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Original Article

MicroRNA-134 functions as a tumor suppressor gene in gastric cancer

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Abstract: MiR-134 can function as an oncogene or a tumor suppressor gene depending on cell type. However, the function of miR-134 in gastric cancer remains unclear to date. This study aims to evaluate the function of miR-134 in gastric cancer and investigate its effect on the sensitivity of gastric cancer cells to 5-FU. The expression of miR-134 assayed by real-time PCR was significantly lower in gastric cancer tissues than in noncancerous tissues. Overexpression of miR-134 significantly inhibited the proliferation and growth in vivo, as well as promoted the apoptosis of gastric cancer cells by targeting KRAS. Finally, the up-regulation of miR-134 enhanced the sensitivity of gastric cancer cells to 5-FU. In conclusion, miR-134 suppresses tumor development in gastric cancer by targeting KRAS and enhances cell sensitivity to 5-FU. Our results encourage researchers to use 5-FU in combination with miR-134 to treat gastric cancer.

Keywords: 5-FU, KRAS, gastric cancer, miR-134

Introduction

Gastric cancer is one of the most common cancers and accounts for the most frequent causes of cancer-related deaths worldwide [1]. The incidence, pathogenesis, and therapeutic options have undergone significant changes in the last decades, but the prognosis for patients with gastric cancer remains poor, especially in more advanced stages. Surgery is the mainstay of treatment of this disease, while chemotherapy plays an extremely important role in the comprehensive treatment of gastric cancer [2]. Conventional chemotherapy drugs, such as cisplatin, paclitaxel and 5-fluorouracil (5-FU), have long-term clinical application in many cancers, including gastric cancer [3]. Unfortunately, numerous gastric cancer patients who initially respond to cisplatin or paclitaxel therapy become drug resistant and relapse. Therefore, the identification of new biomarkers, treatments, and therapeutic targets for human gastric cancer is required [4]. Therefore, there is an urgent requirement for novel prognostic markers and therapeutic targets for gastric cancer to effectively decrease mortality.

MicroRNAs (miRNA) are a class of small RNA molecules that regulate the translation and degradation of mRNAs. MiRNAs bind to complementary sequences in the 3'-untranslated regions (UTRs) of their target mRNAs to promote mRNA degradation or translational repression [5]. In recent years, more attention has been paid to the role of miRNAs in drug resistance, with accumulating evidence that aberrant miRNA expression can affect drug resistance by modulating the expression of relevant target proteins, including drug transporters, cell apoptosis mediators, and cell-cycle-related components [6]. For example, miR-134, which is located in the 14q32.31, was initially identified in cloning research of rat; since this identification, reports have shown that abnormal expression of miR-134 is associated with tumor formation, cell proliferation and even chemoresistance [7]. miR-134 expression was significantly decreased in lung cancer tissues, and that miR-134 affects the fluorouracil sensitivity of lung cancer by decreasing the expression of dihydropyrimidine dehydrogenase [8]. Furthermore, in multi-resistant small cell lung cancer cell, miR-134 expression was decreased signifi-

cantly, and that increasing the expression of miR-134 in drug-resistant cells can significantly increase therapeutic sensitivity to cisplatin, etoposide and doxorubicin [9]. Moreover, miR-134 targets the oncogene SirT1 or KRAS in endothelial and pancreatic ductal adenocarcinoma cells but targets the tumor suppressor gene PTEN. MicroRNA-134 modulates resistance to doxorubicin in breast cancer cells by down-regulating the expression of ABCC1 which is known to encode the multidrug resistance-associated protein 1 [10]. However, the function of miR-134 in gastric cancer remains unclear to date.

The present study aims to provide insights into the association of miR-134 expression with gastric cancer cells growth. Results show that miR-134 is down-regulated in human gastric cancer and that miR-134 over-expression can inhibit tumor cells proliferation and reduce 5-FU cell resistance in gastric cancer cell lines. Our in vitro and in vivo data also prove that miR-134 functions as a tumor suppressor in human gastric cancer progression. The results of this study may serve as a basis to explain the function of miR-134 in gastric cancer.

Materials and methods

Patients and tissue samples

We collected pairs of matched gastric cancer and noncancerous tissue samples from 80 patients (male 53, female 27) who underwent surgical resection at the Cancer Institute and Hospital of Hebei; the Chongmin Hospital of Nanjing; First affiliated hospital of Henan University; Hebei Cancer Hospital between 2009 and 2012 after obtaining informed consent from all patients and receiving approval from the Institutional Ethics Review Committee. The gastric cancer cell lines BGC-823, SNU-16, MGC-803, SGC-7901, MKN-28 and normal human epithelial cells, BESA-2B were cultured in RPMI1640 or Dulbecco's modified Eagle's medium, with 10% FBS, 100 U/ml penicillin as well as streptomycin.

RNA extraction, cDNA synthesis, and real-time PCR assays

Total RNA from tissues and cells was extracted by Trizolmethod (Ambion, USA) completely following the instructions. Single strand cDNA was synthesized by M-MLV (Ambion, USA) using 2 μg of total RNA as template. Oligo (dT)18 was used for mRNA reverse transcription while stem loop used for miRNA. Real time-PCR (RT-PCR) was performed by Bio-rad CFX96 (Bio-rad, USA) using SYBR mix (Tiangen, China). The PCR condition was: 95°C × 30 s, followed by 40 cycles of 95°C × 5 s, 60°C × 34 s. For mRNAs, GAPDH was used as normalized control. For miRNAs, U6 snRNA was used for miRNA control. The relative expression of miR-134 was computed by 2-ΔΔCT method. Primer sequences were as follows KRAS-FW: 5'-gcaatgagggaccagtacatgag-3', KRAS-RW: 5'-gtattgtcggatctccctcacca-3' miR-134-FW: 5'-cgtgctacagtcctggtgtgag-3', miR-134-RW: 5'-tactccatgacgcagcagttgt-3'.

Plasmid construction

The miR-134 precursor sequence was generated by annealing. MiR-134-precursor-F and miR-134-precursor-R extension was digested by BamHI and BglII. The products were then inserted into the BamHI-BglII fragment of the pcDNA-GW/EmGFP-miR vector (Gene Pharma, China). A negative control was also constructed [11].

MiRNA transfection

The miR-134 and scramble mimics were designed and synthesized by GenePharma (GenePharma, China). All mimics were transfected by DharmaFECT1 Reagent (Dharmacon, USA) into the gastric cancer cells to a final concentration of 10 nM. Three independent replication experiments were performed for each transfection [12].

Cell proliferation and apoptosis assay

Cell proliferation assay was performed by the CCK8 method (DOJINDO, Japan). Briefly, approximately 5000 miR-134 mimic-transfected cells and scramble cells were seeded into 96-well plates and then cultured. Proliferation rates were determined at 0, 12, 24, 48, 60 and 72 h after transfection by adding 10 μl of CCK8 reagent [13]. Apoptosis assays were tested in cells with or without miR-134 overexpression using Apoptosis Detection kit I (BD Biosciences, USA) and C6 Flow Cytometer (USA).

Animal experiment

Experiments involving animals were performed according to the Guide for the Care and Use of

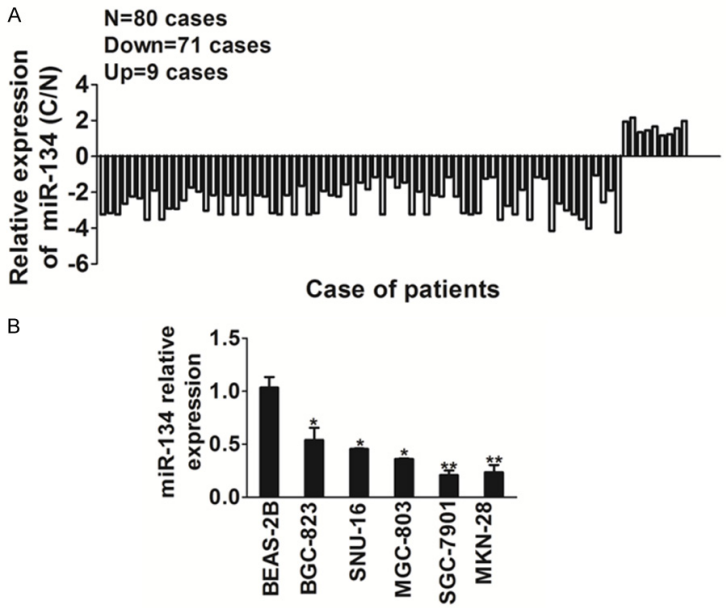

Figure 1. MiR-134 expression in gastric cancer clinical samples and cells. A. MiR-134 expression was detected by real-time PCR in 80 pairs of gastric cancer tissues compared with their matched adjacent non-neoplastic tissues. A total of 71 samples showed down-regulated miR-134. B. The expression levels of miR-134 in normal epithelial cell line (BEAS-2B) and 5 gastric cancer lines. Data were collected from three independent experiments and were average ± SD. values. $*P < 0.05$, $**P < 0.01$ compared with BEAS-2B cells.

Laboratory Animals and the institutional ethical guidelines for animal experiments. Scramble-transfected and miR-134-overexpressing MKN-28 or SGC-7901 cells (6×10^6 cells) were inoculated s.c. into the dorsal flanks of BALB/c nude mice (female, Nu/Nu, six week old). The mice were purchased from the Animal Centre of Henan University and raised under pathogen-free conditions. The tumor volume was measured for 5 weeks. All mice were killed, s.c. tumours were resected, and tumour weights were recorded.

Immunohistochemistry

Mouse tumour tissues were embedded into paraffin sections, treated for 2 h at 65°C and then deparaffinized. Before applying the primary antibodies at 4°C overnight, we carried out the antigen retrieval step. The slides were incubated with a secondary antibody for 2 h at 25°C and then conjugated to HRP (1:100; Zhongshanjinqiao, China). Liquid DAB+ Substrate (zsgb-bio, China) was used to detect HRP activity [14].

Luciferase miRNA target reporter assay

The full length of the 3'UTRs of KRAS, who has complementary sequences in their 3'UTR predicting by Targetscan on line software, were amplified by PCR and inserted into the pMIR-REPORTTM Luciferase Reporter Vector (Ambion, USA). Mutations of the predicted seed regions in the mRNA sequences were created using primers including the mutated sites. HEK-293T cells were transfected with pRL-TK luciferase reporters (50 ng/well, 24-well plates, 1×10^5 cells per well), pGL-3firefly luciferase (10 ng/well), and mimic-134 (50 nmol/L, GenePharma, China) or scramble (50 nmol/L) using Lipofectamine 2000 (Invitrogen, USA). Luciferase activities were measured according to the manuscript of the Dual Luciferase Reporter Assay (Promega, USA).

Western blot

Proteins were separated on 10% SDS-PAGE and then transferred to 0.45 μm PVDF membranes (Amersham, UK). The membranes were incubated with 5% non-fat dried milk overnight at 4°C and with anti-KRAS antibody (CST, USA) at 1:1000 dilution for 2 h at room temperature. After washing twice with TBST, the membranes were incubated with goat anti-rabbit antibody (zsgb-bio, China) at 1:5000 and 1:50000 dilutions for 2 h.

Statistics

Each of the experiments was at least performed triplicate. Student's t-test (two-tailed) and the x^2 test were performed, and statistically significant level was set at $\alpha = 0.05$ two-side). Mean ± SD is displayed in the figures.

Results

Expression of miR-134 in clinical gastric cancer patients and cell lines

The expression of miR-134 in 80 patients (53 males/27 females, mean age of 51 years) was

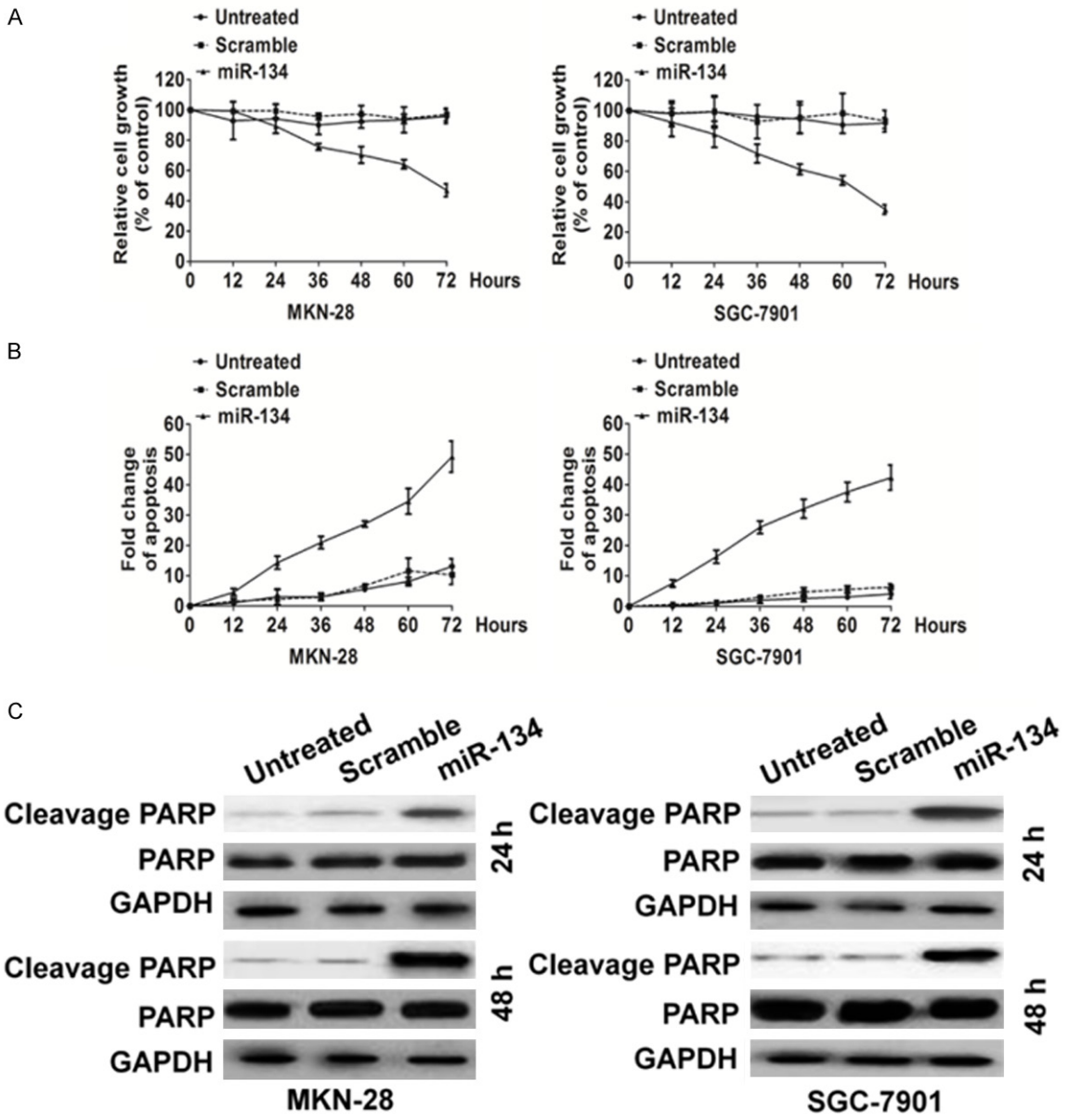

Figure 2. miR-134 over-expression regulates gastric cancer cell growth. A. miR-134 over-expression inhibited MKN-28 and SGC-7901 gastric cancer cell proliferation. The growth index was assessed after 0, 12, 24, 36, 48, 60 and 72 h. Data were collected from three independent experiments and were average ± SD. values. B. miR-134 over-expression promoted cell apoptosis in MKN-28 and SGC-7901. Data were collected from three independent experiments and were average ± SD. values. C. Western blot analysis showed that cleaved PARP was higher in miR-134 transfection cells than in scramble transfection MKN-28 and SGC-7901 cells.

detected by RT-PCR. The expression of miR-134 was down-regulated in 71 (88%) of the 80 gastric cancer samples compared with adjacent tissues (**Figure 1A**). Meanwhile, miR-134 was up-regulated in 9 (11%) of the 80 gastric cancer samples. In addition, the expression of miR-134 in five gastric cancer cell lines was determined. As shown in **Figure 1B**, the relative expression levels for miR-134 in these gastric cancer cells were significantly decreased, especially in MKN-28 and SGC-7901 cells when compared with that of the normal epithelial cell line BEAS-2B.

miR-134 inhibits gastric cancer cells proliferation in vitro

The results of MTT growth assays at 0, 12, 24, 36, 48, 60 and 72 h after miR-134 and scramble transfection were shown in **Figure 2A**.

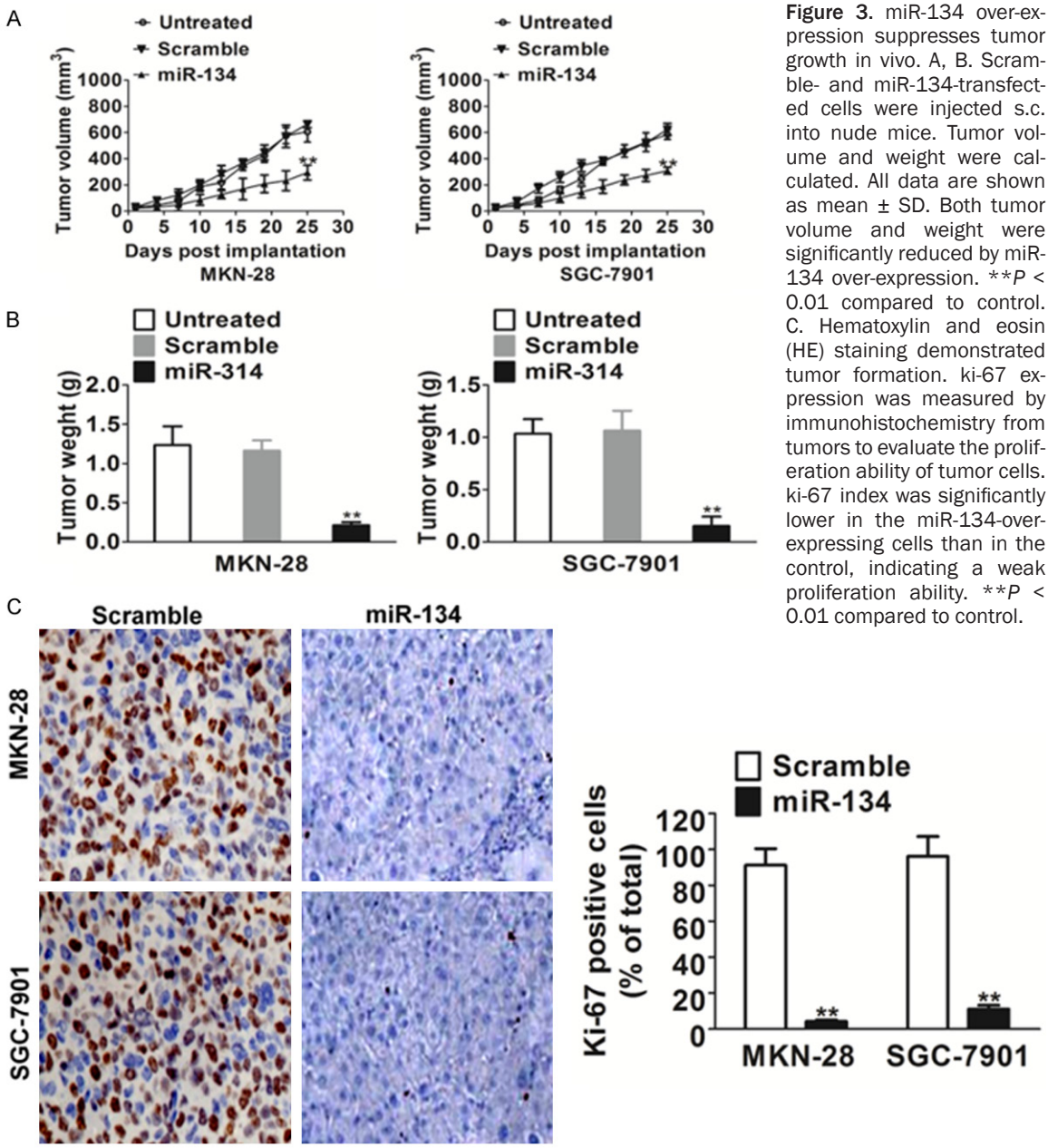

Figure 3. miR-134 over-expression suppresses tumor growth in vivo. A, B. Scramble- and miR-134-transfected cells were injected s.c. into nude mice. Tumor volume and weight were calculated. All data are shown as mean ± SD. Both tumor volume and weight were significantly reduced by miR-134 over-expression. $^{**}P < 0.01$ compared to control. C. Hematoxylin and eosin (HE) staining demonstrated tumor formation. ki-67 expression was measured by immunohistochemistry from tumors to evaluate the proliferation ability of tumor cells. ki-67 index was significantly lower in the miR-134-over-expressing cells than in the control, indicating a weak proliferation ability. $^{**}P < 0.01$ compared to control.

Compared with scramble transfection, miR-134 transfection significantly inhibited the proliferation of gastric carcinoma cell lines MKN-28 and SGC-7901 cells. We also investigated the effect of miR-134 on the apoptosis of MKN-28 and SGC-7901 cells by DNA fragmentation assay and PARP cleavage assay. Similarly, miR-134 transfection induced the apoptosis in both MKN-28 and SGC-7901 cells (**Figure 2B**). Meanwhile, the expression of cleavage PARP was significantly slight in MKN-28 and SGC-7901 cells transfected with scramble when compared to miR-134 transfection (**Figure 2C**). These results indicate that miR-134 can suppress gastric cancer cell survival by inducing apoptosis.

miR-134 inhibits gastric cancer cells growth in vivo

To further verify these findings, an in vivo model was constructed. Scramble-transfected and miR-134 over-expressing MKN-28 cells or SGC-7901 cells were inoculated s.c. into the dorsal flanks of athymic nude mice. After 25 days, tumor growth was significantly slower in the mice inoculation with miR-134-overexpressing cells than in the control mice (**Figure 3A**). In

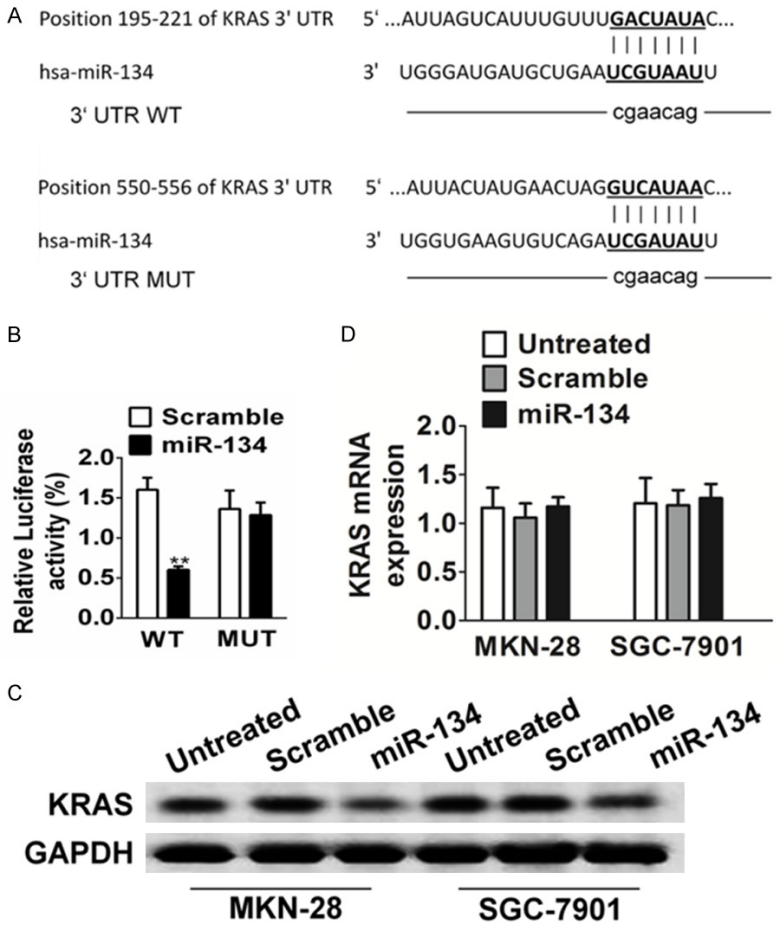

Figure 4. KRAS is a direct target of miR-134. A. Sequence of the miR-134 binding sites within the human KRAS 3'-UTR and a schematic of the reporter construct shown the KRAS 3'-UTR sequence and the mutated KRAS 3'-UTR sequence. B. Luciferase activity of the KRAS reporter in the presence of miR-134 or scramble. Data were collected from three independent experiments and were average ± SD. values. $^{**}P < 0.01$, compared to scramble transfected cells. C. Immunoblotting of KRAS in MKN-28 and SGC-7901 cells transfected with miR-134 or scramble, which showed that the miR-134 reduced the KRAS protein level, indicating that KRAS can be a direct target of miR-134. D. Relative expression of KRAS in MKN-28 and SGC-7901 cells not transfected or transfected with miR-134 or scramble for 24 h, indicating that the miR-134 did not inhibit KRAS mRNA expression.

agreement with the tumor growth curve, the weights of tumors induced by scramble transfection were significantly higher than those induced by miR-134 over-expression (**Figure 3B**). Similarly, immunohistochemical analysis was performed to measure the protein levels of ki-67 in the tumor tissues. Lower ki-67 index was obtained in the miR-134-transfected cell tissues than in the controls (**Figure 3C**). This result indicates that miR-134 over-expression can limit the proliferation of gastric cancer cells in vivo.

miR-134 targets KRAS in gastric cancer

To identify target genes of miR-134, we applied several algorithms that predict the mRNA targets of miRNAs-TargetScan, PicTar and miRanda-mirSVR. The candidate target genes were predicted based on the representation of miR-134 recognition sites in their 3'-UTRs. As predicted, complementarity can be found between miR-134 and KRAS 3'-UTR (**Figure 4A**). To test whether or not KRAS is a target of miR-134, the plasmid pMIR-reporter containing the wild-type 3'-UTR region of KRAS downstream of the luciferase coding region was constructed. HEK-293T cells were co-transfected with reporter plasmid (KRAS) and scramble. Luciferase activity was markedly reduced by approximately 36% in the miR-134-overexpressing cells. The same assay was performed for another reporter plasmid containing mutated KRAS 3'-UTR in miR-134 binding sites. As expected, luciferase assays showed that the luciferase activity of KRAS was decreased in miR-134-expressing cells as compared with vector control cells, whereas that of the control plasmid remained unchanged (**Figure 4B**). These results indicate that miR-134 represses the 3'-UTR of KRAS and that the expression of KRAS is directly regulated by miR-134.

MKN-28 and SGC-7901 cells were transfected with miR-134 to further investigate the interaction between miR-134 and KRAS. Western blot was conducted to measure the level of KRAS protein. The protein expression of KRAS was down-regulated in the miR-134-treated MKN-28 and SGC-7901 cells but not in the scramble

or untreated cells (**Figure 4C**). The mRNA expression of KRAS was determined by real-time PCR. No significant difference was observed between miR-134-treated and scramble-treated or untreated MKN-28 and SGC-7901 cells (**Figure 4D**). These results suggest-that miR-134 directly recognizes the 3'-UTR of KRAS mRNA and inhibits KRAS translation. Thus, miR-134 down-regulation in gastric cancer inhibits KRAS suppression, which consequently decelerates tumourigenesis.

Up-regulation of miR-134 enhances the sensitivity of gastric cancer cells to 5-FU

Previous research demonstrated that miRNA dysregulation is related to the chemo-resistance of gastric cancer. However, to the best of our knowledge, the relationship between miR-134 expression and gastric cancer sensitivity has yet to be explored. Therefore, we treated scramble- or miR-134-transfected MKN-28 and SGC-7901 cells with 0, 5, 10, 15, 20 and 25 μg/ml of 5-FU. The results of MTT assay indicated that miR-134 up-regulation significantly decreased the cell viability of MKN-28 and SGC-7901 cells in response to 5-FU in a dose-dependent manner (**Figure 5A**). Moreover, 5-FU up-regulated miR-134 in the two cell lines. The expression level of miR-134 increased with 5-FU in a dose-dependent manner (**Figure 5B**). To confirm this effect, we evaluated the protein expression levels of KRAS and found the protein levels of KRAS significantly were reduced (**Figure 5C**).

Discussion

MicroRNAs (miRNA), a novel class of regulatory molecules, have been frequently indicated to be dysregulated in diverse human cancers and play important roles in tumourigenesis [15]. MiRNAs have been documented to function both as tumor suppressor genes and oncogenes, regulating many cellular events by targeting specific target genes. Therefore, miRNAs may serve as new biomarkers to predict clinical outcomes in the future [16]. Recent studies have been reported that miR-134 plays a potential role as a tumor suppressor in many kinds of cancers. However, there are no results referring to the role of miR-134 in gastric carcinoma at present.

In this study, we found that the expression of miR-134 is downregulated in gastric cancer samples compared with normal tissues. Moreover, over-expression of miR-134 significantly inhibited gastric cancer cell growth and increased sensitivity to 5-FU. The present study provided insights into the association of miR-134 expression with cell proliferation and apoptosis in gastric cancer. We evaluated miR-134 expression in gastric cancer patients. Results showed that 88% of gastric cancer clinical samples showed significantly lower miR-134 expression than adjacent normal tissues. This result suggests that miR-134 may function as a tumor suppressor. In vitro and in vivo studies proved that miR-134 can also serve as a tumor suppressor gene in human gastric cancer progression. The results of the assays on cell proliferation and apoptosis may be used to further understand the mechanism by which miR-134 contributes to gastric cancer tumourigenesis and progression.

In our study, KRAS oncogene has been experimentally validated as the novel target of miR-134. RAS family play a pivotal role in the transduction of several growth or differentiation factor stimuli. It has been reported that the expression levels of KRAS are related to the malignant degree of cancers, including glioma, breast cancer, melanoma, and other cancers [17]. Recently, accumulating evidence has indicated that expression levels of the RAS family can be regulated by miRNAs. For example, over-expression of miR-145 can inhibits tumor angiogenesis and growth by targeting KRAS and VEGF [18]. Firstly, luciferase reporter assay confirmed that miR-134 directly recognize the 3'-UTR of KRAS transcripts. Secondly, KRAS expression was significantly abolished in gastric cancer cells which miR-134 stably-expressed. In the present study, miR-134 over-expression decreased KRAS levels in two gastric cancer cell lines. This result indicates that KRAS also serves as a target of miR-134 in gastric cancer. No significant changes in KRAS mRNA level were observed. This result indicates that miR-134 may function as a post-transcriptional regulation factor of KRAS. In conclusion, miR-134 is frequently down-regulated in gastric cancer and acts as a tumor suppressor in gastric cancer cells by targeting KRAS. MiR-134 up-regulation enhances the sensitivity of gastric cancer cells to 5-FU. These results provided insights into the functions of miR-134 in 5-FU chemoresistance and the rational development of new targeted combinations against gastric cancer.

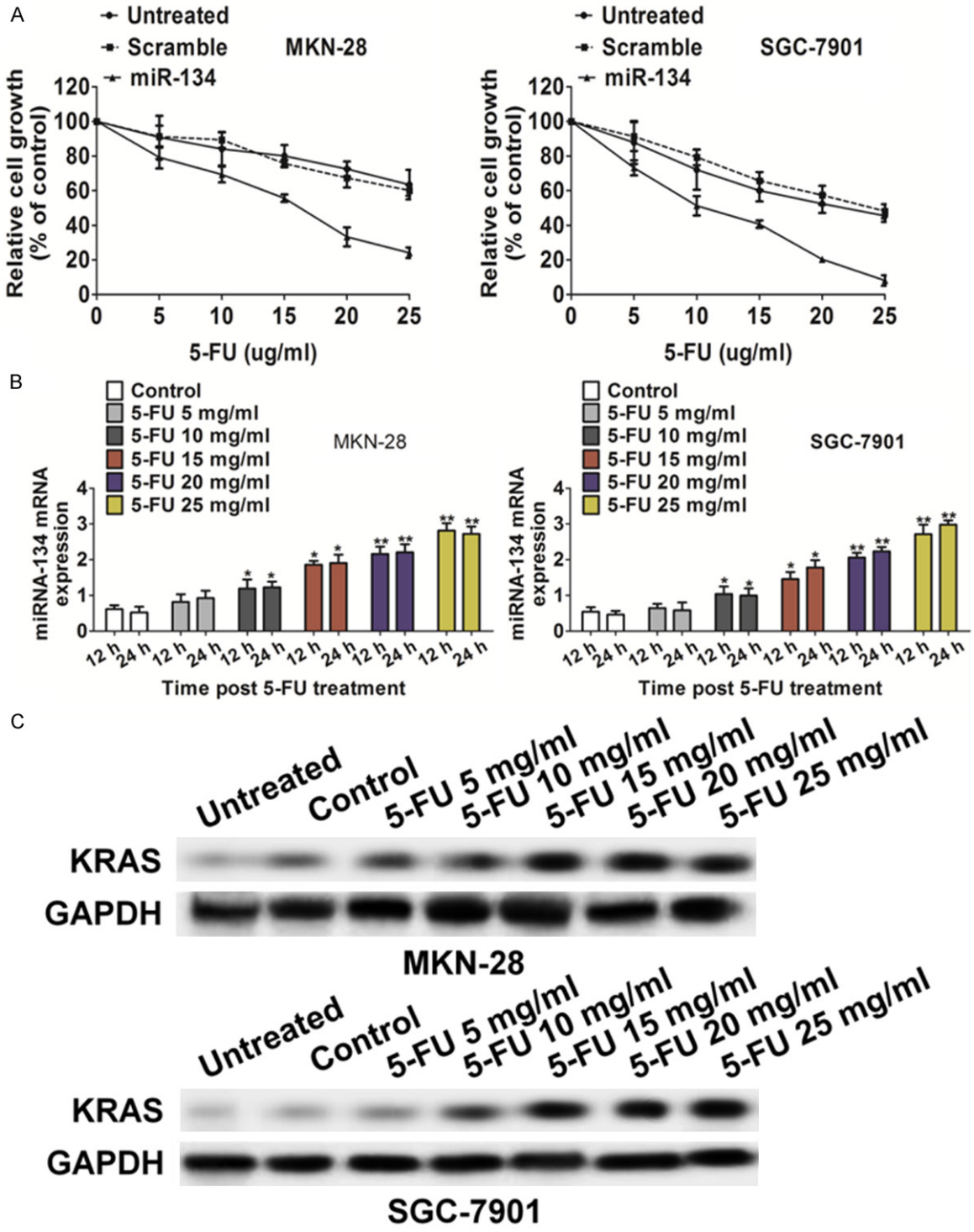

Figure 5. Effect of miR-134 up-regulation on the sensitivity of cells to 5-FU. A, B. Effects of various concentrations of 5-FU on MKN-28 and SGC-7901 cells for 24 h as assessed by MTT assay, indicating that miR-134 up-regulation can significantly increases the sensitivity of cells to 5-FU. C. Western blotting assay shown miR-134 up-regulation facilitate KRAS protein expression.

This study is the first to report that miR-134 over-expression can reduce 5-FU cell resistance in gastric cancer cell lines. Therefore, combining 5-FU with miR-134 regulation may serve as a potential approach for gastric cancer therapy. High 5-FU doses can cause toxic effects, such as nephrotoxicity and ototoxicity. The results of this study showed that the sensi-

tivity of gastric cancer cells to 5-FU significantly increased. Therefore, we speculate that using 5-FU in combination with miR-134 as a potential approach for gastric cancer therapy may achieve low-dose 5-FU application and reduce 5-FU toxic effects.

Acknowledgements

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Disclosure of conflict of interest

None.

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