Upregulated circulating miR-150 is associated with the risk of intrahepatic cholangiocarcinoma

SHOU LI WANG1*, JIKAI YIN1*, TAO LI2, LIUAN YUAN1, DONG WANG1, JIA XING HE1, XILIN DU1 and JIAN GUO LU1

1Department of General Surgery, Tangdu Hospital of The Fourth Military Medical University, Shaanxi 710038; 2Department of General Surgery, General Hospital of Beijing Military Command, Beijing 100700, P.R. China

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Abstract. During the last decade, microRNAs (miRNAs) have been identified as potential biomarkers and therapeutic targets for multiple malignancies; yet, few studies exist on intrahepatic cholangiocarcinoma (ICC). In the present study, a miRNA microarray was applied to determine the significant miRNAs involved in ICC. miR-150 was found to be significantly downregulated in ICC. We further enrolled 15 ICC patients who received radical resection to test these findings in plasma. Using quantitative reverse transcription-polymerase chain reaction (qRT-PCR), we examined and quantified the expression levels of miR-150 in tumor tissues, peritumoral noncancerous tissues and blood samples of 15 ICC patients. The diagnostic value of plasma miR-150 for differentiating patients with ICC from the age- and gender-matched controls was analyzed. For plasma samples, compared with normal controls, the level of miR-150 expression was found to be upregulated (P<0.010) in ICC patients. While differentiating ICC from normal controls, receiver operator curve (ROC) analysis of plasma miR-150 revealed the area under the curve (AUC) of 0.764 (P<0.010) with sensitivity of 80.6% and specificity of 58.1%. The diagnostic value of carbohydrate antigen 19-9 (CA19-9) and the combination of miR-150 and CA19-9 were also evaluated. We found that the combination of these two markers improved the power of screening ICC. Moreover, on the basis of the plasma miR-150 level, 15 ICC patients were divided into a low or high expression group. We found that plasma miR-150 is a potential diagnostic biomarker for ICC.

Introduction

Intrahepatic cholangiocarcinoma (ICC), arising from cholangiocytes of small intrahepatic bile ducts or bile ductules, is the second most common type of primary hepatic malignancy second to hepatocellular carcinoma (HCC) among all liver malignancies. Although ICC accounts for ~10-15% (1) of liver cancers, its incidence and mortality has increased drastically over the past several decades in China and even worldwide (2,3). In most cases, there is no particular clinical symptom for the early onset in ICC, and no specific or practical laboratory markers for early diagnosis. Although radical surgical resection is associated with an estimated 5-year survival rate of ~35% in ICC patients (4), the overall prognosis for ICC has remained very poor for decades (5-8). To date, limited serum markers for ICC, such as carbohydrate antigen 19-9 (CA19-9) (9), carcinogenic embryonic antigen (CEA) (10), cancer antigen 125 (CA-125) (11) and serum cytokeratin 19 fragment (CYFRA 21-1) (12) have been reported to be useful in diagnostic procedures, but the sensitivity and specificity of these markers remain far lower than expected. For diagnosing ICC, the sensitivity and specificity of CA19-9 are 62 and 63%, respectively (13). Thus, these biomarkers cannot be used for early qualitative detection but only for auxiliary diagnosis. There is still an urgent need for the identification of new biomarkers for the early diagnosis of ICC in clinical practice.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules, which play a crucial role in the regulation of gene expression at the post-translational level (14). miRNAs are critically involved in diverse cellular pathways, such as cell proliferation, differentiation and apoptosis (15). Dysregulation of miRNAs has been observed in various tumors including ICC (16-18). Many studies have revealed definite associations between miRNAs and HCC, such as miR-200c, miR-141 and miR-126 that could be used to distinguish primary HCC vs. metastatic tumors in liver. Yet, few studies have focused on the involvement of miRNAs in ICC. For example, miR-31 was found to suppress the expression of RASA1 and promote oncogenesis in ICC cells (18); miR-124 may participate in the migration and invasion of ICC cells by targeting SMYD3 (19). Meanwhile, most of these studies were carried out only on cell lines. According to the vital function of miRNAs, potential
diagnostic, and prognostic roles in other malignancies, we hypothesized that certain miRNAs may have diagnostic and/or prognostic significance in ICC. The aim of this study was to identify potential miRNAs as biomarkers for the screening or diagnosis of ICC in the early stage. We applied miRNA microarray to detect miRNA expression profiling in ICC tissues and adjacent normal tissues. Among the 10 differentially expressed miRNAs, miR-150 showed consistently lower expression levels in ICC. Subsequently, we compared the expression profiles of miR-150 in ICC tissues, adjacent normal tissues, and plasma samples to investigate the correlation between miR-150 and clinical characteristics. To our knowledge, this study is the first one to identify the diagnostic value of miR-150 in ICC.

Materials and Methods

Patients and samples. The study population in the present study was enrolled at the Department of General Surgery of Tangdu Hospital (The Fourth Military Medical University, Xi’an, China) during the period from January 2012 to October 2013. All subjects provided informed consent. The ICC patients were diagnosed based on clinical and imageology, and received radical resection in accordance with clinical practice guidelines of The National Comprehensive Cancer Network (NCCN) for Hepatobiliary Cancers. The final diagnosis was confirmed by pathological diagnosis. Meanwhile, 15 age- and gender-matched patients treated in our department who were cancer-free, were used as controls in the present study. Epidemiological and clinicopathological information for all patients was obtained from the in-hospital medical records. The differences in demographic and clinical characteristics between the 2 groups are shown in Table I. In total, we utilized 15 ICC cancerous tissues, 15 peritumoral normal tissues, 15 paired blood samples from the ICC patients and the controls. The study was approved by the Institute Research Ethics Committee of Tangdu Hospital.

miRNA microarray. miRNA microarray was performed in 3 pairs of tissue specimens which were collected from 1 male and 2 female ICC patients, using a service provider (LC Sciences, Houston, TX, USA). The assay starting with 4 to 8 µg total RNA was 3'-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining. Hybridization was performed overnight on a µParaflo microfluidic chip using a micro-circulation pump (Atactic Technologies, Houston, TX, USA). On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to target miRNA or control RNA and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection probes were made by in situ synthesis using photo-generated reagent chemistry. Hybridization used 100 liters 6X SSPE buffer containing 25% formamide at 34°C. After RNA hybridization, tag-conjugating Cy3 dye was circulated through the microfluidic chip for dye staining. Fluorescence images were collected using a laser scanner (GenePix 4000B; Molecular Devices, Sunnyvale, CA, USA) and digitized using Array-Pro image analysis software (Media Cybernetics, Bethesda, MD, USA). Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (locally-weighted regression).

RNA isolation, complementary DNA synthesis, and quantification. According to the manufacturer's instructions, total RNAs including miRNAs were extracted from tissues and plasma samples using the miRNeasy® Mini Kit (Qiagen, German) and RN24-BLOODmisi (Aidlab Biotechnologies, Beijing, China), respectively. For tissues, every 25 µg sample was ground to powder, and mixed with 700 µl QIAzol lysis reagent, then disrupted and homogenized at room temperature for 5 min. After being mixed with 140 µl chloroform, the upper aqueous phase was added with 525 µl 100% ethanol, and then 700 µl of the sample was pipetted into the RNeasy® Mini column and centrifuged at 10,000 rpm for 15 sec at room temperature. The miRNA was eluted with 40 µl RNase-free water. The isolation of plasma RNA was carried out using a similar procedure according to the RN24-BLOODmisi instructions. The purity of the RNA solution was detected by measuring its absorbance at 260 and 280 nm using a NanoDrop® Lite spectrophotometer (Thermo Scientific, Waltham, MA, USA). Pure RNA solution has an A260/A280 ratio of 1.89-2.07. All RNA preparations were stored at -80°C.

The reverse transcription reactions were carried out on a PTC-200 Peltier thermal cycler (Bio-Rad Laboratories, Shanghai, China) at 42°C for 60 min and then at 70°C for 5 min, using a RevertAid First Strand cDNA synthesis kit (Thermo Scientific) according to the manufacturer's protocol. The reaction solution volume was 20 µl, including 1 µl gene-specific primer, 4 µl 5X reaction buffer, 1 µl Ribolock RNase inhibitor, 2 µl dNTP Mix, 1 µl RevertAid M-MuLV reverse transcriptase, 2.5 µl total RNA solution and 8.5 µl RNase-free water.

To detect the expression level of mature miR-150, quantitative qPCR was carried out using a Maxima SYBR-Green qPCR Master Mix (Thermo Scientific) and ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. A housekeeping gene, hsa-miR-U6, was used as the endogenous control for the tissue and plasma samples. Every 25 µl of the qPCR reaction solution including 12.5 µl 2X Maxima SYBR-Green qPCR Master Mix, 1.5 µl forward primer, 1.5 µl reverse primer, 0.05 µl 10X ROX Reference Dye, 2.5 µl cDNA and 6.95 µl RNase-free water. Amplification was carried out by ABI 7500 with a cycling profile of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. Reverse transcription primers, forward primers and reverse primers were provided by RiboBio Biotech (Gangzhou, China). Every sample, including the negative control sample without cDNA template, was performed in triplicate. The ΔCt method was used to quantify the gene expression level as reported previously (20).

Statistical analysis. Statistical analysis was performed using Statistical Program for Social Sciences (SPSS) software 21.0 (IBM-SPSS, Cary, NC, USA), and graphs were generated using Graphpad Prism 6.0. The difference in miRNA expression levels between groups was calculated using the Student's t-test or the Mann-Whitney U test. The clinicopathological data are represented as means ± SD or frequencies, and differences
between groups were calculated by the t-test, Mann-Whitney U test or Fisher's exact test. The receiver operating characteristic (ROC) curve and the areas under the ROC curve (AUC) were used to evaluate the diagnostic value of miR-150 for differentiating ICC patients from controls. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical features of the patients. Fifteen ICC patients (including 12 males) and 15 age- and gender-matched normal controls were recruited in the present study. The age ranged from 45 to 76 years with a mean value of 57 years. No significant difference was observed in the distribution of age and gender, and there were no significant differences in terms of ALT (P=0.056), AST (P=0.841), ALP (P=0.360), CA19-9 (P=0.134) between the 2 groups. There was significant difference in serum ALB level (P=0.010) between the 2 groups. Despite the difference in ALB level, the 2 groups were fully comparable. As for the statistical analysis of AFP and CA19-9, there were no significant differences between the 2 groups. The distributional difference in original data of AFP and CA19-9 was caused by the maximum value of AFP 609 ng/ml and CA19-9 10,000 ng/ml in 2 patients, respectively.

Expression of miR-150 in tissues and plasma samples. To evaluate the clinical relevance of miR-150 in human ICC, we measured the miR-150 expression in the ICC tumor tissues (CT group), peritumoral normal tissues (NT group), ICC plasma samples (PS group) and matched plasma samples (MPS group) by means of quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The miR-150 expression level in the CT group was significantly lower than that in the NT group (mean ΔCt value: -6.004±1.612 vs. -4.256±1.524, P<0.01; Fig. 2). In addition, compared with the MPS group, the miR-150 expression level in the PS group was upregulated, with a mean ΔCt value of -0.507 vs. -2.079 (P<0.01; Fig. 3). Discrepant expression of miR-150 between ICC tissues and ICC plasma. Discrepant expression profiles of miR-150 between the tumor tissues and plasma samples were noted. The miR-150 expression level in the CT group was significantly lower than that in the NT group (average ΔCt value: -6.004±1.612 vs. -4.256±1.524, P<0.01; Fig. 2). In addition, compared with the MPS group, the miR-150 expression level in the PS group was upregulated, with a mean ΔCt value of -0.507 vs. -2.079 (P<0.01; Fig. 3).
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Figure 2. The miR-150 expression level in the cancerous tissue (CT group) vs. noncancerous tissue (NT group). The average ΔCt value of miR-150 in the CT group was -6.004±1.612, which was significantly lower than that of -4.256±1.524 in the NT group. ***P<0.001.

Figure 3. miR-150 expression level in plasma samples of intrahepatic cholangiocarcinoma (PS group) vs. matched plasma samples (MPS group) of the control. The average ΔCt value of miR-150 in PS group was -0.507±1.473, which was significantly higher than that of -2.079±1.301 in the MPS group. ***P<0.001.

Figure 4. ROC curve of plasma miRNA-150 for differentiating ICC patients from the controls. At the cut-off value of -2.097 for plasma miR-150, the AUC value was 0.791 and the 95% confidence interval ranged from 0.630 to 0.952. The optimal sensitivity and specificity were 93.3 and 53.3%, respectively.

ROC curves for the combination of miR-150 and CA19-9 to quantify the diagnostic power. Plasma miR-150 was able to identify ICC patients from the controls with a discriminatory accuracy of 0.791 (95% CI=0.630-0.952, P=0.007) (Fig. 4). At the cut-off value of -2.097 for plasma miR-150, the optimal sensitivity and specificity were 93.3 and 53.3%, respectively. In the same study population, CA19-9 had an AUC of 0.747 (95% CI=0.551-0.943, P=0.021) (Fig. 5) with a sensitivity of 66.7% and a specificity of 100%, which is consistent with previous studies. Similarly, the diagnostic value of the combination of miR-150 and CA19-9 was analyzed. We found that combina-

The diagnostic value of miR-150, CA19-9, and combination of miR-150 and CA19-9 for ICC. ROC curves were applied to evaluate the diagnostic power of plasma miR-150 and CA19-9, and the discriminatory accuracy was calculated in AUC values. Meanwhile, logistic regression was used to construct
tion of these 2 biomarkers yielded a higher discriminatory accuracy of 0.920 (95% CI=0.817-1.000) (Fig. 6).

Plasma miR-150 correlates with the clinicopathological features of the ICC patients. We analyzed the relevance between plasma miR-150 expression level and clinical features (Table II). Based on the expression level of miR-150, 15 ICC patients were divided into 2 groups: low expression group (n=8), containing the ones with a plasma miR-150 level less than the mean value of -0.507, and a high expression group (n=7) with a plasma miR-150 level higher than the mean value of -0.507. No significant differences were noted between the 2 groups.

Discussion

Intrahepatic cholangiocarcinoma is consistently asymptomatic in the early stages. More importantly, current available tumor markers lack suitable sensitivity and specificity for ICC at early onset. In terms of the treatments for ICC, surgical resection remains the only effective strategy to achieve a possible cure in ICC. Yet, radical surgical resection always relies on the hope that the disease is diagnosed in an early stage. ICC is also a contraindication for liver transplantation due to the poor prognosis (24,25). As a result, the identification of new diagnostic and prognostic biomarkers for ICC with proper sensitivity and specificity is urgently needed.

Previous studies have revealed that miRNAs in blood circulation are usually combined with the protein stably, not in free form, and dysregulation of miRNAs in blood samples may be potential diagnostic markers in diverse diseases (26,27), especially in the field of malignant neoplasms, such as pancreatic cancer (28), hepatocellular cancer (29), neuro-oncology (28), colorectal cancer (30), and gastric cancer (31). Dysregulation of miRNA expression in ICC has not been studied extensively as in many other cancers, perhaps due to the rarity of this pathological entity. Mitchell et al (27) was the first to identify the presence of circulating tumor-associated miRNAs in plasma and showed that circulating miRNAs may have an important value for cancer diagnosis. Those findings suggest that circulating miRNAs could be non-invasive diagnostic markers for cancers and also in ICC. Dysregulation of miRNA-150 was found to have 2 contrary roles in malignant tumors. Some studies revealed that miR-150 promotes tumorigenesis in various cancers. For example, miR-150 exerts its oncogenic function through downregulation of the expression of the pro-apoptotic purinergic P2X7 receptor in epithelial cell cancer (32) and by targeting the pro-apoptotic gene EGR2 in gastric cancer (33). On the contrary, other studies revealed that miR-150 may act as a tumour-suppressor miRNA. For

Table II. Clinicopathological characteristics of the ICC patients categorized according to the plasma miR-150 expression level.

<table>
<thead>
<tr>
<th>Clinical factors</th>
<th>Low group (&lt; -0.507 (ΔCt))</th>
<th>High group (&gt; -0.507 (ΔCt))</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>59±8</td>
<td>54±12</td>
<td>0.441</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>5/3</td>
<td>7/0</td>
<td>0.123</td>
</tr>
<tr>
<td>RBC (x10^9/l)</td>
<td>4.31±0.50</td>
<td>4.23±0.65</td>
<td>0.799</td>
</tr>
<tr>
<td>WBC (x10^9/l)</td>
<td>6.65±3.32</td>
<td>7.02±3.48</td>
<td>0.837</td>
</tr>
<tr>
<td>PLT (x10^9/l)</td>
<td>155.50±87.23</td>
<td>165.28±76.32</td>
<td>0.822</td>
</tr>
<tr>
<td>Hb (x10^9/l)</td>
<td>132.38±16.31</td>
<td>134.71±14.50</td>
<td>0.776</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>45.25±28.67</td>
<td>52.86±48.22</td>
<td>0.712</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>37.00±21.70</td>
<td>45.29±31.75</td>
<td>0.561</td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>149.63±146.32</td>
<td>168.14±123.23</td>
<td>0.797</td>
</tr>
<tr>
<td>gGT (U/l)</td>
<td>16.03±6.02</td>
<td>112.37±165.20</td>
<td>0.174</td>
</tr>
<tr>
<td>TB (µmol/l)</td>
<td>6.95±4.68</td>
<td>83.53±133.16</td>
<td>0.179</td>
</tr>
<tr>
<td>IB (µmol/l)</td>
<td>9.07±3.14</td>
<td>28.73±34.67</td>
<td>0.185</td>
</tr>
<tr>
<td>Operation time (min)</td>
<td>328.13±102.71</td>
<td>340.00±204.25</td>
<td>0.887</td>
</tr>
<tr>
<td>Blood loss (ml)</td>
<td>1275.00±1158.51</td>
<td>1482.86±1199.47</td>
<td>0.739</td>
</tr>
<tr>
<td>TNM stage (I/II/III)</td>
<td>2/2/4</td>
<td>2/3/2</td>
<td>0.669</td>
</tr>
</tbody>
</table>

Based on the expression level of miRNA-150, 15 ICC patients were divided into 2 groups: low expression group (n=8), with a plasma miR-150 level less than the mean value of -0.507, and the high group. There were no correlations between the miRNA-150 expression level and clinicopathological features, such as age, gender and clinical stage; WBC, white blood cell count; RBC, red blood cell count; PLT, platelets; Hb, hemoglobin; ALB, albumin; TB, total bilirubin; ALT, alanine transaminase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutamyl transpeptidase; AFP, α-fetoprotein; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9.
example, miR-150 expression was found to be downregulated in esophageal squamous cell carcinoma (34), and miR-150 inhibited the growth and malignant behavior of pancreatic cancer cells by targeting MUC4 (35). We believe that whether miRNAs function as oncogenes or tumor suppressors is dependent on the cell and tumor type (36). Under different cellular microenvironments, miR-150 may carry out different functions. However, the role of miR-150 in ICC has yet to be elucidated.

Our study found that the expression level of miR-150 was significantly lower in the ICC tissues than their peritumoral noncancerous tissues in the miRNA microarray analysis. Afterwards, the miR-150 expression profile was further determined in the tissue and plasma samples of 15 ICC patients and another 15 age- and gender-matched controls. The tissue expression level of miR-150 was significantly lower in the ICC tissues than that in the peritumoral noncancerous tissues. The expression level of miR-150 in blood was significantly higher in the ICC patients than that in the controls. Notably, we found contrary expression profiles of miR-150 between the tumor tissues and blood samples. These results suggest that miR-150 may be involved in the pathogenesis of ICC as a tumor-suppressor miRNA. Chang et al. (37) showed that miR-150 is downregulated by c-myc which is always highly expressed in ICC (38). This might be the reason for the downregulated miR-150 found in the ICC tissues. However, Pigati et al. (39) reported that the difference in extracellular miRNA and cellular miRNA profiles may suggest the existence of a cellular selection mechanism of miRNA release. Based on the same hypothesis, we believe that miR-150 may be an exocrine miRNA released by peritumoral noncancerous cells such as cholangiocytes or hepatocytes and may serve as an important negative feedback regulating agent. Thus, cholangiocarcinoma cells may selectively secrete or release cellular miRNAs such as miR-150 into the plasma. As a result, miR-150 was found to be upregulated in blood. Yet, the exact mechanism for the difference between tissue and plasma miRNA profiles is not clear and needs to be further studied.

The ROC curve showed that the sensitivity of plasma miR-150 to discriminate ICC patients from the normal controls was 93.3%, and the specificity was 53.3% with the AUC of 0.791. In the same population studied, the sensitivity and specificity of plasma CA19-9 were 66.7% and 100% respectively with AUC of 0.747 at the cut-off value of 28.915 ng/ml. When we further combined miR-150 and CA19-9, the sensitivity and specificity of the diagnosis were 80.0% and 100%, respectively, while the AUC value increased to 0.920, which is much better than miR-150 or CA19-9 alone. As for CA19-9, increased levels of CA19-9 also have been observed in patients with benign diseases such as bacterial cholangitis or cholecodolithiasis. Lack of sufficient sensitivity, specificity, and accuracy limits the use of a single blood-based biomarker in clinical practice. Combining miR-150 with CA19-9 made the sensitivity, specificity, and accuracy much more reliable than these variables with miR-150 or CA19-9 alone. This is more suitable for clinical use. Our results showed that miR-150 could be used as an effective biomarker for diagnosing ICC and more reliable when combined with CA19-9.

For better understanding of the clinical implications of plasma miR-150, we also examined the correlations between the plasma miR-150 expression level and clinical features. There were no significant differences between the low expression group and high expression group. This observation suggests that the miR-150 expression level was not affected by albumin (ALB), total bilirubin (TB), alanine (ALT), aspartate aminotransferase (AST) and other clinical indices. Therefore, miR-150 exists stably in blood samples from ICC patients.

In conclusion, the plasma miR-150 was found to be dysregulated in the ICC patients, and may be a potential biomarker for diagnosing ICC. Yet, it was more effective when combined with CA19-9. We also found that miR-150 was downregulated in ICC tissues but upregulated in ICC plasma. The limitation of the present study was the relatively small sample size. Future prospective trials using larger ICC patient cohorts are needed. Functional assays are also needed to reveal the potential biological roles of miR-150 in the carcinogenesis and/or progression of ICC.

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References


