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PINCH expression and its clinicopathological significance in gastric adenocarcinoma

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Abstract.

Objective: Particularly interesting new cysteine-histidine rich protein (PINCH) is an important component of the local adhesion complexes and upregulated in several types of malignancies, and involved in the incidence and development of tumours. PINCH expression is also independently correlated with poorer survival in patients with colorectal cancer. However, there is no study of PINCH in gastric cancer, therefore, the aim of this project was to investigate PINCH expression and its clinicopathological significance in gastric adenocarcinoma.

Patients and methods: PINCH expression was immunohistochemically examined in normal gastric mucous (n = 30) and gastric adenocarcinoma (n = 73), from gastric cancer patients.

Results: PINCH expression in the associated-stroma of gastric cancers was heterogeneous, and its positive rate (75%) was higher than that of normal gastric mucosa (43%, $x^2 = 9.711$, $p = 0.002$). The stronger staining was observed at the invasive edge of tumour when compared to the inner area of tumour. The rate of positive PINCH (88%) in the cases with lymph node metastasis was higher than that (52%) in the cases without metastasis ($x^2 = 11.151$, $p = 0.001$). PINCH expression was not correlated with patients' gender, age, tumour size, differentiation and invasion depth ($p>0.05$).

Comclusion: PINCH protein might play an important role in the tumourigenesis and metastasis of gastric adenocarcinoma.

Key words: PINCH, gastric adenocarcinoma, metastasis, immunohistochemistry.

1. Introduction

Gastric cancer remains one of the most poorly controlled malignancies in the People Republic of China and is the first leading cause of cancer-related deaths. Adenocarcinoma is the most common type of the stomach cancers. Surgical resection remains the first treatment. Yet, nearly 80% of surgically resected patients with advanced stomach cancer die as a result of recurrent or metastatic disease within 5 years [1]. Therefor, it is necessary to investigate the mechanism of tumourigenesis and metastasis of gastric adenocarcinoma.

Recent studies have suggested that an important role for the interaction of tumour cells and tumour-associated stroma cells in the regulation of tumourigenesis, progression and metastasis of tumours [2]. The adhesion of cells to the extracellular matrix (ECM) is essential for cell migration, division and cell-cell signalling. Variations in this interaction between the tumour and surrounding tissues may facilitate the malignant transformation and invasion of tumour cells [3]. Particularly interesting new cysteine-histidine rich protein (PINCH) is a newly discovered adapter protein, which links the ECM to the support structures within the cell and is expressed in many human tissues, and consists primarly of five LIM (double zinc finger) domains. The PINCH gene is located on chromosome 2q12.2, and the PINCH protein can interact directly with integrin-linked kinase (ILK) and Nck-2 protein, ILK is involved in the intergrin signaling and associated with the first LIM domain of PINCH, while Nck-2 is involved in growth factor signaling and associated with the forth LIM domain of PINCH, so the PINCH is associated with integrin signaling and growth factor signaling, and is regarded as a key convergence point [4-6].

It is shown that PINCH mRNA is expressed in most types of normal tissues, and located in the cytoplasm and cell matrix adherens junction [6]. PINCH expression has

been shown to be upregulated in many types of malignancies, including cancers in the head-neck, skin, breast, lung, prostate, esophagus, colon, rectum, and pancreas. In these tumours PINCH expression localizes to the peri-tumoural stroma cells, particularly at the tumour's invasive edges, a region where signaling in the integrin and growth factor pathways is known to occur. And in further studies on the clinicopathological significance of PINCH expression in the series of the patients with those different types of malignant tumours, the PINCH expression has shown to be increased in high-grade colorectal cancer and in oral squamous cell carcinoma with lymph node metastasis, and predict worse survial in the patients with colorectal cancer independently of tumour stage, growth pattern and differentiation [3,7-12]. However, to our knowledge, no study has been performed in gastric adenocarcinoma yet, therefore, in the present study, we examined PINCH protein expression in normal gastric mucosa and gastric adenocarcinoma, and further to analyse the relationship of PINCH expression in gastric adenocarcinoma with clinicopathological variables including patients' gender, age, tumour size, invasion depth, lymph node status and grade of differentiation.

2. Materials and methods

2.1. Materials

Formalin fixed paraffin-embedded tissue samples were obtained from 73 gastric adenocarcinoma patients who underwent surgical resection at the First Hospital of Hebei Medical University (Shijiazhuang, Hebei Province, China), during 2002-2006. The study included 30 distant normal mucosa specimens which were matched with the primary tumors taken from the margin of distant resection. Among the primary tumours, 48 cases had lymph node metastasis. The patients' gender, age, tumour size, invasion depth, lymph node status and differentiation were obtained from surgical and/or pathological records at the hospital. The mean age of the patients was 63 years old (range 34-79 years). According to the WHO classification [13], tumour differentiation was graded as grade I (high differentiation: 17 cases), grade II (moderate differentiation: 20 cases) and grade III (low differentiation: 36 cases). In statistical analysis, we considered grade I and grade II as well differentiated group, and grade III as poorly differentiated group. All pathological slides including normal specimens and tumours were confirmed by two pathologists (Zhu ZL and Wang ZM).

2.2. Immunohistological staining and evaluation

The preparation, specificity and reliability of the rabbit polyclonal PINCH antibody used in the study were described previously [7,14]. Five-um sections from paraffin-embedded tissue blocks were deparaffinized, hydrated and rinsed in distilled

H_2O. In order to expose masked epitopes, the sections were boiled in citrate buffer (pH 9.0) in a high pressure cooker for 20 min, and then kept at room temperature for 30 min, followed by phosphate-buffered saline (PBS, pH 7.4) wash. The activity of endogenous peroxidase was blocked in 3% H_2O_2 in methanol for 10 min, and then the sections were washed three times in PBS. After blocking with 1.5% horse serum in PBS for 10 min, the sections were incubated with the primary PINCH antibody (a gift from Prof. Ann Rearden, Department of Pathology, University of California, CA) at 2μg/ml at 4°C overnight. Then, a biotinlated anti-rabbit IgG antibody (Fuzhou Maixim Biology Technology Limited Company, Fuzhou, Fujian Provence, China) was applied for 30 min followed by an incubation of an avidin-biotin-peroxidase complex (Fuzhou Maixim Biology Technology Limited Company) for 30 min. The sections were rinsed in PBS between the incubations. The peroxidase reaction was developed using diaminobenzidine (Beijing Zhongshan Biology Technology Limitted Company, Beijing, China) for 8 min. After counterstaining with hemotoxylin, the sections were dehydrated and mounted. The colorectal cancer sections known for positive PINCH were included as negative or positive controls. For negative controls, PBS were used instead of the primary antibody. In all runs, there was no staining in the negative controls, and the positive controls showed clear staining.

PINCH immunostaining was evaluated by two independent pathologists (Zhu ZL and Wang ZM) in a blind fashion without knowledge of any clinicopathological information. Cytoplasmic staining of fibroblasts and myofibroblasts in the stroma was considered PINCH positive. According to the percentage of positive staining, we graded PINCH expression as negative (no positive cells), weak (<20% positive cells), moderate (20-50% positive cells) and strong positive (>50% positive cells), irrespective of the staining intensity [12].

2.3. Statistical analysis

The statistical analyses were performed using SPSS version 11.5. The Chi-square test was used to examine the relationship of the frequencies of PINCH expression in normal gastric mucosa and gastric adenocarcinoma, and the relationship between PINCH expression in cancer and clinicopathological variables. All p-values cited were two-sided and p-values <5% were judged as statistically significant.

3. Results

3.1 PINCH expression in normal mucosa and primary tumour

We examined PINCH protein expression in normal gastric mucosa and gastric adenocarcinoma, and found that the expression of PINCH was in the cytoplasm of fibroblasts and myofibroblasts in the stroma, but not in the normal epithelial cells and tumour cells (Fig. 1A). Among 30 normal specimens, 17 cases was negative (57%), and 13 cases was positive, including 8 (27%) cases with weak, 3 (10%) moderate and only 2 (6%) strong staining, positive staining was more obviously in the stroma arounding the gastric gland neck and gastric pits (Fig. 1B). Among 73 cancers, there were 18 (25%) negative for PINCH, but there were 55 (75%) positive for PINCH, including 10 (14%) weak, 7 (9%) moderate and 38 (52%) strong expressed cases (Fig. 1C). The PINCH staining in tumour-associated stroma was heterogeneous. In addition, we also observed that PINCH expression was especially strong in the stroma at the invasive edges of 24 tumours compared to in the inner-tumour stroma (Fig. 1C).

In statistical analysis, taking into account similar clinicopathological features and facilitating statistical analysis, we considered negative as negative staining group, and weak, moderate and strong positive as positive staining group. In order to avoid artificial effects, cells on the margins of sections and areas with poorly presented morphology were not counted.

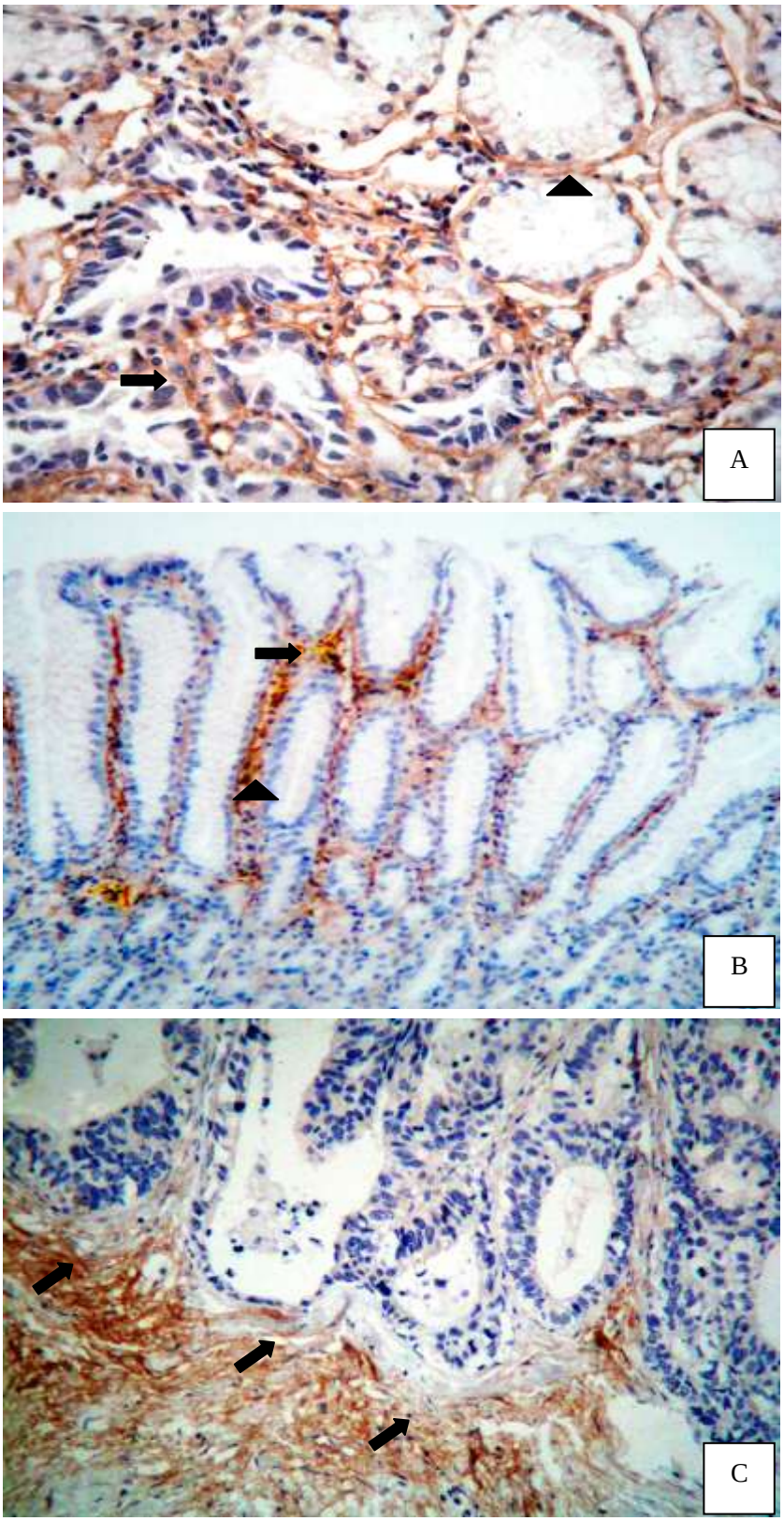

Fig. 1

As shown in Fig. 2 of PINCH expression in normal mucosa and cancer, the rate of positive expression in cancer was 75% (55/73), which was significantly higher than that in the normal mucosa, 43% (13/30, $X^2 = 9.711$, $p = 0.002$).

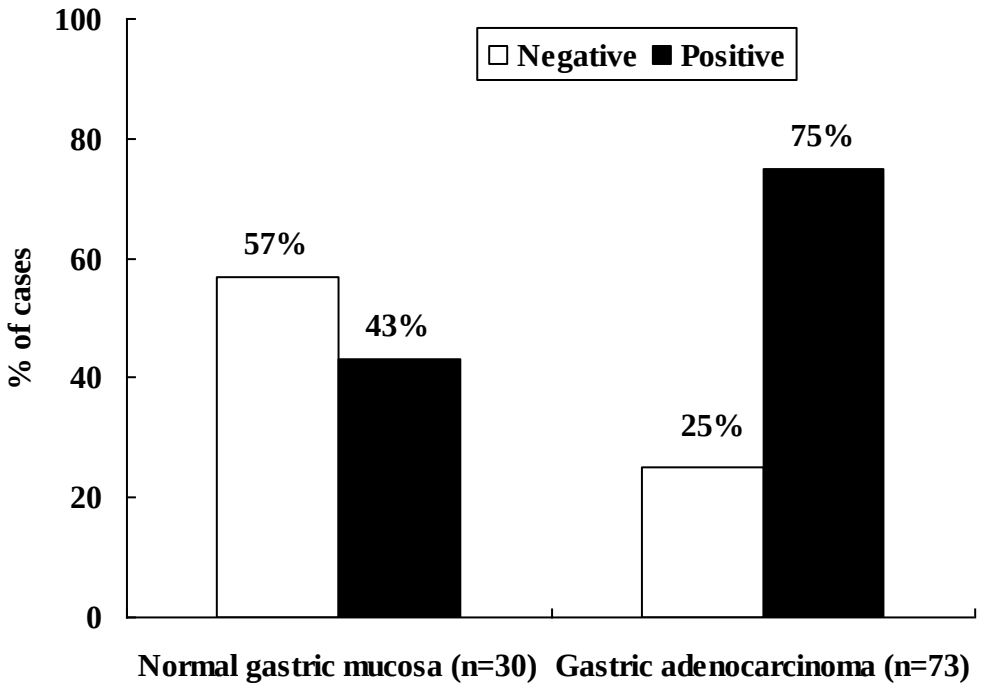

Fig. 2

Furthermore, we also observed the expression of PINCH in the endothelial cells, and found no any positive staining both in the normal mucosa and in the tumours.

3.2 PINCH protein expression in relation to clinicopathological variables

Table 1 shows the relationship between PINCH expressions in tumours with the clinicopathological variables. The cases with lymph node metastasis appeared a higher frequency of PINCH positive expression than those without metastasis in the lymph node (88% Vs. 52%, $X^2 = 11.151$, $p = 0.001$). PINCH expression was not

significantly correlated with patients' gender (p = 0.554), age (p = 0.154), tumour size (p = 0.451), differentiation (p = 0.308) and invasion depth (p = 0.300).

Table 1. The relationship between PINCH protein expression and clinicopathological variables in the patients with gastric adenocarcinoma

Variables	N	PINCH expression		X^2	P value
		Negative (%)	Positive (%)		
Gender					
Male	55	15 (27)	40 (73)	0.349	0.554
Female	18	3 (17)	15 (83)		
Age (years)					
⩽60	39	7 (18)	32 (82)	2.029	0.154
>60	34	11 (32)	23 (68)		
Tumour size (cm)					
⩽5	39	11 (28)	28 (72)	0.567	0.451
>5	34	7 (21)	27 (79)		
Differentiation					
Well	37	11 (28)	26 (70)	1.039	0.308
Poorly	36	7 (19)	29 (81)		
Lymph node status					
Non-metastasis	25	12 (48)	13 (52)	11.151	0.001
Metastasis	48	6 (12)	42 (88)		
Invasion depth					
Serosal	59	13 (22)	46 (78)	1.139	0.300
Mucosa and musal	14	5 (36)	9 (64)		

4. Discussion

The interaction between tumour and stroma has been recognized as an important factor influencing tumour growh and progression [15,16]. For a long time, the majority of cancer researchers have been focusing on alterations of genes and proteins in cancer cells themselves that result in either gain-of-function in oncogenes or loss-of-function in tumour-suppressor genes. However, in fact, stromal variables within or around tumours including stromal cells and various proteins have also important impacts on tumour development and progression. PINCH, identified in 1994 as an evolutionary conserved protein expressed in the tumour-associated stroma cells. The PINCH gene is located to chromosome 2q12.2 and encodes an adaptor protein belonging to the LIM family, consisting of five LIM domains [4].

Reviewing previous studies by our and other research groups on PINCH gene and its expression in tumours, Wang-Rodriquez et al. [7] first examined the expression of PINCH protein by immunohistochemistry in a series of cancers including breast, prostate, skin, lung and colorectal cancers. The results showed that, apart from skin tissue, the rest tumours expressed more PINCH than the corresponding normal tissues. Shortly after, this evidence has been conformed by our research group in oral and esophageal squamous cell carcinoma, colorectal cancer and gliomas, and a higher frequency of PINCH expression in the tumours than in the corresponding normal tissues [8-12, 17]. In the present study, of the 30 normal gastric mucosa, 13 (43%) had PINCH positive staining, and of the 73 primary tumours, 55 (75%) had positive staining. The frequency of the PINCH staining was showed to be significantly increased from normal mucosa to primary tumour. However, it is more significant that,

of 13 cases normal mucosa with PINCH staining, the site of the staining is mainly located in the stroma arounding the gastric gland neck and gastric pits where the glandular epithelial cells have more strong capacity of proliferation and repair, and can differentiate into gastric epithelial cells and intestinal epithelial cells. This result further demonstrate that PINCH may involved in specific protein-protein interactions and mediated essential cellular process including cellular proliferation, differentiation and migration [6]. Taken together, the observation by others of greater PINCH expression in the tumours, and our previous demonstration as well as the result in the present study, suggest that PINCH expression in the tumours may be an important role in the tumourigenesis.

In the present study, we observed the expression of PINCH in gastric adenocarcinoma and found that the PINCH staining in tumour-associated stroma was heterogeneous. In addition, we also found that PINCH expression of 24 tumours was stronger at the invasive edges than in inner area of tumours, and the cases with lymph node metastasis had a much higher frequency of PINCH positive staining than those without metastasis. These results support the idea that PINCH, through involvement in the tumour-stromal interaction, promotes tumour invasiveness [18].

In our previous reports, we not only found the similar pattern of PINCH strong expression at the invasive edges of different types of tumours (including oral and esophageal squamous cell carcinoma, colorectal cancer and glioma) when compared to the inner areas of the tumours, but also found that strong PINCH expression is related to poorly differentiated glioma and oral squamous cell carcinoma with lymph node metastasis, and independently predict unfavorable prognosis of colorectal cancer patients [8-12]. More recently, Sun’s research group also reported that the intensity of stromal immunohistochemical staining for PINCH was increased from normal

mucosa to primary tumour and from primary tumour to lymph node metastasis in colorectal cancer. They also showed that strong staining for PINCH in normal adjacent mucosa related to worse survival, furthermore, in poorly differentiated tumours, PINCH staining at the tumour invasive margin was significantly related to survival, while in better differentiated tumours it was not [18]. Unfortunately, we did not have the follow-up data of the patients in the present study, therefore it was not available for us to examine the relationship of the PINCH expression with the patients' survival. However, in order to compare with the results of Sun's research group, we re-examied all slices according to Sun's score method for PINCH staining [18]. The staining intensity was determined for normal mucosa, and the entire tumour area (including the inner tumour area and the invasive margin of the tumours), and was scored as negative, weak, moderate or strong, irrespective of the percentage of positive cells. Because of the clinicopathological similarities we also combined the negatively, weakly and moderately stained groups as one group (weak group), and compared it to the strong group in the statistical analysis. The results showed that, of the 30 normal mucosa samples, 71% were weakly stained and 29% were strongly stained; of the 73 primary tumour samples 42% had weak staining and 58% had strong staining. The frequency of the strong PINCH staining was shown to be significantly increased from normal mucosa to primary tumour (X^2= 6.448, p = 0.011). Meanwhile, we also found that 29 (40%) primary tumours (including 20 well differentiated tumours and 9 poorly differentiated tumours) had stronger staining at the invasive margin, and the strong PINCH expression at the primary tumour invasive margin was also related to the tumour differentiation (X^2= 6.433, p = 0.011). Besides, we did not find association of PINCH expression with other clinicopathological factors including gender, age, tumour size and invasion depth. These results were

consistent with Sun's reports described previously [18]. Therefore, we speculated that due to gastric cancer and colorectal cancer belong to adenocarcinoma of digestive tract, so they may share some common features. Taken the above results together, PINCH protein may play a role in the invasion and metastasis of the tumours, and prediction of the patients' survival.

PINCH expression was not related to tumour differentiation, in link with the finding of our previous study in esophageal squamous cell carcinoma [12]. Reviewing the previous reports by previous researchers, the relationship of PINCH expression with tumour differentiation were controversial. PINCH expression was increased in colorectal cancers with better differentiation [9], but decreased in glioma with worse differentiation [10]. These different results might be explained by several reasons such as different case number and clinicopathological features, methods and critera for determining positive expression of PINCH as well as statistic methods in these studies.

We did not find PINCH expression was related to tumour invasive depth, although the invasive depth was related to lymph node metastasis, the latter was related to the positive PINCH expression. One possible explation for this might be that the non-association of the PINCH with tumour invasive depth in the present study was due to a limited number of the samples.

Furthermore, we also observed the expression of PINCH in the endothelial cells and found no any positive staining both in the normal mucosa and in the tumours. Gao et al. reported the expression of PINCH in the endothelial cells and its positively association with angiogenesis determined by CD31 in colorectal cancer [9]. PINCH belongs to the family of cell-extracellular matrix adaptor proteins which involved in an interaction with ILK, ILK is implicated in the promotion of tumour angiogenesis

by stimulating vascular endothelial growth factor observed in vitro and in vivo study [19]. Thus, the presence of PINCH in endothelial cells of the tumour vasculature suggests that the PINCH protein is upregulated in tumour angiogenesis, which is particularly important for tumour growth and metastasis.

In addition, by comparing PINCH expression and clinicopathological significance in gastric adenocarcinoma with esophageal squamous cell carcinoma studied earlier at our laboratory [12], we found that the two types of tumours shared certain features although they arise from different cells. For example, PINCH expression was up-regulated in the tumours compared to the matching normal mucosa, and the strong PINCH expression was related to the cases with lymph node metastasis. While PINCH expression was not related to patients' gender, age, tumour size and differentiation both in the two types of tumours.

After PINCH originally identified, the features and relationship with tumour of PINCH has been studied for a long time [6, 20-22]. However, how is PINCH involved in the tumorigenesis and development? Recently, several studies have shown that PINCH, through its LIM1 domain and LIM4 domain, mediating the formation of the complex between ILK and Nck-2, participating the integrin and growth factor signal transduction pathway, is implicated in many critical physiological and pathological processes [20, 23-26]. Through the complex formation with Nck-2, increased PINCH expression may be associated with an up-regulation of the growth factor signaling pathways. Growth factors are important regulators of the tumour-stromal interaction, and for some carcinomas an increase in growth factor receptors in stromal cells is thought to be an essential part in tumour to stroma signaling and hence tumour growth and progression [18, 26-28].

5. **Conclusion**

The results suggest that PINCH seems to play an important role in the tumorigenesis and development of gastric adenocarcinoma although the corresponding mechanism is unclear yet. In order to elucidate the pivotal role of PINCH in gastric adenocarcinoma, it is necessary to perform multi-center and larger sample studies, including not only the protein but also the DNA/RNA analysis in vivo and vitro, in the future.

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Figure legends

Fig.1. A. Positive PINCH immunohistochemical staining in the stroma of normal gastric mucosa (▲) and of gastric adenocarcinoma (➡). There was no PINCH immunostaining in normal epithelial cells and tumour cells. B. In normal gastric mucosa, PINCH immunostaining was mainly present in the stroma arounding the gastric gland neck (▲), and gastric pits (➡). C. Expression of PINCH protein at the invasive margin (arrow) was much stronger than in the inner tumour area.

Fig.2. Frequency of PINCH immunohistochemical staining in normal gastric mucosa and gastric adenocarcinoma.

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