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ORIGINAL ARTICLE

The Expression of Notch 1 and Notch 3 in Gallbladder Cancer and Their Clinicopathological Significance

Luyao Liu[1] · Zhu-lin Yang[1] · Chunwei Wang[1] · Xiongying Miao[1] · Zhiyu Liu[1] · Daiqiang Li[2] · Qiong Zou[3] · Jinghe Li[4] · Lufeng Liang[5] · Guixiang Zeng[6] · Senlin Chen[7]

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Abstract Gallbladder cancers (GBCs) are highly malignant gastrointestinal cancers. The biological makers for the prognosis and targeting therapy of GBCs have not been established. The protein expression of Notch 1 and Notch 3 in 46 squamous cell/adenosquamous carcinomas (SC/ASCs) and 80 adenocarcinomas (AC) was measured using immunohistochemistry. Positive Notch 1 and Notch 3 expression in both SC/ASC and AC was significantly associated with large tumor size, invasion, metastasis, and low surgical curability ($P < 0.05$ or $P < 0.01$). Univariate Kaplan-Meier analysis showed that positive Notch 1 and Notch 3 expression was significantly associated with mean survival of SC/ASC and AC patients ($P < 0.01$ or $P < 0.001$). Multivariate Cox regression analysis showed that positive Notch 1 and Notch 3 expression, as well as low differentiation, large tumor size, high TNM stage, invasion, lymph node metastasis, and surgical curability are independent poor-prognostic factors in both SC/ASC and AC patients. Positive Notch 1 and Notch 3 expression is closely correlated with severe clinicopathological characteristics and poor prognosis in both SC/ASC and AC patients.

Keywords Gallbladder cancer · Prognosis · Notch 1 · Notch 3 · Adenocarcinoma · Squamous cell/adenosquamous carcinomas

✉ Zhu-lin Yang
zhulinyang@yahoo.com

[1] Research Laboratory of Hepatobiliary Diseases, Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, People's Republic of China

[2] Department of Pathology, Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, People's Republic of China

[3] Department of Pathology, Third Xiangya Hospital, Central South University, Changsha, Hunan 410013, People's Republic of China

[4] Department of Pathology, Basic School of Medicine, Central South University, Changsha, Hunan 410078, People's Republic of China

[5] Department of Hepatobiliary and Pancreatic Surgery, Hunan Provincial People's Hospital, Changsha, Hunan 410007, People's Republic of China

[6] Department of Pathology, Loudi Central Hospital, Loudi, Hunan 417011, People's Republic of China

[7] Department of Pathology, Hunan Provincial Tumor Hospital, Changsha, Hunan 410013, People's Republic of China

Introduction

Gallbladder cancers (GBCs) are rare, but aggressive gastrointestinal cancers. More than 90 % of GBCs are adenocarcinoma (AC), and less than 10.4 % of GBCs are squamous cell carcinoma (SC) and adenosquamous carcinoma (ASC) subtype [1]. The clinical and pathological features and biological behavior of SC/ASC are unclear due to its low incidence. The current understanding of the characteristics of SC/ASC subtype GBC is based on a small number of clinical case reports and case series analysis [1–6]. In the early stage of SC/ASC, the main symptoms are right upper quadrant pain and discomfort, which are not specific to the disease, making it hard to diagnose. Generally, patients who were diagnosed early and accepted radical surgery have better prognosis. However, most patients with GBC were diagnosed at a late stage with metastasis and invasion to other organs and tissues. It is commonly believed that SC/ASC tumor proliferates faster and is more malignant than AC. SC/ASC is more prone to invading the surrounding organs, but has less regional lymph node and distant organ metastasis compared to AC [1–6]. However, previous reports are controversial or inconsistent. Radical

resection is still the preferred method for SC/ASC treatment, but the effectiveness of surgery largely depends on early diagnosis. In addition, postoperative radiotherapy and chemotherapy exhibit limited efficacy on the prognosis of SC/ASC patients. Biomarkers for the early diagnosis, prognosis, and targeting therapy of gallbladder cancers are currently being investigated; however, few are ready for clinical application.

Table 1 Comparison of clinicopathological characteristics, Notch1 and Notch3 expression between SC/ASC and AC

Clinicopathological Characteristics	SC/ASC (n = 46)	ASC(n = 80)	χ^2	P
Sex				
male	19(41.3)	26(32.5)	0.986	0.352
female	27(58.7)	54(67.5)		
Age (year)				
≤ 45	3(6.5)	16(20.0)	4.143	0.042
>45	43(93.5)	64(80.0)		
Differentiation				
well	16(34.8)	27(33.8)		
moderately	24(52.2)	25(31.3)	8.515	0.014
poorly	6(13.0)	28(35.0)		
Tumor size				
≤ 3 cm	20(43.5)	50(62.5)	4.280	0.039
>3 cm	26(56.5)	30(37.5)		
Gallbladder stone				
no	18(39.1)	42(52.5)	2.093	0.148
yes	28(60.9)	38(47.5)		
TNM stage				
I + II	12(26.1)	21(26.3)		
III	20(33.5)	38(47.5)	0.287	0.866
IV	14(30.4)	21(26.3)		
Lymph node metastasis				
no	17(37.0)	30(37.5)	0.004	0.952
yes	29(63.0)	50(62.5)		
Invasion				
no	16(34.8)	31(38.8)	0.197	0.658
yes	30(62.5)	49(61.3)		
Surgery				
radical	14(30.4)	26(32.5)		
palliative	18(39.1)	28(35.0)	0.215	0.898
biopsy	14(30.4)	26(32.5)		
Mean survival time (month)	10.07(4–25)	10.34(3–27)	0.014	0.906
Notch1				
-	19(41.3)	28(35.0)	0.496	0.489
+	27(58.7)	52(65.0)		
Notch3				
-	17(37.0)	27(33.8)	0.132	0.708
+	29(63.0)	53(66.2)		

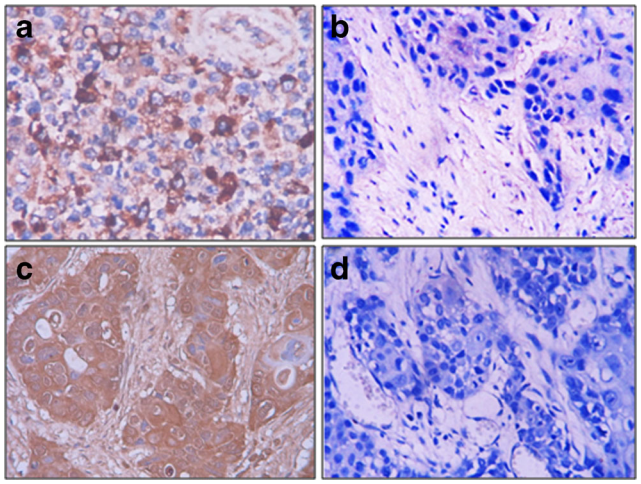

Fig. 1 Immunohistochemical staining of Notch 1 and Notch 3 expression in SC/ASC. **a** Positive Notch 1 expression in poorly differentiated SC. **b** Negative Notch 1 expression in moderately differentiated SC. **c** Positive Notch 3 expression in moderately differentiated SC. **d** Negative Notch 3 expression in moderately differentiated SC. Magnification × 400

Notch signaling is a relatively conserved cell interaction mechanism involved in cell proliferation, apoptosis, and differentiation. Abnormal Notch signaling has been found to be closely related to the development of a variety of human tumors [7–10], and can have oncogenic or tumor suppressive functions depending on the tissue or cell types. Four Notch receptors (Notch 1–4) and five Notch ligands have been identified to play roles in the notch signaling pathway [11]. Studies have reported that Notch 1 can stimulate tumor growth [12, 13]. Overexpression of Notch1 is associated with high malignancy, rapid growth, prone to metastasis and invasion, as well as poor prognosis of some epithelial cancers, such as breast cancer [14], lung cancer [10], gastric cancer [15], colorectal

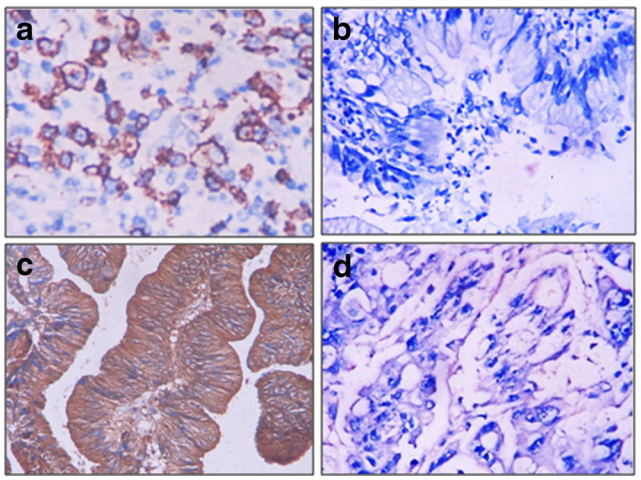

Fig. 2 Immunohistochemical staining of Notch 1 and Notch 3 expression in AC. **a** Positive Notch 1 expression in poorly differentiated AC. **b** Negative Notch 1 expression in well differentiated AC. **c** Positive Notch 3 expression in well differentiated AC. **d** Negative Notch 3 expression in moderately differentiated AC. Magnification × 400

cancer [16], pancreatic cancer [17], cervical cancer [18], ovarian cancer [19] and bladder cancer [20]. Notch1 expression was also reported to play a role as a tumor suppressor in esophageal squamous cell carcinoma, and activation of Notch 1 can induce cancer cell growth arrest and apoptosis [21]. Notch 1 signal inhibits the growth of human tongue squamous cell tumor accompanied by arresting cells at G0 - Gl phase and inducing apoptosis [22]. Overexpression of Notch 1 can inhibit hepatocellular cell proliferation and induce apoptosis [23]. However, the expression of Notch 1 in GBCs and its clinicopathological significances have not been reported.

Notch 3 was first discovered in neuroepithelium, and its intracellular segment of transcription activation domain is obviously shorter than that of Notch 1 and Notch 2 and exhibits a weaker transcriptional activation than other Notch receptors [24]. Recent studies demonstrated that Notch 3 is highly expressed in gastric cancer [25], colorectal cancer [26], hepatocellular carcinoma [27], pancreatic cancer [28], non-small cell lung cancer [29], cervical squamous cell carcinoma [30], ovarian cancer [31] and prostate cancer [32]. These tumors with high Notch 3 expression were more poorly differentiated, grew rapidly, and have lymph node metastasis, invasion, and poor prognosis. Inhibiting Notch 3 expression can inhibit tumor cell proliferation, which may play an important role in the development of malignancies in the epithelium of cancers mentioned above, but the mechanism is not fully understood. In addition, Notch 3 expression has been demonstrated to be closely related to pro-angiogenesis effects in tumors [33]. However, the expression of Notch 3 has not been reported in GBC.

In this study, the expression of Notch 1 and Notch 3 protein was investigated in 46 SC/ASC and 80 AC patients, and their clinical significance was analyzed.

Materials and Methods

Tumor Tissue Collection

Eighty AC and 46 SC/ASC tumor tissues were collected from January 1995 to December 2009 at 3 affiliate hospitals and 4

Table 2 Association of Notch1 and Notch3 expression and the clinicopathological characteristics in patients with SC/ASC

Pathological characteristics	Case No.	Notch1			Notch3		
		Pos. No.(%)	χ^2	*P* value	Pos. No. (%)	χ^2	*P* value
Pathological types							
SC	26	17(65.4)	1.104	0.293	15(57.5)	0.735	0.391
ASC	20	10(50.0)			14(70.0)		
Differentiation							
well	16	7(43.8)	5.695	0.059	8(50.0)	2.236	0.307
Moderately	24	14(58.3)			16 66.7)		
poorly	6	6(100.0)			5(83.3)		
Tumor size							
≤ 3 cm	20	7(35.0)	8.195	0.004	98(40.0)	8.065	0.005
>3 cm	26	20(76.9)			21 (80.8)		
Gallbladder stone							
no	18	9(50.0)	0.922	0.337	12(66.7)	0.167	0.683
yes	28	18(64.3)			17(60.7)		
TNM stage							
I + II	12	3(25.0)			4(33.3)		
III	20	13(65.0)	8.229	0.016	14(70.0)	6.412	0.045
IV	14	11(78.6)			11(78.6)		
Lymph node metastasis							
no	17	5(29.4)	9.538	0.002	6(35.3)	8.912	0.003
yes	29	22(75.9)			23(79.3)		
Invasion							
no	16	6(37.5)	4.546	0.033	6(37.5)	6.870	0.009
yes	30	21(70.0)			23(76.7)		
Surgery							
radical	14	4(28.6)	7.535	0.023	5(35.7)	6.857	0.032
palliative	18	13(72.2)			13(72.2)		
biopsy	14	10(71.4)			11(78.6)		

education hospitals of Central South University. AC and SC/ASC subtype of gallbladder cancers were diagnosed by histological and pathological examinations. SC/ASC was diagnosed when most malignant cells are squamous cells but less than 10 % are adenocarcinoma cells according to the criteria of World Health Organization (WHO). The clinical grade was determined according to standard TNM classification of malignant tumor 7th edition. Radical resection was performed in 14 SC/ASC and 26 AC patients, palliative surgery was performed in 18 SC/ASC and 28 AC patients, and only biopsy was performed on the primary tumor in 14 SC/ASC and 26 AC patients. All patients received no adjuvant or neoadjuvant treatment before tumor tissues were collected. The average age of 46 SC/ASC patients was 55.8 ± 9.6 years and the average age of 80 AC patients was 53.8 ± 9.9 years.

Among the 46 SC/ASC tumors, 16 were well-differentiated, 24 were moderately-differentiated, and 6 were poorly-differentiated. Thirty patients with SC/ASCs had invasion of surrounding tissues and organs, including liver (24 cases), duodenum (1 case), hepatic flexure (1 case), pancreas (2 cases) and greater omentum (2 cases), while 29 had regional lymph node metastasis (any intraoperative and pathologic findings). Among the 80 AC tumors, 27 were well-differentiated, 25 were moderately-differentiated, and 28 were poorly-differentiated. Forty-nine AC patients had invasion, including liver (39 cases), duodenum (2 case), hepatic flexure (3 case), pancreas (3 cases) and greater omentum (2 cases), while 50 had regional lymph node metastasis. Two-year survival information was obtained through letters and phone calls. Patients that survived longer than 2 years were regarded as censored cases. Among the 46 SC/ASC patients, 13 patients survived ≥1 year (4 cases survived >2 years) and 33 patients survived <1 year with an average survival time of 10.07 ± 0.78 months. Among 80 AC patients, 57 survived <1 year and 23 survived ≥1 year (9 cases survived >2 years) with an average survival time of 10.34 ± 0.63 months. This study was pre-approved by the Ethics Committee for Human Research of Second Xiangya Hospital, Central South University.

Immunohistochemistry Staining

Immunohistochemical staining of Notch 1 and Notch 3 protein expression was performed using EnVision™ Detection

Table 3 Association of Notch1and Notch3expression with the clinicopathological characteristics of AC

Clinicopathological characteristics	Case No.	Notch1			Notch3		
		Pos. No.(%)	χ^2	*P* value	Pos. No. (%)	χ^2	*P* value
Differentiation							
well	27	11(40.7)	12.364	0.002	11 (40.7)	12.968	0.002
moderately	25	17(68.0)			18(72.0)		
poorly	28	24(85.7)			24(85.7)		
Tumor size							
≤ 3 cm	50	27(54.0)	7.092	0.006	27(54.0)	8.948	0.004
>3 cm	30	25(83.3)			26(86.7)		
Gallbladder stone							
no	42	25(59.5)	1.166	0.280	27(64.3)	0.153	0.696
yes	38	27(71.1)			26(68.4)		
TNM stage							
I + II	21	9(42.6)			8(38.1)		
III	38	25(65.8)	8.495	0.018	27(71.1)	11.392	0.004
IV	21	18(85.7)			18(85.7)		
Lymph node metastasis							
no	30	12(40.0)	13.187	0.000	12(40.0)	14.792	0.000
yes	50	40(80.0)			41(82.0)		
Invasion							
no	31	14(45.2)	8.756	0.004	14(45.2)	10.067	0.003
yes	49	38(77.6)			39(79.6)		
Surgery							
radical	26	11(42.3)	12.184	0.002	11(42.3))	12.432	0.002
palliative	28	18(64.3)			19(67.9)		
biopsy	26	23(88.5)			23(88.5)		

kit (Dako Laboratories, CA, USA) as previously described. Briefly, 4 μM sections were prepared from routinely paraffin-embedded tumor tissues. After deparaffinization and incubation with 3 % H_2O_2 for 15 min, sections were incubated with rabbit anti-human Notch 1 and Notch 3 antibodies (1:50 dilution, Abgent Company, CA, USA) for 1 h at room temperature, followed by HRP-conjugated anti-rabbit secondary antibody (Abgent) for 30 min. After DAB substrate treatment, sections were counter-stained with hematoxylin and soaking in xylene. The negative control was made by using anti-Green Fluorescent Protein (GFP) antibody (Sigma-Aldrich Shanghai Trading Co Ltd., Shanghai, China) to replace the primary antibody. The positive control was breast cancer sections with positive Notch 1 and Notch 3 expression (Beijing Zhongshan Biotechnology Company, Beijing, China). The percentage of positive cells was calculated from 500 cells in 10 random fields. Tumors with positive staining cells in the cytoplasm ≥25 % were considered positive, while tumors with positive staining cells <25 % were considered negative [34].

Statistical Analysis

Data was analyzed using SPSS 15.0 (the statistical package for the Social Sciences Version 15.0). The relationship of Notch 1 or Notch 3 expression with histological or clinical factors was analyzed using χ2 or Fisher's exact test. Kaplan-Meier test and Cox proportional hazards model were used for univariate survival analysis.

Results

Clinicopathological Characteristics of AC and SC/ASC and Notch 1 and Notch 3 Expression in AC and SC/ASC Tumors

Table 1 shows the comparison of clinicopathological characteristics and Notch 1 and Notch 3 expression between SC/ASC and AC. A significantly higher percentage of AC patients were over the age of 45 compared to SC/ASC patients ($P < 0.05$). A significantly higher percentage of SC/ASC patients had tumor size larger than 3 cm ($P < 0.05$) compared to AC patients. No significant differences in patient's sex, with or without gallstones, TNM stage, lymph node metastasis, invasion of the surrounding organs and tissues, surgical approach (radical, palliative, and biopsy) and the average survival time of patients were observed between AC and SC/ASC patients ($P > 0.05$). Immunohistochemistry revealed that Notch 1 and Notch 3 positive reactions were mainly localized in the cytoplasm of SC/ASCs (Fig. 1) and ACs (Fig. 2). Among 46 SC/ASCs, 27 (58.7 %) and 29 (63.0 %) had Notch 1 and Notch 3 positive expression, respectively. Of the 80 ACs, 52 (65.0 %) and 53 (66.2 %)cases had positive Notch 1 and Notch 3 expression, respectively. There were no significant differences in Notch 1 and Notch 3 expression between AC and SC/ASC patients ($P > 0.05$).

Table 4 Associations of the clinicopathological characteristic, Notch1, and Notch3 expression with the mean survival of patients with SC/ASC

Group	Case No.(n)	Mean survival (month)	Chi-square	*P*
Sex				
male	19	10.74(6–24)	0.767	0.381
female	27	9.85(4–24)		
Age				
≤ 45	3	15.67(8–24)	2.023	0.155
>45	43	9.84(4–25)		
Pathological types				
SC	26	10.19(4–24)	0.223	0.637
ASC	20	10.25(4–24)		
Differentiation				
well	16	13.81(5–24)		
moderately	24	8.92(4–18)	19.125	0.000
poorly	6	5.83(4–9)		
Tumor size				
≤ 3 cm	20	14.35(7–24)	31.337	0.000
>3 cm	26	7.04(4–11)		
Gallbladder stone				
yes	28	11.50(4–24)		
TNM stage				
I + II	12	17.00(9–24)		
III	20	9.20(7–15)	51.139	0.000
IV	14	5.86(4–8)		
Lymph node metastasis				
no	17	14.24(4–24)	16.219	0.000
yes	29	7.86(4–15)		
Invasion				
no	16	15.75(9–24)	32.271	0.000
yes	30	7.27(4–12)		
Surgery				
radical	14	16.64(10–24)		
palliative	18	8.50(6–12)	50.165	0.000
biopsy	14	6.00(4–8)		
Notch1				
-	19	13.16(4–24)	12.088	0.001
+	27	8.15(4–22)		
Notch3				
-	17	14.47(6–24)	17.064	0.000
+	29	7.72(4–15)		

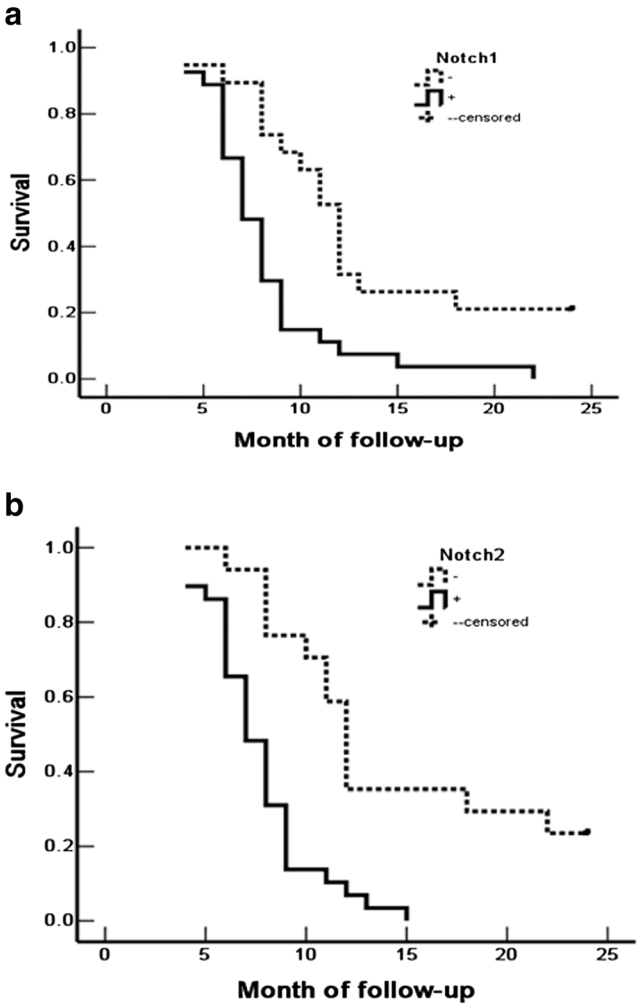

Fig. 3 Notch 1 and Notch 3 expression and survival in patients with SC/ASC. **a** Kaplan-Meier plots of overall survival in patients with SC/ASC and with positive and negative Notch 1 expression. **b** Kaplan-Meier plots of overall survival in patients with SC/ASC and with positive and negative Notch 3 expression

Correlation Analysis of Notch 1 and Notch 3 Expression and the Clinicopathological Characteristics in SC/ASC Patients

The percentage of positive Notch 1 and Notch 3 expression was significantly lower in SC/ASC patients with small tumor size, TNM stage I and II, no invasion, no lymph node metastasis, and radical resection compared to the patients with large tumor size, TNM stage II and IV, lymph metastasis, invasion and radical surgery ($p < 0.05$ or $P < 0.01$). No significant differences in Notch 1 and Notch 3 expression were observed in SC/ASC patients of different sex, age, pathological type, differentiation, and having or having no gallbladder stone (Table 2).

Correlation Analysis of Notch 1 and Notch 3 Expression and the Clinicopathological Characteristics in AC Patients

The percentage of cases with positive Notch 1 and Notch 3 expression was significantly higher in AC patients with poor differentiation, large tumor size, high TNM stage, lymph node metastasis, invasion, and no radical surgery compared to cases with well differentiated tumor, small tumor size, low TNM stage, no lymph node metastasis, no invasion, and radical section of tumors. No significant differences in Notch 1 and Notch 3 expression were observed in AC patients with different sex, age, with or without gallbladder stone (Table 3).

The Correlation of Notch 1 and Notch 3 Expression with Survival in Patients with SC/ASC Tumors

The Kaplan-Meier survival analysis in 46 patients with SC/ASC (Table 4, Fig. 3) revealed that patients with positive Notch 1 and Notch 3 expression survived significantly shorter mean time than patients having negative Notch 1 ($P = 0.001$)

Table 5 Multivariate Cox regression analysis of survival rate in SC/ASC patients

Groups	Factors	B	SE	wald	*P*	RR	95 % CI	
							Lower	Upper
Pathological type	SC/ASC	0.300	0.383	0.615	0.433	1.350	0.638	2.858
Differentiation	well/moderately/poorly	1.503	0.426	12.428	0.000	4.494	1.949	10.364
Tumor size	≤3 cm/>3 cm	2.489	0.797	9.752	0.002	12.048	2.526	57.457
Gallbladder stone	no/yes	0.780	0.484	2.599	0.107	2.181	0.845	5.628
Lymph node metastasis	no/yes	1.522	0.480	10.049	0.002	4.580	1.788	11.736
TNM stage	I + II/III/IV	1.352	0.441	9.400	0.002	3.867	1.629	9.180
Invasion	no/yes	2.542	0.782	10.573	0.001	12.700	2.745	58.767
Surgery	radical/palliative/biopsy	1.058	0.413	6.545	0.011	2.880	1.281	6.476
Notch1	−/+	1.143	0.435	6.894	0.009	3.135	1.336	7.357
Notch3	−/+	1.383	0.482	8.223	0.004	3.989	1.549	10.268

and Notch 3 ($P < 0.001$) expression. The SC/ASC patients with high differentiation, small tumor size, low TNM stage, no lymph node metastasis, invasion, and non radical surgery survived significantly longer ($P < 0.05$ or $P < 0.01$) (Table 4). Cox multivariate analysis showed that poor differentiation, large tumor size (≥ 3 cm), TNM III or IV stage, invasion, lymph node metastasis, and no resection were negatively associated with survival. Positive Notch 1 and Notch 3 expression was negatively correlated with mean survival of SC/ASC patients (Table 5).

The Correlation of Notch 1 and Notch 3 Expression with Survival in Patients with AC Tumors

The Kaplan-Meier survival analysis in AC (Table 6, Fig. 4) patients revealed that patients with positive Notch 1 and Notch 3 expression survived significantly shorter mean time than patients with negative Notch 1 ($P < 0.001$) and Notch 3 ($P < 0.001$) expression. The large tumor size, low differentiation, high TNM stage, lymph node metastasis, invasion, and no radical surgery were also significantly associated with shorter mean survival time in AC patients ($p < 0.001$). Cox multivariate analysis of AC patients revealed that differentiation, tumor size (≥ 3 cm), TNM stage, invasion, lymph node metastasis, and positive Notch 1 and Notch 3 expression were negatively correlated with mean survival (Table 7), while Notch 1 and Notch 3 expression are independent risk factors for AC patients (Table 7).

Table 6 Associations between clinicopathological characteristics, Notch1, and Notch3 expression and the mean survival of patients with AC

Groups	Case No.(n)	Mean survival (month)	Chi-square	*P* value
Sex				
male	26	9.58(3–24)	2.567	0.109
female	54	11.30(3–24)		
Age (year)				
≤ 45	16	10.81(4–24)	0.003	0.956
>45	64	10.72(3–24)		
Differentiation				
well	27	15.07(5–24)		
moderately	25	10.60(4–24)	32.501	0.000
poorly	28	6.68(3–14)		
Tumor size				
≤ 3 cm	50	13.70(6–24)	68.283	0.000
>3 cm	30	5.80(3–10)		
Gallbladder stone				
no	42	10.19(3–24)	0.246	0.620
yes	38	11.34(4–24)		
TNM stage				
I + II	21	18.96(5–24)		
III	38	9.29(6–15)	105.825	0.000
IV	21	5.14(3–7)		
Lymph node metastasis				
no	30	16.27(4–24)	42.372	0.000
yes	50	7.42(3–14)		
Invasion				
no	31	16.68(7–24)	55.535	0.000
yes	49	6.98(3–11)		
Surgery				
radical	26	18.31(10–24)		
palliative	28	8.64(6–11)	113.141	0.000
biopsy	26	5.42(3–9)		
Notch1				
-	28	16.00(7–24)	30.615	0.000
+	52	7.90(3–18)		
Notch3				
-	27	16.59(6–24)	34.735	0.000
+	53	7.76(3–16)		

Discussion

Gallbladder cancers exhibit a highly aggressive nature and poor prognosis. Although radical resection is effective for treating early stage disease, most GBCs are diagnosed at an advance stage without the chance for radical surgery. Currently, radiotherapy and chemotherapy have no significant benefits on patient's prognosis. Biomarkers for the prognosis and targeting therapy of gallbladder cancers are not clinically available. This study measured Notch 1 and Notch 3 expression in 46 SC/ASC and 80 AC patients and evaluated their clinical significance. Our study suggests that Notch 1 and Notch 3 are predictive markers for poor prognosis and markers for disease prognosis.

Gallbladder cancer is an uncommon cancer in the digestive system, with the AC subtype accounting for more than 85 % of gallbladder cancers. Most ACs are well and moderately differentiated (28). SC/ASC is a rare subtype of GBC with an incidence of less than 10.4 %. SC/ASC and AC tumors are thought to have similar clinical manifestations, such as insidious onset and early non-specific clinical symptoms. Early diagnosis is very difficult. Late GBC can exhibit persistent upper abdominal pain, mass, jaundice, and other clinical manifestations (1–6). In this study, 73.8 % of SC/ASC patients had TNM III and IV stage diseases, which is the same as AC (73.8 %). Most of the literature suggests that the squamous cell carcinoma has higher proliferation capacity compared to adenocarcinoma. Thus, SC/ASC tumor is relatively malignant and has poor prognosis, but has lower metastatic potential than AC (1–6). This study showed that 56.5 % of SC/ASC had a tumor size larger than 3 cm which was significantly

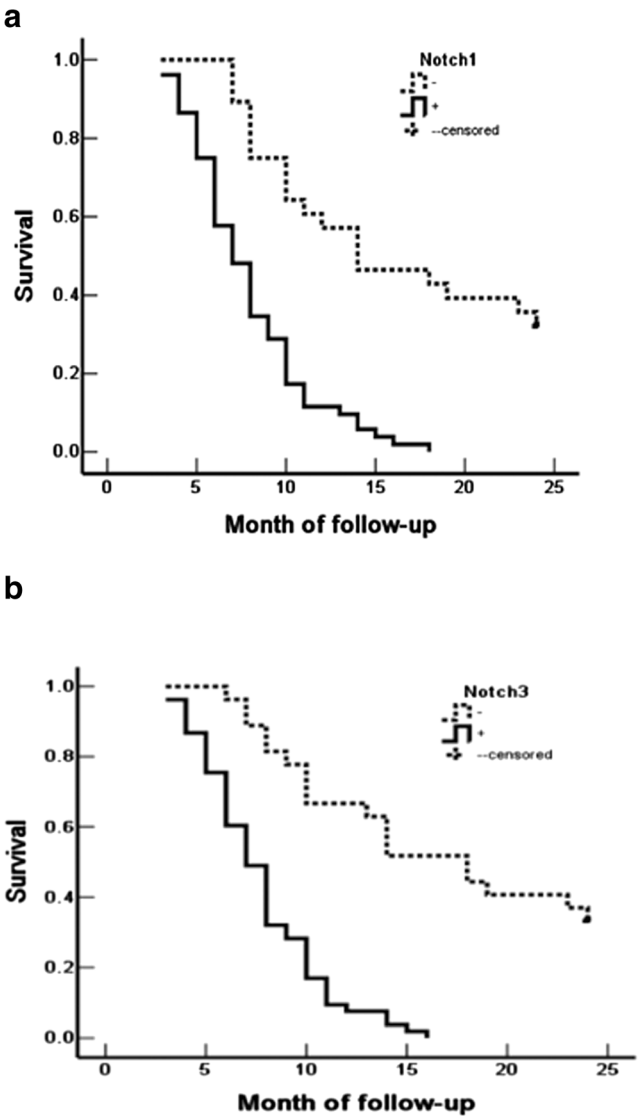

Fig. 4 Notch 1 and Notch 3 expression and survival in patients with AC. **a** Kaplan-Meier plots of overall survival in patients with AC and with positive and negative Notch 1 expression. **b** Kaplan-Meier plots of overall survival in patients with AC and with positive and negative Notch 3 expression

higher than that in AC (37.5 %). However, there was no significant difference in the incidence of lymph node metastasis and invasion to surrounding tissues and organs. The prognosis of SC/ASC and AC is very poor. Most patients with TNM stage III and IV disease die within 2.5 years after diagnosis and the 5-year survival rate is less than 4 %, but 30.4 % of SC/ASC and 32.5 % of AC patients were diagnosed at clinical stage I and II, and these patients received radical resection. The 5-year survival in patients with TNM stage I was up to 60 %. Our study also revealed that tumor differentiation, lymph node metastasis, and invasion were patients' prognostic factors. Thus, early diagnosis is very important. This study also showed that there were no significant differences in most of the clinical and pathological characteristics between these AC and SC/ASC subtypes of GBCs.

Through the Notch1 receptor, Notch signaling pathway regulates cell apoptosis, proliferation and differentiation, as well as the morphological development of multicellular animals. More and more studies have demonstrated that pathological changes of Notch 1 signal are associated with a number of tumors. For example, high Notch 1 expression has been revealed to be associated with the histological grade of tumors, TNM stage, invasion and metastasis, as well as poor prognosis in patients with breast cancer [14], ovarian cancer [9, 19], thyroid cancer [35], and colorectal cancer [16]. Notch 3 also plays an important role in the development of tumors. High Notch 3 expression was observed in the tumor tissues of head and neck cancer [36], non-small cell lung cancer [29], glioma [37], malignant melanoma [38], pancreatic cancer [29], prostate cancer [32], gastric cancer [25], colon cancer [26], abnormal hepatocellular carcinoma [27], cervical cancer [30], and ovarian cancer [31]. In these tumors, high Notch 3 expression was associated with high malignancy, rapid progression, prone to metastasis and invasion as well as poor prognosis. However, Notch 1 and Notch 3 expression in SC/ASC and AC gallbladder cancers has not been reported. Our study found that Notch 1 and Notch 3 were highly expressed in both AC

Table 7 Multivariate Cox regression analysis of survival rate in AC patients

Groups	Factors	B	SE	wald	*P*	RR	95 % CI	
							Lower	Upper
Differentiation	well/moderately/poorly	0.857	0.346	6.117	0.013	2.356	1.195	4.646
Tumor size	≤3 cm/>3 cm	0.974	0.419	5.413	0.020	2.648	1.166	6.015
Gallbladder stone	no/yes	0.340	0.266	1.633	0.201	1.404	0.834	2.365
TNM stage	I + II/III/IV	1.293	0.426	9.209	0.002	3.645	1.581	8.405
Lymph node metastasis	no/yes	1.022	0.468	4.772	0.029	2.777	1.111	6.946
Invasion	no/yes	0.998	0.477	4.383	0.036	2.714	1.066	6.912
Surgery	radical/palliative/biopsy	1.196	0.443	7.301	0.007	3.308	1.389	7.881
Notch1	−/+	1.274	0.478	7.105	0.008	3.575	1.401	9.122
Notch3	−/+	1.316	0.487	7.304	0.007	3.729	1.436	9.687

and SC/ASC tumors. Positive Notch 1 and Notch 3 expression was significantly associated with poor differentiation, large tumor size, high TNM stage, metastasis, invasion, and poor prognosis in both SC/ASC and AC patients, but no significant differences were observed between AC and SC/ASC tumors.

In conclusion, high expression of Notch 1 and Notch 3 protein in AC and SC/ASC subtypes of gallbladder cancers are important biological markers of disease severity and poor prognosis. Targeting inhibition of Notch 1 and Notch 3 expression may be an option for developing therapeutic agents.

Compliance with Ethical Standards

Conflict of Interest All authors declared no conflict of interest.

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