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Low miR-29c expression is a prognostic marker in hepatocellular carcinoma

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ABSTRACT. A previous study has revealed that miR-29c functions as a tumor suppressor in hepatocellular carcinoma (HCC), but the clinical significance and prognostic value of miR-29c in HCC have not been investigated. Paired human HCC tissues and adjacent noncancerous tissues were obtained from 91 patients, between 2008 to 2014. Quantitative real-time PCR (qRT-PCR) was used to analyze miR-29c expression. Kaplan-Meier survival plots and log-rank tests were used to assess differences in the overall survival of different subgroups of HCC patients. It was observed that miR-29c expression was remarkably decreased in HCC tissues relative to that in normal hepatic tissues ($P < 0.001$). The low miR-29c level was significantly associated with histologic grade ($P = 0.001$), microvascular invasion ($P = 0.005$), and tumor stage ($P < 0.001$). Kaplan-Meier analysis showed that decreased miR-29c expression correlated with shorter overall survival ($P = 0.002$). Multivariate Cox regression analysis showed that decreased miR-29c

expression (hazard ratio = 2.19, 95%CI = 1.361-6.779, P = 0.025) was independently associated with poor survival in HCC. Our findings demonstrate that miR-29c expression is significantly downregulated in HCC patients and that miR-29c can act as an independent predictor of unfavorable clinical outcome.

Key words: Expression; Prognostic marker; Hepatocellular carcinoma; MiR-29c

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common neoplasm worldwide, and is one of the leading causes of cancer-related death (Siegel et al., 2015). The prognosis of patients with HCC has improved significantly, largely owing to the development of effective surgical techniques and diagnostic methods over recent years. However, long-term prognosis is still unsatisfactory largely owing to rapid tumor growth, high invasive potential, and high recurrence rates (Imamura et al., 2003). Therefore, prolonging the survival of HCC patients of HCC is an urgent challenge.

MicroRNAs (miRNAs) represent a class of endogenous, highly conserved, small non-coding RNAs that are approximately 22 nucleotides in length (Barshack et al., 2010). MiRNA dysregulation is implicated in the development and progression of practically all tumor types (Hammond, 2006). The miR-29 family is composed of members with conserved miRNA sequences including miR-29a, miR-29b, miR-29c, and miR-29d. The expression levels of a number of miR-29 family members are found to be reduced in various cancers (Inoue et al., 2014; Kurinna et al., 2014; Cui et al., 2015; Duhachek-Muggy and Zolkiewska, 2015; Hu et al., 2015). In HCC, the role of miR-29c has been investigated. Bae et al. (2014) found that miR-29c functioned as a tumor suppressor by directly targeting oncogenic SIRT1 in HCC. The study by Wang et al. (2015) suggested that miR-29c might function as a tumor suppressor that played a crucial role in the development of liver carcinoma by targeting WIP1, therefore possibly representing a target molecule for therapeutic intervention. However, the clinical significance and prognostic value of miR-29c have not been investigated.

MATERIALS AND METHODS

Tumor samples and patient information

Human HCC tissues and their paired adjacent noncancerous tissues were obtained sequentially at the time of surgery from 91 patients at the Department of General surgery, Qilu Hospital, Shandong University, from 2008 to 2014. The ethics committee of Qilu Hospital, Shandong University approved the use of tissues for this study, and each patient gave prior written informed consent and approval. Histological grade and differentiation were evaluated independently by three pathologists according to the WHO classification system. None of the patients received any pre-operative chemotherapy or radiotherapy. A comprehensive set of clinicopathological data was obtained including age, gender, size of the primary tumor, tumor differentiation status, T stage, lymph node metastasis, and distant metastasis.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was used to analyze the expression of miR-29c in 91 pairs of HCC and adjacent noncancerous tissues. Briefly, 10 ng RNA was subjected to reverse transcription using a miRNA cDNA Synthesis Kit (TaKaRa Co., Ltd., Dalian, China). The cDNA was then amplified using miRNA sequence-specific primers (Takara Co., Ltd.) and a SYBR-green RT-PCR Kit (TaKaRa Co., Ltd.) on a Light Cycler 480 system according to the manufacturer's instructions. The PCR cycle profile was as follows: 95°C for 30 s followed by 35 cycles of 95°C for 10 s and 60°C for 25 s. Small nucleolar RNA U6 was used as an endogenous control for normalization of the input RNA. With the same amount of RNA for the control PCR, the threshold cycle (Ct) range for the control U6 small RNA ranged from 11 to 13 cycles. All qRT-PCRs were performed in triplicate.

Statistical analysis

The difference in miR-29c expression levels between HCC samples and matched normal tissue samples or between subgroups classified according to the different clinicopathogenetic features was analyzed using the Mann-Whitney U-test. Kaplan-Meier curves were constructed to determine overall patient survival rates. Kaplan-Meier survival plots and log-rank tests were used to assess the differences in overall survival of different subgroups of HCC patients. The association between HCC prognosis and clinical parameters was first analyzed by univariate analysis, and those that differed significantly were subjected to multiple logistic regressions, with a forward stepwise procedure to identify the independent risk factors for prognosis in HCC. All statistical analyses were performed using the Statistical Package for Social Science program (SPSS for Windows, version 18.0). $P < 0.05$ was considered statistically significant.

RESULTS

miR-29c expression in HCC is lower than in the matched adjacent non-cancerous hepatic tissues

To understand the expression and significance of miR-29c in HCC, we first analyzed miR-29c expression levels in 91 cancerous tissues and their paired adjacent non-cancerous hepatic tissues, using real-time RT-PCR. As shown in Figure 1, miR-29c expression was remarkably decreased in HCC tissues relative to that in normal hepatic tissues ($P < 0.001$). Based on the median value of miR-29c expression in HCC tissues, the patients were divided into two groups: cases with low miR-29c expression ($N = 45$) and cases with high miR-29c expression ($N = 46$).

Relationship between miR-29c expression and clinicopathological factors

To identify whether the expression of the miR-29c in HCC tissues was associated with clinical features of the disease, we analyzed the association between miR-29c expression and various clinicopathological factors in all 91 HCC patients. Low miR-29c levels were significantly associated with histologic grade ($P = 0.001$), microvascular invasion ($P = 0.005$), and tumor stage ($P < 0.001$, shown in Table 1). These results supported the idea that altered miR-29c expression may be an important regulator of aggressive biological behavior in HCC.

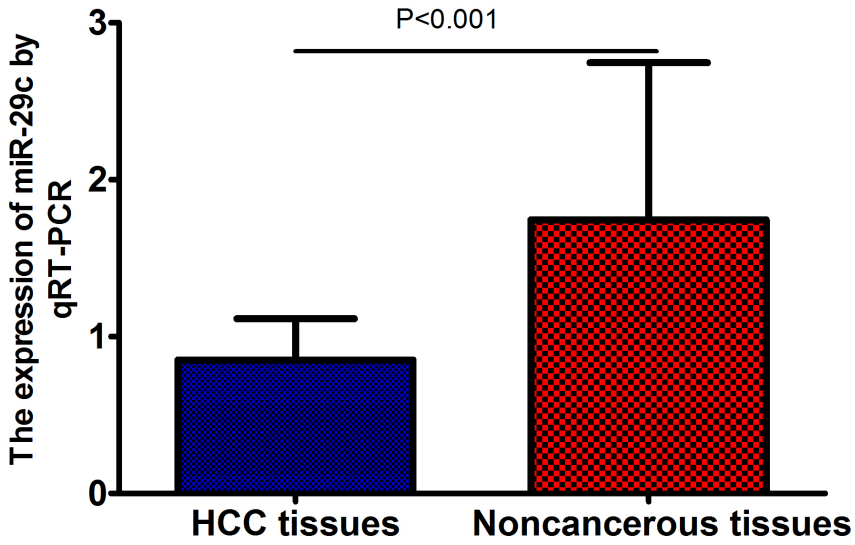

Figure 1. miR-29c expression levels in HCC and matched adjacent non-cancerous hepatic tissues.

Table 1. Association between miR-29c expression and clinicopathological parameters in 91 patients with HCC.

Characteristics	Number of patients	Low miR-29c level		High miR-29c level		P value
		N	%	N	%	
Gender						
Male	56	24	42.86	32	57.14	0.134
Female	35	21	60.00	14	40.00	
Age (years)						
≥55	51	27	52.94	24	47.06	0.528
<55	40	18	45.00	22	55.00	
Histologic grade						
Well/moderately	67	26	38.81	41	61.19	0.001
Poorly	24	19	79.17	5	20.83	
Tumor size (cm)						
≥5	42	23	54.76	19	45.24	0.403
<5	49	22	44.90	27	55.10	
Tumor nodes						
Multi	35	19	54.29	16	45.71	0.522
Single	56	26	46.43	30	53.57	
HBsAg status						
Positive	78	40	51.28	38	48.72	0.551
Negative	13	5	38.46	8	61.54	
Serum AFP (ng/dl)						
≥200	55	31	56.36	24	43.64	0.134
<200	36	14	38.89	22	61.11	
Microvascular invasion						
Yes	33	23	69.70	10	30.30	0.005
No	58	22	37.93	36	62.07	
Tumor stage						
I+II	54	18	33.33	36	66.67	<0.001
III+IV	37	27	72.97	10	27.03	

Relationship between miR-29c expression in HCC tissues and postoperative survival

Kaplan-Meier analysis showed that decreased miR-29c expression was correlated with shorter overall survival (P = 0.002) in 91 HCC patients (shown in Figure 2). Multivariate Cox regression analysis showed that histologic grade (Hazard Ratio (HR) = 3.829, 95%CI = 1.285-12.917, P = 0.002), tumor stage (HR = 3.781, 95%CI = 1.239-10.081, P = 0.003), and decreased miR-29c expression (HR = 2.19, 95%CI = 1.361-6.779, P = 0.025) were independently associated with poor survival in HCC (shown in Table 2).

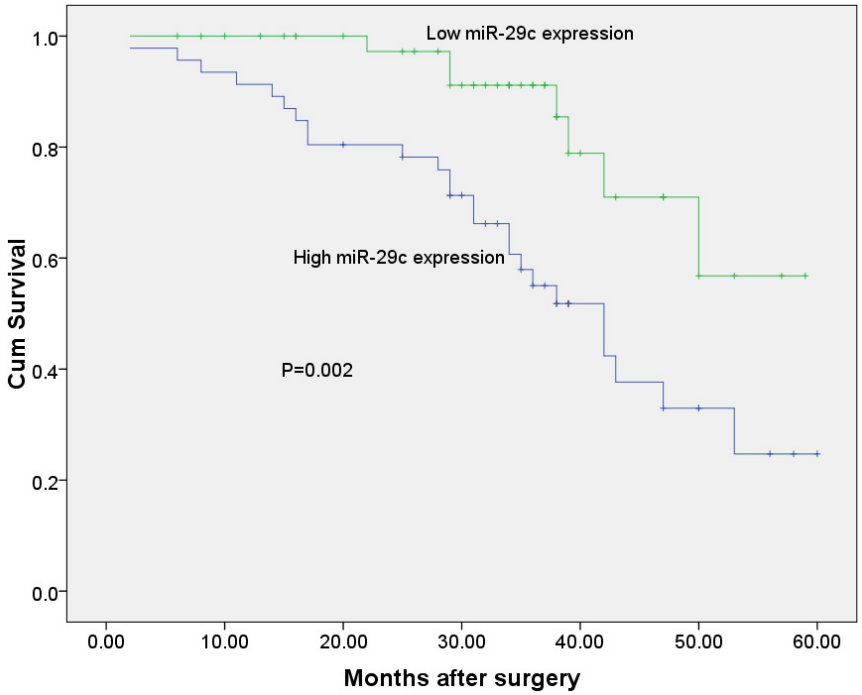

Figure 2. Kaplan-Meier curves stratified according to expression levels of miR-29c.

Table 2. Multivariate Cox regression analyses of overall survival in 91 HCC patients.

Parameter	HR	95%CI	P value
Gender	1.372	0.478-2.191	0.571
Age	2.192	0.771-3.289	0.185
Histologic grade	3.829	1.285-12.917	0.002
Tumor size	2.718	0.715-4.196	0.142
Tumor nodes	1.263	0.562-2.293	0.467
HBsAg status	2.374	0.627-3.338	0.118
Serum AFP	2.172	0.783-3.864	0.091
Microvascular invasion	3.283	0.839-5.339	0.062
Tumor stage	3.781	1.239-10.081	0.003
miR-29c expression	2.192	1.361-6.779	0.025

DISCUSSION

Although HCC is the sixth most common neoplasm worldwide, its poor prognosis makes HCC the third leading cause of cancer-related mortality, which is responsible for 600,000 deaths annually. In some countries, especially in China, HCC accounted for 70-85% of primary liver cancer cases, with the disease burden expected to increase in the coming years (Bosetti et al., 2014; Sherman, 2014).

MiRNAs have been shown to play important roles in variety of oncogenic activities, such as cell proliferation, invasion, angiogenesis, and metastasis (Bartel, 2004). Recent studies have suggested that the expression of many miRNAs is deregulated in a variety of cancers. A number of miRNAs have been suggested to play important roles in HCC development. Thus, exploring and understanding the aberrantly expressed miRNAs may help to reveal the mechanisms underlying HCC carcinogenesis and progression (Wong et al., 2015; Zhang et al., 2015).

The miR-29 family is a family of miRNAs with conserved sequences including miR-29a, miR-29b, miR-29c, and miR-29d. Recently, the expression of many miR-29 family members was found to be reduced in various cancers. For example, Jiang et al. (2015) found that miR-29c was significantly decreased in pancreatic cancer tissues compared to normal pancreatic tissues. MiR-29c directly suppressed the following upstream Wnt regulators: frequently rearranged in advanced T-cell lymphomas 2 (FRAT2), low-density lipoprotein receptor-related protein 6 (LRP6), Frizzled-4 (FZD4), and Frizzled-5 (FZD5). Furthermore, transforming growth factor-β (TGF-β) inhibited miR-29c expression, leading to Wnt activation (Jiang et al., 2015). Fan et al. (2014) found that the expression of miR-29c was downregulated in bladder cancer tissues compared with normal tissues and the low expression of miR-29c was associated with tumor stage ($P = 0.002$). In addition, ectopic over-expression of miR-29c in T24 cells could significantly inhibit cell proliferation, decrease motility, suppress the G1/S cell cycle transition, and induce apoptosis. Furthermore, it could cause a decrease in AKT and GSK-3β phosphorylation. Thus, miR-29c could be an active player in the disease state of bladder cancer and may be a promising tumor suppressor in bladder cancer (Fan et al., 2014). miR-29c was also significantly downregulated in glioma cell lines and primary human glioma tissues, compared to normal human astrocytes and matched non-tumor tissues ($P < 0.05$). Overexpression of miR-29c dramatically reduced cell proliferation and caused the cessation of cell cycle. Reduction in cell proliferation was found to be due to G1 phase arrest since cyclin D1 and cyclin E are diminished, whereas p27 and p21 are upregulated upon miR-29c overexpression, indicating that miR-29c may be a tumor suppressor involved in glioma progression (Fan et al., 2013).

The role of miR-29c has also been investigated in HCC. Bae et al. (2014) found that miR-29c functioned as a tumor suppressor by directly targeting oncogenic SIRT1 in HCC. Another study by Wang et al. (2015) suggested that miR-29c might function as a tumor suppressor that plays a crucial role in the development of liver carcinoma by targeting WIP1, therefore possibly representing a target molecule for therapeutic intervention. However, the clinical significance and prognostic value of miR-29c have not been investigated in detail. In the present study, to understand the expression pattern of miR-29c in HCC and its significance, we first examined miR-29c expression levels in 91 paired cancerous tissues and adjacent non-cancerous hepatic tissues, using real-time RT-PCR. We found that miR-29c expression was remarkably decreased in HCC tissues relative to that in normal hepatic

tissues. To identify whether the expression of miR-29c in HCC tissues was associated with the clinical features of the disease, we analyzed the association between miR-29c expression and various clinicopathological factors in all 91 HCC patients. The low levels of miR-29c were significantly associated with histologic grade, microvascular invasion, and tumor stage. These results supported the idea that altered miR-29c expression may be an important regulator of aggressive biological behavior in HCC. Kaplan-Meier analysis showed that decreased miR-29c expression correlated with shorter overall survival. Multivariate Cox regression analysis showed that histologic grade, tumor stage, and decreased miR-29c expression were independently associated with poor survival in HCC. In conclusion, our study described the clinical significance of miR-29c in HCC patients for the first time. Our findings demonstrated that miR-29c expression was significantly down-regulated in HCC patients and that miR-29c could act as an independent predictor of unfavorable clinical outcomes.

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