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Effects of miR-155 on proliferation and apoptosis by regulating FoxO3a/BIM in liver cancer cell line HCCLM3

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Abstract. – OBJECTIVE: MiR-155 has been shown to be up-regulated in hepatocellular carcinoma (HCC) patients. B-cell lymphoma-2 (Bcl-2) interacting mediator of cell death (BIM) regulates cell proliferation and apoptosis, as its down-regulation is involved in HCC onset. Transcriptional factor FoxO3a mediates BIM expression and is related to HCC pathogenesis. Bioinformatics analysis showed targeted regulation of FoxO3a by miR-155. This study aims to investigate whether miR-155 plays a role in mediating FoxO3a/BIM signal pathway and HCC occurrence.

PATIENTS AND METHODS: HCC patients were collected for tumor and adjacent tissues, in which microRNA-155 (miR-155) and FoxO3a e[illegible]pressions were examined. *In vitro* cultured [illegible]CLM3, HepG2 and L-02 cells were tested for [illegible]al apoptotic rate by flow cytometry and [illegible] compared for miR-155 and FoxO3a express[illegible] Dual-luciferase reporter gene assay demon[illegible]strated the targeted relationship bet[illegible]en miR-155 and FoxO3a. HCCLM3 cells [illegible] with miR-155 inhibitor and/or FoxO[illegible]MD[illegible] Cell apoptosis and proliferation were [illegible]ed by using flow cytometry and MTT as[illegible] respectively. Western blot and [illegible]ctrom[illegible]assay were employed to quan[illegible] FoxO3[illegible]BIM expressions, and caspase [illegible]tivit[illegible]

RESULTS: Compared t[illegible] tis[illegible]s, HCC tissues had significa[illegible]ly [illegible] miR-155 and significantly lower F[illegible]3a exp[illegible]n ($p<0.05$). HCCLM3 and HepG[illegible] had sig[illegible]cantly lower FoxO3a expres[illegible] basal apoptotic rate compared to L02 cells, w[illegible]iR-155 level was significantly h[illegible]er ($p<0.05$)[illegible]iR-155 targeted and inhibited [illegible]UT[illegible]f FoxO3a, increasing BIM expression, [illegible]sp[illegible]-3, and caspase-9 activities, and en[illegible] cell [illegible]optosis and weakening proliferation[illegible]

CON[illegible]ONS: [illegible] tissues elevated the miR-[illegible] a[illegible] suppressed the FoxO3a expressio[illegible] MiR[illegible]eted and inhibited FoxO3a exp[illegible] to suppress the BIM, depress caspas[illegible]d caspase-9 activities, therefore inhibiting [illegible]CC cell apoptosis and facilitating proliferation.

Key Words:
Hepatocellula[illegible] microRNA-155, FoxO3a, Cell apoptosis, [illegible] prolifer[illegible].

In[illegible]uction

P[illegible]ver [illegible]cer is a common malignant t[illegible]or [illegible]ic[illegible]nd is the fifth popular cancer [illegible]h third [illegible]st mortality, only next to pul[illegible]ry cancer and gastric carcinoma[1]. Hepatoc[illegible] carcinoma (HCC) is the major pathological [illegible]ype of liver cancer, occupying about 80%-90% of all cases[2]. FoxO3a is one important men[illegible]er of forehead transcriptional factor family. As [illegible]e definitive tumor suppressor gene, FoxO3a [illegible]s critical roles in inhibiting cell proliferation, arresting cell cycle, and inducing cell apoptosis[3]. FoxO3a down-regulation or inactivation has been confirmed to be related with occurrence, progression, and drug resistance of multiple tumors including colorectal cancer[4], breast cancer[5], and pancreatic carcinoma[6]. B-cell lymphoma-2 (Bcl-2) interacting mediator of cell death (BIM) is key mediator for mitochondrial apoptosis signal pathway, as it can activate Bax with cross-interaction with Bcl-2/Bax to initiate mitochondria-dependent endogenous apoptotic signal transduction[7]. FoxO3a also exerts anti-tumor effects via regulating expression of downstream molecule BIM, and inhibiting cell proliferation and facilitating cell apoptosis[8]. Increasing evidence[9,10] showed significantly lower FoxO3a expression or function in HCC tumor tissues, indicating possible role of FoxO3a abnormality in HCC pathogenesis. MicroRNA is one type of non-coding single-stranded RNA with 22-25 nucleotides length that is recently identified in eukaryotes. It regulates more than one-third of human genes via targeted degradation of mRNA or inhibition of translation,

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thus participating in tissue/organ development, cell proliferation, apoptosis, and differentiation, with increasing attentions in tumor occurrence by expressional or functional abnormality[11]. MiR-155 locates in human chromosome 21q21 and is coded by one highly conserved region in 3rd exon of B-cell integration cluster (BIC) gene[12]. Several researches show abnormal expression of miR-155 in multiple tumor tissues[13-15], indicating the close correlation with tumor occurrence, progression, and prognosis. Previous reports[16,17] showed significantly elevated miR-155 expression in HCC tumor tissues, indicating its possible oncogene role in HCC onset. Bioinformatics analysis showed satisfactory targeted complementary correlation between miR-155 and 3'-UTR of FoxO3a. This study thus investigated if miR-155 played a role in regulating FoxO3a expression and affecting proliferation and apoptosis of liver cancer cells.

Patients and Methods

Major Reagent and Materials

High migrated liver cancer cell line [illegible] low migrated liver cancer cell line [illegible]2, and normal human hepatocyte L02 were p[illegible]sed from Xinyu Bio-tech Co. Ltd. (Nanjing, [illegible] Dulbecco's modified eagle medium (DM[illegible] fetal bovine serum (FBS) a[illegible]cillin-str[illegible]tomycin were purchased fr[illegible] BRL. Co. Ltd. (Grand Island, NY, [illegible]). TR[illegible] traction kit was purcha[illegible]m [illegible]ch (St. Louis, MO, USA). [illegible]NE siRNA transfection reagent [illegible] purc[illegible]m Roche (Basel, Switzerlan[illegible] One-Step [illegible]e RT-PCR Master Mi[illegible] transcription kit was purchased from [illegible]er Scientific (Waltham, MA, US[illegible] SYB[illegible]e was purchased from Ta[illegible]a (Dalian, C[illegible] rabbit anti-human FoxO[illegible]onoclonal antibody (Catalogue No. ab15478[illegible]d rab[illegible]nti-human GAPDH monoclonal [illegible]ody [illegible]alogu[illegible]No. ab128915) were purcha[illegible]cam [illegible]nbridge, MA, USA). Rabbit anti [illegible]PIM[illegible]yclonal antibody (Catal[illegible]No. sc-[illegible]s purchased from San[illegible]otechnol[illegible] (Santa Cruz, CA, USA). [illegible]bbit [illegible]man histone H3.1 polyclonal anti[illegible]dy ([illegible]. 9728) was purchased from [illegible]aling Technology Inc. (Danvers, MA, U[illegible]seradish peroxidase (HRP) conjugated goat a[illegible] IgG (Catalogue No. 111-035-003) was purc[illegible]d from Jackson ImmunoResearch (West Grove, PA, USA). Caspase-9 and caspase-3 activity assay kits were [illegible]eyotime Biotech. (Shanghai, C[illegible]). Annex[illegible]I apoptosis kit was purcha[illegible]rom Beyoti[illegible]iotechnology (Shanghai, [illegible]ual-lucif[illegible]e reporter assay system and [illegible]mo[illegible]plasmids were all purcha[illegible]from Pr[illegible]dison, WI, USA).

Clinical I[illegible]at[illegible]

A total o[illegible] patie[illegible]who received treatment [illegible]he fi[illegible]ospital of Sun Yat-sen U[illegible]rsity from [illegible]er 2015 to May 2016 wer[illegible]ited. Tumor sa[illegible]ples and adjacent tissu[illegible]ted during the surgery. There are 2[illegible]ales a[illegible]ales, aging between 37 and 71 [illegible]rs (average [illegible]0.2 years). There were 10, 9, [illegible]nd 20 cases at TN[illegible] stage I, II, and III. All sample collections have obtained informed consents from patient[illegible] This study has been approved by the Ethical [illegible]mittee of our Institution.

[illegible]re

[illegible]CLM3, HepG2, and L-02 cells were kept in high glucose DMEM medium containing 10% [illegible]nd 1% penicillin-streptomycin, in a culture [illegible]ber at 37°C with 5% CO_2. Culture medium was changed every two days. Cells at log-growth phase with good status were used in further experiments.

Dual Luciferase Reporter Gene Assay

Using HEK293 DNA as the template, full-length fragment of 3'-UTR of FoxO3a gene was amplified. PCR products were collected from agarose gel, and were ligated into pGL-3M plasmid after enzymatic digestion. The recombinant plasmid was then used to transform DH5α competent cells. Primary screening was performed with PCR. Positive clones with correct sequences were selected for transfection further experiments. X-tremeGENE siRNA Transfection Reagent was used to co-transfect pGL3-FoxO3a-3'UTR-wt (or pGL3FoxO3a-3'UTR-mut) and miR-155 mimic into HEK293 cells. After 48 h continuous incubation, dual-luciferase activity was examined.

Construction of Over-expression Plasmid and Cell Transfection

Primers were designed based on mRNA sequence of FoxO3a obtained in Gene Bank (Forward, 5'-ATCCTCGAGATGGCAGAGGCACCGGCTTCC-3'; Reverse, 5'-ATGGATCCTCAGCCTGGCACCCAGCTCTG-3'). Using cDNA as the template, FoxO3a gene fragment was amplified.

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After gel extraction and purification, PCR products were ligated into pMD18-T vector with EcoR1 and Sal1 digestion to transform JM109 competent cells. By positive selection using ampicillin-containing culture medium, positive clone was further amplified to extract recombinant plasmid. With confirmation by DNA sequencing, X-tremeGENE siRNA Transfection Reagent was used to transfect inhibitor NC, miR-155 inhibitor, negative controlled plasmid (NC-pMD18-T) and over-expression plasmid (FoxO3a-pMD18-T) into HCCLM3 cells in five group: inhibitor NC, miR-155 inhibitor, NC-pMD18-T, FoxO3a-pMD18-T, and miR-155 inhibitor + FoxO3a-pMD18-T group. 72 h later cells were collected for assay.

qRT-PCR for Gene Expression Assay

TRIzol reagent was used to extract total RNA by lysis, chloroform extraction, isopropanol precipitation, 75% ethanol elution, and RNAse free water rehydration. Following manual instruction, ReverTra Ace qPCR RT Kit synthesized cDNA from RNA by reverse transcription. Using cDNA as the template, PCR amplification was carried u[illegible] function of Taq DNA polymerase. Prime[illegible]ces used were: miR-155P_F: 5'-ACGCTCA[illegible]AA TGCTAATCGTGATA-3'; miR-155P_R: 5'-[illegible]CC ATGTTGTCCACTGTCTCTG-3'; U6P_F: [illegible] TGGAACGATACAGAGAAG ATT-3'; U[illegible] 5'-GGAACGCTTCACGAATTT[illegible] FoxO3aP_F: 5'-TCGCG CACCAATTCCA[illegible]oxO3aP_R: 5'-TCGCTGTGGCTGAGTG[illegible]TC-3'; [illegible] 5'-ATCTCAGAGCAATGG[illegible]CC-3'; [illegible] 5'-ATTCGTGGGTGGTC[illegible] β-actinP_F: 5'-GAACCCTAAGGC[illegible]AC-3'; β-actinP_R: 5'-TGTCACGCACGA[illegible] CC-3'. [illegible] system, we added 4.5 [illegible]BR Green Mi[illegible]re, 1.0 µl forward primer[illegible]rse primer, 1.0 µl cDNA, and 2.5 µl [illegible]H_2O. [illegible]tion conditions were: 95°C for 5 min, followed [illegible]C 5 min and 60°C for 60 s[illegible]

Western [illegible]ot As[illegible]

Total [illegible]ere e[illegible]cted by routine methods. 60 [illegible]in s[illegible]es were loaded and separated in 10% [illegible]decyl sulphate-polyacry[illegible]el electr[illegible]esis (SDS-PAGE) (3 h), a[illegible]re [illegible]nsferred to polyvinylidene fluoride [illegible]DF) [illegible]mersham Biosciences, Pi[illegible] [illegible] (wet method, 250 mA cur[illegible]in). The membrane was blocked in 5% defatt[illegible]owder for 60 min, followed by primary anti[illegible]anti-FoxO3a at 1: 200, anti-BIM at 1: 200, anti-Histone H3.1 at 1: 200, or anti-GAPDH at 1: 500) incub[illegible]ernight. By phosphate buffered s[illegible] Twee[illegible]PBST) washing (3 times), HR[illegible]belled seco[illegible]y antibody (anti-mouse o[illegible]bbit at 1: [illegible]0 dilution) was added for [illegible]cuba[illegible] under room temperature. After PB[illegible]or three times, enhanced [illegible]miluminesc[illegible]CL, Thermo Scientific P[illegible]e, Ro[illegible]rd, IL, USA) reagent was added fo[illegible]min[illegible] incubation. The membrane was t[illegible]in the[illegible]k and scanned for data a[illegible]lysis [illegible]ant[illegible]One software.

Flow [illegible]metry for C[illegible] Apoptosis

C[illegible] collected in ethylenediam[illegible]etra[illegible]d (EDTA)-free trypsin, and were washed t[illegible]hosphate buffered saline [illegible]S). Total of 100 [illegible] binding buffer was used [illegible] re-suspend in 1-5 $\times 10^5$ cells. The mixture was [illegible]hen mixed wi[illegible] 5 µl Annexin V-FITC and 5 µl [illegible]I, and was in[illegible]ated in the dark for 20 min. Gal[illegible] flow cy[illegible]try (Beckman Coulter Inc., Brea, [illegible] used to test cell apoptosis.

MTT Assay for Cell Proliferation Activity

[illegible]CCLM3 cells from all transfected groups [illegible]eded into 96-well plate. After fully atta[illegible]o the wall, cells were continuously cultured for 72 h. Cells at different time points (24 h, 48 h, and 72 h post-transfection) were collected to test proliferation activity by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. In brief, 4 h before quenching incubation, 10 µl MTT solution was added into each test well. After 4 h continuous culture at 37°C, the original culture medium was discarded, with twice PBS washing. Total of 150 µl dimethyl sulphoxide (DMSO) was then added into each well for 10 min vortex until the complete resolving of crystal violet. Absorbance (A) values were measured at 450 nm in a microplate reader.

Caspase-3 and Caspase-9 Activity Assay

Common procedures for caspase-3 and caspase-9 activity were: standard dilutions of 0, 10, 20, 50, 100, and 200 µM pNA were prepared. Absorbance values at 405 nm wavelength (A405) were measured by a micro-plate reader to plot a standard curve with pNA concentration against A405 value. Culture medium was collected. Attached cells were digested with trypsin, and were collected into culture medium for 4°C centrifugation for 5 min at 600 ×g. The supernatant was carefully removed and washed out by PBS. Total of 100 µl lysis buffer was added for every 2×10^6 cells. Cells

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were lysed at 4°C for 15 mn, and were centrifuged at 18000 ×g with 4°C for 10 min. Supernatants were saved in pre-cold tubes for further use.

For caspase-3 activity assay, 2 mM Ac-DEVD-pNA was placed on ice, mixed with buffer and test samples, with 10 µl Ac-DEVD-pNA. The mixture was incubated at 37°C for 2 h. The A405 value was measured when color changed significantly. In assaying for caspase-9 activity, 2 mM Ac-LEHD-pNA was placed on ice, mixed with buffer and test samples, with 10 µl Ac-LEHD-pNA. The mixture was incubated at 37°C for 2 h. The A405 value was measured when color changed significantly.

Statistical Analysis

SPSS18.0 software (SPSS Inc., PASW Statistics for Windows, Chicago, IL, USA) was employed for data analysis. Measurement data were presented as mean ± standard deviation. Comparison o[illegible] measurement data betw[illegible] s performed in Student's *t*-tes[illegible] statistica[illegible] ificance was defined when $p<$ [illegible].

Result[illegible]

Elevated [illegible] 155 [illegible] Decreased FoxO3a [illegible] in H[illegible] Tissues

qRT-PCR [illegible] owed [illegible] nificantly higher miR-15[illegible] pres[illegible] umor tissues compared [illegible] djacent tiss[illegible] n further higher level in s[illegible] tissues compared to those in stage I-II tu[illegible] 1A). Stage III HCC tumor tissues a[illegible] had [illegible] O3a mRNA expression than [illegible] ge I-II tum[illegible] ich also had higher levels [illegible] han adjacent tissues (Figure 1B). Western blot results also showed lower FoxO3a protein expression in both [illegible] plasm and nucleus of HCC tumor tissues fro[illegible] ge I-II compared to those in adja-

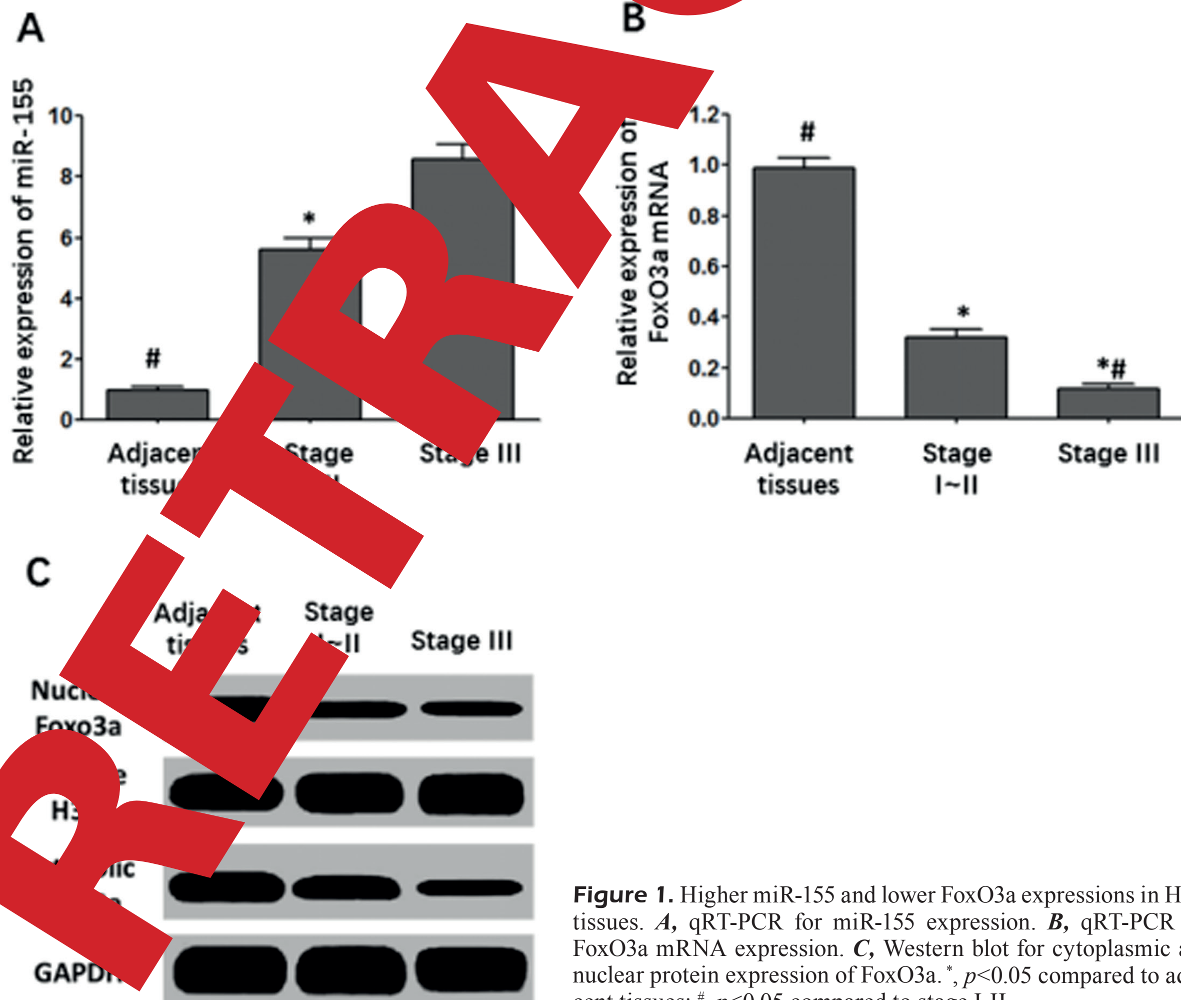

Figure 1. Higher miR-155 and lower FoxO3a expressions in HCC tissues. ***A,*** qRT-PCR for miR-155 expression. ***B,*** qRT-PCR for FoxO3a mRNA expression. ***C,*** Western blot for cytoplasmic and nuclear protein expression of FoxO3a. *, $p<0.05$ compared to adjacent tissues; #, $p<0.05$ compared to stage I-II.

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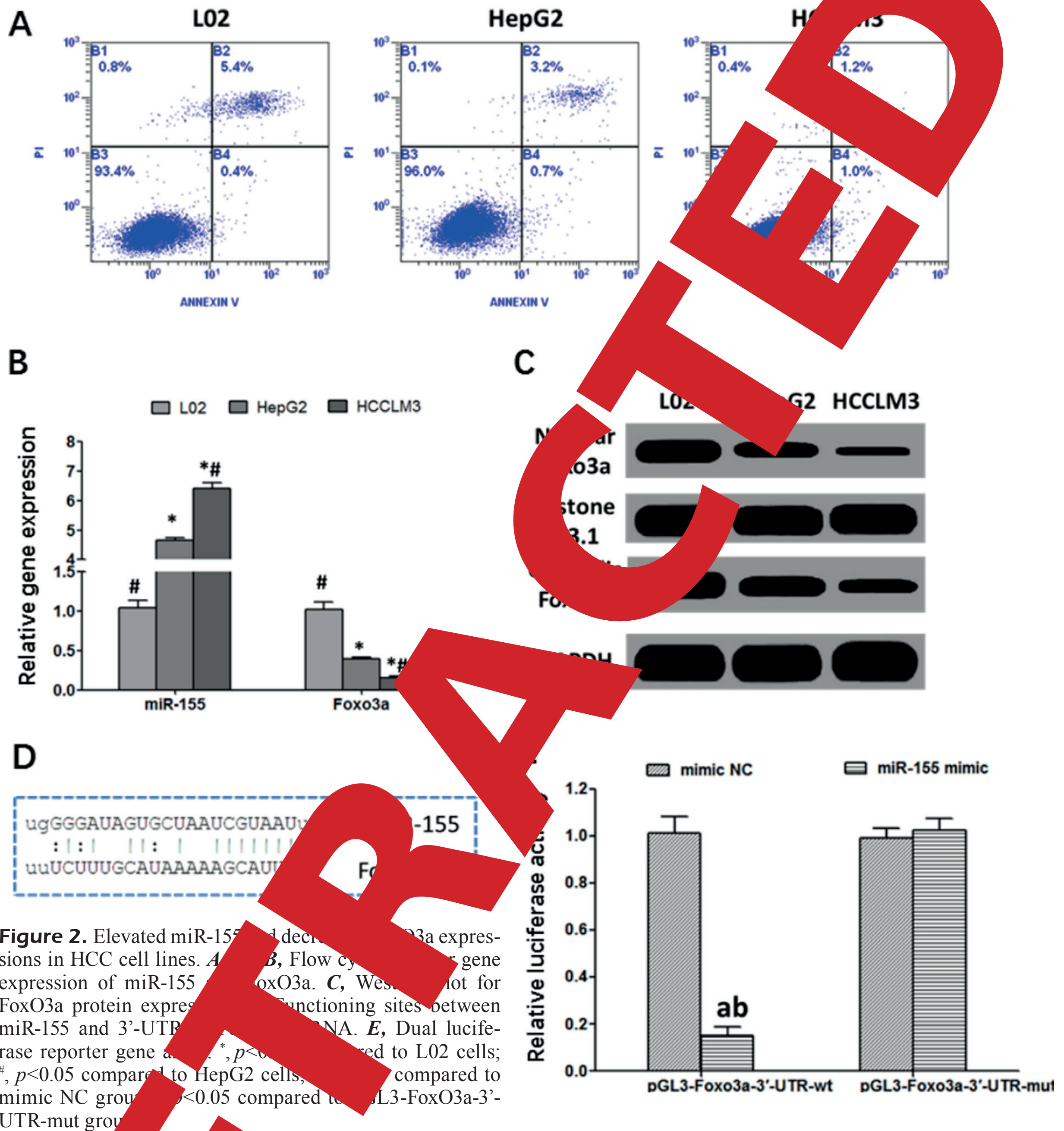

Figure 2. Elevated miR-155 [illegible] expressions in HCC cell lines. *A* [illegible] *B,* Flow cy[illegible] gene expression of miR-155 [illegible]oxO3a. *C,* Wes[illegible]lot for FoxO3a protein expres[illegible] Functioning sites between miR-155 and 3'-UTR [illegible]NA. *E,* Dual luciferase reporter gene a[illegible]. *, *p*<[illegible]red to L02 cells; #, *p*<0.05 compared to HepG2 cells, [illegible] compared to mimic NC group; [illegible]<0.05 compared to [illegible]L3-FoxO3a-3'-UTR-mut group.

cent tissues. [illegible] HCC [illegible]mor tissues also had remarkably lowe[illegible]ic or nuclear FoxO3a pro[illegible]s than st[illegible]II tumors (Figure 1C).

[illegible]vate[illegible] Expression and [illegible] FoxO3a Expression in HCC C[illegible]

Flo[illegible]etry results showed significantly lower basa[illegible]ptotic rate in HCC cell lines HCCLM3 and HepG2 compared to normal human hepatocyte L02, with higher apoptotic rate in highly malignant cell line MHCC97-H compared to that in low malignant cell line HepG2 (Figure 2A). qRT-PCR results showed significantly higher miR-155 expression in HCCLM3 and HepG2 cells compared to L02 cells, whilst FoxO3a mRNA level was significantly lower (Figure 2B). Western blot results showed significantly lower FoxO3a proteins in both cytoplasm and nucleus of HCCLM3 and HepG2 cells compared to L02

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cells (Figure 2C). These results suggested the potential correlation between abnormally elevated miR-155 in HCC and lower FoxO3a, less apoptosis and enhanced malignant phenotype. Bioinformatics analysis indicated targeted complementary binding sites between miR-155 and 3'-UTR of FoxO3a mRNA (Figure 2D). Dual-luciferase reporter gene assay showed that transfection of miR-155 significantly depressed relative luciferase activity in HEK293 cells expressing wild-type FoxO3a-3'-UTR plasmids (Figure 2E), indicating that miR-155 could be targeted 3'-UTR of FoxO3a mRNA to regulate its expression.

MiR-155 Down-regulation Facilitated HCCLM3 Cell Apoptosis and Weakened Proliferation

Using HCCLM3 cells as the research objects, transfection of miR-155 inhibitor and/or FoxO3a over-expressing vector (FoxO3a-pMD18T) significantly elevated both cytoplasmic and nuclear expression of FoxO3a proteins in HCCLM3 cells (Figure 3A). It enhanced expression of downstream targeted gene BIM significantl[illegible] 3A and 3B), potentiating caspase-9 an[illegible] activities (Figure 3C), increasing ce[illegible]ptosis (Figure 3E) and weakening proliferatio[illegible]vit[illegible] (Figure 3D).

Discussi[illegible]

HCC is one of the m[illegible]la[illegible]u-mors in China. Both its in[illegible]nd mortality are in the front line [illegible]ncers. [illegible] features including high mal[illegible]ncy, high m[illegible]c rate, high recurrent ra[illegible]low survival [illegible]e, all of which severely l[illegible]eatment efficacy of HCC, causing u[illegible]avorab[illegible]on both survival and prog[illegible]is of patients. [illegible]evious study[18] showed hi[illegible] than 50% recurrent rate in HCC patients a[illegible] surge[illegible]Moreover, most patients are already[illegible]rmi[illegible]age w[illegible]n diagnosis of HCC. There[illegible]y dia[illegible]is and treatment are of critical i[illegible]e f[illegible]nproving prognosis of HC[illegible]ients[19].

[illegible]d transc[illegible]on factor O subfamily [illegible]xO) [illegible]group of highly conserved tran-[illegible]ripti[illegible]d is widely involved in regu-[illegible] multiple biological processes including e[illegible] development, cell proliferation, differentia[illegible]ptosis, and cell cycle. FoxO transcriptiona[illegible]ctor family includes four members, including FoxO1, FoxO3a, FoxO4, and FoxO6, which share identical D[illegible] domain. These four family me[illegible]s, howev[illegible] coded by different genes f[illegible] different chi[illegible]somes. FoxO3a is the most [illegible]udied tra[illegible]iptional factor so far. FoxO3a [illegible] o[illegible]onserved transcriptional f[illegible]r family [illegible]chromosome 6q21, and [illegible]odes [illegible]e prote[illegible]olecule consisting of 67[illegible]mino[illegible]s. FoxO3a protein own unique for[illegible] DN[illegible] bindi[illegible]omain with highly conse[illegible]re, w[illegible] consists of three α-helix[illegible]ee β p[illegible] wing-like structures. F[illegible]3a is one [illegible]e tumor suppressor gen[illegible] regulates m[illegible]iple signal pathways an[illegible]behaviors as the key point, exert[illegible] critica[illegible] inhibiting cell proliferation, [illegible]esting cell c[illegible]d inducing apoptosis[3]. The [illegible]xpression and abnormal activation of FoxO3a are closely correlated with tumor occurrence, vascular metas[illegible]s, drug resistance, and metastatic homeos[illegible]. As one tumor suppressor gene, [illegible]xO3a [illegible]xert its anti-tumor roles including [illegible]ell proliferation and facilitating cell apo[illegible]sis via regulating the expression of downstream pro-apoptotic factors such as p27Kip1[20] [illegible]IM[8]. Increasing studies[9,10] showed the po[illegible]l role of FoxO3a expression in HCC onset. MiR-155 locates in human chromosome 21q21, and is coded by one highly conserved region in 3rd exon of BIC gene[12]. Previous investigations[16,17] showed significantly elevated miR-155 expression in HCC tumor tissues, indicating its potential tumor-facilitating role in HCC pathogenesis. Bioinformatics analysis results showed satisfactory targeted complementary relationship between miR-155 and 3'-UTR of FOxO3a. Therefore, this study investigated if miR-155 played a role in regulating FoxO3a expression and affecting proliferation and apoptosis of HCC cells.

Results of this work showed significantly elevated miR-155 expression in HCC tumor tissues, with even higher expression level in more advanced TNM stage. Compared to adjacent tissues, HCC tumor tissues had significantly lower cytoplasmic and nuclear FoxO3a protein expression, which may be correlated with clinical stage. These results showed that FoxO3a transcriptional activity in nucleus of HCC tissues was remarkably decreased, largely contributed by the lower level of total FoxO3a proteins, indicating the possible role of miR-155 in inhibiting FOxO3a expression and facilitating HCC onset. Lu et al[9] showed significantly lower FoxO3a expression in HCC tumor tissues and the close correlation with pathological grade and tumor size. Xie et

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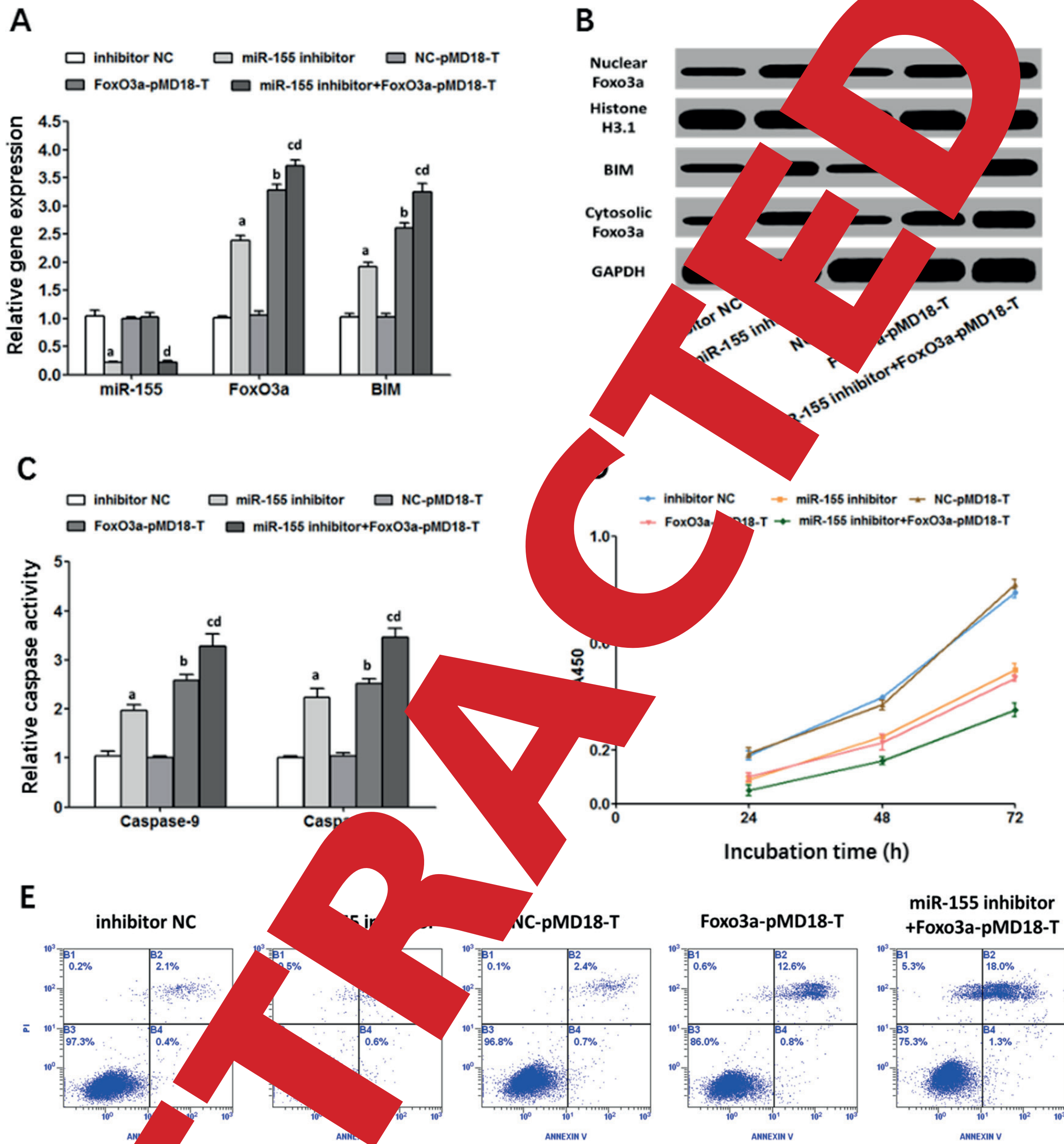

Figure 3. D[illegible]ased [illegible]55 expression facilitated HCCLM3 cell apoptosis and enhanced their proliferation ability. ***A,*** qRT-PCR f[illegible]e exp[illegible]on. ***B,*** W[illegible]stern blot for protein expression. ***C,*** Spectrometry for Caspase activity. ***D,*** MTT assay for cell prolif[illegible]***E,*** Flow[illegible]metry for cell apoptosis. [a], $p<0.05$ comparing between miR-155 inhibitor and inhibitor NC groups. [b], p[illegible]parin[illegible]ween FoxO3a-pMD18-T and NC-pMD18-T groups. [c], $p<0.05$ comparing between miR-155 inhibitor + FoxO3[illegible]inhibitor NC groups. [d], $p<0.05$ comparing between miR-155 inhibitor + FoxO3a-pMD18-T and N[illegible]8-T gro[illegible]

[illegible] showe[illegible]red to normal liver tissues, [illegible]tissue[illegible]ad remarkably lower expres-si[illegible]nd activity of FoxO3a, plus significant corre[illegible]h patient's prognosis. This study observed r[illegible]ably lower FoxO3a expression in HCC tumor tissues, as consistent with Lu et al[9] and Xie et al[10]. Moreover, compared to HepG2, highly malignant HCC cell line HCCLM3 had significantly decreased FoxO3a expression, indicating its probable correlation with enhanced malignant features of liver cancer cells. Guan et al[21] showed significantly higher miR-155 expres-

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sion in HCC tumor tissues, with correlation with vascular metastasis, Edmonson grade and clinical stage. Moreover, those patients having relatively higher miR-155 had lower one-year recurrence-free survival (RFS) compared to those with lower miR-155 expression, indicating that miR-155 up-regulation was correlated with unfavorable prognosis. Han et al[22] also revealed higher miR-155 level in HCC tumor tissues compared to normal liver tissues, as higher expression level indicated lower RFS or overall survival rate. Zhang et al[16] showed significant correlation between abnormally elevated miR-155 with tumor size, Edmonson grade, TNM stage and 5-year survival rate. Results of this study showed significantly elevated miR-155 expression in HCC tumors, as consistent with Guan et al[21] and Han et al[22]. Han et al found abnormally higher miR-155 expression level in HCC cells HepG2 and SMMC-7721[22], as similar to our observation showing higher miR-155 in liver cancer cells. Dual-luciferase reporter gene assay showed that transfection of miR-155 mimic could significantly decrease relative luciferase activity inside HEK293 cells, c[illegible]ing that FoxO3a was the targeted gene of m[illegible] der its regulation. Further test results s[illegible]d that transfection of miR-155 inhibitor and/o[illegible]O3a over-expression plasmids all significantly [illegible]ted FoxO3a expression in HCCLM3 cells, i[illegible]asing expression of downst[illegible]o-apopto[illegible] protein BIM, enhancing cas[illegible] caspase-[illegible] activity, increasing cell [illegible]ptosis [illegible] ning proliferation activ[illegible]rba[illegible] et al[23] showed that chemothe[illegible]nt melatonin could significantly s[illegible]ress p[illegible]lation of FoxO3a in HepG2 [illegible], enhancing [illegible]r transcription activity [illegible]pression of downstream pro-apoptot[illegible] to potentiate cell apoptosis. Kong [illegible] al[24] [illegible] miR-155 could enhance act[illegible]y of PI3K/G[illegible]ta-catenin signal pathy[illegible] via targeted inhibition on P85α expressio[illegible] orde[illegible] facilitate EMT process and invasio[illegible]lity [illegible]CC c[illegible]. Tang et al[25] found that m[illegible]d targ[illegible]nd inhibit FBXW7 expression [illegible]e [illegible]*tro* proliferation ability [illegible] *vivo* t[illegible]sis ability of HCC cells. [illegible][16] showed [illegible]t miR-155 could facilitate [illegible]er ca[illegible]roliferation and cycle progression [illegible] tar[illegible]on on ARID2 expression to [illegible] their apoptosis. Previous studies mostly [illegible]d the correlation between FoxO3a or miR-[illegible] liver cancer. This study, however, revealed t[illegible]ole of miR-155 up-regulation in suppressing FoxO3a expression, antagonizing HCC cell apoptosis and enha[illegible]tosis, all of which have not bee[illegible]orted yet.

Conc[illegible]

HCC tissu[illegible]ave significan[illegible]higher miR-155 expressi[illegible]plus [illegible]er FoxO3a expression. So, miR-15[illegible]eas[illegible]xpres[illegible] of downstream pro-apoptoti[illegible]BIM v[illegible]rgeted inhibition on Fox[illegible]expr[illegible]ken activity of caspase[illegible]nd caspase-[illegible]inhibiting HCC cell apo[illegible]nd enhancing [illegible]ll proliferation.

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Co[illegible]t of Interest

The Authors declare that they have no conflict of interest.

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