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MicroRNA-34A inhibits the growth, invasion and metastasis of gastric cancer by targeting PDGFR and MET expression

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Synopsis

Within the family of RTKs (receptor tyrosine kinases), PDGFR (platelet-derived growth factor receptor) has been implicated in carcinogenesis and tumour development. miRNAs (microRNAs), which can target the mRNAs (messenger RNAs) of cancer-associated genes, are abnormally expressed in various cancers. In this study, our aim was to identify the miRNAs that target PDGFR-α/β and to study the functions of these miRNAs. miR-34a was predicted to target PDGFR, and luciferase reporter assays showed that miR-34a could directly target PDGFR. Meanwhile, we found that miR-34a was down-regulated in gastric cancer tissues and was associated with metastasis. Our findings showed that miR-34a could inhibit gastric cancer cell migration, invasion and proliferation, but these tumourigenic properties were only partially restored when PDGFR-α/β was overexpressed. In subsequent experiments, we found that the overexpression of both PDGFR and MET could completely restore the gastric cancer tumourigenic properties. Moreover, the cancer-associated cell signalling pathway was studied, and we found that miR-34a could inhibit Akt [PKB (protein kinase B)] phosphorylation, which was restored by the overexpression of both PDGFR and MET. In conclusion, miR-34a may act as a potential tumour suppressor in gastric cancer and is associated with the mechanisms of gastric cancer metastasis; miR-34a can inhibit gastric cancer tumourigenesis by targeting PDGFR and MET through the PI3K (phosphoinositide 3-kinase)/Akt pathway.

Key words: miR-34a, PDGFR, MET, gastric cancer

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INTRODUCTION

Gastric cancer remains a major cancer burden across the globe. In 2008, approximately 989000 new cases (7.8 % of global cancer totals) and 738000 deaths (9.7 % of global cancer totals) occurred, making gastric cancer the fourth most common malignancy and the second leading cause of cancer death worldwide [1]. This high death rate is due to widespread cancer invasion and metastasis. Tumour angiogenesis is essential for the growth and metastasis of gastric cancer, and it is mediated by numerous stimulatory and inhibitory factors [2]. The isoforms of PDGFR (platelet-derived growth factor receptor) and its ligand, PDGF, play critical roles in tumour angiogenesis. The PDGFR/PDGF system includes two receptors (PDGFR-α and PDGFR-β) and four ligands (PDGFA, PDGFB, PDGFC and PDGFD). PDGF binds to PDGFR and induces receptor dimerization and autophosphorylation, which lead to the activation of intracellular signalling pathways [3].

PDGFR and its ligand PDGF are involved in carcinogenesis and tumour development. Guo et al. [4] found that the overexpression of PDGF-B and PDGFR-β is correlated with cancer progression and the lymphogenous metastasis of gastric carcinoma [4]. PDGFR-α overexpression has been observed in metastatic medulloblastoma patient samples, compared with non-metastatic patient samples, and the disruption of PDGFR-α function inhibited the metastatic potential of medulloblastoma cells *in vitro* [5]. Mathey et al. [6] also found that PDGFR-β

could be important as an antiangiogenic agent and has since become a component of the standard treatment in ovarian cancer. Furthermore, PDGFR expression levels are associated with the angiogenesis, invasion and metastasis of colon cancer [7–9]. A study showed significantly increased PDGFR-β mRNA (messenger RNA) levels in locally advanced rectal tumours compared with the corresponding normal mucosa [10]. PDGFR is also thought to provide a favourable microenvironment for the growth and survival of cancer cells [11,12]. In a recent study, Gialeli et al. [13] found that the PDGF/PDGFR axis is of paramount importance in the tumour microenvironment, and inhibition of PDGF receptor activation represents a major target for future anticancer therapies. Therefore we concluded that the growth, invasion and metastasis of tumours may be inhibited by attenuating PDGFR expression.

miRNAs (MicroRNAs) are non-coding RNA molecules, approximately 21–23 nucleotides in length, which regulate gene expression at the post-transcriptional level [14–16]. miRNA expression profiling analyses have revealed a global down-regulation of mature miRNA levels in primary human tumours relative to normal tissues [17,18]. Thus, miRNAs may function as tumour suppressors or oncogenes, and deregulated miRNA expression might contribute to tumour cell metastasis. PDGFR expression can be inhibited by some miRNAs in tumours. For example, miR-34c is down-regulated in lung tumours, compared with normal lung tissue; miR-34c inhibits lung cancer proliferation, migration and invasion by targeting PDGFR-α/β [19]. miR-34a can affect the growth of proneural glioma cells *in vitro* and *in vivo* by targeting PDGFR-α [20]. PDGFR can also be regulated by miRNAs in non-tumour cells; Zhang,J. et al. identified miR-9 as an activation-induced regulator of PDGFR-β expression in cardiomyocytes [21]. However, it is not clear which miRNA can regulate PDGFR-α/β expression in gastric cancer. In this study, we identified miRNA that can directly affect PDGFR-α/β expression in gastric cancer. Meanwhile, the functions and features of this miRNA were systematically examined.

MATERIALS AND METHODS

Human tissue specimens and cell lines

This study utilized fresh tissues, including 41 human gastric cancer samples and 41 samples from adjacent normal mucosal tissues that were collected from 41 patients who underwent surgery at the Second Affiliated Hospital of Chongqing Medical University between 2012 and 2013.This study was conducted according to the 'Biomedical Research Involving Human Ethics Review (Tentative)' regulation of the Ministry of Health and the Declaration of Helsinki on Ethical Principles for Medical Research Involving Human Subjects. All samples were obtained with the informed consent of the patients, and the experiments were approved by the Institutional Review Board of the Second Affiliated Hospital of Chongqing Medical University. All parti-

cipants provided written informed consent to participate in this study.

The SGC-7901, HGC-27, AGS, MKN-45 and N87 cell lines were obtained from the ATCC (American Type Culture Collection; Manassas, VA, U.S.A.), and the GES-1 cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cell lines were cultured in RPMI-1640 medium (Hyclone) supplemented with 10 % (w/v)FBS and incubated at 37 °C with 5 % (v/v) CO_2.

Primers

miRNA primers were purchased from the TaKaRa Bio Group (TaKaRa Bio). The following sequences of miRNAs were used in this study: miR-34a:UGGCAGUGUCUUAGC-UGGUUGU-3′,miR-421: AUCAACAGACAUUAAUUGG-GCGC, miR-24: UGGCUCAGUU- CAGCAGGAACAG, miR-29a: UAGCACCAUCUGAAAUCGGUUA, miR-29b: UAG-CACCAU- UUGAAAUCAGUGUU, miR-29c: UAGCACC-AUUUGAAAUCGGUUA, miR-519d: CAAAGUGC- CUCC-CUUUAGAGUG, miR-93:CAAAGUGCUGUUCGUGCAGG-UAG, miR-106a: AAAAGU- GCUUACAGUGCAGGUAG, miR-106b: UAAAGUGCUGACAGUGCAGAU, miR-17: CAAAGU- GCUUACAGUGCAGGUAG, miR-20a: UAAAGU-GCUUAUAGUGCAGGUAG, miR-20b: CAAA- GUGCUCA-UAGUGCAGGUAG, miR-449a: UGGCAGUGUAUUGUUAG-CUGGU, miR-449b: AG- GCAGUGUAUUGUUAGCUGGC, miR-34c: AAUCACUAACCACACGGCCAGG and RNU6B: CGCAAGGAUGACACGCAAAUUCGUGAAGCGUUCCAU-AUUUUU.

RNA isolation and miRNA detection

Total miRNA was extracted from cultured cells and human tissue specimens using RNAiso for Small RNA (TaKaRa Bio) according to the manufacturer's instructions. PolyA tails were added to miR-34a and U6 using the miRNA Reaction Buffer Mix (TaKaRa Bio), and cDNA was synthesized from 5 ng of total RNA using the miRNA Prime ScriptRT Enzyme Mix (TaKaRaBio). Real-time PCR was performed on a CFX96™ Real-Time PCR Detection System (Bio-Rad) using SYBR® Premix Ex Taq™ II (TaKaRa Bio). The following PCR conditions were used: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The data were normalized against the expression of the U6snRNA. After amplification, melting curve analysis was performed to ensure the specificity of the products.

Oligonucleotide transfection

miRNAs and cont-miR mimics were synthesized by Sangon Biotechnology (Sangon), and the mimic co-transfections were performed with Lipofectamine 2000 (Invitrogen). Twenty-four h after transfection, the cells were plated for proliferation, migration and invasion assays. The cells were harvested for RNA and protein analyses 48 h after transfection.

pcDNA expression plasmids and plasmid transfection

The ORF sequences of PDGFR-α/β and MET were amplified from genomic DNA isolated from the AGS cell line and were then subcloned into the GV230 vector (GeneChem Corporation, Shanghai, China).The plasmid was transfected into AGS or MKN45 cells using Lipofectamine 2000 (Invitrogen).Twenty-four h after transfection, the cells were used for a rescue experiment.

Luciferase reporter assay

A PsicheckTM-2 Dual-Luciferase miRNA target expression vector was used for the 3′ UTR luciferase assays (Sangon Biotech). The target genes of miRNA-34a were selected on the basis of the online miRNA target database, http://www.microrna.org/microrna/home.do. The 3′UTR sequence of the PDGFR-α/β gene was amplified by PCR, and the resulting amplicon was cloned into the PsicheckTM-2 Vector to produce a wild-type reporter. The following primer sequences were used to amplify the wild-type 3′ UTRs of PDGFR-α/β: PDGFR-α forward: 5′- TCTAGACCGGCCTGA- GAAACACTATTTGTG-3′, reverse: 5′-TCTAGAACATGAACAGGGGCATTCGTAATACA -3′;PDGFR-β: forward: 5′-TCTAGAAA- AGAGGGCAAA-TGAGATCACCTCCTGCA-3′, reverse 5′-TCTAGATATTGAG-AACCCACTCTC- CCTCCTTGGA-3′. The mutant reporter construct was generated using the Site-directed Gene Mutagenesis Kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. There are two binding sites in the PDGFR-α 3′ UTR and one binding site in the PDGFR-β 3′ UTR; therefore, we designed two primer sequences for the mutant 3′ UTR of PDGFR-α and one primer sequence for the mutant 3′ UTR of PDGFR-β. For the one mutant 3′ UTR of PDGFR-α, the following primer sequences were used: forward: 5′-ACTGCCAAAACATTTATG-ACAAGCTGTATCGCCTCG-3′ and reverse: 5′-CGAG-GCGATACAGCTTGTCATAAATGTTTTGGCAGT. For the other mutant 3′ UTR of PDGFR-α, the following primer sequences were used: forward: 5′-ACTGCCAAAACAT-TTATGACAAGCTGT- ATGGTCGTTTATATTT-3′ and reverse: 5′-AAATATAAACGACCATACAGCTTGTCATAA-ATGTTTTGGCAGT-3′. For the mutant 3′ UTR of PDGFR-β, the following primer sequences were used: forward: 5′-ATGGGGGTATGGTTTTGTCAGACCTAGCAGTGAC-3′ and reverse: 5′-GT- CACTGCTAGGTCTGACAAAACCATACCC-CCAT-3′. For the luciferase assay, Lipofectamine 2000 was used to co-transfect AGS cells with the miR-34a mimics and the PsicheckTM-2 Dual-Luciferase miRNA target expression vectors containing the wild-type or mutant target sequences.

Cell viability assays

The transfected cells were seeded in 96-well plates at a density of 1×10^4 cells/well. MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-$2H$-tetrazolium bromide] solution (20 μ of 5 mg/ml MTT) was added to each well (for a total volume of 250 μl),

and the plates were incubated for 4 hat (hours after treatment) 37 °C. Following the removal of the culture medium, the remaining crystals were dissolved in DMSO, and the absorbance at 570 nm was measured.

Migration and invasion assays

For the transwell migration assays, 1×10^4 cells were plated in the top chamber containing a non-coated membrane (24-well insert; 8 mm pore size; BD Biosciences). For the invasion assays, 2×10^5 cells were plated in the top chamber containing a Matrigel-coated membrane (24-well insert; 8 mm pore size; BD Biosciences). For both assays, the cells were plated in the serum-free medium, and medium supplemented with 10 % (v/v) serum was used as a chemoattractant in the lower chamber. The cells were incubated for 16 h at 37 °C in a tissue culture incubator with 5 % (v/v) CO_2. After 16 h, the non-migrated/non-invading cells were removed from the upper sides of the transwell membrane filter inserts using cotton-tipped swabs. The migrated/invaded cells on the lower sides of the inserts were stained with Giemsa, and the cells were counted.

Antibodies

The antibody against GAPDH was purchased from Santa Cruz Biotechnology. Antibodies against phospho-Akt, PDGFR-α, PDGFR-β and MET were purchased from Abcam, and total Akt antibodies were obtained from BD Biosciences. HRP (horseradish peroxidase)-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology.

Immunoblot analysis

Total protein was extracted from the transfected cells using RIPA lysis buffer (Beyotime), according to the manufacturer's instructions. After the whole cell protein extracts were quantified using the BCA (bicinchoninic acid) protein assay, equivalent amounts of cell lysates were resolved by 10 % SDS/PAGE and were transferred onto polyvinylidene fluoride membranes. The membranes were blocked in 5 % (w/v) non-fat dried skimmed milk in TBST for 1 h at 4 °C and then incubated with primary antibodies. After incubation with HRP-conjugated secondary antibodies, the protein bands were visualized using an enhanced chemiluminescence reagent (Millipore). The following antibody dilutions were used: anti-GAPDH, 1:500; anti-phospho-Akt, 1:2000; anti-PDGFR-α/β and anti-MET, 1:1500; anti-total Akt 1:500; and HRP-conjugated IgG, 1:7000.

Statistical analysis

The SPSS 17.0 software was used for the statistical analysis. The data are presented as the mean $\pm$ S.D.. Group comparisons were performed using Student's t test. Differences were considered significant if $P < 0.05$.
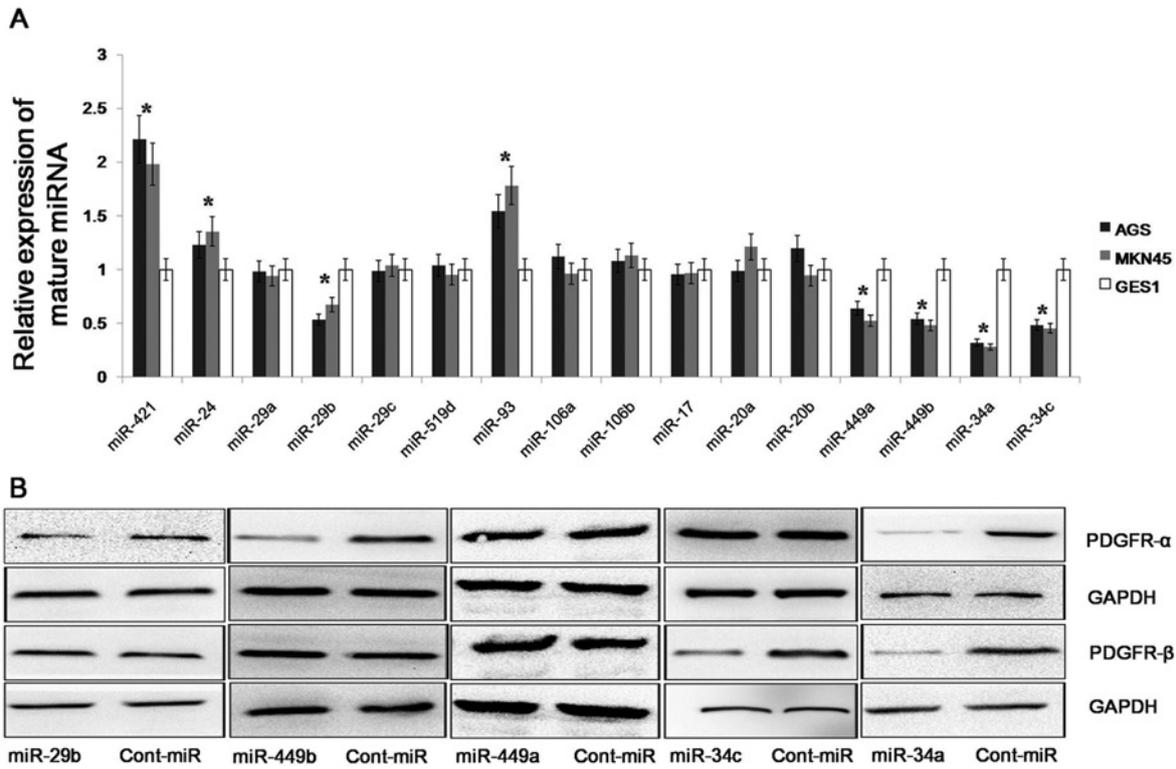
Figure 1   miRNA selection using qRT-PCR and Western blot analyses
(A) The expression levels of 16 miRNAs in gastric cancer cell lines. The expression of these 16 miRNAs was detected using qRT–PCR analysis, and the expression levels of the mature miRNAs were normalized to the levels of the U6 small nuclear RNA. (B) The expression of PDGFR-α/β in gastric cancer cell lines. The expression of PDGFR-α/β was examined using Western blot analysis after the forced expression of miR-29a/b/c, miR-449a/b and miR-34a/c. The data represent the mean $\pm$ S.D.;*$P < 0.01$ compared with the controls.

RESULTS

miRNA selection

To be included in our subsequent analyses, the miRNAs targeting PDGFR-α and PDGFR-β had to be concordantly predicted by Miranda (http://www.microrna.org). Based on the January 2012 release, we found a total of 32 miRNAs that potentially target PDGFR-α and a total of 76 miRNAs that potentially target PDGFR-β. There were 16 miRNAs that could target both PDGFR-α and PDGFR-β. To further investigate the expression of these 16 miRNAs in gastric cancer, we examined their expression in the gastric cancer cell lines AGS and MKN45 and in normal gastric mucosa cells (GES1) using qRT-PCR analysis (Figure 1A). We found that the levels of miR-421, miR-24 and miR-93 were increased in the gastric cancer cell lines compared with the GES1 cells, and the levels of miR-29b, miR-449a/b and miR-34a/c were decreased in the gastric cancer cell lines compared with the GES1 cells. To further identify miRNAs that target PDGFR-α/β, we examined the expression of PDGFR-α/β after the forced expression of miR-29a/b/c, miR-449a/b and miR-34a/c, and we found that the expression levels of both PDGFR-α

and PDGFR-β were decreased only after the overexpression of miR-34a (Figure 1B). Thus, we can predict that miR-34a targets PDGFR-α/β.

miR-34a is down-regulated in gastric cancer tissues

To explore the roles of miR-34a in human gastric cancer development, we detected its expression levels in 41cases of human gastric cancer and 41 cases of adjacent normal mucosa tissues. According to the qRT-PCR analysis, the levels of miR-34a expression were significantly decreased in the tumour tissues compared with the adjacent normal mucosa tissues (Figure 2A). To determine whether miR-34a expression was associated with gastric cancer metastasis, we further examined the miR-34a expression levels in 41 archived primary gastric tumours. These tumours were divided into two groups. The tumours in one group had been resected from 25 patients with lymph node metastases, and the tumours in the other group had been resected from 16 patients without metastases. According to the qRT-PCR analysis, the miR-34a expression levels were significantly lower in the patients with metastases than in the patients without metastases (Figure 2B). These results suggest that miR-34a is down-regulated in gastric
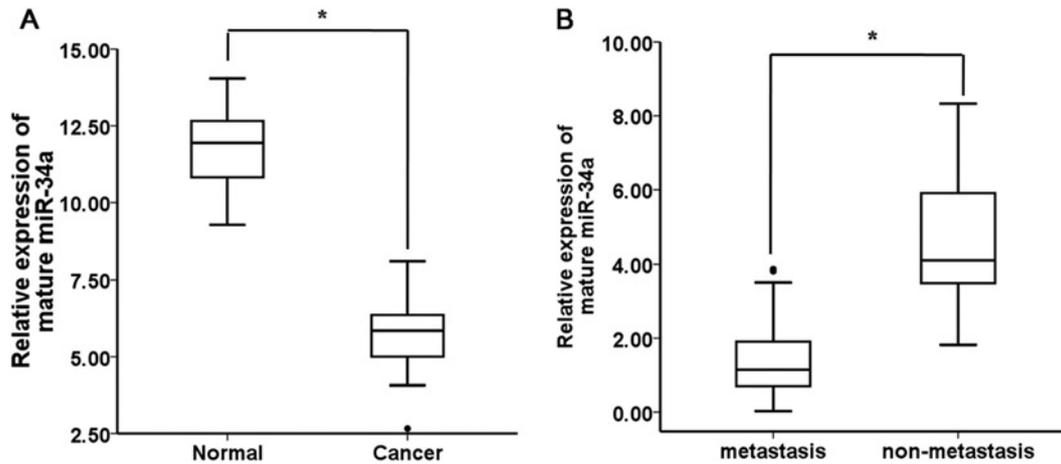
Figure 2   miR-34a is down-regulated in gastric cancer tissues and cell lines
(A) The expression levels of mature miR-34a in gastric cancer (n = 41) and adjacent normal mucosa tissues (n = 41) were determined using quantitative PCR analysis. The data are shown separately in human samples; *, $P < 0.01$. (B) Mature miR-34a expression levels in metastatic (n = 25) and non-metastatic (n = 16) gastric cancers. The data are shown separately in human samples; * $P < 0.01$.
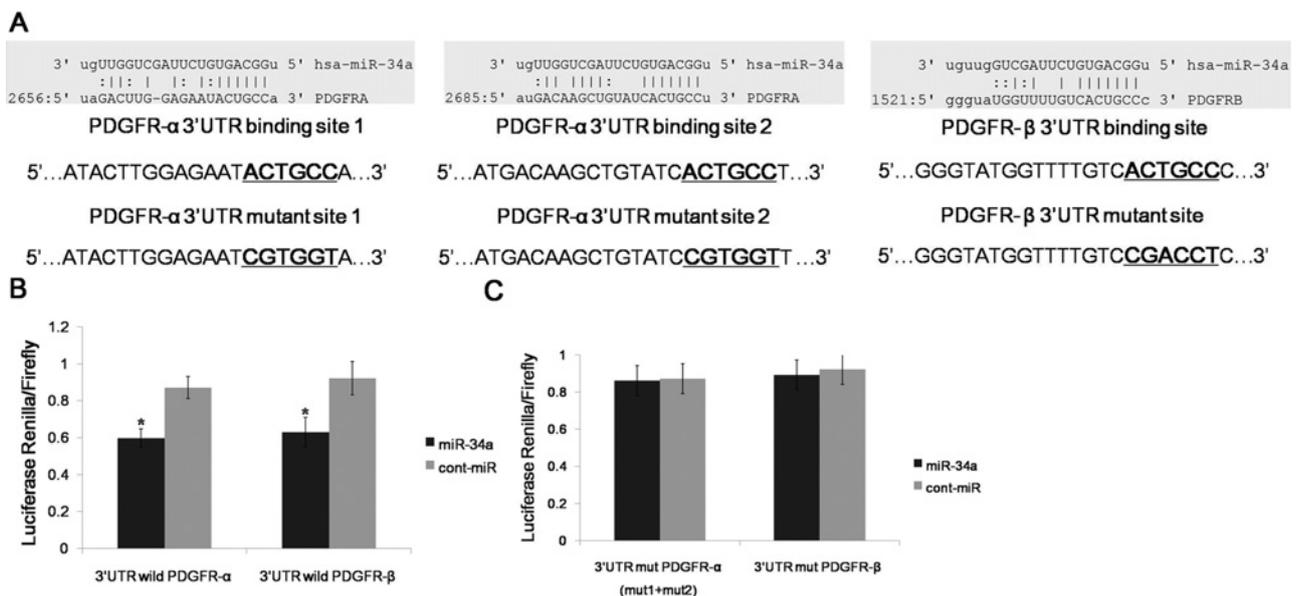

Figure 3   miR-34a can directly target PDGFR-α/β
(A) The wild-type and mutated sequences of the two predicted miR-34a binding sites within the PDGFR-α 3′ UTR and one predicted miR-34a binding site within the PDGFR-β 3′ UTR are shown. (B) Co-transfection of miR-34a and the wild-type PDGFR-α/β 3′ UTR caused a significant decrease in luciferase activity compared with the controls. (C) Co-transfection of miR-34a and the mutant PDGFR-α/β 3′ UTR plasmids (including all mutants) did not cause a decrease in luciferase activity. The data represent the mean $\pm$ S.D.;*$P < 0.01$ compared with the controls.

cancer and that decreased miR-34a expression is associated with gastric cancer metastasis.

miR-34a directly targets PDGFR-α/β

We predicted that the miR-34a targeted PDGFR-α/β using prediction tools and Western blot analysis. To further confirm

that miR-34a directly targets PDGFR-α/β, we performed luciferase reporter assays to examine whether miR-34a interacts directly with PDGFR-α/β. We identified two potential binding sites for miR-34a in the 3′ UTR of the PDGFR-α mRNA and one potential binding site for miR-34a in the 3′ UTR of the PDGFR-β mRNA (Figure 3A). To determine whether PDGFR-α/β is regulated by miR-34a through direct binding to the 3′
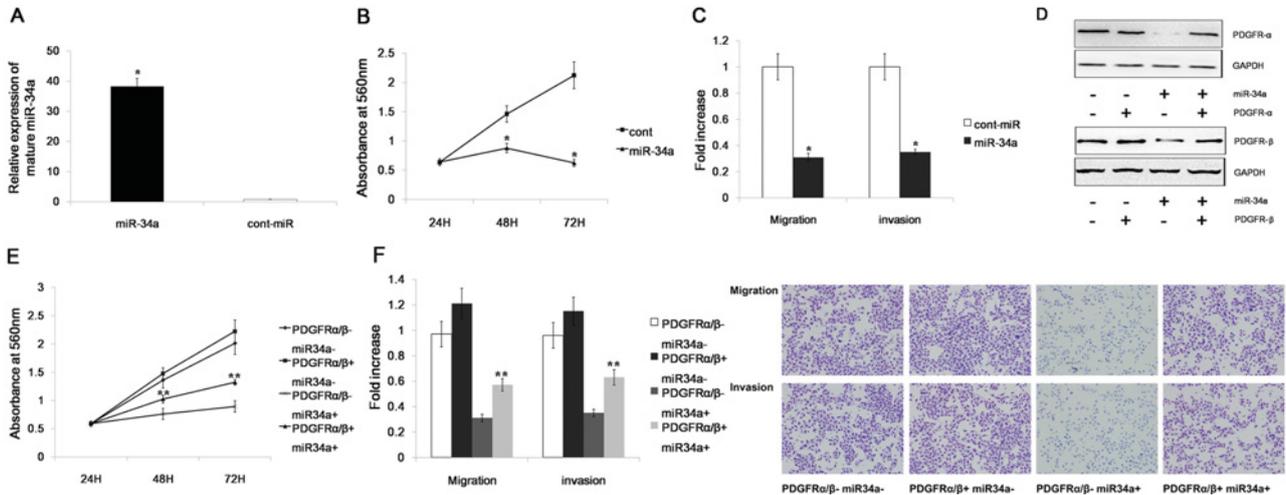
Figure 4 miR-34a inhibits gastric cancer cell migration, invasion and proliferation, in part, by targeting PDGFR
(A) Transient transfection of miR-34a mimics significantly increased miR-34a expression in the AGS cell line. (B) The proliferation of AGS cells was significantly reduced after miR-34a transfection compared with cont-miR transfection.(C) miR-34a significantly reduced the migration and invasion of the AGS cells compared with the controls. (D) Immunoblot analysis of PDGFR expression in AGS cells transfected with miR-34a mimics or cont-miR with or without PDGFR restoration. PDGFR-α/β expression was significantly decreased after transfection with miR-34a mimics, but PDGFR-α/β expression was restored by the overexpression of PDGFR-α/β. (E,F) Gastric cancer cell migration, invasion and proliferation were partially restored after PDGFR restoration. The data represent the mean $\pm$ S.D.; *$P < 0.05$ compared with the controls. ** $P < 0.05$ compared with other groups.

UTR of PDGFR-α/β, we constructed a series of 3′ UTR fragments, including the full-length wild-type PDGFR-α 3′ UTR, a binding site 1 mutant, a binding site 2 mutant, the full-length wild-type PDGFR-β3′ UTR, and a PDGFR-β binding site mutant. We found that co-transfection of miR-34a and the wild-type PDGFR-α/β 3′ UTR caused a significant decrease in luciferase expression compared with the controls (Figure 3B). However, co-transfection of miR-34a and the mutant PDGFR-α/β3′ UTR did not cause a decrease in luciferase expression (Figure 3C). These results suggest that miR-34a can directly target PDGFR-α/β.

miR-34a inhibits gastric cancer cell migration, invasion and proliferation partially by targeting PDGFR

To determine the functional significance of miR-34a overexpression in gastric cancer, we transfected the AGS gastric cancer cell line with miR-34a mimics. miR-34a was significantly overexpressed in the AGS cell line after transfection of the miR-34a mimic compared with the cont-miR-transfected cells (Figure 4A). The cells with forced miR-34a expression exhibited significantly decreased proliferation compared with the cells with forced expression of cont-miR(Figure 4B). Transwell migration and Matrigel invasion assays demonstrated that miR-34a significantly reduced the migration and invasion of AGS cells (Figure 4C). As PDGFR is frequently up-regulated in gastric cancer and promotes cell migration and invasion, and as miR-34a can directly regulate

the expression of PDGFR, we next ascertained whether the reduction of PDGFR expression could explain the inhibition of gastric cancer cell migration, invasion and proliferation observed after the forced expression of miR-34a.We therefore forced the expression of miR-34a in AGS cells that were transfected with a construct containing the PDGFR-α/β coding sequence but lacking the 3′ UTR of the PDGFR-α/β mRNA. As a result, this construct yielded a PDGFR-α/β mRNA that was resistant to miR-34a. The restoration of PDGFR-α/β expression was confirmed through immunoblot analysis (Figure 4D).We found that gastric cancer cell migration, invasion and proliferation were partially restored in the AGS cells after the forced expression of miR-34a and the restoration of PDGFR-α/β (Figures 4E and 4F). However, the inability of PDGFR-α/β restoration to completely restore these tumourigenic qualities indicates that there may be other oncogenes regulated by miR-34a in gastric cancer cells. Therefore we conclude that miR-34a regulates gastric cancer cell migration, invasion and proliferation, in part, by targeting PDGFR.

miR-34a inhibits gastric cancer tumourigenesis by targeting PDGFR and MET

We predicted 304 targets for miR-34a using the following prediction tools: Miranda (http://www.microrna.org), TargetScan (http://www.targetscan.org/) and miRDB (mirdb.org/miRDB/). In these 304 targets, we found dozens of genes associated with tumours, such as notch1, SATB2, E2F5 and MET. Some studies showed that miR-34a is associated with MET in some tumours
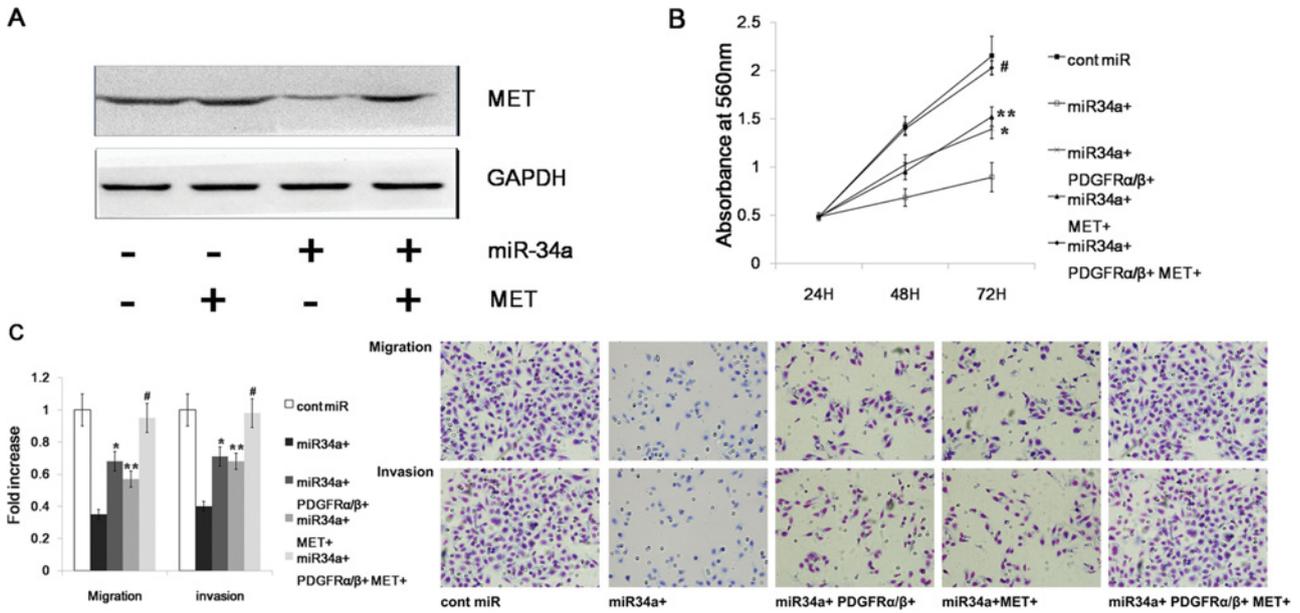
Figure 5   miR-34a can inhibit gastric cancer migration, invasion and proliferation by targeting PDGFR and MET
(A) MET expression was significantly decreased after transfection with miR-34a mimics, but MET expression could be restored by overexpressing MET. (B,C) The tumourigenic qualities of MKN45 cells were inhibited by the forced expression of miR-34a and were partially restored by the overexpression of MET or PDGFR. MKN45 gastric cancer cell migration, invasion and proliferation were completely restored only after overexpressing both PDGFR and MET.

[22,23]. Thus, we inferred that miR-34a might curb gastric cancer tumourigenesis by targeting MET and PDGFR. We constructed plasmids containing the MET coding sequence but lacking the 3′ UTR of the MET mRNA. The restoration of MET expression was confirmed through immunoblot analysis (Figure 5A). The forced expression of PDGFR or MET could partially restore gastric cancer cell migration, invasion and proliferation, and these parameters were completely restored after overexpressing both PDGFR and MET (Figures 5B and 5C).

miR-34a regulates the phosphorylation of Akt via PDGFR and MET

PDGFR and MET act as RTKs (receptor tyrosine kinases) and play pivotal roles in promoting cellular growth and proliferation by transducing extracellular stimuli to intracellular signalling circuits [24,25]. A prominent component of the intracellular signalling machinery is the PI3K (phosphoinositide 3-kinase)/Akt (PKB)/mTOR (mammalian target of rapamycin) [PI3K/Akt(PKB)/mTOR] pathway [26,27]. Because PDGFR and MET are downstream targets of miR-34a, we assumed that miR-34a could decrease the phosphorylation of Akt by targeting PDGFR and MET. Thus, we examined the phosphorylation of Akt after overexpressing miR-34a and found that the phosphorylation of Akt was significantly decreased. The phosphorylation of Akt was restored after overexpressing both PDGFR and MET (Figure 6). Therefore, we conclude that miR-34a regulates the phosphorylation of Akt via PDGFR and MET.

DISCUSSION

We found 16 miRNAs that can target both PDGFR-α and PDGFR-β using prediction tools, and only the expression of miR-29b, miR-449a/b and miR-34a/c were decreased in gastric cancer cell lines compared with normal GES1 cells. Thus, these miRNAs may target both PDGFR-α and PDGFR-β. Further study showed that only miR-34a could reduce the expression of both PDGFR-α and PDGFR-β. miR-34a is associated with various cancers. miR-34a inhibits breast cancer proliferation, the ratio of cells in S phase and tumour formation by targeting LMTK3 (lemur tyrosine kinase 3) [28]. Ma et al. also found that miR-34a not only reduced cell proliferation, but also inhibited the tumourigenicity of colon cancer cells by targeting PAR2 (proteinase-activated receptor 2) [29]. In HCC cell lines, miR-34a reduced cell viability, promoted cell apoptosis and enhanced sorafenib-induced apoptosis and toxicity by inhibiting Bcl-2 expression [30]. Moreover, the forced expression of miR-34a inhibited cell growth and induced apoptosis in pancreatic cancer cells with a concomitant down-regulation of the Notch-1 signalling pathway [31]. In this study, we found that miR-34a expression was decreased in tumour tissues compared with adjacent normal mucosa tissues. Meanwhile, miR-34a expression levels were lower in patients with metastases than in patients without metastases. This finding shows that miR-34a may act as a potential tumour suppressor in gastric cancer and is associated with the mechanisms of gastric cancer metastasis.

Overexpression of miR-34a could reduce PDGFR expression, and luciferase reporter assays also revealed that miR-34a could
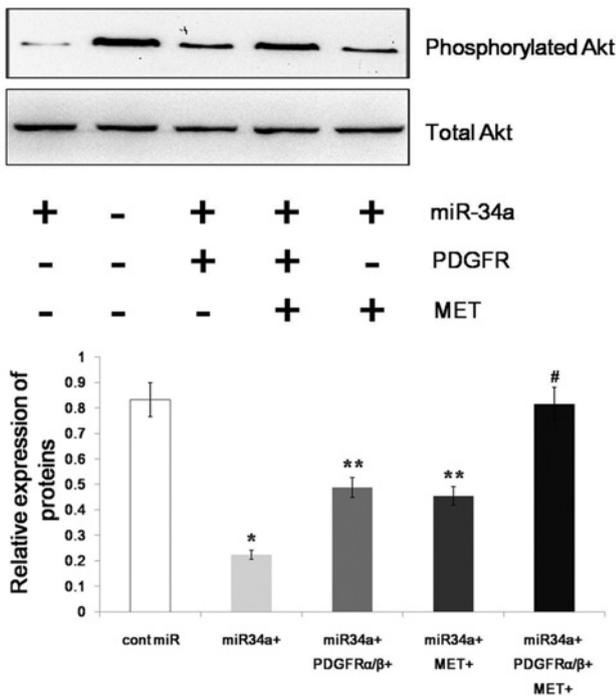
Figure 6 miR-34a regulates the phosphorylation of Akt by targeting PDGFR and MET

The phosphorylation of Akt was inhibited by the forced expression of miR-34a and was partially restored by the overexpression of MET or PDGFR. Overexpressing both MET and PDGFR could completely restore the phosphorylation of Akt. The data represent the mean $\pm$ S.D.; * $P < 0.01$, **$P < 0.05$, #$P > 0.05$ compared with the controls.

interact with the PDGFR-α/β 3′ UTR. These findings indicate that miR-34a directly targets PDGFR-α/β. Overexpressed PDGFR is closely correlated with tumour invasion and patient prognosis in gastric cancer [32]. Thus, we hypothesized that miR-34a regulates PDGFR and can inhibit gastric cancer cell migration, invasion and proliferation; our results confirmed this hypothesis. To our surprise, gastric cancer cell tumourigenic qualities could not be completely restored by PDGFR-α/β overexpression, and gastric cancer cell migration, invasion and proliferation were only partially restored after PDGFR-α/β overexpression. Thus, there may be other oncogenes regulated by miR-34a in gastric cancer cells.

Using a bioinformatics approach and a literature review, we found that MET was the most likely target gene of miR-34a. The receptor tyrosine kinase for hepatocyte growth factors, MET, is a membrane receptor for the HGF (hepatocyte growth factor) [33]. MET tyrosine kinase is known to promote the survival of many cell types following exposure to various apoptotic inducers, including serum starvation, death receptor activation or genotoxic stress [34]. In gastric cancer, MET overexpression is an independent prognostic factor and potential drug target, and MET overexpression could predict which patients may benefit from targeted therapy with MET inhibitors [35]. Chi et al. also found that the expression of c-Met was significantly associated with

gastric cardia adenocarcinoma differentiation, TNM and metastasis [36]. Thus, we hypothesized that miR-34a inhibits gastric cancer tumourigenic qualities by targeting MET and PDGFR. Our experiments showed that overexpressing both PDGFR and MET completely restored the gastric cancer tumourigenic qualities, but the forced expression of PDGFR or MET alone only partially restored the gastric cancer tumourigenic qualities. These findings showed that miR-34a could inhibit gastric cancer tumourigenic qualities by targeting PDGFR and MET expression.

Studies have shown that PDGFR-α/β regulates tumour migration, invasion and proliferation via the PI3K/Akt pathway [37–39]. MET can also induce gastric cancer tumourigenesis abilities through the activation of the PI3K/AKT pathway [24]. In this study, we found that miR-34a significantly attenuated the phosphorylation of Akt, and the phosphorylation of Akt was completely restored after overexpressing both PDGFR and MET. The forced expression of PDGFR or MET only partially restored the phosphorylation of Akt. This result suggests that miR-34a regulates the phosphorylation of Akt via PDGFR and MET in gastric cancer.

In conclusion, our results showed that miR-34a expression was decreased in gastric cancer and was associated with the metastasis of gastric cancer. Thus, miR-34a may act as a potential tumour suppressor in gastric cancer and is associated with the mechanisms of gastric cancer metastasis. Furthermore, miR-34a can inhibit gastric cancer tumourigenesis by targeting PDGFR and MET through the PI3K/Akt pathway. Thus, further studies into the anticancer mechanisms of miR-34a may contribute to the development of new therapeutic strategies for gastric cancer.

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