MicroRNA-200a promotes esophageal squamous cell carcinoma cell proliferation, migration and invasion through extensive target genes

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Abstract. Despite investigations into microRNA (miRNA) expression in esophageal cancer (EC) tissue, miRNAs that participate in EC pathogenesis and their subsequent mechanisms of action remain to be determined. The present study aimed to identify important miRNAs that contribute to EC development, and to assess miRNA biomarkers that could be used in EC diagnosis, prognosis and therapy. Bioinformatics analysis was performed to reanalyze EC tissue miRNA expression microarray dataset GSE113776, which was followed by *in vitro* verification of miRNA functions using reverse transcription-quantitative PCR, western blot analysis and a dual-luciferase reporter assay. Out of 93 miRNAs extracted, only miR-200a was significantly increased in EC tissues. Transfection of KYSE150 esophageal squamous cell carcinoma (ESCC) cells with miR-200a mimics significantly increased their proliferative, migratory and invasive ability, whereas the opposite cell behaviors were observed in ESCC cells transfected with a miR-200a inhibitor. A total of six miR-200a target genes [catenin β1 (CTNNB1), cadherin-1 (CDH1), PTEN, adenomatous polyposis coli (APC), catenin α1 (CTNNA1) and superoxide dismutase 2 (SOD2)] were selected for further analysis based on Gene Ontology terms and Kyoto Encyclopedia of Genes and Genomes pathway analysis, protein-protein interaction network map data and protein expression in esophageal tissue. These target genes were downregulated under miR-200a expression and upregulated in the presence of the miR-200a inhibitor. The association between miR-200a and the 3'-untranslated region of target genes in ESCC cells was confirmed using a dual-luciferase reporter assay. In conclusion, the present study demonstrated that miR-200a may participate in the promotion of ESCC cell proliferation, migration and invasion, and provided novel evidence for the direct interaction between miR-200a and CTNNB1, CDH1, PTEN, APC, CTNNA1 and SOD2, which may contribute to the observed altered cell behavior.

Introduction

Esophageal cancer (EC) is one of the most common types of malignant tumor worldwide and it is associated with a high incidence (5.9 per 100,000) and patient mortality (5 per 100,000) (1). China exhibits one of the highest incidence rates of EC (12.5 per 100,000) and a high patient mortality (~9.1 per 100,000), with a total of ~50% new EC cases identified in 2012 (2,3). Esophageal squamous cell carcinoma (ESCC) is the major histological type of EC, accounting for > 90% of new diagnoses (4,5). However, clinical practices, including the diagnosis and treatment of EC remain challenging worldwide, thus there is an urgent requirement to identify novel biomarkers that can be used to improve the diagnosis and treatment of EC.

MicroRNAs (miRNAs) are widely reported to participate in the pathogenesis of tumor development, and are often used as biomarkers to monitor disease progression (4). miRNAs are small, single-stranded non-coding RNA molecules of ~22 nucleotides in length that can inhibit the expression of target mRNAs through binding to complementarity sites in the 3'-untranslated region (UTR) to induce RNA silencing and post-transcriptional regulation (5,6). Each miRNA can target multiple genes, thus functioning in complex regulatory networks involved in a variety of biological processes, including cell proliferation, migration and invasion, especially within tumors (7-9). In EC, it was reported that miRNA (miR)-100 significantly inhibited EC cell proliferation, migration and invasion through targeting the C-X-C chemokine receptor type 7 (10). miR-373 is highly expressed in EC tissues and was demonstrated to increase cell proliferation, migration and invasion through directly targeting metalloproteinase inhibitor 3, with opposite results occurring following miR-373 silencing (11). In a cohort of 102 patients, miR-451a, miR-144-3p and miR-144-5p expression levels in EC tissues were reported to be significantly lower compared

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with normal tissues (12). Furthermore, low expression levels of miR-144-3p and miR-144-5p is an independent risk factor for the occurrence of EC (12). Multiple other miRNAs, including miR-133a, miR-138, miR-375 and miR-593 serve as tumor suppressors, whereas miR-16, miR-21, miR-31, miR-34b, miR-208, miR-223, miR-373 and miR-423 exhibit oncogenic abilities (13-18). It has been reported that miR-106b, miR-204, miR-371-3p, miR-574-3p, miR-886-3p, miR-1203, miR-1303 and miR-1909 were differentially expressed between patients with and without tumor relapse following surgery (13). miR-21 and miR-375 have previously been used as diagnostic and prognostic biomarkers of EC (14) and miR-100 has been suggested as a promising treatment owing to its tumor suppressor role in EC (10). These studies provide reasoning to identify additional miRNAs that may contribute to EC, alongside determining their mechanism of action in EC pathogenesis. The present study used bioinformatics analysis to reanalyze the miRNA expression microarray dataset of EC tissues (GSE113776), with the aim of identifying novel miRNAs to use as biomarkers for this disease. Since miRNAs demonstrated potential to be used for treatment and as biomarkers of EC according to the research cited above, the current study aimed to further investigate their functions *in vitro* through identifying key miRNAs that contribute to the development of EC, and to identify possible miRNA biomarkers for use in EC diagnosis, prognosis and therapy.

Materials and methods

Bioinformatics analysis. The miRNA profile dataset GSE113776 was downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). Considering the identical tissue variation and potential mutual effects or interactions in ESCC and neuroendocrine carcinoma (NEC), the GSE113776 dataset contained profiled miRNAs expressed in paired NEC and normal tissues based on an Agilent-041686 Unrestricted Human miRNA Microarray platform was used. Since only one sample was collected for sequencing in each group, no related research was cited in the dataset summary. R version 3.6 software (RStudio, Inc.) was used to analyze and visualize the dataset. To select miRNAs, the cut-off value of absolute fold change (|FC|) was set to 2. In addition, differentially expressed miRNAs (DEMs) that are associated with EC development were extracted from The Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov) to intersect with the selected miRNAs. Verification of DEM expression was based on the miRNA profile dataset GSE112840 of ESCC (19).

miRNA target gene extraction and associated pathway enrichment. Using the miRNA databases TargetScanHuman (http://www.targetscan.org), miRTarBase (http://mirtarbase. mbc.nctu.edu.tw) and mirRDB (http://mirdb.org), intersections were filtered as the ready-for-test genes. Representative immunohistochemistry images of these genes in normal esophageal tissues were identified using the Human Protein Atlas (http://proteinatlas.org) to determine their protein expression. The Database for Annotation, Visualization and Integrated Discovery (DAVID; https://david.ncifcrf. gov) was used for pathway enrichment of the filtered genes and ClueGO and Search Tool for Recurring Instances of Neighbouring Genes (STRING; https://string-db.org) were used for visualization, which was performed using Cytoscape version 3.7.0 (https://cytoscape.org/) software.

Cell culture and transfection. The human ESCC cell line KYSE150 was purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. KYSE150 cells were incubated in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% FBS (HyClone; GE Healthcare Life Sciences) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Inc.), and maintained in a humidified incubator at 37°C with 5% CO_2. A total of $5x10^4$ KYSE150 cells/well were seeded in a 6-well plate and were transfected with 50 nM miR-200a mimic (5'-UAACACUGUCUGGU AACGAUGU-3'), miR-200a inhibitor (5'-UAACCUCAUGGU GUACGAAUGU-3') or scramble control sequences (5'-UUG UACUACACAAAAGUACUG-3') (Shanghai GenePharma Co., Ltd.) separately for 24 h at 37°C using 10 nM Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturers' protocol. Cell proliferation and Transwell assays were subsequently performed and transfected cells were harvested at 48 h for reverse transcription-quantitative PCR (RT-qPCR) or western blot analysis.

Cell proliferation assay. Following transfection, a total of $1x10^4$ KYSE150 cells/well were seeded into 96-well plates to assess cell viability. Cells were cultured for 2 days post-transfection in RPMI-1640 medium prior to the addition of 10 μl Cell Counting kit-8 (CCK-8) solution (WST-8, Dojindo Molecular Technologies, Inc.) to each well. Following continuous incubation for 2 h at 37°C, cell viability was determined by measuring the absorbance at 450 nm using an ELISA reader (Tecan Group, Ltd.) at 0, 12, 24 and 48 h.

Migration and invasion assays. Migratory and invasive abilities of transfected KYSE150 cells were examined using Transwell permeable supports (Corning, Inc.). Following cell transfection for 24 h at 37°C, cells were subsequently cultured in 200 μl serum-free RPMI-1640 medium before being transferred into 24-well plates separated into upper and lower chambers. A total of $1x10^5$ KYSE150 cells/well were plated in the upper chambers of Transwell plates with serum-free RPMI-1640 medium, of which membranes were or were not precoated with Matrigel (BD Biosciences) for the invasion and migration assay, respectively. A total of 800 μl RPMI-1640 medium supplemented with 10% FBS was plated in the lower chambers. After 24 h incubation in wells without Matrigel or 48 h with Matrigel for the migration and invasion assay, respectively, cells in the lower chambers were fixed for 20 min with absolute methanol and subsequently stained with 0.1% crystal violet solution (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature. Stained cells were counted in three randomly selected fields using an inverted microscope (magnification, x200). ImageJ version 1.49 software (National Institutes of Health) was used for image analysis and quantification. Experiments were performed in triplicate.

RT-qPCR. Total RNA was extracted from KYSE150 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA

was reverse transcribed into cDNA using PrimeScript RT Reagent kit purchased from Takara Biotechnology Co., Ltd., and the concentration of RNA was determined as described in a previous study (20). qPCR was subsequently performed in 96-well reaction plates using the ABI StepOnePlus™ Real-Time PCR system (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Briefly, in each well, a total of 1 μl cDNA template, 0.2 μl primer (0.1 μl forward and 0.1 μl reverse primer), 3.6 μl diethyl pyrocarbonate-H_2O and 5 μl SYBR® Green dye were mixed. The primers (Sangon Biotech Co., Ltd.) used for the qPCR are presented in Table SI. The following thermocycling conditions were used for the qPCR: Initial denaturation at 95°C for 30 sec; followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 10 sec and extension at 72°C for 30 sec. Expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to the internal reference gene, U6 for miR-200a expression or GAPDH for miR-200a target genes (21).

Western blotting. Total protein was extracted from KYSE150 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) and protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA). Total protein was quantified using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc.) and western blot analysis was performed according to previous protocols (22). A total of 30 μg protein/lane was separated by 6 or 12% SDS-PAGE. The separated proteins were subsequently transferred onto a PVDF membrane (Merck KGaA) and blocked for 1 h at room temperature using 5% non-fat milk. The membranes were incubated overnight at 4°C with the following primary antibodies diluted in 5% milk: Anti-catenin β1 (CTNNB1; 1:1,000; cat. no. sc-59737; Santa Cruz Biotechnology, Inc.), anti-cadherin-1 (CDH1; 1:500; cat. no. sc-71009; Santa Cruz Biotechnology, Inc.), anti-PTEN (1:100; cat. no. sc-73420; Santa Cruz Biotechnology, Inc.), anti-adenomatous polyposis coli (APC; 1:100; cat. no. sc-393704; Santa Cruz Biotechnology, Inc.), anti-catenin α1 (CTNNA1; 1:500; cat. no. sc-47753; Santa Cruz Biotechnology, Inc.), anti-superoxide dismutase 2 (SOD2; 1:200; cat. no. sc-130345; Santa Cruz Biotechnology, Inc.) and anti-GAPDH (1:3,000; cat. no. sc-47724; Santa Cruz Biotechnology, Inc.). Following the primary antibody incubation, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (1:5,000; cat. no. A0216; Beyotime Institute of Biotechnology) for 45 min at room temperature. Protein bands were visualized using the ECL luminol reagent (PerkinElmer Inc.) and a ChemiDoc image analyzer (Bio-Rad Laboratories, Inc.). Protein expression was quantified using ImageJ software (version 1.49v; National Institutes of Health) and normalized to the internal reference gene GAPDH.

Dual-luciferase reporter assay. The luciferase reporter assay was performed as previously described (22). The 3'-UTR sequence of miR-200a target genes (CTNNB1, CDH1, PTEN, APC, CTNNA1 and SOD2) was separately amplified and inserted into the luciferase reporter vector pGL3-enhancer (Promega Corporation). The primers of the 3'-UTR of miR-200a target genes were designed and are presented in Table SII. A total of 1x10⁴ KYSE150 cells/well were seeded

into 24-well plates and incubated for 24 h at 37°C. Wild-type or mutant miR-200a target gene 3'-UTR vectors, combined with the miR-200a mimic, were subsequently co-transfected into KYSE150 cells using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The construction of the miR-200a target genes' 3'-UTR wild-type or mutant reporter genes were performed using the miRNA databases (Fig. S1). Following incubation for 48 h at 37°C, KYSE150 cells were lysed and firefly and *Renilla* luciferase activity was detected using a Luciferase assay system (Promega Corporation), according to the manufacturer's protocol. Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Statistical analysis. Data were analyzed using GraphPad Prism version 6.0 software (GraphPad Software, Inc.). Statistical differences between two groups were compared using a Student's t-test, whereas three groups were compared using a one-way ANOVA with Bonferroni correction post hoc test. Data were presented as the mean ± SEM from 3 independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Heat map of miRNA expression and identification of DEMs in a paired NEC sample. ESCC and neuroendocrine carcinoma (NEC) are from the identical sites in esophageal tissues, indicating a potentially similar mechanism. A heat map was constructed to identify the expression of miRNAs in esophageal tissues using a Heat map package in R. For the miRNA y dataset GSE113776, 494 miRNAs were detected (Fig. 1A). A total of 28 miRNAs were selected, of which the absolute expression value threshold was |FC|>2 (Fig. 1B). In addition, 93 DEMs that were associated with the development of EC were identified from TCGA (Table I). One miRNA was observed to overlap between the selected miRNAs and DEMs from TCGA, which was miR-200a (Fig. 1C). In addition, under the cross-verification with the GSE112840 dataset, miR-200a was confirmed to be upregulated in ESCC compared with the control (Fig. 1D), which suggests the critical role of miR-200a in ESCC.

Effects of miR-200a on the proliferation, migration and invasion of ESCC cells. KYSE150 ESCC cells were transiently transfected with miR-200a mimics or inhibitors and the effects of miR-200a on ESCC cell proliferation were assessed using a CCK-8 assay. RT-qPCR was used to confirm the transfection efficiency of the mimic and inhibitor in KYSE150 ESCC cells; the miR-200a mimic significantly increased miR-200a expression levels compared with the scramble control, whereas the miR-200a inhibitor significantly decreased miR-200a expression levels compared with the scramble controls in ESCC cells (Fig. 2A). The viability of ESCC cells was significantly increased in miR-200a mimic-transfected cells compared with the scramble control at 24 and 48 h, whereas the inhibition of miR-200a expression significantly decreased ESCC cell viability compared with the scramble control at all of the time points (Fig. 2B). To evaluate the effects of miR-200a on cell migration and invasion, a Transwell assay was performed
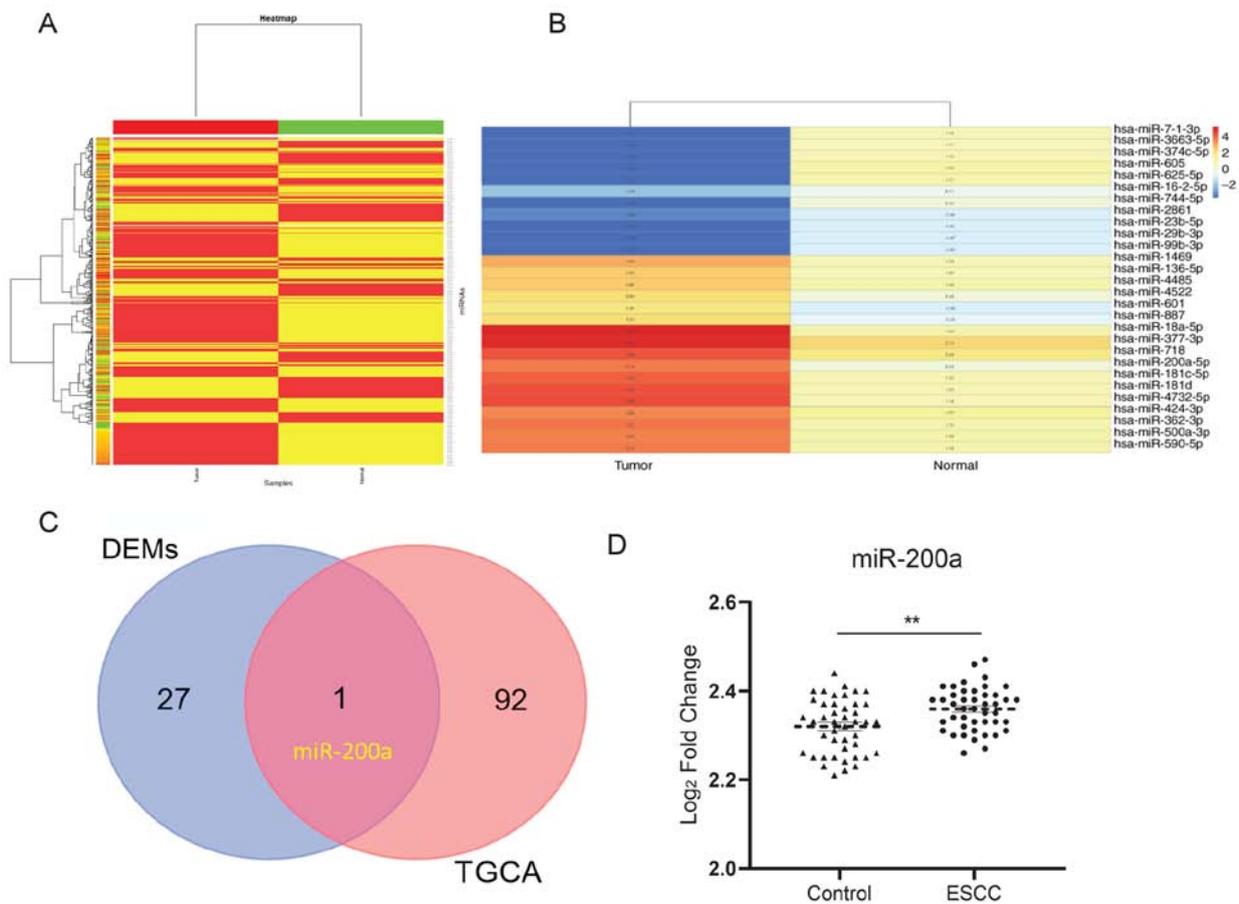
Figure 1. Heat map of miRNA expression in esophageal cancer and the identification of key miRNAs. (A) A total of 494 miRNAs were detected in neuroendocrine carcinoma esophageal tissues in microarray dataset GSE113776. The miRNA expression in the tumoral tissue is presented on the left and expression in the normal tissue is presented on the right. Red indicates high expression; yellow indicates low expression of miRNAs. (B) Identification of 28 miRNAs of which the expression threshold of tumor/normal ratio was indicated as >2 on the heat map. (C) The 28 miRNAs from the tissue microarray and 93 differentially expressed miRNAs identified from TCGA were compared, and miR-200a was demonstrated to be shared between the two groups. (D) Expression levels of miR-200a between ESCC and control sample using the miRNA microarray dataset GSE112840 on ESCC tissue. **P<0.01. DEM, differentially expressed miRNA; ESCC, esophageal squamous cell carcinoma; miR/miRNA, microRNA; TCGA, The Cancer Genome Atlas.

in ESCC cells. miR-200a mimics transfection significantly increased the migratory and invasive ability of ESCC cells compared with the scramble control, whereas the miR-200a inhibitor-transfected cells exhibited significantly reduced migratory and invasive capacity compared with the scramble control (Fig. 2C).

Identification of target genes and pathway enrichment analysis. As miR-200a was the only miRNA selected from the two groups, the current study aimed to identify target genes that miR-200a may regulate in ESCC. The miRNA databases TargetScanHuman, miRTarBase and mirDB were searched and the intersections were filtered using 54 genes. DAVID was used for pathway enrichment and ClueGO and STRING were used for visualization. The results of the GO analysis indicated that the main biological processes affected by miR-200a were embryonic heart tube development and transcriptional regulation, and the main cellular components regulated were transcription factor complexes and cell-cell adherens junctions (Table II). The most significant molecular functions associated with miR-200a were DNA binding, transcription factor binding and cell-cell adhesion. The results of the KEGG pathway analysis reported that basal cell carcinoma pathways

in cancer and carbon metabolism were the most significantly enriched pathways (P<0.05), whereas the Wnt and the Hippo signaling pathways were classed as critical pathways despite not being significant (P=0.05 and P=0.06, respectively; Table II). Subsequently, miR-200a target genes were assigned to ClueGO to assess the association between miR-200a target genes and GO biological process, molecular function and cellular component terms, which yielded the terms 'regulation of transcription', 'positive regulation of core promoter region' and others (Fig. 3A) and GO terms distribution associated with the number of genes and percentage (Fig. 3B). According to the relationships, the protein-protein interaction map of target genes was created using STRING, and the network visualization was performed using Cytoscape (Fig. 3C). In the network, CTNNB1, CDH1, TP53, PTEN, CDK1, APC, CTNNA1, FYN and SOD2 were the most prominent, as these genes exhibited multiple associations with other genes.

Interactions of miR-200a and its target genes. As previously described, CTNNB1, CDH1, TP53, PTEN, CDK1, APC, CTNNA1, FYN and SOD2 were identified as the most significant genes targeted by miR-200a in the network. Representative immunohistochemistry images of these genes

Table I. miRNAs identified from the tissue microarray and TCGA.

Origin	Count	Elements
miRNAs profiling	28	hsa-miR-136 hsa-miR-1469 hsa-miR-16 hsa-miR-181c hsa-miR-181d hsa-miR-18a hsa-miR-200a hsa-miR-23b hsa-miR-2861 hsa-miR-29b hsa-miR-362 hsa-miR-3663 hsa-miR-374c hsa-miR-377 hsa-miR-424 hsa-miR-4486 hsa-miR-4522 hsa-miR-4732 hsa-miR-500a hsa-miR-590 hsa-miR-601 hsa-miR-605 hsa-miR-625 hsa-miR-7-1 hsa-miR-718 hsa-miR-744 hsa-miR-887 hsa-miR-99b
TCGA	93	microRNA 663b microRNA 770 microRNA 520b microRNA 485 microRNA 1270 AC116165.1 microRNA 1302-3 microRNA 498 AC005631.1 microRNA 3156-3 AC100757.1 microRNA 516a-1 microRNA 920 AC233702.1 microRNA 519d microRNA 5787 microRNA 515-2 microRNA 6511a-3 microRNA 181b-1 microRNA 4697 AL391261.1 microRNA 6511a-1 AC005071.4 microRNA 1208 AC106782.1 AF254983.1 AJ271736.1 AC010203.1 microRNA 600 microRNA 6511a-2 AL354833.1 microRNA 4300 microRNA 1468 AL078621.1 microRNA 513c microRNA 3142 AC011453.4 microRNA 412 microRNA let-7f-1 microRNA 221 AC245033.1 microRNA 6859-3 AL354820.1 microRNA 300 microRNA 519b microRNA 182 AC106788.1 microRNA 514a-2 microRNA 622 microRNA 507 AC126544.1 microRNA 205 microRNA 15a Z83819.1 microRNA 409 microRNA 6859-4 AC090825.1 microRNA 2682 microRNA 410 microRNA 154 microRNA 411 microRNA 548x microRNA 519a-1 microRNA 6859-1 microRNA 548i-4 microRNA 1197 microRNA 495 AL161651.1 microRNA 892a microRNA 3680-2 AC133555.1 AC092375.1 microRNA 509-2 AC105339.2 AC023310.1 AC024937.1 microRNA 570 microRNA 517b AC092017 microRNA 6080 microRNA 509-3 microRNA 3147 AC215219.2 microRNA 466 microRNA 129-2 microRNA 548i-1 AC011467.1 microRNA 1-1 microRNA 520d AC011453.3 microRNA 518f microRNA 758

were obtained from the Human Protein Atlas and were used to determine the protein expression of each gene in normal esophageal tissues. These proteins were highly expressed in normal tissues according to the Human Protein Atlas (Fig. S2). Accordingly, the highly expressed genes (CTNNB1, CDH1, PTEN, APC, CTNNA1 and SOD2) were selected for subsequent analysis. To detect the effects of miR-200a on the target genes, CTNNB1, CDH1, PTEN, APC, CTNNA1 and SOD2 mRNA and protein expression levels were examined in KYSE150 ESCC cells transfected with a miR-200a mimic or inhibitor. RT-qPCR and western blot analysis demonstrated that target genes and their protein levels were significantly decreased in cells transfected with miR-200a mimic compared with the scramble-transfected cells, whereas expression levels were significantly increased in cells transfected with the miR-200a inhibitor compared with the control (Fig. 4A). A dual-luciferase reporter assay was performed to evaluate the direct interaction between miR-200a and the 3'-UTR of the target genes. The results indicated that KYSE150 ESCC cells transfected with the miR-200a mimic significantly suppressed the luciferase activity of the wild-type reporters containing the 3'-UTR of all target genes (CTNNB1, CDH1, APC, PTEN, CTNNA1 and SOD2) compared with the scramble control; this inhibition disappeared when the miR-200a target site was mutated (Fig. 4B).

Discussion

ESCC is the main histological type of EC, accounting for >90% of new cases (23); the majority of new ESCC cases occur in Asia, especially in China (2). Clinical treatment for

ESCC remains challenging, and novel diagnosis and treatment strategies are urgently required. miRNAs have been widely reported to be associated with tumor development, and are often regarded as disease biomarkers; it has been demonstrated that miRNAs affect ESCC cell behaviors, including cell proliferation, migration and invasion (7-9). However, miRNAs function in a complicated regulatory network, which involves a number of different biological processes (24). The present study analyzed the miRNA expression microarray of NEC (with its incidence being rare but of similar effects with ESCC) tissues from dataset GSE113776 of the GEO database. A total of 28 miRNAs were selected according to the inclusion criteria. miR-200a was selected following the identification of an intersection between the 28 selected miRNAs and 93 DEMs using the TCGA. Subsequent *in vitro* experiments demonstrated that miR-200a may be involved in promoting ESCC cell proliferation, migration and invasion. Furthermore, the results provided evidences that miR-200a interacts with its target genes, CTNNB1, CDH1, PTEN, APC, CTNNA1 and SOD2, which may contribute to the abnormal cell behaviors in EC.

A number of previous studies have performed high-throughput sequencing, while a total of 140 DEMs have been extracted, with 113 upregulated and 27 downregulated miRNAs being identified in ESCC tissue (25). In a previous study, only five miRNAs (miR-103-1, miR-18a, miR-324, miR-369 and miR-320b-2), which have previously been associated with survival rates (25), were studied. In another study, out of a total of 136 DEMs identified in EC, the top five DEMs were revealed to be miRNA-21, miRNA-93, miRNA-196a-1, miRNA-196a-2 and miRNA-4746 (26). In addition, within
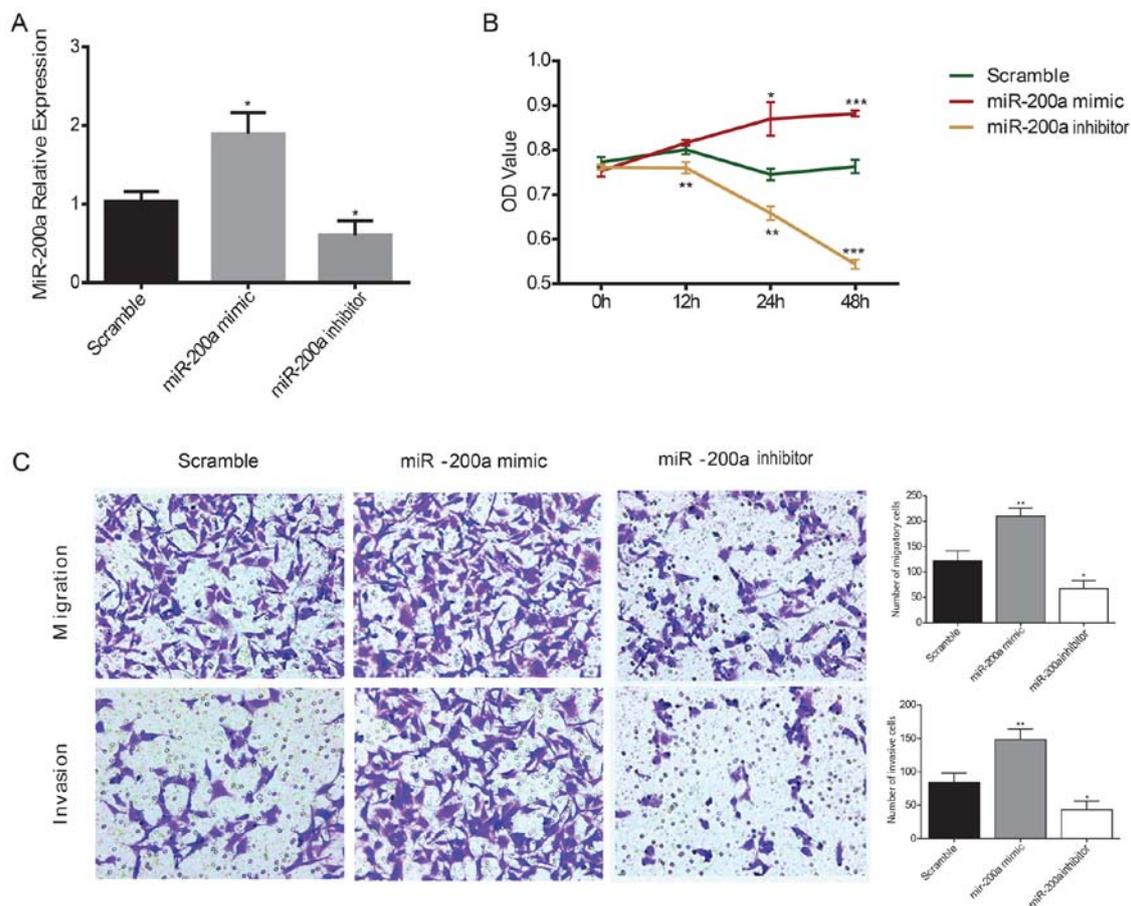
Figure 2. miR-200a promotes ESCC cell proliferation, migration and invasion. (A) Relative miR-200a expression levels in ESCC cells following transfection with scramble control miRNA, miR-200a mimic or inhibitor was measured using reverse transcription-quantitative PCR. (B) The viability of ESCC cells following transfection with scramble control miRNA, miR-200a mimic or inhibitor was measured using a Cell-Counting kit-8 assay after 48 h of transfection. (C) Migratory and invasive ability of ESCC cells were examined using a Transwell assay following transfection with scramble control miRNA, miR-200a mimic or inhibitor for 48 h. Magnification, x200. Data are presented as the mean ± SEM from three independent experiments. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ vs. Scramble. ESCC, esophageal squamous cell carcinoma; miR, microRNA; OD, optical density.

a cohort of 102 patients with EC, miR-451a, miR-144-3p and miR-144-5p were identified; other miRNAs including miR-133a miR-138, miR-375 and miR-593 were observed to serve as tumor suppressors, whereas miR-16, miR-21, miR-31, miR-34b, miR-208, miR-223, miR-373 and miR-423 exhibited oncogenic properties (13-18). Meanwhile, researchers identified miR-28-5p, miR-34a-5p and miR-186-5p as the significant biomarkers of ESCC (27). Furthermore, previous studies explored the underlying miRNAs that can play key roles in ESCC development. For instance, miRNA-10b can promote ESCC cell proliferation, migration, invasion and colony formation, as well as metastasis; miRNA-548 and miRNA-576 enhance the migration and invasion of ESCC cells; miRNA-1 can suppress the proliferation, migration and invasion of ESCC cells (28-30). Based on these studies, a number of miRNAs have been reported to serve a role in EC; however, to the best of our knowledge, no previous studies have predicted the potential role of miR-200a in EC pathogenesis using bioinformatics. In the present study, a total of 28 miRNAs were identified in the NEC microarray dataset GSE113776, including miR-200a. Additionally, a number of important miRNAs, including miR-181c-5p, hsa-miR-500a-3p, hsa-miR-601 and hsa-miR-605 were matched with identified DEMs in previous research (25,26).

To the best of our knowledge, no previous bioinformatics study has identified miR-200a as a crucial miRNA, and this may be due to the fact that a single sample of NEC tissue was used. The GSE113776 dataset may not have been investigated owing to the difficulty of using statistical analysis to assess it. However, this disadvantage permitted the identification of novel miRNAs because the high expression of miRNAs in the tissue may have excluded the significance of the less expressed, but critical miRNAs. In addition, previous studies may not have focused on the most important miRNAs, but instead on determining DEMs. Owing to the single sample comparison in the present study, miR-200a was identified and its was demonstrated to be upregulated in ESCC according to the miRNA profile GSE112840 (19). The importance of miR-200a was confirmed by matching with TGCA records and RT-qPCR verification. It is hypothesized that different types of cells exhibit mutual effects or interactions with each other (31-34); NEC, squamous cell carcinoma and sarcoma exhibit metastatic and site-transfer-direction (32,33), and definitive chemoradiotherapy promoted the conversion of NEC to squamous cell carcinoma (34). In addition, NEC, squamous cell carcinoma and adenocarcinoma can coexist (31,35,36). Although miR-220a was singled out from NEC tissues, the present study demonstrated the effects of miR-200a in ESCC

Table II. GO analysis and KEGG pathway enrichment analyses of miR-200a target genes.

Category	Term	Count	P-value
GO term-biological processes	Embryonic heart tube development	3	<0.001
	Negative regulation of transcription from RNA polymerase II promoter	9	0.001
	Positive regulation of transcription from RNA polymerase II promoter	10	0.002
	Transcription from RNA polymerase II promoter	7	0.004
	Response to zinc ion	3	0.005
	Regulation of transcription, DNA-templated	11	0.01
	Response to drug	5	0.01
	Cell proliferation	5	0.02
	Prostate gland growth	2	0.02
	Canonical Wnt signaling pathway	3	0.02
	Embryonic foregut morphogenesis	2	0.02
	Positive regulation of skeletal muscle tissue development	2	0.03
	Negative regulation of cell proliferation	5	0.03
	Positive regulation of sequence-specific DNA binding transcription factor activity	3	0.04
	Epithelial tube branching involved in lung morphogenesis	2	0.04
GO term-cellular components	Nucleus	29	<0.001
	Transcription factor complex	5	0.001
	Nuclear transcription factor complex	2	0.03
	Cell-cell adherens junction	4	0.05
	Mitochondrial matrix	4	0.05
GO term-molecular function	RNA polymerase II core promoter proximal region sequence-specific DNA binding	10	<0.0001
	Nucleic acid binding	12	0.0001
	Transcription factor activity, sequence-specific DNA binding	10	0.001
	Transcription regulatory region DNA binding	5	0.003
	Transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	5	0.005
	Metal ion binding	13	0.01
	Transcription coactivator activity	4	0.04
	Transcription factor binding	4	0.05
	DNA binding	10	0.05
	Cadherin binding involved in cell-cell adhesion	4	0.05
KEGG_Pathway	Signaling pathways regulating pluripotency of stem cells	4	0.006
	Basal cell carcinoma	3	0.009
	Biosynthesis of amino acids	3	0.01
	Pathways in cancer	5	0.02
	Carbon metabolism	3	0.04
	Wnt signaling pathway	3	0.05[a]
	Hippo signaling pathway	3	0.06[a]

[a]According to the literature review, these pathways are critical even though P>0.05. GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

cells; however, the direct association between ESCC and NEC and adenocarcinoma was not assessed due to the lack of tissue.

The alterations of ESCC cell behaviors following miR-200a overexpression are not well determined. The role of miRNAs depends on the type of miRNAs. It is reported that miR-100 inhibits EC cell proliferation, migration and invasion, (10), while miR-373 enhances cell proliferation, migration and invasion (11). The present study demonstrated that the upregulation of miR-200a could promote ESCC cell proliferation, migration and invasion. This was a similar role to the effects of miR-373 in decreasing the proliferation, migration and invasion of ESCC cells. In addition to the effects of miR-200a in ESCC, miR-200a has been studied in a number of tumor types, including breast, ovarian, gastric, colorectal and pancreatic

Figure 3. Pathway enrichment analysis and identification of key target genes of miR-200a. miR-200a target genes from the intersection of miRNAs databases TargetScanHuman, miRTarBase and mirDB were input into ClueGO (version 2.54) for (A) pathway enrichment and (B) target genes related GO term distribution. (C) A protein-protein interaction network of target genes was evaluated using STRING according to the gene-interaction degree (in grey line), and the networks visualization was performed using Cytoscape software with the size of circle (from green to blue) representing the interaction count. [*]P<0.05, [**]P<0.01 for enrichment P-value from χ^2 test. GO, gene ontology; miR, microRNA; STRING, Search Tool for the Retrieval of Interacting Genes. GRHL1, grainyhead like transcription factor 1; HHIP, hedgehog interacting protein; WNT16, Wnt family member 16; ZNF675, zinc finger protein 675; FOXC1, forkhead box C1; WWTR1, WW domain containing transcription regulator 1; CTNNB1, catenin β1; SOX17, SRY-box transcription factor 17; PLAG1, PLAG1 zinc finger; PTEN, phosphatase and tensin homolog; OTX1, orthodenticle homeobox 1.

cancers (37-41); miR-200a is also associated with cell proliferation, migration and invasion in these tumor types (37-41).

The present study investigated the putative target genes of miR-200a, as miRNAs function in the development of ESCC
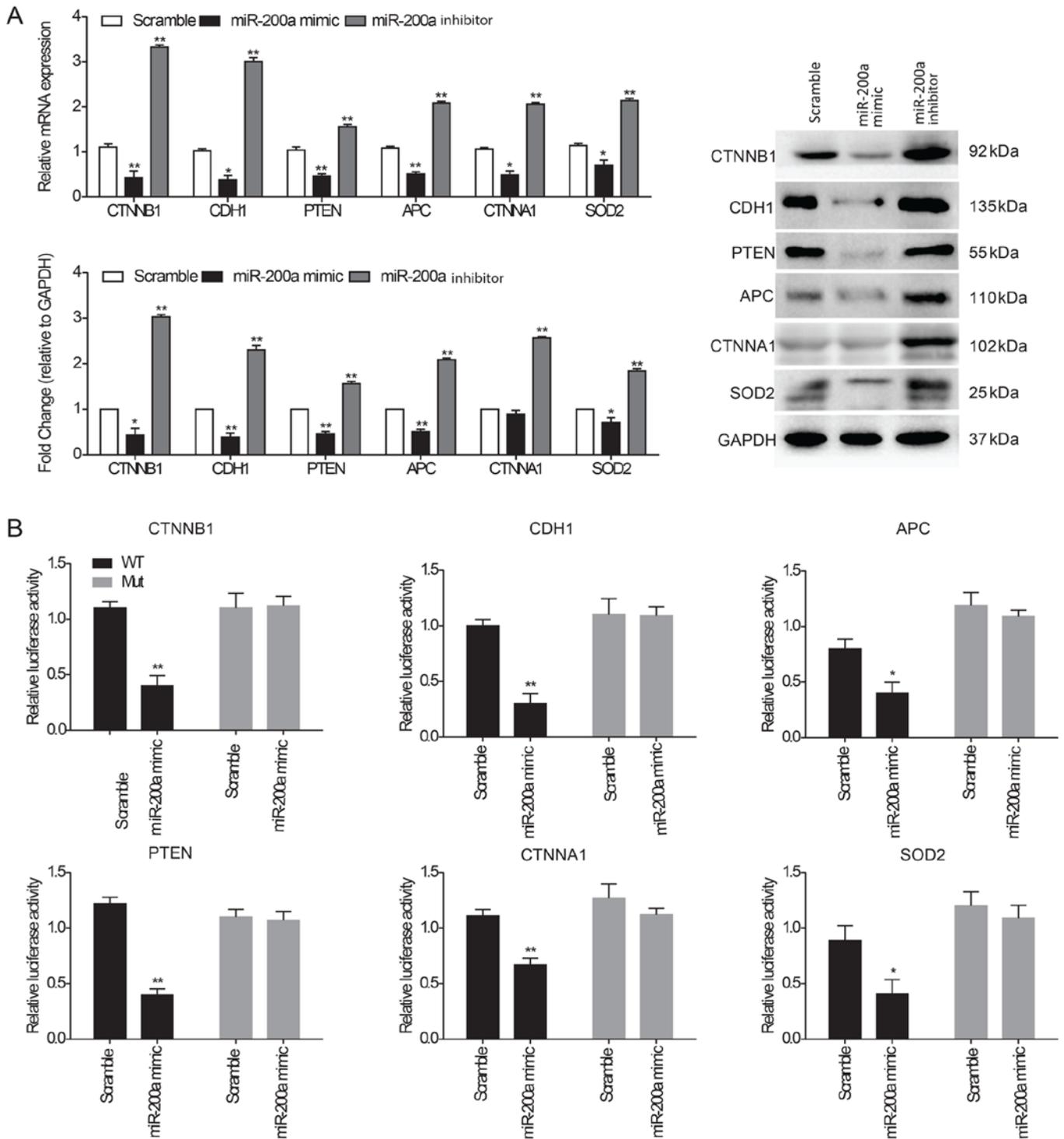
Figure 4. Interaction of miR-200a and its target genes. (A) CTNNB1, CDH1, PTEN, APC, CTNNA1 and SOD2 mRNA and protein expression levels were determined in KYSE150 ESCC cells following transfection with scramble control miRNA, miR-200a mimic or inhibitor using reverse transcription-quantitative PCR and western blot analysis. GAPDH was used as an internal loading control for both analyses. (B) Dual-luciferase assay using wild-type or mutated 3'-UTR sequences of target genes co-transfected with miR-200a mimics into ESCC cells. The cells were evaluated for luciferase activity following 48 h incubation. Data are presented as the mean ± SEM of three independent experiments. *P<0.05 and **P<0.01 vs. Scramble. APC, adenomatous polyposis coli protein; CDH1, cadherin-1; CTNNA1, catenin α1; CTNNB1, catenin β1; ESCC, esophageal squamous cell carcinoma; SOD2, superoxide dismutase; UTR, untranslated region.

via binding to the UTR region of target genes: miRNA-10b targeting FOXO3, micRNA-548 and miRNA-576 down-regulating NRIP1 and miRNA-1 binding to Notch2 (28-30). Bioinformatics analysis revealed that the main biological processes that miR-200a was involved in included embryonic development and transcription regulation, the main cellular components were transcription factor complexes and cell-cell adherence junctions and the main molecular functions were related to DNA binding, transcription factor binding and cell-cell adhesion, which showed the similar results

with the findings of previous studies enriched from other miRNAs (25,26,42). The results of KEGG pathway enrichment, except for the suggestions of basic pathological pathways, also indicated that miR-200a may serve a role in the Wnt signaling pathway and the Hippo signaling pathway, which was consistent with previous findings (43-47). According to target gene prediction and their expression in normal tissues from the Human Protein Atlas, miR-200a was identified to target CTNNB1, CDH1, PTEN, APC, CTNNA1 and SOD2. CTNNB1 is a crucial downstream component of the Wnt signaling pathway, forming a complex to promote phosphorylation on N-terminal serine and threonine residues in the absence of Wnt, while acting as a coactivator for Wnt responsive genes in the presence of the Wnt ligand (48,49). Cadherins are calcium-dependent cell adhesion proteins that interact in a homophilic manner during cell communications and CDH1 is associated with cell-cell adhesions regulation, mobility and proliferation (50). The major function of CTNNB1 is to regulate cell adhesion by participating in the E-cadherin/catenin adhesion complex (51). CTNNA1, which is an isotype of the catenin protein, is associated with a number of cadherins, including E- and N-cadherins, and stabilizes E-cadherin/catenin adhesion complexes (52). APC, which is correlated with its phosphorylation state, participates in the Wnt signaling pathway as a negative regulator and promotes the rapid degradation of CTNNB1 (53,54). PTEN is a dual-specificity protein phosphatase, which mediates the phosphorylation of N-terminal tyrosine, serine and threonine residues (55). The dephosphorylation of tyrosine-phosphorylated focal adhesion kinase inhibits focal adhesion formation, cell migration and integrin-mediated cell spreading (56). SOD2 has been reported to delay tumor cell growth in a number of tumor types (57,58); its activity affects different stages of the cell cycle and its downregulation may stimulate cell cycle progression (59-61). In addition, it has been demonstrated through immunohistochemical staining that the protein expression levels of CTNNB1, CDH1, TP53 and PTEN are decreased in ESCC tissues compared with normal tissues (62-64), while the results of the present study showed the high expression of CTNNB1, CDH1, TP53 and PTEN in normal tissue with IHC staining (Fig. S2). Previous studies reported that miR-200a regulated CTNNB1, CDH1 and PTEN, but not APC, CTNNA1 and SOD2 in tumors. miR-200a was demonstrated to downregulate CTNNB1 and inhibit nasopharyngeal carcinoma cell growth, migration and invasion (65). miR-200a suppressed CDH1 and resulted in the induction of EMT, which has critical functions in tumor cell migration and invasion (66). miR-200a inhibited the expression of PTEN and promoted the invasion and migration of ovarian cancer cells (67). Thus, determining the regulation of miR-200a on APC, CTNNA1 and SOD2 during EC oncogenesis may be useful in the future. Since these target genes have been reported to be associated with the development of a number of types of cancer by influencing the cell cycle and cell adhesion, it is suggested that miR-200a may alter the cell behaviors of ESCC due to the role that the downstream genes have in cell proliferation, migration and invasion pathways.

In conclusion, the present study demonstrated that miR-200a participated in promoting ESCC cell proliferation, migration and invasion, and provided novel evidence for the

direct interaction between miR-200a and CTNNB1, CDH1, PTEN, APC, CTNNA1 and SOD2, which may be responsible for this observed cell behavior.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

BY performed the experiments and wrote the manuscript. YL and LL performed the bioinformatics analysis. HD contributed to the interpretation of the data and helped write and revise the manuscript. LX conceived and designed the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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