Micro RNA-4651 Serves as a Potential Biomarker for Prognosis When Selecting Hepatocellular Carcinoma Patients for Postoperative Adjuvant Transarterial Chemoembolization Therapy

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Our previous reports have shown that microRNA-4651 is a potential early diagnostic and prognostic marker for hepatocellular carcinoma. We aimed to investigate whether microRNA-4651 modified postoperative adjuvant transarterial chemoembolization (pa-TACE) to improve the prognosis of hepatocellular carcinoma. A hospital-based retrospective study, including 302 patients with advanced-stage hepatocellular carcinoma who received tumor resection or tumor resection plus pa-TACE as an initial therapy, was conducted to assess the effects of microRNA-4651 on pa-TACE treatment. MicroRNA-4651 expression in tumor tissues was tested using the TaqMan-PCR technique. The sensitivity of tumor cells to doxorubicin (an anticancer drug used in pa-TACE procedure) was analyzed by the half-maximal inhibitory concentration (IC50). Upregulated microRNA-4651 expression in tumor tissues can improve the therapeutic response of patients with hepatocellular carcinoma on pa-TACE (hazard ratios [95% confidence intervals] = 0.32 [0.22-0.46] for death risk and 0.39 [0.28-0.56] for tumor-recurrence risk, respectively), but downregulated expression cannot. Functional analyses–displayed microRNA-4651 mimics decreased while its inhibitor increased the IC50 of tumor cells to doxorubicin (0.65 [0.61-0.69] versus 2.17 [1.98-2.37] μM). Cytochrome P450 2W1 was shown as a possible target of microRNA-4651. Additionally, dysregulation of microRNA-4651 also affected the clinical pathological features of hepatocellular carcinoma and was an independent prognostic factor for this cancer. *Conclusion:* These results indicate that increasing microRNA-4651 expression may be beneficial for pa-TACE in improving hepatocellular carcinoma prognosis. (*Hepatology Communications* 2018;2:1259-1273).

Hepatocellular carcinoma is highly prevalent in China, especially in the Guangxi Zhuang Autonomous Region.[1] The incidence and mortality of this tumor is increasing because of high incidence of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection and/or high exposure of aflatoxin B1.[2-4] Although advances in curative treatments, such as surgical resection and liver transplantation, significantly improve life expectancy for these patients with early-stage hepatocellular

Abbreviations: AFP, alpha-fetoprotein; CI, confidence interval; ES, Edmondson and Steiner grading system; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HMRE, high microRNA-4651 expression; HR, hazard ratio; IC, inhibitory concentration; LMRE, low microRNA-4651 expression; miR-4651, microRNA-4651; mRNA, messenger RNA; MVD, microvessel density; OS, overall survival; pa-TACE, postoperative adjuvant transarterial chemoembolization; RFS, recurrence-free survival; TACE, transarterial chemoembolization; TUNEL, transferase-catalyzed DNA nick-end labeling.

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carcinoma, this type of therapies are not beneficial for most tumor cases because they are diagnosed at advanced stages.[5-9] In the past decades, transarterial chemoembolization (TACE) has become an increasingly important palliative treatment for hepatocellular carcinoma. TACE treatment is initially regarded as an effective therapy for unresectable hepatocellular carcinoma such as advanced-stage cancer.[10-13] Recently, this therapy is being used as an attractive adjuvant therapy (e.g., postoperative TACE [pa-TACE]) for resectable hepatocellular carcinoma with the hope that it may decrease tumor recurrence and improve survival of patients.[10,14] On the basis of the currently available clinic evidence, however, pa-TACE treatment does not display anticancer effects on hepatocellular carcinoma cases with some specific genetic profiles.[15,16] Thus, it is vital to identify what kinds of hepatocellular carcinoma and characteristics can gain a benefit from pa-TACE treatment.

MicroRNAs are an evolutionarily conserved class of small noncoding single-stranded RNA, typically consisting of 18-24 nucleotides.[17] First, they are transcribed by the RNA polymerase enzyme II into a kind of primary production called the primary microRNA, and then processed into their precursors: duplex structure by Drosha and Dicer RNase enzymes. Finally, the duplex structure is unwound into mature microRNAs by helicases. Functionally, microRNAs almost evolve throughout their lifespan by regulating gene expression.[18-21] To date, more than 1,800 microRNAs have been identified in the mammalian genome (miRDatabase). Some of them, such as microRNA-629, microRNA-1268a, and microRNA-24, have been reported to have early diagnostic and prognostic potential for hepatocellular carcinoma.[22-26] Our previous studies have shown that a higher serum level of microRNA-4651, a short-strand RNA encoded by the microRNA-4651 gene (located at the 75915197th to 75915269th base of chr7), is found in patients with aflatoxin B1-positive hepatocellular carcinoma.[27,28] This increasing serum level displays diagnostic and prognostic potential for aflatoxin B1-related hepatocellular carcinoma. The aims of this study were to explore whether microRNA-4651 in the cancerous tissues modified pa-TACE treatment, thus improving the prognosis of hepatocellular carcinoma.

Materials and Methods

PATIENTS

This study is a hospital-based retrospective study from the Guangxi area, a high-incidence area of hepatocellular carcinoma in China.[2-4] According to the inclusion and exclusion criteria on cases listed in Table 1, a total of 302 patients with advanced-stage

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TABLE 1. INCLUSION AND EXCLUSION CRITERIA FOR CASES WITH HEPATOCELLULAR CARCINOMA

Inclusion and Exclusion Criteria in the Present Study
Inclusion criteria for cases
(1) Cases with a pathologically diagnosed and BCLC C-stage hepatocellular carcinoma
(2) Cases receiving partial liver resect or partial liver resect plus pa-TACE as initial treatment according to CMCH (solitary or multiple tumors mainly located in one lobe of the liver; no extrahepatic metastases; Child-Pugh A-stage liver function; and no contraindication for laparotomy)
(3) Cases not receiving radiotherapy or chemotherapy before surgical operative treatment
(4) Cases understanding the objective of the study and providing informed consent
(5) Cases having ability to complete the necessary investigations and questionnaires
(6) Cases with available cancerous tissue samples and clinical-pathological data (including 5-year follow-up data)
Exclusion criteria for cases
(1) Cases with hepatocellular carcinoma but not confirmed by histopathological examination
(2) Cases receiving chemotherapy or radiotherapy before surgical operative treatment
(3) Cases rejected, dropped out, or lost information
Inclusion criteria for TACE analysis
(1) Cases with a pathologically diagnosed and BCLC C-stage hepatocarcinoma
(2) Cases having good liver function (Child-Pugh stage A)
(3) Cases with multiple tumors more than 5 cm or tumor involving a first or second branch of the portal or hepatic veins
(4) The tumor with multiple lesions localized in one lobe of liver, or the main tumor localized in one lobe only with a small solitary lesion in contralateral lobe, or tumor involving a first or second branch of the portal or hepatic vein, which could be safely resected without grossly remaining tumors, and the patient was judged to have well preserved liver function to survive the operation
(5) Cases underwent partial hepatectomy, and agreed to pa-TACE treatment
(6) Cases without contraindication for TACE
Exclusion criteria for TACE analysis
(1) Patients with non-hepatocarcinoma on postoperative histopathological examination, serious concurrent medical illness, intractable ascites, tumor recurrence within 4 works after the operation
(2) Women cases who were pregnant or breastfeeding
(3) Cases rejected, dropped out, or lost information
(4) Cases with contraindication for TACE
(5) Cases with history of chemotherapy or radiotherapy treatment before surgical operative treatment

Abbreviations: BCLC, the Barcelona Clinic Liver Cancer staging system; CMCH, Chinese Manage Criteria of Hepatocellular Carcinoma.

hepatocellular carcinoma were recruited in the affiliated hospitals of the Youjiang Medical University for Nationalities and Guangxi Medical University between January 2005 and December 2009. In this study, the response rate for patients with hepatocellular carcinoma was approximately 98.4%. Among these cases, 150 of them received tumor resection plus pa-TACE as their initial treatment, according to the inclusion and exclusion criteria of the TACE analyses given in Table 1, and were defined as the TACE group; the others only underwent tumor resect, as their initial treatment was defined as the non-TACE control group (n = 152).

All clinicopathological data, including gender, age, ethnicity, HBV and HCV infective status, liver cirrhosis, tumor grade and stage, and treatment information, were obtained as described previously.[22] Surgically removed fresh samples with hepatocellular carcinoma of all patients were collected for testing microRNA-4651 expression. In this study, HBV and HCV infective status was defined according to the hepatitis B surface antigen (HBsAg) and anti-HCV, respectively. Tumor grade was elucidated using the Edmondson and Steiner grading system (ES) for hepatocellular carcinoma, whereas liver cirrhosis was evaluated by pathological examination.[29] This study was approved by the Institutional Ethics Committee of Youjiang Medical University for Nationalities and was carried out in accordance with the approved guidelines (No. 20041225).

PA-TACE PROCEDURE

In the present study, the pa-TACE procedure was accomplished as the postoperative adjuvant part of the initial therapy procedure for eligible patients with hepatocellular carcinoma (Table 1), which started 4 weeks after the removal of tumors. This postoperative adjuvant therapy contains an injection consisting of a mixture of doxorubicin (65 mg/m^2), cisplatin (7 mg/m^2), and lipiodol, as previously described.[22] Adverse reactions related to pa-TACE were graded according to the World Health Organization criteria.

MICROVESSEL DENSITY EVALUATION

Microvessel density (MVD) in tumor tissues was evaluated according to our previously published immunohistochemistry.[23,27,30] In this study, the MVD was divided into two groups: low density (number of microvessels ≤ 50/×200 magnifications) and high density (number of microvessels > 50/×200 magnifications).

SURVIVAL FOLLOW-UP

For survival analysis, all patients with hepatocellular carcinoma were followed up as described in our previous studies.[16,27,31] In brief, survival data, including overall survival (OS) and tumor recurrence-free survival (RFS) status, were confirmed according to patient or family contact and clinic records, and the last follow-up day was set on July 31, 2017. The duration of OS was defined as during the date of the initial treatment and the date of death or last known date alive, whereas RFS was defined as during the date of the initial treatment and the date of tumor recurrence or last known date alive.

MICRORNA-4651 EXPRESSION ASSAY

The microRNA-4651 expression in cancerous tissues was tested using our previously published TaqMan quantitative reverse-transcription PCR method.[27] Briefly, RNA was first extracted from fresh tumor samples. The corresponding complementary DNAs (cDNAs) were next synthesized and used to determine microRNA-4651 expression by the TaqMan-PCR procedure (with an endogenous control U6). To analyze, microRNA-4651 expression levels were divided into two groups: low microRNA-4651 expression group (LMRE) ($2^{-\Delta Ct} \leq 2.90$) and high microRNA-4651 expression group (HMRE) ($2^{-\Delta Ct} > 2.90$), according to the average value among cases with hepatocellular carcinoma.

CELLS AND CULTURE CONDITIONS

Human liver cancer cell line SMMC-7721 was obtained from the Cell Bank of Shanghai Institute of Cell Biology of the Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Gibco-Invitrogen Corp., Carlsbad, CA) at 37°C in a 5% CO_2 atmosphere. Cells were determined to be free of Mycoplasma, and all experimental analyses were done with cells in logarithmic growth.

CELLULAR SENSITIVITY ASSAY

The cellular sensitivity on antitumor drugs was determined using our previously published half-maximal inhibitory concentration (IC50) method.[32] Briefly, SMMC-7721 cells were transfected with mature microRNA-4651 mimics, its inhibitor, or null control (GenePharma, Shanghai, China) using Lipofectamine 2000 (ca#11668-027, Invitrogen). In the present study, transfection efficiency was elucidated using the LV200 system and according to the following valid criteria: the number of positive transfected cells being more than 40% of total cells. Forty-eight hours after transfection, cells were treated with doxorubicin (a kind of anticancer drug used in the pa-TACE procedure) at 15 different concentrations (0.01 μM-40 μM), and then a cell counting kit (CCK-8) assay (cat# CK04, DojindoCorp., Japan) was used to calculate the IC50 value.

CELL DEATH ASSAY

Cell death was analyzed using a terminal deoxynucleotidyl transferase-catalyzed DNA nick-end labeling (TUNEL) assay and flow cytometry assay according to our previously published method.[32] Briefly, 48 hours after transfection with microRNA-4651 mimics, its inhibitor, or null control, cancer cells were treated with doxorubicin (at final concentrations of 1.25 μM) and analyzed using the TUNEL Apoptosis Detection Kit (cat# 40307ES20, Shanghai YISEN Bio Tech., China) or Annexin V-FITC/PI Apoptosis Detection Kit (cat# 40302ES60, YISEN). In TUNEL analysis, live and nonlive cells exhibited red fluorescent in fluorescence microscopy (at 620 nm), whereas death cells (primarily apoptosis cells) exhibited green fluorescent in fluorescence microscopy (at 520 nm).

GENE EXPRESSION PROFILING ASSAY

To explore possible target genes of microRNA-4651, the gene expression profile analyses were accomplished using the SMMC-7721 cells transfected with mature microRNA-4651 mimics or null control as in the previously described methods.[31] Briefly, total RNAs were first extracted and the corresponding cDNAs were synthesized. Next, relative expression levels of genes were tested using DirectHyb HumanHT-12 Version 4.0 (cat#BD-103-0204, Illumina) and the corresponding Bio-Array Environment software.

CYTOCHROME P450 2W1 (CYP2W1) EXPRESSION ANALYSES

Forty-eight hours after SMMC-7721 transfected with mature microRNA-4651 mimics, its inhibitor, or

null control cells were used for analyzing the amount of CYP2W1 messenger RNA (mRNA) and protein. CYP2W1 mRNA and protein were respectively quantitated using real-time quantitative PCR and western blot methods as previously described.[33]

STATISTICAL ANALYSIS

The differences between the TACE group and the non-TACE group in clinicopathological characteristics were tested using the Pearson chi-squared test. The differences between patients with HMRE and with LMRE in adverse reactions related to pa-TACE were tested using the Pearson chi-square test or Fisher's probabilities test. The associations between microRNA-4651 expression and clinicopathological characteristics of patients with hepatocellular carcinoma were analyzed with nonconditional logistic regression using ORs and 95% confidence intervals (CIs). The effects of pa-TACE treatment and microRNA-4651

TABLE 2. CLINICAL PATHOLOGICAL FEATURES OF HEPATOCELLULAR CARCINOMA CASES WITH OR WITHOUT PA-TACE TREATMENT

		pa-TACE treatment, n (%)			
Variables	Cases, n (%)	No	Yes	χ^2	*P*
Total	302 (100.0)	152 (100.0)	150 (100.0)		
Age (years)*				0.32	0.57
≤48	172 (57.0)	89 (58.6)	83 (55.3)		
>48	130 (43.0)	63 (41.4)	67 (44.7)		
Sex				1.39	0.24
Male	201 (66.6)	106 (69.7)	95 (63.3)		
Female	101 (33.4)	46 (30.3)	55 (36.7)		
Ethnicity				2.58	0.11
Han	163 (54.0)	89 (58.6)	74 (49.3)		
Zhuang	139 (46.0)	63 (41.4)	76 (50.7)		
HBsAg				1.14	0.29
Negative	96 (31.8)	44 (28.9)	52 (34.7)		
Positive	206 (68.2)	108 (71.1)	98 (65.3)		
anti-HCV				1.77	0.18
Negative	269 (89.1)	139 (91.4)	130 (86.7)		
Positive	33 (10.9)	13 (8.6)	20 (13.3)		
Smoking status				0.12	0.73
No	231 (76.5)	115 (75.7)	116 (77.3)		
Yes	71 (33.5)	37 (24.3)	34 (22.7)		
Drinking status				2.28	0.13
No	224 (74.2)	117 (70.4)	117 (78.0)		
Yes	78 (25.8)	45 (29.6)	33 (22.0)		
AFP (ng/mL)				0.53	0.47
≤20	111 (36.8)	54 (35.5)	57 (38.0)		
>20	191 (63.2)	98 (64.5)	93 (62.0)		
Liver cirrhosis				0.11	0.74
No	74 (24.5)	36 (23.7)	38 (25.3)		
Yes	228 (75.5)	116 (66.3)	112 (74.7)		
ES grade				0.66	0.42
Low	160 (53.0)	77 (50.7)	83 (55.3)		
High	142 (47.0)	75 (49.3)	67 (44.47)		
MVD				0.02	0.88
Low	106 (35.1)	54 (35.5)	52 (34.7)		
High	196 (64.9)	98 (64.5)	98 (65.3)		

*Age is grouped according to the average age of patients with hepatocellular carcinoma (48.01 ± 10.03 years).

expression on hepatocellular carcinoma prognosis were evaluated using Kaplan-Meier survival analysis models (with the log-rank test) and Cox regression models (including univariate and multivariate models). Hazard ratios (HRs) and 95% CIs were used to determine the prognostic potential of clinicopathological variables (including pa-TACE and microRNA-4651 expression). All statistical tests were performed using the Statistical Package for Social Science version 18 (SPSS Institute, Chicago, IL), and a *P* value of less than 0.05 was considered statistically significant.

Results

BASELINE CHARACTERISTICS

Of the 302 cases with BCLC-C stage hepatic carcinoma in the final analysis, 201 (66.6%) were male and 101 (33.4%) were female. The mean age ± SD was 48.01 ± 10.03 years (range 30-74 years). A total of 206 (68.2%) patients were detected for serum HBsAg positive, and 228 (75.5%) featured liver cirrhosis. The baseline clinicopathological data were well matched between the TACE group and non-TACE group (Table 2). Results from survival analyses showed that pa-TACE treatment can approve the prognosis of hepatocellular carcinoma (TACE group versus non-TACE group, median survival time = 24.00 [21.13-26.87] months versus 11.00 [8.77-13.23] months for OS and 20.00 [16.95-23.05] months versus 8.00 [6.12-9.88] months for RFS, respectively) (Fig. 1).

MICRORNA-4651 EXPRESSION AFFECTING HEPATOCELLULAR CARCINOMA PROGNOSIS

To elucidate the clinical value of microRNA-4651 expression in the tissues with hepatocellular carcinoma, we first explored the correlation between microRNA-4651 expression and clinicopathological features of cases using logistic regression models (Table 3). We found that microRNA-4651 expression was significantly associated with tumor dedifferentiation (OR = 2.46, 95% CI = 1.52-3.96), serum alpha-fetoprotein (AFP) level (OR = 2.07, 95% CI = 1.28-3.34), and MVD (OR = 3.20, 95% CI = 1.96-5.24), but not to other features (Table 3). Considering that both differentiation degrees and MVD of hepatocarcinoma are two important determinants for chemoresponse and prognosis, we further investigated the

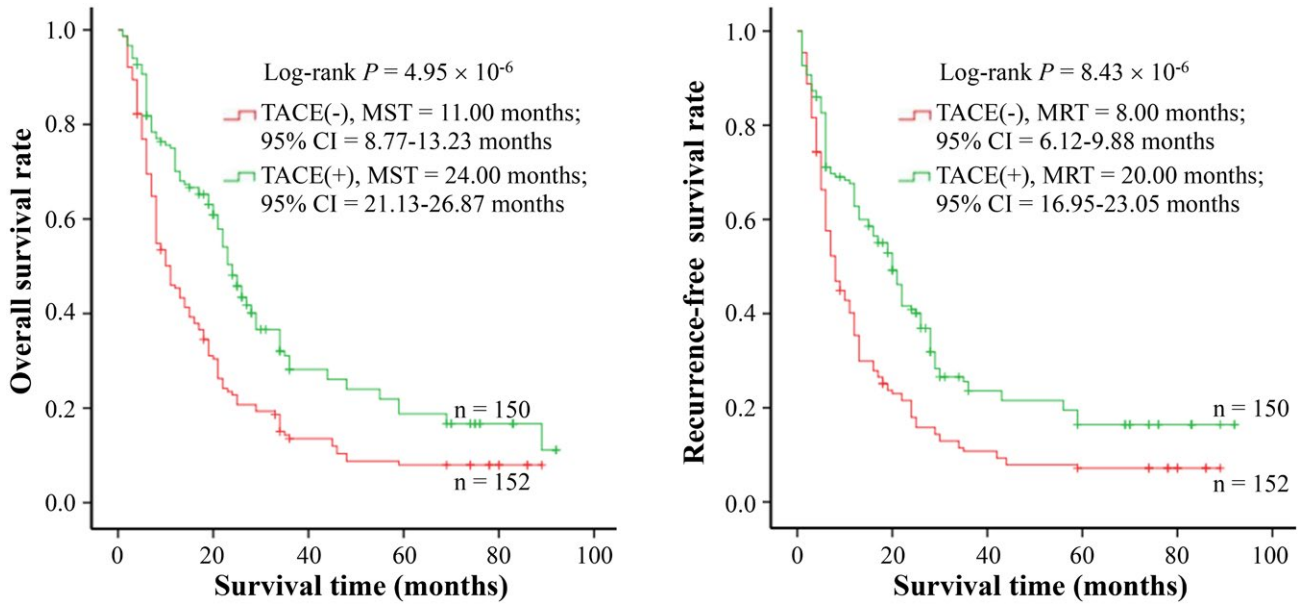

FIG. 1. TACE treatment significantly correlates with hepatocellular carcinoma prognosis. TACE treatment is associated with OS (left) and tumor RFS (right) of hepatocellular carcinoma. Cumulative hazard function was plotted by Kaplan-Meier's methodology, and the *P* value was calculated with two-sided log-rank tests. Abbreviations: MRT, median tumor recurrence-free survival time; MST, median overall survival time.

TABLE 3. CORRELATION BETWEEN MIR-4651 EXPRESSION AND CLINICAL PATHOLOGICAL FEATURES OF HEPATOCELLULAR CARCINOMA

Variables	MiR-4651 Expression, n (%)		OR (95% CI)	P_{trend}
	Low	High		
Total	124 (100%)	178 (100%)		
Age (years)				
≤48	72 (58.1)	100 (56.2)	Reference	
>48	52 (41.9)	78 (43.8)	1.04 (0.65-1.67)	0.86
Sex				
Male	84 (67.7)	117 (65.7)	Reference	
Female	40 (32.3)	61 (34.3)	1.09 (0.67-1.78)	0.73
Ethnicity				
Han	62 (50.0)	101 (56.7)	Reference	
Zhuang	62 (50.0)	77 (43.3)	0.77 (0.48-1.22)	0.26
HBsAg				
Negative	41 (33.1)	55 (30.9)	Reference	
Positive	83 (66.9)	123 (69.1)	1.15 (0.70-1.88)	0.59
anti-HCV				
Negative	113 (91.1)	156 (87.6)	Reference	
Positive	11 (8.9)	11 (12.4)	1.49 (0.69-3.21)	0.31
Smoking status				
No	94 (75.8)	137 (77.0)	Reference	
Yes	30 (24.2)	41 (23.0)	0.94 (0.55-1.61)	0.82
Drinking status				
No	93 (75.0)	131 (73.6)	Reference	
Yes	31 (25.0)	47 (26.4)	1.05 (0.62-1.79)	0.85
AFP (ng/mL)				
≤20	58 (46.8)	53 (29.8)	Reference	
> 20	66 (53.2)	125 (70.2)	2.07 (1.28-3.34)	2.83×10^{-3}
Liver cirrhosis				
No	33 (26.6)	41 (23.0)	Reference	
Yes	91 (73.4)	137 (77.0)	1.26 (0.74-2.15)	0.40
ES grade				
Low grade	82 (66.1)	78 (43.8)	Reference	
High grade	42 (33.9)	100 (56.2)	2.46 (1.52-3.96)	2.26 × 10–4
MVD				
Low	63 (50.8)	43 (24.2)	Reference	
High	61 (49.2)	135 (75.8)	3.20 (1.96-5.24)	3.69×10^{-6}

correlation between microRNA-4651 (miR-4651) expression and these two clinicopathological features using the Spearman's rank correlation model. Results showed that miR-4651 expression was positively associated with tumor differentiation ($r = 0.22$, $P = 1.17 \times 10^{-4}$) (Fig. 2A) and MVD ($r = 0.28$, $P = 1.25 \times 10^{-6}$) (Fig. 2B).

Next, we investigated whether microRNA-4651 expression modified the prognosis of hepatocellular carcinoma using several survival models, including the Kaplan-Meier survival model, and univariate and multivariate Cox regression models (Fig. 3 and Tables 4 and 5). Results from the Kaplan-Meier survival model revealed that those patients having HMRE in their tumor tissues featured a shortened median survival time (10 months for OS and 7 months for RFS) compared with those with LMRE (29 months for OS and 24 months for RFS) (Fig. 3). Cox regression analyses also proved that the dysregulation of microRNA-4651 expression significantly increased the death risk

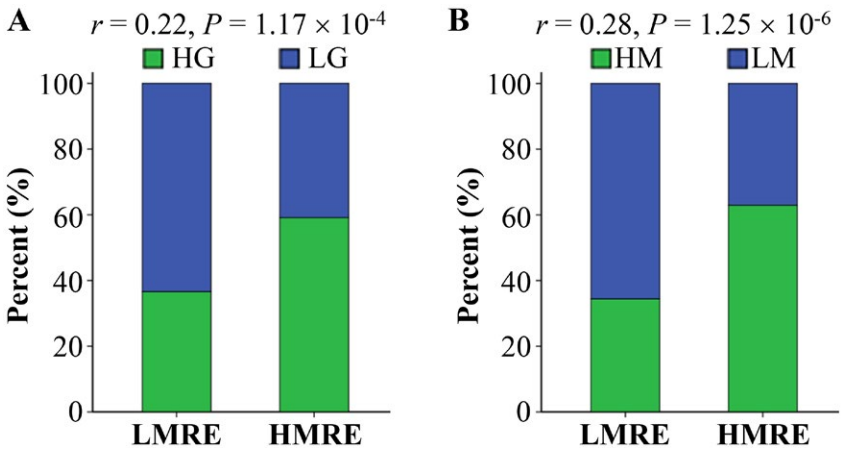

FIG. 2. The miR-4651 expression is significantly associated with the differentiation and MVD of hepatocellular carcinoma. The miR-4651 expression in cancerous tissues from 302 patients with hepatocellular carcinoma was tested using the TaqMan-PCR technique. To analyze, the levels of miR-4651 expression were divided into two groups: low expression group (relative level ≤ 2) and high expression group (relative level > 2), according to the average expression. The levels of miR-4651 expression in the cancerous tissues are positively correlated with the degree of differentiation (A) and MVD (B) of the tumor. Abbreviations: HG, high differentiation grade; HM, high MVD; LG, low differentiation grade; LM, low MVD.

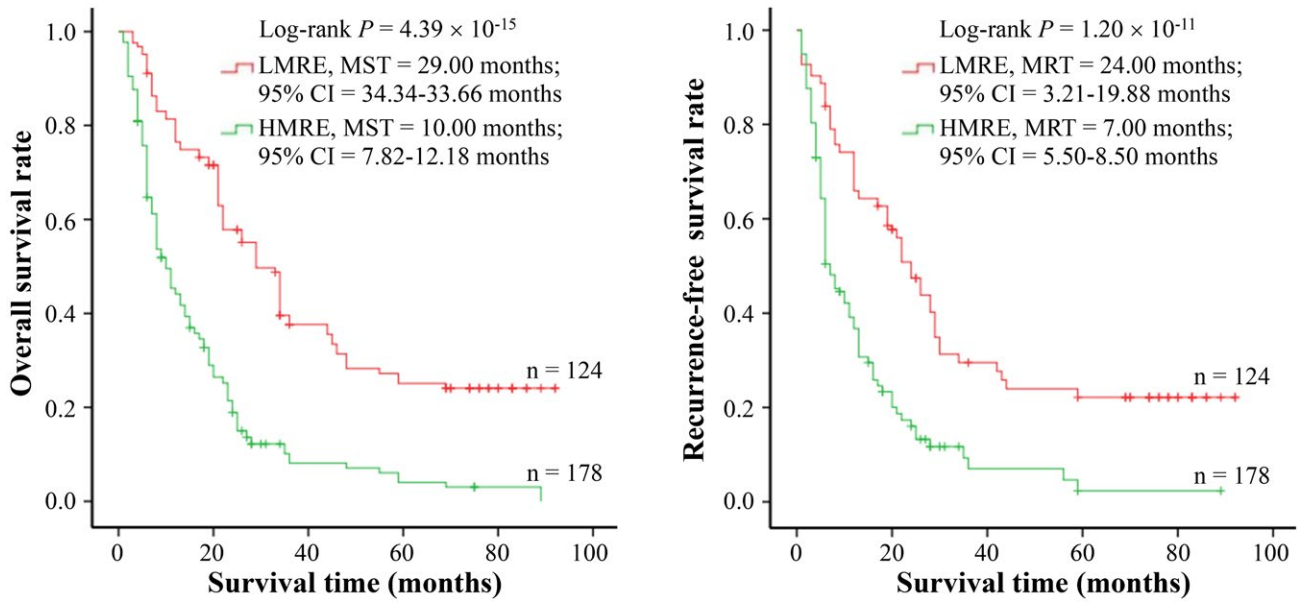

FIG. 3. miR-4651 expression significantly correlated with the prognosis of hepatocellular carcinoma. miR-4651 expression is associated with the OS (left) and tumor RFS (right) of hepatocellular carcinoma. Cumulative hazard function was plotted by Kaplan-Meier's methodology, and the *P* value was calculated with two-sided log-rank tests. Abbreviations: MRT, median tumor recurrence-free survival time; MST, median overall survival time.

(HR [95% CI] = 2.80 [2.13-3.68]) and tumor-recurrence risk (HR [95% CI] = 2.39 [1.83-3.13]) (Table 4). Furthermore, this increasing risk is independent of other known clinicopathological variables such as tumor MVD (Table 5). Taken together, these data are indicative of the prognostic potential of microRNA-4651 expression in tumor tissues for hepatocellular carcinoma.

TABLE 4. UNIVARIATE ANALYSES IDENTIFYING MIR-4651 EXPRESSION AS ONE OF THE SIGNIFICANT PROGNOSTIC PREDICTORS FOR SURVIVAL OF PATIENTS WITH HEPATOCELLULAR CARCINOMA

	OS		RFS	
Variables	HR (95% CI)	P_{trend}	HR (95% CI)	P_{trend}
Age (48 vs. < 48 years)	0.91 (0.71-1.17)	0.47	0.93 (0.72-1.19)	0.56
Gender (female vs. male)	0.90 (0.69-1.16)	0.42	0.87 (0.67-1.14)	0.32
Ethnicity (minority vs. Han)	0.81 (0.63-1.04)	0.10	0.83 (0.64-1.06)	0.14
Smoking (yes vs. no)	0.78 (0.58-1.05)	0.78	0.78 (0.58-1.04)	0.10
Drinking (yes vs. no)	0.94 (0.71-1.26)	0.94	0.88 (0.66-1.17)	0.37
HBsAg (positive vs. negative)	1.27 (0.96-1.68)	0.10	1.13 (0.85-1.45)	0.18
anti-HCV (positive vs. negative)	1.00 (0.67-1.49)	0.98	1.01 (0.68-1.19)	0.97
AFP (≤ 20 vs. > 20 ng/mL)	1.19 (0.92-1.55)	0.19	1.26 (0.98-1.64)	0.08
Liver cirrhosis (yes vs. no)	1.24 (0.92-1.67)	0.16	1.13 (0.85-1.52)	0.40
ES grade (high vs. low)	1.61 (1.25-2.08)	2.10×10^{-4}	1.58 (1.23-2.03)	3.58×10^{-4}
MVD (high vs. low)	1.48 (1.13-1.94)	4.08×10^{-3}	1.39 (1.09-1.68)	0.04
pa-TACE treatment (no vs. yes)	0.57 (0.44-0.73)	1.14×10^{-5}	0.58 (0.45-0.74)	2.04×10^{-5}
MiR-4651 expression (high vs. low)	2.80 (2.13-3.68)	1.58×10^{-13}	2.39 (1.83-3.13)	1.59×10^{-10}

TABLE 5. INDEPENDENT PROGNOSTIC FACTORS OF OS AND RFS FOR PATIENTS WITH HEPATOCELLULAR CARCINOMA BY MULTIVARIATE ANALYSES

	OS		RFS	
Variables	HR (95% CI)	P_{trend}	HR (95% CI)	P_{trend}
Age (48 vs. < 48 years)	0.79 (0.70-1.22)	0.58	0.89 (0.67-1.16)	0.38
Gender (female vs. male)	0.92 (060-1.04)	0.10	0.88 (0.67-1.17)	0.39
Ethnicity (minority vs. Han)	0.84 (0.64-1.11)	0.22	0.86 (0.66-1.13)	0.28
Smoking (yes vs. no)	1.32 (0.98-1.79)	0.07	0.85 (0.58-1.25)	0.42
Drinking (yes vs. no)	0.71 (0.46-1.10)	0.12	0.84 (0.58-1.26)	0.38
HBsAg (positive vs. negative)	0.79 (0.54-1.16)	0.23	1.13 (0.85-1.51)	0.41
anti-HCV (positive vs. negative)	1.00 (0.68-1.46)	0.99	0.77 (0.51-1.18)	0.23
AFP (≤ 20 vs. > 20 ng/mL	0.95 (0.72-1.25)	0.73	1.06 (0.81-1.39)	0.69
Liver cirrhosis (yes vs. no)	1.01 (0.73-1.38)	0.98	0.94 (0.70-1.28)	0.71
ES grade (high vs. low)	1.45 (1.10-1.92)	7.91×10^{-3}	1.48 (1.12-1.94)	5.16×10^{-3}
MVD (high vs. low)	2.28 (1.95-2.73)	1.09×10^{-4}	1.94 (1.53-2.49)	0.03
pa-TACE treatment (no vs. yes)	0.52 (0.39-0.68)	3.39×10^{-6}	0.58 (0.44-0.76)	7.17×10^{-5}
MiR-4651 expression (high vs. low)	2.79 (2.07-3.77)	2.54×10^{-11}	2.30 (1.73-3.07)	1.46×10^{-8}

MICRORNA-4651 EXPRESSION DIFFERENTLY MODIFIES PA-TACE TREATMENT IN IMPROVING HEPATOCELLULAR CARCINOMA PROGNOSIS

Considering that microRNA-4651 expression increases MVD in tumor, we investigated whether microRNA-4651 expression changed the therapeutic effects of pa-TACE on patients with hepatocellular carcinoma (Fig. 4). Among those cases with HMRE in their tumor tissues, pa-TACE treatment could prolong the OS time of patients with hepatocellular carcinoma (median time, 23.00 months; 95% CI, 19.55-26.45 months) and decrease the death risk (HR [95% CI] = 0.32 [0.22-0.46], $P = 1.23 \times 10^{-9}$) (Fig. 4A, left). For RFS, increasing microRNA-4651 expression can improve the ability of pa-TACE to decrease the tumor-recurrence risk (HR [95% CI] = 0.39 [0.28-0.56], $P = 2.37 \times 10^{-7}$) and increase the median RFS time (median time, 15.00 months; 95% CI, 10.05-19.95 months) (Fig. 4A, right). However, similar results were not observed among those with LMRE in their tumor tissues (Fig. 4B). Altogether, these findings indicate that the different expression of microRNA-4651 in the tissues with hepatic carcinoma may differentially affect the effects of pa-TACE treatment on hepatocellular carcinoma.

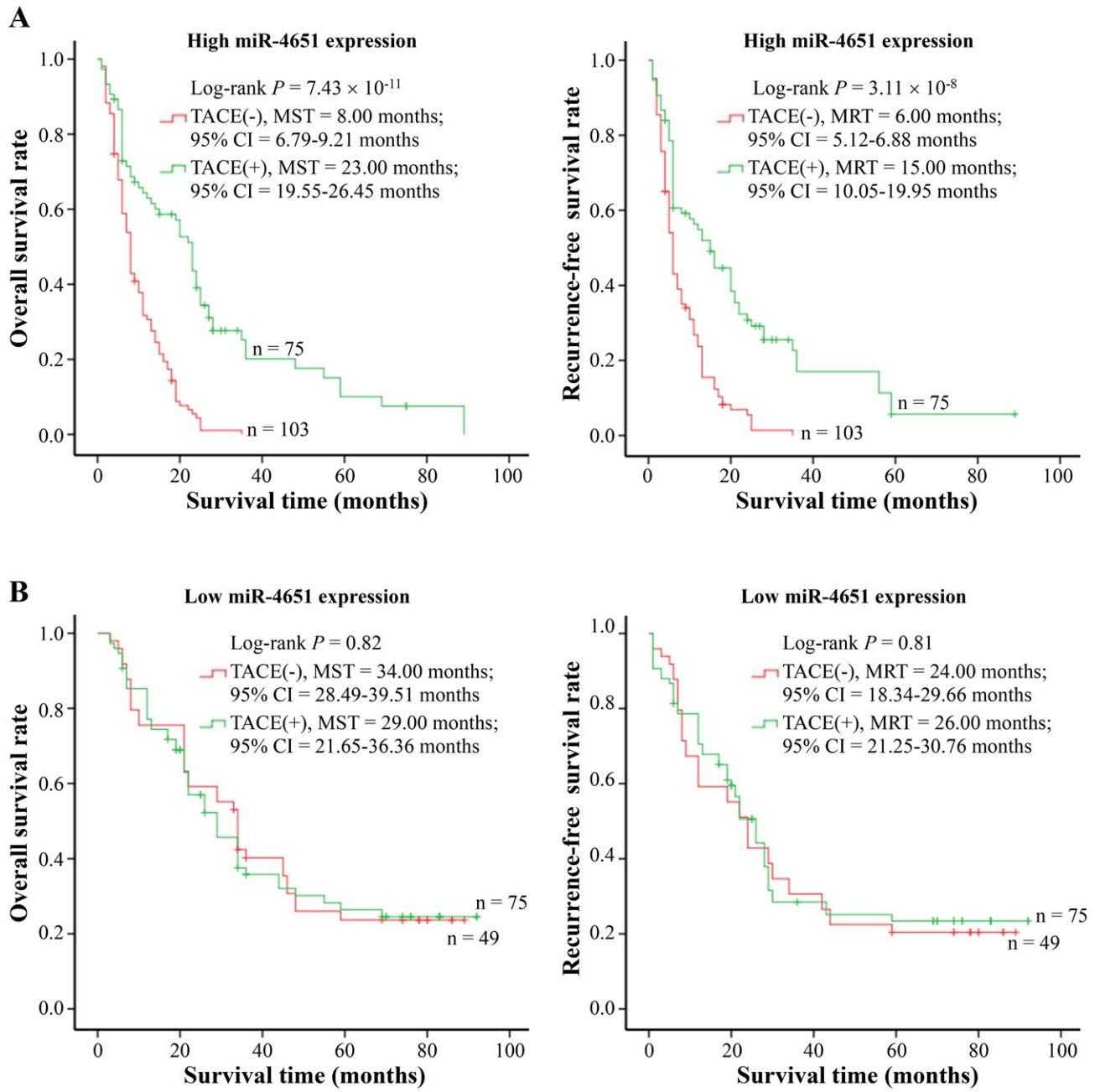

FIG. 4. Survival analysis of TACE treatment in the strata of miR-4651 expression. Among the hepatocellular carcinoma cases with high miR-4651 expression, TACE treatment improved hepatocellular carcinoma OS and tumor RFS (A), but not among patients with low miR-4651 expression (B). Cumulative hazard function was plotted using Kaplan-Meier's methodology, and the *P* value was calculated with two-sided log-rank tests. Abbreviations: MRT, median tumor recurrence-free survival time; MST, median overall survival time.

MICRORNA-4651 EXPRESSION DOES NOT CORRELATE WITH ADVERSE REACTIONS TO PA-TACE

The adverse reactions of patients with hepatocarcinoma, who received pa-TACE treatment, are provided in Table 6. Overall, pa-TACE was well tolerated. The most significant adverse reactions related to pa-TACE were nausea/vomiting and transient hepatic toxicity. However, the different distribution was not observed in hepatocellular carcinoma patients with HMRE and with LMRE in pa-TACE-caused adverse reactions.

TABLE 6. MICRORNA-4651 EXPRESSION AND ADVERSE REACTIONS INDUCED BY PA-TACE TREATMENT

Adverse Reaction	Grade of Adverse Reactions (n_{HMRE}/n_{LMRE}) G-1	G-2	G-3	$P_{G-1}/P_{G-2}/P_{G-3}$
Nausea/vomiting	31/28	3/5	0/0	0.62/0.72/–
Pain	12/10	2/4	0/0	0.64/0.68/–
Alopecia	3/3	0/0	0/0	1.00/–/–
Leukopenia	2/2	1/1	0/0	1.00/1.00/–
Increase in ALT/AST	21/22	10/7	7/10	0.86/0.44/0.44
Increase in GGT	10/13	6/4	0/0	0.29/0.51/–
Decrease in albumin	4/2	0/0	0/0	0.68/–/–
Increase in bilirubin	13/9	8/5	0/0	0.36/0.38/–

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, -glutamyl transpeptidase; n_{HMRE}/n_{LMRE}, number of hepatocarcinoma patients with HMRE/ number of hepatocarcinoma patients with LMRE; $P_{G-1}/P_{G-2}/P_{G-3}$, *P* value for distribution different of the first-grade adverse reaction between patients with HMRE and with LMRE/*P* value for distribution different from the second-grade adverse reaction between patients with HMRE and with LMRE/*P* value for distribution different from the third-grade adverse reaction between patients with HMRE and with LMRE.

This suggests that microRNA-4651 expression may be not correlated with adverse reactions to pa-TACE.

MICRORNA-4651 EXPRESSION IMPROVES THE SENSITIVITY OF HEPATOCELLULAR CARCINOMA TO DOXORUBICIN

To find possible reasons for microRNA-4651 expression to change the therapeutic values of pa-TACE therapy on hepatocellular carcinoma, we tested whether the microRNA-4651 expression was correlated with the sensitivity of SMMC-7721 (a kind of hepatocellular carcinoma cell line) to doxorubicin (an anticancer drug used in the TACE procedure) using IC50 and the cell death index. Results from the IC50 index analyses showed that those cancer cells with microRNA-4651 mimics had a decreasing IC50 value compared with those without mimics (0.65 [0.61-0.69] versus 1.24 [1.15-1.34] μM) (Fig. 5A). Conversely, the inhibitor of microRNA-4651 can increase the IC50 value of cancer cells (2.17 [1.98-2.37] μM) (Fig. 5).

Next, we analyzed the effects of microRNA-4651 on doxorubicin-induced death of hepatocellular carcinoma cells using TUNEL and flow cytometry analyses. Results from TUNEL analyses exhibited that microRNA-4651 mimics increased, whereas its inhibitor decreased, the death of tumor cells ($P < 0.01$) (Fig. 6A). Representative plots showed this effect (Fig. 6B). Additionally, flow cytometry analyses displayed that hepatocellular carcinoma cells with microRNA-4651 mimics featured a higher death rate than those without mimics (68.42% versus 21.34%) (Fig. 6C).

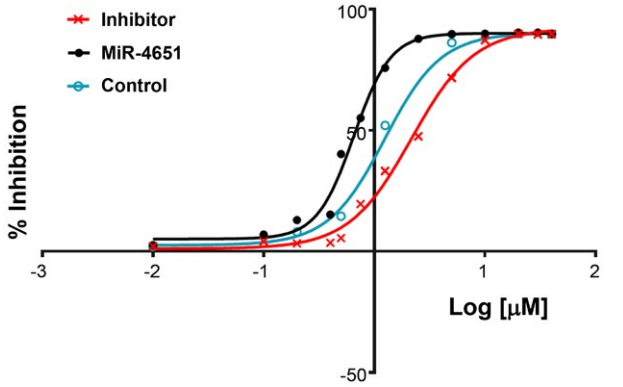

FIG. 5. The miR-4651 expression-modifying effects of doxorubicin treatment on hepatocellular carcinoma cells SMMC-7721 *in vitro*. SMMC-7721 cells were transfected with normal saline (Control), miR-4651 mimics (miR-4651), or miR-4651's inhibitor (Inhibitor). The sensitivity of cells to doxorubicin was evaluated by IC50. IC50 values were calculated by nonlinear regression analysis using the GraphPad Prism software Version 6.0 (GraphPad Software, Inc., San Diego, CA). Abbreviations: DOX, doxorubicin.

CYP2W1 EXPRESSION CORRELATES WITH MICRORNA-4651

To find possible target genes, we created an expression profile assay using microNRA-4651 mimics-transfected SMMC-7721 cells. Among the top 30 differently expressed genes, CYP2W1 was dramatically downregulated in those cells transfected with mature microRNA-4651 mimics compared with those without microRNA-4651 mimics (Fig. 7A). The real-time quantitative PCR and western blot analyses further proved that microRNA-4651 can downregulate the expression of this gene (Fig. 7B,C).

Discussion

Through a hospital-based retrospective study, we investigated the effects of microRNA-4651 expression

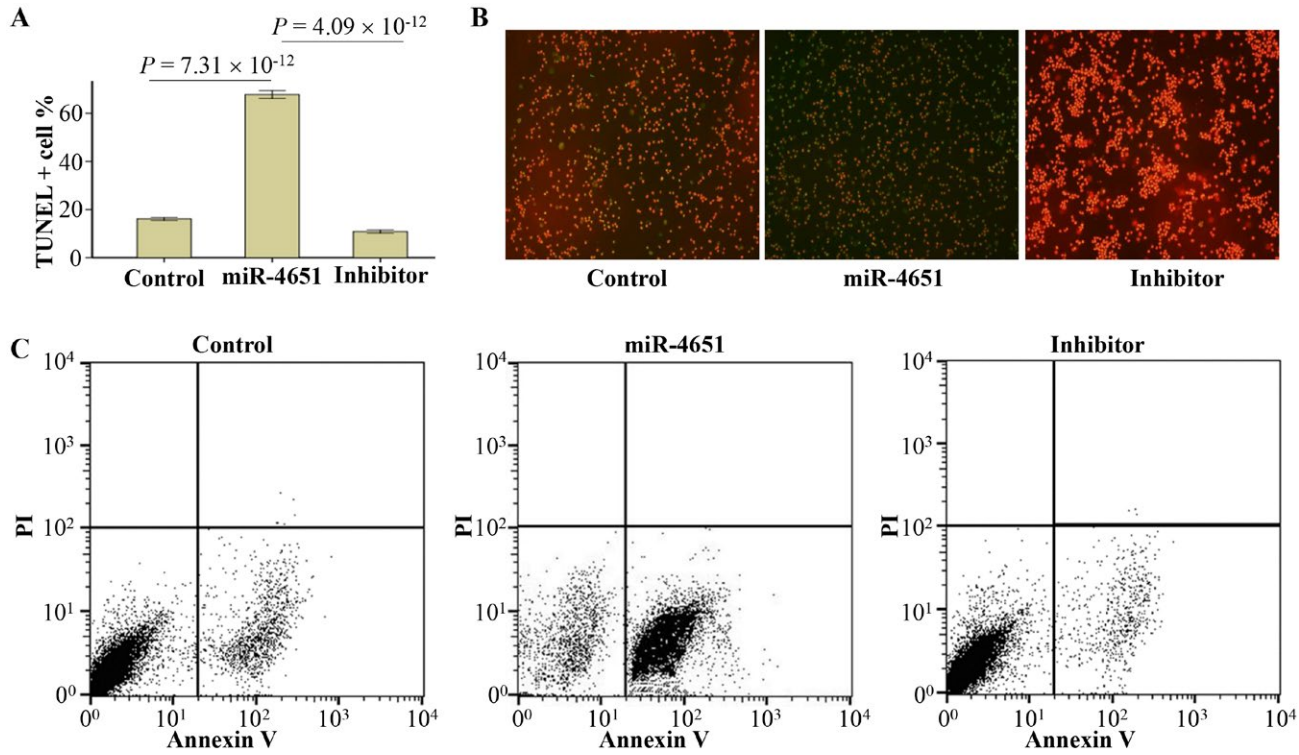

FIG. 6. The miR-4651 expression-modifying doxorubicin-induced death of hepatocellular carcinoma cells SMMC-7721 *in vitro*. SMMC-7721 cells were transfected with normal saline (Control), mature miR-4651 mimics (miR-4651), or miR-4651's inhibitor (Inhibitor). (A) TUNEL staining was used to analyze the doxorubicin-induced cell deaths. In the fluorescence microscopy, cells (including live and nonlive cells) exhibited red fluorescent, whereas death cells exhibited green fluorescent. TUNEL-positive cells (green) were counted in at least 300 cells in randomly chosen fields. The data were expressed as a percentage of TUNEL-positive cells to total cells and analyzed using one-way ANOVA test with Bonferroni correction. (B) The representative fluorescent plots showed the effects of miR-4651 on doxorubicin-induced cell deaths. (C) Flow cytometry analyses were used to analyze the effects of miR-4651 on doxorubicin-induced cell death.

on pa-TACE treatment in improving hepatocellular carcinoma prognosis. We discovered that upregulated microRNA-4651 expression in tumor tissues improved the therapeutic response of patients with hepatocellular carcinoma on pa-TACE treatment (HR = 0.32 and 95% CI = 0.22-0.46 for OS; and 0.39 and 0.28-0.56 for RFS, respectively), but downregulated expression did not. Furthermore, this dysregulation of microRNA-4651 expression displayed its potential for modifying the response of tumor cells to doxorubicin. These results are indicative of microRNA-4651 expression as a valuable biomarker for selecting what kinds of cases should receive pa-TACE treatment.

Increasing evidence has proven that pa-TACE treatment can prolong the life of patients with hepatocellular carcinoma and decrease tumor recurrence compared with surgery alone.[10,34] For example, Tao et al. analyzed the antitumor effects of pa-TACE treatment in patients featuring portal vein thrombus of hepatocellular carcinoma and found that pa-TACE treatment (21.91 months) as well as radiotherapy (14.53 months) significantly prolong the median OS time of cases compared with hepatectomy alone (8.99 months).[34] The results from a meta-analysis (including 25 retrospective studies, 2 prospective studies, and 10 unclassified-type studies) also showed that those hepatocellular carcinoma cases receiving hepatic resection plus pa-TACE had better OS and RFS than those only doing hepatic resection (HR [95% CI] = 0.85 [0.72–1.00] for OS and 0.83 [0.73-0.94] for RFS, respectively).[10] Subgroup analyses based on relevant randomized controlled trials (including 14 studies) further proved that pa-TACE treatment can also improve the OS and RFS of higher-stage hepatocellular carcinoma, such as tumors with vessel invasion and large size.[10] However, many studies have suggested that pa-TACE

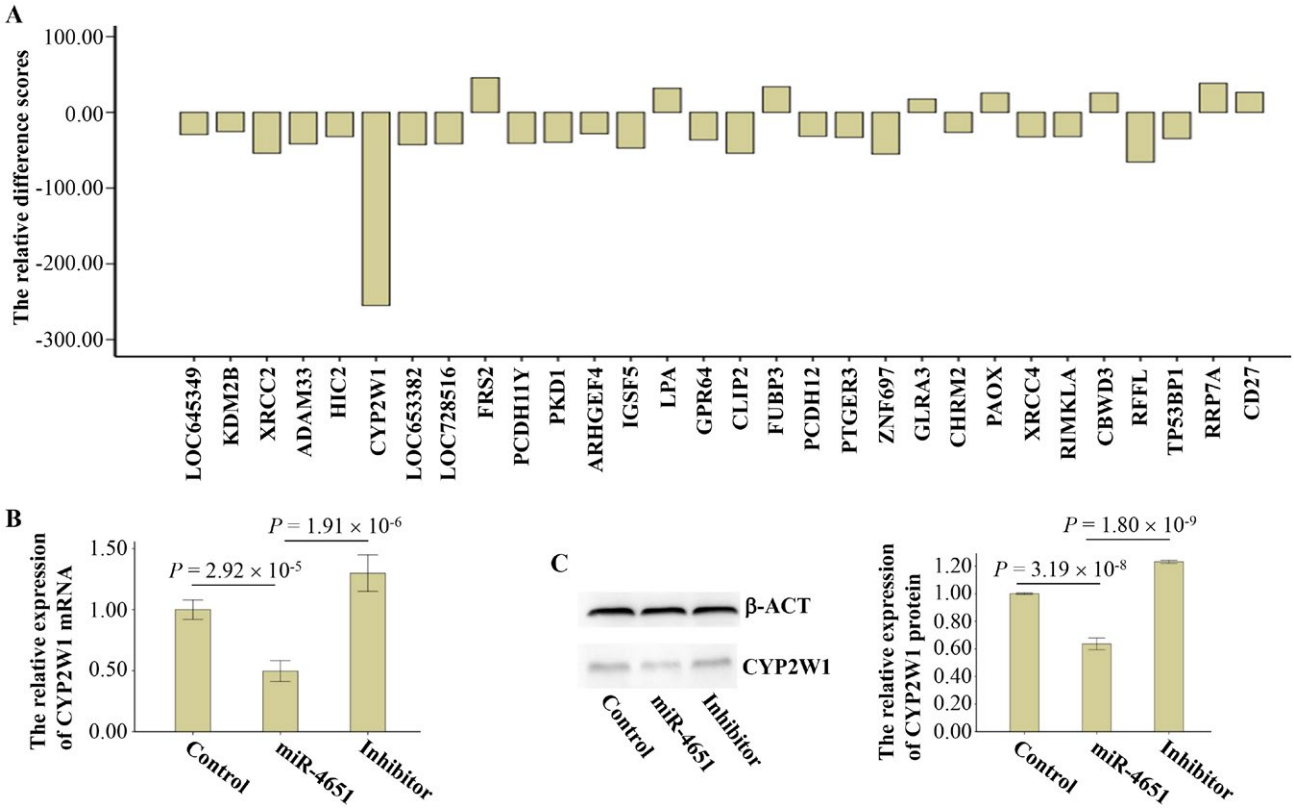

FIG. 7. CYP2W1 expression significantly correlated with miR-4651 in the hepatocellular carcinoma cells SMMC-7721 *in vitro*. (A) Relative expression changes in the top 30 expressed genes as SMMC-7721 cells transfected with mature miR-4651 mimics or its null control. The relative different scores of gene expression in miR-4651 relative to control were calculated using the Bio-Array Environment software. (B) Relative expression of CYP2W1 mRNA was tested using the real-time quantitative PCR method, and data were analyzed using one-way ANOVA test with Bonferroni correction. (C) The level of CYP2W1 protein was tested using western blot analysis (left). The relative level of protein was calculated using the corresponding gray value relative to the control's, and data were analyzed using one-way ANOVA test with Bonferroni correction (right).

displays different therapeutic effects on hepatocellular carcinoma with different genetic profiles. For example, different genetic features such as different expression of polycomb chromobox 4 and x-ray repair complementing 4 among individuals with hepatocellular carcinoma have been proven to modify MVD, and ultimately lead to unsuccessful pa-TACE treatment.[30,32] Genetic changes in a disintegrin and metalloproteinase with thrombospondin motifs 5, microRNA-1268a, microRNA-196a, and microRNA-499a may change individual's response to pa-TACE treatment and result in treatment failure.[15,31]

In this study, we are particularly concerned with the association between microRNA-4651 expression in cancerous tissues and pa-TACE treatment, primarily because our previous reports showed a higher serum mciroRNA-4651 level among patients with aflatoxin B1-related hepatocellular carcinoma, and this upregulation significantly correlated with tumor angiogenesis and prognosis.[27,28] This is indicative of its acting as an oncogene or having roles like oncogenes. Our present data indicate that microRNA-4651 expression in cancerous tissues significantly modifies the response of hepatocellular carcinoma cases to pa-TACE treatment. We also found that this marker was significantly associated with clinicopathological characteristics, such as tumor differentiation and angiogenesis, and was an independent prognostic factor as well as pa-TACE treatment. Altogether, these findings suggest that the levels of microRNA-4651 expression in tumor tissues may have a function that changes the sensitivity of cancerous cells on antitumor drugs used in pa-TACE procedure. The following sensitivity analyses further proved this function. Therefore, microRNA-465 can

act as a biomarker for whether hepatocellular carcinoma cases should receive pa-TACE treatment.

Another potential pathway might involve regulating CYP2W1 expression. CYP2W1 is an important member of the cytochrome P450 superfamily, and its encoding product is a kind of monooxygenase that is involved in drug metabolism and synthesis of cholesterol, steroids, and other lipids.[35] Recently, increasing evidence has shown that the dysregulation of CYP2W1 may affect the carcinogenesis of some tumors such as liver cancer, breast cancer, and colon cancer and modify the anticancer drug metabolism.[36-39] In this study, we finished a gene expression profile assay and the following valid analyses, and found mature microRNA-4651 mimics can decrease the level of CYP2W1 expression. Bioinformatic analyses proved that five potential target sites for microRNA-4651 are at the 3′-untranslated region of CYP2W1. These results suggest that the downregulation of CYP2W1 targeted by microRNA-4651 may be an important pathway for this microRNA to modify the effects of pa-TACE treatment.

In summary, this study describes microRNA-4651 expression in tissues with hepatocellular carcinoma and its association with the therapeutic efficiency of pa-TACE treatment. We determined that microRNA-4651 could act as a significant biomarker for predicting the therapeutic significance of pa-TACE treatment. Furthermore, microRNA-4651 expression was also an independent prognostic biomarker for hepatocellular carcinoma. On the basis of these findings, analyzing the levels of microRNA-4651 expression in cancerous tissues may promote the formation of a hepatic carcinoma management strategy. However, the present study was limited in its ability to evaluate the relationship between microRNA-4651 expression and pa-TACE treatment because of its relatively small sample size. Although we investigated the sensitivity of hepatocarcinoma cells to doxorubicin used in pa-TACE treatment through in vitro assays, we did perform further functional and mechanical analyses. Additionally, we only analyzed the correlation between miR-4651 and hepatocarcinoma prognosis; other microRNAs, such as miR-1268a, might modify this effect. Therefore, prospective studies in combination with functional analyses will be required to fully evaluate the utility of micrRNA-4651 in selecting patients for pa-TACE treatment.

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