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Original Article

MicroRNA-143 shows tumor suppressive effects through inhibition of oncogenic K-Ras in pituitary tumor

Jimin Zhang[1], Dongzhou Ma[1], Hui Liu[2], Jingtao Wang[1], Jinbiao Fan[1], Xiushan Li[1]

[1]Department of Neurosurgery, Affiliated Hospital of Hebei University of Engineering, Handan 056002, Hebei Province, China; [2]Department of Neurosurgery, Jizhong Energy Fengfeng Group Hospital, Handan 056200, Hebei Province, China

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Abstract: MicroRNAs (miRNAs) are a class of small non-coding RNA molecules, about 21-25 nucleotides in length. Accumulating evidence demonstrated that dysregulation or dysfunction of miRNAs are involved in various diseases, including cancer. MiR-143, recently has been reported to function as an important tumor suppressor in prostate cancer, pancreatic ductal adenocarcinoma and other kinds of cancers, but rarely systematically studied in pituitary tumor. In the present study, we firstly found that miR-143 was significantly down-regulated in pituitary tumor tissues and cell lines (GH3 and MMQ). Then, subsequent studies revealed that miR-143 inhibits cell proliferation and promotes apoptosis in both GH3 and MMQ cells. In addition, K-Ras, one of the most important oncogenes involved in many kinds of cancers, was found to be suppressed by miR-143 in pituitary tumor. Furthermore, overexpression of K-Ras greatly reversed the suppressive effect of miR-143 on pituitary tumor cells. In summary, our study demonstrated that miR-143 functions as a tumor suppressor and directly targets K-Ras in human pituitary tumor.

Keywords: MiR-143, pituitary tumor, K-Ras, apoptosis

Introduction

Pituitary tumor is one of the most common intracranial tumors that occur in the pituitary gland, and can be generally classified as clinically functioning and non-functioning pituitary adenomas (NFPAs) which accounts for ~40% of all pituitary tumor [1, 2]. Functioning tumors secrete excess anterior pituitary hormones such as growth hormone (GH), adrenocorticotropic hormone (ACTH), prolactin (PRL), etc, and could result in hormone-specific clinical syndromes [3, 4]. The NFPAs, instead of producing excess functional hormones, usually cause hypopituitarism and neurological symptoms. Most adenomas are benign, approximately 35% are invasive and just 0.1% to 0.2% are carcinomas [5]. However, the invasive pituitary tumors are lethal to patients, because neither surgical section or radiation is effective to these tumors [6]. Hence, identifying early diagnostic biomarkers of pituitary tumors and illustrating its underlying molecular mechanisms are of great importance.

MicroRNAs (miRNAs) are a class of naturally occurring, small non-coding RNA molecules, about 21-25 nucleotides in length [7]. They bind through partial sequence homology to the 3' untranslated region of target messenger RNAs and function through either blocking translation or promoting degradation of messenger RNA [8, 9]. MicroRNAs participate in a variety of cellular processes, including cell growth, apoptosis, development, differentiation, and endocrine homeostasis [10-12]. Accumulating evidence demonstrated that dysregulation or dysfunction of miRNAs is involved in the carcinogenesis and pathogenesis of various human cancers, including pituitary tumor [13-15]. Previous studies demonstrated that miR-143 could function as an important tumor suppressor in many carcinoma, such as prostate cancer and osteosarcoma [16-18]. As with pituitary tumor, although Amaral FC etc. reported that miR-143 expression was down-regulated in ACTH-secreting pituitary tumors [19], its function and effect remains unclear.

K-Ras is one of the three members of the Ras oncogene family, discovered in the early 1980s [20]. It is a very important molecular switch that activates the RAS/RAF/MEK/ERK pathway, which is one of the most important signaling pathways in cancer. The K-Ras oncogene has been implicated in the carcinogenesis of many human cancers, and downregulation of K-Ras could suppress tumor cell growth and increase chemo-sensitivity [21-24]. Recently, several miRNAs were reported to suppress K-Ras expression and function as tumor suppressors including miR-143 [24-26].

In terms of pituitary tumor, there are only a few studies that identified several miRNAs and their related target oncogenes [13, 14, 27, 28]. Here, our results revealed that miR-143 could suppress pituitary tumor by down-regulating K-Ras, and leading to inhibition of cell proliferation and promotion of apoptosis. These findings support the importance of miRNAs in suppression of pituitary tumor, and provide a useful biomarker and potential therapeutic target.

Materials and methods

Tissue samples and cell lines

A total of 20 pituitary tumor and 6 normal tissue samples were obtained from patients in Affiliated Hospital of Hebei University of Engineering. Informed consent for this study was obtained from all patients, and this study was approved by the Research Ethics Committee of the Affiliated Hospital of Hebei University of Engineering. All the samples were immediately snap frozen in liquid nitrogen after surgery and stored at -80°C before extraction of RNA.

GH3 and MMQ cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml). The cells were incubated at 37°C in a humidified incubator with 5% CO_2.

Extraction of RNA and RT-PCR (quantitative reverse transcription polymerase chain reaction)

Total RNAs were extracted from tissues and cells using the TRIzoL reagent (Applied Biosystems, Foster City, CA). To detect mRNA, cDNA was synthesized using Reverse Transcription Kit (Applied Biosystems). Then Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses for genes were conducted with the SYBR Green PCR Master mix (Applied Biosystems) on an ABI 7900 System (Bio-Rad). Here human GAPDH was amplified in parallel as an internal control. To calculate the relative expression levels, the $2^{\Delta\Delta Ct}$ method was used. The primers (Invitrogen) were designed as follows: MiR-143: collar primers, 5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACtgagcta-3'; forward, 5'-AGTGCGTGTCGTGGAGTC-3'; reverse, 5'-GCCTGAGATGAAGCACTGT-3'. k-Ras: forward, 5'-ATGACTGAATATAAACTTGTGG; reverse, 5'-TTACATAATTACACACTTTGTC.

Plasmid construction and cell transfection

The full length wt-K-Rassequence was subcloned into the pcDNA3.1 vector (Invitrogen, USA). The mut-K-Ras vector with mutation in major miR-143 binding sites were mutated by site-directed mutagenesis (Stratagene), was constructed as a mutant control. Wt-K-Ras or mut-K-Ras ectopic over-expression was achieved through pcDNA3.1-wt-K-Ras transfection using lipofectamine2000 (Invitrogen, USA), with an empty pCDNA3.1 vector used as a control. The expression levels of K-Ras were measured by quantitative PCR. Plasmid vectors (pcDNA3.1-BANCR and pcDNA3.1) were transfected into 293T- or GH3 or MNQ cells, respectively. The siRNAs (miR-143 mimics, NC mimics and miR-143 inhibitor) were respectively transfected into 293T- or GH3 or MNQ cells, separately. After transfection for 48 h, cells were harvested for further cell proliferation, apoptosis assays, and quantitative PCR.

Cell viability assay

Cells were seeded in the 96 well plates 24 h after transfection at a density of 1500 cells per well. Then, the cell viability assay was conducted using Cell Counting Kit-8 (CCK8; Dojindo) based on the manufacturer's introduction. The absorbance at 450 nm was measured and recorded. Experiments were repeated three times.

Detection of apoptosis by flow cytometry

For detection of apoptosis, the cells were cultured in low-serum medium and harvested

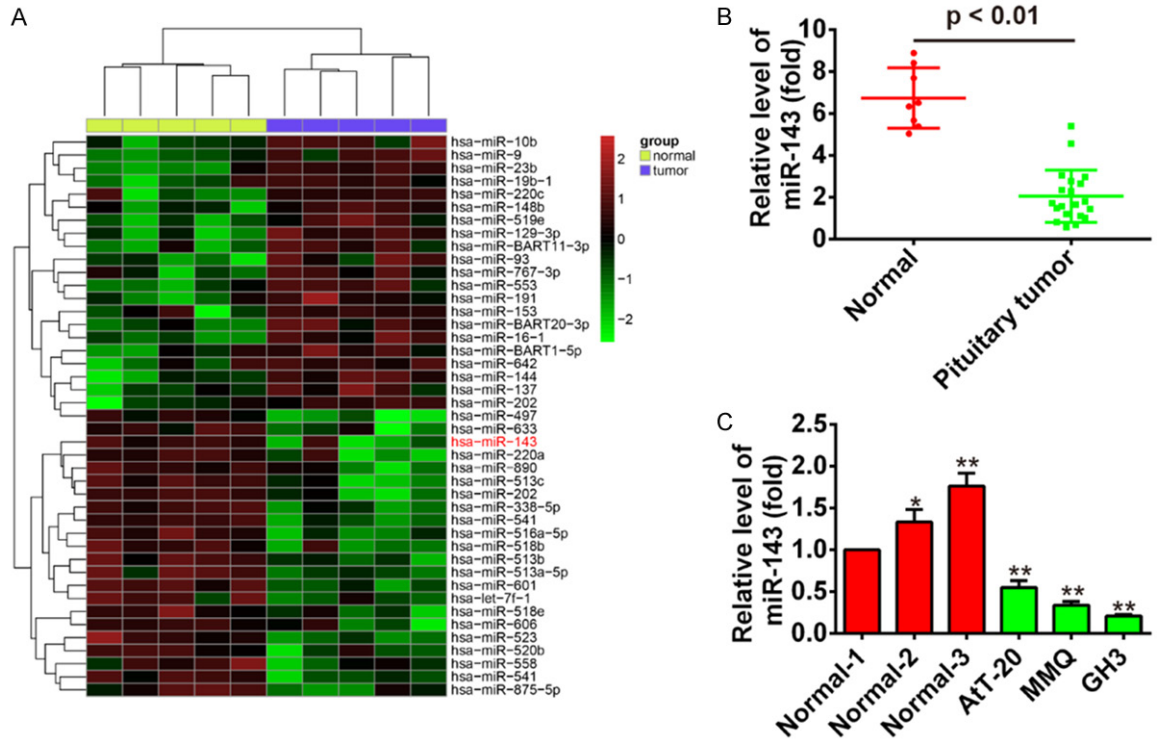

Figure 1. Expression of miR-143 was down-regulated in pituitary tumor tissues and cells. A. Summary heat map showing potential miRNAs predicted to be differently expressed between pituitary tumor tissues and normal tissues. The vertical dendrogram indicates patient sample with closest miRNA expression patterns. The horizontal dendrogram indicates miRNA with closest expression pattern across all patient samples. The heat map colours range from orange-red (high intensity values) to green (low intensity values). B. Expression of miR-143 was down-regulated in pituitary tumor tissues. C. Expression of miR-143 was down-regulated in pituitary tumor cells, including GH3, MMQ and AtT-2. *P < 0.05, **P < 0.01 vs Normal-1 group.

after transfection for 48 h. Cells were subsequently stained with Annexin V-FITC (eBioscience, USA) and PI for 30 min as described by the manufacturer. Apoptosis cells were analyzed by FACS.

Western blot analysis

Protein was extracted from cells using 1× radio immunoprecipitation assay lysis buffer (Santa Cruz Biotechnology), then subjected to SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Membranes were blocked for 2 h in 5% skimmed milk and probed with antibody against human K-Ras (Abcam, Cambridge, UK, 1:1000 dilution) or β-actin (Santa Cruz Biotech, Santa Cruz, CA, 1:1000 dilution) or cleaved-caspase-3 (Abcam, Cambridge, UK, 1:2000 dilution) or totao-caspase-3 (Abcam, Cambridge, UK, 1:2000 dilution) overnight at 4°C, and then with peroxidase-conjugated secondary antibody (Santa Cruz Biotech, Santa Cruz, CA, 1:1000 dilution), visualized by chemiluminescence (GE, Fairfield, CT, USA).

Statistical analysis

All values shown in this paper were expressed as mean ± SD and processed by the SPSS software (version 22.0). Student's t-test was run to analyze the significance of the differences among the groups, and *p* values less than 0.05 were considered as statistically significant.

Results

MiR-143 expression was downregulated in both tissues and cell lines

To better understand miRNA expression alteration in pituitary adenomas, we analyzed the entire miRNAome in pituitary adenomas and in normal pituitary samples by gene microarray and verified by Real-Time PCR. As shown in **Figure 1A**, 43 miRNAs are differentially ex-

pressed between normal pituitary and pituitary adenomas. Surprisingly, we found that miR-143 was significantly downregulated in pituitary tumor tissues. As far as we known, miR-143 has been reported to be downregulated and exert tumor suppressive function in several malignancies such as breast cancer [29], lung cancer [30], gastric cancer [31], colorectal cancer [32], and renal cell carcinoma [33], but never involved in pituitary tumor. Then we assessed miR-143 levels using a qRT-PCR assay in 20 pituitary tumor tissues, 6 normal pituitary tissues, 3 normal cell lines and 3 pituitary tumor cell lines.

As shown in **Figure 1B**, the results showed that the expression levels of miR-143 were significantly lower in carcinoma tissue than those in the corresponding adjacent noncancerous tissues (**P < 0.01). The miR-143 expression in three pituitary tumor cell lines was also clearly downregulated (**Figure 1C**). The GH3 and MMQ cell lines, which possessed the lower levels of miR-143 expression among all tested cell lines, were chosen for further studies. As one new potential biomarker, the roles of miR-143 and its related molecular mechanisms in carcinogenesis of pituitary tumor are unclear and need to be investigated.

miR-143 mimics inhibit pituitary tumor cell proliferation and promotes apoptosis

To examine the effects of miR-143 on cell proliferation and apoptosis, we overexpressed miR-143 by transient transfection of miRNAs mimics and mimics NC, respectively, and their expression was further confirmed using qRT-PCR. As shown in **Figure 2A**, the expression level of miR-143 in transfected cells of miR-143 mimics was significantly higher compared with non-transfected cells (P < 0.01). Consistently, the cell viability of miR-143 transfected cells is obviously reduced (**Figure 2B** and **2C**). Then we further explored cell apoptosis in these cells. In our study, related cells were stained with Annexin V-FITC and PI and analyzed by flow cytometry. According to our results, cell apoptosis rate in transfected cells of miR-143 mimics was visibly increased (**Figure 2D**). Taken together, over-expression of miR-143 mimics could induce cell apoptosis and inhibit cell proliferation of both GH3 and MMQ cell lines.

K-Ras is a direct target of miR-143

Based on previous studies, miR-143 could function as an important oncogene suppressor in kinds of tumors. In our study, miR-143 could also inhibit cell proliferation and promote apoptosis, however its potential target and regulatory mechanism remains unknown. To find out potential target messenger RNAs, we carried out bioinformatics analysis of miR-143. K-Ras was identified as one of the potential targets of miR-143 and caught our attention. The predicted binding of miR-143 with K-Ras 3' untranslated region is illustrated in **Figure 3A**.

As we know, activation of the K-Ras oncogene has been implicated in the carcinogenesis of many human cancers, including pituitary tumor [34]. To further confirm that K-Ras is the direct target of miR-206 in pituitary tumor, pcDNA-wt-K-Ras and pcDNA-mut-K-Ras plasmids were constructed and transfected into 293-T cells, separately. The reporter assay revealed that transfection of miR-143 mimics triggered a marked decrease of luciferase activity in the wt-K-Ras plasmid transfected 293-T cells but made no difference on the luciferase activity in mut-K-Ras transfected 293-T cells, and miR-143 inhibitor could greatly increase luciferase activity of wt-K-Ras (**Figure 3B**). In addition, transfection of miR-143 mimics could significantly reduce expression level of mRNA and protein of wt-K-Ras in GH3 cells, but transfection of miR-143 inhibitor could increase that (**Figure 3C**, **3D**). These data indicates that K-Ras should be a direct target of miR-143 in pituitary tumor.

K-Ras overexpression rescued the inhibitory effect of miR-143 on pituitary tumor cells

To further test whether miR-143 function as tumor suppressor by targeting K-Ras in pituitary tumor, miR-143 mimics alone and/or with pcDNA-K-Ras plasmid were transfected into GH3 and MMQ cells for analysis. MTT assay showed that the tumor suppressor effect of miR-143 could be ameliorated by overexpression of K-Ras in both GH3 and MMQ cell lines (**Figure 4A** and **4B**). It was also found that cells transfected with miR-143 mimics and K-Ras together showed lower apoptosis rate compared with groups transfected with miR-143 mimics only in both GH3 and MMQ cell lines, indicating that K-Ras significantly reversed the

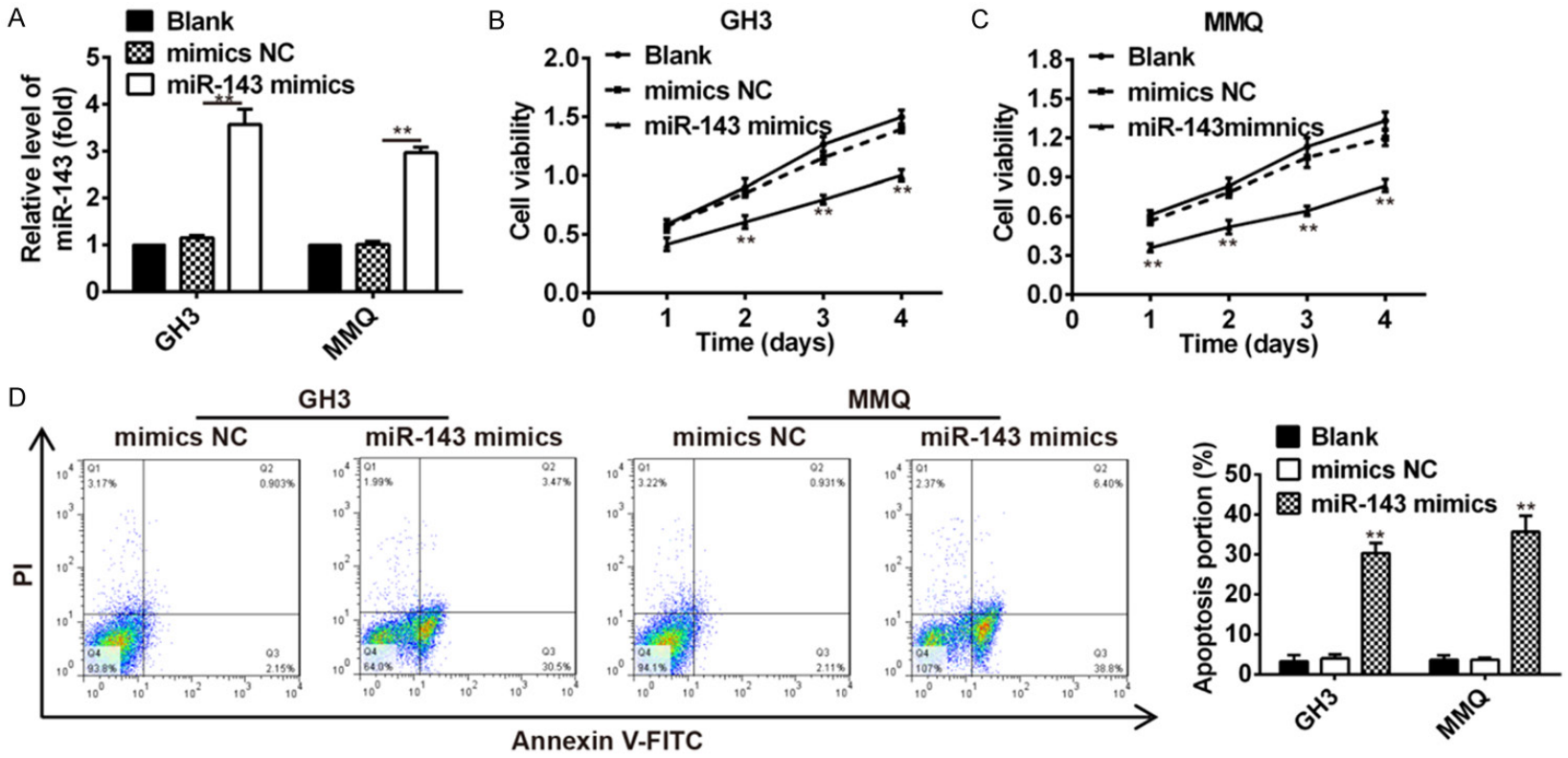

Figure 2. MiR-143 inhibited cell proliferation and promoted apoptosis in pituitary tumor cells. A. Over-expression of miR-143 mimics and NC mimics in GH3 and MMQ cells. B. Cell viability was measured by MTT assays in GH3 cells transfected with miR-143 mimics or mimics NC. The results (mean ± SD) were from three independent experiments (**P < 0.01 vs blank group). C. Cell viability was measured by MTT assays in MMQ cells transfected with miR-143 mimics or mimics NC. The results (mean ± SD) were from three independent experiments (**P < 0.01 vs blank group). D. Apoptosis of GH3 and MMQ cells after transfection with miR-143 mimic or mimics NC were determined by flow cytometric analysis. Prior to flow cytometry, the cells were stained with annexin V-fluorescein isothiocyanate. Shown in the right panel were the incidences of apoptotic cells (**P < 0.01 vs blank group).

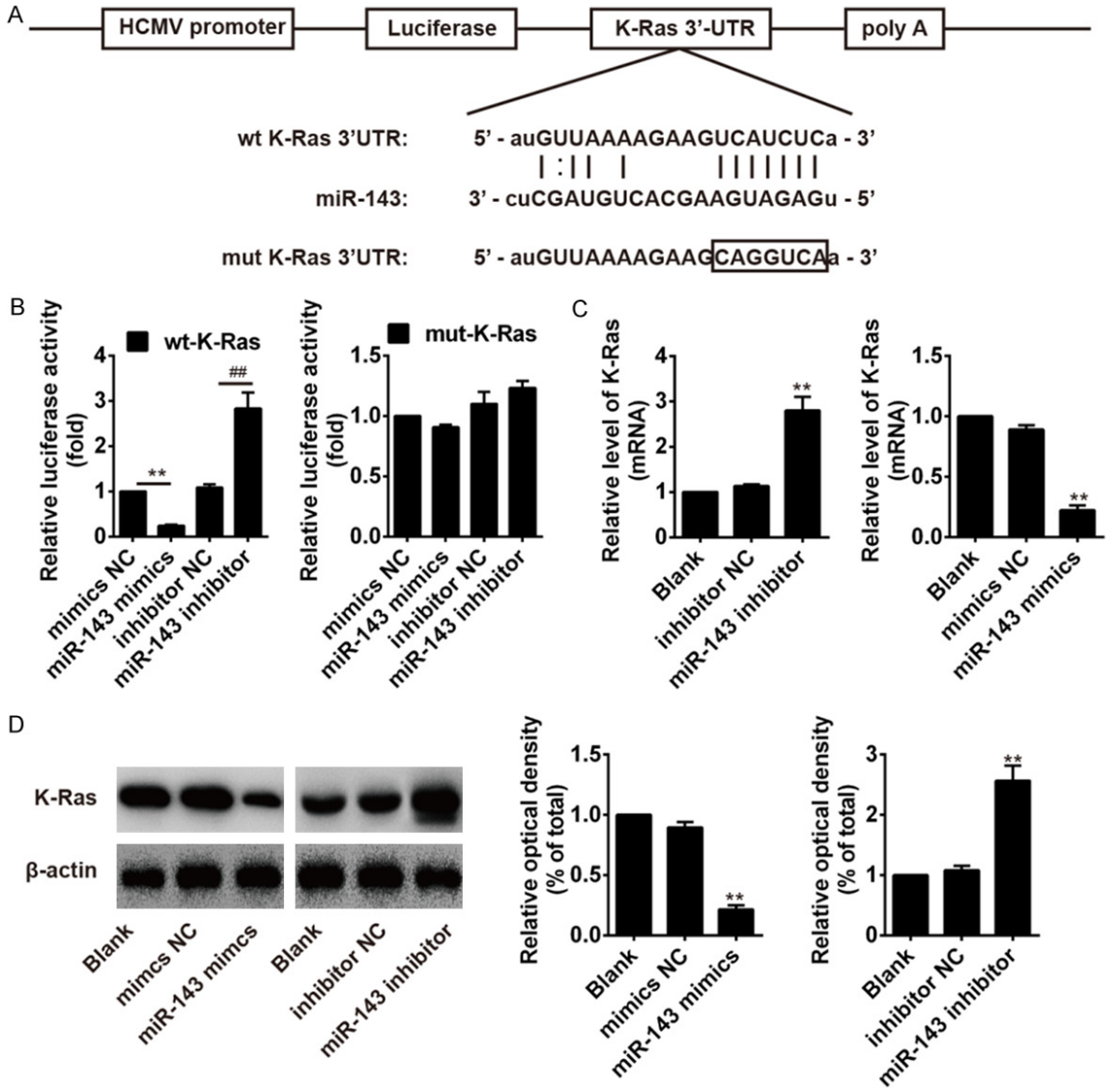

Figure 3. K-Ras is a target of miR-143. A. Predicted miR-143 binding sites in K-Ras 3'UTR. Mut K-Ras indicates the K-Ras 3'UTR with mutation in major miR-143 binding sites. B. The firefly luciferase reporter activity in 293-T cells transfected with mimics NC or miR-143 mimics or inhibitor NC or miR-143 inhibitor after transfection with pCDNA-wt-K-Ras 3'-UTR reporter or pCDNA-mut-K-Ras 3'-UTR reporter plasmid (**P < 0.01 vs mimics NC; ##P < 0.01 vs inhibitor NC). C. The mRNA level of K-Ras in GH3 cells transfected with inhibitor NC or miR-143 inhibitor or mimics NC or miR-143 mimics was measured by qRT-PCR (**P < 0.01). D. The Western blot showed that transfection of miR-143 decreased K-Ras protein expression, while transfection of miR-143 inhibitor increase its expression (mimics NC vs miR-143 mimics, inhibitor NC vs miR-143 inhibitor, **P < 0.01 vs blank group).

stimulative effect of miR-143 on pituitary tumor cell apoptosis (**Figure 4C** and **4D**), and rescued the up regulation of cleaved-cascapse-3 (**Figure 4E** and **4F**). These findings suggest that miR-143 inhibited proliferation signaling primarily due to directly down-regulating K-Ras, and overexpression of K-Ras can rescue the inhibitory effect of miR-143 on pituitary tumor development.

Discussion

In this study, we found that miR-143 was significantly down-regulated in pituitary tumor tissues and cell lines (GH3 and MMQ). In addition, miR-143 mimics inhibits pituitary tumor cell proliferation and promotes apoptosis in GH3 and MMQ cell lines. In line with bioinformatics analysis, K-Ras was identified as a direct target

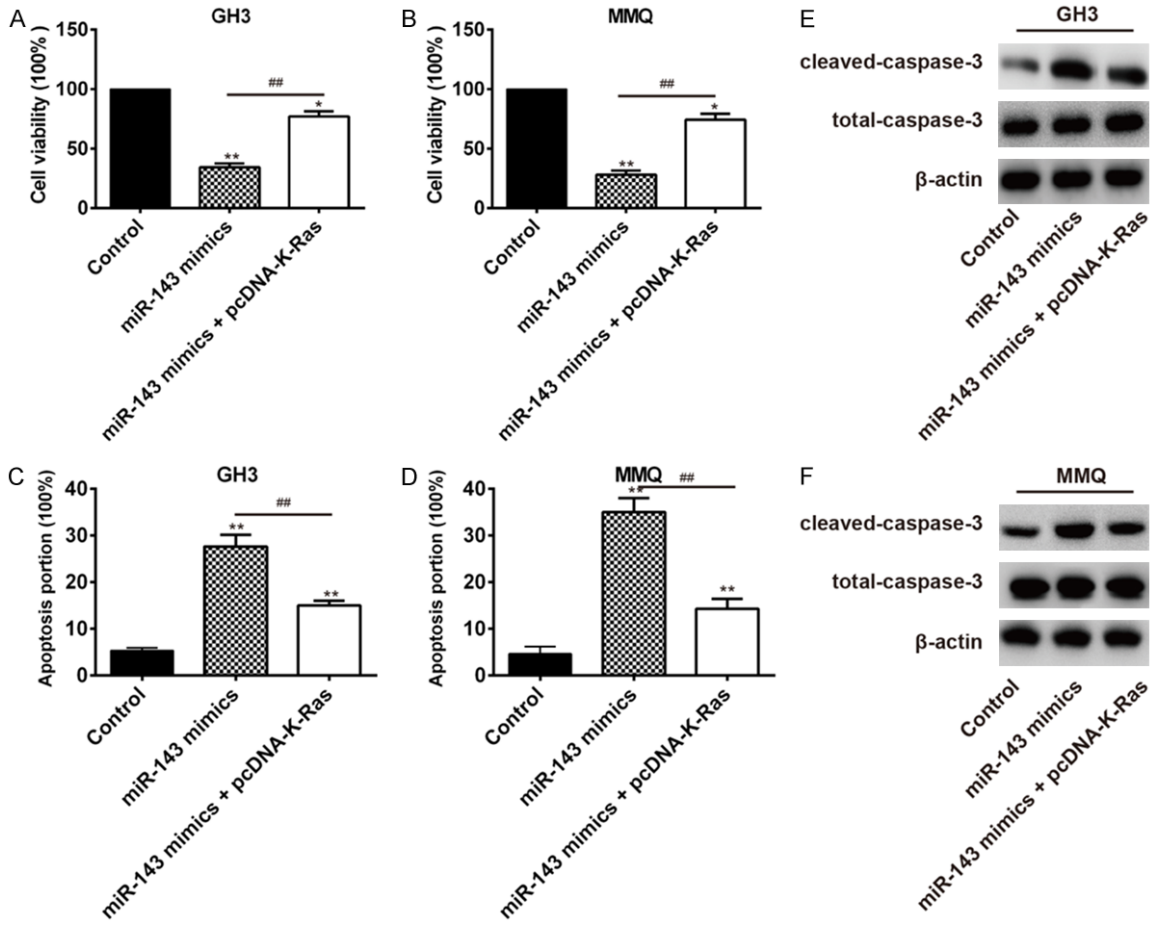

Figure 4. K-Ras overexpression rescued the inhibitory effect of miR-143 on GH3 and MNQ cells. A. Cell viability was measured by MTT assays in GH3 cells transfected with miR-143 mimics or miR-143 mimics plus pcDNA-K-Ras. The results (mean ± SD) were from three independent experiments (**P < 0.01 vs control group; ##P < 0.01 vs miR-143 mimics group). B. Cell viability was measured by MTT assays in MNQ cells transfected with miR-143 mimics or miR-143 mimics plus pCDNA-K-Ras. The results (mean ± SD) were from three independent experiments (**P < 0.01 vs control group; ##P < 0.01 vs miR-143 mimics group). C. Apoptosis of GH3 cells after transfection with control or miR-143 mimic or miR-143 mimics plus pCDNA-K-Ras were determined by flow cytometric analysis. Shown were the incidences of apoptotic cells. Prior to flow cytometry, the cells were stained with annexin V-fluorescein isothiocyanate (**P < 0.01 vs control group; ##P < 0.01 vs miR-143 mimics group). D. Apoptosis of MNQ cells after transfection with control or miR-143 mimic or miR-143 mimics plus pCDNA-K-Ras were determined by flow cytometric analysis. Shown were the incidences of apoptotic cells. Prior to flow cytometry, the cells were stained with annexin V-fluorescein isothiocyanate (**P < 0.01 vs control group; ##P < 0.01 vs miR-143 mimics group). E, F. The Western blot showed that transfection of miR-143 mimics increased cleaved-caspase-3 in GH3 and MNQ cells, while ectopic over-expression of K-Ras rescued this effect.

of miR-143 in pituitary tumorigenesis by subsequent luciferase reporter assay and Real-time PCR. Further experiment confirmed that miR-143 inhibited proliferation signaling primarily due to directly down-regulated K-Ras, and overexpression of K-Ras can rescue the inhibitory effect of miR-622 on pituitary tumor development. Here, we revealed that miR-143 functions as a tumor suppressor in human pituitary tumor through inhibition of K-Ras.

Recently, many miRNAs have been reported as promising biomarkers for the diagnosis or prediction of the prognosis in kinds of cancer. CH Gattolliat et al. reported that expression of two miRNAs, miR-487b and miR-410, shows predictive value beyond the classical high-/low-risk stratification, and thus they could be clinically useful markers of relapse in human neuroblastoma [35]. For oral squamous cell carcinoma (OSCC), in 2014 Feiou Lin et al. identified miR-

206 as one potent biomarker, for it is frequently down-regulated in OSCC and function as a tumor suppressor which directly targets K-Ras [26]. For human pituitary tumor, microRNA-106b was found to promote pituitary tumor cell proliferation and invasion through PI3K/AKT signaling pathway, and hence would likely to become one potential diagnostic biomarker or therapeutic target for treatment of pituitary tumor [36]. Our study revealed that miR-143 is frequently down-regulated in pituitary adenomas and hence suggesting a promising biomarker for pituitary adenomas.

Previous studies demonstrated that miR-143 could function as an important tumor suppressor or was significantly decreased in many types of cancer. For example, Ma Z et al. found that over-expression of miR-143 induces the apoptosis of prostate cancer LNCap cells and significantly inhibited its proliferation by down-regulating Bcl-2 expression [37]. And it was also reported that miR-143 could inhibit Bcl-2 expression, leading to Caspase 3 activation, and thus inducing apoptosis and suppress in osteosarcoma [38]. In addition, Ansari MH et al. claimed that expression miR-143 was greatly down-regulated in most of the examined human esophageal cancer tissues and may be potential biomarker in esophageal squamous cell carcinoma [39]. However, function of miR-143 has never been mentioned in human pituitary tumor. For the first time, we found that miR-143 among other microRNAs was significantly down-regulated in pituitary tumor tissues by RNA screen and it may be involved in pituitary adenomas development.

In the current study, we examined effects of transfection of miR-143 mimics on cell proliferation and apoptosis of GH3 and MMQ cells and found that miR-143 inhibit cell proliferation and promote apoptosis. In addition, our results revealed that K-Ras acted as a direct target of miR-143 in pituitary adenomas and rescue experiment further confirmed our conclusion. It is noteworthy that K-Ras oncogene has been implicated in the carcinogenesis of many human cancers. It can turn on the downstream RAF protein kinases and then activates the RAS/RAF/MEK/ERK pathway, which is one of the most important signaling pathways in cancers [39-42]. Recent studies showed that it act as an important target of several miRNAs in different cancers, such as human oral squamous cell carcinoma [24], glioblastoma [23] and nasopharyngeal carcinoma [43], etc. Here, we firstly found that the expression level of K-Ras was up-regulated by downregulation of miR-143 in pituitary adenomas.

In conclusion, this study demonstrated for the first time that miR-143 is frequently down-regulated in pituitary adenomas and inhibits cell proliferation and promotes apoptosis in GH3 and MMQ cell lines by down-regulating K-Ras. These findings suggest that miR-143 is a promising tumor-suppressing biomarker and may be useful for diagnosis of pituitary adenomas. However, the underlying mechanism of suppression of miR-143 in pituitary adenomas remains to be completely elucidated in future.

Disclosure of conflict of interest

None.

Address correspondence to: Xiushan Li, Department of Neurosurgery, Affiliated Hospital of Hebei University of Engineering, No. 81 Congtai Road, Congtai District, Handan 056002, Hebei Province, China. Tel: +86-0310-5282181; E-mail: Xiushan_lixs@163.com

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