Proceeding

Cellular senescence protein p16^{INK4a} of cancer-associated fibroblasts (CAFs) as a prognostic indicator for cholangiocarcinoma patients

โปรตีน p16^{INK4a} ในกระบวนการการแก่ของเซลล์ไฟโบรบลาสต์ที่สัมพันธ์กับมะเร็งเป็นตัวพยากรณ์โรคสำหรับผู้ป่วยมะเร็งท่อน้ำดี

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Background and Objective: The tumor suppressor protein p16^{INK4a} (inhibitor of CDK4/6) is a cyclin-dependent kinase inhibitor that plays an important role in cell cycle and cellular senescence. The senescent fibroblast expressing p16^{INK4a} contributes to cancer development and poor prognosis of patients. Therefore, our approach focus on the expression of p16^{INK4a} in cancer associated fibroblasts (CAFs) in cholangiocarcinoma (CCA) patient tissues and its clinicopathological correlation.

Method: The immunohistochemistry and immunofluorescence assays were used to investigate the expression of p16^{INK4a} in forty six samples of CCA tissues. Pearson Chi-square test was used for analyzing of p16^{INK4a} expression and clinicopathological data of CCA patients.

Result: Our results showed that high expression of p16^{INK4a} protein was presented in CAFs surrounding tumor tissues of 24 cases (52.2%) of CCA patients. The positive expression of p16^{INK4a} (p=0.041) was significantly associated with the non-papillary type.

Conclusion: The high expression of p16^{INK4a} was found in CAFs, which referred to senescence-CAFs. Senescent-CAFs was significantly correlated with non-papillary type, which is the unfavorable feature of CCA patients.

Keywords: p16^{INK4a}, Cancer associated fibroblasts, Cholangiocarcinoma

Introduction

Cholangiocarcinoma (CCA) is a cancer arising from bile ducts both inside and outside the liver. CCA is one of the most common cancers in Thailand, especially in the northeast region, where the incidence of *Opisthochis viverini* (Ov) infection is high.[1] at present, only surgical resection of all detectable tumor leads to an improvement in 5-year survival. The protein p16^{INK4A}, a cyclin-dependent kinase inhibitor, plays important roles in biological functions,

including the inhibition of cell cycle progression and the induction of cellular senescence.[2] Although functions of $p16^{INK4a}$ have been studied in various types of tumor cells an attention to surrounding stromal cells, including fibroblasts is still little. It has been widely known that the activated fibroblasts, also known as cancer-associated fibroblasts (CAFs), are one of the major cellular components in the stroma of cancer tissues. CAFs have important roles, including the support of tumor growth, angiogenesis, metastasis and drug resistance through the paracrine effect.[3] In this study, we aimed to examine the expression pattern of $p16^{INK4a}$ in CAFs residing in human CCA tissues. The statistical correlation between $p16^{INK4a}$ and the clinicopathological data of CCA patients was analyzed.

Materials and Methods

Human CCA tissues

Human CCA tissues have been collected from the CCA patients who admitted to Srinagarind

hospital, Khon Kaen university and kept at the Cholangiocarcinoma Research Institute (CARI), Faculty of Medicine, Khon Kaen university. The paraffin embedded tissues were obtained from the Cholangiocarcinoma Research Institute. The study protocol was approved by the Ethic Committee for Human Research, Khon Kaen university (HE571283).

Immunohistochemical analysis

Immunohistochemical staining was performed to identify the expression of $p16^{INK4a}$ in human CCA tissues. The tissue sections were incubated with the rabbit anti-CDKN2A/$p16^{INK4a}$ antibody (Abcam, USA) with a dilution of 1:100 and HRP conjugated anti-rabbit secondary antibody (Dako, USA). The stained sections were reviewed under a bright-field microscope. The expression of $p16^{INK4a}$ protein was analyzed semi-quantitatively and the data was interpreted for the cytoplasmic and nuclear staining separately. Tumor tissues were given a score according to intensity of nuclear and cytoplasm was scored as negative = 0, weak = 1, moderate = 2, and strong = 3 and the percentage of the stained cells at different staining intensities. H-score calculated by using the formula 1 × (% of 1 + cells) + 2 × (% of 2 + cells) + 3 × (% of 3 + cells).

Immunofluorescence staining

Immunofluorescence staining was performed to localize the expression of $p16^{INK4a}$ and alpha smooth muscle actin (α-SMA) proteins in human CCA tissues. The tissue sections were incubated with two primary antibodies, including rabbit anti-CDKN2A/$p16^{INK4a}$ antibody (Abcam, USA) with a dilution of 1:100 and mouse anti-alpha SMA antibody (Abcam, USA) with a dilution of 1:100 followed by secondary antibodies, including Alexa Flour™ 488 goat anti-rabbit antibody (Invitrogen, USA) with a dilution of 1:200 and Alexa Flour™ 555 goat anti-mouse antibody (Invitrogen, USA) with a dilution of 1:200). This was carried out in darkness. The tissue sections were additionally incubated with 4,6-diamidino-2-phenylindole (DAPI) staining solution with

dilution of 1:2000 (Invitrogen, USA). The stained tissue sections were reviewed under a confocal laser scanning microscope (Carl-Zeiss/LSM800).

Statistical analysis

The Statistics Package for the Social Science; SPSS software version 16.0 was used for the statistical analysis. The correlation between the expression of $p16^{INK4a}$ tumor suppressor protein in CAFs and clinicopathological data of CCA patients was analyzed by Pearson Chi-square test. The results are considered to be statistically significant at *p-value* < 0.05.

Results

The expression of $p16^{INK4a}$ protein in CAFs

The expression of $p16^{INK4a}$ protein in CAFs was identified by H-scoring immunohistochemical grading based on intensity and frequency represented in brown. The results showed that high expression of $p16^{INK4a}$ was 24 cases (52.2%) and low expression of $p16^{INK4a}$ was 22 cases (47.8%) observed in CAFs surrounding bile duct epithelial tissues as shown in **Figure 1**. The immunofluorescence staining of $p16^{INK4a}$ and α-SMA proteins were localized in the senescence-CAFs of human CCA tissues (**Figure 2**).

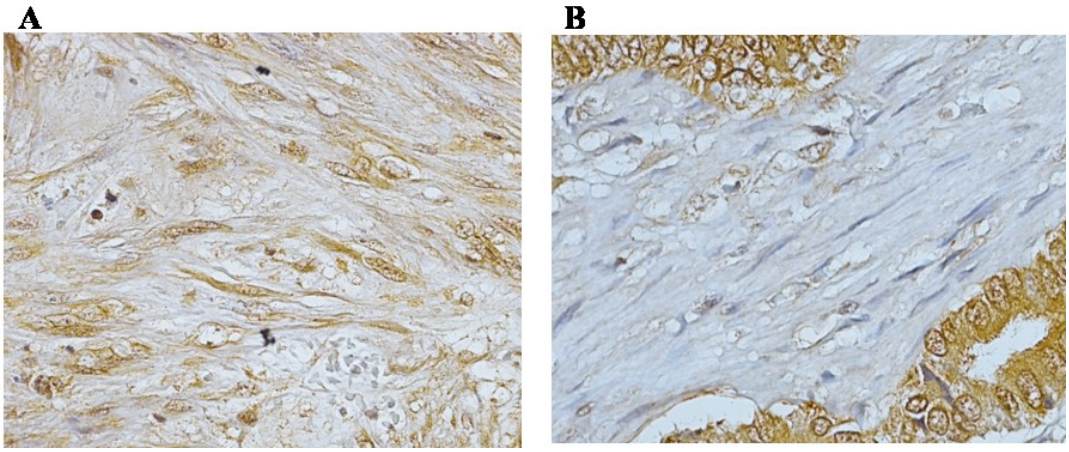

Figure 1 Representative immunohistochemical staining of $p16^{INK4a}$ protein expression in CAFs from human CCA tissues (x400). **A** high expression of $p16^{INK4a}$ in CAFs, **B** low expression of $p16^{INK4a}$ in CAFs

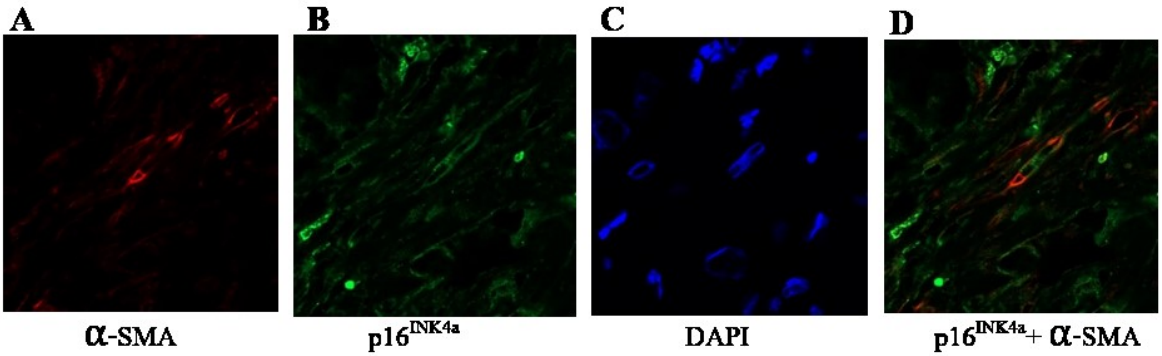

Figure 2 Representative immunofluorescene double staining of $p16^{INK4a}$ and **α**- in CAFs from human CCA tissues (x1000). **A** Immunofluorescence staining of **α**-SMA, **B** Immunofluorescence staining of $p16^{INK4a}$ staining, **C** DAPI staining, **D** Overlay image of **A** and **B** showing CAFs expressing $p16^{INK4a}$.

The correlation between $p16^{INK4a}$ protein and clinicopathological data of CCA patients

Among 46 human CCA tissues, 29 cases (63.0%) were male and 17 cases (37.0%) were female. The age of patients ranged from 42 to 77 years old (the median age = 61 years). In this study, the clinicopathological data of CCA patients including histological types, stages, and margin were categorized into groups. The histological types were classified as the non-papillary type of 25 (54.3%) cases and the papillary type of 21 (45.6%) cases. Stages were classified as the early stage of 15 (32.6%) cases and the advanced stage of 31 (67.4%) cases. Margin was classified as R0 of 31 (67.4%) cases and the R1 + R2 of 15 (32.6%). Pearson Chi-square test showed a significant correlation between high expression of $p16^{INK4a}$ tumor suppressor with non-papillary type (p= 0.019). Age, gender, lymph node metastasis, overall metastasis, stage, margin and recurrence did not show any association with the expression of $p16^{INK4a}$ protein (**Table 1**).

Table 1 Correlation between the expression of $p16^{INK4a}$ protein and clinicopathological data of CCA patients.

Factors	N=46	$p16^{INK4a}$ tumor suppressor protein		
		Low	High	p-value
Age (years)				
< 61	21	9	12	0.536
≥ 61	25	13	12	
Gender				
Male	29	16	13	0.193
female	17	6	9	

Table 1 Correlation between the expression of $p16^{INK4a}$ protein and clinicopathological data of CCA patients. (per)

Factors	N=46	$p16^{INK4a}$ tumor suppressor protein		
		Low	High	p-value
Histological type				
Non-papillary	25	8	17	0.019*
Papillary	21	14	7	
Lymph node metastasis				
Absent	24	13	11	0.369
Present	22	9	13	
Overall metastasis				
Absent	22	12	10	0.382
Present	24	10	14	
Stage				
Early stage	15	6	9	0.460
Advanced stage	31	16	15	
Margin				
R0	31	14	17	0.603
R1+R2	15	8	7	
Recurrence				
Absent	32	15	17	0.845
Present	14	7	7	

* p-value less than 0.05 was considered statistically significant.

Discussion and Conclusions

CAFs have important roles, including the support of tumor growth, angiogenesis, metastasis and treatment resistant through the paracrine effect.[3] The expression of $p16^{INK4a}$ protein contributes to the increase in risk of carcinoma and correlates with poor prognosis.[4] This study demonstrated that the expression of $p16^{INK4a}$ protein presented in senescent CAFs of CCA tissues. The high expression of $p16^{INK4a}$ in CAFs was significantly associated with CCA patients with a non-papillary type. It has been report that the non-papillary type of CCA is correlated with poor prognosis.[5] Therefore, senescent CAFs which expressed high level of $p16^{INK4a}$ might be a prognostic factor for predicting a clinical outcome of CCA patients.[5,6]

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