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Proto-oncogene, Pim-3 with serine/threonine kinase activity, is aberrantly expressed in human colon cancer cells and can prevent Bad-mediated apoptosis

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We previously observed that Pim-3 with serine/threonine kinase activity, was aberrantly expressed in malignant lesions of endoderm-derived organs, liver and pancreas. Because Pim-3 protein was not detected in normal colon mucosal tissues, we evaluated Pim-3 expression in malignant lesions of human colon, another endoderm-derived organ. Pim-3 was detected immunohistochemically in well-differentiated (43/68 cases) and moderately differentiated (23/41 cases) but not poorly differentiated colon adenocarcinomas (0/5 cases). Moreover, Pim-3 proteins were detected in adenoma (35/40 cases) and normal mucosa (26/111 cases), which are adjacent to adenocarcinoma. Pim-3 was constitutively expressed in SW480 cells and the transfection with Pim-3 short hairpin RNA promoted apoptosis. In the same cell line, a pro-apoptotic molecule, Bad, was phosphorylated at Ser^{112} and Ser^{136} sites of phosphorylation that are representative of its inactive form. Ser^{112} but not Ser^{136} phosphorylation in this cell line was abrogated by Pim-3 knockdown. Furthermore, in human colon cancer tissues, Pim-3 co-localized with Bad in all cases (9/9) and with phospho-Ser^{112}Bad in most cases (6/9). These observations suggest that Pim-3 can inactivate Bad by phosphorylating its Ser^{112} in human colon cancer cells and thus may prevent apoptosis and promote progression of human colon cancer. (*Cancer Sci* 2007; 98: 321–328)

Pim-3 was originally identified as the depolarization-induced gene, *KID-1*, in PC12 cells, a rat pheochromocytoma cell line.[1] Because KID-1 shows a high sequence similarity with the proto-oncogene Pim family that expresses serine/threonine kinase activity, it was renamed as Pim-3.[2] Accumulating evidence indicates the potential involvement of other Pim family members, Pim-1 and Pim-2, in various types of carcinogenesis.[3–6] Indeed, Deneen and colleagues demonstrated that Pim-3 gene transcription was enhanced in EWS/ETS-induced malignant transformation of NIH 3T3 cells.[7] Moreover, we revealed that Pim-3 was selectively expressed in malignant lesions but not normal tissues of endoderm-derived organs, liver[8] and pancreas.[9] These observations suggest the potential involvement of Pim-3 in carcinogenesis, particularly in endoderm-derived organs.

Apoptosis is an important mechanism during normal crypt formation in another endoderm-derived organ, colon.[10] The molecular mechanism of colon cancer progression is known to involve several key events and mutation of a number of crucial genes.[11] A partial suppression of apoptosis occurs as a result of the inactivation of both alleles of the *APC* gene and the suppression of apoptosis allows APC-deficient cells to develop adenomatous polyps.[12] Further suppression of apoptosis occurs as these cells develop additional genetic mutations and phenotypic changes.[13] Thus, the dysregulation of apoptosis is crucially involved in colon carcinogenesis.

Accumulating evidence indicates that Pim kinases can phosphorylate substrates that regulate apoptosis, and that activated Pim kinases might contribute to the pathogenesis of a wide variety of malignancies.[14] Similarly, as observed on other members of Pim family,[15,16] we demonstrated that aberrantly expressed Pim-3 can inactivate a pro-apoptotic BH3-only Bcl-2-like molecule, Bad, by phosphorylating its serine residue and eventually prevent apoptosis in human pancreatic cancer cell lines.[9] These observations suggest that Pim-3 may contribute to carcinogenesis by negatively modulating apoptosis.

Our previous northern blotting analysis failed to detect Pim-3 mRNA in normal human endoderm-derived organs including liver, pancreas and colon.[8] Given that Pim-3 can prevent apoptosis, we assumed that Pim-3 might be aberrantly expressed in malignant lesions of the colon and might contribute to colon carcinogenesis. To address this hypothesis, we examined Pim-3 expression in human colon cancer tissues and found that Pim-3 protein was detected in a substantial portion of human colon cancer samples, particularly at the less advanced stages of cancer development. Moreover, the ablation of endogenous Pim-3 in the colon cancer cell line reduced the amount of phosphorylated Bad and eventually promoted apoptosis. Furthermore, we demonstrated that Pim-3 was co-localized with Bad in cancer cells in human colon cancer tissues.

Materials and methods

Antibodies. The following antibodies were used: mouse anti-Bad and rabbit anti-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit anti-heat shock protein (Hsp)60 antibodies (StressGen Biotechnologies, Victoria, Canada); Alexa Fluor 488 donkey anti-rabbit IgG, and Alexa Fluor 594 donkey anti-mouse IgG (Molecular Probes); rabbit anti-phospho-Ser^{112}Bad, anti-phospho-Ser^{136}Bad, and anti-phospho-Ser^{155}Bad antibodies (Cell Signaling Technology, Beverly, MA, USA); ImmunoPure peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies (Pierce Biotechnology, Rockford, IL, USA).

Cell culture. A well-differentiated colon cancer-derived cell line, SW480[17] was maintained in RPMI-1640 medium (Sigma Chemical); while a moderately differentiated colon cancer-derived cell line, HT29,[18] and a poorly differentiated cell line, HCT116[19] were maintained in McCoy's 5 A Modified Medium (Invitrogen, Carlsbad, CA, USA). The media were supplemented with 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA, USA) and the cells were incubated in a humidified incubator at 37°C in 5% CO_2.

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Colon tissue samples. Colon tissue specimens were obtained with informed consent from individuals as shown in Table 2. As controls, colon tissues were also obtained from four patients with diverticulitis but without any malignant lesions. These samples were collected and paraffin-embedded at the Toyama University Hospital with approval from the Human Subjects Research Ethical Committee of Toyama University, Faculty of Medicine. An additional human colon tissues was obtained from a patient upon surgery for colon cancer with his informed consent and with approval from the Human Subjects Research Ethical Committee of Kanazawa University Hospital. We separated the tumor and normal tissue, which was at least 2 cm apart from the edge of tumor foci and was judged histologically to be free from adenocarcinoma cells, and stored them immediately at −80°C until protein extraction.

Preparation of polyclonal anti-Pim-3 antibodies. White Japanese rabbits were immunized with a synthetic, keyhole limpet hemocyanin-conjugated Pim-3 peptide (CGPGGVDHLPVKILQPAKAD), that corresponds to the amino acid residues between 13 and 32 in human Pim-3. This region is well conserved in human and murine Pim-3, but shows a high diversity with other Pim family members, Pim-1 and Pim-2.[8] An equal volume of the antigen (2 mg/mL) and an adjuvant, Titer Max Gold (CytRx, Atlanta, GA, USA), were mixed and emulsified by sonication. Rabbits were immunized 11 times (2-week interval), by s.c. injection of the antigen mixture into their backs. Immunoglobulin (Ig)G fractions were purified from serum utilizing a Protein G Sepharose 4 Fast Flow column (Amersham Biosciences AB, Uppsala, Sweden) according to the manufacturer's instructions. The resultant IgG fractions were further purified using HiTrap NHS-activated HP colum (Amersham Biosciences AB) conjugated with the peptide used for the immunization. The concentration of the affinity-purified antibody was determined by measuring the absorbance at 280 nm.

Immunohistochemical analysis of human colon cancer tissues. Paraffin-embedded human colon adenocarcinoma tissues were deparaffinized in xylene, rehydrated through graded concentrations of ethanol and washed with phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked with 3% H_2O_2 (DakoCytomation, Carpinteria, CA, USA) for 5 min. For antigen retrieval, sections were heated in 10 mM sodium citrate buffer (pH 6.0), followed by blocking with Non-Specific Staining Blocking reagent (DakoCytomation). The slides were incubated with rabbit polyclonal anti-Pim-3 IgG (3 μg/mL) or with normal rabbit IgG overnight at 4°C. The slides were further incubated with horseradish peroxidase (HRP)-labeled antirabbit polymer EnVision + System (DakoCytomation) at room temperature for 30 min. Immune complexes were visualized with Peroxidase Substrate DAB kit (Vector Laboratories, Burlingame, CA, USA), counterstained with hematoxylin, dehydrated and coverslipped. Positive cells exhibit either a supranuclear or cytoplasmic staining pattern. A researcher without a prior knowledge of the clinical samples evaluated the proportion of Pim-3-positive cells in colon carcinoma tissues in a semiquantitative manner, without assessing the intensities, as follows; (–) absent positive cells; (+–) positive cells in less than 5% of total; (+) positive cells with 5–25%; (++) positive cells with 25–50%; and (+++) positive cells with greater than 50%.

Immunofluorescence analyses. Cells were seeded on Laboratory-Teck chamber slides (Nalge Nunc International, Naperville, IL, USA) and incubated at 37°C with 5% CO_2. Forty-eight hours later, they were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100/PBS for 10 min. Immunostaining was performed by incubating the slides with rabbit polyclonal anti-Pim-3 (3 μg/mL), or with the combination of mouse anti-Bad (1:30) and rabbit anti-Hsp60 antibodies (1:200), followed by incubation with Alexa Fluor 488 donkey anti-rabbit and/or Alexa Fluor 594 donkey anti-mouse (1:100) fluorescent antibodies for 1 h at room temperature. As a negative control, slides were incubated with normal donkey serum instead of the primary antibodies. In some experiments, after washing with PBS, cells were incubated with propidium iodide (Annexin V-FITC kit, Bender MedSystems, GmbH, Vienna, Austria; 1 μg/mL) for 1 min. Images were captured on a Carl Zeiss LSM510 confocal microscope (Carl Zeiss Microimage, Thornwood, NY, USA).

Immunoprecipitation and western blotting. Human colon tissues were minced into small pieces and homogenized in RIPA Lysis buffer (Santa Cruz Biotechnology), supplemented with protease inhibitors. The lysates were sonicated and centrifuged at 20 800 *g* at 4°C for 15 min. The resultant cell lysates were used for western blotting with anti-Pim-3 IgG as previously described.[9] Cell lysates from colon cancer cell lines were subjected to immunoprecipitation with anti-Pim-3 IgG followed by western blotting analysis with anti-Pim-3 IgG as previously described.[9] Cell lysates immunoprecipitated with control rabbit IgG were used as a negative control, while those from HEK293 cells transfected with human Pim-3 cDNA were used as a positive control. In some experiments, mouse anti-Bad antibody (1:100) was used instead of anti-Pim-3 antibody in western blot analyses following immunoprecipitation with anti-Pim-3 antibody.

Transfection with short hairpin RNA (shRNA). SW480, HT29 and HCT116 cells were transfected with shRNA for Pim-3 (5′-GCACGUGGUGAAGGAGCGG-3′) and Scramble shRNA (5′-GCGCGCUUUGUAGGAUUCG-3′), inserted into the pSilencer 3.1-H1 Neo expression vector (Ambion, Austin, TX, USA), using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions. In our preliminary experiments, Pim-1 and Pim-2 mRNA was faintly detected in SW480, HT29 and HCT116 cells. However, we further confirmed that the transfection of Pim-3 shRNA reduced the mRNA expression of Pim-3, but not Pim-1 and Pim-2 (data not shown).

Cell cycle and cell apoptosis analysis. At the indicated time intervals following the transfection with either Pim-3 or Scramble shRNA, the cells were harvested and fixed with 70% ethanol at −20°C. The fixed cells were incubated with 50 μg/mL propidium iodide (Molecular Probes) and 1 μg/mL RNase A for 30 min at room temperature. DNA content was then analyzed on a FACS Calibur system (Becton Dickinson, Bedford, MA, USA). The distribution of cells in each cell-cycle phase was determined by using cell ModFitLT Software (Becton Dickinson). In some experiments, phosphatidylserine exposure level was determined by staining the cells with human Annexin V-FITC Kit (Bender MedSystem GmbH, Vienna, Austria), according to the manufacturer's instructions. At least 20 000 stained cells were analyzed on a FACS Calibur system for each determination.

Determination of Bad proteins after Pim-3 shRNA transfection. At the indicated time intervals following shRNA transfection, whole cell lysates were prepared by using CelLytic-M mammalian Cell Lysis/Extraction Reagent containing complete protein inhibitor cocktail as previously described.[9] Aliquots (50 μg) of the obtained supernatants were separated by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto an Immobilon-P Transfer membrane. After being saturated with 3% bovine serum albumin (BSA), the membrane was incubated with rabbit anti-Pim-3, mouse anti-Bad, rabbit anti-phopho-Ser^{112}Bad, antiphospho-Ser^{136}Bad, or anti-phopho-Ser^{155}Bad antibodies followed by the incubation with ImmunoPure peroxidase-conjugated anti-mouse or anti-rabbit IgG. The blotted membrane was then treated with the Super Signal West Dura Extended Duration Substrate and signals were detected by LAS-3000 mini charge-coupled device (CCD) camera.

Statistical analysis. The data on human colon carcinoma tissues were analyzed statistically by using Spearman's correlation analysis to analyze the rank data. $P < 0.05$ was accepted as statistically significant.

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In other experiments, data were expressed as mean ± SD and analyzed by using one-way ANOVA, followed by Fisher's protected least significant difference test. Differences were considered significant, when $P < 0.05$.

Results

Enhanced Pim-3 protein expression in human colon cancer tissues. Pim-3-positive cells were not detected in colon tissues that lacked any malignant lesions (data not shown), or normal colon epithelium distant to adenocarcinoma (Fig. 1A-a). In contrast, a substantial number of Pim-3-positive cells were detected in normal epithelium adjacent to adenocarcinoma tissues (Fig. 1A-b) or adenoma tissues (Fig. 1A-c), as well as adenocarcinoma (Figs 1A-d,e). The positive reaction was not observed when control IgG was used as the primary antibody instead of anti-Pim-3 antibodies (Fig. 1B), indicating the specificity of the reaction. Pim-3 protein was detected mainly in the cytoplasm of adenoma and adenocarcinoma cells (Fig. 1A-c–e). Moreover, western blotting analysis detected Pim-3 protein in tumor but not normal colon tissue derived from a patient with well-differentiated colon carcinoma (Fig. 1C), further indicating the specificity of the used antibody. Although some adenocarcinoma tissues consisted mostly of Pim-3-positive cells (Fig. 1A-d), positive rates were the highest in adenoma (Table 1). Poorly differentiated adenocarcinoma ($n = 5$) did not show any positive staining (Fig. 1A-f), while well- and moderately differentiated adenocarcinoma exhibited similar Pim-3 staining (Figs 1A-c,d, Table 2). Moreover, tumor sizes and invasive depth did not have any significant effects on the extent of Pim-3 staining (Table 2). In contrast, the presence of lymph node or liver metastatic foci resulted in decreased Pim-3 expression, compared with adenocarcinoma without a distant metastasis (Table 2). When 60 samples from a different group were simultaneously analyzed for Pim-3 expression in both primary foci and metastatic lesions, Pim-3 expression in primary foci did not show any significant correlation with that in metastatic foci (Table 3). These observations suggest that Pim-3 expression was enhanced more frequently at the early phase rather than the later stages of colon carcinogenesis when distant metastasis occurs.

Constitutive Pim-3 expression in human colon cancer cell lines. Pim-3 expression in colon cancer tissues prompted us to examine Pim-3 expression in colon cancer-derived cell lines, SW480, HT29 and HCT116, which are derived from well-, moderately, and poorly differentiated cancer cells. Reverse transcription polymerase chain reaction (RT–PCR) analysis detected Pim-3 mRNA in these cell lines (data not shown). Immunoblotting analysis demonstrated that SW480 and HT29 contained similar but greater amounts of Pim-3 than HCT116 (Fig. 2a,b). An immunofluorescence analysis further demonstrated a lower Pim-3 expression in HCT119, compared with SW480 and HT29 (Fig. 2c), consistent with a lower incidence of Pim-3 expression in human poorly differentiated colon cancer (Table 2). Because Pim-3 can inactivate a pro-apoptotic BH3-only protein, Bad by phosphorylating Ser^{112},[9] we examined the phosphorylation states of Bad in these cell lines. Bad was constitutively phosphorylated at Ser^{112} and to a lesser degree, Ser^{136} and Ser^{155} in these cell lines. Furthermore, the amounts of total Bad and phospho-Ser^{112}Bad, but not other phosphorylated forms of Bad, were higher in SW480 and HT29 than HCT116 cells (Fig. 2a,b).

Enhanced apoptosis of human colon cancer cell lines resulting from the ablation of endogenous Pim-3 protein. In order to clarify the role of endogenous Pim-3 in cell survival, we ablated Pim-3 protein by the transfection into SW480 of Pim-3 shRNA. Transfection of Pim-3 shRNA, but not Scramble shRNA, into SW480 cells markedly diminished Pim-3 protein by 48 h post-transfection (Fig. 3a). The transfection of Pim-3 shRNA resulted in a higher ratio of sub-G1 cell populations with reduced G1 and G2/M

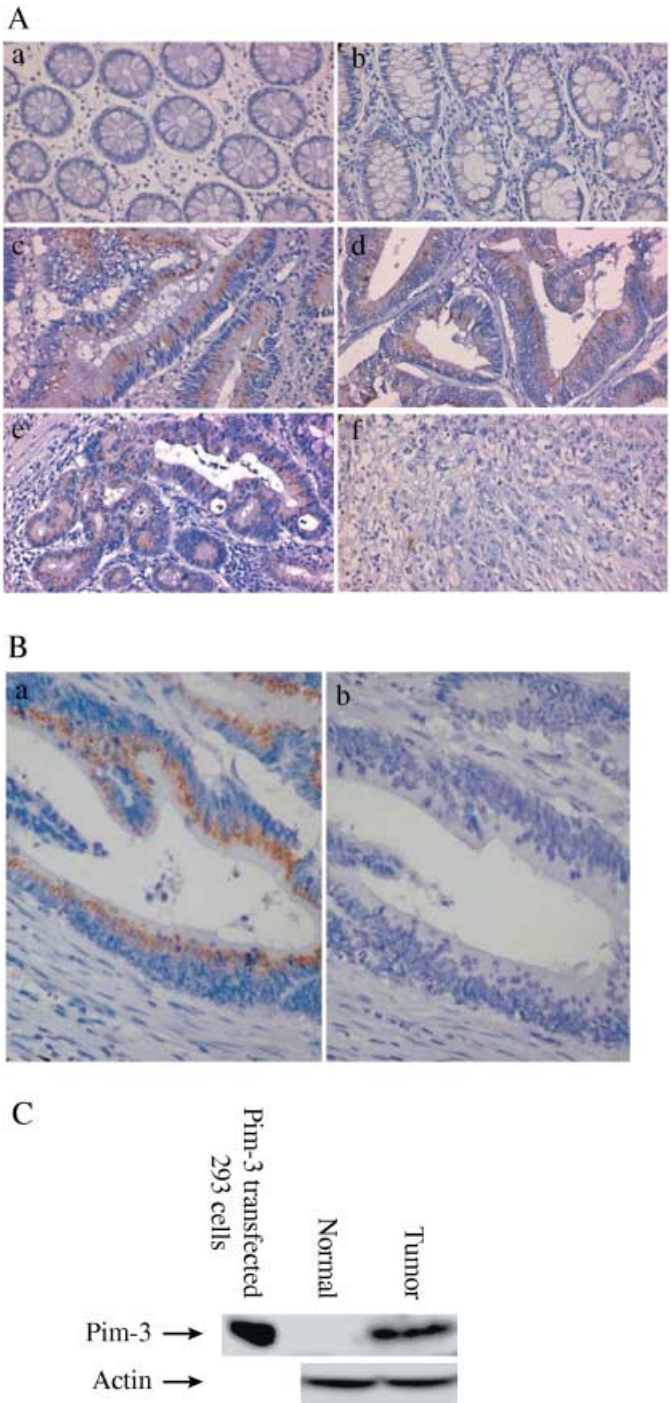

Fig. 1. Aberrant expression of Pim-3 protein in human colon cancer tissues. Immunostaining with anti-Pim-3 antibodies was performed on human normal colon tissue distant to adenocarcinoma (A-a), normal colon tissue adjacent to adenocarcinoma (A-b), adenoma tissues (A-c and B), well-differentiated adenocarcinoma (A-d), moderately differentiated adenocarcinoma (A-e), and poorly differentiated adenocarcinoma (A-f) were immunostained with either anti-Pim-3 antibodies (A and B-a) or normal rabbit immunoglobulin (Ig)G (B-b) as described in Materials and Methods. Representative results are shown here. (Original magnification, ×400.) (C) Western blotting analysis of Pim-3 protein in human colon cancer tissues. Cell lysates from colon cancer and normal tissues (3 mg) were subjected to an immunoblotting with anti-Pim-3 antibody as described in Materials and Methods. HEK293 cells transfected with human Pim-3 cDNA were used as a positive control.

Table 1. Pim-3 expression in colorectal mucosa, adenoma, and adenocarcinoma

Groups	*n*	Pim-3 expression					Positive Rate (%)
		–	±	+	++	+++	
Non-neoplastic mucosa adjacent to adenocarcinoma	111	85	10	15	1	0	23.4*
Adenoma adjacent to adenocarcinoma	40	5	7	25	3	0	87.5**
Adenocarcinoma	131	60	9	48	10	4	54.2***

Proportion of Pim-3-positive cells were evaluated in mucosa and adenoma, that are adjacent to adenocarcinoma, as well as the adenocarcinoma area, as described in Materials and Methods. *, compared with adenoma, $P = 0.001$, Spearman's rank correlation coefficient (Rs) = 0.596; **, compared with adenocarcinoma, $P = 0.008$; $Rs = 0.202$; ***, compared with mucosa, $P = 0.001$, $Rs = 0.347$.

Table 2. Pim-3 positive samples were analyzed in correlation with clinicopathological features of colorectal adenocarcinoma

Clinicopathological features	*n*	Pim-3 expression					Positive rate (%)	Rs	*P*-value
		–	+–	+	++	+++			
Sex								0.057	0.544
Female	46	17	5	18	5	1	63.0		
Male	68	31	4	25	5	3	54.4		
Age								0.073	0.439
< 60	19	10	1	6	0	2	47.4		
= 60	95	38	8	37	10	2	60.0		
Tumor size								0.135	0.154
< 4 cm	46	16	2	22	5	1	65.2		
= 4 cm	68	32	7	21	5	3	52.9		
Invasive depth								0.046	0.626
Above submucosa	16	4	2	9	1	0	75.0		
Muscularis propria	13	6	0	7	0	0	53.8		
Below subserosa	85	38	7	27	9	4	55.3		
Lymph node metastatic foci								0.195	0.038
–	52	17	4	22	7	2	67.3		
+	62	31	5	21	3	2	50.0		
Liver metastatic foci								0.459	0.001
–	95	30	8	43	10	4	68.4		
+	19	18	1	0	0	0	5.3		
Differentiation								0.121	0.200
Well-differentiated	68	25	7	28	5	3	63.2		
Moderately differentiated	41	18	2	15	5	1	56.1		
Poorly differentiated	5	5	0	0	0	0	0		

Table 3. Pim-3 expression in primary foci was analyzed in correlation with metastasis

Primary foci	*n*	Metastases					Positive rate (%)	Rs	*P*-value
		–	+–	+	++	+++			
–	34	23	4	7	0	0	32.4	0.188	0.151
+–	4	1	3	0	0	0	75.0		
+	20	10	7	3	0	0	50.0		
++	2	0	1	1	0	0	100		
+++	0	0	0	0	0	0	0.0		
Total	60	34	15	11	0	0	43.3		

populations, compared to the cells transfected with Scramble shRNA (Fig. 3bi–iii). Moreover, Pim-3 shRNA transfectants contained a markedly higher ratio of apoptotic cells as evidenced by enhanced phosphatidylserine externalization (Fig. 3biv–vi). Similar phenomena were observed when HT29 cells were transfected with Pim-3 shRNA (data not shown). We next examined the effects of Pim-3 ablation on Bad phosphorylation. The ablation of Pim-3 reduced the amount of phospho-Ser^{112}Bad but not the amounts of total Bad and phospho-Ser^{136}Bad (Fig. 3a). Several lines of evidence indicate that Bad was translocated from cytoplasm to mitochondria upon its dephosphorylation.[20] Hence, we explored the intracellular localization of Bad upon dephosphorylation of Ser^{112} but Ser^{136} Bad arising from Pim-3 ablation. We employed anti-Hsp60 antibodies to detect mitochondria, because Hsp60 is exclusively present in mitochondria.[21] Bad was not co-localized with Hsp60 in untreated cells but Pim-3 ablation induced the co-localization of Bad with Hsp60 (Fig. 3c), suggesting its mitochondrial localization. Similar phenomena were observed on another colon cancer cell line, HT29 (data not shown). Thus, these observations suggest that Pim-3, aberrantly expressed in human colon cancer cells, can inactivate the potent pro-apoptotic factor, Bad, and thereby prevent its translocation to mitochondria, by phosphorylating Bad Ser^{112}.

Co-localization of Pim-3 with Bad protein in primary human colon cancer. In order to address whether there was evidence to suggest that Pim-3 might phosphorylate Bad in primary human colon cancer cells, we performed a double-color immunofluorescence analysis on human colon cancer tissues. Pim-3 protein was detected in tumor cells in 9/10 human colon cancer samples that we examined. In all these Pim-3-positive samples, Bad protein was co-localized with Pim-3 proteins in both the horizontal (*xy* axis) and orthogonal planes (*xz* and *yz* axes) (Fig. 4a). Pim-3 proteins did not co-localize with glyceraldehyde-3-phosphate dehydrogenase

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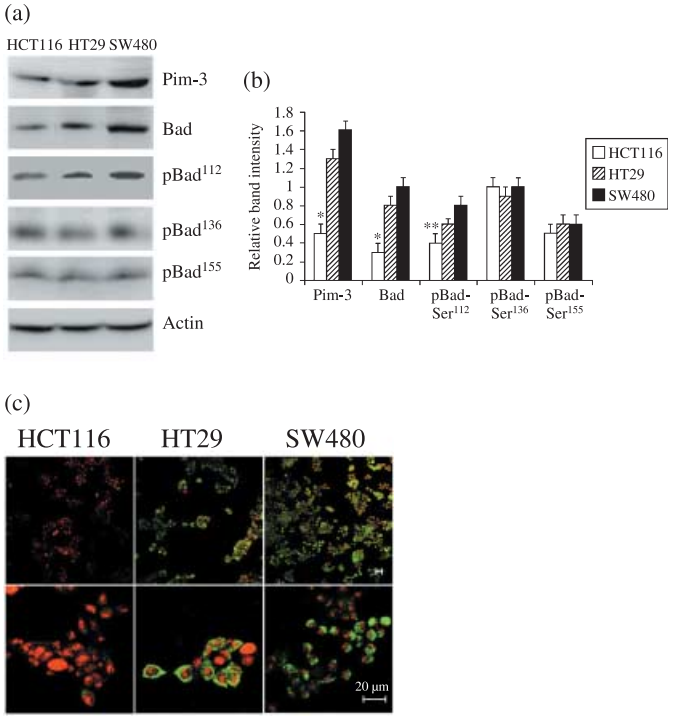

Fig. 2. Constitutive Pim-3 expression in human colon cancer cell lines, SW480, HT29 and HCT116. (a) Immunoblotting analysis of Pim-3 and various forms of Bad proteins in human colon cancer cell lines. Cell lysates were obtained from human colon cancer cells. Aliquots (2 mg) were subjected to an immunoprecipitation with anti-Pim-3 antibodies or control rabbit immunoglobulin (Ig)G, followed by an immunoblotting with anti-Pim-3 antibodies as described in Materials and Methods. Other aliquots (50 μg) were immunoblotted with antibodies for various forms of Bad proteins and β-actin. Representative results from three independent experiments are shown here. (b) Quantification of Pim-3 and various forms of Bad proteins in colon cancer cells. The band intensities of Pim-3 and various forms of Bad in Fig. 2A were measured with NIH Image Analysis Software Ver. 1.62 (NIH, Bethesda, MD, USA) and were normalized to those of β-actin. Asterisk indicates **, $P < 0.01$ compared with SW480 cells. (c) Immunofluorescence analysis of Pim-3 protein expression in human colon cancer cell lines. A double-color immunofluorescence analysis was carried out on human colon cancer cell lines, HCT116 (left row) and HT29 cells (middle row) and SW480 (right row) with the combination of anti-Pim-3 antibodies and propidium iodine as described in Materials and Methods. Green and red indicate Pim-3 protein and nuclei, respectively. Representative results from three independent experiments are shown here. (Original magnification, upper panel, ×200; lower panel, ×800. Bars, 20 μm.)

(GAPDH), a protein that is abundantly present in the cytoplasm (data not shown). Moreover, an immunoprecipitation with anti-Pim-3 antibodies co-precipitated Bad in a human colon cancer tissue (Fig. 4b). These observations would further indicate the co-localization of Pim-3 with Bad in human colon cancer cells. Finally, in 6/9 Pim-3-positive samples, Pim-3 protein co-localized with phospho-Ser^{112}Bad in both the horizontal (*xy* axis) and orthogonal planes (*xz* and *yz* axes) (Fig. 4c). Thus, Pim-3 may phosphorylate Bad at Ser^{112} even in primary colon cancer, thereby regulating apoptosis of the cancer cells.

Discussion

Orderly apoptosis of colon epithelial cells is essential for normal colon crypt formation.[10] A partial deregulation of apoptosis occurs due to the inactivation of both alleles of the *APC* gene, the first key event in colon carcinogenesis.[12] Further suppression of apoptosis occurs as these cells develop additional genetic mutations and phenotypic changes.[13] However, the molecular mechanisms underlying apoptosis deregulation in colon carcinogenesis remain elusive. Accumulating evidence suggests that the regulation of apoptosis of colon cancer cells involves several kinases including Erk1/2, p38 mitogen-activated kinase, and Akt.[22–26] Pim kinases can phosphorylate a similar range of substrates as Akt and thus can provide survival signals to various types of tumor cells.[14] Our observation that Pim-3 expression was enhanced in the malignant lesions of endoderm-derived organs, the liver[8] and pancreas,[9] prompted us to evaluate Pim-3 expression in colon carcinoma tissue. Indeed, Pim-3 was expressed in a substantial portion of primary colon cancer tissues. Moreover, we have determined that aberrantly expressed Pim-3 may counteract apoptosis by phosphorylating a pro-apoptotic molecule, Bad.

Another member of the Pim family, Pim-1, becomes constitutively active without any alteration in conformation, due to the absence of any regulatory domains.[27] Pim-3 shows a high sequence identity with Pim-1, even in the kinase domain and lacks any regulatory domains.[8] Thus, when aberrantly expressed in colon cancer cells, Pim-3 can be active without any further modification. Recently, the human *Pim-3* gene has been assigned to chromosome 22q13. A comparative genomic hybridization analysis demonstrated that the gain at 22q13.3 represented the minimal chromosomal changes found specifically in colon cancer tissues with microsatellite instability.[28] However, we failed to detect Pim-3 gene amplification in several human colon cancer cell lines including SW480 and HT29 (unpublished data). Thus, it is not likely that constitutive enhanced expression of Pim-3 in colon cancer cells is a result of *Pim-3* gene amplification.

Several lines of evidence suggest that a transcription factor, Stat3, is constitutively activated in colon cancer[29–31] and that both Pim-1 and Pim-2 can be a transcriptional target of Stat3.[32,33] Moreover, we also observed that the transfection of a dominant negative form of Stat3 reduced Pim-3 expression in SW480 cells (unpublished data), suggesting that Stat3 can regulate Pim-3 gene transcription. Of interest is that Pim-1 can synergistically induce Stat3-mediated cell cycle progression and anti-apoptotic process.[32] Aberrantly expressed Pim-3 may also synergistically augment the Stat3-induced malignant phenotype of colon cancer as Pim-3 has a remarkable sequence identity with Pim-1. If this is indeed the case, blocking Pim-3 activity may reduce Stat3 activity and thus inhibit colon cancer progression.

Akt shows a similar substrate specificity as the Pim kinases including Pim-3 and is presumed to have an essential role in the regulation of apoptosis.[14] Activation of Akt can contribute to the pathogenesis of a wide variety of malignancies. Indeed, Roy *et al*. demonstrated that activated Akt was detected in a substantial portion of colon adenoma (57% positive) and adenocarcinoma (57% positive) but not in normal colonic epithelium and hyperplastic polyps.[25] Based on these observations, these authors claimed that Akt overexpression may be an early event during colon carcinogenesis. Pim-3 exhibited a similar staining pattern as Akt. Pim-3 was also detected in normal colon mucosal tissues that are adjacent to adenocarcinoma but do not exhibit any morphological abnormalities. Thus, Pim-3 overexpression might be an earlier event than that of Akt during the development of the malignant phenotype. Moreover, the incidence of Pim-3 expression was higher in adenoma than adenocarcinoma. We previously observed that precancerous lesions in liver showed a higher incidence of Pim-3 expression than hepatocellular carcinoma lesions.[8] Thus, it is tempting to speculate that aberrant Pim-3 expression might generally have a crucial role in the early phase but not later phase of carcinogenesis.

Genetic analysis has revealed that somatic missense mutations in the gene of *Bad*, a pro-apoptotic BH3-only protein, occur in a small proportion of colon adenocarcinoma samples.[34] It was postulated that somatic mutation of *Bad* may contribute to colon

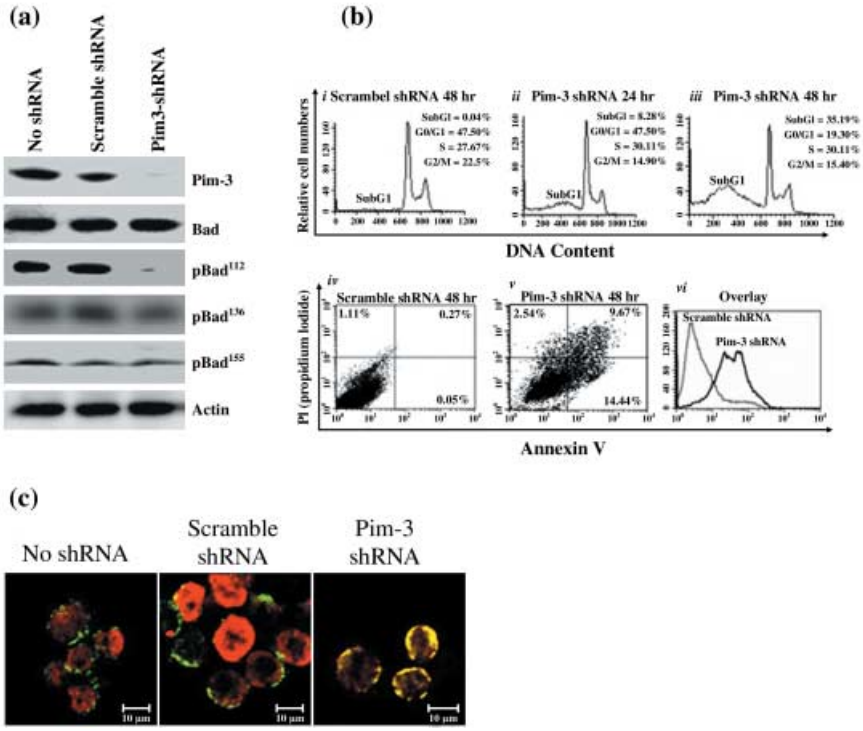

Fig. 3. Effects of ablation of endogenous Pim-3 protein on Bad phosphorylation and apoptosis. (a) Cell lysates were obtained from human colon cancer cells, SW480, that were transfected with Pim-3 shRNA, Scramble shRNA, or no shRNA, and the resultant lysates were subjected to immunoblotting as described in Materials and Methods. Representative results from three independent experiments are shown here. Similar results are obtained using HT29 cells. (b) A human colon cancer cell line, SW480, was either transfected with Scramble shRNA (i,iv) or Pim-3 shRNA (ii,iii,v,vi). Cells were harvested 48 h after transfection and subjected to staining with propidium iodide (PI) (i–iii) or combined staining with PI and annexin V (iv–vi) as described in Materials and Methods. The proportion of cells in each cell cycle phase was determined (i–iii) as described in Materials and Methods. The number in each quadrant (iv,v) indicates the proportion of the cells present in the quadrant. The intensities of annexin-V staining are shown in (vi), by overlaying the data in (iv) and vice versa. Representative results from three independent experiments are shown here. Similar results were obtained using HT29 cells. (c) Immunofluorescence analysis of Bad intracellular localization in human colon cancer cells. SW480 cells, transfected with Pim-3 shRNA, Scramble shRNA or no shRNA, were seeded onto chamber slides. Thirty-two hours after the transfection, a double-color immunofluorescence analysis was performed as described in Materials and Methods. Green, red, and yellow represent Hsp60, Bad, and co-localization of Hsp60 and Bad, respectively. Representative results from three independent experiments are shown here. (Original magnification, ×1600; bar, 10 μm.)

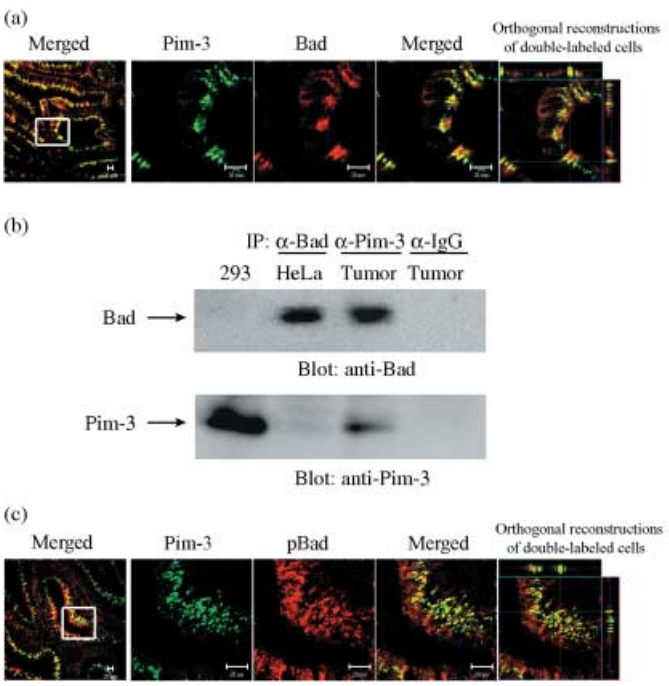

Fig. 4. Association of Pim-3 with Bad in human colon cancer tissues. (a) Human colon cancer samples were immunostained with either the combination of anti-Pim-3 and anti-Bad antibodies as described in Materials and Methods. The fluorescent images were digitally merged in the left and right two panels. The box in the left panel indicates the site, where a picture with a higher magnification was taken. Representative results from 10 individual samples are shown here. (Original magnification, left panel, ×200; other panels, ×800. Bars, 50 μm.) (b) Cell lysates were obtained from a human colon cancer tissue, HeLa cells and HEK293 cells transfected with human Pim-3 cDNA. The cell lysates from a human colon cancer tissues were subjected to immunoprecipitation with anti-Pim-3 or control rabbit IgG. HeLa cells express Bad, but not Pim-3, and HEK293 cells express Pim-3, but not Bad. Hence, Hela cells a were subjected to immunoprecipitation with anti-Bad. The resultant precipitated were further subjected to immunoblotting with anti-Bad (upper panel) or anti-Pim-3 (lower panel) antibodies as described in Materials and Methods. (c) Human colon cancer samples were immunostained with either the combination of anti-Pim-3 and antiphosho-Ser^{112}Bad antibodies as described in Materials and Methods. The fluorescent images were digitally merged in the left and right two panels. The box in the left panel indicates the site, where a picture with a higher magnification was taken. Representative results from 10 individual samples are shown here. (Original magnification, left panel, ×200; other panels, ×800. Bars, 50 μm.)

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carcinogenesis due to decreased apoptosis-inducing activities of a mutant *Bad*, when compared with wild-type Bad. The pro-apoptotic activity of Bad is regulated by its phosphorylation at serine residues. Unphosphorylated Bad binds and eventually inactivates anti-apoptotic family members, primarily Bcl-X_L but also Bcl-2.[35–37] Bad can be inactivated by the phosphorylation at either Ser^{112} or Ser^{136}. Phosphorylated Bad loses its capacity to bind to anti-apoptotic molecules such as Bcl-X_L and Bcl-2 and its movement from the surface of mitochondria to the cytosol requires the 14-3-3 protein. Upon liberation from Bad, Bcl-X_L and Bcl-2 can maintain mitochondrial membrane potential and subsequently prevent apoptosis.[38,39] In this report, we demonstrated that Bad is constitutively phosphorylated at Ser^{112} and to a lesser degree, Ser^{136} in colon cancer cell lines. Moreover, the ablation of Pim-3 reduced phosphorylation of Ser^{112} similarly observed on human pancreatic cancer cell lines.[9] Furthermore, our *in vitro* kinase assay revealed that Pim-3 phosphorylates Bad Ser^{112} (unpublished data), which represents an inactive form of Bad.[15,16] Considering that Bad mutants with lower pro-apoptotic activities were detected in colon carcinomas,[34] Pim-3-mediated phosphorylation and subsequent inactivation of Bad protein may also contribute to colon carcinogenesis by preventing the apoptosis process.

The Akt consensus phosphorylation site contains arginine at the −5 and −3 in relation to the phosphorylation site,[40] similar to that required for Pim-1 activity.[32] Although both Bad Ser^{136} and Ser^{112} conform to this motif, Akt preferentially phosphorylates Bad Ser^{136}.[38] In colon cancer cell lines, Ser^{112} and to a lesser extent, Ser^{136} are constitutively phosphorylated and the ablation of Pim-3 reduced the phosphorylation of Ser^{112} but not Ser^{136}. Of interest is that the inhibition of Akt induced apoptosis of colon cancer cells, without dephosphorylating Bad Ser^{112} and Ser^{136}.[41] Our *in vitro* kinase assay revealed that Pim-3 phosphorylates Bad Ser^{112} (unpublished data) and we observed that the amount of phosphor-Ser^{112}, but not phosphor-Ser^{136}, Bad was proportional to the amount of Pim-3 in human colon cancer cell lines. Moreover, we previously observed that the transfection of Pim-3 enhanced the phosphorylation of Ser^{112}, but not Ser^{136}, Bad in human pancreatic cell lines.[9] Thus, it is probable that Pim-3 directly phosphorylates Bad Ser^{112} and inactivates Bad, thereby preventing the apoptosis of colon cancer cells. Thus, the inhibition of Pim-3 may induce apoptosis of colon cancer cells in a distinct way from the inhibition of Akt. Moreover, Pim-3 may offer an advantage as a molecular target, due to its selective expression in malignant lesions of liver,[8] pancreas[9] and colon, in contrast to the expression of Akt in most normal organs.

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