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The role of microRNA-1274a in the tumorigenesis of gastric cancer: Accelerating cancer cell proliferation and migration via directly targeting FOXO4

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ABSTRACT

MicroRNAs (miRNAs) are a series of 18–25 nucleotides length non-coding RNAs, which play critical roles in tumorigenesis. Previous study has shown that microRNA-1274a (miR-1274a) is upregulated in human gastric cancer. However, its role in gastric cancer progression remains poorly understood. Therefore, the current study was aimed to examine the effect of miR-1274a on gastric cancer cells. We found that miR-1274a was overexpressed in gastric cancer tissues or gastric cancer cells including HGC27, MGC803, AGS, and SGC-7901 by qRT-PCR analysis. Transfection of miR-1274a markedly promoted gastric cancer cells proliferation and migration as well as induced epithelial–mesenchymal transition (EMT) of cancer cells. Our further examination identified FOXO4 as a target of miR-1274a, which did not influence FOXO4 mRNA expression but significantly inhibited FOXO4 protein expression. Moreover, miR-1274a overexpression activated PI3K/Akt signaling and upregulated cyclin D1, MMP-2 and MMP-9 expressions. With tumor xenografts in mice models, we also showed that miR-1274a promoted tumorigenesis of gastric cancer in vivo. In all, our study demonstrated that miR-1274a prompted gastric cancer cells growth and migration through dampening FOXO4 expression thus provided a potential target for human gastric cancer therapy.

1. Introduction

It has been reported that approximately 989,600 new cases of gastric cancer worldwide and 738,000 gastric-cancer-specific deaths was found in 2008 [1]. Deaths occurred in developing nations occupies 70%, approximately 40% reporting in China, along with the high incidence of gastric cancer in Asia, Eastern Europe and South America [1]. Upon diagnosis, locally advanced or metastatic disease can be detected in two-thirds of gastric cancer patients [2]. Unfortunately, up to one half of the patients would bore a recurrent disease after curative surgery. The median survival time for these patients is only 6–9 months [3]. Old age, smoking, alcohol consumption, above normal body weight, unhealthy diet, low economic status, pernicious anemia, other chronic gastric diseases and *Helicobacter pylori* infection are the risk factors that contribute to gastric cancer induction [4,5].

MicroRNAs (miRNAs) are a novel series of 18–25 nucleotides length non-coding RNAs, which repress protein translation through binding to target mRNAs. MiRNAs can regulate gene expressions through sequence-specific pairing of miRNAs with 3'-untranslated regions of the target messenger RNA (mRNA) [6,7]. Importantly, the discovery of microRNAs (miRNAs) has led to the identification of potential therapeutic ways and revolutionized biomarker for diagnostic and prognostic implications. MiRNAs may function as tumor suppressors or oncogenes and alterations in mirna expression may play a critical role in tumorigenesis and cancer progression [8]. Ueda et al. [9] identified that 22 miRNAs were upregulated and 13 downregulated in gastric cancer versus non-tumor mucosa upon the analysis of 237 miRNAs in 353 gastric samples with microarray. Human miR-1274a was proposed based on the identification of small 18-nucleotide RNA fragments and was identified as tRNALys mimics [10]. This miRNA has been shown to be involved in the pathogenesis of multiple diseases. After H1N1 infection, the bronchial epithelial cells expressed increased

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miR-1274a [11]. Recent study found that miR-1274a expression was upregulated in cutaneous malignant melanoma via the microarray analysis [12].

Forkhead box (Fox) proteins are a family of transcriptional factors containing a common DNA-binding domain (DBD) characterlized by forkhead box or winged helix domain [13]. Fox subfamilies FOXO comprises four highly related members FOXO1, FOXO3, FOXO4, and FOXO6, which can bind to the consensus sequence, TTGTTTAC or BBTRTTTTD, or interact with other transcription factors to modulate target genes expressions [14–17]. The FOXO4 has attracted increasing interest due to its role in cancer carcinogenesis [18]. Low expression of FoxO4 was demonstrated to be correlated with decreased expression of E-cadherin and elevated expression of vimentin in non-small cell lung cancer [19]. Kwon I-K et al. [20] has reported that through activating FOXO4 in colon cancer cells, cGMP-dependent protein kinase(PKG) could reduced expression of β-catenin and lead to TCF activity inhibition. In another research, Su L et al. [21] found that the expression of FOXO4 was decreased significantly in most gastric cancer tissues and in various human gastric cancer cell lines.

MiR-1274a was demonstrated to be upregulated in human gastric cancer tissues and predicted to target FOXO4 [22]. In the current study, we aimed to delineate the effect of miR-1274a on gastric cancer cells and examine whether FOXO4 is a regulated object of miR-1274a. We showed that miR-1274a was overexpressed in gastric cancer cells and directly targeted FOXO4, which might contribute to the tumorigenesis of gastric cancer.

2. Materials and methods

2.1. Clinical tissue samples

Paired gastric cancer tissues and non-tumor gastric tissues were collected from routine therapeutic surgery. Our experiments involving human tissues and human cancer cells were approved by the Ethical Committee of The First Affiliated Hospital of Zhengzhou University and were carried out in according to the declaration of Helsinki. Written informed consent was obtained from each patient.

2.2. Cell lines culture

Gastric cancer cell lines HGC27, MGC803, AGS, and SGC-7901 were provided by the Cell Resource Center of the Chinese Academy of Sciences (Shanghai, PR China). Human normal epithelial GES-1 cells were maintained in our laboratory. AGS cells were grown in Ham's F12 (Gibco, USA). Gastric cancer cells were cultured in RPMI1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA). All cell lines were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Real-time quantitative PCR

Total miRNA from gastric tissues and cultured cells was extracted using a mirVana miRNA Isolation Kit (AM1560, Life technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA using a TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA,USA); Expression levels of miR-1274a were quantified using a miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems) according to the manufacturer's instructions. Relative expression levels of miR-1274a were calculated using $2^{-[(\text{Ct of miR}-1274a) - (\text{Ct of U6})]}$ and normalized to U6 small nuclear RNA expression. For the analysis of FOXO4 mRNA expression, the primers were designed according to human FOXO4 mRNA sequence in GenBank (NM_005938.3). Forward: 5′-AGG CCA CCG GCA AAA GCT CTT -3′; Reverse: 5′-CTT CCG TCC ACG AAG CAG -3′. Expression data were normalized to human β-actin. (Forward: 5′-GAG CTA CGA GCT GCC TGA CG -3′; reverse: 5′-CCT AGA AGC ATT TGC GGT GG -3′).

2.4. Plasmid construction and transfection

miR-1274a precursor and the negative control pre-miRNA were synthesized by GenePharma (Shanghai, China). For the transfection of pre-miR1274a or negative control pre-miRNA, Lipofectamine 2000(Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's instructions.

2.5. Dual-luciferase reporter assay

The cDNA containing the predicted miR-1274a binding sequences of FOXO4 were amplified and subcloned into pGL3 luciferase promoter vector (Promega, Madison, WI, USA). As a control, the pGL3-FOXO4-MUTANT was also constructed using the Quickchange mutagenesis kit (Strata-gene, Heidelberg, Germany). HEK-293T cells were transfected with 0.1 lg of pGL3-AEG-1 or pGL3-mutAEG-1 vectors along with 40 nM pre-miR-1274a or control pre-miR using lipofectamine transfection reagent (Invitrogen, Carlsbad, CA, USA). The luciferase activities were measured using the dual-luciferase reporter assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

2.6. Cell migration assay

Cell migration was detected by the Transwell assay. Briefly, gastric cancer cells (2×10^4) transfected with control or miR-1274a mimic were seeded in per upper chambers in serum-free media without the Matrigel membrane, whereas the lower chambers were loaded with DMEM containing 5% FBS. After 24 h, the non-migrating cells on the upper chambers were removed by a cotton swab. The numbers of Cancer cells were counted after they were fixed and stained with crystal violet.

2.7. MTT assay

Gastric cancer cells(2×10^6) transfected with control or miR-1274a mimic were seeded in 96-well plates and then stained at the indicated time points with 100 μL sterile MTT for 4 h at 37 °C. The culture medium was removed and 150 μL DMSO (Sigma–Aldrich) was added. Absorbance was measured at 490 nm. All experiments were performed for triple times.

2.8. Western blot

Total proteins in the cancer cells were extracted and then separated by SDS-PAGE, and western blot analysis was performed according to standard procedures. Detection of β-actin on the same membrane was used as a loading control. Specific antibodies for ZEB1(1:1000, E-20,sc-10572,Santa Cruz Biotech, Santa Cruz, CA, USA),E-cadherin(1:1500, DECMA-1, sc-59778, Santa Cruz Biotech), vimentin (1:1500, RV202, sc-32322,Santa Cruz Biotech), N-cadherin(1:1500, D-4, sc-8424,Santa Cruz Biotech), FOXO4(1:2000, EPR5442, ab128908, Abcam, Cambridge, UK), phosphorylated Akt(p-Akt)(1:2000,EP2109Y, ab81283,Abcam), cyclin D1(1:1500,DCS-6, sc-20044, Santa Cruz Biotech), matrix metalloproteinase (MMP)-2(1:1500,8B4, sc-13595, Santa Cruz Biotech)and MMP-9(1:1500,6-6B, sc-12759, Santa Cruz Biotech)were employed for the immunodetection of the corresponding proteins. Goat anti-mouse IgG

(1:10,000, Sigma, St. Louis, MO, USA) followed by enhanced chemiluminescence (ECL, Amersham Pharmacia, NJ, USA) was used.

2.9. Xenograft tumor assay

MGC-803 cells (3×10^6 cells) transfected with vectors overexpressing miR-542-3p or the control vectors (Gene-Pharma, Shanghai, China) diluted in 200 ll of PBS were inoculated subcutaneously into the right flank of nude mice. The tumor volume was measured every 2 days by a vernier caliper and calculated using the formula: volume = length × width^2 × π/6. About 3 weeks after inoculation, mice were euthanized by subcutaneous injection with sodium pentobarbital (40 mg/kg) and the tumors were weighed.

2.10. Statistical analysis

Data were presented as means ± SD. Differences between two groups were analyzed by Student's t test, while differences between multiple groups were analyzed by ANOVA. Data were considered to be statistically significant when $p < 0.05$.

3. Results

3.1. High expression of miR-1274a is found in gastric cancer tissues and gastric cancer cells

Previously, miR-1274a levels have been shown to be upregulated in human gastric cancer tissues [22]. To examine miR-1274a expression ingastric cancer tissues of our study, 78 samples were collected and processed to miR-1274a expression analysis by qRT-PCR. Compared with the normal tissue, miR-1274a expression was significantly elevated in cancer tissues (Fig. 1A). Furthermore, the expression of miR-1274a in a series of gastric cancer cell lines was also detected. In line with the result showed in cancer tissues, enhanced miR-1274a expression was observed in multiple gastric cancer cells including HGC27, MGC803, AGS, and SGC-7901compared with gastric epithelial cell GES-1 (Fig. 1B). These results confirmed that miR-1274a expression was upregulated in either gastric cancer tissues or gastric cancer cells.

3.2. MiR-1274a contributes to gastric cancer cellsproliferation and migration

To investigate the role of miR-1274a in gastric cancer progression, control (ctrl) or miR-1274a mimic was transfected into HGC27 and MGC803 cells of which the proliferation and migration were evaluated. The results of qRT-PCR and MTT assay showed that miR-1274a mimic but not ctrl mimic transfection raised miR-1274a expression (Fig. 1C) as well as promoted cell proliferation (Fig. 1D). Moreover, miR-1274a mimic remarkably accelerated cell migration as detected by Transwell assay (Fig. 1E). These results demonstrated that miR-1274a promoted gastric cancer cells proliferation and migration.

3.3. MiR-1274a induces epithelial–mesenchymal transition (EMT) of gastric cancer cells

To further explore the association of miR-1274a with cancer clinical outcomes, we next examined EMT of HGC27 and MGC803 cells with miR-1274a overexpression. HGC27 andMGC803 cells were transfected with ctrl or miR-1274a mimic as above and the expressions of ZEB1, E-cadherin, vimentin and N-cadherin were analyzed by immunoblot. As was showed in the figure, miR-1274a mimic led to elevated expressions of ZEB1, E-cadherin and decreased expressions of vimentin and N-cadherin in HGC27 and MGC803 cells than the control (Fig. 2A, B). These results revealed induction of EMT of gastric cancer cells by miR-1274a.

3.4. FOXO4 is a target of miR-1274a in gastric cancer cells

FOXO4 expression level was dramatically decreased in lymph node-positive colorectal carcinoma tissues [23] and gastric cancer [21]. The results of bioinformatics analysis by miRanda (http://www.microrna.org/) and TargetScan (http://www.targetscan.org) showed that miR-1274a directly bound to the 3′ UTR of FOXO4 (Fig. 3A). Therefore, we next investigated the effect of miR-1274a on FOXO4 expression. HGC27 and MGC803 cells were transfected with ctrl or miR-1274a mimic as above and FOXO4 mRNA and protein expressions were analyzed. Compared with the control, miR-1274a mimic transfection did not change the expression of FOXO4 mRNA (Fig. 3B) but resulted in downregulated protein level of FOXO4 (Fig. 3C). Furthermore, to confirm whether miR-1274a may modulate FOXO4 expression via the direct binding, we co-transfected miR-1274a mimic with luciferase plasmids containing FOXO4 3′ UTR (pGL3-FOXO4) or containing FOXO4 3' UTR mutant (pGL3-FOXO4-MUTANT) into HEK293T cells. The relative luciferase activity of pGL3-FOXO4 but not pGL3-FOXO4-MUTANT was significantly decreased in the presence of miR-1274a mimic (Fig. 3D). Hence, our data demonstrated that the modulation of FOXO4 expression by miR-1274a was sequence-specific.

3.5. MiR-1274a activates PI3K/Akt signaling in gastric cancer cells

FOXO4 has been reported to suppress invasion in vivo and lymph node (LN) metastasis in vitro through counteracting PI3K/Akt signaling [24]. Subsequently, we investigated the activation of PI3K/Akt in gastric cancer cells with miR-1274a overexpression. MGC803 cells were transfected with ctrl or miR-1274a mimic and the expression of phosphorylatedAkt(p-Akt) was analyzed. The results showed that an increasing expression of p-Akt was observed in cancer cells transfected with miR-1274a mimic but not in control or those with ctrl mimic (Fig. 4A). Moreover, expressions of PI3K/Akt downstream targets including cyclin D1, MMP-2 and MMP-9 were also examined. MiR-1274a mimic transfection significantly up-regulated the expressions of cyclin D1, MMP-2 and MMP-9 (Fig. 4A). Our data demonstrated that miR-1274a may prompt PI3K/Akt signaling in gastric cancer cells.

3.6. MiR-1274a promotes tumorigenesis of gastric cancer in vivo

Additionally, MGC803 cells transfected with ctrl or miR-1274a mimic were subcutaneously injected into the back region of nude mice at a single site. After the transplantation, the volumes of the xenografts were measured every 2 days. Compared with the control, miR-1274a overexpression significantly increased xenografts growth (Fig. 4B). Furthermore, tumor-bored mice were sacrificed and the xenografts were harvested and weighted after 28 days of the transplantation. The results showed that miR-1274a overexpression remarkably enhanced tumor weight compared to the control (Fig. 4C).

4. Discussion

Dysregulation of miRNAs is considered as a leading factor for many diseases [25,26]. MiR-1274a was demonstrated to be increased inchronic objective pulmonary disease (COPD) samples compared with smokers without airflow limitation [27]. MiR-1274a is up-regulated by sorafenib and mediates the repression effect of sorafenib on hepatocellular carcinoma [28]. Furthermore, An J et al. [29] found that miR-1274a was amplified in related genomic region

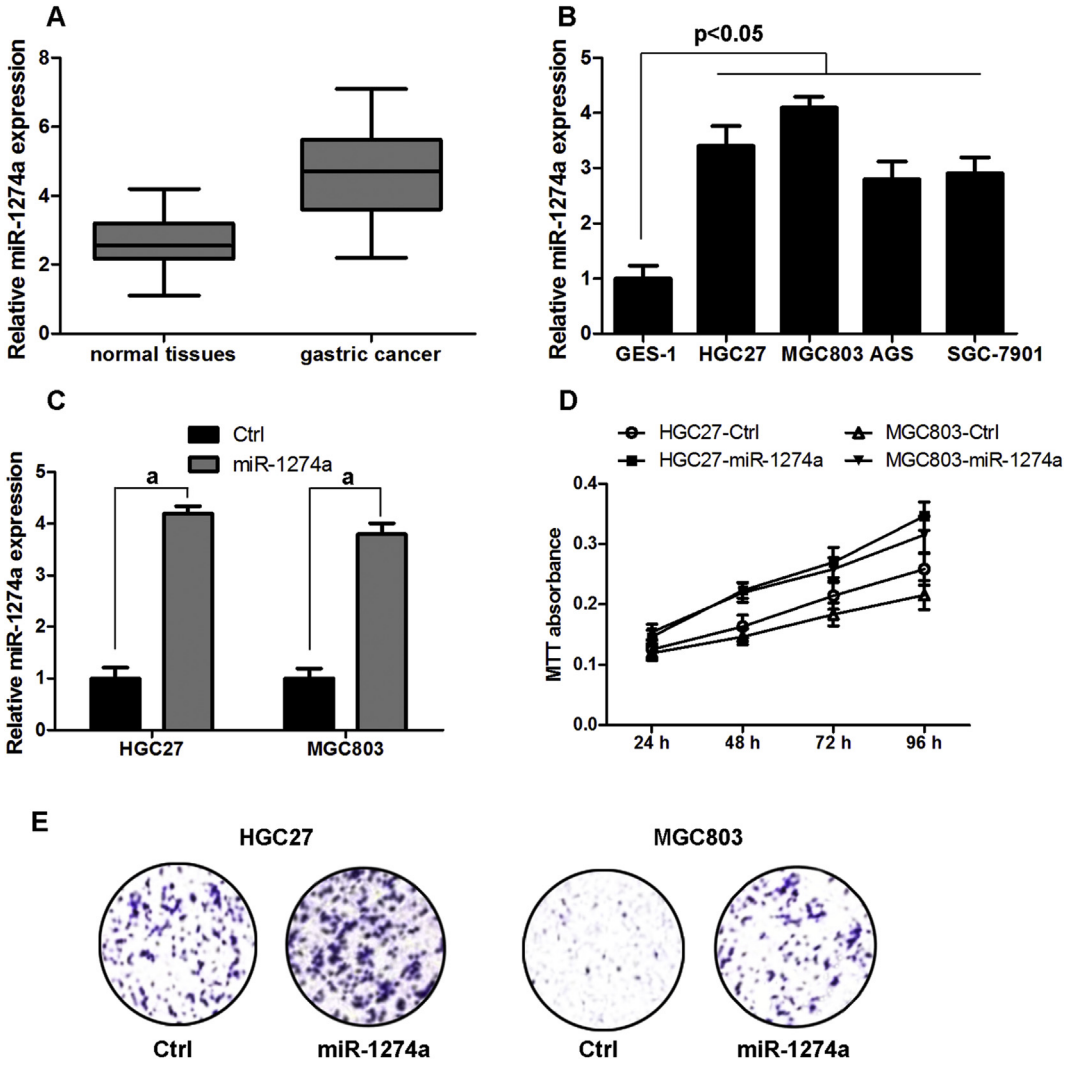

Fig. 1. MiR-1274a expression and its role in gastric cancer proliferation and migration. (A) 78 samples of gastric cancer were collected and the miR-1274a expression was analyzed qRT-PCR. (B) MiR-1274a expression in multiple gastric cancer cells including HGC27, MGC803, AGS, and SGC-7901 as well as in gastric epithelial cell GES-1 was analyzed qRT-PCR. (C) MiR-1274a expression inHGC27 and MGC803 cells after control (Ctrl) or miR-1274a mimic transfection. (D) The proliferation of HGC27 and MGC803 cells was determined by MTT assay after control(Ctrl) or miR-1274a mimic transfection. (E) Migration of HGC27 and MGC803 cells was counted by Transwell assay after control(Ctrl) or miR-1274a mimic transfection. [a]P<0.05 vs Ctrl.

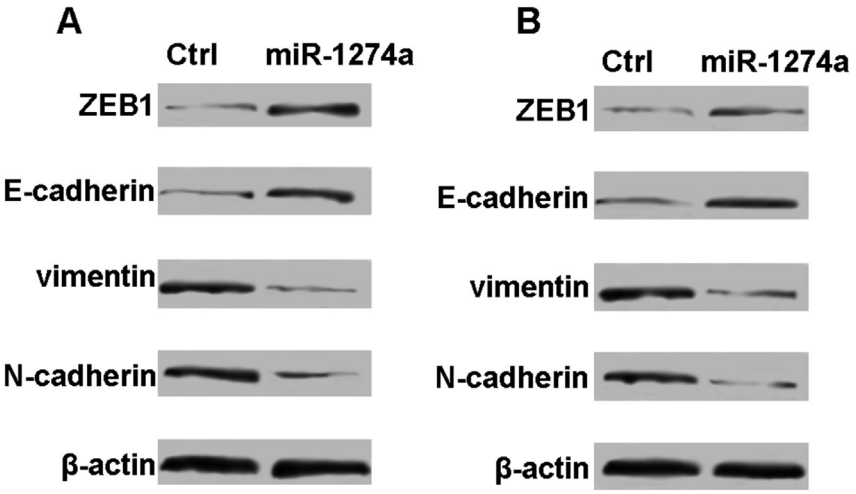

Fig. 2. MiR-1274a induces epithelial–mesenchymal transition(EMT) of gastric cancer cells. HGC27 (A) and MGC803 cells (B) were transfected with ctrl or miR-1274a mimic and the levels of ZEB1, E-cadherin, vimentin and N-cadherin protein were analyzed by Western blot.

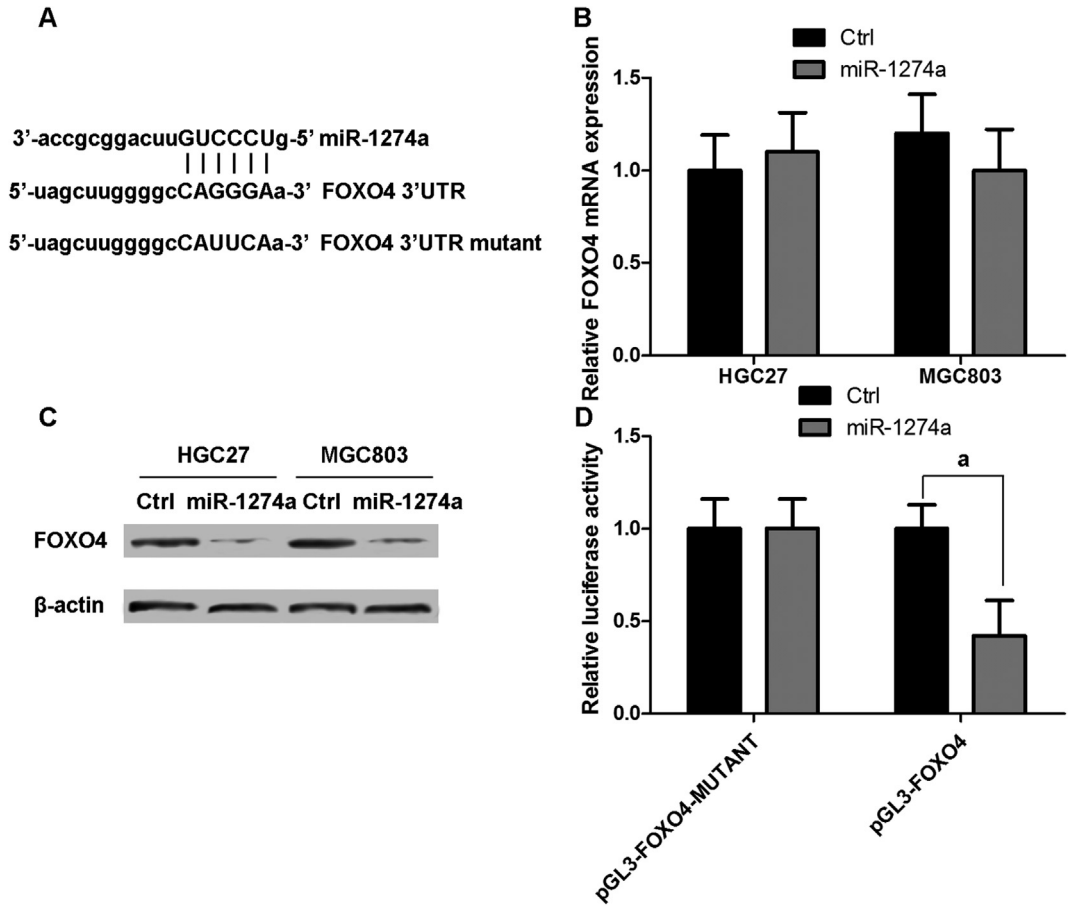

Fig. 3. FOXO4 is a target of miR-1274a in gastric cancer cells. (A) MiR-1274a directly bound to the 3′ UTR of FOXO4 by bioinformatics analysis. HGC27 and MGC803 cells were transfected with ctrl or miR-1274a mimic and FOXO4 mRNA (B) and protein (C) expressions were analyzed. (D) MiR-1274a mimic was co-transfected with luciferase plasmids containing FOXO4 3′ UTR (pGL3-FOXO4) or containing FOXO4 3′ UTR mutant (pGL3-FOXO4-MUTANT) into HEK293T cells. The relative luciferase activity was measured using the dual-luciferase reporter assay kit. [a]P<0.05 vs Ctrl.

and overexpressed in gastric cancer samples. In line with this previous report, our data showed that miR-1274a expression was augmented in gastric cancer tissues and multiple gastric cancer cells like HGC27, MGC803, AGS, and SGC-7901.

In the current study, through miR-1274a mimic construction and transfection, miR-1274a was overexpressed in MGC803 cells. The results of MTT assay showed that miR-1274a significantly promoted the proliferation of cancer cells. Moreover, miR-1274a overexpression resulted in accelerated rate of cell migration in MGC803 cells. The data may reveal the role of miR-1274a in gastric cancer cell progression via prompting cell proliferation and migration. The importance of EMT-related reversible plasticity is clearly emphasized during cancer progression [30]. EMT phenotypeis demonstrated to occur in drug-resistant human cancer cells and the acquisition of the EMT phenotype is associated with drug resistance and cancer cell metastasis [31]. Murai T et al. [32] analyzed the EMT status of 116 gastric cancer patients by calculating the vimentin/E-cadherin mRNA expression ratio in cancerous tissue and predicted the EMT status as an independent prognostic factor. Indeed, our results showed that miR-1274a induced EMT of MGC803cells though upregulation of ZEB1, E-cadherin expressions and downregulation of vimentin and N-cadherin expressions. These results suggested that miR-1274a might be involved in EMT of gastric cancer.

Activation of the FOXO family of proteins is associated with cell cycle arrest and the induction of apoptosis [33,34]. The notion that FOXO proteins are bona fide tumor suppressor is supported by accumulated evidence [35]. In a study, deletion of FOXO1, FOXO3A and FOXO4 led to a progressive cancer-predisposed condition characterized by thymic lymphomas and haemangiomas [16]. Evidence has also emphasized the role of FOXO4 in cancer progression. Overexpression of FOXO4 resulted in an increase in doxorubicin-mediated cytotoxicity, which was further exacerbated by overexpression of a solely nuclear localized FOXO4 mutant [36]. Moreover, up-regulating FOXO4 inhibited the growth and metastasis of gastric cancer cell lines in vitro and led to dramatic attenuation of tumor growth, as well as liver and lung metastasis in vivo [21]. In our study, FOXO4 protein expression was modulated by miR-1274a in MGC803 cells which directly bound to the 3' UTR of FOXO4. The results indicated that miR-1274a might induce gastric cancer tumorigenesis via directly inhibiting FOXO4 expression.

Mechanically, FOXO4 loss is supposed to be associated with increased PI3K/Akt-mediated metastatic invasiveness, and restoration of FOXO4 expression might suppress prostate cancer metastatic aggressiveness [24]. Activation of PI3K/Akt signaling pathway is found in either gastric cancer cells or tumor tissue from patients with advanced gastric cancer [37,38]. Our further investigation found that miR-1274a triggered PI3K/Akt signaling and upregulated the expressions of cyclin D1, MMP-9 and MMP-2 in MGC803 cells. Additionally, our in vivo experiment showed that miR-1274a overexpression significantly increased xenografts growth and enhanced tumor weight in nude mice.

In summary, our data demonstrated that gastric cancer tissues and cell lines acquired upregulating miR-1274a expression, which

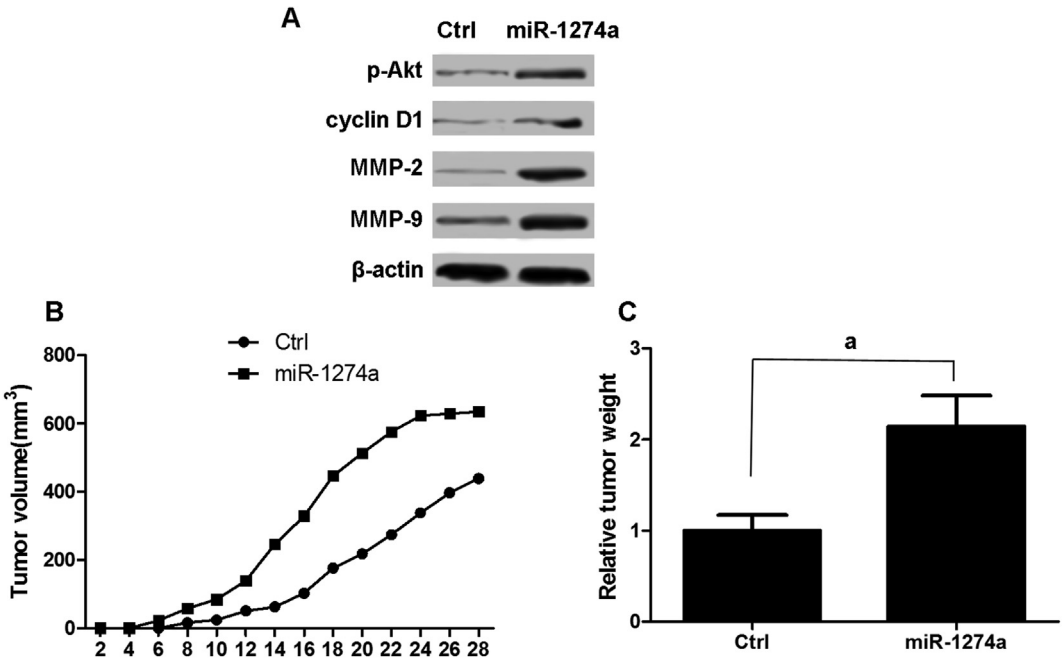

Fig. 4. MiR-1274a activates PI3K/Akt signaling in vitro and promotes tumorigenesis of gastric cancer in vivo. (A) MGC803 cells were transfected with ctrl or miR-1274a mimic and the expression of phosphorylated Akt(p-Akt), cyclin D1, matrix metalloproteinase (MMP)-2 and MMP-9 was analyzed. MGC803 cells transfected with ctrl or miR-1274a mimic were subcutaneously injected into the back region of nude mice at a single site. (B)After the transplantation, the volumes of the xenografts were measured every 2 days. (C) Tumor-bored mice were sacrificed and the xenografts were harvested and weighted after 28 days of the transplantation. [a]P<0.05 vs Ctrl.

accelerated cancer cells proliferation and migration as well as EMT though directly targeting FOXO4 expression.

Conflict of interest

The authors declare no conflict of interest.

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