PINCH mRNA Overexpression in Colorectal Carcinomas Correlated with *VEGF* and *FAS* mRNA Expression

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Abstract. *Background: Particularly interesting new cysteine-histidine-rich protein (PINCH) was found to be up-regulated in the stroma of colorectal carcinomas (CRCs) in our previous studies and was involved in angiogenesis through activation of fibroblasts in extracellular matrix (ECM) in response to tumors. Here, we examined PINCH mRNA expression in colorectal cancer and investigated its relationship with the clinicopathological features and proliferation cell nuclear antigen (PCNA), vascular endothelial growth factor (VEGF) and FAS. Materials and Methods: The primary cancer tissues, adjacent noncancerous tissues and the proximal and distant margins of normal mucosa were collected from 81 colorectal cancer patients during surgery. PINCH, PCNA, VEGF and FAS mRNA expression was examined by reverse transcriptional PCR (RT-PCR). Results: PINCH mRNA expression was significantly increased in primary tumors compared with that in adjacent noncancerous tissues, and the proximal and distant margins of normal mucosa ($p<0.0001$). Expression of PINCH mRNA in colon cancer tended to be higher than expression in rectal cancer ($p=0.051$). Tumors which had infiltrated through the wall of the colorectum trended to have higher PINCH mRNA expression ($p=0.073$). PINCH mRNA expression in primary tumors was positively related to the expression of PCNA mRNA ($r=0.534$, $p=0.010$), VEGF mRNA ($r=0.431$, $p=0.022$) and FAS mRNA ($r=0.542$, $p=0.012$). Conclusion: PINCH mRNA was overexpressed in colorectal cancer and associated with PCNA mRNA, VEGF mRNA and FAS mRNA expression. PINCH mRNA was involved in the development of colorectal cancer and might play a role in the epithelial mesenchymal transition in the rectum differently than in the colon, through the adenomatous polyposis coli (APC)/catenin pathway.*

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Key Words: Cysteine-histidine-rich protein (*PINCH*), colorectal carcinoma, epithelial mesenchymal transition (EMT), vascular endothelial growth factor (*VEGF*), *FAS*.

Colorectal cancer (CRC) is the third most common cancer worldwide, with an estimated one million new cases and a half million deaths each year (1). Although the 5-year mortality rate of colorectal cancer has declined over the last three decades, it is still necessary to identify more effective prognostic biomarkers and therapeutic targets. Particularly interesting new cysteine-histidine-rich protein (*PINCH*) is a widely expressed, evolutionarily conserved protein that consists of five Lin-1, Isl-1, Mec-3 (LIM) domains and an auto-epitope homologous to 'senescent cell antigen' (2). *PINCH* is located on chromosome 2q12.2, and the protein functions as an adapter protein for signal transduction in the integrin and growth factor pathways (3-5). *PINCH* mRNA is expressed in most normal tissues (2). *PINCH* protein has been shown to be markedly overexpressed in the tumor-associated stroma in a variety of common human cancer types compared with the corresponding normal tissues, including breast, prostate, lung, skin, and colon cancer (6). In our previous studies, the expression of *PINCH* protein was not only up-regulated in the stroma of the primary tumors and lymph node metastases of colorectal cancer, but also in mucinous and signet-ring cell carcinomas compared to non-mucinous carcinomas (7). However, there was no information available on *PINCH* mRNA expression in the primary tumor of colorectal cancer.

To understand the role of *PINCH* mRNA in colorectal adenocarcinoma, we examined the expression of *PINCH* mRNA in primary tumors, the non-cancerous mucosa samples

which were 2 cm away from tumors, and the distant normal mucosal samples of proximal and distal margins, and analyzed the relationship between *PINCH* mRNA expression and the clinicopathological variables of colorectal cancer, including patient's gender, age, tumor location, histological type, grade of differentiation, invasive depth, lymph node metastasis and Dukes' stage. Furthermore, we analyzed the relationship between *PINCH* mRNA expression and proliferation cell nuclear antigen (*PCNA*), vascular endothelial growth factor (*VEGF*) and *FAS* mRNA expression.

Materials and Methods

Patients. Fresh cancer samples were obtained from 81 patients with colorectal cancer during surgeries between 2007 and 2008 and immediately placed in liquid nitrogen at Tangshan Gongren Hospital. The adjacent non-cancerous mucosa specimens 2 cm away from tumors, and normal mucosal specimens of proximal and distal margins were collected at the same time. All the patients gave signed informed consent. None of the patients had received preoperative radiotherapy or chemotherapy. The patient's gender, age, tumor location, histological type, grade of differentiation, invasive depth, and lymph node metastasis were obtained from surgical and pathological records. Patients with incomplete clinical records were excluded from certain analyses. The mean age of the patients was 57.6 years (range from 31-90 years). The histology of the tumors was as follows: non-mucinous adenocarcinoma (n=51), mucinous/ signet-ring cell carcinomas (n=24). Tumors were graded as well (n=5), moderately (n=53) and poorly (n=20) differentiated. All slides of normal mucosa specimens and tumors were examined by two pathologists (Z.Y. Zhang and Y. M. Hu). In addition, cases including primary tumors, adjacent noncancerous mucosa specimens and normal mucosal specimens of proximal and distal margins were chosen randomly for the detection for *PCNA* mRNA (n=24), *FAS* mRNA (n=24) and *VEGF* mRNA (n=29).

Total RNA extraction. Total RNA was extracted from tissue samples using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Fresh tissue was homogenized in 1 ml TRIzol to isolate total RNA.

Reverse transcription-polymerase chain reaction. Total RNA (2 μg) was reverse transcribed into cDNA using the M-MLV Reverse Transcription System (Promega, San Luis Obispo, CA, USA). A DNA-free template control (containing water) was included and each sample was reverse transcribed in duplicate. To quantify the expression of the target gene, 10 μl samples of PCR products were separated by 1.5% agarose gel electrophoresis and quantified by Gel Imaging System (TFP-M/WL, Vilber Lourmat, France) after ethidium bromide (10 mg/l) staining. The mRNA expression was estimated by the densitometric ratio of the target gene to β-actin. The primers for *PINCH, PCNA, FAS* and *VEGF*, and the conditions of RT-PCR are shown in Table I.

Statistical analysis. All data are presented as mean±SD and analyzed by SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Unpaired and paired Student's *t*-test and one-way ANOVA were used to test the relationship between *PINCH* mRNA expression and other clinicopathological variables. Pearson's correlation test analyzed the relationship between *PINCH* mRNA expression and *PCNA, FAS* and *VEGF* mRNA expressions. A *p*-value <0.05 was considered as statistically significant.

Table I. *Primers and thermal cycle conditions used (n=35 cycles).*

Gene		Primer sequence (5'-3')	Product (bp)
β-*Actin*	Forward	GGAAATCGTGCGTGACATTA	378
	Reverse	GGAGCAATGATCTTGATCTTC	
PINCH	Forward	TGTGCCAAGTGTGAGAAACC	199
	Reverse	GCAGG TAGAACAGGCAAAGC	
PCNA	Forward	CTTTTCTGTCACCAAATTTGTACC	206
	Reverse	AACTGCATTTAGAGTCAAGACCC	
VEGF	Forward	GGCAGAAGGAGGAGGGCAGA	246
	Reverse	CCTATGTGCTGGCCTTGGTGAG	
FAS	Forward	CTGCCAAGAAGGGAAGGAGT	189
	Reverse	GGTGCAAGGGTCACAGTGTT	

Step	Temperature	Time (min)
Reverse transcription	42˚C	60
Initial PCR activation step	95˚C	
PCR amplification		
Denaturation	95˚C	0.5
Annealing	52˚C	0.5
Extension	72˚C	1
Final extension	72˚C	10

Results

The expression of PINCH mRNA in primary tumor, adjacent non-cancerous mucosa, and distant normal mucosa of proximal and distal margins. The expression of *PINCH* mRNA in primary tumors was notably higher than its expression in adjacent non-cancerous mucosa, and distant normal mucosa of proximal and distal margins (all $p<0.0001$, Figure 1A and B). Similar results were obtained by electrophoresis and densitometric analysis. As shown in Figure 1A, *PINCH* signal in the primary tumor lane was significantly stronger than in lanes loaded with PCR products from the adjacent noncancerous tissue or the proximal and distant margin of normal mucosa. There was no significant difference of *PINCH* mRNA expression between adjacent non-cancerous mucosa and distant normal mucosa of proximal or distal margins ($p>0.05$, Figure 1B).

The relationship between PINCH mRNA expression in primary tumor and other clinicopathological variables. As shown in Table II, the expression of *PINCH* mRNA in colon carcinomas tended to be higher than that in rectal carcinomas (0.816±0.51 *vs*. 0.635±0.293, $p=0.051$). Tumors infiltrating through the wall of the colorectum tended to have higher *PINCH* mRNA expression (0.554±0.324 *vs*. 0.747±0.417, $p=0.073$). There was no correlation between *PINCH* mRNA

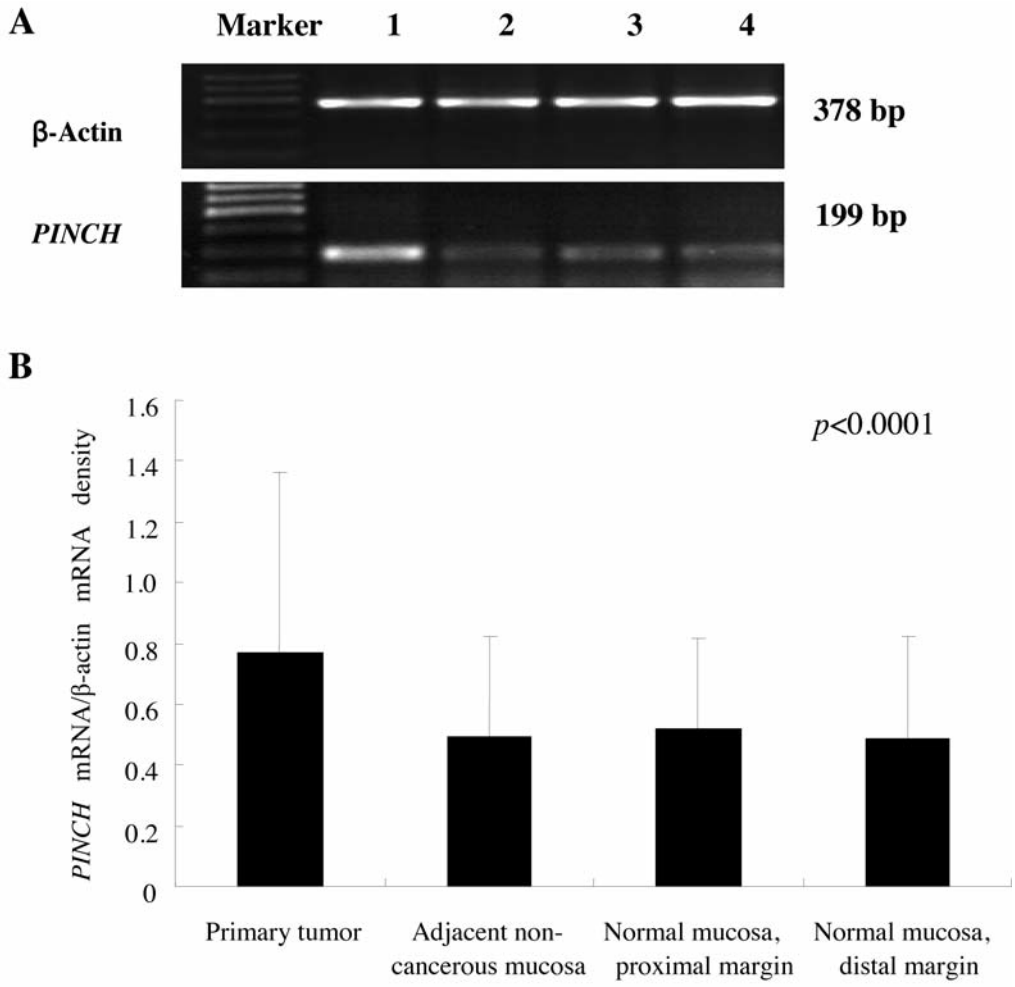

Figure 1. *RT-PCR of PINCH mRNA expression in primary tumor, adjacent noncancerous mucosa, and proximal and distant margins of normal mucosa. A: Lane M, 1, 2, 3 and 4 were marker, tumor, adjacent noncancerous mucosa, proximal, and distant margins of normal mucosa, respectively. β-Actin (378 bp) was used as control. B: Quantfication of PINCH (199 bp) (A) mRNA expression from cancer was stronger than that from adjacent noncancerous mucosa, and that from the proximal and distant margins of normal mucosa.*

expression and other clinicopathological variables including gender (p=0.319), age (p=0.823), histological type (p=0.727), tumor differentiation (p=0. 924), lymph node metastasis (p=0.569) and Dukes' stage (p=0.171).

The relationship between the mRNA expressions of PINCH, and PCNA, VEGF and FAS in colorectal cancer. The mRNA expressions of *PCNA, VEGF* and *FAS* in primary tumors was higher than that in the adjacent non-cancerous mucosa, and distant normal mucosa of proximal and distal margins (p<0.0001) (Figure 2). *PINCH* mRNA expression was positively related to the expression of *PCNA* mRNA (r=0.534, p=0.01), *VEGF* mRNA (r=0.431, p=0.022) and FAS mRNA (r=0.542, p=0.012, Figure 3). There was no significant difference of *PCNA, VEGF* and *FAS* mRNA expressions between adjacent non-cancerous mucosa and distant normal mucosa of proximal and distal margins (p>0.05, data not shown).

Discussion

It has been widely accepted that the initiation and progression of tumors are influenced by the behavior of tumor microenvironments comprising the extracellular matrix (ECM), the newly formed vasculature, inflammatory cells and fibroblasts (8, 9). Tumor-associated fibroblasts (activated fibroblasts and myofibroblasts) have a well-recognized role as a source of paracrine (cell to cell) growth factors that influence the growth, migration and invasion of carcinoma cells in the carcinogenic process. Activated fibroblasts are responsible for the synthesis, deposition and remodeling of ECM in tumor stroma (8). *PINCH* is

Table II. *PINCH mRNA expression in relation to clinicopathological variables of colorectal cancer.*

Variables	n	Mean±SD	T(F)-Value	*p*-Value
Gender			1.002	0.319
Male	49	0.678±0.378		
Female	31	0.772±0.459		
Age (years)			0.225	0.823
<60	29	0.720±0.429		
≥60	50	0.699±0.404		
Tumor location			1.985	0.051
Colon	35	0.816±0.51		
Rectum	45	0.635±0.293		
Histological type			0.351	0.727
Non-mucinous	51	0.682±0.349		
Mucinous/signet-ring cell	24	0.716±0.445		
Differentiation			0.079	0. 924
Well	5	0.634±0.39		
Moderately	53	0.689±0.445		
Poor	20	0.714±0.259		
Invasive depth			1.814	0.073
Mucosa+muscularis	16	0.554±0.324		
Subserosa or beyond	63	0.747±0.417		
Lymph node metastasis			0.572	0.569
No	41	0.689±0.407		
Yes	39	0.742±0.419		
Dukes' stage			1.383	0.171
A+B+C	72	0.736±0.424		
D	8	0.525±0.200		

expressed in activated fibroblasts and myofibroblasts and has been implicated as a 'molecular switch' for multiple protein-protein interactions mediating integrin signals within focal adhesions (3, 10). PINCH protein expression was found to be up-regulated in the tumor-associated stroma in a variety of human cancer types compared with the corresponding normal tissues (6). PINCH protein expression was higher in oral squamous cell carcinomas, esophageal squamous cell carcinomas, gliomas and colorectal cancer than that in the normal tissues in our previous studies (7, 11-14). In the present study, the *PINCH* mRNA was also overexpressed in primary tumor compared with that in the adjacent non-cancerous mucosa, and distant normal mucosa of proximal and distal margins. It seems that *PINCH* mRNA is also involved in the tumorigenesis of colorectal cancer.

In the present study, *PINCH* mRNA expression tended to be higher in cases where the tumor cells infiltrated throughout the wall. The infiltrative growth pattern is associated with recurrence and unfavorable prognosis in colorectal cancer. This demonstrates that *PINCH* mRNA appears to be related to aggressive features during the development of colorectal cancer. The expression of *PINCH* mRNA was somewhat related to the location of primary tumor in the present study: tumors in the colon trended to have higher *PINCH* mRNA expression. Different genetic abnormalities have been found in colorectal carcinomas from different sites. Colon cancer and rectal cancer are different entities with molecular and biological differences. The relationship between the expression of *PINCH* mRNA and tumor location lends further evidence to this. A family history of colorectal cancer appeared to affect relative risk of colon cancer more strongly than relative risk of rectal cancer (15). Rectal carcinomas exhibited significantly more nuclear β-catenin than colon carcinomas, which is a critical mediator of the WNT signaling pathway negatively regulated by *adenomatous polyposis coli* (APC) (16). Additionally, compared with colon cancer, rectal cancer is less KRAS-dependent and the APC gene-restricting pattern is more common (17). Therefore, the APC/catenin pathway in rectal cancer was suggested as the predominant one. Huang *et al*. found SW480 colon cancer cells to overexpress the gene encoding thymosin β4 (Tβ4), which was accompanied by a loss of E-cadherin, as well as a cytosolic accumulation of β-catenin, two most prominent markers of epithelial mesenchymal transition (EMT). Integrin-linked kinase (ILK) up-regulation in Tβ4- overexpressing SW480 cells seemed to be attributed mainly to a stabilization of this kinase by complexing with *PINCH* more efficiently (18). In this context, we suggest that the expression of *PINCH* mRNA in the rectum might indirectly be regulated by carcinogenetic pathway, *e.g*. by APC/WNT pathway, differently from that in the colon. However, more experimental evidence is required for this.

PINCH belongs to a family of cell ECM adhesion proteins involved in regulating cellular proliferation, differentiation and survival by interaction with ILK, participating in integrin-mediated intracellular and growth factor signaling pathways. Moreover, recent studies have raised the concept of the co-evolution of tumor cells with tumor-associated stroma. The stromal environment of tumors appears to be a leading factor, and not just a supporting one in the initiation of tumors (19). The tumor microenvironment and interactions between tumor and stromal cells have a reciprocal relationship in tumor development and progression. EMT is essential for normal embryonic development, but also for progression of non-invasive adenomas into malignant, metastatic carcinomas. Alterations in cell-cell adhesion, cell-substrate interaction, ECM degradation and cytoskeleton organization are major events that occur during EMT (20). In one of our previous studies, endosialin overexpressed on fibroblasts in the tumor-associated stroma in colorectal cancers as well as PINCH protein overexpressed on the fibroblasts in the stroma. But we found a few cases with endosialin expressed on tumor cells. This hinted the interactions of endosialin between the tumor microenvironment and tumors (21). Thus, we further hypothesize that *PINCH* might play a role in the EMT in the rectum different from that in the colon through the WNT and

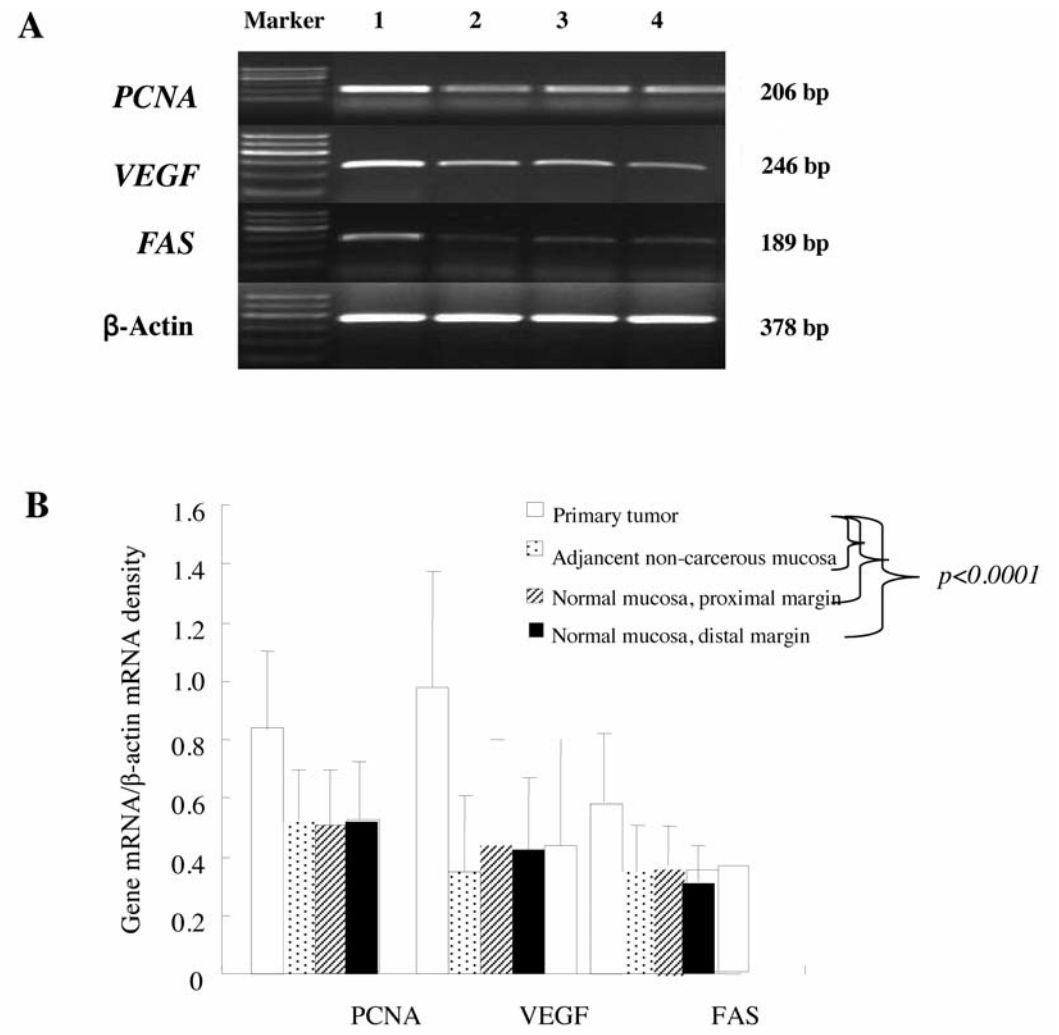

Figure 2. *RT-PCR of PCNA, VEGF and FAS mRNA expression in primary tumor, adjacent noncancerous mucosa, and proximal and distant margins of normal mucosa. A: Lane M, 1, 2, 3 and 4 were marker, tumor, adjacent noncancerous mucosa, and proximal and distant margins of normal mucosa, respectively. β-Actin (378 bp) was used as control. B: Quantfication of PCNA (206 bp) (A), VEGF (246 bp) and FAS (189 bp) mRNA expression from cancer was stronger than that of adjacent noncancerous mucosa, and that of the proximal and distant margins of normal mucosa.*

APC pathway. If therapies target on *PINCH*, they might block the signal transduction both in stroma and tumor cells, providing more efficient strategies for therapy of patients with rectal cancer.

It has been determined that poor differentiation, abundant necrosis, and increased *PCNA* and *VEGF* expression represent a more malignant potential of tumors (22). There was also a positive relationship found between *PCNA* and apoptosis (23). *FAS* is an important receptor to induce apoptosis, activated by *FAS* ligand in the target cell surface. *FAS*/*FASL* are closely related to the infiltration and metastasis of tumor. *VEGF* is highly expressed in several types of human solid tumors including colorectal, breast, lung and prostate carcinoma through improved tumor neovascularity and the proliferation of tumor cells (24). It has been shown *in vitro* and *in vivo* that ILK is implicated in the promotion of tumor angiogenesis by stimulating *VEGF* expression (25).

PINCH expression was particularly intense in stromal cells at invasive edges that can facilitate cancer invasion (6, 13). In previous study, endothelial cells that stained for CD31 co-stained for *PINCH*, indicating that some endothelial cells in the tumor vasculature expresses *PINCH* protein (13). We showed that strong expression of *PINCH* was associated with high microvessel density in stroma of colorectal tumors (26). All these data demonstrate that *PINCH* seems to be involved in angiogenesis through activation of fibroblasts in the ECM in response to tumors. In

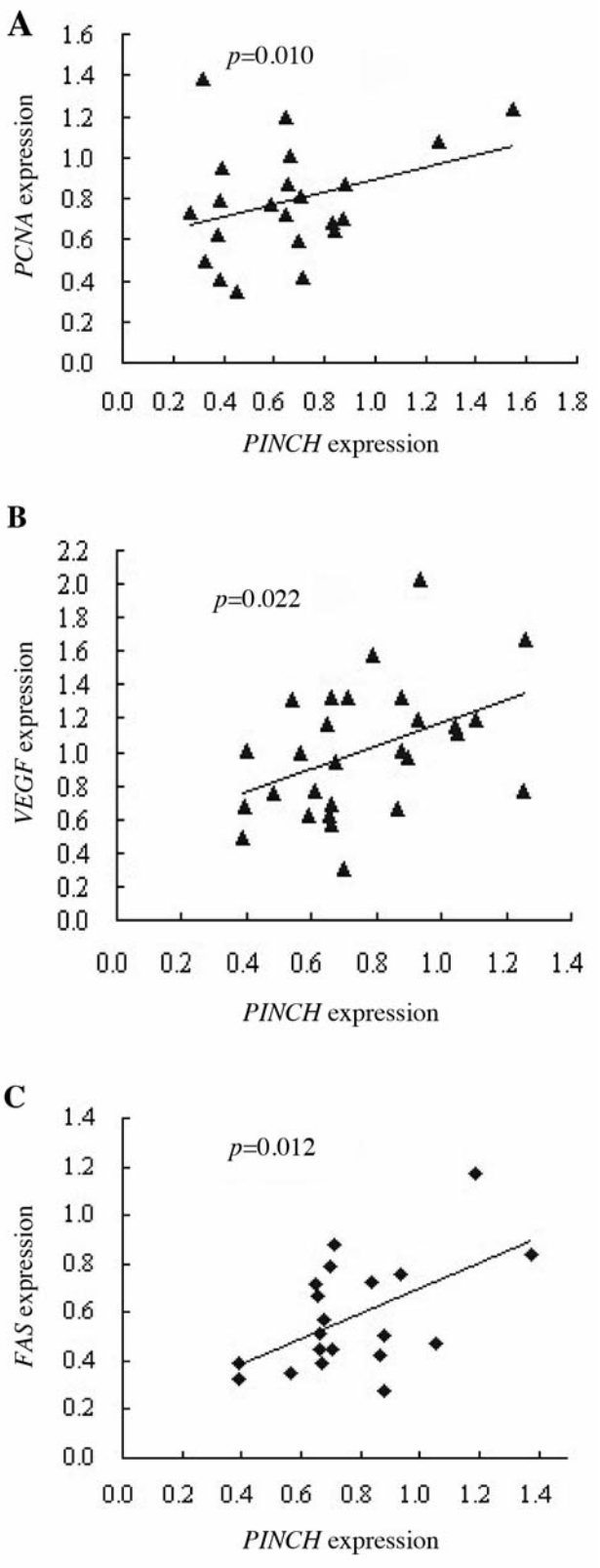

Figure 3. *The relationship between mRNA expressions of PINCH and PCNA, VEGF and FAS. PINCH mRNA expression was positively related to PCNA (A), VEGF (B) and FAS(C) mRNA expressions.*

the present study, *PINCH* mRNA overexpression in primary tumors was positively related to *PCNA, VEGF* and *FAS* mRNA expression, indicating *PINCH* might participate in tumor development associated with the regulation of proliferation, angiogenesis and apoptosis.

Conclusion

Our findings suggest *PINCH* mRNA expression may play a role in the EMT in the rectum different from in the colon through the APC/catenin pathway.

Competing Interests

The Authors declare that they have no competing interests.

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