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Downregulation of microRNA-214 and overexpression of FGFR-1 contribute to hepatocellular carcinoma metastasis ☆

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ABSTRACT

miR-214 is one of the most significantly downregulated microRNAs (miRNAs) in hepatocellular carcinoma (HCC). Fibroblast growth factor receptor 1 (*FGFR-1*) is a *miR-214* target gene implicated in the progression of HCC. However, the roles of *miR-214* and *FGFR-1* in HCC are not fully understood. Here, we analyzed the expression of miR-214 and FGFR-1 in 65 cases of HCC and paired non-neoplastic tissue specimens using real-time PCR and Western blot (WB), respectively. Our data indicated that *miR-214* was downregulated and *FGFR-1* was overexpressed in HCC compared to the paired non-neoplastic tissues. The low *miR-214* expression was correlated with portal vein invasion ($p = 0.016$) and early recurrence ($p = 0.045$) in HCC patients. Moreover, the low *miR*-214 expression was correlated with high positive rate of FGFR-1 in HCC cases ($p = 0.020$). *Our data further demonstrated that miR-214 overexpression in* SK-HEP1 *and* HepG2 *cells downregulated FGFR-1 expression and inhibited liver cancer cell invasion.* The Luciferase assay results further demonstrated the targeted regulation of *FGFR*-1 by miR-*214*. *In conclusion, our data indicate that the downregulation of miR-214 in* HCC *and the upregulation of its target gene FGFR-1 is associated with* HCC *progression.* Therefore, *miR-214* and *FGFR-1* are potential prognostic markers and therapeutic targets in HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths worldwide [1,2]. This dismal outcome has been attributed to intrahepatic metastasis, a major hallmark of HCC. Tumor cells invade the major branches of portal vein, resulting in intrahepatic metastasis. To provide survival benefit for patients with HCC, medical practitioners perform surgical resection/liver transplantation as the standard treatment modality resulting in an overall five-year survival rate of <5% [3,4]. Therefore, there is a urgent need to understand the molecular mechanisms underlying HCC metastasis, and to identify novel therapeutic targets.

Deregulation or dysfunction of microRNAs (miRNAs) is involved in cancer development and related to clinical outcomes of cancer patients, including patients with HCC [5–10]. Several miRNAs, including miR-224, miR-106b, miR-21 [11–13], are overexpressed in HCC and involved in HCC progression. However, very few downregulated miRNAs have been reported to be associated with HCC development and progression. Wang, et al. [14] reported that *miR-214* is one of the most significantly downregulated miRNAs in HCC patients. β-catenin and hepatoma-derived growth factor (HDGF), the target genes of miR-214, inhibit HCC cell growth and invasion [15,16]. However, other potential target genes of *miR-214* and their molecular function in HCC progression remain largely unknown. Fibroblast growth factor receptor 1 (FGFR1) is a potential target gene of miR-214 and overexpressed in HCC. FGFR1 overexpression increased cancer cell growth and infiltration and was involved in angiogenesis, which promoted the progression of HCC [17,18]. In this study, FGFR-1 was characterized as one of the functional downstream targets of miR-214 and contributed to the progression of HCC.

2. Material and methods

2.1. Patients and samples

Clinicopathological data were collected as described in our previous study [2]. Sixty-five patients with primary HCC treated in Cancer Hospital of Tianjin Medical University between 2006 and 2008 were selected according to the previous criteria including

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confirmed diagnosis by pathology; no preoperational chemotherapy or TAE; first hepatectomy of patients; negative incisal margins; and cases with complete clinicopathologic data [2]. Among the 65 patients, there were 47 males (72.3%) and 18 females (27.7%). HBV was positive in 55 patients (84.6%). The percentage of AFP (>100 ng/ml) was 72.3%. One tumor was detected in 72.3% (47/65) of the patients and multiple tumors ($\geqslant$2) were found in 27.7% (18/65) of the patients. Histological evaluation showed that 46.2% (30/65) of the tumors were classified as grade 1; 53.8% (35/65) of the tumors were classified as grades 2 and 3. The patients were followed-up at the outpatient clinic in the same hospital as described before [2]. The mean time of follow-up was 29.6 months (3–months). HCC and non-neoplastic tissues were collected and stored at −80 °C until analysis. Informed consent of liver specimen collection was obtained from the patients. The study protocol was approved by the Ethics Committee of Tianjin Medical University and the investigations were conducted according to the Declaration of Helsinki Principles.

2.2. Cell culture and transfection

Human HCC cell lines expressing FGFR-1, SK-HEP1 and HepG2 were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with heat-inactivated 10% fetal bovine serum (FBS; Invitrogen) at 37 °C in a humidified incubator containing 5% CO_2.

For transfection, 2×10^5 cells were seeded into each well of a six-well plate and incubated overnight. The cells were then transfected with pre-miR miRNA precursor molecule *pre-214* (*pre-miR-214*; Applied Biosystems) or the plasmid pDONR223-FGFR1 (Addgene, Cambridge, MA, USA) at a final concentration of 100 nM with Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transfection specificity was verified using the pre-miR miRNA precursor molecule negative Control #1 (control pre-miR; Applied Biosystems). The expression levels of *miR-214* and *FGFR-1* were quantified 24 h after transfection, and the cells were subjected to Western blot analysis.

2.3. Quantitative RT-PCR test of miR-214 and Western blot analysis of FGFR-1

The procedures of RNA extraction and realtime-PCR were performed as described before [19]. The expressions of *miR-214* and its control RNU44 were detected using a TaqMan miRNA assay system (Applied Biosystems, Foster City, CA, USA). The median miRNA intensity value of 65 patient samples was used as the threshold. The patients were divided into two groups according to *miR-214* expression: (1) below median group expressing low *miR-214* and (2) above median group expressing high *miR-214.*

Western blot was performed as previous report [19]. Primary antibodies *FGFR-1* and horseradish-peroxidase-coupled secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The background was subtracted, and the signals of the detected bands were normalized to the amount of the loading control β-actin band (Cell Signaling Technology, Inc. Danvers, MA, USA) band. The protein levels were quantified using ImageJ software (National Institute of Mental Health, Bethesda, MD, USA. http://rsb.info.nih.gov/ij).

2.4. 3′ UTR luciferase reporter assay

The human *FGFR-1* 3′ UTR luciferase reporter construct (*FGFR-1*-3′ UTR WT) was generated by cloning *FGFR-1* mRNA 3′ UTR sequence into downstream of pMIR-report construct (Ambion, Foster City, CA, USA). The *FGFR-1* 3′ UTR sequence was generated by PCR using primer *FGFR-1* 3′ UTR forward SpeI: AAAACTAGTCTTCCTGTCCAAACTCCATCC -3′ and primer *FGFR-1* 3′ UTR reverse SacI: 5′-CACCTCGGATCTTTTGTATTTAGCAGTAATCCAGC -3′. The *miR-214* target site-mutation *FGFR-1* 3′ UTR luciferase reporter 1 (*FGFR-1*-3′ UTR mutation) construct was generated by conducting direct-site mutagenesis with mutation primers, which induce the *miR-214* binding site to mutate from AATTTACTTCTGCCACCTG CTGG to AATTTACTTCTGCCACGACGAGG.

SK-HEP1 cells were co-transfected with an *miR-214* plasmid and a wild-type or mutant *FGFR-1* 3′ UTR luciferase reporter construct. Luciferase activities were determined using a Dual-Glo luciferase. Data were normalized by dividing firefly luciferase activity by *Renilla* luciferase activity.

2.5. In vitro invasion assays

SK-HEP1 and HepG2 cell invasion assays were performed using 24-well Matrigel invasion chambers (BD Biosciences, CA, USA). The lower chambers were filled with 0.75 ml of DMEM medium containing 10% FBS. A cell suspension of 1×10^5 in 0.5 ml of DMEM medium was added to each well in the upper chamber. After the cells were incubated for 24 h at 37 °C in a humidified incubator with 5% CO_2, the invasive cells attached to the lower surface of the membrane insert were fixed in 10% formalin at room temperature for 5 min and stained with 0.05% crystal violet. The non-invading cells that remained on the upper surface of the membrane were removed by scraping. The number of invasive cells on the lower surface of the membrane was then counted under a microscope.

2.6. Statistical analysis

The correlation between *miR-214* and disease-free survival time was evaluated by calculating the Spearman rank correlation coefficient. Mean ± SD of clinicopathological valuables were calculated. Differences in the means were analyzed using one-way ANOVA or Student's *t*-test. We also used the Kaplan–Meier method and the log-rank test for univariate survival analysis; we used the Cox proportional hazards regression model for multivariate analysis. SPSS version 16.0 (IBM) was used to perform statistical analysis. Two-tailed *p* values <0.05 were considered statistically significant.

3. Results

3.1. miR-214 expression and its correlation with clinicopathological characteristics in HCC patients

We examined *miR-214* expression in 65 HCC and matched non-neoplastic tissue specimens using real-time PCR technology (Fig. 1A). The relative *miR-214* expression in HCC samples (1.13 ± 0.49) was significantly lower when compared to matched non-neoplastic tissues (1.65 ± 0.92; $p < 0.01$; Fig. 1B).

We next analyzed the correlations between miR-214 expression and clinicopathological characteristics, including age, sex, HBV infection, Child-Pugh grade, AFP, tumor number, tumor size, histological grade, portal vein invasion, recurrence, and *FGFR-1* expression (Table 1). The patients were divided into two groups based on the median value of the *miR-214* expression: 32 cases with low *miR-214* expression and 33 cases with high *miR-214* expression. The patients with low *miR-214* expression had higher ratio of portal vein invasion ($p = 0.016$) and higher recurrence rate ($p = 0.045$) than those with high miR-214 expression. Although the *p* values did not reach statistical significance, the patients with low *miR-*

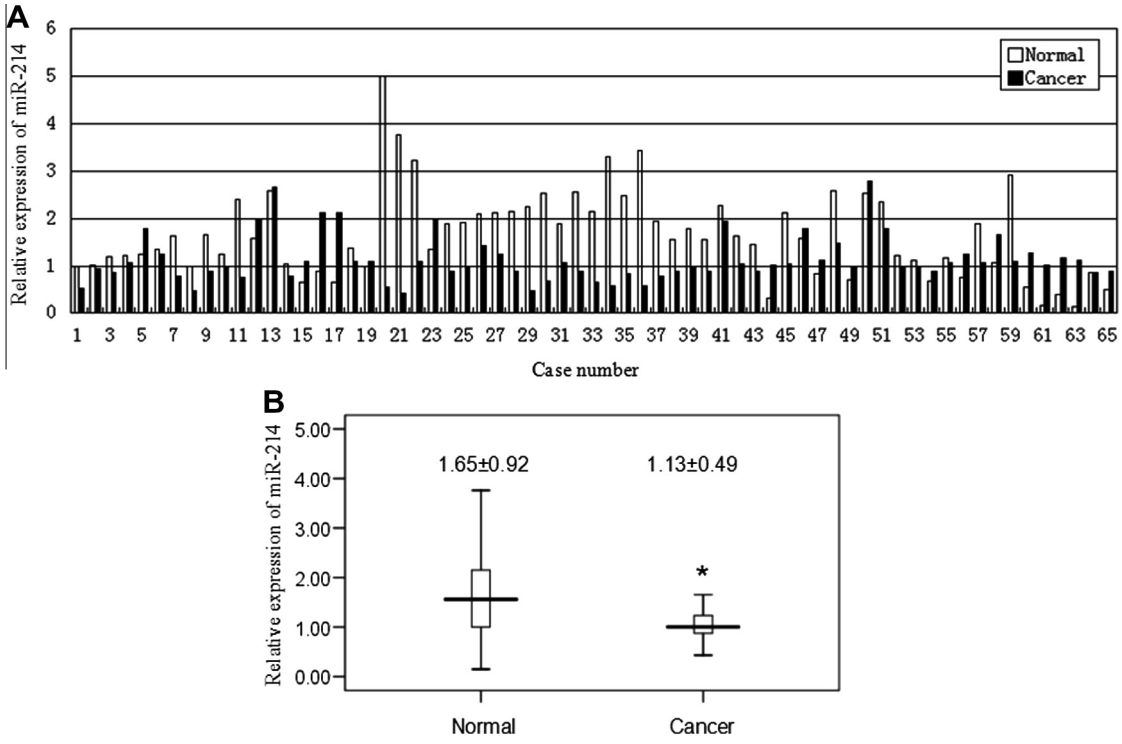

Fig. 1. *miR-214* expression of in non-neoplastic and tumor tissues of 65 HCC patients. Relative *miR-214* expression was detected by real-time PCR in each paired non-neoplastic and HCC tissues (A). Relative *miR-214* expression in HCC tissues ($n = 65$, 1.13 ± 0.49) was significantly lower than that in non-neoplastic liver tissues (1.65 ± 0.92) (B) $^{*}p < 0.05$.

Table 1
Univariate analysis of miR-214 expression and clinicopathological data of patients with HCC.

Variables	*n*	Low miR-214 expression	High miR-214 expression	*p*
Age				
<51 years	26	14	12	0.383
⩾51 years	39	18	21	
Gender				
Male	47	26	21	0.332
Female	18	6	12	
HBV				
Positive	55	29	26	0.105
Negative	10	3	7	
Child-Pugh grade				
A	39	18	21	0.383
B,C	26	14	12	
AFP				
<100 ng/ml	18	7	11	0.172
⩾100 ng/ml	47	25	22	
Tumor number				
<2	47	21	26	0.071
⩾2	18	11	7	
Tumor size				
<5 cm	26	14	12	0.383
⩾5 cm	39	18	21	
Tumor grade				
1	30	12	18	0.056
2, 3	35	20	15	
Portal vein invasion				
No	39	16	23	0.016
Yes	26	16	10	
Recurrence				
No	37	17	20	0.045
Yes	28	15	13	
FGFR-1 expression				
Low	36	20	16	0.020
High	29	12	17	

214 expression exhibited a higher tendency to suffer from multiple lesions ($p = 0.071$) and poor differentiation ($p = 0.056$).

In order to evaluate the prognostic significance of miR-214 expression, we performed survival analyses. Among the 65 HCC patients, recurrence was observed in 28 cases (43.1%) during the follow-up period. The median disease-free survival periods of patients with low and high *miR-214* expressions were 22.0 and 28.0 months, respectively. The *miR-214* expression was positively correlated with disease-free survival time ($r = 0.547$, $p < 0.001$) (Fig. 2A). Kaplan–Meier and Log-rank analyses showed that the patients with low *miR-214* expression had worse disease-free survival rate than those with high miR-214 expression ($p = 0.028$; Fig. 2B). Multivariate survival analysis demonstrated that multiple tumors (hazard risk [HR] = 2.706, $p = 0.023$), portal vein invasion (HR = 2.111, $p = 0.049$) and low *miR-214* expression (HR = 2.037, $p = 0.032$) were independent prognostic markers suggesting poor survival for patients with HCC (Table 2). All of these data manifested that low miR-214 expression was associated with cancer progression, aggressive behavior and poor outcome in HCC, suggesting the role of miR-214 as a tumor suppressor in HCC.

Table 2
Cox regression survival analysis in HCC patients.

Variables	HR (95% CI)	*p*
Age (⩾51 years *vs* <51 years)	0.985 (0.955–1.016)	0.350
Gender (female *vs* male)	0.606 (0.255–1.439)	0.256
HBV (positive *vs* negative)	0.651 (0.227–1.867)	0.425
Child-Pugh grade (A vs B,C)	1.856 (0.917–3.757)	0.086
AFP (⩾100 ng/ml *vs* <100 ng/ml)	0.884 (0.553–5.397)	0.347
Tumor number (⩾2 *vs* <2)	2.706 (1.150–6.372)	0.023
Tumor size (⩾5 cm *vs* <5 cm)	0.875 (0.732–1.046)	0.143
Tumor grade (2,3 *vs* 1)	1.469 (0.720–2.997)	0.290
Portal vein invasion (yes *vs* no)	2.111 (0.901–3.872)	0.049
FGFR-1 expression (high vs low)	1.188 (0.997–1.415)	0.054
miR-214 expression (low *vs* high)	2.037 (1.064–3.906)	0.032

Abbreviation: HR, hazard risk; CI, confidence interval; HBV, hepatitis B virus; AFP, Alpha fetoprotein.

3.2. miR-214 regulated FGFR-1expression

FGFR-1 protein expression was examined in HCC and paired non-neoplastic tissues with Western blot assay and quantified using ImageJ software (Fig. 3A). FGFR-1 expression was upregulated significantly in cancer cells when compared to paired non-neoplastic tissues, suggesting that FGFR-1 was important in HCC progression. Moreover, the tumors with low miR-214 expression showed higher FGFR-1 expression than those with high miR-214 expression ($p = 0.020$). Their expression also showed a negative correlation ($r = -0.304$, $p = 0.014$, Fig. 3B).

The negative correlation between FGFR-1 and miR-214 expression in our HCC samples and the potential *miR-214* binding site in *FGFR1*-3′ UTR suggested *FGFR1* might be a target gene of miR-214. We performed luciferase reporter assay to examine whether miR-214 directly targeted *FGFR1*. We cloned the 3′-UTR of *FGFR1* into the pMIR vector and generated pMIR-*FGFR1* construct. Co-transfection of pMIR-*FGFR1*, pRL-TK and pre-miR-214 resulted in a 61.2% reduction in luciferase activity compared with that after co-transfection with pre miR-Ctrl (Fig. 3C). In contrast, miR-214 failed to

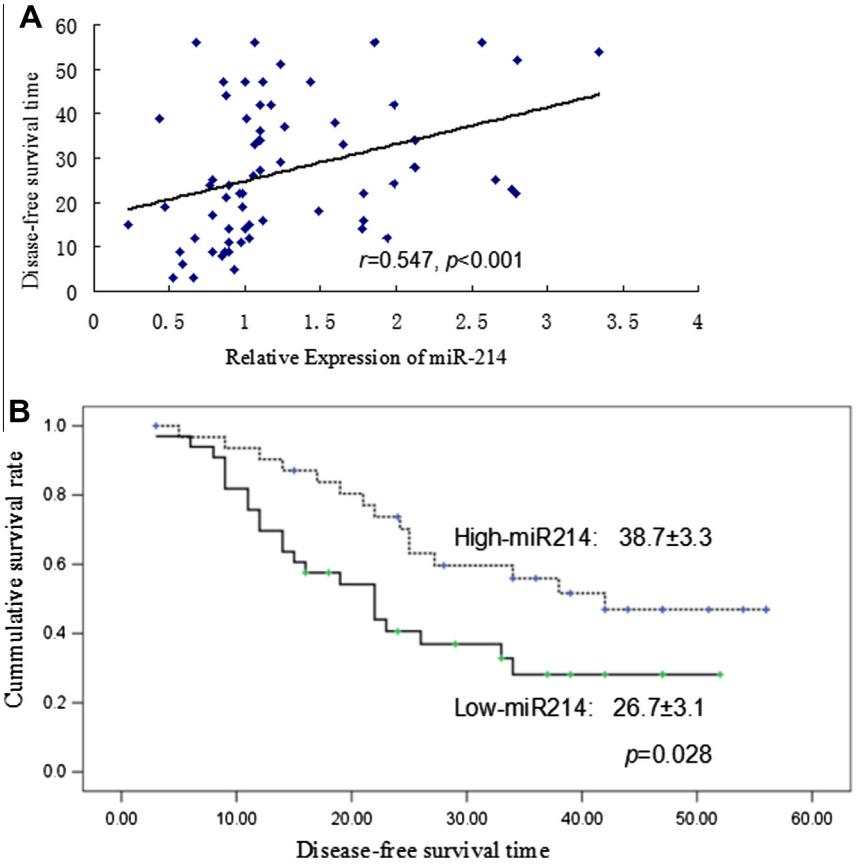

Fig. 2. Kaplan–Meier analysis of the disease-free survival of 65 patients with HCC expressing *miR-214* expression. (A) *miR-214* expression was positively correlated with the disease-free survival of patients with HCC ($r = 0.547$, $p < 0.001$). The median value (1.07) of *miR-214* level was chosen as the cutoff point to distinguish *miR-214* low-expression tumors ($n = 32$) from *miR-214* high-expression cases ($n = 33$). (B) The prognosis in later group was significantly better than that of former one ($p = 0.028$) (Kaplan–Meier log-rank survival analysis).

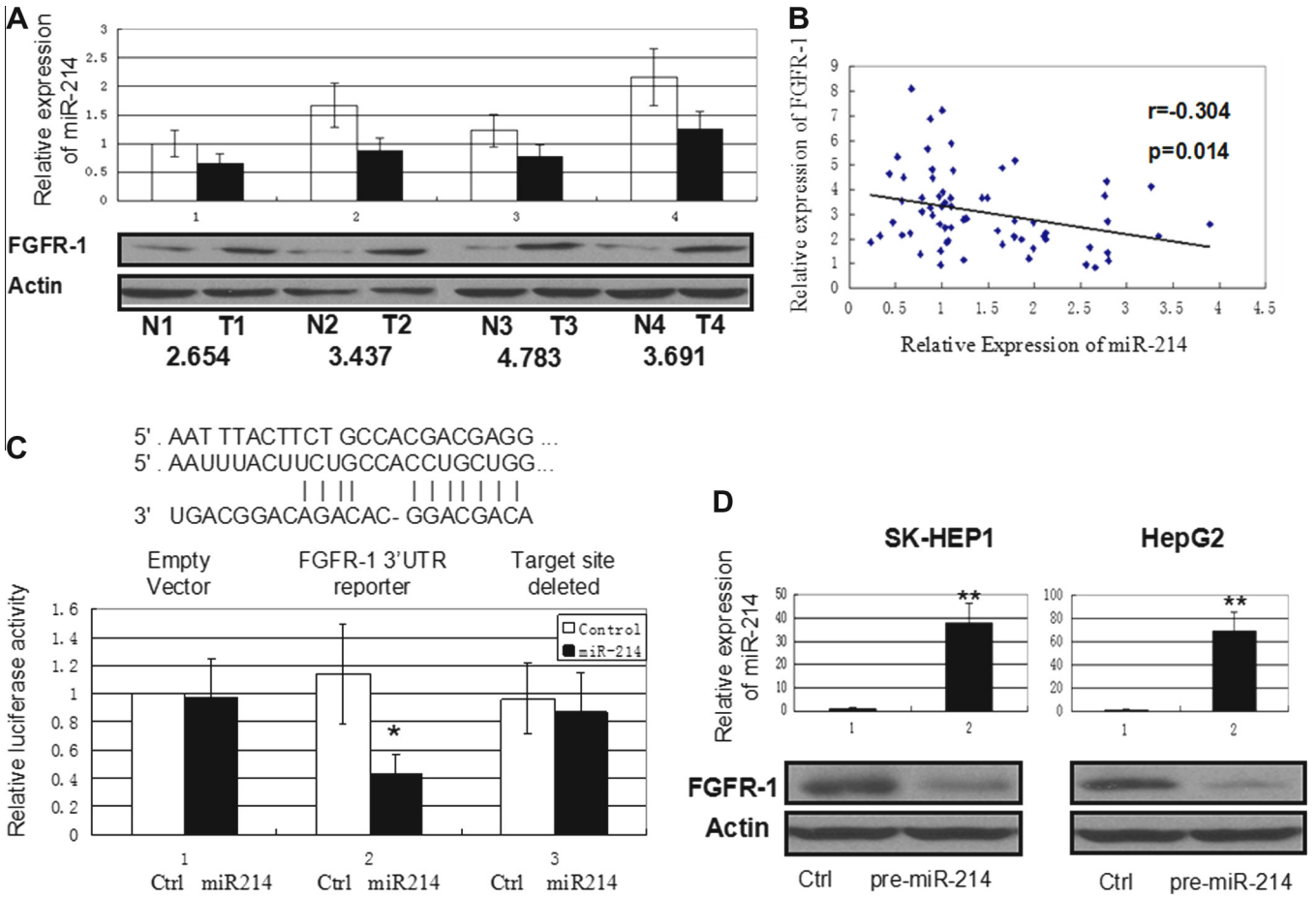

Fig. 3. Correlation between *miR-214* and *FGFR-1* expressions in HCC and paired non-neoplastic tissues. (A) *miR-214* and *FGFR-1* expressions were detected by real-time RT-PCR and Western blot, respectively. *FGFR-1* expressions were quantified with ImageJ software in four-paired HCC and non-neoplastic tissues. (B) The negative correlation between *miR-214* and *FGFR-1* expressions. (C) *miR-214* directly targets human *FGFR-1*, 3′-UTR of FGFR-1 mRNA is partially complementary to *miR-214*. Effect of *miR-214* on Luciferase Luc-*FGFR-1*-3′ UTR luciferase activity and Luc- *FGFR-1*-3′ UTR mutation. This assay was conducted using SK-HEP1 cells and demonstrated that *FGFR-1* is a direct *miR-214* target. (D) Transfection of pre-miR-214 into SK-HEP1 and HepG2 resulted downregulation of FGFR-1. $^{*}p < 0.01$. N: non-neoplastic tissue; T: tumor.

inhibit the luciferase activity when we co-transfected pRL-TK, pre-miR-214 and pMIR-*FGFR1*-3′ UTR mutation (Fig. 3B), suggesting that miR-214 could directly target the 3′ UTR of *FGFR1*. Transfection of pre-miR-214 into SK-HEP1 and HepG2 also resulted downregulation of FGFR-1 (Fig. 3D).

3.3. miR-214 inhibited HCC cell invasion through the regulation of FGFR-1expression

To determine whether miR-214 could downregulate FGFR-1 expression and inhibit cell invasion, we transfect SK-HEP1 and HepG2 cells with pre-miR-214. As shown in Fig. 4A, the *in vitro* invasion assay indicated that the relative invasiveness of the HCC cell lines transfected with *pre-miR-214* was reduced by approximately 37% and 53%, respectively ($p < 0.05$). To demonstrate that miR-214 exerts its effect on invasion through FGFR-1, we performed a rescue experiment, where miR-214 was cotransfected with a FGFR-1 expression vector into HepG2. The ectopic expression of FGFR-1 mitigated significantly the effect of miR-214 on invasion (Fig. 4B). The *in vitro* results further demonstrated that *miR-214* could inhibit HCC metastasis by downregulating *FGFR-1*.

4. Discussion

miR-214 downregulation occurs in a large part of HCC tissues [14]. In the HCC cases with complete clinical data in the present study, *miR-214* was downregulated in cancer tissues. Low *miR-214* expression was significantly associated with portal vein invasion and early recurrence, which are important clinical determinants for the prognosis of patients with HCC. Low *miR-214* expression was possibly correlated with reduced disease-free survival of HCC patients. Hence, *miR-214* expression level should be determined in HCC tissues and may be used as a novel approach to predict the prognosis of HCC patients.

Although miRNA profiles reveal prospective characteristics of cancer, the functions and real targets of miRNAs remain unknown. The predicted targets of the majority of miRNAs based on sequence homology are comprehensively validated by *in vitro* and *in vivo* experiments. Target scan and Pictar showed that *FGFR-1* is an important target of *miR-214* with a high context score. *FGFR-1* expression in HCC increased significantly compared with that of non-neoplastic tissue and negatively correlated with *miR-214* expression indicating *FGFR-1* may be a regulatory target of *miR-214*.

FGFR-1 signaling is important because *FGFR-1* expression is low in a normal liver epithelium and high in a human liver cancer epithelium. Previous studies showed the association of *FGFR-1* with a more aggressive phenotype in prostate cancer and breast cancer [20–22]. The ectopic expression of *FGFR-1* in the prostate epithelium affects the expressions of matrix degrading enzymes, including matrilysin (MMP-7) [23] indicating that *FGFR-1* is involved in matrix turnover and cell invasion. These data suggested that

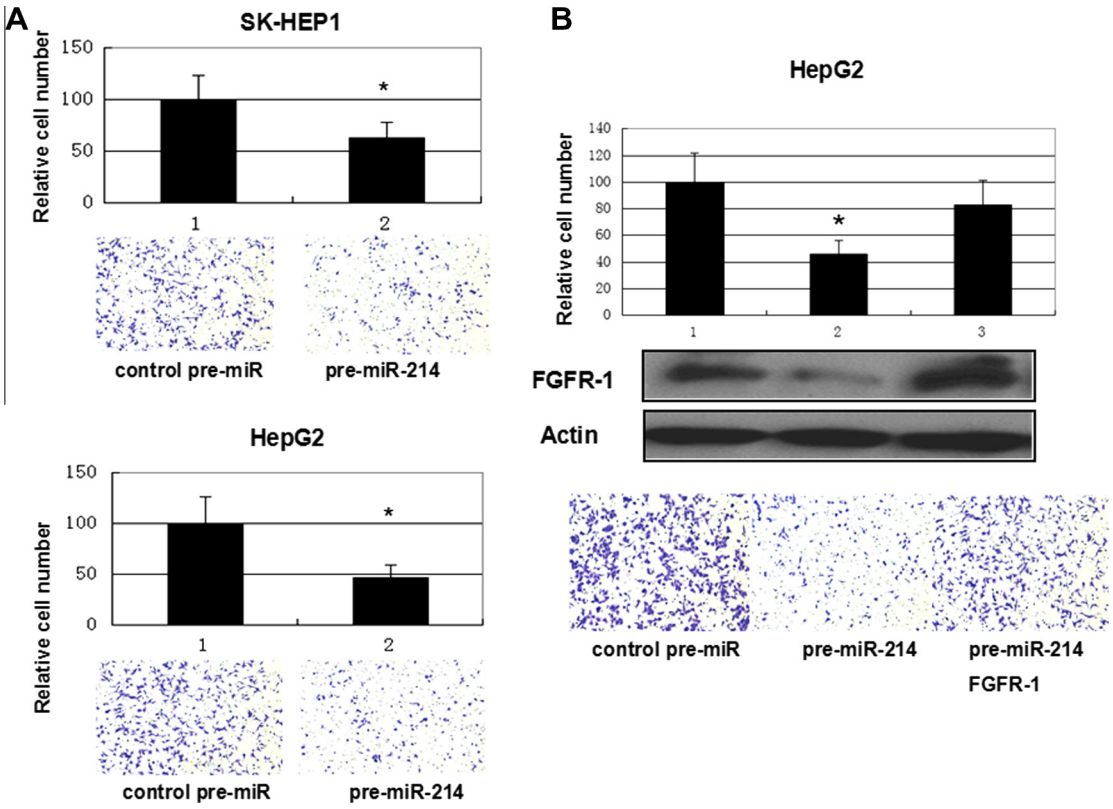

Fig. 4. *miR-214* modulated *FGFR-1* expression and cell invasive potential of SK-HEP1 and HepG2. (A) The relative invasiveness of the HCC cell lines transfected with *pre-miR-214* was specifically reduced by approximately 37% and 53%, respectively. (B) miR-214 was cotransfected with a FGFR-1 expression vector into HepG2. The ectopic expression of FGFR-1 mitigated significantly the effect of miR-214 on invasive inhibition. $^{*}p < 0.05$, $^{**}p < 0.01$.

FGFR-1 protein may be important in regulating cytoskeletal dynamics; as a result, *FGFR-1* protein may function in cancer cell invasion and metastatic behavior. However, the mechanism of *FGFR-1* expression in cancer cells remains unknown. In this study, *miR-214* downregulation may play an important function in regulating *FGFR-1*.

miR-214 can bind to *FGFR-1* at conserved sites with a high context score. The luciferase assay in HCC cell lines demonstrated that *FGFR-1* could be regulated directly by *miR-214*. Considering that HCC exhibits heterogeneity and *FGFR-1* is regulated by other mechanisms, we found that *FGFR-1* is significantly associated with *miR-214*. The ability of overexpressed *FGFR-1* to counteract the pro-invasion effects of *miR-214* unequivocally shows the importance of this negative relationship in HCC metastasis. The functional analysis of *miR-214* and *FGFR-1* in animal models possibly provides information of their metastatic function and reveals the importance of clinical treatment for patients with HCC. This objective would be our future research aim.

It was previously reported that *miR-214* downregulation contributes to intrahepatic cholangiocarcinoma metastasis by targeting Twist [24]. *miR-214* can also suppress growth and invasiveness of cervical cancer cells by targeting GALNT7 [25]. These results are consistent with those in other studies, indicating that single miRNA can target multiple mRNAs, named 'targetome', to regulate gene expression post-transcriptionally [26]. Considering this presumption, we recommend further studies to identify this targetome and the functions of *miR-214* in cancer development. Further studies should also determine why *miR-214* is downregulated in HCC and other cancers [27,28]. The current view suggests that miRNA expression is mainly controlled at the transcriptional level. Numerous transcription regulators including Myc, E2F, p16, and STAT3 that influence the transcription and production of miRNAs have been identified [29,30].

In conclusion, the study provided a model of tumor progression, in which downregulated *miR-214* and subsequent overregulation of FGFR1 promoted the aggressiveness of HCC. These results suggested that *miR-214* and its downstream effectors could be useful prognostic markers and/or therapeutic targets in HCC.

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