ML-IAP (Livin) and Human Cancers: A Recent Review

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Article ID: WMC002159
Article Type: Review articles
Submitted on: 05-Sep-2011, 01:27:12 AM GMT  Published on: 05-Sep-2011, 06:09:44 PM GMT
Article URL: http://www.webmedcentral.com/article_view/2159
Subject Categories: CANCER
Keywords: Livin, Cancer, IAP
How to cite the article: Kanwar J R, Sun X, Cheung C H. ML-IAP (Livin) and Human Cancers: A Recent Review. WebmedCentral CANCER 2011;2(9):WMC002159
Source(s) of Funding:
This work is supported by grant from the National Cheng Kung University, Tainan, Taiwan R.O.C.

Competing Interests:
The authors declare no competing interests.
ML-IAP (Livin) and Human Cancers: A Recent Review

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Abstract

A relatively new member of the inhibitor of apoptosis protein (IAP) family, ML-IAP (Livin), is found to be involved in the apoptotic resistance of tumor cells. Studies on cell lines and patients have suggested the possibility that Livin is a cancer-specific protein. On the other hand, down-regulation of Livin expression by siRNA has been shown to decrease cancer cell viability and drug resistance in vitro and in vivo. Since detailed molecular mechanisms of Livin have been discussed elsewhere, this review will mainly look at the expression of Livin in various cancer cell lines and clinical samples. This review will further discuss the possibility of targeting Livin in cancers in future clinical situations.

Introduction

The family of cell apoptosis inhibitor proteins (IAPs) is highly conserved among yeasts, invertebrates and in the vertebrates. They are characterized by 70 amino acid baculoviral repeats (BIR) and have wide spread roles in anti-apoptotic functions. These include NAIP, c-IAP1 (MIHB, HIAP-2), c-IAP2 (HIAP-1, MIHC, API2), XIAP (hILP, MIHA, ILP-1), Survivin, BRUCE (apollon), ILP-2 and Livin (ML-IAP, KIAP). Livin is one of the novel IAP family members. It has low caspase binding activity as compared to its fellow IAP members because of its lone BIR domain, but it can inhibit apoptosis by inhibiting an inhibitor of IAPs, SMAC/DIABLO (second mitochondria-derived activator of caspase/direct IAP binding protein with low pl). Thus Livin can play a crucial role in apoptosis, which can be implicated in designing diagnostic tools and/ or anti cancer therapeutics. Similar to Survivin, Livin expression has been reported in various tumor cells and developmental tissues but not in normal adult tissues, making it a probable candidate as a cancer-specific IAP family protein. Thus, great interest has been shown in using Livin as a direction for cancer treatment these recent years.

Expression of Livin in Human Cancers

Expression of Livin was first identified in melanoma, where the highest levels were observed in two human melanoma-derived cell lines, G361 and SK-Mel29. Similar to survivin, the expression of Livin was not observed in most normal non-cancerous cells. Kasof and Gomes’s study showed that although it was expressed in normal placenta tissue, developmental tissues, and in several cancer cells, expression of Livin was not detectable by Northern blot in most normal adult tissues. Similar study also revealed 12 of 15 (80%) tumor-derived RCC cell lines scoring positive for Livin mRNA expression. Taken together, the over-expression of Livin seems to be cancer cell specific.

Melanomas

Livin has been named ML-IAP or melanoma IAP, as it was identified high expression of α- and β-isforms in a number of melanoma lines. Immunohistochemistry, in situ hybridisation and RT-PCR assays were explored to observe Livin expression in human melanoma tissues and the difference in expression between melanoma and melanocytic naevus were investigated in 48 patient samples containing 34 melanomas and 14 dermal naevi. The results showed Livin expression rate in archived melanoma tissues was around 50–70%, with no difference between primary and secondary melanomas. A small number of dermal naevi also expressed Livin.

Neuroblastoma

Based on the fact that melanoma and neuroblastoma are neural crest–derived tumors, Findley and co workers, using immunohistochemistry and reverse transcriptase–polymerase chain reaction (RT-PCR), have examined expression of livin in primary neuroblastoma tumor tissue and neuroblastoma cell lines and attempted to correlate expression of the gene with clinical and biological features of the tumor. The study consists of analyzing autopsies of patients with neuroblastoma and neuroblastoma cell lines by immunoperoxidase using an anti-Livin monoclonal antibody. 26.5% of the neuroblastoma tumor tissues showed high Livin expression, 53% showed low intermediate expression, and 20.5% were negative. Similarly, neuroblastoma cell lines showed high and
intermediate Livin expression. The results indicates expression of Livin in primary and cultured neuroblastoma cells and high Livin expression may identify a subset of neuroblastoma patients with a particularly poor prognosis among those with MycN amplified tumors.

**Lung Cancer**

Several studies were conducted on the expression of Livin in lung cancer. In a clinical study done by Yagihashi’s group, auto-antibodies to Livin were detected in patients with lung cancer. The study revealed that sera from 19 of 37 lung cancer patients (51.3%) were positive by ELISA using recombinant livin protein. For the subgroup positivity according to clinical stage, 0 of 6 were from stage I and II patients, 8 of 11 were from stage III patients, and 12 of 20 were from stage IV patients. In another study on lung cancer done by Hariu et al., the expression of Livin was detected in 6 of 8 (75%) adenocarcinoma, 5 of 7 (70%) squamous cell carcinoma, and 2 of 2 (100%) large cell carcinoma specimens. In Crnkovic-Mertens et al.’s study, non-small cell lung cancer tumor tissues were examined by RT-PCR for Livin expression. Expression of livin was detected in 8 of 13 tumor specimens (61.5%) and only 1 of 7 normal tissue samples (14.3%). In vitro, Livin was also found to be expressed in lung cancer cell lines, but not in normal tissues. In addition, Livin mRNA was found to be expressed in two of four lung adenocarcinoma cell lines (LNY-1 and A549), one of two squamous cell carcinoma cell lines (SBC-2). In a more specific study done on non-small cell lung cancer cells, Crnkovic-Mertens’s group determined Livin expression in 10 cell lines originated from adenocarcinomas or squamous cell carcinomas of the lung. Expression of Livin was quantified using RT-PCR and 6 of 10 (60%) cell lines were scored positive for Livin mRNA expression.

**Gastrointestinal Cancer**

Patients with gastrointestinal cancer were also screened in another clinical study done by Yagihashi’s group. Auto-antibodies were again examined with ELISA using full-length recombinant Livin protein. Sera from 17 of 35 gastrointestinal cancer patients (47%) were reactive to Livin, while 1 control serum from the healthy donors was reactive. For the subgroup results of gastrointestinal cancer, there were 3 of 5 positive sera from biliary tract cancer patients, 3 of 6 from gastric cancer, 6 of 11 from colorectal cancer, 1 of 3 from hepatoma, 2 of 7 from pancreatic cancer, and 2 of 3 from esophageal cancer.

**Esophageal Carcinoma**

In Chen et al.’s clinical study, the role of Livin in esophageal cancer was determined, as well as its relationship to different clinical stages. Expression of Livin measured by both immunohistochemical (IHC) assay and Western blotting showed that expression of Livin in clinicopathologic stage two, three, and four was significantly higher than that of stage one (P<0.01). Moreover, optical density value and positive cell percentage for both tests in clinicopathologic stage two, three, and four were significantly higher than that of stage one (P<0.01), with stage four being significantly higher than stage two and three (P<0.01). The mRNA level of Livin was tested using RT-PCR to further evaluate Livin contribution to pathogenesis of esophageal carcinoma. Results indicated that the up-regulation of Livin gene transcription matched with the protein level of Livin that was significantly increased along with the progression of esophageal carcinoma.

**Leukemia**

With 222 patients having a median age at diagnosis of 65.5 months, a study done by Choi et al.’s group identified the clinical relevance of Livin expression in childhood acute lymphoblastic leukemia (18). Livin mRNA was found to be expressed in 57 of 222 patients (25.7%), most of which were higher in patients with favorable prognostic factors. Moreover, Livin expression rate was higher in patients with a favorable (leukemic blast <25%) day 7 bone marrow response than in patients with an unfavorable (leukemic blast ≥25%) response (35.0% versus 8.7%, P<0.001). Thus, results showed the association between Livin expression and favorable response to chemotherapy.

**Osteosarcoma**

A study done by Nedelcu’s group determined the expression of Livin among tumor specimens of 29 high-grade central osteosarcoma patients, with a complete clinical follow-up for a minimum of 5 years. Livin was detected in 17 of the 29 cases (59%), with expression of Livin in the cytoplasm of all 17 and in the nucleus of only 3 cases (10%). Although no statistically significant difference between patients whose tumors expressed cytoplasmic Livin and the non-expressing group was seen over 5 years, however, for the 3 patients with nuclear Livin-positive tumors, 5 year overall survival rates were significantly poorer compared to individuals whose tumors were negative for nuclear expression (P<0.0002). Mean survival rate for patients with nuclear Livin-positive tumors was 255 days, while the remaining patients
had a mean survival rate of 3,057 days.

**Mesothelioma**

Interestingly, Kleinberg’s group revealed that Livin expression was absent in malignant mesothelioma despite presence of other IAPs. Immunoblotting of 10 mesothelioma effusions showed expression of XIAP in 9 of 10 cases and survivin in 4 of 10 cases but no Livin in any cases. In addition, immunohistochemistry in 112 malignant mesothelioma cases revealed both cytoplasmic and nuclear expression of various IAPs, but none of which was Livin. The uniform absence of Livin suggests that Livin may have no role in the biology of malignant mesothelioma.

**Molecular Structures and Functions of Livin**

Since Livin is widely expressed in tumor tissues, it is important to determine its molecular functions in cancer cells. The Livin gene is located on chromosome 20 at band q13. It contains five introns, six exons, and a total cDNA coding sequence of 897 bp. Research on non-small cell lung cancer cells has demonstrated Livin as a target of the b-catenin/T cell factor (TCF) signaling pathway. Transcription of Livin is activated by b-catenin through the formation of a complex with the TCF transcription factor family. Thus, the activity of Livin promoter is increased considerably by activation of b-catenin and could be blocked by a dominant negative form of DTCF4. Structurally, it is comprised of 280 amino acids with a single Baculoviral IAP Repeat (BIR) domain and COOH-terminal RING finger domain. This structure makes it unique to many other IAPs, which may contain up to 3 tandem BIR repeats. Similar to other IAPs, Livin generally interacts with downstream caspases such as caspase-3, caspase-7, and caspase-9, initiating their inactivation and degradation. The BIR domain forms a novel zinc-fold responsible for anti-apoptotic activity and interaction with caspases, while the RING domain has a varying role depending on the IAP and/or apoptotic stimulus. Mutagenesis study revealed that residue substitution of aspartate-138 into alanine in the BIR domain completely abolished the ability of Livin to bind caspases-9 in vitro. Interestingly, given the structural similarity between Livin and XIAP, Livin inhibits the activity of caspases-9 less potently than XIAP. Livin also interferes with apoptosis through indirect interactions. The indirect interaction is through its competition with XIAP in binding to SMAC/DIABLO (second mitochondria-derived activator of caspase/direct IAP binding protein with low pl), a protein that abrogates the ability of IAPs to inhibit cell death upon co-expression with them. Structure analysis reveals that aspartate 138 in the BIR domain of Livin is a critical residue for the binding of SMAC. Physical interaction between Livin’s BIR domain and amino-terminal residues of SMAC/DIABLO inhibits Livin’s anti-apoptotic activity (Fig. 1). However, since SMAC can only interact with one IAP, Livin’s higher binding affinity to SMAC than that of XIAP’s BIR 3 domain therefore releases the reportedly stronger inhibitor, XIAP, to inhibit caspase activity.

Recently, a splicing variant (truncated version) of Livin has been identified. The wild-type (full length) Livin is renamed as Livin α and its splicing variant is named as Livin β. Both the full length and truncated version of Livin consist of identical amino-acid sequences with the exception of 18 amino acids deletion present in the β-isiform. Despite various structural analyses suggesting that Livin’s BIR domain inhibits the activity of caspases, conflicting results have been reported for both Livin α and β in functional analyses. For example, over-expression of Livin α by gene transfection showed inhibitory effect of caspases-3 in menadione-treated cells but also promoted etoposide-induced caspases-3 activity in the same cell line. In addition, over-expression of Livin α could not protect cells from apoptosis induced by Fas. Interestingly, Livin α and β may interfere apoptosis at different situations. Livin α was shown to protect cells from apoptosis initiated by staurosporine, while apoptosis initiated by etoposide was blocked by Livin b in the same cell line. However, similar anti-apoptotic activity between Livin α and β was shown in the Jurkat T cell line after triggering apoptosis via tumor necrosis factor and CD95 receptors. Furthermore, one study suggests that Livin β is able to modulate the cytotoxicity induced by Natural killer (NK) cells. Jurkat cells transfected with Livin β were able to escape from the NK cell-mediated apoptosis under co-culture situations. In contrast, cells transfected with either Livin α or an empty vector were not able to escape from the NK cell-mediated apoptosis. The same study also reveals that in cells with Livin undergoing caspase-mediated cleavage to become a truncated protein (tLivin), the Livin a produced tLivin is pro-apoptotic upon apoptotic stimuli such as etoposide and NK cell cytotoxicity. Upon induction of apoptosis in Livin-expressing cell lines, cleavage of both isoforms of Livin occurred via caspase activity, becoming tLivin. As a result, isoform a shortened from a 39,000 to a 30,000 fragment, while isoform b shortened from a 37,000 to a 28,000 fragment, but the BIR domain and COOH-terminal RING finger domain of the cleaved Livin remained intact. A test on Livin showed its
protection from apoptosis induced by anti-CD95/Fas antibody in Jurkat cells when compared to non-Livin expressing cells. Yet, after cleavage, cells expressing tLivin showed a much higher rate of apoptosis compared to non-Livin expressing cells. Therefore, cleavage of Livin was shown to not only eliminate its anti-apoptotic activity but also produced a subunit with a marked pro-apoptotic effect. Similar results have also been discovered recently in vivo, where the NK cells induced cleavage of Livin to its pro-apoptotic truncated protein that in turn inhibited tumor growth. Further study on this subject revealed that cleaved Livin b is a stronger pro-apoptotic factor than that of Livin a. More importantly, the pro-apoptotic factor and sub-cellular localization can be disrupted by a single point mutation of the N-terminal glycine residue of tLivin, and the RING domain mediates the cytoplasmic distribution of the full-length Livin. Thus, anti-apoptotic function may be restored upon deletion of either the N-terminal region or the RING domain of tLivin, while its pro-apoptotic function is induced when tLivin is localized and its 11 N-terminal amino acids is joined to the RING domain. These studies suggest Livin to be switched from an anti-apoptotic molecule to a pro-apoptotic molecule.

Targeting Livin in Cancers

Livin’s role as an inhibitor of downstream caspases to cause apoptotic resistance also raises interest in targeting it for cancer treatment. More analyses were thus done to evaluate the possibility of using Livin as a therapeutic target. Immune-mediated approach was among one of the possibilities suggested in targeting Livin. As mentioned previously, anti-Livin antibodies in the serum of patients with gastrointestinal cancer, lung cancer, and breast cancer have been detected, indicating Livin’s role as a major tumor-associated antigen that can be presented by major histocompatibility complex (MHC) class I molecules. In another study on lung cancer, Livin peptides were constructed to induce cytotoxic T lymphocytes (CTLs) in a HLA-restricted manner. Peptide-specific CTLs were successfully induced from four of five patients with Livin positive lung cancer, and these CTLs showed cytotoxicity against Livin lung cancer cell lines. A similar study was also done in melanoma, where responses occurred in 14 of 45 (~35%) patients’ peripheral blood lymphocytes and in 6 of 14 (~43%) patients’ tumor-infiltrating lymphocytes. However, responses against the Livin peptides were not restricted to melanoma patients but also present among peripheral blood T cells in 4 of 32 healthy individuals, revealing its lack of accuracy. Along with the lack of studies in constructing new Livin peptides over recent years for immunotherapy, the immune-mediated approach for anti-Livin therapy is still in question.

Anti-Livin therapies were also done using antisense sequences. In one study, an antisense construct designed to include the entire coding region of Livin was used against the protein to trigger apoptosis in cancer cell lines expressing Livin mRNA. Transfections were performed in HeLa and G361 cells, both of which expressed Livin, as well as SW480 cells, which had no detectable levels of Livin expression. Results showed that the antisense construct decreased the viability of cells by 80-90% in HeLa and G361 cells relative to the control, while antisense showed little effect on the viability of SW480 cells. Moreover, increase in DNA fragmentations and DEVD-like caspase activities were detectable as well. The effect of Livin antisense oligonucleotides on viability, which correlated to Livin expression, was demonstrated. In a recent study, treatment on several human melanoma cell lines with an 11-base DNA oligonucleotide homologous to the telomere 3’ overhang sequence (T-oligo) also induced apoptosis in vivo. MM-AN cells briefly exposed to T-oligo in culture and injected into the flank or tail vein of SCID mice led to a tumor volume and metastases number reduction of 85-95% in treated mice when compared to control mice. In addition, intrasplenically or systematically administered T-oligos selectively inhibited the growth of previously established MM-AN tumor nodules in the flank and peritoneal cavity by 85-90% without detectable toxicity. Another study also evaluated melanoma cell viability upon down-regulation of Livin mRNA using antisense oligonucleotides. Using real-time PCR, treatment with the most potent antisense sequences M706 and M711 showed a reduction in Livin mRNA expression of SK-MEL 28 melanoma cells by 47% and 52%, respectively. Treatment with different concentrations of oligonucleotide M706 also showed that it reduced cell viability in a dose-dependent manner, with viability of the SK-MEL28 cell line decreased by 39% compared to untreated cells at 600nM. Furthermore, oligonucleotide M706 resulted in a two-fold increase of 85-90% without detectable toxicity. Another study also demonstrated that antisense could reduce cell viability significantly along with chemotherapy.

The possibility of using Livin as a therapeutic target
was also evaluated by using siRNA. One study silenced the Livin gene expression of HeLa and MeWo melanoma cells by transfecting small interfering RNAs (siRNAs) pSUPER-Livin-1 and pSUPER-Livin 2, thereby increasing caspase-3 activity and inducing apoptosis. Moreover, combination treatments of siRNA with pro-apoptotic stimuli such as, doxorubicin, UV-irradiation, and TNFa were applied and resulted in strong increases in apoptotic rate in Livin-positive cells. Another study targeted Livin in renal carcinoma cell lines using siRNA and also evaluated the apoptotic response of these cells toward different pro-apoptotic stimuli, such as UV irradiation or chemotherapeutic drugs. Strong increase in number of apoptotic cells in Caki-1 and 769-P cell lines was visualized when compared to control transfectants in response to UV irradiation and drug 5-fluorouracil, respectively. In contrast, cell lines that lack detectable Livin expression were not affected by these treatments. siRNA-mediated silencing of Livin gene expression was also performed on malignant melanoma LiBr cell lines, leading to cell cycle arrest and apoptosis. The apoptosis index of cells transfected with siRNA for the Livin gene was significantly higher than the un-transfected control. The protein level of procaspase-3 was also down-regulated by siRNA and the cleaved form of caspase-3 was increased in cells transfected with siRNA when compared to the blank control. Using flow cytometry, the percentage of cells blocked in the G_0/G_1 phase due to siRNA increased to 69.41%±4.41% (P<0.05), while cells in the S phase decreased to 18.59%±2.65% (P<0.05).

Such results were also seen in tumor cell lines LoVo and SPCA-1, in which the specific down-regulation of Livin expression significantly inhibited in vitro cell proliferation and in vivo tumorigenicity. Livin knockdown via RNAi also led to cell arrest of the cell lines in the G_0/G_1 phase of cell cycle, eventual apoptosis, and chemosensitivity enhancement. Combination treatments of knocking down endogenous Livin expression and conventional chemotherapy in non-small cell lung cancer cell lines A549 and 103H was demonstrated recently as well. Decreases in IC_{50} values of adriamycin and cisplatin were shown to be statistically significant in cells transfected with Livin siRNA, thus indicating an enhancement in chemosensitivity. These studies have shown therapeutic benefits in the chemosensitizing effect of siRNA-mediated down-regulation of Livin expression in human cancer. Since Livin is over expressed in cancer cells, the antibodies to Livin can be immobilized in preparing cancer target specific nanoparticles for cancer specific drug delivery and for better imaging systems to improve the imaging system in cancer patients. Magnetic nanoparticles can be used to monitor the cancer load in these patients with Livin specific delivery to cancers cells.

**Conclusions**

In conclusion, the evident relationship between Livin and cancer has been shown over the past decade. However, studies with larger sample size and higher accuracy and specificity may be required before clarifying its diagnostic or prognostic role. Further use of Livin antibodies can be immobilized on nanoparticles to target the cancer cells for cancer cell specific drug delivery and monitoring the cancer patients. On the other hand, future studies taking into the account of isoform-specific expression analyses, along with more investigation on the Livin expression, should benefit the clinical significance of Livin. We suggest the potential of Livin as a therapeutic target with more in vivo studies and studies on its molecular mechanisms.

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