Abstract. As esophageal squamous cell carcinoma (ESCC) is one of the most frequently diagnosed cancers in Asia, it is crucial to uncover its underlying molecular mechanisms that support its development and progression. Several articles have reported that microRNA (miR)-485-5p inhibits the malignant phenotype in a number of cancer types, such as lung, gastric and breast cancer, but to the best of our knowledge, its function in ESCC has not been studied in depth until the present study. It is of great significance to probe the regulatory action and underlying mechanism of miR-485-5p in ESCC. In brief, this study identified that miR-485-5p expression in ESCC tissues was significantly lower than that in normal tissues. The decrease in miR-485-5p expression was associated with a larger tumour size and poor histology and stage. The expression of miR-485-5p was relatively high in Eca 109 and TE-1 cells, but relatively low in KYSE 30. The overexpression of miR-485-5p inhibited cell proliferation, migration and invasion in vitro, whereas miR-485-5p knockdown did the opposite. Flotillin-1 (FLOT-1) can facilitate the malignant phenotype in various cancer types. The present study found that in ESCC tissue, the protein expression of FLOT-1 was negatively correlated with miR-485-5p expression. Further experiments showed that miR-485-5p directly targeted the 3'-untranslated region of FLOT-1. The overexpression of miR-485-5p significantly suppressed the mRNA and protein expression levels of FLOT-1, whereas knockdown had the reverse effects. Furthermore, overexpression of miR-485-5p restrained epithelial-mesenchymal metastasis (EMT)-related factors at both the mRNA and protein levels. At the same time, it also inhibited the growth of ESCC and restrained the EMT in vivo. In summary, miR-485-5p was found to be an inhibitor of ESCC and may have potential as a novel target candidate for ESCC treatment.

Introduction

Cancer is anticipated to be the leading cause of death worldwide in the 21st century and is expected to be the main barrier to increasing life expectancy (1). Esophageal cancer (EC) ranks seventh according to incidence (572,000 new cases), and sixth in terms of mortality (509,000 deaths) (1), while it ranks third (477,900 new cases) and fourth (375,000 deaths) in terms of incidence and mortality in China (2). EC primarily consists of two cell types, esophageal squamous cell carcinoma (ESCC) in addition to esophageal adenocarcinoma. ESCC is the most common (90%) histological subtype of EC (3); ~70% of cases occur in men (1). Over the past decade, the early stage detection and overall survival rates have improved, benefiting from the increased uptake of early referral schemes, novel endoscopic therapies and perioperative treatment strategies in some developed countries. However, the overall survival among patients with EC remains low; the 5-year survival is 10-15% among all patients, although it increases to 40% among patients who undergo curative surgery (4,5). Therefore, the molecular mechanism of EC underlying its development and progression is of great significance.

It is commonly known that the microRNA (miRNA/miR) family, which are composed of 18-25-nucleotide short non-coding RNAs, can regulate the expression of target mRNAs by binding to the 3'-untranslated regions (3'-UTRs). These miRNAs can downregulate the expression of target mRNA, which negatively modulates the gene expression or mRNA degradation at the posttranscriptional level (6-8). For example, miR-1254 downregulates E3 ubiquitin-protein ligase SMURF1 to reduce cell proliferation, migration and Matrigel
invasion in gastric cancer cell lines (9), while miRNA-146a downregulates VEGF to reduce cancer metastasis in hepatocellular carcinoma (10). To date, miRNAs have been regarded as vital factors in cancer progression, including tumour proliferation, migration, invasion, metastasis and radiosensitivity (11-15). It has been found that miR-485-5p, which regulates different targets in various human cancers, has the ability to function as a suppressor tumour gene. miR-485-5p downregulates the expression of tumour protein D54 (16) and paired box 3 (17) to inhibit the proliferation and invasion of glioma cells. However, it can also reduce the O-GlcNAcylation of polycomb complex protein BMI-1 (18) and inhibit proliferation by targeting CD147 in colorectal cancer (19). Han et al. (20) reported that O-linked N-acetylglucosamine transferase could be downregulated by the tumour suppressor miR-485-5p to inhibit the progression of ESCC, while in the present study another target was found, flotillin-1 (FLOT-1).

Flotillins are a group of ubiquitously expressed, evolutionarily conserved, membrane-associated scaffolding proteins located on microdomain lipid rafts containing two homologous isoforms, FLOT-1 and FLOT-2, which are involved in various procedures, including cell proliferation, migration, cell adhesion, survival, differentiation, endocytosis, signal transduction, membrane trafficking and T-cell activation (21-27).

In the present study, it was verified that miR-485-5p expression was reduced in ESCC. Cell proliferation, locomotion, invasion and epithelial-mesenchymal metastasis (EMT) were blocked by the overexpression of miR-485-5p. As FLOT-1 is a direct target of miR-485-5p, and various reports have verified FLOT-1 plays an important role in promoting cancer progression (21-27), it was concluded that miR-485-5p suppresses ESCC by targeting FLOT-1 and inhibiting the EMT.

Materials and methods

Subjects and tissue specimens. The Ethics Committee of The Fourth Hospital of Hebei Medical University (Shijiazhuang, China) approved this study (approval no. 2019053). Written informed consent was obtained from all subjects or guardians. In this study, ESCC and adjacent tissues (>1 cm away from the edge of the tumor) were surgically excised from 80 patients with ESCC between August 2015 and August 2018. None of the patients had previously received chemo- or radiotherapy. After surgery, the samples were dipped in RNAlater™ (Ambion; Thermo Fisher Scientific, Inc.) and stored in a -80˚C freezer. The specimens were clinically and histologically diagnosed by the Department of Thoracic Surgery and Pathology, The Fourth Hospital of Hebei Medical University. The lymph node metastasis and staging of patients were determined according to the AJCC Esophageal Cancer Staging System, Eighth Edition (28).

Cell lines and culture. ESCC cell lines Eca 109, KYSE 170, KYSE 180 and TE-1 were obtained from the Shanghai Institutes for Biological Sciences. KYSE 30, KYSE 510, TE-12 and YES-2 cell lines were provided by Professor Masatoshi Tagawa (Department of Molecular Biology and Cancer Biology, Chiba University, Chiba, Japan). All cells were cultured in air containing 5% CO₂ at 37°C in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Biological Industries). 1% penicillin and streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.).

Transfection. KYSE 30, Eca 109 and TE-1 cell lines (at 70% density), were transfected with 100 nM miR-485-5p mimics (cat. no. miR10002175-1-5; Guangzhou RiboBio Co., Ltd.), miRNA mimic negative controls (mimics-NC; cat. no. miR10000001-1-5; Guangzhou RiboBio Co., Ltd.), miR-485-5p inhibitor (cat. no. miR20002175-1-5; Guangzhou RiboBio Co., Ltd.), miRNA inhibitor negative controls (inhibitor-NC; cat. no. miR20000001-1-5; Guangzhou RiboBio Co., Ltd.), FLOT-1 plasmid (2 µg/well; YouBio) and pcDNA 3.1 (2 µg/well; YouBio) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The time interval between transfection and subsequent experimentation was 48 h.

RNA extraction and reverse transcription-quantitative (RT-q)PCR. According to the protocols reported previously (29), RNA extraction and RT-qPCR were carried out. Total RNA was extracted from cells or tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). According to the manufacturers' instructions, the GoScript™ Reverse Transcription System (Promega Corporation) was used to synthesize cDNA from 2 µg total RNA. It is worth noting that the reverse transcription primer of U6 and miR-485-5p replacing random primer were used to perform target-specific reverse transcription. Meanwhile, cDNA, which was used to quantitate the expression of mRNA, was reverse transcribed as described previously (29). qPCR was performed in triplicate with a 1/4 dilution of cDNA using the SYBR-Green PCR Kit (Promega Corporation) with the 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 sec, and annealing and extension at 60°C for 1 min. Gene specific qPCR primers were as follows: U6 snRNA reverse transcription primer, 5'-CGCTTCCAGGTTTGCGTCTGTA-3'; U6 snRNA forward, 5'-GCTTCCGGCAACATAATGAAAT-3' and reverse, 5'-CGCTTCAAGAATTTGCGTCTGTA-3'; miR-485-5p reverse transcription primer, 5'-GTCGTATCCATGCTCTGCAGTGGGAGTGCCC-3'; miR-485-5p forward, 5'-GGAGAGCGTCTGGGATGTA-3' and reverse, 5'-GCCTTCGCGTGGATGTA-3'; n-cadherin forward, 5'-CTCTCCAGTTGAGCTGTTTTA-3' and reverse, 5'-AGGCTCTTTTGACACCGCTCTC-3'; vimentin forward, 5'-GGAGACC TTGGGCGTTGAAAC-3' and reverse, 5'-TCCAGACGGTTGCTAGGTT-3'; zinc finger E-box-binding homeobox 1 (ZEB1) forward, 5'-TTGTAGCGGCTGGATTGTTT-3' and reverse, 5'-AGACGATAGTTGGTCGGAGC-3'; FLOT-1 forward, 5'-CCATCTCAGTCACTGGCATT-3' and reverse, 5'-CCGCAACACTCTCGTCCTGGTCT-3' and GAPDH forward, 5'-GGACACTTGACCTGCGGCTCAGTT-3' and reverse, 5'-GTA GCCCAGATGCCGGTGA-3'.

All primers were purchased from Geneay Biotech Co., Ltd. The relative expression levels of miRNA and mRNA were normalized to U6 and GAPDH expression, respectively. The
relative mRNA expression levels were calculated using the 2/[ΔΔCt] method (30).

**Semi-quantitative PCR.** After RNA extraction from ESCC cell lines and target-specific reverse transcription to cDNA, as aforementioned, cDNA was diluted with nucleic acid free water with a 1:4 dilution. qPCR was performed with the diluted cDNA using the GoTaq® G2 Master Mixes (cat. no. M7822; Promega Corporation) with the GeneAmp™ PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec; and a final extension at 72°C for 15 min. The agarose gels were 2% with 0.1% ethidium bromide. The gels were visualized using GBOX EF2 gel doc system (Syngene). Sequences of reverse transcription, forward and reverse primers were the same as aforementioned for RT-qPCR.

**Cell proliferation assay.** After cell transfection with miR-485-5p mimics, mimics-NC, miR-485-5p inhibitor and inhibitor-NC for 24 h, an MTS assay (Promega Corporation) was performed to examine proliferation. Harvesting and subculturing of KYSE 30, Eca 109 and TE-1 cells in 96-well plates (2,500 cells/well) were then performed. Cells were incubated for 0, 24, 48, 72 and 96 h, and cell proliferation was assessed using the MTS assay at each desired time point according to the manufacturer’s instructions. MTS reagent (20 µl/well) was added into each well and cells were incubated at 37°C for 2 h in the dark, and the absorbance was measured at 492 nm by a microplate reader. The experiment was performed in triplicate.

**Cell migration and invasion assays.** Cell migration and invasion were assessed by migration and invasion assays in a Transwell chamber (Corning, Inc.). Matrigel was used to coat invasion were assessed by migration and invasion assays in a Transwell chamber (Corning, Inc.). Matrigel was used to coat

in humidified air containing 5% CO₂ at 37°C with serum-free RPMI-1640. The wounds were imaged using a light microscope at the same location at 0, 12 and 24 h, and the percentage of the wound area was calculated (wound area/total area). The experiment was carried out three times.

**Immunohistochemistry (IHC).** All specimens (5 µm) were fixed in 4% formalin at 25°C for 24 h, and then embedded in paraffin. Antigen retrieval was achieved by boiling the specimens in Tris-EDTA buffer (cat. no. C1038; Beijing Solarbio Science & Technology Co., Ltd.) at 120°C for 3 min, followed by blocking endogenous peroxide and protein activity with endogenous peroxidase blocker (reagent I, streptavidin-avidin-biotin detection system; cat. no. SP-9001, ZSGB-BIO) for 20 min in the dark at 25°C. After blocking with normal goat serum (reagent II, streptavidin-avidin-biotin detection system; cat. no. SP-9001, ZSGB-BIO) at 25°C for 30 min, sections were incubated with primary antibodies specific for FLOT-1 (1:100; cat. no. 18634; Cell Signaling Technology, Inc.), E-cadherin (1:100; cat. no. ab40772; Abcam), Ki-67 (1:100; cat. no. 27309-1-AP; ProteinTech Group, Inc.), N-cadherin (1:50; cat. no. 22018-1-AP; ProteinTech Group, Inc.) and Vimentin (1:100; cat. no. 10366-1-AP; ProteinTech Group, Inc.) overnight at 4°C. Next, sections were incubated with the secondary antibody (reagent III, streptavidin-avidin-biotin detection system; cat. no. SP-9001, ZSGB-BIO) at 37°C for 30 min, followed by incubation with a HRP-labelled streptavidin solution (reagent IV, streptavidin-avidin-biotin detection system; cat. no. SP-9001, ZSGB-BIO) at 25°C for 30 min. Sections were washed with PBS after each step. After visualization of the positive antigen antibody reaction by incubation with 3,3-diaminobenzidine-tetrachloride (DAB, cat. no. ZLI-9018; ZSGB-BIO) at 25°C for 5 min, sections were counterstained with haematoxylin at 25°C for 3 min and evaluated by light microscopy at x40 and 200 magnification.

The expression of FLOT-1 was evaluated using IHC scores by choosing five views with high power lenses at random. The total number of tumour cells and the number of positive cells were calculated. The percentage of positive cells in the total number of tumour cells was calculated. The corresponding scores of positive cell percentages are shown as follows: i) 0, 0-25% positive cells; ii) 1, 26-50% positive cells; iii) 2, 51-75% positive cells; and iv) 3, 76-100%. In addition to the percentages of positive cells, FLOT-1 expression was also scored according to the dyeing strength: i) 0, no claybank; ii) 1, light claybank; iii) 2, medium claybank; and iv) 3, dark claybank. FLOT-1 expression in tissues was ranked according to the following scores: i) 0, -; ii) 1-2, +; iii) 3-4, ++; and iv) 5-6, ++++. All the sections were interpreted by two pathologists.

**Dual-luciferase reporter assay.** TargetScan (http://www.targetscan.org/vert_72/) was employed to predict the targets of miR-485-5p. The FLOT-1 mRNA 3'-UTR target sequence was cloned by Creative Biogene. The wild-type (WT) or mutant (MUT) miR-485-5p binding sequence of FLOT-1 was inserted into the pmirGLO plasmid (YouBio) to establish the recombinant luciferase reporter plasmids (FLOT-1 WT and FLOT-1 MUT). 293T cells (Procell Life Science & Technology Co., Ltd.) were co-transfected with pmirGLO, FLOT-1 WT
or FLOT-1 MUT and miR-485-5p mimics or mimics-NC with Lipofectamine® 2000. Following 48 h of incubation, the firefly and Renilla luciferase activities of 293T cells were examined using the Dual-Luciferase® Reporter Assay System (cat. no. E1910; Promega Corporation) following the manufacturer’s protocols. The relative luciferase activity was determined by normalizing the firefly luciferase activity against the Renilla luciferase activity.

Western blotting. Total protein was obtained from Eca 109 or KYSE 30 cells using RIPA buffer and PMSF (Beijing Solarbio Science & Technology Co., Ltd.). Protein concentration was determined using a BCA Protein Assay kit (cat. no. SI255483; Pierce; Thermo Fisher Scientific, Inc.). Proteins (80 µg/lane) were separated via SDS-PAGE on 10% gels, and then separated proteins were electrophoretically transferred onto PVDF membranes (EMD Millipore). Membranes were then blocked with 5% skimmed milk at room temperature for 1 h. The membrane was incubated with antibodies against FLOT-1 (1:1,000; cat. no. 18634; Cell Signaling Technology, Inc.), E-cadherin (1:1,000; cat. no. ab40772; Abcam), ZEB1 (1:500; cat. no. 21544-1-AP; ProteinTech Group, Inc.), N-cadherin (1:500; cat. no. 22018-1-AP; ProteinTech Group, Inc.), Vimentin (1:500; cat. no. 10366-1-AP; ProteinTech Group, Inc.) and GAPDH (1:10,000; cat. no. AP0063; Bioworld Technology, Inc.) at 4°C for 12 h. Next, the membranes were incubated at room temperature for 1 h with a fluorochrome-labelled secondary anti-rabbit IgG (1:10,000; cat. no. 926-32211; LI-COR Biosciences). The membranes were visualized using the Odyssey® Infrared Imaging System (LI-COR Biosciences). GAPDH served as a loading control. Western blot analysis was carried out three times, independently.

Lentiviral transfection. Eca 109 cells were cultured in 6-well plates. HitransG P (40 µl/well; GeneChem, Inc.) together with lentivirus-miR-485-5p (MOI 10) or lentivirus-vector (5 µl/well; GeneChem, Inc.) labeled with enhanced green fluorescent protein (EGFP) were added into the wells. HitransG P and lentivirus-miR-485-5p or lentivirus-vector were removed 12-18 h later. Then, cells were cultured in humidified air containing 5% CO₂ at 37°C for 72 h. Millesimal puromycin was used to select cells for 24 h. The cells were observed under a fluorescence microscope, if the EGFP-labeled cells were nearly at 100%, the lentiviral transfection was successful.

Tumour xenograft in animals. A total of 12 male athymic Balb/c nude mice (4-6 weeks, 20-25 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animal experiments were performed with approval from the Animal Care Committee of The Fourth Hospital of Hebei Medical University (approval. no. 110324020104285428). The mice were housed at The Fourth Hospital of Hebei Medical University Experiment Animal Centre (humidity, 50%; temperature, 25°C; light cycle, 12 h light/12 h dark; ad libitum access to food and water). The mice were randomly divided into two groups with six mice per group. Eca 109 cells were stably transfected with lentivirus-miR-485-5p or lentivirus-vector. Cells were suspended in PBS (5x10⁶ cells/200 µl) and injected into the right flanks of mice subcutaneously. Tumour volumes were monitored with a calliper every 5 days. Following which, the mice were sacrificed by spinal dislocation on day 32. Finally, the tumours were removed and weighed and fixed in formalin for IHC analysis, as described above.

Statistical analysis. Statistics were calculated using GraphPad Prism (v5.0) software (GraphPad Software, Inc.) and SPSS v22.0 (IBM Corp.). The experimental data are presented as the mean ± standard deviation from at least three separate experiments. For comparison among the paired samples, paired t-tests were performed. For comparison among the unpaired samples, an unpaired Student’s t-test was performed. One-way ANOVA followed by Dunnett’s post hoc test was used to examine the significant differences in the experimental data between multiple groups. The relationship between clinicopathological results and the expression of miR-485-5p was examined using a Pearson’s chi-squared test. The correlation between miR-485-5p and FLOT-1 expression was analysed using Spearman’s correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-385-5p is downregulated in ESCC tissues. The relative expression of miR-485-5p in 80 pairs of ESCC and para-carcinoma tissues was detected via RT-qPCR. As shown in Fig. 1A, human ESCC tissues exhibited lower expression of miR-485-5p than adjacent control tissues.

It was also demonstrated that the expression of miR-485-5p was strongly associated with the clinicopathological features of ESCC. Based on the expression of miR-485-5p, 80 ESCC samples were split into two groups. There were 44 cases in the high expression group and 36 cases in the low expression group. Decreased miR-485-5p expression was associated with a larger tumour size and poor differentiation and stages III/IV. Moreover, a decrease in miR-485-5p was more common in male patients (Table I). These data indicated that the expression of miR-485-5p was downregulated in ESCC tissues.

Furthermore, the expression of miR-485-5p was examined in ESCC cell lines (KYSE 30, KYSE 170, KYSE 180, KYSE 510, Eca 109, TE-1, TE-12 and YES-2) by semi-quantitative PCR. miR-485-5p expression was verified to be relatively high in Eca 109 and TE-1 cells, but relatively low in KYSE 30 cells, as shown in Fig. 1B. Thus, these three cell lines were used for the following experiments.

KYSE 30, Eca 109 and TE-1 cell lines were transfected with miR-485-5p mimics and inhibitor to explore the role of miR-485-5p in the biological functions of ESCC cells. The ratio of cell line transfection was validated by RT-qPCR. As indicated in Fig. IC, the expression of miR-485-5p was significantly upregulated in mimics group and downregulated in the inhibitor group, compared with the control groups.

miR-485-5p inhibits the proliferation, migration and invasion of ESCC cell lines. The influence of miR-485-5p on the proliferative capacity of ESCC cells was verified using an MTS assay. Compared with Eca 109, KYSE 30 and TE-1 cells transfected with the inhibitor-NC, the proliferation rates of cells transfected with miR-485-5p inhibitor were increased. Whereas, the inhibition rates of Eca 109, KYSE 30 and TE-1 cells transfected with miR-485-5p mimics were decreased in
contrast to cells transfected with mimics-NC. The proliferation rate was significantly reduced in Eca 109, KYSE 30 and TE-1 cells transfected with miR-485-5p mimics compared with the mimics-NC group, while the opposite effect was observed in the inhibitor group (Fig. 2A).

Further investigation demonstrated the inhibitory role that miR-485-5p played in ESCC cells. The migration ratios of Eca 109, KYSE 30 and TE-1 cells transfected with miR-485-5p mimics were reduced, whereas the migration ratios of Eca 109, KYSE 30 and TE-1 cells transfected with miR-485-5p inhibitor were significantly increased (Fig. 2B and C). Moreover, compared with the negative control, the invasion ratio was also decreased in Eca 109, KYSE 30 and TE-1 cell lines overexpressing miR-485-5p, but increased in the miR-485-5p knockdown group (Fig. 2D and E).

Next, a wound healing assay was performed to confirm these results. Compared with the negative control, the migratory rates of Eca 109, KYSE 30 and TE-1 cells transfected with mimics at 24 h were decreased. The overexpression of miR-485-5p reduced the migratory rate of ESCC cells (Fig. 2F).

Table I. Association between clinicopathological characteristics of patients with esophageal squamous cell carcinoma and miR-485-5p expression.

| Clinicopathological characteristics | miR-485-5p expression | P-value |
|-----------------------------------|-----------------------|---------|
|                                   | High | Low |         |
| Age, years                        | 24   | 16  | 0.369   |
| ≥65                               | 20   | 20  |         |
| <65                               | 36   | 21  |         |
| Sex                               | 8    | 15  |         |
| Male                              | 28   | 13  |         |
| Female                            | 16   | 23  |         |
| Size, cm³                         | 10   | 23  | 0.014†  |
| ≥10                               | 28   | 13  |         |
| <10                               | 22   | 17  | 0.805   |
| Lymph node metastasis             |      |     |         |
| Present (N1-N3)                   | 22   | 17  |         |
| Absent (N0)                       | 22   | 19  |         |
| Histological type                 |      |     | 0.001†  |
| Poor                              | 14   | 25  |         |
| Well to moderate                  | 30   | 11  |         |
| Stage                             |      |     | 0.006†  |
| I/II                              | 27   | 11  |         |
| III/IV                            | 17   | 25  |         |

°P<0.05; †P<0.01. miR, microRNA.

In addition, to further verify whether FLOT-1 is a direct target of miR-485-5p, a dual-luciferase reporter assay system was utilized. MUT and WT FLOT-1 3’UTR (site-directed mutations were contained in the former) were cloned into pmiRGLO reporter plasmids. pmiRGLO-WT-FLOT-1 3’UTR and miR-485-5p mimics were co-transfected into 293T cells. As shown in Fig. 3E, the overexpression of FLOT-1 WT + miR-485-5p mimics group compared with the FLOT-1 WT + mimics-NC group. Moreover, the overexpression of miR-485-5p did not have any
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To determine the relationship between the expression of miR-485-5p and FLOT-1 protein expression levels using Spearman's correlation analysis. miR-485-5p expression was measured via reverse transcription-quantitative PCR, and FLOT-1 protein levels were measured by IHC.

Table II. Negative correlation between miR-485-5p and FLOT-1 protein expression levels using Spearman's correlation analysis. miR-485-5p expression was measured via reverse transcription-quantitative PCR, and FLOT-1 protein levels were measured by IHC.

| miR-485-5p expression | FLOT-1 expression | r-value | P-value |
|-----------------------|-------------------|---------|---------|
| Negative (-/-)        | High              | 14      | 5       |
|                       | Low               |         |         |
|                       |                   | -0.560  | P<0.01  |
| Positive (++/+++)     |                  | 6       | 15      |

FLOT-1, flotillin-1; miR, microRNA; IHC, immunohistochemistry.

Overexpression of miR-485-5p inhibits the EMT. EMT is an important biological process that promotes the invasion and metastasis of cancer cells. It has been reported that miR-485-5p can reverse EMT in non-small cell lung cancer cells (32). As the present study confirmed that miR-485-5p overexpression could inhibit the migration and invasion of ESCC cells, it was of significance to determine the association between miR-485-5p and EMT in ESCC cells by evaluating the mRNA and protein expression levels of EMT-associated factors. The RT-qPCR and western blotting results revealed that E-cadherin expression in Eca 109 and KYSE 30 cells transfected with miR-485-5p mimics was increased, whereas Vimentin, N-cadherin and ZEB1 expression levels were decreased (Fig. 4A-C).
miR-485-5p suppresses the growth of ESCC in vivo. To illustrate the influence of miR-485-5p on tumour growth in vivo, Eca 109 cells transfected with lentivirus-miR-485-5p were injected into the flanks of nude mice, while cells transfected...
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with lentivirus-NC were utilized as the negative control (Fig. 5A and B). As demonstrated in Fig. 5C-E, the lentivirus-miR-485-5p group exhibited an observable decline in tumour volume and weight compared with the control group. On day 32, the volume and weight of tumours transfected with lentivirus-miR-485-5p were 1,324.5±201.39 mm$^3$ and 0.45±0.11 g, respectively, while those of the negative control were 2,482.83±555.13 mm$^3$ and 0.76±0.15 g, respectively. Moreover, the expression levels of FLOT-1, Ki-67 and EMT-related proteins were investigated by IHC. The protein
expression levels of Ki-67, FLOT-1, N-cadherin and Vimentin, compared with those in the control group, were all obviously decreased in the lentivirus-miR-485-5p transfection group, while E-cadherin was elevated (Fig. 6). In summary, miR-485-5p played roles as a tumour suppressor and apoptosis promotor of ESCC in vivo.

Discussion

The first miRNA was discovered in 1993 when the heterochronic gene lin-4 from Caenorhabditis elegans was identified as a small non-coding RNA (33). Over the past decades, as an increasing number of miRNAs have been identified, investigators have gradually realized that miRNAs are a cluster of short non-coding RNAs that can inhibit the stability of mRNA structures, decrease the translation ratio of proteins, disrupt the regulation of a number of biological processes, and play a role in cancer development (34). miRNAs have an impact on the biological behaviour of cancer cells, including proliferation, migration, invasion, cell cycle and apoptosis, and play a role in tumour occurrence and development (34-38).

miR-485-5p has been demonstrated to be a functional tumour suppressor. Duan et al (39) reported that miR-485-5p functions as a tumour suppressor in gastric cancer by targeting
7,8-dihydro-8-oxoguanine triphosphatase. Gao et al (40) discovered that miR-485-5p blocks the WW domain-binding protein 2/Wnt signalling pathway to inhibit the progression of hepatocellular carcinoma. The inhibitory function of miR-485-5p has also been reported in thyroid cancer (41,42), cholangiocarcinoma (43), lung cancer (32,44), osteosarcoma (45), breast cancer (46) and EC (20). Han et al (20) discovered that miR-485-5p, which can downregulate the expression of O-linked N-acetylglucosamine transferase, represses the proliferation and invasion of EC cells. The present study showed that miR-485-5p was reduced in human ESCC, and that overexpression of miR-485-5p could suppress the proliferation, migration and invasion of ESCC cell lines. Through bioinformatics prediction, FLOT-1 was identified as a potential target of miR-485-5p.

Previous studies have revealed that the dysregulation of FLOT is involved in various cancers. Several reports have demonstrated that the overexpression of FLOT-1 increases the ratio of tumourigenicity as well as cell proliferation by activating FOXO3a transcriptional activity in breast cancer (47), whereas the upregulation of FLOT-1 adjusted by NF-κB and Wnt/β-catenin promotes cell invasion, motility and lymph metastasis of the pelvis in cervical carcinoma (48). Furthermore, Liu et al (49) verified that highly expressed FLOT-2 facilitates proliferation, migration and invasion in melanoma. Studies have found that FLOT-1 facilitates the signalling of tumour necrosis factor-α (TNF-α) receptor and is relevant to ESCC progression (50). Guo et al (31) verified that FLOT-1 promotes the malignant phenotype of lung adenocarcinoma in vitro, including cell proliferation, migration and invasion. Moreover, Jang et al (51) reported that the sumoylation of FLOT-1 promotes EMT in metastatic prostate cancer.

Previous studies have verified that FLOT-1 facilitates malignant phenotypes, such as cell proliferation, migration, invasion and EMT in vitro, while in the present study, the results of bioinformatics prediction showed that FLOT-1 could be a potential target of miR-485-5p. It was verified that the expression of FLOT-1 could be directly reduced by the upregulation of miR-485-5p. It was verified that the expression of FLOT-1 could be directly reduced by the upregulation of miR-485-5p in ESCC. As shown in the present study, miR-485-5p overexpression inhibited cell proliferation, migration, invasion and EMT in ESCC, thus it is reasonable to conclude that miR-485-5p can inhibit these cell behaviours by repressing FLOT-1 expression. There are some limitations in this article. It was verified that miR-485-5p expression was
higher in ESCC tissues than that in paracancerous tissues, however miR-485-5p expression levels in ESCC cell lines were not compared with a normal esophagus cell line. Although other studies reported FLOT-1 facilitates malignant phenotypes in various cancer types, the present study did not verify its functions in ESCC directly. Overall, understanding the underlying actions of miR-485-5p in the pathogenesis of ESCC will increase the knowledge of the biological basis of tumour progression, which will increase the possibility of developing a novel diagnostic marker and original therapeutic strategy for ESCC.

Acknowledgements
Not applicable.

Funding
This project was supported by the National Natural Science Foundation of China (grant nos. 81973520 and 81673642).

Availability of data and materials
All data generated or analysed during this study are included in this published article.

Authors' contributions
RiZ and YS conceived the hypothesis, designed and performed the experiments. XZ and RuZ conducted the data collection, treated the animals, supervised the findings of this work, aided in interpreting the results, and provided the funds and critical revision of the manuscript. YS and LZ confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate
This project was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University (Shijiazhuang, China). Written informed consent was obtained from all subjects or guardians.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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