OncoTargets and Therapy

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ORIGINAL RESEARCH

RETRACTED ARTICLE: Long Non-Coding RNA EGOT Promotes the Malignant Phenotypes of Hepatocellular Carcinoma Cells and Increases the Expression of HMGA2 via Down-Regulating miR-33a-5p

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Background: Chronic hepatitis C virus (HCV) infection is an important risk factor for hepatocellular carcinoma (HCC). EGOT is a long non-coding RNA (lncRNA) induced after HCV infection that increases viral replication by antagonizing the antiviral response. Interestingly, EGOT also acts as a crucial regulator in multiple cancers. However, its role in HCC remains unclear.
Methods: Real-time PCR (RT-PCR) was used to detect the expression of EGOT in HCC samples and cell lines. CCK8 assay and colony formation assay were performed to evaluate the effect of EGOT on proliferation. Scratch healing assay and transwell assay were used to detect the changes of migration and invasion. Flow cytometry was used to detect the effect of EGOT on apoptosis. The interaction between EGOT and miR-33a-5p was determined by bioinformatics analysis, RT-PCR, and dual-luciferase reporter assay. Western blot was used to confirm that high mobility group protein A2 (HMGA2) could be modulated by EGOT.
Results: Compared with normal liver tissues, the expression level of EGOT in HCC tissues was significantly up-regulated. EGOT markedly regulated viability, migration and invasion of HCC cells. The expression level of EGOT was negatively correlated the expression level of miR-33a-5p. It is also confirmed that EGOT could specifically bind to miR-33a-5p and could reduce its expression, in turn, up-regulate the expression of HMGA2.
Conclusion: Our data imply that EGOT may be a novel therapeutic target for HCC, and highlights the key role of EGOT/miR-33a-5p/HMGA2 in the progression of this deadly disease.
Keywords: HCV, HCC, EGOT, miR-33a-5p, HMGA2

Introduction

Hepatic carcinoma (HCC), one of the common tumors of the digestive system, is featured by insidious onset, rapid development and high mortality. It is reported that HCC is the fifth largest type of cancer in the world and the third common cause of death related to cancer.[1] In China, nearly 400,000 people die of HCC each year.[2] The pathogenic factors include environmental factors, genetic variation, eating habits, etc., among which the most important ones are hepatitis B virus (HBV) and/or hepatitis C virus (HCV) infection.[3] At present, the treatment strategies of HCC include surgery, chemotherapy, target therapy, etc., but the prognosis of patients with HCC is still unsatisfactory due to postoperative recurrence and metastasis.[4,5] Therefore, it is of significance to study the potential biological mechanisms in the progression of HCC for the prevention and treatment of this disease.

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Long non-coding RNAs (lncRNAs) are a class of RNA molecules that are more than 200 nucleotides in length without protein-coding ability. Although many lncRNAs are unable to translate proteins, they play an important role in various biological processes.[6,7] A large number of studies have shown that lncRNAs can affect the progression of many types of tumor.[8–10] For example, lncRNA OR3A4 promotes the growth, invasion and metastasis of gastric cancer by up-regulating the expression of guanine-nucleotide-binding protein beta polypeptide 2-like 1;[11] the up-regulated expression of lncRNA ZFAS1 is associated with the epithelial-mesenchymal transition (EMT) of breast cancer cells;[12] and lncRNA HCAL, as ceRNA of lysosomal-associated transmembrane protein 4B, promotes the growth and metastasis of HCC.[13] In addition, studies have shown that EGOT promotes the development and progression of gastric cancer;[14] and it is a lncRNA induced by HCV infection, and increases viral replication by counteracting antiviral responses.[15] However, the role of EGOT in HCC is currently unclear.

MicroRNAs (MiRs) are a group of highly conserved endogenous non-coding RNA molecules, about 21 to 25nt in length. They play an important role in cell differentiation, metabolism, proliferation, and apoptosis.[16] For example, miR-212 downregulates the methyl CpG binding protein MeCP2, and promotes the development and progression of gastric cancer;[17] and miR-143 is underexpressed in TW01 nasopharyngeal carcinoma cells under the action of TGF-β1 cytokines.[18] Studies have shown that the tumor suppressor miR-33a-5p is a potential biomarker for early diagnosis of lung cancer;[19] and miR-33a-5p inhibits EMT of non-small cell carcinoma, and is a prognostic factor for patients.[20] The role of miR-33a-5p in the development and progression of HCC requires further research. Previous articles have revealed that lncRNA directly interacts with miRNAs and regulates their expression. For example, in osteosarcoma, overexpression of lncRNA DANCR inhibits the expression of miR-33a-5p;[21] and in tongue squamous carcinoma, lncRNA CASC15 targetedly inhibits miR-33a-5p.[22] However, the interaction between EGOT and miR-33a-5p has not been elucidated.

High mobility group protein A2 (HMGA2) gene, a small non-histone chromosomal protein, is located in 12q13-15. It can bind to chromatin to change its structure or directly interacts with its related proteins and participates in enhancer formation to regulate the transcription of genes, which in turn affects embryogenesis, tissue development and tumorigenesis.[23] HMGA2 is overexpressed in many epithelial malignancies, such as breast cancer, lung cancer, pancreas cancer and oral squamous cell carcinoma.[24–27] Studies have shown that HMGA2 can affect tumor angiogenesis and interfere with the cell cycle, promote tumor cells to obtain stem cell characteristics and undergo EMT, thereby maintaining the ability of tumor cells to proliferate, differentiate, invade, metastasize and self-renew.[28,29] The targeted regulation of HMGA2 expression by miR-33a-5p has been demonstrated in a previous study: miR-33-5p promotes osteoblast differentiation by directly targeting the 3′UTR of HMGA2.[30]

Through bioinformatics analysis, we found that there was a mutual binding site between EGOT and miR-33a-5p, indicating that the upregulation of the former in tumor tissues may inhibit the expression of the latter. This study was designed to detect the expression level of EGOT in HCC tissues and cells, and explore its effects on the expression levels of miR-33a-5p and HMGA2, providing a new theoretical basis for the clinical treatment of HCC.

Materials and Methods

Pathological Tissue Collection

Fresh tissue samples of 52 patients with HCC who underwent HCC removal from the Department of Hepatobiliary Surgery of Wuhan Fourth Hospital from December 2013 to December 2015 were selected. No patients received adjuvant therapies such as chemotherapy or radiotherapy before surgery. Specimens of the control group were obtained from paracancerous tissues of the same patient (at least 3cm from the surgical margin), and no cancer cells were found after postoperative pathological examination. All specimens were placed in liquid nitrogen at −196°C immediately after removal. All patients provided written informed consent, and that this was conducted in accordance with the Declaration of Helsinki.

Cell Culture

The normal liver cell line L02 and the HCC cell lines Hep3B, Huh7, and SMMC-7721 were provided by the Clinical Management Center of Wuhan Fourth Hospital. All cells were cultured in DMEM medium (Thermo) added with 10% fetal bovine serum (Gibco Thermo Fisher Scientific), and 1% penicillin/streptomycin (Invitrogen), and placed in an incubator at 5% CO_2 and 37°C. The medium was changed once every 2 days until the cells were spread over the bottom of the flask for passage. The cells were

passaged after 0.25% trypsin digestion, and taken in the logarithmic growth phase for the experiment. And the use of the cell lines was approved by the Clinical Ethics Committee of Wuhan Fourth Hospital.

Cell Transfection

The cells were rinsed with PBS buffer (Thermo), for 3 times, trypsinized for 2min, and transferred to a sterile 15mL centrifuge tube. After that, they were centrifuged and counted, and seeded at 4×10^5 cells per well in 6-well plates. When the fusion rate was about 70%, the transfection reagent was diluted at a concentration of 3μL/L with serum-free medium, and incubated at 37°C for 20 min. The siRNAs were diluted at a concentration of 50μmol/L with serum-free medium, respectively, incubated at room temperature for 5 min, mixed with the same volume of the transfection reagent, and continued to be cultured in a 37°C incubator. After 12 hrs, the state of the transfected cells was observed, and the serum-free medium was changed to the complete medium to continue culture. After 48 hrs of further culture, the RNA was extracted to verify the transfection efficiency. The overexpressed EGOT and the control plasmids were transfected into the HCC cell lines in the same manner. Stably transfected cells were s[illegible] using Geneticin.

RNA Extraction and Real-Time PCR

Total RNA was extracted from frozen [illegible]sues an[illegible]lls were with Trizol reagent (Invitrogen). [illegible]verse [illegible]scription was carried out using MMLV reve[illegible] transcripta[illegible] (invitrogen) to generate a first strand [illegible]DNA. Quantitative real-time polymerase chain reacti[illegible] (qRT-PCR) was performed on the ABI 7500 Real-[illegible]me PCR System (TransGen Biotech) using the SYBR pr[illegible]ix EX TAQ II (Takara, Dalian, China) according to th[illegible] manu[illegible]urer's i[illegible]tructions. The conditions for qRT-P[illegible]R we[illegible]as foll[illegible]s: pre-denaturation at 95°C for 10min, [illegible]5°C fo[illegible]15s, 60°C for 15s, 45 cycles, and the fluorescen[illegible]ignal temperature was 60°C. GAPDH was used as inte[illegible]l reference to detect the expression of EGOT and miR-33a-5p, and the 2(-ΔΔCt) method was used for statistical analysis. Each experiment was repeated and measured for three times. The information for specific primer sequences was shown in Table 1.

Table 1 Primers Used in This Study

Name	Primer Sequences
LncRNA EGOT	Forward:5′-CACTGCACAGGGAAACACAAA-3′ Reverse:5′-ACCCTGTTCATAAGCCCTGATG-3′
miR-33a-5p	Forward:5′-GGTGCATTGTAGTTGCATTGC-3′ Reverse:5′-GTGCAGGGTCCGAGGTATTC-3′
GAPDH	Forward:5′-AGCCACATCGCTCAGACAC-3′ Reverse:5′-GCCCAATACGACCAAATCC-3′

CCK8 Assay

The proliferation ability of the cells was examined by the CCK-8 assay. The procedures were performed according to the instructions of the CCK-8 kit (Beyotime Biotechnology). Cells were seeded in 96-well c[illegible]ure pla[illegible] at a volume of (1~2) $\times 10^3$ cells per well. [illegible] volume o[illegible]ach well was 100μL, and a blank cont[illegible]l (wit[illegible]medium o[illegible]y) was established and cultured fo[illegible], 2, 3 and 4 [illegible]ys. [illegible]μL LCCK8 was added to each well, [illegible]d cultu[illegible]was continued for 1 hrs at 37°C before it was st[illegible]pped. [illegible]control w[illegible]s were adjusted to zero, and the abs[illegible]ance (OD v[illegible]e) [illegible] each well was measured at 450nm [illegible] a mic[illegible]plate reader. The relative OD ratio was used to in[illegible]cate the cell p[illegible]liferation ability. The average value of 3 [illegible]lls was taken for each group, and the proliferation curve [illegible]as drawn. T[illegible]e experiment was repeated for 3 times.

Co[illegible]ny Formation Assay

[illegible]lls in logarithmic growth phase were seeded into 6-well plates. 1000 cells were seeded per well. After 14 days of culture, the culture solution was removed. They were rinsed three times with PBS, fixed with methanol for 20min, stained with 1% methylene blue for 40min, rinsed twice with deionized water, and air-dried. The number of colonies was calculated under a microscope. Three replicate wells were set in each group, and the experiment was repeated for 3 times.

Flow Cytometry

Apoptosis was analyzed by flow cytometry. Cells in logarithmic growth phase were seeded in 96-well plates at 1×10^4 cells/well, cultured for 24 hrs, rinsed twice with PBS, fixed with 70% ethanol, and stored at 4°C overnight. They were rinsed with PBS once to adjust the cell density to 1×10^6/mL. The propidium iodide staining solution was added to adjust the final concentration to 0.05mg/mL and stained at 4°C for 30min. Then, the apoptosis was analyzed by flow cytometer. Three replicate wells were set in each group.

Wound Healing Assay

The migration ability of the cells was examined using the wound healing test. Cells (2×10^5) were added to 12-well plates

and cultured for 48 hrs. A 200μL sterilizing tip was used to scribe straight lines, forming a direct scratch in the middle of the confluent monolayer to measure the initial distance (0 time) of the scratch under the microscope. The distance of the scratch was measured after culturing for 24 hrs. Each experiment was repeated and measured for three times.

Transwell Assay

Cell migration and invasion were detected by transwell assay. Cells were trypsinized. 10^5 cells were placed in a 1.5mL EP tube, and 200μL serum-free medium was added to resuspend the cells, which was then placed in a transwell chamber. DMEM medium containing 10% FBS was added to the lower chamber, and placed in a 37°C and 5% CO_2 incubator for 24 hrs. Then, the transwell chamber was taken. The cells in the chamber were wiped with a cotton swab, and the remaining cells were gently rinsed off with PBS. After fixation and staining, 8 random fields under the microscope were photographed and counted. All experiments were repeated for three times.

Dual Luciferase Reporter Gene Assay

Luciferase reporter gene assays were performed using a dual-luciferase reporter assay system (Promega, Madison, W USA). The target fragments of wild type EGOT and mutated EGOT were constructed and integrated into pGL3 vector (Promega, Madison, WI, USA) to construct pGL3-LncEGOT-wild type (LncEGOT-wt) and pGL3-LncEGOT-mutated (LncEGOT-mut) reporter vector. EGOT-wt or EGOT-mut was co-transfected into HEK 293 cells with miR-33a-5p mimics or a negative control. After 48 hrs of transfection, luciferase activity was determined according to the manufacturer's instructions. All experiments were repeated three times.

Western Blot

RIPA lysis buffer (Beyotime, China) with protease inhibitor mixture (Roche) was used to prepare breast cancer cell lysates. Protein samples were added for SDS-PAGE and transferred to a nitrocellulose membrane. After being blocked with 5% fat-free milk, primary antibody HMGA2 (diluted 1:1000, Cell Signaling Technology) and anti-GAPDH (diluted 1:2000, Santa Cruz) antibody were used to detect the membrane. After being washed, the membrane was incubated with horseradish peroxidase-conjugated (HRP) secondary antibody (1:2000, Santa Cruz Biotechnology) for 1 hr. Then the membrane was placed on an automatic developing device (ChemiDocXRS Imaging System) to develop and calculate the gray value.

In vivo Studies

All animal experiments were approved by Huazhong University of Science and Technology, and carried out by Institutional Animal Care and Use Committee guidelines (Huazhong University of Science and Technology). Four-week-old male BALB/c athymic nude mice were maintained and used for xenotransplantation experiments. Hep3B cells (2×10^7/mL) from control group and EGOT overexpression group were used. After trypsinization, the cells were centrifuged, and washed for three times with PBS, and then resuspended in PBS. The cell suspension was inoculated to the right (EGOT overexpression group) and left (control group) side of each mouse. The longest and shortest diameters of the tumor mass were measured every 3 days using a caliper. The formula was performed to calculate the tumor volume: volume = (0.5×length×width^2). In the lung metastasis study, 1×10^6 cells in every group were injected into caudal vein of 10 mice, respectively. 15 days later, mice were sacrificed and lung colonization was quantified through pathological examination.

Data Analysis

Data analysis was performed using SPSS18.0 statistical software. All data were expressed as mean ± SD. Student t test was used for statistical analysis. $P<0.05$ was considered statistically significant.

Results

EGOT Was Highly Expressed in HCC Tissues and Cell Lines

First of all, we used qRT-PCR to detect the expression of EGOT in HCC and adjacent normal tissues. The results showed that the expression of EGOT was significantly up-regulated in HCC tissues compared with that in normal tissues, and the expression of EGOT in HCV-related HCC tissues was especially higher (Figure 1A). In addition, compared that in normal liver cell line L02, EGOT expression was significantly elevated in HCC cell lines (Figure 1B). Subsequently, we further analyzed the level of EGOT expression in HCC tissues and clinicopathologic indicators and found that the higher expression of EGOT was associated with larger tumor size and portal vein tumor thrombus (Table 2). These results indicated that EGOT might exert oncogenic role in HCC.

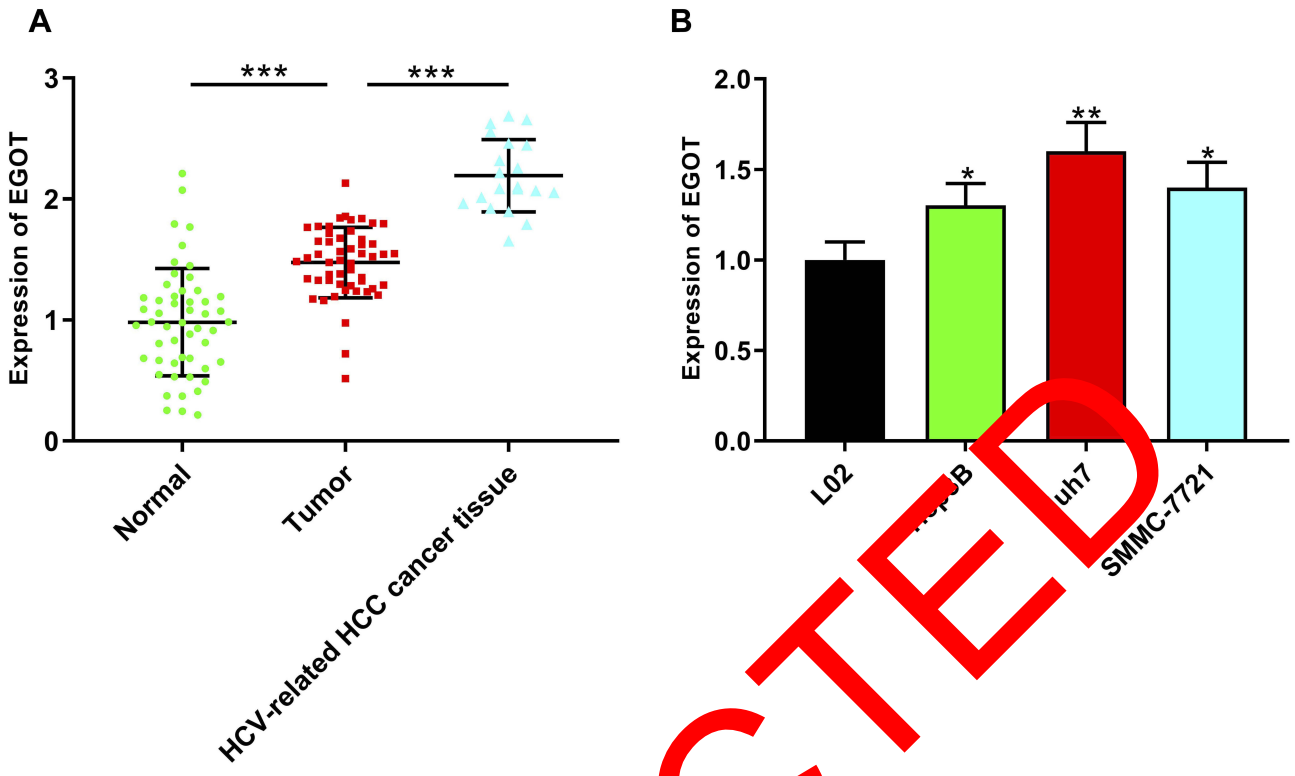

Figure 1 EGOT was highly expressed in HCC tissues and cell lines. (**A**) qRT-PCR was used to detect the expression level of EGOT in normal tissues, HCC tissues and HCV-related HCC tissues. (**B**) qRT-PCR was used to detect the expression level of EGOT in normal liver cell L02 and different HCC cell lines including Hep3B, Huh7, SMMC-7721 cells. *P<0.05, **P<0.01, ***P<0.001.

Down-Regulation of EGOT Inhibited Proliferation, Migration and Invasion of HCC Cells

Next, to investigate the effects of EGOT on proliferation, migration and invasion of HCC cells, we constructed Huh7 cells with EGOT knocked down by lentivirus carrying targeted shRNAs. To avoid off-target effects, two shRNAs were used in this study (Figure 2A). CCK-8 and colony formation assays showed that knockdown of EGOT significantly inhibited the proliferation of Huh7 cells (Figure 2B and C). Wound healing and transwell experiments revealed that knockdown of EGOT significantly reduced migration and cell invasion of Huh7 cells (Figure 2D–F). Additionally, flow cytometry analysis showed that knockdown of EGOT significantly promoted the apoptosis of Huh7 cells (Figure 2G). The above results implied that the down-regulation of EGOT had obvious inhibitory effects on the growth and metastasis of HCC cells.

Up-Regulation of EGOT Promoted Proliferation, Migration and Invasion of HCC Cells

Since EGOT was relatively lowly expressed in Hep3B cells, we chose the Hep3B cell line to perform gain of function analysis. We constructed Hep3B cells with overexpressed EGOT by lentivirus carrying EGOT plasmid (Figure 3A). CCK-8 and colony formation assays showed that overexpression of EGOT significantly promoted the proliferation of Hep3B cells (Figure 3B and C). Wound healing and transwell experiments revealed that overexpression of EGOT significantly increased Hep3B cell migration and invasion (Figure 3D–F). Flow cytometry analysis showed that overexpression of EGOT significantly inhibited the apoptosis of Hep3B cells (Figure 3G). Having observed that EGOT modulates the malignant phenotypes of HCC cells in vitro, using Hep3B cells and nude mice, we further constructed a tail vein injection model and a subcutaneous xenograft model to validate our demonstrations in vivo. In line with observations of in vitro study, in EGOT overexpression group, 8 of 10 mice showed severer lung metastasis, whose incidence is significantly higher than that in the control group (Figure 3H and I), and overexpression of EGOT significantly promoted tumor growth in vivo (Figure 3J). Collectively, the above results indicated that the up-regulation of EGOT had significant promoting effects on the growth and metastasis of HCC cells.

Table 2 Association Between EGOT Expression in HCC Tissues and Clinicopathological Characteristics

Parameters	Group	n	EGOT Expression		*P* value
			Low	High	
Gender	Male Female	38 14	17 9	21 5	0.211
Age (years)	≤60 >60	20 32	11 15	9 17	0.569
Tumor size	≤5cm >5cm	14 38	11 15	3 23	0.012*
AFP	<20 ≥20	23 29	10 16	13 13	0.402
Histological grade	Well/moderate Poorly	43 9	20 6	23 3	0.271
Clinical stage	I/II III	30 22	17 9	13 13	0.262
Tumor number	Solitary Multiple	46 6	25 1	21 5	0.083
Drinking state	Yes No	23 29	10 16	13 13	0.402
Smoking state	Yes No	22 30	10 16	12 14	0.574
PVTT	Yes No	20 32	6 20	14 12	0.023*
Microvascular invasion	Yes No	43 9	19 7	24 2	0.67

Note: *Presents the statistic difference less than 0.05.
Abbreviation: PVTT, portal vein tumor thrombus.

EGOT Directly Acted on miR-33a-5p

Since the cytoplasmic lncRNA is considered to be a small RNA sponge, its targeted inhibition of specific miRNA activity suggests that EGOT may play its role in this way. We searched the starbase database for potential target miRNAs of EGOT. As shown (Figure 4A), EGOT contained a conserved target site for miR-33a-5p. Next, qRT-PCR was used to determine the expression of EGOT and miR-33a-5p in HCC tissues. 20 cases were randomly selected from 52 cases of HCC tissues mentioned above for analysis, and a correlation coefficient between EGOT and miR-33a-5p was calculated as: $R^2 = 0.43$ ($P < 0.05$, Figure 4B). A negative regulation between EGOT and miR-33a-5p was initially confirmed. Dual luciferase reporter assays showed that compared with that of the control group, overexpression of miR-33a-5p significantly reduced the luciferase activity of the EGOT luciferase reporter vector, whereas had no significant effects on the luciferase activity in EGOT mutation group (Figure 4C), which proved that miR-33a-5p was a targeted miRNA for EGOT. In addition, the expression level of miR-33a-5p was significantly increased after down-regulating the EGOT of HCC cells Huh7 and Hep3B (Figure 4D), and the expression level of miR-33a-5p was significantly decreased after up-regulating EGOT (Figure 4E). The regulatory relationship between EGOT and miR-33a-5p was further confirmed.

EGOT Modulated the Expression of HMGA2

qRT-PCR results showed that compared with that of the control group, HMGA2 expression on mRNA level was significantly down-regulated after knockdown of EGOT in Huh7 cells (Figure 5A). Conversely, HMGA2 expression was significantly upregulated after overexpression of EGOT in Hep3B cells (Figure 5B). We also demonstrated that in HCC samples, there is a positive correlation between EGOT and HMGA2 mRNA (R^2=0.644, P<0.05, Figure 5C). Additionally, Western blot assays showed that compared with that of the control group, the expression of HMGA2 on protein level was significantly increased after overexpression of EGOT in Hep3B cell line, and it was significantly down-regulated after knockdown of EGOT in Huh7 cell line (Figure 5D). We also detected the expression level of EGOT, miR-33a-5p and HMGA2 in the tumor tissues from nude mice tumorigenicity assay. Consistent with the in vitro data, EGOT overexpression increased the expression level of EGOT and HMGA2 in tumor tissues, while reduced the expression level of miR-33a-5p (Figure 5E–G). Collectively, these data indicated that EGOT could regulate the expression of HMGA2 in HCC.

EGOT Increased the Expression of HMGA2 by Inhibiting the Function of miR-33a-5p, and Promoting Proliferation and Metastasis of HCC Cells

It has been validated that HMGA2 was a target of miR-33a-5p,[30] and to determine whether EGOT regulates proliferation and metastasis of HCC cells via miR-33a-5p/HMGA2 axis, we transfected the miR-33a-5p mimics into cells with overexpressed EGOT. The results of qRT-PCR showed that transfection of miR-33a-5p mimics in Huh7 cells reduced the expression level of EGOT, and increased the expression

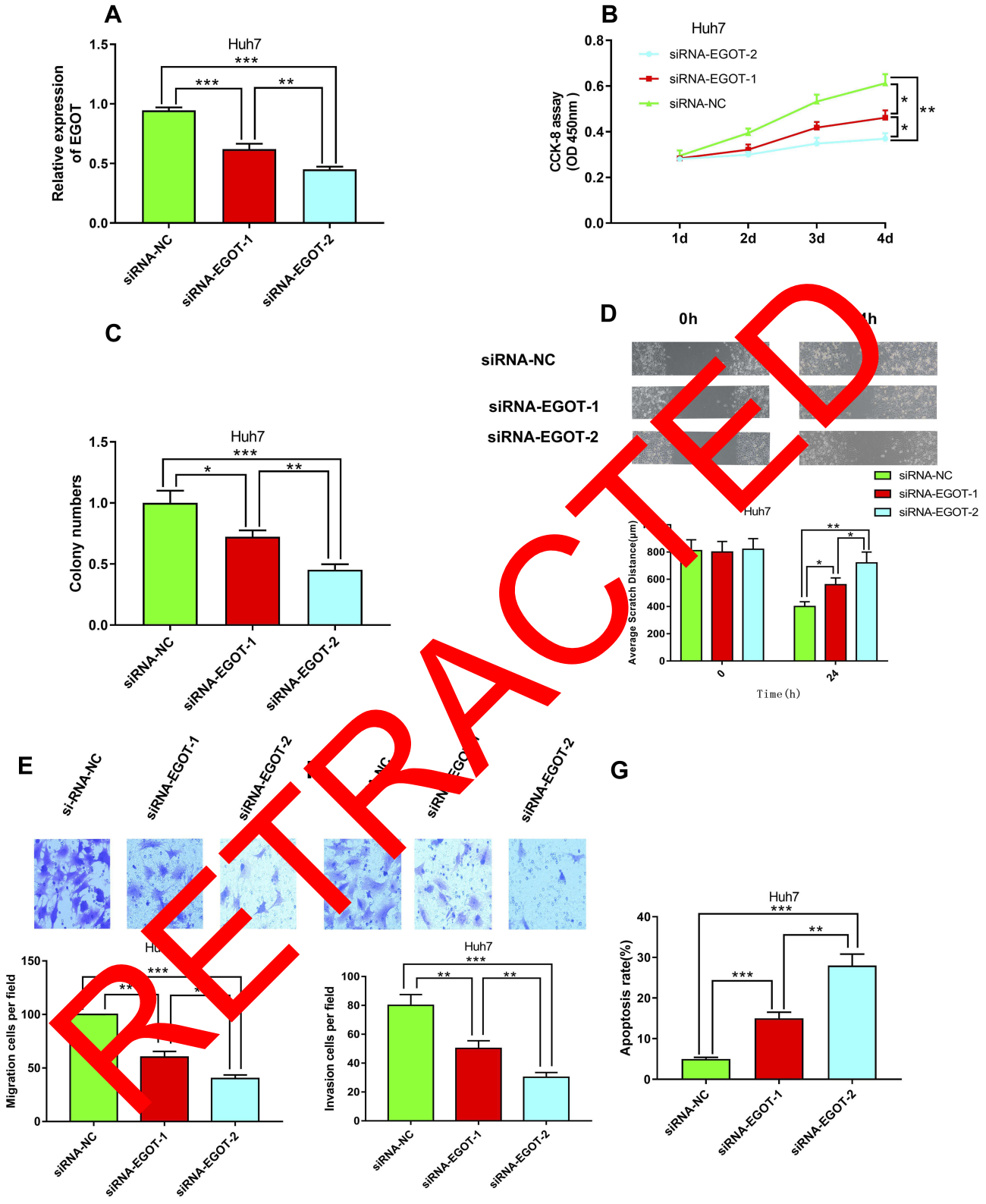

Figure 2 Knockdown of EGOT inhibited the malignant phenotypes of HCC cell line Huh7. (**A**) qRT-PCR was used to verify the efficiency of knockdown of EGOT by RNA interfering. (**B**) CCK-8 method was used to detect the proliferation of Huh7 cells after EGOT knockdown. (**C**) Colony formation assay was conducted to evaluate the ability of colony formation of Huh7 cells after EGOT knockdown. (**D**) Wound-healing assay was used to examine the motility of Huh7 cells after EGOT knockdown. (**E, F**) Transwell assays were used to detect the migration and invasion of Huh7 cells after EGOT knockdown, respectively. (**G**) The apoptosis of Huh7 cells after EGOT knockdown was analyzed by flow cytometry. *P<0.05, **P<0.01, ***P<0.001.

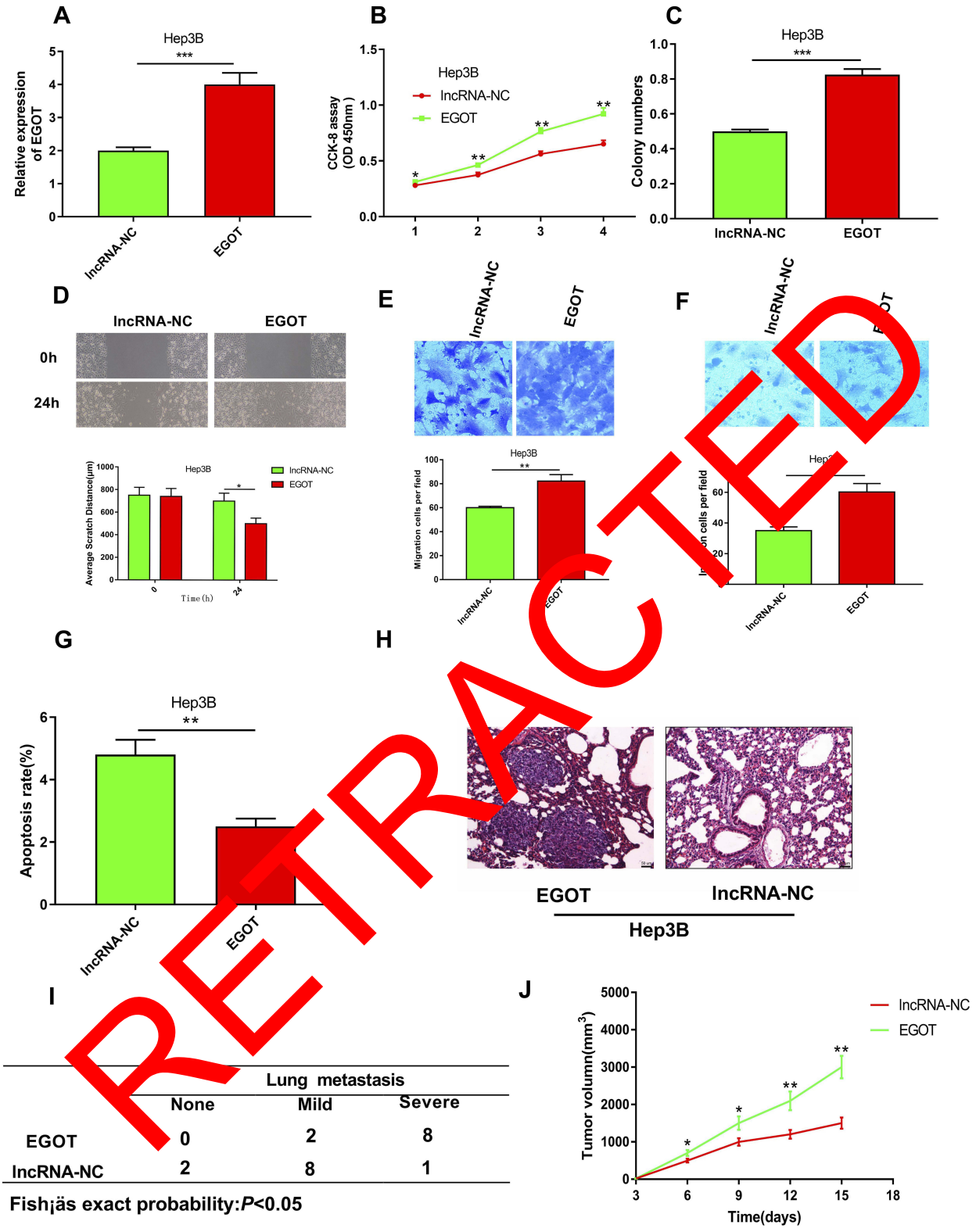

Figure 3 Overexpression of EGOT promoted the malignant phenotypes of HCC cell line Hep3B. (**A**) qRT-PCR was used to verify the efficiency of overexpression of EGOT in Hep3B cells. (**B**) CCK-8 method was used to detect the proliferation of Hep3B cells after EGOT overexpression. (**C**) Colony formation assay was conducted to evaluate the ability of colony formation of Hep3B cells after EGOT overexpression. (**D**) Wound-healing assay was used to examine the motility of Hep3B cells after EGOT overexpression. (**E, F**) Transwell assays were used to detect the migration and invasion of Hep3B cells after EGOT overexpression, respectively. (**G**) The apoptosis of Hep3B cells after EGOT overexpression was analyzed by flow cytometry. (**H, I**) Incidence and severity of lung metastasis in mice pulmonary metastasis model with EGOT overexpressed Hep3B cell and control cell. (**J**) Tumor volume in nude mice xenograft model with EGOT overexpressed Hep3B cell and control cell. **P*<0.05, ***P*<0.01, ****P*<0.001.

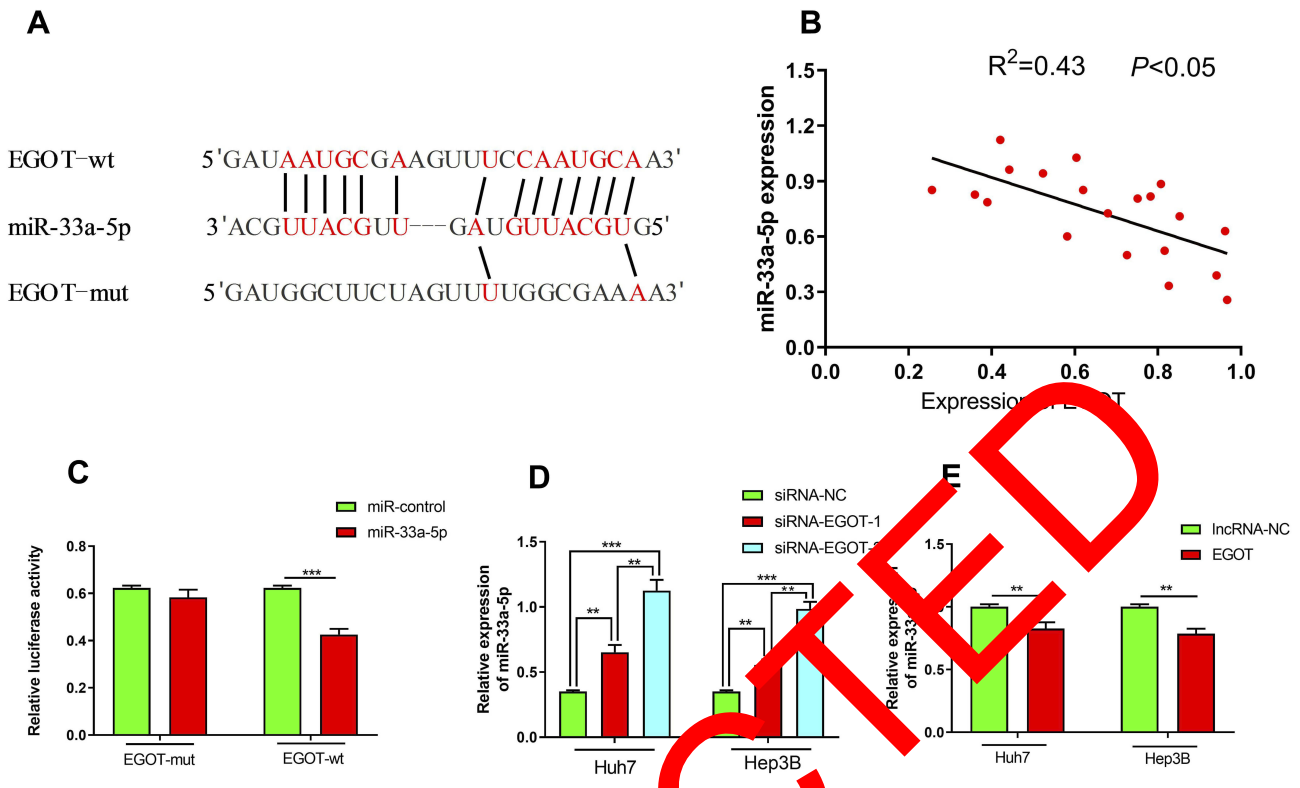

Figure 4 miR-33a-5p was a target of EGOT. (**A**) The potential target site of miR-33a-5p and EGOT was shown as a schematic representation. (**B**) An inverse correlation was found between the expression levels of miR-33a-5p and EGOT in HCC samples. (**C**) Dual-luciferase reporter assay showed that miR-33a-5p can only reduce the luciferase activity of wide type EGOT sequence. (**D, E**) qRT-PCR was used to detect the changes of miR-33a-5p after EGOT was knocked down or overexpressed in HCC cell lines Huh7 and Hep3B. ***P*<0.01, ****P*<0.001.

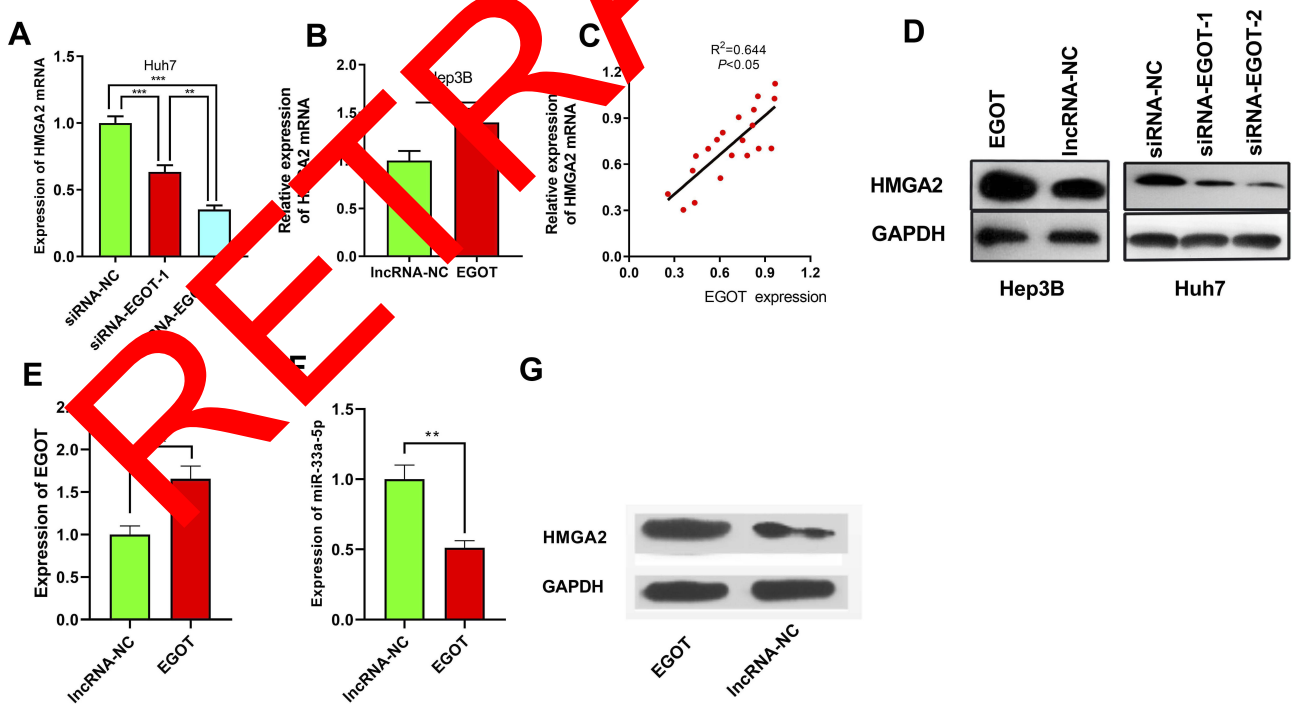

Figure 5 EGOT could modulate the expression level of HMGA2. (**A, B**) qRT-PCR was used to detect the changes of HMGA2 mRNA after EGOT was knocked down or overexpressed in HCC cell lines Huh7 and Hep3B. (**C**) A positive correlation was found between the expression levels of EGOT and HMGA2 mRNA in HCC samples. (**D**) Western blot was used to detect the changes of HMGA2 protein after EGOT was overexpressed or knockdown in HCC cell lines Huh7 and Hep3B. (**E**–**G**) qRT-PCR and Western blot were used to detect the expression level of EGOT, miR-33a-5p and HMGA2, respectively, in the tumor tissues of nude mice from EGOT overexpression group and control group. **P*<0.05, ***P*<0.01, ****P*<0.001.

level of miR-33a-5p and HMGA2 (Figure 6A–C). CCK-8 and colony formation assays demonstrated that transfection of miR-33a-5p mimics reduced the proliferation of Huh7 cells, whereas overexpression of EGOT inhibited the action of miR-33a-5p (Figure 6D and E). Wound healing and transwell assays confirmed that transfection of the miR-33a-5p mimics reduced the migration and invasion of Huh7 cells, which could be counteracted by overexpression of EGOT (Figure 6F–H). Flow cytometry showed that transfection of miR-33a-5p mimics increased the apoptosis ability of Huh7 cells compared to that of Huh7 HCC cells with overexpressed EGOT (Figure 6I). Based on the data above, we concluded that EGOT affects the progression of HCC by regulating miR-33a-5p/HMGA2.

Discussion

HCV infection is an important factor in the tumorigenesis and progression of HCC. 10% to 25% of HCC are associated with HCV infection worldwide.[31] Studies have shown that HCV is a single positive stranded RNA that does not integrate with the host chromosome in vivo. It may induce HCC carcinogenesis through chronic inflammation of hepatocytes and oxidative stress, or through affecting the cell cycle to cause damage or mutation of host DNA.[32] It was found that EGOT, a negative regulator of antiviral response, is induced by activation of RIG-I and PKR in HCV-infected cells, which is beneficial for HCV replication and increases the possibility of cirrhosis and HCC.[15] Interestingly, in this study, we found that EGOT was significantly up-regulated in HCV-associated HCC tissues compared to that of normal tissues, implying the crucial role of EGOT in the tumorigenesis of HCV-associated HCC.

The tumor-suppressing and cancer-promoting functions of lncRNA have been widely concerned. For example, overexpression of lncRNA SVUGP2 inhibits the proliferation, migration and invasion of HepG2 and Hep3B HCC cells.[33] It is reported that EGOT plays an oncogenic role in gastric cancer and could be used as a diagnostic and prognostic biomarker.[14] In the present study, we observed that EGOT was significantly highly expressed in HCC cells than that in adjacent normal tissues, which may be associated with elevated markers of advanced malignancies. Functional experiments with up- or down-regulation were used to explore the effects of EGOT on the biological behavior of HCC cells. We demonstrated that EGOT promoted the proliferation, migration and invasion of HCC cells, suggesting that EGOT exerts a cancer-promoting effect in the development of HCC. To our best knowledge, this is the first study focusing on the function of EGOT in HCC.

MicroRNAs participate in many biological processes through regulation target gene.[34] Studies have shown that miR-33a-5p inhibits the proliferation of lung adenocarcinoma cells, enhances the anti-tumor effect of celastrol, and increases the sensitivity to celastrol by targeting mTOR.[35] miR-33a-5p is also reported to be down-regulated in HCC tissues and linked to chemoresistance.[36] In this study, we observed that miR-33a-5p had tumor suppressive effects on HCC cells, which was consistent with previous reports.[19,20,36] Several studies have found that lncRNAs, as endogenously competitive RNAs, regulate miRNA expression levels. To further investigate the downstream molecular mechanism of EGOT to regulate HCC growth, we found that miR-33a-3p was a target of EGOT, and overexpression of EGOT inhibited the expression of intracellular miR-33a-5p in HCC cells. In addition, transfection of miR-33a-5p mimics counteracted the promoting effect of EGOT on proliferation and metastasis of HCC cells. The above studies indicate that miR-33a-5p exerts a tumor suppressor effect in HCC cells, and EGOT exerts a cancer-promoting effect partly by targeted inhibition of it.

Through three AT-hooks, HMGA2 can be ligated to the AT-rich sulcus in DNA to affect transcription of the target gene by altering the structure of the DNA.[23] In addition, HMGA2 is highly expressed and significantly associated with tumor prognosis, tumor grade and metastasis in many cancers such as breast cancer, lung cancer, ovarian cancer, oral squamous cell carcinoma, and pancreatic cancer.[23] The role of HMGA2 in HCC has also been addressed in previous studies. It is reported that hepatitis B virus X protein promotes EMT of HCC cells by targeting HMGA2;[37] Meanwhile, a recent study have shown that the HMGA2-sh-3p20 fragment in the 3′UTR of HMGA2 mRNA promotes the growth of HCC cells by up-regulating HMGA2.[38] However, whether EGOT affects the expression of HMGA2 has not been addressed. In this study, we initially confirmed the positive correlation between EGOT and HMGA2 in clinical samples. Furthermore, we inhibited the expression of miR-33a-5p by overexpression of EGOT, while the expression of HMGA2 was increased accordingly. Therefore, we concluded that EGOT/miR-33a-5p/HMGA2 axis could be present in the development and progression of HCC.

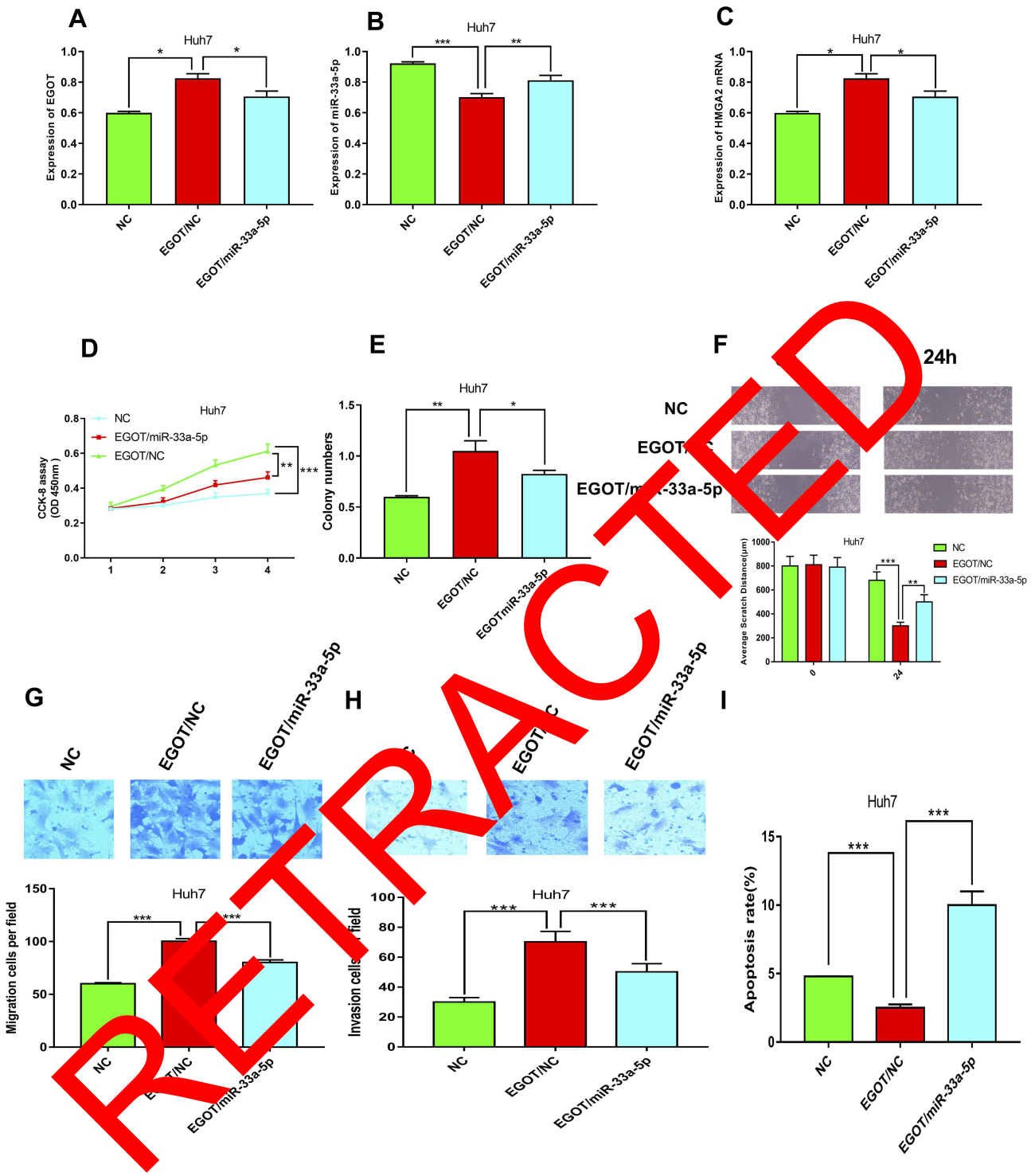

Figure 6 miR-33a-5p reverse the effects of EGOT on the expression of HMGA2 and malignant phenotypes of HCC cells. (**A**–**C**) qRT-PCR was used to detect the expression level of EGOT, miR-33a-5p and HMGA2 after transfection. (**D**) CCK-8 method was used to detect the proliferation of Huh7. (**E**) Colony formation assay was conducted to evaluate the ability of colony formation of Huh7 cells. (**F**) Wound-healing assay was used to examine the motility of Huh7 cells. (**G, H**) Transwell assays were used to detect the migration and invasion of Huh7 cells, respectively. (**I**) The apoptosis of Huh7 cells was analyzed by flow cytometry. **P*<0.05, ***P*<0.01, ****P*<0.001.

In summary, we demonstrated that EGOT promoted the proliferation, migration and invasion of HCC. In addition, we have also found that miR-33a-5p functions as a tumor suppressor in HCC cells. Mechanically, EGOT promoted cancer progression by inhibition of miR-33a-5p to increase HMGA2 expression. Our study explores new mechanisms in the development of HCC and provides a novel theoretical basis for the diagnosis and treatment of HCC.

Ethics Statement

Our study was approved by the Clinical Ethics Committee of Wuhan Fourth Hospital.

Data Sharing Statement

The data used to support the findings of this study are available from the corresponding author upon request.

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Disclosure

The authors report no conflicts of interest in this work.

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