Upregulation of microRNA-32 is associated with tumorigenesis and poor prognosis in patients with hepatocellular carcinoma

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Abstract. MicroRNA-32 (miR-32) is associated with tumor progression and poor prognosis in certain malignant tumors. However, the function and clinical relevance of miR-32 in human hepatocellular carcinoma (HCC) has not yet been elucidated. The present study aimed to investigate the expression and prognostic value of miR-32 from liver samples in patients with HCC. The expression of miR-32 was analyzed in HCC and healthy tissues using Gene Expression Omnibus datasets. Reverse transcription-quantitative polymerase chain reaction was used to analyze the levels of miR-32 mRNA in 154 HCC liver samples, 33 of which were paired with adjacent non-tumor tissues. The overall survival (OS) rate in patients with HCC was evaluated using Kaplan-Meier survival analysis, and the factors that may affect the prognosis and survival of patients with HCC were analyzed using univariate (log-rank test) and multivariate Cox proportional hazard models. The present results demonstrated that miR-32 expression levels were significantly upregulated in HCC liver biopsies compared with normal tissues (P<0.05). miR-32 expression was significantly associated with the number of foci and tumor diameter (P<0.05). In addition, Kaplan-Meier analysis revealed that patients with low miR-32 expression had longer OS and disease-free survival compared with those with high miR-32 expression (P<0.01). Altogether, to the best our knowledge, the present study is the first study to indicate the association between increased miR-32 expression with HCC progression and poor prognosis in patients. This suggests that miR-32 may have potential prognostic value and may be used as a tumor biomarker for the diagnosis of patients with HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver malignancy (1). Currently, HCC ranks as the third leading cause of cancer-associated mortality worldwide and the sixth leading cause of human cancer (2-4). During the past few decades, a number of HCC treatments have been developed. However, the therapeutic effects are not optimal due to tumor recurrence, metastasis and, in particular, a low survival rate (5). Therefore, novel treatment options based on an improved understanding of the molecular mechanisms of HCC need to be investigated.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules (20-24 nucleotides), processed as miRNA precursors with a hairpin structure (6-8). The specific involvement of miRNAs in different tumor types and associations with prognosis are of interest. miRNAs are highly conserved and tissue-specific. Previous studies have shown that miRNA expression varies in different types of cancer, indicating that miRNAs may be involved not only in tissue function and cell specificity, but also in complex gene regulation (9-11).

In recent years, a plethora of studies have suggested that dysregulation of miRNA-32 (miR-32) is associated with tumor progression and poor prognosis in different types of tumors. miR-32 has been shown to be upregulated in colorectal cancer (CRC) (12), renal cell carcinoma (13) and prostate cancer (14), and downregulated in non-small cell lung cancer (NSCLC) (15), osteosarcoma (16), gastric cancer (17) and

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oral squamous cell carcinoma (18). Yan *et al* (19) reported that miR-32 levels were upregulated in HCC tissue, and cell proliferation, invasion and migration were upregulated by targeting phosphatase and tensin homolog (PTEN). These results indicate that miR-32 may be a potential biomarker for HCC diagnosis. However, the clinical relevance and function of miR-32 in HCC remains elusive.

The present study aimed to investigate the expression levels of miR-32 in HCC tissues by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In addition, the association of miR-32 and patient clinicopathological characteristics was analyzed.

Materials and methods

Ethics statement. The present study was approved by the Ethics Committee of Jilin University (approval no. 20151101; Jilin, China). Each patient provided written informed consent prior to participation in the present study.

miRNA expression in HCC from Gene Expression Omnibus (GEO) datasets. miRNA expression levels were investigated in HCC tissues and normal tissue samples in the GEO datasets using the NCBI Platform (http://www.ncbi.nlm.nih. gov/). A total of three original datasets were downloaded (GEO accession no. GSE31383, GSE21362 and GSE22058), and differentially expressed miRNAs in HCC samples and adjacent non-tumor tissue were analyzed. Fold-change (FC) ≥2 or ≤0.5 and P<0.01 were used as basic screening parameters for cluster analysis. Hierarchical clustering was performed using the Multiple Experiment Viewer (MeV) 4.7.1 software program (http://www.tm4.org/).

Clinical specimens. A total of 154 HCC primary tumor tissues obtained from patients with HCC (123 male, 30 female and 1 unknown) with a median age of 65.4 years (range, 45-81 years), who underwent surgical resection between July 2004 and October 2013 at China-Japan Union Hospital, Jilin University (Changchun, China), were analyzed. In total, 33 HCC samples were paired with adjacent non-tumor tissues. No patient received chemotherapy or radiotherapy prior to surgery. Follow-up data and statistics were recorded for all patients until September 2014.

RNA extraction and RT-qPCR for miRNA. Total RNA was isolated from HCC and normal tissue specimens using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. RNA concentration was determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.), and purity was identified in 1.5% denaturing agarose gels. TaqMan probe-based qPCR was carried out using TaqMan MicroRNA Reverse Transcription kit (no. 4366597) and Universal Master Mix II (no. 4440048) (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the protocol of the manufacturer. The specific primers are as follows: for hsa-miR-32, forward, 5'-GCACATTCATCATAC ACGCCG-3' and reverse, 5'-GACCACTGAGGTTAGAGC CA-3'; for U6, forward, 5'-CTCGCTTCGGCAGCACATATA CT-3' and reverse, 5'-ACGCTTCACGAATTTGCGTGTC-3'. Thermocycling conditions for RT reaction were as follows:

Initial reaction at 16°C for 30 min, followed by 42°C for 30 min, 85°C for 5 min, with a final reaction at 4°C. Thermocycling conditions for PCR were as follows: Initial denaturation at 94°C for 10 min, followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, with a final extension at 72°C for 10 min. Each reaction was independently tested in duplicate a minimum of three times. U6 small nuclear RNA was used as an internal control, and the $2^{-\Delta\Delta Cq}$ method (20) was used to analyze miR-32 expression levels.

Statistical analysis. All statistical analyses were performed using the IBM SPSS statistical software (version 22.0; IBM SPSS, Armonk, NY, USA). χ^2 test and t-test were used to examine the associations between clinical characteristics and miR-32 expression. Overall survival (OS) curves were constructed using Kaplan-Meier survival analysis, and results were compared using the log-rank test. In order to estimate independent prognostic factors associated with survival, univariate and multivariate survival analyses were performed using the Cox regression model. P<0.05 was considered to indicate a statistically significant difference.

Results

Cluster analysis of miRNA expression in HCC using MEV4.7.1 software. miRNA expression was investigated in HCC and normal tissues (n=73). Raw data were retrieved using the search terms 'GSE#21362' in the GEO dataset. A total of 15 different miRNAs with P<0.01 and FC ≥1.4 were identified. The MEV4.7.1 clustering software was used to analyze 15 different miRNAs (Fig. 1A). The analysis indicated that miR-32 expression in HCC tissues was significantly higher compared with non-tumor tissues (P<0.001; Fig. 1B).

Next, the raw data were downloaded from the GEO database (GEO accession no. GSE22058). In the present study, the genome-wide expression profiles of miRNAs from paired tumor and adjacent non-tumor tissues from a cohort of 96 patients with HCC in Hong Kong were evaluated. A total of 15 dysregulated miRNAs were screened out using FC ≥3 or ≤0.3 and P<0.01 (Fig. 1C). It was revealed that miR-32 expression was also significantly higher in HCC compared with non-tumor tissues (P<0.001; Fig. 1D).

The same method was then used to analyze the 'GSE31383' GEO dataset (Fig. 1E). Approximately 57 miRNAs were tested in HCC samples (n=9) and normal tissue (n=10). A total of 17 dysregulated miRNAs were screened with P<0.01. miR-32 expression in HCC tissue was significantly higher (P=0.01) compared with non-tumor tissue (Fig. 1F).

Altogether, differentially expressed miRNAs were screened using three different raw datasets (GEO accession no. GSE31383, GSE22058 and GSE21362). Notably, 17 miRNAs were identified, including miR-32, which were common in all datasets (P<0.01; Fig. 1G). These results indicated that miR-32 is increased in HCC compared with non-tumor tissues, and therefore miR-32 may be a potential biomarker for diagnosis of HCC.

Increased expression of miR-32 in HCC. miR-32 expression was evaluated in tumor samples (n=33) compared with adjacent non-tumor tissues (n=33) by RT-qPCR. Results of
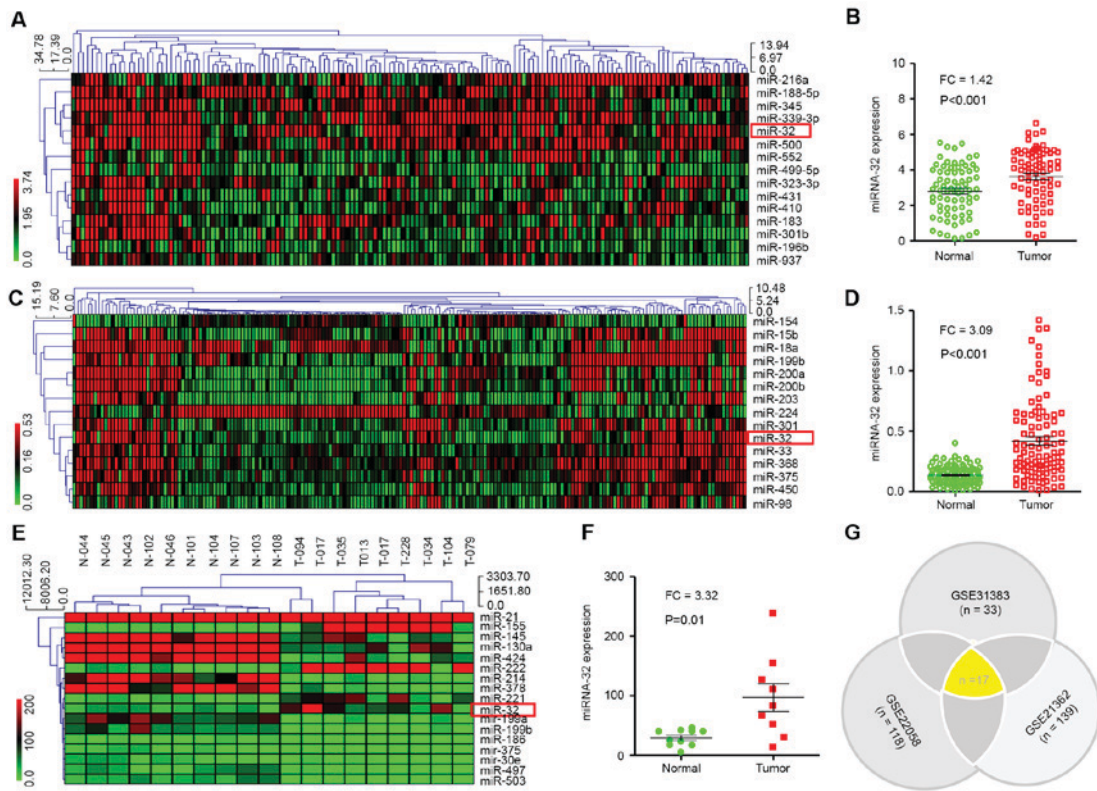
Figure 1. Analysis of miR-32 expression in patients with HCC was performed using GEO datasets, and MEV4.7.1 clustering software was used to analyze different miRNAs. (A) Clustering analysis was performed using the MEV4.7.1 based on 15 dysregulated miRNAs using FC ≥1.4 and P<0.01. (B) miR-32 expression levels in HCC tissues vs. normal tissues were analyzed using the GEO database (GEO accession no. GSE21362). (C) Clustering analysis was performed with the MEV4.7.1 software. A total of 15 dysregulated miRNAs were screened using FC ≥3 or ≤0.3 and P<0.01. (D) The expression levels of miR-32 in HCC (n=96) and normal tissues (n=96) derived from the GEO database (GEO accession no. GSE22058) were analyzed. (E) Clustering analysis was performed using MEV4.7.1 based on 17 dysregulated miRNAs and screened using FC ≥3 or ≤0.3 and P<0.01. (F) miR-32 expression in HCC tissues (n=9) was significantly higher compared with non-tumor tissues (n=10). Datasets derived from the GEO database (GEO accession no. GSE22058). (G) A Venn diagram was generated using three GEO datasets. A total of 17 common miRNAs were identified, including miR-32. HCC, hepatocellular carcinoma; miR, microRNA; FC, fold-change; GEO, Gene Expression Omnibus; miRNA, microRNA.
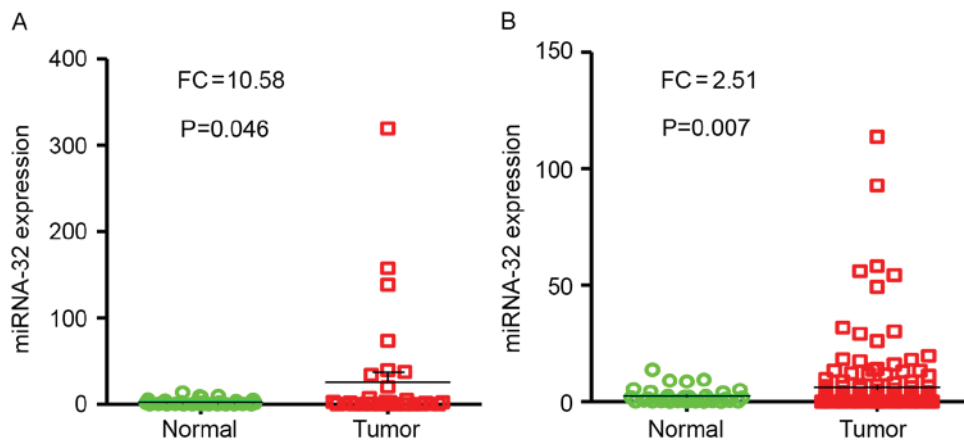

Figure 2. miR-32 expression in normal liver and HCC tissues. (A) miR-32 expression levels in HCC vs. paired adjacent non-tumor tissues (n=33). (B) miR-32 expression levels in 154 HCC samples and 33 non-tumor tissues. miR, microRNA; HCC, hepatocellular carcinoma.

the present study revealed that miR-32 expression levels were significantly higher in HCC tumor specimens compared with adjacent non-neoplastic tissues (P<0.05; FC, 10.58; Fig. 2A).

miR-32 expression levels were examined in tumor samples (n=154), and levels of expression were compared with paired adjacent non-tumor tissues (n=33) from patients with HCC using RT-qPCR. Notably, results demonstrated that the levels of miR-32

expression were significantly higher in HCC tumor specimens compared with normal tissues (P=0.007; FC, 2.51; Fig. 2B).

Association between miR-32 expression and clinicopathological characteristics of patients with HCC. The association between miR-32 expression levels and the individual clinicopathological characteristics were evaluated in

Table I. miR-32 expression and clinicopathological characteristics of patients with hepatocellular carcinoma.

Factor	n	miR-32 expression		P-value	Overall survival, months		P-value
		Low	High		Mean	95% CI	
Age, years							
≥60	84	42	42	0.996	45.98	39.11-52.84	0.472
<60	70	35	35		37.97	32.42-43.52	
Sex							
Male	123	61	62	0.957	41.33	37.14-47.54	0.101
Female	30	15	15		42.46	31.48-51.44	
Unknown	1	1	0				
Tumor differentiation							
Poor	11	4	7	0.541	20.91	5.77-36.04	0.015
Good	125	65	60		44.42	39.30-49.55	
Unknown	18	8	10				
Tumor diameter, cm							
≥5	58	21	37	0.012	36.51	30.79-42.22	0.009
<5	96	56	40		46.01	39.63-52.39	
Number of foci							
Multiple	81	32	49	0.022	34.41	27.86-40.96	0.014
Single	67	39	28		51.96	46.57-57.34	
Unknown	6	6	0		-	-	

CI, confidence interval; miR-32, microRNA-32.

patients with HCC. As shown in Table I, miR-32 expression was positively associated with the number and diameter of foci (P<0.05). However, no significant association was observed between miR-32 expression and other clinical characteristics, including age, sex and tumor differentiation (P>0.05).

Association between clinical characteristics and HCC prognosis. In order to analyze whether clinical factors, including sex, age, diameter, tumor differentiation and number of foci affect HCC prognosis, Kaplan-Meier survival curves were plotted and compared using a log-rank test (Table I). It was observed that tumor diameter was significantly associated with decreased duration of OS (P=0.009; Fig. 3A) and disease-free survival (DFS) (P=0.025; Fig. 3B) in patients with HCC. In addition, tumor differentiation was significantly associated with decreased duration of OS (P=0.015; Fig. 3C) and DFS (P=0.01; Fig. 3D) in patients with HCC. Similar results were obtained regarding the number of foci and duration of OS (P=0.014; Fig. 3E) and DFS (P=0.046; Fig. 3F).

As shown in Table II, univariate analysis using the Cox regression model revealed that miR-32 expression levels [hazard ratio (HR)=2.51; confidence interval (CI): 1.48-4.27; P=0.001], number of foci (HR=36.78; CI: 8.98-150.68; P<0.001) and tumor diameter (HR=1.95; CI: 1.17-3.24; P=0.011) were positively associated with poor prognosis (P<0.05). However, clinicopathological characteristics, including age, sex and tumor differentiation exhibited no association with HCC prognosis (P>0.05).

miR-32 upregulation is a prognostic marker for survival in patients with HCC. To determine the prognostic value of miR-32 expression in HCC, Kaplan-Meier survival analysis was used to evaluate the associations between miR-32 expression and OS and DFS. The results revealed that high miR-32 expression was associated with poorer OS, whereas low miR-32 mRNA levels were associated with increased OS. Therefore, miR-32 expression was significantly associated with decreased duration of OS (P<0.001; Fig. 4A) and DFS (P=0.001; Fig. 4B) in patients with HCC.

Kaplan-Meier survival analysis indicated that patients with HCC with high miR-32 expression and large tumor size (≥5 cm) had significantly decreased duration of Kaplan-Meier survival analysis indicated that patients with HCC with high miR-32 expression and large tumor size (≥5 cm) had significantly decreased duration of OS (P<0.001; Fig. 4C) and DFS (P=0.001; Fig. 4D). In addition, low miR-32 expression and multiple tumor foci were associated with poorer OS and DFS (Fig. 4E and F).

Discussion

HCC ranks as the sixth most common type of cancer worldwide. HCC treatment includes radical surgery or chemotherapy, but the postoperative recurrence rate is high (21). Therefore, the prognosis of HCC currently remains unsatisfactory. The toxicity of chemotherapeutic drugs and cancer-associated tumor resistance are matters of concern, and improvements in therapy are required. In the past few years, the development of new molecular biology techniques
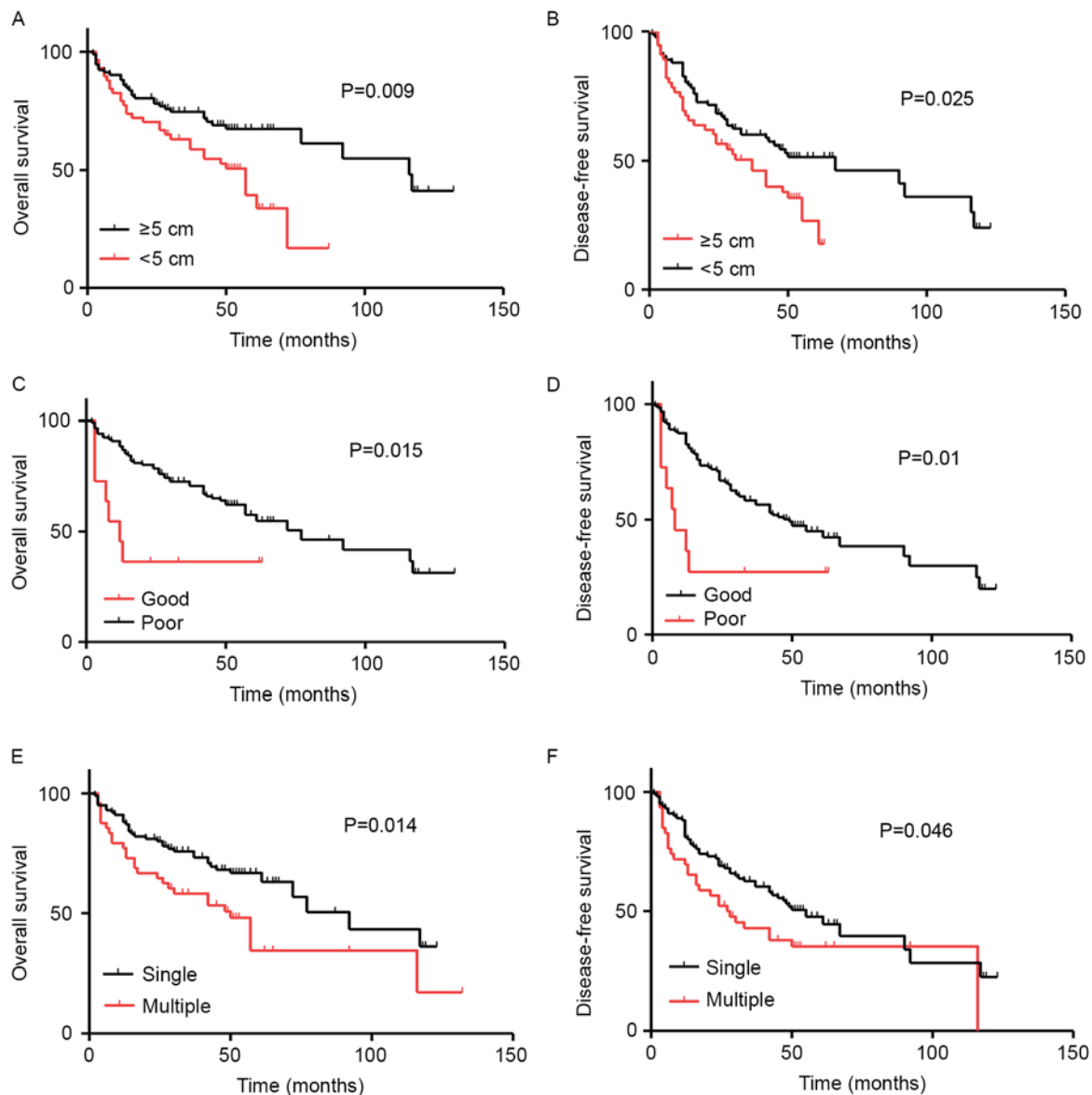
Figure 3. Univariate survival analysis of different clinical parameters with OS and DFS in patients with HCC. Kaplan-Meier plots indicating the associations between: (A) OS and HCC tumor diameter; (B) DFS and HCC tumor diameter; (C) OS and tumor differentiation; (D) DFS and tumor differentiation; (E) OS and number of foci and (F) DFS and number of foci. HCC, hepatocellular carcinoma; OS, overall survival; DFS, disease-free survival.

have enabled targeted HCC therapy (22). miRNAs are regarded as ideal biomarkers, since the diagnostic and therapeutic sensitivity of miRNAs is associated with clinical outcome compared with other biomarkers, including metabolites, antibodies and nucleic acids (23-25).

Recently, a study confirmed that the differences in miRNA expression levels in HCC tissues compared with normal tissues are not only statistically significant, but also associated with HCC diagnosis, prognosis and treatment (26). Li *et al* (27) reported that six different miRNAs were significantly upregulated in HCC samples compared with non-tumor tissues. Notably, it was revealed that miR-375 alone exhibited diagnostic value for HCC (27). Sukata *et al* (28) demonstrated that miR-98, let-7f and let-7a are potential early diagnostic markers for liver cancer. Pineau *et al* (29) revealed that downregulation of miR-221 in HCC performs an important role in tumorigenesis and drug resistance by inducing apoptosis.

miR-32 is able to function as a tumor suppressor and as a tumor promoter in different types of cancer (30). Due to the tissue specificity of miR-32, its biological function has been thoroughly studied. miR-32 functions as a tumor suppressor in osteosarcoma and NSCLC (16,31). In another study, Wu *et al* (12,32) reported that miR-32 is also involved in development of CRC, in part, due to suppression of PTEN. Furthermore, upregulation of miR-32 was associated with specific clinicopathological characteristics of patients with CRC. Therefore, miR-32 is considered to be a putative molecular marker of poor prognosis in patients with CRC.

Next, raw datasets from the GEO database were analyzed. In the raw datasets, it was revealed that miR-32 expression levels were significantly higher in HCC tumor specimens compared with non-neoplastic tissues (33-35). Indeed, using FC ≥2 or ≤0.5 and P<0.01 for screening in cluster analysis revealed that miR-32 was a common denominator of the three

Table II. Cox regression model analysis for OS based on the clinicopathological characteristics of patients with hepatocellular carcinoma.

A, Univariate analysis

Factor	HR	95% CI	P-value
Sex (male vs. female)	0.526	0.24-1.16	0.111
Age, years (≥60 vs. <60)	1.21	0.42-1.08	0.481
Tumor differentiation (poorly vs. moderately/well)	0.9	0.60-1.36	0.632
Tumor diameter, cm (≥5 vs. <5)	1.95	1.17-3.24	0.011
Number of foci (multiple vs. single)	36.78	8.98-150.68	<0.001
miR-32 expression (high vs. low)	2.51	1.48-4.27	0.001

B, Multivariate analysis

Factor	HR	95% CI	P-value
Sex			
Age, years			
Tumor differentiation			
Tumor diameter, cm	4.47	2.13-9.35	<0.001
Number of foci	4.63	2.20-9.76	<0.001
miR-32 expression			

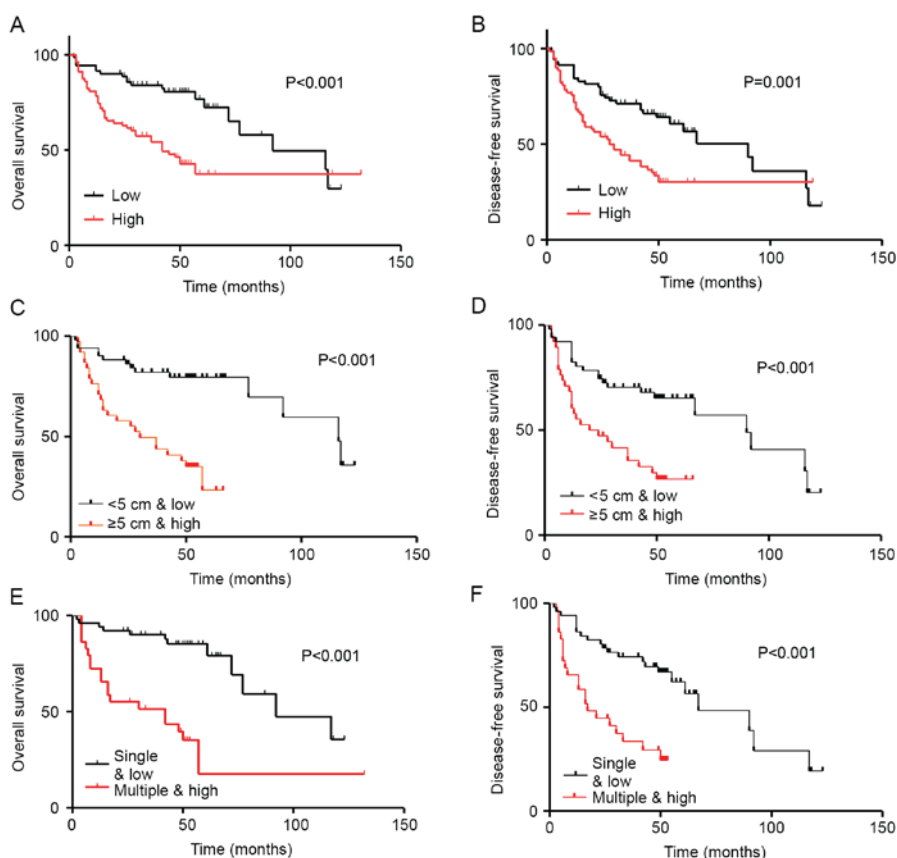HR, hazard ratio; CI, confidence interval; miR-32, microRNA-32.

Figure 4. Survival analysis of miR-32 expression and clinical parameters in patients with HCC. Univariate survival analysis of (A) OS and (B) DFS in HCC as determined by Kaplan-Meier plots based on miR-32 expression. Multivariate analysis of (C) OS and (D) DFS by Kaplan-Meier survival analysis was based on diameter and miR-32 expression. Multivariate analysis of (E) OS and (F) DFS by Kaplan-Meier survival analysis was based on number of foci and miR-32 expression. HCC, hepatocellular carcinoma; OS, overall survival; DFS, disease-free survival; miR-32, microRNA-32.

datasets. In a series of *in vitro* experiments performed by Yan *et al* (19), it was reported that miR-32 induces cell migration, proliferation and invasion in HCC by targeting PTEN. However, the clinical relevance between miR-32 and HCC currently remains unknown.

In the present study, it was revealed that miR-32 expression in HCC samples was significantly higher compared with normal tissues. The association between miR-32 and the clinical parameters was also examined in patients with HCC. High miR-32 expression was associated with tumor diameter and the number of foci, indicating that the upregulation of miR-32 has a crucial role in the progression of liver cancer. In order to investigate the potential prognostic value of miR-32, the association between the expression of miR-32 and OS in patients with HCC was analyzed. Kaplan-Meier survival analysis revealed that patients with high miR-32 expression levels had decreased OS time. Furthermore, Cox analysis demonstrated that miR-32 was an independent prognostic indicator of HCC. Therefore, levels of miR-32 expression may be an optimal indicator and risk factor for reduced OS and DFS in patients with HCC.

In summary, the present study demonstrated the clinical and prognostic significance of miR-32 in HCC. High miR-32 expression may be an optimal indicator of poor HCC prognosis. Altogether, the present analysis indicated that high miR-32 expression may be a potential biomarker for patients with HCC.

Acknowledgements

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