Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Effects of interfering plasmid on the pathogenicity of gastric cancer by cell proliferation and apoptosis testing

xingliang Geng
Peking Union Medical College Hospital
Weidong Li
Peking Union Medical College Hospital
guoyang liao (mybelieze@126.com)
Chinese Academy of Medical Sciences and Peking Union Medical College

Research

Keywords: miRNA-340, gastric cancer, proliferation, migration

Posted Date: June 10th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-33049/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Abstract

Background

To study the effects of recombinant interfering plasmids (pCDNA3.1-miR340) on the pathogenicity of gastric cancer by assessing cell proliferation and apoptosis.

Methods

Microarrays were used to analyse the function of pCDNA3.1-miR340. Reverse transcription polymerase chain reaction (RT-PCR) was used to evaluate miRNA-340 expression in different patients, while the pCDNA3.1-miR340 plasmid was constructed for use in wound-healing and migration assays. Expression of the miRNA-340 target, RhoA, was assessed by Western blotting.

Results

miRNA-340 expression was significantly higher in patients with gastric cancer. Treatment with pCDNA3.1-miR340 significantly slowed tumour growth compared to treatment with empty plasmid. Additionally, when miRNA-340 expression was reduced, apoptosis decreased significantly, while RhoA protein expression increased 1.90-2.02-fold in SGC-7901 cells.

Conclusions

pCDNA3.1-miR340 had a positive therapeutic effect on the pathogenicity of gastric cancer.

Background

Gastric cancer is the fifth leading type of cancer and the third leading cause of death from cancer worldwide. Collectively, the disease has a morbidity rate of 7% and a death rate of 9% [1, 2]. In 2012, 950,000 people suffered from gastric cancer, resulting in 723,000 deaths [1, 2]. Although death rates have decreased in many countries since the 1930s, morbidity remains high in East Asia and Eastern Europe [3, 4].

The initial symptoms of gastric cancer include heartburn, upper abdominal pain, nausea, and loss of appetite, while more advanced symptoms include weight loss, yellow skin and eyes, vomiting, difficulty swallowing, and blood in the stool [5, 6]. The cancer may spread from the stomach to other tissues of the body, including the liver, lungs, bones, abdominal lining and lymph nodes [7, 8].

The most common cause of gastric cancer is infection with the bacterium *Helicobacter pylori*, which occurs in more than 60% of cases [4, 9]. Other common causes include consuming pickled vegetables

and smoking, while 10% of patients have a family history of the disease. Approximately 1-3% of patients inherit genetic syndromes from their parents, including hereditary diffuse gastric cancer [9]. Current research primarily focuses on the links with gene expression; however, few studies have considered the effects of microRNAs on gastric cancer cells [10].

Micro-RNAs (miRNA) are small, non-coding RNA molecules that function in RNA silencing and post-translational regulation. Gene silencing occurs via mRNA degradation or translation inhibition. If complete complementarity exists between the miRNA and its target mRNA, the mRNA is often degraded. However, incomplete complementarity between miRNA and its target typically leads to translation inhibition [11, 12].

miRNA-340 is a tumour suppressor in many human cancers, including breast, colorectal, and gastric cancers [13, 14]. The expression of this miRNA was analysed in 116 paraffin-embedded breast cancer samples to evaluate the clinical and prognostic significance of expression, and it was suggested that miRNA-340 might play a role in cancer progression [13]. To extend such studies, the current study constructed a recombinant interfering plasmid, pCDNA3.1-miR340, and assessed its effects on the pathogenicity of gastric cancer using cell proliferation and apoptosis assays.

Aim of work:

This study was to analyzed the effects of recombinant interfering plasmids (pCDNA3.1-miR340) on the pathogenicity of gastric cancer by assessing cell proliferation and apoptosis.

Patients And Methods

Patients and diagnoses

Gastric cancer tissues were collected from 70 patients in the Department of General Surgery, First People's Hospital of Yunnan, from November 2015 to March 2019. The patient cohort had an average age of 56.5 years (range: 26-80 years) and was comprised of 39 males and 31 females. Twenty-four patients had a tumour diameter > 4 cm. Patient data are listed in **Table 1**.

Dissected tissues were washed with saline immediately following isolation, frozen in liquid nitrogen, and stored at -80°C. All samples were classified as moderately differentiated tumours, according to the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) TNM staging system for gastric cancer (7th Edition) [15].

Total RNA extraction

Total RNA was extracted using the miRNeasy Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA concentration and purity were calculated by the A260/A280 ratios, with all samples yielding purity values of 1.8-2.1.

Reverse transcription polymerase chain reaction (RT-PCR)

Complementary DNA (cDNA) synthesis was performed using the RT2 First Strand cDNA Synthesis Kit (Takara, Tokyo, Japan), and qPCR was performed using SYBR Green PCR Master Mix (Promega, Madison, WI, USA) on IQ5.0 equipment (PCR system; Bio-Rad, Hercules, CA, USA). The primer sequences used to target miR340 were as follows:

F: 5'-TTATAAAGCAATGAGACTGATT-3',

R: 5'-TCAGTCTCATTGCTTTATAATT-3'.

Microarray hybridisation

Double stranded cDNA was initially labelled and hybridised to the microarray (Arraystar, Rockville, MD, USA). Following hybridisation and washing, slides were scanned with an Axon Gene Pix 4000B micro-array scanner (Molecular Devices, Sunnyvale, CA, USA).

Cell lines

SGC-7901 and BGC823 cells were isolated from gastric cancer patients and obtained from the American Type Culture Collection (ATCC). All culture conditions followed those described by the ATCC. KMB17 cells were purchased from the Kunming Medical Biology Institute which were stored at the Department of Clinical Laboratory, First People's Hospital of Yunnan.

Recombinant plasmid construction

Nucleic acids were isolated from KMB17 cells following 48 h culture using a phenol-chloroform extraction protocol. The miRNA-340 gene was amplified by PCR using primers that introduced 5' BamHI and 3' NotI restriction sites. PCR products were cloned into pCDNA3.1 to yield the recombinant interfering plasmid, pCDNA3.1-miR340 (Kunming Medical Biology Institute, Chinese Academy of Sciences).

Wound-healing assay

The wound-healing assay was used “to detect motility and migration changes in SGC-7901 and BGC823 cells, respectively. Approximately 3×10^5 cells were seeded in each well of a 6-well culture plate. Cells were transfected with pCDNA3.1-miR340, or empty vector control, and incubated for 12 h at 37°C. Cells were then scratched in the centre of each well using a P-20 pipette tip, washed three times in phosphate buffer saline (PBS), and cultured in serum-free medium for 0 h, 6 h, 12 h and 24 h under continual monitoring using an inverted microscope.

Migration assays

Cells (3×10^5 per well) were cultured for 12 h in 6-well cell culture plates containing trans-well inserts (8 μm pore size; Corning; New York, NY, USA) coated with fibronectin (10 μg/mL). Following the 12 h incubation, the culture medium was separated into two layers by the transwell insert, with the upper layer being serum-free and containing 0.1% BSA, while the bottom layer contained 20% foetal bovine serum (FBS) in Dulbecco’s modified Eagle’s medium (DMEM). Following additional 24 h incubation, the bottom of the transwell insert was stained with Giemsa and the migration rates were calculated.

Annexin V/propidium iodide (PI) staining

Apoptosis was calculated by Annexin V/PI co-staining. Serum-free media containing L-mimosine (400 lM) was added to ensure G1 synchronisation. After 24 h incubation, media containing 10% FBS was added. Following an additional 12 h incubation, cells were fixed in 75% ethanol, re-suspended in 100 μL Annexin-V-Fluos labelling solution, 10 μL annexin reagent, and 10 μL PI solution in 150 μL incubation buffer (according to the manufacturer’s instructions; Roche, Basel, Switzerland). Samples were incubated in the dark at room temperature for 7 min followed by the addition of 100 μL incubation buffer. Fluorescence was observed by fluorescence-activated cell sorting (FACS) and the percentage of positive cells was determined based on fluorescence intensity using FAC Station Software (BD Biosciences, San Jose, CA, USA). An isotope control was included in the quadrant analysis. Experiments were performed in triplicate.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot

KMB17 and SGC-7901 cells were harvested and centrifuged at 1,000 g for 10 min (Thermo Fisher Scientific, Waltham, MA, USA). Cell pellets were re-suspended in lysis buffer (Beyotime, Jiangsu, China) followed by the addition of a proteinase inhibitor cocktail (1%) (Sigma-Aldrich, St. Louis, MO, USA), 25 mM NaF and 1 mM Na_3VO_4. The mixture was frozen to -80°C and thawed four times. The mixture was

then centrifuged at 10,000 g for 30 min at 4°C (Thermo) and the supernatant containing RhoA was collected and separated using SDS-PAGE (8%). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), and blocked for 2 h at 25°C using 5% BSA Tris-HCl and 0.05% Tween-20. Following blocking, the membrane was incubated with monoclonal anti-RhoA primary antibodies and rabbit anti-mouse monoclonal secondary antibodies. GADPH was used as the loading control and proteins were detected using an enhanced chemiluminescence system (ECL; BestBio, Shanghai, China).

Statistical analysis

Statistical analyses were performed using SPSS software (ver. 20.0; Chicago, IL, USA). Statistically significant differences were determined by one-way analysis of variance, while the chi-square test was used to calculate the significance of differences in detection rates between two groups. A *P*-value of < 0.05 was considered significant.

Results

Identification of the recombinant plasmid

The results of the electrophoresis of the recombinant plasmids by a 3% agarose gel. It was at correct size, with a high concentration of supercoiled plasmid. After sequencing, there were no base mutations or deletions in the recombinant plasmid pCDNA3.1-miR340. Additional, the concentration of the modified recombinant plasmids was 285 ng/μl by UV spectrophotometer for further use.

Expression profiles of miRNA-340

To explore differences in the expression of miRNAs in gastric cancer, microarray analysis of 96 miRNAs was performed on the gastric cancer tissues from all 70 patients. Notably, miRNA-340 expression was significantly reduced in gastric cancer tissues compared to healthy controls (Fig. 1A). This finding was confirmed by quantitative RT-PCR (**$P < 0.01$, Fig. 1B). There was a close relationship between miRNA-340 reducing expression with the incidence of the gastric cancer. And the meaningful finding of miRNA-340 was through the microarray hybridisation method, which would help scientists find more meaningful miRNA for other types of cancer.

Effects of pCDNA3.1-miR340 interference in cells

Wound-healing and migration assays were used to test the effects of recombinant pCDNA3.1-miR340 treatment on cancer cells. The healing rate of the treatment group was slower than that of the normal tumour group, while treatment also reduced the rate of healing in SGC-7901 and BGC823 cells compared to cells receiving empty control plasmid. No significant difference was observed in KMB17 cells upon

treatment with pCDNA3.1-miR340. Migration assays supported the suggestion that pCDNA3.1-miR340 interfered with the motility and migration of SGC-7901 and BGC823 cells, but not KMB17 cells (Fig. 2).

Analysis of apoptosis rates

Apoptotic ratios were calculated for SGC-7901 cells transfected with pCDNA3.1-miR340. Based on cell staining with Annexin V-FITC and PI, it was observed that pCDNA3.1-miR340 accelerated the cell cycle (Fig. 3A) and decreased apoptosis (Fig. 3B).

Western blot

RhoA expression was increased 1.90-2.02-fold in SGC-7901 cells upon treatment with pCDNA3.1-miR340. That target protein of miRNA340 could play more function on cancer growth. On the other hand, we constructed that plasmid could block RhoA expression for treatment of gastric cancer (Fig. 4).

Discussion

Gastric cancer is the third leading cause of cancer-related death worldwide. Moreover, gastric tumour development has a genetic basis [15]. miRNAs regulate gene expression by binding to complementary 3'-UTRs of specific mRNAs and inhibiting translation or inducing degradation. Thus, miRNAs act at the post-transcriptional level [16, 17]. Previous studies showed that miRNAs exhibited anti-cancer functions by modulating cell proliferation, apoptosis, metabolic pathways, and signal transduction [18]. More recent evidence has also suggested that miRNAs may be involved in gastric cancer development and progression.

miRNA-340 expression may suppress malignant cell growth since it was observed that reduced miRNA-340 expression contributed to tumorigenesis and progression [19]. Wu and colleagues reported that loss of miRNA-340 expression was associated with lymph node metastasis, high tumour histological grade, clinical stage, and shorter overall survival in breast cancer patients [20]. Additionally, Sun *et al.* revealed that miRNA-340 expression inhibited the growth of colorectal cancer cells and was associated with poorer prognosis [21]. However, few studies have detailed changes in miRNA-340 expression in gastric cancer cells. The current study found that miRNA-340 expression was lower in gastric cancer cells; based on this observation, a recombinant interfering plasmid, pCDNA3.1-miR340, was constructed to assess its effects on SGC-7901 and BGC823 cells. Treatment with pCDNA3.1-miR340 inhibited SGC-7901 and BGC823 cell growth, suggesting this miRNA has an anti-proliferative effect on gastric cancer cells. Cell cycle arrest was one of the prerequisite conditions for cell apoptosis, which could increase apoptosis in gastric cancer cells. Thus, additional studies are necessary to determine the mechanisms by which pCDNA3.1-miR340 inhibits cell growth.

RhoA is a small GTPase belonging to the Rho family of proteins. It is associated with cytoskeleton regulation, where it exerts effects on the formation of actin stress fibres and actomyosin contractility [22]. Additionally, loss of RhoA function is attributable to failed gastrulation and cell migration. Notably, this

study found that pCDNA3.1-miR340 inhibited RhoA expression in gastric cancer cells, but not healthy cells.

Conclusion

We could draw the conclusion that pCDNA3.1-miR340 had a positive therapeutic effect on the pathogenicity of gastric cancer, which could be used for treatment with patients in the future.

List Of Abbreviations

RT-PCR, Reverse transcription polymerase chain reaction;

miRNA, Micro-RNAs;

ATCC, American Type Culture Collection;

DMEM, Dulbecco's modified Eagle's medium ;

PI, propidium iodide ;

FACS, fluorescence-activated cell sorting ;

PVDF, polyvinylidene difluoride.

Declarations

Ethics approval and consent to participate

The guardians of all study participants provided written informed consent. The study was conducted according to the declaration of Helsinki, implemented by guidelines of the World Health Organization. The experiment was conducted under the supervision of the ethics committee of the Kunming Medical University (No. KMMU201601047091).

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and material

We declared that materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them.

Competing interests

No.

Funding

This study was supported by the International Cooperation Project (2011DFR30420); Scientific Research Projects of Health Care (200802023), National Science, and Technology Major Project (2013ZX10004003-003-002), Innovation team in Yunnan Province (2015HC027), and Medical Science and Technology Innovation Project of Chinese Academy of Medical Sciences (2016-I2M-3-026).

Authors' contributions

Xingliang Geng, performed the research,

Weidong Li, analysed data,

Guoyang Llao,designed the study, and wrote the paper.

Acknowledgements

No.

References

1. Fernandez S, Risolino M, Mandia N, Talotta F, Soini Y, Incoronato M, Condorelli G, Banfi S, Verde P. miR-340 inhibits tumor cell proliferation and induces apoptosis by targeting multiple negative regulators of p27 in non-small cell lung cancer. Oncogene. 2015;34:3240–50.
2. Hwang HW, Mendell JT. MicroRNAs in cell proliferation, cell death, and tumorigenesis. Br J Cancer 2007; 96 Suppl: R40-44.
3. Han S, Roman J. Peroxisome proliferator-activated receptor γ: a novel target for cancer therapeutics? Anticancer Drugs. 2007;18:237–44.
4. Theocharis S, Margeli A, Kouraklis G. Peroxisome proliferator activated receptor-gamma ligands as potent antineoplastic agents. Curr Med Chem Anticancer Agents. 2003;3:239–51.
5. Nana-Sinkam SP, Croce CM. Clinical applications for microRNAs in cancer. Clin Pharmacol Ther. 2013;93:98–104.

6. Shen J, Stass SA, Jiang F. MicroRNAs as potential biomarkers in human solid tumors. Cancer Lett. 2013;329:125–36.
7. Osman A. MicroRNAs in health and disease-Basic science and clinical applications. Clin Lab. 2012;58:393–402.
8. Amiel J, de Pontual L, Henrion-Caude A. MiRNA, development and disease. Adv Genet. 2012;80:1–36.
9. Majid S, Dar AA, Saini S, Shahryari V, Arora S, Zaman MS, Chang I, Yamamura S, Chiyomaru T, Fukuhara S, Tanaka Y, Deng G, Tabatabai ZL, Dahiya R. MicroRNA-1280 inhibits invasion and metastasis by targeting ROCK1 in bladder cancer. PLoS One. 2012;7:e46743.
10. Dong H, Luo L, Hong S, Siu H, Xiao Y, Jin L, Chen R, Xiong M. Integrated analysis of mutations, miRNA and mRNA expression in glioblastoma. BMC Syst Biol. 2010;4:163.
11. Rugge M, Meggio A, Pennelli G, Piscioli F, Giacomelli L, De Pretis G, Graham DY. Gastritis staging in clinical practice: the OLGA staging system. Gut. 2007;56:631–6.
12. Rugge M, Genta RM, OLGA Group. Staging gastritis: an international proposal. Gastroenterology. 2005;129:1807–8.
13. Inoue M, Tsugane S. Epidemiology of gastric cancer in Japan. Postgrad Med J. 2005;81:419–24.
14. Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, Sibley RK. Helicobacter pylori infection and the risk of gastric carcinoma. N Engl J Med. 1991;325:1127–31.
15. Edge SB, Compton C. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Ann Surg Oncol. 2010;17:1471–4.
16. Zhou X, Wei M, Wang W. MicroRNA-340 suppresses osteosarcoma tumor growth and metastasis by directly targeting ROCK1. Biochem Biophys Res Commun. 2013;437:653–8.
17. Babae N, Bourajjaj M, Liu Y, Van Beijnum JR, Cerisoli F, Scaria PV, Verheul M, Van Berkel MP, Pieters EH, Van Haastert RJ, Yousefi A, Mastrobattista E, Storm G, Berezikov E, Cuppen E, Woodle M, Schaapveld RQ, Prevost GP, Griffioen AW, Van Noort PI, Schiffelers RM. Systemic miRNA-7 delivery inhibits tumor angiogenesis and growth in murine xenograft glioblastoma. Oncotarget. 2014;5:6687–700.
18. Liu X, Choy E, Hornicek FJ, Yang S, Yang C, Harmon D, Mankin H, Duan Z. ROCK1 as a potential therapeutic target in osteosarcoma. J Orthop Res. 2011;29:1259–66.
19. Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ. Helicobacter pylori infection and the development of gastric cancer. N Engl J Med. 2001;345:784–9.
20. Wu ZS, Wu Q, Wang CQ, Wang XN, Huang J, Zhao JJ, Mao SS, Zhang GH, Xu XC, Zhang N. MiR-340 inhibition of breast cancer cell migration and invasion through targeting of oncoprotein c-Met. Cancer. 2011;117:2842–52.
21. Sun Y, Zhao X, Zhou Y, Hu Y. MiR-124, miR-137 and miR-340 regulate colorectal cancer growth via inhibition of the Warburg effect. Oncol Rep. 2012;28:1346–52.

22. Kiss C, Li J, Szeles A, Gizatullin RZ, Kashuba VI, Lushnikova T, Protopopov AI, Kelve M, Kiss H, Kholodnyuk ID, Imreh S, Klein G, Zabarovsky ER. Assignment of the ARHA and GPX1 genes to human chromosome bands 3p21.3 by in situ hybridization and with somatic cell hybrids. Cytogenet Cell Genet. 1997;79:228–30.

Tables

Table 1. Details of miRNA-340 expression for 38 primary gastric cancer patients

Clinical feature		Number	Number of patients with high versus low miRNA-340 expression		*P*-value
			High	Low	
Gender	Male	28	13 (28.57%)	25 (71.42%)	0.7205
	Female	10	9 (40%)	6 (60%)	
Age (y)	> 60	33	10 (56%)	24 (47%)	0.4417
	≤ 60	5	2 (49%)	3 (51%)	

Figures

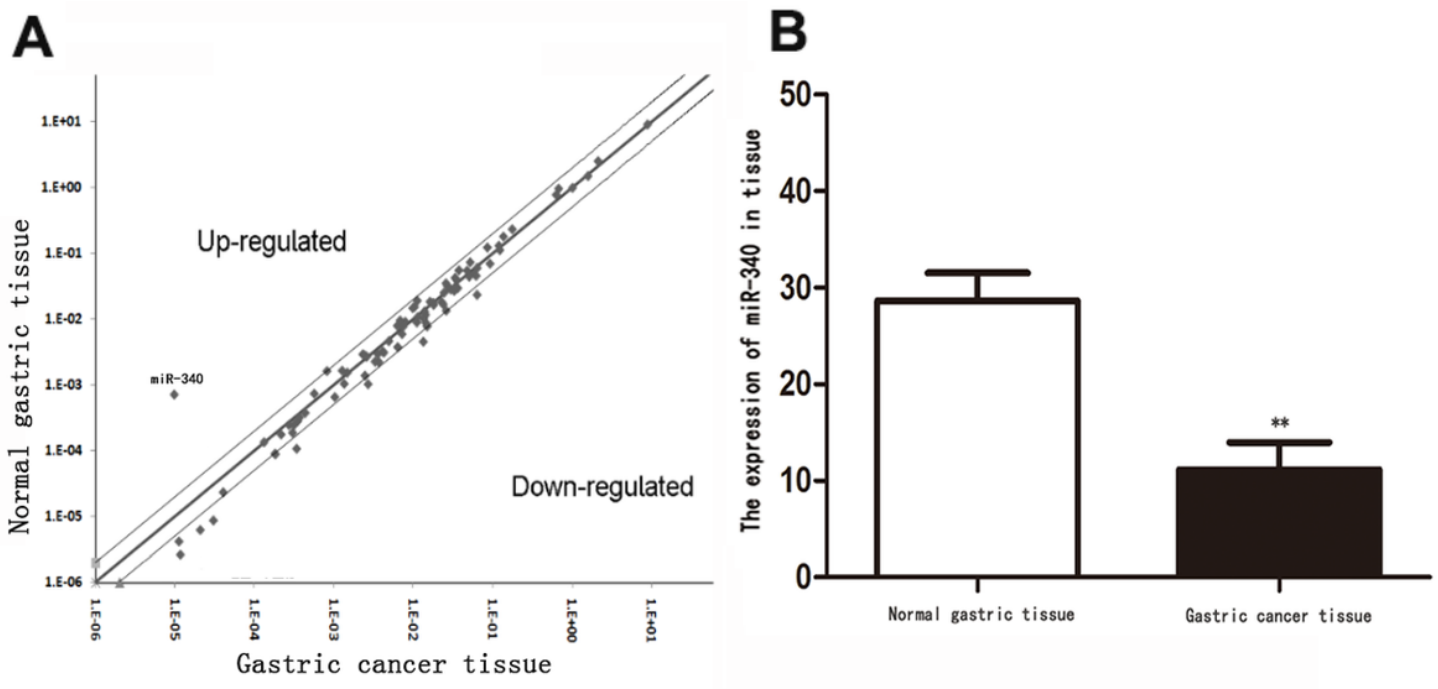

Figure 1

Expression patterns of miRNA-340 in gastric cancer cells. The expression of miRNA-340 was significantly reduced in gastric cancer cells, as shown by (A) microarray analyses and (B) quantitative reverse transcription polymerase chain reaction (**P < 0.01).

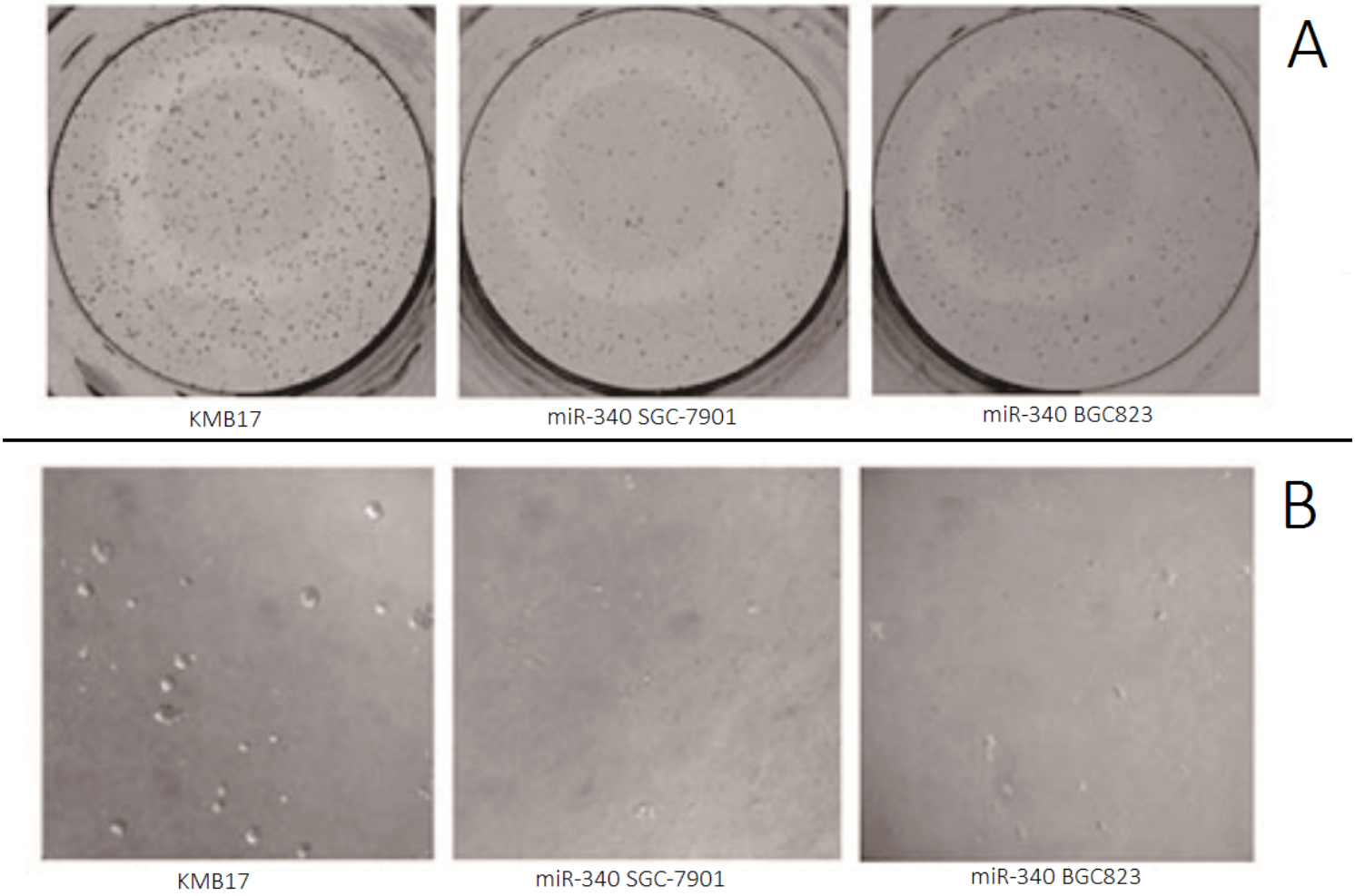

Figure 2

Interference effects of pCDNA3.1-miR340 in different cell lines (*P < 0.05).

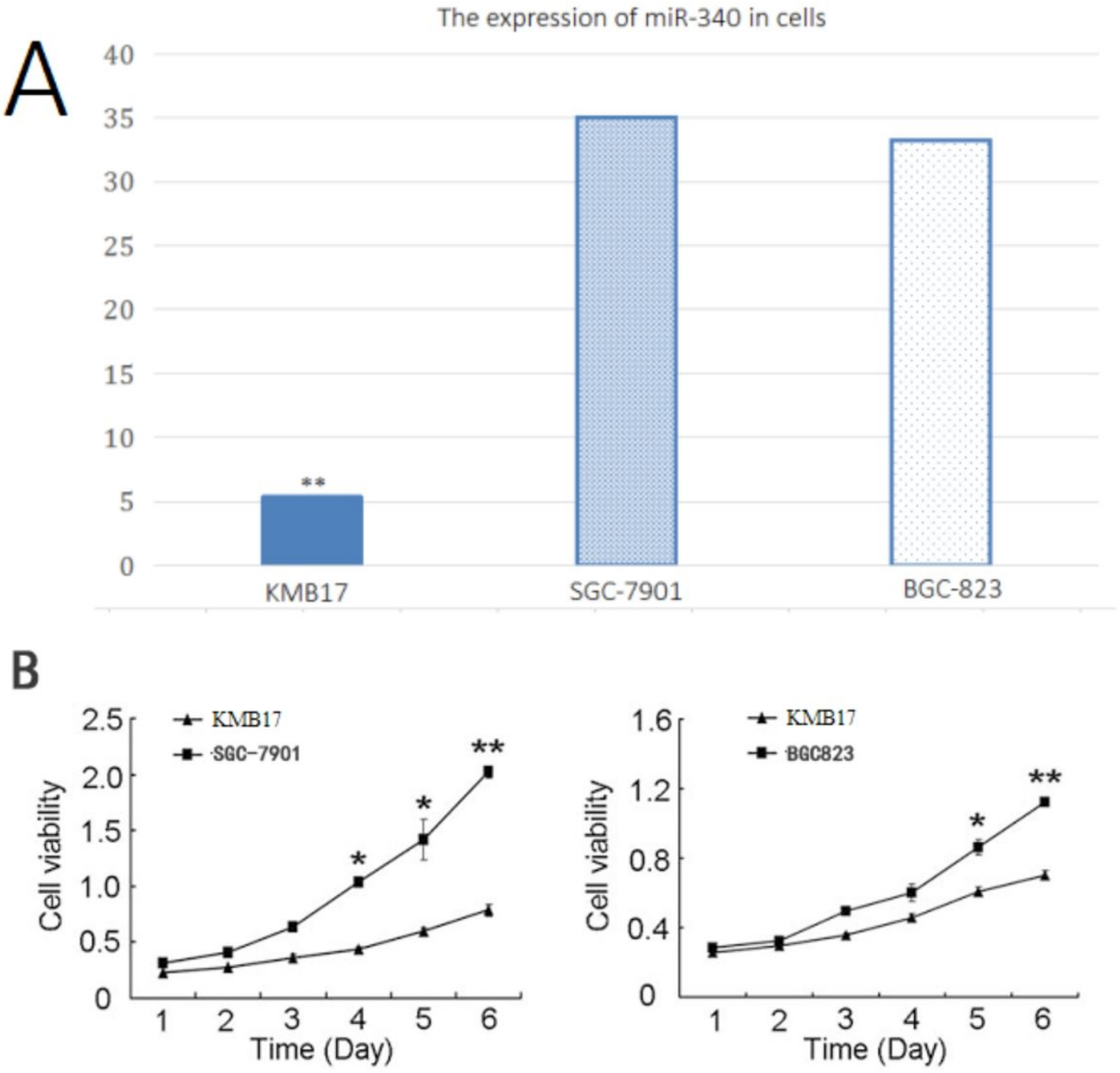

Figure 3

The interfering plasmid, pCDNA3.1-miR340, significantly delayed tumour growth and increased apoptosis. SGC-7901, BGC823 and KMB17 cells treated with pCDNA3.1-miR340 had (A) accelerated cell cycles and (B) reduced apoptosis.

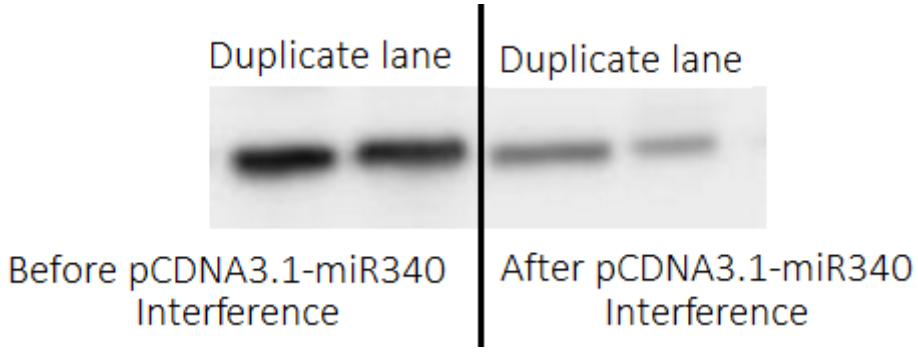

Figure 4

The results of western blotting for RhoA expression both before and after pCDNA3.1-miR340 interference, with duplicate lane for each group.