Serum miR-335 Level is Associated with the Treatment Response to Trans-Arterial Chemoembolization and Prognosis in Patients with Hepatocellular Carcinoma

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Key Words
MicroRNA-335 • Trans-arterial chemoembolization • Prognosis

Abstract
Aim: To identify the role of serum MicroRNA-335 (miR-335) in determining the treatment response to Trans-arterial chemoembolization (TACE) in patients with hepatocellular carcinoma (HCC) and their prognosis after TACE. Methods: A total of 125 HCC patients were enrolled in this study. All these patients underwent TACE and the treatment response was evaluated. All patients were followed for prognosis analyses. Serum miR-335 levels immediate before and 30 days after TACE were determined. Results: HCC patients had significantly lower miR-335 levels than hepatitis patients and healthy controls. Lower serum miR-335 levels were closely associated with more progressive clinical features, including a higher mean serum AFP level, more vascular invasion, cirrhosis and larger tumor size. Response rates were higher in patients with high miR-335 compared to those with low miR-335 level. Patients with lower serum miR-335 levels had significantly poorer prognosis than patients with higher serum miR-335 levels. Conclusion: Our data suggest that serum miR-335 can be used as a molecular marker to predict the treatment response and clinical outcome in HCC patients receiving TACE.

Introduction
Hepatocellular carcinoma is one of the most common cancers worldwide [1, 2]. Many patients are diagnosed at late stage and miss the optimal timing for tumor resection [3-5].

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Trans-arterial chemoembolization is a new treatment for HCC, which is currently considered as the standard care for patients with unresectable HCC [6-8]. However, the treatment response to TACE in HCC patients varies widely and there is no reliable marker to predict patient's response to TACE [9]. Identification of patients who are sensitive or resistant to TACE is important for individualized treatment.

MicroRNAs (miRNAs) is a class of noncoding RNA molecules (usually 18–25 nucleotides in length), which regulates approximately 30 % of all protein-coding RNAs [10]. Recently, the role of miRNAs in tumorigenesis and metastasis has attracted increasing interest [11-13]. Specifically, miR-335 has been shown to act as a suppressor of tumor metastasis by regulating several classic signal pathways [14-16]. Increased miR-335 expression predicts a favorable prognosis in primary gallbladder carcinoma [17]. In HCC cell lines, the expression levels of miR-335 is significantly correlated with its host gene (MEST). In samples from HCC patients, the miR-335 expression level is dramatically lower in tumor tissues than in neighboring non-tumor tissues [18].

In this study, we aimed to investigate the role of serum miR-335 in predicting the treatment response and prognosis in HCC patients receiving TACE. Our finding suggests that serum miR-335 may be used as a molecular marker to predict the TACE response and clinical outcome in HCC patients.

Materials and Methods

This study enrolled a total of 125 patients newly diagnosed with HCC (Stage II, III and IV) in our institution from Aug 2006 to Mar 2010. All these patients underwent TACE as primary treatment. The diagnosis of HCC was based on the diagnostic criteria for HCC used by the European Association for the Study of the Liver. The clinical tumor stages were determined according to the TNM classification system of the International Union against Cancer (Sixth Edition). We enrolled 125 patients with hepatitis B or C infection but no evidence of HCC and 125 healthy volunteers as controls. Ethical approval was obtained from the Hospital Research Ethics Committee and written informed consent was obtained from all participants.

Transarterial chemoembolization procedure and follow-up

All the HCC patients in this study received TACE treatment. Briefly, a selective 5-French catheter was introduced and the tip of the catheter was advanced into the right or left hepatic artery, or into the tumor-feeding branches. The emulsion of lipiodol and chemotherapeutic agents was infused. The treatment regimen was comprised of carboplatin 300 mg (Bristol-Myers Squibb, New York, USA), epirubicin 50 mg (Pharmorubicin, Pfizer, USA), and mitomycin C 8 mg (Zhejiang Hisun Pharmaceutical Co. Ltd., Taizhou, China) mixed with 5 mL ethiodized oil. Subsequently, embolization was performed with injection of absorbable gelatin sponge particles (1–2 mm in diameter; Gelfoam; Hanzhou ALC Ltd, Hangzhou, China) through the catheter to reach stasis in the tumor-feeding artery. The treatment regimen was used consistently in this study, regardless of tumor number and size. Patients were observed carefully after treatment and analgesia was given if necessary.

After successful procedure, all HCC patients were closely followed until May, 2013. The median duration of follow-up was 12.7 months (range 1.0–56 months). The Response Evaluation Criteria in Solid Tumors (RECIST) was used to measure tumor response: CR (complete response), disappearance of all target lesions; PR (partial response), at least a 30% decrease in the sum of the longest diameter of the target lesions; SD (stable disease), neither PR nor progressive disease; PD (progressive disease), at least a 20% increase in the sum of the longest diameter of the target lesions, or the appearance of new lesions or metastasis [23]. Overall survival (OS) was defined as the interval between treatment and death or last observation of surviving patients. Time to progression (TTP) was defined as the interval between treatment and disease progression or last observation of patients without disease progression.

Serum miR-335 detection

Blood samples from HCC patients were withdrawn immediately before and 30 days after TACE treatment. Blood samples were also obtained from hepatitis patients and healthy controls at the same
time. Real-time quantitative RT-PCR for miRNA was performed to detect miR-335 expression in serum. Total RNA was extracted using a QIAamp RNA Blood kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. RNU6B were used as a control gene to exclude any possible heterogeneous expression. The miR-335 and RNU6B-specific cDNA were synthesized from total RNA using gene-specific primers according to the TaqMan MicroRNA assays protocol (Applied Biosystems, Foster City, CA, USA). The primer sequences were as following: for miR-335: forward 5’-GGC GTC AAG AGC AAT AAC GAA-3’, reverse 5’-GTG CAG GGT CCG AGG TAT TC-3’; for RNU6B: forward 5’-GGC TTC GGC AGC ACA TAT AC-3’, reverse 5’-TTC ACG AAT TTG CGT GTG AT-3’. Relative quantification of target miRNA expression was evaluated using the comparative cycle threshold (CT) method. The raw data were presented as the relative quantity of target miRNA, normalized with respect to RNU6B. Each sample was examined in triplicate.

Statistical analysis

SPSS16.0 software was used for the statistical analysis. The clinicopathological features were compared by using Student’s t test or ANOVA. Logistic regression analysis was performed to analyze various combinations of clinical parameters and miRNA. The p value was bilaterally tested, and values less than 0.05 were regarded as statistically significant.

Results

The clinical and demographical characteristics of all participants are listed in Table 1. There is no significant difference in age, gender distribution, smoker and alcohol users among HCC patients, hepatitis patients and healthy controls. However, HCC patients had significantly higher serum ALT and AFP levels and higher prevalence of cirrhosis compared to hepatitis patients.

Figure 1 shows the serum miR-335 level before and after TACE. Before TACE, the expression level of miR-335 in the serum of HCC patients (0.89±0.49) was significantly lower than that from hepatitis patients (1.89±0.56) and healthy controls (1.95±0.76, P<0.001, Table 1. The clinical and demographical characteristic of all participants. (0.89±0.49) was significantly lower than in serum from hepatitis patients (1.89±0.56) and healthy controls (1.95±0.76, P<0.001). Thirty days after TACE, there is an increase in serum miR-335 in HCC (1.12±0.59), but still significantly lower than hepatitis patients (1.88±0.67) and healthy controls (1.98±0.35).

| Viable                     | Healthy controls | Hepatitis B and C patients | HCC patients receiving TACE | P value |
|----------------------------|------------------|----------------------------|-----------------------------|---------|
| Age (years)                | 52.8 (33.7-56.8) | 52.5 (22.6-73.6)           | 53.0 (29.6-65.5)            | NS      |
| Gender (male, %)           | 93               | 90                         | 95                          | NS      |
| Smoker                     |                  |                            |                             |         |
| Never                      | 76               | 66                         | 68                          | NS      |
| Ever                       | 26               | 30                         | 24                          |         |
| Current                    | 23               | 29                         | 33                          |         |
| Alcohol user               |                  |                            |                             |         |
| No                         | 99               | 85                         | 60                          | 0.001   |
| Yes                        | 26               | 40                         | 65                          |         |
| ALT (U/L)                  | 32 (3-44)        | 76 (38-223)                | 57 (22-132)                 | 0.035   |
| AFP (mg/L)                 | 11 (0-19)        | 24.2 (9-132)               | 54.3 (18-2324)              | <0.001  |
| Serum miR-335 (before TACE)| 1.95±0.76        | 1.89±0.56                  | 0.89±0.49                   | <0.001  |
| Serum miR-335 (after TACE) | 1.98±0.35        | 1.88±0.67                  | 1.32±0.59                   | <0.001  |
| Cirrhosis (absent/present) | -                | 98/27                      | 42/83                       | <0.001  |
| Tumor size (cm)            | -                | 3.5 (2.5-6.6)              | -                           |         |
| Tumor number (single/multiple) | -              | 90/35                      | -                           |         |
| Vascular invasion (absent/present) | -          | 72/33                      | -                           |         |
Fig. 1. The serum miRNA-335 before (A) and after TACE (B). Figure 1A shows the serum miR-335 level before TACE. The serum miR-335 level in HCC patients was significantly lower than that from hepatitis patients and healthy controls (P=0.001). Figure 1B shows that the serum miR-335 level in HCC patients was slightly elevated after TACE, but still significantly lower than hepatitis patients and healthy controls.

Table 2. The clinicopathologic features between HCC patients with high and low miR-335 levels

| Variable                  | Low miR-335 (n=64) | High miR-335 (n=61) | P value |
|---------------------------|---------------------|----------------------|---------|
| AFP (μg/L)                | 232 (188-886)       | 686 (123-2324)       | <0.001  |
| Cirrhosis (absent/present)| 11/43               | 31/40                | 0.005   |
| Tumor size (cm)           | 4.1 (2.8-6.6)       | 3.4 (2.5-5.4)        | 0.03    |
| Vascular invasion (absent/present) | 25/39 | 47/14 | <0.001 |

Table 3. The treatment response between HCC patients with high and low miR-335 levels

| miR-335 Status                  | CR+PR (n=34) | SD+PD (n=91) | P value |
|---------------------------------|--------------|--------------|---------|
| Low miR-335 (before TACE)       | 14           | 42           | 0.385   |
| High miR-335 (before TACE)      | 20           | 49           |         |
| Low miR-335 (after TACE)        | 10           | 50           | 0.009   |
| High miR-335 (after TACE)       | 24           | 41           |         |

Fig. 1. Thirty days after TACE, the mean serum miR-335 level in HCC patients was slightly elevated (1.12±0.59), but still significantly lower than hepatitis patients (1.88±0.67) and healthy controls (1.98±0.35, P<0.001).

Using the mean value of serum miR-335 as cut-off value, we compared the associations of serum miR-335 before and after TACE with the clinicopathologic features of HCC. Our data indicate that the serum miR-335 levels before TACE were not associated with the HCC clinical
features (data not shown). In contrast, thirty days after TACE, we found that HCC patients with low miR-335 levels (n=64) exhibited more aggressive features, including higher serum AFP level, more vascular invasion, more cirrhosis and larger tumor size (Table 2), compared to those with high miR-335 levels (n=61).

Table 4. The independent prognostic factor for HCC prognosis by Cox proportional hazards analysis

|       | Viable | TTP       | OS      |
|-------|--------|-----------|---------|
|       | HR(95% CI) | P value | HR(95% CI) | P value |
| AFP   | 2.34(1.33-3.87) | <0.001 | 2.08(1.05-3.56) | <0.001 |
| Serum miR-335(after TACE) | 0.34(0.12-0.87) | <0.001 | 0.64(0.33-0.93) | <0.001 |
| Vascular invasion | 2.45(1.42-4.26) | <0.001 | 1.89(1.23-3.79) | <0.001 |
Among all HCC patients subject to TACE procedure, 34 patients gained good responses (CR+PR) and 91 patients did not respond well to TACE (SD+PD). We next conduct ROC analyses to see the ability of serum miR-335 in discriminating the treatment response to TACE in HCC patients. We found that the serum miR-335 levels after TACE, rather than the levels before procedure, could distinguish good response from poor response to TACE treatment (AUC=0.922, specificity=87%, sensitivity=77%, P<0.001, cut-off value=1.34, Fig. 2). Using the cut-off value, all HCC patients were divided into high mir-335 group and low mir-335 groups. As is shown in Table 3, high levels of miR-335 post-TACE were prevalent among patients with good treatment response. The serum miR-335 levels before procedure did not correlate with the response to treatment.

We further performed the Kaplan-Meier analysis to compare the effect of serum miR-335 on the clinical outcome in these patients indicated by TTP and OS (Figure 3A and 3B). The median duration of follow-up was 12.7 months (range 1.0–56 months). We found that patients with lower serum miR-335 levels had significantly shorter OS (median: 11.5 months) or TTP (median: 5.3 months) than patients with higher serum miR-335 levels (median: OS: 21.9 months; TTP: 13.2 months, both P<0.001 by log rank analyses).

To determine the prognostic role of miR-335, we conducted the COX analyses. In univariate analysis, serum miR-335, serum AFP and vascular invasion were associated with OS and TTP (Table 4). These variables were then entered into the multivariate Cox proportional hazards analysis; and our data revealed that serum miR-335 level was a favorable prognostic factor for both TTP (HR = 0.34, 95%CI: 0.2-0.87, P< 0.001) and OS (HR = 0.64, 95%CI:0.33-0.93, P<0.001) in HCC patients receiving TACE.

Discussion

The present study demonstrated that the expression levels of serum miR-335 in patients with HCC correlated with response to TACE and clinical outcome. Low miR-335 levels were significantly associated with poor treatment response and inferior survival. We also found serum miR-335 levels constituted an independent determinant of both OS and TTP in these patients even when considered in the context of other validated clinical prognosticators.

Some miRNAs play important roles in regulating the biological behavior of cancer cells. For example, miR-224 mediates celastrol-induced inhibition of migration and invasion of hepatocellular carcinoma cells [13]. MiR-127 post-transcriptionally suppresses cell growth in hepatocellular carcinoma cells [11]. Abnormal expression of a series of miRNA, including miR-21, miR-155 and miR-17-5p/miR-20b, is observed in primary lung cancer and secondary lung tumors from HCC, which can be normalized by percutaneous radiofrequency ablation [12].

Deregulation of miRNAs can contribute to HCC development by affecting cell proliferation, apoptosis, invasion or metastasis [19-26]. Both miRNA signatures and a single miRNA have been shown to provide new molecular approaches for diagnosis and prognosis of patients with HCC. Of note, it has been reported that serum miR-200a or miR-201 may prognosticate disease outcome in HCC patients treated by TACE [27, 28]. In addition, the first liposome-formulated miRNA mimic is currently being studied in a multicenter Phase I Clinical Trial in patients with unresectable primary liver cancer [29].

MiR-335 could act as an oncogene or tumor-suppressor in several types of cancers. It functions as a tumor suppressor in breast cancer and inhibits metastatic cell invasion [30]. On the other hand, miR-335 has been reported to be upregulated in malignant astrocytomas, as a putative oncogene that may confer growth advantage to astrocytoma cells [31, 32]. Moreover, miR-335 has been found to predict sensitivity to anticancer treatment: it was downregulated in cisplatin- and paclitaxel-resistant ovarian cancer cells, thereby suggesting its involvement in the development of chemoresistance [33, 34]. However, in the setting of HCC treated by TACE, the role of miR-335 has not been fully clarified.
The result presented here shows decreased miR-335 expression levels in patients with HCC, which is consistent with a recent report indicating that miR-335 is reduced in HCC via aberrant promoter hypermethylation [35]. Besides, we found that HCC patients with low miR-335 levels depicted an unfavorable clinicopathologic features (high AFP values, vascular invasion, cirrhosis and large tumor volume), and those patients also had a poor response to TACE. These findings alone could explain the worse clinical long-term outcome in patients with lower miR-335 expresser status. Additionally, after adjusting for other confounding clinical parameters, the association between miR-335 and OS/TTP still remained significant, suggesting that miR-335 may have strong and independent prognostic impact in HCC. To our knowledge, this is the first study showing the link between serum miR-335 levels and therapeutic efficacy of TACE among HCC patients.

Several limitations should be addressed in this study. First, a prospective study in larger population from different countries would be needed to confirm the predictive values of miR-335 in HCC patients. Second, evaluation of additional microRNAs in HCC patients awaits further study. Third, Future investigations are needed to elucidate biological effects of miR-335 in HCC after TACE.

In conclusion, we provide the first evidence that the expression of a single microRNA, miR-335, is associated with clinical outcome of HCC patients treated with TACE. Furthermore, expression levels of miR-335 could provide prognostic information in those patients independently from a comprehensive panel of other established clinical predictors. Thus, establishment of standardized methods of microRNA quantification may be crucial for stratification of treatment modalities in HCC patients. Targeted treatments that increase endogenous levels of miR-335 might represent novel therapeutic options.

**Disclosure Statement**

None.

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