

Prognostic value of hsa-mir-299 and hsa-mir-7706 in hepatocellular carcinoma

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Abstract. This study aimed to investigate the expression of microRNA (miRNA) 299 and miRNA-7706 in patients with hepatocellular carcinoma (HCC), and to explore their effects on proliferation of SK-HEP-1 HCC cells. Expression of miRNA-299 and miRNA-7706 in tumor tissue (HCC group) and adjacent healthy tissue (>30 mm away from the tumor tissue) of 179 patients with HCC was determined by real-time polymerase chain reaction (qRT-PCR). miR-299 mimics and miR-7706 mimics were transfected into SK-HEP-1 HCC cells by RNA transfection. The proliferation and invasion of SK-HEP-1 cells were detected by CCK-8 kit and Transwell kit, respectively. Compared with adjacent tissues, expression levels of miRNA-299 and miRNA-7706 in HCC group were significantly downregulated. Analyses on the correlation between the expression of miRNA-299 and miRNA-7706 and clinical factors showed that expression levels of miRNA-299 and miRNA-7706 were significantly correlated with pathological stages and lymph node metastasis. ROC curve analysis showed that the areas under the curve were 0.837 and 0.845 for miRNA-299 and miRNA-7706 in the prediction of HCC, respectively. Survival analysis showed that the 5-year overall survival rate of patients with high expression levels of miRNA-299 and miRNA-7706 was significantly different from that of patients with low expression levels ($P=0.016$). Compared with cells transfected with scramble mimics, proliferation and invasion abilities of SK-HEP-1 cells transfected with miR-299 mimics and miRNA-7706 were significantly weakened. Results suggested that downregulation of miRNA-299 and miRNA-7706 can inhibit the proliferation of HCC cells and can be used as a new target for the treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignant cancer in the world (1,2). HCC is also the third most common cause of cancer-related death (3,4). The early diagnosis of HCC is usually difficult and there is a lack of effective treatments, so the prognosis of patients with HCC is usually poor (5). The development of HCC is a multi-step and multi-stage pathology process with multiple genes involved (6,7). To date, no specific and sensitive tumor markers have been clinically used for early diagnosis of HCC. Therefore, this study was carried out to identify markers for the diagnosis of HCC.

MicroRNA (miRNA) is a group of endogenous single-stranded non-coding RNA with a length of about 17-25 nucleotides. miRNAs mainly target the 3' untranslated region (3'UTR) of target genes to degrade mRNA or inhibit the translation of mRNA (8-10). miRNAs also participate in the formation of organs, cell proliferation and apoptosis, and tumorigenesis (11,12). It has been found that miRNAs are closely related to the development of different types of cancer. Differential expression of some miRNAs may affect the growth and metastasis of tumors (13).

We screened the differential expression genes of patients with HCC through the TCGA database and found that miRNA-299 and miRNA-7706 were differentially expressed in HCC patients (data not shown). This finding has not been reported before. Therefore, this study was carried out to investigate the expression of miRNA-299 and miRNA-7706 in HCC and their effects on SK-HEP-1 cells, so as to promote the early diagnosis and prognosis of HCC.

Materials and methods

Sample collection. Tumor tissues and adjacent healthy tissues were collected from 179 HCC patients who were treated in The Third Affiliated Hospital of Sun Yet-sen University (Guangzhou, China) from June 2010 to February 2015. Those patients included 127 males (70.9%) and 52 females (29.1%) with a mean age of 47.2 ± 8 years. None of the patients received radiotherapy and chemotherapy before surgery. TNM staging was performed in accordance with the 2009 seventh edition of the UICC (Union for International Cancer Control). HCC TNM staging: 36 cases of stage I, 69 cases of stage II, and

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Table I. Primers of miRNA-199, miRNA-7706 and endogenous control.

Items	Primer sequences
miRNA-299	
Reverse transcription primers	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACAAGCGGTT-3'
Sense	5'-TCAAGCTTTGAAGCGCCTGTGC-3'
Antisense	5'-GCCTAAGGAGGGTCCGAGGTATTC-3'
miRNA-7709	
Reverse transcription primers	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACTCTCGGCA-3'
Sense	5'-TCAAGCTTTGAAGCGCCTGTGC-3'
Antisense	5'-GCCTAAGGAGGGTCCGAGGTATTC-3'
U6	
Sense	5'-GGAACGCTTCACGAATTTG -3'
Antisense	5'-ATTGGAACGATACAGAGAAGATT-3'

Table II. Correlation between the expression of miRNA-299 and miRNA-7706 and clinical factors.

Clinical factors	N (%)	Expression level of miRNA-299	P-value	Expression level of miRNA-7706	P-value
Age (years)			0.684		0.841
<60	72 (40.2)	0.94±0.24		1.12±0.41	
≥60	107 (59.8)	0.87±0.31		1.24±0.47	
Sex			0.714		0.614
Male	127 (70.9)	0.67±0.51		0.91±0.47	
Female	52 (29.1)	0.88±0.45		0.98±0.51	
Pathological stage			0.039		0.042
I	58 (32.4)	0.94±0.22		1.22±0.44	
II	79 (44.1)	0.81±0.39		0.98±0.39	
III	42 (23.5)	0.73±0.16		0.81±0.33	
TNM stage			0.057		0.061
I	36 (20.1)	0.94±0.29		1.14±0.41	
II	69 (38.5)	0.92±0.32		1.07±0.37	
III/IV	74 (41.4)	0.85±0.34		0.92±0.46	
Lymph node metastasis			0.031		0.044
Positive	89 (49.7)	0.73±0.23		0.84±0.32	
Negative	90 (50.3)	0.91±0.18		1.27±0.39	
History of smoking			0.062		0.784
Yes	100 (55.8)	0.82±0.33		1.09±0.36	
No	79 (44.2)	0.87±0.36		1.01±0.44	

74 cases of stage III-IV. Pathological types of all specimens were confirmed as HCC. This study was approved by the Ethics Committee of The Third Affiliated Hospital of Sun Yet-sen University, and all patients signed informed consent.

Reagents and instruments. SK-HEP-1 cells were purchased from Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (Shanghai, China). Total RNA extraction reagent TRIzol, miScript Reverse Transcription kit, PCR kit, fetal bovine serum, DMEM, PCR amplification primers and transfection reagent were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). miRNA-299,

miRNA-7706 and negative control scramble mimics were purchased from GenePharma Co., Ltd. (Shanghai, China). 7900HT fluorescence quantitative PCR instrument was purchased from ABI (Thermo Fisher Scientific, Inc.).

Primer design. miRNA-199 and miRNA-7706 reverse transcription primers and PCR specific primers were designed and synthesized by GenePharma Co., Ltd. (Table I).

Experimental methods

Cell culture. SK-HEP-1 cells were cultured with DMEM containing 10% fetal bovine serum and 100 g/l penicillin-

streptomycin double antibiotics in an incubator (37°C, 5% CO₂). SK-HEP-1 and SK-HEP-1 cells were collected at logarithmic growth phase. After digestion with 0.25% trypsin, 3x10⁵ cells were transferred to each well of 6-well plates. Cells were cultured in medium containing no antibiotics before transfection. Transfection mixture was prepared according to the instructions of Lipofectamine™ 2000 kit. Transfection mixture was added to each well of 6-well plate and was shaken. After incubation at 37°C for 6 h, culture medium and transfection reagent were removed. Finally, 2 ml medium containing 10% fetal bovine serum was added into each well for further culture.

Total RNA extraction. Total RNA was extracted from the cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the instructions. After RNA extraction, the concentration of each sample RNA was determined by ultraviolet spectrophotometer. All RNA samples were stored at -80°C before use.

Reverse transcription. Reverse transcription was performed using miScript Reverse Transcription kit and 1 µg of total RNA was used to synthesize cDNA. Reverse transcription conditions: 42°C for 3 min, 42°C for 60 min and 95°C for 3 min. All cDNA samples were stored at -80°C before use.

RT-PCR. Real-time fluorescence quantitative PCR was performed on 7900HT fluorescence quantitative PCR instrument using cDNA and SYBR premix Ex Taq™ II PCR kit. Reaction system was: 10 µl of SYBR premix Ex Taq™ II PCR mix, 1 µl of cDNA, 0.5 µl of each primer and 8 µl of RNase-free water. Reaction conditions were: 95°C for 5 min, followed by 45 cycles of 95°C for 15 sec, 60°C for 45 sec and 72°C for 25 sec. U6 was used as an endogenous control (Table II). Results were analyzed using the 2^{-ΔΔCt} method.

Cell proliferation assay. miRNA-299, miRNA-7706 and scramble mimics were transfected into 1x10⁶ SK-HEP-1 cells, followed by incubation for 24 h. After that, SK-HEP-1 cells were inoculated into 96-well plates with 6x10³ cells per well, followed by incubation for 3-5 h. DMEM (100 µl) was added after cell adhesion, followed by incubation in an incubator (37°C, 5% CO₂) for 6 days. CCK-8 (10 µl) was added daily, and OD values at 450 nm were measured using a microplate reader.

Transwell invasion assay. Matrigel was kept at 4°C overnight (12 h). Matrigel (50 µl; 1:10 dilution) was added to the upper chamber, followed by incubation at 37°C for 6 h. After intervention with serum-free medium for 48 h, cells were digested with trypsin and resuspended in DMEM containing 1% fetal bovine serum to adjust the cell density to 1x10⁶/ml. Cell suspension (100 µl) was transferred into the upper chamber, while the lower chamber was filled with 500 µl of culture medium containing 10% FBS. After incubation for 24 h in an incubator, Matrigel and the cells that failed to invade were removed. The membrane was fixed for 30 min, and staining with 0.1% crystal violet was performed for 15 min. The results were observed under an inverted microscope. Each experiment was repeated three times.

Statistical analysis. Statistical analysis was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Measurement data were expressed as mean ± SD. ANOVA was used for comparisons between multiple groups and SNK test was used for post hoc test. Student's t-test was used for

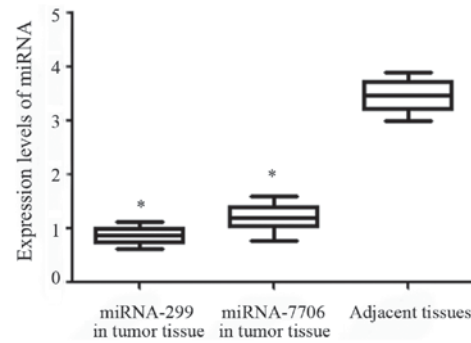


Figure 1. Expression of miRNA-299 and miRNA-7706 in HCC tissues and adjacent tissues. Expression of miRNA-299 and miRNA-7706 in different tissues was determined by RT-PCR. Expression levels of miRNA-299 and miRNA-7706 in HCC tissues were significantly lower than those in adjacent tissues (*P<0.05). HCC, hepatocellular carcinoma.

comparison of clinical factors. Correlation analyses were performed by Pearson's correlation analysis. Survival analysis was performed using Kaplan-Meier curve method. P<0.05 was considered to indicate a statistically significant difference.

Results

miRNA-299 and miRNA-7706 expression in HCC tissues and adjacent tissues. The relative expression levels of miRNA-299 and miRNA-7706 in tumor tissue were 0.71±0.41 and 1.2±0.57, respectively, which were significantly lower than those in adjacent healthy tissue (P<0.05) (Fig. 1).

Correlation between miRNA-299 and miRNA-7706 expression and clinical factors. Expression levels of miRNA-299 and miRNA-7706 were correlated with pathological stage and lymph node metastasis (P<0.05). With the increase in pathological stage, expression levels of miRNA-299 and miRNA-7706 significantly decreased (P=0.039; P=0.042). Patients with lymph node metastasis showed significantly reduced expression levels of miRNA-299 and miRNA-7706 (P=0.031; P=0.044). No significant correlation were found between expression levels of miRNA-299 and miRNA-7706 and other clinical and pathological factors including age, sex, TNM stages and history of smoking (P>0.05) (Table II).

Diagnostic value of miRNA-299 and miRNA-7706 for HCC. Diagnostic value of miRNA-299 and miRNA-7706 for HCC was analyzed by ROC curve. As shown in Fig. 2, AUC=0.804, AUC=0.781; confidence interval 95% CI: 0.724-0.842, 95% CI: 0.754-0.876.

Correlation between expression of miRNA-299 and miRNA-7706 and prognosis of HCC patients. Patients were divided in to two groups according to the median value of the expression levels of miRNA-299 and miRNA-7706. Follow-up study showed that survival rate of miRNA-299 and miRNA-7706 high expression group was significantly higher than that of low expression group (P<0.05, P=0.027). As shown in Figs. 3 and 4, Kaplan-Meier survival curve analysis showed that expression levels of miRNA-299 and miRNA-7706 were correlated with the prognosis of patients with HCC.

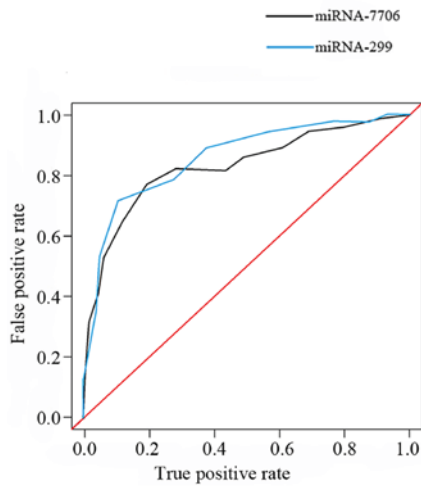


Figure 2. ROC curve analysis of the diagnostic values of miRNA-299 and miRNA-7706 for HCC. The areas under the curve of miRNA-299 and miRNA-7706 were 0.804 and 0.781, respectively. HCC, hepatocellular carcinoma.

Effects of miRNA-299 mimics and miRNA-7706 mimics transfection on expression of miRNA-299 and miRNA-7706 in HCC cells. After transfection of miRNA-299-mimics-SK-HEP-1 and miRNA-299-scramble-mimics-SK-HEP-1 into SK-HEP-1 cells, expression levels of miRNA-299 were 7.89 ± 2.87 and 1.17 ± 0.45 , respectively. After transfection of miRNA-7706-mimics-SK-HEP-1 and miRNA-7706-scramble-mimics-SK-HEP-1 into SK-HEP-1 cells, expression levels of miRNA-7706 were 11.58 ± 2.25 and 1.53 ± 0.57 . After transfection of miRNA-299-mimics-SK-HEP-1 and miRNA-7706-mimics-SK-HEP-1, expression levels of miRNA-299 and miRNA-7706 in SK-HEP-1 cells were significantly increased ($t=4.01$, $P<0.05$; $t=7.50$, $P<0.05$).

Effects of miRNA-299 and miRNA-7706 on proliferation of SK-HEP-1 cells. miRNA-299-mimics-SK-HEP-1, miRNA-7706-mimics-SK-HEP-1, miRNA-299-scramble-mimics-SK-HEP-1 and miRNA-7706-scramble-mimics-SK-HEP-1 were transfected into 1×10^6 SK-HEP-1 cells, followed by incubation for 24 h. After that, SK-HEP-1 cells were inoculated into 96-well plates with 6×10^3 cells per well, followed by incubation in an incubator (37°C , $5\% \text{CO}_2$) for 6 days. CCK-8 ($10 \mu\text{l}$) was added daily, and OD values at 450 nm were measured using a microplate reader. GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to plot the proliferation curve of SK-HEP-1 HCC cells after transfection (Fig. 5).

Effects of miRNA-299 and miRNA-7706 on invasion of SK-HEP-1 cells. Compared with SK-HEP-1 cells transfected with scramble mimics, invasion ability of SK-HEP-1 cells was significantly weakened after transfection with miRNA-299 and miRNA-7706 mimics ($P<0.05$) (Fig. 6).

Discussion

In this study, expression of miRNA-299 and miRNA-7706 in tumor tissue and adjacent healthy tissue of HCC patients was

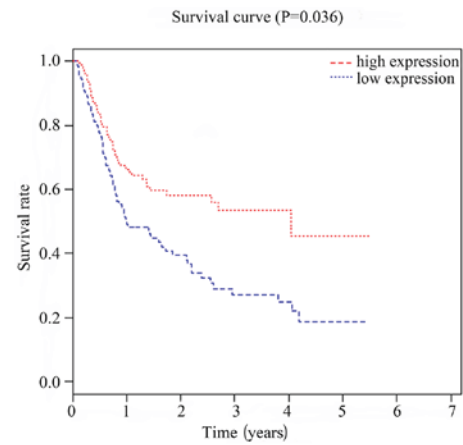


Figure 3. Correlation between miRNA-299 and prognosis of HCC patients. Kaplan-Meier survival curve analysis showed that the survival rate of miRNA-299 high expression group was significantly higher than that of low expression group ($P=0.027$). HCC, hepatocellular carcinoma.

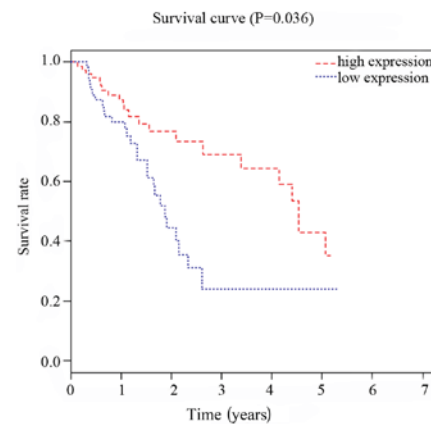


Figure 4. Correlation between miRNA-7706 and prognosis of HCC patients. Kaplan-Meier survival curve analysis showed that the survival rate of miRNA-7706 high expression group was significantly higher than that of low expression group ($P=0.027$). HCC, hepatocellular carcinoma.

determined by qRT-PCR. Studies have found that the expression level of miRNA-299 is low in a variety of tumor tissues, such as endometrial rectal, colon and ovarian cancers (14-16). However, studies on miRNA-7706 are relatively insufficient. Expression pattern and function of two miRNAs in HCC still haven't been reported. Analysis on the correlation between expression levels of miRNA-299 and miRNA-7706 and patients' clinical data showed that expression levels of miRNA-299 and miRNA-7706 were significantly reduced in patients with lymph node metastasis. Other clinical and pathological factors such as age, sex, TNM grade and history of smoking showed no significant correlation with miRNA-299 and miRNA-7706 expression. Expression levels of miRNA-299 and miRNA-7706 in tumor tissues were significantly lower than that in adjacent tissues. At the same time, expression levels of those two miRNAs in HCC cells SK-HEP-1 were also lower than that in control cells. These results indicate that miRNA-299 and miRNA-7706 can inhibit tumor cell proliferation. This is consistent with the finding of Göhring *et al* (17) that miRNA-299 can inhibit the proliferation and invasion of tumor cells. Based on those

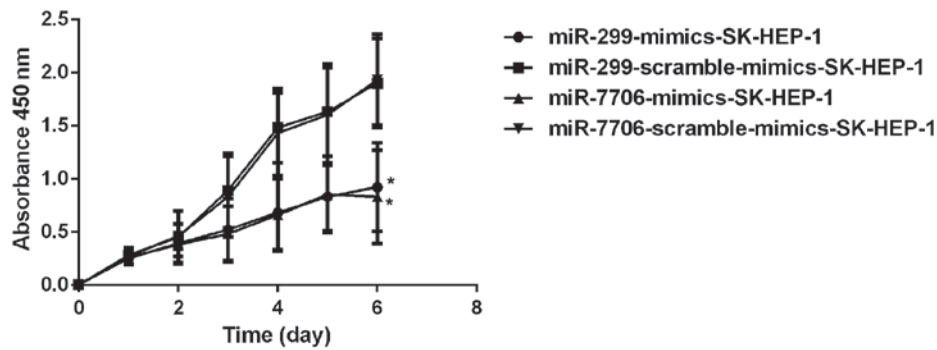


Figure 5. Effects of miRNA-299 and miRNA-7706 on the proliferation of SK-HEP-1 cells. CCK-8 method was used to detect cell proliferation after miRNA-299 mimics and miRNA-7706 mimics transfection. After 6 days of culture, proliferation of SK-HEP-1 was significantly inhibited after transfection with miRNA-299-mimics-SK-HEP-1 and miRNA-7706-mimics-SK-HEP-1 ($P < 0.05$).

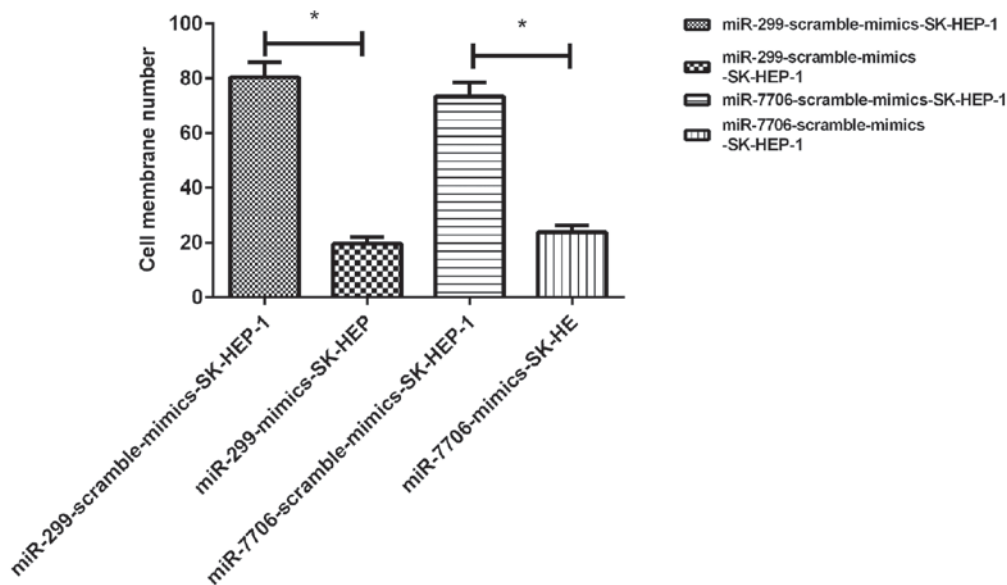


Figure 6. Effects of miRNA-299 and miRNA-7706 on the invasion of SK-HEP-1 cells. Results of Transwell assay showed that invasion of SK-HEP-1 cells was significantly reduced after transfection with miRNA-299 mimics and miRNA-7706 mimics ($P < 0.05$).

studies, we speculate that miRNA-299 and miRNA-7706 can also regulate cell proliferation and invasion in HCC. In this study, results of CCK-8 cell proliferation assay and Transwell cell invasion assay showed that miRNA-299 and miRNA-7706 could significantly inhibit the proliferation and invasion of SK-HEP-1 cells. Many studies (18-20) found that inhibition of tumor cell proliferation and invasion can significantly inhibit tumor growth, which is consistent with the results in our study. ROC curve analysis showed that AUCs of miRNA-299 and miRNA-7706 AUC were 0.804 and 0.781, respectively, which is consistent with the finding reported by Ren *et al* (21), indicating that two miRNAs can be used as markers for clinical diagnoses. Besides that, analyses on the correlation between the expression of miRNA-299 and miRNA-7706 and clinical factors showed that expression levels of miRNA-299 and miRNA-7706 have no significant correlations with age, sex, TNM stage and history of smoking of patients ($P < 0.05$), indicating that miRNA-299 and miRNA-7706 can potentially be new markers for HCC. Kaplan-Meier survival curve analysis showed that the survival rate of patients in miRNA-299 and miRNA-7706 high expression group was significantly higher than that of low

expression group, indicating that patients with low expression levels of those two miRNAs tend to have short-term survival. In this study, we detected the expression of miRNA-299 and miRNA-7706 in HCC. Correlation analysis was performed to explore the correlations between the expression of those two miRNAs and clinical factors, which were indicated by the P-value. Chi-square test showed that pathological stage and lymph node metastasis were significantly correlated with the expression of those two miRNAs in different stages. Those data may provide new indexes for clinical staging and prediction of lymph node metastasis. In order to provide references for clinical practice, effects of those two miRNAs on proliferation and invasion of HCC cells were explored. We found that those two miRNAs could effectively inhibit the proliferation and invasion of cancer cells. ROC analysis showed that those two miRNAs has promising clinical diagnostic value for HCC, which is expected to be applied clinically in future.

Results of our study showed that miR-299 and miR-7706 have similar functionality in hepatoma carcinoma cell line SK-HEP-1. However, our study also has some shortcomings. Most patients in this study were from local region. Therefore

regional difference may affect the results of our study. In addition, this study is also limited by the small sample size. The interaction between miRNA-299 and miRNA-7706 is still unknown. Therefore, our future studies will focus on the interactions between those two miRNAs and their roles in other diseases.

In conclusion, expression levels of miRNA-299 and miRNA-7706 were significantly reduced in tumor tissue of HCC patients, suggesting that they may play important roles in the development of HCC. Detection of expression levels of miRNA-299 and miRNA-7706 may provide references for the diagnosis of HCC. Our study provided new insights for the diagnosis and treatment of HCC.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

MD and FW wrote the manuscript and was responsible for cell culture. HC collected the sample. YL performed reverse transcription. JZ contributed to RT-PCR. ZZ and HY helped cell proliferation assay and Transwell assay. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of The Third Affiliated Hospital of Sun Yet-sen University (Guangzhou, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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