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# PINCH Protein Expression in Normal Endometrium, Atypical Endometrial Hyperplasia and Endometrioid Endometrial Carcinoma

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## PINCH protein expression in normal endometrium, atypical endometrial hyperplasia and endometrioid endometrial carcinoma

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### Abstract

Background: Particularly interesting new cysteine-histidine rich protein (PINCH), as an adapter protein of LIM family for signal transduction in the integrin and growth factor pathway, is upregulated in the stroma of several common types of cancers and involved in promoting tumor progression. In the present study, we examined PINCH expression in the normal endometrium, atypical endometrial hyperplasia and endometrioid carcinoma and further studied the relationships of PINCH expression with clinicopathological variables in the cancer patients. Methods: PINCH expression was examined by immunohistochemistry in 23 normal endometrial samples, 18 atypical endometrial hyperplasias and 48 endometrioid endometrial carcinomas. **Results:** The PINCH expression in the stroma of cancer (71%) was significantly increased compared to either normal endometrium (17%, P<0.0001) or atypical hyperplasia (39%, P=0.017), along with 9 cancers that had stronger PINCH expression at the invasive margin of the cancers compared to the inner cancers. PINCH expression in cancer was higher in the patients having hypertension (P=0.041) and estrogen exposure time >30 years (P=0.021). Besides, the PINCH expression was not related to menopausal status, gravid status, blood sugar/lipid, family background of cancer, histologic grade, myometrial invasion, cervical involvement, lymph nodal metastases, growth pattern, estrogen and progestogen receptor (P>0.05). Conclusion: The results suggest that PINCH seems to play a role in the tumorigenesis and development of endometrial cancer, which at present is unknown, and merits further study.

**Key word**: PINCH; endometrioid endometrial carcinoma; atypical hyperplasia; immunohistochemistry

### Introduction

PINCH (particularly interesting new cysteine-histidine rich protein) protein is the member of LIM family (protein for lin-11 isl-1 mec-3) which comprises an array of five LIM domains [1,2]. The PINCH gene is located on chromosome 2q12.2 and widely expresses in the specific cells of tumor-associated stroma including fibroblasts, myofibroblasts and endothelial cells as an adapter protein [3]. The PINCH protein through integrin-linked kinase (ILK) - mediated integrin signaling complexes that is involved in cell adhesion, which regulates cell shape change and migration [4]. The PINCH interacts with Nck-2 which implicated in growth factor signal transduction pathways [5]. At present, it has been confirmed that PINCH expression is markedly upregulated in the stroma associated with many common types of cancers including breast, prostate, lung, skin, colorectal, oral and esophageal squamous cell cancers, especially abundant in the stroma at the invasive margin of tumors compared to the inner tumors [6-10]. Therefore increased PINCH may augment signal transduction in tumor-stroma, involving in tumorigenesis and tumor progression.

Endometrial cancer is the most common gynecologic malignancy. Worldwide each year, 142,000 women are diagnosed, and 42,000 women die from this disease [11]. Endometrial cancers are histologically divided into endometrioid and non-endometrioid, the both are different in etiology and the course of malignancy [12]. Endometrioid adenocarcinomas are the most common histological type and comprise 75% of all cases. They are estrogen dependent and derived from atypical endometrial hyperplasia [13]. However, to our knowledge, there is no study about the relationship between PINCH and endometrial cancer yet. In the present study, we examined PINCH expression in the normal endometrium, atypical endometrial

hyperplasia and endometrioid carcinoma by immunohistochemistry, and further studied the relationships of PINCH expression with clinicopathological variables in the cancer patients.

### Materials and methods

#### Materials

For immunohistochemistry, formalin-fixed paraffin-embedded tissue blocks were obtained from 48 patients of endometrioid endometrial carcinoma with integral clinical data at the First Hospital of Hebei Medical University (Shijiazhuang, China) from 2005 to 2007. Patients were diagnosed according to the FIGO (International Federation of Gynecology Obstetrics) Surgical Staging System for Endometrial Cancer (2000). The study also included 18 atypical endometrial hyperplasia and 23 normal endometrium (11 proliferative phase, and 12 secretory phase). The study was approved by the ethical committee at the First Hospital of Hebei Medical University, Shijiazhuang, Hebei Province, P. R. China.

All pathological diagnoses were reconfirmed by two pathologists (Zhang ZY and Zhu ZL). The median age of the patients was 36, 40.5, and 56 years old (range, 22 to 60, 26 to 77, and 33 to 74 years old) for normal endometrium, atypical endometrial hyperplasia and endometrial cancer, respectively. The patients with endometrial cancer did not receive radiotherapy, chemotherapy and/or other anti-cancer therapy before surgery. All subjects of the study did not receive administration of the non-steroidal anti-inflammatory drugs and hormone therapy 3 months before surgery.

### Immunohistochemistry

The preparation, specificity, and reliability of the rabbit polyclonal PINCH antibody used in the study were as described previously [6,14]. Five-micrometer continuous sections from

paraffin-embedded tissue were deparaffinized, hydrated and rinsed in distilled H<sub>2</sub>O. 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<sub>2</sub>O<sub>2</sub> 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 (kindly obtained from Prof. A. Rearden, Department of Pathology, University of California, La Jolla, CA, USA) at 2µg/ml at 4°C overnight. Then, a biotinlated anti-rabbit IgG antibody (Fuzhou Maixin Biology Technology, Fuzhou, China) was applied for 30 min followed by an incubation of an avidin-biotin-peroxidase complex (Beijing Zhongshan Biology Technology, Beijing, China) for 30 min. The sections were rinsed in PBS between the incubations. The peroxidase reaction were developed using diaminobenzidine (Beijing Zhongshan Biology Technology) for 8 min. After counterstaining with hemotoxylin, the sections were dehydrated and mounted. The breast cancer sections known for PINCH positive were included as positive or negative controls. The negative control was designed in every staining procedures, I.e., PBS instead of the primary antibody. In all staining procedures, the positive controls showed clear staining, and there was no staining in the negative controls.

The sections were microscopically examined and scored independently by the two pathologists (Zhang ZY and Zhu ZL) without any information on the clinicopathological data. We randomly selected 10 high power fields (10×40) without repetition and overlapping in each case. To avoid artificial effects, cells in areas with necrosis, with poor morphology or at the margins of sections were not counted. In cases with discrepant results by the two pathologists,

a consensus score was reached after re-examination. The expression of the PINCH, if positive, was present in the cytoplasm and the membrane of fibroblasts/myofibroblasts in the stroma and the epithelial cells of normal endometrium, atypical endometrial hyperplasia and tumor cells. We scored PINCH staining for both intensity and percentage. The intensity of the staining was graded as 0 point for negative, 1 for weak, 2 for moderate or 3 for strong, regardless of the staining percentage. The percentage of the staining was classified as 0 point for <5% staining, 1 for 6-25%, 2 for 26-50%, and 3 for > 50%, regardless of the staining intensity. The final score was defined as the sum of the staining intensity and the percentage in each case, i.e., 0 to 6 points. In the statistical analysis, we considered 0 to 2 points as negative group, and  $\geq$ 3 points as positive group.

### Statistical analysis

All data were tested by using SPSS13.0 statistics program. The Chi-square method and the Fisher's exact test were used to examine the relationship of PINCH expression in endometrial carcinoma, atypical endometrial hyperplasia and normal endometrium, and the relationships between PINCH expression in cancer and clinicopathological variables. All *P*-values were cited two-sided, and P < 0.05 was considered as statistically significant.

### Results

# The expression of PINCH in the stroma of normal endometrium, atypical endometrial hyperplasia and endometrial carcinoma

The expression of the PINCH was examined in normal endometrium, atypical endometrial hyperplasia and endometrial carcinoma from surgical resection specimens. The PINCH protein was localized in the cytoplasm and the membrane of fibroblasts and myofibroblasts in the stroma and epithelial cells of normal endometrium, atypical endometrial hyperplasia and tumor cells, and the staining in the stroma was often heterogeneous and granulous, especially in the cancer (Fig. 1). Among 48 cancers, 34 cancers had PINCH positive expression in the stroma, in which PINCH expression presented markedly heterogeneous, with a great variation of both the numbers of positive cells and the staining intensity in the different region of the same section. In 9 of 48 cancers, there was stronger PINCH expression at the invasive margin of the tumor compared to that in the inner tumor, but the rest tumors had similar expression at the invasive margin and the inner tumor.

As shown in Table 1, the frequency of PINCH positive expression in the stroma of normal endometrium, atypical endometrial hyperplasia and endometrial cancer was 17%, 39% and 71%, respectively. The PINCH expression in cancer was significantly increased compared to either normal endometrium (P<0.0001) or atypical hyperplasia (P=0.017). However, there was no significant difference in the expression between normal endometrium and atypical hyperplasia (P=0.164), neither between proliferative and secretory phase in normal endometrium (P=0.093).



Figure 1. PINCH expression was negative in the stroma of normal endometrium (thick arrow, A), positive in the stroma of atypical endometrial hyperplasia (thick arrow, B) and endometrioid carcinoma (thick arrow, C). The negative control in a endometrioid carcinoma showed negative PINCH expression (D). There was no PINCH expression in the epithelial cells of normal endometrium, atypical endometrial hyperplasia and tumor cells (thin arrows,  $SP \times 200$ ).

Groups	n	PINCH expression		D 1
		Negative (%)	Positive (%)	P value
Normal endometrium	23	19 (83)	4 (17)	
Proliferative phase	11	11 (100)	0	0.093 <sup>a</sup>
Secretory phase	12	8 (67)	4 (33)	
Atypical endometrial hyperplasia	18	11 (61)	7 (39)	0.164 <sup>b</sup>
				0.017 <sup>c</sup>
Endometrioid carcinoma	48	14 ( 29)	34 (71)	<0.0001 <sup>d</sup>

*Table 1 PINCH expression in the stroma of normal endometrium, atypical endometrial hyperplasia and endometrioid carcinoma.* 

<sup>a</sup> proliferative *vs*. secretory.

<sup>b</sup> atypical endometrial hyperplasia *vs.* normal endometrium.

<sup>c</sup> atypical endometrial hyperplasia *vs.* carcinoma.

<sup>d</sup> carcinoma *vs*. normal endometrium.

# The expression of PINCH in the epithelial cells of normal endometrium, atypical endometrial hyperplasia and tumor cells

We also observed that PINCH expressed in the epithelial cells of normal endometrium, atypical endometrial hyperplasia and tumor cells, and the frequency of the PINCH positive expression was 61%, 67% and 42%, respectively. There was no statistical difference among the three groups, neither between proliferative and secretory phase in normal endometrium ((P>0.05, Table 2). We further analyzed the relationship of the PINCH immunostaining of tumor cells with patients' clinicopathological features, and revealed that it was no statistical association (the data were not shown).

*Table 2 PINCH expression in the epithelial cells of normal endometrium, atypical endometrial hyperplasia and tumor cells.* 

Groups	n.	PINCH expression		
		Negative (%)	Positive (%)	r value
Normal endometrium	23	9 (39)	14 (61)	
Proliferative phase	11	6 (54)	5 (46)	0.214 <sup>a</sup>
Secretory phase	12	3 (25)	9 (75)	
Atypical endometrial hyperplasia	18	6 (33)	12 (67)	$0.754^{b}$
				$0.070^{\circ}$
Endometrioid carcinoma	48	28 (58)	20 (42)	0.130 <sup>d</sup>

<sup>a</sup> proliferative *vs*. secretory.

<sup>b</sup> atypical endometrial hyperplasia vs. normal endometrium.

<sup>c</sup> atypical endometrial hyperplasia *vs*. carcinoma.

<sup>d</sup> carcinoma *vs*. normal endometrium.

### The relationships between PINCH expression in the stroma and clinicopathological

### features in patients with endometrial carcinoma

The Table 3 shows that relationship of PINCH expression in the stroma with

clinicopathological features in patients with endometrioid carcinoma. PINCH expression in cancer of the patients with estrogen exposure time > 30 years (82%, P=0.021) and hypertension (82%, P=0.041) was markedly higher than that of estrogen exposure time < 30 years (50%) and normotensive (55%). There were only two cases having metastasis in the lymph nodes, and the both had positive PINCH expression, we did not do statistical analysis because of too few cases. Besides, there was no association of PINCH expression with the patients' age, gravid status, blood sugar/lipid, family background of cancer, histologic grade, myometrial invasion, cervical involvement, growth pattern, estrogen and progestrone receptor (P>0.05, Table 3).

Variables	n.	PINCH expression			
		Negative (%)	Positive (%)	P value	
Menopausal status				0.506	
Premenopausal	12	4 (33)	8 (67)	-	
Postmenopausal	34	8 (24)	26 (76)		
Gravid status				0.237	
No pregnancy	5	3 (60)	2 (40)		
1 child	7	1 (14)	6 (86)		
≥2 children	34	10 (29)	24 (71)		
Blood pressure (mmHg)				0.041	
<140/90	20	9 (45)	11 (55)		
≥140/90	28	5 (18)	23 (82)		
Blood sugar (mmol/l)				0.369	
<6.1	28	6 (21)	22 (79)		
≥6.1	18	6 (33)	12 (67)		
Blood lipid				0.654	
Normal	13	4 (31)	9 (69)		
High	10	4 (40)	6 (60)		
Estrogen exposure time (years)				0.021	
<30	18	9 (50)	9 (50)		
≥30	28	5 (18)	23 (82)		
Family background of cancer				0.452	
No	41	13 (32)	28 (68)		
Yes	6	1 (17)	5 (83)		
FIGO stage*				0.893	
I	34	10 (29)	24 (71)		
II	8	2 (25)	6 (75)		
III	5	1 (20)	4 (80)		
IV					
Histologic grade					
G1	1	0	1		
G2	25	7 (28)	18 (72)		
G3	5	1 (20)	4 (80)		
Myometrial invision				0.975	
No	3	1 (33)	2 (67)		
Superficial myometrial invasion	33	9 (27)	24 (73)		
Deep myometrial invasion	11	3 (31)	8 (69)		
Cervical involvement				0.933	
No	35	10 (28)	26 (72)		
Yes	11	3 (27)	8 (73)		
Lymph nodal metastases					
No	35	12 (34)	23 (66)		
Yes	2	0	2		
Growth pattern				0.170	
Infiltration	24	5 (21)	19 (79)		
Expansive	23	9 (39)	14 (61)		
Estrogen receptor**					
-	1	0	1		
+	1	0	1		
++	3	1 (33)	2 (67)		
+++	3	1 (33)	2 (67)		
Progesterone receptor**					
-	1	0	1		
+	1	0	1		
++	3	1 (33)	2 (67)		
+++	3	1 (33)	2 (67)		

Table 3 The relationship between PINCH expression in the stroma of endometrioid carcinoma and the clinicopathological features

\*FIGO: International Federation of Gynecology Obstetrics \*\*Estrogen and Progesterone receptor determined by using immunohistochemistry

### Discussions

By using the same PINCH antibody, several groups have done a series of studies in oral, esophageal squamous cell and colorectal cancer, and the results have shown that PINCH was strongly expressed in tumor-associated stroma compared to the corresponding normal tissues, particularly at the invasive margin of the tumor compared to the inner tumor [7-10]. These studies have further shown that the abundant PINCH expression in the stroma of primary tumor was associated with high-graded tumor, recurrence and nodal metastasis [7-8,10, 15], and furthermore predicted a worse outcome in the patients [7]. In the present study, we first including premalignant lesion, atypical hyperplasia, and found that the PINCH expression in the stroma increased from normal endometrium (17%) to atypical hyperplasia (39%) to cancer (71%). Although the increased PINCH expression of atypical hyperplasia did not reach statistical significance when compared to that of normal endometrium, it showed an upward trend. Moreover, in some cancers, PINCH showed stronger expression at the invasive margin of the tumor compared to the inner tumor. In review of the previous studies, none of them has examined PINCH expression in the premalignant lesions. In the present study, we observed increased PINCH expression in tumor-associated stroma not only occurred in cancer, also appeared in the precancerous stage, atypical endometrial hyperplasia. It seems that PINCH protein was involved in the process of tumorigenesis/progression from normal mucosa to atypical hyperplasia and to carcinoma in the endometrium. In a certain extent, PINCH is upregulated in tumor-associated stroma, particularly at invasive margins, and may be a marker for stroma manifesting the ability to facilitate invasion.

We further studied the clinicopathological significance of PINCH expression in the stroma

of endometrial cancer by examining the relationships between PINCH expression with clinicopathological variables. The results showed that the PINCH associated with hypertension and lifetime exposure to endogenous estrogen. Estrogen is closely associated with endometrioid carcinoma and premalignant lesions, and long time and sustained unopposed estrogen environment, either endogenous or exogenous is the major and direct inciting factor for the development of cancer. Hypertension is also a high risk factor for developing endometrioid carcinoma [16]. We have also noticed that there were only two cases who had pelvic node metastases, and the both showed PINCH positive. Since it is too few cases to draw a conclusion. which the reasons for this non-significance may be still due to the small size of the sample. There was no association of PINCH and hormone receptor by the cancers, either progesterone or estrogen receptor, and the other clinicopathological variables. One of the reasons may be due to the small size of the cases. Furthermore, we could not perform an analysis on the relationship of PINCH expression with survival due to the lack of follow-up data of the patients.

We also found that the PINCH expressed in the normal epithelial, atypical and tumor cells. The PINCH positive rate in the tumor cells seemed to be lower than that in the atypical and normal epithelial, but the difference was not statistical significant. Previous other studies did not show that PINCN expression was positive in normal epithelium and tumor cells except gliomas [7-9,15]. It seemed that PINCH expression in endometrial epithelial and tumor cells may be not an accidental phenomenon, and it may be associated with the specificity of endometrial tissue in a matter of intracellular and extracellular matrix (ECM) interaction. The PINCH forms a ternary complex with integrin-linked kinase (ILK) and  $\alpha$ -parvin. The

PINCH-ILK- $\alpha$ -parvin (PIP) complex is an essential signaling platform, serving as a convergence point for integrin and growth-factor signaling and regulating cell adhesion, spreading, and migration [17-18]. The LIM 1 domain of PINCH binds to the aminoterminal ankyrin repeat domain of ILK and can modulate ILK activity that mediates cell-extracellular matrix adhesion and transduces bi-directional signals between the ECM and intracellular signaling pathways [18]. The LIM 4 domain of PINCH binds to the SH2-SH3 protein Nck2 that links the PDGF receptor and other receptor tyrosine kinases via Rho effectors (e.g. Pak) to cytoskeleton rearrangement [5,19-20]. PINCH-2 was identified as a second member of the PINCH family in 2002, and therefore, PINCH was renamed to PINCH-1 [21]. Despite the sequence similarity, PINCH-2 and -1 are encoded by different genes. Furthermore, PINCH-2, unlike PINCH-1, is dispensable for embryonic development and restricted expression [22]. However, PINCH-2 binds ILK through a site that is identical to that of PINCH-1[21]. A previous study by others showed that treatment with transforming growth factor (TGF)- $\beta$ 1 can elevate the level of PINCH-2, resulting in concomitant displacement of PINCH-1 from the PIP complex, and compromised podocyte spreading [23]. Furthermore, PINCH-2 does not bind to Rsu-1 as PINCH-1 does. The ras suppressor, Rsu-1, binds to the PIP complex based on its interaction with the LIM 5 domain of PINCH-1 [24-25]. In the present study, we used the PINCH-1 antibody like the previous studies [6-10,14-15]. This paradox about downregulated PINCH-1 expression in tumor cells of endometrioid carcinoma may be associated with PINCH-2, and merits further study and explores the inherent regulating mechanism of PINCH, both PINCH-1 and PINCH-2.

Previous studies also showed that the most common tumor suppressor gene of endometrioid

carcinoma, PTEN is involved in interaction of PINCH-ILK by the Pi3 kinase pathway [26]. Thus the role of PINCH as a crucial adapter protein between integrin and growth factor signal transduction pathways, likes other molecular targeted agents, and merits further study in the treatment of endometrial cancer and other common malignant tumors after surgical resection, hormonal therapy, radiation and chemotherapy [27-29].

In conclusion, PINCH protein expression markedly increased in the tumor-associated stroma in endometrioid carcinoma compared to normal endometrium and atypical endometrial hyperplasia, especially at invasive margin of tumor. Furthermore, the PINCH was positive associated with hypertension and estrogen exposure time. Therefore, PINCH does seem to play a role in the tumorigenesis and development of endometrial cancer, which at present is unknown, and merits further study.

### **Conflict of interest**

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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