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KLF4-Mediated Negative Regulation of IFITM3 Expression Plays a Critical Role in Colon Cancer Pathogenesis

Dawei Li^{1,4}, Zhihai Peng¹, Huamei Tang², Ping Wei^{3,4}, Xiangyu Kong⁵, Dongwang Yan¹, Huang Fei¹, Qiang Li⁵, Xiangdong Le⁵, Qi Li^{4,5}, and Keping Xie⁵

¹ Department of General Surgery, Shanghai Jiaotong University Affiliated First People's Hospital, Shanghai 200080, People's Republic of China

² Department of Pathology, Shanghai Jiaotong University Affiliated First People's Hospital, Shanghai 200080, People's Republic of China

³ Institutes of Biomedical Sciences, Shanghai Fudan University, Shanghai 200080, People's Republic of China

⁴ Department of Neurosurgery, The University of Texas MD Anderson Cancer Center, Houston, Texas

⁵ Department of Gastrointestinal Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas

Abstract

Purpose—IFITM3, an interferon-inducible gene, is overexpressed in human colorectal cancer. In this study, we sought to determine the clinical significance and underlying mechanisms of its dysregulated expression in human colon tumor specimens and murine models of this disease.

Experimental Design—IFITM3 expression in a tissue microarray of tumor and matched normal colon tissue specimens and lymph node metastasis specimens obtained from 203 patients with colon cancer was measured immunohistochemically.

Results—IFITM3 was expressed at higher levels in colon tumors and, particularly, nodal metastases than in normal colon tissue. A Cox proportional hazards model showed that IFITM3 expression was an independent prognostic factor for disease-free survival in patients with colon cancer. Knockdown of IFITM3 expression by a specific small interfering RNA significantly suppressed the proliferation, colony formation, migration, and invasion of colon cancer cells *in vitro* and tumor growth and metastasis in a xenograft model. Restored expression of KLF4, a putative tumor suppressor, downregulated IFITM3 expression in colon cancer cells *in vitro*. Two KLF4-binding sites in the IFITM3 promoter bound specifically to KLF4 protein in a chromatin immunoprecipitation assay and promoter mutagenesis analyses. Specific deletion of KLF4 led to IFITM3 overexpression in colon mucosa in *Villin-Cre⁺;Klf4^{fl/fl}* mice. An inverse correlation between loss of KLF4 expression and IFITM3 overexpression was evident in human colon tumors.

Conclusion—these clinical and mechanistic findings indicate that IFITM3 is a direct transcriptional target of KLF4 and that dysregulated KLF4 expression leads to aberrant IFITM3 expression, thus contributing to colon cancer progression and metastasis.

Corresponding Author: Zhihai Peng, Department of General Surgery, Shanghai First People's Hospital, 85 Wujin Road, Shanghai, 200080, The People's Republic of China. Phone: 8621-63240090-3102; Fax: 8621-63241377; pengpzh@hotmail.com; or Keping Xie, Department of Gastrointestinal Medical Oncology, Unit 426, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-792-2828; Fax: 713-745-1163; kepxie@mdanderson.org.

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Keywords

IFITM3; KLF4; colon cancer; progression; metastasis

Introduction

Colon cancer is one of the most common cancers worldwide (1). For metastatic colon cancer, the 5-year overall survival (OS) rate is about 10% (2). A mechanistic understanding of colon cancer initiation, recurrence, and metastasis is therefore an important goal. Genome-wide gene-expression analysis of colon tumor and corresponding normal colon tissue cells is crucial to understanding how oncogenes and tumor suppressor genes alter the complex cellular molecular context of colon cancer and thus drive colon tumor progression (3).

In previous experimental and bioinformatic genome-wide gene-expression analyses, we found that expression of the IFITM3 gene was aberrantly higher in colon tumors than in matched normal colon mucosa (4). Researchers first isolated IFITM3 from a genetic screen aimed at identifying the genes involved in the acquisition of germ-cell competence (5). Investigators proposed that epiblast cells having the highest levels of expression of IFITM3 initiated germ-cell specification and that homotypic association discriminated germ cells from their somatic neighbors. Studies then showed that IFITM3 belongs to a family of five murine genes (along with IFITM1, IFITM2, IFITM5, and IFITM6) clustered within a 68-kb genomic region on chromosome 7 (6). The IFITMs are short, two-transmembrane-domain proteins (5 to 18 kDa) with high core sequence similarity but divergent N and C termini. The human homologues (IFITM1, IFITM2, and IFITM3) are clustered on chromosome 11 within an 18-kb genomic sequence (7–9). These genes respond to treatment with type I and II interferons and encode for interferon-induced transmembrane proteins involved in the homotypic cell adhesion functions of interferons (8, 10).

Expression of the IFITM3 gene is implicated in diverse cellular processes, including immune-cell regulation, somitogenesis, germ-cell homing and maturation, and bone mineralization (10–13). Recent studies identified possible roles for IFITM genes involved in carcinogenesis. For example, researchers first isolated the IFITM3 gene from tumor tissue and severely inflamed mucosa in the colons of patients with ulcerative colitis, describing it as a preferential marker for ulcerative colitis-associated colon cancer (14, 15). Another study showed that IFITM1 and IFITM3 were expressed at higher levels in astrocytoma cells than in normal astrocytes in mice (16). Furthermore, authors reported that upregulation of IFITM1 expression played a critical role in both the precancerous stage and carcinogenesis in patients with gastric mucosa infected with *Helicobacter pylori* and cervical cancer (17–19). Also, IFITM1 overexpression has promoted and knockdown of IFITM1 expression has significantly suppressed the invasiveness of head and neck tumor cells (20). However, the precise function and underlying mechanisms of IFITM3 in colon cancer pathogenesis remain unclear.

Furthermore, the expression and regulation of IFITM1, IFITM2, and IFITM3 is not clear. One recent study identified IFITMs as potential targets of Wnt/ β -catenin signaling in carcinogenesis in the intestinal epithelium (21). Importantly, KLF4, a critical tumor repressor in the initiation and progression of gastrointestinal cancers (22, 23), directly interacts with Wnt/ β -catenin signaling. However, whether KLF4 critically regulates IFITM expression is unknown. In the present study, we sought to determine the role of IFITM3 and molecular mechanisms underlying dysregulated expression of it in colon cancer progression.

Materials and Methods

Human tissue specimens and patient information

For tissue microarray (TMA) construction and immunohistochemical analysis, we used human colon tumor specimens obtained from 203 patients with colon cancer that were preserved in the Colon Cancer Tissue Bank at Shanghai Jiaotong University Affiliated First People's Hospital (Shanghai, People's Republic of China). The primary colon cancer in these patients was diagnosed (and later confirmed by at least two pathologists) and the patients were accepted for colectomy at Affiliated First People's Hospital from 2001 to 2003. The 203 formalin-fixed, paraffin-embedded specimens were selected to represent all of the stages and histological types of colon cancer. Tumor staging for the specimens was carried out according to the American Joint Committee on Cancer staging criteria (24). The patients' disease-free survival (DFS) and overall survival (OS) durations were defined as the interval from initial surgery to clinically or radiologically proven recurrence or metastasis and from initial surgery to death, respectively. The follow-up period for this analysis concluded on June 29, 2008. The use of human specimens was approved by proper institutional review boards.

TMA construction and immunohistochemistry

After screening hematoxylin- and eosin-stained slides for optimal tumor tissue and tumor-adjacent tissue up to 2 cm from the tumor, TMA slides were constructed (in collaboration with Shanghai Biochip). Two cores were taken from each formalin-fixed, paraffin-embedded tumor and matched normal colon tissue specimen as well as at least one lymph node metastasis core using punch cores that measured 2 mm in greatest dimension from the nonnecrotic areas of tumor, lymph node metastasis, and matched normal colon tissue specimens. Among the 203 study patients were 66 from whom primary colon tumor and matched lymph node metastasis specimens were obtained. These specimens were included in the TMA. Sections (4 μ m thick) of formalin-fixed, paraffin-embedded colon tumor specimens were prepared and processed for immunostaining using a rabbit polyclonal antibody against human KLF4 (H-180 [1:200 dilution]; Santa Cruz Biotechnology), a monoclonal antibody against IFITM3 (H00010410-M01 [1:1000 dilution]; Abnova), and a rabbit polyclonal antibody against human proliferating cell nuclear antigen (Ki67 [1:200 dilution]; Santa Cruz Biotechnology).

Cell lines and culture conditions

The human colon cancer cell lines HCT116 and SW480 were purchased from the American Type Culture Collection. All cells were maintained at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Real-time reverse transcription-polymerase chain reaction

Total RNA was isolated from cell cultures or tissues using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from 1 μ g of total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Real-time reverse transcription-polymerase chain reaction (PCR) analysis of expression of the IFITM3 gene was performed using 2 μ L of cDNA and the SYBR Green Master Mix (Bio-Rad) as recommended by the manufacturer for the IFITM3 primers 5'-caaggaggagcagcagg-3' (forward) and 5'-ttgaacagggaccagacg-3' (reverse). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Each PCR product was run in triplicate for the target and internal control genes.

Western blot analysis

Whole-cell lysates were prepared from the colon cells as described previously (25). Standard Western blot analysis of the lysates was performed with an antibody against KLF4 (Santa Cruz Biotechnology) or against IFITM3 (Abnova) and a second anti-IgG antibody (Amersham Life Sciences). The membranes were then stripped and blotted with an anti- β -actin antibody (Sigma Chemical Co.) and used as loading controls. The probe proteins were detected using an enhanced chemiluminescence system (Amersham Life Sciences) according to the manufacturer's instructions.

Transient transfection of colon cancer cells

To induce overexpression of KLF4 in HCT116 and SW480 cells, the cells were transfected with adenovirally infected KLF4 (Ad-KLF4) or enhanced green fluorescent protein (EGFP [Ad-EGFP]) as described previously (26). To inhibit IFITM3 expression in these cells, they were transfected with a pool of IFITM3 small interfering RNA (siRNA) oligonucleotides (Santa Cruz Biotechnology; 50 nmol/L) or control siRNA oligonucleotides (Santa Cruz Biotechnology; 50 nmol/L). Also, the cells treated with Oligofectamine reagent alone were included as mock controls.

Cell proliferation assay

For an MTT assay, transfected cells (3×10^3) were seeded in a 96-well culture plate and subsequently incubated with MTT reagent (0.5 mg/mL) at 37°C for 2 h and then with dimethyl sulfoxide at 37°C for 1 h. The MTT assay was performed 24, 48, and 72 h after transfection. The results were plotted as the mean $\pm$ standard deviation from three separate experiments with eight replicates per experiment for each experimental condition.

Soft agar assay

Colon cancer cells were suspended in 0.3% agar medium (DMEM containing 10% FBS) and then plated on a 0.6% agar base layer at a concentration of 1.5×10^3 cells per six-well plate. The cells were incubated in a humidified atmosphere (5% CO₂) at 37°C. The colonies that were 50 μ m in diameter or larger were counted 10 days after cell plating.

Cell migration and invasion assay

Cell migration and invasion assays were conducted using a modified 24-well Boyden chamber with a membrane that was uncoated or coated with Matrigel (BD Biosciences), respectively. Briefly, 24 h after transfection of both HCT116 and SW480 cells either with a control (mock or control siRNA-treated) or IFITM3 siRNA, the cells were harvested and suspended in DMEM at a concentration of 8×10^4 /mL. Cells prepared in 500 μ L of DMEM were loaded in the upper wells, and a medium containing 20% FBS was placed in the lower wells as a chemoattractant stimulus. Migrated cells on the bottom surface of the filter were fixed, stained with H&E, and counted under a microscope in three randomly selected fields at a magnification of 200 $\times$.

Cell immunofluorescence

HCT116 and SW480 cells transfected with Ad-KLF4 or Ad-EGFP were cultured on Falcon chamber slides (BD) at up to 50–60% confluence before being fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100. The cells were then immersed three times in phosphate-buffered saline, incubated with indicated primary antibodies overnight at 4°C and corresponding Alexa Fluor-conjugated secondary antibodies (Invitrogen) for 1 h at room temperature, and mounted using a mounting medium containing 4',6-diamidino-2-phenylindole. Microscopic images of cells were obtained using an Axio Observer A microscope (Zeiss).

IFITM3 promoter constructs, site-specific mutagenesis, and IFITM3 promoter activity analysis

A 0.5-kb IFITM3 promoter was cloned into pGL3 basic luciferase reporter vectors. Site-specific mutagenesis of the IFITM3 promoter was performed using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The primers used to generate the mutant vector were as follows: site 1, 5'-gggcctggagtggtgaatcctcagcgcaggcctg-3' (sense) and 5'-caggcctgcgctgaggattccacactccaggccc-3' (antisense); site 2, 5'-cagctgctggcctggattctggagacccccaaca-3' (sense) and 5'-tggtgggggtctccagaatccaggccagcagctg-3' (antisense). The mutation was confirmed via DNA sequencing. The IFITM3 promoter activity was normalized by cotransfection with a β -actin/Renilla luciferase reporter containing a full-length Renilla luciferase gene (25). We quantified both firefly and Renilla luciferase activity using a dual-luciferase assay system (Promega) 24 h after transfection.

Chromatin immunoprecipitation assay

HCT116 and SW480 cells were seeded to about 80% confluence in 15-cm culture dishes and transfected with Ad-KLF4 or Ad-EGFP. Twenty-four hours later, a chromatin immunoprecipitation (ChIP) assay was performed using a ChIP assay kit (Cell Signaling Technology) according to the manufacturer's instructions. Briefly, DNA cross-binding proteins were cross-linked with DNA and lysed in sodium dodecyl sulfate lysis buffer. The lysate was sonicated to shear DNA to around 500 bp. Anti-KLF4 antibodies were then added, and the samples were incubated overnight at 4°C. Immunoprecipitation using a normal rabbit IgG as a negative control was performed, as well. Resulting immunocomplexes were precipitated for 2 h with protein A-Sepharose beads, and DNA was recovered using phenol-chloroform extraction. Next, the DNA was subjected to PCR analysis to amplify a 512-bp region (−438 to +74 bp) of the IFITM3 promoter using the primers 5'-tgagggttatgggagacg-3' and 5'-gggttactgggatggttc-3'. The PCR products were resolved electrophoretically on a 2% agarose gel and visualized using ethidium bromide staining.

Animal Experiments

For orthotopic model of colon cancer, female athymic BALB/c nude mice were purchased from The Jackson Laboratory. The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used when they were 8 weeks old. To produce cecal tumors, 5×10^5 cells in 50 μ l of Hank's balanced salt solution were injected into the cecum wall of nude mice. Mice were sacrificed 35 days after tumor implantation. Cecal tumors were weighted and hepatic metastases were determined as described previously (27). For Colon-tissue-specific KLF4-knockout mice, the derivation and use of *Klf4-LoxP* and *villin-Cre* mice were described previously (28). These *Klf4-LoxP* mice had loxP in the introns flanking exons 2 and 3. The mice had a C57BL6 genetic background. These animals were mated with *Villin-Cre* mice, which were obtained from The Jackson Laboratory (004586, B6.SJL-Tg(*Vil-cre*)997Gum/J), which have expression of Cre under the control of a 12.4-kb regulatory region of the murine Villin promoter. *Tg(Vil-Cre)* mice also had a C57BL6 genetic background. Pairs of mice heterozygous for the floxed *Klf4* allele and positive for *Villin-Cre* were intercrossed to produce offspring homozygous for the floxed allele and containing the *Villin-Cre* transgene and *Klf4^{fl/fl}* (control) offspring. The animals were housed and maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International in accordance with the current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and National Institutes of Health.

Statistical analysis

The two-tailed χ^2 test was used to determine the significance of the difference among the covariates. Survival durations were calculated using the Kaplan-Meier method. The log-rank test was used to compare the cumulative survival rates in the patient groups. A Cox proportional hazards model was used to calculate univariate and multivariate hazard ratios for the study variables. The IFITM3 expression level, patient age, disease stage (American Joint Committee on Cancer system), and tumor differentiation and distant metastasis were included in the model. The significance of the *in vitro* data was determined using the Student *t*-test (two-tailed). In all of the tests, *P* values less than 0.05 were considered statistically significant. The SPSS software program (version 12.0; SPSS Inc.) was used for statistical analyses.

Results

IFITM3 overexpression and its direct association with colon cancer aggressiveness

We first investigated the expression of IFITM3 protein in the 203 primary colon tumor and paired adjacent normal colon mucosa specimens as well as the 66 lymph node metastasis specimens in a TMA. We observed IFITM3-positive staining in the cytoplasm of the cancer cells with IFITM3-negative or weak IFITM3-positive staining in adjacent normal colon cells (Fig. 1A, Supplementary Table 1). We confirmed the presence of aberrant upregulation of IFITM3 expression at both the mRNA and protein level in colon tumor and normal colon tissue specimens using real-time PCR and Western blotting (Fig. 1B).

We further analyzed the relationship between clinicopathological features and IFITM3 expression levels in colon cancer cases. IFITM3 expression was positively correlated with disease stage ($P < 0.001$), pT classification ($P < 0.001$), regional lymph node metastasis ($P = 0.004$), and distant metastasis ($P = 0.009$) (Supplementary Table 2). In contrast, IFITM3 expression was inversely correlated with OS and DFS rate (Fig. 1C, Supplementary Tables 3 and 4). Moreover, in the subgroups of stages III and IV patients who underwent standard postoperative chemotherapy (Supplementary Table 5), there were significant differences in OS and PFS between IFITM3-negative and IFITM3-positive subgroups (Supplementary Tables 3 and 4). These findings strongly indicated that IFITM3 expression plays a critical role in colon cancer development and progression and is a valuable biomarker for this disease.

Association of IFITM3 overexpression with decreased KLF4 expression and increased Ki67 expression

Prior studies have shown that loss of KLF4 expression contributed to colon carcinogenesis, while the underlying mechanisms remain to explore (21–23). It is unknown whether loss of KLF4 in fact is in part responsible for IFITM3 overexpression. To identify the molecular mechanisms underlying IFITM3 overexpression and its impact on colon cancer pathogenesis, we performed immunostaining for KLF4 and Ki67 in normal colon tissue, colon tumor, and nodal metastasis specimens (Fig. 2A, Supplementary Tables 6 and 7). The levels of IFITM3 expression in the primary tumors were higher than those in the normal colon tissue, whereas the levels of IFITM3 expression in the nodal metastases were higher than those in the primary tumor and normal tissue specimens (Fig. 2B, left panel). We observed a significant inverse correlation between the levels of IFITM3 and KLF4 expression (Fig. 2B, middle panel), whereas the level of IFITM3 expression correlated directly with that of Ki67 expression (Fig. 2B, right panel; Fig. 3A). These results indicated that IFITM3 expression was significantly associated with loss of KLF4 expression and increased colon cancer cell proliferation.

Inhibition of colon cancer cell growth by siRNA-induced downregulation of IFITM3 expression

To determine the role of IFITM3 in colon cancer cell proliferation, we treated HCT116 and SW480 cells with IFITM3 siRNA. We confirmed the efficacy of knockdown of IFITM3 expression using Western blotting (Fig. 3B). We determined the cells' viability using an MTT assay and colony-formation ability using a soft agar assay. We found that knockdown of IFITM3 expression significantly inhibited the growth of cancer cells (Fig. 3C). This result was consistent with the finding showing a direct correlation between increased IFITM3 expression and a high proliferation index in colon tumor specimens. These data suggested that overexpression of IFITM3 contributes to hyperproliferation of colon cancer cells.

Attenuation of the migratory and invasive ability of colon cancer cells by IFITM3 silencing

Because IFITM3 expression was correlated with both distant and nodal metastasis of colon cancer (Supplementary Tables 8 & 9), we investigated the role of IFITM3 in the migration and invasion of colon tumors. We found that the levels of both migration and invasion of IFITM3 siRNA-transfected HCT116 and SW480 cells were significantly lower than those of control cells ($P < 0.001$) (Fig. 4). Consistent with those in vitro findings, knockdown of IFITM3 expression suppressed the growth and metastasis of HCT116 cells in nude mouse model, whereas overexpression of IFITM3 did the opposite (Figure 5A & 5B).

Genetic disruption of KLF4 and IFITM3 overexpression in murine colon mucosa

To further investigate the relationship between IFITM3 and KLF4 expression, we generated colon-specific KLF4-knockout mice, confirming the gene deletion using histological analysis (Fig. 5C), which showed an absence of goblet cells in colon mucosa, and Western blot analysis (Fig. 5D). We then measured the expression of both KLF4 and IFITM3 in KLF4-knockout ($KLF4^{-/-}$), KLF4-heterogeneous ($KLF4^{+/-}$), and KLF4-wild-type ($KLF4^{+/+}$) mice using both Western blot and immunohistochemical analysis. The $KLF4^{+/+}$ mouse had the highest KLF4 but lowest IFITM3 expression levels (Figs. 5D and 5E). Therefore, data from our studies of both human colon tumor specimens and this mouse model demonstrated that decreased KLF4 expression was significantly correlated with increased IFITM3 expression.

Negative regulation of IFITM3 expression by KLF4

To identify the molecular mechanisms of negative regulation of IFITM3 expression by KLF4, we first examined the effects of KLF4 overexpression on IFITM3 expression in HCT116 and SW480 cells. Restored KLF4 expression significantly repressed IFITM3 expression at both the mRNA (Fig. 6A1) and protein (Figs. 6A2 and 6A3) level. Furthermore, to determine the role of KLF4 in regulation of IFITM3 transcription, we generated an IFITM3 promoter and mutations of IFITM3 in two KLF4-binding sites in HCT116 and SW480 cells using site-specific mutagenesis (Fig. 6B1). Transfection of KLF4 significantly inhibited the IFITM3 promoter activity (Fig. 6B2), whereas mutations of the IFITM3 promoter reporter in either (Mut1 or Mut2) or both (Mut3) KLF4-binding sites significantly increased this activity (Fig. 6B3). These results suggested that the KLF4-binding sites were negative regulatory elements in the IFITM3 promoter. Finally, we performed a ChIP assay to determine how KLF4 directly interacts with the IFITM3 promoter. We transduced both HCT116 and SW480 cells with Ad-EGFP and Ad-KLF4. We then immunoprecipitated the chromatin using an anti-KLF4 antibody and amplified the regions from -438 to +74 bp flanking the putative KLF4-binding site using specific PCR primers. As shown in Fig. 6C, anti-KLF4 antibodies but not control IgG amplified the predicted-size DNA fragments from the precipitates of the cells. These results suggested that

endogenous KLF4 bound to the IFITM3 promoter in both HCT116 and SW480 cells and that enforced KLF4 expression increased the binding of KLF4 to the IFITM3 promoter.

Discussion

In this study, we discovered four lines of evidence supporting a critical role for IFITM3 in colon cancer pathogenesis. First, we observed a direct correlation of elevated IFITM3 expression with colon tumor progression. IFITM3-positive staining of colon cancer cells could be used to identify a greatly increased risk of recurrence and metastasis in patients after colectomy. Second, overexpression of IFITM3 increased the proliferation and metastasis of human colon cancer cells, indicating that targeting of IFITM3 is a potential therapeutic strategy for colon cancer. Third, genetically enforced KLF4 overexpression led to decreased IFITM3 expression in colon cancer cells, and we observed a negative correlation between KLF4 expression and IFITM3 expression in both human colon tumor samples and a KLF4-knockout mouse model. Fourth, KLF4 directly regulated the expression of the IFITM3 gene at the transcriptional level via binding to the IFITM3 promoter. Therefore, loss of KLF4 expression during the initiation and development of colon tumors contributed to abnormal IFITM3 expression and activation; the latter may be a novel molecular marker for poor prognosis and contribute to colon tumor pathogenesis and aggressive colon cancer biology.

Numerous studies have indicated the importance of IFITM3 expression in the oncogenesis of several malignancies, including colorectal cancer (16, 21). In addition, authors have reported upregulated IFITM3 expression in early and late intestinal neoplasms (4, 14, 15). However, the predictive significance of IFITM3 in colon cancer remains unclear. In the present study, we specifically investigated the expression of IFITM3 in a TMA containing tumor specimens, matched normal colon tissue specimens, and lymph node metastasis specimens obtained from 203 patients with colon cancer. We observed the strongest IFITM3-positive staining in the nodal metastasis specimens. We also found that IFITM3 expression was significantly correlated with an advanced cancer biology, which was indicated by invasion depth, lymph node metastasis, and distant metastasis. Also, IFITM3-positive staining was associated with poor OS and DFS rates. Therefore, we provide the first evidence that IFITM3 can be used as a novel biomarker for outcome in patients with colon cancer after colectomy. This means that at the time of initial diagnosis of colon cancer, IFITM3 expression can be used not only to design optimal, individualized treatment but also to distinguish patients who would benefit from close monitoring after surgery from those who would not.

Researchers have suggested that IFITM genes function in a variety of contexts, including immune cell regulation, somitogenesis, and germ cell development (8, 10–13). However, the precise roles of IFITM3 in carcinogenesis remain to be elucidated. Herein we provide evidence indicating that IFITM3 expression may affect the proliferation, migration, and invasiveness of colon cancer cells. Consistent with our findings, investigators have established that expression of IFITM1 promotes head and neck tumor invasion in the early stages of disease progression by mediating the expression of molecules downstream, including matrix metalloproteinases 12 and 13 (20). Recently, Lange and colleagues reported that targeted IFITM3 mutations have no detectable effects on development of the germ line or generation of live young mice (29), which contrasts with a previous study showing that IFITM3 mediates the migration of early primordial germ cells (9, 11). The different roles of IFITM3 in the development of the germ line and tumorigenesis may depend primarily on the involvement of different signaling pathways in these processes. Our current results suggested that IFITM3 could regulate both colon cancer cell growth and

invasion. Whether IFITM3 is more important in regulating cell growth than cell migration and the underlying mechanism remain to be determined further.

Up to now, the mechanisms responsible for IFITM3 overexpression in malignances have been unknown. However, a study showed that in conditional Apc mutant mice, deletion of Apc alleles, which leads to the formation of colon adenomas, rapidly induced IFITM3 expression (21). That study also showed that induction of wild-type APC resulted in a strong reduction of β -catenin signaling and IFITM3 expression in HT29 colon cancer cells (21). Thus, IFITM3 is a potential therapeutic target of activated β -catenin signaling in carcinogenesis in the intestinal epithelium. Nevertheless, the molecular mechanism of IFITM3 overexpression underlying the progression of colon cancer remains undefined. In a previous study, we found that loss of KLF4 expression plays a critical role in the pathogenesis and development of digestive system tumors, including gastric and pancreatic cancer (26, 30). In the present study, we investigated whether expression of the IFITM3 gene is a downstream molecule of KLF4 in colon cancer cases. Our results indicated that KLF4 could bind directly to the IFITM3 promoter and downregulate transcriptional activation of IFITM3. Also, we observed an inverse correlation between KLF4 expression and IFITM3 expression in human colon tumor specimens and a murine model of colon cancer. KLF4 has been known to be a highly expressed transcription factor in the adult intestine and critical for intestinal differentiation. Researchers have observed loss of KLF4 expression in many malignances and that restoration of KLF4 expression can antagonize the development and progression of intestinal tumors (31). Consistently, a recent study has clearly demonstrated that expression of KLF4 is a prognostic predictor for colon cancer (32) and a haploinsufficiency of *Klf4* promotes adenomatous polyposis coli dependent intestinal tumorigenesis (31). In addition, KLF4 directly interacts with the C-terminal transactivation domain of β -catenin and inhibits Wnt/ β -catenin signaling in intestinal tumors (33). Thus, KLF4 can mediate IFITM3 gene expression via both direct transcriptional inhibition and attenuation of the Wnt/ β -catenin signaling pathway. It warrants further investigations on how the interactions between Wnt/ β -catenin and KLF4 regulate the expression and function of IFITM3 and contribute to colon carcinogenesis. Therefore, improved understanding of the mechanism of KLF4-mediated inhibition will lead to novel therapies for colorectal cancer.

In summary, this study provided critical insight into the role of the IFITM3 gene in the progression of colon cancer. We showed that IFITM3 expression plays important roles in colon tumor progression by downregulating the proliferation, migration, invasion, and metastatic potential of tumor cells. The frequent upregulation of IFITM3 expression in human colon cancer cells highlights its potential as a novel therapeutic target for this cancer, which is closely associated with deregulated Wnt signaling and KLF4 signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

IFITM3 expression in colon cancer cells and its impact on patient survival. **A**, TMA sections were prepared for immunostaining using a specific antibody against IFITM3. Representative photos of IFITM3 protein expression in colon tumors are shown (magnification: **A1**, 20 $\times$; **A2**, 100 $\times$; **A3**, 400 $\times$). The majority of the adjacent normal colon tissue cells (N) were negative for IFITM3 expression, whereas colon tumor cells (T) were strongly positive for IFITM3 expression. **B**, results of analysis of IFITM3 mRNA expression using real-time PCR (**B1**) and IFITM3 protein expression using Western blotting (**B2**). N, normal cells; T, tumor cells. **C**, the OS and DFS rates were estimated using the Kaplan-Meier method. Both rates in patients with IFITM3-positive primary tumors were significantly worse than those in patients with IFITM3-negative primary tumors ($P = 0.002$ for OS and $P = 0.005$ for DFS).

Figure 2.

Inverse association of IFITM3 expression with KLF4 expression. Three sets of consecutive TMA sections were prepared for immunostaining using specific antibodies against IFITM3, KLF4, and Ki67. **A**, representative photos of IFITM3 and KLF4 protein expression in normal colon, colon tumor, and nodal metastasis specimens (original magnification: 400× for the inserts, 100× for all others). **B**, IFITM3 expression levels were significantly higher in lymph node metastasis specimens than in primary colon tumor specimens, whereas IFITM3 expression levels in both primary colon tumor and lymph node metastasis specimens were significantly higher than those in normal colon tissue specimens (**B1**, $P < 0.001$ [χ^2 test]). The IFITM3 expression levels were inversely correlated with the KLF4 expression levels (**B2**: $P < 0.001$, $r = -0.575$) and directly correlated with the Ki67 expression levels (**B3**: $P < 0.001$, $r = 0.325$) in primary colon tumors as analyzed using the Pearson χ^2 test.

Figure 3.

The effect of IFITM3 expression on colon cancer cell proliferation. **A**, representative photos of IFITM3 and Ki67 protein expression in primary colon tumor and nodal metastasis specimens (original magnification: 400× for the inserts, 100× for all others). **B**, HCT116 and SW480 cells were transfected with IFITM3 siRNA (Si-IFITM3) or a control siRNA (Si-Control). Total protein lysates were harvested from the cell cultures, and the levels of IFITM3 expression in the lysates were determined using Western blot analysis. **C**, soft agar assay (left panels) and MTT assay (right panels) results for HCT116 and SW480 cells transfected with IFITM3 siRNA (Si-IFITM3) or a control siRNA (Si-Control) or left untransfected (Mock). This representative experiment was one of three with similar results.

Figure 4.

Regulation of colon cancer cell migration and invasion by IFITM3. The migration and invasiveness of HCT116 and SW480 cells transfected with IFITM3 siRNA (Si-IFITM3) or a control siRNA (Si-Control) or left untransfected (Mock) were determined as described in Materials and Methods. The untreated cell cultures were given arbitrary migration and invasiveness percentages of 100%. A, representative photos of cell migration and invasion. B, cell migration and invasion percentages. * $P < 0.001$ in a comparison of the Si-IFITM3-treated group with the Mock and Si-Control-treated groups. This representative experiment was one of three with similar results.

Figure 5.

Influence of altered IFITM3 expression on colon tumor growth and metastasis and IFITM3 expression in colon tissue specimens obtained from *KLF4*^{-/-} mice. **A & B**, HCT116 cells with mock transfection, control siRNA, IFITM3 siRNA, and IFITM3 expression vector were orthotopically implanted in the cecum of nude mice (n=10), tumors were weighted and liver metastases were determined (**A**). Gross tumors in the cecum (upper panel) and removed from the cecum (lower panel) were also shown (**B**). **C**, hematoxylin- and eosin-stained sections of colon mucosa obtained from *Villin-Cre*⁺;*Klf4*^{+/+} (*KLF4*^{+/+}) and *Villin-Cre*⁺;*Klf4*^{fl/fl} (*KLF4*^{-/-}) mice. Unlike in *KLF4*^{+/+} mice, goblet cells were nearly absent from *KLF4*^{-/-} colon epithelium. **D**, Western blot analysis of IFITM3 and KLF4 protein expression in total protein lysates prepared from colon mucosa obtained from *Villin-Cre*⁺;*Klf4*^{+/+} (*KLF4*^{+/+}), *Villin-Cre*⁺;*Klf4*^{fl/fl} (*KLF4*^{+/+}), and *Villin-Cre*⁺;*Klf4*^{fl/fl} (*KLF4*^{-/-}) mice. **E**, immunostained consecutive sections of colon tissue specimens obtained from *KLF4*^{+/+}, *KLF4*^{+/+}, and *KLF4*^{-/-} mice. The representative photos show IFITM3 and KLF4 protein expression (original magnification: 400× for the inserts, 100× for all others).

Figure 6.

Downregulation of IFITM3 expression by KLF4 in colon cancer cells. **A**, HCT116 and SW480 cells were transduced with Ad-KLF4 (KLF4) or control Ad-EGFP (EGFP) at a multiplicity of infection (MOI) of 5 and incubated for 24 h. Total RNA and protein lysates were harvested for determination of the levels of IFITM3 and KLF4 expression using real-time PCR (**A1**) and Western blotting (**A2**). For immunofluorescence, the cells were transduced with Ad-CMV (with empty expression cassette) as control (**A3**). **B**, Schematic structure of the IFITM3 promoter (**B1**). The IFITM3 promoter reporter was transfected into HCT116 and SW480 cells in triplicate with transduction of Ad-EGFP or Ad-KLF4 at an MOI of 5 (**B2**). The IFITM3 promoter reporter (WT) and mutations of it in KLF4-binding site 1 (Mut1), KLF4-binding site 2 (Mut2), or both (Mut3) were transfected into HCT116 cells in triplicate (**B3**). The relative IFITM3 promoter activities were measured 24 h after transfection, and the activities in the treated groups were expressed as the fold or percentage of that in their respective control groups. **C**, HCT116 (**C1**) and SW480 (**C2**) cells were transduced with Ad-KLF4 (KLF4) or control Ad-EGFP (EGFP) at an MOI of 5, and chromatin was extracted from the cells 24 h after transduction. A ChIP assay was performed using a specific anti-KLF4 antibody and oligonucleotides flanking the IFITM3 promoter regions containing putative KLF4-binding sites. The nucleotide positions and sequences of the PCR forward and reverse primers flanking those sites in the ChIP assay are described in Materials and Methods. IP, immunoprecipitation.