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Expression and significance of the polar regulation-associated protein in cholangiocarcinoma*

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Abstract ***Objective:*** To investigate the expressions of atypical protein kinase C ι subtype (aPKC-ι) and E-cadherin in cholangiocarcinoma, and analyze molecular mechanisms of the invasion and metastasis of cholangiocarcinoma. ***Methods:*** The expressions of aPKC-ι and E-cadherin in 9 specimens of benign bile duct tissues and 35 specimens of cholangiocarcinoma were detected by EnVision immunohistochemistry, and their correlations to the clinicopathologic characteristics and invasion of cholangiocarcinoma were analyzed. ***Results:*** The positive rate of aPKC-ι was significantly higher in cholangiocarcinoma than in benign bile duct tissues (68.6% vs. 11.1%, $P = 0.006$), while the positive rate of E-cadherin was significantly lower in cholangiocarcinoma than in benign bile duct tissues (37.1% vs. 88.9%, $P = 0.016$). aPKC-ι expression was negatively correlated to E-cadherin expression ($r = –0.287$, $P < 0.05$). aPKC-ι expression was positively and E-cadherin expression was negatively correlated to the differentiation and invasion of cholangiocarcinoma ($P < 0.05$). ***Conclusion:*** The expressions of aPKC-ι and E-cadherin may reflect the differentiation and invasive potential of cholangiocarcinoma. As a polar regulation-associated protein, aPKC-ι may play an important role in the invasion and metastasis of cholangiocarcinoma.

Key words bile duct neoplasm; atypical protein kinase C; E-cadherin; immunohistochemistry

Cholangiocarcinoma is a kind of tumors from bile duct cells with high malignancy degree, the incidence rate is increasing in people. It characters on difficulty diagnosed, high malignancy, early metastasis and poor prognosis. Accordingly, the key point of prophylaxis and treatment to cholangiocarcinoma is to analyze the molecular mechanism of its invasion and metastasis. Lately researches indicated that atypical protein kinase C (aPKC) which could regulate the cell polarity expresses abnormally in some malignant tumors and its expression lever has some relationship with genesis, development and prognosis of tumors [1–5]. In this research, we detected the expression of aPKC-ι that is a new subtype of aPKC and the expression of E-cadherin to tissues with cholangiocarcinoma, studied its relation to clinical pathology and prognosis, evaluated whether it is worthy to be used to predict the prognosis, and analyzed the molecular mechanism of cholangiocarcinoma invasion and metastasis.

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Materials and methods

Patients and clinicopathological information

Between January 2000 and December 2004, 35 consecutive patients underwent surgical resection of cholangiocarcinoma at the Department of General Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, China. Of the 35 cases, 17 were males and 24 females with the age ranging from 24 to 75 years old (median 51 years). Nine having benign bile duct tissues which including 5 cyst of bile ducts and 4 chronic cholangitis tissues were chosen for control.

Experimental materials and immunohistochemistry methods

All samples (44 cases) were routinely dealt with formalin (10%) fixation, paraffin imbedding and sections (4 μm), baked (65 ℃) for 4 hours, dewaxed by xylene and hydrated by passage through a graded series of ethanol (once each at 100%, 80%, 70%, and 50%). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol (15 min). After each following step, sections were washed with 0.01 M phosphate buffered saline (PBS), three times for 6 minutes. Antigen retrieval

was carried out using pressure cooking in citrate buffer (pH 7.0, 97–100 ℃) for 15 min, cooled down for 20 min. The tissue sections were then covered with anti-aPKC ι polyclonal antibody (Abgent Biotechnology, USA; CA, 1:20 dilution) for 60 min and were incubated 30 minutes with protein block serum (Dako, Carpenteria, California, USA). After washing, the sections were stained with diaminobenzidine liquid system. The sections were then counterstained with Mayer's haematoxylin and mounted. Staining for anti-E cadherin polyclonal antibody (Boster, Wuhan; CA, 1:50 dilution) was carried out as described previously. For each case, a corresponding section was incubated in PBS (0.01 mol/L) the primary antibody as a negative control for non-specific staining, and without anti-aPKC ι or anti-E cadherin polyclonal antibody as blank control.

Interpretation of immunohistochemical staining

Immunohistochemical aPKC-ι staining was graded as positive if buffy staining was seen in the cell membrane and/or cytoplasm, and immunohistochemical E-cadherin staining was graded as positive if buffy staining was seen in the cell membrane. It counted scores by the positive cells rate and staining intensity. The positive cells rate was graded as follows: every section randomly observed by counting 10 different fields with the high power field (counting by actual observed fields unless no more than 10), less than 5% (score 0); the positive cells rate was 6%–35%, 36%–70% and more than 70% respectively (scores 1, 2, and 3). Staining intensity was graded as follows: no staining (score 0); pale yellow staining (score 1); buffy staining (score 2); strongly brown staining (score 3). Total scores were added in above two parameters for each section, no more than scores 4 was regarded as low expression, and more than scores 4 was regarded as high expression.

Statistical methods

The analyses were performed using the software package SPSS, version 12.0 for Windows. Fisher's exact and Chi-square tests were used to evaluate the association between aPKC-ι and E-cadherin expressions and clinicopathologic parameters. The correlation between increased aPKC-ι and E-cadherin expressions was evaluated by Pearson test. P-value < 0.05 was considered statistically significant.

Results

The expression of aPKC-ι in cholangiocarcinoma

aPKC-ι was expressed in benign bile duct tissues. The staining pattern included a membranous staining of the luminal surface, cytoplasmic staining, or both (Fig. 1a). In cholangiocarcinoma tissues, the staining pattern included a cytoplasmic strong staining, membranous weak staining of the luminal surface (Fig. 1b). Low expression of aPKC-ι was detected in 8 of 9 (88.9%) benign bile ducts. aPKC-ι was highly expressed in 27 of 35 (68.6%) cholangiocarcinomas ($\chi^2 = 7.434$, $P = 0.006$).

The expression of E-cadherin in cholangiocarcinoma

E-cadherin was highly expressed in benign bile duct tissues. The staining pattern included a membranous strong staining of the luminal surface (Fig. 2a). In cholangiocarcinoma tissues, the staining pattern included a membranous weak or no staining of the luminal surface (Fig. 2b). High expression of E-cadherin was detected in 8 of 9 (88.9%) benign bile ducts. E-cadherin was lowly expressed in 22 of 35 (62.9%) cholangiocarcinomas (χ^2 =

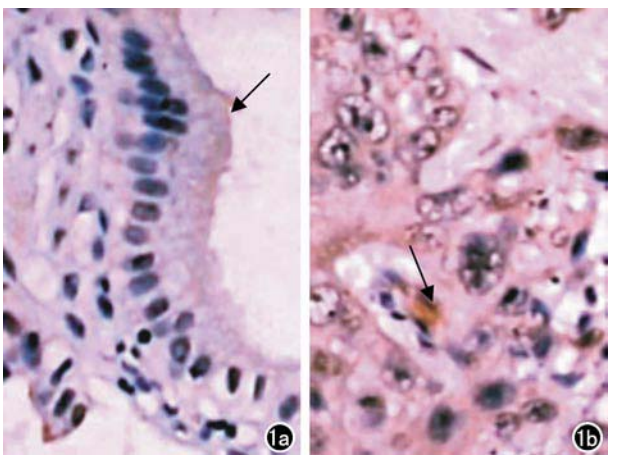

Fig. 1 Expression of aPKC-ι in bile duct tissues (EnVision × 400). (a) Low expression of aPKC-ι in cyst of bile duct; (b) High expression of aPKC-ι in poorly differentiated cholangiocarcinoma

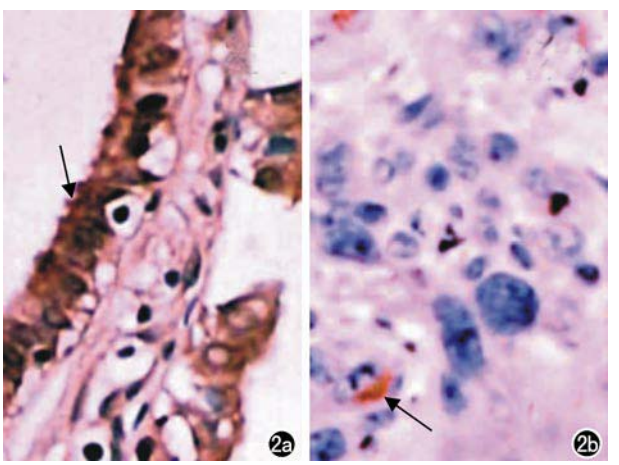

Fig. 2 Expression of E-cadherin in bile duct tissues (EnVision × 400). (a) High expression of E-cadherin in cyst of bile duct; (b) Low expression of E-cadherin in poorly differentiated cholangiocarcinoma

Table 1 Correlation of aPKC-ι and E-cadherin expressions to clinicopathologic characteristics of cholangiocarcinoma

Group	Cases (n = 35)	aPKC-ι		P value	E-cadherin		P value
		Low	High		Low	High	
Differentiation							
Well-moderately	18	9	9		8	10	
Poorly-undifferentiated	17	2	15	0.027	14	3	0.035
Infiltrate muscular layer							
Negative	10	7	3		2	8	
Positive	25	4	21	0.004	20	5	0.002
Lymph node metastasis							
Negative	24	11	13		12	12	
Positive	11	0	11	0.006	10	1	0.027
Clinical TNM stage							
I–II	14	8	6		5	9	
III–IV	21	3	18	0.011	17	4	0.012

5.750, $P = 0.016$).

Correlation of aPKC-ι and E-cadherin expressions to clinicopathologic characteristics of cholangiocarcinoma

We found positive significant association of aPKC-ι staining with pathological differentiation, infiltrate status, lymph nodal metastasis and clinical TNM staging ($P < 0.05$; Table 1). Correlation analysis revealed that the expression of E-cadherin was negatively related to the progress of the tumor pathological differentiation, infiltrate status, lymph nodal metastasis and clinical TNM staging ($P < 0.05$; Table 1). In these cases, there was a negative relationship between the expressions of aPKC-ι and E-cadherin by Spearman's test ($r = –0.287$, $P < 0.05$).

Discussion

Cholangiocarcinoma is a kind of tumors with high malignancy degree. It's a complicated biological process in early metastasis [6]. In the process it's a initialized step that malignant cells invasive growth to neighbourhood tissues. Research showed that malignant cells invasive growth toward adjacent tissues had close relation to disfunction of adhering between cells together with the establishment of repolarization state that profited malignant cell locomotion [6–8].

Atypical protein kinase C (aPKC) has been discovered recently, including aPKC-ζ, aPKC-λ and aPKC-ι [6]. Research indicated that the compounds formed by aPKC and other proteins that could regulate the cell polarity convey to intercellular binding sites from cytoplasm and align to the tight junctions between epithelial cells, which contributed a lot to intercellular junction and establishment and maintenance of adhere sites [7]. In the polarizing process of mammalian epithelial cells, the compounds of aPKC were transported to the binding site of intercells form kytoplasm, and located on tightly binging site of inter-epithelium [8]. aPKC/PAR6 is key factor for cell locomotion which microtubulin organizer center (MTOC) and Golgi's apparatus directed shift to apical, which extends via signal transduction access of CDC42/aPKC/PAR6 that forms head pseudopodium and rearrangement of actin and microtubulin in cystoskeleton, such as APC (adenomatous polyposis coli). That is vital for cell polarization reconstruction [1, 8]. Lately aPKC-ι's gene amplification and high expressions in small cell lung cancer (SCLC) and ovarian cancer (OC) were reported by Mills G. and Field P. respectively. The high expression of activated aPKC-ι lead to inaction of the Par3-Par6-aPKC complex which plays a critical role in the establishment and maintenance of epithelial cell polarity, tight junctions, and adherens junctions, loss of apical-basal cell polarity is required for epithelial-mesenchymal transition (EMT), which is a critical step in cellular motility and invasiveness [1, 3].

In the polarizing process of mammalian epithelial cells, polarization regulatory proteins, such as carcinogenic factor aPKC, pTEN and RhoGTP enzyme, E-cadherin, β-catenin signal transduction access and so on, are all correlated to the genesis and development of the tumors of human beings [9, 10]. Among the total, abnormal or absent expression of E-cadherin is all correlated to invasion and metastasis of the tumor cells [11]. E-cadherin is very important for enhancement of intercellular adhering and maintenance of structural integrity and polarity, and it has inhibitory action to invasion and metastasis of tumor cells. Furthermore, it participates in intracellular signal transduction and can transfer information and stabilize cells [12]. Many studies on stomach, mammary gland, lung etc, indicated that absent expression of E-cadherin triggered cancer cell to release and assigned invasion characteristics [13].

Our experimental results demonstrated that aPKC-ι expressed lowly in benign bile duct tissues mostly (8/9, 88.9%), with high expression of only one specimen of cyst

of bile duct. Nevertheless, it expressed higher evidently in specimens of cholangiocarcinoma (27/35, 68.6%), mostly in cytoplasm; E-cadherin expressed highly in benign bile duct tissues mostly (8/9, 88.9%), while it expressed lower evidently in cholangiocarcinoma (13/35, 37.1%), mostly impaired membrane expression; furthermore, the expressions of aPKC-ι and E-cadherin correlated to tumor's differential extent, infiltration and aversion of lymphoid node. It suggests that abnormal expressions of aPKC-ι and E-cadherin correlates firmly to invasion and metastasis of cholangiocarcinoma. The results also showed that there was a negative relationship between the expressions of aPKC-ι and E-cadherin in cholangiocarcinoma, indicating that there is an interregulatory mechanism between the abnormal expressions of aPKC-ι and E-cadherin in the process of invasion and metastasis of cholangiocarcinoma, whereas the specific mechanism is still not clear, and its precise relation and mutual effect mechanism need further investigation. Therefore, as one kind of polarization regulatory proteins, aPKC-ι plays an important role in the process of cholangiocarcinoma cells' invasion and metastasis, providing the foundation for further analysis of mechanism of action of aPKC-ι in regulating the polarization and invasion of cholangiocarcinoma cells.

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