miR-221-3p promotes hepatocellular carcinogenesis by downregulating O6-methylguanine-DNA methyltransferase

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ABSTRACT

This study aimed to investigate the influence of miR-221-3p and O\textsuperscript{6}-methylguanine-DNA methyltransferase (MGMT) interaction in human hepatocellular carcinoma (HCC), thereby revealing a novel molecular mechanism of hepatic carcinogenesis involving miR-221-3p and MGMT. Fluorescence qPCR and immunoblot assays were performed to determine the expression of RNA and protein in HCC tissues and cell lines. We also employed the firefly and Renilla luciferase assay to verify the target relationship between miR-221-3p and MGMT mRNA. Assessments including the MTT assay, wound-healing assay, transwell assay, colony foci formation experiment, and flow cytometric experiment were carried out to determine the viability, migration, invasion, proliferation, cell cycle progression, and apoptosis of SMMC-7721 and BEL-7404 cell lines with the modulated expression of miR-221-3p and MGMT. Compared to healthy tissues and cell line HL7702, miR-221-3p was significantly upregulated but MGMT was significantly downregulated in carcinomas and cancerous cell lines. Forced miR-221-3p overexpression was found to enhance the proliferation, migration, invasion, and clonogenicity of cell lines, but it suppressed cell apoptosis. Findings also revealed that forced miR-221-3p overexpression had little effect on cell cycle progression. After MGMT was confirmed to be a target gene of miR-221-3p, it was found that the forced upregulation of miR-221-3p downregulated MGMT mRNA and protein levels significantly. MiR-221-3p was identified as an HCC promoting factor, and it specifically inhibited the expression of the MGMT. Besides, the upregulation of miR-221-3p had a positive influence on HCC pathogenesis by inhibiting MGMT expression.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer mortality globally. In 2018, about 781,631 individuals worldwide died of liver cancer.\textsuperscript{1} HCC represents 75%–85% of primary liver cancer cases, and it can result from chronic infection, aflatoxin-contaminated foodstuffs, heavy alcohol intake, obesity, smoking, and type 2 diabetes.\textsuperscript{2} While research suggests that early diagnosis and treatment can prolong the chances of survival of HCC patients,\textsuperscript{3} the survival of advanced or metastatic HCC patients cannot be prolonged with conventional chemotherapy, which is the most commonly used therapy today. This limitation is, in part, because the mechanism that influences HCC is not yet clear. Besides, this mechanism is complicated in that it involves several genes and proteins that induce abnormal phenotypes, including cellular cycle, apoptosis, proliferation, cell migration, and invasion.\textsuperscript{4,5} Researchers have intensified efforts to investigate genes and proteins associated with HCC, but they are yet to make substantive progress given that the specificity and sensitivity of genes and proteins as therapy targets are limited.\textsuperscript{7,8,9,10} Hence, it is crucial to identify novel HCC pathogenesis factors.

miRNAs belong to a non-coding small RNA family that includes miRNAs, snoRNAs, and exRNAs. They are single-stranded and 22 ~ 28 nucleotides in length and are responsible for interrupting the protein translation process by pairing up with 3′UTRs of mRNAs. In recent years, scientists have paid more attention to miRNAs‘ involvement in cell proliferation, apoptosis, migration, cell cycle, invasion, and other biological processes.\textsuperscript{11,12,13,14,15} Researchers have also observed that different miRNAs have different biological functions. To name a few, miR-105 played a negative role in the proliferation of HCC cells through the PI3K/AKT signaling pathway.\textsuperscript{16} miR-221 was found to be overexpressed in hepatic carcinoma tissues compared with healthy hepatic tissues.\textsuperscript{17} This RNA molecule could also improve the hepatocellular cancer cells’ growth ability, as indicated by the increased ratio of cells in the synthesis phase of the cell cycle.\textsuperscript{18} In 2008, a study reported that miR-221-3p bound to the 3′UTRs of cyclin-dependent kinase inhibitor genes p27Kip1 and p57Kip2, thus enhancing cell proliferation in HCC.\textsuperscript{18} Cyclin-dependent kinase inhibitor proteins was also discovered to inhibit cyclin-dependent kinases, thereby limiting cell cycle progression and acting as tumor suppressors. The DNA damage-inducible transcript 4 (DDIT4) is a gene that is ontologically related to protein binding. In 2010, Pinau et al.\textsuperscript{19} found that DDIT4 was the target of miR-221-3p. Besides, O\textsuperscript{6}-methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme that can suppress and eliminate guanine-alkyl groups influenced by alkylating agents, which are potent carcinogens.\textsuperscript{20} In several experiments, MGMT promoter methylation has been found to inactivate MGMT, which is commonly observed in human neoplasms such as head
and neck cancers, pulmonary cancers, and glioma.\textsuperscript{21,22} Nonetheless, only a few studies have reported the target relationship between miR-221-3p and MGMT.

In the current study, we carried out bioinformatics analysis and cellular experiments to determine whether miR-221-3p could directly bind to MGMT mRNA. To investigate the influence of the interaction of miR-221-3p and MGMT on HCC, we conducted several cytological and molecular biological experiments such as the RT-qPCR, western blot, MTT assay, and transwell assay. By carrying out these experiments, we are optimistic that our results may provide more insights into a new therapy target axis in HCC.

Materials and methods

Liver cancer samples and cell culture

Materials for this study were collected at the Guangxi Medical University Affiliated Tumor Hospital, and they included snap-frozen healthy hepatic tissues (n = 10), snap-frozen hepatic carcinomas (n = 20), and snap-frozen adjacent tissues (n = 20). All the tissues used in this study were based on the guidelines approved by the Ethics Committee. HL-7702 (cultured with RPMI 1640), Hep G2 (cultured with DMEM), BEL-7404 (cultured with RPMI 1640), Huh-7 (cultured with DMEM), SMMC-7721 (cultured with RPMI 1640) and 293 T (cultured with DMEM) cell lines were purchased from the Shanghai Institute of Life Sciences Cell Resource Center (China). The HL-7702 cell line was the human normal hepatocyte cell line, while HepG2, BEL-7404, Huh-7, and SMMC-7721 cell lines were the human hepatoma cell lines. 293 T cell line was used specifically for the dual luciferase assay. These cell lines were authenticated using short tandem repeat (STR) authentication, a service that was provided by Shanghai GENECHEM Co., Ltd (China). Ten STR loci were detected, and the matches were more than 80%. The phenotypes of SMMC-7721 and BEL-7404are showed in Supplementary Figure 1. All the cell culture media were complemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic-antimycotic solution (Gibco). Before the experiments were performed, all the cells were kept at 37°C with 5% CO\textsubscript{2} in an incubator.

Sample preparation and RNA isolation

Hepatic carcinoma, adjacent and normal hepatic tissues, were collected in sterile containers and immediately frozen in liquid nitrogen to avoid RNA degradation. The total RNAs were obtained from tissues and cells using TRIzol reagent (Tiangen Biotech, Beijing, China). 600–3,750 ng/µL total RNA was obtained from the 700 µL of tissue samples. The quality and quantity of the isolated total RNAs were evaluated using the spectrophotometric reading method.

qRT-PCR

Equal amounts of RNA were converted into cDNA, and RT-qPCR was performed using the Power SYBR Green PCR Master Mix. Afterward, U6 expression was used to normalize the expression of miR-221-3p,\textsuperscript{23} and GAPDH expression was utilized to normalize the expression of MGMT. The primer sequences of miR-221-3p were F: 5’-GGCATGAACCTG GCATACAA-3’ and R: 5’-TTCGGTAGCTGAACCC -3’. The primer sequences of MGMT were F: 5’-ATGAA ACACACACGTGA-3’ and R: 5’-AATAGAGGAGGG CAGCGTT-3’. The comparative C\textsubscript{T} method (2^{-ΔΔCt}) was used to calculate relative RNA expression.\textsuperscript{24}

Cell transfection

SMMC-7721 and BEL-7404 cell lines were chosen for all the following experiments except for the firefly luciferase reporter assay. The two cell lines were transfected with several molecules, including miR-221-3p mimic, miR-221-3p inhibitor, negative control RNA, and MGMT overexpression plasmids. After culturing the cells to 70% confluence, the transfection was performed. The cells in the control group were not transfected, however. To transfect the experimental cells, MGMT Human Tagged ORF Clone was purchased from OriGene (Cat#: RC229131, Shanghai, China). Apart from the overexpression plasmids, all the other molecules were designed and purchased from GENECHEM (Shanghai, China).

MTT assay

The selected BEL-7404 and SMMC-7721 cell lines were seeded in 96-well plates in RPMI 1640 with 10% FBS. After 12 h, both cell lines were washed with phosphate-buffered saline (PBS) twice and transfected with the molecules. The MTT reagent purchased from Sigma (USA) was later used to detect cell proliferation every day for five days. Next, 20 µL MTT reagents were incubated in each well for 14 min. Optical absorbance was then read at 490 nm.

Cell cycle and apoptosis assays

The cell cycle profiles were analyzed by flow cytometry, and the percentage of cells in different phases were calculated. BEL-7404 and SMMC-7721 cell lines in various groups were harvested and fixed with 80% ethanol. Then, the fixed cells were incubated with 0.1 mg/ml propidium iodide (PI) dye (ebioscience, USA) and 0.1 mg/ml RNase A for 50 min at 37°C without light. The BD-LSRFortessa flow cytometer (BD Biosciences) was used to measure the cell cycle, and the data were analyzed using ModFit LT\textsuperscript{TM} Trial and Reader software version 4.0 (USA).

Like in cell cycle flow cytometric assay, in cell apoptosis assay, the ethanol-fixed cells were resuspended in 1.25 µL of Annexin V-FITC and 10 µL of PI staining solutions (mixed in 500 µL 1x binding buffer, ebiosciences, USA), and kept in the dark for less than half an hour. Cells stained with FITC were defined as early apoptotic cells, cells stained with both FITC and PI were defined as late apoptotic cells. Both early and late apoptotic cells were regarded as apoptotic cells.

Wound-healing assay

The transfected cells were seeded in 12-well plates at a density of 30,000 cells per well and then cultured under 5% CO\textsubscript{2} at 37°C. At 90% confluence, the cell monolayers of each well were scraped to
make cell-free areas with pipet tips. After washing them in PBS, the serum-free medium was used to culture cells under 5% CO₂ at 37°C. The migration of cells into the scratch was observed at 24 h using an inverted microscope system (model IX83, Olympus, Japan). The wound distance was measured using Adobe Photoshop, a graphics editor. The migration rate was defined as the ratio of migration distance at 24 h to the wound width at 0 h.

**Transwell invasion assay**

The matrigel (BD Bioscience, USA) was diluted in RPMI-1640 media in a volume ratio of 1:3 before it was added to transwell inserts (BD Bioscience, USA) overnight for incubation at 37°C. The cell suspension in 100 µL serum-free medium was then added to the upper chambers mentioned above for 2-day incubation. The cells on the downsize of the upper chambers were fixed in 4% paraformaldehyde for 30 min. After washing the cells with PBS and staining with 0.1% crystal violet for 20 min, the stained cells were counted at 200× magnification.

**Colony foci formation assay**

The transfected cells at the log phase were harvested and digested by mild trypsinization (Sigma, USA). Next, they were washed in PBS three times and seeded in a 6-well plate at a cell concentration of 400 ~ 1,000 cells per well. After 14 days of growth in 10% FBS RPMI 164 media, the colonies were fixed with 4% paraformaldehyde (Santa Cruz Biotechnology, USA) for 30 min, stained for 1 h using 1 mL/well crystal violet solution, and photographed using an inverted microscope (model IX83, Olympus, Japan). Then, the colony foci number was determined to assess the cells’ ability to form colonies.

**Immunoblot assay**

The transfected cells were cultured for 3 days and were analyzed with western blot in order to determine MGMT protein expression. Equal quantities (40 µg) of proteins extracted from BEL-7404 and SMMC-7721 cells were separated on 12% SDS gels by electrophoresis and then transferred onto nitrocellulose blotting membranes (Invitrogen, USA). After that, 5% nonfat milk was dissolved in Tris-buffered saline with 0.1%. TWEEN 20 was later used to block the blotting membranes for 1 h. Then, the blocked membranes were washed with TBST (TRIS-Buffered Saline, 0.1% TWEEN* 20 Detergent) four times and incubated at 4°C for 20 h with the primary antibodies against MGMT (Cat#: ab39253, Abcam, UK) and GAPDH (Cat#: ab9484, Abcam, UK). The primary antibodies were subsequently washed off with TBST, and the membranes were incubated with Rabbit Anti-Mouse IgG secondary antibody (Cat#ab6728, Abcam, UK) for 90 min. Using TBST to wash PVDF membranes four times, the membranes were exposed according to the Pierce™ ECL Western Blotting Substrate protocol (Thermo Fischer, USA).

**Immunohistochemistry**

Hepatic carcinoma, adjacent and normal hepatic tissues were collected at biopsy, fixed in 4% formaldehyde, embedded in paraffin, and cut into 10 slices for immunohistochemical staining. The xylene-deparaffinized tissue slices were rehydrated in ethanol and incubated with 3% hydrogen peroxide to remove endogenous peroxidase. After washing them three times, the tissue slices were incubated overnight with primary antibodies against MGMT (Cat#: ab39253, Abcam, UK) at 4°C. The secondary antibody labeled by horseradish peroxidase (HRP) was incubated with the slices for another 20 min. Then, the tissue slices were stained with DAB-Peroxidase Substrate Solution (Cat#: 34002, Thermo Fischer, USA). The sections were later counterstained with Mayer’s hematoxylin solution (Sigma, USA). The whole tissue slices were then observed at ×100 magnification to identify a ‘hot spot.’ For further analysis, the field was shifted to ×400 magnification. Three pathologists who had no idea about the study results read the IHC pictures based on a 5-tier scoring system (see Figure 4a).

**Firefly luciferase reporter assay**

RNA hybrid, microRNA, Pictar, and TargetScan were used to predict the targets of miR-221-3p. The sequence of human MGMT mRNA 3’-UTR contains the complementary sequence motif of miR-221-3p, which was amplified with PCR. The specific primers of MGMT were designed as 5’-ACCGTTTTCGAGCGAGTACTT-3’ (MGMT-3’UTR-F) and 5’-GGTGAACGAGCTTTGCTGGA-3’ (MGMT-3’UTR-R). The mutated human MGMT mRNA 3’-UTR did not include the binding site of miR-221-3p, which was amplified using PCR. The primers of mutated human MGMT mRNA 3’-UTR were designed as 5’-CGCTGCTGTCTGACTGTTGCTG-3’ (MGMT-3’UTR-Mut-F) and 5’-CATATGCAGCTAGCAGCTGGTGTTTC-3’ (MGMT-3’UTR-Mut-R). Containing the firefly luciferase reporter, the GV272 vector (5010 bp in length) was purchased from GENECHEM (Shanghai, China). The promoter of the GV272 vector was increased from 48 bp to 250 bp (Figure 5b). The constructed GV272-MGMT or GV272-MGMT-Mut was transfected into the 293 T cell line. The cells were then seeded in 24-well plates and transfected with 0.1 µg of either GV272-MGMT or GV272-MGMT-Mut per well together with 0.01 µg of pRL-TK vectors (Promega, USA), which contained Renilla luciferase and 0.4 µg of the miR-221-3p mimics or negative control (NC) of mimics. The transfection was done using X-tremeGENE HP (Cat. No. 06366236001, Sigma, USA) in a final volume of 0.5 mL. After 1-day transfection, the Dual-Luciferase Reporter Assay System (Promega, USA) was employed to measure the firefly and Renilla luciferase intensities. Results were reported as firefly luciferase intensity/Renilla luciferase intensity, and each transfection was done in triplicate.

**Statistical analysis**

Data of this study were reported as mean±SD. Statistical differences between the blank or negative control group (in case of tissue examinations, the comparison was made to healthy tissue) and experimental groups were analyzed using a one-way analysis of variance method by SPSS software 19.0. P(probability)<0.05 was defined as statistically significant.
Results

Hepatocellular carcinomas and hepatocellular carcinoma cells showed upregulation of miR-221-3p

To reveal the expression of miR-221-3p in HCC, we detected the level of miR-221-3p in liver carcinomas (n = 20), adjacent-to-tumor tissues (n = 20), and healthy liver tissues (n = 10) and 5 cell lines (HL-7702, BEL-7404, SMMC-7721, Huh-7 and Hep G2) using real-time qPCR. As illustrated in Figure 1a, the expression level of miR-221-3p in tumors was 1.33-fold of that in healthy tissues. Compared with HL-7702 cell line (normal human hepatic cells), human hepatoma cells BEL-7404, SMMC-7721, Huh-7, and Hep G2 showed significant upregulation of miR-221-3p, especially SMMC-7721 and BEL-7404 cell lines (up to about 3.8- and 3.5-fold of healthy hepatic cells, respectively) (Figure 1b). BEL-7404 and SMMC-7721 cell lines were chosen for subsequent experiments because of this significant upregulation of miR-221-3p.

Because we wondered whether the aberrant upregulation of miR-221-3p played a crucial role in HCC prognosis, we used the pan-cancer data analysis algorithm of StarBase (http://starbase.sysu.edu.cn/) to analyze the prognosis value of miR-221-3p in HCC. The result showed that HCC patients (n = 369) with a high level of miR-221-3p exhibited poorer prognosis than those with a low level of miR-221-3p, with a slight statistical significance (hazard ratio = 1.41, log-rank p = .053) (Figure 1c).

Overexpression of miR-221-3p induced cell viability, migration, and invasion

In the MTT assay, cell viability was detected in SMMC-7721 and BEL-7404 cell lines after transfecting with exogenous miR-221-3p mimic or miR-221-3p inhibitor for 5 days. The optical density (OD) values at 490 nm wavelength were illustrated. Compared with the negative control (NC) group, the miRNA
mimic transfection caused a 1.17- and 1.11-fold increase in cell viability at day 4 and day 5, respectively. However, the miRNA inhibitor transfection caused a 1.81- and 1.85-fold decrease in cell viability at day 4 and day 5 in SMMC-7721 cells, respectively (Figure 2a). BEL-7404 cells displayed similar results as SMMC-7721 cells. Meanwhile, SMMC-7721 and BEL-7404 cells transfected with miR-221-3p mimic, miR-221-3p inhibitor, or negative control RNA were photographed at 0 h and 24 h after the scratch was leveraged to analyze the ability of migration. In SMMC-7721 and BRL-7404 cell lines, the migration rate of cells transfected with miR-221-3p mimic was enhanced by 1.1- and 1.73-fold, and the migration rate of cells transfected with miR-221-3p inhibitor was reduced by 69% and 59% compared with the NC group (Figure 2b). Moreover, the transwell invasion assay results demonstrated that the invasion ability was enhanced by 1.22- and 1.37-fold with the treatment of miR-221-3p mimic; however, it was reduced by 30% to 50% with the treatment of miR-221-3p inhibitor (Figure 2c). As for the colony foci formation assay, the miR-221-3p mimic-transfected cells formed more clones, whereas the miR-221-3p inhibitor-transfected cells resulted in fewer clones (Figure 2d). The statistical analysis results suggested that miR-221-3p was possibly hepatocellular carcinogenic.

miR-221-3p inhibition induced cell apoptosis but had no effects on cell cycle in vitro

Both SMMC-7721 and BEL-7404 cells transfected with miR-221-3p inhibitor exhibited a 1.25-fold increase in the apoptosis rate when compared with the NC group (Figure 3a). In the SMMC-7721 cell line, the transfection of miR-221-3p mimic and inhibitor resulted in a higher ratio of cells in the G2/M phase (1.2- and 1.36-fold of the NC group, respectively), whereas no changes were observed for the percentage of cells in G1 phase and S phase compared with the NC group (Figure 3b). Similarly, the percentage of BEL-7404 cells transfected with miR-221-3p inhibitor was 1.26-fold of the NC group in the G2/M phase but not in G1 and S phase (Figure 3b). The data showed that miR-221-3p downregulation promoted the apoptosis of hepatic carcinoma cells. However, it did not influence HCC cell apoptosis by changing cell cycle progress.

MGMT was the target of miR-221-3p and was downregulated in hepatic carcinoma

microRNA target prediction software applications such as RNA hybrid, microRNA, Pictar, and TargetScan were used to explore the targets of miR-221-3p. The software predicted that MGMT mRNA 3‘ UTR contained a binding site for miR-221-3p (Figure 4a). To validate the binding relationship between MGMT mRNA 3‘ UTR and miR-221-3p, the wild-type or mutated 3‘ UTR sequences of MGMT were respectively inserted into the firefly luciferase reporter plasmids. Afterward, the constructed vectors were transfected into 293 T cells together with miR-221-3p mimic or NC RNA to conduct a dual-luciferase reporter gene assay. As indicated in Figure 4b, the miR-221-3p overexpression reduced the luciferase activity by approximately 50% when it was co-transfected with wild-type MGMT mRNA 3‘UTR. Nevertheless, it showed little effect in luciferase activity when co-transfected with the mutated 3‘ UTR sequence of MGMT mRNA. Through immunohistochemical staining, the expression of MGMT protein in carcinomas was downregulated, as indicated by a lower average IHC score (Figure 4c). We then confirmed the transfection efficiency of miR-221-3p mimic and inhibitor on MGMT expression in hepatic carcinoma cells. As presented in Figure 4d, the result from RT-PCR showed that the transfection of miR-221-3p mimic into the SMMC-7721 and BEL-7404 cells led to significantly low levels of MGMT mRNA (42% and 50% decrease, respectively). The western blot assay produced a result that was similar to the RT-PCR assay: the transfection of miR-221-3p mimic into both cell lines decreased the level of MGMT protein (Figure 4e). By performing Gene Expression Profiling Interactive Analysis (GEPIA), we found that MGMT expression at stage II and III was lower than that at stage I of HCC (p = .00191, F-test was used). GEPIA acquires data from the TCGA open database (n = 377). MGMT expression was negatively correlated with staging; however, at stage IV, the level of MGMT expression was higher than that at stage II and III. According to the TNM system, at stage II and III, the tumor is larger, and it grows into nearby tissues or lymph nodes. At stage IV, the tumor reached other organs. We thought that MGMT might try to repair the DNA at stage IV to prevent the aggressive progression of HCC (figure 4f). However, MGMT expression gradually increased at stage IV. We also found that a lower level of MGMT was an indicator of inferior prognosis results for HCC patients (Figure 4g). The data obtained from the dual luciferase assay, RT-PCR, immunohistochemical experiment, western blot, GEPIA analysis, and prognosis analysis proved that MGMT was not only the target of miR-221-3p but also a tumor suppressor gene of HCC.

Overexpression of MGMT inhibited miR-221-3p-induced cell viability and migration

To further validate the effect of miR-221-3p on HCC by directly targeting MGMT, MTT and transwell assays were performed by co-transfecting miR-221-3p mimic and MGMT ORF clone in hepatic carcinoma cells. MTT experimental results revealed that forced MGMT overexpression attenuated the promoting effect of miR-221-3p on cell viability in the SMMC-7721 cell line (Figure 5a). A similar result was observed in BEL-7404 (Figure 5a). As for the effect of MGMT overexpression on cell migration, the transwell assay results showed that SMMC-7721 and BEL-7404 cell lines co-transfected with miR-221-3p mimic and MGMT ORF clone resulted in a 1.21- and 1.26-fold decrease in the number of invading cells per field, respectively, compared with the cells that were transfected with miR-221-3p mimic (Figure 5b). These data further proved that by targeting MGMT, miR-221-3p could promote the viability, migration, and invasion of hepatocellular carcinoma cells.

Discussion

To confirm that the target gene of miR-221-3p plays a vital role in defining novel therapeutic targets, we verified its target relationship with its target gene MGMT and then inspected the potential mechanism involving miR-221-3p and MGMT in HCC. We also observed in this study that miR-221-3p was
Figure 2. The effects of miR-221-3p on viability, migration, invasion, and proliferation in SMMC-7721 and BEL-7404 cell lines. (a) The cell viability was detected by MTT assay after transfection with miR-221-3p mimic, miR-221-3p inhibitor, negative control RNA for continuing five days. All the OD values were read at 490 nm. (b) Wound healing assay was used to assess the effect of miR-221-3p on migration rate. SMMC-7721 and BEL-7404 cells were transfected with miR-221-3p mimic, miR-221-3p inhibitor, and negative control RNA. Migration rate was defined as the ratio of migrating distance at 24 h to cell spacing at 0 h. (c) The ability of invasion was detected by transwell assay in SMMC-7721 and BEL-7404 cells in different groups. SMMC-7721 and BEL-7404 cells were transfected, as stated in Methods. The X-axis represents different groups depending on the transfection reagents, and the Y-axis represents the invasion cell number. (d) Colony foci formation results of transfected SMMC-7721 and BEL-7404 cells. The X-axis represents different groups, and the Y-axis represents the number of clones. All the bars in the figure represent mean ± SD from three independent experiments. * p < .05 and ** p < .001, compared with con (blank control) group.
Figure 3. The effects of miR-221-3p on apoptosis and cell cycle in SMMC-7721 and BEL-7404 cells. (a) Flow cytometric assay was conducted to detect cell apoptosis after the transfection of SMMC-7721 and BEL-7404 cells with miR-221-3p mimic, miR-221-3p inhibitor, and negative control RNA. (b) The cell cycle was measured using the flow cytometric assay. The SMMC-7721 and BEL-7404 cells were transfected with the same molecules as mentioned above. The bars come from three independent experiments. Values are mean ± SD. *p < .05 and **p < .001, compared with CON (blank control) group.
Figure 4. MGMT was the target of miR-221-3p and it’s down-regulated in HCC tissues and cells. (a) The targeting relationship between miR-221-3p and MGMT mRNA was predicted through RNA hybrid, microRNA, Pictar, and TargetScan. The binding scheme was illustrated. (b) The information of GV272 vector that was used to build GV272-MGMT or GV272-MGMT-Mut vectors for transfection in 293 T cell line. The relative luciferase activity was measured using a dual luciferase reporter gene assay. The 293 T cells were co-transfected with pRL-TK vectors containing Renilla luciferase and 0.4 μg of the miR-221-3p mimic or negative control (NC). (c) The immunohistochemical staining was used to measure the expression of MGMT protein in the tumors, adjacent liver tissues, and normal liver tissues. The positive percentage equals the ratio of positive cases to total cases. -: negative; +: positive. (d) The expression of MGMT mRNA was detected by RT-qPCR in SMMC-7721 and BEL-7404 cells transfected with miR-221-3p mimic, miR-221-3p inhibitor, negative control RNA. The bars represent mean ± SD of mRNA relative expression from three independent qPCR experiments. * p < .05 and ** p < .001, compared with CON (blank control) group. (e) The expression of MGMT protein was detected using western blot. In SMMC-7721 and BEL-7404 cells after transfection with miR-221-3p mimic, miR-221-3p inhibitor, and negative control RNA. (f) The expressions of MGMT at different stages of HCC was plotted using GEPIA data (an online gene profiling analysis tool). (g) The overall survival for MGMT in HCC patients. The survival curve comparing the patients with high and low expression of MGMT in HCC was also plotted using GEPIA. Log-rank p = .0021.
upregulated in human hepatic tumors and cell lines. As well as augmenting the viability, migration, and invasion of HCC cells, miR-221-3p inhibited cell apoptosis without affecting the cell cycle progression of hepatocellular carcinoma cells. According to the prediction of the bioinformatics software, MGMT mRNA 3’UTR contained a complementary site of miR-221-3p. In short, we found that miR-221-3p reduced the transcription and translation of MGMT genes to promote the viability, migration, and invasion of HCC cells.

A number of researchers have proved that miRNAs have a close relationship with cancer progression. Based on the evidence from the literature, miRNAs play essential roles in
breast cancer, malignant glioma, esophageal cancer, and pancreatic cancer.25,26,27,28 Apart from promoting tumor development and the progression of tumor cells, miR-221 has been reported to inhibit cell apoptosis in HCC.29,30 miR-221-3p and miR-221-5p are the mature spliceosomes of miR-221, and miR-221-3p can be a more significant participant in various physiological processes because it is broadly conserved. Based on this fact, we selected miR-221-3p as the miRNA of interest to investigate its function in HCC cells. Forced miR-221-3p overexpression in our cell models revealed that miR-221-3p contributed to tumorigenesis in HCC. Our findings agree with the results of several previous reports, in which miR-221-3p has been demonstrated to be an onco-miRNA in liver carcinogenesis.17,31,32,33

What’s more, several studies have shown that miR-221-3p, as an oncogenic miRNA, promoted the clonogenicity and invasiveness of gastric carcinoma and cervical cancer cells significantly.34,35,36 Also consistent with the research conducted by Yuan et al.,37 our results indicated that a positive impact on cell proliferation occurred when the miR-221-3p expression was upregulated in our experimental cell line models. As for apoptosis, some contradictory reports have been found. Dai et al.38 for instance, used thapsigargin (1 mM) to induce the endoplasmic reticulum stress in HepG2 and SMMC-7721 cell lines. They found that miR-221 mimics enhanced ER stress-induced apoptosis. Gramantieri et al.,39 on the other hand, discovered that miR-221 upregulation increased the cell ratio in S phase, thereby leading to the proliferation of HCC-derived cell line Hep3B. Compared with the studies conducted by Dai et al. and Gramantieri et al., a different influence of miR-221-3p on apoptosis or proliferation in hepatic carcinoma cells occurred. This might result from different cell lines or endoplasmic reticulum stress. We used flow cytometry not only to detect the apoptosis rate of SMMC-7721 and BEL-7404 cells but also to explore the positive or negative impact of miR-221-3p on apoptosis of hepatic carcinoma cells. Our result revealed that the cell apoptosis percentage reduced in cells associated with the upregulation of miR-221-3p. This revelation suggests that miR-221-3p suppressed HCC cell apoptosis. We also used flow cytometry to detect the cell cycle. Our findings indicated that miR-221-3p did not affect the HCC cell cycle even though a recent study reported that the downregulated miR-221 led to cell-cycle arrest in HCC.39 Furthermore, it was reported that migration and invasion were enhanced in HCC cells that were exposed to miR-221 mimic.40 Our data also support this finding. When performing wound-healing assay, transwell assay and colony formation assay, our data suggested that the upregulation of miR-221-3p significantly promoted cell migration, cell invasion, and colony formation in HCC.

Given that miRNAs have enormous effects on cancers by regulating their target genes, the study of miRNAs and their target genes has become an attractive area of research for scientists as it provides new possibilities for curing cancers. For example, upregulating miR-122 suppressed the expression of its target gene, ubiquitin-specific peptidase 53 (P53), to induce apoptosis in bile duct carcinoma cells.41 In their research, Hu et al. found that miR-221 augmented osteosarcoma cell growth, migration, and invasion by limiting cyclin-dependent kinase inhibitor 1B (p27, Kip1).42 In our study, we confirmed that the MGMT mRNA had a target relationship with miR-221-3p. We also found that MGMT mRNA and protein level was upregulated and accompanied by miR-221-3p suppression, but they were downregulated along with the upregulation of miR-221-3p. The regulation of miR-221-3p on MGMT mRNA significantly affected the migration, invasion, and colony foci formation of HCC cells (SMMC-7721 and BEL-7404 cell lines).

As promoter methylation has been found to serve as an essential mechanism for gene silencing, Gu et al. quantified the strong association between MGMT promoter methylation and non-small cell lung cancer-genesis using a meta-analysis protocol. Their analysis indicated that the MGMT gene was frequently silenced in non-small cell lung cancer tissues because promoter methylation resulted in the loss of function of MGMT. Eighteen studies carried out from 2001 to 2011 were included, and further prospective studies were carried out to confirm the association.43 After analyzing the high methylation frequency and loss-of-function of MGMT in 82 gastric cancer tissues using western blotting, Yousuf et al. found that the loss of MGMT protein concomitantly with MGMT promoter hypermethylation was observed in 37 cases.44 In the study performed by Zekri et al.,45 the methylation of MGMT promoter was found with a significantly high methylation index in HCC tissues than in corresponding adjacent healthy tissues. These findings indicated that MGMT expression might be downregulated in HCC tissues. Our data also suggested that the expression level of MGMT protein in hepatic tumors was significantly lower than that in healthy liver tissues. A lower level of MGMT was a poor indicator of HCC prognosis. This result has been supported by the findings of Matsukura S et al., who showed that the downregulation of MGMT was a poor prognostic factor using a Cox proportional-hazard regression model.46

Nonetheless, the results of this research have been compromised by limitations such as lack of in vivo assays. To some extent, insufficient animal model experiments degraded the generalization of this research. According to GEPIA analysis, the expression of MGMT at stage II and III of HCC was lower than that of other stages of HCC. Nonetheless, the MGMT expression at stage IV was a little higher than that at stage III. It seemed that MGMT might be trying to recover the DNA at stage IV in order to prevent further HCC progression. Nevertheless, this needs further investigation.

Conclusion

Our study confirms the targeting relationship between MGMT mRNA and miR-221-3p in human HCC cells. In other words, this research demonstrates how the interaction between MGMT mRNA and miR-221-3p plays a crucial role in HCC cells. Based on our findings, miR-221-3p can promote the viability, migration, and invasion of HCC cells by suppressing MGMT transcription and translation. This interaction between miR-221-3p and MGMT mRNA can be regarded as a novel
gene therapy target for HCC. In sum, our research not only explains human liver carcinogenesis but also provides insights into the prognosis biomarker for HCC.

Disclosure statement
The authors declare that there is no conflict of interest.

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