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PINCH expression and its significance in esophageal squamous cell carcinoma

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Abstract. Particularly interesting new cysteine-histidine rich protein (PINCH), as a newly discovered protein of LIM family members, may play a role in signal transduction of integrin and growth factor, and involved in the incidence and development of tumors. PINCH protein is overexpressed in tumor-associated stroma of several types of tumors. However, there is no study of the PINCH in esophageal cancer, therefore we investigated PINCH expression in esophageal squamous cell carcinomas and its clinicopathological significance in the patients. PINCH expression was immunohistochemically examined in 20 normal esophageal samples and 64 esophageal squamous cell carcinomas. The results showed that PINCH expression in the stroma of cancers was heterogeneous, and its positive rate (56%) was higher than that of normal esophageal mucosa (5%, $p < 0.0001$). The stronger staining was observed at the invasive edge of tumor when compared to the inner area of tumor. The rate of positive PINCH (90%) in the cases with lymph node metastasis was higher than that (41%) in the cases without metastasis ($p < 0.0001$). PINCH expression was not correlated with patients' gender, age, tumor location, size and differentiation ($p > 0.05$). The results suggest that PINCH protein may be a marker of tumor associated-stroma involving tumor development, and predicting the ability of invasion and metastasis of esophageal squamous cell carcinoma.

Keywords: PINCH, esophageal squamous cell carcinoma, immunohistochemistry

1. Introduction

Adapter proteins play important roles in the formation, compartmentalization and stabilization of signaling complexes via interaction between protein domains [1,2]. Particularly interesting new cysteine-histidine rich protein (PINCH) is a newly discovered adapter protein, which is widely expressed and evolutionarily conserved, and consists primarily 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 [3].

It is shown that PINCH mRNA is expressed in many types of normal tissues, and located in the cytoplasm and cell matrix adherens junction [3]. The expression of PINCH in colorectal, lung, prostate, breast and skin cancer was examined by Western blot and immunohistochemistry, the results suggest that the PINCH expression is involved in the invasion and metastasis of the tumors, and the PINCH was noted to be especially

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abundant in the stroma of the invasive tumor margin, a region where signaling in the integrin and growth factor pathways is known to occur [4]. In further studies on the clinicopathological significance of the PINCH expression in the series of the patients with different types of malignant tumors, the PINCH expression has shown to be increased in high-graded gliomas and in oral squamous cell carcinoma with lymph node metastasis, and predict worse survival in the patients with colorectal cancer independently of tumor stage, growth pattern and differentiation [5–8]. However, to our knowledge, no study has been performed in esophageal cancer yet, therefore, in the present study, we examined PINCH protein expression in normal esophageal mucosa and esophageal squamous cell carcinoma, and further explored its clinicopathological significance in the patients.

2. Materials and methods

2.1. Material

Formalin-fixed paraffin-embedded tissue was obtained from the Department of Pathology of The First Hospital of Hebei Medical University, China. There were 64 esophageal squamous cell carcinomas, among them 7 cases in the upper, 36 in the middle and 21 in the lower section of the esophagus. The patients' gender, age, tumor location, size, lymph node status and differentiation were obtained from surgical and/or pathological records at the hospital. The mean age of the patients was 59.5 years old. According to the WHO classification, tumor differentiation was graded as grade I (high), grade II (moderate) and grade III (low). In addition, there were 20 normal esophageal mucosa samples which were obtained from distant margin of the surgical segment of the esophageal squamous cell carcinomas. All pathological slides including normal specimens and tumors were confirmed by two pathologists (Zhu ZL and Wang ZM).

2.2. Immunohistochemistry

The preparation, specificity and reliability of the rabbit polyclonal PINCH antibody used in the study were described previously [4,9]. 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_2in 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 2 μg/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 or/and purified rabbit IgG (Vector Labs) were used instead of the primary antibody. In all runs, there was no staining in the negative controls (Fig. 1A), 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. The intensity of the staining was graded as negative (no positive cells), weak ($<$20% positive cells), moderate (20%–50% positive cells) and strong positive ($>$50% positive cells). In statistical analysis, we considered negative and weak positive as negative group, and moderate and strong as positive group. In order to avoid artificial effects, cells on the margins of sections and areas with poorly presented morphology were not counted.

2.3. Statistical analysis

The Chi-square method was used to examine the relationship of the frequencies of PINCH expression in normal esophageal mucosa and cancer, 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.

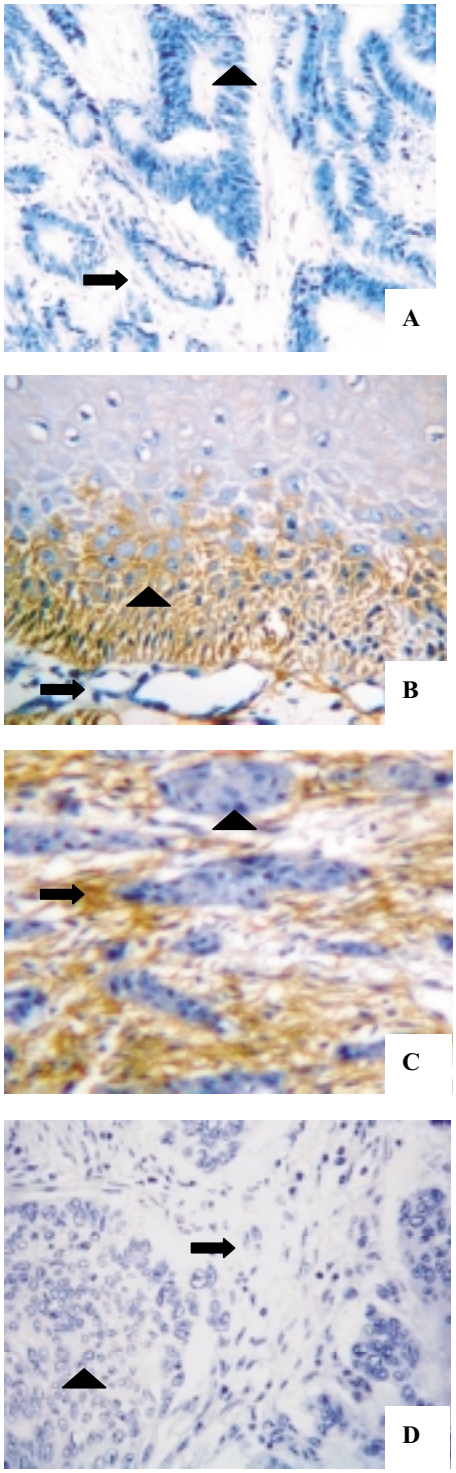

Fig. 1. A negative control (a colon cancer known for positive PINCH), where the primary PINCH was replaced by purified rabbit IgG, showed no staining of the PINCH in the stromal cells (➡) and tumor cells (▲) (A). The positive PINCH expression in the cells junction (▲) of the under one-third of epithelia layer of normal esophagus mucosa, but not in the stromal cells (➡) (B). The PINCH positive expression in the tumor-associated stroma (➡), but not in the tumor cells (▲) of esophageal squamous cell carcinoma (C). PINCH negative in the tumor-associated stroma (➡) and tumor cells (▲) of esophageal squamous cell carcinoma (D).

Table 1
The relationship between PINCH protein expression and clinicopathological variables in the patients with esophageal squamous cell carcinomas

Variables	*no.*	PINCH		P value
		Negative (%)	Positive (%)	
Gender				0.60
Male	50	21 (42)	29 (58)	
Female	14	7 (50)	7 (50)	
Age (years)				0.70
$\leqslant$50	19	9 (47)	10 (53)	
$>$50	45	19 (42)	26 (58)	
Location				0.99
Upper	7	3 (43)	4 (57)	
Middle	36	16 (44)	20 (56)	
Lower	21	9 (43)	12 (57)	
Tumor size (cm)				0.85
$\leqslant$3	26	11 (42)	15 (58)	
$>$3	38	17 (45)	21 (55)	
Grade				0.98
I	20	9 (45)	11 (55)	
II	39	17 (44)	22 (56)	
III	5	2 (40)	3 (60)	
Lymph node status				0.0002
Non-metastasis	44	26 (59)	18 (41)	
Metastasis	20	2 (10)	18 (90)	

3. Results

Among 20 normal esophageal mucosa samples, one case was positive for PINCH expression, in which the positive expression was only observed in epithelial cells junction in the under one-third of epithelial layer (Fig. 1B). While the rest samples, either epithelial or stroma cells, were negative for PINCH.

Among 64 cancers, 36 cases (56%) were positive for PINCH in the tumor-associated stroma (Fig. 1C), and 28 cases (44%) were negative for PINCH in the tumor-associated stroma (Fig. 1D). The staining was heterogeneous, with a great variation of both the numbers of positive cells and the staining intensity in the same case. We also observed that PINCH expression was especially strong in the stroma at the invasive edges of 13 tumors compared to in the inner-tumor stroma. The remaining 28 (44%) tumors showed negative for PINCH expression.

The cases with lymph node metastasis appeared a higher frequency of PINCH positive expression than those without metastasis in the lymph node (90% Vs 41%, $p = 0.0002$, Table 1). Lymph node metastasis was related to poorer differentiation (10% of the metastases in grade I, 33% in grade II, and 100% in grade III, $p = 0.0005$). The PINCH expression was not significantly correlated with patients' gender ($p = 0.60$), age ($p = 0.70$), tumor location ($p = 0.99$), size ($p = 0.85$) or differentiation ($p = 0.98$) shown in Table 1.

4. Discussion

Wang-Rodriquez et al. [4] first examined the expression of PINCH protein in the 33 breast, 22 prostate, 8 skin (4 basal cell carcinomas and 4 squamous carcinomas), 6 lung (3 Adenocarcinomas and 3 squamous carcinomas) and 5 colorectal cancers. The results showed that, apart from skin tissue, the rest tumors examined expressed more PINCH than normal tissues. This evidence has been confirmed in oral squamous cell carcinomas, colorectal cancers and gliomas, where the tumors had a higher frequency of PINCH expression than the corresponding normal tissues [5–8]. In the present study, 56% of 64 cancers were PINCH positive. In contrast, only 5% of 20 normal esophageal mucosa samples showed weakly positive expression of PINCH and the rest were negative. It seems that PINCH expression was also involved in the development of esophageal squamous cell carcinoma.

Sun's research group further analyzed clinicopathological significance of PINCH expression in the larger series of the patients including 57 patients with oral squamous cell carcinoma, two subgroups of colorectal cancer patients ($n = 174$, $n = 141$) and 82 patients with glioma, and found that the strong PINCH expression was even notably at the invasive edges of different types of tumors when compared to the inner areas of the tumors. More importantly, they found that strong PINCH expression is related to poorly differen-

tiated glioma and oral squamous cell carcinoma with lymph node metastasis, and independently predict unfavorable prognosis of colorectal cancer patients [5–8]. In the present study, we found that some tumors had stronger PINCH expression at the invasive edges than in inner area of tumor, and the cases with lymph node metastasis had a much higher frequency of PINCH positive expression than those without metastasis (90% Vs 41%). Unfortunately, we did not have the follow-up data of the patients, therefore it was not available for us to examine the relationship of the PINCH expression with patients' survival. However, taken the above results together, PINCH protein may play a role in the invasion and metastasis of the tumors, and prediction of the patients' survival.

The previous results regarding the relationship of PINCH expression with tumor differentiation were controversial. PINCH expression was increased in colorectal cancers with better differentiation [6], but decreased in gliomas with worse differentiation [7], and had no relationship with differentiation in oral squamous cell carcinoma [8]. In the present study, PINCH was not related to tumor differentiation, although the differentiation was related to lymph node metastasis, the latter was related to the positive PINCH expression. PINCH was likely to be involved in metastasis but not differentiation of esophageal cancer. We could not exclude a possibility that the non-association of the PINCH with tumor differentiation in the present study was due to a limited number of the cases.

We further compared PINCH expression and its clinicopathological significance in esophageal squamous cell carcinoma with oral squamous cell carcinoma studied earlier at our laboratory [8], and found that the two types of the tumors shared certain features, for example, the PINCH expression was increased in the tumors compared to the corresponding normal mucosa, especially the increased expression at the invasive edge of the tumor compared to the inner area of the tumor, and the strong PINCH expression was related to the cases with lymph node metastasis. While PINCH expression was not related to patients' gender, age, tumor location, size and differentiation in the either of the two types of the tumors. The mouth and esophagus belong to the upper digestive tract, and the both types of the tumors arise from of the squamous epithelial cells. Regarding the lesion location, most of the tumors are in the vulnerable to the site of attrition: oral cancers are particularly prevalent in the edge of tongue geography and cheek, and esophageal cancers in the site of esophageal stenosis. They also share some common risk factors to tumor development, such as smoking and drinking, rough, cold and hot food, as well as virus infection. The average age of the patients with either type of the tumors is among 50–60 years old, along with a higher incidence in men than women. Although the prognosis of the oral cancer and esophageal cancer is affected by many factors such as tumor location, size, growth pattern, histological grade and stromal reaction, the most important factor is the lymph node status. In tongue cancer, the 5-year survival rate of the patients without or with lymph node metastasis was 87.5% and 26.7%, and, in buccal cancer, the 5-year survival rate of the patients without or with lymph node metastasis was 63.1% and 18.5%. In esophageal cancer, the 5-year survival rate of the patients without or with lymph node metastasis was 67.5% and 25% [10–12].

PINCH protein has been observed to present in the endothelial cells and tumor-associated myofibroblasts, and was positively associated with angiogenesis determined by CD31 in colorectal cancers([5], unpublished data). PINCH is a family of cell-extracellular matrix adhesion proteins involved in an interaction with ILK, participating in integrin mediated intracellular and growth factor signaling pathways. ILK is implicated in the promotion of tumor angiogenesis by stimulating vascular endothelial growth factor observed in vitro and in vivo study [13]. Thus, these results indicate that PINCH seems to be involved in angiogenesis through the activation of fibroblasts in response to tumor.

PINCH is an adapter protein and consists of five LIM domains [4]. PINCH is associated with integrin signal transduction pathway, and can regulate cell adhesion, cell shape and cell migration. However, PINCH dose not directly combine with integrin, in fact, through the first double zinc-finger domain of LIM structure combine with ANK sequence of N-terminal of ILK, while the C-terminal of ILK can combine with cytoplasmic domain of integrin, so that they formed a signal complex to participate in the integrin signaling pathways [14]. Recently, studies have shown that PINCH protein is also involved in signal transduction systems of growth factor. By virtue of the fourth double zinc-finger domain of PINCH, it can combine with the Nck-2. Nck-2 is a Src homology adapter protein which involved in growth factor signaling system [15]. It can be seen that, through the first double zinc-finger domain and the fourth double zinc-finger domain, PINCH can combine the ILK and Nck-2, in integrin and growth factor signal transduction pathway. PINCH plays a critical effect such as a cross intersection points, equivalent to a "three-way" role. Therefore, PINCH protein may play

an important role in the incidence and development of tumors.

Why is PINCH protein expression in the tumor-associated stroma intensive, particularly evidence in the invasive edge? This is probably due to a combination of PINCH and ILK, which regulate the basic function such as the interaction between cells and extracelluar matrix. Fibronectin, a major component of the extracellular matrix is regulated by ILK, in the adjustment process, PINCH protein is needed, therefore, the intensive expression of PINCH protein in the tumor-associated stroma could be correlated with the accumulated fibronectin here [16]. During tumor development, the fibronectin formed by mobile of the tumor-associated stroma and other extracellular matrix deposited can form an edge area which can provide a suitable interface platform for mobile of tumor cells [17,18]. PINCH protein is present in the fibroblasts and myofibroblasts of tumor-associated stroma. Both types of cells can probably provide a "scaffold role" or a "bridging role" for the invasion and metastasis of tumor. The results also suggested that search for effective drugs against such cells to cut off or prevent the tumor invasion and metastasis can provide a new idea or therapeutic target in anti-tumor therapy.

5. Conclusion

PINCH protein may be a marker of tumor-associated stroma involving tumor development and predicting the ability of invasion and metastasis of esophageal squamous cell carcinoma.

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