Relationship between expression of Livin and the biological behavior of human oral squamous cell carcinoma

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Received June 2, 2014; accepted August 28, 2014

DOI: 10.3892/or.2014.3510

Abstract. Livin is one of the most important members of the inhibitor protein (IAP) family. It is overexpressed in several types of tumors and may have prognostic significance. The purpose of this study was to investigate Livin expression in human oral squamous cell carcinomas (OSCCs) and to determine whether Livin affects tumor cell behavior in OSCC cell lines and thus evaluate its potential usefulness in serving as a possible target for molecular-targeted therapy in a preclinical model. The expression of Livin protein was investigated in human OSCC tissues through immunohistochemistry and western blot analysis. To evaluate the impact of Livin knockdown on the behavior of human OSCC cell lines, invasion, migration, proliferation and apoptosis assays using small-interfering RNA (siRNA) were performed. RT-PCR and western blot analysis were used to assess alteration of Livin expression at the mRNA and protein levels. The results revealed that expression of Livin was increased in the human OSCC tissues compared with the adjacent normal mucosa. In addition, immunoreactivity of Livin was expressed in 8 OSCC tissues (44.4%). Knockdown of Livin resulted in significantly reduced cell invasion, migration and proliferation in the human OSCC cells. Livin knockdown induced cell apoptosis in the human OSCC cells. Moreover, Livin inhibited apoptosis by suppressing the activity of caspases in the human OSCC cells. In conclusion, Livin is associated with invasive and oncogenic phenotypes such as tumor cell invasion, tumor cell migration, tumor cell proliferation, and resistance to apoptosis in human OSCC cells.

Introduction

Head and neck cancer refers to a group of biologically similar cancers that start in the lip, oral cavity, nasal cavity, paranasal sinuses, pharynx and larynx. Ninety percent of head and neck cancers are squamous cell carcinomas, originating from the epithelium of these regions. Head and neck squamous cell carcinomas (HNSCCs) represent approximately 2.4% of all cancers diagnosed and were implicated in 7,600 deaths in 2009 (1). Although early-stage head and neck cancers, particularly laryngeal and oral cavity cancers, have high cure rates, up to 50% of head and neck cancer patients present with advanced disease (2). The patients with locally advanced disease have a 5-year survival rate of approximately 50%, and 10% of patients who suffer from distant metastatic disease have a 5-year survival rate of approximately 25% (3). Despite the advancement achieved in surgical methods and radiation therapy, the prognosis of head and neck cancers has not shown a satisfactory improvement (1-6). Therefore, in order to achieve more effective treatment of patients with HNSCC, particularly patients with advanced disease, new treatment modalities such as molecular-targeted therapy are clearly needed.

The family of inhibitor of apoptosis protein (IAP) consists of a group of structurally related proteins with anti-apoptotic properties (7,8). Currently, eight human IAP members have been identified, including cIAP1, cIAP2, NAIP, Survivin, XIAP, BRUCE, ILP-2 and Livin (9-14). Livin is a novel member of the IAP family. In contrast to other members of the IAP family, such as cIAP1, cIAP2, XIAP and NAIP, it is expressed at low levels in normal adult tissues, but is overexpressed in a wide variety of human malignancies, such as colon cancer, leukemia, bladder cancer, neuroblastoma, malignant pleural mesothelioma, pancreatic cancer, osteosarcoma, renal cell carcinoma, gastric cancer, breast carcinoma and lung cancer (15-29). It has been reported that Livin may be involved in the progression of tumors, and high levels of this molecule are correlated with tumor progression and patient outcome (22,30). However, the impact and expression of Livin in the progression of HNSSC are still unknown, and this issue has not been reported in any studies, except Livin in nasopharyngeal carcinoma (32).
In order to analyze the pathobiological significance of Livin in human oral squamous cell carcinomas (OSCCs), we examined expression of Livin and investigated the potential relevance between its expression and various clinicopathological variables in a well-defined series of OSCCs. We also determine whether Livin affects tumor cell invasion, migration, proliferation and apoptosis in OSCC cell lines. This study demonstrated Livin expression and its association with tumor progression in OSCC and may provide the basis for applying Livin in the molecular-targeted therapy for OSCCs.

Materials and methods

Patients and tumor specimens. To evaluate Livin protein expression, paraffin-embedded tissue sections were collected from 18 patients who underwent definitive surgery for OSCC at Chonnam National University Hwasun Hospital (Jeonnam, Korea) between March 2006 and August 2009. All 18 patients underwent appropriate definitive surgery. In 6 patients, surgery was followed by radiation therapy alone or cisplatin-based concurrent chemoradiation therapy.

Patient characteristics including age, gender, tumor location, stage, depth of tumor invasion, lymph node metastasis, distant metastasis, treatment, recurrence, survival and follow-up information were obtained by review of the hospital records. All patients were staged according to the 7th edition of the American Joint Committee on Cancer (AJCC) staging system (33). Overall survival was calculated from the first surgical date to the date of death or the date of last follow-up.

Immunohistochemistry. Livin protein expression was investigated immunohistochemically in formalin-fixed, paraffin-embedded tissue blocks obtained from 18 OSCC patients. Tissue sections (5 µm) were cut from each paraffin block, mounted, and dried on glass slides. Tissues were deparaffinized, rehydrated, and retrieved with retrieval buffer. Endogenous peroxidase activity was blocked with peroxidase-block, mounted, and dried at room temperature. Cells transfected with Livin siRNA or negative control siRNA were seeded at 3x10^5 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 chemotactrant was loaded into the lower chamber. After incubation for 24 h, cells on the top surface of the filter were wiped off with cottonballs. Cells that had migrated to the bottom surface of the Transwell were stained with Diff Quik solution (Sysmex, Kobe, Japan) and counted in five random squares in the microscopic field of view. Results are expressed as the mean ± standard error of the number of cells/field in three individual experiments.

Cell invasion assay. Cell invasion was measured using a Transwell invasion apparatus (Costar, Cambridge, UK). Transwell filters (8.0-µm pore size) were coated overnight with 1% gelatin solution on both the top and bottom surfaces and dried at room temperature. Cells transfected with Livin siRNA or negative control siRNA were seeded at 3x10^5 cells in 120 µl of media, and 7 µg/ml fibronectin (Calbiochem, La Jolla, CA, USA) as the chemoattractant was loaded into the lower chamber. After incubation for 24 h, cells on the top surface of the filter were wiped off with cottonballs. Cells that had migrated to the bottom surface of the Transwell were stained with Diff Quik solution (Sysmex, Kobe, Japan) and counted in five random squares in the microscopic field of view. Results are expressed as the mean ± standard error 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 1.5x10^5 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 inverting microscope. The distance between gaps was normalized to 1 cm after capture of three random sites.

Cell viability. Cell viability was determined with the EZ-CyTox (tetrazolium salt, WST-1) cell viability assay kit (Daeil Lab Inc., Seoul, Korea). After applying the WST-1 reagent at 37°C, cell viability was measured using a microplate reader (Infinite M200; Tecan, Austria GmbH, Vienna, Austria) with Magellan V6 data analysis software (Tecan). Triplicate wells were used for each experiment, and all experiments were conducted at least in triplicate.

Cell apoptosis assay. Apoptosis was determined by Annexin V-fluorescein isothiocyanate (FITC) assay. Cells transfected with Livin siRNA or negative control siRNA were collected using trypsin, washed twice in phosphate-buffered saline (PBS) twice 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 added and re-suspended in 400 ml of binding buffer. Cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Data analysis was performed using standard Cell Quest software (Becton Dickinson).
Protein isolation and western blot analysis. Cells and tissues 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, Rockford, IL, USA). Resolved proteins (10-20 µg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the resolved proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% BSA in TBST at room temperature for 1 h, the PVDF membranes were sequentially blotted with the primary antibodies: polyclonal anti-human Livin, cleaved caspase-3, cleaved caspase-7, cleaved poly-ADP ribose polymerase (PARP) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and polyclonal anti-human glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology) at 4˚C overnight. After rinsing in TBST, each membrane was incubated with the anti-rabbit or anti-mouse HRP-conjugated secondary antibody (Santa Cruz Biotechnology) at room temperature for 1 h. Blots were detected with chemiluminescence (ECL) HRP substrate (Millipore) and recorded by a Ras-4000 image reader (Fujifilm, Tokyo, Japan).

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR). The RNA from cells was isolated using TRIzol reagent (Invitrogen), reverse transcribed, and amplified using specific primers for Livin and GAPDH. Primer sequences were: Livin 5'-CACACAGGCCCCATCAGGAC AAG-3'/5'-ACGGCACAAAGACGATGGAC-3' and GAPDH 5'-ACCACAGTCCATGCCATCAG-3'/5'-TCCACACCCCTG TTGCTGTA-3'. The size of the amplified products was 456 bp for Livin α and 403 bp for Livin β. for the 18 patients with OSCC enrolled in this study, the 3-year overall survival rate was 72%. The 3-year overall survival was 80% in the negative Livin group, and 63% in the positive Livin group. Livin expression was not associated with patient overall survival (p=0.54) (Fig. 2).

Statistical analysis. Experimental differences between the Livin knockdown group and the control group were tested with the Student's t-test. The statistical software program SigmaPlot Software (version 6.0; Systat Software, San Jose, CA, USA) was used. Survival curves were calculated using the Kaplan-Meier method, and comparison of the curves was performed using the log-rank test. The statistical software program used was Statistical Package for the Social Sciences (SPSS) version 18.0 (Microlab Software Inc., Chicago, IL, USA) was used. A p-value <0.05 was considered to indicate a statistically significant result.

Results

Expression of Livin is increased in the OSCC tissues compared with normal mucosa. The patient data are summarized in Table I. The study group of patients included 12 males and 6 females. The mean age was 59.8±10.8 years (mean ± standard deviation), with a range from 41 to 79 years. The mean follow-up period was 50.5 months, with a range from 11.2 to 84.7 months. Immunohistochemical analysis revealed that 8 (44.4%) of the 18 OSCC tissues overexpressed Livin relative to the adjacent normal mucosa. A pattern of strong nuclear staining was evident (fig. 1A). In contrast, there was no staining in another OSCC tissue sample as negative control (fig. 1B). The number of positive tumor cells for each tissue was recorded. Three tissues (16.7%) showed 2+ staining and five tissues (27.8%) had 1+ staining. Ten tissues (55.6%) had negative staining.

Correlations between Livin protein expression and clinicopathological characteristics of the human OSCC cases. Correlations between Livin protein expression and clinicopathological characteristics in human OSCC are shown in Table II. Livin protein expression was not associated with age, gender, tumor location, stage, T stage, N stage, distant metastasis and recurrence (p>0.05) (Table II). For the 18 patients with OSCC enrolled in this study, the 3-year overall survival rate was 72%. The 3-year overall survival was 80% in the negative Livin group, and 63% in the positive Livin group. Livin expression was not associated with patient overall survival (p=0.54) (Fig. 2).

Knockdown of Livin suppresses tumor cell invasion, migration and proliferation in human OSCC cells

Expression and knockdown of Livin in a human OSCC cell
RT-PCR analysis demonstrated Livin mRNA expression in the OSCC cell line (Fig. 3A). Western blot analysis confirmed high Livin expression in the PCI 50 cells (Fig. 3B). Knockdown of Livin results in significantly reduced cell invasiveness in human OSCC cells. In the cell invasion assay, the number of invasive Livin siRNA-transfected PCI 50 cells was 1029.0±28.7, whereas the number of invasive cells in the negative control siRNA-transfected PCI 50 cells was 511.0±24.1. The difference between the two groups was statistically significant (p<0.01) (Fig. 4). Livin knockdown resulted in significantly reduced cell invasiveness in the human OSCC cells.

Knockdown of Livin results in significantly diminished cell migration in human OSCC cells. In the cell migration assay, the artificial wound gap became significantly narrower in the plates of the negative control siRNA-transfected PCI 50 cells.
Figure 3. Livin knockdown by small-interfering RNA (siRNA) in the PCI 50 cell line. (A) The mRNA expression of Livin was decreased in the Livin small-interfering RNA (siRNA)-transfected PCI 50 cells (L) when compared with the negative control siRNA-transfected PCI 50 cells (C). (B) The protein expression of Livin was decreased by Livin knockdown in the PCI 50 cells. GAPDH was used as the loading control.

Figure 4. Effect of Livin knockdown on cell invasion in the PCI 50 cell line. The number of invading cells was significantly less in the Livin small-interfering RNA (siRNA)-transfected PCI 50 cells (L) when compared with the negative control siRNA-transfected PCI 50 cells (C). The stained invasive cells were counted and are represented as a bar graph between groups (mean ± SEM, n=3; *p<0.01).

Figure 5. Effect of Livin knockdown on cell migration in the PCI 50 cell line. Cell migration was markedly decreased in the Livin small-interfering RNA (siRNA)-transfected PCI 50 cells (L) when compared with the negative control siRNA-transfected PCI 50 cells (C) at 12 and 24 h. Cell migration is displayed as relative healing distance measured in three random sites. Values indicate mean ± SEM of three independent experiments (*p<0.01).
when compared with that of the Livin siRNA-transfected PCI 50 cells at 12 and 24 h (p<0.01) (Fig. 5). After 24 h, the wound gaps were filled in the plates of the negative control siRNA-transfected OSCC cells. In contrast, the wound gaps were wide open in the plates of Livin siRNA-transfected OSCC cells after 24 h. The results clearly showed that Livin knockdown resulted in significantly diminished cell migration in the human OSCC cells.

Knockdown of Livin results in significantly decreased cell proliferation in human OSCC cells. The cell viability was assessed at 1, 2, 3 and 4 days in the negative control siRNA-transfected PCI 50 cells and Livin siRNA-transfected PCI 50 cells to assessed the effect of Livin on cell proliferation (Fig. 6). The number of proliferating cells was significantly decreased in the Livin siRNA-transfected PCI 50 cells when compared to this number in the negative control siRNA-transfected PCI 50 cells at 2, 3 and 4 days (p=0.03, p=0.01, and p=0.003, respectively). The results clearly showed that Livin knockdown resulted in significantly decreased cell proliferation in human OSCC cells.

Knockdown of Livin induces cell apoptosis in human OSCC cells. To determine the impact of Livin on cell apoptosis, responses of Livin siRNA-transfected OSCC cells and negative control siRNA-transfected OSCC cells were compared by an Annexin V apoptosis assay. Livin-knockdown OSCC cells displayed increased apoptosis, compared with the control cells (Fig. 7A). The proportions of early and late apoptotic PCI 50 cells induced by transfection of Livin siRNA (37.4 and 7.1%, respectively) were greater than that induced following transfection with negative control siRNA (11.6 and 1.2%, respectively) (Fig. 7A). Additionally, an increase in cleaved caspase-3, cleaved caspase-7 and cleaved PARP, which are key enzymes of apoptosis, was noted in the Livin-knockdown OSCC cells (Fig. 7B). These results suggest that Livin knockdown-induced apoptosis was associated with the modulation of apoptosis regulatory proteins such as caspase-3, caspase-7 and PARP.

Discussion

The IAP family consists of a group of intracellular proteins that are essential for the regulation of apoptosis (19,34). The IAPs consist of one baculovirus IAP repeat (BIR) domain and a COOH-terminal RING finger domain that suppresses apoptosis induced by a variety of stimuli (29). Apoptosis plays an important role in oncogenesis and tumor regression (35). Loss of apoptotic regulation can lead to a variety of diseases including cancer. IAPs bind directly and potentially inhibit a complex array of cysteine aspartyl-specific proteases, caspase-3, caspase-7, which are responsible for apoptosis and which are induced by diverse pro-apoptotic stimuli (34).

Livin is one of the potent members of the IAP family. Livin is undetectable in most normal tissues but is upregulated in a wide variety of human cancers (15-29). Livin expression contributes to tumor progression, a poor prognosis and is correlated with more aggressive tumor behavior, such as decreased response to radiotherapy and chemotherapy and reduced survival time in many human cancers (18,22,23,36). These findings represents a potential novel therapeutic target for the treatment of human cancers.

However, whether Livin is related to head and neck cancer has not been investigated. Upon review of the English language literature, there is only one report of Livin expression in biopsy samples of nasopharyngeal carcinoma. Xiang et al reported that Livin was expressed in 48.75% of...
nasopharyngeal carcinoma cases (32). In the present study, Livin was expressed in 8 (44.4%) of 18 OSCC tissues. This result is similar to that for nasopharyngeal carcinoma. In addition, OSCC tissues exhibited overexpression of Livin relative to the adjacent normal mucosa. This suggests that Livin may be involved in the development of OSCC. In addition, Livin is specifically overexpressed in cancer tissues, but almost absent in most normal adult tissues with the exception of the placenta (37,38). This molecule represents a possible target for developing drugs that selectively eliminate cancer cells.

OSCC is characterized by a marked propensity for local invasion and lymphatic metastasis. Understanding the molecular mechanisms that mediate SCC invasion and metastasis may enable identification of novel therapeutic targets for management of tumor dissemination. Livin promotes the invasion, growth and apoptotic resistance in a variety of human cancer cells (14,39-41). In addition, Livin expression may be essential for survival of certain cancer cells (17,21,23,41,42). In this study, Livin knockdown inhibited tumor cell invasion, migration and proliferation in OSCC cells. These results indicate participation of Livin in tumor progression and metastasis in OSCCs, and support immunohistochemical data showing an association of Livin expression with cervical lymph node metastasis in human OSCC tissues. In addition, Livin expression was found to be significantly associated with cell proliferation in the human OSCC tissues.

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 (second mitochondrial-derived activator of caspase/direct IAP binding protein with low pl) (14,39-41). In the present study, Livin knockdown induced cell apoptosis in human OSCC cells. As knockdown of Livin suppression could lead to apoptotic cell death in human OSCC cells, suppression of Livin should have a benefit in cancer treatment. These results suggest that knockdown of Livin could provide a potential therapeutic strategy to induce apoptosis in OSCC cells and to significantly improve antitumor responses.

To determine the mechanisms of cell apoptosis in Livin siRNA-transfected OSCC cells, the change in apoptosis-related proteins in Livin siRNA-transfected OSCC cell lysate was evaluated. This study showed that the expression of cleaved caspase-3, -7 and PARP was upregulated in human OSCC cells after knockdown of Livin. Therefore, Livin inhibits apoptosis by suppressing the activity of caspases in human OSCC cells.

Finally, we assessed the expression of Livin and its prognostic relevance in a well-defined series of human OSCC tissues with complete clinicopathological data including survival. No significant correlation was found between Livin expression and various clinicopathological parameters including age, gender, tumor location, stage, T stage, N stage, distant metastasis, and recurrence. Furthermore, Livin expression was not correlated with survival. However, the 3-year overall survival was 80% in the negative Livin group and 63% in the positive Livin group. Although there was no statistically significant differences between Livin expression and survival, Livin expression had a trend toward predicting a reduced survival rate. This result may reflect the relatively small sample size. In addition, the steps involved in OSCC development and progression are not dependent on Livin expression alone but may be regulated by many biological processes including invasion and metastasis. Further studies are required to clarify the impact of Livin on the biologic and prognostic significance in OSCC.

In summary, knockdown of Livin inhibited tumor cell invasion, migration and proliferation in human OSCC cells. Knockdown of Livin induced cell apoptosis in human OSCC cells. Livin inhibited apoptosis by suppressing the activity of caspases in human OSCC cells. Livin was not related with the clinicopathologic parameters of patients with OSCC. However, Livin expression was predictive of a poor 3-year overall survival rate.

In conclusion, Livin is associated with invasive and oncogenic phenotypes such as tumor cell invasion, tumor cell migration, tumor cell proliferation, and resistance to apoptosis in human OSCC cells.

References


