MicroRNA-940 promotes cell proliferation and invasion of glioma by directly targeting Kruppel-like factor 9

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Abstract. MicroRNA-940 (miR-940) has been extensively studied in the pathogenesis of numerous types of human cancer; however, the expression pattern, roles and molecular mechanisms underlying the regulatory actions of miR-940 in glioma remain unknown. The present study aimed to further investigate miR-940 by studying its expression, roles and mechanisms of action in glioma. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to detect miR-940 expression in glioma tissues and cell lines. The regulatory effects of miR-940 in glioma cell proliferation and invasion were determined using MTT and cell invasion assays. Bioinformatics analyses was performed to identify the potential target of miR-940, which was further confirmed by luciferase reporter assay, RT-qPCR and western blot analysis. In the present study, significantly increased miR-940 expression levels were observed in glioma tissues and cell lines compared with normal brain tissues and normal human astrocytes, respectively. Decreased miR-940 expression levels attenuated glioma cell proliferation and invasion in vitro. Kruppel-like factor 9 (KLF9) was predicted as a potential target of miR-940. Further assays demonstrated that miR-940 negatively regulated KLF9 expression in glioma cells by directly targeting the 3′-untranslated regions of KLF9. Additionally, KLF9 expression was downregulated in glioma tissues and was inversely correlated with miR-940. Furthermore, KLF9 knockdown was able to rescue the effects of miR-940 on glioma cell proliferation and invasion. The results of the present study suggest that miR-940 may function as an oncogene in glioma by targeting KLF9 and may be a considered a therapeutic target for the treatment of gliomas.

Introduction

Glioma is the most common type of malignant brain tumour in adults and accounts for >70% brain tumours (1). Regarding the degree of malignancy, glioma may be divided into four grades (grades I-IV) (2). Glioma is characterized by rapid growth, cellular heterogeneity, angiogenesis, extensive invasion, hypoxia and necrosis (3,4). At present, the predominant treatments for gliomas include surgical resection followed by chemotherapy and radiotherapy. Significant developments in the diagnosis and therapy for gliomas have been made; however, the therapeutic outcomes of patients with glioma remains unsatisfactory (5). The median duration of survival of patients with glioma is ~15 months post-diagnosis (6). In addition, the therapeutic methods available for the treatment of gliomas are restricted by poor understanding of the formation and progression of this cancer (7). Therefore, thorough understanding of the molecular mechanisms underlying the pathogenesis of glioma is essential to identify novel, effective and targeted therapies for patients with this fatal disease.

MicroRNAs (miRNAs) comprise a series of endogenous, short single-stranded non-coding RNAs (19-25 molecules) that primarily serve as gene regulators (8). These molecules negatively regulate gene expression by directly binding to partial complimentary sites in the 3′-untranslated regions (3′-UTRs) of their target genes, resulting in mRNA degradation or inhibition of translation (9). In total, >1,000 miRNAs have been identified in the human genome, and these miRNAs have been estimated to regulate ~30% human protein-coding genes (10). Numerous miRNAs have recently been demonstrated to be aberrantly expressed in glioma, including miR-613 (11), miR-219 (12), miR-936 (13) and miR-141-3p (14). Dysregulation of miRNA contributes to tumorigenesis and tumour development by regulating numerous diverse biological processes, including cell proliferation, the cell cycle, apoptosis, differentiation, metastasis, chemoresistance and radioresistance (15-17). Therefore, miRNAs may be developed as diagnostic and therapeutic targets for the management of malignancies.

miR-940 has been well studied in the pathogenesis of a number of types of human cancer (18-22); however, the expression pattern, roles and molecular mechanisms of the regulatory actions of miR-940 in glioma remain unknown. The present study aimed to further understand the roles of miR-940 by...
studying its expression and mechanisms of action in glioma. In the present study, significant upregulation of miR-940 in glioma tissues and cell lines was observed. Additionally, inhibition of miR-940 decreased cell proliferation and invasion in glioma; Kruppel-like factor 9 (KLF9) was additionally proposed to be a novel and direct target of miR-940 in glioma in the present study. These results not only provide novel insight into the underlying mechanisms associated with the occurrence and development of glioma; however, may additionally contribute to the identification of novel therapeutic targets for glioma.

Materials and methods

Human tissue specimens. A total of 23 pairs of glioma and adjacent normal brain tissues were obtained from patients (14 males, 9 females; age range, 47-72 years) diagnosed with glioma and treated via surgical resection at The First Affiliated Hospital of Xi’an Medical University (Xi’an, China) between October 2015 to March 2017. None of the patients underwent radiotherapy or chemotherapy prior to surgical resection. The present study was approved by the Ethics Committee of The First Affiliated Hospital of Xi’an Medical University. Written informed consent was provided by all participants.

Cell lines, culture conditions and transfection. Normal human astrocytes (NHAs) were purchased from ScienCell Research Laboratories, Inc. (San Diego, CA, USA) and cultured in astrocyte medium (ScienCell Research Laboratories, Inc.) according to the manufacturer’s protocols. A total of four glioma cell lines (U251, U87, T98G and LN229) were acquired from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin and 1% streptomycin (all obtained from HyClone; GE Healthcare Life Sciences, Logan, UT, USA) as described below. The U87 cell line originated from the American Type Culture Collection (Manassas, VA, USA), and is likely to be glioblastoma, but of unknown origin. This condition did not affect the interpretation of the results of the present study as glioblastoma multiforme is the subtype of glioma. U251 and U87 cell lines exhibited relatively higher miR-940 expression among the four glioma cell lines; thus, these two cell lines were chosen for further functional assays. The cell lines were maintained at 37°C in an incubator with 5% CO₂.

miR-940 inhibitor and the corresponding negative control miRNA inhibitor (NC inhibitor) were synthesised by Shanghai GenePharma Co., Ltd. (Shanghai, China). Small interfering RNA (siRNA) against the KLF9 (KLF9 siRNA) and negative control siRNA (NC siRNA) were chemically produced by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The sequences were as follows: miR-940 inhibitor, 5’-GGGAG CGGGGGUGGUCCU-3'; NC inhibitor, 5’-CAGUACUUU UUGUAGUACAA-3'; KLF9 siRNA, 5’-CAGUUCGCG UGUGCGCGUG-3' and NC siRNA, 5’-UUCGCUAACG GUACGUUU-3'. U251 and U87 cells were seeded into 6-well plates with a density of 8x10⁵ cells per well, and transfected with miR-940 inhibitor (100 pmol) or NC inhibitor (100 pmol), or KLF9 siRNA (100 pmol) or NC siRNA (100 pmol) using Lipofectamine 2000™ (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer’s protocol. Successful transfection was determined through detecting the expression of miR-940 and KLF9 siRNA, respectively. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and the cell invasion assay were conducted following 48 h post-transfection. The MTT assay and western blot analysis were performed at 24 and 72 h post-transfection, respectively.

RNA isolation and RT-qPCR. A TRIzol® Reagent Kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from tissue samples or cell lines. To analyse miR-940 expression, RT was conducted using a TaqMan MicroRNA RT Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.); qPCR was performed using a TaqMan MicroRNA assay (Applied Biosystems; Thermo Fisher Scientific, Inc.). The temperature protocol for RT-PCR was as follows: 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. The thermocycling conditions for qPCR were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 60 sec. To examine the mRNA expression levels of KLF9, cDNA was synthesized from total RNA using a SYBR Premix Ex Taq™ II Kit (Takara Biotechnology Co., Ltd., Dalian, China) followed by qPCR with a SYBR Premix Ex Taq™ (Takara Biotechnology Co., Ltd.). The temperature protocol for RT-PCR was as follows: 37°C for 15 min and 85°C for 5 sec. The thermocycling conditions for qPCR were as follows: 5 min at 95°C, followed by 40 cycles of 95°C for 30 sec and 65°C for 45 sec. U6 and GAPDH were used as endogenous controls to normalize the expression levels of miR-940 and KLF9 mRNA, respectively. The primers were designed as follows: miR-940, forward 5’-CACACATCCGTCGCGGTAGG-3', reverse, 5’-CTA CAGAATGCCCGCGTCTGC-3'; U6, forward 5’-GCTTCC GCAGCACATATACTAAAT-3', reverse, 5’-GCCTTCACG AAAATTGCCTGTAC-3'; KLF9, forward 5’-AACATGCTT GCTGGAAAGTC-3', reverse, 5’-TCACAAAGCGTGGC CAGCG-3' and GAPDH, forward 5’-GGAGCGAGATCCCC CAAAAT-3' and reverse, 5’-GGCTTGGTGTCACTATCTTC CATGG-3'. Relative gene expression was calculated using the 2⁻ΔΔCt method (23).

MTT assay. An MTT assay was conducted to evaluate the effects of miR-940 on glioma cell proliferation. Transfected cells were seeded into 96-well plates at a density of 3,000 cells/well and incubated at 37°C for various durations (0, 24, 48 and 72 h). At each time point, 20 µl MTT solution (Beyotime Institute of Biotechnology, Haimen, China) was added to each well, which was further incubated at 37°C for 4 h. The supernatant was subsequently discarded, and 200 µl dimethyl sulfoxide was added into each well to dissolve the formazan crystals. Cell proliferation was determined by detecting the optical density at a wavelength of 490 nm with an enzyme-linked immunosorbent assay reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell invasion assay. A cell invasion assay was performed to detect the invasive abilities of gliomas cell. Transfected U251 and U87 cells were collected and suspended in FBS-free DMEM. A total of 1x10⁵ suspended cells were seeded into the
upper chambers (Corning, Inc., Corning, NY, USA) coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The lower chambers were covered with 500 µl DMEM supplemented with 10% FBS. After 48 h incubation, non-invasive cells that did not invade via the pores were gently removed with cotton swabs. The invasive cells were fixed with 4% polyoxymethylene at room temperature for 20 min, stained with 0.5% crystal violet at room temperature for 20 min; cells were analyzed under an inverted light microscope (magnification, x200; CKX41; Olympus Corporation, Tokyo, Japan). The number of invasive cells was counted in at least five randomly selected fields.

Bioinformatics analysis. Online miRNA target prediction software, including TargetScan 7.1 (www.targetscan.org/) and miRanda (www.microrna.org) were used to predict the putative target genes of miR-940.

Dual-luciferase reporter assay. The 3'-UTR of KLF9 containing putative wild-type (Wt) or mutated (Mut) miR-940-binding sequences was cloned and inserted into the pmirGLO Dual-Luciferase miRNA target expression vector (Promega Corporation, Madison, WI, USA), and labelled as pmirGLO-KLF9-3'-UTR Wt or pmirGLO-KLF9-3'-UTR Mut, respectively. Cells were seeded into 24-well plates with a density of 1.0x10^5 cells per well one night prior to transfection. Cells were co-transfected with miR-940 inhibitor or NC inhibitor and pmirGLO-KLF9-3'-UTR Wt or pmirGLO-KLF9-3'-UTR Mut, using Lipofectamine 2000™ (Thermo Fisher Scientific, Inc.). Following transfection for 48 h, transfected cells were collected and subjected to luciferase activity analysis using a Dual-Luciferase reporter assay system (Promega Corporation) according to the manufacturer's protocol. The firefly luciferase activity was normalized to Renilla luciferase activity.

Western blot analysis. Total protein of tissues and transfected cells was isolated using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology), and was quantified using a bichinonic acid protein assay Kit (Beyotime Institute of Biotechnology). An equal amount of protein (30 µg) was separated by 10% SDS-PAGE, which were subsequently transferred to a nitrocellulose membrane.
U87 cells to decrease endogenous expression levels. After 48 h transfection, RT-qPCR analysis was used to assess transfection efficiency. The results of the present study demonstrated a significant downregulation of miR-940 in U251 and U87 cells following transfection with miR-940 inhibitor compared with cells transfected with NC inhibitor (Fig. 2A; P<0.05). MTT and cell invasion assays were conducted to evaluate the effects of miR-940 downregulation on glioma cell proliferation and invasion, respectively. The results demonstrated that inhibition of miR-940 expression significantly decreased proliferation (Fig. 2B; P<0.05) at 48 and 72 h and invasion (Fig. 2C; P<0.05) of U251 and U87 cells compared with cells transfected with NC inhibitor. These results suggested the possible role of miR-940 in the progression of glioma.

**KLF9 is a direct target gene of miR-940 in glioma.** To investigate the underlying molecular mechanism of miR-940 in glioma, the present study performed bioinformatics analyses to identify the potential targets of miR-940. KLF9 was predicted to be a target of miR-940 (Fig. 3A). To confirm whether miR-940 directly targets the 3'-UTR of KLF9, a dual-luciferase reporter assay was conducted using U251 and U87 cells co-transfected with miR-940 inhibitor or NC inhibitor and pmirGLO-KLF9-3'-UTR Wt or pmirGLO-KLF9-3'-UTR Mut. As presented in Fig. 3B, the downregulation of miR-940 increased the luciferase activity of cells transfected with pmirGLO-KLF9-3'-UTR Wt; however, luciferase activity of pmirGLO-KLF9-3'-UTR Mut was notably unaffected in U251 and U87 cells. To further understand the regulation...
of KLF9 mediated by miR-940, RT-qPCR and western blot analysis was conducted to measure KLF9 expression levels in U251 and U87 cells transfected with miR-940 inhibitor or NC inhibitor. As presented in Fig. 3C and D, KLF9 mRNA (P<0.05) and protein (P<0.05) expression levels in miR-940 inhibitor-transfected U251 and U87 cells were significantly upregulated compared with cells transfected with NC inhibitor. Collectively, these results suggested that KLF9 may be a direct target gene of miR-940 in glioma.

**Inverse correlation between miR‑940 and KLF9 expression levels in glioma tissues.** KLF9 expression was measured in 23 paired glioma tissues and adjacent normal brain tissues to demonstrate the association between miR-940 and KLF9. As presented in Fig. 4A, KLF9 mRNA expression levels in glioma tissues were significantly decreased compared with adjacent normal brain tissues (P<0.05). Furthermore, KLF9 mRNA expression levels in glioma tissues were inversely correlated with miR-940 expression levels in glioma tissues (Fig. 4D; R=-0.5567; P=0.0058). These results suggested that KLF9 may be a direct target of miR-940 in glioma tissues.

**KLF9 knockdown reverses the effects of miR‑940 on glioma cell proliferation and invasion.** As aforementioned, the downregulation of miR-940 suppressed cell proliferation and invasion in glioma and KLF9 was identified as a direct target of miR-940. Therefore, the present study aimed to determine whether alterations in cell proliferation and invasion following miR-940 knockdown directly occur as a result of KLF9 upregulation. U251 and U87 cells were transfected with miR-940 inhibitor in the presence of KLF9 siRNA or NC siRNA. Western blotting demonstrated that the increased expression levels of KLF9 due to miR-940 inhibitor were suppressed via
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The inhibition of proliferation (Fig. 5B; P<0.05) and invasion (Fig. 5C; P<0.05) associated with miR-940 downregulation was reversed by KLF9 knockdown. The oncogenic roles of miR-940 in glioma cell proliferation and invasion may be, at least in part, attributable to the upregulation of KLF9.

Discussion

In the present study, miR-940 expression levels were upregulated in glioma tissues and cell lines. Downregulation of miR-940 decreased the proliferation and invasion of glioma cells; KLF9 was confirmed as a direct target gene of miR-940 in glioma. In addition, the expression levels of KLF9 were downregulated in glioma tissues and were negatively correlated with that of miR-940 in the present study. Furthermore, KLF9 knockdown partially reversed the oncogenic effects of miR-940 on glioma cell proliferation and invasion. The results of the present study suggest that miR-940 may be further developed as a promising therapeutic target for the treatment of NSCLC.

miR-940 was previously demonstrated to be aberrantly expressed in a number of types of human cancer. miR-940 was downregulated in hepatocellular carcinoma, and this downregulation was significantly associated with Edmondson grade, tumor microsatellite or multiple tumors, vascular invasion, recurrence and metastasis (18,24). Patients with hepatocellular carcinoma and low miR-940 expression levels exhibited poorer prognosis compared with high expression levels (18). miR-940 was additionally demonstrated to be downregulated in prostate (20), breast (21) and ovarian cancer (22); however, miR-940 expression was observed to be upregulated in gastric cancer tissues, cell lines and plasma (25). High miR-940 expression levels were strongly correlated with the advanced N stage of gastric cancer (19). Additionally, patients with hepatocellular carcinoma and high miR-940 expression levels exhibited shorter median times of recurrence compared with patients with low expression levels (19). Upregulation of miR-940 was additionally observed in pancreatic cancer tissues and cell lines (26). These results suggested that the expression profile of miR-940 exhibits tissue specificity and may be developed as a biomarker for the detection and prognosis of particular cancer types.

Dysregulation of miR-940 has been associated with tumor initiation and the progression of numerous types of human cancer (18-21). Upregulation of miR-940 suppressed cell growth and promoted apoptosis of hepatocellular carcinoma (18). Rajendiran et al (20) observed that ectopic expression of miR-940 decreased cell migration and invasion of prostate cancer. Hou et al (21) demonstrated that
miR-940 overexpression prohibited the proliferative and migratory abilities of triple-negative breast cancer cells. Conversely, Wang et al. (22) identified that restoration of miR-940 expression restricted cell proliferation and induced apoptosis in ovarian cancer. However, miR-940 served oncogenic roles in gastric cancer by promoting cell metastasis in vitro and in vivo (19). Yang et al. (26) proposed that upregulation of miR-940 increased cell proliferation and invasion of pancreatic cancer. These results demonstrated the tissue specific biological roles of miR-940 in various types of human malignancies.

Numerous targets of miR-940 have been identified, including estrogen-related receptor γ in hepatocellular carcinoma (18), migration and invasion enhancer 1 in prostate cancer (20), protein kinase C-δ in ovarian cancer (22), zinc finger protein 24 in gastric cancer (19), and glycogen synthase kinase 3β (26) and secreted frizzled related protein 1 (26) in pancreatic carcinoma. In the present study, KLF9 was identified as a direct target gene of miR-940 in glioma. Bioinformatics analysis predicted that KLF9 is a potential target of miR-940. Additionally, luciferase reporter assays demonstrated that downregulation of miR-940 increased the luciferase activity of Wt; however, not the mutated plasmids, suggesting that miR-940 may directly target the 3’-UTR of KLF9. In addition, inhibition of miR-940 exhibited a significant upregulation in the expression of KLF9 at the mRNA and protein expression levels. Furthermore, KLF9 was downregulated in glioma tissues and was negatively correlated with miR-940 expression levels. Finally, KLF9 knockdown may counteract the effects of miR-940 on glioma cell proliferation and invasion.

Figure 5. KLF9 knockdown reverses the effects of miR-940 inhibitor on glioma cell proliferation and invasion. U251 and U87 cells were co-transfected with miR-940 inhibitor and KLF9 siRNA or NC siRNA. At different times following transfection, cells were collected for subsequent experiments. (A) Following 72 h post-transfection, cells were harvested and subjected to western blotting to determine KLF9 expression levels. (B) Cell proliferation and (C) invasion of indicated cells was detected via MTT and cell invasion assays, respectively (magnification, x200). *P<0.05 vs. NC inhibitor; #P<0.05 vs. miR-940 inhibitor+KLF9 siRNA. KLF9, Kruppel-like factor 9; miR, microRNA; siRNA, small interfering RNA; NC, negative control; OD, optical density.
Collectively, miR-940 may serve oncogenic roles in glioma by directly targeting KLF9.

KLF9, located on human chromosome 9q13, is a member of the KLF family (27). The expression of KLF9 was decreased in numerous types of human malignant tumors, including pancreatic ductal adenocarcinoma (28), esophageal squamous cell carcinoma (29), hepatocellular carcinoma (30) and prostate cancer (31). Increasing evidence suggests the significant role of KLF9 in carcinogenesis and cancer progression; KLF9 serves as tumor suppressor and regulates a variety of cellular processes, including cell proliferation, apoptosis, migration and invasion (29,31-33). Additionally, low levels of KLF9 expression have been detected in glioma tissues and cell lines (34). Decreased KLF9 expression promotes cell proliferation in vitro and tumor growth in vivo (34). These results suggest that KLF9 may be a promising target for patients with glioma.

In conclusion, miR-940 was overexpressed in glioma tissues and cell lines in the present study. Decreased miR-940 expression levels may inhibit cellular proliferation and invasion of glioma cells by directly targeting KLF9. The results of the present study suggested a theoretical basis for the application of miR-940/KLF9 in the treatment of patients with glioma.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

SW and DZ designed the present study, DZ, PH and LJ performed RT-qPCR, and the MTT and luciferase reporter assays. YW and ZY conducted the cell invasion assay and western blot analysis. All authors have read and approved the final draft.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The First Affiliated Hospital of Xi’an Medical University (Xi’an, China). Written informed consent was provided by all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


