Abstract. – OBJECTIVE: Cholangiocarcinoma (CCA) is the second most common primary malignant hepatobiliary cancer worldwide. The circular RNA (circRNA) Cdr1as has been found to play critical roles in various cancers. However, the relationship between circRNA Cdr1as and CCA is still ambiguous. The aim of this study was to explore the expression level of Cdr1as in CCA and to further investigate the clinicopathological and prognostic value of Cdr1as.

PATIENTS AND METHODS: We investigated the circRNA Cdr1as expression in 54 paired tumor and adjacent normal tissues of CCA patients by using quantitative Real-time PCR, and evaluated the relevance between Cdr1as expression level and the clinicopathological features. We further assessed the association of Cdr1as expression with overall survival and its prognostic efficacy.

RESULTS: The expression of circRNA Cdr1as in tumor tissues was higher than that in adjacent normal tissues. The overexpression of Cdr1as was closely associated with the advanced TNM stage, lymph node invasion, and postoperative recurrence. The overall survival of CCA patients with high Cdr1as expression was worse than that of the CCA patients with low Cdr1as expression. According to multivariate analysis, the Cdr1as expression could be considered as an independent prognostic biomarker for cholangiocarcinoma with acceptable sensitivity and specificity.

CONCLUSIONS: Our finding suggested that circRNA Cdr1as may serve as a potential vicious molecular biomarker to predict the aggressive tumor progression and worse prognosis for CCA patients.

Key Words: Cholangiocarcinoma, CircRNA, Cdr1as, Prognosis, Biomarker.

Introduction

Cholangiocarcinoma (CCA), arising from biliary epithelial cells, accounts for the second most common malignancy of primary hepatobiliary neoplasms and is characterized by poor prognosis and growing incidence worldwide[1-2]. Without typical symptoms or effective preventive measures and resistance to traditional chemotherapy/radiotherapy, the radical surgical resection is still the only possible curative therapy for this lethal disease[3,4]. However, the postoperative morbidity and mortality were not satisfied, and the efficacy of CCA treatment was limited to less than 30% 5-year survival rate[5]. Therefore, the discovery of novel biological markers and therapeutic targets will be highly beneficial for the future treatment of CCA including early diagnosis and improved prognosis.

Plentiful efforts have focused on the research of noncoding RNAs (ncRNAs) to inform deep insights into cancers. Several studies[6-9] have demonstrated that ncRNAs played important roles in cell proliferation, metastasis and drug resistance of various cancers. The newly found circular RNAs (circRNAs), a unique class of endogenous noncoding RNAs mainly composing of transcript from exons which are formed by non-colinear reverse splicing and featuring stable structure, are widely expressed and characterized as abundant, conserved and tissue-specific molecules in mammalian cells[10-12]. Compared to linear RNAs, circRNAs have the remarkable characteristic of non-canonical splicing to form the covalently closed loop without free 5'.
or 3' ends\textsuperscript{13,14}. There is increasing evidence that circRNAs have been involved and played critical roles in various pathophysiological processes of human body through regulating genes expression\textsuperscript{15-17}. Other researches\textsuperscript{18-20} have ulteriorly demonstrated that circRNAs were dysregulated and closely associated with the occurrence and progression in many carcinomas, including breast cancer, gastric cancer, and colorectal carcinoma. Meanwhile, detecting the expression of circRNAs, which have been recognized as oncogenes or tumor suppressors, could be used to assist the clinical diagnosis and predict the prognosis for cancer patients. According to studies\textsuperscript{21-23}, circRNAs have been becoming the novel diagnostic/prognostic biomarkers and may serve as the potential therapeutic targets for cancers prevention and treatment. However, the expression and functional roles of circRNAs in cholangiocarcinoma remains unclearly.

In this study, we focused on circRNA Cdr1as (Cdr1 antisense RNA) which had been demonstrated to be the inhibitor and sponge for miR-7 in the embryonic zebrafish. The overexpression of circRNA Cdr1as could cause midbrain defects, which was similar with the phenotypes found in the knockdown of miR-7\textsuperscript{24,25}. Also, recent studies\textsuperscript{26-29} showed that circRNA Cdr1as was dysregulated and promoted the carcinogenesis in many cancers. Our work aimed to investigate the Cdr1as capability of being a molecular biomarker that might assist in evaluating the disease severity and assessing the prognosis for CCA patients.

**Patients and Methods**

**Patients and Tissues Samples**

A total of 54 paired tumor and adjacent normal tissues were obtained from CCA patients at The Second Affiliated Hospital of Harbin Medical University from September 2011 to March 2013. The patients were all pathologically diagnosed with cholangiocarcinoma, and none of the recruited patients received pre-/post-operative chemotherapy or radiotherapy. The resected tissues samples were obtained and immediately stored at -80°C unit use. The patients’ detailed clinicopathological characteristics were collected, and each of the patients received same medical care and regular follow-up after hospital discharge. This study was approved by the Ethical Committee of the Second Affiliated Hospital of Harbin Medical University and written informed consent was obtained from each enrolled patient.

**RNA Extraction and cDNA Synthesis**

Total RNA was extracted from tissues using the TRIzol reagent (Invitrogen Co, Carlsbad, CA, USA) according to manufacturer’s protocol. The NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was also applied to measure RNA concentration and purity. The extracted RNA was reverse transcribed into cDNA using the reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions.

**Quantitative Real-Time PCR (qRT-PCR)**

To detect the circRNA Cdr1as expression, we performed qRT-PCR with SYBR Green PCR kit (Takara, Dalian, Liaoning, China) based on the manufacturer’s protocol by using ABI 7500 System (Applied Biosystems, Foster City, CA, USA). GAPDH was monitored as the endogenous control. The primers were synthesized by GenePharma Bio (GenePharma, Shanghai, China). The sequences of primers were as follows (5’-3’): Cdr1as primer: TCAACTGGCT-CAATATCCATGTC (forward) and ACCTTGA-CACTATCCATGTC (reverse), GAPDH primer: CCCATCACCATCTTCCAGGAG (forward) and GTTGTCATGGATGACCTTGCC (reverse). The relative expression of Cdr1as was calculated using the $2^{-\Delta \Delta C_t}$ method.

**Statistical Analysis**

Statistical analysis was performed with the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The statistical differences between groups were analyzed by Student’s $t$-test or chi-square test. Kaplan-Meier method was performed to calculate the overall survival and the differences were evaluated using the log-rank test. The univariate and multivariate analysis were performed using the Cox proportional-hazards regression model. The prognostic efficacy of Cdr1as expression as a potential biomarker was assessed by receiver operating characteristics (ROC) curve analysis and the association of Cdr1as expression with overall survival was evaluated by Pearson correlation analysis. $p < 0.05$ was considered as statistically significant.
Results

CircRNA Cdr1as Expression is Upregulated in CCA

By performing qRT-PCR, the expression level of circRNA Cdr1as in 54 paired CCA tumor tissues and adjacent normal tissues were detected. As shown in Figure 1, the Cdr1as expression in tumor tissues was significantly higher than that in adjacent normal tissues. This results showed that the expression of circRNA Cdr1as was upregulated in cholangiocarcinoma and might promote the carcinogenesis.

Correlation Between Cdr1as Expression and Clinicopathological Features of CCA Patients

According to the median expression of Cdr1as, we categorized the CCA patients into low Cdr1as expression group (n=30) and high Cdr1as expression group (n=24). The clinicopathological features of low and high Cdr1as expression group patients were summarized in Table I. The data showed that the overexpression of Cdr1as was observed to be closely correlated with advanced TNM stage, lymph node invasion, and postoperative recurrence. However, there was no as-

Figure 1. The expression of circRNA Cdr1as was upregulated in cholangiocarcinoma. A-B, The expression level of Cdr1as in tumor tissues was significantly higher than that in adjacent normal tissues. C, Comparison of Cdr1as expression levels between tumor tissues and adjacent normal tissues from 54 CCA patients. ***p < 0.0005.

Table I. Relation between Cdr1as expression and clinicopathological features of CCA patients.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group</th>
<th>Total</th>
<th>Low</th>
<th>High</th>
<th>p-value</th>
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<tr>
<td>Gender</td>
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<td>33</td>
<td>19</td>
<td>14</td>
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<tr>
<td></td>
<td>Female</td>
<td>21</td>
<td>11</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>&lt; 60</td>
<td>20</td>
<td>12</td>
<td>8</td>
<td>0.622</td>
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<td>≥ 60</td>
<td>34</td>
<td>18</td>
<td>16</td>
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<td>Tumor location</td>
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<td>4</td>
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<td>10</td>
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<tr>
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<td>20</td>
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<td>9</td>
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<td>Histological type</td>
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<td>25</td>
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<td></td>
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<td>2</td>
<td></td>
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<tr>
<td></td>
<td>Papillary carcinoma</td>
<td>3</td>
<td>2</td>
<td>1</td>
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<td>I+II</td>
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<td>7</td>
<td>0.044</td>
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<td></td>
<td>III+IV</td>
<td>30</td>
<td>13</td>
<td>17</td>
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<td>Lymph node invasion</td>
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<td>10</td>
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<td></td>
<td>Negative</td>
<td>27</td>
<td>20</td>
<td>7</td>
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<tr>
<td>HBV infection</td>
<td>Positive</td>
<td>26</td>
<td>15</td>
<td>11</td>
<td>0.766</td>
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<tr>
<td></td>
<td>Negative</td>
<td>28</td>
<td>15</td>
<td>13</td>
<td></td>
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<tr>
<td>Serum CA19-9</td>
<td>&gt; 37 u/ml</td>
<td>28</td>
<td>15</td>
<td>13</td>
<td>0.382</td>
</tr>
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<td></td>
<td>≤ 37 u/ml</td>
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<td>9</td>
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<tr>
<td>Recurrence</td>
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<td>14</td>
<td>18</td>
<td>0.036</td>
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<tr>
<td></td>
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Association between Cdr1as expression and other clinicopathological variables. The relative Cdr1as expression of CCA patients with the parameters including lymph node invasion (negative/positive), different TNM stage, and postoperative recurrence (absent/present) was showed in Figure 2.

Association of Cdr1as Expression with Overall Survival of CCA Patients

As shown in Figure 3, Kaplan-Meier analysis showed that the overall survival of CCA patients with high Cdr1as expression was significantly worse than that of patients with low Cdr1as expression. Figure 4 showed that the relative Cdr1as expression was negatively associated with the overall survival of CCA patients \( (R = -0.5006, \ p = 0.0001) \). Meanwhile, Cox regression model analysis was utilized to evaluate whether the Cdr1as expression could be used to predict the overall survival of CCA patients. Univariate analysis revealed that lymph node invasion, TNM stage, postoperative recurrence, and Cdr1as expression were risk factors for CCA patients’ overall survival. The circRNA Cdr1as expression could be considered as an independent prognostic factor for CCA patients by multivariate analysis (Table II).

Prognostic Potential of Cdr1as in CCA

Furthermore, we performed receiver operating characteristic (ROC) curve analysis to assess the prognostic value of circRNA Cdr1as expression in predicting the overall survival for CCA patients. As shown in Figure 5, the value of area under curve (AUC) was 0.740 (95% CI: 0.606-0.875), and the sensitivity and specificity were 83.3% and 58.3%, respectively.

Discussion

As the pathogenesis of cholangiocarcinoma is still concealed and few effectively curative therapies can be applied except for surgical resection,
A novel prognostic biomarker for cholangiocarcinoma: circRNA Cdr1as

The molecular research contributed to novel prognostic biomarkers and therapeutic targets to improve the outcomes of this lethal disease.\textsuperscript{30-32} With the development of high-throughput techniques and the deepening of cancer research, vast sums of ncRNAs (miRNAs, lncRNAs, and circRNAs) have been discovered dysregulated in human neoplasms and involved in diverse malignant biological properties, including proliferation, anti-apoptosis, drug resistance and metastasis, making powerful contribution to the molecular diagnosis and prognosis assessment for cancers.\textsuperscript{33-36}

CircRNAs is a unique class of endogenous noncoding RNAs mainly composing of transcript from exons which are formed by non-colinear reverse splicing and featuring stable structure. The remarkable feature of circRNAs, compared with linear RNAs, is the covalently closed loop without 5’ or 3’ ends formed by non-canonical splicing. Recent studies\textsuperscript{37-39} revealed that the widely dysregulated expression of circRNAs was also closely related to various cancers, including gastric cancer, colorectal cancer, and pancreatic adenocarcinoma. However, the expression and function of circRNAs in cholangiocarcinoma are still poorly explored. The circRNA Cdr1as, a maternally expressed noncoding RNA firstly discovered in the embryonic zebrafish midbrain and located on chromosome Xq27.1 in humans, has been identified as the super sponge of miR-7 which was associated with an expanding list of primary human tumors. Nevertheless, there is no evidence of the expression profile and potential clinical significance of circRNA Cdr1as linked to CCA.

In this report, we firstly investigated the expression and prognostic potential of the circRNA Cdr1as in cholangiocarcinoma. We observed that the expression of Cdr1as was significantly upregulated in tumor tissues than that in adjacent normal tissues. The overexpression of Cdr1as was closely associated with lymph node invasion, advanced TNM stage, and postoperative recurrence, suggesting that circRNA Cdr1as could probably be involved in the oncogenesis and metastasis of cholangiocarcinoma. Furthermore, the overall survival of CCA patients with high Cdr1as expression was much worse and the relative Cdr1as expression was negatively associated with the overall survival. We also applied univariate and multivariate analysis to identify the prognostic value of Cdr1as expression; the results showed that the circRNA Cdr1as expression could be considered as an independent prognostic biomarker for CCA patients. In addition, the ROC curve analysis indicated that the expression detection of Cdr1as had an acceptable prognostic efficacy in predicting the

Table II. Univariate and multivariate analysis for overall survival of CCA patients.

<table>
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<th>Parameters</th>
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<td>95% CI</td>
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<td>Gender</td>
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<td>0.794-2.587</td>
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<td>Age</td>
<td>1.022</td>
<td>0.990-1.056</td>
</tr>
<tr>
<td>HBV infection</td>
<td>1.361</td>
<td>0.766-2.418</td>
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<tr>
<td>Serum CA19-9</td>
<td>1.382</td>
<td>0.742-2.574</td>
</tr>
<tr>
<td>Lymph node invasion</td>
<td>2.731</td>
<td>1.496-4.984</td>
</tr>
<tr>
<td>TNM stage</td>
<td>2.166</td>
<td>1.197-3.919</td>
</tr>
<tr>
<td>Recurrence</td>
<td>2.378</td>
<td>1.279-4.421</td>
</tr>
<tr>
<td>Cdr1as expression</td>
<td>2.435</td>
<td>1.342-4.420</td>
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</table>

Figure 5. Receiver operating characteristic (ROC) curve analysis of the Cdr1as expression for the overall survival.
overall survival of CCA patients (sensitivity of 83.3% and specificity of 58.3%). The subsequent research work should be focused on the exact underlying regulatory mechanisms of circRNA Cdr1as in cholangiocarcinoma by more in vitro and in vivo studies.

Conclusions

Our study demonstrated that the expression of circRNA Cdr1as was upregulated in cholangiocarcinoma and Cdr1as can serve as a novel independent prognostic biomarker for CCA patients. The dysregulation of circRNA Cdr1as and its underlying molecular mechanisms in cholangiocarcinoma need to be explored in further researches.

Acknowledgements

National Natural Science Foundation of China (Grant No. 81602088, 81170426), Health and Family Planning Commission Research Project of Heilongjiang Province (Grant No. 2016-049), Heilongjiang Postdoctoral Science Foundation (Grant No. LBH-Z16096), Innovative Science Foundation of Harbin Medical University (Grant No. 2016LCZX09) and China Postdoctoral Science Foundation (Grant No. 2017M621305).

Conflict of Interest

The Authors declare that they have no conflict of interests.

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A novel prognostic biomarker for cholangiocarcinoma: circRNA Cdr1as


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