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ORIGINAL ARTICLE

Reanalysis of microRNA expression profiles identifies novel biomarkers for hepatocellular carcinoma prognosis

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Abstract The aim of our study is to identify microRNAs (miRNAs) that have significance in the prognosis and pathogenesis of hepatocellular carcinoma (HCC). The miRNAs differentially expressed in HCC were examined by using a human miRNA microarray dataset, and then the acquired candidates were screened by another microarray dataset. As a result, we got 25 miRNAs which were aberrantly expressed in cancer and meanwhile predicated distinct prognosis. Among them, miR-139-5p was down-regulated in HCC and its low expression in cancer tissue meant poor prognosis. Additionally, we demonstrated that its low expression was also related to several clinicopathologic characteristics such as vein invasion, BCLC stage, p-AKT expression, and pIGFR1 expression.

In vitro, it has been discovered that treatment of HCC cells with a miR-139-5p mimic lead to inhibition of cell growth and migration. Moreover, luciferase assay showed that KPNA4 was not the direct target of miR-139-5p. Ectopic expression of miR-139-5p has not repressed the expression of KPNA4, but inhibited the nuclear import of NF-κB and phosphorylation of Akt. In conclusion, for the first time, we identify 25 deregulated miRNAs that are associated with prognosis and prove that miR-139-5p functions as a tumor suppressor in HCC and its low expression predicts poor prognosis.

Keywords microRNA · Hepatocellular carcinoma · Prognosis · miR-139-5p · NF-κB

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Introduction

Primary liver cancer is one of the most frequently diagnosed malignant tumors and hepatocellular carcinoma (HCC) is the major histological subtype, accounting for more than 70 % of total liver cancer. HCC is one of the leading causes of cancer death worldwide [1]. High mortality rate is attributed to late diagnosis and unsatisfactory treatment. Therefore, there is still a need to develop novel diagnostic and prognostic biomarkers and investigate the inner pathogenesis to seek new cures [2].

MicroRNAs (miRNAs) are short non-coding RNAs that regulate post-transcriptional translation of genes. It has been reported that over one third of human genes may be miRNA targets [3]. Deregulated miRNAs may cause a variety of diseases, including cancers. Researches suggest that miRNAs have the potential to serve as biomarkers or therapy targets of cancers. In the case of HCC, plasma miR-21 level could be useful for differentiating cancer patients from hepatitis patients or healthy volunteers [4]. Low miR-26 expression in tumor tissues predicts worse prognosis but a better response

to interferon therapy [5]. Additionally, miR-34 has been proved to be a regulator of tumor suppression, and its mimic named MRX34 has entered phase I clinical trials [6]. Although a growing body of researches focus on the implication of miRNAs in HCC, a comprehensive investigation remains necessary.

MicroRNA microarray analysis is a high-throughput way to identify novel differential expressed miRNAs. This technique has been useful in biomarker discovery. Two independent researches have been conducted to identify potential miRNAs used for prognosis evaluation in HCC [7, 8], but there is no obvious intersection between them. Accordingly, we attempted to reanalyze the existing microarray datasets so as to seek new clues. By using GEO datasets, we have identified 25 miRNAs which not only exhibit altered expression but also predict different clinic prognosis in hepatocellular carcinoma. Among them, miR-139-5p was selected for further study. Its lower expression indicated more severe vascular invasion, higher BCLC stage, more p-AKT, and pIGFR1 expression. Functional assays showed that miR-139-5p acted as a tumor suppressor by regulating cell proliferation and migration in HCC cell lines. Furthermore, we found that ectopic expression of miR-139-5p could inhibit the nuclear import of NF-κB and phosphorylation of Akt. These results provide a foundation for further study on the prognostic biomarkers of HCC, and indicate that miR-139-5p has the potential as a therapy agent of HCC.

Material and methods

Cell culture, culture conditions, and HCC samples

Huh7 and SMMC-7721 cell lines were purchased from the Chinese Academy of Sciences Committee Type Culture Collection cell bank. All of the cells were maintained in DMEM medium (Hyclone, USA) supplemented with 10 % FBS (Hyclone, USA) and 100 U penicillin/streptomycin at 37 °C and 5 % CO_2 in a humidified chamber. Eight pairs of HCC samples and paired non-tumor tissues used in RT-qPCR were collected from patients who underwent surgery in Renmin Hospital of Wuhan University. All patients have signed informed consent. This study was approved by the Ethics Review Board of our hospital.

Transient infection

Synthetic miR-139-5p mimic, negative control mimic (miR-NC) were purchased from Biomics Biotechnologies (Nantong, China). Huh7 cells and SMMC-7721 cells were seeded in 6-well plates and then cultivated overnight. Next, 100 pmol of miRNA mimic was dissolved in 200 μl of serum-free, antibiotic-free, medium. Similarly, 5 μl of Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) was

mixed with 200 μl of the same medium. Then, they were added together and placed at room temperature for 20 min. The 400 μl of transfection solutions were then added to each well containing 1.6 ml of medium. Six hours later, the cultures were replaced with 2 ml fresh complete medium.

Cell proliferation

Huh7 and SMMC-7721 cells were plated in 96-well plates at a concentration of 5000 cells per well and incubated for 24 h after transfection. Every 24 h, 200 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added into each well and then the cells continued to be cultured for 4 h. Then, dimethyl sulfoxide (DMSO) was used to dissolve the crystals. The absorbance was measured at 570 nm. The growth curve was constructed by using OD570 nm as ordinate and time as abscissa. Experiments were performed in triplicate.

In vitro migration assays

Transwell chambers with 8-μm filters (Corning) were used for the assay. Transfected cells were resuspended in serum-free medium, and 200 μl of the cell suspension (5×10^4 cells) was added to the upper chamber. Then, 600 μl of complete medium was added to the bottom chamber. The chamber was then cultivated for 48 h. The cells that had not migrated were removed from the upper face of the membrane. The cells adhered to the lower face were fixed with 20 % methanol and then stained with Hematoxylin. Cell migration was determined by counting six random microscopic fields. The data are presented as mean ± standard errors (SEM).

Western blot

Seventy-two hours after transduction with miR-139-5p mimics or miR-NC, protein was isolated from adherent cells by using Nuclear and Cytoplasmic Protein Extraction Kit or Cell lysis buffer for Western and IP (Beyotime, China). Protein concentration was determined by using the BCA protein assay. Protein was separated by 12 % SDS-PAGE and transferred to nitrocellulose membranes. The primary antibodies were showed as follows: p65 (Cell Signal Tech, USA), p-P65 (Cell Signal Tech, USA), KPNA4 (ProteinTech, China), AKT (Cell Signal Tech, USA), and p-AKT (Invitrogen, USA). Signals were detected with ECL Plus (Beyotime, China) and exposed to a film. GAPDH was used as the loading control.

Quantitative RT-PCR

Total RNA from hepatic cancer tissues was extracted by using Trizol reagent (Aidlab, China). cDNA was reversely

transcribed with M-MLV Reverse Transcriptase (Genecopoeia, USA). qRT-PCR was conducted according to the instructions of miScript SYBR Green PCR Kit (QIAGEN, Germany). The primers for miR-139-5p were as follows: the loop primer: 5′-GTCGTATCCAGTGC AGGGTCCGAGGTATTCGCACTGGATACGAC CAACCTCA-3′; the forward primer: 5′-TGCG CTGGAGACGCGGCCCTGTT-3′; the reverse primer: 5′-CCAGTGCAGGGTCCGAGGTATT-3′. The expression level of miR-139-5p was normalized to the expression level of U6. The results were analyzed by using $2\Delta\Delta Ct$ method.

Table 1 Differential expression miRNAs correlated with prognosis in HCC

MicroRNAs	MiRNAs correlated with prognosis of HCC patients		Deregulated miRNAs between HCC and non-cancer tissues		Summary
	Putative function	P value[a]	Expression level in HCC	P value[b]	
miR-105	Protective	0.0022	High	0.0405	No
miR-139-5p	Protective	<0.0001	Low	<0.0001	Yes
miR-142-3p	Protective	0.0436	Low	<0.0001	Yes
miR-145*	Protective	0.0001	Low	0.0002	Yes
miR-181a*	Protective	0.0405	Low	<0.0001	Yes
miR-15b	Protective	0.0051	High	0.0152	No
miR-181b	Protective	0.0122	Low	0.0222	Yes
miR-183	Protective	0.0118	High	0.0002	No
miR-186	High-risk	0.0250	High	0.0459	Yes
miR-18b*	Protective	0.0385	Low	0.0048	Yes
miR-26a	High-risk	0.0081	Low	<0.0001	No
miR-224	Protective	0.0152	High	<0.0001	No
miR-23a*	Protective	0.0368	Low	0.0037	Yes
miR-29b	Protective	0.0202	High	0.0288	No
miR-29c	Protective	0.0018	Low	0.0248	Yes
miR-30a	Protective	0.0210	Low	0.0003	Yes
miR-31	Protective	0.0019	Low	0.0012	Yes
miR-422a	Protective	0.0048	Low	<0.0001	Yes
miR-496	Protective	0.0246	High	0.0451	No
miR-509-3p	High-risk	0.0181	High	0.0242	Yes
miR-522	High-risk	0.0021	High	0.0441	Yes
miR-548b-3p	High-risk	0.0075	High	0.0323	Yes
miR-584	High-risk	0.0041	High	0.0494	Yes
miR-601	High-risk	0.0150	High	0.0343	Yes
miR-675	High-risk	0.0003	High	0.0003	Yes
miR-661	High-risk	0.0005	Low	0.0497	No
miR-9*	High-risk	0.0265	High	0.0183	Yes
miR-99b	High-risk	0.0412	Low	0.0006	No
miR-200b	Protective	0.0166	Low	<0.0001	Yes
miR-106b	Protective	0.0004	High	<0.0001	No
miR-34b	Protective	0.0019	Low	0.0306	Yes
miR-301b	Protective	0.0111	High	<0.0001	No
miR-28-5p	Protective	0.0090	High	0.0209	No
miR-330-3p	High-risk	0.0094	High	0.0337	Yes
miR-612	High-risk	0.0128	High	<0.0001	Yes
miR-769-5p	High-risk	0.0028	High	0.0025	Yes
miR-146b-3p	Protective	0.0038	Low	0.0002	Yes

[a] Analyzed by the Log-rank test in the GEO dataset GSE31384

[b] Analyzed by pared t test in the GEO dataset GSE21362

Luciferase reporter assay

The 3′-UTR segment of KPNA4 mRNA containing the miR-139-5p-binding sites was amplified by PCR and inserted into the pmirGLO vector (Promega, USA) to obtain the wild-type plasmid pmirGLO-WT-KPNA4. Similarly, the corresponding mutational plasmid pmirGLO-mut-KPNA4 was generated. The sequence of miR-139-5p was cloned into the pLVX-ShRNA2 vector to obtain the plasmid pLVX-ShRNA2-miR-139-5p. Next, plasmid pLVX-ShRNA2-miR-139-5p was co-transfected in HEK293 cells with pmirGLO-WT-KPNA4 or pmirGLO-mut-KPNA4 by using Lipofectamine 2000. After 48 h, luciferase activity was measured by using a dual-luciferase reporter assay kit (Beyotime, China).

Statistical analysis

GraphPad Prism5.0 was used in the current research. The miRNA profile of GSE31384 [7], GSE21362 [9], GSE36915 [10], GSE20596 [11], and GSE14520 [12] was downloaded from Gene Expression Omnibus. The differential expression of miRNAs in tumor and non-tumor tissue were compared by t test. Overall survival analysis was performed in the tumor samples by Kaplan-Meier method, the Log-rank test. The relationship between miRNA expression and clinic characteristic was analyzed by chi-square test. All cellular studies were repeated for three times, and results were analyzed by t test. Statistical tests and p values were two-sided. Differences were considered significant with a value of $P < 0.05$.

Results

Screening of differential expression miRNAs correlated with prognosis in HCC

To screen out the deregulated miRNAs associated with distinct prognosis, firstly, we analyzed the association between

Fig. 1 MiR-139-5p is downregulated in HCC and its high expression predicts better prognosis. **a**, **b** MiR-139-5p is downregulated in HCC compared with non-cancer tissues in the datasets GSE21362 and GSE36915. **c** High miR-139-5p levels increase the overall survival of HCC patients in the dataset GSE31384. **d** The miR-139-5p expression level is negatively associated with cancer T stage, and lower miR-139-5p expression is found in cancers with higher T stage in the dataset GSE36915. **e** Expression of miR-139-5p in cancer tissues and paried adjacent tissues collected from postoperative HCC patients. The *bars* represent the mean values (mean ± SEM).*, **, *** represent $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively
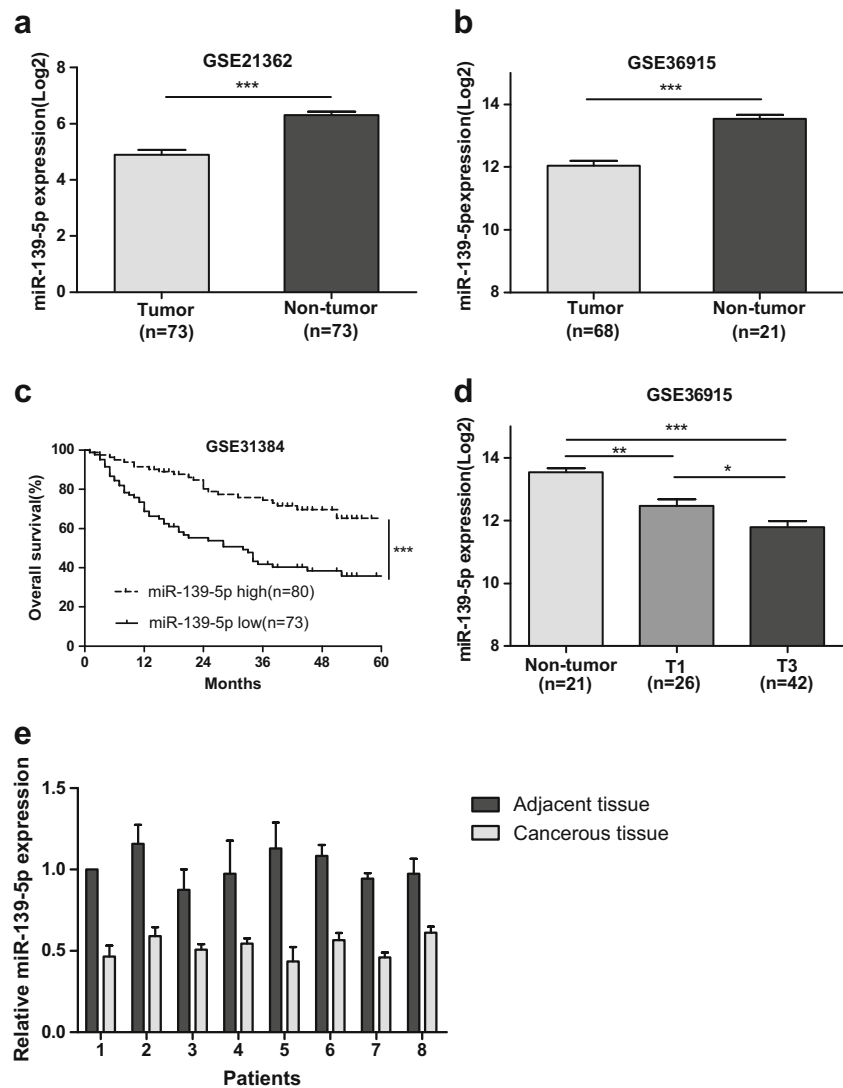
the expression level of every miRNA and the overall survival of cancer patients by using a GEO dataset GSE31384 encompassing 166 cases of HCC tissues. We categorized 166 cases into 2 groups based on miRNAs expression levels (low = bottom half, high = top half). The miRNAs correlated with prognosis were selected, and then we further investigated whether the acquired miRNA candidates existed the differential expression in another GEO dataset GSE21362. As a result, we got 37 miRNAs meeting above standards (Table 1). Fourteen miRNAs were low-expressed in HCC tissue compared with adjacent non-tumor tissues; at the same time, their high expression in cancer tissues predicted better prognosis ($P < 0.05$). In contrast, another 11 miRNAs were high-expressed in HCC tissues, and their low expression in cancer tissues meant better prognosis ($P < 0.05$). The rest of miRNAs which were regarded as protective factors but were up-regulated in HCC tissues or regarded as high-risk factors but were down-regulated in cancer tissues were not included.

miR-139-5p is down-regulated in HCC and its high expression predicts better prognosis

Among the 25 miRNAs, we selected miR-139-5p to make the next investigation. As shown in Fig. 1a, c, compared with non-cancer tissues, miR-139-5p was low-expressed in cancer tissues and its high expression predict better prognosis. To validate the results, we investigated its expression by using another GEO dataset GSE36915 encompassing 68 HCC and 21 non-tumor liver tissues (Fig. 1b). Indeed, its low expression in cancer tissues was confirmed. What is more, we found that miR-139-5p level was negatively associated with the T grade in HCC (Fig. 1d). Next, we collected eight pairs of primary liver cancer

Table 2 Correlation between miR-139-5p expression and clinicopathologic characteristics of patients with HCC (GSE20596)

Characteristics	No. of patient	miR-139-5p expression		Chi-square value	P value
		High	Low		
Age (years)					
≤55	18	6	12	0.0453	0.8314
>55	61	22	39		
Gender					
Male	52	21	31	1.6240	0.2026
Female	27	7	20		
AFP, ng/ml					
≤300	53	20	33	2.7610	0.0966
>300	14	2	12		
Vascular invasion					
Yes	45	10	35	7.8440	0.0051
No	32	17	15		
BCLC stage					
0	8	7	1	10.4300	0.0153
A	55	17	38		
B	7	2	5		
C	8	2	6		
ICH: pRPS6					
Yes	38	14	24	0.0080	0.9300
No	45	17	28		
ICH: pIGFR1					
Yes	15	2	13	4.8810	0.0272
No	61	27	34		
ICH: pAkt					
Yes	24	3	21	8.1780	0.0042
No	59	27	32		
ICH: CTNNB1					
Yes	28	9	19	0.4895	0.4841
No	55	22	33		

BCLC Barcelona Clinic Liver Cancer, *ICH* immunohistochemistry, *AFP* α-fetoprotein

tissues and detected its expression in carcinoma tissues and paired adjacent tissues by using RT-qPCR. Results showed that miR-139-5p was low-expressed in cancer tissues (Fig. 1e).

The expression level of miR-139-5p in cancer is associated with several clinicopathologic characteristic

As mentioned above, miR-139-5p was associated with T grade, so we further investigated the clinic implication of miR-139-5p on cancer progression. We assessed the relationship between its expression and clinicopathologic characteristics in the GEO dataset GSE20596 encompassing 89 HCC patients (Table 2). Two groups were categorized based on the average expression level of miR-139-5p (low = lower than the average, high = higher than the average). Results demonstrated that low expression of miR-139-5p was related to more severe vein invasion ($P = 0.0051$), higher BCLC stage ($P = 0.0153$), more p-AKT ($P = 0.0042$), and pIGFR1 expression ($P = 0.0272$). However, there was no relationship between its expression and sex, age, AFP level ($P > 0.05$).

Transient infection of miR-139-5p mimic inhibits proliferation and migration of HCC cells

Since miR-139-5p played an important role in HCC, we further assessed its function in vitro. We transduced Huh7 and

SMMC-7721 cell lines with miR-139-5p mimic and negative control mimic, respectively. Cell proliferation from these two groups were examined by MTT assay. The results showed that cell proliferation was significantly inhibited in cells transfected with miR-139-5p mimic compared with the control cells (Fig. 2a, b). Next, we investigated the impact of miR-139-5p on the ability of cell migration. Transwell assay indicated that cells transfected with miR-139-5p mimic exhibited significantly reduced rate of migration (Fig. 2c, d).

miR-139-5p regulates the nuclear translocation of NF-κB via AKT signaling rather than in a KPNA4-dependent manner

To further investigate the inner mechanism, we predicted the potential target gene of miR-139-5p by computational analysis in three databases including TargetScan, miRanda, and miRwalk. One candidate identified was KPNA4, which had one miR-139-5p-binding site in the 3′-UTR region (Fig. 3b). Through analyzing the GEO datasets GSE14520, we proved that KPNA4 was high-expressed in HCC tissues (Fig. 3a). It has been demonstrated that KPNA4 is associated with NF-κB nuclear import [13, 14]. So we supposed that miR-139-5p conversely regulated the expression of KPNA4 and thus affected the nuclear import of NF-κB. However, luciferase reporter assay revealed that ectopic expression of miR-139-5p

Fig. 2 Transient infection of miR-139-5p mimic inhibits proliferation and migration of HCC cells. **a**, **b** Effect of miR-139-5p upregulation on the proliferation of Huh7 cells (**a**) and SMMC-7721 cells (**b**) was determined by MTT assay. **c**, **d** Transwell assay of Huh7 cells (**c**) and SMMC-7721 cells (**d**) migration after treatment with miR-139-5p, negative control, respectively. **e**, **f** Quantification of migrated cells is shown for Huh7 cells (**e**) and MMC-7721 cells (**f**). The *bars* represent the mean values (mean ± SEM).*, **, *** represent $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively
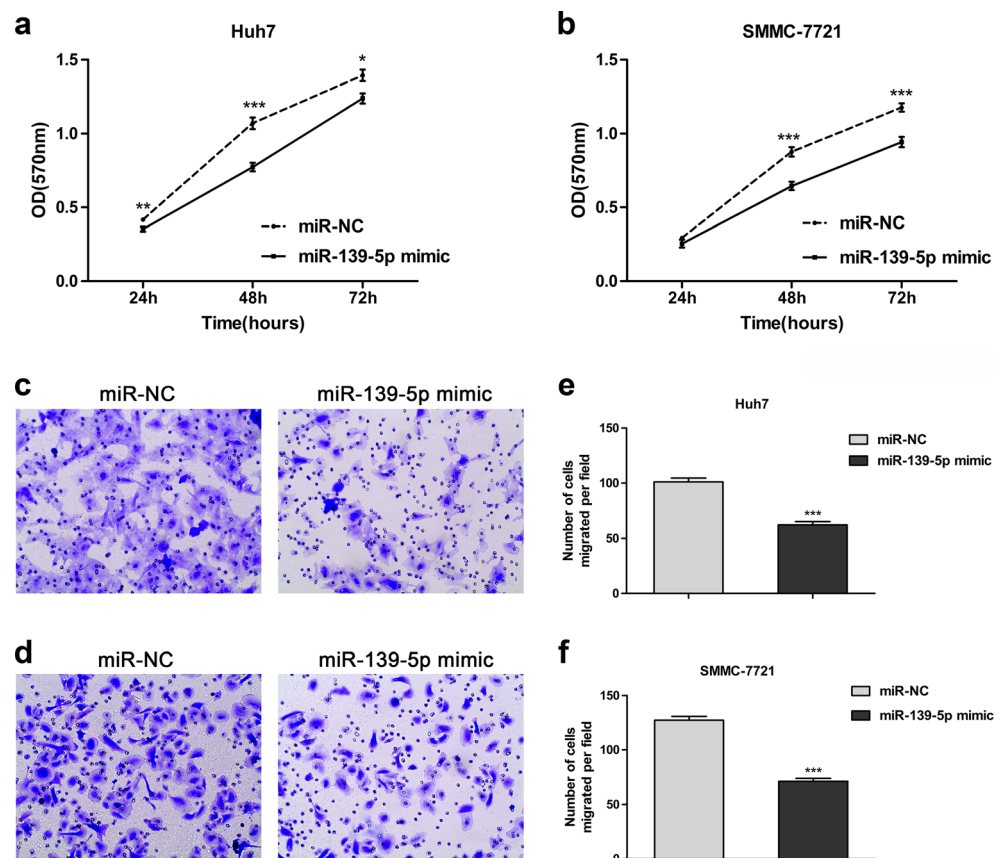
Fig. 3 miR-139-5p regulates the nuclear translocation of NF-κB via AKT signaling rather than in a KPNA4-dependent manner. **a** KPNA4 is up-regulated in HCC compared with paired non-cancer tissues in the datasets GSE14520. **b** the potential miR-139-5p binding site at the 3′UTR region of KPNA4 was computationally predicted. **c** Luciferase activity assay demonstrated that KPNA4 was not the direct target of miR-139-5p. **d, f** Western blot analysis showed that ectopic expression of miR-139-5p blocked nuclear import of NF-κB. **e, g** Western blot analysis demonstrated that up-regulation of miR-139-5p inhibited Akt/NF-κB pathway activation. The *bars* represent the mean values (mean ± SEM).*, **, *** represent $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively
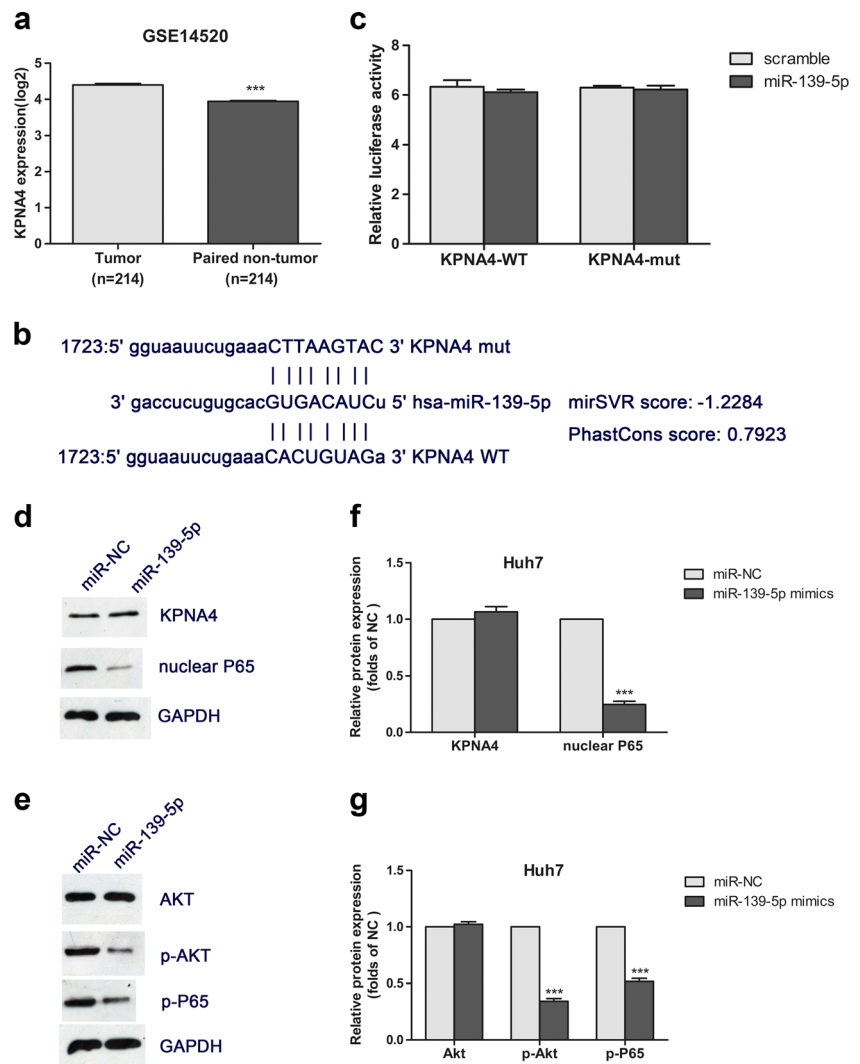
failed to decrease the luciferase activity of pmirGLO-wt-KPNA4 and pmirGLO-mu-KPNA4 (Fig. 3c). Western blot analysis also showed that transfection of miR-139-5p mimics had no impact on the expression of KPNA4. However, the nuclear import of NF-κB was blocked (Fig. 3d, f). As mentioned above, the expression of miR-139-5p was inverse with p-AKT, so we investigated the impact of miR-139-5p on AKT/NF-κB pathway. Results indicated that ectopic expression of miR-139-5p decreased the level of p-AKT obviously and blocked the nuclear import of NF-κB (Fig. 3d, e, g). These data supported that miR-139-5p up-regulation inhibited the nuclear translocation of NF-κB via AKT signaling rather than in a KPNA4-dependent manner.

Discussion

In this study, for the first time, we identified 25 miRNAs which were aberrantly expressed in cancer and associated with prognosis. Among them, miR-139-5p was further proved to be a tumor suppressor, and its expression level was related to vascular invasion, BCLC stage. miR-139-5p repressed nuclear translocation of NF-κB via AKT signaling rather than in a KPNA4-dependent manner.

Many studies have demonstrated that miRNAs exhibit altered expression levels in cancers and may play potential roles as diagnostic and prognostic biomarkers of cancer [15]. Among the 25 miRNAs, the expression of several miRNAs in HCC including miR-139-5p [16], miR-142-3p [17], miR-29c [18], miR-31 [19], miR-30a [20], miR-422a [21], miR-200b [22], and miR-675 [23] are consistent with other literature reports. However, the expression of some miRNAs in HCC such as miR-522 [24] and miR-186 [25] lacks relevant literature support, but their expression in other cancer tissues display the same patterns as our results. In addition, miRNAs like miR-29c, miR-31, and miR-30a are proved to be associated with prognosis [18–20]; these cited literatures prove our miRNAs profile is worthy of further exploration. Because we

have not set the change fold when comparing the expression level of miRNAs, the miRNA list might contain false positives and need to be further validated by experiments. Comparing with other researches [7, 8], we do not discover the similar miRNA list which underline the differential expression and prognosis simultaneously.

Our functional assays showed that miR-139-5p acted as a tumor suppressor by regulating cell proliferation and migration in HCC cell lines. In fact, several other literatures have indicated that it took part in some pathological courses including cell proliferation, apoptosis, migration, and invasion [16, 26, 27]. In addition, it could also mediate chemo-sensitivity of breast cancer cells to docetaxel [28]. Comparing with other studies, we firstly demonstrated that the expression level of miR-139-5p was inversely associated with vein invasion, BCLC stage, p-AKT expression, and pIGFR1 expression. Moreover, AKT and IGFR1 activation play an important role in tumorigenesis, tumor progression, and metastasis. Coincidentally, IGFR1 is proved to be the target of miR-139-5p [29]. In addition to its prognosis significance, miR-139-5p is also reported to serve as an early diagnostic biomarker in tongue squamous cell carcinoma [30]. In another research on lung cancer, circulating miR-139-5p is used to distinguish cancer patients from healthy smokers [31]. However, whether it is useful for early diagnosis of HCC remains to be further studied.

Since miR-139-5p plays an important role in the pathologic process of cancers, its inner mechanism intrigues us. We discovered that up-regulation of miR-139-5p could repress nuclear translocation of NF-κB, which was KPNA4-independent and associated with down-regulation of AKT activation. In a word, miR-139-5p functions as a tumor suppressor by inhibiting Akt/NF-κB pathway. In fact, two literatures have also proved that miR-139-5p could regulate Akt signaling [32, 33]. IGF-1R and IRS1 are validated as the direct target of miR-139-5p and regarded as the intermediate between miR-139-5p and Akt/NF-κB pathway. In addition to Akt signaling, it has been reported that miR-139-5p could regulate Notch1 pathway and vascular development [34]. In view of these, we consider that the implication of miR-139-5p in HCC is significant. It is worthy of deep investigation in the future.

Compliance with ethical standards

Conflict of interest None.

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