Serum microRNA-29a is a promising novel marker for early detection of colorectal liver metastasis

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Background: Colorectal cancer (CRC) metastasis occurs in various organs, most frequently in liver. Serological examination including tumor and biochemical markers for liver function evaluation is routinely performed, though its accuracy is not high. MicroRNAs (miRNAs) have been implicated in a variety of human diseases including cancer, and have many characteristics of an ideal biomarker most notably their inherent stability and resilience. Recently, several studies have indicated that circulating miRNAs hold much potential as novel noninvasive biomarkers for cancer and other disease processes. The objective of this study was to investigate the potential of serum miRNAs as novel biomarkers for CRC with liver metastasis. Methods: This study was divided into three phases: (I) 3 candidate serum miRNAs were detected by using real-time RT-PCR, corresponding 38 CRC patients with liver metastasis and 36 CRC patients without metastasis. (II) Marker validation by real-time RT-PCR on a similar cohort of age- and sex-matched CRC patients without (n = 20) and with liver metastasis (n = 20). (III) We examined the correlation between the expressions of candidate serum miRNAs with clinical parameters of CRC patients. Results: Serum miR-29a was significantly higher in colorectal liver metastasis (CRLM) patients than in CRC patients. This marker yielded a receiver operating characteristic curve area of 80.3%. At a cutoff value of 0.155, the sensitivity was 75% and the specificity was 75% in discriminating metastatic from non-metastatic patients. In addition, increased levels of miR-29a expression were also observed in colorectal tumors from CRLM patients compared with CRC patients. No significant difference was observed in the levels of serum miR-92a between metastatic and non-metastatic patients. Conclusions: These findings suggest that serum miR-29a has strong potential as a novel noninvasive biomarker for early detection of CRC with liver metastasis.

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1. Introduction

Colorectal cancer (CRC) is the 3rd most common malignancy worldwide and the second most lethal cancer type in the developed world [1], and up to 50% of affected patients will develop metastases during the course of their disease [2]. About 25% of CRC patients have liver metastasis at the time of diagnosis and another 25–30% of them will present with liver metastasis in the following 2–3 years [3]. Despite studies which show longer survival rates for metastatic patients after hepatic resection, at least two-thirds of patients experience recurrence. Thus early diagnosis of liver metastases of CRC leads to timely treatment, which favors a better prognosis.

Laparoscopy has not been advocated as a screening test for colorectal liver metastasis (CRLM) due to its invasiveness. Fine needle aspiration cytology also has not been advocated as a screening test, because of its high risk of complications [4]. Imaging modalities, such as contrast enhanced computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography CT (PET-CT), may establish the diagnosis of CRLM [5]. However, it is more difficult to make the clinical diagnosis of early CRLM due to the absence of typical symptoms or signs. To reduce metastases-related mortality, the development of new noninvasive diagnostic markers for CRLM is of great significance.

MicroRNAs (miRNAs) are a class of small noncoding RNAs that have central roles in the regulation of gene expression [6]. The discovery that miRNA expression is frequently dysregulated in cancer warrants investigation to further elucidate their precise role in malignancy [7]. Most studies of miRNA expression profiling have been done using samples from the tissues of origin. Recently, several studies have indicated the diagnostic and prognostic utility of circulating miRNAs. One of the first studies measuring miRNA levels in serum was reported by Lawrie et al. [8], who showed that sera levels of miR-21 were associated with relapse-free survival in patients with diffuse large B-cell lymphoma. Subsequently, circulating miRNAs have been postulated as novel biomarkers for CRC with liver metastasis.

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for cancer and other disease processes [9–16]. Circulating miRNAs have also been shown to be predictive of CRC compared with advanced adenomas and healthy subjects or gastric cancer, inflammatory bowel disease and normal subjects [17,18]. For example, in a training population of 25 CRC patients and 20 healthy controls, the expression levels miR-17-3p and miR-92 were found to be elevated in the plasma of CRC patients. Ng et al. then analyzed a validation cohort of 90 CRC patients and 50 healthy controls and found that the expression of miR-92 in plasma could distinguish CRC patients from healthy control patients with 70% specificity and 89% sensitivity [18]. In another study, Huang et al. studied the miRNA profiles in the blood stream of early stage colon cancer patients and identified circulating miRNAs which distinguished CRC from healthy controls with 83% sensitivity and 84.7% specificity, adenomas from healthy controls with 73% sensitivity and 79% specificity [17]. To date, there have been no reports on the role of circulating miRNAs in CRLM. We hypothesized that levels of specific cancer-associated miRNAs in circulation would differ between CRC patients with and without liver metastasis. If this hypothesis held true, it would signify a major breakthrough in CRC management, bringing us ever closer to finding a novel, sensitive, and noninvasive biomarker for CRLM.

2. Materials and methods

2.1. Patients and samples

Following ethical approval and written informed consent, venous blood samples were collected from 38 CRLM patients and 36 age- and sex-matched CRC patients without metastasis for at least 3 years and placed in a serum separator tube (Table 1). In addition, a similar cohort of age- and sex-matched CRC patients without (n = 20) and with metastasis (n = 20) were identified from whom colorectal tumor tissues were collected (Table 1). All patients had histologically confirmed CRC with or without liver metastasis and their relevant demographic and clinicopathological details were obtained from our prospectively maintained database. Serum carcinoembryonic antigen (CEA) was tested in all subjects using the automat (Modular E170, Roche Diagnostics, Germany). Clinical stage of CRC was evaluated on the basis of the TNM classification system.

2.2. Serum preparation and RNA extraction

Total RNA was extracted from colorectal tumor tissue using Trizol (Invitrogen) following the manufacturer’s instructions. The blood was centrifuged at 1600 rpm for 5 min and the serum aliquoted into 1.7 ml Eppendorf tubes, followed by a 15 min high speed centrifugation at 12,000 rpm to completely remove cell debris. For RNA isolation from serum, 250 μl of serum was homogenized in 750 μl of Trizol LS (Invitrogen). Then 200 μl of chloroform was added to the sample and the mixed solution was centrifuged. After an additional chloroform extraction and precipitation with isopropanol, the pellet was washed twice by centrifugation with 70% ethanol. The RNA pellet was dried for 10 min at room temperature and dissolved in 30 μl of diethylpyrrocarbonte (DEPC)-treated water. DNase treatment (Qiagen) was carried out to remove any contaminating DNA. In general, we obtained 600 ng of RNA from 1 ml of serum.

2.3. miRNA targets

The expression of 3 serum miRNAs was chosen on the basis of their reported relevance to CRC (Table 2) [17,18]. MiR-16, a reliable endogenous control for investigating serum miRNA levels in recent studies [10,17], was also detected.

2.4. Reverse transcription (RT) and quantitative PCR (qPCR)

RT and qPCR kits made specifically for accurate miRNA analysis were used to evaluate expression of the chosen miRNAs from serum and tissue samples. The 15 μL RT reactions were performed using a TaqMan® microRNA Reverse Transcription Kit (Applied Biosystems, USA) and incubated for 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C, and then maintained at 4 °C. For real-time PCR, 1.33 μL diluted RT products were mixed with 10 μL of 2 × Taqman PCR master mixture (No AmpErase UNG), 1 μL TaqMan MicroRNA Assay and 7.67 μL of nucleoside-free water in a final volume of 20 μL according to manufacturer instructions. All reactions were run on the ABI 7300 (Applied Biosystems, USA) using the following conditions: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Real-time PCR was done in triplicate, including no-template controls. Relative expression of miRNA was calculated using the comparative CT (2−ΔΔCT) method [19] with miR-16 as the endogenous control to normalize the data [20]. The cycle threshold (CT) is defined as the number of cycles required for the FAM signal to cross the threshold in real-time PCR. ΔCT was calculated by subtracting the CT values of miR-16 from the CT values of the chosen miRNA. ΔΔCT was then calculated by subtracting mean ΔCT of the control samples from ΔCT of tested samples. Fold change of miRNA was calculated by equation 2−ΔΔCT.

Table 1
Summary of clinical details of CRC patients used for miRNA analysis.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Non-metastatic serum cohort (n = 36) n (%)</th>
<th>Metastatic serum cohort (n = 38) n (%)</th>
<th>Non-metastatic tissue cohort (n = 20) n (%)</th>
<th>Metastatic tissue cohort (n = 20) n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, year</td>
<td>56.6</td>
<td>58.6</td>
<td>55.9</td>
<td>56.8</td>
</tr>
<tr>
<td>Range</td>
<td>28–76</td>
<td>32–82</td>
<td>35–75</td>
<td>30–78</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Man</td>
<td>22 (61)</td>
<td>21 (55)</td>
<td>11 (55)</td>
<td>10 (50)</td>
</tr>
<tr>
<td>Woman</td>
<td>14 (39)</td>
<td>17 (45)</td>
<td>9 (45)</td>
<td>10 (50)</td>
</tr>
<tr>
<td>Primary tumor (T)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>10 (28)</td>
<td>2 (5)</td>
<td>7 (35)</td>
<td>5 (25)</td>
</tr>
<tr>
<td>III</td>
<td>22 (61)</td>
<td>30 (79)</td>
<td>9 (45)</td>
<td>9 (45)</td>
</tr>
<tr>
<td>IV</td>
<td>4 (11)</td>
<td>6 (16)</td>
<td>4 (20)</td>
<td>6 (30)</td>
</tr>
<tr>
<td>Regional lymph nodes (N)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>14 (39)</td>
<td>4 (11)</td>
<td>7 (35)</td>
<td>5 (25)</td>
</tr>
<tr>
<td>1</td>
<td>15 (42)</td>
<td>18 (47)</td>
<td>9 (45)</td>
<td>10 (50)</td>
</tr>
<tr>
<td>2</td>
<td>7 (19)</td>
<td>16 (42)</td>
<td>4 (20)</td>
<td>5 (25)</td>
</tr>
<tr>
<td>Carcinoembryonic antigen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>5.64</td>
<td>16.80</td>
<td>7.24</td>
<td>11.12</td>
</tr>
<tr>
<td>p53 status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>29 (80)</td>
<td>32 (84)</td>
<td>17 (85)</td>
<td>16 (80)</td>
</tr>
<tr>
<td>Negative</td>
<td>7 (20)</td>
<td>6 (16)</td>
<td>3 (15)</td>
<td>4 (20)</td>
</tr>
</tbody>
</table>
Table 2

<table>
<thead>
<tr>
<th>miRNAs of interest</th>
<th>Previous association with CRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-29a</td>
<td>Increased expression in plasma CRC patients and advanced adenomas compared with that of healthy controls [17]</td>
</tr>
<tr>
<td>miR-92a</td>
<td>Increased expression in plasma CRC patients [17,18] and advanced adenomas [17] compared with that of healthy controls</td>
</tr>
<tr>
<td>miR-17-3p</td>
<td>Increased expression in plasma and tumor tissue of CRC patients compared with that of healthy controls [18]</td>
</tr>
<tr>
<td>miR-16</td>
<td>Reliable endogenous control for investigating serum miRNA levels in recent studies [10]</td>
</tr>
</tbody>
</table>

2.5. Statistical analysis

Due to the magnitude and range of relative miRNA expression levels observed, results data were log transformed for analysis. Data are presented as mean ± SD. There was no evidence against normality for the log10 transformed data as confirmed using the Kolmogorov–Smirnov test. Student’s t test was used to evaluate expression differences of the chosen miRNAs between cases and controls. Receiver operating characteristic (ROC) curves were constructed and the area under the curve (AUC) was calculated to evaluate the specificity and sensitivity of predicting cases and controls. The relationship between the miRNAs expression and prognosis was studied by Kaplan–Meier survival analysis. All statistical tests were two-sided, and a probability level of P < 0.05 was considered to be statistically significant. Data analysis was done using SPSS 11.0 software (SPSS, Inc.).

3. Results

3.1. Patient characteristics

A total of 114 participants including 58 metastatic patients and 56 non-metastatic CRC patients were recruited into this study. All non-metastatic patients were metastasis free for at least 3 years. Patient characteristics and clinicopathological parameters were summarized in Table 1. There were no significant differences of age between non-metastatic patients (56.4 ± 11.4) and metastatic patients (58.0 ± 12.6) (P = 0.353, ANOVA). The sex distribution in CRC group was 33:23 (male:female) and in CRLM group was 31:27 (P = 0.344, χ² test).

3.2. Detection of miRNA in serum of non-metastatic and metastatic CRC patients

To explore the potential of using circulating miRNAs as novel biomarkers for CRLM, we investigated the levels of 3 candidates miRNAs and one reliable endogenous control (Table 2: miR-29a, miR-92a, miR-17-3p, and miR-16) in serum samples of metastatic CRC patients (n = 38) as well as non-metastatic patients (n = 36). No significant difference was found in terms of the levels of miR-16 between metastatic and non-metastatic patients. Using miR-16 as normalization control [10,17], expression levels of miR-29a in serum was significantly higher in metastatic patients than in non-metastatic patients (Fig. 1A). ROC curve analyses revealed that serum miR-29a was a valuable biomarker for differentiating metastatic patients from non-metastatic CRC patients with an AUC of 0.803 (95% CI: 0.660–0.946) (Fig. 2A). At the cutoff value of 0.155 for miR-29a, the optimal sensitivity and specificity were 75% and 75%, respectively, which is much more sensitive than serum CEA (60% sensitivity and 34% specificity) (Fig. 2C). The odds ratio for cases with miR-29a > 0.155 being associated with metastasis was 3.500 (95% CI: 1.274–9.617).

No significant difference was observed in the levels of miR-92a between metastatic patients and non-metastatic patients (Fig. 1B, P = 0.672). At the cutoff value of −0.0557 for miR-92a, the optimal sensitivity and specificity were 42% and 61%, respectively (Fig. 2B), which is similar with serum CEA (60% sensitivity and 34% specificity) (Fig. 2C). The odds ratio for cases with miR-92a > −0.0557 being associated with metastasis was 1.115 (95% CI: 0.446–2.785).

As for miR-17-3p, the detection rates were <50% in both metastatic and non-metastatic serum samples by RT-qPCR, thus, this miRNA was not chosen in further analytic studies.

3.3. Relationship of serum and tissue miRNA expression

Given that serum miR-29a was significantly elevated in metastatic patients, we proceeded to investigate miR-29a expression in colorectal tumor tissue in a similar cohort of age- and sex-matched metastatic (n = 20) and non-metastatic CRC patients (n = 20). Tumor expression of miR-29a was also significantly higher in metastatic group compared with that in non-metastatic group: (2.04) versus (1.93), P = 0.035 (Fig. 3A). Controlling for age and sex, miR-29a expression in tumor tissue showed a significant positive correlation with serum miR-29a levels.

There was no significant correlation in mean miR-92a expression between tumor tissue and serum, a trend of increasing tumor miR-92a expression for those metastatic patients was observed (Fig. 3B).

Fig. 1. Expression levels of miR-29a (A) and miR-92a (B) in CRC patients without (n = 36) or with (n = 38) liver metastasis. Using miR-16 as normalization control, expression levels of miR-29a in serum was significantly higher in metastatic patients than in non-metastatic patients (P < 0.05).

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**Fig. 2.** Receiver operating characteristics (ROC) curve analysis using serum miR-29a (A) and miR-92a (B) for discriminating metastatic patients from non-metastatic CRC patients. Serum miR-29a yielded an AUC (the areas under the ROC curve) of 0.803 (95% CI: 0.660–0.946) with 75% sensitivity and 75% specificity (A), and serum miR-92a yielded AUC of 0.497 (95% CI: 0.366–0.628) with 42% sensitivity and 61% specificity (B), while serum CEA yielded AUC of 0.603 (95% CI: 0.411–0.795) with 60% sensitivity and 34% specificity of (C).

**Fig. 3.** The miR-29a (A) and miR-92a (B) expression in colorectal tumor tissues from CRC patients without metastasis (n = 20) and with metastasis (n = 20). Tumor expression of miR-29a was significantly higher in metastatic group compared with non-metastatic group (P < 0.05).

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3.4. Relationship of serum miRNAs to clinicopathological parameters

Next, we examined the correlation between the expression of miR-29a and miR-92a in serum with clinical parameters. The levels of miR-29a demonstrated a significant elevation in CRC patients with advanced tumor T stage (T IV vs. T II, P = 0.014, Fig. 4A). No significant association was found between the two miRNAs and tumor N stage or P53 status (P > 0.05, B–F), while the levels of two miRNAs demonstrated an elevation trend in patients of P53 negative (C and F), and miR-92a is expressed at non-significant higher levels in more advanced tumor T stage (D).

Fig. 4. The levels of miR-29a demonstrated a significant elevation in CRC patients with advanced tumor T stage (T IV vs. T II, A). No significant association was found between the two miRNAs and tumor N stage or P53 status (P > 0.05, B–F), while the levels of two miRNAs demonstrated an elevation trend in patients of P53 negative (C and F), and miR-92a is expressed at non-significant higher levels in more advanced tumor T stage (D).

4. Discussion

Liver is the most common site of distant metastatic spread from CRC. Serological examination including tumor and biochemical markers for liver function evaluation is routinely performed, though its accuracy is not high [21]. The level of carcinoembryonic antigen (CEA) is elevated in 63% of CRLM patients, while the activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) is increased in about 30% of CRLM patients [22]. Thus, there is need for new noninvasive diagnostic markers for CRLM.

Results from recent studies revealed that circulating miRNAs are potential diagnostic biomarkers and prognostic factors in cancers and other disease processes [9–18]. For example, more than 100 circulating miRNAs can be identified in the blood of healthy individuals and this profile differs significantly from that of patients with CRC who have several tumor-specific miRNAs [10]. Chen and colleagues demonstrated 69 miRNAs in the serum of CRC patients that were not present in the serum of healthy controls. Moreover, they identified a unique expression profile of 14 serum miRNAs for CRC that were not present in another cancer group (lung cancer) [9]. Although the clinical significance of these findings has yet to be elucidated in detail, they demonstrate the potential of circulating miRNAs as non-invasive diagnostic or prognostic markers for cancer.

To our knowledge, this observational study is the first report of serum miRNAs in CRLM patients and our results demonstrate that cancer-associated miRNAs in serum can potentially serve as novel
noninvasive biomarkers for CRLM. The first miRNA we selected to study was miR-29a, which can act as either oncogene or tumor suppressor. For example, miR-29a has been found to be under-expressed in lung cancers [23], while Gebeshuber et al. revealed that miR-29a was up-regulated in mesenchymal, metastatic Ras+XT cells relative to epithelial EpRas cells, and could suppress the expression of tristetraprolin [24]. In a recent study conducted by Resnick et al., miR-29a was also significantly over-expressed in the serum from ovarian cancer patients [15]. In addition, the up-regulation of miR-29a was also observed in the plasma of CRC patients, the sensitivity and the specificity were 69.0% and 89.1%, differentiating CRC from controls [17]. In our study, we demonstrate that miR-29a is significantly increased in the serum of CRLM patients in comparison to non-metastatic CRC patients, and can discriminate CRLM patients from non-metastatic CRC patients with high specificity and sensitivity (Fig. 2A), which is more sensitive than serum CEA (60% sensitivity and 34% specificity) (Fig. 2C). More importantly, the miR-29a expression in a similar cohort of CRLM patients and non-metastatic CRC patients shows a similar significant increase in colorectal tumor tissue.

miR-92a is part of the miR-17-92 gene cluster, located on chromosome 13q13. The over-expression of miR-92a has been observed in several diseases, suggesting its important role in tumorigenesis. Recently, Ng et al. reported for the first time that miR-92a in plasma could distinguish CRC patients from healthy control patients with 70% specificity and 89% sensitivity [18]. In addition, the up-regulation of miR-92a was also observed in the plasma of CRC patients, the sensitivity and the specificity were 84.0% and 71.2%, differentiating CRC from controls [17]. In our study, however, no significant difference was observed in the levels of miR-92a between metastatic patients and non-metastatic patients (Fig. 1B, \( P = 0.672 \)). The inconsistent results may due to the different samples or different diseases.

Although the diagnostic efficiency of serum miR-29a may not be optimal, a panel of serum miRNA markers may improve the sensitivity and specificity of this assay for CRC screening. Patients with increased serum miRNAs might prompt more accurate and specific clinical examinations.

In conclusion, serum miR-29a appears to be a novel biomarker for early detection of CRLM. Our data provide the basis for further investigations of miR-29a, preferably in large prospective studies, to validate its clinical utility (individually or as part of a panel) as a non-invasive, economic screening tool for CRLM.

References


