X-Linked Inhibitor of Apoptosis Protein Expression Level in Colorectal Cancer Is Regulated by Hepatocyte Growth Factor/C-Met Pathway via Akt Signaling

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Abstract

Purpose: The inhibitor of the apoptosis protein (IAP) family members, such as the X-linked IAP (XIAP), survivin, and livin, are essential for cell survival and antiapoptosis in colorectal cancer cells. We *hypothesized* that the hepatocyte growth factor (HGF) activation in colorectal cancer via c-Met receptor regulates IAP proteins through Akt signaling.

Experimental Design: The level of IAPs and C-Met mRNA expression was assessed using a quantitative real-time reverse transcriptase-PCR (RT-PCR) assay on colorectal normal mucosa (n = 13), adenomas (n = 6), and colorectal cancer tumors (n = 50). The role of HGF/C-Met pathway through Akt and XIAP was investigated by small interfering RNA (siRNA) and quantitative RT-PCR analysis of colorectal cancer lines.

Results: Of the IAPs, only XIAP showed significant correlation to tumor development and progression. XIAP mRNA level in primary colorectal cancer was significantly higher than that in colorectal normal mucosa (P = 0.01); liver metastases was significantly higher than primary colorectal cancer tumors (P = 0.04); and primary colorectal cancer N1/N2 cases were significantly higher than N0 cases (P = 0.008). HGF stimulation of colorectal cancer lines enhanced XIAP mRNA expression but not other IAPs. Activation of XIAP expression by HGF was inhibited by siRNA targeting Akt1 and Akt2.

Conclusions: Activation of C-MET enhances XIAP through the Akt pathway. XIAP up-regulation was shown to be correlated to colorectal cancer tumor progression. The Akt-XIAP pathway may be a potential molecular target for regulating colorectal cancer progression.

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Colorectal carcinoma is one of the most common gastrointestinal malignancy worldwide and the second leading cause of cancer-related deaths in the United States (1). Tumor invasion, regional lymph node metastasis, and distant organ metastasis are important factors for determining colorectal cancer prognosis. Recent molecular studies of tumor-related signal pathways have elucidated sequential mechanisms associated with the various tumor progression events (2, 3).

C-Met, a proto-oncogene, is suggested to be associated with colorectal cancer progression (4, 5). The C-Met protein contains a tyrosine kinase domain, which is found to initiate various cell function regulating signals via the Akt signaling pathway (6–8). Hepatocyte growth factor (HGF), also known as scatter factor, produced by stromal fibroblasts or other cell types, such as hepatocytes, can function in a paracrine manner on cancer cells via the C-Met receptor (4). The activation of C-Met by the HGF/scatter factor ligand can induce proliferation, motility, adhesion, and invasion of tumor cells (9–11). HGF/scatter factor activation of the C-Met tyrosine kinase pathway has been thought of as one of the key factors influencing the events of tumor progression. Several studies have reported overexpression of C-Met mRNA/protein in colorectal cancer (5, 12, 13). We previously reported that higher C-Met mRNA expression by quantitative real-time reverse transcriptase-PCR (RT-PCR) assay was correlated with aggressive primary tumor invasion and presence of regional lymph node metastasis (5).

Cell death is regulated by antiapoptotic proteins, such as Bcl-2 family and the inhibitor of the apoptosis protein (IAP) family (14–16). Bcl-2 inhibits cytochrome *c* release from mitochondria, whereas proteins of the IAP family act downstream to prevent processing of caspase-9 by cytochrome *c* and the apoptotic protease activating factor 1 apoptosome complex (14, 15). The IAP family members, X-linked IAP (*XIAP*), survivin, and livin, have been shown in colorectal cancer cells (17–19).

XIAP is located on chromosome Xq25 and encodes a cytoplasmic protein of 497 amino acids, 57 kDa (20, 21). XIAP prevents processing of procaspase-3, procaspase-6, and procaspase-7 by binding or blocking the activity of caspase-9 (17). The expression of *XIAP* has been shown to be up-regulated in

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many human carcinomas (22, 23). *Survivin*, an IAP, is located on chromosome 17q25 and encodes a 17-kDa protein (15, 24). Although the mechanism of inhibiting apoptosis by survivin has not been conclusively defined, it has been reported that survivin blocks the intrinsic mitochondrial pathway of apoptosis by interacting with caspase-9 and preventing apoptosomes in cells from receiving an apoptotic stimulus (25). *Survivin* can also bind the effector cell death proteases, caspase-3 and caspase-7, to inhibit caspase activity and cell death (26). Overexpression of *survivin* has been shown to have oncogenic potential; it overrides the G_2-M phase cell cycle checkpoint to enforce progression of cells through mitosis and affects microtubule stability of the mitotic spindles (15, 27). *Livin*, located on chromosome 20q13, is another major IAP member (28). Livin, similar to other IAP members, can inhibit apoptosis by preventing apoptotic protease activating factor 1 and cytochrome *c* from activating caspase-9 (28).

Recent studies suggested that HGF/scatter factor activation of the C-*Met* tyrosine kinase pathway may be influencing antiapoptosis and cell survival of tumor cells (6). We *hypothesized* that HGF activation of those colorectal cancer overexpressing C-*Met* receptor may regulate IAP family proteins via the Akt signaling pathway. IAP expression in colorectal cancer specimens may be related to tumor clinicopathology characteristics of colorectal cancer development, and tumor progression. In this study, we assessed mRNA expression of *XIAP*, *survivin*, and *livin* and compared them to C-Met in colorectal normal mucosa, colorectal adenoma, and colorectal cancer specimens. The regulatory role of HGF/C-Met pathway in antiapoptotic protein expression through IAPs expression via Akt was investigated in *in vitro* studies.

Materials and Methods

Clinicopathology of primary colorectal cancer. Primary colorectal cancer tumors were obtained form 39 patients. The clinicopathology characteristics were as follows: 19 males and 20 females, with a mean age of 71.2 ± 12.5 with a range of 46 to 95. American Joint Committee on Cancer stage primary tumor distribution was as T1 = 3 (8%), T2 = 9 (23%), T3 = 24 (62%), and T4 = 3 (8%). American Joint Committee on Cancer regional lymph node status was N0= 22 (56%), N1 = 11 (28%), and N2 = 6 (15%). American Joint Committee on Cancer staging was stage I = 12 (31%), stage II = 10 (26%), and stage III = 17 (44%). Histologic grade of primary colorectal cancer was well differentiated = 6 (15%), moderate differentiated = 23 (59%), and poor/undifferentiated = 10 (26%).

Cell lines. Three established colorectal cancer cell lines, HT-29, DLD1, and SW480, were obtained from the American Type Culture Collection (Manassas, VA). All established cell lines were grown in RPMI 1640 supplemented with 100 mL/L heat-inactivated fetal bovine serum, penicillin, and streptomycin (Life Technologies, Grand Island, NY) in T75-cm^2 flask as previously described (29). Total RNA was extracted from cells when cell cultures reached 70% to 80% confluence as previously described (30).

Patients and specimens. Patients with colorectal tumors undergoing surgery were accrued from the John Wayne Cancer Institute at Saint Johns Health Center (Santa Monica, CA) and Century City Hospital (Los Angeles, CA). All patients in the study were consented according to the guidelines set forth by the John Wayne Cancer Institute and other participating center's human subjects Institutional Review Board committee. We studied a total of 56 colorectal tumors (6 colorectal adenoma, 39 primary colorectal cancers, and 11 liver metastases of colorectal cancer) from 54 patients randomly selected by the database coordinator between 1999 and 2002. Thirteen colorectal normal mucosa were obtained from patients with primary colorectal cancer at surgery. All colorectal tumor specimens and colorectal normal mucosa used in the quantitative RT-PCR analysis were collected immediately after colorectal cancer resection, processed under nucleic acid sterile conditions as previously described (31), and stored at −80°C if not immediately processed. Tumor specimens were coded and assayed in a blinded manner.

Small interfering RNA transfection. The Akt small interfering RNA (siRNA) was obtained from Cell Signaling Technology (Beverly, MA). The Akt siRNA inhibits expression of both Akt1 and Akt2. Silencer-negative control siRNA (Ambion, Inc., Austin, TX) was used as control to show that transfection does not induce nonspecific effects on gene expression. Lamin siRNA was used as a positive control. DLD1 and SW480 cells were transfected with the Akt siRNA or negative control siRNA by lipofection (LipofectAMINE Reagent, Invitrogen, Carlsbad, CA). Cells (10^5) were plated on a 24-well culture plates and incubated in 0.4 mL of Opti-MEM with 4 μL of LipofectAMINE and Akt siRNA or negative control siRNA at a final concentration of 50 nmol/L for 10 hours. After incubation, medium was changed to RPMI 1640 containing 10% fetal bovine serum. At 48 hours after transfection, the medium was changed to serum-free RPMI 1640 to render the cells quiescent for 4 hours, and the cells were stimulated with recombinant human HGF (40 ng/mL; R&D Systems, Minneapolis, MN) for 3 hours. After the stimulation with the HGF, total cellular RNA was extracted and processed for quantitative RT-PCR. siRNA for human C-Met (Dharmacon Research, Inc., Lafayette, CO) was transfected into DLD1 cells (10^5) with LipofectAMINE (Invitrogen, Carlsbad, CA). Assays were done as described for Akt siRNA. For proliferation studies, cells were grown in six-well cell culture plates, transfected with C-Met siRNA, and then treated with HGF 24 hours. Controls were cells not transfected with siRNA, cells only transfected with siRNA and not treated with HGF, cells not transfected with siRNA and HGF treated, cells transfected with lamin siRNA and HGF treated, and cells transfected with a scrambled siRNA and HGF treated. For counting cells were harvested by trypsinization and then counted with a hemacytometer in duplicates under the microscope. The assays were done twice.

RNA isolation. Total cellular RNA from cell lines, normal epithelium, and colorectal tumor specimens was extracted and purified using Tri-Reagent (Molecular Research Center, Cincinnati, OH) as previously described (30, 31). All RNA extractions were done in designated sterile laminar flow hood using RNase/DNase-free laboratory ware. RNA was quantified and assessed for purity by UV spectrophotometry and RIBOGreen detection assay (Molecular Probes, Eugene, OR). Tissue processing, RNA extraction, and quantitative RT-PCR assay setup were done in separate designated rooms to prevent cross-contamination, as previously reported (30, 31).

Synthesis of primers and probes. Using the Oligo Primer Analysis Software, version 6.0 (National Biomedical systems, Plymouth, MN), we selected primer and probe sequences to optimally hybridize and amplify target cDNA for quantitative RT-PCR assay. To avoid possible amplification of contaminating genomic DNA, primers were designed so that each PCR product covered at least one intron. Fluorescence resonance energy transfer probe sequences were as follows: XIAP, 5′-ACTGAAGAGCAGCTAAGGCGCCTG-3′; *survivin*, 5′-CCGGAGCGGATGGCCGAGGCTGGC-3′; livin, 5′-TGAGCTGCCCACACCCAGGAGAG-3′; C-Met, 5′-TGGGAGCTGATGACAAGAGGAG-3′; Akt1, 5′-AGCATGGAGTGTGTGGACAGCGAG-3′; Akt2, 5′-TGAAGTCGCTCACACAGTCACCGAG-3′; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5′-CAGCAATGCCTCCTGCACCACCAA-3′. *GAPDH* was used as an internal reference housekeeping gene for the quality of all specimen mRNA assessed, as previously described (30). All specimens assessed were determined to have high integrity of mRNA for PCR studies.

Quantitative real-time reverse transcriptase-PCR assay. All reverse transcriptase reactions were done using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) with oligo-dT priming as

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previously described (30, 31). The quantitative RT-PCR assay was done in the iCycler iQ Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using cDNA from 250 ng of total RNA for each reaction (30). Samples were amplified with a precycling hold at 95°C for 10 minutes followed by 45 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute for *XIAP, survivin*, C-Met, and *GAPDH* (annealing at 58°C for livin), and extension at 72°C for 1 minute.

A standard curve for quantifying mRNA copy number was established by amplifying nine aliquots of templates with known copy numbers (10^0 to 10^8 copies; refs. 5, 30). The known templates are plasmids constructed with cDNA synthesized by reverse transcription, amplified by PCR, run on 2% agarose gel electrophoresis, and then extracted using the QIAquick gel extraction method (Qiagen, Valencia, CA). The cDNA was ligated into the pCR II-TOPO cloning vector (Invitrogen, San Diego, CA), clones were transformed into *Escherichia coli* DH5-α cells, and cultures were expanded (5, 30). Plasmids containing the target gene were purified and quantified for use in the quantitative RT-PCR setup. To confirm the correct size of the inserted PCR product, plasmids were digested with specific restriction enzymes, and the cDNA clone PCR products were then verified on gel electrophoresis.

The PCR amplification of the serially diluted cDNA standard templates of each marker showed a logarithmic signal increase (5, 30). Standard curves were generated for each marker by using the threshold cycle (C_t) of templates in known numbers of copies. The C_t of each sample was plotted on the standard curve, and the mRNA copy number was calculated by the iCycler iQ Real-time Detection System Software (Bio-Rad Laboratories) as previously described (5). For each assay, the standard templates, three established colorectal cancer cell lines (HT-29, SW480, and DLD-1; ref. 5), four standard normal controls (normal healthy donor peripheral blood lymphocytes from noncancer patients; refs. 5, 30), and reagent controls (reagent alone without RNA or cDNA) for quantitative RT-PCR assay were included. All assays were optimized for individual markers, and reproducibility verified with patients' specimens. Each assay was repeated at least twice to verify the results, and the mean mRNA copy number was used for analysis. The mRNA expression of each gene was normalized with *GAPDH* mRNA expression. Final data analysis was done using the ratio of mRNA copy number of target mRNA to *GAPDH* mRNA copy number.

Immunohistochemistry. Expression of *XIAP, survivin, livin*, and C-*Met* in colorectal cancer cells was assessed by immunohistochemistry as previously described (5). Formalin-fixed paraffin-embedded tissue blocks (≤5 years old) retrieved from Surgical Pathology were used in the study. Freshly cut 5-μm sections were mounted on slides and then deparaffinized in xylene. The slides were incubated in Target Retrieval Solution (DAKO, Carpinteria, CA) at 95°C for 20 minutes for *survivin* and *livin*; for C-*Met*, the slides were incubated in 0.01 mol/L sodium citrate and heated in a microwave oven for 12 minutes. The sections were incubated and kept at 4°C overnight with monoclonal mouse anti-human *XIAP* antibody (BD Biosciences, San Diego, CA) at a dilution of 1:200, polyclonal goat anti-human *survivin* antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human livin polyclonal IgG antibody (1:100; Alpha Diagnostics International, Inc., San Antonio, TX), or polyclonal rabbit anti-human C-Met antibody (1:200; Zymed Laboratories, San Francisco, CA). Pathologically verified metastatic malignant melanoma specimens were used as positive controls (5). Negative control slides were treated with nonimmunized goat or rabbit or mouse IgG under equivalent conditions as the assay specimens and with no primary antibody. For the secondary developing reagents, a labeled streptavidin-biotin kit (DAKO) was used. Slides were developed with diaminobenzaminidine and counterstained with hematoxylin. Histopathology was reviewed by two reviewers.

Statistical analysis. To investigate the association between each mRNA expression and clinicopathologic variables, ANOVA and Student's *t* test were used. Spearman correlation coefficient analysis was used to assess the relation among each marker mRNA expressions. All *P*s = 0.05, using two-sided analysis, were considered statistically significant.

Results

Akt small interfering RNA effect on colorectal cancer lines. Initially, we assessed the efficacy of Akt siRNA to down-regulate Akt in colorectal cancer lines with high expression of C-Met. The inhibitory effect of Akt siRNA for Akt mRNA expression on colorectal cancer lines, DLD1 and SW480, both of which overexpressed C-Met mRNA (data not shown) was assessed. The Akt siRNA was designed to inhibit expression of both Akt1 and Akt2. After transfection with Akt siRNA, DLD1 and SW480 cells expressed significantly less Akt1 and Akt2 mRNA when compared with nontransfected DLD1 or SW480 cells (Figs. 1 and 2). The transfection with the negative control siRNA had no effects on Akt1 or Akt2 mRNA expression levels of the colorectal cancer cell lines (Figs. 1 and 2). The study verified the inhibitory effect of the Akt siRNA on Akt1 and Akt2 mRNA expression in DLD1 and SW480 cells.

Hepatocyte growth factor effect on inhibitor of apoptosis protein mRNA expression. HGF stimulation of C-Met expressing cells has been suggested to promote cell proliferation and anti-apoptosis. Our hypothesis was that HGF stimulation of C-Met

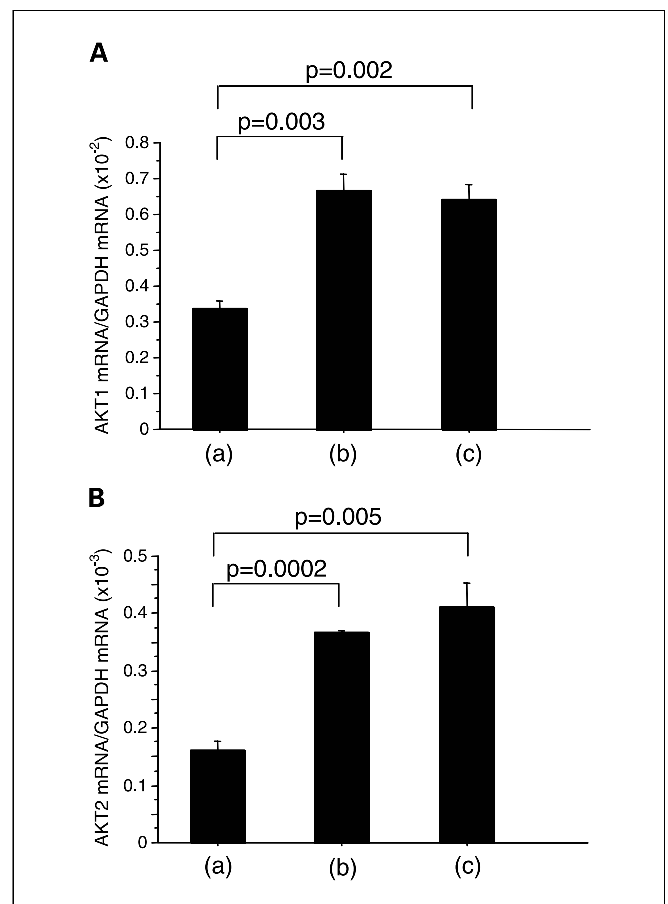

Fig. 1. Effect of Akt mRNA expression level after Akt siRNA transfection of DLD1 cells. *A,* Akt1 mRNA levels. *B,* Akt2 mRNA levels. *a,* Akt siRNA; *b,* negative control siRNA; *c,* mock vector (no siRNA) treatment.

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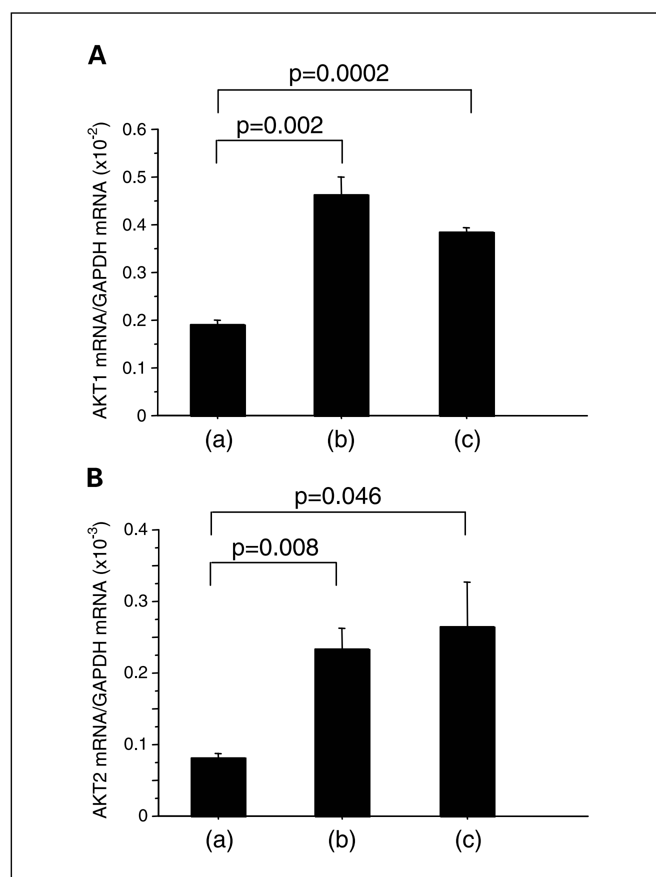

Fig. 2. Effect of Akt mRNA expression level after Akt siRNA transfection of SW480 cells. *A,* Akt1 mRNA. *B,* Akt2 mRNA. *a,* Akt siRNA; *b,* negative control siRNA; *c,* mock vector treatment.

expressing colorectal cancer cells up-regulated the level of IAPs to promote antiapoptosis and allow for survival. The mRNA expression levels of the IAPs, *XIAP*, *survivin*, and *livin* in colorectal cancer lines stimulated with HGF were assessed. Among the three IAP family members, only *XIAP* mRNA expression significantly ($P < 0.05$) increased after HGF stimulation in both DLD1 and SW480 cells (Fig. 3A and Fig. 4A). *Survivin* or *livin* mRNA expression did not show any significant changes after HGF stimulation (Fig. 3B and C and Fig. 4B and C). The experiments showed that HGF stimulation of C-Met-bearing cells can up-regulate IAPs however only XIAP significantly.

We then set out to determine if the up-regulation of *XIAP* after HGF stimulation involved Akt. Transfection with Akt siRNA into DLD1 and SW480 cells significantly decreased XIAP mRNA expression level compared with transfection with the negative control siRNA or mock vector (vector with no siRNA; Fig. 3A and Fig. 4A). This indicated that Akt expression can regulate *XIAP* up-regulation. In Akt siRNA-transfected cells, *XIAP* mRNA expression was not affected by HGF stimulation. These results confirmed that Akt is a critical link in the HGF-C-Met activation pathway of *XIAP*. Moreover, the expression levels of *survivin* or livin in the Akt siRNA-transfected and the respective negative control siRNA-transfected cell lines remained unchanged. The results suggested that *survivin* and *livin* are not directly involved in the HGF/C-Met activation pathway via Akt. To determine that HGF stimulation is specific through C-Met, C-Met mRNA expression was down-regulated by siRNA. C-Met siRNA was transfected into cell lines to determine the specificity of HGF treatment on C-Met-expressing cells. Expression of *GAPDH*, C-Met, XIAP, Akt1, and Akt2 were determined following HGF treatment of C-Met siRNA-transfected cells. Relevant controls were as described above. There was down-regulation of gene expression for C-Met (100%), *XIAP* (96%), Akt1 (82%), and Akt2 (80%) after siRNA C-Met transfection plus HGF treatment compared with HGF treatment

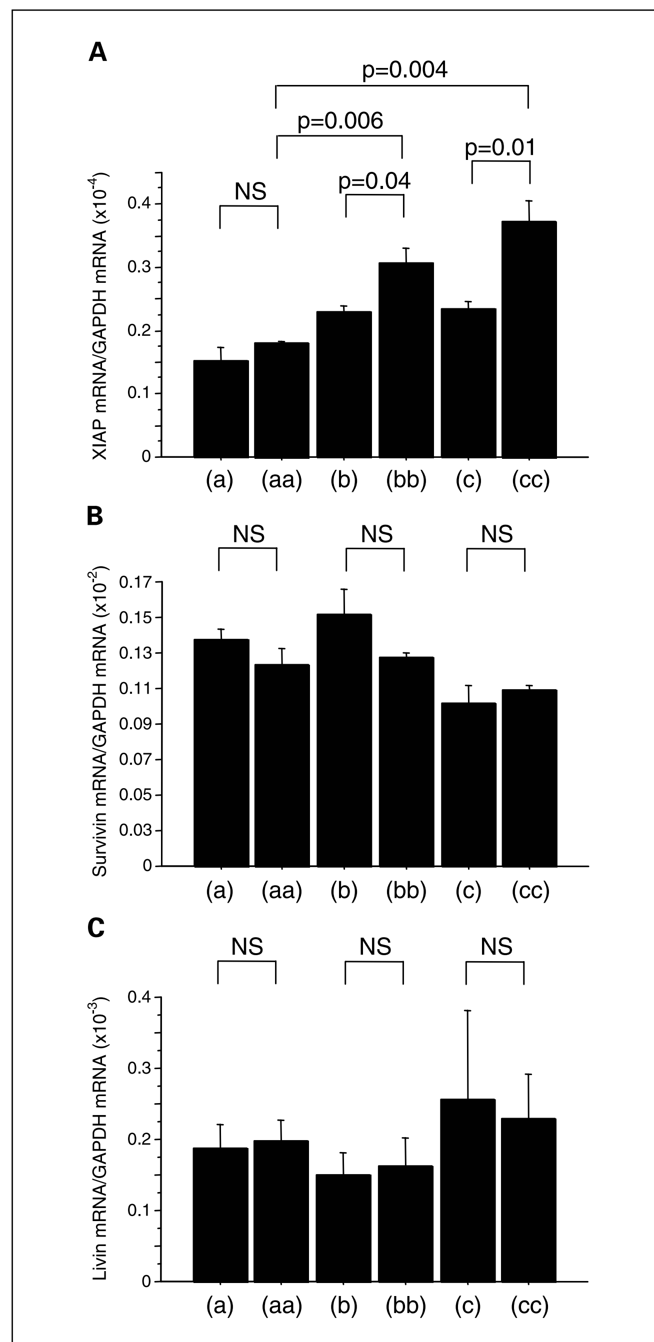

Fig. 3. IAP mRNA level after HGF stimulation and Akt siRNA transfection on DLD1 cells. *A,* XIAP mRNA. *B,* survivin mRNA. *C,* livin mRNA. *a,* Akt siRNA, HGF (−); *aa,* Akt siRNA, HGF (+); *b,* control siRNA, HGF (−); *bb,* control siRNA, HGF (+); *c,* mock HGF (−); *cc,* mock HGF (+).

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alone. Overall C-Met siRNA transfection of cell lines abrogated the up-regulation of *Akt* and *XIAP* gene expression after HGF stimulation.

Additional experiments were done to determine the effect of siRNA C-Met transfection on HGF treatment on cell growth. DLD-1 cells after HGF treatment showed an increase of 15% cell growth in 24 hours. However, this growth was abolished in C-Met siRNA-transfected cells treated with HGF. Control cell cultures did not show any significant changes. The studies with C-Met siRNA support the pathway mechanism of the HGF/C-Met/Akt/XIAP activation as we hypothesized. The results indicate that HGF activates the Akt pathway and induces XIAP expression in colorectal cancer cell lines that overexpress C-Met. Inhibition of Akt1 and Akt2 expression by siRNA suppressed XIAP mRNA expression, which is activated through the HGF/C-Met/Akt pathway.

C-Met mRNA expression in normal and adenoma and colorectal cancer tumors. We examined human normal, benign, and colorectal cancer specimens to determine if the *in vitro* observation in cell lines of IAP expression, particularly XIAP up-regulation, was related to colorectal cancer tumor development and progression. To assess C-Met expression, we used a quantitative mRNA assay as a biomarker surrogate of gene expression in tissues. Initially, C-Met expression was analyzed to assess the overall expression levels relative to colorectal normal mucosa, colorectal adenoma, and colorectal cancer specimens. C-Met mRNA expression was detectable by quantitative RT-PCR in 12 of 13 (92%) normal colon mucosal tissues, 5 of 6 (83%) adenomas, and in 50 of 50 (100%) colorectal cancer specimens. The C-Met mRNA expression level in colorectal cancer specimens was significantly higher than that of colorectal normal mucosa tissues (colorectal normal mucosa versus all colorectal cancer specimens, $P < 0.0001$; colorectal normal mucosa versus T1/T2 tumor, $P = 0.03$). Colorectal adenoma showed different levels of C-Met mRNA expression (colorectal adenoma versus all colorectal cancer, $P = 0.06$). C-Met mRNA expression levels significantly correlated with the depth of tumor invasion (T level: T1/T2 versus T3/T4, $P = 0.02$; Fig. 5A). However, the C-Met expression in liver metastases was not significantly different from primary colorectal cancer tumors. C-Met mRNA copy levels in N1/N2 cases were significantly higher than N0 cases ($P = 0.05$; Fig. 5A). The analyses showed that C-Met overexpression was related to primary tumor development and progression. Identifying this pattern then allowed us to proceed to determine if IAP expression followed a similar pattern of up-regulation.

Inhibitor of apoptosis protein mRNA expression in colorectal cancer tumors. We next assessed mRNA expression levels of IAP family members, *XIAP*, *survivin*, and *livin* in colorectal normal mucosa, colorectal adenoma, and colorectal cancer tumors. Quantitative mRNA expression of the specific genes was used as a biomarker surrogate of expression levels of tissues. The *XIAP* mRNA expression was detectable by quantitative RT-PCR assay in 69 of 69 (100%) tissue specimens that included colorectal normal mucosa, colorectal adenoma, and colorectal cancer tumors. The levels of *XIAP* mRNA in colorectal cancer was significantly higher than that of 13 specimens of colorectal normal mucosa (colorectal normal mucosa versus all colorectal cancer specimens, $P = 0.01$; colorectal normal mucosa versus T3/T4 tumor, $P = 0.02$). There was a significant trend of *XIAP* mRNA up-regulation related to

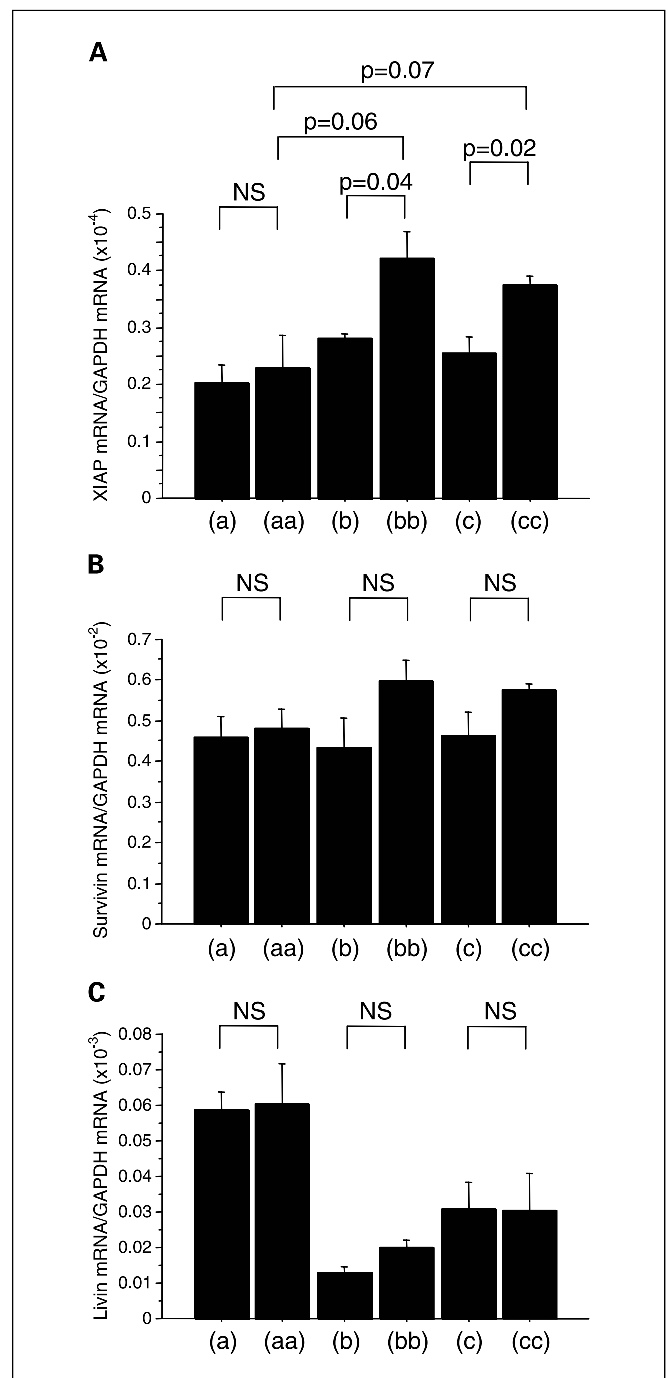

Fig. 4. IAP mRNA level after HGF stimulation and Akt siRNA transfection on SW480 cells. *A,* XIAP mRNA. *B,* survivin mRNA. *C,* livin mRNA. *a,* Akt siRNA, HGF (−); *aa,* Akt siRNA, HGF (+); *b,* control siRNA, HGF (−); *bb,* control siRNA, HGF (+); *c,* mock HGF (−); *cc,* mock HGF (+).

development of colorectal normal mucosa, colorectal adenoma, and colorectal cancer. In liver metastases, *XIAP* mRNA levels was significantly higher than primary colorectal cancer tumors (liver metastasis versus T1/T2 tumor, $P = 0.006$; liver metastasis versus T3/T4 tumor, $P = 0.04$; Fig. 5B). Overall, *XIAP* mRNA levels in colorectal cancer N1/N2 cases were significantly higher than colorectal cancer N0 cases ($P = 0.008$; Fig. 5B). These analyses indicated that *XIAP* expression up-regulation was correlated

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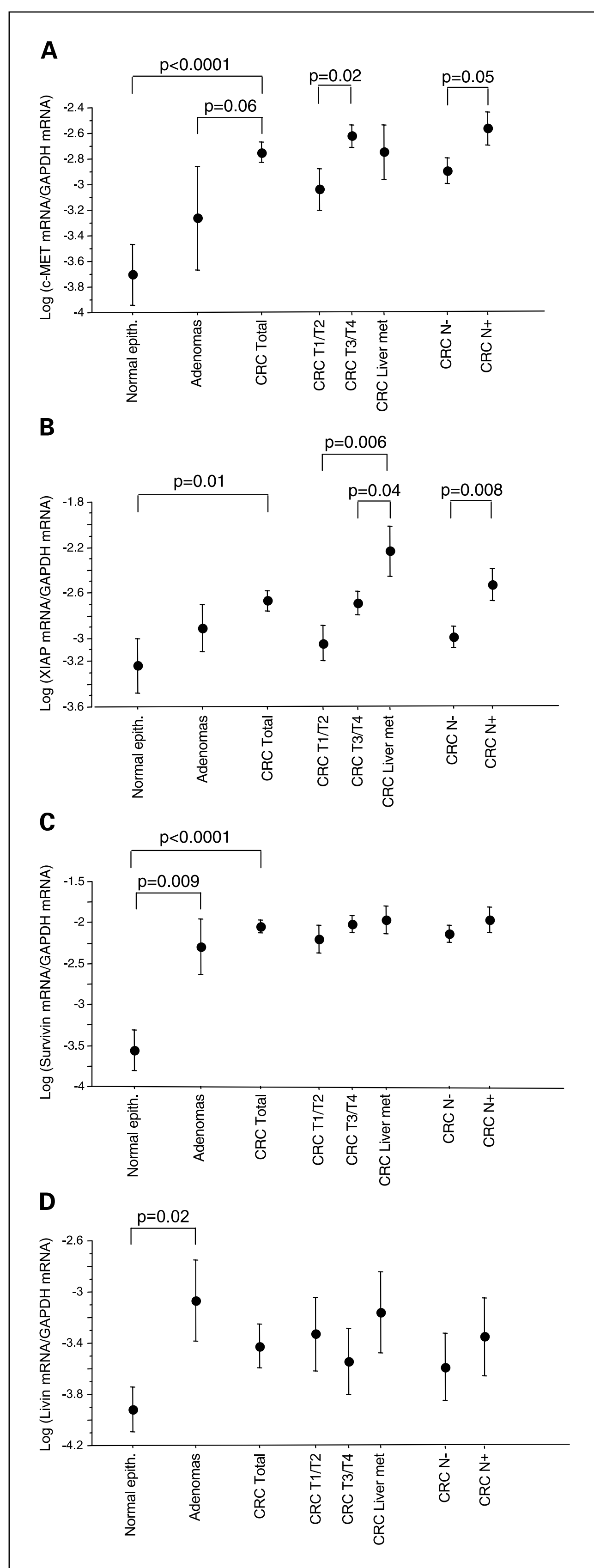

Fig. 5. C-Met and IAP mRNA levels in colon specimens. *A,* C-Met mRNA. *B,* XIAP mRNA. *C,* survivin mRNA. *D,* livin mRNA. Normal epithelium; colorectal cancer (*CRC*) liver metastases; N−, lymph node metastasis (−); N+, lymph node metastasis (+).

to progression of colorectal normal mucosa, colorectal adenoma, to colorectal cancer. The analyses also showed that XIAP mRNA levels of colorectal cancer tumors were significantly related to progression of pathology stage.

Other IAP family members survivin and livin were then examined in colorectal normal mucosa, colorectal adenoma, and colorectal cancer tissues to determine if an up-regulation trend similar to that of *XIAP* also exists. Survivin mRNA expression was detectable by quantitative RT-PCR assay in 66 of 69 (96%) specimens. The *survivin* mRNA expression level in colorectal normal mucosa was significantly lower than that from colorectal adenoma and colorectal cancer (colorectal normal mucosa versus all colorectal cancer, $P < 0.0001$; colorectal normal mucosa versus colorectal adenoma, $P = 0.009$; colorectal normal mucosa versus T1/T2 tumor, $P = 0.0002$; Fig. 5C). However, there were no differences in *survivin* mRNA expression among colorectal adenoma, colorectal cancer primary tumors, and liver metastases. *Survivin* mRNA expression level did not correlate with either T level or N factor (Fig. 5C). This indicated that there was no observable trend of increase in *survivin* expression as related to colorectal cancer and pathology stage. *Livin* mRNA expression was detectable by quantitative RT-PCR assay in 61 of 69 (88%) specimens. The livin mRNA expression level in colorectal normal mucosa was significantly lower than that from colorectal adenoma (colorectal normal mucosa versus colorectal adenoma, $P = 0.02$; Fig. 5D). However, there were no significant differences in livin mRNA expression among colorectal adenoma, colorectal cancer primary tumors, and liver metastases. *Livin* mRNA expression level did not correlate with T level or N factor (Fig. 5D). The results showed that livin mRNA expression does not significantly change with colorectal cancer progression.

To determine whether our *in vitro* observations on C-Met activation were related to *XIAP* up-regulation, we assessed the correlation of expression of these genes in colorectal cancer tumors. The C-Met mRNA expression level was significantly correlated with *XIAP* mRNA expression level in all colorectal tissues ($P < 0.0001$) and colorectal cancer tumors ($P = 0.0005$; Fig. 6A and B). There was a significant correlation of C-Met mRNA expression with survivin mRNA expression when data from all colon tissues were assessed together. However, results from colorectal cancer alone did not show significant correlation of C-Met mRNA expression to *survivin* mRNA level (data not shown). The C-Met mRNA expression level did not correlate with *livin* mRNA expression level (data not shown). These analyses strongly suggest up-regulation of C-Met correlates with *XIAP* up-regulation in colorectal cancer tissues. The results also suggest that *survivin* and *livin* are not directly related to C-Met activation.

Immunohistochemistry analysis of C-Met protein expression. Immunohistochemistry was done on respective primary colorectal cancer in which quantitative RT-PCR was done to determine concordance of the results. C-Met, *XIAP*, *survivin*, and *livin* proteins in colorectal cancer and colorectal normal mucosa were evaluated (data not shown). Very weak or no immunostaining for C-Met, *XIAP*, *survivin*, and *livin* protein was observed in normal epithelial cells of the colorectal normal mucosa. In contrast, primary colorectal cancer cells showed a range of immunoreactivity in the cytoplasm. Colorectal cancer tumors with high C-Met, *XIAP*, *survivin*, and *livin* mRNA copies showed strong immunostaining for each protein. In particular,

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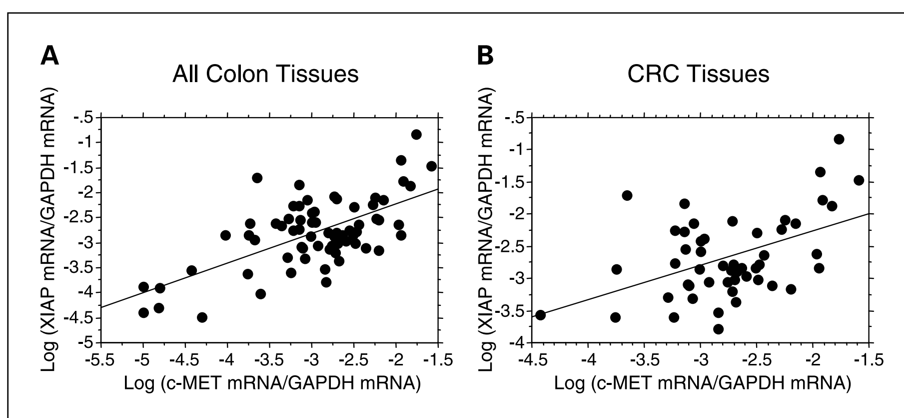

Fig. 6. Correlation between C-Met and XIAP mRNA expression. *A,* all colon specimens (n = 69). *B,* colorectal cancer (*CRC*) specimens (n = 50).

C-Met and XIAP expression showed a similar strong immunostaining pattern in colorectal cancer tumors. These immunohistochemistry results confirmed our findings that mRNA expression of XIAP correlated with that of C-Met.

Discussion

HGF plays a major role in tumor proliferation, migration, invasion, and metastasis via the C-Met pathway in primary colorectal cancer tumor. Tumorigenic activity of C-Met depends on deregulation of the HGF/C-Met signaling pathway. Constitutive activation of tyrosine kinase receptors can be obtained by C-Met overexpression and activation by ligand (4). Recent studies have shown that HGF/C-Met signaling pathway activates Akt by phosphorylation (6). Akt signaling is known to be involved in cell survival and antiapoptosis (6). IAP protein expression correlates with not only antiapoptotic potentials but also tumor proliferation, metastasis, and poor prognosis in various carcinomas (32 – 34). We hypothesized that activation of the HGF/C-Met pathway may accelerate the antiapoptotic mechanisms of colorectal cancer tumors by increasing IAP expression via Akt. We determined that there is a link of C-Met activation and IAPs by assessing colorectal cancer lines with specific gene knockout with siRNA and quantitative gene expression analysis. It was found that HGF stimulation can enhance XIAP mRNA expression levels but not *survivin* or *livin*. The activation of XIAP expression was shown to be inhibited by siRNA targeting Akt1 and Akt2. Patient tissues were then assessed and determined that both *XIAP* and C-Met mRNA expression was significantly higher in colorectal cancer tumors than in colorectal normal mucosa and colorectal adenoma. *XIAP* expression levels correlated with colorectal cancer tumor's pathology stage. XIAP mRNA expression was significantly higher in metastatic tumors in liver than in primary tumors; this supports that up-regulation of *XIAP* may play a role in promoting antiapoptosis and aggressive tumors. Moreover, only *XIAP* expression among the IAP family was significantly correlated to development of colorectal normal mucosa, colorectal adenoma, and colorectal cancer.

Among the three IAP members, only *XIAP* mRNA expression was significantly increased by HGF stimulation in colorectal cancer cell lines and was blocked by inactivation of Akt by Akt siRNA. The results suggest that the transcription of *XIAP* is regulated by HGF/C-Met pathway via Akt1 and Akt2 activation in colorectal cancer cells. *XIAP* plays an important role in cell survival and antiapoptosis against antitumor drugs in colorectal cancer cells. Moreover, the findings on *XIAP* up-regulation of expression in colorectal cancer specimens support our *in vitro* results. We showed *XIAP* mRNA overexpression as a potential molecular marker associated with liver and lymph node metastasis. *XIAP* is likely to be involved in both colorectal cancer development and tumor progression including metastasis. Overexpression of this gene would facilitate antiapoptosis and promote cell survival.

HGF has been known to activate Akt via the C-Met signaling pathway (8, 35). Recent studies have shown that activated Akt (phosphorylated Akt) negatively regulates apoptotic pathways (36, 37). In particular, Dan et al. (37) showed similar results to our *in vitro* study; our results and other studies indicate that *XIAP* is a direct posttranscriptional downstream target of Akt and an important mediator of Akt on cell survival. siRNA against Akt1 and Akt2 lowered the amount of active (phosphorylated) Akt *in vitro* (37 – 39). In recent studies, other groups have shown by *in vitro* studies the role of HGF activation via C-Met on Akt and *XIAP* (40, 41). Our *in vitro* results support that HGF/C-Met pathway activates antiapoptosis via Akt and *XIAP* activation. In support of this, we showed that C-Met siRNA transfection of colorectal cancer cells significantly abrogated C-Met expression and HGF effect on up-regulation of *XIAP*, Akt1, and Akt2. Other growth factors may be involved in regulating XIAP (42). Further studies are needed to assess other growth factors and signaling pathways independent of the HGF/C-Met pathway.

The transcription level of other IAP members, such as *survivin*, was not significantly affected by HGF stimulation and Akt siRNA. The results suggest that signal pathways other than HGF/C-Met pathway may affect survivin transcription levels (43, 44). Kim et al. (43) reported that *survivin* transcription is regulated by APC/T-cell factor/β-catenin pathway, which is activated in development of colorectal cancer. *Survivin* mRNA expression levels in colorectal cancer tumors were significantly higher than normal epithelium. However, *survivin* mRNA expression level in colorectal cancer tumors did not correlate with any clinicopathologic factors. Interestingly, adenomas also expressed survivin mRNA similar to colorectal cancer. Activation of *survivin* may play a more important role in the development of colorectal cancer than in tumor progression. Yang et al. (17) reported that coexistence of high levels of *XIAP* and survivin plays antiapoptotic functions in tumor cell lines. Our results also suggest that *XIAP* and survivin may cooperatively regulate cell survival and anti-apoptosis in colorectal cancer cells.

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Livin is expressed in colorectal cancer tumors, but its role in colorectal cancer tumors however, its functional role, is not well known (28). In this study, *livin* mRNA expression was not significantly changed by HGF stimulation or Akt inactivation by Akt siRNA. The *livin* mRNA expression level in colorectal cancer tumors did not correlate with any clinicopathologic factors, suggesting its limited role in colorectal cancer progression.

In summary, we have shown that *XIAP* expression is related to colorectal normal mucosa, colorectal adenoma, and colorectal cancer progression, and that *XIAP* up-regulation is through the HGF/C-Met/Akt pathway. The studies showed that IAP up-regulation, particularly *XIAP*, plays an important role in colorectal cancer development and progression. We showed *XIAP* mRNA overexpression as a potential molecular marker associated with lymph node metastasis and C-Met mRNA expression. Our *in vitro* results showed that *XIAP*, among the three IAP members, was the main downstream target of Akt, which was activated by HGF/C-Met signaling. To our knowledge, this is the first study to determine the three IAP mRNA expression levels in colorectal normal mucosa, colorectal adenoma, and colorectal cancer tissues. The *XIAP* mRNA expression in primary colorectal cancer may be a useful molecular indicator for regional lymph node metastasis and tumor progression. Further studies on the biological behavior of the colorectal cancer tumors involving *XIAP* may lead to the development of new targeted therapy inhibiting this HGF/C-Met signal pathway.

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References

1. Jemal A, Thomas A, Murray T, Thun M. Cancer statistics, 2002. CA Cancer J Clin 2002;52:23–47.
2. Goel A, Arnold CN, Boland CR. Multistep progression of colorectal cancer in the setting of microsatellite instability: new details and novel insights. Gastroenterology 2001;121:1497–502.
3. Zhou W, Goodman SN, Galizia G, et al. Counting alleles to predict recurrence of early-stage colorectal cancers. Lancet 2002;359:219–25.
4. Trusolino L, Comoglio PM. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. Nat Rev Cancer 2002;2:289–300.
5. Takeuchi H, Bilchik A, Saha S, et al. C-Met expression level in primary colon cancer: a predictor of tumor invasion and lymph node metastases. Clin Cancer Res 2003;9:1480–8.
6. Xiao GH, Jeffers M, Bellacosa A, Mitsuuchi Y, Vande Woude GF, Testa JR. Anti-apoptotic signaling by hepatocyte growth factor/Met via the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways. Proc Natl Acad Sci U S A 2001;98:247–52.
7. Coltella N, Manara MC, Cerisano V, et al. Role of the MET/HGF receptor in proliferation and invasive behavior of osteosarcoma. FASEB J 2003;17:1162–4.
8. Shinomiya N, Gao CF, Xie Q, et al. RNA interference reveals that ligand-independent met activity is required for tumor cell signaling and survival. Cancer Res 2004;64:7962–70.
9. Jiang WG, Lloyds D, Puntis MC, Nakamura T, Hallett MB. Regulation of spreading and growth of colon cancer cells by hepatocyte growth factor. Clin Exp Metastasis 1993;11:235–42.
10. Uchiyama A, Essner R, Doi F, et al. Interleukin 4 inhibits hepatocyte growth factor-induced invasion and migration of colon carcinomas. J Cell Biochem 1996;62:443–53.
11. Matsumoto K, Nakamura T, Kramer RH. Hepatocyte growth factor/scatter factor induces tyrosine phosphorylation of focal adhesion kinase (p125FAK) and promotes migration and invasion by oral squamous cell carcinoma cells. J Biol Chem 1994;269:31807–13.
12. Di Renzo MF, Olivero M, Giacomini A, et al. Overexpression and amplification of the met/HGF receptor gene during the progression of colorectal cancer. Clin Cancer Res 1995;1:147–54.
13. Wielenga VJ, van der Voort R, Taher TE, et al. Expression of C-Met and heparan-sulfate proteoglycan forms of CD44 in colorectal cancer. Am J Pathol 2000;157:1563–73.
14. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2002; 2:647–56.
15. Altieri DC. Validating survivin as a cancer therapeutic target. Nat Rev Cancer 2003;3:46–54.
16. Raff M. Cell suicide for beginners. Nature 1998;396: 119–22.
17. Yang L, Cao Z, Yan H, Wood WC. Coexistence of high levels of apoptotic signaling and inhibitor of apoptosis proteins in human tumor cells: implication for cancer specific therapy. Cancer Res 2003;63:6815–24.
18. Yagihashi A, Asanuma K, Tsuji N, et al. Detection of anti-livin antibody in gastrointestinal cancer patients. Clin Chem 2003;49:1206–8.
19. Endo T, Abe S, Seidlar HB, et al. Expression of IAP family proteins in colon cancers from patients with different age groups. Cancer Immunol Immunother 2004;53:770–6.
20. Rajcan-Separovic E, Liston P, Lefebvre C, Korneluk RG. Assignment of human inhibitor of apoptosis protein (IAP) genes xiap, hiap-1, and hiap-2 to chromosomes Xq25 and 11q22-q23 by fluorescence *in situ* hybridization. Genomics 1996;37:404–6.
21. Stehlik C, de Martin R, Kumabashiri I, Schmid JA, Binder BR, Lipp J. Nuclear factor (NF)-κB-regulated X-chromosome-linked IAP gene expression protects endothelial cells from tumor necrosis factor α-induced apoptosis. J Exp Med 1998;188:211–6.
22. Ferreira CG, van der Valk P, Span SW, et al. Expression of X-linked inhibitor of apoptosis as a novel prognostic marker in radically resected non-small cell lung cancer patients. Clin Cancer Res 2001;7:2468–74.
23. Parton M, Krajewski S, Smith I, et al. Coordinate expression of apoptosis-associated proteins in human breast cancer before and during chemotherapy. Clin Cancer Res 2002;8:2100–8.
24. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 1997;3:917–21.
25. O'Connor DS, Grossman D, Plescia J, et al. Regulation of apoptosis at cell division by p34cdc2 phosphorylation of survivin. Proc Natl Acad Sci U S A 2000;97:13103–7.
26. Tamm I, Wang Y, Sausville E, et al. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res 1998;58:5315–20.
27. Li F, Ambrosini G, Chu EY, et al. Control of apoptosis and mitotic spindle checkpoint by survivin. Nature 1998;396:580–4.
28. Kasof GM, Gomes BC. Livin, a novel inhibitor of apoptosis protein family member. J Biol Chem 2001;276: 3238–46.
29. Miyashiro I, Kuo C, Huynh K, et al. Molecular strategy for detecting metastatic cancers with use of multiple tumor-specific MAGE-A genes. Clin Chem 2001;47:505–12.
30. Takeuchi H, Kuo C, Morton DL, Wang HJ, Hoon DS. Expression of differentiation melanoma-associated antigen genes is associated with favorable disease outcome in advanced-stage melanomas. Cancer Res 2003;63:441–8.
31. Bilchik AJ, Saha S, Wiese D, et al. Molecular staging of early colon cancer on the basis of sentinel node analysis: a multicenter phase II trial. J Clin Oncol 2001;19:1128–36.
32. Lu CD, Altieri DC, Tanigawa N. Expression of a novel antiapoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinomas. Cancer Res 1998;58:1808–12.
33. Kato J, Kuwabara Y, Mitani M, et al. Expression of survivin in esophageal cancer: correlation with the prognosis and response to chemotherapy. Int J Cancer 2001;95:92–5.
34. Cao C, Mu Y, Hallahan DE, Lu B. XIAP and survivin as therapeutic targets for radiation sensitization in preclinical models of lung cancer. Oncogene 2004;23:7047–52.
35. Okano J, Shiota G, Matsumoto K, et al. Hepatocyte growth factor exerts a proliferative effect on oval cells through the PI3K/Akt signaling pathway. Biochem Biophys Res Commun 2003;309:298–304.
36. Sonoda Y, Matsumoto Y, Funakoshi M, Yamamoto D, Hanks SK, Kasahara T. Anti-apoptotic role of focal adhesion kinase (FAK). Induction of inhibitor-of-apoptosis proteins and apoptosis suppression by the overexpression of FAK in a human leukemic cell line, HL-60. J Biol Chem 2000;275:16309–15.
37. Dan HC, Sun M, Kaneko S, et al. Akt phosphorylation and stabilization of X-linked inhibitor of apoptosis protein (XIAP). J Biol Chem 2004;279:5405–12.
38. Sugatani T, Hruska KA. Akt1/Akt2 and Mammalian target of rapamycin/bim play critical roles in osteoclast differentiation and survival, respectively, whereas akt is dispensable for cell survival in isolated osteoclast precursors. J Biol Chem 2005;280:3583–9.
39. Stahl JM, Sharma A, Cheung M, et al. Deregulated Akt3 activity promotes development of malignant melanoma. Cancer Res 2004;64:7002–10.
40. Uddin S, Hussain AR, Al-Hussein KA, Manogaran PS, Wickrema A, Gutierrez MI, Bhatia KG. Inhibition of phosphatidylinositol 3′-kinase/AKT signaling promotes apoptosis of primary effusion lymphoma cells. Clin Cancer Res 2005;11:3102–8.
41. Shinomiya N, Gao CF, Xie Q, et al. RNA interference reveals that ligand-independent MET activity is required for tumor cell signaling and survival. Cancer Res 2004;64:7962–70.
42. Pardo OE, Lesay A, Arcaro A, et al. Fibroblast growth factor 2-mediated translational control of IAPs blocks mitochondrial release of Smac/DIABLO and apoptosis in small cell lung cancer cells. Mol Cell Biol 2003;23:7600–10.
43. Kim PJ, Plescia J, Clevers H, Fearon ER, Altieri DC. Survivin and molecular pathogenesis of colorectal cancer. Lancet 2003;362:205–9.
44. Zhang T, Otevrel T, Gao Z, Ehrlich SM, Fields JZ, Boman BM. Evidence that APC regulates survivin expression: a possible mechanism contributing to the stem cell origin of colon cancer. Cancer Res 2001;61:8664–7.