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Inhibitors of Apoptosis Proteins Expression and Their Prognostic Significance in Colorectal Carcinoma

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Background: The expression of the inhibitor of apoptosis proteins (IAPs) family has not been fully investigated in colorectal carcinomas. This study investigated IAP expression in colorectal carcinomas and assessed their prognostic significance. **Methods:** Livin, XIAP, and SMAC/DIABLO expression was assessed by immunohistochemistry in 159 colorectal carcinomas. Correlations between protein expression and clinicopathological features were evaluated. The survival data analysis was estimated according to the Kaplan-Meier method. **Results:** Increased expression of IAPs in cancer tissues compared to surrounding nonneoplastic counterparts was observed in 67 cases (42.1%) for Livin, 50 cases (31.4%) for XIAP, and 68 cases (42.8%) for SMAC. A significant correlation was found between Livin expression and tumor differentiation, and SMAC expression and tumor location. The recurrence-free and overall survival of patients with low Livin expression were inferior to those of patients with high Livin expression ($p=0.054$ and 0.095, respectively). High XIAP expression was significantly associated with shorter progression-free survival ($p=0.041$). **Conclusions:** Our study demonstrated that altered expression of IAP family members, including Livin, XIAP, and SMAC, is frequent in colorectal carcinoma. This result suggests that high Livin expression and low XIAP expression may be a favorable prognostic implication related to patient survival.

Key Words: Inhibitor of apoptosis proteins; X-linked inhibitor of apoptosis protein; DIABLO protein, human; Colorectal neoplasms; Prognosis

Colorectal carcinoma (CRC) remains one of the leading causes of cancer mortality despite remarkable advances in cancer diagnostics and treatment.[1] Similar to other malignancies, deranged apoptotic cell death is one of the important mechanisms of carcinogenesis and tumor progression of CRC.[2] This process is mediated by a group of proteins called inhibitor of apoptosis proteins (IAP), which have potential anti-apoptotic functions by inhibiting caspase activities.[3,4] Previous studies have suggested that IAP overexpression results in survival of neoplastic cells and therapeutic resistance to cytotoxic agents.[3] Overexpressed IAPs have been documented in a variety of solid tumors and cancer cell lines.[3]

IAPs are characterized by the presence of one or more repeats of a highly conserved 70 amino acid domain, termed the baculoviral IAP repeat (BIR). Livin is a novel member of the IAP family, originally identified in melanoma.[3] Recently, Livin expression has been found to be overexpressed in various cancers, including bladder cancer, lung cancer, acute lymphoblastic leukemia, and neuroblastoma.[5-8] Livin is selectively expressed in the most common human neoplasms and appears to be involved in tumor cell resistance to chemotherapeutic agents.[4] Several *in vitro* and *in vivo* studies have demonstrated that downregulation of Livin expression increases the apoptotic rate, reduces tumor growth potential, and sensitizes tumor cells to chemotherapeutic drugs.[9,10]

To date, little is known about Livin expression in CRC and its association with clinical implications. The aim of the present study was to investigate the expression of Livin along with its closely related IAP member, XIAP, and an endogenous antagonist of IAP proteins, SMAC/DIABLO, in a series of 159 CRC cases.

MATERIALS AND METHODS

Patient selection and sample collection

Primary tumor samples were collected from 159 patients diagnosed with colorectal adenocarcinoma at Chonnam National University Hwasun Hospital. The patients had undergone a colectomy between April 2004 and December 2004. The cases were identified retrospectively from clinicopathological data. The availability of adequate tissue material and clinical follow-up data were the only inclusion criteria. The following clinicopathological characteristics were evaluated for their relevance to protein expression or long-term survival: age (>60 yr vs ≤60 yr), gender, tumor size (>4.5 cm vs ≤4.5 cm), tumor location (proximal vs distal), tumor differentiation (high grade vs low grade), tumor extent (T1, T2 vs T3, T4), lymph node metastasis, distant metastasis, and history of chemoradiation treatment after surgery. No patient received chemoradiation treatment preoperatively. The low-grade group consisted of well and moderately differentiated adenocarcinomas, whereas the high-grade group consisted of poorly differentiated adenocarcinomas, including mucinous carcinomas. Tumor extent and staging was determined using the American Joint Committee on Cancer tumor, node and metastasis system.[11] The patient's progression-free survival (PFS) and overall survival (OS) were calculated from the date of surgery. The survival endpoint was the date of last follow-up or progression and death. This study was approved by the Institutional Review Board of Chonnam National University Hospital.

Immunohistochemistry

Immunohistochemisty for Livin, XIAP, and SMAC/DIABLO was performed using formalin-fixed, paraffin-embedded tissue sections and the avidin-biotin complex method. Briefly, representative paraffin blocks were cut consecutively at 4 μm thickness, deparaffinized in xylene, and treated with 0.3% hydrogen peroxide in methanol for 20 minutes to block endogeneous peroxidase activity. Sections were subjected to pressure-cooking for 7 minutes in 10 mM citrate phosphate buffer (pH 6.0) and then incubated with primary antibodies for 60 minutes at room temperature. Antibodies to Livin (1:200, Active Motif, San Diego, CA, USA), XIAP (1:100, BD Biosciences, San Jose, CA USA), and SMAC/DIABLO (1:100, R&D Systems, Minneapolis, MN, USA) were used. Anti-mouse immunoglobulin G (Sigma, St. Louis, MO, USA) labeled with biotin was used as a secondary antibody and was incubated for 7 minutes at 45°C. Avidin-biotin peroxidase complex staining using diaminobenzidine (Sigma) as the chromogen was performed with a Streptavidin-Horseradish Peroxidase Detection system (Ventana, Biotek Solutions, Tucson, AZ, USA). Negative controls were treated similarly with the exception of primary antibodies. Tissue from melanoma, esophageal cancer, and lung cancer samples were used as positive controls. Immunohistochemical staining was completed in duplicate for samples that did not agree. A high level of agreement was achieved using this procedure. Assessment for staining intensity was performed as follows: low, weak staining in cytoplasm and/or the nucleus compared to that of non-tumoral epithelial cells; and high, readily appreciable or dark brown staining distinctly marking the tumor cell cytoplasm and/or nucleus. Immunohistochemical staining was re-evaluated for cases showing disagreement between observers. Two pathologists reviewed the cases together and then reached an agreement for inconclusive samples. Tumor cell staining intensity was evaluated in coded slides by two of the authors, and the evaluation was performed twice without any knowledge of the clinical details.

Statistical analyses

Statistical analyses were performed with the SPSS ver. 15.0 (SPSS Inc., Chicago, IL, USA). The relationship between protein expression and categorical variables was compared by the χ^2 test, or Fisher exact probability test, when appropriate. Survival curves were estimated using the Kaplan-Meier method. The distribution of survival was comparatively studied using the log-rank test. Independent prognostic factors were determined using the Cox's proportional hazard model. The level of significance was set at $p<0.05$.

RESULTS

Clinicopathological findings

Clinicopathological findings from the 159 cases selected for this study were reviewed. The average age of onset of the patients was 61.7 years (range, 21 to 85 years); the subjects included 95 males and 64 females. The patients were followed for an average time of 47.8 months (range, 1 to 81 months) to determine clinical outcomes. Among the 159 resected cases, the primary tumor size varied from ≤4.5 cm in 76 cases to >4.5

cm in 83 cases. The locations of the cancers were the proximal colon from the cecum to the splenic flexure in 35 cases and the distal colon from the splenic flexure to the rectum in 124 cases. Twenty-one of 159 tumors showed high grade differentiation. The tumor extent was limited (T1 or T2) in 27 cases and advanced (T3 or T4) in 132 cases. Tumor metastasis to the lymph nodes was observed in 78 of 159 cases, and tumor metastasis to distant organs was observed in 18 cases (Table 1).

Expression of IAPs and correlation with clinicopathological factors

The intensity of IAP expression in CRCs was measured by immunohistochemistry. Livin, XIAP, and SMAC positive immunoreactivity was observed almost exclusively in the cytoplasm. In non-tumoral mucosa cells, Livin, XIAP, and SMAC exhibited mild cytoplasmic localization (Fig. 1A). However, they were significantly overexpressed in cancer tissues compared to nonneoplastic counterparts (Fig. 1D). Cases in which protein expression in tumor tissues was comparable to or weaker than surrounding nontumoral tissues were categorized into a low expression group (Figs. 1B, C, 2A, C), and cases with higher protein expression in tumor tissues than in the surrounding counterparts were categorized into a high expression group (Fig. 2B, D). High Livin, XIAP, and SMAC expression was found in 67 tumors (42.1%) of 159 samples and in 50 (31.4%) and 68 (42.8%) of 159 CRC cases, respectively (Table 2).

Table 1. Clinicopathological profiles of the 159 patients with colorectal cancer

Patient characteristics		No. of cases	%
Age (yr)	≤60	61	38.4
	>60	98	61.6
Sex	Male	95	59.7
	Female	64	40.3
Tumor size (cm)	≤4.5	76	47.8
	>4.5	83	52.2
Tumor location	Ascending	29	18.2
	Transverse	6	3.8
	Descending	10	6.3
	Rectosigmoid	114	71.7
Differentiation	Well	90	56.6
	Moderate	48	30.2
	Poor	21	13.2
Tumor extent	T1	7	4.4
	T2	20	12.6
	T3	123	77.3
	T4	9	5.7
Lymph node metastasis	Absent	81	50.9
	Present	78	49.1
Distant metastasis	Absent	141	88.7
	Present	18	11.3
Stage	1	19	11.9
	2	58	36.5
	3	64	40.3
	4	18	11.3
Chemoradiation treatment	Absent	36	22.6
	Present	123	77.4

IAP immunoreactivity and clinicopathological characteristics

The association between IAP protein expression and clinicopathological features was also examined in patients with CRC. A significant correlation was found between high Livin expression and low-grade tumor differentiation ($p = 0.031$). Livin expression tended to be high in tumors located distally, but the difference was not statistically significant ($p = 0.066$) (Table 2). Low SMAC expression was highly associated with tumors located distally ($p = 0.007$). XIAP expression did not show any significant correlation with the clinicopathological factors.

Analysis for recurrence-free survival (RFS) and OS

Kaplan-Meier curves were plotted using the staining results to determine the prognostic importance of Livin, XIAP, and SMAC expression in colorectal cancer. Livin-high patients survived significantly longer than Livin-low patients in terms of both RFS (median survival period, 62.5 months vs 54.1 months, respectively) and OS (median survival period, 64.3 months vs 56.4 months, respectively), although survival benefits showed a statistically borderline significance ($p = 0.054$ and $p = 0.095$, respectively) (Figs. 3A, 4). XIAP-low patients demonstrated a significantly longer PFS than XIAP-high patients (median survival period, 62.1 months vs 49.9 months, respectively, $p = 0.041$) (Fig. 3B), although no significant difference in OS was observed between the XIAP-low group and the XIAP-high group (median survival period, 61.7 months vs 56.4 months, respectively, $p = 0.364$). In contrast, no difference was observed in the PFS and OS rates between patients with high or low SMAC expression ($p = 0.506$ and $p = 0.775$, for PFS and OS, respectively). In univariate survival analyses, the following clinicopathological factors had a statistically significant affect on PFS and OS in patients with CRC: tumor extent, lymph node metastasis, distant metastasis, tumor differentiation, and tumor size ($p < 0.05$ for all and data not shown).

Tables 3 and 4 show the results of the multivariate analysis using the Cox proportional hazards model for PFS and OS. The

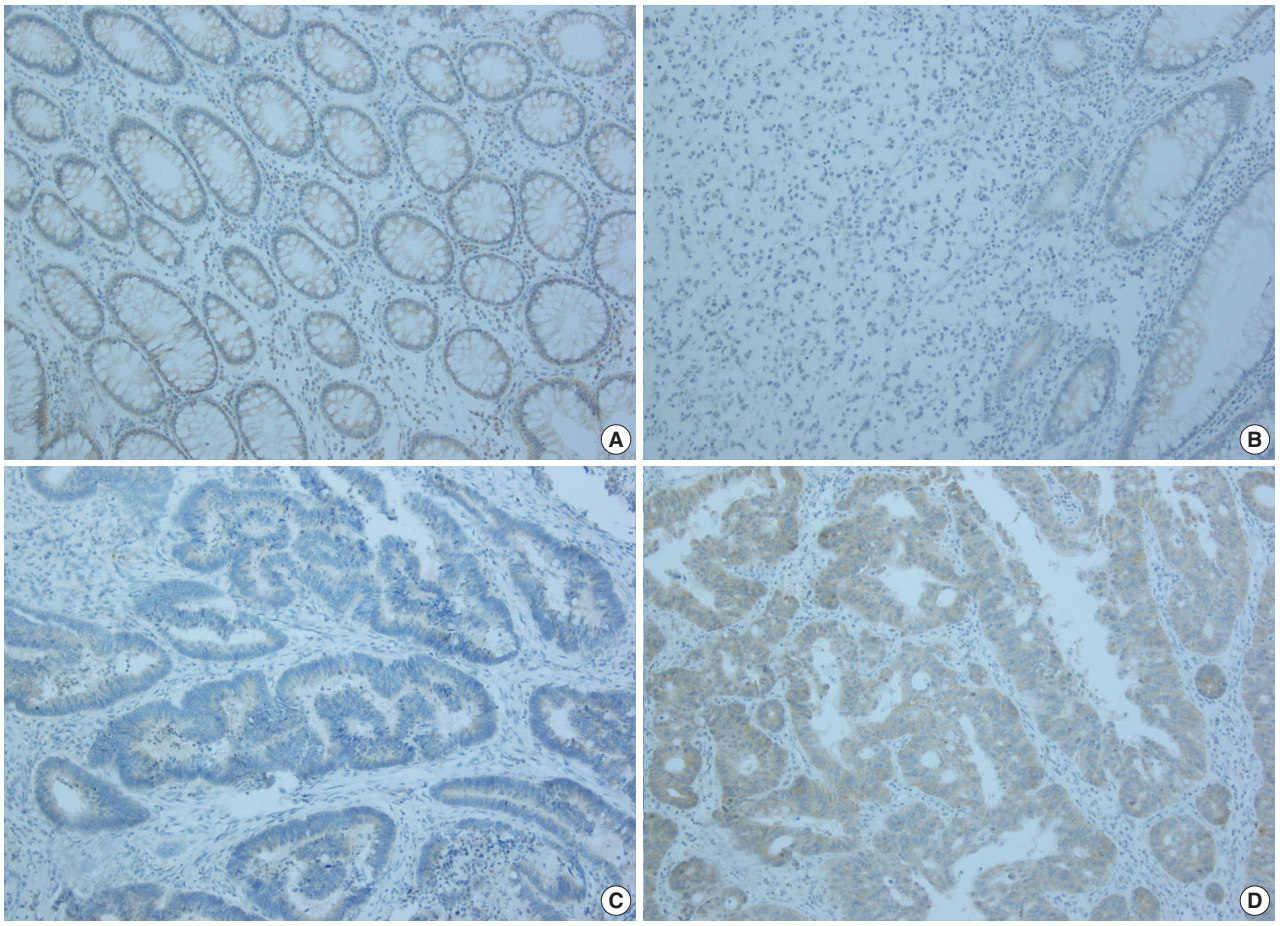

Fig. 1. Immunohistochemical analysis of Livin protein expression in colorectal carcinoma tissues. Livin expression is localized in the cytoplasm of tumor cells. Epithelial cells are weakly positive in the nontumoral mucosa (A). Livin protein expression is graded as negative (B), weakly positive (C), and strongly positive (D).

Table 2. Correlation between inhibitor of apoptosis proteins (IAP) expression and preoperative clinicopathological parameters of colorectal cancer

Patient characteristics			Livin			XIAP			SMAC		
			Low (n=92)	High (n=67)	p-value	Low (n=109)	High (n=50)	p- value	Low (n=91)	High (n=68)	p-value
Age (yr)	≤60	61	38	53	0.372	41	20	0.774	37	24	0.491
	>60	98	54	44		68	30		54	44	
Sex	Male	95	53	42	0.519	66	29	0.761	54	41	0.903
	Female	64	39	25		43	21		37	27	
Tumor size (cm)	≤4.5	76	43	33	0.754	56	20	0.182	48	28	0.148
	>4.5	83	49	34		53	30		43	40	
Tumor location	Proximal	35	25	10	0.066	22	13	0.411	13	22	0.007
	Distal	124	67	57		87	37		78	46	
Differentiation	Low grade	138	75	63	0.031	96	42	0.481	79	59	0.993
	High grade	21	17	4		13	8		12	9	
Tumor extent	T1 or T2	27	17	10	0.556	22	5	0.112	16	11	0.815
	T3 or T4	132	75	57		87	45		75	57	
LN metastasis	Absent	81	43	38	0.214	57	24	0.615	45	36	0.520
	Present	78	49	29		52	26		46	32	
Distant metastasis	Absent	141	79	62	0.190	98	43	0.47	80	61	0.724
	Present	18	13	5		11	7		11	7	
Stage	1 or 2	77	41	36	0.253	55	22	0.449	43	34	0.732
	3 or 4	82	51	31		54	28		48	34	

LN, lymph node.

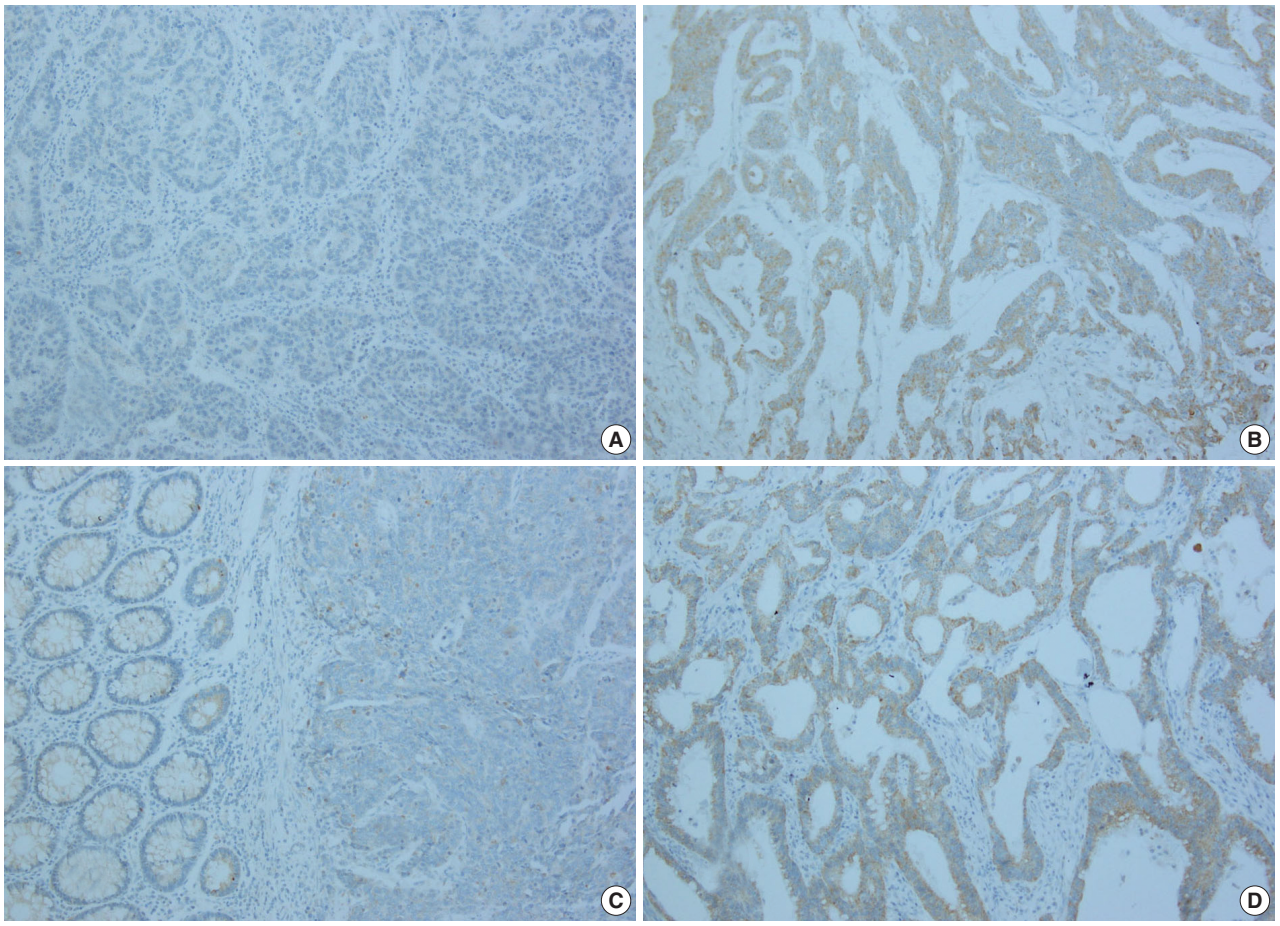

Fig. 2. Immunohistochemical analysis of XIAP and SMAC in colorectal carcinoma tissues. Subcellular distribution is dominantly cytoplasmic. Cases in which protein expression in tumor tissues is comparable to or weaker than surrounding nontumoral tissues are categorized into a low expression group (A), and cases with higher protein expression in tumor tissues than in the surrounding counterparts are categorized into a high expression group (B) for XIAP. Cases are grouped similarly for SMAC (C, D).

covariates included in the model were IAP expression, age, gender, stage, tumor differentiation, tumor location, tumor size, and chemoradiation treatment. The factors were simplified into two groups for better statistical discrimination in the Cox regression analysis. The results revealed that tumor size and stage were independent prognostic factors for both RFS and OS. Additionally, high XIAP expression had a statistically significant borderline influence on progression and high-grade tumor differentiation on death.

DISCUSSION

Eight IAPs have been identified in human cells to date, including 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).[3] Increasing evidence has suggested that the IAP family of proteins is essential for regulating apoptosis and nuclear factor-κB (NF-κB) signal transduction.[3]

Livin is a recently identified member of the IAP family and has been recognized as a potential modulator of apoptosis and survival in cancer.[3] Livin is not detectable in most adult tissues except the heart, lung, spleen, ovary, testes, and placenta but is present in transformed cells and in a variety of cancers.[12,13] Livin antagonizes apoptotic activity by inhibiting caspases-3, -7, and -9.[12]

Wang *et al.*[10] reported that *Livin* gene downregulation using siRNA led to apoptosis and chemosensitivity enhancement in tumor cells, whereas other researchers have demonstrated that Livin overexpression renders malignant cells resistant to chemotherapy.[9] Livin increases selectively in gastric cancer tissues compared with surrounding nontumoral tissues.[14] Wang *et al.*[15] showed that knockdown of Livin expression by siRNA rendered tu-

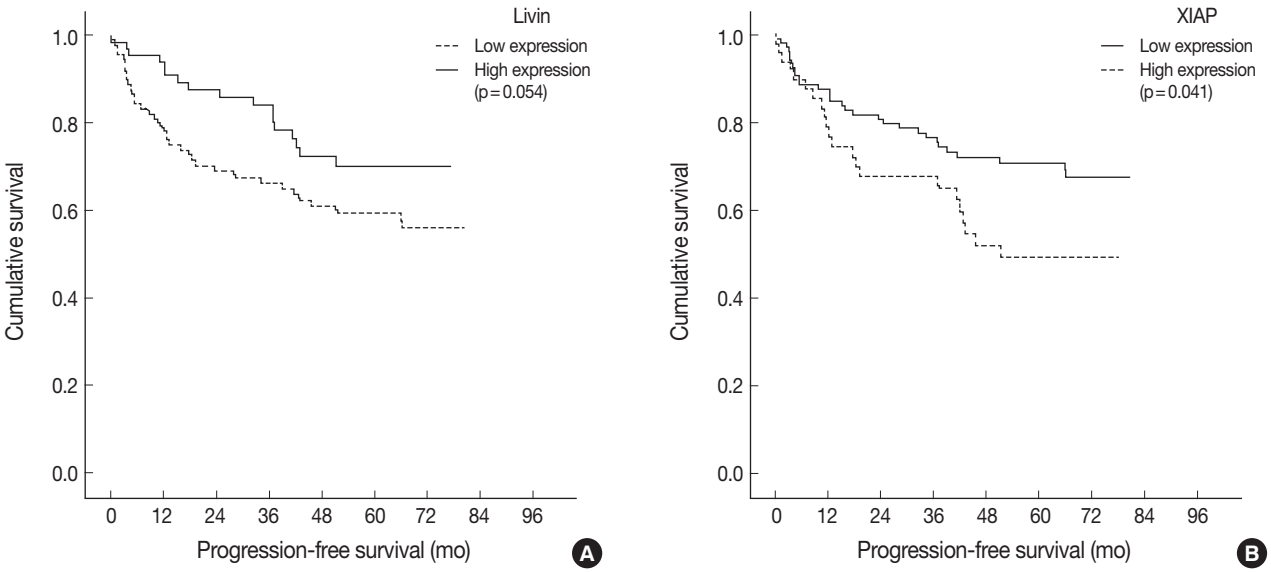

Fig. 3. Kaplan-Meier estimates of progression-free survival. High Livin expression is associated with longer survival rates than low Livin expression (p=0.054) (A). Low XIAP expression is significantly associated with extended survival (p=0.041) (B).

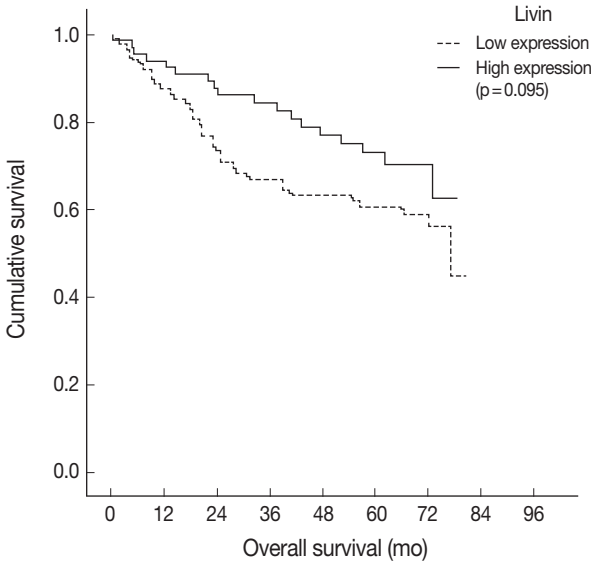

Fig. 4. Kaplan-Meier estimates of overall survival. High Livin expression is associated with longer survival rates than those of low Livin expression (p=0.095).

Table 3. Multivariate analyses of prognostic variables for recurrence-free survival

	Hazard ratio	95% CI	p-value
Age >60 yr	1.275	0.721-2.255	0.402
Female sex	0.669	0.373-1.199	0.177
Differentiation (high grade)	1.553	0.764-3.156	0.223
Location (distal colon)	0.679	0.328-1.403	0.296
Tumor size >4.5 cm	1.876	1.031-3.411	0.039
Chemoradiation treatment	1.836	0.726-4.643	0.199
Stage (advanced)	3.415	1.726-6.757	0.000
High Livin expression	0.608	0.326-1.134	0.118
High XIAP expression	1.707	0.925-3.149	0.087
High SMAC expression	1.009	0.544-1.873	0.977

CI, confidence interval.

Table 4. Multivariate analyses of prognostic variables for overall survival

	Hazard ratio	95% CI	p-value
Age >60 yr	1.133	0.632-2.031	0.674
Female sex	0.914	0.519-1.610	0.755
Differentiation (high grade)	1.897	0.941-3.824	0.073
Location (distal colon)	0.739	0.365-1.494	0.400
Tumor size >4.5 cm	2.173	1.184-3.991	0.012
Chemoradiation treatment	0.683	0.327-1.424	0.309
Stage	2.669	1.372-5.193	0.003
High Livin expression	0.731	0.397-1.349	0.316
High XIAP expression	1.346	0.722-2.510	0.350
High SMAC expression	0.844	0.452-1.575	0.594

CI, confidence interval.

mor cells from a colorectal cell line sensitive to chemotherapeutic agents. Nevertheless, no reports are available on the relationship between Livin expression and survival outcomes in patients with CRC in the English literature. The present study showed a strong trend for better PFS and OS in patients with high Livin expression than in patients with low Livin expression. Additionally, high Livin expression correlated with low-grade tumor differentiation. This result appeared to be contrary to the general understanding of IAPs, as Livin is a member of the IAP fam-

ily and is supposed to be associated with a poor prognosis by prolonging cancer cell survival. However, the prognostic implication of Livin expression varied according to tumor type. Livin expression level is unrelated to patient survival in nasopharyngeal carcinoma[16] and hepatocellular carcinoma.[17] In patients with neuroblastoma, high Livin expression decreases survival only when combined with amplified *MYCN*, whereas Livin expression alone has no effect on survival.[8] In contrast, a favorable prognosis was reported in patients with Livin-positive malignant pleural mesotheliomas[18] and similarly in childhood acute lymphoblastic leukemia.[5]

The *Livin* gene encodes two splicing variants called Livin-α and Livin-β.[13,19] These two isoforms play different roles in the antiapoptotic capacity of tumor cells and may represent a regulatory balance between apoptosis and its counterpart.[4] Clinically, Livin-α is getting more attention because it is more closely related to tumor behavior than Livin-β. Increased Livin-α expression is related to the proportion of bladder tumors with a high risk of relapse, possibly by regulating the G1-S cell cycle transition.[7,20] In contrast, some researchers have reported that Livin-α displays a paradoxical pro-apoptotic effect in response to certain stimuli depending on subcellular localization and mutation at the cleavage site, which eliminates this pro-apoptotic effect of Livin-α.[13,21] Although Ashhab *et al.*[13] showed high levels of both α- and β-isoforms in colon carcinoma cell lines, differences in the distribution of Livin according to its splicing variants remain to be clarified.

XIAP is unique in that it directly binds and inhibits activated caspases.[22] For example, XIAP sequestrates caspase-9 through its BIR3 domain and suppresses caspase-3 and caspase-7 via its BIR2 domain with its N-terminal linker.[22] In addition, XIAP promotes NF-κB activation by enhancing NF-κB translocation from the cytoplasm into the nucleus.[23]

Although Endo *et al.*[24] reported that XIAP mRNA expression is not different between normal and cancerous colorectal tissues, we observed higher XIAP expression in cancerous tissues than that in surrounding normal tissues by immunohistochemistry. Increased XIAP expression has been reported in a variety of human tumors as well, including esophageal carcinoma, clear cell renal carcinoma, ovarian carcinoma, lymphoma, and pancreatic cancer.[25] We correlated low levels of XIAP expression with a favorable PFS in our CRC series. A previous report showed that a XIAP-high group had significantly shortened PFS and OS in CRC.[26] Shi *et al.*[25] and Augello *et al.*[17] demonstrated similar results in patients with hepatocellular carcinoma.

Recently, second mitochondria-derived activator of caspase/direct IAP binding protein with low pI (SMAC/DIABLO) has been identified as one of main antagonists of IAP proteins and is involved in the balance and regulation of apoptotic stimuli.[17] It has been demonstrated that Livin is inhibited by SMAC, which is released from mitochondria along with cytochrome *c* after initiation of the intrinsic apoptotic cascade. SMAC appears to function as a general IAP inhibitor by binding to IAP-family members and neutralizing their antiapoptotic activities via its N-terminal residues, which recognize a surface groove on the BIR3 domains.[3]

In cells expressing Livin, the ability of IAPs to inhibit cell death is reversed by SMAC coexpression or adding SMAC peptides.[27] Livin has very high-affinity for SMAC but is significantly inferior to XIAP in terms of caspase inhibition. It has been proposed that the inhibitory effect of Livin on caspase-3 and caspase-9 is minor, whereas the anti-apoptotic effect of Livin could be ascribed to its antagonizing the XIAP-SMAC interaction rather than direct inhibition.[28] Because SMAC has a pro-apoptotic effect, its expression should be considered a favorable prognostic marker in malignancies. Unfortunately, only a limited number of studies are available to date. Only one study has correlated SMAC expression with prognosis in patients with CRC. Endo *et al.*[29] proposed that the decrease in SMAC/DIABLO expression is an independent factor determining a poorer prognosis of patients with CRC. In the present study, SMAC expression was correlated with proximal tumor location but did not show any significant survival difference according to expression level.

It is presumed that most IAP members would exert a similar influence on the clinical behavior of malignant tumors, considering the significant homology among IAP genes. However, the current results imply that there could be different biological roles for each IAP member according to tumor type. Although both Livin and XIAP presumably antagonize apoptotic activities within tumor cells, their effects on survival were opposite; high Livin expression was associated with a better prognosis, whereas high XIAP expression was associated with a worse prognosis. This observation suggests that Livin and XIAP may have additional molecular functions in addition to their apparent roles in apoptotic cascades in terms of biological balance within cells with both anti- and pro-apoptotic consequences.[21] How altered IAP expression affects tumor progression remains to be clarified. For a thorough understanding of the role of Livin in carcinogenesis, future investigations are needed to investigate the expression and subcellular localization of the protein and the relationship between the Livin-α and -β isoforms.[17]

In conclusion, our results demonstrated frequent alterations in the expression of IAP family members in a large series of CRC. The IAP expression profiles suggested that Livin and XIAP may play a substantial role as effective prognostic markers in CRC. Moreover, because of the preferential expression of Livin and XIAP in CRC tissues rather than in nontumoral tissues, these data suggest that targeting the pathways encompassing Livin and XIAP may be useful in a group of selective patients with altered gene expression.

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