Identification of miR-589-5p as a Potential Prognostic Biomarker of Hepatocellular Carcinoma

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Research

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Abstract

Background: MicroRNAs (miRNAs) are small endogenous non-coding RNAs with 22 nucleotides approximately. Numerous studies have reported that microRNAs may hold the potential to serve as biomarkers in diagnosing cancer and predicting prognosis. miR-589-5p is a well-documented oncogenic miRNA implicated in several human cancer types. However, the potential value of miR-589-5p as a biomarker to facilitate early diagnosis and predict prognosis in hepatocellular carcinoma (HCC) patients remains obscure.

Methods: miR-589-5p expression was examined by in situ hybridization (ISH) in 10 adjacent normal tissues (ANT), 21 liver fibrosis tissues, 113 HCC tissues, including 22 HCC with grade I, 37 HCC with grade II, 49 HCC with grade III and 5 hepatocholangiocellular carcinoma tissues. Statistical analysis and Kaplan–Meier survival analysis was performed to evaluate the clinical correlation between miR-589-5p expression and clinicopathological features and survival prognosis in HCC patients.

Results: miR-589-5p expression was dramatically upregulated in HCC tissues by in situ hybridization (ISH). Overexpression of miR-589-5p was not only positively correlated with poor tumor differentiation degree, increased AFP levels, advanced clinical stage, distant metastatic status and venous invasion, but also predicted poor overall and relapse-free survival in HCC patients. Importantly, the expression levels of miR-589-5p measured by real-time PCR in high-ISH scores tissues were dramatically upregulated compared with those in low-ISH scores tissues, and a strong and positive correlation of miR-589-5p expression levels in real-time PCR with ISH staining index was demonstrated in HCC tissues.

Conclusion: our results support the notion that miR-589-5p might hold a promising value as a novel biomarker to facilitate early diagnosis and predict prognosis in HCC patients.

Introduction

Hepatocellular carcinoma (HCC) is one of the most devastating malignancies, which contributes to enormous cancer-related deaths, making HCC the third-leading cause responsible for cancer-related deaths worldwide [1]. Surgical resection is the primary therapeutic strategy with potentially curative possibility. However, for the majority of HCC patients, surgery could not be considered because of the advanced stage at the time of diagnosis. Therefore, exploration of novel clinical biomarker to predict and diagnose HCC appears to be of clinically paramount importance, which will facilitate early detection and prevention of HCC so as to improve the survival time in HCC patients.

MicroRNAs (miRNAs) that are a type of small endogenous noncoding RNAs implicated in various biological functions, such as apoptosis, cell cycle and proliferation, cell differentiation and immune response [2, 3]. miRNAs exert their functions by binding to specific sequences in the 3' untranslated region (3'UTR) of downstream target genes, leading to mRNA degradation and/or translational inhibition at post-transcriptional levels [2]. Numerous studies have demonstrated that aberrant expression of miRNAs is extensively reported to be implicated in the tumorigenesis, progression and metastasis in a

variety of human cancer types [4–8]. Importantly, accumulating evidence has reported that miRNAs hold the potential clinical applicable value for early diagnosis and recurrence monitoring of cancer, including HCC. For example, miR-155 was found to be upregulated in HCC tissues, and patients with high miR-155 levels had shorter overall survival (OS, log rank $P < 0.001$) and recurrence-free survival (RFS, log rank $P < 0.001$) [9]. Furthermore, increased level of miR-21 in the serum of HCC patients has been reported to be used to distinguish HCC from patient with chronic hepatitis and healthy controls. It was noteworthy that the sensitivity and specificity of miR-21were superior to that of AFP as a biomarker in diagnosing HCC [10]. Therefore, these findings support the crucial role of miRNAs in early detection and diagnosis of HCC, which will be helpful and time-saving to perform surgical tumor resection given early detection and diagnosis of HCC.

miR-589-5p, as one of the originally identified miRNAs, has been demonstrated to be dramatically upregulated in clinical HCC tissue samples, as assessed by our own HCC tissues and multiple independent publicly available HCC datasets, including The Cancer Genome Atlas (TCGA), E-GEOD-31384 and E-GEOD-36918 in our previous study, which was significantly correlated with poorer overall and relapse-free survival in HCC patients [11]. Since expression of miR-589-5p is examined and analyzed by real-time PCR in our previous study as well as in other study [12], a more reliable and commonly used clinical technique is required to further determine the clinical significance of miR-589-5p in HCC patients.

In the current study, our results reveal that the staining intensity of miR-589-5p was strongly enhanced in HCC tissues by ISH technique, which was positively associated with poor differentiation degree, AFP levels, clinical stage, distant metastatic status and venous invasion. And more importantly, high levels of miR-589-5p predicted poor overall and relapse-free survival in HCC patients. Furthermore, a strong and positive correlation of miR-589-5p expression levels in real-time PCR with in situ hybridization staining index was demonstrated in HCC tissues. Taken together, our findings indicate that miR-589-5p may be used as a potential biomarker for early detection and poor prognosis in HCC patients.

Materials And Methods

Patients and tumor tissues

113 paraffin embedded, archived HCC tissues were obtained during surgery at the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China) between 2006 and 2010, and the clinicopathological characteristics of HCC patients were summarized in Table 1. Patients were diagnosed based on clinical and pathological evidence, and the specimens were immediately snap-frozen and stored in liquid nitrogen tanks. For the use of these clinical materials for research purposes, prior patients' consents and approval from the Institutional Research Ethics Committee were obtained.

Table 1
The clinicopathological characteristics in 113 hapatocellular carcinoma patients

Parameters	Number of cases	Parameters	Number of cases
Gender		Age (years)	
Female	37	< 60	52
Male	76	≥60	61
AFP		Differentiation	
< 400	47	High/moderate	45
≥400	66	Poor	68
Clinical stage		T stage	
I	24	$T_1 - T_2$	59
II – IV	89	$T_3 - T_4$	54
N stage		M stage	
N_0	43	M_0	54
N_1	70	M_1	59
Survival status		Tumor size (5 cm)	
Alive	29	< 5	47
Dead	69	≥5	66
Venous invasion			
Negative	54		
Positive	59		

In Situ Hybridization (ish)

ISH was performed on HCC tumors using locked nucleic acid (LNA) probes for miR-589-5p (Exiqon, Vedbaek, Denmark) as described previously [13]. Briefly, paraffin-embedded HCC tumors were deparaffinized, treated with proteinase K, and fixed in paraformaldehyde. The digoxigeninlabeled LNA probe was hybridized overnight. Slides were rinsed and incubated with anti-digoxigenin, a horseradish peroxidase (HRP)-linked antibody for 2 hr. The detection reaction was performed using the DAB Ready-to-Use Kit. The ISH scores were given by the two independent investigators, and were averaged for further comparative evaluation of the miR-589-5p expression. Tumor cell proportion was scored as follows: 0 (no positive tumor cells), 1 (< 10% positive tumor cells), 2 (10–35% positive tumor cells),

3 (35–70% positive tumor cells), and 4 (> 70% positive tumor cells). The staining intensity was graded according to the following criteria: 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellow brown), and 3 (strong staining, brown). The ISH score was calculated as the product of staining intensity score and the proportion of positive tumor cells. Using this method of assessment, we evaluated miR-589-5p expression in HCC samples by determining the staining intensity (SI), with scores of 0, 1, 2, 3, 4, 6, 8, 9, or 12.

Statistical analysis

All statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Associations between miR-589-5p score and clinicopathological characteristics of the patients were analyzed using the Chi-squared test. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. $P < 0.05$ was considered significant.

Results

miR-589-5p is upregulated in hepatocellular carcinoma

To investigate the potential prognostic significance of miR-589-5p as a tumor biomarker, we first examined miR-589-5p expression levels in 10 adjacent normal tissues (ANT), 21 liver fibrosis tissues, 113 HCC tissues, including 22 HCC with grade I, 37 HCC with grade II, 49 HCC with grade III and 5 hepatocholangiocellular carcinoma using in situ hybridization (ISH). As shown in Fig. 1A, we found that miR-589-5p was mainly detected in the cytoplasma of HCC cells. Furthermore, the staining intensity of miR-589-5p in non-malignant tissues, including ANT and fibrosis was relative low with ISH score from 0–2; however, the case number of HCC tissues with higher ISH scores was much higher than that of non-malignant tissues (Fig. 1B). Average expression levels of miR-589-5p in non-malignant tissues and malignant tissues were further analyzed. As shown in Fig. 1C, the staining intensity of miR-589-5p was strongly upregulated in malignant tumor tissues compared with that in non-malignant tissues. In-depth analysis revealed that average ISH score of miR-589-5p was slightly increased in grade I HCC tissues and dramatically upregulated in grade II and grade III HCC tissues, as well as in hepatocholangiocellular carcinoma tissues, but no significant difference of miR-589-5p expression between in ANT and in liver fibrosis tissues (Fig. 1D). Therefore, these findings demonstrate that miR-589-5p is overexpressed in clinical HCC samples.

Overexpression of miR-589-5p correlates with advanced clinicopathological characteristics and poor prognosis in HCC patients

The correlation of miR-589-5p expression levels with clinicopathological characteristics in HCC patients was further explored. Statistical analysis showed that miR-589-5p expression was significantly and positively correlated with poor tumor differentiation degree, increased AFP levels, advanced clinical stage, distant metastatic status and venous invasion in HCC patients (Table 2). Kaplan–Meier survival analysis

revealed that high levels of miR-589-5p predicted poorer overall survival compared with low levels of miR-589-5p in HCC patients (Fig. 2A). Furthermore, HCC patients with high miR-589-5p levels exhibited early relapse compared with those with low miR-589-5p levels (Fig. 2B). Therefore, our results indicate that overexpression of miR-589-5p predicts poor clinicopathological characteristics and prognosis in HCC patients.

Table 2
The correlation between miR-589-5p and clinicopathological characteristics in 113 patients with hepatocellular carcinoma.

Parameters	Number of cases	miR-589-5p ISH		*P* values
		Low	High	
Gender				
Female	37	22	15	0.415
Male	76	39	37	
Age (years)				
< 60	52	27	25	0.685
≥60	61	34	27	
AFP				
< 400	47	33	14	0.004*
≥400	66	28	38	
Differentiation				
High/moderate	45	30	15	0.028*
Poor	68	31	37	
T stage				
$T_1 - T_2$	59	32	27	0.955
$T_3 - T_4$	54	29	25	
N stage				
N_0	43	25	18	0.487
N_1	70	36	34	
M stage				
M_0	54	36	18	0.010*
M_1	59	25	34	
Clinical stage				
I	24	19	5	0.005*
II - IV	89	42	47	

Parameters	Number of cases	miR-589-5p ISH		*P* values
		Low	High	
Tumor size (cm)				
< 5	47	29	18	0.184
≥5	66	32	34	
Venous invasion				
Negative	54	35	19	0.027*
Positive	59	26	33	

Clinical ISH scores of miR-589-5p is positively associated with mRNA levels of miR-589-5p in clinical HCC samples

Our previous study has demonstrated that recurrent gains-induced miR-589-5p overexpression was observed in HCC tissues, which activated STAT3 signaling by targeting multiple negative regulators of STAT3 signaling pathway, including SOCS2, SOCS5, PTPN1 and PTPN11, finally promoting the cancer stem cell characteristics and chemoresistance in HCC patients [11]. To confirm the expression levels and clinical significance of miR-589-5p in HCC patients, a more reliable clinical technique, in situ hybridization, was used to examine the staining intensity of miR-589-5p in clinical HCC samples, and we found that the staining intensity of miR-589-5p was strongly upregulated in malignant tumor tissues compared with that in non-malignant tissues (Fig. 1). Then, the median staining index (SI = 3) of miR-589-5p in all HCC tissues was used as the cutoff value to stratify high and low expression of miR-589-5p. According to this assessment, 61 HCC tissues were defined as low-ISH score tissues with SI < 4 and 52 was defined as high-ISH score tissues with SI ≥ 4. As shown in Fig. 3A, the expression levels of miR-589-5p measured by real-time PCR in high-ISH tissues were dramatically upregulated compared with those in low-ISH tissues. Importantly, a strong and positive correlation of miR-589-5p expression levels in real-time PCR with staining scores of miR-589-5p ISH was demonstrated in HCC tissues (Fig. 3B), indicating a positive linear correlation between staining scores of miR-589-5p ISH and miR-589-5p mRNA expression. Collectively, our results in the current study in combined with our previous findings [11] support the idea that miR-589-5p might hold a promising value as a novel biomarker to facilitate early diagnosis and predict prognosis in HCC patients.

Discussion

In the current study, our results demonstrated that miR-589-5p expression was dramatically upregulated in HCC tissues using in situ hybridization, which was correlated with poor tumor differentiation degree, increased AFP levels, clinical stage, distant metastatic status and venous invasion in HCC patients. Importantly, HCC patients with high miR-589-5p score exhibited shorter overall survival and early recurrence-free survival compared with those with low miR-589-5p score. Therefore, our results indicate

that miR-589-5p may be used as a potential clinical diagnostic biomarker to facilitate early detection and diagnosis of HCC.

In the vast majority of human cancer types, miR-589-5p was reported to be down-regulated and function as a tumor-suppressive miRNA, including endometrial carcinoma [14], prostate cancer [15], non-small cell lung cancer [16] and acute myeloid leukemia [17]. However, in the HCC context, several lines of evidence have reported that miR-589-5p was remarkably upregulated in clinical HCC tissues, and overexpression of miR-589-5p significantly contributed to the progression and aggressiveness of HCC to varying mechanisms [11, 12]. These studies suggested that the oncogenic or tumor suppressive roles of miR-589-5p vary in different types of cancer. Importantly, high levels of miR-589-5p were positively associated with shorter overall survival in HCC patients [11, 12] and early recurrence-free survival [11]. However, overexpression of miR-589-5p was mainly detected using quantitative real-time PCR (qRT-PCR) technique, and expression levels of miR-589-5p were influenced by multiple factors, including tissue storage duration, RNA extraction method, the specificity of PCR primers and so on, which to some extent influenced the clinical significance of miR-589-5p in diagnosis and prognosis of HCC. Thus, a more reliable and commonly used clinical technique, such as in situ hybridization, is required to further determine the clinical significance of miR-589-5p in HCC patients. In the current study, through in situ hybridization technique, we further examined expression score of miR-589-5p in 113 HCC tissues, and found that miR-589-5p score was dramatically upregulated in HCC tissues compared with that in non-malignant tissues. Further investigation showed that high score of miR-589-5p was observed in HCC patients with poor tumor differentiation, increased AFP levels, advanced clinical stage, distant metastatic status and venous invasion. Importantly, high miR-589-5p score predicted poor overall survival and early recurrence-free survival in HCC patients compared with those with low miR-589-5p score. Collectively, our results indicate that miR-589-5p may hold a promising applicable value to facilitate early diagnosis and predict prognosis in HCC patients from a clinical perspective.

More and more momentum has shed light on metastatic phenotype of cancer, which contributes to the vast majority of cancer-related deaths [18, 19]. For HCC, metastatic behavior is also commonly observed in this type of tumor characterized by the abundant blood flow, where aggressiveness of HCC is mediated by both intrahepatic metastases, as well as distant target organ metastasis to the lungs, bones, brain and adrenal gland [20, 21], which significantly contributes to poorer prognosis in HCC patients [22]. Notably, our results in this study presented that overexpression of miR-589-5p was associated with poor prognosis in HCC patients. In addition, statistical analysis further showed that overexpression of miR-589-5p was positively correlated with venous invasion and M stage (distant metastasis). This finding supported the notion that miR-589-5p may be implicated in metastatic HCC, which further contributes to poor prognosis in HCC patients. However, the prognostic significance and functional role of miR-589-5p in metastatic HCC remain to be further elucidated in the following work in the future.

Paradoxically, Zhang and the colleagues have reported that miR-589-5p was reduced in $CD90^+$ MHCC97H and MHCC97L compared with $CD90^-$ cells, which predicted poor overall and disease-free survival in HCC patients. Furthermore, Zhang et al found that miR-589-5p functioned as a tumor-suppressive miRNA to

inihibit cancer stem cell-like traits and tumorigenesis of HCC cells [23]. In our previous study, our results demonstrated that miR-589-5p was upregulated in HCC tissues and overexpression of miR-589-5p was associated with poor prognosis in HCC patients, which was further validated in Xu's study [12], as well as several independent publicly available HCC datasets, including TCGA, E-GEOD-36918 and E-GEOD-31384 [11]. Similarly, our finding of the current study further determined that miR-589-5p was overexpression in HCC tissues compared with that in adjacent tumor normal tissue using in situ hybridization. Mechanistically, the findings from our previous study showed that amplification was the primary mechanism responsible for miR-589-5p overexpression in HCC tissues [11], clarifying that miR-589-5p is mainly upregulated in HCC tissue. The discrepancy about the differential expression levels and different prognostic role of miR-589-5p expression in HCC patients in different studies may be explained by the different sample number analyzed: there were 136 HCC tissue samples used in our previous study [11] and TCGA dataset (n = 372); while only 40 HCC tissue samples were used in Zhang's study. Therefore, our results in combination with Xu's study indicate that overexpression of miR-589-5p is closely correlated with poor prognosis in HCC patients.

Conclusion

In summary, our results in the current study in combined with our previous findings [11] demonstrate that miR-589-5p is overexpressed in HCC tissues by different methods of measurement, which predicts poor survival and early relapse in HCC patients. Therefore, our results imply that miR-589-5p might hold a promising value as a novel biomarker to facilitate early diagnosis and predict prognosis in HCC patients.

Abbreviations

miRNAs; microRNAs; HCC:hepatocellular carcinoma; ISH:in situ hybridization; OS:overall survival; RFS:recurrence-free survival; TCGA:The Cancer Genome Atlas; LNA:locked nucleic acid; ANT:adjacent normal tissues; SI:staining index.

Declarations

Acknowledgements

Not applicable

Authors' contributions

Jianting Long and Baoxian Liu developed ideas and drafted the manuscript. Zhijia Yao and Chunlin Jiang conducted the experiments and contributed to the analysis of data. Huiwen Weng contributed to the analysis of data. Shi Fang contributed to the analysis of data and revised the manuscript. Heping Li edited the manuscript. All authors contributed to revise the manuscript and approved the final version for publication.

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Availability of data and materials

All the data and material are available.

Ethics approval and consent to participate

Prior patients' consents and approval from the Institutional Research Ethics Committee were obtained.

Consent for publication

Approved.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

Figure 1

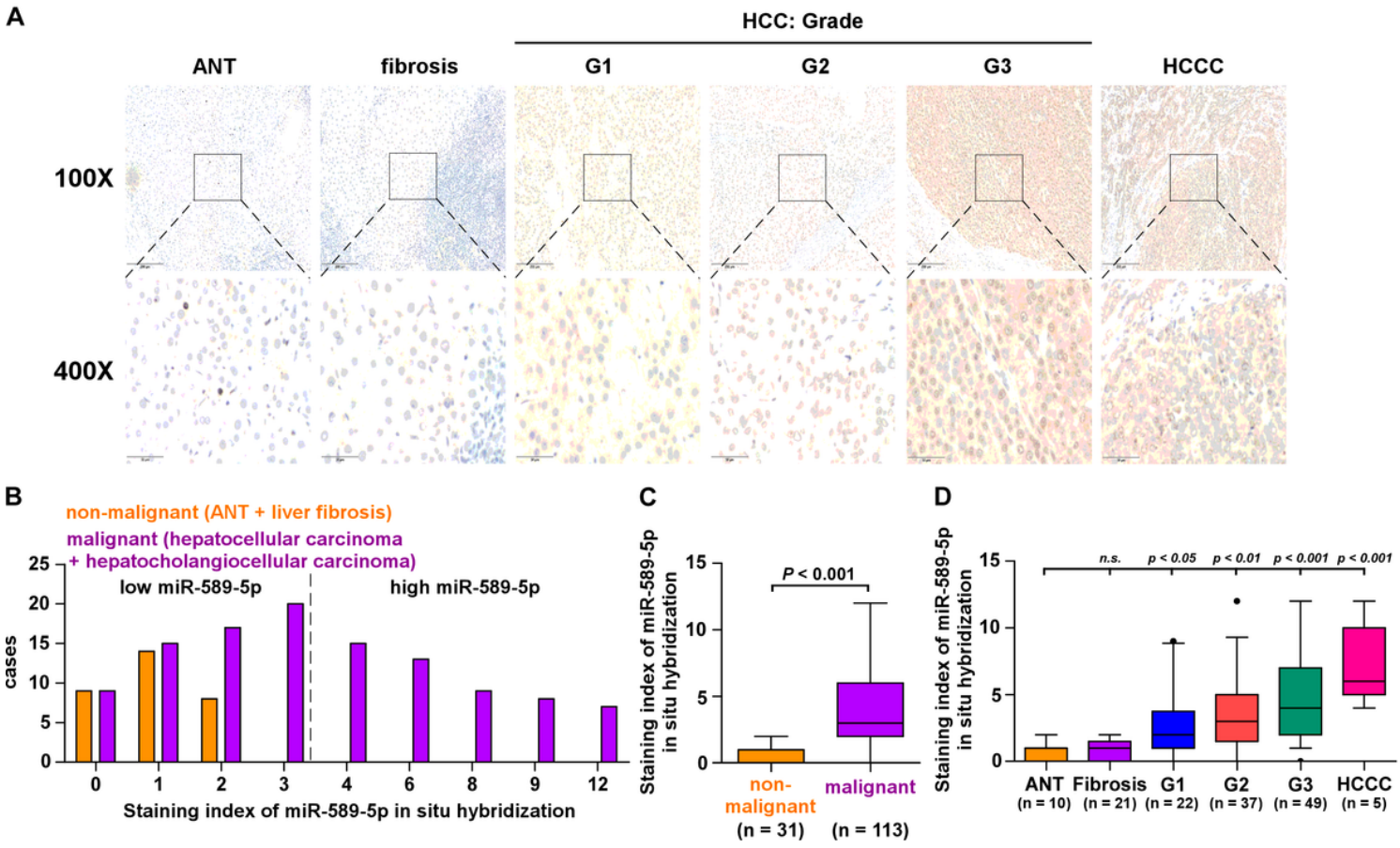

Figure 1

miR-589-5p is upregulated in hepatocellular carcinoma. (A) Representative images of low-power (100×, top) and high-power (400×, bottom) fields of miR-589-5p expression by in situ hybridization (ISH) in

adjacent normal tissue (ANT, n = 10), liver fibrosis tissues (n = 21), HCC tissues with grade I (G1, n = 22), HCC tissues with grade II (G2, n = 37), HCC tissues with grade III (G3, n = 49) and hepatocholangiocellular carcinoma tissues (HCCC, n =5). Scale bars, 200 mm for 100× magnification and 50 mm for 400× magnification. (B) The number of ANT, liver fibrosis tissues and malignant HCC tissues in different staining index groups of ISH. (C) Staining index of miR-589-5p in 31 non-malignant tissues, including 10 ANT and 21 liver fibrosis tissues, and 113 HCC tissues by ISH. (D) Staining index of miR-589-5p by ISH in 10 ANT, 21 liver fibrosis tissues, 22 HCC tissues with grade I, 37 HCC tissues with grade II, 49 HCC tissues with grade III and 5 hepatocholangiocellular carcinoma tissues.

Figure 2

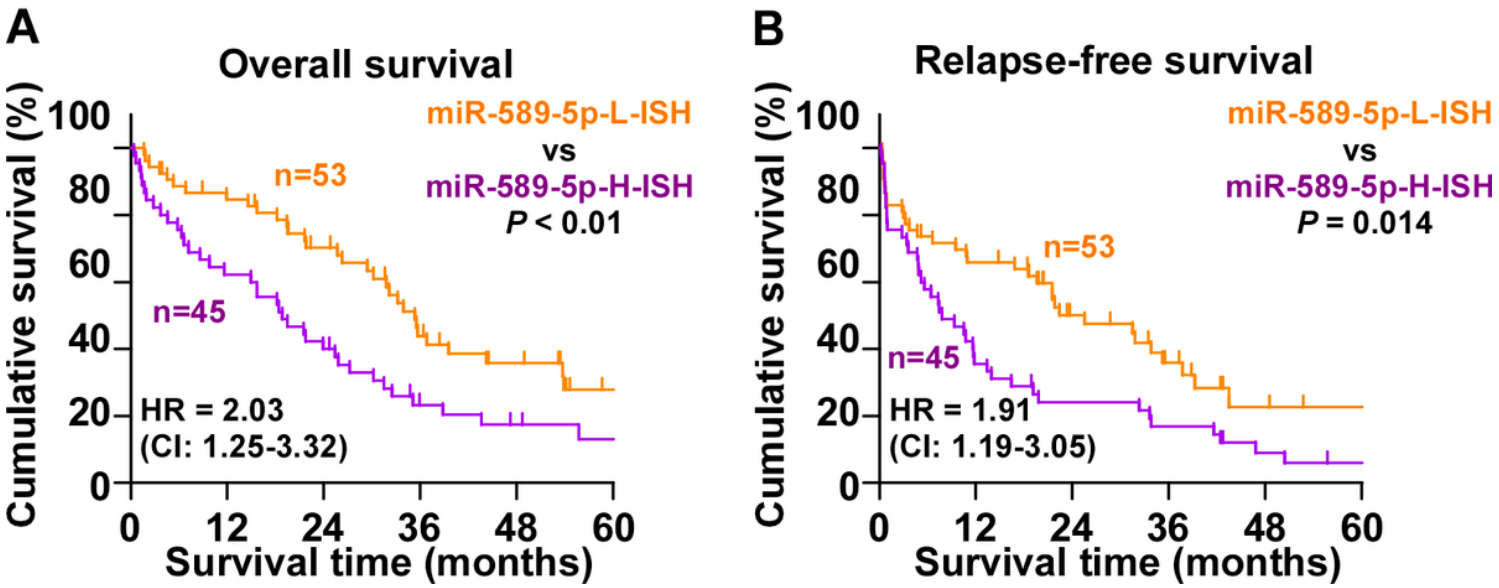

Figure 2

Overexpression of miR-589-5p predicts poor overall and relapse-free survival in HCC patients. (A) Kaplan–Meier analysis of overall curves of patients with HCC in high miR-589-5p ISH (n=45) and low miR-589-5p ISH (n=53). P < 0.01, log-rank test. The data shown in scatter plot and bar graph were determined by the median with interquartile range and median with standard deviation. (B) Kaplan–Meier analysis of relapse-free curves of patients with HCC in high miR-589-5p ISH (n=45) and low miR-589-5p ISH (n=53). P =0.014, log-rank test. The data shown in scatter plot and bar graph were determined by the median with interquartile range and median with standard deviation.

Figure 3

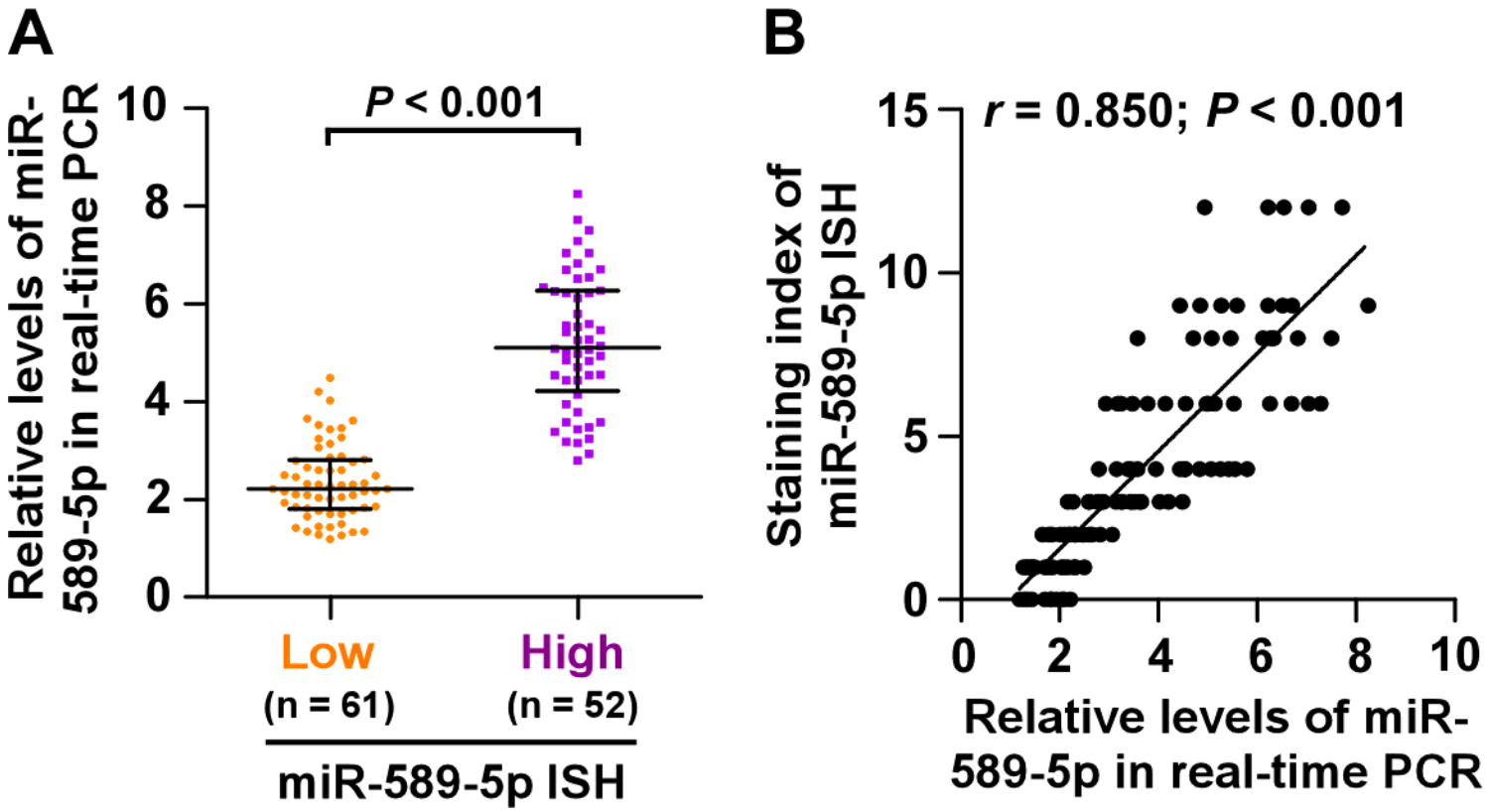

Figure 3

Clinical ISH scores of miR-589-5p is positively associated with RNA levels of miR-589-5p in clinical HCC samples. (A) Real-time PCR analysis of miR-589-5p in 52 HCC tissues with high ISH scores compared with 61 HCC tissues with low ISH scores. The median ISH score in HCC tissues was used to stratify low and high ISH scores. (B) Correlation of miR-589-5p expression level with ISH scores with expression levels of miR-589-5p by real-time PCR in HCC tissues.