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Original Article

Over-expression of *microRNA-25* promotes cell proliferation and induces cell apoptosis in patients with hepatocellular carcinoma

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Abstract: Backgrounds: microRNAs (miRNAs) have been confirmed to play an important role in the occurrence and development of cancers. The purpose of this study was to investigate the effects of *miR-25* on the cell growth of HCC and its prognostic role. Methods: The expression of *miR-25* was detected by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis. The relationship between *miR-25* expression and clinical factors was also analyzed. The proliferation and apoptosis assay were conducted to compare the influences of *miR-25* expression on cell growth. Kaplan-Meier analysis was used to evaluate the overall survival of patients with different *miR-25* expression. The prognostic value was estimated via Cox regression analysis. Results: *miR-25* was over-expression in HCC tissues compared to adjacent normal tissues. And its expression was influenced by AFP significantly. The cell proliferation of HCC cells was promoted by *miR-25* while the apoptosis of it was inhibited. Patients with high *miR-25* expression had a shorter overall survival than those with low expression through Kaplan-Meier analysis. Cox regression analysis showed high expression of *miR-25* was closely related to the prognosis of HCC. Conclusions: *miR-25* was increased in HCC patients and it could promote cell proliferation as well as suppress cell apoptosis. Moreover, it might serve as an independent marker in the prognosis of HCC.

Keywords: Hepatocellular carcinoma, *miR-25*, proliferation, apoptosis, prognosis

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignant cancer and the second leading cause of cancer-related to death worldwide [1, 2]. Its incidence trends to rise year by year, especially in Asia and Africa [3]. Surgical resection is the main treatment for HCC [2]. However, even undergo surgery, the recurrence of HCC is still frequently [4]. So the therapeutic method has changed from surgical removal to comprehensive treatment which is not only include surgical removal but contain other therapies such as interventional therapy and chemotherapy. As HCC is resistant to chemo and radiation therapies, and it is also hard to be found in early stage, HCC still has a poor prognosis [5, 6]. Future tumor development, recurrence of the primary lesion or metastatic spread also makes prognostication to be very difficult for patients with HCC [7]. Therefore, the exploration about the molecular mechanism for the tumorigenesis of HCC is indispensable for developing effective therapy.

microRNAs (miRNAs) are a kind of endogenous, small, non-coding RNAs with a length of 18-25 nucleotides [8]. They are linked with many physiological processes of various of diseases such as cell cycle, apoptosis, hematopoietic cell differentiation, metabolism, neural development and metastasis [9-11]. miRNA also controls the expression of its target gene via binding to the 3'-Untranslated Regions (3'-UTR) of a target-mRNA at post transcriptional level [9]. Recent years, the aberrant expression of miRNAs has been confirmed to be related to tumor stage, invasion, metastasis, resistant to chemotherapeutic and so on. *miR-25,* a member of miR-106b-25 cluster, is a small RNA with 22 nucleotides in length and located within intron 13 of the minichromosome maintenance protein 7

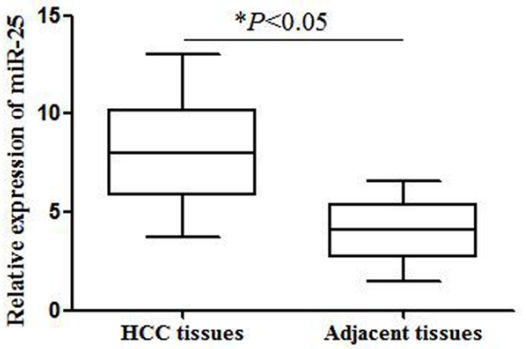

Figure 1. The expression of *miR-25* in HCC tissues and adjacent normal tissues. *miR-25* expression was higher in HCC tissues than in adjacent normal tissues ($P<0.05$).

(MCM7) gene on chromosome 7q22.1 [12]. Previous studies demonstrated that the expression of *miR-25* was increased in many human malignancy such as pediatric brain cancer, medulloblastomas, prostate cancer, hepatocellular carcinoma, gastric cancer, colorectal cancer, lung adenocarcinoma etc [13-18]. However, the function of it in the tumor development and its clinical significance in HCC is still unclear.

In this study, we detected the expression of *miR-25* in HCC patients and analyzed its relationship with clinicopathological characteristics of HCC patients. Then the aberrant expression of *miR-25* on the cell proliferation and apoptosis were investigated. Finally, we explored the prognostic value of *miR-25* in HCC by Kaplan-Meier and Cox regression analysis.

Materials and methods

Patients and tissue samples

This study was conducted at Affiliated Hospital of Hebei University and approved by the Ethnic Committee of the hospital. 118 patients who were diagnosed as HCC were collected and none of them had received chemotherapy or radiotherapy before surge-ry. Written informed consent was obtained from each patient involved in advance.

Fresh clinical HCC tissues and adjacent normal tissues from patients with HCC were obtained, respectively. Then the samples were frozen by liquid nitrogen immediately. Finally, the samples were stored at -80°C for RNA extraction. The detailed clinical formation including age, sex, tumor size, AFP, HbsAg, neoplasm metastasis and therapeutic method were recorded in a database. A 5-years' follow-up was performed via a telephone call or questionnaire letters. Patients who were died from unexpected events or other disease were excluded from our study.

Cell culture and cell transfection

Human HCC cell lines HepG2 and normal liver cell lines LO2 were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and penicillin (200 U/ml) at 37°C with 5% CO_2.

The *miR-25* mimics, *miR-25* inhibitor (anti-*miR-25*), miR control (NC) and negative miR inhibitor (anti-miR-NC), were purchased from Ambion, and transfected at a final concentration of 30 nM with Lipofectmine 2000 (Invitrogen).

QRT-PCR analysis

Total RNA was extracted from the HCC tissues and adjacent normal tissues using mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA), respectively. Then reverse transcription was conducted by TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) to synthesize the first chain of cDNA. Finally, RT-PCR reaction was performed in the Applied Biosystems 7900 Fast Real-Time PCR system (Applied Biosystems, Foster City, California, USA). And RNU44 was used as internal control. The data are processed by the comparative cycle threshold (CT) method to evaluate the relative quantification of *miR-25* expression. Each sample was examined in triplicate.

Cell proliferation assay

Cells were seeded into 96-well plates (1.0×10^4 cells per well). Cell viability was assessed by cell-counting kit-8 assay (Beyotime Institute of Biotechnology, Shanghai, China). The absorbance of each well was monitored by a spectrophotometer (Thermo, Shanghai, China) at 450 nm. Each sample was in triplicate.

Cell apoptosis assay

After transfection, the apoptosis in cultured cells was analyzed using annexin V labeling.

Table 1. Association between the expression of *miR-25* and clinical characteristics

Clinicopathological characteristics	n	miR-25 expression		X^2	*P*
		high	low		
Sex				1.264	0.261
female	48	27	21		
male	70	32	38		
Age				0.311	0.577
≤50	51	24	27		
>50	67	35	32		
Tumor size				1.464	0.691
<3 cm	17	7	10		
3-5 cm	41	23	18		
5-10 cm	31	14	17		
>10 cm	29	15	14		
AFP				10.033	0.018
<20 ng/mL	12	2	10		
20-400 ng/mL	18	6	12		
>400-1000 ng/mL	39	21	18		
>1000 ng/mL	49	30	19		
HbsAg				0.136	0.712
yes	62	32	30		
no	56	27	29		
Neoplasm metastasis				2.175	0.140
yes	62	27	35		
no	56	32	24		
Therapies				0.458	0.795
TACE	37	18	19		
comprehensive therapy	56	27	29		
conservative treatment	25	14	11		

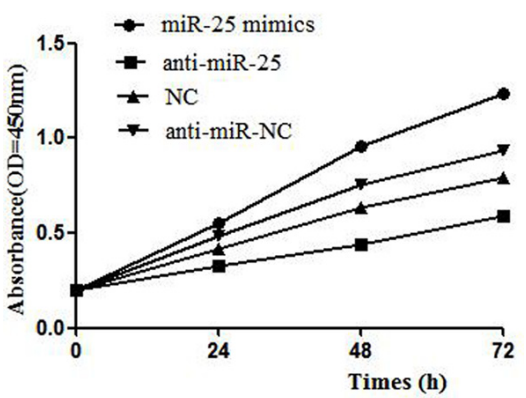

Figure 2. Up-regulation of *miR-25* promoted the cell proliferation in HCC cells.

Firstly, Logarithmic phase cells were collected with three compound perforations. Then washed the cells using binding buffer, added staining buffer to resuspend the precipitate. 100 μl cell suspension was taken for dyeing with Five micro-litre Annexin V-APC. After incubation for 15 min in dark, the mixture was analyzed using FACS Calibur (BD, USA).

Statistical analysis

The software of SPSS version 13.0 for Windows (SPSS Inc, IL, USA) was adopted for statistical analysis. All data were presented at Median ± SD. The differences between two groups were analyzed by a student's t test while among three groups were estimated by one way ANOVA. The relationship between the expression of *miR-25* and the clinicopathological characteristics were analyzed via chi-square test. Association between *miR-25* expression and overall survival of patients with HCC was evaluated with Kaplan-Meier analysis. Cox regression analysis was performed to identify the factors that might be related to the prognosis of HCC. The differences were considered to be statistical significant when $P<0.05$.

Results

Expression of miR-25 in patients with HCC and healthy controls

The expression level of *miR-25* in HCC tissues, adjacent normal tissues and healthy controls were detected by qRT-PCR analysis. The result showed that the expression level of *miR-25* was obviously higher in HCC tissues than in adjacent tissues and healthy controls (7.978±2.512 vs. 4.022±1.499) which revealed it might be an oncogene (**Figure 1**, $P<0.05$).

Relationship between miR-25 expression and clinicopathological characteristics

To evaluate the association between the expression of *miR-25* and clinical features, the

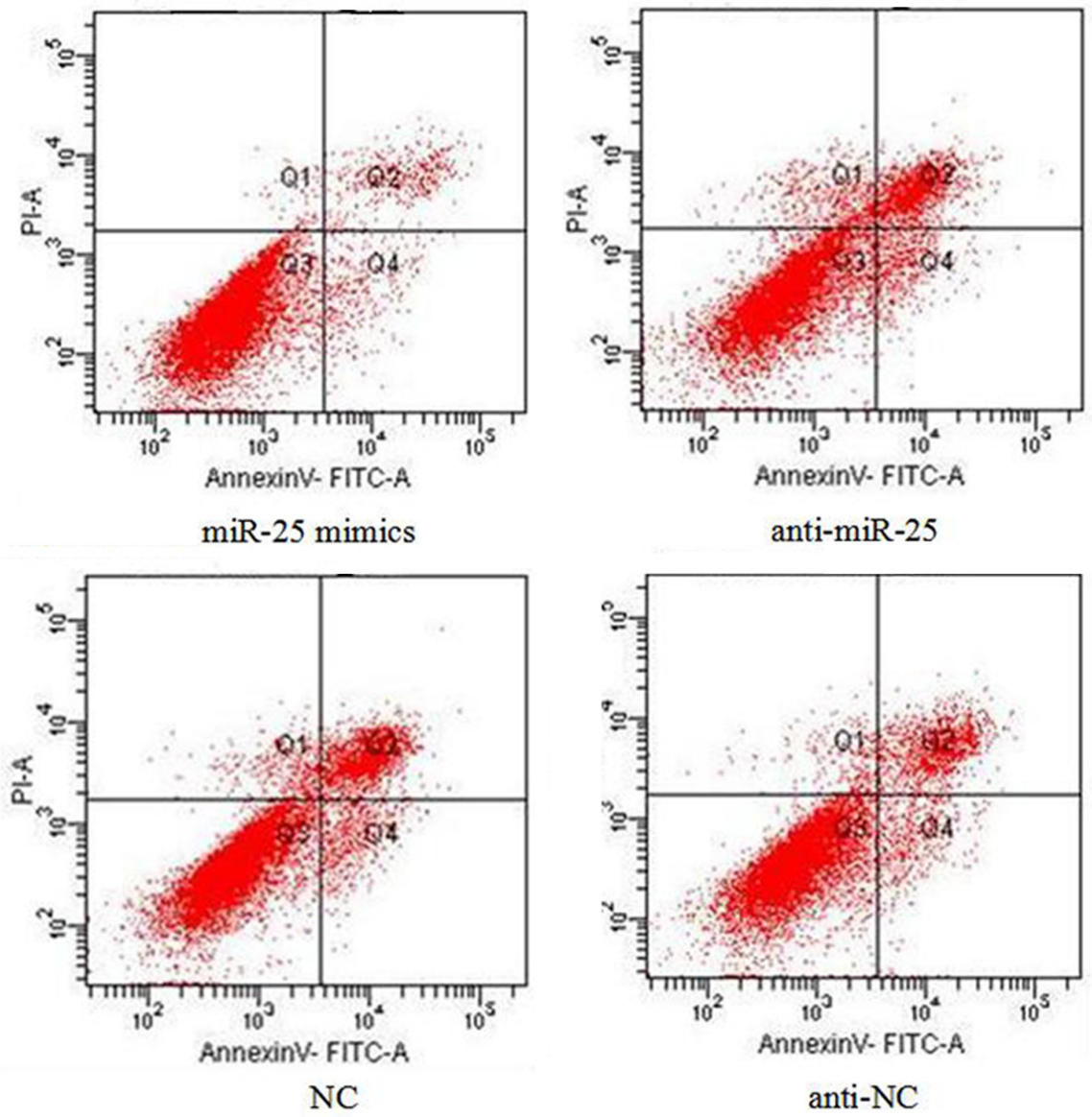

Figure 3. Up-regulation of *miR-25* reduced cell apoptosis in HCC cells.

HCC patients were divided into high expression group (high-*miR-25*) and low-expression group (low-*miR-25*) according to the median expression of *miR-25* (7.978). As shown in **Table 1**, the expression of *miR-25* was tightly associated with AFP (P=0.018). However, there was no relationship between *miR-25* expression and age, sex, tumor size, HbsAg, therapies and neoplasm metastasis.

Up-regulation of miR-25 promoted cell proliferation and inhibited cell apoptosis

To detect the functional roles of *miR-25* in HCC, proliferation and apoptosis assay were conducted after the cells transfected with pre-*miR-25*, anti-*miR-25*, pre-miR-nc, and anti-miR-nc, respectively. The outcome showed the cell proliferation was promoted in pre-*miR-25* cells, whereas down-regulation of *miR-25* expression had the reverse effects (**Figure 2**). As respect to apoptosis of cells, we used flow cytometry analysis and it manifested that the apoptosis was inhibited by the up-regulation of *miR-25* (**Figure 3**).

Prognostic performance of miR-25 in patients with HCC

During the follow-up, there were 24 patients were censored. Kaplan-Meier analsis demon-

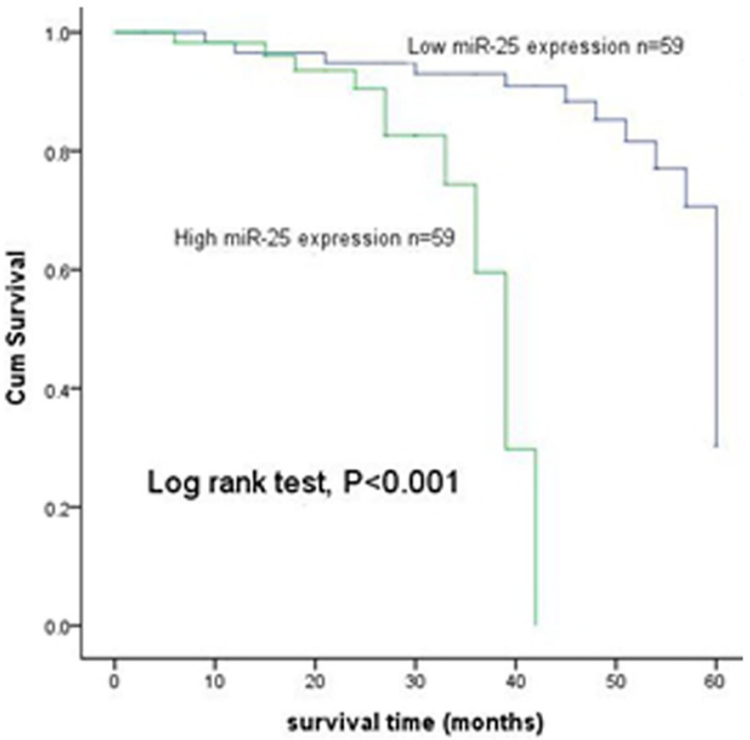

Figure 4. Kaplan-Meier analysis for estimating the relationship between *miR-25* expression and overall survival of patients with HCC. Patients with high *miR-25* expression had a longer overall survival than those with low expression (log rank test, *P*<0.001).

Table 2. Cox regression analysis adjusted for clinical factors for estimating the prognostic value of *miR-25* in patients with HCC

Parameter	Risk ratio	95% CI	P
Low miR-25 expression	-	-	-
High miR-25 expression	7.976	2.261-28.132	0.001

strated that the overall survival of patients with high *miR-25* expression was shorter than those with low *miR25* expression (**Figure 4**, log-rank test, *P*<0.001). Besides, multivariate analysis adjusted for clinicopathological characteristics with Cox regression analysis showed high *miR-25* expression (HR=7.976, 95% CI=2.261-28.132, *P*=0.001) was closely related to the prognosis of HCC (**Table 2**). Moreover, it might be independent prognostic indicator in HCC.

Discussion

HCC is a particularly deadly type of cancers with a poor prognosis which accounts for about 6% of all new cancers diagnosed worldwide and each year more than 500,000 new patients are diagnosed with HCC in the world [19, 20]. Its 5-year survival rate is very low [2]. Cirrhosis, hepatitis B, and C infection, sustained alcohol use, age, and male gender were considered to be the most important risk factors for this disease [21, 22]. Furthermore, tumor microenvironment, inflammation, oxidative stress, and hypoxia act in concert with various molecular events are main factors that can promote HCC initiation, progression, and metastasis [23]. Therefore, it is essential to explore the pathogenesis and find some accurate bio-markers in HCC.

Recently, there were many studies indicated that a number of miRNAs are dysregulated in HCC, while their aberrant expressions are associated with tumorigenesis, metastasis, prognosis or diagnosis. For instance, miR-148b was found to be down-regulated and could be a diagnostic and prognostic marker in HCC via the study of Ziari et al [24]. Li et al., considered the down-regulation of miR-325 could promote the cell invasion and proliferation by targeting high mobility group box 1 [25]. miR-107 was increased in HCC and its up-regulation was a promoter for the cell proliferation via targeting Axin2 [26]. Besides, let-7a, miR-21, miR-221, miR-222, miR-224, miR-122a, miR-125a, miR-139, miR-145, miR-150 were also abnormal expression and might act as different roles in HCC [27]. miR-25 was considered to be abnormal expressed in many cancers. Kim et al., found *miR-25* was significantly up-regulated in human stomach cancer tissues compared with the adjacent tissues [28]. miR-25 was verified not only highly expressed in ovarian cancer but could inhibit apoptosis of ovarian cancer cells by targeting Bim in the view of Zhang et al [29]. Zhao et al. detected that miR-25 was significantly increased and promoted cell proliferation, migration and invasion by targeting reversion-inducing-cysteine-rich protein with kazal motifs (RECK) in human GC tissues [30]. Li et al. found that the expres-

sion of *miR-25* was significantly down-regulated in colon cancer and provided the first evidence for *miR-25* to be an independent prognostic factor for patients with colorectal cancer [31]. Although miR-25 was reported to be over-expression and correlated with the prognosis of HCC [32], its roles in the cell proliferation and apoptosis remain uncovered.

In current study, we detected the expression of *miR-25* in HCC tissues and adjacent normal tissues. The up-regulated trend of *miR-25* was consistent to the result of previous works. This might indicated that *miR-25* might be an oncogene. Then we further explored its relationship with clinical factors. And the expression of AFP was proved to be associated with the expression of *miR-25* which revealed it might be involved in the development of HCC.

Subsequently, we investigated the influences of *miR-25* on the HCC development through proliferation and apoptosis assay. It was shown that the up-regulation of *miR-25* contributed to the cell proliferation while it suppressed the cell apoptosis in HCC. This outcome proved *miR-25* promoted the progression of HCC and the detection of its expression might help predict the malignant degree in future. Besides, we explored its prognostic value in HCC via Kaplan-Meier and Cox regression analysis which indicted it could be an independent prognostic marker. And the view was agreed with the previous study [32].

In conclusion, *miR-25* is increased in HCC patients and the expression level is related to AFP. Moreover, *miR-25* may serve as a promoter for cell proliferation and inhibitor for cell apoptosis. In addition, *miR-25* could be an independent prognostic marker in clinical practice of HCC.

Disclosure of conflict of interest

None.

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