ORIGINAL PAPER

MicroRNA-148b expression is decreased in hepatocellular carcinoma and associated with prognosis

Zhiyong Zhang · Wei Zheng · Jun Hai

Abstract MicroRNAs are small non-coding RNA molecules that have been shown to regulate the expression of genes linked to cancer. MicroRNA-148a (miR-148a) has recently been found to be involved in many critical processes in human malignancies. The present study is to clarify the expression pattern and prognostic role of miR-148b in human hepatocellular carcinoma (HCC). The expression of miR-148b was detected in 156 cases of HCC and 36 cases of normal control specimens by real-time PCR. Results showed that miR-148b expression was significantly decreased in HCC compared with that in normal control. It was also demonstrated that aberrant miR-148b expression was associated with vein invasion and TNM stage of HCC. Kaplan–Meier analysis showed that decreased miR-148b expression was associated with poor overall survival of patients. A multivariate survival analysis also indicated that miR-148b could be an independent prognostic marker. These results proved that miR-148b expression was decreased in HCC and associated with tumor invasion and progression. The present study also provides the first evidence that miR-148b could be an independent prognostic factor for patients with HCC, indicating the potential role of miR-148b as a prognostic marker in clinical practice, and the inhibition of miR-148b may even become a new therapeutic method for the treatment of HCC.

Keywords miR-148b · Hepatocellular carcinoma · Real-time PCR · Prognosis

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Introduction

MicroRNAs (miRNA) are a class of evolutionarily conserved, single-stranded, non-protein coding RNAs (20–22 nucleotides) that are estimated to regulate 30 % of all genes in animals by binding to specific sites in the 3′ untranslated regions (3′UTR), resulting in posttranscriptional repression, cleavage or destabilization [1, 2]. Beyond the involvement in diverse biological processes, it has been well demonstrated that deregulation or dysfunction of miRNAs can be detected in conditions such as cancer, cardiovascular diseases and disorders of central nervous system [3–5]. Recent studies showed that miRNAs play essential roles in the biology of various human cancers, including cell differentiation, proliferation, apoptosis, invasion and angiogenesis [6, 7]. In hepatocellular carcinoma (HCC), accumulating evidence also suggested that miRNAs participate in HCC angiogenesis, invasion and metastasis [8]. In HCC, multiple miRNAs with aberrant expression have been identified, among which miR-21, miR-25, miR-181a and miR-182 were found to be overexpressed and to play oncogenic roles, while miR-23, miR-101, miR-139 and miR-141 were found to be decreased, indicating the tumor suppressive roles of these miRNAs [9–16]. Due to the diverse and crucial roles of miRNAs in HCC, clarifying the functional and clinical significance of a specific miRNA may provide relevant insights into efficacious disease management.

MiR-148a is a member of the miR-148/152 family, which consisted of miR-148a, miR-148b and miR-152 [17]. The pre-miR-148/152 family members can be processed into the mature members of the miR-148/152, which are involved in various biological processes through complementary binding between the seed sequence and the 3′UTR of target mRNAs. The mature members of

miR-148/152 family have been proved to be expressed in various tumors and normal tissues during growth, development and tumorigenesis, indicating the critical role of miR-148/152 family in these processes. It was reported that miR-148a was significantly upregulated in the plasma of multiple myeloma and high levels of miR-148a were related to shorter relapse-free survival times [18]. While in gastrointestinal cancers, miR-148a and miR-152 were downregulated in cancer tissue and cancer cell lines [17]. Moreover, low expression of miR-148a was also found to be significantly associated with lymph node metastasis in gastric cancer [19]. These findings indicated that the miR-148/152 family might play complicated and diverse roles in human malignancies. In HCC, miR-148a was found to be upregulated in hepatitis B-associated HCC, while miR-152 was downregulated in HBV-related HCC tissues compared with adjacent non-cancerous hepatic tissues [20, 21]. However, to our knowledge, the expression pattern and function of miR-148b have not been addressed yet.

In the present study, we investigated the expression level of miR-148b in clinical HCC specimens and normal tissues. We also analyzed the association of miR-148b with clinicopathological characteristics and overall survival of the patients.

Materials and methods

Patients and specimens

The present study was approved by the Ethics Committee of Shaanxi Provincial People's Hospital. Patients involved had provided written informed consent. Fresh HCC specimens were collected from 156 patients who underwent surgery between 2006 and 2008 in Shaanxi Provincial People's Hospital. None of these patients had received chemotherapy prior to surgery. In addition, non-cancerous normal control samples from 36 patients who underwent surgery for reasons other than malignancy. The histomorphology of all the tissue specimens was confirmed by the Department of Pathology, Shaanxi Provincial People's Hospital. After surgical resection, the specimens were put immediately into liquid N_2 for 10 min and then into a -70 °C ultra-freezer. Patients' clinical information, such as age, sex and differentiation status, was collected and stored in a database. A complete follow-up was available for at least 5 years. Overall survival is defined as the time elapsed from the surgery to the death of the patients with HCC. The follow-up information of all the participants was updated every 3 months by a telephone call and questionnaire letters. The death of the participants was ascertained by a report from the family and verified by the review of public records.

Quantitative real-time PCR

To detect miRNA level by real-time PCR, total RNA was purified from all the 156 cases of HCC and 36 cases of normal control specimens using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). Only those total RNA samples with an OD A260/A280 ratio close to a value of 2.0, which indicates that the RNA is pure, were subsequently analyzed. The miR-148b and U6 internal control-specific cDNAs were synthesized from the total RNA using gene-specific primers according to the TaqMan microRNA assay protocol (Applied Biosystems, Foster City, CA, USA). The reverse transcription products were then amplified and detected by real-time PCR using a TaqMan microRNA assay specific for hsa-miR-148b. Each sample was examined in triplicate, and the raw data are presented as the relative quantification of miR-148b expression evaluated by the comparative cycle threshold (CT) method, normalized with respect to U6. The mean normalized miR-148b expression $\pm$ the standard deviation (SD) was calculated from triplicate analyses. The real-time PCR was performed with an ABI 7500 system (Applied Biosystems), and the comparative $2^{-\Delta\Delta Ct}$ analysis was conducted using SDS 2.2.2 software (Applied Biosystems).

Statistical analysis

The difference in the expression of miR-148b between HCC and the normal specimen was analyzed with Student's t test. Associations between miR-148b expression and clinicopathological characteristics were analyzed via Mann–Whitney U test or Kruskal–Wallis test, as appropriate. The survival curves were estimated using the Kaplan–Meier method, and differences in the survival distributions were evaluated with a log-rank test. A Cox proportional hazards modeling of the factors potentially related to survival was performed to identify those factors that might have a significant influence on survival. Differences with a P value of 0.05 or less were considered to be statistically significant.

Results

The expression of miR-148b was decreased in HCC

Clinicopathological characteristics of all patients with HCC recruited are shown in Table 1. Real-time PCR assay results showed that the relative expression of miR-148b in these HCC specimens normalized to U6 was 1.36 ± 0.15 (mean $\pm$ SD), while the relative expression of miR-148b in 36 cases of normal control specimens was 1.96 ± 0.21. The difference in miR-148b expression between HCC and

Table 1 Relationship between miR-148b expression and clinico-pathological characteristics

Characteristic	n	miR-148b expression		P value
		Low	High	
Total	156	98	58	
Gender				0.778*
Male	100	62	38	
Female	56	36	20	
Age				0.501*
≤50	86	52	34	
>50	70	46	24	
Tumor size(cm)				0.127*
≤5	85	58	27	
>5	71	40	31	
Vein invasion				0.035*
Negative	120	70	50	
Positive	36	28	8	
Liver cirrhosis				0.775*
Negative	12	8	4	
Positive	142	90	54	
Hepatitis B virus infection				0.972*
Negative	24	15	9	
Positive	132	83	49	
Histologic grade				0.882†
Well	23	15	8	
Moderate	82	50	32	
Poor	51	33	18	
TNM stage				0.006*
I + II	91	49	42	
III + IV	65	49	16	
Serum AFP level (ng/ml)				0.144*
≤25	60	42	18	
>25	96	56	40	

* P value when expression levels were compared using Mann–Whitney U test

† P value when expression levels were compared using Kruskal–Wallis test

normal tissue was statistically significant, indicating that miR-148b expression was decreased in HCC ($P < 0.001$). The decreased expression pattern suggested that miR-148b might play a tumor suppresser role in HCC. To facilitate further analysis of the association of miR-148b expression with clinicopathological characteristics and prognosis, we manually defined HCC specimens with a level of miR-148b expression lower than 1.75 (mean-SD for the expression of miR-148b in normal control specimens) as low miR-148b expression group, while specimens with miR-148b expression level no lower than 1.75 were defined as high miR-148b expression group. Accordingly, 98 cases of HCC were classified as low miR-148b expression group, while 58 cases were classified as the high expression group.

The expression of miR-148b in HCC was related to tumor progression

As miR-148b expression was shown to be decreased in HCC, which suggested that it might play a tumor suppresser role in these tumors. We further investigated the association of miR-148b expression with clinicopathological characteristics of these patients to explore the potential role of miR-148b in tumor progression. Results showed that aberrant miR-148b expression was significantly associated with vein invasion and TNM stage of HCC. Concretely, low levels of miR-148b expression were more frequently detected in tumors with vein invasion (Table 1, $P = 0.035$). Besides, low levels of miR-148b expression were also more frequently detected in TNM stage III and stage IV tumors (Table 1, $P = 0.006$). These results showed that decreased expression of miR-148b in HCC was associated with significantly aggressive pathologic features, indicating that miR-148b might inhibit HCC progression. However, miR-148b expression was not found to be associated with the gender ($P = 0.778$), age ($P = 0.501$), tumor size ($P = 0.127$), liver cirrhosis ($P = 0.775$), hepatitis B virus infection ($P = 0.972$), histologic grade ($P = 0.882$) or serum AFP level ($P = 0.144$).

The expression of miR-148b in HCC determines prognosis

Considering that miR-148b expression in HCC was proved to be significantly associated with tumor progression, we next evaluated its prognostic role in these patients. For all 156 patients recruited, during the entire follow-up period, 98 of 156 patients (62.8 %) with HCC had died with the median overall survival time of 39 months. Kaplan–Meier survival analysis and log-rank test demonstrated a significant difference in the outcomes of patients who were divided into two groups based on their miR-148b expression level. Specifically, patients with low expression of miR-148b had significantly worse overall survival rates compared with those who had cancers with low miR-148b expression (log-rank test $P < 0.001$, Fig. 1). The median survival time of patients with low levels of miR-148b expression was 32 months (95 % CI 23–41), while the median survival time of those patients with high levels of miR-148b expression was 48 months (95 % CI 41–55). When the unadjusted hazard ratio (HR) was considered with high levels of miR-148b expression as reference, those patients with low levels of miR-148b expression had a 1.83-fold higher risk of death (95 % CI 1.19–2.83, $P = 0.006$). As far as clinicopathological characteristics of
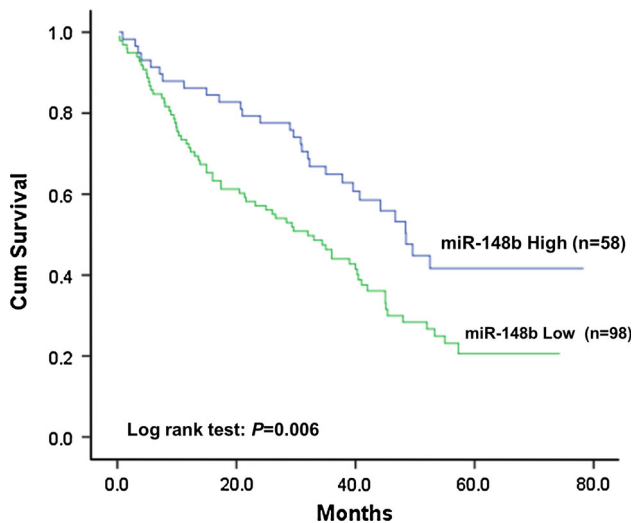
Fig. 1 Kaplan–Meier postoperative survival curve for patients with HCC and miR-148b expression

patients were considered, tumor size, TNM stage, histologic grade and vein invasion were also found to be associated with overall survival of HCC for those patients with large tumor, advanced stage, undifferentiated tumor and vein invasion had worse overall survival rates and higher risk of death. However, sex, age, liver cirrhosis, hepatitis B virus infection or serum AFP level had no prognostic value regarding the overall survival.

Because miR-148b expression was found to be associated with the overall survival in univariate survival analysis, we further evaluated whether miR-148b could serve as an independent prognostic marker for patients with HCC by multivariate Cox proportional hazards model analysis. In Cox proportional hazards model adjusted for sex, age, tumor size, TNM stage, histologic grade and vein invasion, miR-148b expression was found to be independently associated with overall survival of patients with HCC. The adjusted HR of the low miR-148b expression group was 1.86 (95 % CI 1.23–2.98, $P = 0.002$), indicated that patients with low miR-148b expression had poor overall survival. These results proved that miR-148b could be an independent prognostic factor of overall survival for patients with HCC, without consideration of sex, age, tumor size, TNM stage, histologic grade or vein invasion (Table 2).

Discussion

Hepatocellular carcinoma is the sixth most common malignant tumor and the third most common cause of cancer-related deaths worldwide [22]. According to global cancer statistics, there are about 626,000 new HCC cases and nearly 600,000 HCC-related deaths each year all over the world [22, 23]. The majority of HCC arise from chronic infections with hepatitis B virus or hepatitis C virus, with other non-viral causes playing much lesser roles [24, 25]. Patients with HCC do not have overt symptoms until the tumor is in its advanced stages, making treatment difficult and ineffective [26, 27]. In most cases, surgical resection and liver transplantation are the only curative treatment methods for these patients [28, 29]. Frequent tumor metastasis and recurrence after surgical therapy lead to the dismal outcome of patients with HCC with the 5-year survival rate of patients with HCC to be approximately 35 % [30, 31]. Although a growing number of novel treatment strategies have been developed for HCC, such as molecular targeted therapy and gene therapy, satisfactory therapeutic outcomes have not been achieved. Under this circumstance, prognosis predication and following accurate patient stratification are of great importance to optimize personalized therapy [32]. However, traditional staging systems may not be effective to predict prognosis for individual patients [33]. Considering that the survival rate of HCC is still low, identifying new prognostic markers and modifying staging systems, in particular by biomarkers that reflect tumor aggressiveness, are likely to improve the prognostic assessment of HCC patients and could therefore fulfill a clinical need of personalized prescription.

Till now, more than 1,900 miRNAs have been addressed in human, which are estimated to regulate over 60 % of the genes in mammals [34]. Due to its various regulations on gene expression, miRNAs participate in multiple cellular functions, such as proliferation, apoptosis, differentiation, cancer carcinogenesis and progression [35–37]. However, individual miRNA in different cancer types has a large number of different target genes, which may result in different functions. Thus, identifying the function of an individual miRNA may enable deeper insight into the regulation of gene expression and the complexity of cancer progression.

In the present study, we investigated miR-148b expression by real-time PCR in 156 specimens of HCC and 36 cases of normal control. Based on the relative expression analysis, we investigated the association of miR-148b with clinicopathological characteristics and prognosis of patients with HCC. Results showed that the expression level of miR-148b was decreased in HCC compared with that in normal control specimens. The expression pattern of miR-148b found in our study is consistent with previous investigation which showed that miR-148b expression was downregulated in the liver cancer cell lines HepG2, MHCC97L and MHCC97H relative to the hepatic cell line L02 [38]. Previously, miR-148b has been found to have different roles in various tumors and acts as oncogene or tumor suppressor. And our results provided the first evidence that miR-148b expression was decreased in human

Table 2 Univariate and multivariate analysis of prognostic factors in HCC

	Unadjusted HR[a] (95 % CI)	P	Adjusted HR[b] (95 % CI)	P
miR-148b				
High	–		–	
Low	1.83 (1.19–2.83)	0.006	1.86 (1.23–2.98)	0.002
Sex				
Female	–		–	
Male	1.12 (0.56–2.26)	0.740	1.14 (0.72–1.78)	0.578
Age				
<60	–		–	
≥60	1.47 (0.76–2.83)	0.253	1.39 (0.86–2.28)	0.183
Tumor size (cm)				
≤5	–		–	
>5	1.65 (1.08–1.97)	0.026	1.59 (1.04–1.86)	0.043
Vein invasion				
Negative	–		–	
Positive	1.95 (1.24–3.08)	0.004	1.84 (1.21–2.83)	0.008
TNM stage				
I + II	–		–	
III + IV	4.96 (2.72–9.04)	<0.001	4.05 (1.80–9.10)	0.001
Histologic grade				
Well + moderate	–		–	
Poor	2.06 (1.12–3.78)	0.020	2.14 (1.06–4.28)	0.032

HR hazard ratio, *95 % CI* 95 % confidence interval

[a] Hazard ratios in univariate models

[b] Hazard ratios in multivariable models

HCC. These results revealed that miR-148b may play a tumor suppresser role in HCC carcinogenesis.

In addition, we also found that miR-148b expression was closely associated with HCC vein invasion and the TNM stage, as results revealed that low level of miR-148b expression was more frequently to be detected in tumors with vein invasion or advanced TNM stage. These results indicated the possible participation of miR-148b in invasion and progression of HCC. This association is consistent with previous findings in gastric and colorectal cancers, which also found that miR-148b could suppress cell proliferation, migration and invasion in these tumors [39, 40]. Based on the present results, together with the evidence above, it is thus proposed that miR-148b may play a tumor suppresser role in HCC progression.

As miR-148b was found to be associated with vein invasion and TNM stage of HCC, considering that invasion and stage are crucial factors affecting prognosis. We further evaluated the prognostic role of miR-148b in HCC by Kaplan–Meier analysis and log-rank test. Results showed that patients with low levels of miR-148b expression had worse overall survival compared with those with high levels of miR-148b expression. The statistically significant association indicated that miR-148b could be a prognostic marker for patients with HCC. To further evaluate the independently prognostic value of miR-148b in HCC, we performed a Cox proportional hazards model adjusted for sex, age and other factors related to survival of HCC. Results proved that decreased miR-148b expression constituted a marker of poor overall survival independent of adjusted prognostic factors; therefore, miR-148b might be utilized to identify high-risk individual patients with HCC who are good candidates to receive more aggressive treatment. Based on available evidences, the positive association of miR-148b with progression and prognosis of HCC may be at least partly caused by its targets, such as cholecystokinin-2 receptor (CCK2R) which can suppress tumor cell growth, invasion and metastasis [39, 40].

In conclusion, our investigation showed for the first time that miR-148b expression was decreased in HCC and associated with tumor progression. The present study also provided the first evidence that miR-148b expression could be an independent prognostic factor for patients with HCC. The results suggested that miR-148b may be used not only

for identifying HCC patients with a higher risk of death but also for providing valuable clues to understanding the possible mechanism of miR-148/152 family and HCC progression.

Conflict of interest   None.

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