Plasma miR-185 is decreased in patients with esophageal squamous cell carcinoma and might suppress tumor migration and invasion by targeting RAGE

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Jing R, Chen W, Wang H, Ju S, Cong H, Sun B, Jin Q, Chu S, Xu L, Cui M. Plasma miR-185 is decreased in patients with esophageal squamous cell carcinoma and might suppress tumor migration and invasion by targeting RAGE. Am J Physiol Gastrointest Liver Physiol 309: G719–G729, 2015. First published August 27, 2015; doi:10.1152/ajpgi.00078.2015.—The receptor for advanced-glycation end products (RAGE) is upregulated in various cancers and has been associated with tumor progression, but little is known about its expression and regulation by microRNAs (miRNAs) in esophageal squamous cell carcinoma (ESCC). Here, we describe miR-185, which represses RAGE expression, and investigate the biological role of miR-185 in ESCC. In this study, we found that the high level of RAGE expression in 29 pairs of paraffin-embedded ESCC tissues was correlated positively with the depth of invasion by immunohistochemistry, suggesting that RAGE was involved in ESCC. We used bioinformatics searches and luciferase reporter assays to investigate the prediction that RAGE was regulated directly by miR-185. Besides, overexpression of miR-185 in ESCC cells was accompanied by 27% (TE-11) and 49% (Eca-109) reduced RAGE expression. The effect was further confirmed in RAGE protein by immunofluorescence in both cell lines. The effects were reversed following cotransfection with miR-185 and high-level expression of the RAGE vector. Furthermore, the biological role of miR-185 in ESCC cell lines was investigated using assays of cell viability, Ki-67 staining, and cell migration and invasion, as well as in a xenograft model. We found that overexpression of miR-185 inhibited migration and invasion by ESCC cells in vitro and reduced their capacity to develop distal pulmonary metastases in vivo partly through the RAGE/heat shock protein 27 pathway. Interestingly, in clinical specimens, the level of plasma miR-185 expression was decreased significantly (P = 0.002) in patients with ESCC [0.500; 95% confidence interval (CI) 0.248–1.676] compared with healthy controls (2.410; 95% CI 0.612–5.671). The value of the area under the receiver-operating characteristic curve was 0.73 (95% CI 0.604–0.855). In conclusion, our findings shed novel light on the role of miR-185/RAGE in ESCC metastasis, and plasma miR-185 has potential as a novel diagnostic biomarker in ESCC.

MicroRNA-185; plasma; receptor for advanced-glycation end products; metastasis; esophageal squamous cell carcinoma

THE RECEPTOR FOR ADVANCED-GLYCATION END PRODUCTS (RAGE), a member of the immunoglobulin superfamily, was isolated originally from bovine lung endothelium based on its ability to bind advanced-glycation end products (AGE) (22). The human gene for RAGE is located in the Class III region of the major histocompatibility complex on chromosome 6 (30), and the protein was first cloned by Neeeper et al. in 1992 (25). In a regulated manner, RAGE is generally expressed at lower levels in a wide range of differentiated adult cells (22). Several clinical studies have shown, however, that higher levels of RAGE are involved in various cancers, including gastric (40), glioma (3), colorectal (5), pancreatic (1), and prostate cancers (8), as well as other types. Thus we expected that RAGE expression would have to be important in the development of esophageal squamous cell carcinoma (ESCC) although fewer data are available for this tumor (13).

RAGE activates multiple signaling pathways and produces various cellular responses in different cancers, including enhancement of cell proliferation (40), cell survival, autophagy (1), invasion and metastasis (5), angiogenesis (3), and limitation of apoptosis (8); thus, RAGE helps in the progression of a cancer and complicates it. Expression of RAGE is regulated, in part, by a number of ligands, including AGE, S100/calgranulin (37), the high-mobility group box protein 1 (4), and, in part, by non-protein-coding RNA molecules, such as microRNAs (miRNAs ~23 nucleotides long). The miRNAs are endogenous noncoding RNA molecules that regulate the gene expression negatively, mainly through direct interaction with the 3’-untranslated regions (3’UTR) of their corresponding mRNA targets (10). It has been estimated that miRNAs regulate more than 70% of the total human genes at the posttranscriptional level, indicating that miRNAs have pivotal roles in physiological and pathological processes (23). There is increasing evidence that the deregulation of miRNAs is involved in a wide range of diseases, including human cancers (10). These data highlight the important roles of miRNAs in tumor development and provide new insights into the molecular mechanisms underlying carcinogenesis. However, the role of RAGE-related miRNAs in ESCC remains unclear.

We show that high levels of RAGE are regulated in ESCC by miR-185, which suppressed tumor invasion and migration of ESCC cells significantly in vitro and reduced their capacity to develop distal pulmonary metastases in vivo partly through the RAGE/heat shock protein (HSP) 27 pathway. Finally, we found that plasma miR-185 was decreased in patients with ESCC, suggesting that miR-185 could serve as a diagnostic and therapeutic reagent for ESCC.

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PLASMA MIR-185 AND RAGE IN ESOPHAGEAL SQUAMOUS CELL CARCINOMA

MATERIALS AND METHODS

Clinical samples. A total of 92 individuals, including the controls, were enrolled in this study. Set 1 contained 29 patients with ESCC (21 men and 8 women; age 37–80 yr, mean 62 yr). All patient tumor tissues and adjacent normal tissues were obtained from resection before receiving any other therapy. Set 2 contained another 28 patients with ESCC (19 men and 9 women; age 50–82 yr, mean 62 yr) and 35 healthy controls (Supplemental Table 1; supplemental material for this article is available online at the American Journal of Physiology Gastrointestinal and Liver Physiology website). Plasma samples were collected via puncture of the cubital vein into tubes containing EDTA as an anticoagulant before any therapeutic procedure, including surgery, chemotherapy, or radiotherapy. All samples were centrifuged for 15 min at 1,000 g, aliquoted, and stored at −70°C. Hemolyzed specimens were not used for the preparation of plasma, and the plasma was prepared by keeping the blood at 4°C for dissolution before centrifuging at 2,500 g for 15 min to separate plasma and platelets. Analysis of all samples was done within 6 mo postcollection. All patients were diagnosed clinically and histopathologically and were classified by the tumor node metastasis (TNM) system (UICC, 2009). This study was approved by the Local Ethics Committee of the Affiliated Hospital of Nantong University, Nantong, China, and all participants provided informed, written consent.

Cell lines and cell culture. Human ESCC cell lines Eca-109 (Academy of Life Science, Shanghai, China) and TE-11 (Jennio Biological Technology, Guangzhou, China) were grown in RPMI 1640 medium (GIBCO/Invitrogen, Carlsbad, CA), supplemented with 10% (vol/vol) FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 5% (vol/vol) CO2 atmosphere at 37°C.

Plasmids and transfection. Cells were transiently transfected with an miR-185 mimic or a negative control (Invitrogen) and high-level expression of RAGE vector or a control (pcDNA3.1) (Genesi, Wuhan, China) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. RNA and proteins were harvested at 48 or 72 h posttransfection. For the generation of stable transfectants, an miR-185 lentivirus-expressing vector and a negative control were purchased from Genechem (Shanghai, China). 

RNA extraction and qRT-PCR. Control and cancer samples were interdigitated. miRNA from 400 μl of plasma was extracted using the mirVana protein and an RNA isolation system (Ambion, Carlsbad, CA). Total RNA was extracted from cell pellets using the TRizol reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA and miRNA were reverse transcribed using the RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific, Pittsburgh, PA).

The primers used to detect RAGE and 18S rRNA were as follows: RAGE: forward 5'-CGG CTG GTG TTT CCA ATA A-3', reverse 5'-TGT TCC TTC ACA GAT AAT C-3'; 18S rRNA: forward 5'-GTT CTT AGT TGG TGG AGC GAT TT-3', reverse 5'-GGC TGA ACG CCA CTT GTC C-3'. Bulge-loop miRNA qRT-PCR primer sets (1 reverse transcription primer and a pair of qRT-PCR primers for each set) specific for miR-185 and U6 were designed by RiboBio (Guangzhou, China). The 2-μl reverse transcription product was amplified using 9 μl of SYBR Green Master (Roche, Basel, Switzerland), 2 μl of gene-specific forward/reverse primers, and 5 μl of nuclease-free water in a final volume of 20 μl. The qRT-PCR was run on an ABI 7500 system (Applied Biosystems, Grand Island, NY), and the reaction mixtures were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min.

The relative fold expression change was calculated using the ΔΔCt method.

Cell viability. For the transiently transfected miR-185 mimic and the control, cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan). Cells (100 μl, 2 × 10^4 cells/ml) were seeded into 96-well plates. Cell growth was measured after the first, third, and fifth seeding. Following the manufacturer’s instructions, 10 μl of MTT solution (5 mg/ml) was added into 100 μl of culture medium and incubated for 4 h at 37°C, and then 100 μl of DMSO was added. The optical density at 490 nm (OD490) was measured. Each experiment was done in quadruplicate.

Cell migration and invasion assay. Cell migration assays were done using Transwell chambers with 8-μm pores (Corning, NY); then 600 μl RPMI 1640 containing 15% (vol/vol) FBS was added to the lower compartment as the chemotactic factor. Tumor cells (100 μl, ~2 × 10^5/ml) in serum-free RPMI 1640 were added to the upper compartment of the chamber. After incubation for 24 h, the nonmigrating cells were removed with a cotton swab. Cells that migrated to the underside of the membrane were stained with Giemsa (Bioworld Technology, St. Louis Park, MN), imaged, and counted under a microscope (Olympus, Tokyo, Japan). Each experiment was done in triplicate.

Cell invasion assays were done in the same way. A 50-μm sample of rehydrated and diluted Matrigel (BD Biosciences, San Jose, CA) was added into the upper compartment of the chamber and incubated at 37°C for 2 h. After 48 h, the number of invasive cells was calculated.

Ki-67 staining. Cell proliferation was assayed by 3,3′-diaminobenzidine (DAB) staining with anti-Ki-67 (1:100) antibody (Immunoway, Newark, DE). The proliferation index was determined by Allred’s score as described previously (14).

Immunofluorescence. Western blot analysis, and immunohistochemistry. Eca-109 and TE-11 cells grown on coverslips were fixed with 4% (vol/vol) formaldehyde for 30 min at 4°C, treated with 0.3% (vol/vol) Triton X-100/10% (vol/vol) normal FBS for 30 min and then incubated overnight at 4°C with the first antibody. After being rinsed with PBS, the cells were incubated with FITC-conjugated secondary antibodies, and then cells were incubated for 10 min with Hoechst 33342 dye (1 mg/ml) after incubation with secondary antibodies. The stained cells were detected under a microscope (Olympus). All assays were done three times in duplicate. Western blot and immunohistochemistry were done and quantified as described previously (14). Antibodies to RAGE polyclonal antibody (10 μg/ml) (R&D Systems, Minneapolis, MN), phospho-focal adhesion kinase (FAK, 1:50), phospho-HSP27 (1:50) (Bioworld Technology, St. Louis Park, MN), and matrix metalloproteinase (MMP)-2 (1:50) (Proteintech, Chicago, IL) were used to detect the individual proteins.

 Luciferase assays. A firefly luciferase expression vector, psiCHECK-2, was used in the luciferase reporter assay. To construct the wild-type (WT) 3′-UTR of RAGE (psiCHECK2-RAGE-3′-UTR-WT) plasmid, the WT 3′-UTR fragment of the RAGE mRNA, which contains two miR-185-binding sites, was amplified and cloned into the Xhol and NotI sites downstream of the luciferase reporter gene in psiCHECK-2. Mutant 3′-UTR of RAGE (psiCHECK2-RAGE-3′-UTR-MUT) carried the mutated sequences in two miR-185-binding sites. 293T cells were cotransfected with 50 nM miR-185 mimic or the negative control and 100 ng of psicHECK2-RAGE-3′-UTR-WT or psicHECK2-RAGE-3′-UTR-MUT using Lipofectamine 2000 (Invitrogen). Cells were collected 48 h after transfection and analyzed with the Dual-Luciferase Reporter Assay System (Promega, San Luis Obispo, CA).

Study in vivo. To evaluate the effect of miR-185 on tumorigenicity, Eca-109 cells (2.0 × 10^6 cells), transfected with miR-185 lentivirus-expressing vector or control, were injected subcutaneously into Balb/c nude mice (10 per group). Tumor diameter (mm) was measured every 2 days for 3 wk. Tumor volume (mm^3) was estimated by measuring the longest and shortest diameters of the tumor. The tumor specimens were fixed in formalin, embedded in paraffin wax, and cut into slices.
For pulmonary metastasis assays, Eca-109 cells (1 × 10⁶/ml) transfected with the negative control (Agomir-control, 5 µM; RiboBio, Guangzhou, China) or miR-185 (Agomir-185, 5 µM; RiboBio), respectively, were suspended in 200 µl of PBS for each mouse (3 per group). The cells were injected into nude mice through the lateral tail vein. After 3 wk, the mice were killed, and lung tissue was dissected and fixed with phosphate-buffered neutral formalin before embedding in paraffin. Each paraffin block was cut into five sections and stained in hematoxylin and eosin (H and E), followed by analysis of the number of micrometastasis tumor cells. All experimental procedures were approved by the Animal Ethics Committee of Nantong University.

Statistical analysis. The SigmaPlot 11.0 software package was used for all statistical analysis. Statistical significance \( P \) was determined by the Kruskal-Wallis one-way ANOVA on ranks. The level of statistically significant difference was set at \( P < 0.05 \).

RESULTS

The expression of RAGE is increased in patients with ESCC. To investigate the relationship of RAGE expression and clinicopathological features in patients with ESCC, we detected the expression of RAGE by immunohistochemistry using 29 pairs of paraffin-embedded ESCC tissues and adjacent normal tissues. The results showed that RAGE expression was mainly at cytoplasmic and membranous locations (Fig. 1, A–D). The Allred score, which contains proportional scores and intensity scores of RAGE, was 2 (95% confidence interval (CI) 2–2), 4 (95% CI 2–6), and 4 (95% CI 2–6) in patients with stage I, II, and III ESCC, respectively \( (P = 0.216) \) (Fig. 1E). However, the Allred score of RAGE was significantly higher \( (P = 0.030) \) when the tumor was infiltrated into the muscularis externa (4; 95% CI: 2–6) compared with the submucosa (2; 95% CI 2–2) \( (P = 0.001) \) compared with the negative control; by contrast, miR-185 caused no significant change to the expression of RAGE significantly \( (P < 0.001) \) compared with the negative control. These data demonstrated a specific translational inhibitory effect of miR-185 on the 3'-UTR of RAGE through direct interaction.

To further examine the potential negative regulatory effect of miR-185 on endogenous RAGE, we used transient transfection with the miR-185 mimic or negative control into TE-11 (which displayed the lowest level of miR-185 among the cell expression in ESCC compared with the adjacent normal tissues was correlated positively with the depth of invasion.

RAGE as a target of miR-185. To associate miRNAs with the regulation of RAGE expression, a bioinformatics search with a common database (microRNA.org) was used for potential miRNAs targeting mRNA of RAGE. The database predicted miR-185 and miR-182 as potential miRNAs to target RAGE. mirSVR score \( (\leq -0.1) \); the lower the score, the greater the stability of miRNA-mRNA) and phastCons score \( (\geq 0) \), the higher the score, the better the conservation) were the choice criteria. By contrast, we selected miR-185 from the target candidates as the most attractive for further analyses. This result was confirmed by another database (TargetScan). To test the prediction, we first constructed luciferase reporters with either psiCHECK2-RAGE-3'-UTR-WT or psiCHECK2-RAGE-3'-UTR-MUT including the predicted miR-185 recognition sites. Two putative binding sites for miR-185 were found in the 3'-UTR of RAGE at 200–206 bp and 216–222 bp \( (P = 0.714) \) (Fig. 2A), and we evaluated their respective luciferase activity after incubation with either negative control or an miR-185 mimic in 293T cells. The results showed that miR-185 inhibited the expression of the transcript containing the WT 3'-UTR of RAGE significantly \( (P < 0.001) \) compared with the negative control; by contrast, miR-185 caused no significant change to the expression of the transcript containing the mutant 3'-UTR of RAGE \( (P = 0.714) \) (Fig. 2B). These data demonstrated a specific translational inhibitory effect of miR-185 on the 3'-UTR of RAGE through direct interaction.
lines tested) and Eca-109 cells (which is a common cell line used in studies of ESCC). Expression of RAGE mRNA in TE-11 and Eca-109 transfection with the miR-185 mimic was only 27% and 49% of that ESCC cells transfection with negative control, respectively (\(P < 0.001\)). The effect was further confirmed in RAGE protein in both cell lines by immunofluorescence (Fig. 2C). Moreover, we cotransfected TE-11 cells with the miR-185 mimic as well as high-level expression of a RAGE vector. We examined the effects on RAGE gene expression by Western blot. Cotransfection of the miR-185 mimic and an empty vector control resulted in down-regulation of RAGE. The effects were reversed following cotransfection of the miR-185 mimic and high-level expression of the RAGE vector (Fig. 2D). The above data indicated that expression of endogenous RAGE is negatively regulated, at least in part, by miR-185.

Overexpression of miR-185 suppresses ESCC migration and invasion in vitro. We next sought to verify the effects of miR-185 in the development of ESCC. We examined the impact of excessive miR-185 on the proliferation of human ESCC cells. The successful transfection of an miR-185 mimic was confirmed by miRNA qRT-PCR analysis (relative miR-185 level in TE-11: mimic-185 group mean \(\pm\) SD = 7.06 \(\pm\) 0.07 vs. negative control group mean \(\pm\) SD = 1.31 \(\pm\) 0.07; relative miR-185 level in Eca-109: mimic-185 group mean \(\pm\) SD = 7.96 \(\pm\) 0.92 vs. negative control group mean \(\pm\) SD =
1.30 ± 0.17; P < 0.001). Compared with the control, cell viability, as determined by the CCK-8 assay, was lower for the miR-185 mimic in transfected TE-11 and Eca-109 cells, but the difference did not reach statistical significance (Fig. 3A). Furthermore, stably transfected miR-185 lentivirus-expressing vector into Eca-109 cells was used. The successful overexpression of miR-185 was confirmed by miRNA qRT-PCR analysis (relative miR-185 level in Eca-109: miR-185 lentivirus vector group mean ± SD = 90.61 ± 11.50 vs. negative control group mean ± SD = 1.00 ± 0.12; P < 0.001). The overexpression of miR-185 had no obvious impact on ESCC cell proliferation as detected by MTT analysis (Fig. 3B). The lack of effect of miR-185 on the proliferation of Eca-109 and TE-11 cells was demonstrated also by DAB staining for Ki-67, which is expressed specifically in proliferating cells. Compared with control-transfected cells, the Allred scores of Ki-67-positive cells ranged from 7.7 to 6.0 in TE-11 cells (P = 0.288) and from 7.7 to 6.7 in Eca-109 cells (P = 0.101) after transfection with the miR-185 mimic (Fig. 3C). Given that the RAGE is increased in ESCC and was correlated positively with the depth of invasion, we asked whether miR-185 could have an important role in ESCC cell invasion and migration. As expected, we found that transfection with the miR-185 mimic suppressed the migratory (average migrated cells: mimic-185 group mean ± SD = 52.00 ± 16.62 vs. negative control group mean ± SD = 490.00 ± 26.90; P < 0.001) and invasive (average invaded cells: mimic-185 group mean ± SD = 26.00 ± 3.21 vs. negative control group mean ± SD = 66.00 ± 7.21; P < 0.001) capacity of TE-11 cells significantly (Fig. 3D). The suppression of migration (average migrated cells: mimic-185 group mean ± SD = 164.00 ± 13.53 vs. negative control group mean ± SD = 493.00 ± 40.51; P < 0.001) and invasion

Fig. 3. The role of miR-185 in ESCC cell proliferation and metastasis in vitro. A: Eca-109 and TE-11 cells were transfected with either the miR-185 mimic or a control and then analyzed for cell viability by the Cell-Counting Kit 8 assay. B: Eca-109 cells were transfected with either the miR-185 lentivirus-expressing vector or a control and then analyzed for cell viability by the Cell-Counting Kit 8 assay. C: Eca-109 and TE-11 cells were transfected with either the miR-185 mimic or a control and then by 3,3'-diaminobenzidine staining for Ki-67. D: migratory and invasive assay in TE-11 cells. E: migratory and invasive assay in Eca-109 cells. **P < 0.001.
average invaded cells: mimic-185 group mean ± SD = 16.00 ± 2.65 vs. negative control group mean ± SD = 54.00 ± 4.58; P < 0.001) by miR-185 overexpression was confirmed in Eca-109 cells (P <0.001) (Fig. 3E). These observations indicated that miR-185 inhibits metastasis in ESCC by regulating the migratory and invasive ability of ESCC cells in vitro.

The overexpression of miR-185 suppressed ESCC metastasis in vivo. Given that miR-185 suppressed the migration and invasion of ESCC cells markedly in vitro, we further investigated the biological role of miR-185 in vivo. miR-185 lentivirus-expressing vector stably transfected cell line Eca-109 cells were inoculated into the skin of nude mice. At 3 wk postinoculation, we found that the volume and weight of the tumor formed by miR-185 overexpressing Eca-109 cells (volume mean ± SD = 1,476.51 ± 225.79 mm³; weight mean ± SD = 1.86 ± 0.47 g) developed no significant difference from those formed by miR control-transfected cells (volume mean ± SD = 1,250.37 ± 355.91 mm³; weight mean ± SD = 1.49 ± 0.48 g) (P = 0.800, Fig. 4, A and B; P = 0.097, Fig. 4C).

Furthermore, we tested tumor tissue samples from nude mice by immunohistochemistry and found that metastasis-related protein MMP-2 (Allred score of miR-185 group mean ± SD = 4.500 ± 0.577 vs. negative control group was mean ± SD = 7.750 ± 0.500; P < 0.001), but not proliferation-related protein Ki-67 (Allred score of miR-185 group mean ± SD = 5.750 ± 0.750 vs. negative control group was mean ± SD = 6.250 ± 0.500; P = 0.207), was downregulated in tissue samples from miR-185-transfected nude mice (Fig. 4D). To further determine whether miR-185 inhibition reduces the distal metastases of ESCC cells in vivo, we performed the pulmonary metastasis assays via BALB/c nude mice models with another miR-185 enhancer. The Eca-109 cells were collected after transfection with negative control (5 μM) or miR-185 (5 μM) for 24 h and then were injected into nude mice through the lateral tail vein, respectively. The mice were killed after 3 wk. The lungs were removed and subjected to histological and pathological examination. As expected, miR-185 strongly reduced the number of metastatic ESCC cells in vivo.
Fig. 4. The role of miR-185 in ESCC cell proliferation and metastasis in vivo. The Eca-109 cell line was transfected with either the miR-185 lentivirus expressing the vector or a control and then analyzed. A: representative images of tumor in subcutaneous sites were present at 3 wk after the inoculation of nude mice. Tumor volume (B) and tumor weight (C) following subcutaneous injection of Eca-109 cells stable for overexpression of miR-185 are shown. D: matrix metalloproteinase 2 (MMP)-2 and Ki-67. E: nude mice were injected with $2 \times 10^5$ Eca-109 cells containing negative control or miR-185 enhancer, and the number of micrometastasis tumor cells in lung tissues was calculated. The columns represent the mean, and the error bars represent the standard deviation. **$P < 0.001$. 

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the lung tissues of nude mice by the H and E staining (miR-185-treated group: number of micrometastasis per section mean / SD = 4.667 / 0.577 vs. control group: number of micrometastasis per section mean / SD = 9 ± 1; P = 0.003), suggesting that miR-185 repressed the distal metastases of ESCC cells in vivo (Fig. 4E). To examine whether the RAGE/phospho-FAK or RAGE/HSP27 pathway was involved in the molecular mechanism of the miR-185-associated ESCC metastasis, we tested nude mice tumor samples by immunohistochemistry and found that RAGE (Allred score of miR-185 group mean / SD = 3.250 / 0.500 vs. negative control group was mean / SD = 7.500 / 0.577; P < 0.001) was downregulated and that phosphorylation of HSP27 (Allred score of miR-185 group mean / SD = 7.000 / 0.816 vs. negative control group was mean / SD = 2.500 / 2.082; P = 0.007) was upregulated in miR-185-transfected nude mouse tissue samples. However, the expression of phosphorylation of FAK did not differ (Allred score of miR-185 group mean / SD = 0.750 ± 0.957 vs. negative control group was mean / SD = 0.750 ± 0.957; P = 1.000) (Fig. 5). Taken together, these observations suggested that miR-185 suppressed ESCC metastasis by downregulating RAGE in vivo, partly through the RAGE/HSP27 pathway.

The level of plasma miR-185 was decreased in patients with ESCC. To evaluate the diagnostic potential of miR-185, plasma samples from 28 patients with ESCC and 35 healthy controls were examined by miRNA qRT-PCR analysis. The mean Ct values of U6 in the plasma of patients with ESCC and healthy controls were 26.14 (SD 2.40) and 27.34 (SD 2.64), respectively (P = 0.067). Therefore, we used U6 for normalization of miR-185 analyses. The mean Ct values of miR-185 in the plasma of patients with ESCC and healthy controls were 23.64 (SD 2.48) and 24.03 (SD 1.25), respectively. The relative amount of miR-185 was calculated. Compared with healthy controls (2.410; 95% CI 0.612–5.671), the relative level of plasma miR-185 expression was decreased significantly (P = 0.002) in patients with ESCC (0.500; 95% CI 0.248–1.676) (Fig. 6A). The value of the area under the receiver-operating characteristic curve was 0.73 (95% CI 0.604–0.855) (Fig. 6B). We examined the association of plasma miR-185 concentrations with clinicopathological factors, including age, sex, tumor differentiation, lymph node metastasis, distant metastasis, and TNM stage in the 28 patients with ESCC; no significant correlation was found (Table 1). These results suggest that plasma miR-185 is decreased and that it has potential as a novel diagnostic biomarker in ESCC.
DISCUSSION

In earlier studies, dysregulation of RAGE is a common event in several human cancers (22). In this study, we found that RAGE is upregulated frequently and is associated with the depth of invasion in ESCC. miRNAs are considered to regulate gene expression by binding to target mRNAs, which are crucial regulators of basic cellular functions, including proliferation, G1/S transition, invasion, and migration (12, 20, 26). The molecular mechanism by which miRNAs target RAGE that inhibits ESCC remains unclear. For the first time, we found that miR-185 was upstream of RAGE, and the overexpression of miR-185 can suppress ESCC cell migration and invasion in assays in vitro and in vivo partly through the RAGE/HSP27 pathway. Furthermore, miR-185 was downregulated in the plasma of patients with ESCC, suggesting its potential utility as a biomarker. These results suggest that miR-185 could be a novel tumor suppressor with important roles in metastasis in patients with ESCC.

Nasser et al. (24) found that RAGE expression was upregulated in primary breast cancer and in lymph node metastases. They found that RAGE-deficient mice had a reduced propensity for breast tumor growth and metastasis. The possible mechanism was RAGE bound to its ligand S100A7 and mediated its ability to activate ERK, NF-κB, and cell migration linked to breast cancer development (24). In human oral cancer, Ko et al. (15) found that AGEs could increase ERK phosphorylation, enhance cell migration, and promote the expression of RAGE, MMP2, and MMP9. AGE-RAGE interaction can help to explain the poor prognosis of patients with diabetes mellitus with oral cancer (15). Similarly, in this study, we found a positive correlation between highly expressed RAGE and the depth of invasion in ESCC by immunohistochemistry. The molecular mechanism of RAGE high expression in ESCC, however, has not been elucidated.

miRNAs can suppress posttranscriptional protein translation by binding to the 3'UTRs of targeted gene transcripts. The following evidence supports the hypothesis that overexpression of RAGE in ESCC could result from underexpression of a specific miRNA molecule, miR-185. 1) Overexpression of miR-185 decreased the luciferase reporter activity of the WT specific miRNA molecule, miR-185.

Table 1. The level of plasma miR-185 and clinicopathological features in patients with esophageal cancer

<table>
<thead>
<tr>
<th>Variables</th>
<th>n</th>
<th>Plasma miR-185 expression mean (95% CI)</th>
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<td>Age, yr</td>
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<td>≤62</td>
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<td>&gt;62</td>
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<td>0.516 (0.267–2.422)</td>
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<td>The degree of differentiation</td>
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<td>G2</td>
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<td>G3</td>
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<tr>
<td>Positive</td>
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<td>Negative</td>
<td>26</td>
<td>0.440 (0.239–1.408)</td>
<td>0.246</td>
</tr>
<tr>
<td>Positive</td>
<td>2</td>
<td>1.744 (0.638–2.850)</td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>20</td>
<td>0.500 (0.283–1.676)</td>
<td>0.509</td>
</tr>
<tr>
<td>III-IV</td>
<td>8</td>
<td>0.438 (0.087–2.297)</td>
<td></td>
</tr>
</tbody>
</table>

The median age was 62 yr. CI, confidence interval.

miR-185 is reported to be involved in metastasis in other human cancers, including breast cancer and prostate cancer (29, 36). miR-185 can induce cell cycle arrest and repress cell prolifer-
ation in colorectal, prostate, glioma, and breast cancers (19, 21, 31, 32). However, by CCK-8, MTT, and Ki-67 assays, transfection of ESCC cells with two types of overexpression of miR-185 (miR-185 mimic and miR-185 lentivirus-expressing vector) resulted in no marked change of proliferation ability compared with cells transfected with the negative control. In addition, studies in vivo demonstrated no statistically significant reduction in subcutaneous tumor volume or weight following miR-185 overexpression. We favor the hypothesis that miR-185 can target many genes and that different mRNAs have different roles in different kinds of tumors.

The prometastasis signal is mediated preferentially through the regulators FAK and HSP27 (17). FAK might promote cell spreading, motility, invasion, and survival in malignancy. In breast cancer, elevated expression and activity of FAK are often correlated positively with tumor cell metastasis and poor prognosis (38). In vitro, nonphosphorylated HSP27 inhibited Listeria actin-based motility in cell extracts and bound to and sequestered purified actin monomers (7). Inhibition of the phosphorylation of HSP27 decreased sequestration of cellular G-actin and mobilized cellular G-actin for polymerization, ensuring proper actin dynamics to promote the velocity of cell migration (44). On the basis of this study, we suggest a novel mechanism in which the reexpression of miR-185 might down-regulate RAGE expression directly, promoting the phosphorylation of HSP27 instead of FAK and resulting in the suppression of ESCC cell migration and invasion.

Most studies aimed at the identification of diagnostic or prognostic miRNAs are focused on miRNAs frequently expressed differentially in tumor tissue (35, 39, 43). Not all miRNAs expressed in cancer cells have been detected in the circulation (2); only ~70% of the released miRNAs mirror the cellular expression profiles originating in specific cancer cells (27, 33). This study is the first to demonstrate a significant difference in the level of plasma miR-185 between patients with ESCC and healthy controls. No significant correlation was observed in the association of plasma miR-185 concentrations with clinicopathological factors, including age, sex, tumor differentiation, lymph node metastasis, distant metastasis, and TNM stage, which is similar to existing reports (28, 34). A possible explanation is that the resource of miRNAs in blood is complex and that changes in plasma levels are not necessarily correlated with altered miR-185 activity in the tumor. These findings highlight the usefulness of miR-185 as a potential biomarker in ESCC. Nonetheless, the biological stage of ESCC at which miR-185 becomes biologically relevant is uncertain.

In summary, patients with ESCC exhibited significantly decreased concentrations of plasma miR-185 compared with healthy controls. Overexpression of miR-185 resulted in down-regulation of RAGE, suppression of migration, and the invasive ability of ESCC cells, partly by an HSP27-involved pathway, which was confirmed by studies in vivo. The present study has several limitations with respect to clinical detection because results were obtained only for plasma samples. It would be of interest to examine the reciprocal expression of miR-185 in ESCC plasma and tissues. Further studies on serial detection in larger, independent patient populations are needed to make blood miR-185 quantification more appropriate for clinical use. Finally, although we identified RAGE as a target of miR-185 in ESCC, it is possible that miR-185 targets other genes in distinct subsets of ESCC.

**REFERENCES**


**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

Author contributions: R.J., W.C., H.W., S.J., H.C., B.S., Q.J., and M.C. conception and design of research; R.J., W.C., H.W., B.S., Q.J., and M.C. performed experiments; R.J., W.C., H.W., B.S., Q.J., and M.C. analyzed data; R.J., W.C., H.W., B.S., Q.J., and M.C. drafted manuscript; R.J., W.C., H.W., B.S., Q.J., and M.C. approved final version of manuscript.
MicroRNA-185 suppresses proliferation, invasion, migration, and tumorigenicity of human prostate cancer cells through targeting androgen receptor.


MicroRNA-223 functions as an oncogene in human colorectal cancer cells.


MicroRNA-185 suppresses tumor proliferation by directly targeting E2F6 and DNMT1 and indirectly upregulating BRCA1 in triple-negative breast cancer.


MicroRNA-18a in plasma contributes to cancer detection and monitoring in patients with gastric cancer.


MicroRNA expression in breast cancer cells: over-expression of miR-196a.


MicroRNA-375 inhibits tumor growth and metastasis in breast cancer.