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The Angiogenic Switch Molecule, Secreted FGF-Binding Protein, an Indicator of Early Stages of Pancreatic and Colorectal Adenocarcinoma

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Abstract

Tumor angiogenesis has been related to the initiation as well as progression toward more aggressive behavior of human tumors. We will discuss genetic events underlying the initiation and progression of colorectal and pancreatic adenocarcinoma with a particular focus on the modulation of angiogenesis. A secreted fibroblast growth factor (FGF) binding protein (FGF-BP), which is an extracellular chaperone molecule for FGFs, has been shown to enhance FGF-mediated biochemical and biologic events and to be a crucial rate-limiting factor for tumor-dependent angiogenesis. Histochemical and in situ hybridization studies with archival samples show that FGF-BP is induced early during the initiation of colorectal and pancreatic adenocarcinoma. We will discuss the potential of this secreted protein as a serum marker to identify at-risk subjects.

Keywords

FGF-Binding Protein; Colon Cancer; Pancreatic Cancer; Dysplastic Lesions; Cancer Progression

INTRODUCTION

Colorectal cancer is the third most common type of cancer among men and women in the United States. Eighty-five percent of affected patients undergo surgical removal of the primary tumor as first-line treatment. However, disease will recur in approximately half of these patients within 5 years (1). It was estimated that in 2004, there were approximately 100,000 new cases and 57,000 deaths from colorectal cancer in the United States (2). Risk factors include age, a diet rich in fat and cholesterol, inflammatory bowel disease (especially ulcerative colitis), and genetic predisposition, including hereditary polyposis and nonpolyposis syndromes. Colorectal cancer screening tests, such as the fecal occult blood test (FOBT), flexible sigmoidoscopy, and colonoscopy, performed in asymptomatic individuals of age 50 years and older have been shown to achieve accurate detection of early stage cancer and its precursors. Reductions in morbidity and mortality can be achieved through detection and treatment of early stage colorectal cancers and the identification and removal of adenomatous polyps.

GENETIC ALTERATIONS IN COLORECTAL CANCER

Progress has been made in understanding the molecular basis of colorectal cancer predisposition and progression. The vast majority of colorectal cancers are adenocarcinomas, which develop through a series of clinical and histopathologic steps, ranging from a single

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crypt lesion through small benign tumors (adenomatous polyps) to malignant and invasive carcinomas (3). Over the last 15 years, Vogelstein and colleagues discovered a series of genetic alterations that contribute, through their multiplicity over many years, to the eventual initiation and progression of colorectal cancer (4) (Fig 1).

Adenomatous Polyposis Coli (APC) Tumor Suppressor Gene

Germline mutation in the adenomatous polyposis coli (APC) tumor suppressor gene is the earliest event during the transformation process in both sporadic colorectal tumors and familial adenomatous polyposis (FAP), an autosomal dominant disorder causing extensive adenomatous polyps in the colon and early onset colorectal cancer (4-6). Carboxy-terminal truncations of the

APC protein result in impairment of the ability of APC to interact with and downregulate beta-catenin, a transcriptional activator regulated by the Wnt signaling pathway (7-13). The resulting constitutive overexpression of beta-catenin is likely responsible for the activation of growth-promoting oncogenes, such as cyclin D1 or c-myc (14-16). In 1990, Moser and colleagues identified an APC mutant mouse, namely *Apc^{Min/+}*, which carries a mutation in the APC gene similar to that detected in FAP and sporadic colorectal cancers (17). *Apc^{Min/+}* mice do not survive beyond 120 days due to the occurrence of intestinal tumors that start from a rapid development of adenomatous polyps.

K-ras Proto-Oncogene

The *K-ras* pathway mediates the cellular response to extracellular signals, such as growth factors, cytokines, and hormones that regulate cell proliferation, differentiation, and apoptosis (18). Mutation of the *ras* proto-oncogene has been detected in various human cancers, including 95% of pancreatic cancers and up to 50% of large bowel adenocarcinomas (19-21). Mutation of the *K-ras* oncogene has been found in adenomas measuring <1 cm (10%-15%) as well as in larger adenomas and adenocarcinomas (30%-60%) (22-24). Therefore, it has been proposed that a mutation of *K-ras* is likely to represent a hallmark contributing to the transition from intermediate to late adenoma or adenocarcinoma (25).

Deletions of Chromosome Arm 18q

Chromosome arm 18q deletions (mainly 18q21 deletions) represent a later event associated with colon cancer progression through the stages of late adenoma to carcinoma (26). These deletions account for the reduced expression of the tumor suppressor DCC (deleted in colorectal carcinoma) (27-30) as well as SMAD4/DPC4 (deleted in pancreatic carcinoma) (31-33).

p53 Tumor Suppressor Gene

Loss or inactivation of p53 tumor suppressor gene has been reported in a high percentage of colorectal cancers as a late event during disease progression (34). Disruption of p53 by gene targeting in human colon cancer cells results in cell resistance to different chemotherapeutic agents, as opposed to wild type cells (35). Hence, loss of p53 in human colorectal cancers may explain the inefficacy of chemotherapy and may be correlated with decreased survival in patients with colorectal adenocarcinomas (36-39).

PANCREATIC CANCER

Pancreatic cancer accounts for more than 30,000 deaths each year in the United States (40). Although pancreatic adenocarcinoma represents fewer than 2% of new cancer cases in the United States, it is the fifth leading cause of cancer-related death. There is no effective early screening test, and symptoms appear during late-stage disease, when the tumor has

metastasized and invaded surrounding tissues. Therefore, the majority of patients are not suitable for curative resections. Additionally, pancreatic cancer cell resistance to cytotoxic agents and radiation represents a further element that accounts for the poor prognosis (41,42). Pancreatic adenocarcinoma develops through a step-wise progression that initiates with distinct epithelial lesions in the small interlobular ducts known as pancreatic intraepithelial neoplasias (PanINs). PanINs can be flat (PanIN-1A), papillary without atypia (PanIN-1B), papillary with atypia (PanIN-2), or with characteristics of carcinoma in situ (PanIN-3) (43) (Fig. 2). Interestingly, as observed in infiltrating colon adenocarcinomas, PanINs display some genetic changes such as activation of the *K-ras* pathway (44,45), as well as inactivation of tumor suppressor genes, such as p16, p53, DPC4, and BRCA2 (46).

ANGIOGENESIS IN GASTROINTESTINAL CANCERS

Classically, angiogenesis is a process of endothelial sprout formation from preexisting vessels (47). In a more complex picture, angiogenesis is the mechanism mediating the growth and remodeling of a capillary network (48,49). In the adult organism, angiogenesis is commonly restricted to conditions of tissue repair and remodeling (eg, during menstruation and mammary gland involution), and pathologic circumstances, such as wound repair, inflammation, and neoplastic growth where it plays a pivotal role (48,50). Specific angiogenic factors can initiate a multi-step process that begins with vasodilatation, followed by the enhancement of vessel permeability and stroma degradation. Acting as chemotactic and mitogenic agents, angiogenic factors can also induce endothelial cell migration and proliferation. As a result, endothelial cells converge and tightly assemble to generate a new lumen or they intercalate with endothelial cells in a preexisting vessel to give rise to capillary elongation. The newly formed capillary-like vessels undergo final remodeling, in which they rearrange from an irregular into a structured plexus of branching vessels and capillary loops (51). In neoplastic tissues, endothelial cells present various morphologic and physiologic abnormalities and produce anomalous vasculature, characterized by tortuous, elongated, and dilated vessels, atypical enlargement of the vascular lumen, and excessive branching. The newly formed vessels are randomly fused with either arterioles or venules and create an atypical microcirculation. As a result, neoplastic regions are often hypoxic and acidic due to the chaotic and slow blood flow (48,52-55). In addition, tumor vessels are leaky because they present endothelial fenestrae, vesicles and transcellular holes, widened inter-endothelial junctions, and a discontinuous or absent basement membrane (56-58). Tumor vessels may also lack functional perivascular cells (59) and their walls can be formed by a mosaic of alternating endothelial and cancer cells (vasculogenic mimicry) (60).

In 1971, Folkman proposed that new blood vessel formation was required for tumor growth and metastasis (61,62) and proposed that solid tumors cannot grow beyond 1-2 mm³ without a massive blood vessel supply. These microscopic tumor masses can eventually activate an “angiogenic switch” by inducing the sprouting of new capillaries from surrounding mature blood vessels. These blood vessels will infiltrate the tumor mass, initiating the increase in size of the tumor mass and its metastatic perfusion. It is now widely accepted that the angiogenic switch is “off” when the effect of pro-angiogenic molecules is counter-weighted by that of anti-angiogenic molecules, and it is “on” when the concentration of angiogenic inducers prevail on that of the angiogenic inhibitors.

Thus, activation of the angiogenic switch during early stages of tumor development suggests that regulation of the angiogenic process is likely to be a rate-limiting step in the progression from small lesion to extensive disease (49,63, 64). Numerous pro-angiogenic factors involved in tumor-dependent angiogenesis have been described, including, among others, members of the vascular endothelial growth factor (VEGF) family, platelet-derived growth factor (PDGF),

transforming growth factor- α and - β (TGF- α and TGF- β), and members of fibroblast growth factor (FGF) family.

Although pancreatic cancer is not a grossly vascular tumor, it is often characterized by enhancement of tumor-dependent angiogenesis (65). A growing line of evidence has shown that various FGFs, such as FGF-1, FGF-2, FGF-5, FGF-7 (66-69) and FGF receptors (70-73) are upregulated in pancreatic cancer tissue samples and cell lines. These findings suggest that FGF-dependent downstream biologic events are likely to play an important role in the pathobiology of pancreatic cancer.

In colorectal cancer, several studies indicate angiogenesis as a crucial event leading to colon cancer progression. As a matter of fact, colorectal cancer is one of the best-studied models of tumor angiogenesis (74). As in many other tumors, several angiogenic regulators have been recognized in colon cancer, including VEGF, PDGF, thrombospondin, and angiopoietins (74,75). Likewise, overexpression of FGF and FGFRs in colon cancer cells and tissues, as well as increase of FGF-2 serum levels in patients with advanced colon cancer, have been extensively reported (76-81).

FIBROBLAST GROWTH FACTORS AND FIBROBLAST GROWTH FACTOR-BINDING PROTEIN

The FGF family comprises 23 distinct, structurally-related proteins described to date that exert biologic effects in different cells and organ systems, including tumor growth and angiogenesis (82-85). FGFs are heparin-binding proteins, which interact with low affinity heparan sulfate proteoglycans (HSPGs). HSPGs are ubiquitous cell-surface and extracellular matrix (ECM) proteins, which have been shown to protect FGFs from thermal denaturation and proteolysis as well as to increase FGF receptor affinity and facilitate FGF binding to cell surface receptor. In addition, ECM-associated HSPGs modulate FGF bioavailability by generating a local reservoir for the growth factor and allowing a sustained stimulation of endothelial cells (82, 84). Mobilization of FGFs from the ECM storage, and in particular of FGF-1 and FGF-2, occurs via HSPG digestion by heparanases or glycosaminoglycan-degrading enzymes (82, 84).

An alternative mechanism of FGF release from the ECM is mediated by a secreted FGF-binding protein (FGF-BP). FGF-BP was first described in 1991 by Wu et al as a low-affinity heparin binding protein, isolated from A431 human epidermoid carcinoma cells (86). Several studies conducted in our laboratory have contributed to a more extensive characterization of FGF-BP. FGF-BP binds to FGF-1 and FGF-2 in a noncovalent, reversible manner facilitating the release of the growth factor from the ECM and presenting it to FGF receptors (87-90). Moreover, FGF-BP is likely to supplement the role of heparan sulfate in mediating FGF-dependent signaling and mitogenesis (91). Indeed, we and others have shown that heparin, heparan sulfate, and other heparinoids are able to compete with FGF-BP binding to FGF-2 as well as a direct binding of FGF-BP to perlecan, thereby indicating a potential role for FGF-BP in releasing FGF proteins tightly bound to ECM molecules (90,92). Numerous studies conducted in our laboratory have confirmed a role for FGF-BP as an extracellular chaperone for FGFs that enhances FGF-dependent biochemical and biologic functions. In murine NIH-3T3 fibroblasts as well as in bovine GM7373 endothelial cells, FGF-BP was shown to enhance FGF-2-induced MAPK signaling pathway and cell proliferation and we showed recently that the interaction domain with FGF-2 is a short domain in the C-terminus of the FGF-BP protein (90,91,93). Furthermore, FGF-BP was responsible for enhancing FGF-2-mediated angiogenesis in a chick chorioallantoic membrane (CAM) assay (90). Overexpression of FGF-BP in a chicken transgenic model resulted in embryonic lethality due to massive disruption of blood vessel structure and integrity and subsequent hemorrhage (94).

FGF-BP is expressed below the level of detection by Northern blotting in normal adult human tissues. However, its expression is considerably elevated in various tumors, including skin, head and neck, cervical, lung, squamous cell carcinomas, breast, colon, and pancreatic adenocarcinomas (87,88,95-97). Studies with FGF-BP-negative cell lines indicated FGF-BP as a rate-limiting factor for tumor growth and angiogenesis in a nude mouse model (87). Similarly, ribozyme-targeting of endogenous FGF-BP from human squamous cell carcinoma and colorectal cancer cell lines resulted in a significant reduction of tumor growth and angiogenesis (88). These findings support a potential role of FGF-BP as an angiogenic switch in human neoplasia (88).

FIBROBLAST GROWTH FACTOR-BINDING PROTEIN IS AN EARLY MARKER IN GASTROINTESTINAL CANCERS

Our laboratory has recently focused on the analysis of FGF-BP expression and regulation during the progression of gastrointestinal cancers. In an initial study by Ray and colleagues (96), immunohistochemical analysis of normal and pathologic human colon biopsies using a polyclonal anti-FGF-BP antibody showed a remarkable upregulation of FGF-BP in dysplastic lesions, originating at the level of single crypts. Upregulation of FGF-BP protein was detected in a small portion (16%) of 76 apparently normal colon samples. However, expression of FGF-BP was detected in most samples with moderate to severe dysplasia (62 of 85; $P < 0.0001$ normal *versus* dysplastic mucosa) (Fig 3A). Furthermore, coincident with FGF-BP overexpression in dysplastic crypts, an increase of blood vessel density was found by immunostaining of the lamina propria with a CD31 antibody (from 80 ± 7 to 154 ± 9 vessels/field). The fact that specimens from different inflammatory bowel diseases, such as ulcerative colitis or Crohn's disease, did not show a significant FGF-BP upregulation prompted the authors to link FGF-BP overexpression in the onset of colon cancer to an early genetic occurrence.

Analysis of FGF-BP mRNA by ISH in APC^{Min/+} mouse colon specimens showed a significant upregulation of FGF-BP in colon adenomas (21 of 27, >30% of the adenoma surface area). In contrast, little if any FGF-BP expression was noted in adjacent normal intestine (5 of 19; $P < 0.001$, normal *versus* adenomas) (Fig 3B). Interestingly, a prominent correlation between FGF-BP upregulation and cytoplasmic and nuclear beta-catenin staining in dysplastic colon was seen but not in a dextran sulfate-induced model of inflammatory colon disease (98). This finding further corroborated the hypothesis that FGF-BP is likely to be an early response gene during the initiation of colon cancer. Moreover, based on these findings and promoter-reporter studies, FGF-BP was shown to be a direct target of the activation of the Wnt/beta-catenin pathway (Fig 1) and therefore represents an early event for colon cancer initiation.

Tassi and colleagues (97) described the generation and characterization of a series of monoclonal antibodies against human FGF-BP. Monoclonal antibodies were applied for immunohistochemical (IHC) detection of FGF-BP in tissue microarrays containing a series of tissue samples from different stages of both colon and pancreatic cancers. In parallel, detection of FGF-BP mRNA in the same tissue slides was performed by in situ hybridization. High levels of FGF-BP protein and mRNA were found in those samples representing early stages of colon cancers, such as adenomatous polyps (Table 1 and Fig 4). However, a significant upregulation of FGF-BP was also identified in all other stages of colorectal cancer progression, such as invasive adenocarcinoma and metastases, compared to the levels obtained in normal specimens ($P < 0.0001$).

In addition, this report showed for the first time the analysis of FGF-BP expression during different progression stages of pancreatic cancer. Surprisingly, FGF-BP protein and mRNA were found dramatically upregulated during the early stages of malignant transformation, such

as low-grade (PanIN1 and 2) and high-grade PanIN as well as invasive adenocarcinoma. Positive FGF-BP expression was also evidenced in some pancreatitis specimens, whereas very low or no expression was detected in normal pancreatic ducts. Nonadenocarcinoma samples showed expression of FGF-BP in 23% of samples at low levels, although this expression still represented a significant increase relative to normal specimens ($P=0.0044$). A highly significant correlation resulted from comparison between protein and mRNA expression in all samples analyzed.

Based on these results, it is tempting to speculate that FGF-BP acts as an early regulatory gene, activated during the early stages of pancreatic cancer progression, most likely from the development of low-grade to high-grade PanINs (Fig 2). It is also possible that, as a consequence of FGF-BP overexpression in the early stages of colon and pancreatic cancers, an enhancement of tumor-dependent angiogenesis and invasion may occur, due to the increase of FGF-BP-mediated FGF bioavailability.

It is likely that FGF-BP, as a secreted protein, is shed into the circulation. Therefore, generation of a sensitive ELISA assay able to detect FGF-BP in patient sera may represent an important diagnostic screening method for early detection of colon and pancreatic premalignant lesions, thereby allowing early medical intervention.

CONCLUSION

Early events that initiate the stepwise progression of premalignant lesions of colon epithelia and pancreatic ducts to invasive cancer may provide circulating markers for the detection of such lesions. FGF-BP could be such a marker to identify subjects at an increased risk for developing invasive cancers.

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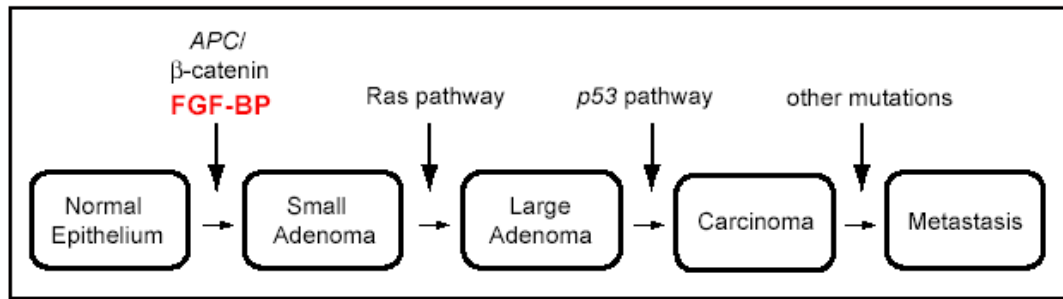


Figure 1. Genetic alterations during the development of colorectal cancer. Significant genetic alterations at different junctures during the transformation of colon epithelia to invasive adenocarcinoma are depicted. Induction of FGF-BP expression is an early event that is driven by mutations and activation of the WNT/beta-catenin pathway (Adapted and modified from Kinzler Vogelstein [4]).

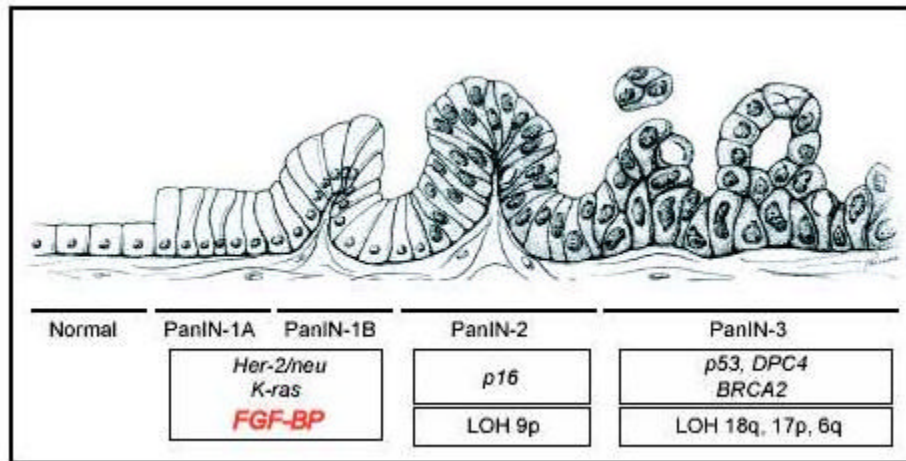


Figure 2. Genetic alterations during malignant transformation of pancreas epithelia. The progression from normal duct epithelium to low-grade and high-grade PanIN (Pancreatic Intraepithelial Neoplasia) and the associated accumulation of genetic alterations are shown (Adapted and modified from Hruban and colleagues [45]).

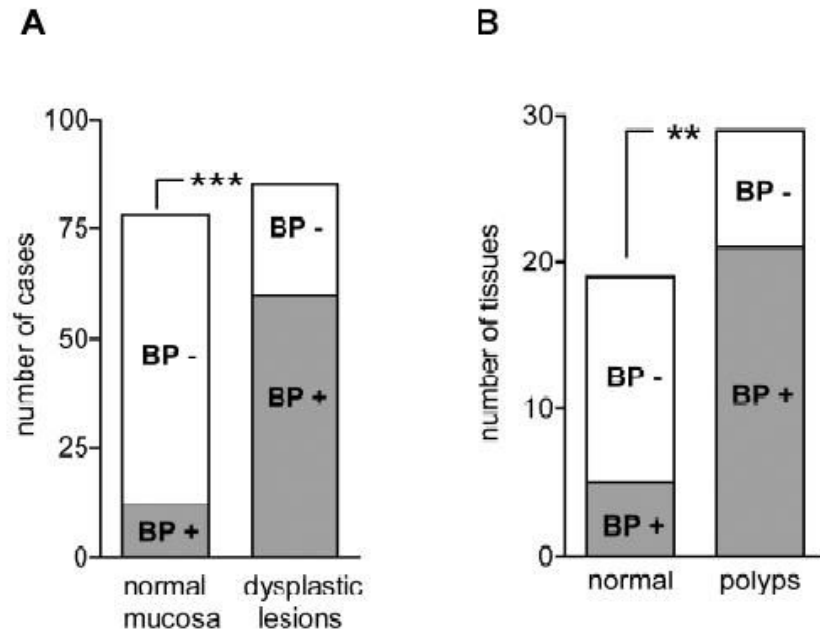


Figure 3. **FGF-BP expression in human and mouse dysplastic lesions.** (A) Frequency of FGF-BP protein expression, analyzed by immunohistochemistry (IHC) with a polyclonal anti-FGF-BP antibody in 161 human specimens $***P < 0.0001$. (B) Frequency of FGF-BP mRNA expression in normal and dysplastic intestinal tissues obtained from B6 APC^{Min/+} mice. FGF-BP expression coincides with the activation of the beta-catenin pathway in this model $**P < 0.001$ (96).

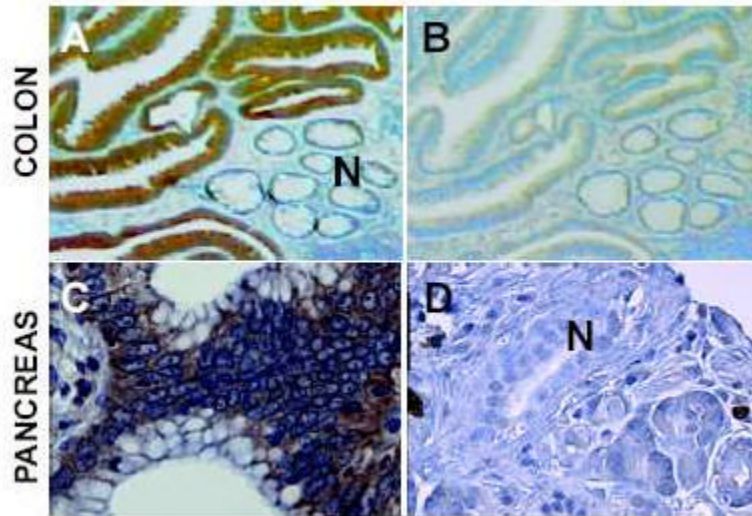


Figure 4.
FGF-BP staining in premalignant human colon and pancreas lesions. (A) Histochemistry with a monoclonal antibody against FGF-BP shows expression (dark brown staining) in dysplastic lesions of the colon next to normal crypt epithelia (N). (B) A serial section from panel A stained with the secondary antibody only. (C) FGF-BP staining in a PanIN lesion. (D) A normal pancreatic duct shows no staining for FGF-BP. Further details in Ref. (97).

Table I

Percentage of FGF-BP protein and mRNA expression analysis (%) in normal and pathologic colon and pancreatic tissues analyzed by IHC (monoclonal antibody) and ISH. n = number of cases analyzed (97).

FGF-BP	Protein (%)	n	mRNA (%)	n
COLON				
Normal crypts	15	92	5	61
Adenomatous polyps	90	29	100	9
Adenocarcinoma	72	46	80	44
Metastases	80	71	91	66
PANCREAS				
Normal ducts	5	71	5	73
Pancreatitis	47	17	40	20
PanIN LOW	43	61	52	58
PanIN HIGH	68	19	79	19
Adenocarcinoma	49	69	60	69
Nonadenocarcinoma	23	35	26	34