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Targeting of miR-20a against CFLAR to potentiate TRAIL-induced apoptotic sensitivity in HepG2 cells

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Abstract. – OBJECTIVE: Elevated expression of caspase-8 (CASP8) and Fas-associating protein with a novel death domain (FADD)-like apoptosis regulator (CFLAR) increases sensitivity against tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced cell apoptosis, but with an unclear mechanism. A previous study showed decreased microRNA-20a (miR-20a) expression in hepatocellular carcinoma (HCC) patient's tumor tissues. Bioinformatics analysis showed potential targeting relationship between the 3-UTR of CFLAR and miR-20a. This study investigated if miR-20a played a role in regulating CFLAR expression and HCC apopto[illegible]

PATIENTS AND METHODS: Expressions[illegible] 20a and CFLAR in model rat HCC tissues wer[illegible]m-pared to normal tissues. HCC patients were als[illegible]-lected for measuring miR-20a and CFLAR exp[illegible] sions between tumor and adjacent tissues. Dual-ciferase reporter gene assay was[illegible]d to evalu-ate the relationship between [illegible]20a[illegible] CFLAR. Cultured HepG2 cells treate[illegible]n 120 [illegible]l TRAIL were mixed with miR-20a m[illegible]and/or s[illegible]AR fol-lowed by measurement of [illegible]se-8 cell apoptosis by flow c[illegible]met[illegible]oliferation by MTT assay and prote[illegible]xpressio[illegible]estern blot.

RESULTS: MiR[illegible]xpression w[illegible]nificantly decreased in r[illegible]sues, while[illegible]AR was over-expresse[illegible]CC[illegible]ts had lower miR-20a level and hi[illegible]her CFLAR l[illegible]tumor tissues. MiR-20a targe[illegible]-UTR of CFL[illegible]inhibit its expres-sion. TR[illegible]emarkably up-regu[illegible]d CFLAR expres-sion.[illegible]t inhib[illegible]miR-20a expression and/or si-len[illegible]CFLAR[illegible]nificantly potentiated caspase-8 and[illegible]s[illegible]activity[illegible]nhanced sensitivity of HepG2[illegible]wards T[illegible]-induced cell apoptosis, [illegible]decre[illegible]ell p[illegible]erative function.

CONCLUS[illegible]CC had lower miR-20 and hig[illegible]CFLAR[illegible]ession. MiR-20a targeted and inh[illegible]d CFLAR expression, facilitated activation o[illegible]d caspase-3, and enhanced sensi-[illegible]y of Hep[illegible]2 cells towards TRAIL-induced apop-[illegible]is, and subsequently reduced cell proliferation.

Key[illegible]ds
MicroRNA-20, CFLAR, Hepatocellular carcinoma, HepG2, TRAIL, Cell apoptosis.

Introduction

Prima[illegible]iver c[illegible]is one of the most common malignant tumo[illegible]clinic. The occurrence [illegible]cancer is ran[illegible]5th among common m[illegible]gnant tumors, and as the 3rd highest mortality only next t[illegible]ulmonary cancer and gastric [illegible]inoma[1]. Hep[illegible]cellular carcinoma (HCC) is th[illegible]ior patho[illegible]ical sub-type of primary liver [illegible]ccupies more than 80% of all cases[2]. [illegible]necrosis factor related apoptosis inducing ligand (TRAIL) selectively induces tumor cell apoptosis but lacks toxicity on normal cells, [illegible]s showing promising application in clinic[3]. TRAIL mainly works on death receptor 4 (DR4) or DR5 on the cell membrane to further recruit downstream signal molecules to form death-inducing signaling complex (DISC), which activates caspase cascade response, leading to initiation of cell apoptosis[4]. Various studies[5-7], however, showed drug resistance in almost all HCC cell lines against TRAIL-induced cell apoptosis. Caspase-8 (CASP8) and Fas-associating protein with a novel death domain (FADD)-like apoptosis regulator (CFLAR) are a negative regulators of cell apoptosis with similar structures as apoptosis initiating protein caspase. Compared with wild-type caspase-8, CFLAR lacks the cysteine structure, which is necessary for catalytic activity, and it is thus, one natural inhibitor for caspase-8[8]. CFLAR plays an inhibitory role in TRAIL-recruiting DISC formation and apoptosis signal transduction[9]. CFLAR was up-regulated in various tumor tissues including prostate cancer,[10] pancreatic carcinoma,[11] colorectal cancer[12] and melanoma[13]. Previous studies[14,15] also showed elevated CFLAR expression in HCC tissues, which affects patient survival and prognosis. Moreover, the up-regulation of CFLAR in HCC cells was found to contribute

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to the decreased sensitivity of HCC cells against TRAIL-induced apoptosis[15,16]. MicroRNA (miR) is one type of non-coding RNA with 21-24 nucleotides in eukaryotes. Via complementary binding to the 3'-UTR of target gene mRNA, miR could regulate gene expression via inhibiting translation of target gene mRNA or direct degradation of mRNA, thus participating in the modulation of various biological processes including cell proliferation, apoptosis and cycle, all of which have drawn lots of research interests regarding the relationship between miRNA and tumor pathogenesis[17]. Previous studies[18,19] also suggested significantly depressed microRNA-20a (miR-20a) expression in HCC tumor tissues, suggesting that miR-20a might be a tumor inhibitor gene for HCC. Further bioinformatics analysis showed complementary binding sites between 3'-UTR of CFLAR and miR-20a. This study thus investigated if miR-20a played a role in regulating CFLAR expression and affecting TRAIL-induced apoptosis sensitivity in HepG2 HCC cells.

Patients and Methods

Patients

A total of 49 HCC patients who were gnosed in General Hospital of Ningxia Medi University from August 2015 t 2016 we recruited. Tumor tissues and ue samples (≥ 5 cm from tumor ed were c cted during surgery. There were les an aged between 39 and 6 ye age years). Sample coll n wa oved by the Ethical Committe General l of Ningxia Medical U and infor onsents were obtaine m a nts.

Reage nd Materials

M prague awley (SD) rats (8-10 weeks age, bod ight 22 0 g) were purchased from Ningxia iversit nimal Center (Shanxi, China). nitrosa e (DEN) was provided by ide l iculture Science Academy of uan, C human liver cancer cell line Her and norm human hepatocytes L02 were p American Type Culture Collection CC) (Manassas, VA, USA); Dulbecco's Modi l Eagle's medium (DMEM) culture medium, fe ine serum (FBS) and penicillin-streptomycin we urchased from Gibco (Rockville, MD, USA). Recombinant human TRAIL factor was purchased from Peprotech Co. Ltd. (Rocky Hill, NJ, USA). Reverse transcription kit PrimeScript™ RT reagent Kit and SYBR Green dye were purchased from TaKaRa (Dalian, Liaoning, China). Cell-Light EdU cell proliferation assay kit was purchased fro (Guangzhou, Guangdong, China). M anti-h man cleaved caspase-3 and caspa were purchased from Santa Cruz Biotech (Cruz, CA, USA). Rabbit anti-human CFLAR w chased from Abcam (Cambridge, M USA). A use and anti-rabbit secondary body were pur from Jackson Immuno arch (Grove, USA). Annexin V/PI totic was pu chased from Yusheng (Sh gha . Caspa acti-vity kit was pu sed fro Visi ilpitas, CA, USA). C e-3 activity purchased from Beyo ng, China). l-Luciferase Reporter ay sy nd pGL3-promoter were purchased from Pepr Co. Ltd. (Rocky Hill, NJ

ablishmen f HCC Model l Histopath gy Examination

imal Welf and Ethics: All personnel involv dy have been approved by the Animal and Welfare Committee. A clean nd appropriate environment was provided for during the whole experiment with food r provided *ad libitum* to minimize unnecessary injury, stress or pain for animals. A total of 30 males SD rats received 0.05% DEN water solution for 8 consecutive months. 17 SD rats had liver cancer (56.7% induction rate). Another 20 SD rats with normal feeding were recruited as control group. Animals were sacrificed for collection of tumor or normal liver tissues, which were fixed in 4% paraformaldehyde, followed by routine dehydration, paraffin embedding and sectioning. Hematoxylin and eosin (H&E) staining were then performed to observe tissue morphology. The successful induction of HCC model mainly depends on the histopathology staining. Normal livers had complete and regularly arranged hepatic lobules, with string-shaped arrangement of hepatocytes, which had similar size. Cells had acidophilic cytoplasm, clear and sharp mono-nucleus in the central region. Normal liver tissues had no stenosis of hepatic sinus, or infiltration of inflammatory cells into lobule and portal areas. HCC tissues had polymorphism, or abnormal morphology. Enlarged nucleus and less cytoplasm were accompanied with mono-nucleus, poly-nuclear mega cells and division of nucleus. HCC cells had nest-like regulation, plus infiltration toward adjacent tissues, with focal hemorrhage or necrosis.

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EdU Labeling for Measuring in Vivo Cell Proliferation

EdU labeling was used to measure *in vivo* cell proliferation inside tissues. In brief, 96 h before sacrificing, animals received EdU dye in phosphate buffered saline (PBS) via intraperitoneal injection (10 mg/kg). 96 h later, rats were sacrificed by cervical dislocation to collect liver tissues for preparing paraffin-based sections (5-7 μm). Tissue sections were rinsed in xylene, dehydrated in gradient ethanol, and rinsed in glycine 2 mg/mL for 10 min. After incubation in 0.5% Triton X-100 in PBS for 10 min, tissue sections were rinsed in PBS for 10 min, followed by adding 100 μL 1 × Apollo staining reaction buffer for 30 min incubation under dark at room temperature. After rinsing in 0.5% Triton X-100 in PBS for 2-3 times (10 min each), methanol rinsing (5 min) and PBS washing (5 min), tissues were observed under a fluorescent microscope.

HepG2 Cell Culture and TRAIL Treatment

Human HCC cell line HepG2 and [illegible] liver cell L02 were cultured in high-g[illegible]se Dulbecco's Modified Eagle Medium (DM[illegible]) containing 10% fetal bovine serum (FBS) a[illegible] 1% penicillin-streptomycin, a[illegible]e incuba[illegible]ted in 37°C chamber with [illegible]ith medium changed every 2 d[illegible]. The [illegible]eriment was performed when [illegible]red c[illegible]hed 60-80% confluence. Cells [illegible]ected[illegible] they were treated w[illegible]0, 20, [illegible]d 80 ng/mL TRAIL for 24 h.

MTT Assay [illegible] Ass[illegible]g Proliferat[illegible]e Activity

Cells [illegible]e seeded into 9[illegible] plate at a density o[illegible]0^4/well. With attachment growth, cells wer[illegible]ated wi[illegible], 30, 60 and 120 ng/mL TRAIL for 4[illegible]0 [illegible](4,5-di[illegible]hylthiazol-2-yl)-2,5-diphenyl[illegible]ium h[illegible]ide (MTT) solution was [illegible]dded[illegible]ch[illegible]. With 4 h continuous culture at 37°C, [illegible]pernatant was completely removed, followed by addition of 150 μl dimethyl s[illegible]SO) for 10 min vortex until the complete resolving of crystal violet. Absorbance (A) value was measured at 450 nm in a microplate reader. Six parallel samples were performed in each treatment group. Relative activity of the cultured cells was calculated by ($A_{\text{drug treatment group}}$-$A_{\text{Blank control}}$)/$A_{\text{Control group}}$ × 100%.

Dual Luciferase Reporter Gene Assay

Using HEK293 genome as the template, full-length fragment of 3'-UTR of CFLAR gene was amplified. Polymerase chain reacti[illegible] products were purified from agaros[illegible], and [illegible]gated into pGL-3M luciferase re[illegible]er plasmid after XbaI/NotI dual digestion[illegible]ombinant plasmid was then used to transform[illegible] competent cells. Positive clone[illegible]h prim[illegible]ree ning were selected for f[illegible]er cell trans[illegible] and following experi[illegible]s. Lipo[illegible]amine 2[illegible] was used to transfe[illegible]EK29[illegible]ells wi[illegible]h 400 ng pGL3-CFLA[illegible]'U[illegible]mid, 25[illegible]ol/L miR-20a mimi[illegible]r miR-[illegible]gati[illegible]ontrol), and 25 ng p[illegible]'K. After [illegible]nsfection, Opti-MEM[illegible] was disca[illegible]d, with the replacem[illegible] of no[illegible] DMEM containing 10% FBS and 1% strepto[illegible]-penicillin. After 48 h [illegible]us incubation[illegible]al-luciferase assay w[illegible]erformed. In brief, cells were washed twice [illegible]BS, followe[illegible]y addition of 100 μL passive [illegible] buffer (PL[illegible] With vortex at room temperature for 20 m[illegible] the mixture was centrifuged at 3[illegible] min. Then, 20 μL cell lysate was mi[illegible]n 100 μL LAR II. Fluorescent value I was measured in a microplate reader. The [illegible]tic reaction was stopped by adding 100 [illegible]L S[illegible]& Glo® reagent (Promega Madison, WI, USA), followed by quantification of fluorescent value II. The relative expression of reporter gene was calculated as the ratio of fluorescent value I/fluorescent value II.

Hep G2 Cell Transfection

Cultured HepG2 cells after treated with 120 ng/mL TRAIL were divided into 6 groups: non-treated group, mimic NC control, miR-20a mimic group, si-NC group, si-CFLAR group, and miR-20a mimic + si-CFLAR group. Lipofectamine 2000 was used to transfect oligonucleotide fragments into HepG2 cells, which were cultured in serum- and antibiotic-free medium. After 6 h, DMEM containing 10% FBS and 1% streptomycin-penicillin was added for 48 h continuous culture in further experiments. Oligonucleotide sequences were: mimic NC, 5'-CAGUA CUUUU GUGUA GUACA A-3'; miR-20a mimic, 5'-UAAAG UGCUU AUAGU GCAGG UAG-3'; si-CFLAR sense, 5'-GCAGT CTGTT CAAGG AGCAT T-3'; si-CFLAR anti-sense, 5'-TGCTC CTTGA ACAGA CTGCTT-3; si-NC sense, 5'-TTCTC CGAAC GTGTC ACGTT T-3'; si-NC anti-sense, 5'-ACGTG ACACG TTCGG AGAAT T-3'.

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qRT-PCR for Gene Expression Assay

Total RNA was extracted from cells by Trizol method. In brief, cells were lysed by 1 mL Trizol, and total RNA was extracted using 200 µL chloroform. The supernatant was collected. RNA was precipitated by 1 mL isopropanol, followed by twice rinsing in 1 ml 70% ethanol. RNA pellet was dissolved in diethyl pyrocarbonate (DEPC) treated water. cDNA was synthesized in a 10 µL system including 1 µg total RNA, 2 µL RT buffer (5 ×), 0.5 µL oligo dT + random primer mix, 0.5 µL RT enzyme mix, 0.5 µL RNase inhibitor, and ddH_2O. The reaction conditions were: 37°C for 15 min, followed by 98°C 5 min. cDNA products were kept at -20°C. Using cDNA as the template, PCR amplification was performed under the direction of TaqDNA polymerase using primers ($miR\text{-}20aP_{RT}$: 5'-GTCGI ATCCA GTGCA GGGTC CGAGG TATTC GCACT GGATA CGACT AC-CTG-3'; $miR\text{-}20aP_F$: 5'-GCGGC GGTAA AGTGC TTATA GTG-3'; $miR\text{-}20aP_R$: 5'-TGCAG GGTCC GAGGT AT-3'; $U6P_F$: 5'-ATTGG AACGA TACAG AGAAG ATT-3'; $U6P_R$: 5'-GGAAC GCTTC AC-GAA TTTG-3'; $CFLARP_F$: 5'-GTCTG CTGAA GTCAT CCATC-3'; $CFLARP_R$: 5'-ACTAC GCC-CA GCCTT TTGG-3'; $\beta\text{-actinP}_F$: 5'-GAACC[illegible]AG GCCAA C-3'; $\beta\text{-actinP}_R$: 5'-TGTCA [illegible]GATTT CC-3'. In a PCR system with 10 µL to[illegible]lume, we added 4.5 µL 2 × SYBR Green Mixtur[illegible] µL of forward/reverse primer (at 2.5 µm/L), 1 [illegible] cDNA, and 3.0 µL ddH_2O. PCR [illegible]ions wer[illegible] 95°C for 15 s, 60°C for 30 s an[illegible]0 s. The reaction was performed o[illegible]o-Rad[illegible]ercules, CA, USA) CFX96 fluor[illegible]t quan[illegible]PCR cycler for 40 cycles to [illegible]

Western Blot

Cells were [illegible] with RIPA[illegible] buffer at 4°C for 20[illegible], fo[illegible] by centrifugation at 12000 × g[illegible] 30 min. 8[illegible] protein samples in the sup[illegible]ant were sepa[illegible] by 8% sodium dodec[illegible]ulfate [illegible]olyacrylamide gel electropho-resi[illegible]DS-PA[illegible], and were transferred to poly-viny[illegible]ride ([illegible]DF) membrane. The membr[illegible]as blo[illegible] in 5% defatted milk [illegible]er fo[illegible]f[illegible]wed by primary antibody (an[illegible]FLAR[illegible]00 dilution, anti-cleaved ca-spa[illegible]3 at 1:200, anti-cleaved caspase-3 at 1:200 o[illegible]n at 1:400) purchased from Santa [illegible]z Biotechnology (Santa Cruz, CA, USA) and [illegible]ubated at 4°C overnight. After phosphate buf-[illegible]saline-tween-20 (PBST) washing (5 min × 3 t[illegible]s), horseradish peroxidase (HRP)-labeled secondary antibody (1:5000 for both anti-mouse and anti-rabbit) purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), was added and incubated for 60 min. After phosphate buffered saline-tween-20 (PBST) rinsing for three times (5 min each), electrochemilum[illegible] (ECL), purchased from Amersham [illegible]scienc[illegible] (Piscataway, NJ, USA) reagent [illegible]dded and incubated for 2 min. The mem[illegible] was then exposed in the dark. Quantity One [illegible]analysis software was used to[illegible]lyze th[illegible]tive grey density of bands.

Assay for Caspase[illegible]d Ca[illegible]se-8 A[illegible]tivity

Caspase-3 Activit[illegible] Assay

Standard dil[illegible]s of 0, [illegible]50, [illegible]and 200 µM pNA we[illegible]pared from [illegible]stock. Absorbance v[illegible]5 nm wavel[illegible]ns were measured by [illegible]micro[illegible]reader to plot a standard curve with pNA conc[illegible]tion against A405 value[illegible]d cells were [illegible]ted by trypsin, and c[illegible]cted into culture medium for 4°C centrifuga-[illegible] at 600 × g f[illegible] min. The supernatant was ca-[illegible]ly removed [illegible] washed out by PBS. 100 µL ly[illegible]uffer was [illegible]d for every 2 × 10^6 cells. Cel-ls w[illegible]C for 15 min, and centrifuged at 1800[illegible]4°C for 10 min. Supernatants were [illegible]ollected for further use. Ac-DEVD-pNA was [illegible]n ice, mixed with buffer and test samples, [illegible]µL Ac-DEVD-pNA. The mixture was incubated at 37°C for 120 min. A405 value was measured when color was changed significantly.

Caspase-8 Activity Assay

Attached cells were digested by trypsin, counted and centrifuged. 50 µL pre-cooled cell lysis buffer was added to each 2 × 10^6 cells. After 10 min iced lysis, the mixture was centrifuged at 10000 × g for 1 min. The supernatant was collected and quantified for protein concentration. 50 µL cell lysis buffer was used to dilute 100 µg proteins, with the addition of 50 µL 2 × reaction buffer (containing 10 mM DTT). 5 µL IETD-pNA substrate (4 mM stock, final concentration at 200 µM) was added for 120 min incubation at 37°C. A405 was measured by a microplate reader.

Flow Cytometry for Detecting Cell Apoptosis

Cells were digested with trypsin and collected through 1000 r/min centrifugation for 5 min. Cells were then washed with PBS twice. 100 µL 1×Binding Buffer was used to re-suspend cells. 5 µL Annexin V-FITC and 5 µL PI staining solution were then added to the mixture. After gentle mixture, this was incubated in the dark for 10

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min, followed by addition of 400 μL 1 × Binding Buffer, and was immediately loaded for online testing in Beckman FC500MCL flow cytometry apparatus.

Statistical Analysis

SPSS18.0 software (SPSS Inc. Chicago, IL, USA) was used for data analysis. Measurement data were presented as mean ± standard deviation (SD). Student *t*-test was used to compare measurement data between groups. Comparison among multiple groups was made by one-way ANOVA with Tukey's post hoc correction. A statistical significance was defined when $p<0.05$.

Results

Elevated CFLAR and Lowered miR-141 Expression in HCC Model Animals

After 8 month drinking of 0.05% DEN-containing water, 17 out of 30 (56.7%) rats developed HCC. Hematoxylin and eosin (H&E) staining showed intact and regular arrangement of hepatic lobular structure in normal rats, while HCC model rats displayed disappearance of norm lobes, disarranged cell arrangement volume, plus inconsistent radial ri ith hepatic cords (Figure 1A). EdU labeling showed significantly more EdU-positive cells C model animals compared with nal grou ica ting active proliferation epatic cells (1B). Further assay sh d rema ly eleva miR-20a expression ICC ues co pared with normal hepa tis ist CF was down-regulated umors (e 1C 1D).

Elevated nd Lower miR-141 Express in H umor Tissues

Further assay reve significantly elevated mi xpression in or tissues of HCC p nts compared with adjacent tumor tissues ure 2B), wh CFLAR mRNA and protein ls were sig antly higher in tumors (Fi-

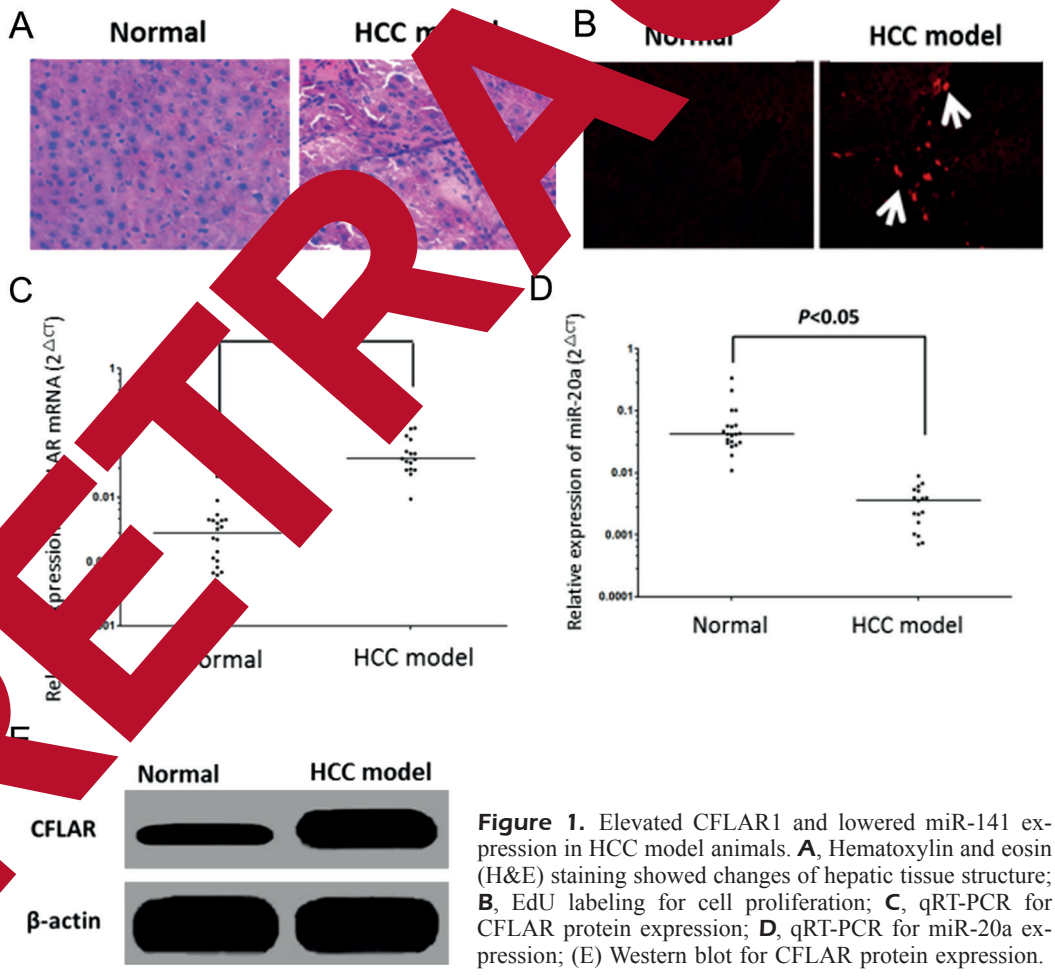

Figure 1. Elevated CFLAR1 and lowered miR-141 expression in HCC model animals. **A**, Hematoxylin and eosin (H&E) staining showed changes of hepatic tissue structure; **B**, EdU labeling for cell proliferation; **C**, qRT-PCR for CFLAR protein expression; **D**, qRT-PCR for miR-20a expression; (E) Western blot for CFLAR protein expression.

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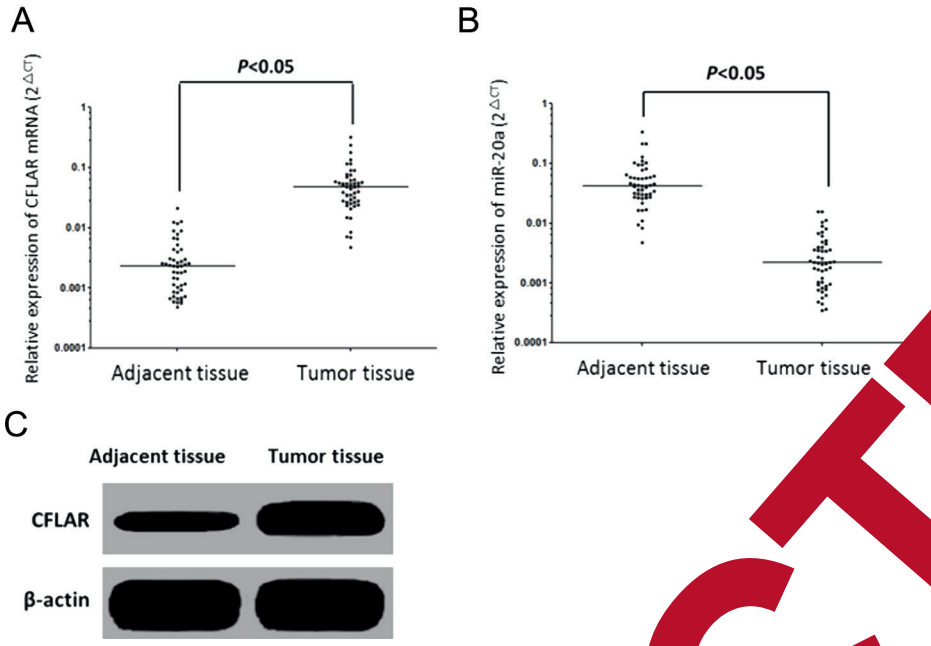

Figure 2. Elevated CFLAR and lowered miR-141 expression in HCC tumor tissues. (**A**) qRT-PCR fo mRNA expressi PCR for miR expressio (**C**) Weste t for CFLAR protein ex

gure 2A and Figure 2C), suggesting possible regulation relationship between miR-20 d CFLAR.

miR-20a Expression in HepG2 Cells Inhibited CFLAR Expression

Flow cytometry analysis sho ignificant lower basal apoptosis rate in lls compared with L02 normal he ytes (re 3A). Compared with L02 cell miR-20 ion of HepG2 cell was sig fica y ( ) whilst CFLAR exp on wa ated (Figure 3B and 3C). CCK ssay reveal k inhibition on HepG2 feration by L treatment, indicati ossi istance of HepG2 cells against TR L (Figure 3 rther assay showed no signi activation of c -3 or caspase-8 even t d with igher concen ations of TRAIL. On other h high level of TRAIL (120 ng/mL) r elevat CFLAR expression in HepG2 Figure). Results showed miR- own- ti ight play a role in elevating C R expr and antagonizing HCC apoptosi Bioinform ics analysis showed targeting r ween miR-20a and the 3'-UTR of LAR mRNA (Figure 3E and 3F). Dual-lucife- e reporter gene assay showed remarkably decre- elative luciferase activity by miR-20a up-regu n (Figure 3G), demonstrating that miR-20a could target the 3'-UTR of CFLAR mRNA to regulate its expression. After transfecting miR-20a mi lls, CFLAR mRNA and protein expressi e significantly decreased, further confirming the targeted inhibition of miR-20a on expression (Figure 3H).

miR-20a Inhibited CFLAR, Enhanced Caspase-8 Activity and Facilitated TRAIL-Induced HepG2 Cell Apoptosis

Western blot results showed HepG2 cells expressed CFLAR at a relatively higher level. High concentration TRAIL treatment (120 ng/mL) significantly elevated CFLAR expression in HepG2 cells, but not increase the enzymatic activity of caspase-8 and caspase-3 (Figure 4A, 4B and 4C). Induction of cell apoptosis and inhibition of cell proliferation were also weakened (Figure 4D and 4E). After transfecting miR-20a mimic and/or si-CFLAR, the expression of CFLAR in HepG2 cells was significantly reduced (Figure 4A), whilst casapase-3 and caspase-8 activity was potentiated (Figure 4A, 4B and 4C), accompanied with remarkably elevated cell apoptosis (Figure 4E) and reduced cell proliferation (Figure 4D).

Discussion

HCC has over 50 per 100.000 occurrences in China, where more than 300.000 people are newly diagnosed as HCC, occupying half of the world patient population, thus severely affecting

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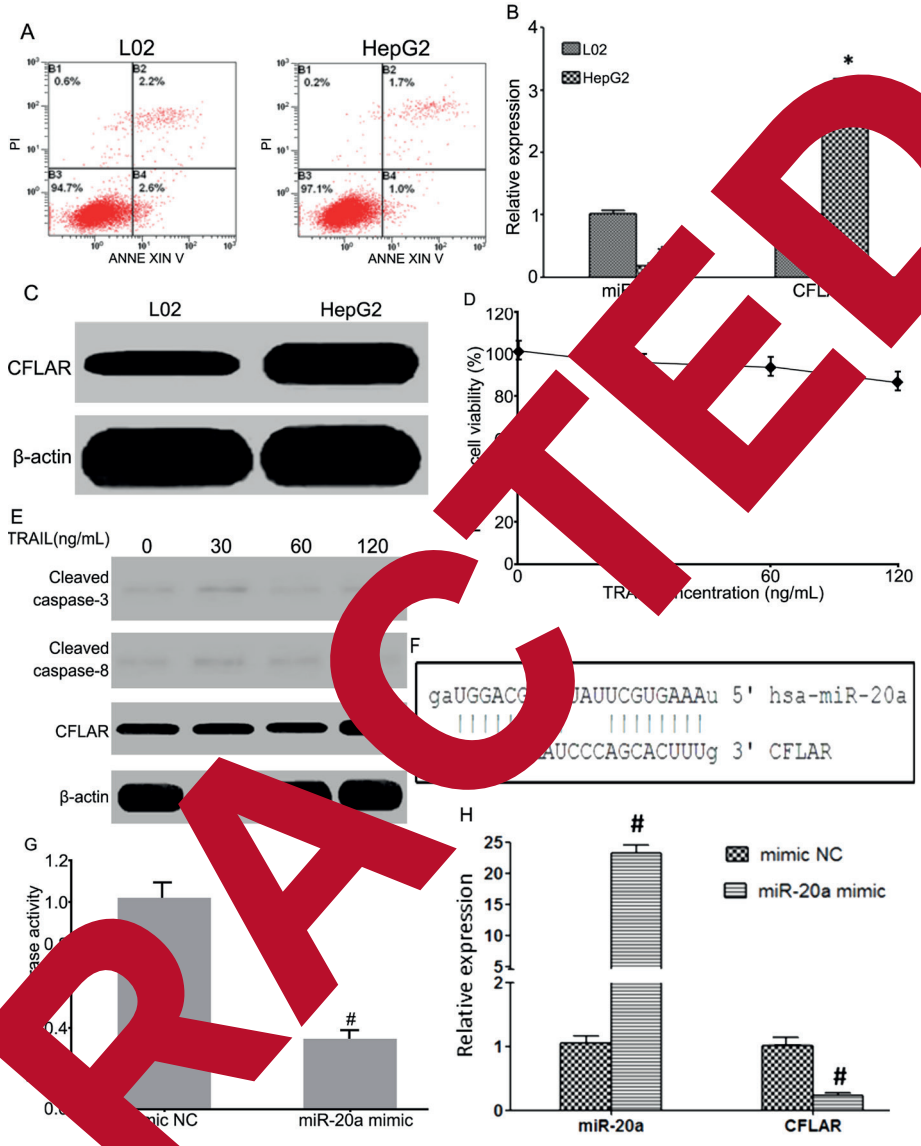

Figure 3. miR-20a expression in HepG2 cells inhibited CFLAR expression. **A**, Flow cytometry for cell apoptosis; **B**, qRT-PCR for gene expression in L02 and HepG2 cells; **C**, Western blotting for CFLAR protein expression in L02 and HepG2 cells; **D**, MTT assay for cell proliferative activity; **E**, Western blot for effect of TRAIL treatment on CFLAR protein expression; **F**, miR-20a targeted on 3'-UTR of CFLAR mRNA; **G**, Dual-luciferase reporter gene assay; (H) qRT-PCR for miR-20a and CFLAR gene expression. *p<0.05 compared with L02 cells; #, p<0.05 compared with mimic NC group.

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the public ...alth[20]. Mo... 600.000 people died fro... CC all over the ..., with 110.000 died i... China, ...cupying 18.3% of world's cases[21]. ...e to i... ous onset, HCC normally has asy... ...re at e... stage. However, with high m... ...cy an... pid disease progression, ...patie... ...ready at advanced or terminal stage ... dmitted, thus causing difficulty for treatment and unfavorable prognosis. R... ...gh significant progressions have been made in the basic and clinical medicine, producing major advances through combined treatment using surgery, radio-, chemo- and immunotherapy obtained, the treatment efficiency, however, is still unsatisfactory. It is surveyed that 1-year and 2-year survival rate of HCC are only 42.8% and 38.8%, respectively[22], with around 20% of 5-year survival rate[23]. Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is one recently discovered member of tumor necrosis factor (TNF). It can induce cell apoptosis via binding and activating death receptor (DR)[24]. In contrast with TNF and Fas in TNF super-family, TRAIL only induces apoptosis of those viral-infected cells, transformed cells and tumor cells, but with minor toxicity and killing effects toward normal cells[25]. After binding to DR4 or DR5 on cell membrane, TRAIL can form trimer of ligand-receptor complex, which further induces binding between death domain (DD) of cytoplasmic domain of DR and DD at C-terminal region of Fas-associated death

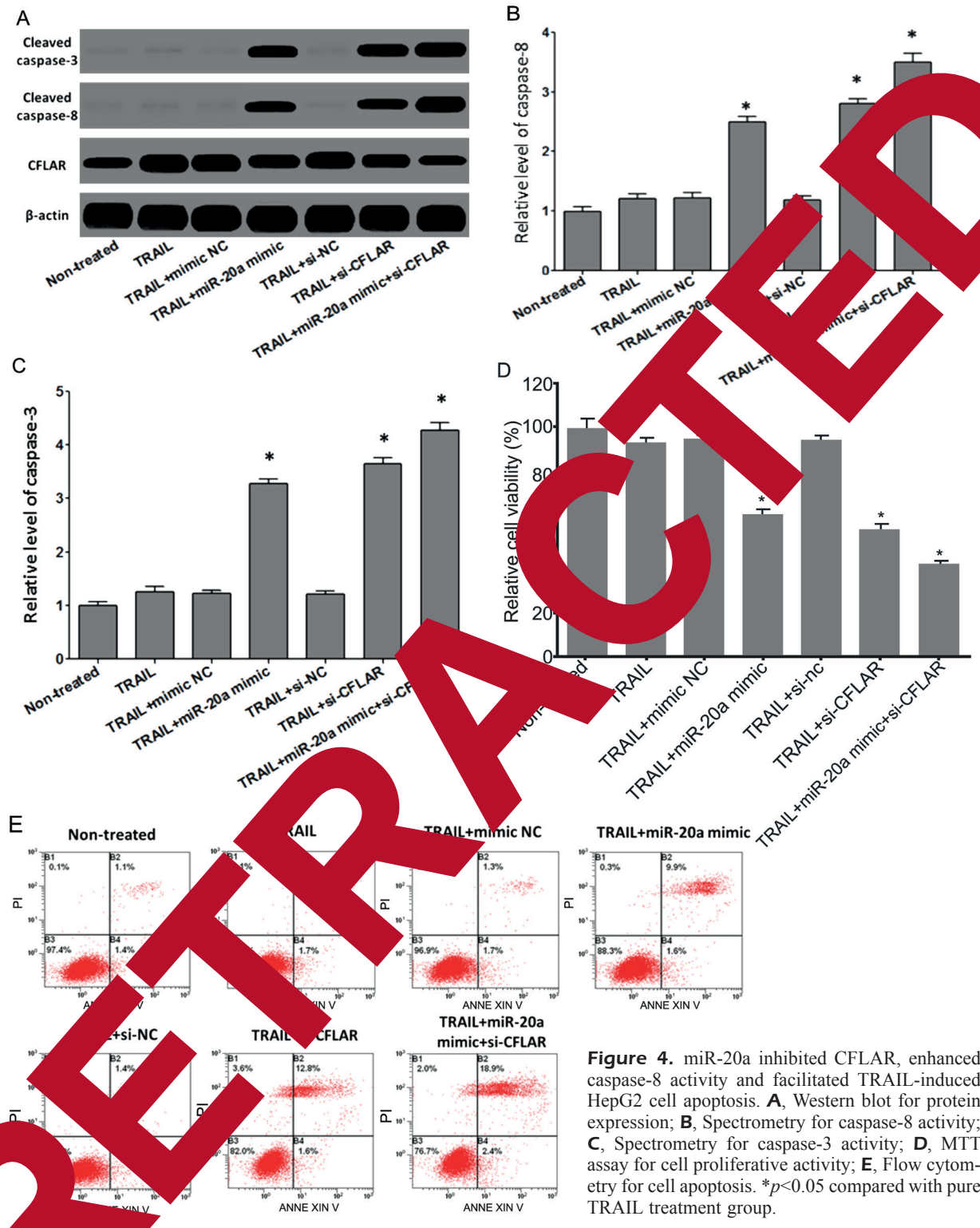

Figure 4. miR-20a inhibited CFLAR, enhanced caspase-8 activity and facilitated TRAIL-induced HepG2 cell apoptosis. **A**, Western blot for protein expression; **B**, Spectrometry for caspase-8 activity; **C**, Spectrometry for caspase-3 activity; **D**, MTT assay for cell proliferative activity; **E**, Flow cytometry for cell apoptosis. *p<0.05 compared with pure TRAIL treatment group.

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main (FADD). FADD further utilizes its death r domain (DED) at N-terminus to bind to pro pase-8 to form DR4/DR5-FADD-Procaspase-8 death inducing signal complex, which facilitates auto-cleavage of procaspase-8 into active apoptosis initiator caspase-8 for caspase cascade reaction, to sequentially activate apoptosis executing protein caspase-3, -6 and -7. This directly degrade intracellular structural and functional proteins for cell apoptosis[4]. CFLAR

is a naturally existed inhibitor for caspase-8, with similar structure and sequence with cysteine-aspartate protein-8 (caspase-8), which has two N-terminal structural domain plus one C-terminal structure domain lacking the cysteine structure necessary enzyme catalytic activity[26]. By competitive binding with caspase-8 to form heterodimer via its N-terminal structural domain, CFLAR can deactivate caspase-8, impede the formation of functional DISC and caspase-8 activation, and quench caspase cascade reaction, thus blocking cell apoptosis which is induced by TNF, TRAIL, FasL signal molecules and related DR[27]. Abnormally elevated CFLAR expression plays a critical role in inducing apoptotic resistance by DR ligand such as TNFα[28] TRAIL[29] and Fas[30]. Previous studies showed the correlation between CFLAR up-regulation and occurrence of various tumors including prostate cancer[10], pancreatic cancer[11] and colorectal carcinoma[12]. Elevated expression of CFLAR is one important reason for tumor cells to invade from drug-induced cell apoptosis as well as from immune surveillance, leading to tumor progression and aggravation. Increasing evidence sh d abnormally elevated expression of CFL HCC tissues and tumor cells, indicating p tential role in inducing resistance of tumor against TRAIL-induced apoptosis[15,16]. Previ studies showed significantly sed mi 20a expression in HCC tu ggesting a potential role of miR-20 the H patho-genesis. Bioinformatics lysis complementary bindi of CFLAR and miR . This investigated if miR-20a playe ole in reg CFLAR expression and TRAIL-i d apoptotic sensitiv f H cells. In DEN-induce HCC mod we observe ificantly elevated CFLAR pression in tu issues compared w normal liver tissues, whilst miR-20a leve as do egulated. Moreover, this study a bs d up-r lation of CFLAR and down-r on of -20a in HCC patients, ating ible le of miR-20a down-regu n in f ng CFLAR expression. Du et a showed 8 .72% positive rate of CFLAR e CC tumor tissues, whilst CFLAR barely detected in adjacent tissues. Also, LAR expression was negatively correlated non-recurrent survival time of patients. Co stent with this, our study found remarkably elevate CFLAR expression in HepG2 cells compared to normal L02 cells, which was also in accordance with a study conducted by Okano et al[15] who observed up-regulation of CFLAR in HCC cell line. Fan et al[18] found significantly down-regulated miR-20a expressio tumor tissues, with more suppresse pressi in recurrent patients, and shorter val period in HCC patients with lower miR pression. Chen et al[19] observed lower miR-20 ession in HCC cell line compare th nor pa tocytes. This study also d lower miR both HCC tumors an l line pared adjacent tissue or n her cyte cell line, which were consi nt ious s es[18,19]. MTT assay sh weak essi fects of TRAIL on H 2 cell proli indicating lower sens HepG2 cells nst TRAIL. Consider abun xpression of CFLAR in HepG2 cells, it is po that CFLAR played a r ducing TRA lated apoptotic re- s ce. Moreover, high concentration (120 nL) TRAIL atment also elevated RIPK1 ession in ac on to basal levels. However, T L did not uce the activation of caspase-8 at a high concentration. Sun et al[16] RAIL could up-regulate CFLAR xpression in HepG2 and Hep3B cells, but with ffects on inducing HCC apoptosis. Our dy tained similar results. Up-regulation of CFLAR also plays a role in inducing apoptotic resistance induced by other reagents, in addition to TRAIL-induced cell apoptosis. As reported by Chen et al[31], CFLAR up-regulation induced the resistance of HepG2 cells against Taxol-induced apoptosis, as knockout of CFLAR expression significantly enhanced the sensitivity of Taxol-induced apoptosis. Dual luciferase reporter gene assay confirmed targeted regulation of CFLAR expression by miR-20a. Further analysis showed that up-regulating miR-20a expression and/or silencing CFLAR expression significantly potentiated caspase-8 and caspase-3 activation, facilitated TRAIL-induced cell apoptosis and enhanced the inhibitory effects of TRAIL on HepG2 cells proliferation. Sun et al[16] showed that siRNA interference of CFLAR expression significantly enhanced the sensitivity of HepG2 cells for TRAIL-induced cell apoptosis, which were consistent with our present study. In a research regarding the correlation between HCC and miR-20a, Fan et al[18] revealed the targeted regulation of miR-20a on Mcl-1 gene expression, which can inhibit proliferation of HCC cells including HepG2 and SMMC-7721, induce cell cycle reorganization, and facilitate cell apoptosis in an-

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ti-tumor process. Chen et al[19] found that miR-20a could exert tumor suppressing gene-like activity via reducing CCND1 expression in HepG2 cells, inhibiting cell proliferation, migration or invasion. The other studies[32,33] also proved that the miR-20a could inhibit the migration, invasion and proliferation of the HCC cell lines. This work revealed the anti-tumor effect of miR-20a in targeted inhibition of CFLAR, and enhancing the sensitivity of HepG2 cells for TRAIL-induced cell apoptosis, thus replenishing our knowledge for the relationship between miR-20a and HCC pathogenesis.

Conclusions

Down-regulation of miR-20a and up-regulation of CFLAR are observed in HCC cells. MiR-20a could inhibit CFLAR expression, and antagonize the inhibitor effects of CFLAR on caspase-8 activity, thus facilitating activation of caspase-8 and -3, sensitizing HepG2 cells for TRAIL-induced cell apoptosis, and subsequently reduced cell proliferation ability.

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