Original Article
Clinicopathological significance of aberrant Notch receptors in intrahepatic cholangiocarcinoma

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Abstract: Notch signaling has been reported to be activated to promote biliary epithelial cell differentiation and tubulogenesis during bile duct development. In this study, clinicopathological significance of aberrant expression of Notch receptors in intrahepatic cholangiocarcinoma (ICC) was investigated. Thus, forty-one ICC specimens were examined by immunohistochemistry using anti-Notch1-4 antibodies, respectively. Expression of Notch receptors was scored by percentage of positive tumor cells and intensity of immunostaining. Clinicopathological parameters and survival data were compared with the expression of Notch receptors, respectively. Expression of Notch receptors was identified in cancer cells, as well as in non-neoplastic cells. Compared with adjacent non-tumor liver tissues, Notch1 and 4 were up regulated, and Notch2 and 3 were relatively weaker. Positive immunostaining of Notch1 in ICC cells was detected in 34 cases (82.9%), Notch2 in 23 (56.1%), Notch3 in 16 (39.0%) and Notch4 in 14 (34.1%). Notch1 was overexpressed in cases with tumor size > 5 cm (P = 0.036). Expression of Notch2 was correlated inversely with histological grade (P = 0.016). Overexpression of Notch4 was more common in cases with serum CA125 > 35 U/ml than cases with CA125 ≤ 35 U/ml (P = 0.048). Expression of Notch3 was not correlated with any other clinicopathological parameters. Moreover, Notch4 was related to poor survival (P < 0.001). To conclude, this study reveals that aberrant expression of Notch receptors 1 and 4 might play important roles during ICC progression.

Keywords: Intrahepatic cholangiocarcinoma, Notch receptors, immunohistochemistry

Introduction

Intrahepatic cholangiocarcinoma (ICC) ranks second in frequency amongst human liver cancers causing 13% of annual cancer-related deaths worldwide and 3% of deaths in Western countries [1]. This life-threatening disease with a dismal outcome is due to rapid tumor growth, tendency to invade adjacent organs and metastasize [2]. Till now, little is known about the mechanism of tumor development and progression of ICC.

Notch signaling has been reported to be an evolutionally highly conserved pathway that regulates physiological processes including cellular differentiation, proliferation, apoptosis and stem cell maintenance in a wide range of organisms [3]. Activation of Notch signaling pathway is mediated by interactions of bordering cells via cell-to-cell contact of membrane-associated Notch receptors and ligands [4].

Binding of Notch receptors (Notch1 through 4) to ligands (Jagged 1 and 2, DLL 1, 3 and 4) triggers receptors to proteolytic cleavages; resulting in release of the Notch intracellular domain (NICD), which translocates to the nucleus and activates transcription of downstream target genes, such as *Hes*, Hey, etc [5]. Recent studies revealed that aberrant Notch signaling is involved in a variety of pathological conditions including cancers. Notch receptors may have a role as an oncogene or a tumor suppressor gene depending upon cell type, though the majority of studies reveal that Notch signaling promotes tumorigenesis [6]. However, there is no report about the overall association between Notch receptors and ICC.

In the present study, we examined the expression of four Notch receptors in human ICC and adjacent non-tumor liver tissues and investigated the correlations between aberrant expression of Notch receptors and clinicopathological

Table 1. Antibodies used for immunohistochemistry

Antibody	Dilution	Company	Non-neoplastic tissues	Aberrant expression in cancer
Notch1	1 : 100	Cell Signaling Technology	Variable	Membrane/cytoplasm/nucleus
Notch2	1 : 400	Abcam	Variable	Membrane/cytoplasm/nucleus
Notch3	1 : 100	Santa Cruz	Variable	Membrane/cytoplasm/nucleus
Notch4	1 : 100	Santa Cruz	Variable	Nucleus
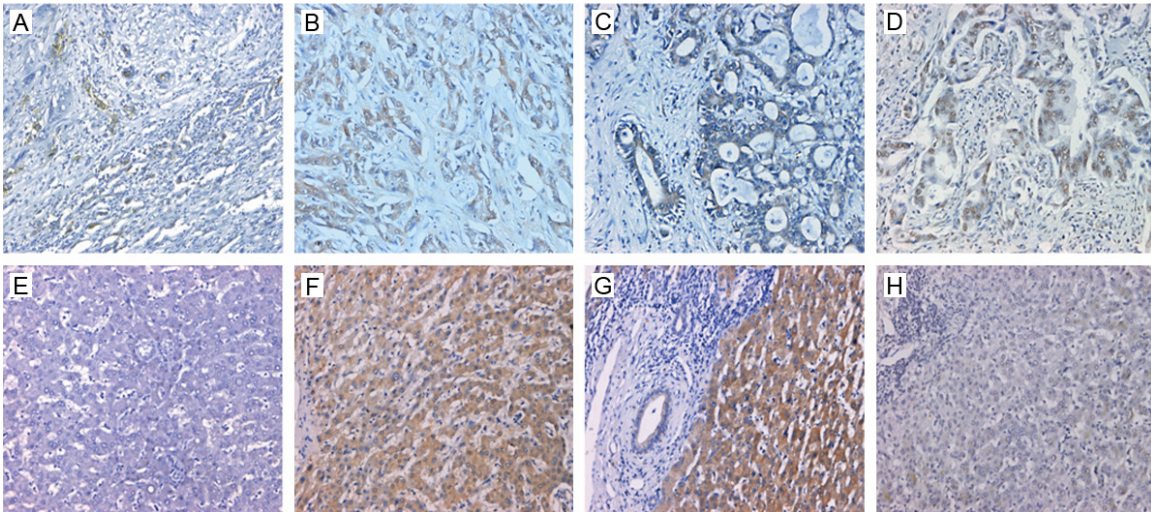
Figure 1. Expression of Notch receptors in ICC (Immunohistochemistry). A, E: Notch receptor 1; B, F: Notch receptor 2; C, G: Notch receptor 3; D, H: Notch receptor 4; A-D: ICCs (200x); E-H: Non-neoplastic tissues (200x).

parameters with survival, to elucidate the possible role of Notch signaling in ICC.

Materials and methods

Patients and specimens

Forty-one patients with surgically resected ICC were included in the present study, from September 2006 till January 2012 at Sun Yat-Sen Memorial Hospital, Guangzhou, China. None of the patients had received chemotherapy or radiation therapy prior to the radical tumor resection. Tissue samples were fixed in 10% formalin and paraffin-embedded for histopathological examination and immunohistochemistry. The project was approved by the Ethical Committee of the Hospital and was in accordance with the Helsinki Declaration of 1975.

Immunohistochemical staining

Immunohistochemical (IHC) staining was carried according to the protocol defined in the PV Two-Step Kit (Zhongshan Goldenbridge Biot-

echnology, Beijing, China) instructions. Briefly, sections of a paraffin-embedded tissue block were deparaffinized twice in xylene for 15 min and rehydrated through graded ethanol solutions. Sections were subsequently heated in a microwave oven twice for antigen retrieval for 8 min. Citrate buffer (10 mmol/L, pH 6.0) was used as the antigen retrieval buffer. Endogenous peroxidases were inactivated by immersing the sections in 0.3% hydrogen peroxide for 15 min. Then the slides were incubated overnight at 4°C in a humidified chamber with Notch1-4 monoclonal antibodies (**Table 1**), respectively. The sections were further incubated with goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate for 30 min at room temperature. Finally, the sections were developed with DAB colour solution (50 µl/section) for 2 min at room temperature. Then hematoxylin (Boster Biotechnology, Wuhan, China) was used as a chromogen (50 µl/section), and the slides were consecutively counter-stained for 30 sec. With the exception of the omission of primary antibodies, negative controls were processed in

Table 2. Expression rates of Notch receptors 1-4 in ICC n (%)

Receptors	Negative	Positive		Total
		Low grade	High grade	
Notch1	7 (17.1)	21 (51.2)	13 (31.7)	41 (100)
Notch2	18 (43.9)	11 (26.8)	12 (29.3)	41 (100)
Notch3	25 (61.0)	14 (34.1)	2 (4.9)	41 (100)
Notch4	27 (65.8)	10 (24.4)	4 (9.8)	41 (100)

the same manner as above. All sections were washed three times in phosphate-buffered saline (PBS, pH 7.4) for 5 min after each step.

Interpretation after IHC

The semi-quantitative method was applied for the immunohistochemical expression of Notch receptors. The percentages of positively stained cells were determined by examination under a microscope of 5 randomly selected foci, which were each composed of > 100 cells. The scoring was based on distribution and intensity according to a previous report [7]. Briefly, the percentage of positive ICC cells with expression of Notch receptors was determined semi-quantitatively and each sample was scored on a scale of 0-4, in which 0: negative, 1: positive staining in 1%-25% of tumor cells, 2: positive staining in 26%-50% of tumor cells, 3: positive staining in 51%-75% of tumor cells, and 4: positive staining in 76%-100% of tumor cells. The intensity of immunostaining was determined as 0: negative staining, 1: weakly positive staining, 2: moderately positive staining, and 3: strongly positive staining. The immunoreactive score of each section was calculated by the sum of these two parameters. The total sum score was graded as negative (sum: 0), low grade (sum: 2-4) and high grade (sum: 5-7).

Statistical analysis

Statistical analysis was conducted with the SPSS software package (version 13.0; SPSS Inc., Chicago, USA). The relation between the expression of Notch receptors in ICC samples and the clinicopathological data was analyzed by two-tailed Chi-square test. Survival durations were calculated using the Kaplan-Meier method. The log-rank test was used to compare cumulative survival in the patient groups. *P* value < 0.05 was considered statistically significant.

Results

Clinicopathological characteristics of patients

Forty-one patients comprised 29 males (70.7%) and 12 females (29.3%), with a range from 29 to 75 years. The mean patient age was 58 years. Well, moderately and poorly differentiated ICCs were showed in 13 (31.7%), 16 (39.0%) and 12 (29.3%) cases, respectively. Fourteen patients (34.1%) showed serum HBs-Ag positive. Four patients (9.8%) showed liver cirrhosis. Twenty-two patients (53.7%) revealed lymph node metastasis. Fourteen cases (34.1%) showed organ invasion. Thirty-six patients showed single tumor (87.8%). Twenty-two patients (53.7%) showed tumor size > 5 cm. Thirty-eight sufferers (92.7%) showed no evidence of bile duct tumor thrombi and 30 patients (73.2%) had no portal vein tumor thrombi, respectively. Three cases (7.3%) showed serum α-fetoprotein (AFP) > 25 ng/ml. Thirty-one cases (75.6%) showed serum CA199 > 35 U/ml. Seventeen cases (41.5%) showed serum CA125 > 35 U/ml.

Expression of Notch receptors

As shown in **Figure 1**, Notch1, 2 and 3 were expressed in membranes, cytoplasm and nuclei of human ICC cells. Notch4 was expressed in nuclei in these cells. Notch1 showed 21 cases (51.2%) of low grade immunoreactivity and 13 cases (31.7%) of high grade immunoreactivity. Notch2: 11 cases (26.8%) of low grade and 12 cases (29.3%) of high grade. Notch3: 14 cases (34.1%) of low grade and 2 cases (4.9%) of high grade. Notch4: 10 cases (24.4%) of low grade and 4 cases (9.8%) of high grade (**Table 2**). These four Notch receptors were also expressed in non-neoplastic biliary epithelial cells with variable intensities, and occasionally in micro-vessels. Notch2, 3 and 4 were also expressed in adjacent liver cells. Compared with adjacent non-tumor liver cells, Notch1 and 4 were up regulated, while Notch2 and 3 were relatively weaker. Notably, in non-neoplastic tissues, Notch4 was expressed in both cytoplasm and nuclei.

Correlation between expression of Notch1-4 and clinicopathological factors with survival

Table 3 summarizes the correlations between expression of Notch receptors and clinicopath-

Table 3. Correlation between expression of Notch receptors 1-4 and clinicopathological parameters

Characteristics		Notch1			Notch2			Notch3			Notch4		
		-	+	P value	-	+	P value	-	+	P value	-	+	P value
Age (years)				0.165			0.147			0.441			0.131
≤ 50	9	3	6		6	3		7	2		8	1	
> 50	32	4	28		12	20		18	14		19	13	
Sex				0.165			0.734			0.734			0.068
Male	29	3	26		12	17		17	12		22	7	
Female	12	4	8		6	6		8	4		5	7	
Cirrhosis				1.000			1.000			0.281			0.280
Yes	4	0	4		2	2		1	3		4	0	
No	37	7	30		16	21		24	13		23	14	
Capsular invasion				0.232			0.754			0.742			0.186
Yes	15	1	14		6	9		10	5		12	3	
No	26	6	20		12	14		15	11		15	11	
Portal vein tumor thrombi				1.000			0.291			0.287			1.000
Yes	11	2	9		3	8		5	6		7	4	
No	30	5	25		15	15		20	10		20	10	
Bile duct tumor thrombi				1.000			0.243			1.000			1.000
Yes	3	0	3		0	3		2	1		2	1	
No	38	7	31		18	20		23	15		25	13	
Lymphatic metastasis				0.419			0.531			0.120			1.000
Yes	22	5	17		11	11		16	6		14	8	
No	19	2	17		7	12		9	10		13	6	
Organ invasion				1.000			0.051			1.000			1.000
Yes	14	2	12		3	11		9	5		9	5	
No	27	5	22		15	12		16	11		18	9	
Tumor number				0.567			0.363			0.362			1.000
Single	36	7	29		17	19		23	13		24	12	
Multiple	5	0	5		1	4		2	3		3	2	
Tumor size				0.036			1.000			0.435			0.754
≤ 5 cm	19	6	13		8	11		10	9		12	7	
> 5 cm	22	1	21		10	12		15	7		15	7	
Tumor stage (*UICC, 2010)				0.651			1.000			0.723			0.275
I + II	11	1	10		5	6		6	5		9	2	
III + IV	30	6	24		13	17		19	11		18	12	
Histological grade				0.165			0.016			0.084			1.000
G1 + G2	29	3	26		9	20		15	14		19	10	
G3 + G4	12	4	8		9	3		10	2		8	4	
HBs-Ag				0.075			1.000			0.332			0.734
Positive	14	0	14		6	8		7	7		10	4	
Negative	27	7	20		12	15		18	9		17	10	
Serum AFP				1.000			0.573			1.000			0.539
≤ 25 ng/ml	38	7	31		16	22		23	15		24	14	
> 25 ng/ml	3	0	3		2	1		2	1		3	0	
CA199				0.164			1.000			0.150			0.447
> 35 U/ml	31	7	24		14	17		21	10		19	12	
≤ 35 U/ml	10	0	10		4	6		4	6		8	2	
CA125				0.679			0.202			1.000			0.048
> 35 U/ml	17	2	15		5	12		10	7		8	9	
≤ 35 U/ml	24	5	19		13	11		15	9		19	5	

Compared via the chi-square test (Fisher's exact test). *UICC: Union for International Cancer Control.
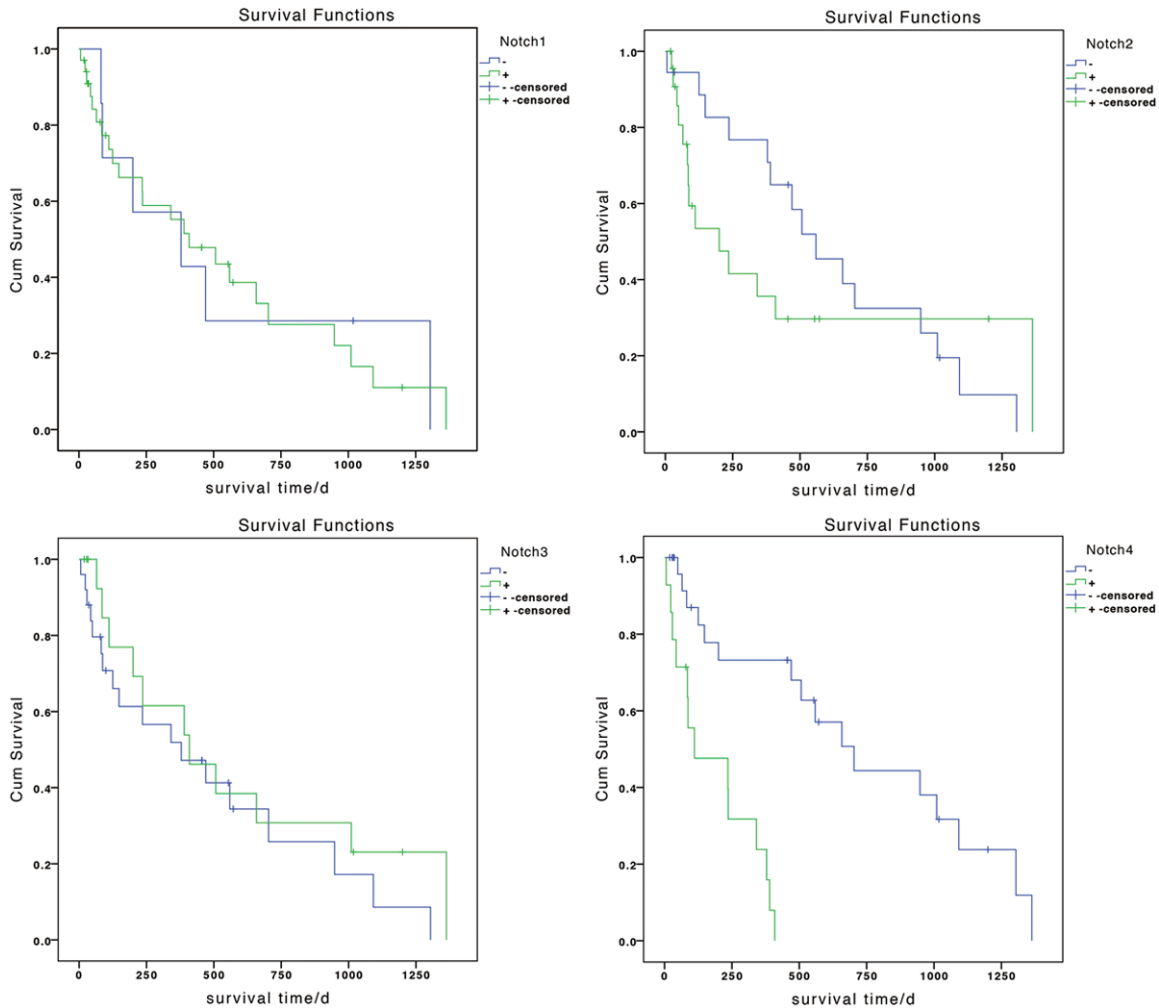
Figure 2. Overall survival curves using the Kaplan-Meier method by log rank test. Median survival was 390 days.

ological parameters, including statistical analyses. Notch1 was overexpress in cases with tumor size > 5 cm (P = 0.036). The level of Notch2 was significantly higher in low histological grade cases than cases with high histological grade (P = 0.016). Notch4 was more common in cases with serum CA125 > 35 U/ml than cases with CA125 ≤ 35 U/ml (P = 0.048). The expression of Notch3 was not correlated with any clinicopathological parameters. None of age, gender, liver cirrhosis, capsular invasion, portal vein tumor thrombi, bile duct tumor thrombi, lymphatic or organ metastasis, tumor number, tumor stage, serum HBs-Ag, serum AFP level and serum CA199 level was correlated with the expression of those receptors. As shown in **Figure 2**, Expression of Notch4 in ICC cells was related to poor survival in a statistically significant manner (P < 0.001). There was no significant correlation between the expres-

sion of Notch1-3 and survival (Notch1: P = 0.936, Notch2: P = 0.446, Notch3: P = 0.363).

Discussion

Notch signaling pathway plays a critical role in cell fate decision, tissue patterning, morphogenesis, and is involved in many malignant tumors [8]. As aforementioned, it is generally accepted that it could behave as either an oncogene or a tumor suppressor gene depending upon cell type [9-14]. Soomin A et al reported that Notch1 and 4 might be markers of poor prognosis in hepatocellular carcinoma [15]. In extrahepatic cholangiocarcinoma and gallbladder carcinoma, it is reported that Notch1 and 3 play a positive role during cancer progression, and DLL4 correlates with poor survival [7]. Three previous reports have connected Notch signaling with ICC. Two papers demonstrated that ICC could originate from hepatocytes in

mice by activating Notch1 [16, 17] and one recent paper showed that Notch1 could induce a migratory effect in ICC by causing an epithelial-mesenchymal transition and activating Rac 1 [18]. Taken together, they indicate that Notch signaling may play a positive role in the development and progression of ICC. However, few studies have dealt with all Notch receptors in this malignancy yet.

In the present study, immunohistochemical analysis showed that Notch receptors 1-4 were all aberrantly expressed in tumor cells of ICC tissues. Notch1, 2 and 3 were expressed in membranes, cytoplasm and nuclei of tumor cells, and Notch4 was expressed only in nuclei. Compared with the adjacent non-tumor liver, Notch1 and 4 were up regulated, whereas Notch2 and 3 were down regulated in ICC. Additionally, we found that the overexpression of Notch1 related to larger tumor size and the overexpression of Notch4 related to higher serum CA125 level, suggesting their up-regulation may be linked to tumor progression. Expression of CA125 (MUC16) has been reported to be a significant independent factor of poor prognosis in ICC-mass forming type [19]. In line with this, we found that high expression of Notch4 was associated with poor overall survival, indicating that it may have a probable role as a poor prognosticator in ICC. These results suggest that the up regulation of Notch receptors 1 and 4 might exert tumorigenic effects in human ICC, reflecting role of oncogene.

Notch2 signaling has been reported to be related to the regulation of biliary epithelial cell differentiation and induction of tubulogenesis during early intrahepatic bile duct development in mice. Mutations of Notch2 lead to Alagille syndrome, a multi-organ disorder involving impaired intrahepatic bile ducts [20]. Herein we found that the up-regulation of Notch2 tended to be related to well histologic differentiation of ICC and it is, to some extent, consistent with the prior report. Taken together that Notch2 was comparatively down regulated in ICC, we supposed that the expression of Notch2 correlated to a higher chance of survival. However, beyond our expectation, P value would be 0.06 if Breslow test wasused to compare cumulative survival. It perhaps discovered that the expression of Notch2 is associated with a poorer out-

look. Of course, this required further elucidation and more cases should be studied for the identification of the biological role in the expression of Notch2.

Notably, our results of immunohistochemistry showed that the expression of Notch4 was seen only in nuclei of tumor cells. However, some previous reports showed that Notch4 was detected in both the nucleus and cytoplasm of tumor cells. The supposed reasons might be that Notch4 signaling was over activated in this kind of malignancy. Almost all the Notch4 receptors were cleaved and the NICD was translocated to the nuclei. In line with this, expression of Notch4 could be found in both cytoplasm and nuclei of non-neoplastic tissues.

Two previous reports demonstrated the relationship between Notch signaling and HBV infection. Trehanpati *et al* verified that HBV infection up-regulated Notch1, TgF-β and FoxP3 expression on intrahepatic T-cells in cirrhosis, resulting in fibrogenesis and disease progression [21]. Pei *et al* confirmed that Notch1 knockdown could regulate the immune balance of Th1/Th2 in chronic hepatitis B patients [22]. In this study, Notch1 tented to be overexpressed in cases with serum HBs-Ag positive (P = 0.075), suggesting that their overexpression may be linked to cancer initiation (hepatitis B virus infection).

However, there was one inherent limitation in the relatively less studied cases for the present study. Another inherent limitation of this study is that all the studied cases were surgically removed cancers, thus we did not include late advanced or early stage cases. This study retrospectively analyzed 41 cases of ICC. Detailed studies of the mechanism are still required to understand the function and significance of Notch signaling in human cancers.

In the context of ICC, the present study implies that the up-regulation of Notch receptors 1 and 4 correlates with cancer progression, and that the overexpression of Notch4 correlates with poor survival. Investigation on a large scale should be performed to understand the contribution of Notch signaling pathway in liver cancer initiation and progression.

Disclosure of conflict of interest

None.

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