

RAPID COMMUNICATION

## Particularly interesting new cysteine-histidine rich protein expression in colorectal adenocarcinomas

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**CONCLUSION:** The expression of PINCH was upregulated in colorectal cancers, and especially at the margin of tumours, and further was related to mucinous and signet-ring cell carcinomas. The results suggest that expression of PINCH may be involved in the tumourigenesis and aggressiveness of colorectal cancers.

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**Key words:** PINCH; Stroma; Colorectal cancer; Immunohistochemistry

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### Abstract

**AIM:** To study the relationship between particularly interesting new cysteine-histidine rich protein (PINCH) expression and clinicopathological factors in Chinese colorectal cancer patients.

**METHODS:** The expression of PINCH was examined by immunohistochemistry in 141 samples of primary colorectal adenocarcinoma and 92 normal samples of colorectal mucosa. Eighty of the cases had both primary tumour and normal mucosa from the same patients.

**RESULTS:** PINCH was expressed in the stroma of normal mucosa and tumours. PINCH expression in tumour-associated stroma was increased compared to normal mucosa in both unmatched cases ( $n = 141$ ,  $X^2 = 85.79$ ,  $df = 3$ ,  $P < 0.0001$ ) and matched cases ( $n = 80$ ,  $X^2 = 45.86$ ,  $df = 3$ ,  $P < 0.0001$ ). Among 135 tumours with visible invasive margin, 86 (64%) showed stronger PINCH expression at the invasive margin than in the intratumoural stroma. The frequency of PINCH strong expression in mucinous and signet-ring cell carcinomas was higher (52%) compared to non-mucinous carcinomas (29%,  $X^2 = 5.13$ ,  $P = 0.02$ ). We did not find that PINCH expression was related to patient's gender, age, tumour location, tumour size, gross status, histological type, differentiation, invasion depth, lymph node status and Dukes' stage ( $P > 0.05$ ).

### INTRODUCTION

Studies have shown the importance of stromal tissue in regulating the physiological processes of the body. Disruption of stromal-epithelial interactions and cell adhesion alters cellular signalling, which influences proliferation, angiogenesis, differentiation, motility, death, genomic integrity and other phenotype in the cells and tissues<sup>[1-3]</sup>. Extra-cellular matrix (ECM) adhesion is a fundamental process that controls a variety of cellular processes including cell shape changes and migration. Cell-ECM interactions are mediated by a selective group of membrane and cytoplasmic proteins at the ECM contact sites. Integrin-linked kinase (ILK) is a multidomain protein that plays an important role at ECM adhesion sites<sup>[2-7]</sup>.

PINCH is a LIM domain adapter protein. The PINCH gene is located on chromosome 2q12.2. PINCH and ILK components function as an adaptor protein connecting the growth factor-signalling pathways with the integrin-signalling pathway. It is found in focal adhesions, large cellular complexes that link extracellular matrix to the actin cytoskeleton interacting with ILK and Nck2. PINCH has been implicated as a platform for multiple protein-protein interactions mediating integrin signalling within focal adhesions<sup>[4,7-11]</sup>.

In the present study, the expression of PINCH was immunohistochemically studied in 141 samples of primary colorectal adenocarcinoma and 92 normal mucosa samples. The aims were to investigate the expression of PINCH in

normal mucosa and primary tumours in Chinese colorectal cancer patients and to identify the relationship between PINCH expression and clinicopathological variables including patient's gender, age, tumour location, tumour size, gross status, histological type, grade of differentiation, invasive depth, metastasis in the lymph nodes and Dukes' stages.

## MATERIALS AND METHODS

### Patients

Tumour samples were collected from 141 patients with colorectal adenocarcinoma diagnosed at Department of Pathology, Tangshan Worker's Hospital, China, between 2000 and 2002. The study also included 92 normal mucosa samples, 80 of which were matched with the tumours, i.e., from the same patients. Normal samples were taken from the margin of the distant resection being histologically free from pre-tumour and tumour. None of the patients received preoperative radiotherapy or chemotherapy. The patient's gender, age, tumour location, tumour size, gross status, histological type, grade of differentiation, invasive depth, lymph node status and Dukes' stages were obtained from surgical and pathological records. The mean age of the patients was 56 years (range, 30–80 years). Tumours of the caecum, ascending and transverse colon were defined as proximal tumours and tumours of the descending and sigmoid colon and rectum were defined as distal tumours. The mean tumour size was 4.9 cm (range, 0.8–20 cm). Tumours were graded as better (well + moderate) and worse (poor) differentiation. All samples were examined by two pathologists.

### Immunohistological staining and evaluation

The preparation, specificity and reliability of the rabbit polyclonal PINCH antibody (obtained kindly from Professor Ann Rearden, Department of Pathology, University of California, La Jolla, CA) used in this study have been described previously [10, 11].

The paraffin-embedded tissue sections (5 µm) were deparaffinised in xylene and rehydrated in graded ethanol. In order to expose masked epitopes, the sections were boiled in 0.01 mol/L Tris-EDTA buffer (pH 9.0) in a high pressure-cooker, kept at room temperature for 30 min and washed with phosphate-buffered saline (PBS, pH 7.4). The activity of endogenous peroxidase was blocked in 0.5% H<sub>2</sub>O<sub>2</sub> in methanol for 10 min and washed with PBS. The sections were incubated with the 2 µg/mL primary PINCH antibody at 4 °C overnight and washed with PBS. The sections were incubated with polymer enhancer for 20 min, then with polymerized HRP-anti mouse/rabbit IgG antibody for 30 min and rinsed in PBS between the incubation steps. After washed with PBS, peroxidase reaction was performed by use of 3,3'-diaminobenzidine for 5 min [Elivision TM plus polyer HRP (mouse/rabbit) IHC kit, Fuzhon Maixin Biology Technology Limited Company, Fuzhou, China]. After counterstained with haematoxylin, the sections were dehydrated and mounted. Normal mucosa and the matched primary tumours were stained in the same run of immunostaining to avoid bias on the pattern and intensity of the staining. Sections known to show

**Table 1 PINCH expression in relation to clinicopathological variables in colorectal cancers**

Variables	<i>n</i>	Strong PINCH <i>n</i> (%)	Weak PINCH <i>n</i> (%)	<i>P</i>
Gender				0.67
Male	71	23 (32)	48 (68)	
Female	70	25 (36)	45 (64)	
Age (years)				0.77
≤56	70	23 (33)	47 (67)	
>56	71	25 (35)	46 (65)	
Tumour location				0.61
Proximal	42	13 (31)	29 (69)	
Distal	99	35 (35)	64 (65)	
Tumour size (cm)				0.64
≤4.9	86	28 (33)	58 (67)	
>4.9	55	20 (36)	35 (64)	
Gross status				0.60
Ulcerated	108	38 (35)	70 (65)	
Polypoid/Lager fungating	33	10 (30)	23 (70)	
Histological type				0.02
Non-mucinous	117	34 (29)	83 (71)	
Mucinous/signet-ring cell	27	14 (52)	13 (48)	
Grade of differentiation				0.59
Better	99	33 (33)	66 (67)	
Worse	42	16 (38)	26 (62)	
Lymph node status				0.11
Non-metastasis	100	30 (30)	70 (70)	
Metastasis	41	18 (44)	23 (56)	
Invasive depth				0.48
Intra-bowel wall	105	34 (32)	71 (68)	
Ultra-bowel wall	36	14 (39)	22 (61)	
Dukes' stage				0.17
A	52	12 (23)	40 (77)	
B	51	19 (37)	32 (63)	
C	34	15 (44)	19 (56)	
D	4	2 (50)	2 (50)	

strong immunostaining for PINCH were used in each run receiving either the primary antibody or PBS as positive and negative controls. In all the staining procedures, the positive controls showed staining clearly and there was no staining in the negative controls.

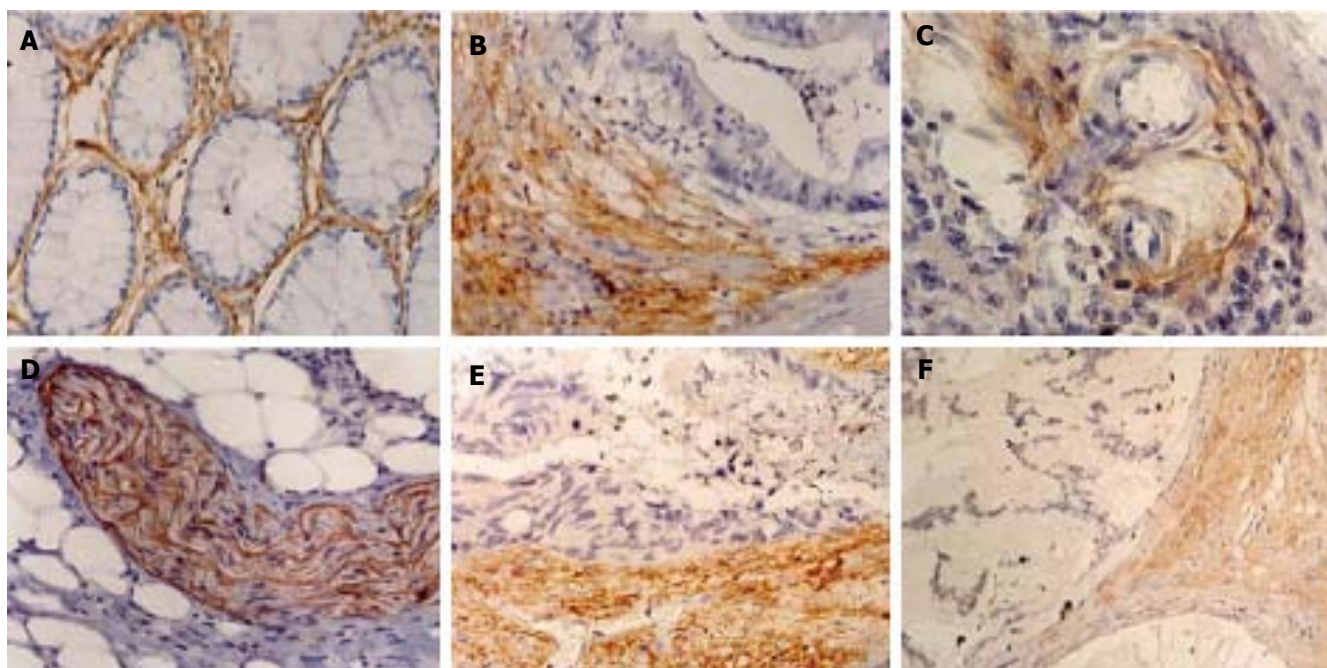
PINCH immunostaining was independently examined by two pathologists in a blinded fashion without knowledge of the clinical and pathological information. To avoid artificial effect, the staining on the margins of sections and areas of poorly presented morphology were not counted. The intensity of PINCH staining in stroma was graded as negative (no positive cells), weak (<5% positive cells), moderate or strong staining. In cases with discrepant results, a consensus score was reached after re-examination.

### Statistical analysis

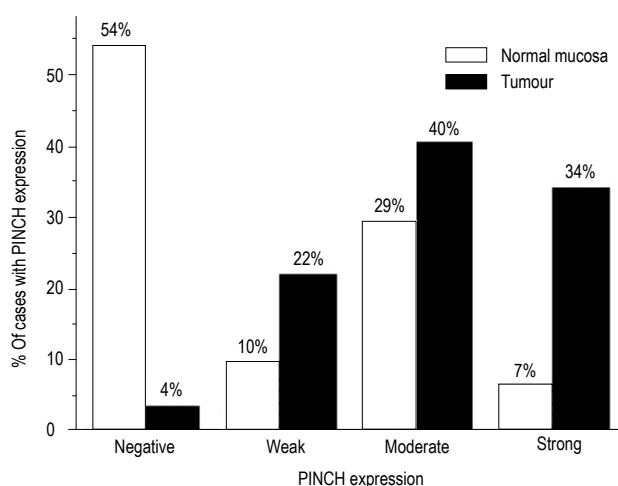
The chi square method was used to test the relationship between the frequencies of PINCH expression in normal mucosa and tumour as well as between PINCH expression in tumour and clinicopathological variables. All *P* values cited were two-sided and *P*<0.05 was considered statistically significant.

## RESULTS

PINCH was expressed in the stroma in both normal



**Figure 1** PINCH immunohistochemical staining in normal mucosa (A), adenocarcinoma (B), tunica adventitia of blood vessel (C), peripheral nerve fascicles (D) and stronger expression of PINCH at the invasion edge than in the inner area (E), and at the invasion edge of mucinous carcinoma (F).



**Figure 2** Frequency of PINCH immunohistochemical staining in 92 normal mucosa and 141 tumour samples.

mucosa and tumour samples, mainly in cytoplasm of fibroblasts and myofibroblasts, a proportion of endothelial cells in the tumour vasculature and peripheral nerves (Figure 1). Among 135 tumours with visible margin, 86 (64%) showed stronger PINCH expression at the invasive edges than in the intratumoural stroma, 31 (23%) showed opposite evidence, the remained 18 (13%) showed the same staining intensity. There was no PINCH expression in normal epithelial and tumour cells (Figure 1).

PINCH expression was negative in 50 (54%), weak in 9 (10%), moderate in 27 (29%) and strong in 6 (7%) in 92 normal mucosa samples, and in 5 (4%), 31 (22%), 57 (40%) and 48 (34%) respectively in 141 tumour samples. The expression was significantly increased in tumour samples compared to normal mucosa samples ( $\chi^2 = 85.79$ ,  $df = 3$ ,

$P < 0.0001$ , Figure 2). Even in the 80 matched cases of normal mucosa samples (51%, 10%, 33% and 6%) and tumours (5%, 20%, 43% and 33%), the significance was still remained ( $\chi^2 = 45.86$ ,  $df = 3$ ,  $P < 0.0001$ ).

Since the clinicopathological features of tumours with negative, weak and moderate staining were similar, they were combined as one group (weak group) to compare with strong group in statistical analyses. Table 1 summarises the expression of PINCH in relation to patient's gender, age, tumour location, tumour size, gross status, histological type, grade of differentiation, invasive depth, lymph node status and Dukes' stage. The result showed that the frequency of strong PINCH expression was higher in mucinous/signet-ring cell carcinomas (52%) than in non-mucinous carcinomas (29%,  $\chi^2 = 5.13$ ,  $P = 0.02$ , Table 1). Figure 1 shows a mucinous carcinoma with PINCH expression. The frequency of strong PINCH expression in Dukes C+C+D tumour (45%) tended to be higher than that in Dukes' A + B tumour (30%,  $\chi^2 = 2.65$ ,  $P = 0.10$ ). It was also shown that patients with lymph node metastasis seemed to have higher PINCH expression (44%) than those without metastasis (30%,  $\chi^2 = 2.50$ ,  $P = 0.11$ ). We did not find other relationships between the expression of PINCH and clinico-pathological variables ( $P > 0.05$ , Table 1).

## DISCUSSION

In the present study, we observed that PINCH presented in fibroblasts, myofibroblasts, a proportion of endothelial cells of the tumour vasculature and peripheral nerves. The expression of PINCH was especially strong in stroma at the invasive edges of tumours compared to the intratumoural stroma, suggesting that PINCH as a biological factor, may be involved in the angiogenesis and invasiveness of tumour. This evidence may partly



explain why strong PINCH expression is associated with a poor prognosis in colorectal cancer patients<sup>[12]</sup>. In the present study, the frequency of strong PINCH expression was significantly higher in mucinous and signet-ring cell carcinomas than in non-mucinous carcinomas, which may also explain why PINCH expression is related to a poor clinical outcome. Studies demonstrated that patients with mucinous colorectal carcinomas have a worse prognosis than those with non-mucinous carcinomas<sup>[13-15]</sup>, indicating that mucins interfere with immunologic recognition of tumour cells by masking antigenic epitopes with sialic acid residues and inhibiting lymphocyte infiltration<sup>[16]</sup>. We have previously reported that there is less inflammatory infiltration in colorectal cancer with strong PINCH expression<sup>[12]</sup>.

PINCH is directly associated with ILK and Nck-2 proteins that are downstream effectors of integrin and growth factor signalling. Some of these growth factors, such as PDGF-mediated tumour-stromal interactions are important to tumour growth. PINCH is required for ILK localisation to integrin-containing adherens junctions where ILK regulates fibronectin matrix assembly<sup>[4, 6, 7, 9-12]</sup>, suggesting that PINCH protein may increase the upregulated growth factor signalling in stromal cells and a marker for stroma angiogenesis and invasion of tumour cells.

PINCH protein is involved in integrin-mediated cell-ECM interactions, where the different mechanisms or different genetic pathways may develop different histological types of tumour by specific classes of carcinogens. In this context, the expression of PINCH may be associated with the phenotype of epithelial cells in the colorectum. The frequency of K-ras mutation and microsatellite instability is higher in mucinous carcinomas than in non-mucinous carcinomas<sup>[17-20]</sup>. In contrast, mucinous carcinomas exhibit significantly less p53 mutation and protein<sup>[20-23]</sup>. These results lead to the hypothesis that K-ras and microsatellite instability may influence mucus production or degradation, resulting in the development of mucinous carcinoma. In contrast to non-mucinous tumours, the development of mucinous carcinomas may be independent from p53 alteration. Thus, PINCH may be another factor involved in the development of mucinous carcinomas.

In conclusion, the expression of PINCH was upregulated in colorectal cancers, and especially at the margin of the tumours, and further was related to mucinous carcinomas. The results suggest that expression of PINCH may be involved in the tumorigenesis and aggressiveness of colorectal cancers.

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