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Hepatocellular carcinoma progression is protected by miRNA-34c-5p by regulating FAM83A

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Abstract. – OBJECTIVE: To elucidate the role of microRNA-34c-5p (miRNA-34c-5p) in the progression of hepatocellular carcinoma (HCC) and the indicated mechanism.

PATIENTS AND METHODS: Relative levels of miRNA-34c-5p and FAM83A in HCC tissues and cell lines were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Their influences on clinical features in HCC patients were analyzed. Kaplan-Meier method was introduced for assessing the relationship between miRNA-34c-5p and overall survival in HCC. After overexpression of miRNA-34c-5p in HepG2 and HB611 cells, we detected proliferative, migratory and invasive abilities by cell counting kit-8 (CCK-8) and transwell assay. The interaction between miRNA-34c-5p and FAM83A was explored by Dual-Luciferase reporter assay. Finally, their co-regulation on HCC cell phenotypes was examined.

RESULTS: MiRNA-34c-5p was downregulated in HCC tissues, especially stage III+IV cases. Its level was correlated to tumor size, tumor number and TNM staging in HCC. Overexpression of miRNA-34c-5p inhibited proliferative, migratory and invasive abilities in HepG2 and HB611 cells. In addition, miRNA-34c-5p targeted on FAM83A and negatively regulated its level. Overexpression of FAM83A could reverse the inhibitory effects of miRNA-34c-5p on malignant phenotypes of HCC cells.

CONCLUSIONS: By negatively regulating FAM83A level, miRNA-34c-5p alleviates the progression of HCC.

Key Words:
MiRNA-34c-5p, FAM83A, HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignant tumors leading to cancer death[1]. At present, liver resection and liver transplantation are the main therapies that can cure HCC. However, postoperative 5-year survival is very low[2,3]. The rates of metastasis and recurrence of HCC are relatively high[4,5]. It is necessary to clarify the molecular mechanisms underlying HCC progression and metastasis, thus developing effective strategies for HCC treatment.

MicroRNAs (miRNAs) are novel, noncoding RNAs that regulate gene expressions at the post-transcriptional level by complementary base pairing to mRNAs[6]. They are extensively involved in regulating cell functions[7,8]. In recent years, a large number of miRNAs have been identified to participate in tumor progression, including HCC[9-11]. MiRNA-34c-5p is previously reported to be lowly expressed in specimens of laryngeal squamous cell carcinoma, and it is linked to the prognosis[12]. In osteosarcoma cells, miRNA-34c-5p contributes to inhibition of proliferative, migratory and invasive abilities[13]. The methylation of miRNA-34c-5p attenuates proliferation and metastasis in colorectal cancer, as well as epithelial-mesenchymal transition (EMT)[14]. The potential function of miRNA-34c-5p in HCC progression, however, remains unclear.

FAM83A contains a domain of unknown function 1669 (DUF1669) at the N terminal. It was initially discovered in 2005 as a potential cancer biomarker[15]. FAM83A is highly expressed in many types of tumors, and it is involved in tumor progression[16-20]. Recently, FAM83A has been detected to be upregulated in HCC samples, serving as an initiator for the carcinogenesis of HCC[21]. This study aims to uncover the co-regulation of miRNA-34c-5p and FAM83A on the malignant progression of HCC. Our findings may provide

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novel targets for clinical treatment of HCC.

Patients and Methods

Sample Collection

HCC specimens (n=50) and paracancerous ones (n=50) were collected from HCC patients in Dongguan People's Hospital, Southern Medical University. Inclusion criteria: (1) patients were clinically diagnosed as HCC; (2) No treatment-related contraindications. Exclusion criteria: (1) patients had other therapies except for TACE, including chemotherapy, liver resection, liver transplantation and ablation surgery; (2) patients were treated by TACE because of other reasons; (3) metastatic patients. All specimens were quickly frozen and stored at -80°C for use. Clinical data of them were recorded. Written informed consent was obtained before sample collection. This investigation was approved by the Ethics Committee of Dongguan People's Hospital, Southern Medical University.

Cell Culture and Transfection

Cell lines were provided by China Center for Type Culture Collection (CCTCC). HCC cell lines (SMMC-7721, HepG2, HB611 and HHCC) and immortalized hepatocytes (L-02) were cultivated in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) at 37°C with 5% CO_2 humidity. Culture medium was daily replaced. Cells were transfected with miRNA-34c-5p mimic, miR-NC, pcDNA-FAM83A or pc-DNA, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 48-72 h.

Cell Counting Kit-8 (CCK-8)

Cells were inoculated in a 96-well plate with 1×10^4 cells/well. At the appointed time points, absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Quantitative RT-PCR (RT-qPCR)

TRIzol method (Invitrogen, Carlsbad, CA, USA) was applied for isolating cellular RNA. Through reverse transcription of RNA, the extracted complementary deoxyribose nucleic acid (cDNA) was used for PCR detection by SYBR Green method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference. Relative mRNA level was calculated by $2^{-\Delta\Delta CT}$ method. The primer sequences were listed as follows: MiRNA-34c-5p: forward 5'-CGGAGGCAGTGTAGTTAGCT-3', reverse: 5'-GTGCAGGGTCCGAGGT-3'; FAM83A: forward: 5'-AAGCACAACAACATCAGAGACCT-3', reverse: 5'-ATGGAAATGTTCTTGAGGTGACTG-3'; GAPDH: forward: 5'-CTGACTTCAACAGCGACACC-3', reverse: 5'-TGTGGTAGCCAAATTCGTTGT-3'. U6 : forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'- AACGCTTCACGAATTTGCGT-3'.

Transwell Assay

1×10^5 cells suspended in 200 μL of serum-free were applied on the upper of transwell chamber (Corning, Corning, NY, USA) pre-coated either with 24 μg/μL Matrigel or not, and 500 μL of medium containing 10% FBS was applied in the bottom. After cell culture for 18-20 h, penetrating cells were fixed in methanol and dyed using 1.0% crystal violet. Stained cells were captured and calculated.

Western Blot

Total proteins in transfected HCC cells were isolated using AmyJet cellular protein extraction kit (Wuhan, China). They were separated in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Non-specific antigens were blocked in 5% skim milk for 1 h, followed membrane incubation with primary and secondary antibodies for the indicated time points: anti-FAM83A (1:1000; Sigma-Aldrich, St. Louis, MO, USA) and anti-GAPDH. Band exposure was finally conducted for grey value analysis.

Dual-Luciferase Reporter Assay

Predicted binding sites in the 3'-untranslated region (3'-UTR) of FAM83A and miRNA-34c-5p, and the mutant sequences were inserted into pGL3 vectors, which were respectively co-transfected in HepG2 cells with miRNA-34c-5p mimic or miR-NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Luciferase activity was detected at 48 h.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) and GraphPad Prism (Version X; La Jolla, CA, USA) were used for data processing. The relationship between miRNA-34c-5p and clinical features in HCC patients was assessed by the χ^2-test. Overall survival in HCC patients expressing high or

low level of miRNA-34c-5p was analyzed by Kaplan-Meier method. $p<0.05$ was considered as statistically significant.

Results

Low Expression of MiRNA-34c-5p in HCC Specimens

Compared with paracancerous tissues, miRNA-34c-5p was markedly downregulated in HCC ones (Figure 1A). Similarly, miRNA-34c-5p was also downregulated in HCC cell lines than normal hepatocytes (Figure 1B). HepG2 and HB611 cells expressed the lowest level of miRNA-34c-5p among the detected HCC cell lines, and they were used in the following experiments. We further analyzed the relationship between miRNA-34c-5p and clinical features in HCC patients. Recruited patients were classified into two groups according to the median level of miRNA-34c-5p in their HCC tissues. As data revealed, miRNA-34c-5p was correlated to tumor size, tumor number and TNM staging in HCC (Table I). Lower level of miRNA-34c-5p was detected in stage III+IV HCC cases compared to stage I+II cases (Figure 1C). Kaplan-Meier curves revealed that low level of miRNA-34c-5p predicted poor prognosis in HCC patients (Figure 1D). It is indicated that miRNA-34c-5p may be related to the progression of HCC.

Overexpression of MiRNA-34c-5p

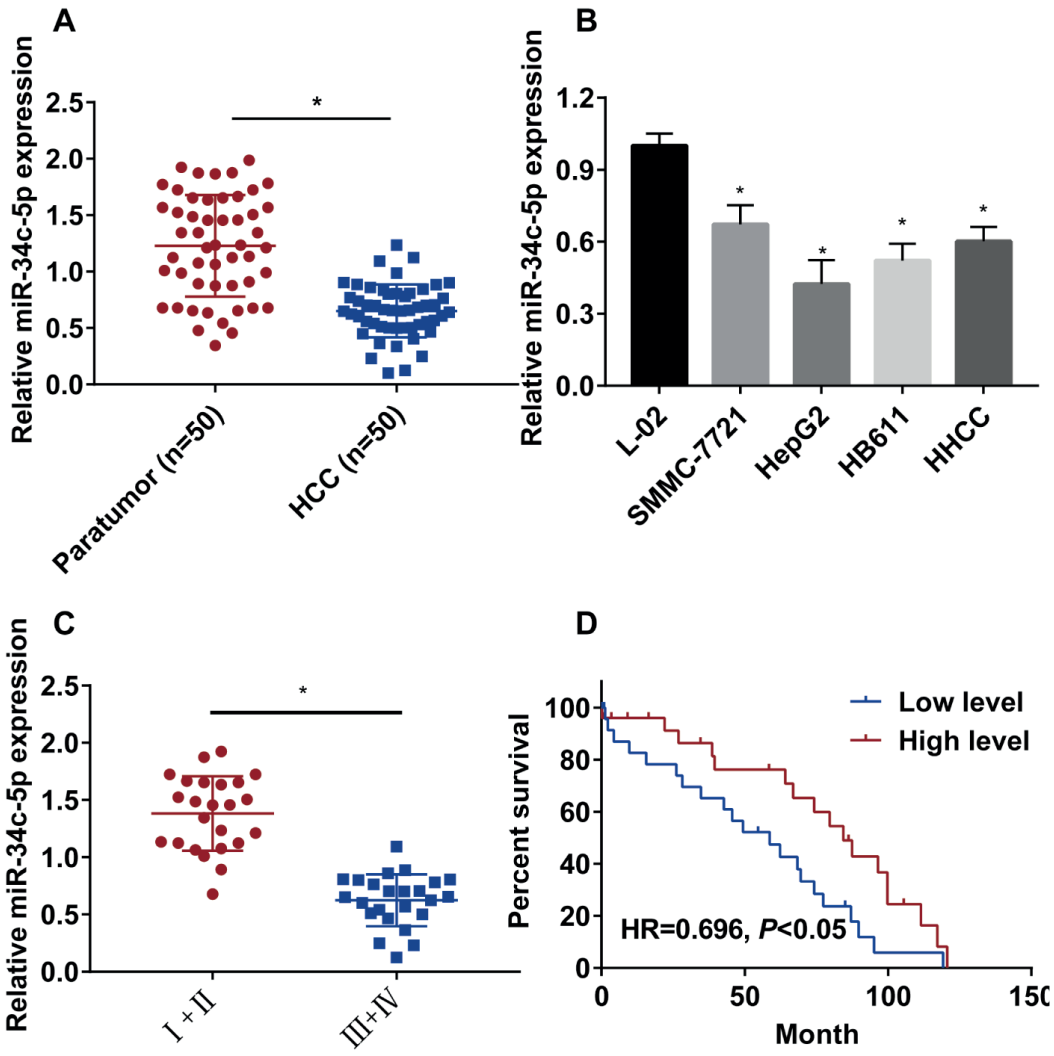

Figure 1. Low expression of miRNA-34c-5p in HCC specimens. **A,** MiRNA-34c-5p was downregulated in HCC tissues than paracancerous ones; **B,** MiRNA-34c-5p was downregulated in HCC cell lines than normal hepatocytes; **C,** MiRNA-34c-5p level was lower in stage III+IV HCC cases than those stage I+II cases; **D,** Overall survival was worse in HCC patients expressing low level of miRNA-34c-5p than those with high level.

Table I. Correlation analysis between miR-34c-5p expression and clinicopathological parameters of HCC patients.

Clinicopathologic features	No. of cases	miR-34c-5p expression		*p*-value
		Low (n=25)	High (n=25)	
Age (years)				0.571
≤60	26	14	12	
>60	24	11	13	
Gender				0.254
Male	28	12	16	
Female	22	13	9	
Tumor nodule number				
Solitary	19	6	13	0.041*
Multiple (≥2)	31	19	12	
Tumor size				0.009*
≤5CM	28	5	14	
>5CM	32	20	11	
TNM stage				
Stage I	22	7	15	0.023*
Stage II-III	28	18	10	
HBV				0.107
Absent	13	4	9	
Present	37	21	16	
Vascular invasion				0.774
Negative	21	10	11	
Positive	39	15	14	

HBV: hepatitis B virus.

Inhibited Proliferative, Migratory and Invasive Abilities in HCC

Transfection of miRNA-34c-5p mimic remarkably upregulated miRNA-34c-5p in HepG2 and HB611 cells (Figure 2A). CCK-8 assay showed that overexpression of miRNA-34c-5p declined viability in HepG2 and HB611 cells (Figure 2B). Migratory and invasive abilities in HCC cells overexpressing miRNA-34c-5p were markedly suppressed than those of controls (Figure 2C). It is concluded that miRNA-34c-5p was able to inhibit proliferative and metastatic abilities in HCC.

MiRNA-34c-5p Targeted and Negatively Regulated FAM83A

Potential binding sites in the 3'-UTR of FAM83A and miRNA-34c-5p were predicted using miRanda (Figure 3A). Subsequently, Dual-Luciferase reporter assay uncovered a decline of Luciferase activity in the wild-type FAM83A vector after overexpression of miRNA-34c-5p, proving the binding between miRNA-34c-5p and FAM83A (Figure 3B). Compared with paracancerous tissues, FAM83A was markedly upregulated in HCC ones (Figure 3C). Both mRNA and protein levels of FAM83A were downregulated in HepG2 and HB611 cells overexpressing miRNA-34c-5p (Figure 3D, 3E).

Overexpression of FAM83A Could Reverse the Role of MiRNA-34c-5p in Regulating HCC Cell Phenotypes

To further elucidate the role of FAM83A in regulating HCC cell phenotypes influenced by miRNA-34c-5p, we designed a series of rescue experiments. Compared with those overexpressing miRNA-34c-5p, FAM83A level was higher in HCC cells co-overexpressing miRNA-34c-5p and FAM83A (Figure 4A). Of note, decreased viability, and numbers of migratory and invasive cells in HepG2 and HB611 cells overexpressing miRNA-34c-5p were partially reversed by co-overexpression of FAM83A (Figure 4B, 4C). We therefore demonstrated that miRNA-34c-5p alleviated malignant phenotypes of HCC cells by targeting FAM83A.

Discussion

HCC is a highly malignant tumor affecting human health in the world, which accounts for 80-90% of liver cancer cases[22]. Although great strides have made on improving clinical diagnosis

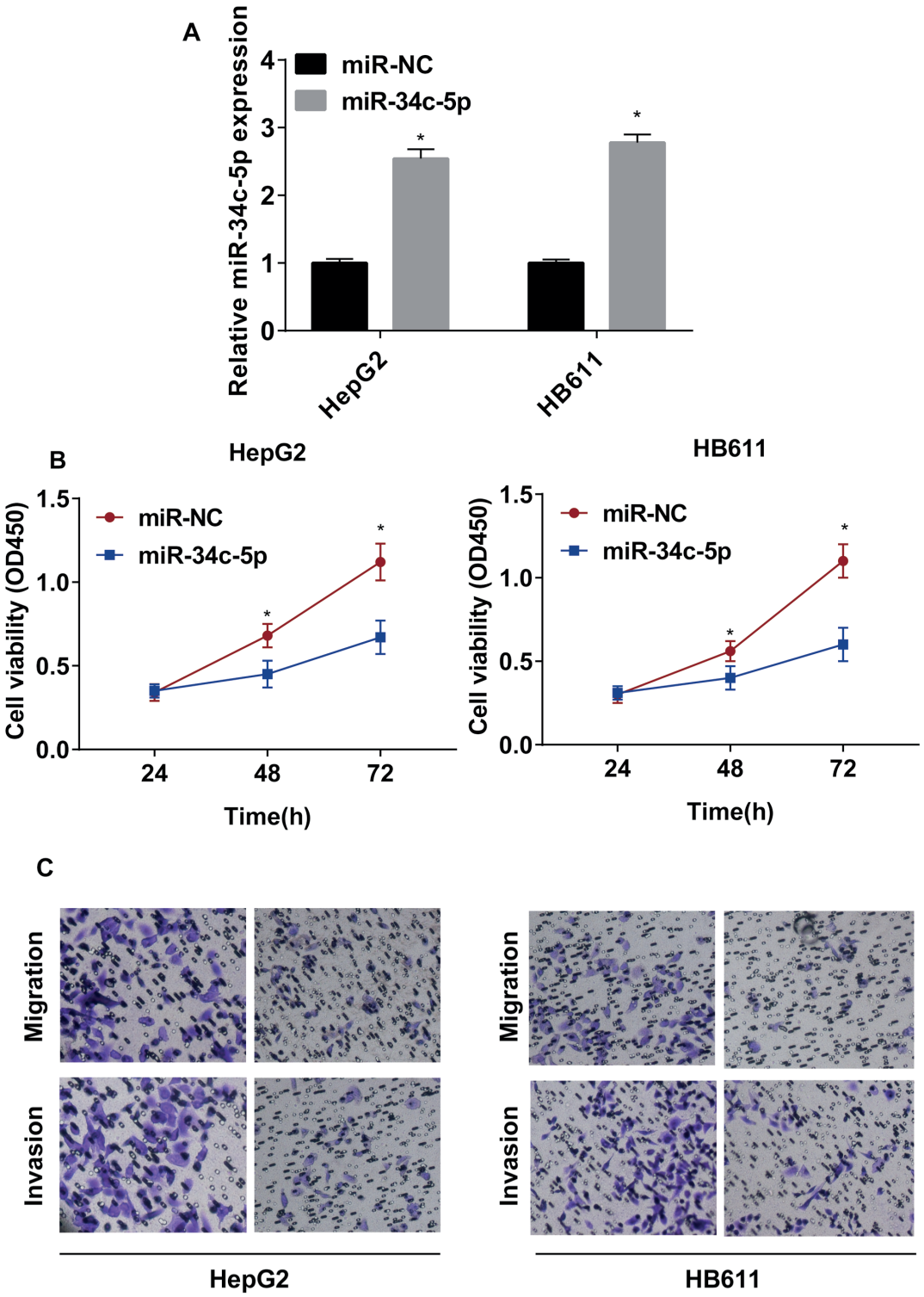

Figure 2. Overexpression of miRNA-34c-5p inhibited proliferative, migratory and invasive abilities in HCC. **A,** Transfection of miRNA-34c-5p mimic significantly upregulated miRNA-34c-5p in HepG2 and HB611 cells; **B,** Overexpression of miRNA-34c-5p reduced viability in HepG2 and HB611 cells; **C,** Overexpression of miRNA-34c-5p reduced migration and invasion in HepG2 and HB611 cells, (magnification: 40×).

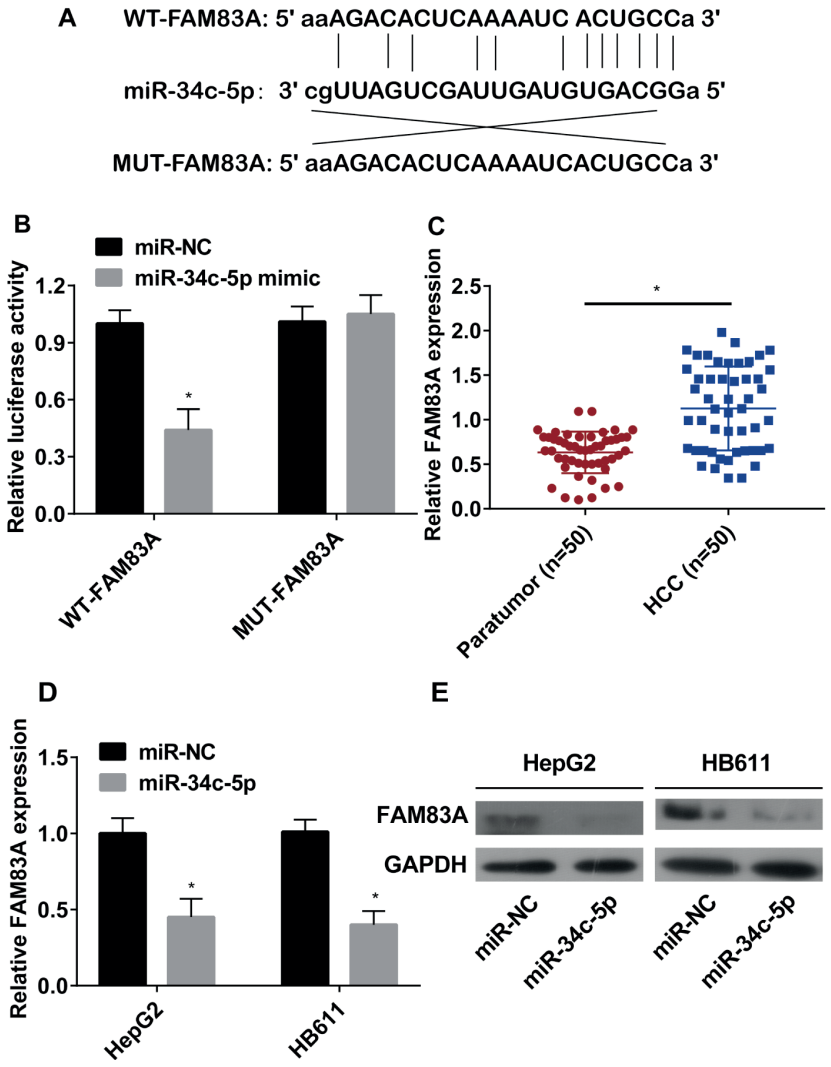

Figure 3. MiRNA-34c-5p targeted and negatively regulated FAM83A. **A,** Binding sites in the 3'-UTR of miRNA-34c-5p and FAM83A predicted using miRanda; **B,** Dual-Luciferase reporter assay verified the binding between miRNA-34c-5p and FAM83A; **C,** FAM83A was upregulated in HCC tissues than paracancerous ones; **D,** Overexpression of miRNA-34c-5p downregulated mRNA level of FAM83A in HepG2 and HB611 cells; **E**, Overexpression of miRNA-34c-5p downregulated protein level of FAM83A in HepG2 and HB611 cells.

and treatment of HCC, the prognosis is far away from satisfy. The 5-year survival of HCC is as low as 7%[23,24]. Effective biomarkers for predicting and treating HCC are urgently required.

MiRNAs are post-transcriptional regulators containing 22-24 nucleotides[25]. Through recognizing and binding 3'-UTR of target mRNAs, miRNAs inhibit mRNA translation or stimulate their degradation[26,27]. Downregulation of miRNAs often leads to overexpression of their target genes. Increasing evidence has shown the critical functions of miRNAs in the occurrence and progression of tumors. Regulatory effects of miRNAs on the progression, metastasis and chemotherapy of HCC provide novel ideas for targeted therapy[28]. Our findings uncovered that miRNA-34c-5p was lowly expressed in HCC tissues and cells. Low level of miRNA-34c-5p predicted poor prognosis in HCC patients. Furthermore, overexpression of miRNA-34c-5p remarkably attenuated proliferative, migratory and invasive abilities in HepG2 and HB611 cells. It is suggested that miRNA-34c-5p could be a tumor suppressor involved in the progression of HCC, which is consistent

with previous findings in osteosarcoma and acute myeloid leukemia[29].

FAM83A is considered to be a potential oncogene in pancreatic cancer, lung cancer and breast cancer[30-33]. Compared with paracancerous tissues, FAM83A was upregulated in HCC tissues. Consistently, Liu et al[34] proposed that upregulated FAM83A in HCC specimens can drive the metastasis. Using the bioinformatic tool, potential binding sites in the 3'-UTR of miRNA-34c-5p and FAM83A were discovered. Subsequently, Dual-Luciferase reporter assay showed that miRNA-

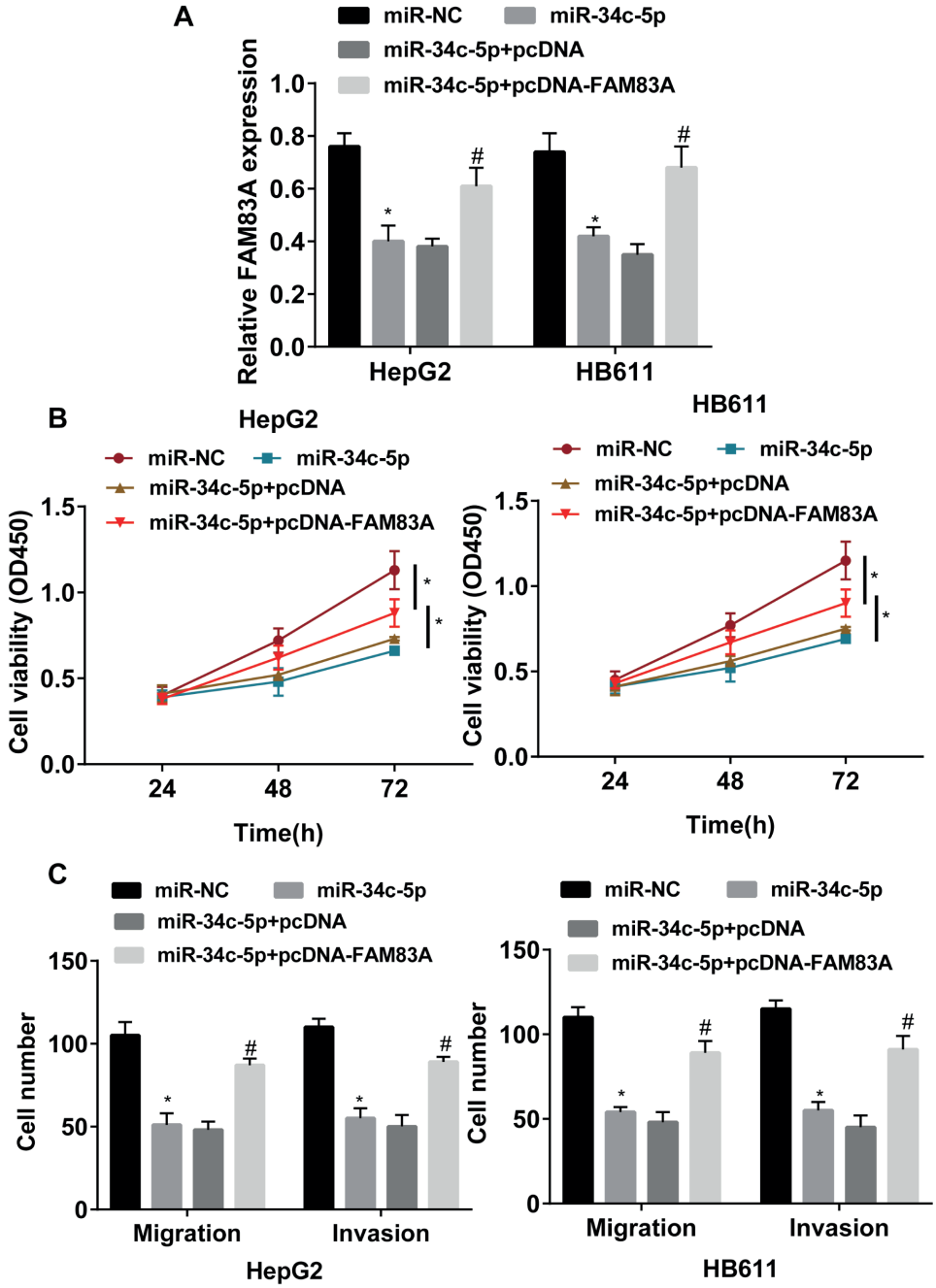

Figure 4. Overexpression of FAM83A could reverse the role of miRNA-34c-5p in regulating HCC cell phenotypes. **A,** FAM83A level was higher in HepG2 and HB611 cells co-overexpressing miRNA-34c-5p and FAM83A than those overexpressing miRNA-34c-5p; **B,** Overexpression of FAM83A reversed the role of miRNA-34c-5p on proliferation in HepG2 and HB611 cells; **C,** Overexpression of FAM83A reversed the role of miRNA-34c-5p on migration and invasion in HepG2 and HB611 cells.

34c-5p could directly target on FAM83A 3'-UTR. MiRNA-34c-5p negatively regulated FAM83A level in HCC cells. Of note, co-overexpression of miRNA-34c-5p and FAM83A was able to reverse the inhibitory effects of overexpressed miRNA-34c-5p on proliferative, migratory and invasive abilities in HCC. To sum up, miRNA-34c-5p alleviates the malignant progression of HCC by binding FAM83A 3'UTR and thus negatively regulating its level. Our findings provide a novel aspect for explaining the molecular mechanisms of HCC progression.

Conclusions

Shortly, by negatively regulating FAM83A level, miRNA-34c-5p alleviates the progression of HCC. FAM83A is believed as a novel diagnostic and therapeutic target for HCC, which is of great clinical significance.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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