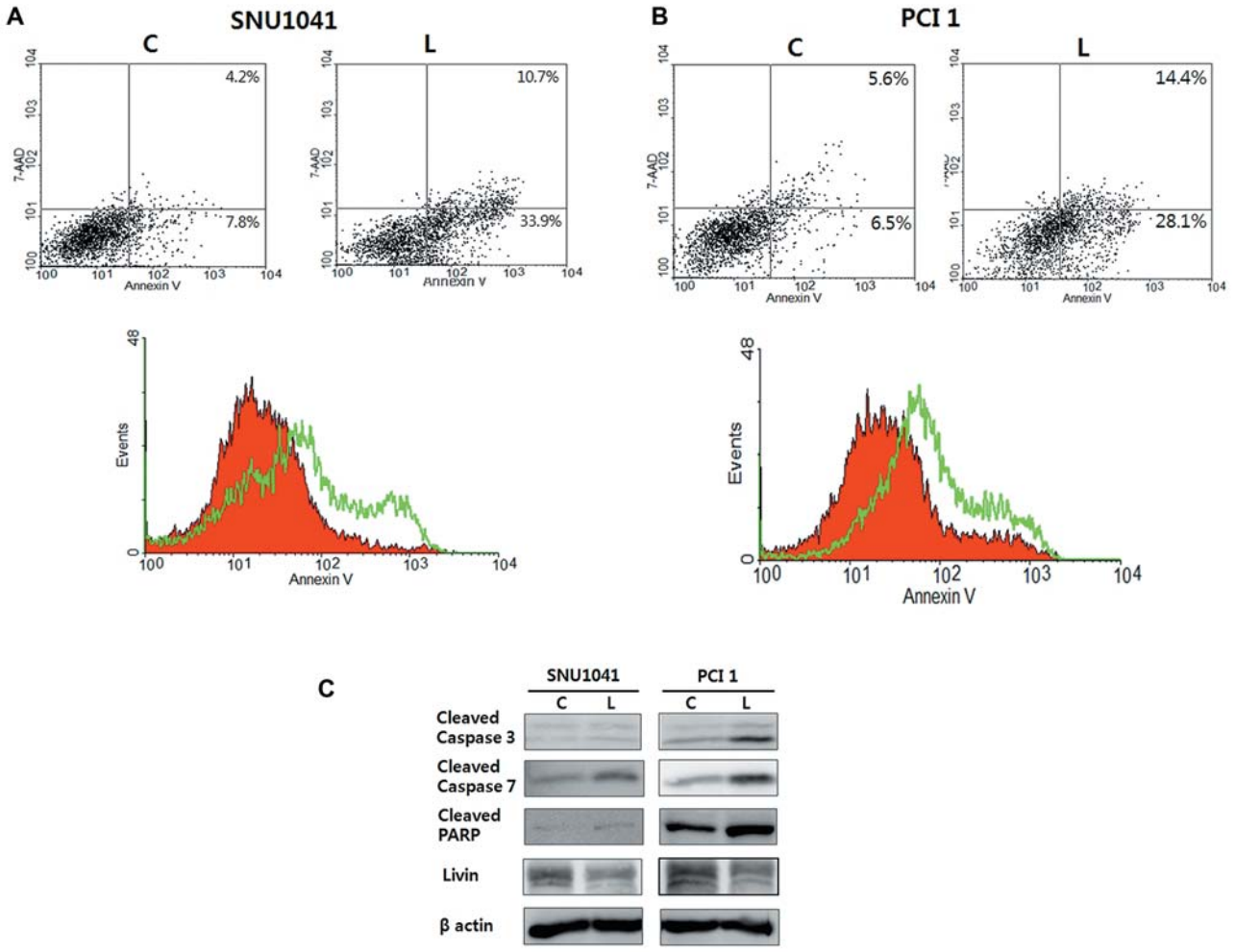

Figure 6. *Effect of Livin-knockdown on cell apoptosis in human laryngohypopharyngeal squamous cell carcinoma cell lines. (A and B) Livin knockdown SNU 1041 and PCI 1 cells (L) displayed increased apoptosis, compared to control cells (C). (C) The effects of Livin knockdown on the mediators of apoptosis. Cleaved caspase -3, -7 and PARP expression was increased in SNU1041 and PCI 1 cells by Livin knockdown.*

current study, Livin-knockdown inhibited cell invasion and migration in LHSCC cells. These results suggest that Livin contributes to alter the invasive phenotypes by increasing tumor cell invasiveness and motility.

In conclusion, Livin was overexpressed in LHSCC tissues. Livin siRNA-mediated knockdown inhibited cell invasion, migration, and apoptosis activity in LHSCC cells. Livin could be useful for therapeutic intervention against tumor progression in LHSCC.

Acknowledgements

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