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MicroRNA-1324 inhibits cell proliferative ability and invasiveness by targeting MECP2 in gastric cancer

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Abstract. – OBJECTIVE: The aim of this study was to investigate the expression level and potential molecular mechanism of microRNA-1324 in gastric cancer (GCa) to provide a new perspective for the diagnosis and treatment of GCa.

PATIENTS AND METHODS: The expression levels of microRNA-1324 and MECP2 in GCa tissues and cell lines were detected using quantitative Real Time Polymerase Chain Reaction (qRT-PCR). The influence of microRNA-1324 and MECP2 on the proliferation or invasiveness of GCa cells were investigated by cell counting kit-8 (CCK-8) and colony formation assay or transwell assay, respectively. Furthermore, the regulatory interplay between microRNA-1324 and MECP2 was verified *via* Dual-Luciferase reporting assay, qRT-PCR, and Western Blot.

RESULTS: QRT-PCR results revealed that microRNA-1324 expression was remarkably down-regulated in GCa tissues and cell lines, while the expression of MECP2 was remarkably up-regulated. Subsequently, we confirmed that miR-1324 could target and bind to MECP2, as well as inhibit its expression. Inhibition of microRNA-1324 remarkably enhanced the proliferative capacity and invasiveness of GCa cells. However, opposite results were observed after inhibiting MECP2 expression. At the same time, flow cytometry revealed that inhibition of microRNA-1324 accelerated cell cycle but inhibited apoptosis. Conversely, opposite results were observed when MECP2 was down-regulated in vitro.

CONCLUSIONS: MicroRNA-1324 was remarkably down-regulated in GCa tissues or cell lines. Meanwhile, it could inhibit MECP2 expression, and promote the proliferation and invasion of GCa cells, eventually participating in the occurrence and development of GCa.

Key Words:
Gastric cancer (GCa), MicroRNA-1324, MECP2, Cell proliferation, Cell invasion.

Introduction

Gastric cancer (GCa) is one of the most common malignant tumors in the digestive system. There are 755,000 new cases of GCa in the world every year[1]. Meanwhile, GCa ranks second in cancer death. The early symptoms of GCa are not evident. About 40% of patients with GCa have been confirmed associated with distant metastasis[2]. At present, the main clinical treatment for GCa includes radiotherapy, chemotherapy, and surgery. However, the overall mortality has not been remarkably reduced. Rapid growth and distant metastasis of advanced GCa tissue is still the main cause of high mortality of GCa[3]. Compared with traditional treatment, gene therapy has the advantages of precise targeting, strong specificity, and small side effects[4]. Although several studies have focused on the mechanism of GCa development, the pathophysiological changes of its pathogenesis still remain unclear[5]. Therefore, further exploration of targeted therapeutic factors for the development of GCa is an important breakthrough in early diagnosis and treatment of GCa and reduction of mortality[6].

MicroRNAs (miRNAs) are a class of non-coding small RNAs with about 21-23 nt in length, which are highly conserved. MicroRNA precursors can produce mature microRNAs under the action of Dicer. Mature microRNAs have been

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reported to participate in the development of tumors by regulating corresponding target genes[7]. MicroRNAs inhibit the translation or directly degrade target mRNAs through non-completely complementary binding to the 3' untranslational region (UTR) of target genes. This may eventually inhibit the expression of target genes[8,9]. MiRNAs are involved in several biological processes, such as cell proliferation, metastasis, and apoptosis[10]. Meanwhile, they regulate a variety of human diseases (including tumors), which are expected to become target genes for tumor therapy. Since microRNA can target and control the expression of hundreds of genes, it has shown good application prospect in the diagnosis and treatment of malignant tumors[11]. MicroRNA-1324 is a newly discovered miRNA in recent years. It has been observed closely correlated with cell growth and metastasis in hepatocellular carcinoma and other diseases[12,13]. However, its expression in GCa and the underlying molecular mechanism remain unclear. Our previous studies have found that microRNA-1324 expression in GCa tissues is significantly higher than that of normal gastric tissues. The expression level of miR-1324 in 60 matched GCa tissues and normal gastric tissues was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The results revealed that microRNA-1324 was lowly expressed in GCa tissues. To further investigate the effect of microRNA-1324 on the proliferation and invasion of GCa cells, MKN45 and MKN28 cells were chosen as the research object. Subsequently, the chemically synthesized microRNA-1324 inhibitor was introduced into GCa cells (MKN45 and MKN28) by liposome transient transfection to reduce microRNA-1324 level *in vitro*. Cell counting kit-8 (CCK-8), colony formation, and transwell invasion assays were performed to explore the influence of microRNA-1324 on the proliferation and invasion characteristics of GCa cells, respectively. Our findings might provide a theoretical basis for the diagnosis and treatment of GCa.

Patients and Methods

Sample Collection

The research was approved by the China-Japan Union Hospital of Jilin University Ethics Committee. Informed consent was obtained from each subject before the study. GCa tissues and normal control tissues were collected from 60 patients diagnosed with GCa. No patient received radiotherapy or chemotherapy before surgery. Collected tissue specimens were stored in liquid nitrogen for use.

Cell Culture

Normal gastric mucosal cell line (GES1_ and human GCa cell lines (MKN45, MKN28, BGC823, and MGC803) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco, Grand Island, NY, USA) medium supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) in an incubator containing 5% CO_2 at 37°C.

Cell Transfection

Cells were first seeded into six-well plates, and cell transfection was performed according to the instructions of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). After incubation for 6 h, the cells were added with complete medium. MicroRNA-1324 mimics, inhibitor, and corresponding negative controls were purchased from GenePharma (Shanghai, China). MECP2 siRNA and corresponding normal controls were purchased from Jikai Gene (Nanjing, China).

RNA Extraction and QRT-PCR

Tissues and cells required for the experiment were first lysed with 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted total RNA was quantified by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and stored in a –80°C refrigerator for use. Subsequently, complementary deoxyribonucleic acids (cDNAs) was reverse transcribed according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Code No. RR036A, Otsu, Shiga, Japan). MiRNA quantitative PCR procedures were performed in accordance with miScript SYBR Green PCR Kit. QRT-PCR was performed on the ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA) by SYBR® Green Master Mix (TaKaRa, Otsu, Shiga, Japan). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal parameters for miRNA and mRNA, respectively. Relative expression of the gene was calculated by the $2^{-\Delta\Delta CT}$ method. Primers used were shown as follows: MicroRNA-1324: Forward: 5'- ACACTCCAGCTGGGCCAGACAGAATTCTATGC-3'; Reverse: 5'-CTCAACTGGTGTCGTGGAGTCGGCAAT-

TCAGTTGAGGAAAGTGC-3'; MECP2: Forward: 5'-TGACCGGGGACCCATGTAT-3'; Reverse: 5'-CTCCACTTTAGAGCGAAAGGC-3'; U6: Forward: 5'-CTCGCTTCGGCAGCACA-3'; Reverse: 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH: Forward: 5'-GCACCGTCAAGGCTGAGAAC-3'; Reverse: 5'-GGATCTCGCTCCTGGAAGATG-3'.

Cell Counting Kit-8 (CCK-8) Assay

Transfected GCa cells were seeded into 96-well plates at a density of 1×10^5/mL. After culture for 0, 24, 48, and 72 h, respectively, 10 μL of CCK-8 reagent was added to each well. Then, cells were incubated for 2 h at 37°C in the dark. Optical density (OD) value at 450 nm was measured by an enzyme-linked immunosorbent assay (ELISA).

Colony Formation Assay

Transfected GCa cells were trypsinized, re-suspended, and counted. Subsequently, GCa cells were evenly plated into 3.5 cm well plates. After culture for 2 weeks, the cells were fixed with 4% paraformaldehyde and stained with crystal violet. Finally, formed colonies were photographed, and the number of colonies was counted and compared.

Cell Invasion

Cell invasion was performed using Matrigel invasion chambers (BD Biosciences; Franklin Lakes, NJ, USA). 1×10^5 of GCa cells were suspended in serum-free medium and inoculated into the upper chamber. Meanwhile, complete medium containing 10% fetal calf serum (FCS) was added to the lower chamber. After 24 h of incubation, the cells adhering to the membrane were fixed and stained with 0.5% crystal violet. Five fields of view were randomly selected for each sample, and the number of invading cells was counted under a microscope.

Flow Cytometry

Cells in each group were first collected, and cell density was adjusted to about 5×10^4/mL. Then, the cells were fixed with 1 mL of 75% ice ethanol pre-cooled at –20°C, and stored in a refrigerator at 4°C overnight. After discarding the supernatant, the cells were added with100 μL of RNaseA, followed by incubation at 37°C for 30 min in the dark. Then, cells were added with 400 μL of Propidium Iodide (PI) and incubated for 4 min at 4°C. Cell cycle was finally detected by flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Cell Apoptosis

Cells were first washed twice with phosphate-buffered saline, trypsinized, and fixed with cold 70% ethanol for 30 min at 4°C. Subsequently, the cell pellet was collected and incubated in a solution containing 5 mL of Annexin V-FITC (fluorescein isothiocyanate) and 1 mL of PI (50 mg/mL). Flow cytometry was finally performed to detect the apoptosis of cells.

Dual-Luciferase Reporter Gene Assay

The binding sites of microRNA-1324 and MECP2 were predicted by the miRDB website. This binding sequence was sent to GenePharma (Shanghai, China) to construct wild-type and mutant plasmids. MicroRNA-1324 mimics and negative controls were purchased from GenePharma (Shanghai, China). Relative fluorescence values of plasmids after transfection were measured by a standardized method. This experiment was repeated for 3 times, and the average value was calculated.

Western Blot Assay

Transfected cells were added with cell lysate containing protease inhibitor phenylmethanesulfonyl fluoride (PMSF; Beyotime, Shanghai, China). The cells were then scraped off on ice and lysed. Subsequently, the lysate was aspirated and centrifuged at 12, 000 r/min for 15 min. The supernatant was collected, and the concentration of extracted protein was determined by the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). 50 μg of total protein was separated by dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with skimmed milk for 1 h, the membranes were incubated with primary antibodies of GAPDH and MECP2 (working concentration 1:1000) overnight at 4°C. On the next day, the membranes were incubated with corresponding secondary antibody at 37°C for 2 h. Immunoreactive bands were detected by the enhanced chemiluminescence (ECL) method.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (SPSS Inc. Chicago, IL, USA) and GraphPad Prism 7 (GraphPad Software Inc., La

Jolla, CA, USA) were used for all statistical analysis. Student's *t*-test was used to compare the difference between groups. Measurement data were expressed as mean ± SD (standard deviation). $p<0.05$ was considered statistically significant.

Results

Expression of MicroRNA-1324 and MECP2 in GCa

We first detected the expression of microRNA-1324 in GCa tissues by qRT-PCR. The results revealed that microRNA-1324 expression was remarkably down-regulated in GCa tissues when compared with normal control tissues (Figure 1A). Many studies have shown that miRNAs can participate in biological processes by inhibiting the expression of target genes. Therefore, we predicted the possible binding of microRNA-1324 through the bioinformatics website. By analyzing the combined scores and biological functions, MECP2 was finally chosen. Subsequent results demonstrated that the expression of MECP2 in GCa tissues was remarkably higher than that of normal control tissues (Figure 1B). Furthermore, we found that microRNA-1324 was remarkably down-regulated in GCa cell lines (Figure 1C), while MECP2 was up-regulated (Figure 1D). These findings were consistent with the results in human tissues. QRT-PCR results demonstrated that the expression of microRNA-1324 was remarkably negatively correlated with MECP2 expression in GCa tissues (R=-0.6475, $p<0.001$) (Figure 1E). All these results suggested that microRNA-1324 and MECP2 might play important roles in the development of GCa.

MicroRNA-1324 Binds to MECP2 and Inhibits the Expression of MECP2

Our findings indicated that microRNA-1324 and MECP2 were differentially expressed in GCa tissues and were negatively correlated. To further explore the binding relationship between microRNA-1324 and MECP2, we predicted the binding site of microRNA-1324 to MECP2. Meanwhile, MECP2 wild-type overexpression plasmid and mutant overexpression plasmid were constructed (Figure 2A). Dual-Luciferase reporter gene assay showed that microRNA-1324 could bind to MECP2 (Figure 2B). To further investigate the regulatory mechanism of microRNA-1324 on MECP2, we overexpressed or inhibited microRNA-1324 level in MKN45 and MKN28 cells by microRNA-1324 mimics or inhibitor transfection, respectively. The mRNA and protein expression of MECP2 was detected by qRT-PCR and Western blot. The results revealed that overexpression of microRNA-1324

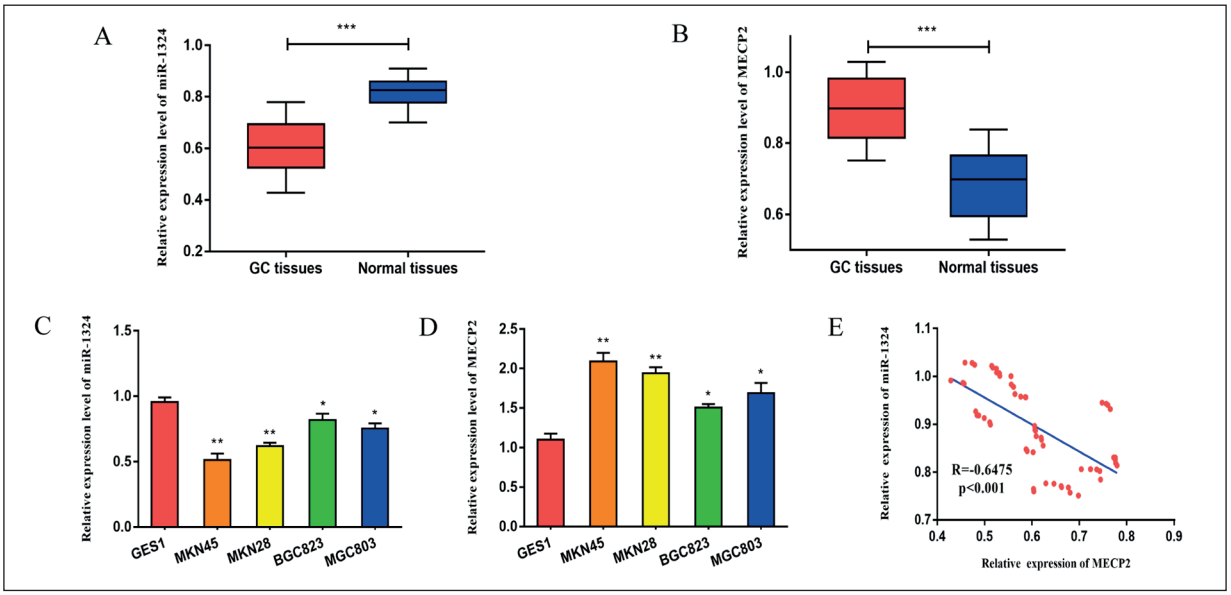

Figure 1. Expression of miR-1324 and MECP2 in GCa. **A,** Expression of miR-1324 in 60 cases of GCa tissues and corresponding normal tissues. **B,** Expression of MECP in 60 cases of GCa tissues and corresponding normal tissues. **C,** Expression of miR-1324 in normal cell lines and GCa cell lines. **D,** Expression of MECP2 in normal cell lines and GCa cell lines. **E,** Correlation between expression of miR-1324 and MECP2 in GCa tissues. Data were presented as mean ± SD; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

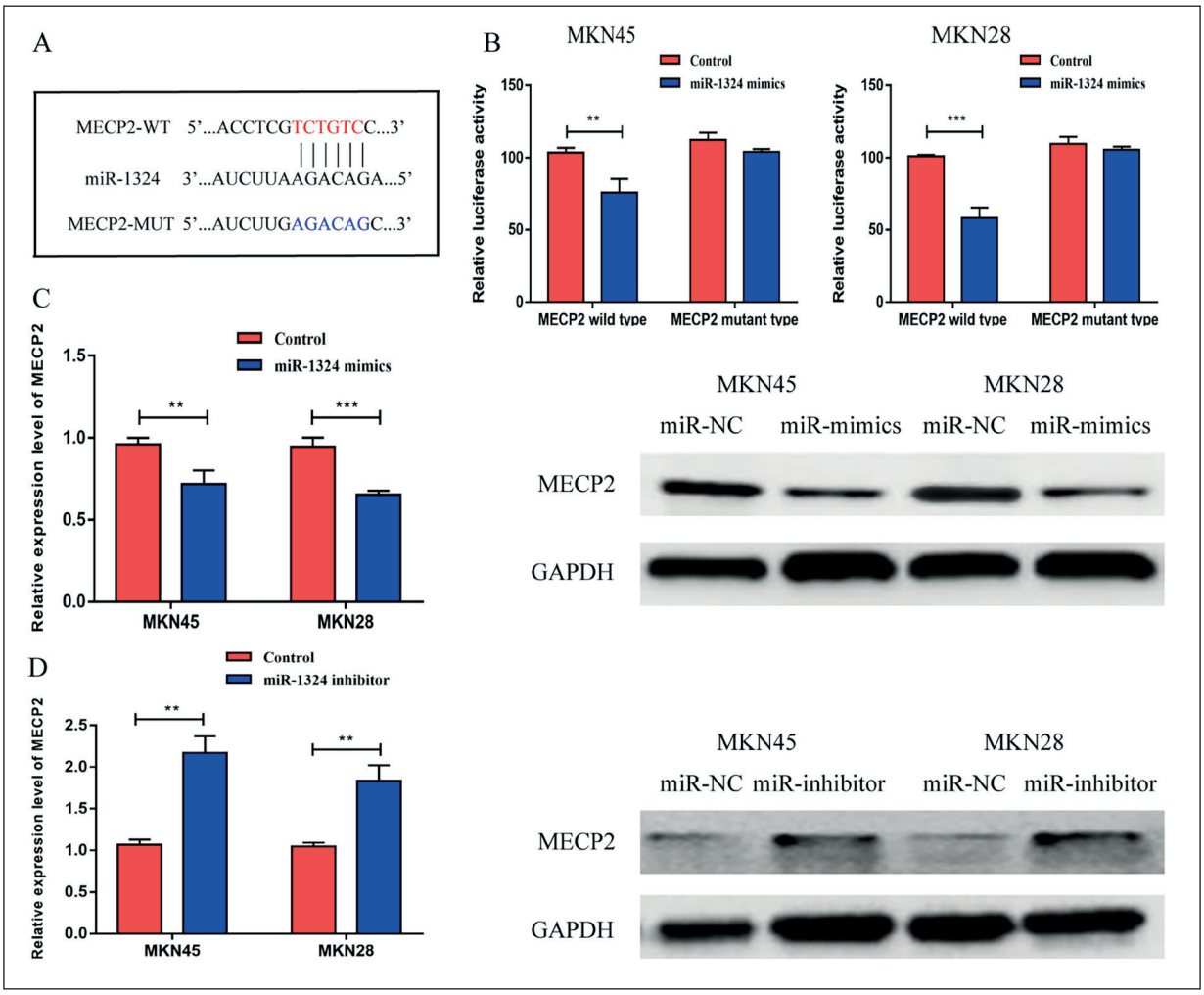

Figure 2. MiR-1324 can bind to MECP2 and inhibit its expression. **A,** Potential binding site for miR-1324 and MECP2. **B,** Dual-Luciferase reporter gene assay verified the binding relationship between miR-1324 and MECP2. **C,** Overexpression of miR-1324 in GCa cells inhibited the mRNA and protein expressions of MECP2. **D,** Inhibition of miR-1324 in GCa cells increased the mRNA and protein expressions of MECP2. Data were presented as mean ± SD; ** $p<0.01$, *** $p<0.001$.

in GCa cells remarkably inhibited the mRNA and protein expressions of MECP2 when compared with normal control group (Figure 2C). However, inhibition of microRNA-1324 in cells remarkably up-regulated the mRNA and protein expressions of MECP2 (Figure 2D). These results indicated that microRNA-1324 might be involved in the progression of GCa by inhibiting MECP2.

Effect of MicroRNA-1324 and MECP2 on Cell Proliferative Ability and Invasiveness

To further investigate the effects of microRNA-1324 and MECP2 on cell function, we examined the expressions of microRNA-1324 and MECP2 in GCa cells (MKN45 and MKN28). Meanwhile, changes in cell proliferative ability and invasiveness were detected. As shown in Figure 3A-3B, the proliferation of cells was remarkably enhanced after inhibition of microRNA-1324. However, the proliferation of cells was significantly weakened after MECP2 inhibition. Similarly, we found that the invasive ability of cells transfected with microRNA-1324 inhibitor was remarkably enhanced. Transfection of MECP2 siRNA partially reversed the effect of microRNA-1324 inhibitor on cell invasion (Figure 3C). The above results indicated that microRNA-1324 could inhibit cell proliferative ability and invasiveness, while MECP2 could promote cell growth and metastasis.

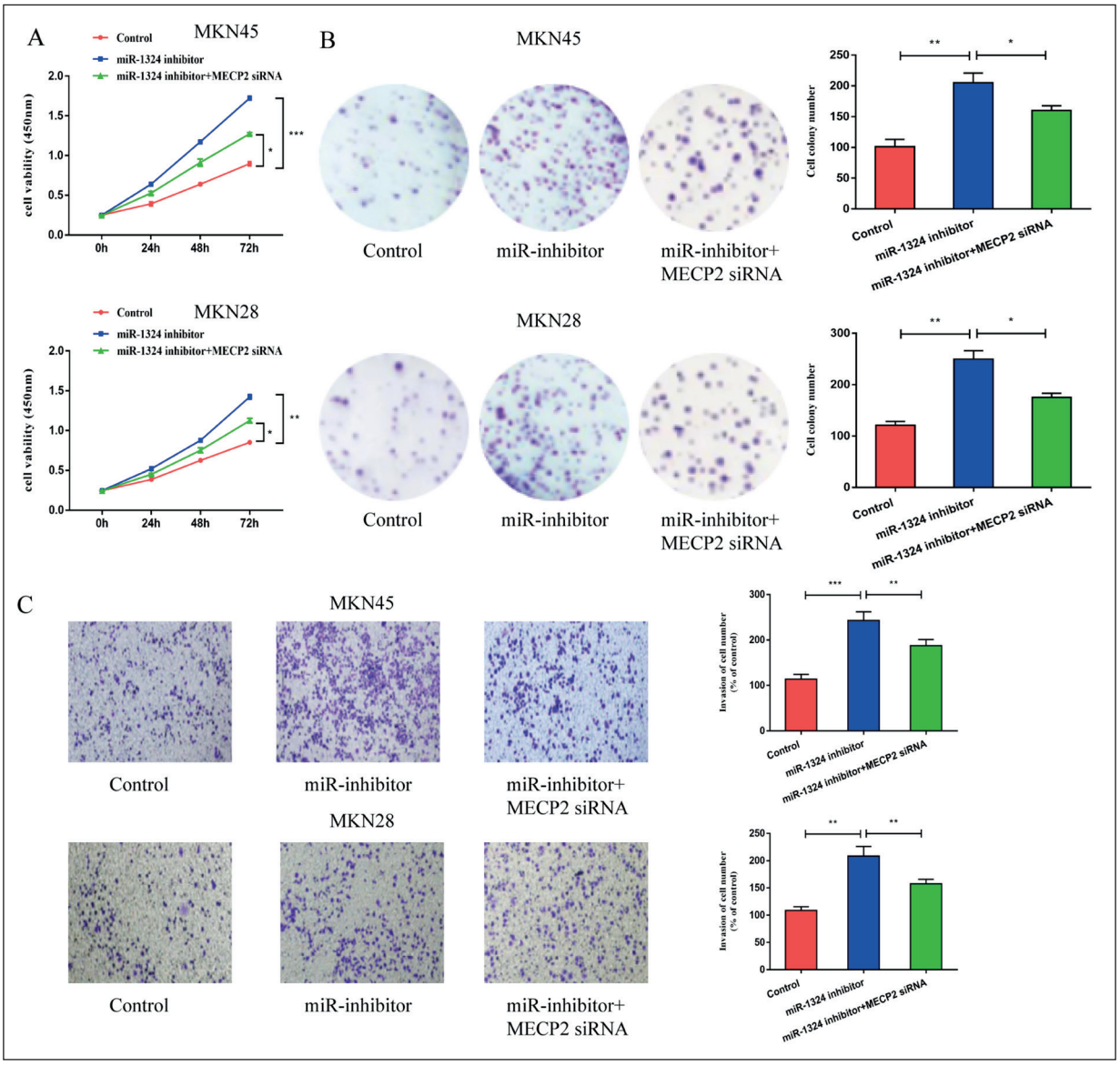

Figure 3. Effect of miR-1324 and MECP2 on cell proliferation and invasion. **A,** Effect of miR-1324 and MECP2 on cell proliferation was examined by CCK-8 assay. **B,** Effect of miR-1324 and MECP2 on cell proliferation was examined by colony formation assay (magnification: 10×). **C,** Effect of miR-1324 and MECP2 on cell invasion was examined by transwell invasion assay (magnification: 40×). Data were presented as mean ± SD; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Effect of MicroRNA-1324 and MECP2 on Cell Proliferative Ability and Invasiveness

Subsequently, we investigated the effects of microRNA-1324 and MECP2 on cell cycle and apoptosis by flow cytometry. The results revealed that cell cycle was remarkably promoted after transfection of microRNA-1324 inhibition in GCa cells. However, cell cycle was inhibited after transfection of MECP2 siRNA (Figure 4A). At the same time, our results illustrated that inhibition of microRNA-1324 in GCa cells reduced the proportion of apoptotic cells, while inhibition of MECP2 could increase cell apoptosis (Figure 4B). These findings indicated that microRNA-1324 might inhibit cell cycle and promote cell apoptosis by inhibiting MECP2.

Discussion

The occurrence and development of GCa is a biological process with multiple genes and fac-

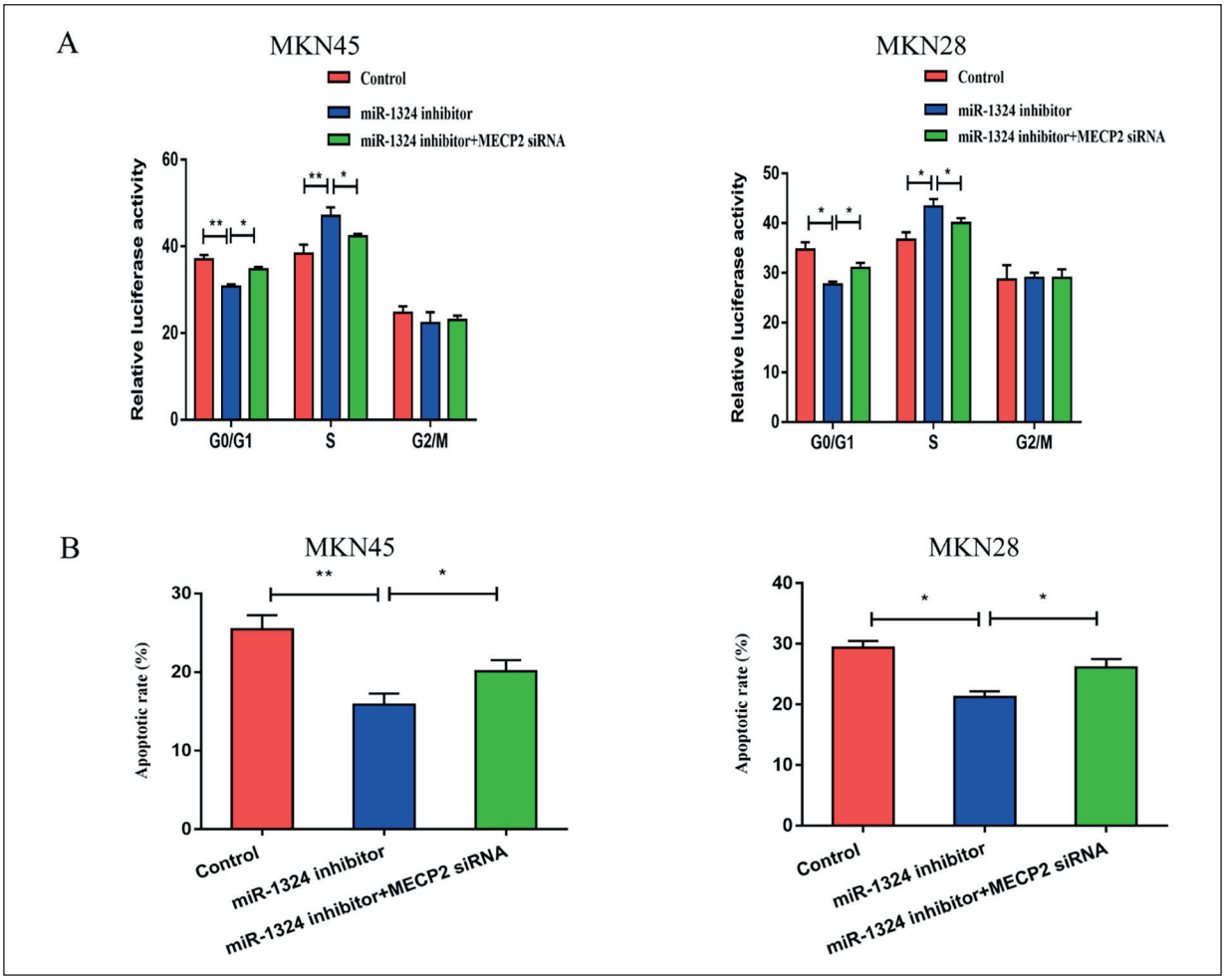

Figure 4. Effect of miR-1324 and MECP2 on cell cycle and apoptosis. **A,** Effect of miR-1324 and MECP2 on cell cycle was examined by flow cytometry. **B,** Effect of miR-1324 and MECP2 on apoptosis was examined by flow cytometry. Data were presented as mean ± SD; * $p<0.05$, ** $p<0.01$.

tors[1]. GCa is characterized by uncontrolled proliferation of cells caused by various reasons, including the inactivation of tumor suppressor genes, activation of proto-oncogenes, and the interaction of related transfer genes. This may eventually lead to the development of GCa[14,15]. Current studies have shown that miRNAs can target hundreds of genes. Meanwhile, miRNAs are widely used in drug target screening and gene function research. They have also shown great application prospects in the diagnosis and treatment of malignant tumors. MiRNAs are a class of non-coding small RNA molecules consisting of 19-25 nucleotides[9]. They play an important role in cell activity, which affects tumorigenesis by regulating many genes in tumor cells[16,17]. MiRNAs can bind to target gene mRNAs through base-pair complementary binding, inhibit protein translation, and regulate downstream signaling pathways to exert biological functions[18,19]. Ultimately, this may affect multiple biological processes, such as proliferation and metastasis of tumor cells. MiRNAs play vital roles in the development of GCa[20-23]. Aberrantly expressed miR-188-5p promotes GCa metastasis by activating Wnt/β-catenin signaling. MiR-664a-3p acts as a cancer-promoting gene in GCa by targeting the Hippo pathway. MiR-876-3p regulates cisplatin resistance and stem cell-like properties of GCa cells by targeting TMED3. MiR-632 promotes GCa progression by accelerating angiogenesis in a TFF1-dependent manner. Based on previous studies, we hypothesized that microRNA-1324 might act as a tumor suppressor gene in GCa. To verify this hypothesis, we first examined the expression of microRNA-1324 in GCa tissues and cell lines qRT-PCR. Next, we

explored its effects on the proliferation, invasion, and cell cycle and apoptosis of GCa cells. QRT-PCR results revealed that microRNA-1324 was lowly expressed in GCa tissues when compared with adjacent normal tissues. Similarly, microRNA-1324 level in GCa cell lines was significantly lower than that of normal control cell lines. Bioinformatics predicted that microRNA-1324 might bind to MECP2 and regulate its expression. Dual-Luciferase reporter gene assay, qRT-PCR, and Western blot demonstrated that microRNA-1324 could target the 3'UTR of MECP2 and inhibit the mRNA and protein expressions of MECP2 in GCa cells. To further validate our speculation, we conducted experiments at the cellular level. The effects of microRNA-1324 and MECP2 on the proliferation, invasion, cell cycle, and apoptosis of GCa cells were detected by CCK-8, transwell invasion assay, and flow cytometry, respectively. Cellular experiments revealed that inhibition of microRNA-1324 remarkably promoted the proliferation and invasion of GCa cells, promoted cell cycle, and inhibited cell apoptosis. Simultaneous inhibition of MECP2 expression could partially reverse the biological effects of microRNA-1324 inhibition on GCa cell proliferation and apoptosis. These results indicated that microRNA-1324 functioned as a tumor suppressor gene in GCa.

Conclusions

We first indicated that microRNA-1324 was remarkably down-regulated in GCa tissues or cell lines. Meanwhile, it could inhibit MECP2 expression and promote the proliferative capacity, as well as invasion of GCa cells, thereby participating in the occurrence and development of GCa. Our findings provided a new perspective for scientific research and clinical treatment of GCa.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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