E2F1 induced miR-652-3p promotes malignancy and metastasis of cancer cells via inhibiting TNRC6A in hepatocellular carcinoma

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Research

Keywords: Hepatocellular carcinoma, E2F1, metastasis, miR-652-3p, TNRC6A

Posted Date: June 9th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1633194/v1

Abstract

Background

Metastasis serves as the leading cause of treatment failure and cancer-related death in HCC. The role of metastasis-associated miRNAs in HCC was elusive.

Methods

Combined analysis of TCGA data and exosomal miRNA sequencing data was performed. Expression of miR-652-3p in HCC was assessed. Function experiments of miR-652-3p and TNRC6A were performed both in vitro and in vivo. MRNA sequencing, PCR and western blot were performed to verify target genes and pathway of miR-652-3p. The lung metastasis and xenograft cancer model in nude mice was established to investigate the effects of miR-652-3p/TRNC6A axis on tumor metastasis in vivo. The potential transcription regulator of miR-652-3p was verified. The relationship of the expression of miR-652-3p/TNRC6A axis and prognosis of HCC patients was analyzed.

Results

Upregulated miR-652-3p was found in the tumor tissues of HCC, especially in metastatic HCC patients. Overexpression of miR-652-3p promoted and knockdown of miR-652-3p suppressed HCC metastasis both in vitro and in vivo. What's more, miR-652-3p promoted HCC metastasis via regulating EMT pathway. Trinucleotide repeat-containing gene 6A protein (TNRC6A) was identified as a direct target of miR-652-3p, whose expression is negatively related with AFP level in HCC, and knockdown of TNRC6A promoted HCC metastasis. And knockdown of TNRC6A restored repressed EMT and HCC metastasis caused by inhibition of miR-652-3p. E2F1 transcriptionally promoted the expression of miR-652-3p through binding in the specific site in its DNA sequence. Besides, clinical results revealed that high expression of miR-652-3p and low expression of TNRC6A was positively correlated to shortened overall survival and disease-free survival in HCC patients.

Conclusions

The present study showed that E2F1 increases the expression of miR-652-3p, and miR-652-3p promotes EMT and HCC metastasis by directly inhibiting the expression of TNRC6A in HCC.

Background

HCC has become one of the most malignant cancers. HCC is the fourth leading cause of cancer-related deaths, which lays a heavy economic burden on the healthcare system in developing and developed countries [1]. The morbidity of HCC varies among different racial groups and geographic regions [2, 3].

The 5-year survival rate of patients with HCC is only 5%-30%, and metastasis is the leading cause of cancer-related death [4, 5]. The frequent metastasis, including intrahepatic and distant metastasis, accounts for the poor survival and high recurrent after treatment in HCC. Therefore, more researches are urgently needed to explore the occurrence and development of HCC metastasis and its underlying mechanism, which may promote the development of early diagnosis and treatment for metastatic HCC.

MiRNAs are approximately 18–24 nucleotide non-coding RNAs that regulate gene expression by complementarily binding to the 3′UTR sequence of their target mRNA, thus leading to the degradation of mRNA or translational repression [6, 7]. Current evidence indicated that miRNAs play essential roles in cancer metastasis [8]. It is known that cancer cells communicate with cells involved in the microenvironment via secreting extracellular vesicles (EVs). EVs mediated cellular communication influences the metastatic process of tumors [9]. As the main contents in EVs, miRNAs play an essential role in regulating cancer development and metastasis. Many studies pointed out that exosomal miR-103 and miR-1247-3p were related to a high risk of metastasis and recurrence in HCC patients [10, 11], and serum exosomal miR-21 and miR-93 were found can be effective indicators of HCC [12, 13]. Lots of studies showed that miR-652-3p might be an oncogenic miRNA in the development of many cancers. For instance, miR-652-3p promoted the proliferation, migration, and invasion ability of bladder cancer cells *in vitro* [14], and miR-652-3p was found to promote the development of non-small cell lung cancer (NSCLC) [15]. In addition, the expression of miR-652-3p has also been reported to be upregulated in human breast cancer, osteosarcoma, and rectal cancer [16–18], but the relationship between miR-652-3p and HCC and its mechanism was not investigated.

Our previous study and other researches have uncovered the essential role of transcription factor E2F1 in regulating proliferation, tumorgenesis, apoptosis, and differentiation in mutiple cancers [19–22]. MiRNAs regulate the expression of E2F transcription factors and E2Fs family members can in turn regulate miRNA's expression as well [19]. For example, tumor-suppressive miR-34a can cause senescence-like growth arrest by downregulation of E2F1 in colon cancer cells [23]. MiR-449a/b, which structurally resembles the p53-inducible miR-34 family, was found to be the direct transcription targets of E2F1 and attenuated the activity of E2F1 [24]. Many microRNAs influence the metastasis of cancer cells by targeting transcription factors, like E2F1. MiR-34a can suppress migration and invasion of HCC cells by regulating the expression of E2F1[25]. MiR-519d-3p could be activated by p53 to directly suppress the expression of E2F1, thus inhibiting the metastasis of prostate cancer [26].

GW182 was first found as an autoantigen from the serum of motor and sensory neuropathy patients [27]. It is a 182-KDa protein that contains multiple glycine-tryptophan repeats, one or more glutamine-rich (Q-rich) regions, and a classical RNA recognition motif (RRM) at the C terminus. GW182 family proteins are shown to be essential in miRNA-mediated gene silencing [28]. There are three GW182 paralogs in vertebrates (TNRC6A, TNRC6B, and TNRC6C) [29]. TNRC6A was found expressed in both prostate carcinoma and esophageal squamous cell carcinoma. In contrast, no evident expression was found in their normal cells [30]. Loss of TNRC6A expression was observed in gastric and colorectal cancers with high microsatellite instability [31]. In this case, TNRC6A may play a different role in different cancers.

However, the exact role of TNCR6A in HCC is still elusive. In this paper, we mainly uncover the biological function of miR-652-3p and its target gene TNRC6A in the HCC cells and its underlying mechanism.

Materials And Methods

Bioinformatics analysis

The TCGA (www.tcga-data.nci.nih.gov/tcga), which containing transcript expression data of LIHC and Tissue Expression of healthy tissue, was used. The Expression of multiple mRNAs was analyzed using above data set. Clinical data including survival information of LIHC patients from TCGA was obtained and its clinical correlations with TNRC6A and miR-652-3p were carried out. Genes were ranked by expression level of TNRC6A, followed by differential expression analysis and Gene Otology (GO) analysis.

Human tissue samples

With informed consent from all patients, the liver tumor tissues and its matched adjacent tissues of HCC patients, were collected from The First Affiliated Hospital of Chongqing Medical University. The ethical guidelines of the 1975 Declaration of Helsinki were followed and protocols were approved by the Ethics Committee of The First Affiliated Hospital of Chongqing Medical University in this study.

miR-652-3p and TNRC6A expression level and survival analysis

The data from TCGA database was used to investigate the expression of miR-652-3p and TNRC6A in HCC and corresponding para-carcinoma tissues. GEPIA database (http://gepia.cancer-pku.cn/) and Starbase database were used to investigate the expression of TNRC6A and miR-652-3p, respectively. The correlations between miRNA (including miR-652-3p) or TNRC6A expression and overall survival (OS), disease-free survival (DFS) were compared by Starbase database (http://starbase.sysu.edu.cn/) and Kaplan–Meier plotter (https://kmplot.com/analysis/), respectively. Kaplan–Meier survival plot was used to perform comparison in two groups.

Cell culture

Human hepatocellular carcinoma cell lines (HCC-LM3, Huh7) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai Institute of Cell Biology). All these cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Biological Industries, Israel) supplemented with 10% fetal bovine serum (FBS) (Moregate Biotech, Australia) at 37°C in a humidified chamber with 5% CO2.

Plasmids, siRNA, miRNA mimics and inhibitors transfection

The miR-652-3p mimics and inhibitors used in this study were listed: The miR-652-3p mimics and miR-652-3p inhibitors (Ruibo biotechnology, Guangdong, China) were purchased from Ruibo bio-company. The mature sequence of has-miR-652-3p: AAUGGCGCCACUAGGGUUGUG, TNRC6A siRNA, and its negative control siRNA (Sunya, China) were purchased from Sunya Biotechnology Company. E2F1

overexpression plasmids (Repobio, China) were purchased from Repobio Company. According to the manufacturer's instructions, all mimics, inhibitors, and siRNA, plasmids were transfected into HCC cells using jetPRIME® transfection reagent (Polyplus Transfection, France).

Cell proliferation and colony formation assays

Transfected HCC cells (2×10^3 cells/well) were seeded into a 96-well plate and incubated overnight for the cell proliferation assay. According to the manufacturer's instructions, cell counting kit-8(CCK8) was used to test cell viability every 24 hours for 72 hours. As for colony formation assay, transfected cells (1000 cells/well) were seeded into 6-well plates and maintained in completed DMEM media with 10% FBS. The culture medium was changed every 3-4 days. After three weeks, the colonies were fixed in 4% Paraformaldehyde Fix Solution, stained with 0.1% crystal violet (Sigma), and the clone number was counted and recorded.

Cell cycle analysis

HCC cells ($2x10^5$) were digested and seeded into 6-well plate. After overnight incubation, cells were transfected with miR-652-3p mimics or negative control mimics and incubated for 48h. Cells were harvested and fixed in 75% ethanol at -20˚C for 24-48h. After fixation, PBS was used to wash cells one time. Then, 200μl DNA PREP Stain (Beckman Coulter, Inc., Brea, CA, USA) was used to resuspend cells, and the suspension was incubated in the darkroom for 30min at room temperature. BD LSR II instrument (BD Biosciences, San Jose, CA, USA) was used to perform cell cycle analysis. Flowjo software version 10 was used for further analysis.

Wound-Healing Assay

The transfected HCC cells were seeded into a 6-well plate and incubated until about 100% confluence. Then, a pipette tip was used to scratch cell surface, and then phosphate-buffered saline (PBS) was used to remove cell debris. To evaluate the healing effect of cells with different treatments, cell images were observed and captured under a microscope at a particular time (the time for Huh7 and HCC-LM3 were 72h and 120h, respectively).

Migration and Invasion Assay

For migration assay, transfected HCC cells (5.0×10^4 cells per well) were digested and seeded into the upper chamber of the Transwell plate (24-well, eight μm pore size, Millipore, USA), cultured in 200μL DMEM without FBS. 800μL DMEM with 10% FBS were used to fulfill the lower chamber. Moreover, for invasion assay, before cell seeding, upper chambers were pre-coated with a 40μL coating medium, which was consisted of 32μL DMEM medium and 8μL Matrigel (BD Biosciences, USA) for 3 hours. Equal amounts of cells were seeded into the upper chamber, and the following were performed as described above. After a particular incubation time, cells were fixed by 4% paraformaldehyde and stained by 0.2% crystal violet, and the number of cells was calculated.

Western Blotting and antibodies

Cells were lysed in the RIPA Lysis buffer (Beyotime Biotechnology, China) containing Protease Inhibitor Cocktail (ThermoFisher, USA). Protein concentration was measured by Bradford assay (Bio-Rad Laboratories, Inc., Hercules, USA). Western blots were performed as previously described [32]. Antibodies for western blot were listed as below: N-Cadherin (13116T, CST, USA), E-Cadherin (3195T, CST, USA), Snail (3879T, CST, USA), GAPDH (10494-1-AP, protein tech, China), and TNRC6A (A6115, ABclonal Technology, China).

RNA extraction and quantitative real-time polymerase chain reaction

TRIzol reagent was used to extract total RNA. HiScript II Q RT SuperMix (Vazyme Biotech, Nanjing, China) and Mir-X™ miRNA First-Strand Synthesis Kit (Takara, Kyoto, Japan) were used for mRNA and miRNA cDNA synthesis, respectively. Furthermore, miRNA reverse primers (universal primers) are also provided in the kit. For mRNA, SYBR Green (Vazyme Biotech, Nanjing, China) was used to perform a real-time quantitative polymerase chain reaction (q-PCR) to measure mRNA expression. The relative expression level of genes was normalized with GAPDH internal controls. For miRNA, TB Green® Premix Ex Taq™ (Tli RNase H Plus) (Takara, Kyoto, Japan) was used to perform qPCR to measure the expression of miRNA. The relative expression level of miRNA was normalized with U6 controls. All q-PCR was performed in triplicate on the Bio-Rad QX100 Droplet Digital PCR system (USA). All primers were obtained from Tsingke Biological Technology (Beijing, China).

mRNA sequencing

Total RNA was extracted by using TRIzol reagent following the instruction of the manufacturer. And further mRNA sequencing was performed by GenePharma Company (GenePharma, China). Briefly, mRNA was purified from total RNA using magnetic beads with Oligo (dT) and fragmented into ~200 bp short fragments, and then cDNA libraries were constructed. RNA-seq was performed on the Illumina HiSeq 2000 platform according to the manufacturer's instructions, and reads were generated.

Luciferase reporter assay

A nucleotide sequence of TNRC6AmRNA 3′UTR containing the binding site for has-miR-652-3p seed sequence was synthesized and inserted to construct psi-TNRC6A-3UTR (WT). The mutation of the above nucleotide sequence lacking the seed sequence was inserted to construct the psi-TNRC6A-3UTR (MUT). Psi-TNRC6A-WT/MUT plasmids and miR-625-3p mimics/NC mimics were co-transfected into HCC-LM3 cells. After 24 hours, cells with different treatments were harvested and lysed by passive lysis buffer. A dual-luciferase reporter system (Promega, USA) using LB 960 Centro (Berthold) was used to measure luciferase activity. The luminescence intensity of Firefly luciferase was normalized to that of Renilla luciferase.

Chromatin Immunoprecipitation (ChIP) assay

The ChIP kit (9003, CST, USA) was purchased from CST company. According to the protocol from manufacturer, the ChIP grade antibody against E2F1 (66515-1-Ig, Proteintech, China) was used for ChIP assay. The ChIP assay was performed as previously described in our previous study [22].

Animal Studies

Male nude mice (4-6 weeks old) were purchased from the Zhejiang Academy of Medical Sciences animal center and used in all experiments. All animal experiments were performed according to the authorized procedures and approved by the ethical committee of the Zhejiang Academy of Medical Sciences animal center. And all animal experiments were carried out conforming to the requirement of the guidelines of the National Institutes of Health (Guide for the Care and Use of Laboratory Animals, 2011). To detect the metastatic ability of HCC cells, 100ml PBS contained 1.0 x 10^6 HCC-LM3 cells treated with antigomiR-652-3p were injected into mice via tail vein to construct lung metastatic models. After that, TNRC6A siRNA or negative control siRNA (5nmol per mice) and polyplus transfection reagent (Polyplus Transfection, France) were injected through the tail vein to knockdown TNRC6A *in vivo* according to the manufacturer's instructions. After eight weeks, the lungs of mice were collected after euthanization, and lung metastatic nodules were calculated. To detect the EMT change *in vivo*, 100ml PBS contained 5.0 x 10^6 HCC-LM3 cells were injected subcutaneously into nude mice to construct HCC model. After that, TNRC6A siRNA or negative control siRNA (5nmol per mice) and polyplus transfection reagent (Polyplus Transfection, France) were injected into tumorsaccording to the manufacturer's instructions. After four weeks, tumors were harvested after euthanization, and IHC was performed.

Statistical analysis

The SPSS 22.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analyses. All data were presented as the mean ± standard deviation. A two-tailed Student's *t*-test was used to assess comparisons between two groups. The Kaplan-Meier method was used to assess the overall survival rate of patients. Statistically significant difference was considered when $P<0.05$.

Results

MiR-652-3p was upregulated in HCC patients

According to our previous study, highly metastatic HCC cells (MHCC-97hm) were constructed from their parental low metastatic HCC cells (MHCC-97h). Based on our previous exo-miRNA sequencing data, the profiles of miRNA expression in highly metastatic HCC cells were different from those in low metastatic parental HCC cells [22] (Figure 1A). 218 exo-miRNAs were upregulated in 97hm exosomes compared to 97h exosomes ($P<0.05$). And metastasis significantly threatens the prognosis of HCC patients. There is global variation of the incidence and mortality in primary HCC, and one of the highest burden was seen in East Asia [1]. To find out the potential miRNA which played an indispensable role in regulating metastasis and cancer development in Asian HCC patients, the miRNA expression profiles in Asian HCC patients from The Cancer Genome Atlas (TCGA) database were analyzed (Figure 1B), 99 miRNA were found

upregulated in HCC tumor tissues compared with para-tumor tissues (P<0.05 and logFC>2). After combining analysis, 23 potential miRNAs were obtained (Figure 1C and 1D). Furthermore, the top 8 miRNAs were ranked according to their fold change in highly metastatic HCC cells (MHCC-97hm), compared with low metastatic parental HCC cells (MHCC-97h) (Figure 1E). Through using the Starbase database (http://starbase.sysu.edu.cn/panMirDiffExp.php), the relationship between the overall survival rate of HCC patients and the miRNA expression level (including miR-652-3p, miR-660-5p, and miR-221-3p) were further analyzed (Figure 1F). Results showed that HCC patients with high expression of miR-652-3p had a lower overall survival rate. No significant difference was observed in the miR-660-5p and miR-221-3p groups. These results indicated that miR-652-3p could be a potential biomarker to predict the prognosis of patients with HCC.

MiR-652-3p promoted HCC metastasis both *in vitro* and *in vivo*

To investigate the biological function of miR-652-3p in HCC cells, miR-652-3p was ectopically overexpressed in Huh7 and HCC-LM3 cells via transfecting miR-652-3p mimics. After transfection, the expression level of miR-652-3p was significantly increased in Huh7 and HCC-LM3 cells (Figure S1A and S1B). However, from the colony formation assay results, overexpression of miR-652-3p did not improve proliferation ability in HCC cells (Figure S2A and S2B). Similar results were seen in the CCK8 assay (Figure S2C). Next, to ensure whether miR-652-3p could regulate the cell cycle of HCC cells, we used a flow cytometry assay to analyze the effect of miR-652-3p overexpression on the cell cycle in HCC. However, no significant difference in cell cycle was observed between the miR-652-3p overexpression group and NC group in Huh7 and HCC-LM3 cells (Figure S2D). These results suggested that miR-652-3p had no impact on the cell proliferation in HCC cells.

Next, transwell assays and wound healing assays were performed to evaluate the effect of miR-652-3p on the migration and invasion ability of HCC cells. And the results from transwell assays (Figure 2A and 2B) and wound healing assays (Figure 2C) showed that overexpression of miR-652-3p increased migration and invasion ability *in vitro*. Next, to fully identify the role of miR-652-3p in the metastatic ability in HCC cells, miR-652-3p inhibitors were used to decrease the expression of miR-652-3p *in vitro* (Figure 2D). And inhibition of miR-652-3p significantly decreased migration (Figure 2E and 2G) and invasion ability (Figure 2F) of HCC-LM3 and Huh7 cells *in vitro*. And inhibition of miR-652-3p suppressed HCC lung metastasis *in vivo* (Figure 2H). Compared with NC group, fewer lung metastatic niches were observed in the antigomiR group (Figure 2I). These results demonstrated that miR-652-3p significantly increased the migration and invasion ability of HCC cells both *in vitro* and *in vivo*.

MiR-652-3p regulated cell adhesion and EMT in HCC

To better understand the underlying mechanism of miR-652-3p in regulating HCC development, mRNA sequences were performed in the miR-652-3p overexpression group and negative control group in HCC-LM3 cells (Figure 3A). And through Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, we found that miR-652-3p regulated focal adhesion pathway and Ras signaling pathway (Figure 3B), which played indispensable roles in promoting cancer metastasis in HCC. Through GSEA analysis,

we found that EMT-associated pathway was enriched in the miR-652-3p overexpression group (Figure 3C). In contrast, no strong correlation was found among the cell cycle-associated pathway and miR-652-3p (Figure 3D), which were in accordance with our above results. And western blots were used to determine the expression of EMT-associated biomarkers. Overexpression of miR-652-3p increased the expression of N-cadherin and Snail while decreasing the expression of E-cadherin (Figure 3E). These results indicated that miR-652-3p might promote HCC metastasis via regulating EMT in HCC.

MiR-652-3p regulated the expression of TNRC6A in HCC cells

To identify potential target genes of miR-652-3p, three databases (miRDB, TargetScan, and miRMAP) were used, and four potential target genes (ISL1, TNRC6A, CAPZB, and NPTN) were eventually obtained through databases analysis (Figure 4A). Q-PCR was utilized to verify the mRNA expression of target genes, and we found that only TNRC6A, CAPZB, and NPTN were significantly downregulated in the miR-652-3p overexpression group (Figure 4B). Nevertheless, there was no significant difference in the mRNA expression of ISL1 between the miR-652-3p overexpression group and NC group. Through correlation analysis in the Starbase database, the expression of miR-652-3p was negatively correlated with the expression of TNRC6A, while the expression of miR-652-3p was positively correlated with the expression of CAPZB and NPTN in 370 cases of HCC (Figure 4C). So we selected TNRC6A as a potential direct target gene of miR-652-3p.

The 3′UTR of the TNRC6A contained a highly conserved and perfect complementary region for binding the seed sequence of miR-652-3p (Figure 4D). We next constructed the psi-TNRC6A-WT and psi-TNRC6A-MUT luciferase reporter plasmids. Overexpression of miR-652-3p significantly decreased the luciferase activity of HCC cells transfected with psi-TNRC6A-WT compared to the miRNA mimic negative control, while the mutation of the binding sites eliminated the effect of miR-652-3p on luciferase activity (Figure 4E). These results revealed that miR-652-3p regulated the expression of TNRC6A by directly binding to the 3′UTR of TNRC6A in HCC cells. Next, western blots were used to detect the protein level in HCC-LM3 and Huh7 cells after transfecting with miR-652-3p mimics. And the results showed that the protein level of TNRC6A was markedly decreased after miR-652-3p overexpression in HCC cells (Figure 4F). All these indicated that TNRC6A was a potential target gene of miR-652-3p, and the expression of TNRC6A was regulated by miR-652-3p.

TNRC6A inhibited migration and invasion of HCC cells

The role of TNRC6A was elusive in cancers including HCC. Firstly, through pan-cancer analysis, decreased TNRC6A expression was found in the tumor tissues in liver cancer(LIHC) and other cancers, compared to their normal tissues(Figure 5A). Alpha-fetoprotein (AFP) is known to be frequently highly expressed in HCC patients [33]. AFP serves as a standard diagnostic marker of liver cancer, promoting cancer cell growth, EMT and metastasis of HCC through regulating the expression of multiple oncogenes (including β-catenin and mTOR) [34-36]. Then, we want to investigate the relationship between the expression of TNRC6A and HCC malignancy, and lower TNRC6A expression was found in AFP high expression group (Figure 5B). Based on the data from TCGA database, we divided all data of HCC into two groups

according to the expression of TNRC6A (TNRC6A high expression group and TNRC6A low expression group), and the gene expression profiles of two groups were analyzed (Figure 5C). Moreover, through Gene Ontology (GO) analysis, we found that TNRC6A regulated cell adhesion pathway, which plays an important role in promoting EMT in cancers (Figure 5D).

Next, to evaluate the regulation effect of TNRC6A on the migration and invasion ability of HCC cells, we used siRNA to knockdown TNRC6A in Huh7 and HCC-LM3 cells transiently. The q-PCR results showed that the expression of TNRC6A was successfully knockdown after transfection (Figure 5E). From the results of transwell assays, knockdown of TNRC6A significantly increased the migration and invasion ability of Huh7 and HCC-LM3 cells (Figure 5F-5H). And the results from wound healing assays also supported the same conclusion that knockdown of TNRC6A promoted migration and invasion ability in HCC cells (Figure 5I and 5J). These results demonstrated that TNRC6A suppressed cancer metastatic ability in cancer cells, and TNRC6A may play an anti-cancer role in the development of HCC.

Inhibition of TNRC6A restored suppressed metastatic ability mediated by inhibition of miR-652-3p in HCC

To further investigate whether the biological function of miR-652-3p was mediated by TNRC6A, we used the miR-652-3p inhibitor and siTNRC6A to co-transfect Huh7 and HCC-LM3 cells, and then transwell assays (Figure 6A and 6B) and wound healing assays (Figure 6C and 6D) were performed. Results showed that inhibition of miR-652-3p suppressed migration and invasion ability in HCC cells, which was in accordance with our above results, and knockdown of TNRC6A restored cancer metastatic ability. Next, to explore whether TNRC6A was involved in regulating EMT-associated pathway mediated by miR-652-3p, western blots were carried out after transfection (Figure 6E and 6F). As expected, inhibition of miR-652-3p decreased mesenchymal biomarkers (N-cadherin and Snail), while knockdown of TNRC6A restored N-cadherin and Snail in HCC-LM3 and Huh7 cells. Inhibition of miR-652-3p increased epithelial biomarkers (E-cadherin), and knockdown of TNRC6A restricted its expression caused by miR-652-3p inhibition. What's more, from the results of lung metastasis assay in nude mice, we also found inhibition of miR-652-3p decreased the number of metastatic nodes in the lung, while knockdown of TNRC6A at the same time increased the number of metastatic nodes (Figure 6G). In addition，through IHC staining in the xenografts with different treatments, we also found that knockdown of TNRC6A decreased E-cadherin expression and increased N-cadherin and Snail expression *in vivo* (Figure 6H and 6I). These results demonstrated that TNRC6A was involved in regulating cancer metastasis, and EMT in HCC was mediated by miR-652-3p both *in vitro* and *in vivo.*

Transcription factor E2F1 promoted the expression of miR-652-3p in HCC

Mounting researches showed that transcription factors (TFs) trigger EMT and promote cancer metastasis through transcriptionally regulating the expression of miRNAs [37, 38]. To identify the potential transcription regulator which can influence the expression of miR-652-3p, JASPR database (http://jaspar.genereg.net/) was used. And E2F1 was found as a potential transcription regulator in promoting transcription of miR-652-3p. Interestingly, we have found that E2F1 promoted EMT and was enriched in highly metastatic HCC cells in our previous study. ChIP assay was used to demonstrate the

two putative E2F1 binding sites in the DNA sequence of has-miR-652-3p (Figure 7A and 7B). As Figure 7C showed, only the second binding site was verified as functional E2F1 binding site (Figure 7C)), and it is confirmed by Southern blot assay (Figure 7D). What's more, miR-652-3p-promoter-luciferase reporter plasmid system was constructed, and E2F1 overexpression plasmid (Figure 7E) was co-transfected into HCC-LM3 cells. After 72 hours, luciferase activities were detected, and results showed that E2F1 transcriptionally activated the expression of miR-652-3p in HCC-LM3 (Figure 7F) and Huh7 cells (Figure S3). And we found that overexpression of E2F1 increased the expression of miR652-3p (Figure 7G). In addition, through using TCGA data, we found that the expression of E2F1 was positively related with the expression of miR-652-3p in HCC (Figure 7H). All these data identified that the transcription factor E2F1 directly binded the specific DNA sequences of has-miR-652-3p and promoted its expression in HCC cells.

High expression of miR-652-3p and low expression of TNRC6A was related to worse prognosis in the HCC patients

To further assess the potential role of E2F1/miR-652-3p/TNRC6A axis in diagnosis and prognosis of HCC patients during clinical practice, the experiments of HCC tissues and corresponding adjacent tissues were performed. The expression of miR-652-3p in our 51 pairs of primary HCC tissues and corresponding adjacent tissues was firstly detected through PCR ($p<0.01$, Figure 8A) and in situ hybridization assay (Figure 8B), and upregulated miR-652-3p was found in HCC tissues. Interestingly, higher expression level of miR-652-3p was detected in the cancer tissues of HCC patients with metastasis, compared with patients without metastasis (Figure 8C), What's more, higher expression of E2F1 was found in cancer tissues of HCC patients with metastasis as well (Figure 8C), which was in accordance with our above results. Through TCGA data, high expression of miR-652-3p was found in the tumor tissues instead of normal tissue in Asian HCC patients (Figure 8D). The expression of miR-652-3p was negatively correlated with the expression of TNRC6A in HCC (Figure 4C). Through using KM plotter database, 166 cases of Asian HCC patients were divided into miR-652-3p high expression group and miR-652-3p low expression group, and high expression of miR-652-3p was correlated with low overall survival rate (Figure 8E). And from the data in TCGA, we found that TNRC6A had a low expression in tumor tissues in HCC (Figure S4A). Moreover, compared with TNRC6A high expression group, low expression of TNRC6A was correlated with poorer overall survival rate (Figure S4B). Through further analysis, we found that TNRC6A may play an essential role in predicting prognosis in Asian HCC patients and all HCC patients worldwide. Through KM plotter database, we found that high expression of TNRC6A was correlated with better overall survival rate (Figure 8F) and disease-free survival rate (Figure 8G) in HCC patients. Moreover, the patients with high expression of miR-652-3p and low expression of TNRC6A had the shortest survival time compared with other groups (Figure 8H). All these results indicated that E2F1/miR-652-3p/TNRC6A pathway played an indispensable role in HCC, which can serve as novel biomarkers in the diagnosis and predicting prognosis in HCC patients (Figure 8I).

Discussion

HCC is one of the most common malignancies worldwide. The 5-year survival rate of HCC is only 5%-30%, and the poor prognosis is mainly ascribed to the propensity for metastasis [4, 5]. Timely diagnosis and active treatment of HCC can significantly prolong the survival of HCC patients. The serum level of AFP is the most common tumor marker of HCC. However, AFP's false positive rate and relatively low sensitivity and specificity restrict its application in early diagnosis [39]. It is urgent to identify novel prognostic biomarkers and treatment targets for metastatic HCC. And for this case, as powerful diagnostic and therapeutic targets of many cancers, miRNAs hold great promise.

MiRNAs were reported to regulate almost all aspects of cancer biology, such as proliferation, invasion, apoptosis, metastasis, angiogenesis, etc. [40]. Our results showed that miR-652-3p promoted migration and invasion ability both *in vitro* and *in vivo* while having no impact on proliferation ability in HCC cells. Through acting as tumor suppressors or oncogenes, deregulated miRNAs resulted in different outcomes in the development of various cancers. The expression of miR-652-3p had been reported to be upregulated in human breast cancer, osteosarcoma, rectal cancer, non-small cell lung cancer, and bladder cancer [14–18], and in this study, we found miR-652-3p was upregulated in the tumor tissues, compared with normal tissues in HCC. As we known, miRNAs can be regulated by transcription factors in multiple cancers. The E2F transcription factor family control the cell cycle progression by regulating the timely expression of genes required for DNA synthesis [41]. E2F1 is one of most powerful transplantation factor of them, which has great influence in not only proliferation and tumorigenesis but also in apoptosis and differentiation [19–21]. E2F1 can increase the miR-224/miR-452 cluster in advanced melanoma and invasive/metastatic cell lines [42]. Our previous research showed that E2F1 promoted cellular and exosomal miRNAs in HCC [22]. In this research, we found that E2F1 transcriptionally activated the expression of miR-652-3p in HCC cells. What’s more, HCC tissue with higher expression of E2F1 had higher miR-652-3p expression.

EMT is an important prerequisite for metastasis and HCC development [43]. Epithelial cells lose their cell-cell adhesion characteristics, and they differentiate into a mesenchymal phenotype [44]. Cadherin-mediated adhesion also plays a significant role in EMT. Downregulation of E-cadherin is the fundamental event in EMT, which enhances the destabilization of adherens junctions [45]. The downregulation of E-cadherin is balanced by the upregulated expression of mesenchymal N-cadherin, which induces a ‘cadherin switch’ that alters cell adhesion [46, 47]. The changes in gene expression of EMT involve master regulators, including SNAIL, TWIST, and zinc-finger E-box-binding (ZEB) transcription factors, whose expression is activated early in EMT [45]. Snail family with its members snail transcriptional repressor 1 (SNAI1) and Slug (SNAI2) are the most significant inducers of EMT in HCC [43]. Through KEGG pathway analysis, we found that miR-652-3p is highly related to the focal adhesion pathway and Ras signaling pathway, and through GSEA analysis, we found that miR-652-3p has a very close relationship with the EMT-associated pathway. The overexpression of miR-652-3p increased the expression of N-cadherin and Snail while decreasing the expression of E-cadherin in HCC. All these indicated that miR-652-3p regulated cancer development and metastasis by regulating cell adhesion and EMT.

TNRC6A plays a crucial role in miRNA-mediated RNA silencing. It directly interacts with Ago proteins and promotes miRNA targets' translational repression and/or degradation [48]. TNRC6A was found expressed in multiple tumors, including prostate carcinomas, esophageal squamous cell carcinomas, gastric cancers, and colorectal cancers [30, 31]. However, a rare study on the effect of TNRC6A in cancer metastatic ability was found. A study revealed that miR-30, frequently overexpressed in multiple human cancers, promoted the development of cancer by suppressing two targets, CHD7 and TNRC6A, in cells and mouse models [49]. TNRC6A was found to be downregulated in moderately differentiated prostate tumors compared to normal glands of the peripheral zone [50], and TNRC6A was detected in tissues of colorectal cancer patients with shorter overall survival and poor prognosis [51]. Our findings showed that TNRC6A was a potential target gene of miR-652-3p, and miR-652-3p could directly regulate the expression of TNRC6A via binding its 3’-UTR. In addition, we found that knockdown of TNRC6A promoted cancer metastatic ability of HCC cells. Moreover, inhibition of TNRC6A restored suppressed metastatic ability mediated by inhibition of miR-652-3p in the HCC both *in vitro* and *in vivo.* Furthermore, high expression of miR-652-3p and low expression of TNRC6A correlated with a worse overall survival rate and disease-free survival rate in HCC patients.

Summarily, miR-652-3p and TNRC6A play a pivotal role in promoting metastasis in HCC, and carcinogenic transcription factor E2F1 promoted the expression of miR-652-3p directly. MiR-652-3p promotes EMT and metastasis of cancer cells in HCC by directly inhibiting the expression of TNRC6A. High expression of miR-652-3p and low expression of TNRC6A indicates poor prognosis in HCC patients. This knowledge could provide a new prognostic biomarker and treatment target for HCC.

Conclusion

In this study, through combining our miRNA sequencing data in HCC cells with different metastatic potential and TCGA data, we found that miR-652-3p was upregulated in HCC tumor tissues. MiR-652-3p promoted HCC metastasis via regulating EMT in HCC. Next, we found that TNRC6A was the direct target of miR-652-3p, which is negatively correlated with AFP and suppressed cancer metastasis in HCC. Knockdown of TNRC6A restored repressed EMT and metastasis in HCC caused by inhibition of miR-652-3p. Carcinogenic transcription factor E2F1 promoted the expression of miR652-3p directly. High expression of miR-652-3p and low expression of TNRC6A indicated poor prognosis in HCC patients. All these demonstrated that E2F1/miR-652-3p/TNRC6A played pivotal roles in promoting cancer malignancy and metastasis in HCC, and miR-652-3p and TNRC6A could be robust biomarkers to predict prognosis in HCC patients.

Abbreviations

HCC
Hepatocellular carcinoma
miRNA
microRNA

EMT
epithelial-mesenchymal transition
TNRC6A
Trinucleotide repeat-containing gene 6A protein
EVs
extracellular vesicles
NSCLC
non-small cell lung cancer
RRM
RNA recognition motif
GO
Gene Otology
OS
overall survival
DFS
disease-free survival
DMEM
Dulbecco's modified Eagle medium
FBS
fetal bovine serum
CCK8
cell counting kit-8
PBS
phosphate-buffered saline
q-PCR
quantitative polymerase chain reaction
ChIP
Chromatin Immunoprecipitation
TCGA
The Cancer Genome Atlas
KEGG
Kyoto Encyclopedia of Genes and Genomes
TFs
Transcription factors
AFP
Alpha-fetoprotein
NC
negative control.

Declarations

Ethics approval and consent to participate

The Ethics Committee of The First Affiliated Hospital of Chongqing Medical University approved the study protocol, which was in accordance with the *Declaration of Helsinki.* All participants provided signed written informed consent documentation. Animal experiments were undertaken following approval of the Animal Committee of the Zhejiang Academy of Medical Sciences animal center and were carried out conforming to the requirement of the guidelines of the National Institutes of Health (Guide for the Care and Use of Laboratory Animals, 2011).

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated/analyzed in the current study are available.

Competing interests

The authors declare that they have no competing interests.

Fundings

This study was supported by a grant from the Medical Health Science and Technology Project of Zhejiang Province (No. 2020KY675) and Natural Science Foundation of Zhejiang Province (LQ20H160037).

Acknowledgements

This study was supported by a grant from the Medical Health Science and Technology Project of Zhejiang Province (No. 2020KY675) and Natural Science Foundation of Zhejiang Province (LQ20H160037).

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Tables

Table 1 The sequence of primer

Name	Forward primer (5’–3’)	Reverse primer (5’–3’)
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
TNRC6A	CAGAACAGATAAAGCCCAGTGT	CTGTAGCTCGCTTGGCATTATTA
CAPZB	CCCAGCAAATCGAGAAAAACCT	CAAGGGAGGGTCATACTTGTTAC
ISL1	GCGGAGTGTAATCAGTATTTGGA	GCATTTGATCCCGTACAACCT
NPTN	GAGGTCATTATTCGAGACAGCC	TTGATCCTGTACTCCATGTTGC
hsa-miR-652-3p	AATGGCGCCACTAGGGTTGTG	
ChIP-site1	TCCGAGTGCAGAAGACGAG	CTTCTTCCCCTCCCTCTCTC
ChIP-site2	TTGCCCTTATCCAGGTTTTG	AGCTTCATTTCCCTGCTCCT

Figures

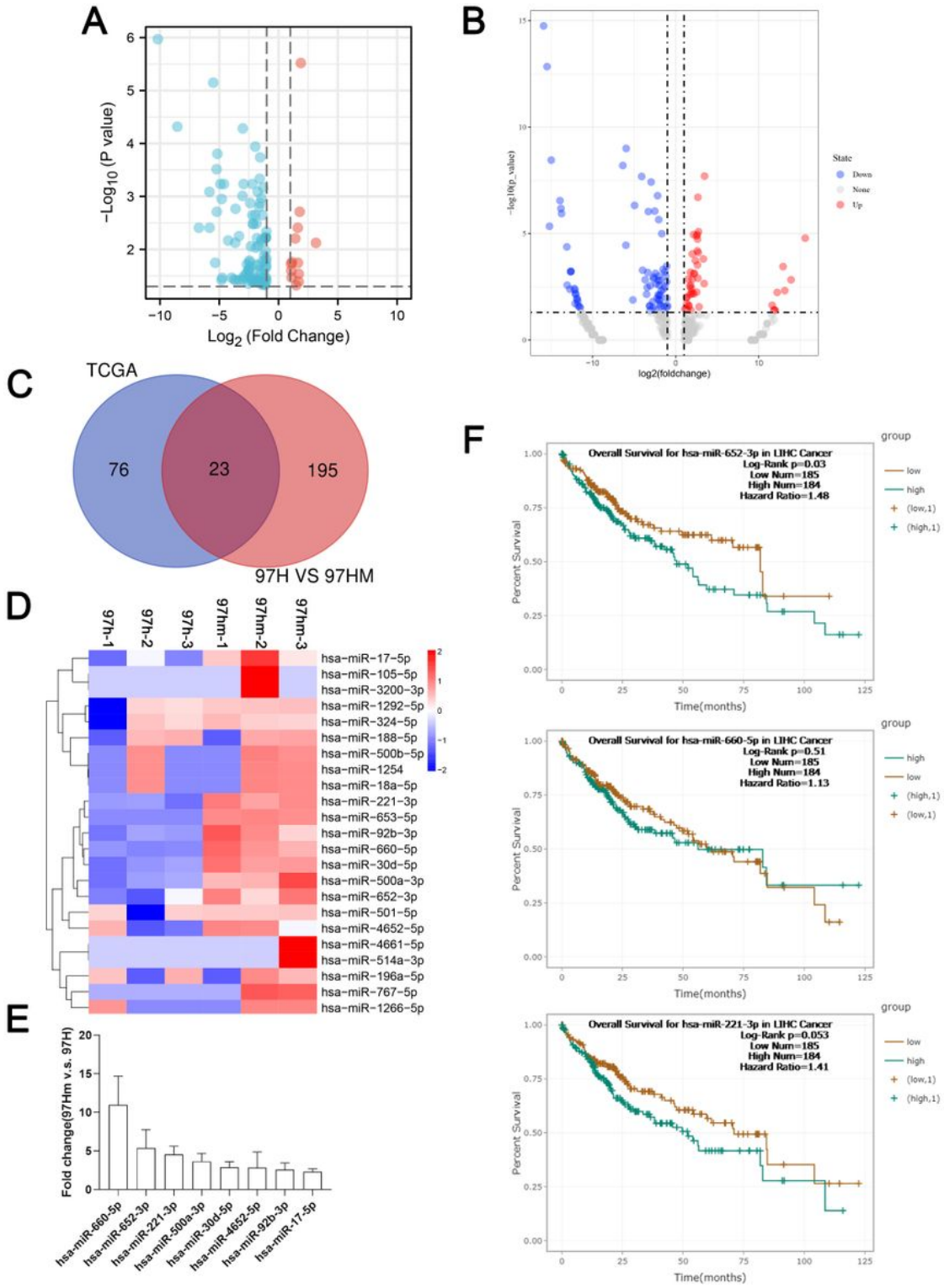

Figure 1

The expression of miR-652-3p was upregulated in HCC.

Volcano map of differential exosomal miRs between high metastatic HCC cells (97hm) and low metastatic HCC cells (97h) (A) and Volcano map of differential miRs between tumor tissue and tumor-adjacent tissue in Asian HCC patients were shown (B). The potential miRs were obtained through

combining analysis (C), and a heat map of these miRs in the exosomes of high metastatic HCC cells (97hm) and low metastatic HCC cells (97h) were presented (D). The top 8 miRs were ranked according to the fold change (E). The relationship between the overall survival rate of HCC and miR-652-3p, miR-660-5p, and miR-221-3p levels was shown. Data were obtained from the Starbase database (F).

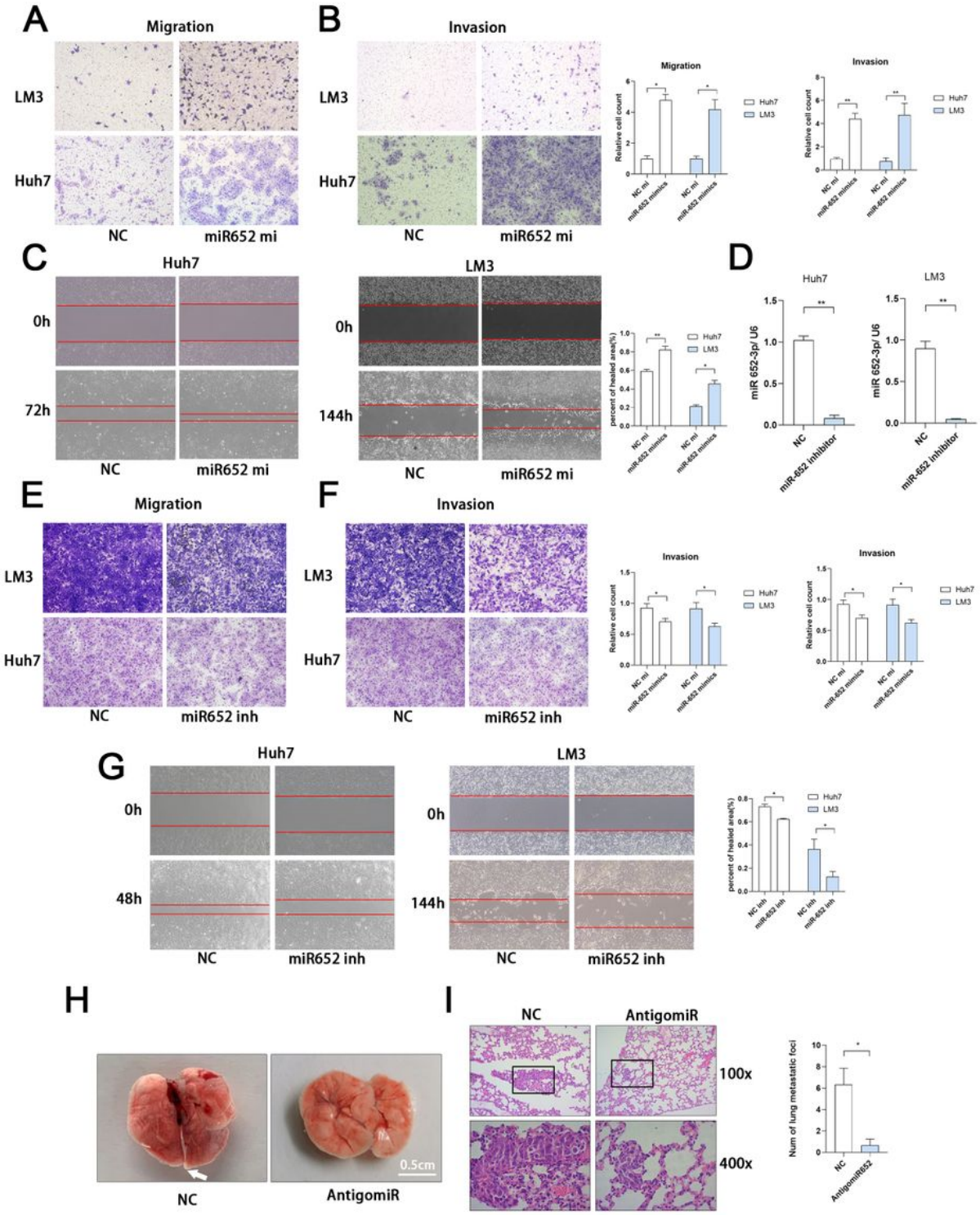

Figure 2

MiR-652-3p promoted the migration and invasion ability of HCC cells.

The effect of miR-652-3p overexpression in promoting migration and invasion ability of HCC-LM3 and Huh7 cells was measured by transwell assays (A, B) and wound healing assays (C). The expression of miR-652-3p in HCC cells with transfection of miR-652-3p inhibitor (D). And the effect of miR-652-3p inhibition in reducing migration and invasion ability of HCC-LM3 and Huh7 cells was measured by transwell assays (E, F) and wound healing assays (G). Compared with NC group, fewer lung metastatic niches were observed macroscopically (H) and microscopically (I) in the antigomiR-652-3p group（n=3）. *P <0.05, **P <0.01.

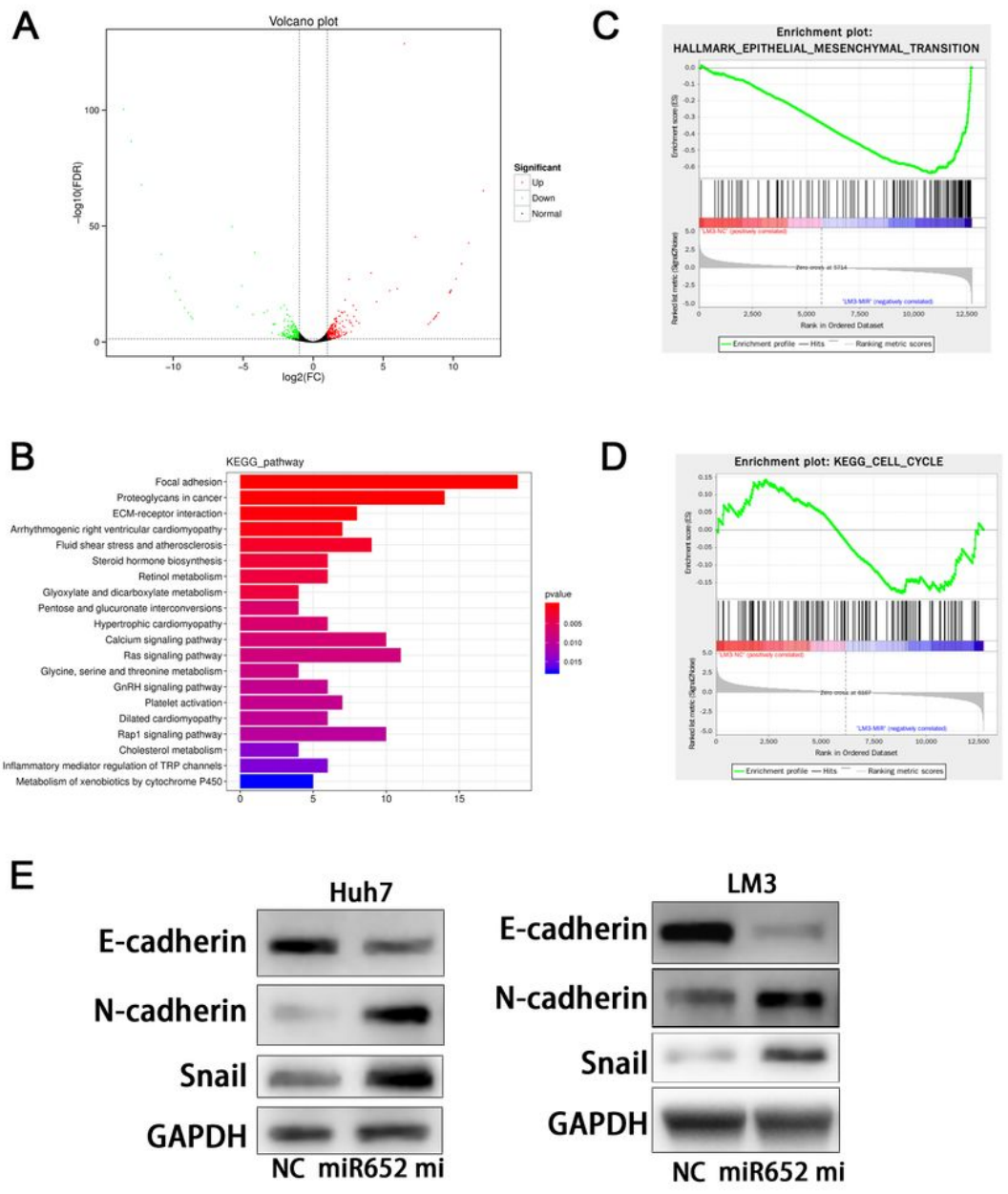

Figure 3

MiR-652-3p regulated cell adhesion and EMT in HCC.

Volcano map of differential mRNA expression level between miR-652-3p overexpression group and negative control group (A) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were shown (B). The EMT-associated pathway was found enriched in the miR-652-3p overexpression

group (C), while no strong correlation was found among the cell cycle-associated pathway and miR-652-3p in GSEA analysis (D). Western blots showed that overexpression of miR-652-3p increased the expression of EMT-associated proteins N-cadherin and Snail while decreasing the expression of E-cadherin (E).

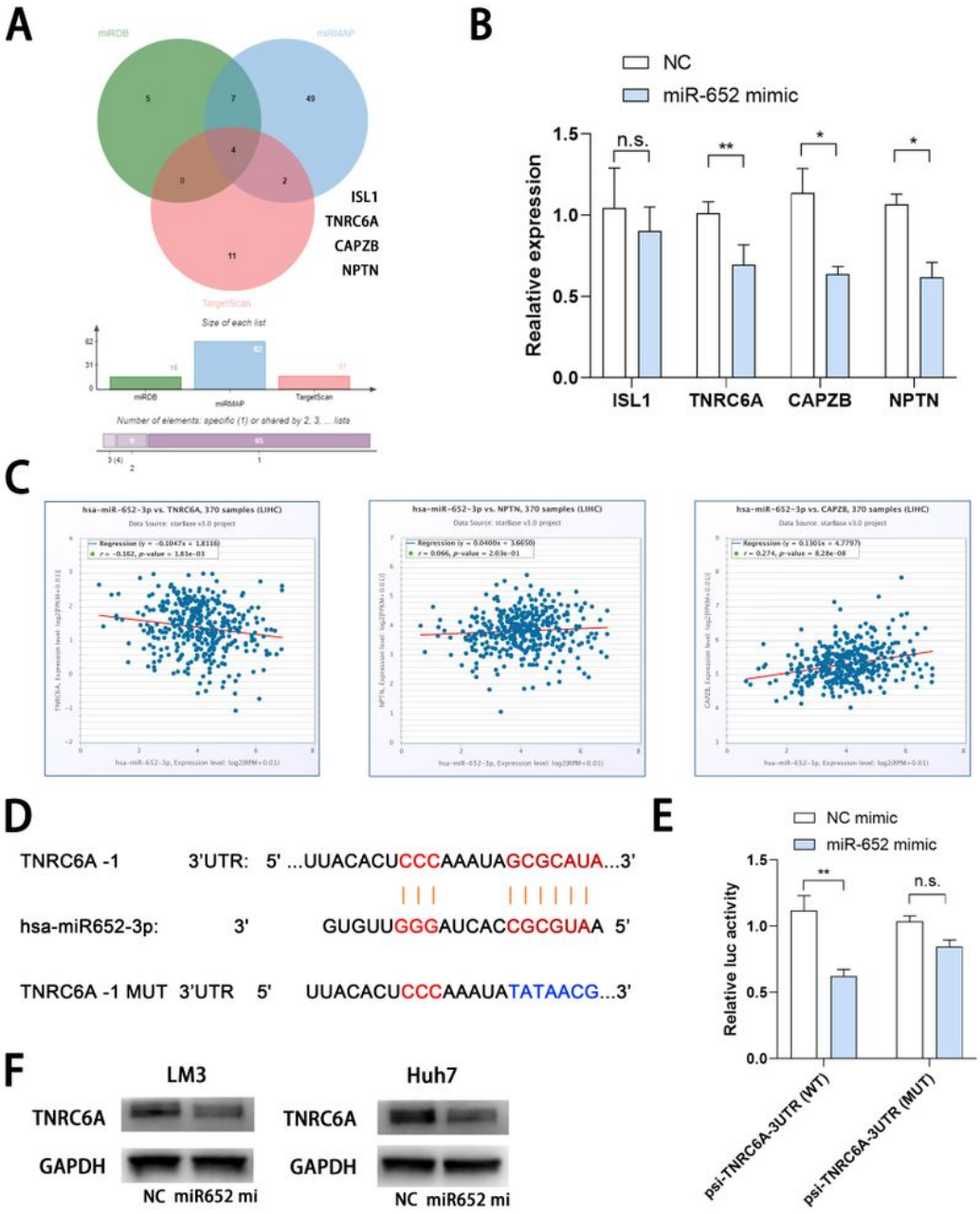

Figure 4

MiR-652-3p regulated the expression of TNRC6A in HCC cells.

Four potential target genes (ISL1, TNRC6A, CAPZB, and NPTN) of miR-652-3p were obtained through analysis of three databases (miRDB, miRMAP, and TargetScan databases) (A). The mRNA expression of four potential target genes between the miR-652-3p overexpression group and NC groups was shown (B). Correlation analysis of the expression of miR-652-3p and mRNA expression of TNRC6A, CAPZB, and NPTN in 370 cases of HCC were shown (C). The sequence alignment between miR-652-3p and TNRC6A mRNA showed the seed sites of miR-652-3p. A nucleotide substitution mutation of the seed sites of TNRC6A was constructed (D). HCC-LM3 was used to perform a Luciferase reporter assay in triplicate. And luciferase activities were measured after transfection of miR-652-3p mimics and negative control, and respective data was shown in the bar graphs, **P <0.01 (E). Western blots showed the protein level of TNRC6A after transfection of miR-652-3p in HCC-LM3 and Huh7 cells (F). n.s.: no significance. *P <0.05, **P <0.01.

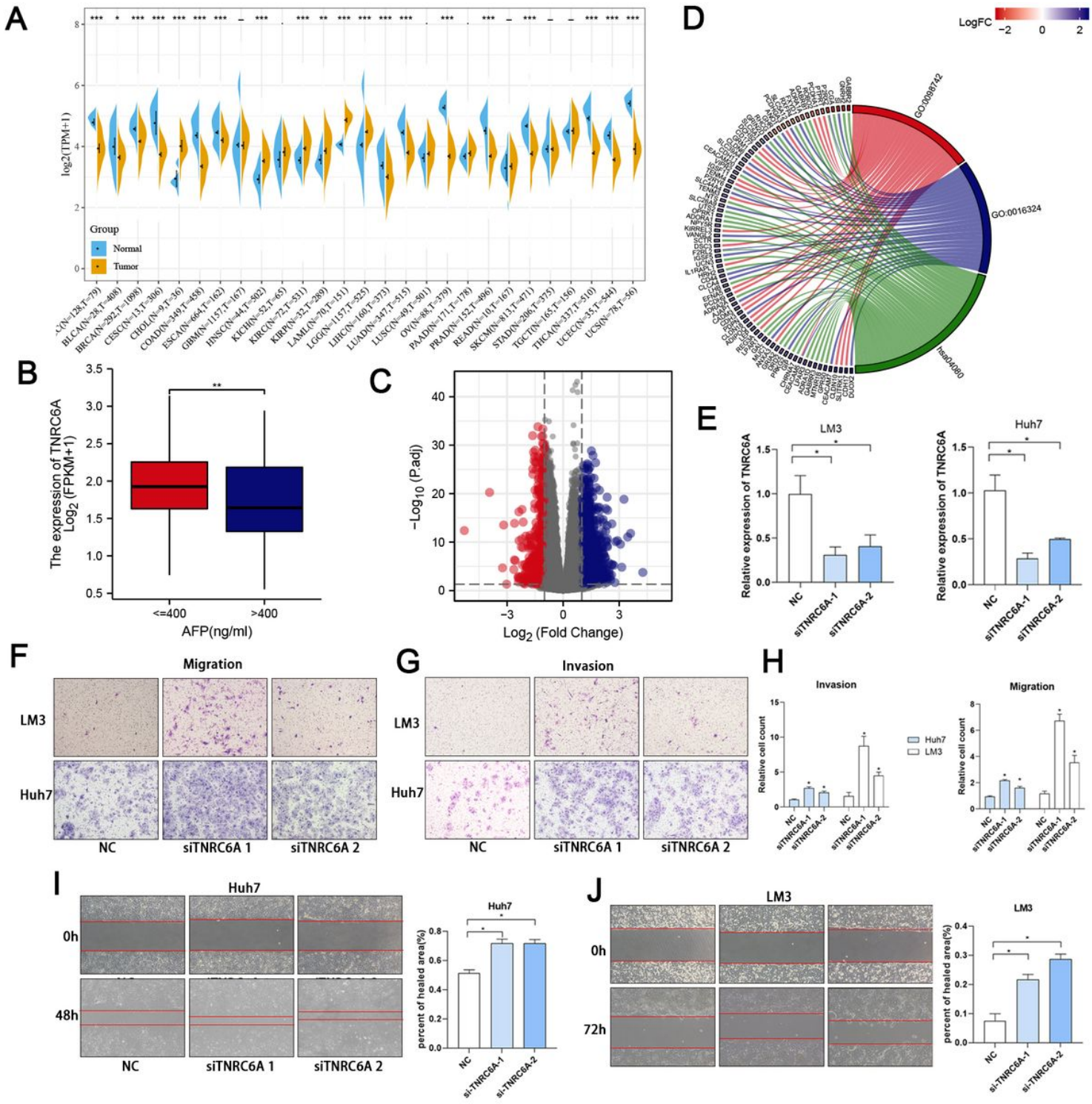

Figure 5

TNRC6A inhibited the migration and invasion ability of HCC cells.

The expression of TNRC6A in different cancers was shown (A). And the relationship of TNRC6A and AFP expression was presented (B). Volcano map of differential mRNA profile of TNRC6A high expression group and TNRC6A low expression group (C), and chordal graph of the relationship among TNRC6A and cell adhesion pathway associated genes was shown according to TCGA data (D). The expression of

TNRC6A after transfected by TNRC6A siRNA in HCC-LM3 and Huh7 cells was shown (E). The effects of TNRC6A knockdown in promoting migration and invasion ability of HCC-LM3 and Huh7 cells were measured by transwell assays (F, G, H) and wound healing assays (I, J). *P <0.05.

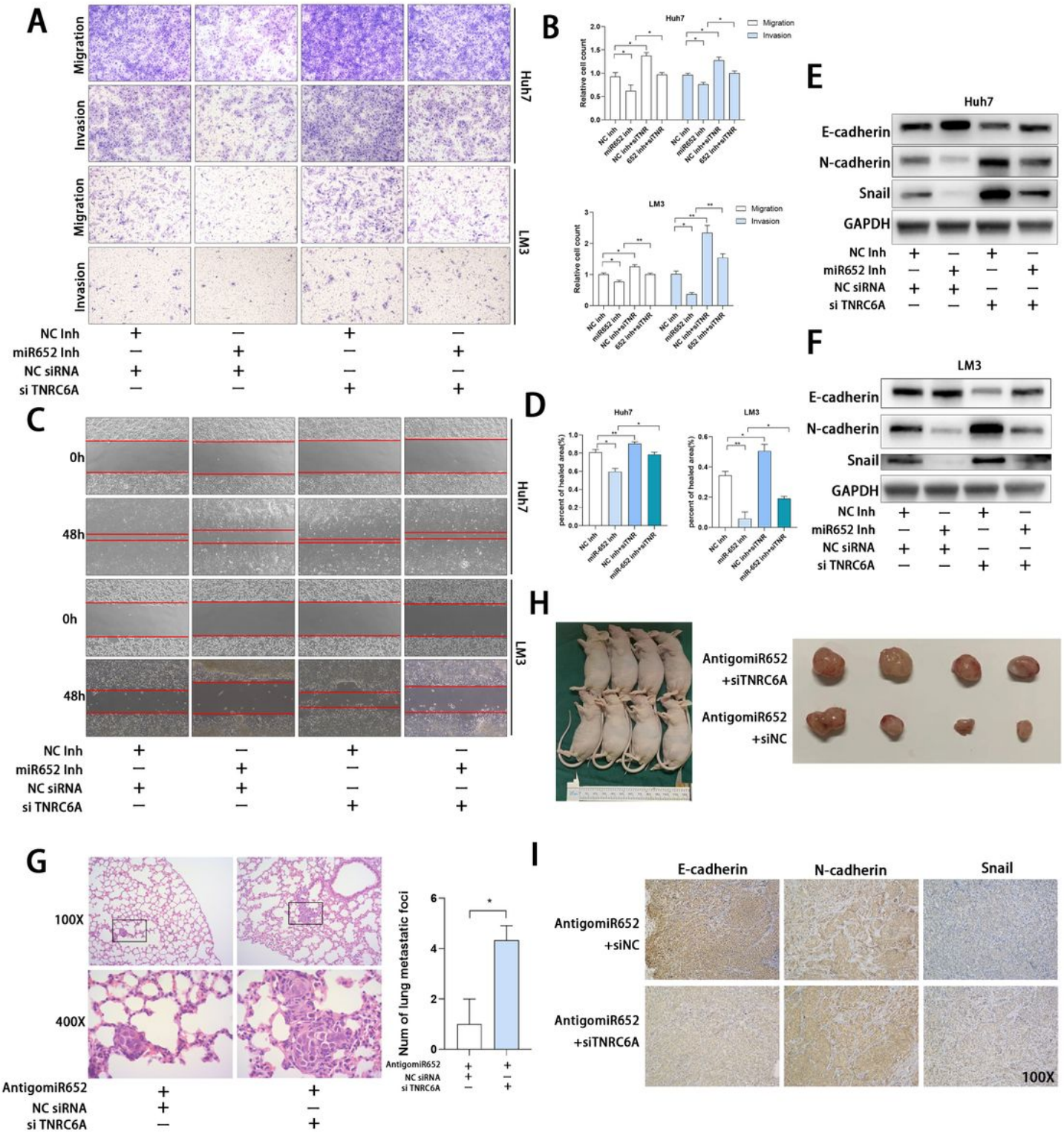

Figure 6

Inhibition of TNRC6A restored suppressed metastatic ability mediated by inhibition of miR-652-3p in HCC.

The results of transwell assays (A, B) and wound healing assays (C, D) were measured after co-transfected with miR-652-3p inhibitor or NC inhibitor and TNRC6A siRNA or NC siRNA in HCC-LM3 and Huh7 cells. The protein levels of E-cadherin, N-cadherin, and Snail in HCC-LM3 and Huh7 cells were shown (E, F). The lung metastatic tumors were observed after HE staining. The numbers of metastases per lung were calculated (n=3) (G). Photographic images of the mice and its xenograft tumors with different treatments (n=4) (H). Representative images from immunohistochemistry assays (E-cadherin, N-cadherin and Snail protein expression) of tumor sections were shown (I). The images were magnified 100 times. *P <0.05, **P <0.01.

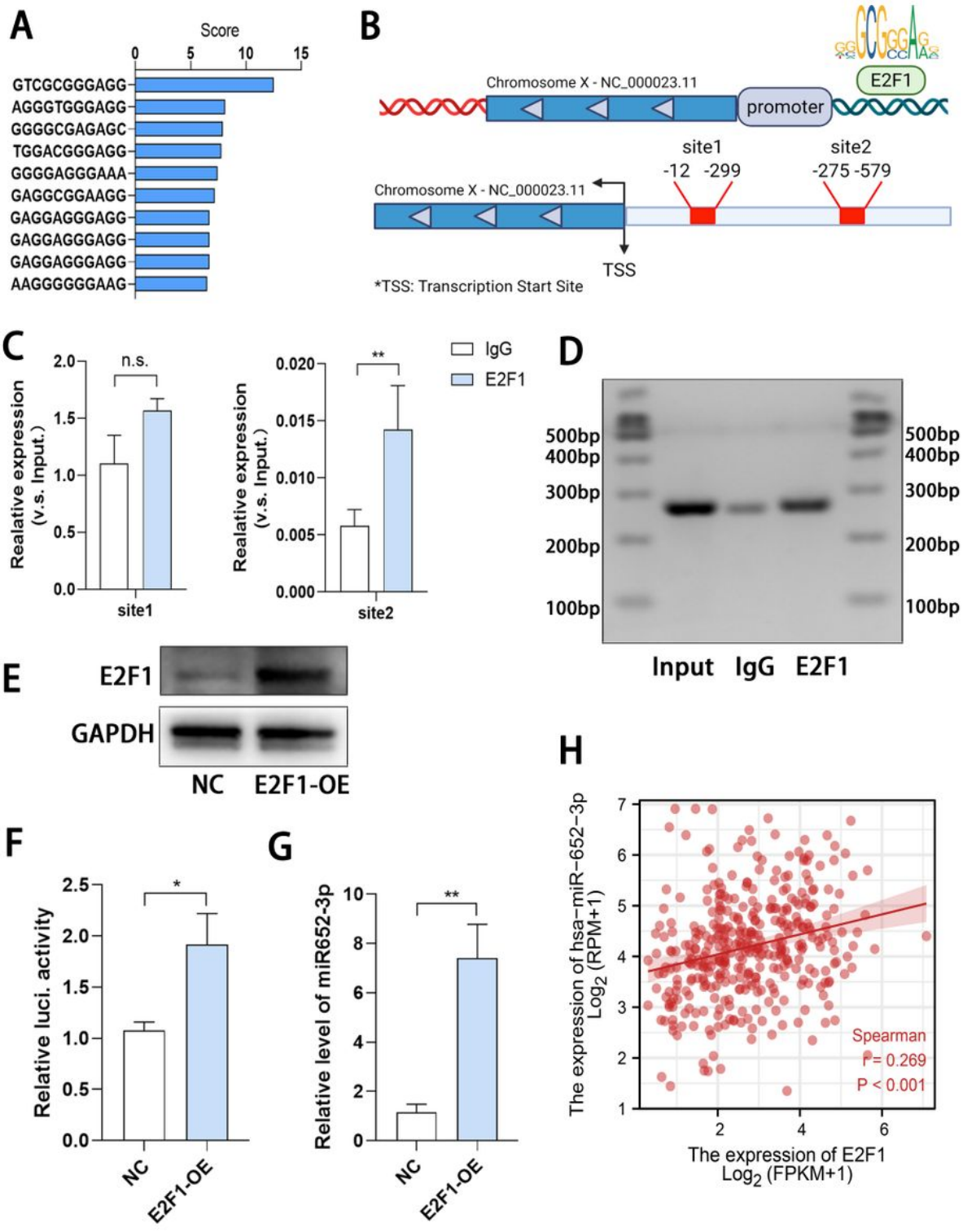

Figure 7

Transcription factor E2F1 promoted the expression of miR-652-3p in HCC.

The score of E2F1 potential binding sequences was shown (A). The diagram showed the selected binding sites of E2F1 in the specific sequence of the host gene of has-miR-652-3p (B). The expression of segments containing E2F1 binding sites was detected by q-PCR after ChIP assay in HCC-LM3 cells (C).

The result of Southern blot after ChIP assay was shown (D). HCC-LM3 cells were transfected with E2F1 overexpression plasmid (E). Relative luciferase activity was detected in HCC-LM3 cells after co-transfection of NC or E2F1 overexpression plasmid and miR-652-promoter-luciferase plasmids (F). The level of miR-652-3p in E2F1 overexpression group and NC group were measured by q-PCR (G). The relationship of E2F1 expression and miR652-3p expression in the patients of HCC from TCGA database was shown (H).

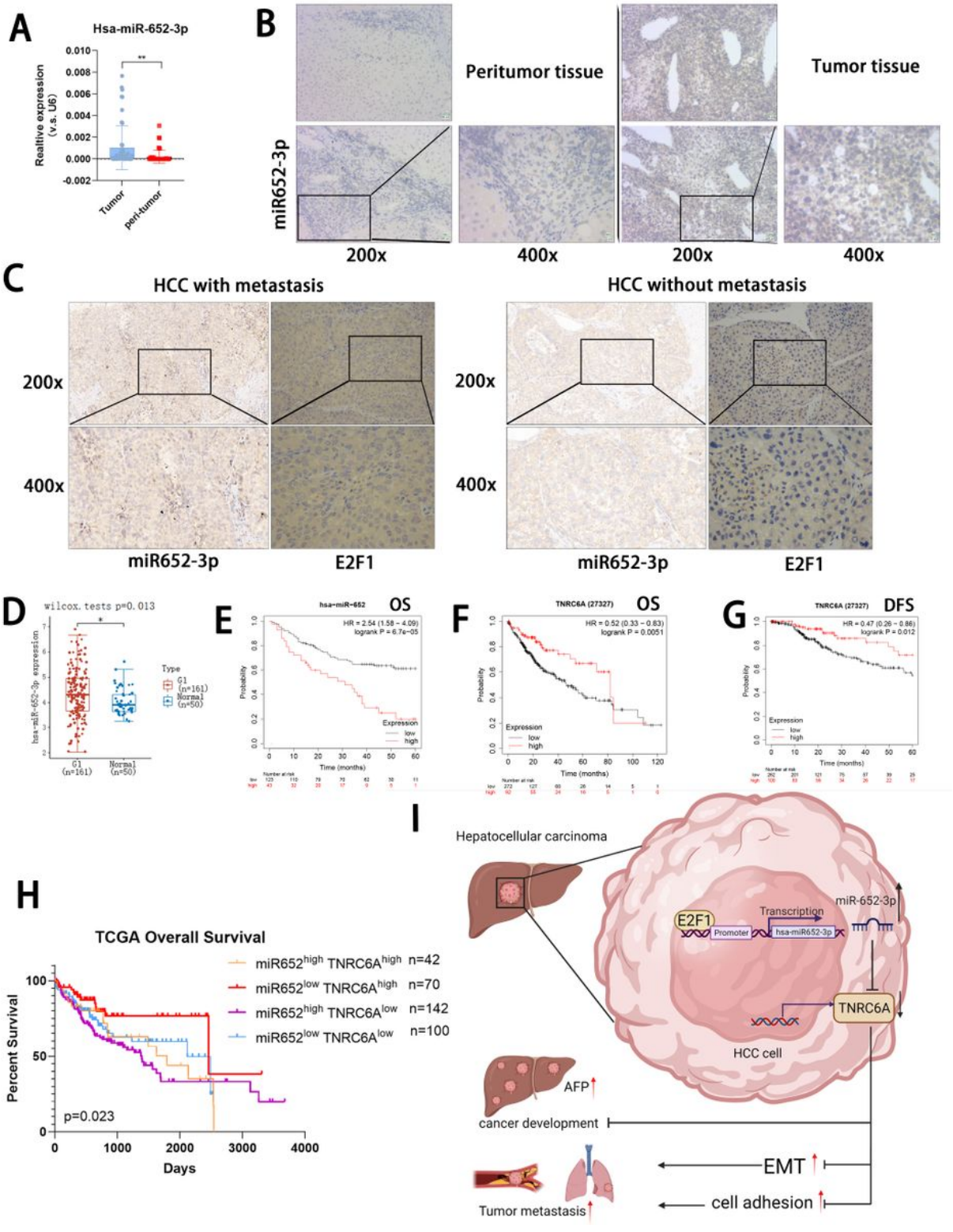

Figure 8

High expression of miR-652-3p and low expression of TNRC6A was related to worse prognosis in the HCC patients.

High expression of miR-652-3p was found in the tumor tissues instead of normal tissues in primary HCC patients (A). In situ hybridization of miR-652-3p in HCC tissue and its match adjacent liver tissue was shown (B). The expression of miR-652-3p and E2F1 in the tumor tissues of HCC patients with or without metastasis was shown (C). High expression of miR-652-3p was found in the tumor tissues instead of normal tissues (D), and high expression of miR-652-3p was correlated with low overall survival rate in Asian HCC patients from TCGA database (E). High expression of TNRC6A was correlated with better overall survival rate (F) and disease-free survival rate (G) in HCC patients from TCGA database. Overall survival analysis based on the co-expression of miR-652-3p and TNRC6A in HCC according to TCGA data (H). The diagram of E2F1/miR-652-3p/TNRC6A in regulating the development and metastasis of HCC was shown.

Supplementary Files

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- supplimentarydata20220424.docx