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ORIGINAL ARTICLE

Downregulation of miR-148b as biomarker for early detection of hepatocellular carcinoma and may serve as a prognostic marker

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Abstract MicroRNAs (miRNAs) have a large number of various target genes in different cancer types, which may result in many biological functions. Thus, identifying the molecular mechanisms of miRNAs may effect on the complexity of cancer progression via regulation of gene. In the current study, we utilized real-time PCR to quantify the diction of miR-148b in trail hepatocellular carcinoma (HCC) specimen and normal tissues. Furthermore, we evaluated the relationship of miR-148b and clinicopathological features with survival of HCC patients. Therefore, we evaluated the level of miR-148b expression in 101 HCC patients and also in 40 normal control cases. The result suggested lower expression in tumor tissue than normal control tissues (0.96±0.14; 1.84±0.20, $P<0.05$). Our findings suggest that the declined expression of miR-148b can considerably be linked to tumor node metastasis (TNM) stage (stages III and IV; $P=0.02$) and vein invasion ($P=0.029$). Nevertheless, miR-148b expression was not related to sex ($P=0.674$), age ($P=0.523$), size of tumor ($P=0.507$), liver cirrhosis, and histologic grade ($P=0.734$). Survival analysis showed that low expression was remarkably related to overall survival ($P=0.012$). Furthermore, multivariate survival test suggested that decline of miR-148b diction was linked to poor survival in HCC patients. Our results suggested that miR-148b is decreased in HCC. Therefore, we concluded that miR-148b may play its role in the prognosis of HCC.

Keywords miRNA · Liver · Pathology · Cancer · PCR

Introduction

MicroRNAs (miRNAs) are small non-protein coding genes of about 19–23 nucleotides which functions in many biological functions including cell fate specification, cellular proliferation, differentiation, and apoptosis through alteration of the target expression by both downregulation and upregulation [1–3]. The correlation within miRNA dictions and tumor prognosis has been documented [4–6]. Furthermore, the role of that miRNAs as either oncogenes or tumor suppressors has been indicated in human carcinogenesis [7]. Application of molecular research can be beneficial to clarify the functional and clinical importance of a specific miRNA, as well as it is significant to serve the therapeutic effect of these mechanisms that miRNAs are involved.

Different studies have previously indicated that miR-148b was downregulated in various cancers including colon, oral, pancreatic, and gastric cancer tissues, indicating that it plays a key role as a tumor-suppressor miRNA [8, 9]. Hepatocellular carcinoma (HCC) is the most common primary liver cancer that is the third cause of cancer-related mortality all over the

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world [10]. Zhang et al. [11] have demonstrated that miR-148b had potential ability to suppressor tumor in HCC patients. They suggested that miR-148b has a prognostic value in clinical evaluations [11].

Furthermore, real-time PCR was applied to quantify the diction rate of miR-148b in clinical HCC specimen and normal tissues. Furthermore, we evaluated the relationship of miR-148b with clinicopathological investigations and survival of HCC cases.

Material and methods

Samples

Taken together, 101 HCC samples were collected from patients who had undergone surgery at Tehran hospitals between March 2011 and April 2014. The current study was confirmed by Research Ethics Committee, Iran. Moreover, we collected non-cancerous normal control tissues from 40 patients who underwent surgery for reasons other than malignancy. All samples were evaluated by two pathologists. The collected sample was snap frozen in liquid (−70 °C) until use. Clinical information of HCC patients were collected and summarized in Table 1. We defined overall survival based on the elapsed time, from the surgery to death. All participants were followed up by a phone call and a questionnaire. The deaths of cases were recorded by the family.

Quantitative real-time PCR

Total RNA was purified from HCC samples and normal control tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Gene-specific primers were used to synthesize the miR-148b and U6 internal control specific cDNAs from the total RNA according to the TaqMan MicroRNA assay (Applied Biosystems, Foster City, CA, USA). Furthermore, real-time PCR was carried out using an Express SYBR® GreenER qPCRs supermix Universal kit (Invitrogen) by system of Rotor-gene 6000 (Qiagen). We used the comparative cycle threshold (CT) method to calculate changes in expression. The relative amount of miR-148b was normalized with respect to U6 RNA. Moreover, 2ΔΔCt method was applied to calculate the fold-change between cancer and normal tissue for miR-148b. It is worth noting that ΔΔCt=ΔCt (tumor sample)–ΔCt (control sample). As a result, by application of the mentioned method, diction rate of miR-148b in tumor and normal samples was evaluated.

Statistical analysis

SPSS software 16.0 (SPSS Inc., Chicago, IL, USA) was applied in the current study. The differences of miR-148b diction in tumor and normal tissue were calculated by applying Student's *t* test. Furthermore, the relationship between gene expression and clinical feature was analyzed by Fisher's test. The survival analysis was performed between tumor and normal samples (the log-rank test and Kaplan-Meier method). In addition, a Cox proportional hazards modeling was performed to identify independent factors linked to patient survival. Differences were significant at $P<0.05$.

Table 1 Correlation between miR-148b expression and clinicopathological features of patients with HCC

Characteristic	Number	miR-148b expression		*P* value
		Low	High	
Gender				0.674
Male	66	37	29	
Female	35	20	15	
Age				0.523
≤60	57	39	18	
>60	44	29	15	
Tumor size (cm)				0.507
≤5	55	35	17	
>5	47	32	15	
Vein invasion				0.029
Negative	81	54	27	
Positive	20	17	3	
Liver cirrhosis				0.684
Negative	8	6	2	
Positive	93	75	26	
Histologic grade				0.734
Well	19	11	8	
Moderate	44	32	12	
Poor	38	25	13	
TNM stage				0.021
I+II	58	40	18	
III+IV	43	39	4	

Results

The miR-148b diction in HCC

By using quantitative real-time PCR, decreased level of expression in tumors was observed in comparison with normal tissues (0.96±0.14; 1.84±0.20, $P<0.05$). Decreased expression indicated that miR-148b might play a tumor suppresser role in HCC.

In our study, HCC patients were divided in to low and high level of expression based on the median expression level of miR-148b. Sixty-two cases were assigned to the low miR-148b expression group; the level of miR-148b expression was

lower than 1.86 (mean-SD for the expression of miR-148b in normal control specimens), while 39 specimens with expression level no lower than 1.86 were assigned to the high-expression group.

Moreover, results suggested that low expression was remarkably detected in tumor node metastasis (TNM) stage (stages III and IV; P=0.021) and vein invasion (P=0.029). Our data recommended that miR-148b might inhibit HCC progression. Nevertheless, miR-148b expression was not related to sex (P=0.674), age (P=0. 523), size of tumor (P= 0.507), liver cirrhosis, and histologic grade (P=0.734, Table 1).

Survival analysis in studied group

By using Kaplan-Meier survival and log-rank analysis, it became clear that shorter overall survival was significantly related to low expression (in log-rank test, P=0.012; Fig. 1). The median survival time of patients with low levels of miR 148b expression was 32.5 months (95 % CI 25–40), but the median survival time of the cases with high rates of miR-148b expression was 46 months (95 % CI 40–52).

We used multivariate Cox proportional hazards model analysis to determine the significance of the miR-148b expression and different factors in the progression of HCC (Table 2).

This analysis was performed to determine the significance of the marker and other factors.

The result indicated that low miR-148b expression, TNM stage, and vein invasion were independently associated with poor survival of patients with HCC, and other factors had no significant prognostic value for survival in HCC (Table 2, HR was calculated to be 2.21; 95% CI 1.[illegible]9.126, P=0.012).

Table 2 Multivariate analysis with a Cox proportional hazards model between clinicopathological factors by Cox regression model

Clinicopathological characteristics	HR	95 % CI	P value
Sex	0.851	0.5113–3.212	0.584
Age	1.211	0.946–3.216	0.625
TNM stage	2.428	1.437–9.174	0.011
Tumor size (cm)	0.641	0.972–3.463	0.628
Vein invasion	2.123	1.654–8.742	0.032
Histologic grade	1.235	0.643–2.176	0.511
miR-148b level	2.212	1.431–9.126	012

Discussion

miRNAs have a large number of various target genes in different cancer types, which may result in many biological functions. Thus, identifying the molecular mechanisms of miRNAs may result in the regulation of gene expression and the complexity of cancer progression. Several studies have indicated that there is a correlation between miRNA expressions and tumor prognosis [6, 12].

In the current study, we utilized real-time PCR to quantify the diction of miR-148b in clinical HCC specimen and normal tissues. These results revealed that miR-148b is

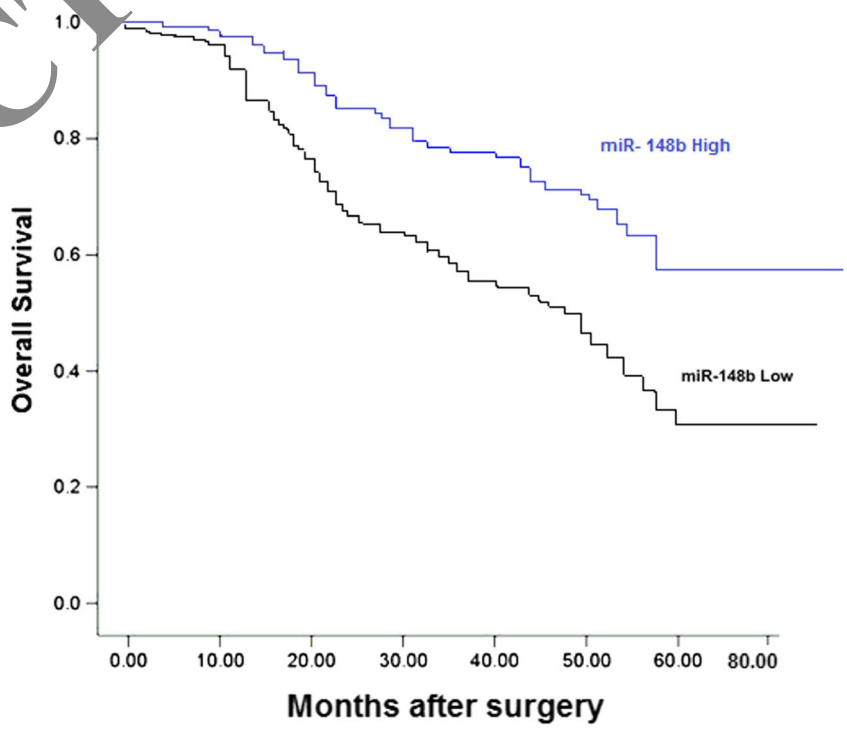

Fig. 1 Correlation between miR-148b expression and survival time in HCC patients. Patients with low expression had significantly shorter overall survival in comparison with high expression (log-rank test, P=0.012)

downregulated in HCC and plays its role as a tumor suppressor. As matter of fact, a reduced expression of miR-148b may be responsible for the progression.

Our result showed the lower expression in tumor tissues in comparison with corresponding normal control tissues. The expression of miR-148b in the current study is in agreement with a previous study that suggested downregulation of miR-148b diction in HCC and its relationship with tumor invasion and progression [11]. Previous studies have found that miR-148b acts as tumor suppressor in many tumors [11, 13, 14]. Liu et al. [14] have found that miR-148b expression is low in non-small cell lung cancer (NSCLC) cells by targeting carcinoembryonic antigen (CEA), which results in CEA overexpression and disease progression in NSCLC [14].

Furthermore, we evaluated the relationship of miR-148b with clinicopathological investigations and survival of cancers cases. In our study, we observed that declined diction of miR-148b was liked to TNM stage (stages III and IV) and vein invasion (P=0.021; P=0.029). Our data recommended that miR-148b might be linked to tumor invasion and progression and is consistent with previous studies that were conducted based on miR-148b in many kinds of tumors [11, 14–18]. Survival analysis and log-rank test suggested that low expression of miR-148b can be related to shorter overall survival than high expression, indicating that this miRNA may act as a marker in the prognosis of HCC. We used multivariate Cox proportional hazards in our study which indicated that downregulation of miR-148b can be related to poor survival of HCC patients. It can be concluded that miR-148b was an independent prognostic marker.

In conclusion, our results revealed that miR-148b is downregulated in HCC and may play a role as an independent prognostic factor in patients with HCC, suggesting that miR-148b has a potential role as a prognostic marker in clinical evaluations.

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Conflicts of interest None

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