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MicroRNA-146a acts as a metastasis suppressor in gastric cancer by targeting WASF2

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ABSTRACT

Previous studies have shown that miR-146a acts as either an oncogene or a tumor suppressor in various cancers. In this study, we investigated the role of miR-146a in gastric cancer cells and its potential target genes. The results showed that miR-146a expression correlated inversely with WASF2 protein expression in gastric cancer cell lines. Overexpression of miR-146a suppressed the migration and invasion of gastric cancer cells, and also the protein level of WASF2. WASF2 was shown to be a direct target gene of miR-146a by luciferase assays. Restoration of WASF2 promoted the migration and invasion of gastric cancer cells, similar to that mediated by miR-146a inhibition. This study has identified an onco-suppressive role of miR-146a in gastric cancer cells by its reduction of WASF2 expression. The newly identified miR-146a/WASF2 axis partially reveals the molecular mechanism underlying the migration and invasion of gastric cancer cells and represents a new potential therapeutic target for gastric cancer.

1. Introduction

Metastases account for 90% of human cancer deaths [1], yet the exact molecular mechanisms of metastases remain unclear. Gastric cancer is the second leading cause of cancer-related death worldwide [2]. Most patients with gastric cancer are diagnosed in the advanced stages of disease and present with extensive invasion and lymphatic metastasis, and only a small percentage of patients receive effective intervention [3,4]. Therefore, it is important to fully explore the molecular mechanisms of gastric cancer progression, which might contribute to the development of novel targeted therapies.

miRNAs are small non-coding RNAs that serve as negative regulators of gene expression [5–8]. Through base-pairing with the 3′-untranslated region (3′-UTR) of their target mRNAs, miRNAs cause gene silencing either by mRNA degradation or translational suppression [9]. miRNAs are involved in a wide range of important biological processes [8]. Accumulating evidence strongly suggests that miRNAs can function as novel oncogenes or tumor suppressors, and the deregulation of specific miRNAs in diverse types of cancer is associated with tumor growth, angiogenesis, apoptosis and metastasis [9,10]. miR-146a plays a major role in the pathogenesis of many human diseases, including cancer, autoimmune disorders, virus infection, and muscle disorders [11]. Recent studies have shown the abnormal expression and paradoxical roles of miR-146a in various human cancer tissues. It functions as an oncogene in cervical cancer and anaplastic thyroid carcinoma, but as a tumor suppressor in pancreatic cancer and breast cancer [12–18]. Tchernitsa and colleagues demonstrated an important role of miR-146a in the lymph node metastasis of gastric cancer by miRNA microarray analysis [19]. Although evidence has shown that miR-146a inhibits the migration and invasion of gastric cancer cells [20], the molecular mechanisms and the related target genes are largely unknown.

WASF2 (WASP family verprolin homologous protein 2) and ROCK1 (Rho-activated protein kinase) are predicted target genes of miR-146a in Targetscan and miRBase, respectively. WASF2 is a downstream effector molecule involved in the transmission of signals from small GTPases to the actin cytoskeleton [21]. It mediates the elongated cell movement mode. ROCK1 is a protein kinase that is a key regulator of the actin cytoskeleton and cell polarity, and it is involved in the rounded cell movement mode [22,23]. These two modes of motility have been observed in invading tumor cells [24].

In this study, we explored the effects of miR-146a on gastric cancer cells, and whether WASF2 and ROCK1 are its functional target genes, to partially clarify the molecular mechanisms of migration and invasion in gastric cancer cells.

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2. Materials and methods

2.1. Cell culture

Human gastric cancer cell lines (MKN-45, SGC-7901, HGC-27 and MGC-803), and HEK293T cells were purchased from the Cell Resource Center, Shanghai Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences. Cells were maintained at 37 °C in a humidified air atmosphere containing 5% carbon dioxide in RPMI1640 (MKN-45, SGC-7901, HGC-27 and MGC-803) or Dulbecco's Modified Eagle's Media (HEK293T) supplemented with 10% FBS.

2.2. miRNA extraction and TaqMan real-time PCR

MicroRNAs were isolated from the gastric cancer cell lines using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The expression of mature microRNAs was assayed using TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA, USA) specific for hsa-miR-146a and RNU6B. Real-time PCR was performed using the ABI 7900 real-time PCR machine. RNU6B was used as an endogenous control. All TaqMan PCRs were performed in triplicate.

2.3. Western blotting

Equal amounts of protein were resolved by 10% SDS–polyacrylamide gel electrophoresis and transblotted onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA). After blocking in 5% nonfat milk, the membranes were incubated with rabbit anti-WASF2 monoclonal antibody (mAb; 1:1000; Cell Signaling Technology) or rabbit anti-GAPDH mAb (1:10,000; Epitomics). The membranes were then incubated with horseradish peroxidase-coupled immunoglobulin G and visualized by chemiluminescence.

2.4. Construction of plasmids

The pre-miR-146a and miR-146a inhibitors were purchased from Guangzhou Fulen Gen Co. The vectors were pEZX-MR03 and pEZX-MR04, respectively. The 3′UTR segment of WASF2 was amplified from normal human cDNA and inserted into the psiCHECK2 vector (Promega, Madison, WI, USA) at the XhoI site immediately downstream the luciferase stop codon. We also generated a mutant construct in the complementary site with the Quik Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The coding sequence of human WASF2 was cloned into the expression vector, pCDH-CMV-MCS-EF1-Puro (System Biosciences, Mountain View, CA, USA). The shRNAs against WASF2 were synthesized by Ribobio and inserted into the pLKO.1-TRC cloning vector (Invitrogen, Carlsbad, CA, USA). All constructs were verified by sequencing.

2.5. Lentivirus production and transduction

A mixture of pEZX-MR03-miR-146a or pEZX-MR03, pEZX-MR04-anti-miR-146a or pEZX-MR04, pCDH-WASF2 or pCDH-CMV-MCS-EF1-Puro, pLKO.1-shWASF2 or pLKO.1-TRC cloning vector, and the adjuvant vectors psPAX2 and pMDG2, were transfected into HEK293T cells using Lipofectamine 2000 reagent to generate lentiviruses. MKN-45 and SGC-7901 cells were infected with the recombinant lentivirus-transducing units plus 8 mg/ml polybrene (Sigma).

2.6. Cell migration and invasion assays

For transwell migration assays, 3×10^4 cells were plated into the top chamber containing a non-coated membrane (24-well insert; pore size, 8 mm; BD Biosciences, Bedford, MA, USA). For invasion assays, each well was layered with 50 μl of a 1:4 mixture of Matrigel/Dulbecco's minimal essential medium. 1×10^5 cells were plated in the top chamber containing a Matrigel-coated membrane. In both assays, cells were plated in medium without serum. Medium supplemented with serum was used as a chemoattractant in the lower chamber. The cells were incubated for 48 h and cells that did not migrate or invade the pores were removed with a cotton swab. Cells on the lower surface of the membrane were fixed with methanol and stained with Giemsa and counted. Each experiment was performed in triplicate.

2.7. Real-time PCR for mRNA

Total RNA from cultured cells was extracted using TRIzol reagent (Invitrogen). Quantitative real-time PCR (qRT-PCR) assays were carried out to detect mRNA expression using the PrimeScript RT Reagent Kit (TaKaRa) and SYBR Premix Ex Taq (TaKaRa) according to the manufacturers' instructions. The levels of the WASF2 transcript were measured using the forward primer, CAAGACACGTAAGGAAGAGTGG, and the reverse primer, CACTGGGTAACTGAATTCTGCTG. GAPDH was used as an internal control and amplified with the forward primer, GTTTCTATAAATTGAGCCCGCAG, and the reverse primer, CGACCAAATCCGTTGACTCC. The cycling parameters were as follows: initial melting at 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s.

2.8. Luciferase assays

HEK293T cells were seeded in 96-well plates at 5000 cells per well the day before transfection. Cells were co-transfected with 120 ng pEZX-MR03 or pEZX-MR03-miR-146a and 30 ng firefly luciferase reporter vector containing the WASF2 3′-UTR (psiCHECK2-UTR-WT) or its mutant 3′-UTR (psiCHECK2-UTR-MUT). Forty-eight hours after transfection, firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay system (Promega). The firefly luciferase activities were used as an internal control for transfection efficiency.

2.9. Statistical analysis

Each experiment was repeated at least three times. Student's *t*-test was used to investigate the significance of the difference between the covariates. All statistical analyses were conducted using SPSS 15.0 software. $P < 0.05$ was considered statistically significant and error bars represent SD.

3. Results

3.1. miR-146a expression correlates inversely with the level of WASF2 protein in gastric cancer cell lines

The expression levels of miR-146a, WASF2 and ROCK1 in a series of gastric cancer cell lines were examined (Fig. 1). As shown in Fig. 1, WASF2 was highly expressed in cell lines with a low level of endogenous miR-146a (MKN-45 and SGC-7901), while its expression level was much lower in cell lines with a high level of endogenous miR-146a (HGC-27 and MGC-803). Statistical analysis showed a significant inverse correlation between miR-146a and WASF2 protein levels ($r = -0.9429$, Spearman, $P = 0.0167$). No significant correlation between miR-146a and ROCK1 was found ($r = -0.8000$, Spearman, $P = 0.3333$; Supplementary Fig. S1).

3.2. miR-146a suppresses the migration and invasion of gastric cancer cells

The expression of miR-146a in cells with high invasive capacity (SGC-7901 and MKN-45) was lower than in those with low invasive capacity (MGC-803 and HGC-27). Because an inverse correlation was found between the expression of miR-146a and WASF2, and because another study has shown that WASF2 promotes invasion and metastasis in many cancers by accelerating actin polymerization through activation of the Arp2/3 complex [25], we postulated that overexpression of miR-146a in gastric cancer cells may inhibit their invasive and metastatic capacities. We established MKN-45 and SGC-7901 cell lines stably expressing miR-146a by lentivirus infection, which had a relative lower level of miR-146a. Successful overexpression of mature miR-146a was confirmed by qRT-PCR (Supplementary Fig. S2). Morphological changes were found in the miR-146a-infected cells, which became round and flat and were less invasive compared with cells transfected with the vector control. We also found that miR-146a overexpression significantly suppressed the migration and invasion of gastric cancer cells in Matrigel chamber assays (Fig. 2A and B). In contrast, the migration and invasion were enhanced when miR-146a was knocked down by transfection of a specific inhibitor into MGC-803 and HGC-27 cells (Fig. 2C and D), which expressed higher level of miR-146a. The knockdown of mature miR-146a was confirmed by qRT-PCR (Supplementary Fig. S2).

3.3. miR-146a post-transcriptionally down-regulates WASF2 expression by directly targeting its 3′-UTR

TargetScan and miRBase bioinformatics analyses of the 3′-UTRs of WASF2 and ROCK1 revealed one or two putative binding sites for miR-146a, respectively. To investigate the potential interaction, human WASF2 and the ROCK1 3′-UTR and their corresponding mutant counterparts were subcloned after the Renilla