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***Observational Study***

**Low** **expression of ARID1A correlates with poor prognosis in****intrahepatic cholangiocarcinoma**

Yang SZ *et al.* Low-expressed ARID1A and intrahepatic cholangiocarcinoma

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**Abstract**

**AIM:** To investigate the relationship between ARID1A expression and clinicopathologic parameters, and its prognostic value for patients with intrahepatic cholangiocarcinoma (IHCC).

**METHODS:** We assessed ARID1A protein and mRNA expression in IHCC tissues and paracarcinomatous (PC) tissues from 57 patients with IHCC, using western blot and quantitative real-time reverse transcription polymerase chain reaction, respectively. We used Fisher’s exact and χ2 tests to analyze relationships between clinicopathological parameters and ARID1A expression, and the Kaplan-Meier method and Cox regression to analyze survival.

**RESULTS:** The mean ARID1A protein level in IHCC tissues was 1.16±0.36 relative units (RU), which was significantly lower than that in PC tissues (1.26 ± 0.21 RU, *P* < 0.01) and NL tissues (1.11 ± 0.31, *P* < 0.001). The mean ARID1A mRNA level in IHCC tissues (1.20 ± 0.18) was also lower than that in PC tissues (1.27 ± 0.15, *P* < 0.001) and normal liver tissues (1.15 ± 0.34, *P* < 0.001). Low ARID1A expression was significantly associated with tumor nodule, vein invasion and recurrence. Median overall survival (OS) and disease-free survival (DFS) for the low ARID1A expression group was 15.0 and 7.0 mo, which were significantly shorter than those for the high ARID1A expression group at 25.0 and 22.0 mo (OS: *P* < 0.01; DFS: *P* < 0.001). Low ARID1A expression was significantly associated with worse OS (HR = 3.967, 95%CI: 1.299-12.118, *P* = 0.016) in multivariate analyses.

**CONCLUSION:** Low expressed ARID1A is associated with poor prognosis in patients with IHCC. ARID1A may be a candidate prognostic biomarker in IHCC.

**Key words:** Intrahepatic Cholangiocarcinoma; Progression; Prognosis; ARID1A; Biomarker

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**Core tip:** We investigated the relationship between ARID1A expression and clinicopathologic parameters, and its prognostic value for patients with intrahepatic cholangiocarcinoma (IHCC). We examined ARID1A protein and mRNA expression in IHCC and paracarcinomatous (PC) tissue from 57 patients with IHCC. The mean ARID1A protein and mRNA expression levels in IHCC tissues were significantly lower than those in PC tissues and normal liver tissues. Low ARID1A expression was significantly associated with tumor nodule, vein invasion and recurrence. Low ARID1A expression was significantly associated with worse overall survival in multivariate analyses. ARID1A may be a candidate prognostic biomarker in IHCC.

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**INTRODUCTION**

Primary liver cancer (PLC) is the fifth most common cancer, with about 600000 annual deaths[1,2]. Intrahepatic cholangiocarcinomas (IHCC) accounts for 5%-10% of all PLCs in western countries[3,4], matched with the hepatocellular carcinoma (HCC), which accounts for about 90% of all PLCs[5]. IHCCs, a rare malignant tumor arising from the biliary tract, is aggressive and related with a very poor prognosis[6]. In Asian countries, the incidence of IHCC is much higher than the incidence worldwide[7]. Although the incidence of IHCC is relatively low, it has been progressively and significantly increasing over the last 30 years[8]. Presently, surgical resection is the only option for treating IHCC. Although surgery improves the median survival compared with conservative therapy alone (1.8 months), the outcome is still poor, with a postoperative median survival of 12.2 months[9,10]. Thus to help develop diagnostic methods for enhanced therapeutic outcome, information of the somatic mutations that contribute to the oncogenesis of IHCC is an important first step.

The AT-rich interactive domain 1A (ARID1A) protein (BAF250) is a member of the switching defective/ sucrose non-fermenting (SWI/SNF) complexes, which function as ATP-dependent chromatin remodelers[11,12]. The ARID1A gene is located at chromosome 1p36, which is related to the regulation of many cellular processes including proliferation, DNA repair, development, differentiation, and tumor suppression[13]. Absence of ARID1A protein or gene expression has been found in the precursor stage of clear cell carcinoma of the ovary, ovarian endometrioid adenocarcinoma, breast cancer and colorectal cancer and correlates with tumor progression in these cancers[14-17]. These findings indicate that ARID1A may be a tumor-suppressor gene.

Although correlations between ARID1A gene mutation and ARID1A protein expression with clinicopathologic parameters and prognosis in HCC have been recently reported[18], no study has examined its correlation with IHCC. Here, we investigated ARID1A gene and protein expression in surgically resected IHCC tumors to observe whether its expression status could be a prognostic biomarker for IHCC.

**MATERIALS AND METHODS**

***Patients and tissue specimens***

IHCC and adjacent paracarcinomatous (PC) liver specimens from 57 IHCC patients and normal liver tissues from 19 hepatic hemangioma patients (controls) were collected in the operation room. Samples were instantly frozen in liquid nitrogen and stored in −80°C until testing. PC tissues were hepatic tissue collected 2–5 cm away from the tumor edge. All patients underwent surgery in the Department of Hepatobiliary Surgery, Yantaishan Hospital, from January 2012 to June 2013. Diagnoses were defined by pathological examination. The study protocol was approved by the Ethics Committee of the Yantaishan Hospital. Informed consent was obtained from each patient.

We collected clinicopathologic parameters, including age, gender, liver function, tumor size, tumor number, histopathological classification, vessel invasion, recurrence, and survival time of patient. Vessel invasion was observed during pathological examination, which indicated tumor infiltration in the portal vein and/or hepatic vein. We monitored recurrence by ultrasound, computed tomography scan, and magnetic resonance imaging.

***Western blot analysis***

IHCC and PC tissues were homogenized and treated with RIPA lysis buffer (Dingguo, Beijing, China), and protein samples were resolved on a 4%–12% acrylamide gradient gel. Samples were transferred to a polyvinylidene fluoride membrane using the iBlot fast transfer electric transfer (Invitrogen, IL, United States). Membranes were blocked at room temperature for 1 h in 5% milk, and incubated with primary antibodies against ARID1A or GAPDH (1:1000, Abcam, MA, United States) at 4 °C overnight. Membranes were then washed with TBST three times, followed by incubation with appropriate secondary antibodies (1:8000, Abcam, MA, United States) at room temperature for 2 h. TBST three times, and exposed to film using the ECL kit (Pierce, CA, United States). ARID1A-specific signals were quantified from X-ray films using a scanner with BandScan 4.30 densitometry software, and expressed as integrated intensity units relative to GAPDH signals[19].The results were analyzed by physicians in a blinded manner.

***Quantitative real-time reverse transcription-polymerase chain reaction***

Total RNA was extracted from IHCC and PC tissues with Trizol. The retroviral reverse transcriptase kit (Takara, Tokyo, Japan) was used to synthesize cDNA with the reaction conditions of 37 °C for 60 min and 95 °C for 3 min. Primers were as follow: sense: 5′- TTAACTCCAGCCACCAAAATGAAC-3′ and antisense: 5′- ATAGAGGCGATAGAGGTCCAGAGG-3′ for the ARID1A gene; and sense: 5′-GAAGGTGAAGGTCGGAGTC -3′ and antisense: 5′-GAAGATGGTGATGGGATTTC -3′ for GAPDH. Real-time polymerase chain reaction (PCR) was performed with the 7500 real-time quantitative PCR instrument (Applied Biosystems, CA, United States) using the following conditions: 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s for 40 cycles. Data were normalized using the GAPDH housekeeping gene and were expressed as 2−ΔCt.

***Patient follow-up***

We obtained follow-up data after discharge for all 57 IHCC patients by direct communication with the patients or their relatives, or by reviewing hospital records. Disease-free survival (DFS) was measured from the date of hepatectomy until tumor recurrence. Overall survival (OS) was measured from the date of hepatectomy until death or the last follow-up point. The last follow-up evaluation was censored on August 31, 2015, or up to the time of death.

***Statistical analysis***

Quantitative values are presented as mean ± standard deviation or median (range). The Student’s t-test was used to evaluate differences in ARID1A protein and mRNA expression between IHCC and PC tissues. Fisher’s exact and χ2 tests were used to analyze the correlation between ARID1A expression level and clinicopathologic parameters in patients with IHCC. Survival curves were plotted by the Kaplan-Meier method and compared using the log-rank test. Survival data were evaluated using Cox proportional hazards model. All tests were two-tailed; *P* < 0.05 was considered significant. The SPSS package 17.0 (SPSS Inc., Chicago, IL, United States) was used for all analyses. The statistical methods of this study were reviewed by Prof. Xiaoqing Liu from Peking Union Medical College Hospital.

**RESULTS**

***Patient characteristics***

The mean age of the IHCC patients was 55.1 ± 9.3 years, and 68.4% (39/57) were male. The mean age of the controls was 51.2 ± 6.6 years, and 57.9% (11/19) were male. Forty patients with IHCC had at least one tumor nodule larger than 3 cm. Tumors were well differentiated in 22 patients, and moderately or poorly differentiated in 17 and 18 patients. The median follow-up time was 20 mo (range: 2–28 mo). IHCC recurred in 33 patients over a median recurrence time of 15.0 mo. During the follow-up, 30 patients died, with a mean survival time of 19.0 ± 6.4 mo.

***ARID1A protein and mRNA expression in IHCC and PC tissues***

The mean ARID1A protein level in IHCC tissues was 1.16 ± 0.36relative units (RU). This was significantly lower than that in PC tissues (1.26 ± 0.21 RU, *P* < 0.01) and normal liver (NL) tissues (1.11 ± 0.31, *P* < 0.001) (Figure 1). The mean ARID1A mRNA level in IHCC tissues (1.20±0.18) was also lower than that in PC tissues (1.27 ± 0.15, *P* < 0.001) and NL tissues (1.15 ± 0.34, *P* < 0.001). (Figure 2)

***Correlations between ARID1A expression and clinicopathological features***

We next divided the IHCC tissues according to ARID1A expression. IHCC tissues with ARID1A protein expression lower than that in NL tissues were defined as low expression tumors. Among the 57 total IHCC cases, 19 cases showed ARID1A low expression and 38 cases showed ARID1A high expression. The correlations of ARID1A mRNA and protein expression with clinicopathological parameters are shown in Table 1. Low ARID1A expression was significantly associated with tumor nodule, vein invasion and recurrence.

ARID1A protein expression in IHCC tissues with multiple tumor nodules was significantly lower (*n* = 18, 0.93 ± 0.34 RU) than in those with solitary tumor nodules (*n* = 39, 1.27 ± 0.32, *P* < 0.001). ARID1A mRNA expression in IHCC tissues with multiple tumor nodule (*n* = 18, 1.13 ± 0.17) was also lower than in those with solitary tumor nodule (1.23 ± 0.16, *P* < 0.05).

ARID1A protein expression in IHCC tissues with vein invasion was significantly lower (*n* = 24, 0.93 ± 0.33 RU) than in those without vein invasion (*n* = 33, 1.33 ± 0.28, *P* < 0.001). ARID1A mRNA expression in IHCC tissues with vein invasion (1.18 ± 0.18) was also lower than in those without vein invasion (1.21 ± 0.17), although without statistical significance.

ARID1A protein levels in IHCC tissues from patients who experienced recurrence during the follow-up (*n* = 33, 1.00 ± 0.33) were significantly lower than in those without recurrence (*n* = 24, 1.39 ± 0.27, *P* < 0.001). ARID1A mRNA levels in IHCC tissues with recurrence (1.20 ± 0.17) were also lower than that in those without recurrence (1.25 ± 0.21), although without statistical significance.

***Association of ARID1A expression with prognosis***

Kaplan-Meier survival curves and log-rank tests showed that low ARID1A protein expression in IHCC tissues was associated with poor prognosis. The median OS for the low ARID1A expression group was 15.0 mo, which was significantly shorter than that of the high ARID1A expression group at 25.0 mo (*P* < 0.01; Figure 3A). The median DFS rates for low ARID1A expression and high ARID1A expression groups were 7.0 mo and 22.0 mo, respectively (*P* < 0.001; Figure 3B). Furthermore, multivariate analysis using the Cox proportional hazards model indicated that ARID1A expression levels, tumor nodule and vein invasion were an independent predictor of DFS in patients with IHCC (Table 2). However, ARID1A expression level was the only independent predictor of OS in a multivariate analysis. Low ARID1A expression was significantly associated with worse OS in patients with IHCC compared with high ARID1A expression (HR = 3.967, 95%CI: 1.299-12.118, *P* = 0.016, Table 2).

**DISSCUSION**

ARID1A/BAF250 is a component of the SWI/SNF family complexes and is extensively expressed in different human tissues[14,20,21]. The SWI/SNF chromatin remodeling complexes are recurrently mutated in various carcinomas[22]. Mutations in several subunits of these complexes have been identified, including in BAF 180, SNF5, BRM/SWI2-related genes, as same as ARID1A[22,23]. ARID1A/BAF250 provides SNF/SWI complex specificity and facilitates protein-protein or protein-DNA molecule interactions. Knockdown of ARID1A gene causes cell cycle arrest in osteoblasts cells, and previous studies have demonstrated a correlation of ARID1A loss and tumorigenesis[11]. Together this supports a potential tumor suppressor function of ARID1A and suggests that ARID1A plays an important role in tumorigenesis and tumor progression.

Guichard *et al*[24] performed high-resolution copy-number analysis on 125 tumor tissues of patients with HCC and whole-exome sequencing on 24 of these tumors. The author found new recurrent modifications in four genes (ARID1A, RPS6KA3, NFE2L2 and IRF2) that had not been formerly described in HCC. In Fujimoto’s research[25], the entire genomes of 27 HCCs were sequenced and analyzed. Twenty-five were associated with hepatitis B or C virus infections, including two groups of multicentric tumors. Statistical and functional analyses showed that ARID1A, ARID1B, ARID2, MLL and MLL3 genes were mutated in about 50% of the tumors. Huang *et al*[18] showed that ARID1A was mutated in 13% HBV-associated HCC specimens. These studies suggested that ARID1A may be involved in advanced HCC.

IHCC is the second most common primary liver cancer after HCC, accounting for 5%–10% of all cholangiocarcinomas[26]. The origin of IHCC is normally demarcated as grade II intrahepatic bile duct epithelium[27,28]. The prognosis and the mortality rate of IHCC is ppoor, as IHCCis usually diagnosed at terminal stages because of absence of appropriate methods for early diagnosis, and surgical treatment only extends the postoperative median survival to 12.2 mo[9,10]. Hence, better understanding of the somatic mutations that contribute to the oncogenesis of IHCC is critical for the development of diagnostic strategies. Through exome sequencing of 32 patients with IHCC, Jiao *et al*[29] found many inactivating mutations in various chromatin-remodeling genes (PBRM1, ARID1A and BAP1) and activating mutations in one of these genes was observed in nearly half of all cancers sequenced. Zou *et al*[30] sequenced carcinoma tissues and paracarcinous tissues in a large cohort of 103 patients with IHCC in China and found that IHCC-specific somatic mutation is correlated with liver inflammation, fibrosis and cirrhosis. The authors identified 25 mutated genes with eight possible driver genes, including KRAS, TP53, IDH1, ARID1A, PTEN, ECE2, EPPK1 and FYN. We observed that ARID1A may be a key point for the oncogenesis of IHCC. However, whether ARID1A status impacts clinical behavior has not been clear. We promoted this retrospective study to investigate the relationship between ARID1A expression and clinicopathologic parameters, and its predictive value for IHCC prognosis.

We found lower ARID1A protein in IHCC tumor tissues compared with PC tissues of patients with IHCC and NL tissues of hepatic hemangioma patients. We also observed significant difference between the expression level of ARID1A mRNA in IHCC samples and PC tissues.

An important result of the current study was that ARID1A protein expression was associated with tumor nodule, vein invasion and tumor recurrence status. These factors are highly correlated with invasion and metastasis of IHCC. Our results indicate that more invasive tumors have lower ARID1A protein expression, suggesting that ARID1A has a suppressive function in IHCC. ARID1A protein expression was not related with sex, age, liver function, tumor size, and tumor differentiation.

Many studies have examined the relevance of ARID1A mutation or protein loss to survival in several carcinomas, although the findings were varied. ARID1A mutation or protein loss was a predictor of worse prognosis in cervical carcinoma[31], and gastric cancer[21]. Other studies found no association between ARID1A mutation or protein loss and survival in endometrial clear-cell carcinoma[32], and ovarian clear cell adenocarcinoma[33]. Other reports suggested that ARID1A mutation or protein loss may be related to survival in endometrial carcinoma[20] and gastric cancer[21]. Our study is the first to explore the relationship between ARID1A expression and IHCC survival. OS and DFS were significantly shorted in the low ARID1A expression group than that of the high ARID1A expression group. These associations need to be verified and the mechanisms clarified in future investigations. In univariate analyses, ARID1A expression, tumor nodule, vein invasion) and recurrence status were found to be significant prognostic factors. In multivariate analysis, all of the above factors were independent prognostic factors of DFS, while only ARID1A expression was an independent prognostic factor of OS. Together our findings suggest that ARID1A may be a candidate prognostic biomarker in IHCC.

This is the first investigation of the correlations between ARID1A gene and protein expression with clinicopathologic features of IHCC. Expressions of ARID1A protein and mRNA of IHCC tissues were lower than that of PC tissues. ARID1A protein levels in IHCC tissues from patients with recurrence during follow-up were significantly lower than in those without recurrence. IHCC tissues with vein invasions had significantly lower ARID1A protein levels than those without vein invasions. ARID1A protein expression in IHCC tissues were correlated with OS and DFS. Based on our results showing low ARID1A expression in IHCC, we speculate that the manipulation of ARID1A expression in IHCC patients might have therapeutic implications. However, ARID1A functions and mechanisms of regulation in normal and IHCC tissues remain unclear and require further study.

**COMMENTS**

***Background***

Primary liver cancer (PLC) is the fifth most common cancer, with about 600000 annual deaths. Intrahepatic cholangiocarcinomas (IHCC) accounts for 5%-10% of all PLCs in western countries, matched with the hepatocellular carcinoma (HCC), which accounts for about 90% of all PLCs.

***Research frontiers***

The authors investigated ARID1A gene and protein expression in surgically resected IHCC tumors to observe whether its expression status could be a prognostic biomarker for IHCC.

***Innovations and breakthroughs***

This is the first investigation of the correlations between ARID1A gene and protein expression with clinicopathologic features of IHCC. Expressions of ARID1A protein and mRNA of IHCC tissues were lower than that of paracarcinomatous (PC) tissues.

***Peer-review***

This study is very interesting. In this study, the authors investigated the relationship between ARID1A expression and clinicopathologic parameters, and its prognostic value for patients with IHCC. The ARID1A protein and mRNA expression in IHCC tissue and PC tissue from 57 patients with IHCC were assessed. The authors found that the low expressed ARID1A is associated with poor prognosis in patients with IHCC, and ARID1A may be a candidate prognostic biomarker in IHCC.

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**Figure 1 ARID1A protein expression in intrahepatic cholangiocarcinoma patients.** Western blotting for ARID1A expression in intrahepatic cholangiocarcinoma (IHCC) tissues PC tissues and NL tissues. GAPDH was used as the internal loading control. IHCC: Intrahepatic cholangiocarcinoma; PC: Paracarcinomatous; NL: Normal liver.



c

**Figure 2 ARID1A mRNA level in intrahepatic cholangiocarcinoma patients.** IHCC: Intrahepatic cholangiocarcinoma; PC: Paracarcinomatous; NL: Normal liver. c*P* < 0.001 *vs* PC and NL.



A 

B

**Figure 3 Overall survival (A) and disease-free survival (B) of patients with intrahepatic cholangiocarcinoma after surgical resection according to ARID1A protein expression in intrahepatic cholangiocarcinoma tissues (*P* < 0.01; log-rank test).**

|  |
| --- |
| **Table 1 Clinicopathological features of patients with IHCC and correlation between protein expression of ARID1A and clinicopathological parameters** |
| **Characteristics** | **ARID1A** | ***P* value** |
|  | **Low expression*****n* = 19** | **High expression*****n* = 38** |  |
| Age |  |  | 0.703 |
| < 50 | 7 | 16 |  |
| ≥ 50 | 12 | 22 |  |
| Sex |  |  | 0.546 |
| Female | 7 | 11 |  |
| Male | 12 | 27 |  |
| Child- classification |  |  | 0.500 |
| A | 15 | 34 |  |
| B | 4 | 4 |  |
| Differentiation |  |  | 0.546 |
| Well | 10 | 12 |  |
| Moderately | 4 | 13 |  |
| Poorly | 5 | 13 |  |
| Tumor size (cm) |  |  | 0.306 |
| > 3 | 15 | 25 |  |
| ≤ 3 | 4 | 13 |  |
| Tumor nodule |  |  | < 0.001 |
| Solitary | 7 | 32 |  |
| Multiple | 12 | 6 |  |
| Vein invasion |  |  | 0.004 |
| Positive | 13 | 11 |  |
| Negative | 6 | 27 |  |
| Recurrence status |  |  | 0.004 |
| Yes | 16 | 17 |  |
| No | 3 | 21 |  |

**Table 2 Multivariate analysis of OS and disease-free survival on ARID1A protein expression in patients with intrahepatic cholangiocarcinoma (Cox proportional hazards model)**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Prognostic factors** | **Overall survival** |  |  | **Disease-free survival** |  |
|  | **Hazard ration****(95%CI)** | ***P* vaule** |  | **Hazard ration****(95%CI)** | ***P* vaule** |
| Tumor nodule |  |  |  |  |  |
| Solitary *vs* Multiple | 0.292 (0.093-0.914) | 0.034 |  | 1.092 (0.328-3.639) | 0.886 |
| Vein invasion |  |  |  |  |  |
| Positive *vs* Negative | 0.181 (0.079-0.413) | < 0.001 |  | 1.207 (0.509-2.859) | 0.669 |
| ARID1A expression |  |  |  |  |  |
| Low *vs* High | 7.240 (2.281-22.980) | < 0.001 |  | 3.967 (1.299-12.118) | 0.016 |