A miR-567-PIK3AP1-PI3K/AKT-c-Myc feedback loop regulates tumour growth and chemoresistance in gastric cancer

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A R T I C L E  I N F O

Article history:
Received 28 March 2019
Received in revised form 19 April 2019
Accepted 2 May 2019
Available online 9 May 2019

Keywords:
miRNA-567
Gastric cancer
Chemoresistance
Tumour growth
Prognostic biomarker

A B S T R A C T

Background: Gastric cancer (GC) ranks the fifth most common cancer, and chemotherapy is one of the most common treatments for GC. However, chemoresistance limits the effectiveness of chemotherapy and leads to treatment failure. This study aims to investigate the biological effect of miR-567 on gastric tumorigenesis and chemoresistance and reveal the possible mechanisms.

Methods: We measured the expression of miR-567 in 37 paired normal and stomach tumour specimens, as well as GC cell lines by Real-time PCR. The functional effects of miR-567 were validated using in vitro and in vivo assays. Dual-luciferase report assays and Chromatin immunoprecipitation (ChIP) assay were conducted for target evaluation, western blot assay was used to confirm the relationships.

Findings: Our data showed that miR-567 was downregulated in gastric tissues and gastric cancer cells compared with normal tissues and gastric epithelial cells. In vitro, Gain- and lose-of-function assays showed miR-567 not only weakened cells proliferative ability, but also sensitized GC cells to 5-FU and oxaliplatin. In vivo, miR-567 overexpression significantly repressed the tumorigenesis of GC cells compared with the vector control. Mechanistic analysis showed that PIK3AP1 activated AKT phosphorylation in GC. Meanwhile, miR-567 directly targeted PIK3AP1 to inactivate PI3K/AKT/c-Myc pathway and c-Myc inversely regulated miR-567 expression, thus forming a miR-567-PIK3AP1-PI3K/AKT-c-Myc feedback loop explaining the function of miR-567.

Interpretation: Our studies revealed that miR-567 acts as a tumour suppressor gene and suppresses GC tumorigenesis and chemoresistance via a miR-567-PIK3AP1-PI3K/AKT-c-Myc feedback loop. These results suggest that miR-567 may serve as a target for chemoresistance and a potential prognostic biomarker for GC.

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1. Introduction

Gastric cancer (GC) is the fifth most incident and the third most common cause of cancer-related death in the world [1]. The development of GC is a complex multistep process, including numerous genetic and epigenetic changes. GC is also affected by environmental factors, such as helicobacter pylori (Hp) infection [2]. In the past few years, advances in surgical techniques, radiotherapy, chemotherapy and targeted molecular therapy have improved the prognosis of GC [3,4]. However, early diagnosis of GC is a significant challenge, and advanced or recurrent GC patients have poor survival rates because of chemother-apy resistance [5–8]. Therefore, gastric cancer is a threat to global health and there is an urgent need for early diagnosis marker as well as solving the chemoresistance problem. Recently, a growing number of miRNAs have been proved to be closely correlated with GC [6], but the specific molecular mechanisms underlying the their function in cancer cells are still under investigation.

microRNAs (miRNAs or miRs), a class of small non-coding RNAs of 19–25 nucleotides in length, are believed to regulate gene expression by binding miRNAs to the 3′ untranslated region (3′UTR) of mRNAs, thereby leading to mRNA degradation or blocking of mRNA translation.

https://doi.org/10.1016/j.ebiom.2019.05.003
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It has been estimated that over 30% of human genes are regulated by miRNAs [13,14]. Acting as tumour suppressor genes or oncogenes, miRNAs are involved in many cellular processes, such as differentiation, proliferation, metastasis and chemoresistance [15–18]. For example, miR-424-5p, miR-125b, miR-21 and miR-17-92 have been recognized as regulators of GC cell tumourigenesis and drug resistance [19–23].

miR-567 was previously reported to target FGF5 and inhibit cell proliferation, migration and invasion in osteosarcoma [24]. Meanwhile, researches have revealed that miR-567 acted as a tumour suppressor gene and inhibited the carcinogenesis of breast cancer [25–28]. However, the biological role of miR-567 and its molecular mechanism underlying GC progression still remains unknown. In our study, we found miR-567 was obviously down-regulated in GC tissue and GC cells compared with adjacent non-cancer tissues and gastric epithelial cells, suggesting it playing a suppressive role in GC tumour progression. Indeed, we discovered that miR-567 inhibits GC cell proliferation and enhanced chemotherapeutic sensitization to 5-FU and oxaliplatin. In addition, our findings showed an atypical miR-567-PIK3AP1-PI3K/AKT-c-Myc feedback loop, which inhibited GC cell proliferation and sensitized GC cells to 5-FU and oxaliplatin. Altogether, these results provide a miR-567-mediated mechanism to modulate GC cell growth and chemoresistance. This pathway suppresses proliferation and sensitizes CRC cells to 5-FU and oxaliplatin. All in all, these results provide a mechanism by which miR-567 modulates CRC cell growth.

2. Materials and methods

2.1. Cell culture and treatment

A series of GC cell lines (GES-1, MKN45, BGC823, AGS, MGC803, BGC803, MKN28) were obtained from Foleibao Biotechnology Development (Shanghai, China). The cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (NBCS) (PAA Laboratories, Inc., Pasching, Austria). All of these cell lines were incubated in a humidified chamber with 5% CO2.
2.2. Clinical samples

Fresh primary GC specimens with paired normal gastric tissues were obtained from the Tumor Tissue Bank of Nanfang Hospital. There are at least 1 cm space between the cancer tissues and the paired normal tissues. In each case, pathological diagnosis was made after elective surgery for GC in Nanfang Hospital during 2009 and 2014. All experiments performed are endorsed by the Ethics Committee of Southern Medical University and complied with the Declaration of Helsinki. No informed consent was required because data were going to be analysed anonymously.

2.3. Animals

All animal experiments were carried out with the approval of the Southern Medical University Animal Care and Use Committee in accordance with the guidelines for the ethical treatment of animals. Nude nude mice were maintained in a barrier facility in racks of Southern Medical University Animal Care and Use Committee in accordance with the Declaration of Helsinki. No informed consent was required because data were going to be analysed anonymously.

2.4. Western blot analysis

Protein expression was assessed by immunoblot analysis of cell lysates (20–40 μg) in RIPA buffer in the presence of rabbit antibodies to GAPDH (1:1000; Santa Cruz, California, USA); rabbit antibody to PI3K (1:1000; Proteintech); rabbit antibodies to P13K, P-PI3K, P-akt(Ser473)(#4060), AKT(#4691) (1:1000; CST, Danvers, MA); mouse antibodies to CCND1 (1:1000; Proteintech); rabbit antibodies to c-Myc (1:1000; Abclone). Relative protein abundance of phosphorated proteins was determined by normalisation with levels of corresponding non-cancer tissues, which is at least 1 cm away. In each case, pathological diagnosis was made after elective surgery for GC in Nanfang Hospital during 2009 and 2014. All experiments performed are endorsed by the Ethics Committee of Southern Medical University and complied with the Declaration of Helsinki. No informed consent was required because data were going to be analysed anonymously.

3. Results

3.1. miR-567 is down-regulated in GC tissues and cell lines

Real-time PCR was used to measure the expression of miR-567 in GC tissues and gastric cell lines. We detected a significant decrease of miR-567 mRNA expression in GC tissues compared with corresponding non-cancer tissues, which is at least 1 cm away from the cancer region (Fig 1A). We further compared the miR-567 expression levels in GC cell lines with normal gastric epithelium cell line GES-1, which is non-malignant and none-tumourigenic cell lines [29,30]. A relatively lower mRNA expression was observed in GC cell lines (Fig 1B).

3.2. Exogenous miR-567 suppresses GC cell proliferation, colony formation, chemoresistance to 5-FU and oxaliplatin in vitro

MGCG03 and BGC823 cell lines were selected for further experiments as they have moderate endogenous expression levels of miR-567. We transfected miR-567 mimic oligonucleotides and anti-miR-567 into MGC803 and BGC823 cell lines to evaluate their effects on cellular behaviours. Real-time PCR was performed to detect the transfection efficiency (Fig. 1C). Subsequently, we examined the influence of miR-567 on GC cells using cell counting kit-8 (CCK-8) assay (Fig. 1D), colony formation assay (Fig. 1E), cell-cycle assay (Fig. 2A& S1) and EdU incorporation assay (Fig. 2B). The results showed that overexpressed miR-567 significantly suppressed cell growth and G1 to S cell cycle transition in MGC803 and BGC823 cells, which suggested that miR-567 inhibited cell proliferation by arresting the tumour cells at G1/G0 phase. By contrast, suppression of miR-567 markedly promoted cell proliferation and induced G1/S transition. GC cell lines overexpressing miR-567 exhibited significantly increased sensitivity to 5-FU and oxaliplatin, while inhibition of miR-567 expression caused the opposite effects (Fig. 2C & D). To further investigate the mechanism behind miR-567 suppression of chemoresistance and cell proliferation, we examined the protein expression of pro-apoptosis and cell cycle biomarkers in GC cells after miR-567 overexpression and knockdown, as shown in Fig. 2E, miR-567 increased the protein expression of cleave caspase-3, cleave caspase-9 and cleave PARP, suggesting miR-567 promoted chemosensitivity by inducing cell apoptosis. Furthermore, we found miR-567 expression was positively correlated with the protein expression of p21, while negatively correlated with the protein expression of CDK4, CDK6 and CyclinD1 in GC cells, indicating miR-567 may suppress GC cell proliferation by regulating the expression of cell cycle marker P21, CDK4, CDK6 and CyclinD1.

3.3. Endogenous overexpression of miR-567 inhibits GC growth and progression in vivo

We used a lentivirus (LV)-based system to investigate the biological function of miR-567 in vivo. LV-miR-567 was used to infect MGC803 cells and establish GC cell lines with stable miR-567 overexpression. Real-time PCR assay confirmed a remarkable increase of miR-567 expression in LV-miR-567-transfected cells compared with LV-miR-NC-transfected cells (Fig. S2; P < .05). We used LV-miR-567-overexpressing MGC803 (MGC803/LV-miR-567) cells and control cells to perform a tumourigenesis assay in nude mice. Tumours in the MGC803/LV-miR-567 group grew more slowly than those in the MGC803/LV-NC group (Fig 2F, left panel). Moreover, tumour
in the MGC803/LV-miR-567 group grew more quickly than those in control group ($P < .01$). Immunohistochemistry (IHC) confirmed that the tumours of MGC803/LV-miR-567 group also showed significantly lower Ki-67 index compared with control group, suggesting that miR-567 overexpression markedly decreased cell proliferation (Fig. 2F, right panel).

**Fig. 3.** PIK3AP1 is the direct target of miR-567 and promotes GC cell proliferation and increases drug sensitivity. (A) Real-time PCR analysis were performed to detect the mRNA expression of candidate genes in MGC803 and BGC823 cells transfected with miR-567 mimic, Student’s $t$-test, mean ± SD. (B) miR-567 and its putative binding sequences in the 3′ UTR of PIK3AP1. A mutation was generated in the complementary site that bound to the seed region of miR-567. Luciferase reporter assay was used to determine miR-567 direct targeting the PIK3AP1 3′ UTR, Student’s $t$-test, mean ± SD, ***$P < .001$. (B) Western blot analysis were performed to detect the protein expression of PIK3AP1 in MGC803 and BGC823 cells, both transfected with miR-567 mimic or anti-miR-567. CCK-8 assay (C), colony formation assay (D), FACS assays (E) and EdU incorporation assays (F) of GC cells were performed after transfected with PIK3AP1 or pENTER vector, Student’s $t$-test, mean ± SD, *$P < .05; **P < .01$. (H) Western blot experiments were used to analyse the expression of pro-apoptosis proteins, cell cycle marker and related proteins in PI3K/AKT pathway after miR-567 knockdown and overexpression in MGC803 and BGC823 cells. (I) Dose-response curves of MGC803 and BGC823 treated with 5-FU for 36 h or oxaliplatin for 24 h, the cells were previously transfected with PIK3AP1 and pENTER. Parametric generalized linear model with random effects, Student’s $t$-test, mean ± SD, *$P < .05; **P < .01; ***P < .001$.}
3.4. PIK3AP1 is the direct target of miR-567

In order to identify the potential target gene of miR-567, we performed a bioinformatic analysis using eight databases (miRWalk, Mircot4, miRanda, miRDB, miRMap, PITA, RNAhybrid and Targetscan). After selecting the cancer-related genes that have not been reported in gastric cancer, we screened out 15 candidate genes from the common target genes predicted in these databases. Subsequently, real-time PCR assay showed that PIK3AP1 showed the most significant fold decrease among these genes after miR-567 overexpression (Fig. 3A). Therefore, PIK3AP1 were identified as a potential target of miR-567.

The analysis showed that miR-567 targeted PIK3AP1 by binding to the 3′-UTR region of PIK3AP1 (Fig. 3B above). Subsequently, dual-luciferase reporter assays were performed to determine whether miR-567 could directly target the 3′-UTR region of PIK3AP1. 3′-UTR fragment (wt) of PIK3AP1 containing miR-567 binding site and their mutant fragments (mut) were cloned into luciferase report vectors psiCHECK-2. The empty psiCHECK-2, wt or mut 3′-UTR vector and miR-567 were co-transfected into MGC803, BGC823 and 293 T cell lines. The result showed that miR-567 markedly attenuated the luciferase activity of wide-type PIK3AP1 3′-UTR, whereas the effect was abrogated after the 3′-UTR binding site of PIK3AP1 was mutated (Fig. 3B below). Moreover, western blot assay showed that both PIK3AP1 protein expression decreased in miR-567-overexpressing GC cells but increased after miR-567 inhibition (Fig. 3C & S3). These findings reveal the potential significance of miR-567 as a determinant of PIK3AP1 expression in GC cells.

3.5. PIK3AP1 promotes GC cell proliferation and chemosensitivity

Given that miR-567 targeted PIK3AP1 and suppressed its expression, we investigated the biological function of PIK3AP1 in GC cells. Data from Kaplan-Meier plotter database were utilized to visualize the association between PIK3AP1 expression and overall survival in patients with GC. The results showed patients with high PIK3AP1 expression in tumours had a trend towards poorer survival when compared with patients showed low PIK3AP1 expression (Fig.54; HR = 1.3, P = .018). CCK8 assay (Fig. 3D), colony formation assay (Fig. 3E), cell-cycle analysis (Fig. 3F & S5) and EdU incorporation assays (Fig. 3G) showed that over-expression of PIK3AP1 significantly enhanced the proliferative ability of MGC803 and BGC823 cells and increased the cells in S phase and G2 phase. Furthermore, PIK3AP1 overexpression induced chemoresistance to 5-FU and oxaliplatin in MGC803 and BGC823 cells (Fig. 3I). As shown in Fig. 4H, western blot assay showed PIK3AP1 decreased the protein expression of cleave caspase-3, cleave caspase-9 and cleave PARP, suggesting PIK3AP1 suppressed chemosensitivity by prevent cell apoptosis. Moreover, PIK3AP1 expression was negatively correlated with the protein expression of p21, while positively correlated with the protein expression of CDK4, CDK6 and CyclinD1 in GC cells, suggesting miR-567 promoted GC cell proliferation by mediated the expression of cell cycle markers.

3.6. miR-567 negatively regulates PI3K/AKT-c-Myc pathway in GC cells

To identify the oncogenic signalling related to PIK3AP1, we applied GSEA to GC cases (GSE57303). The result showed that PIK3AP1 was positively associated with PI3K/AKT signalling in the GC cohort (Fig. 4A; P = .0208). As expected, western blot assay showed PIK3AP1 significantly increase phosphorylation levels of p-Akt, p-Pi3K and Pi3K/AKT downstream target c-Myc, indicating that PIK3AP1 regulated PI3K/AKT signalling pathway (Fig. 4B). Thus, we speculated that miR-567 is an upstream regulator of PI3K/AKT signalling. Indeed, Western blot assay showed low phosphorylation levels of p-Akt and p-Pi3K, with no change in the total protein amount of Akt and Pi3K, and down-regulation of c-Myc in GC cells treated with miR-567 mimic compared...
with control cells. Also, we observed opposite results of p-Akt, p-PI3K and c-Myc expression in GC cells treated with anti-miR-567 (Fig. 4C). Western blot results confirmed that miR-567 also regulated PI3K/AKT-c-Myc pathway in tumours from MCC803/LV-miR-567 cells (Fig. 4D). Furthermore, treatment with LY294002 was sufficient to neutralize the strengthened role of anti-miR-567 in PI3K/AKT-c-Myc pathway (Fig. 4E). In contrast, introduction of PIK3AP1 could rescue to activate the suppressed pathway (Fig. 4F). These results suggested an inhibitory role of miR-567 in GC proliferation, at least in part, by inhibiting PI3K/AKT-c-Myc pathway.

3.7 PIK3AP1 is essential to miR-567-mediated suppression of GC cell behaviour and oncogenic signalling

Rescue experiments showed that transiently transfecting PIK3AP1 into miR-567-overexpressing GC cells significantly restored miR-567-mediated suppression of proliferation and promoted G1/S and S/G2 cell cycle transition (Fig. 5A-D & S6). Furthermore, we observed that PIK3AP1 overexpression rescued miR-567-mediated suppression of CDK4, CDK6, and CyclinD1, but not p21 (Fig. 5E). Meanwhile, CCK-8 assay showed that efficiently attenuated miR-567-induced sensitivity to 5-FU and oxaliplatin (Fig. 5F). Consistent with these result, we observed PIK3AP1 markedly decreased miR-567-induced apoptosis and reduced the protein expression of cleave caspase-3, cleave caspase-9 and cleave PARP (Fig. 5E). These findings indicate that PIK3AP1 overcomes cell proliferation suppression and drug sensitivity induced by miR-567.

3.8 c-Myc inhibits miR-567 expression by binding to its promoter region

To examine whether miR-567 was inversely regulated by c-Myc, we used small-interfering RNAs (siRNA) to suppress c-Myc expression in MGC803 and BGC823 cells. Real-time PCR analysis showed increasing miR-567 expression after c-Myc knock-down, indicating that c-Myc is an upstream regulator of miR-567 (Fig. 6C). Moreover, Subsequent real-time PCR analysis showed that LY294002 and PIK3AP1 siRNAs significantly increased miR-567 expression (Fig. 6A & D), while PIK3AP1 overexpression reduced miR-567 expression (Fig. 6B), indicating PIK3AP1 and PI3K/AKT pathway can inversely regulated miR-567 expression. Taken together, we discover a mechanism that miR-567...
expression is suppressed by c-Myc, PI3K/AKT pathway and PIK3AP1, thereby forming a complex miR-567-PIK3AP1-PI3K/AKT-c-Myc regulatory feedback loop.

Chromatin immunoprecipitation (ChIP) assay was further used to determine whether c-Myc bound to the miR-567 promoter in MGC803 and BGC823 cells. Indeed, DNA from the immunoprecipitated chromatin showed an obvious enrichment of this specific region compared with negative control (IgG) pulldown, and the enrichment as abrogated when cell were treated with LY294002 (Fig. 6E). To investigate the transcriptional regulatory mechanism of miR-567 expression, we used JASPAR (http://jaspar.genereg.net) to analyse a 2-kb region upstream of the miR-567. One putative c-Myc-binding site (from 1504 to 1511) was predicted in human miR-567 promoter region (Fig. 6F). A reduction of the wild-type miR-567 promoter luciferase activity was observed on upregulation of c-Myc in 293 T, BGC823 and MGC803 cell lines (Fig. 6F; P < .001). Our data indicated c-Myc was involved in miR-567 transcription via binding to its promoter, and this process is negatively regulated by LY294002 which decrease c-Myc expression by suppressing PI3K/AKT pathway. Therefore, as the upstream regulators of c-Myc, AKT signalling and PIK3AP1 can inversely regulate miR-567 expression.

Finally, in order to prove our conclusion with more persuasive evidence, we conducted a real-time PCR assay, detecting the mRNA expression of miR-567, PIK3AP1 and c-Myc in 37 GC tissues and 37 paired adjacent normal tissue. Analysis of the results showed that miR-567 expression is negatively correlated with PIK3AP1 and c-Myc expression...
(Fig. 7A & B), but PIK3AP1 is positively correlated with c-Myc expression (Fig. 7C). Thus, the relationship among miR-567, PIK3AP1 and c-Myc is clearly identified.

4. Discussion

Although cancer cell proliferation and chemoresistance are the overwhelming causes of cancer mortality, a comprehensive picture of modular and cellular determinants governing these processes remains largely unknown. Multiple lines of evidence have proven that abnormal expression of miRNAs are linked to cancers tumourigenesis and drug resistance [32,33]. In our study, miR-567 was found to be markedly down-regulated in tumour tissues and GC cells compared with normal tissues and gastric epithelial cells. Subsequent experiments showed that miR-567 not only significantly inhibited cell proliferation in vitro and delayed xenograft tumour growth in vivo, but also sensitized GC cells to 5-FU and oxaliplatin via a miR-567-PIK3AP1-PI3K/AKT-c-Myc feedback loop. In short, our study firstly demonstrates that miR-567 is a novel suppressor gene in GC tumourigenesis and drug resistance and might present as a molecular biomarker for GC progression.

As a downstream target of miR-567 indicated in our study, PIK3AP1 is essential for miR-567-mediated suppression of GC cell behaviour and oncogenic signalling. PIK3AP1 is an adapter protein originally isolated from B cells. After tyrosine-phosphorylated on its four YxxM, PIK3AP1 binds and recruits PI3K to the membrane upon B-cell receptor (BCR) oligomerization to facilitate generation of PIP3 from PIP2, this process is essential for BCR-induced AKT phosphorylation [31,32]. In natural killer (NK) cells, PIK3AP1 plays a similar role in immunoreceptor tyrosine-based activation motif (ITAM)-mediated AKT phosphorylation [33]. These studies suggest PIK3AP1 is the upstream regulator of PI3K/AKT pathway, which is consistent with the GSEA analysis and experimental result in our study. In Fig. 3A, although BBS1, OLR1, PRKAR2B, DIS3, CPSF2 and FZD5 also showed different fold decrease after miR-567 overexpression, PIK3AP1 displayed the most significant fold decrease compared with decrease of other gene. Moreover, previous study and GSEA analysis suggested PIK3AP1 was associated with PI3K/AKT pathway, which was crucial for cell proliferation, metabolism and survival [34,35]. Thus, we speculated that PIK3AP1 played an important role in miR-567-mediated GC tumourigenesis and chemoresistance, and chose PIK3AP1 as the potential target of miR-567. Indeed, subsequent experiments proved that PIK3AP1 was essential to miR-567-mediated suppression of GC tumourigenesis and drug resistance.

In our study, c-Myc inhibited miR-567 expression by binding to its promoter region, thus formed a miR-567-PIK3AP1-PI3K/AKT-c-Myc feedback loop, by which miR-567 suppressed GC tumourigenesis and drug resistance. c-Myc is an oncogenic transcription factor playing a pivotal role in the control of cell proliferation, apoptosis and drug resistance [36–38]. Mutated c-Myc is observed in many cancers and resulted in persistent expression of c-Myc protein, which causes abnormal expression of many genes. A number of candidate c-Myc target genes regulate cell energy metabolism, cell cycle progression (particularly in G1 phase) and chemoresistance [37,38]. Meanwhile, c-Myc has been reported to promote drug resistance to 5-Fu and oxaliplatin in colon cancer stem cells (CSCs) via regulating the expression of ATP-binding cassette transporters [38], suggesting its role in chemoresistance to 5-Fu and oxaliplatin in gastric cancer. Indeed, our study showed that the miR-567-PIK3AP1-PI3K/AKT-c-Myc pathway involving c-Myc is closely associated with resistance to 5-Fu and oxaliplatin. Moreover, AKT activation is associated with the activation of c-Myc expression. The glycogen synthetase kinase 3 (GSK3) and forkhead transcription factor FKHRL1, both the downstream target of AKT signalling pathway, contribute to the upregulation of c-Myc expression [39]. Therefore, blocking PI3K/AKT pathway will lead to the reduction of c-Myc expression. As expected, we observed a markedly decrease of c-Myc protein expression in MGC803 and BGC823 cells after treatment with LY294002 (an inhibitor of the PI3K/AKT signalling pathway). In all, these results provide a mechanism explaining the association between c-Myc and GC tumourigenesis and drug resistance, and emphasize the important role of c-Myc in GC progression.
Studies have shown that a single microRNA may contain multiple transcription factor binding sites, thus being regulated by different transcription factors [40,41]. In our study, miR-567 expression level between different pairs of c-Myc siRNAs or PIK3AP1 siRNAs was not consistent with their knock-down efficiency, for example, si-575 (c-Myc siRNAs) got the best knock-down efficiency but the level of miR-567 do not increase a lot especially in MGC803 cell. This phenomenon confused us and we speculated it was probably because of the off-target effects of siRNA. For example, si-575 might not target c-Myc specifically and reduce the expression of other genes at the same time, which negatively regulated miR-567 expression, thus resulting in the unexpected increase results of miR-567 expression. Nevertheless, in our study, the c-Myc siRNAs and PIK3AP1 siRNAs showed overall efficient knockdown effects on miR-567 expression, proving that c-Myc and PIK3AP1 can inversely regulate miR-567 expression.

5. Conclusions

As summarized in our working model in Fig. 7D, miR-567 inhibits GC cell proliferation and enhances chemotherapeutic sensitisation to 5-FU and oxaliplatin via a positive feedback loop. As an upstream regulator of PIK3AP1, miR-567 blocks PI3K/AKT pathway by directly targeting PIK3AP1, c-Myc is regulated by AKT signalling and binds to the promoter region of miR-567, thus leading to transcription arrest of miR-567. Therefore, we find a novel miR-567-PIK3AP1-PI3K/AKT-c-Myc feedback loop, which explains the mechanism underlying miR-567-mediated tumourigenesis and chemotherapy resistance. Taken together, our study suggests that miR-567 might act as a biomarker and molecular target for the prevention of GC progression.

Acknowledgements

Not applicable.

Ethical approval and consent to participate

All experiments performed are endorsed by the Ethics Committee of Southern Medical University and complied with the Declaration of Helsinki. No informed consent was required because data were going to be analysed anonymously. All animal experiments were carried out with the approval of the Southern Medical University Animal Care and Use Committee in accordance with the guidelines for the ethical treatment of animals. All animal experiments involved ethical and humane treatment under a license from the Guangdong Provincial Bureau of Science.

Consent for publication

All authors consent for publication.

Availability of supporting data

Availability in supplementary data.

Funding sources

This work was supported by the National Natural Science Foundation of China (Nos. 81572813, 81773082, 81702903, 81872423), Guangdong Natural Science Foundation (2017A030310038, 2018B030311036), Fork Ying Tung Education Foundation (161035), Higher Education Fund Project of Guangzhou (2012C070) and Special Funds for the Cultivation of Guangdong College Students’ Scientific and Technological Innovation. (“Climbing Program” Special Funds) (pdjh0102). The funding institutions had no role in the study design, data collection, data analysis, interpretation or writing of the manuscript.

Declaration of interests

The authors have declared that no conflict of interest exists.

Author contributions

L. Z. led study design and prepared the manuscript; F. -F. Z. K., T. L. and X. -Q. Y. carried out the experiments; H. W and J. W performed statistical analysis; W. -D. L assisted in tissue sample collection; R. Z performed data analysis and interpretation; L. -J. X provided data collection. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.05.003.

References

[1] Figueiredo C, Camargo MC, Leite M, Fuentes-Panana EM, Rabkin CS, Machado JC. Pathogenesis of gastric cancer: genetics and molecular classification. Curr Top Microbiol Immunol 2017;400:277–304.
[2] Leal M, Wissneski F, de Oliveira Gigeck C, et al. What gastric cancer proteomic studies show about gastric carcinogenesis? Tumour Biol 2016;37(8):9991–10001.
[3] Newton A, Datta J, Looi-Conn Bonilla A, Karakousis G, Roses R. Neoadjuvant therapy for gastric cancer: current evidence and future directions. J Gastrointest Oncol 2015;6(5):534–43.
[4] Wang X, Wang Y, Qiu M, et al. Postoperative chemoradiotherapy in gastric cancer: a phase I study of radiotherapy with dose escalation of oxaliplatin, 5-fluorouracil, and leucovorin [POLIFORM regimen], Med Oncol 2011;28(Suppl. 1):S274–9.
[5] Shi M, Li K, Maskey N, et al. 15-PGDH expression as a predictive factor response to neoadjuvant chemotherapy in advanced gastric cancer. Int J Clin Exp Pathol 2015;8(6):6910–8.
[6] Shenkar N, Baradaran B, Shani-Habibi D, Kaziem T. Circulating miRNAs: valuable biomarkers for diagnosis and prognosis of gastric cancer. Curr Med Chem 2018;25(6):698–714.
[7] Park J, Kim S, Kim J, et al. Risk factors for early metachronous tumor development after endoscopic resection for early gastric cancer. PLoS One 2017;12(9):e0185501.
[8] Shi W, Gao J. Molecular mechanisms of chemoresistance in gastric cancer. World J Gastrointest Oncol 2016;8(9):673–81.
[9] Zhao Y, Shen X, Tang T, Wu C. Weak regulation of many targets is cumulatively pow- erful - an evolutionary perspective on microRNA functionality. Mol Biol Evol 2017;34(12):3041–6.
[10] Alamoudi A, Alhouny A, Gad H. miRNA in tumour metabolism and why could it be the preferred pathway for energy reprogramming. Brief Funct Genomics 2018;17(1):57–69.
[11] Murphy C, Singewald N. Potential of microRNAs as novel targets in the alleviation of pathological fear. Genes Brain Behav 2018;17(3):e12427.
[12] Djamali-Tchatoucha A, Ntshello K. Expression profile of stress-responsive Arabidopsis thaliana miRNAs and their target genes in response to inoculation with Pectobacterium carotovorum subsp. carotovorum. Pak J Biol Sci 2017;20(3):147–53.
[13] Filipowicz W, Bhattacharyya S, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 2008;9(2):102–14.
[14] Friedman J, Jones P. MicroRNAs: critical mediators of differentiation, development and disease. Swiss Med Wkly 2009;139(33–34):466–72.
[15] Brás-Rosário L, Matsuda A, Pinheiro A, et al. Expression profile of microRNAs regulating proliferation and differentiation in mouse adult cardiac stem cells. PLoS One 2013;8(5):e63041.
[16] Tsukita S, Yamada T, Takahashi K, et al. MicroRNAs 106b and 222 improve hyperglycemia in a mouse model of insulin-deficient diabetes via pancreatic γ-cell proliferation. EBioMedicine 2017;15:163–72.
[17] Palma Flores C, García-Vázquez R, Gallardo Rincón D, et al. MicroRNAs driving invasion and metastasis in ovarian cancer: opportunities for translational medicine (review). Int J Oncol 2017;50(3):1461–76.
[18] Gu J, Fang L, Huang Y, et al. Simultaneous overactivation of Wnt/β-catenin and TGFβ signalling by miR-128-3p confers chemoresistance-associated metastasis in NSCLC. Nat Commun 2017;8:15870.
[19] Zhang J, Liu H, Hou H, et al. Circular RNA LARP4 inhibits cell proliferation and invasion of gastric cancer by sponging miR-424-5p and regulating LAT51 expression. Mol Cancer 2017;16(11):151.
[20] Lei C, Du F, Sun L, et al. miR-143 and miR-145 inhibit gastric cancer cell migration and metastasis by suppressing MYO6. Cell Death Dis 2017;8(10):e3101.
[21] Wu J, Wang J, Jiang X, et al. miR-125b promotes cell migration and invasion by targeting PPP1CA-Rb signal pathways in gastric cancer, resulting in a poor prognosis. Gastric Cancer 2015;18(4):231–40.
[22] Zhang P, Chen L, Yuan X, et al. Exosomal transfer of tumor-associated macrophage-derived miR-21 confers cisplatin resistance in gastric cancer cells. J Exp Clin Cancer Res 2017;36(1):53.
[23] Cioffi M, Trabulo SM, Sanchez-Ripoll Y, et al. The miR-17-92 cluster counteracts quiescence and chemoresistance in a distinct subpopulation of pancreatic cancer stem cells. Gut 2015;64(12):1936–48.

[24] Liu D, Zhang C, Li X, Zhang H, Pang Q, Wan A. MicroRNA-567 inhibits cell proliferation, migration and invasion by targeting FGF5 in osteosarcoma. EXCLI J 2018;17:102–12.

[25] Shao Y, Liang B, Long F, Jiang S. Diagnostic MicroRNA biomarker discovery for non-small-cell lung Cancer adenocarcinoma by integrative bioinformatics analysis. Biomed Res Int 2017;2017:2563085.

[26] Wang F, Wong S, Chan L, Cho W, Yip S, Yung B. Multiple regression analysis of mRNA-miRNA associations in colorectal cancer pathway. Biomed Res Int 2014;2014:676724.

[27] Cava C, Bertoli G, Ripamonti M, et al. Integration of mRNA expression profile, copy number alterations, and microRNA expression levels in breast cancer to improve grade definition. PloS One 2014;9(5):e97681.

[28] Bertoli G, Cava C, Diceglie C, et al. MicroRNA-567 dysregulation contributes to carcinogenesis of breast cancer, targeting tumor cell proliferation, and migration. Breast Cancer Res Treat 2017;161(3):605–16.

[29] Zhou Z, Ji Z, Wang Y, et al. TRIM59 is up-regulated in gastric tumors, promoting ubiquitination and degradation of p53. Gastroenterology 2014;147(5):1043–54.

[30] Okada T, Maeda A, Iwamatsu A, Gotoh K, Kurosaki T. BCAP: the tyrosine kinase substrate that connects B cell receptor to phosphoinositide 3-kinase activation. Immunity 2000;13(6):817–27.

[31] Domínguez-Cáceres M, García-Martínez J, Calabrini A, et al. Prolactin induces c-Myc expression and cell survival through activation of Src/Akt pathway in lymphoid cells. Oncogene 2004;23(44):7378–90.

[32] Luo J, Xiang G, Pan C. Discovery of microRNAs and transcription factors co-regulatory modules by integrating multiple types of genomic data. IEEE Trans Nanobioscience 2017;16(1):51–9.