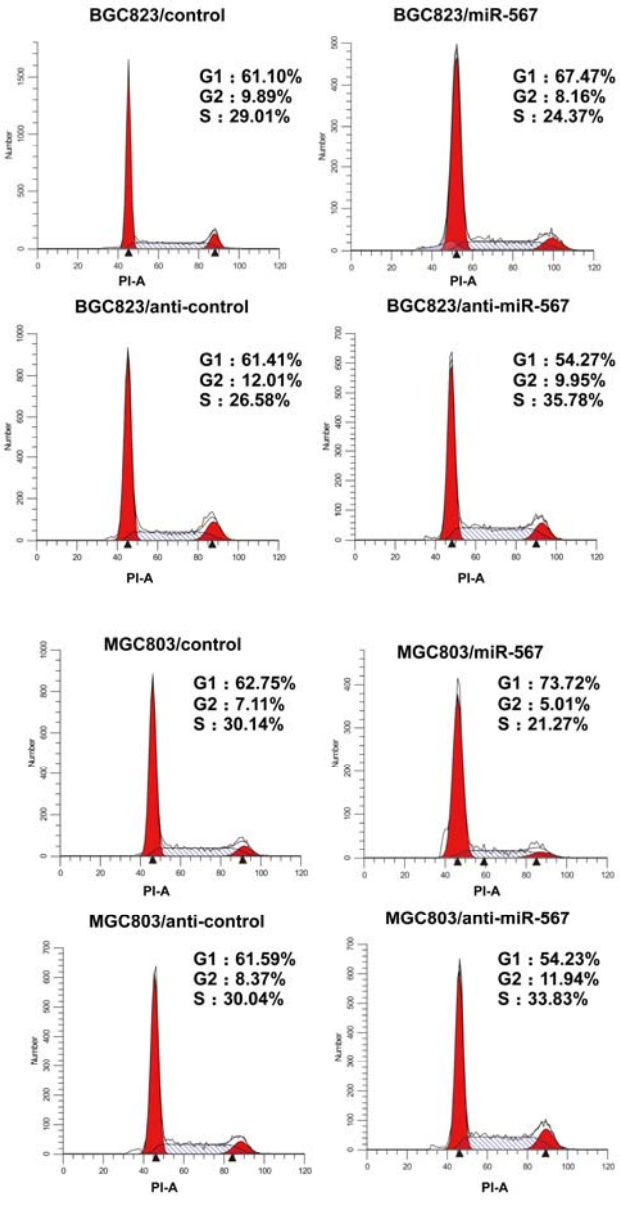
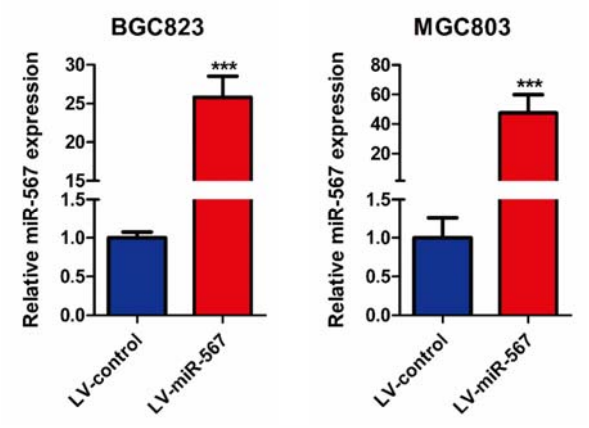
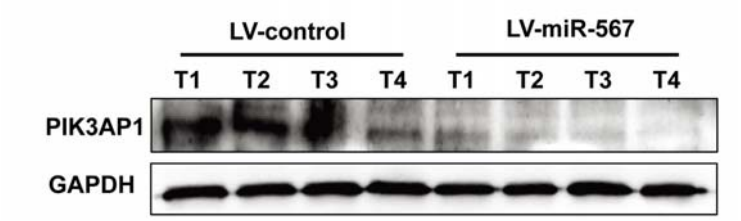
Supplementary Figures



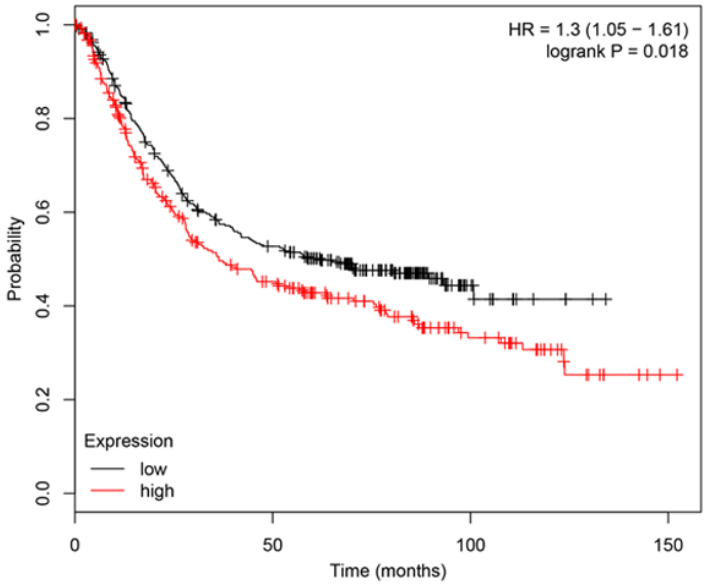
Supplementary Figure 1. Representative figures of cell cycle assay in Fig.2A.



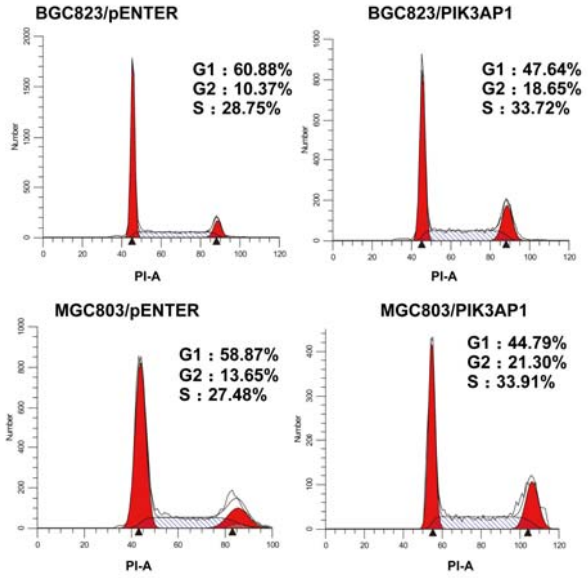
Supplementary Figure 2. Real-time PCR was performed to detect the mRNA expression of miR-567 in MGC803 and BGC823 cells, both transfected with LV-miR-NC or LV-miR-567, Student's t-test, mean ± SD, *** $P<0.001$.



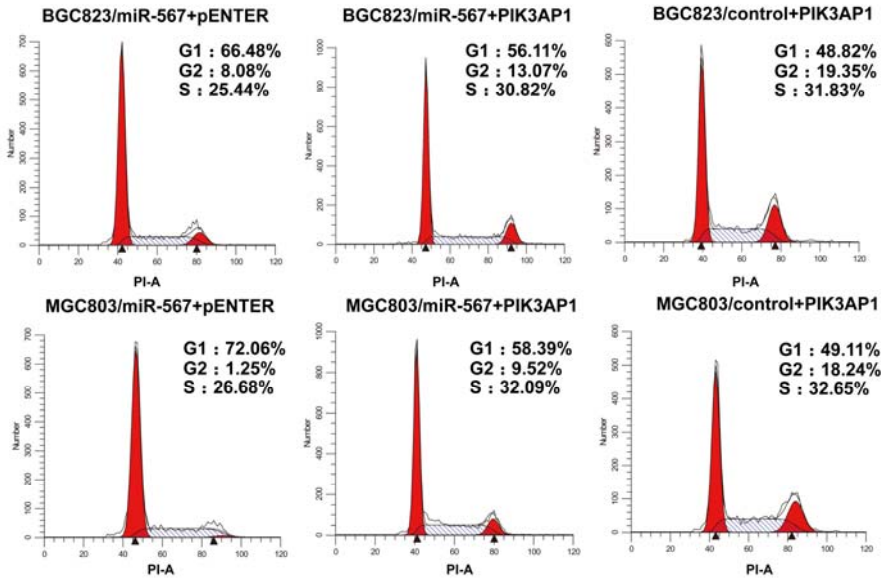
Supplementary Figure 3. Western blot was performed to detect the protein expression of PIK3AP1 in MGC803 cells after treated with LV-miR-NC or LV-miR-567.



Supplementary Figure 4. Kaplan-Meier survival plots showed that higher expression of PIK3AP1 resulted in a worse survival.



Supplementary Figure 5. Representative figures of cell cycle assay in Fig. 3F.



Supplementary Figure 6. Representative figures of cell cycle assay in Fig. 5C.

Supplementary Materials and Methods

RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen). To quantitate PIK3AP1 and c-Myc expression, total RNA was polyadenylated and underwent reverse transcription using PrimeScript™ RT Master Mix (TaKaRa, Dalian, China). Real-time PCR was carried out using a SYBR® Premix Ex TaqTM II (TaKaRa, Dalian, China) on an ABI 7500HT system. To quantitate miR-567 expression, total RNA was polyadenylated and underwent reverse transcription using an NCode miRNA First-Strand cDNA Synthesis kit (Invitrogen). Real-time PCR was carried out using an SYBR Green PCR master mix (Applied Biosystems; Foster City, Calif, USA) on an ABI 7500HT system. *GAPDH* or *U6* snRNA were used as an endogenous control. All samples were normalized to internal controls, and fold changes were calculated through relative quantification (2-ΔΔCT). The primers used are shown in Supplementary Table S1&S2.

Cell proliferation assays and colony formation

Cell proliferation assays were carried out using Cell Counting Kit 8 (CCK8) (Dojindo; Kumamoto, Japan). Cells were plated in 96-well plates at a density of 1 × 104 cells per well and cultured in the growth medium. At the indicated time points, the number of cells in triplicate wells was measured at an absorbance at 450 nm of reduced WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt). For the colony-formation assay, 400 viable cells were placed in each well in 6-well plates and maintained in a complete medium for 2 weeks. Colonies were fixed with methanol and stained with 0.1% (w/v) crystal violet.

Cell cycle analysis and EdU incorporation assay

Cells (1×10^6) were collected, washed with phosphate buffer solution (PBS). The cells were resuspended in 1 ml DNA staining solution (MULTI SCIENCE; Hangzhou, China), then vortex the mix for 10 seconds. The mixture was then incubated at room temperature for 30 min in the dark. Analysis of cell cycle was carried out using flow cytometry.

For EdU incorporation assay, proliferating GC cells were examined using the Cell-Light EdU Apollo 488 or 567 In Vitro Imaging Kit (RiboBio) according to the manufacturer's protocol. Briefly, plate 1×10^5 cells on well of a 6-well plate and incubate overnight, after incubation with 20 μM EdU labeling solution under appropriate growth condition for 2 h, GC cells were fixed with 4% paraformaldehyde for 15min at room temperature, remove paraformaldehyde and wash twice with 1ml of 3% BSA in PBS, then remove wash solution and add 1 ml of in 0.3% Triton X-100 in PBS to each well and incubate for 20 minutes at room temperature. Stained the cells with Apollo fluorescent dyes and protected from light. A total of 5 μgml^{-1} of DAPI were used to stain cell nuclei for 10 min, then wash each well with 1ml PBS. The mean number of EdU-positive cells was counted for each well under a fluorescent microscope in five random fields. Photoshop 6.0 software was applied for cell counting. All assays were independently performed for three times.

Tumor growth assay

For the tumor growth assay, 1×10^6 stable transfected cells were independently injected subcutaneously into the left back of nude mice (n = 4/group). After 4 wk, 150 mg/kg luciferin was intraperitoneally injected into each mouse 10 min before imaging. Caliper IVIS Lumina II (Caliper Life Sciences, Hopkinton, MA, USA) was used for bioluminescence imaging of tumor growth. Mice were sacrificed 28 days after cell

injection, and tumors were dissected and weighted.

Lentiviruses transfection assay

Lentiviruses overexpressing human miR-567 and empty vector were built by Suzhou GenePharma, Co. Ltd. (Suzhou, China). For lentiviral transfection of MGC803 and BGC823 cells, 100 multiplicity of infection LV-miR-567 or empty vector lentiviruses were added to a well containing $5x10^4$ cells, medium and 8 μg/ml polybrene. After 24 h of incubation, transfected cells were selected with 2 μg/ml puromycin (Sigma-Aldrich). Empty vector lentivirus was used as a control. Selected cells were maintained in growth medium with 0.5 μg/ml puromycin.

Immunohistochemistry (IHC)

Dako Envision two-step method of immunohistochemistry was used according to the manufacturer's instructions. The tumour sections (3um) were first deparaffinized and hydrated through graded alcohol to water. Then, antigen retrieval was performed by high-pressure heat in citrate buffer for 5 minutes. After natural cooling, the sections were treated with 3% H2O2 for 15 minutes to eliminate intrinsic peroxidase activity. Then the sections were incubated with primary antibodies overnight at 4℃. The primary antibodies include: mouse antibody to CCND1 (60186-1-Ig, Proteintech, Wuhan, China dilution 1:300), and rabbit antibody to Ki-67 (27309-1-AP, Proteintech, Wuhan, China, dilution 1:1000). After washing with PBS for 3 times, the sections were incubated with the biotinylated secondary antibodies (dako, Denmark; Glostrup) for 60 minutes at room temperature. Sections were visualized with 3,3'-iaminobenzidine DAB and counterstained with hematoxylin for 30 seconds and then dehydrated and mounted in neutral gum.

Luciferase reporter assay

PIK3AP1 was predicted to be directly regulated by miR-567 using TargetScan software. A 400-bp fragment of PIK3AP1 3'UTR amplified by PCR primers was cloned into psiCHECK-2 vectors (named wt). The primers were 5'-CGGTCGCGCTCGAGTGGGACTTGAGCATTT-3' (forward) and 5' ATTTGCGGCCGCGGATCACTGAGGGAACA-3' (reverse). Site-directed mutagenesis of the miR-567 binding site in the PIK3AP1 3'UTR (named mt) was performed using GeneTailor Site-Directed Mutagenesis System (Invitrogen). For reporter assays, wt or mt vector and the control vector psiCHECK-2 vector were cotransfected into BGC823, MGC803 and 293T cells with miR-567 mimics. Luciferase activity was measured at 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA).

To generate a miR-567 promoter vector, a 2,000-bp fragment containing one binding site of c-Myc was PCR-amplified and inserted into a psiCHECK-2 luciferase reporter vector. In addition, c-Myc-binding site mutation vectors were constructed. These psiCHECK-2-derived vector and c-Myc-expressing vectors were cotransfected into 293T or MGC803 and BGC823 cells using Lipofectamine 2,000 Reagent (Invitrogen).

Chromatin immunoprecipitation assay

According to the manufacturer's instructions, ChIP assay was performed to examine whether c-Myc combined to miR-567 promoter by a ChIP assay kit (Millipore, catalog: 17-371). MGC803 and BGC823 cells were firstly fixed with 1% formaldehyde to covalently crosslink proteins to DNA and then chromatin was harvested from the GC cells. Crosslinked DNA was sheared to 200–1,000 base pairs in length with sonication

and then subjected to an immmmunoselection process, which required the use of Anti-c-Myc antibody (Abclone). Finally, PCR was used to measure enrichment of DNA fragments in the putative c-Myc-binding sites in the miR-567 promoter on the basis of the specific primers, the primers were 5'- AGGCAGAGGTCCTAAAGTAT-3' (forward) and 5'- AGGGCTGAGGTCACAAT-3' (reverse).

References

1. Zhao, M., et al., miR-3188 regulates nasopharyngeal carcinoma proliferation and chemosensitivity through a FOXO1-modulated positive feedback loop with mTOR-p-PI3K/AKT-c-JUN. Nat Commun, 2016. 7: p. 11309.

2. Niu, Y., et al., LASP1-S100A11 axis promotes colorectal cancer aggressiveness by modulating TGFβ/Smad signaling. Sci Rep, 2016. 6: p. 26112.

3. Yang, Y., et al., Epithelial-mesenchymal transition and cancer stem cell-like phenotype induced by Twist1 contribute to acquired resistance to irinotecan in colon cancer. Int. J. Oncol., 2017. 51(2): p. 515-524.

Supplementary Table S1. RT-PCR primer sequences for human genes

Gene name	Forward primer	Reverse primer	Product length
PIK3AP1	CCCAGAGGATGCGACATC	GACAGCAGCACCACGACA	197 bp
c-Myc	TACAACACCCGAGCAAGGAC	AGCTAACGTTGAGGGGCATC	189 bp
GAPDH	AAGGTCGGAGTCAACGGATTTG	CCATGGGTGGAATCATATTGGAA	159 bp

Supplementary Table S2. RT-PCR primer sequences for miR-567

Gene name	Primer	Product length
miR-567	AGTATGTTCTTCCAGGACAGAACAAA	197 bp