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Original Article

Positive ADAR1 expression indicated poor prognosis in hilar cholangiocarcinoma

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Abstract: Objective: Adenosine deaminase acting on RNA (ADAR)1 played important roles in the progression of human cancers. However, little was known the roles of ADAR1 in the progression of hilar cholangiocarcinoma (HC). Methods: The expression of ADAR1 was detected by western blotting and immunohistochemistry, the potential relevance to clinicopathological characteristics and prognostic significance of ADAR1 in HC patients were also investigated. Results: The expression protein levels of ADAR1 in HC tissues and cell lines were both significantly higher than those in non-tumor tissues and normal bile duct cell line. Size of tumor, lymph node metastasis, vascular invasion and perineural infiltration were identified as independently risks of ADAR1 expression. Patients with positive ADAR1 expression presented shorter OS and DFS than those without negative ADAR1 expression. Further we found differentiation and ADAR1 expression were identified as the independent factors of the overall survival, while ADAR1 expression were identified as the independent factor of the disease-free survival. Conclusions: ADAR1 performed important functions in the aggressiveness of HC and positive ADAR1 expression indicated poor prognosis for HC patients.

Keywords: Hilar cholangiocarcinoma, ADAR1, prognosis

Introduction

Hilar cholangiocarcinoma (HC), which comprises over 60-70% of cholangiocarcinoma, develops from the right and/or left hepatic ducts at or near the biliary confluence [1, 2]. HC is an fatal tumor with a poor prognosis for lower curative surgery ratio and little effectiveness of adjuvant therapy such as chemotherapy and radiation [3, 4]. The etiology of HC is a complex process that involved changes of a series of molecular biology. Among them, the mechanism of DNA imbalance is one of the research hotspots [5]. RNA editing is a widespread post-transcriptional process among DNA formation [6]. And the most frequent type of RNA editing is the conversion of adenosine to inosine, whcih is catalyzed by the double-stranded RNA specific adenosine deaminase acting on RNA (ADAR) family of proteins [7]. Editing by ADAR1 has been implicated in embryonic hematopoiesis and development of various non-nervous tissues [8]. It is also known that ADAR1 shows different expression levels in cancer tissues and ADAR1 tends to promote the occurrence and progression of cancers. ADAR1 played oncogenic roles through their catalytic deaminase domains in the occurrence and progression of cervical squamous cell carcinoma [9]. While ADAR1 contributed to gastric cancer development and progression via activating mTOR/p70S6K/S6 ribosomal protein signaling axis [10]. Song et al. found ADAR1 expression was associated with tumour-infiltrating lymphocytes, and low ADAR1 expression demonstrated the best disease-free survival (DFS) in triple-negative breast cancer with lymph node metastasis [11]. However, little was konwn ADAR1's role in HC progression. In present study, the protein expression values of ADAR1 in HC tissues and cell lines, as well as their associations with the overall survival (OS) and DFS, were assessed.

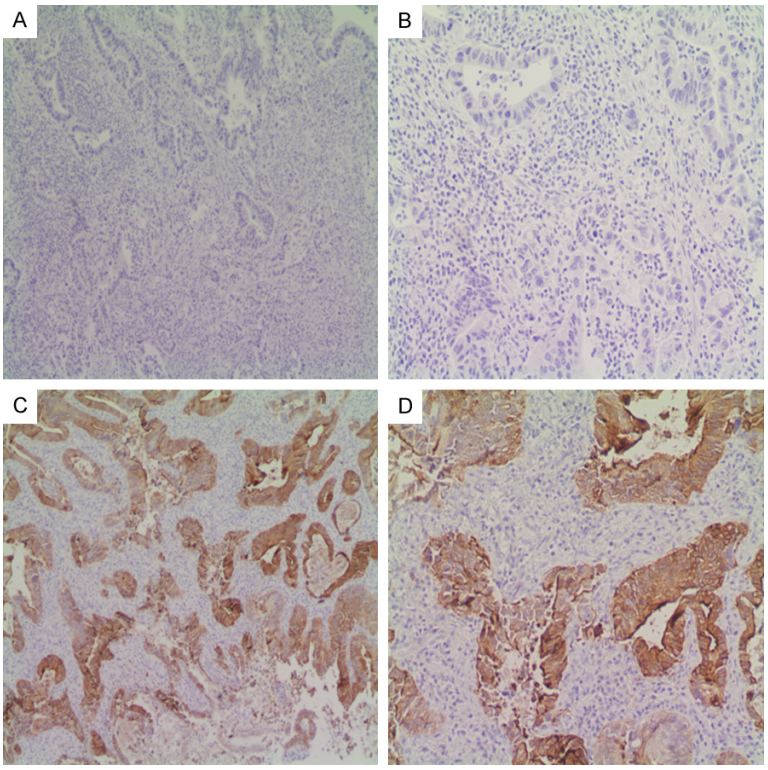

Figure 1. A, B. ADAR1 is negatively expressed. C, D. ADAR1 is positively expressed.

Patients and methods

Ethics statement

This research was approved by the Ethics Committee of Tianjin Nankai Hosptal, and written informed consent was obtained from each patient involved in the study.

Patients and information collection

Patients with HC and who underwent curative surgery at Tianjin Nankai Hosptal between April 2009 and December 2014 were collected. The eligibility criteria for this study were as follows: (1) Histologically proven HC, (2) Patients without distant metastasis or peritoneal dissemination that was confirmed during the operation (3) The patients were not subjected to radiation, chemical or biological treatment before potentially curative surgery was performed, (4) Adjuvant chemotherapy or radiotherapy was not routinely administered to the patients, (5) Availability of complete follow-up data. Fresh HC tissues and non-tumor tissues were collected from 10 HC patients between January 2017 and July 2017 to investigate protein ex-pression values.

Cell lines

HC cell lines FRH-0201 and QBC939 were purchased from the fourth military medical university (Chongqin, China). Normal bile duct cell line HIB-EC was purchased from Biowit Technologies, Ltd. (Shenzhen, China). Cell lines were maintained at 37°C in a humidified atmosphere at 5% CO_2 and 95% air in RPMI 1640 (Thermo Electron Corporation, Beijing, China) with 10% (v/v) FBS (Life Tech, Mulgrave Victoria, Australia) and penicillin-streptomycin (10,000 IU/mL penicillin and 20 mg/mL streptomycin; Roche, Swiss).

Western blotting

Total protein extracts were separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel electrophoresis and were electrotransferred to PVDF membranes. After blocking nonspecific binding sites for 60 min with 5% nonfat milk, the membranes were incubated overnight at 4°C with a primary rabbit antihuman ADAR1 (ab126745, 1:1,000, abcam) The membranes were then washed 3 × 15 min with PBS-T and probed with a horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin antibodies (1:2,000 dilution; ZhongShan Biotechnology) for 60 min at room temperature. The membranes were then washed three times with PBS-T for 10 min. The immunocomplexes was visualized by enhanced chemoluminescence system (Cell Signaling, USA). The intensity of the protein bands was determined by densitometry using AlphaEase-FC software (Alpha Innotech, USA). To confirm equal loading, GAPDH antibody was served as a loading control.

Immunohistochemistry analysis

Formalin-fixed tissues were embedded in paraffin, sectioned at 4 μm, and mounted on silane-coated slides for immunohistochemistry

Table 1. Clinicopathological characteristics of HC patients

Variables	Cases (n, %)
Gender	
Male	66 (73.3%)
Female	24 (26.7%)
Age	
≤ 60	52 (57.8%)
> 60	38 (42.2%)
Size of tumor	
≤ 4	57 (63.3%)
> 4	33 (36.7%)
Bismuth-Corlette classification	
Type I-II	33 (36.7%)
Type III	32 (35.6%)
Type IV	25 (27.8%)
Differentiation	
Well/Moderate	28 (31.1%)
Poor	62 (68.9%)
Tumor stage	
T1	14 (15.6%)
T2	20 (22.2%)
T3	56 (62.2%)
Lymph node metastasis	
Yes	44 (48.9%)
No	46 (51.1%)
Vascular invasion	
Yes	19 (21.1%)
No	71 (78.9%)
Perineural infiltration	
Yes	12 (13.3%)
No	78 (86.7%)

analysis. The sections were deparaffinized with dimethylbenzene and rehydrated through 100, 95, 90, 80, and 75% ethanol. Antigen retrieval treatment was done at 95°C for 20 min in 0.01 mol/L sodium citrate buffer (pH 6.0), and endogenous peroxidases were blocked using 3% hydrogen peroxide for 15 min at room temperature. They were washed in PBS and blocked with 10% goat serum (ZhongShan Biotechnology, China) for 30 min, then incubated with rabbit anti-human ADAR1 polyclonal antibody (ab126745, 1:50, abcam) in a humidified chamber at 4°C overnight. Following three additional washes in PBS, the sections were sections were incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Finally, the visualization signal was developed with 3,3'-diaminobenzidine solution, and all of the slides were counterstained with 20% hematoxylin. The slides were dehydrated and mounted on cover slips. For negative controls, PBS was used in place of primary antibody.

Immunohistochemical assessment

The staining score in cells in each slide was assessed according to the staining intensity and the percentage of the positive cells. The final staining score of more than 3 was defined as positive expression. The staining intensity was scored as 0 (negative), 1 (very weak), 2 (weak), 3 (medium) and 4 (strong). The extent of staining was scored as 0 (0-10%), 1 (10%-30%), 2 (30%-50%), 3 (50%-75%) and 4 (> 75%) according to the percentage of positive-staining cells in relation to the total cancer cells [9].

Follow-up

After curative surgery, all patients were followed every 3-6 months for 2 year, then every year or until death. If recurrence was suspected in some specific patients, further assessments such as abdominal CT or magnetic resonance cholangiopancreatography were used to make a definitive diagnosis. The follow-up of all patients who were included in this study was completed in September 2017.

Statistical analysis

Continuous variables were described using mean ± standard deviation, differences in the different variable were estimated paired-sample t-test. Qualitative correlation analysis was performed by χ^2 test. Multivariate correlation analysis was conducted by logistic regression. The survival was compared through the Kaplan-Meier method and log-rank tests. Cox proportional hazards regression model was used to analyze the independent prognostic factors. For all analyses, $P < 0.05$ was considered statistically significant. All statistical analyses were carried out using SPSS software 18.0.

Result

Patient characteristics

Based on inclusion, 90 HC patients were eligible for this study. ADAR1 expression in paraffin specimens were of HC patients were observed

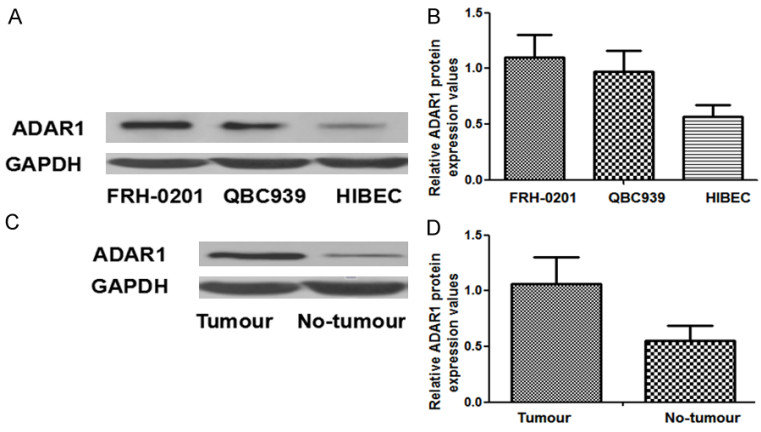

Figure 2. A. Western blot analysis of ADAR1 in cell lines; B. Relative ADAR1 protein expression values in cell lines (t-test); C. Western blot analysis of ADAR1 in HC tissues and normal tissues; D. Relative ADAR1 protein expression values in GC tissues and normal tissues (t-test).

in the cytoplasmby immunohistochemical staining (**Figure 1**). There were 35 positive ADAR1 expression patients in present study. The other clinicopathological characteristics were shown in **Table 1**.

Protein expression

The protein expression values of ADAR1 were detected in HC tissue, non-tumor tissue, HC cell lines and normal bile duct cell line. ADAR1 expression values in HC cell lines FRH-0201 (1.10 ± 0.23) and QBC939 (0.97 ± 0.19) were significantly higher than those normal bile duct cell line HIBEC (0.54 ± 0.11) ($P < 0.05$). While the relative protein expression values of ADAR1 in HC tissues were higher than those innormal tissues (1.06 ± 0.24 vs 0.55 ± 0.16) ($P < 0.05$) (**Figure 2**).

Associations between the status of ADAR1 and clinicalpathological characteristics

The ADAR1 expression in different gender, age, size of tumor, location, differentiation, Bismuth-Corlette classification, tumor stage, lymph node metastasis, vascular invasion and perineural infiltration were analyzed. Chi-square test showed size of tumor, tumor stage, lymph node metastasis, vascular invasion and perineural infiltration were related with ADAR1 expression. Logistic regression found size of tumor (hazard ratio (HR) = 2.217; P = 0.001), lymph node metastasis (HR = 2.159; P = 0.012) vascular invasion (HR = 0.241; P = 0.049) and perineural infiltration (HR = 0.061; P = 0.005) were identified as independently risks of ADAR1 expression (**Tables 2** and **3**).

Survival analysis

Univariate analysis showed significant relationships between the OS and size of tumor, differentiation, tumor stage, lymph node metastasis and ADAR1 expression ($P < 0.05$), not gender, age, Bismuth-Corlette classification vascular invasion and perineural infiltration ($P > 0.05$). Furthermore, differentiation (HR = 2.007; P = 0.019) and ADAR1 expression (HR = 1.987; P = 0.022) were identified as the independent factors of the OS. Meanwhile, the relationships between the DFS and clinicopathological factors were anaylzed. Univariate analysis showed that differentiation, tumor stage, lymph node metastasis and ADAR1 expression were prognostic factors for DFS. ADAR1 expression (HR = 2.034; P = 0.029) were identified as the independent factor of the DFS following the multivariate analysis (**Table 4**). Patients with positive ADAR1 expression presented shorter OS and DFS than those without negative ADAR1 expression (**Figure 3**).

Discussion

Genetic alterations and dysregulated epigenetic modifications play important roles in progression of cancers [12]. Recently, we found that RNA editing might play improt roles in the process of RNA transcription, adenosine to inosine RNA editing is a posttranscriptional process mediated by ADAR enzymes, which changes the primary sequence of RNA and are interpreted as guanosine by the translational machinery [13]. ADARs are implicated in development of diseases. There are three members of ADAR family: ADAR1, ADAR2 and ADAR3. ADAR 1 has 2 major isoforms: a shorter ADAR1p110 and a longer interferon-inducible ADAR1p150, which are synthesized from different translation-initiating methionines [14, 15]. ADAR 1 binds and edits double-stranded RNA secondary structures found mainly within untranslated regions of many transcripts. Editing of pri-miRNAs and small RNAs by ADAR1 may

Table 2. Relationships between ADAR1 and clinicopathological factors by χ^2 test

Variables	ADAR1 expression		*p* value
	Positive (n, %)	Negative (n, %)	
Gender			0.744
Male	25 (71.4)	41 (74.5)	
Female	10 (28.6)	14 (25.5)	
Age			0.923
≤ 60	20 (57.1)	32 (58.1)	
> 60	15 (42.9)	23 (41.9)	
Size of tumor			0.006
≤ 4	16 (45.7)	41 (74.5)	
> 4	19 (54.3)	14 (25.5)	
Bismuth-Corlette classification			0.823
Type I-II	12 (34.2)	21 (38.1)	
Type III	12 (34.2)	20 (36.3)	
Type IV	11 (31.6)	14 (25.6)	
Differentiation			0.678
Well/Moderate	10 (28.6)	18 (32.7)	
Poor	25 (71.4)	37 (62.3)	
Tumor stage			0.017
T1	1 (2.8)	13 (23.6)	
T2	7 (20.0)	13 (23.6)	
T3	27 (77.2)	29 (52.8)	
Lymph node metastasis			0.002
Yes	10 (28.6)	34 (61.8)	
No	25 (71.4)	21 (38.2)	
Vascular invasion			0.015
Yes	12 (34.2)	7 (12.7)	
No	23 (65.8)	48 (87.3)	
Perineural infiltration			0.001
Yes	10 (28.6)	2 (3.6)	
No	25 (71.4)	53 (96.4)	

Table 3. Relationships between ADAR1 and clinicopathological factors by Logistic regression

Variables	Multivariate		
	HR value	*p* value	(95% HR)
Size of tumor	2.217	0.001	1.987-3.126
Tumor stage	1.530	0.322	0.887-1.987
Lymph node metastasis	2.159	0.012	1.876-2.654
Vascular invasion	0.241	0.049	0.0345-0.968
Perineural infiltration	0.061	0.005	0.0456-0.798

interfere with miRNA biogenesis at the precursor stage and thereby alter the changes of molecular biological [16]. ADAR1 was reported to play essential roles in hematopoietic stem cell maintenance and the development of non-nervous tissues. ADAR1 had fundamental roles in the regulation of cancer cell phenotype and lead to the development of the cancers. In cervical cancer, the expression level in squamous cell carcinoma tissues was higher than the CIN and non-cancerous tissues, further they found the OS rate of ADAR1 positive patients was significantly lower compared with that of patients with negative ADAR1 expression, and they assumed ADAR1 might play important roles in the occurrence, progression and prognosis of cervical squamous cancer [9]. Meanwhile, other studies found patients with ADAR1 gene amplification had poor outcomes in lung cancer, ADAR1 overexpression enhances the editing frequencies of target transcripts such as NEIL1 and miR-381 and they thought ADAR1 as an oncogene affects downstream RNA editing and patient prognosis in lung cancers [17]. In esophageal squamous cell carcinoma, patients with overexpression of ADAR1 displayed a poor prognosis. In vitro and in vivo found adenosine to inosine editing events mediated by ADAR1 drive the development of esophageal squamous cell carcinoma [18]. Patients with ADAR1 overexpression in hepatocellular carcinoma was a risk of liver cirrhosis and postoperative recurrence and had poor prognoses [19]. However, the carcinogenic role of ADAR1 in HC remained unknown.

In present study, we investigated ADAR1 expression in HC and its correlation with clinicopathological characteristics of patients, including OS and DFS. We first investigated the ADAR1 protein expression in HC tissues and cell lines, and we found the protein expression levels of ADAR1 in HC tissues and cell lines were both significantly higher than those in non-tumor tissues and normal bile duct cell line. These observations sug-

Table 4. Univariate and multivariate analysis of factors influencing OS and DFS

Variables	OS			DFS		
	Univariate	Multivariate analysis		Univariate	Multivariate analysis	
	p value	HR value (95% HR)	*p* value	*p* value	HR value (95% HR)	*p* value
Gender	0.256			0.678		
Male						
Female						
Age	0.130			0.805		
≤ 60						
> 60						
Size of tumor	0.034	1.380 (0.887-1.765)	0.228	0.126		
≤ 4						
> 4						
Bismuth-Corlette classification	0.204			0.208		
Type I-II						
Type III						
Type IV						
Differentiation	0.005	2.007 (1.498-2.254)	0.019	0.023	1.713 (0987-2.003)	0.077
Well/Moderate						
Poor						
Tumor stage	0.005	1.394 (0.765-1.675)	0.088	0.017	1.325 (0.567-1.698)	0.157
T1						
T2						
T3						
Lymph node metastasis	0.002	1.448 (0.897-1.765)	0.199	0.002	1.618 (0.794-1.956)	0.114
Yes						
No						
Vascular invasion	0.180			0.055		
Yes						
No						
Perineural infiltration	0.321			0.359		
Yes						
No						
ADAR1 expression	< 0.001	1.987 (1.564-2.365)	0.022	< 0.001	2.034 (1.459-2.654)	0.029
Positive						
Negative						

gested that ADAR1 may functionas an oncogene in HC and ADAR1 may be a new biomarker in identifying the HC tumour. Further the correlations between ADAR1 expression with clinicopathological characteristics were analyzed. Our findings indicated the associations of ADAR1 with the size of tumor, lymph node metastasis, vascular invasion and perineural infiltration. Large size of tumor, more lymph node metastasis, vascular invasion and perineural infiltration predicted positive ADAR1 expression, which indicating that ADAR1 may affect the invasion, metastasis and progression of HC, ADAR1 was associated with aggressive pathological features in HC patients. However, the mechanism is not clear, which needs for further study. Last we assessed the prognostic value of ADAR1 expression in HC, and ADAR1 expression was identified as the independent factors of the OS and DFS. Patients with positive ADAR1 expression presented shorter OS and DFS than those without negative ADAR1 expression, which indicated that patients with positive ADAR1 expression may be a high-risk group with poor survival and ADAR1 may be applied as potential target for HC treatment.

In conclusion, our study demonstrated that ADAR1 was upregulated in HC, ADAR1 was a novel biomarker in identifying HC and evaluating the prognosis.

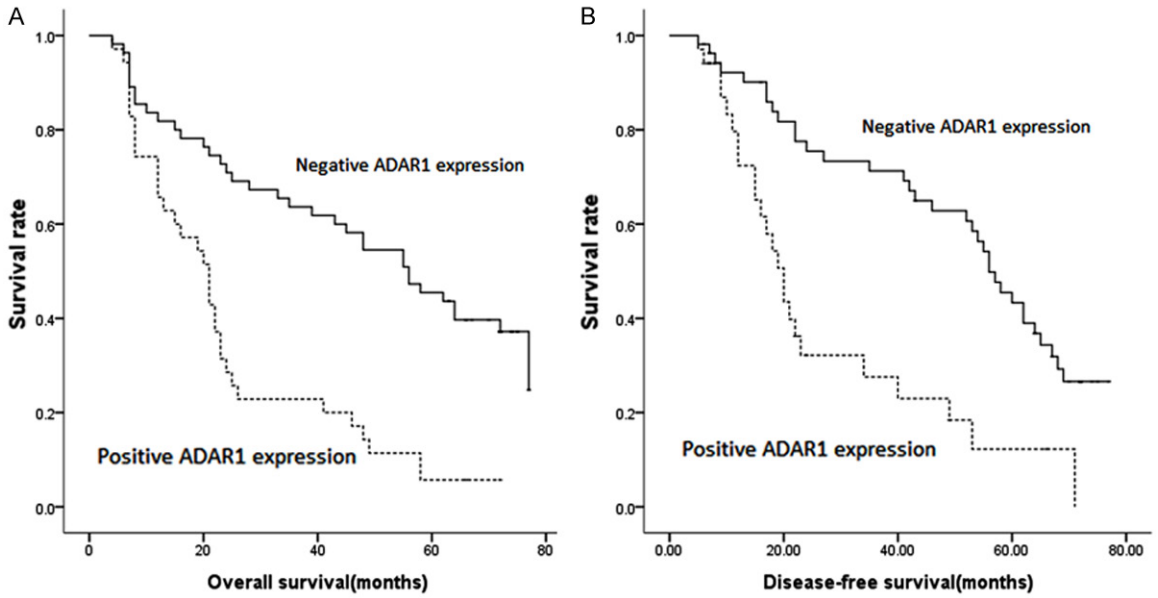

Figure 3. A. Overall survival curve of HC patients according to ADAR1 expression (negative or positive) (P = 0.022). B. Disease-free survival curve of HC patients according to ADAR1 expression (negative or positive) (P = 0.029).

Disclosure of conflict of interest

None.

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