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MiRNA-485-5p, inhibits esophageal cancer cells proliferation and invasion by down-regulating O-linked N-acetylglucosamine transferase

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Abstract. – OBJECTIVE: Previous reports suggest that miRNA-485-5p is dysregulated and contributes to tumorigenesis in some cancer types. Nevertheless, the biological role of miRNA-485-5p in esophageal cancer (EC) is not well understood. Additionally, we found that the expression of miR-485-5p in EC tissues was aberrant.

PATIENTS AND METHODS: Quantitative RT-PCR (qRT-PCR) was used to demonstrate the expression of miRNA-485-5p in EC cell lines. Cell counting kit-8 (CCK-8) assay and transwell assay indicated that miRNA-485-5p overexpression inhibited cell proliferation, migration, and invasion in EC cell lines. Additionally, Western blotting, dual-luciferase reporter assay, and rescue assay predicted that O-linked N-acetylglucosamine transferase (OGT) was a direct target of miRNA-485-5p. Moreover, we showed that miRNA-485-5p regulated EC tumorigenesis by down-regulating OGT expression *in vitro* and *in vivo*.

RESULTS: The upregulation of miR-485-5p (fold change = 44 and 26 in ECA109 and TE-1, respectively; $p<0.001$) was showed by qRT-PCR. Compared with the control groups, the expression miR-485-5p significantly suppressed the proliferation, migration, and invasion of EC cells. The bioinformatic analysis predicted that the 3' untranslated region (UTR) of OGT contains one miR-485-5p target sequences. Western blotting and dual-luciferase reporter assay showed that activation of OGT 3'UTR was increased by co-transfection with miR-485-5p. Finally, CCK-8 assay predicted that the rescue effects of OGT expression on miR-485-5p induced inhibition of cell growth and tumor weight in Eca109 and TE1 cells.

CONCLUSIONS: Our results suggest that miRNA-485-5p is a suppressor of EC tumorigenesis and could serve as a novel candidate for therapeutic applications in EC treatment.

Key Words:
MiR-485-5p, Esophageal cancer, Tumorigenesis, Proliferation, Invasion.

Introduction

MicroRNAs (miRNAs) have been identified as promising therapeutic targets in several human cancers. MiRNAs are approximately 18-24 nucleotides in length. MiRNAs negatively regulate gene expression at the post-transcriptional and translational level by binding to complementary sequences in the 3'-untranslated regions (3'-UTR) of the target messenger RNAs (mRNAs). MiRNAs control many important cell functions including cell angiogenesis, proliferation, apoptosis, and invasion and are dysregulated in many human cancers[1-6]. Previous reports have

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confirmed that miR-485-5p is a tumor suppressor in the pathogenesis of various human cancers. MiR-485-5p inhibits cell proliferation in hepatocellular carcinoma, prevents metastasis and EMT of lung adenocarcinoma by down-regulating Flot2, and its overexpression suppresses spontaneous metastasis of breast cancer cells *in vivo*. Overexpression of miR-485-5p is significantly down-regulated in gastric cancer tissues compared with normal tissues and it is a novel biomarker for the overall survival of gastric cancer patients. These previous results suggest that miR-485-5p might function as a tumor suppressor and its down-regulation may contribute to esophageal tumorigenesis. However, the role of miR-485-5p in esophageal tumorigenesis has not been definitively shown[7-12]. In this study, we show that miR-485-5p expression is diminished in esophageal cancer (EC) cells. In addition, in EC cells, miR-485-5p significantly inhibits cell proliferation, migration, and invasion by directly downregulating O-linked N-acetylglucosamine transferase (OGT). The results from our study indicate that miR-485-5p has a tumor suppressor activity in EC cells and might serve as a novel therapeutic target for esophageal tumor.

Patients and Methods

Cell Lines

The human EC cell lines ECA-109, EC-8712, TE1, TE4, HET-1A were ordered from American Type Culture Collection (ATCC; Manassas, VA, USA). Human embryonic kidney (HEK)-293T cells were purchased from Shanghai Institutes for Biological Sciences Cell Resource Center. All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and penicillin/streptomycin 1%. Cells were incubated with 5% CO_2 at 37°C.

Lentiviral Infection, Plasmid Construction, and Transfection

The mature miR-485-5p sequence was obtained from the miRBase database. The GV248 vector (ubi-MCS-PGK-EGFP-puro) (Obio Technology Corp., Ltd., Shanghai, China) was linearized using the restriction enzyme EcoRI (New England Biolabs, Ipswich, MA, USA). A fragment containing the mature miR-485-5p sequence was introduced into the GV238 (ubi-MCS-puro) vector's multiple cloning sites. HEK-293T packaging cells were used to generate plasmid-containing viral particles using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The human OGT complementary deoxyribonucleic acid (cDNA) was cloned into the pcDNA3.1 vector (Obio Technology Corp., Ltd., Shanghai, China). A scrambled control vector was also constructed. For plasmid transfection, Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) was used according to the instructions.

RNA Extraction, Reverse Transcription, and Real-Time PCR

Total ribonucleic acid (RNA) was isolated from cell lines using a mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the instructions. MiR-485-5p expression level was obtained using quantitative real-time PCR assay with high-specificity miR-485 qRT-PCR Detection Kit (Stratagene Corp, La Jolla, CA, USA). To determine the alteration of OGT mRNA expression after transfection of miR-485-5p into cells, the human OGT cDNA synthesis was performed using an RNA PCR kit (TaKaRa, Otsu, Shiga, Japan), and qPCR was carried out using the SYBR Premix Ex Taq II kit (TaKaRa, Otsu, Shiga, Japan) according to the instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. QPCR was performed using the Applied Biosystems 7500HT Fast Real-Time PCR System, and the threshold cycle (CT) number was determined using the ABI 7500 qPCR System SDS software v2.0.1. All reactions were performed in triplicate. Relative quantification of miR-485-5p and OGT expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

miR-485-5p F: 5'-CCAAGCTTCACCCATTCCTAACAGGAC-3', R: 5'-CGGGATCCGTAGGTCAGTTACATGCATC-3'.

GAPDH F: 5'-GGAAGGTGAAGGTCGGAGTCA-3', R: 5'-GTCATTGATGGCAACAATATCCACT-3'.

Cell Proliferation Assay

Cell proliferation was evaluated by performing a cell counting kit-8 (CCK-8) assay using a cell counting kit (CCK-8; Solarbio, Beijing, China). Cells were seeded onto a 96-well plate and cultured at 37°C. Cells were added to each well using 10 μL of the CCK-8 solution. Plates were incubated at 37°C for 2 h, and the absorbance at 450 nm was measured. Proliferation rates were determined at days 1, 2, 3, 4, and 5 post-transfections.

Cell Migration and Invasion Assays

Cell migration and invasion assays were performed using 24-well polycarbonate membrane transwell chambers with 8-μm pores (Corning Incorporated, Corning, NY, USA). Cells were plated in serum-free DMEM on the upper side of the membrane with (for the cell invasion assay) or without (for the cell migration assay) Matrigel (BD Bioscience, Franklin Lakes, NJ, USA). DMEM with 10% FBS was added to the lower chambers. After incubation at 37°C for 24 h, the nonmigratory or non-invasive cells on the upper side of the membrane were removed. Cells that migrated or invaded to the lower membrane surface were fixed and stained with 0.1% crystal violet. Migration and invasion were quantified by counting cells in 6 random fields.

Dual-Luciferase Reporter Assay

HEK-293T cells were plated on 24-well plates and 3'-UTR plasmids were co-transfected. A Renilla luciferase vector (Promega, Madison, WI, USA) was cotransfected to normalize for transfection efficiency. After 48 h of transfection, firefly and Renilla luciferase activity were determined by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Relative firefly luciferase activity was measured by normalizing the Renilla luciferase activity.

Western Blotting

Total protein from cultured cells was extracted, and protein concentrations were determined using a BCA protein assay kit (Beyotime, Shanghai, China). Protein samples were fractionated using SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and blocked for 1 h with 5% non-fat milk. Membranes were then incubated at 37°C with primary antibodies: rabbit anti-human OGT (1:3000; CST, Danvers, USA) and mouse anti-human β-actin antibodies (1:3000, CST, Danvers, USA) were used to detect β-actin as a control. Protein expression levels were detected with enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) detection solution.

In vivo Assay

Four-week-old male/female BALB/c nude mice were injected subcutaneously in the right flank with 3×10^6 TE1 cells. After 7 days, the mice were randomly selected for treatment with miR485-5p or as controls. Viral particles were locally injected into the tumor mass once a week for continuous 4 weeks. Tumor weight was measured after weeks. This study was approved by the Animal Ethics Committee of Shandong University Affiliated Shandong Cancer Hospital and Institute.

Statistical Analysis

All experiments were performed in triplicate and the data are expressed as mean ± standard deviation (mean ± SEM). One-way analysis of variance (ANOVA) was performed for serial analysis, whereas two treatment groups were compared by the unpaired Student's *t*-test. Statistical Product and Service Solutions (SPSS) 17.0 Software (SPSS Inc., SPSS Statistics for Windows, Chicago, IL, USA) was used for all statistical analysis. A p-value <0.05 was considered statistically significant. LSD test was used to post-hoc test ANOVA.

Results

MiR-485-5p is Expressed at Low Levels in EC Cell Lines

To ascertain the role of miR-485-5p in cell lines ECA-109, EC-8712, TE1, TE4, and HET-1A, we first analyzed miR-485-5p expression levels by quantitative RT-PCR (qRT-PCR). We found that compared to its expression in normal human EC cells, miR-485-5p expression was markedly diminished in ECA-109, EC-8712, TE1, TE4, HET-1A (Figure 1).

MiR-485-5p Inhibits EC Cell Proliferation

We overexpressed miR-485-5p in ECA-109 and TE-1 EC cell lines and examined the ef-

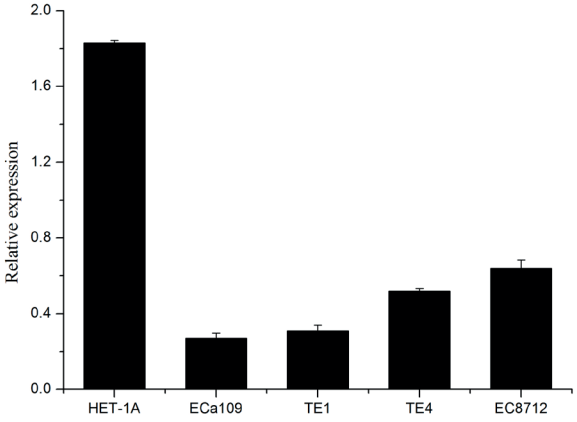

Figure 1. The average relative expression of miR-485-5p in normal and EC cells. GAPDH was used as an internal control for gene expression analysis. Data are shown as mean ± SD (n = 3) $p<0.05$.

fects on cell proliferation. As shown in Figure 2A, using qRT-PCR, we confirmed miR-485-5p overexpression. The CCK-8 proliferation assay showed that exogenous miR-485-5p overexpression significantly suppressed the proliferation of ECA-109 and TE1 cells (Figure 2B).

MiR-485-5p Inhibits the Migration and Invasion of EC Cells

We evaluated the potential roles of miR-485-5p in EC cells migration and invasion using transwell migration and invasion assays. As shown in Figure 3, miR-485-5p overexpression inhibited both cell migration and invasion in ECA-109 and TE1.

OGT is a Direct Target of MiR-485-5p

We set out to identify the targets that are directly regulated by miR-485-5p in EC cells. The miR-485-5p target genes were predicted using the Target Scan algorithm and the sequence alignment confirmed that the seed sequence of miR-485-5p is complementary to the 3'UTR of OGT (Figure 4A). We then performed luciferase reporter assays to determine whether OGT expression was directly regulated by miR-485-5p. As shown in Figure 4B, luciferase activity in cells co-transfected with pGL3-OGT and 3'-UTR-wt/miR-485-5p was lower than that in cells co-transfected with pGL3-OGT and 3'-UTR-wt/NC. No substantial difference in luciferase activity was observed between cells co-transfected with pGL3-OGT and 3'-UTR mut/miR-485-5p, compared to cells co-transfected with pGL3-OGT and 3'-UTR-mut/NC. To further understand the regulation of OGT by miR-485-5p, we performed Western blot analyses to identify how OGT protein expression was altered upon miR-485-5p overexpression. We found that OGT protein expression was significantly reduced following miR-485-5p overexpression in ECA-109 and TE1 cells, suggesting that OGT is

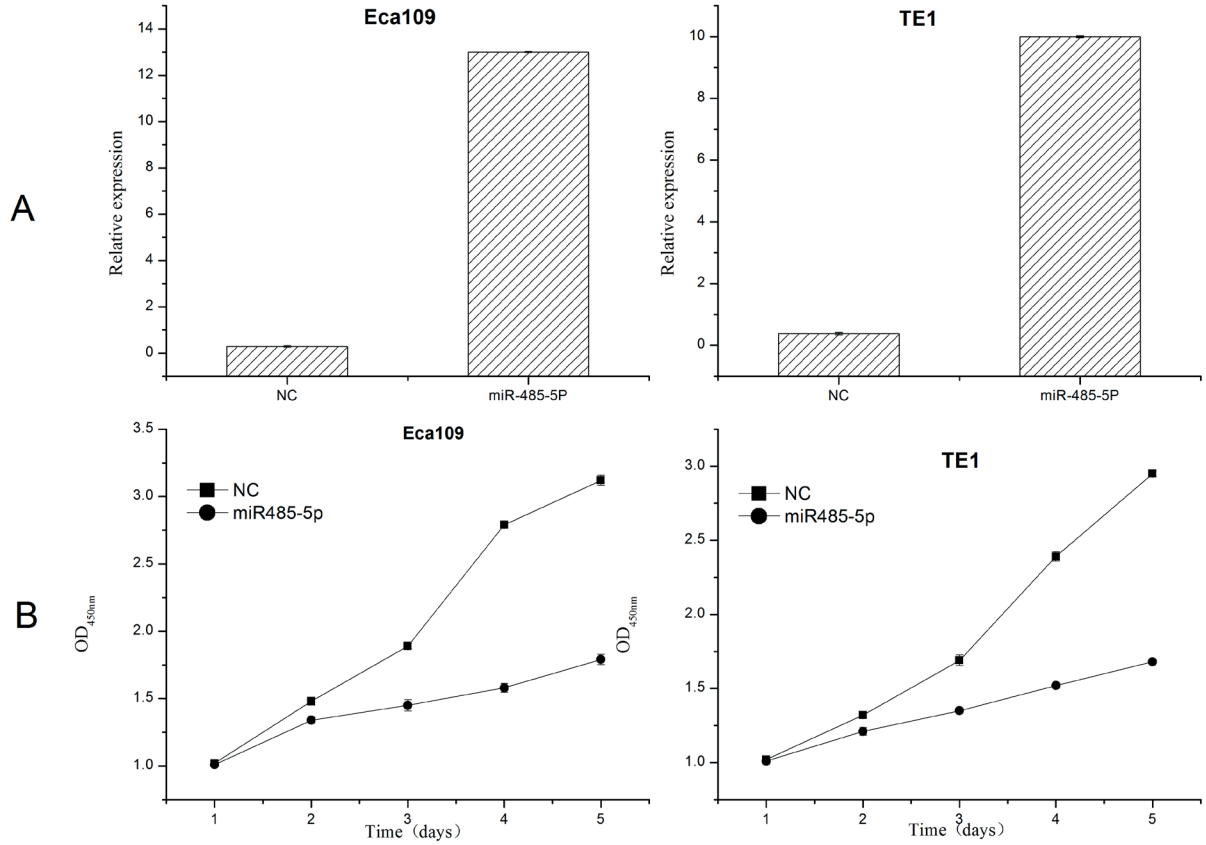

Figure 2. MiR-485-5p inhibits EC cells proliferation. ***A,*** Confirmation of miR-485-5p overexpression by qRT-PCR in Eca109 and TE1 cells transfected with miR-485-5p. GAPDH was used as an internal control for gene expression analysis. ***B,*** Results of a CCK-8 assay to assess the effects of miR-485-5p on cell proliferation in Eca109 and TE1 cells. Data are shown as mean ± SD (n = 3) $p<0.05$.

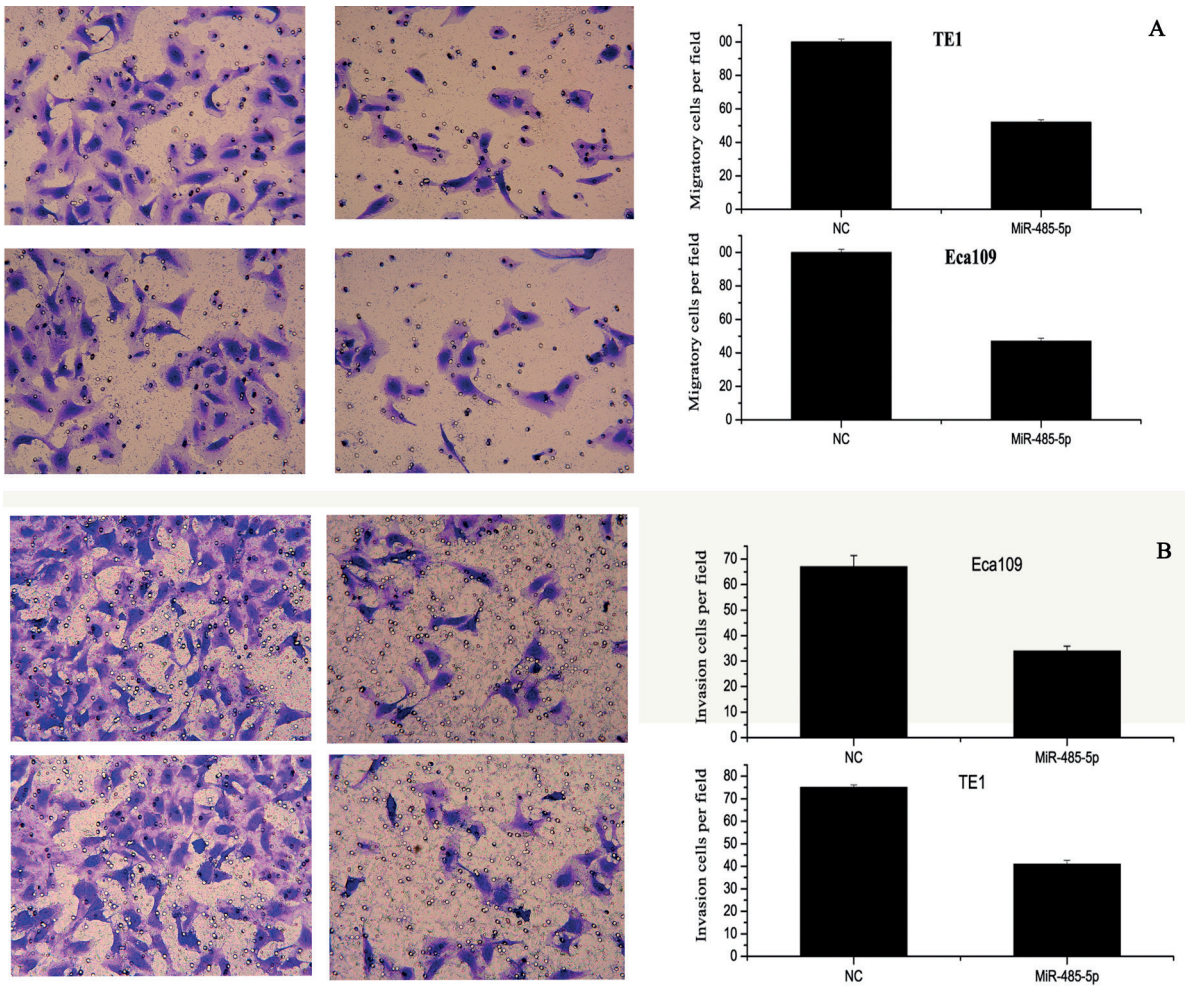

Figure 3. MiR-485-5p inhibits the migration and invasion of EC cells. ***A,*** Transwell migration assays using Eca109 and TE1 cells transfected with miR-485-5p or miR-NC. ***B,*** Transwell invasion assays using Eca109 and TE1 cells transfected with miR-485-5p or miR-NC. Data are shown as mean ± SD (n = 3) $p<0.05$. (200x).

negatively regulated by miR-485-5p in EC cells (Figure 4C).

MiR-485-5p Regulates EC Cell Proliferation by Down-Regulating OGT

We have thus far demonstrated that miR-485-5p suppresses EC cell tumorigenicity and that OGT is a direct target of miR-485-5p in EC cells. Our next goal was to determine the role of the OGT in EC cells using rescue experiments with OGT. We first co-transfected ECA-109 and TE-1 cells with miR-485-5p and pcDNA3.1-OGT constructs, or empty vector (pcDNA3.1), and confirmed transfection efficiency by Western blot (Figure 5A). Next, we performed CCK-8 assays in ECA-109 and TE-1 cells, which indicated that OGT overexpression partly reversed the inhibition of cell proliferation by miR-485-5p (Figure 5B). At last, tumor weight in response to miR-485-5p and OGT overexpression was detected in TE1 cells (Figure 5C). When the OGT was overexpressed, the weight of the tumor increased almost 2.5 times compared to miR-485-5p overexpressed only.

Discussion

Many studies[13-25] have found that miRNAs can function either as oncogenes or tumor suppressors in human cancer by controlling target gene expression. In this work, we found that miR-485-5p expression was markedly decreased in EC cells compared with normal cells. We explored

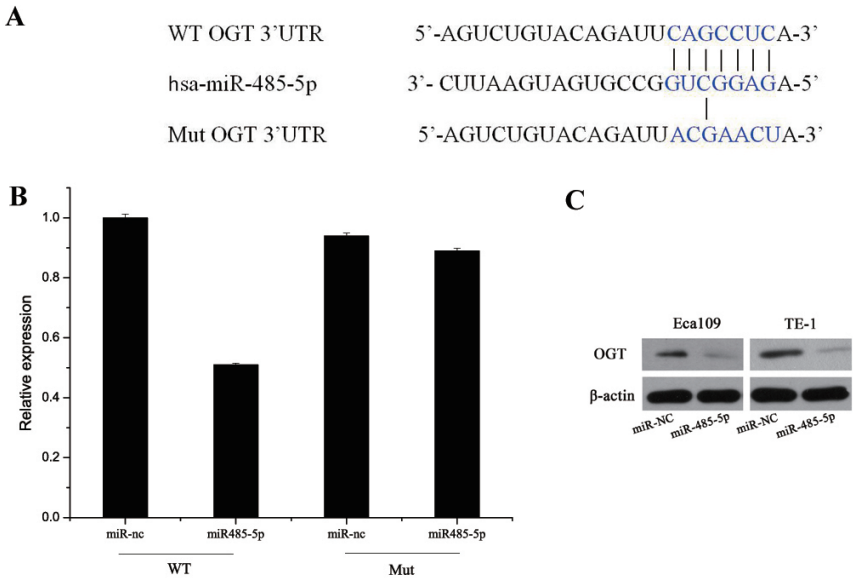

Figure 4. Identification of OGT as a direct target of miR-485-5p. Schematic demonstration of miR-485-5p target site in the 3'-UTR of the OGT. ***A,*** Luciferase assay results from HEK-293T cells co-transfected with the OGT-3'-UTR-wt reporter plasmids and either miR-485-5p or miR-NC. ***B,*** Western blot analysis of OGT levels following miR-485-5p overexpression in Eca109 and TE1 cells. Data are shown as mean ± SD (n = 3) $p<0.05$.

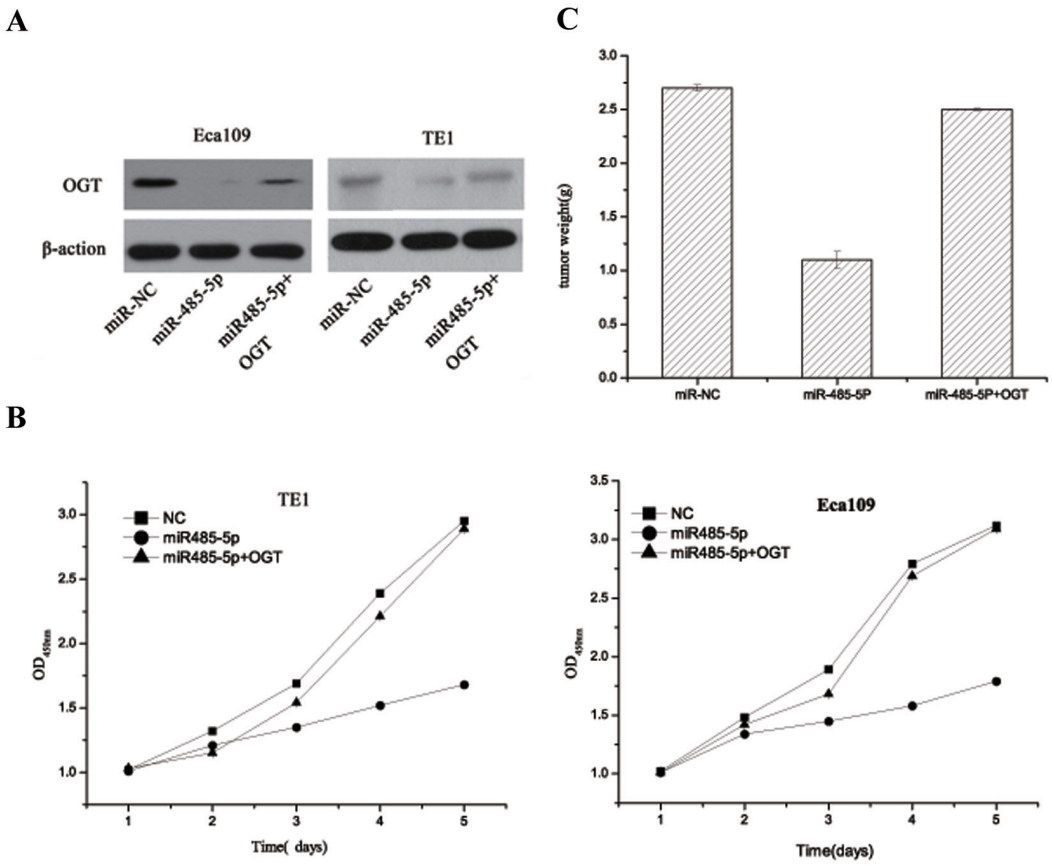

Figure 5. Identification of OGT as a direct target of miR-485-5p. ***A,*** Western blot analysis of OGT levels following miR-485-5p overexpression in Eca109 and TE1 cells. ***B,*** CCK-8 assay to evaluate the rescue effects of OGT expression on miR-485-5p induced inhibition of cell growth in Eca109 and TE1 cells. ***C,*** Tumor weight in response to miR-485-5p and OGT overexpression in TE1 cells. Data are shown as mean ± SD (n = 3) $p<0.05$.

the effects of miR-485-5p on cell proliferation, migration, and invasion in EC cells and found that miR-485-5p overexpression significantly subdued these cellular activities. Our results suggest that miR-485-5p might adversely regulate EC tumorigenesis as a tumor suppressor. To identify the mechanism by which miR-485-5p regulates EC growth, we predicted its target genes using the TargetScan algorithm and found that miR485-5p negatively regulated the expression of OGT, a target gene identified in EC cells. In addition, we established that the OGT negative regulation was important for miR-485-5p-mediated growth inhibition in EC cells. Previous reports[26-28] have indicated that OGT promotes tumorigenesis in various human cancers. In the present study, we found that OGT can partially reverse the repression of cell proliferation, migration, and invasion induced by miR-485-5p EC cells.

Conclusions

We demonstrated that miR-485-5p inhibited tumor growth, migration, and invasion in EC cells by directly regulating the expression of OGT. Our study showed that miR-485-5p acts as a tumor-suppressive miRNA in EC and suggested that miR-485-5p might serve as a novel therapeutic target for treating these malignant cancers.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Acknowledgements

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