MicroRNA-205 suppresses the growth of adrenocortical carcinoma SW-13 cells via targeting Bcl-2

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Abstract. Compared to adrenocortical adenoma (ACA), adrenocortical carcinoma (ACC) has very poor prognosis and limited treatment options. Also conventional methods to distinguish ACC from ACA can be difficult. At this time, no molecular pathological markers are reliable enough to distinguish either tumor. Recently, increasing data have indicated miRNAs to be crucial regulators in the tumor-related processes. In the present study, we found that miR-205 expression is significantly suppressed in ACC tissues compared with ACAs, and that this induces apoptosis and impairs proliferation of ACC SW-13 cells *in vitro* as well as inhibits tumor growth *in vivo*. Using bioinformatic predictions, Bcl-2 was identified to be a target of miR-205 via 3'-untranslated region (3'UTR) interactions, which was confirmed by luciferase assay, qRT-PCR, immunohistochemical assay and western blotting showing that mRNA and protein expression of Bcl-2 were negatively related to miR-205. Further investigation into the mechanism found that activation of Bcl-2 cleaved Bax, releasing caspase-9 and -3 that are involved in the intrinsic apoptosis pathway, eventually inducing SW-13 cell apoptosis. In conclusion, miR-205 suppresses the growth of ACC SW-13 cells via targeting the anti-apoptotic gene Bcl-2.

Introduction

Adrenocortical carcinoma (ACC) is a rare but aggressive endocrine malignancy with an incidence of ~1-2/million annually and a 5-year survival rate of 16-44% (1-3). Currently, there is limited information concerning the molecular pathology and pathogenesis of ACC and there is no effective therapy. In addition, distinguishing ACC from adrenocortical adenoma (ACA) using conventional histology and imaging can be difficult. Presently, no molecular pathological markers are able to reliably distinguish between the two cancers. Thus, local invasion or distant metastasis is the only absolute demonstration of malignancy (4). Given the rarity and poor survival rate of ACC and the high prevalence of ACA in the general population (5), as well as the different treatments used (6), the use of biomarkers to confirm the type of adrenocortical tumors (ACT) is required. These markers are also therapeutic targets and prognostic indicators for ACC.

MicroRNAs (miRNAs) are 19-25 nucleotide small, non-coding RNAs involved in 30% of human gene expression via binding to target mRNAs (7,8). Previous data showed that small RNAs are misregulated in human tumors and can serve as either oncogenes or tumor suppressor genes (9-11). Functional studies suggest that aberrant miRNA expression is a key to cancer development, including cell proliferation, apoptosis and differentiation (12). Due to specific expression patterns and tremendous regulatory capacity, miRNAs are being assessed as potential biomarkers to help diagnose and treat different types of cancers, including ACC. However, limited miRNA expression analyses have been performed on ACC.

We noted downregulation of miR-205 expression in ACC samples compared to ACAs. Overexpression of miR-205 not only induced apoptosis and impaired proliferation of ACC SW-13 cell *in vitro*, but also inhibited tumor growth *in vivo*. Furthermore, miR-205 inhibited Bcl-2 mRNA and protein expression via 3'-untranslated region (3'UTR) interaction; this activated the intrinsic apoptosis pathway in SW-13 cells. Therefore, Bcl-2 is a critical anti-apoptotic gene involved in the intrinsic apoptotic pathway (13), and it is a target via which miR-205 inhibits SW-13 cell proliferation.

Materials and methods

Cell line, tissues and mice. A SW-13 cell line was purchased from the Cell Repository of the Chinese Academy of Science and originated from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Leibovitz-15 (L-15) media at 37˚C without CO_2. Then, 32 cases of ACC and ACA tissue samples were obtained from the Guangzhou General Hospital of People's Liberation Army. Clinical characteristics of the study cohort are summarized

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Table I. Clinical characteristics of the study cohort.

Characteristics	ACC	ACA
No. of patients	11	21
Age (average ± SD)	53±12	48±16
Gender (female/male)	8/3	14/7
Syndrome		
Cushing's	7	7
Subclinical Cushing's	0	1
Conn's	0	3
Non-functioning	4	10

ACC, adrenocortical carcinoma; ACA, adrenocortical adenoma; SD, standard deviation.

Table II. Primers used in the present study.

Primers		Sequence
miR-205	F	5'-ACACTCCAGCTGGGTAGGTAGTTTCATGTTGTT-3'
miR-205	R	5'-CTCAACTGGTGTCGTGGA-3'
U6	F	5'-CTCGCTTCGGCAGCACA-3'
U6	R	5'-AACGCTTCACGAATTTGCGT-3'
Bcl-2	F	5'-ATTTTGTAGTCACCCACCTCTAAGG-3'
Bcl-2	R	5'-CATCTCCCTTCACAGCAGAACTTAAC-3'
β-actin	F	5'-GGGAAATCGTGCGTGACATTAAGG-3'
β-actin	R	5'-CAGGAAGGAAGGCTGGAAGAGTC-3'

F, forward; R, reverse.

in Table I. Tumors were classified as ACC when the Weiss criteria were ≥3. Tumors were classified as benign when the Weiss criteria were <3 (14). Female nude mice 4 weeks of age were provided by the Animal Center of Southern Medical University.

Quantitative real-time PCR (qRT-PCR). According to the manufacturer's protocol, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and first strand cDNA was synthesized using miScript Reverse Transcription kit (Qiagen). miR-205 expression was quantified by qRT-PCR using TaqMan microRNA assays (Applied Biosystems, Carlsbad, CA, USA) and U6 was used for normalization. Bcl-2 expression was quantified by qRT-PCR using SYBR-Green assays (Applied Biosystems) and β-actin was used for normalization. Real-time PCR was performed on the ABI 7500 Sequence Detection System (Applied Biosystems), and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All experiments were performed in triplicate independently. The sequences of primers are depicted in Table II.

miRNA mimics and transfection. miR-205 mimics, the negative control pre-miR (miR-NC) and the miR-205 inhibitors provided by RiboBio (Guangzhou, China) were transfected into SW-13 cells using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's manual.

Cell proliferation assay. SW-13 cells transiently transfected with miR-205, miR-NC and miR-205 inhibitors were cultured in 96-well plates ($4x10^3$ cells/well) for 24 h. Cell proliferation was measured via MTT assay (KeyGen, China) and further verified using a 5-ethynyl-2'-deoxyuridine (EdU) assay (RiboBio) following the manufacturer's instructions. SW-13 cells incorporating EdU were observed under fluorescent microscopy. Cell proliferation was measured using the following formula: SW-13 cell proliferation = number of EdU-positive cells/all cells x 100.

Cell apoptosis assay. Apoptosis of transfected SW-13 cells was measured using the Annexin V/Propidium Iodide (PI) Detection kit (KeyGen) following the manufacturer's instructions. Data were analyzed by flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA). In addition, SW-13 cell apoptosis was measured via TUNEL assay. Cells were stained with TUNEL (Sigma, St. Louis, MO, USA) to quantify apoptotic nuclei. At least five visual fields were observed under a fluorescent microscope for each sample.

Plasmid construction and stable overexpression of miR-205. The pre-hsa-miR-205 sequence was synthesized with the following primers by PCR: 5'-CCCAAGCTTCTGGGTGG CTGTTTTGAAAAC-3' (F), and 5'-CCGCTCGAGGAAGCA CGCACACTCCAGATG-3' (R), and subcloned into the pcDNA3.1(+) plasmid (Invitrogen) following the digestion of *Eco*RI and *Bam*HI to generate the recombinant plasmid pcDNA3.1(+)-miR-205. SW-13 cells were cultured to reach 60-80% confluence, and were then transfected with pcDNA3.1(+)-miR-205 according to the Lipofectamine 2000 instructions into 24-well plates. Then, transfected cells were sorted by G418 (200 mg/l) and cultured. The expression of miR-205 was measured by qRT-PCR.

Tumor formation in nude mice. Stably transfected SW-13 cells ($1x10^8$) were subcutaneously injected into the flank of each nude mouse (3 groups of 6 mice). All animal experiments were performed following the NIH Guide for the Care and Use of Laboratory Animals. Tumor volume (V) was calculated using the formula: $(L \times W^2) \times 0.5$ (L, length; W, width) with a Vernier caliper.

miRNA target prediction. The predicted target genes and their conserved sites of the seed region binding with each miRNA were investigated using TargetScan (http://www.targetscan.org) and miRSVR (http://www.microrna.org).

Plasmid construction and luciferase reporter assay. mRNA of wild-type Bcl-2-3'UTR (3,906-3,928 nt; GenBank accession no. NM_000633) containing binding sequence complementary to miR-205 was digested by *Xba*I and *Fse*I and cloned downstream of the pGL3-REPORT luciferase vector (Promega, Madison, WI, USA). Based on the wild-type plasmid, site-specific mutagenesis generated mutated Bcl-2-3'UTR

complementary to miR-205. For the luciferase reporter assay, SW-13 cells were co-transfected with wild-type or mutated Bcl-2-3'UTR and miR-205 transiently, and the luciferase was measured with a Dual-Luciferase Assay System (Promega) 48 h later. Each reporter plasmid was transfected at least three times.

Western blotting. After being stably transfected with plasmid or miR-205, proteins extracted from SW-13 cells were subjected to SDS-PAGE and transferred to PVDF membranes. The membrane was incubated with specific primary antibodies against Bcl-2, Bax (Santa Cruz Biotechnology, Santa Cruz, CA, USA), caspase-9 and -3 (Cell Signaling Technology, Danvers, MA, USA) at 4˚C overnight. After washing with phosphate-buffered saline (PBS), the blotted membrane was incubated with HRP-conjugated anti-mouse or anti-rabbit IgG (1:2,000) (RiboBio) at room temperature for 2 h. β-actin was used as an internal control. The signal was detected as previously described (15).

Immunohistochemical (IHC) assay. IHC staining of xenograft tumor slices was performed according to published methods. Then, 3-μm-thick slices were incubated with diluted primary antibody against Bcl-2 (Santa Cruz Biotechnology) at 4˚C overnight. After removing the residual primary antibody, HRP-polymer-conjugated secondary antibody was applied at 37˚C for 1 h. Next, slices were counterstained with hematoxylin. Three fields were selected for quantification of percentages of positive tumors and the staining intensity.

Statistical analysis. Data are presented as means ± SEM. Differences among groups were detected with the Student's t-test and ANOVA. P-values <0.05 were considered to indicate a statistically significant result.

Results

Expression of miR-205 decreases in ACC tissues. To understand the role of miR-205 in ACC, expression of miR-205 was measured in both ACC (n=11) and ACA tissues (n=21) using qRT-PCR. We noted less expression of miR-205 in ACC tissues compared to ACAs (P=0.008) (Fig. 1). Thus, miR-205 has a tumor-suppressor role in ACC.

miR-205 reduces proliferation and induces apoptosis of SW-13 cells. SW-13 cells were transfected with miR-205 mimics, miR-NC and miR-205 inhibitors and miR-205 expression was measured by qRT-PCR (Fig. 2A). In an MTT assay, significantly decreased proliferation was observed over time in cells expressing miR-205 compared with cells expressing miR-NC. miR-205 inhibitors promoted cell proliferation as well (P<0.05, Fig. 2B). In addition, an EdU assay was used to measure effectiveness of miR-205 on SW-13 cell proliferation. EdU, acting as a thymidine analogue replaces thymine (T) in replicating DNA (16). We observed that SW-13 cells incorporating EdU in the miR-205 group were significantly decreased when compared to the miR-NC group. In contrast, miR-205 inhibitors increased SW-13 cell DNA replication (P<0.05, Fig. 2C). Thus, SW-13 DNA replication was inversely related to miR-205 expression.

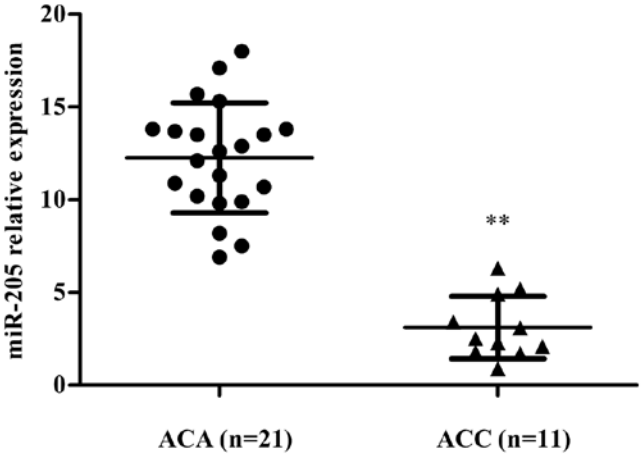

Figure 1. miR-205 expression in ACC and ACA tissues (**P<0.01). ACC, adrenocortical carcinoma; ACA, adrenocortical adenoma.

To identify the cause of reduced cell proliferation, we measured apoptosis using Annexin V/PI and TUNEL assays. The proportion of apoptotic SW-13 cells transfected with miR-205 was greater than the miR-NC group. miR-205 inhibitors decreased apoptotic proportions according to the Annexin V/PI assays (P<0.05, Fig. 2D). Similarly, SW-13 apoptosis was significantly increased due to miR-205 and was reduced due to miR-205 inhibitor according to TUNEL assay data (Fig. 2E, P<0.05). Thus, inhibition of SW-13 cell proliferation by miR-205 was associated with increased apoptosis.

miR-205 inhibits tumor growth in vivo. To investigate the effect of miR-205 on tumor formation, we established SW-13 cells stably overexpressing miR-205 by the eukaryotic expression vector pcDAN3.1(+). Then, miR-205 expression was measured by qRT-PCR (Fig. 3A). Tumors in the subcutaneous nude mouse model were palpable in ~7 days in the miR-NC and untreated groups, whereas tumors in the miR-205 group were palpable ~2 weeks after inoculation. All mice developed tumors at the end of the experiment (Fig. 3B). The mean tumor volume of the miR-205 group was reduced by >80% when compared to the miR-NC group (P<0.05, Fig. 3C). miR-NC and untreated animals were not different with respect to tumor volume. Thus, elevated miR-205 in SW-13 cells reduced their ability to form tumors and miR-205 suppresses proliferation of SW-13 cells in the animal model.

miR-205 induces SW-13 cell apoptosis by targeting the 3'UTR of Bcl-2. To learn how miR-205 induces SW-13 cell apoptosis, bioinformatics databases were used to predict several target genes of miR-205, including Lin28, Bak1, Cbx7 and Bcl-2. Bcl-2, an anti-apoptotic gene that is important to numerous tumors. We hypothesized that miR-205 promotes apoptosis and inhibits proliferation of SW-13 cells by targeting the Bcl-2 gene.

First, the 3'UTR of Bcl-2 containing either a wild-type or mutant binding sequence complementary to miR-205 was cloned into a luciferase reporter plasmid (Fig. 4A). miR-205 reduced luciferase activity of wild-type Bcl-2 (~80%) in SW-13 cells (P<0.05, Fig. 4B), yet it failed to repress the mutated one. To estimate whether miR-205 downregulated

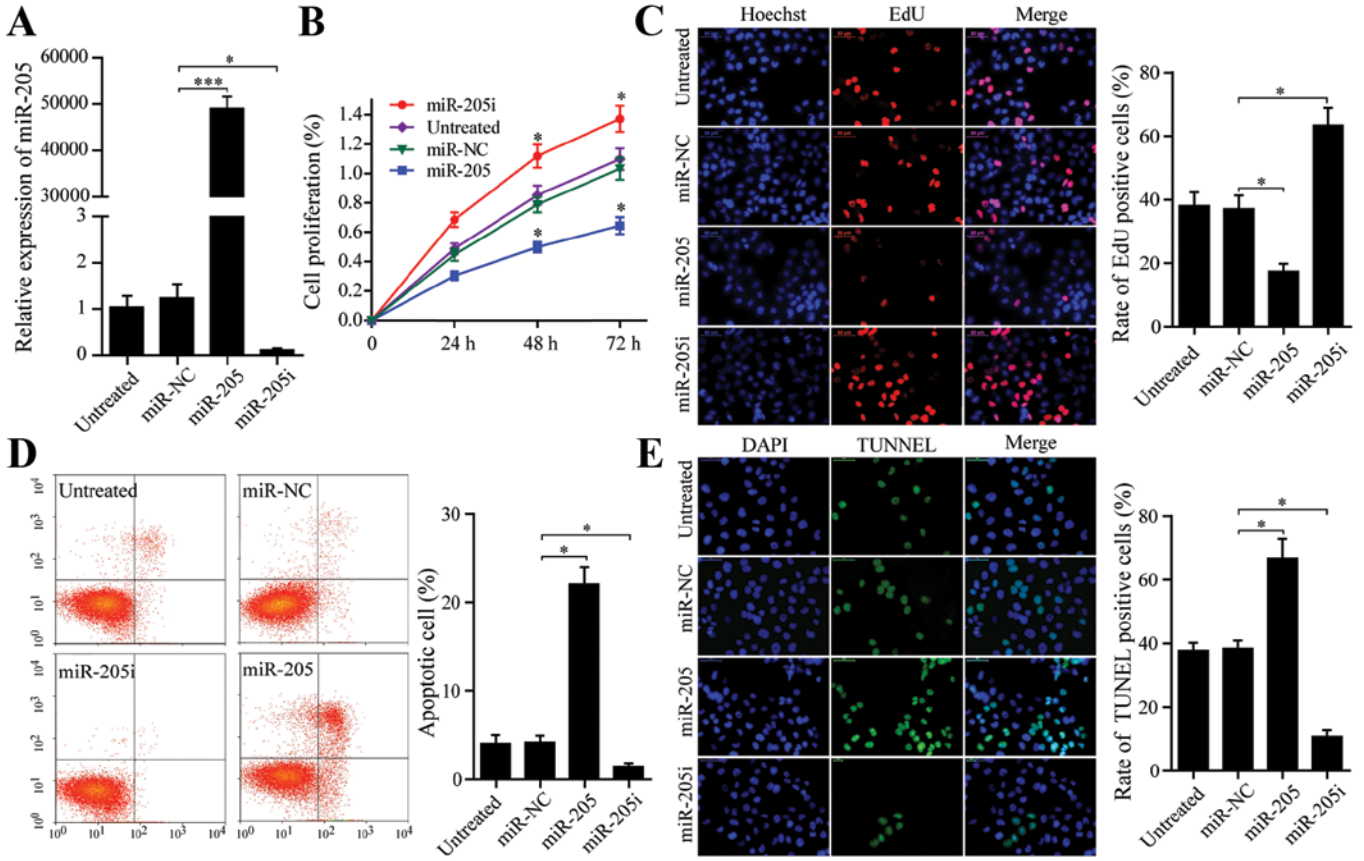

Figure 2. (A) Relative expression of miR-205 in transiently transfected SW-13 cells. (B and C) Cell proliferation measured by MTT and EdU assays. (D and E) Cell apoptosis measured by Annexin V/PI and TUNEL assays. Data are based on experiments performed independently in triplicate. (*P<0.05, ***P<0.001; miR-NC, negative control pre-miR; miR-205i, miR-205 inhibitors).

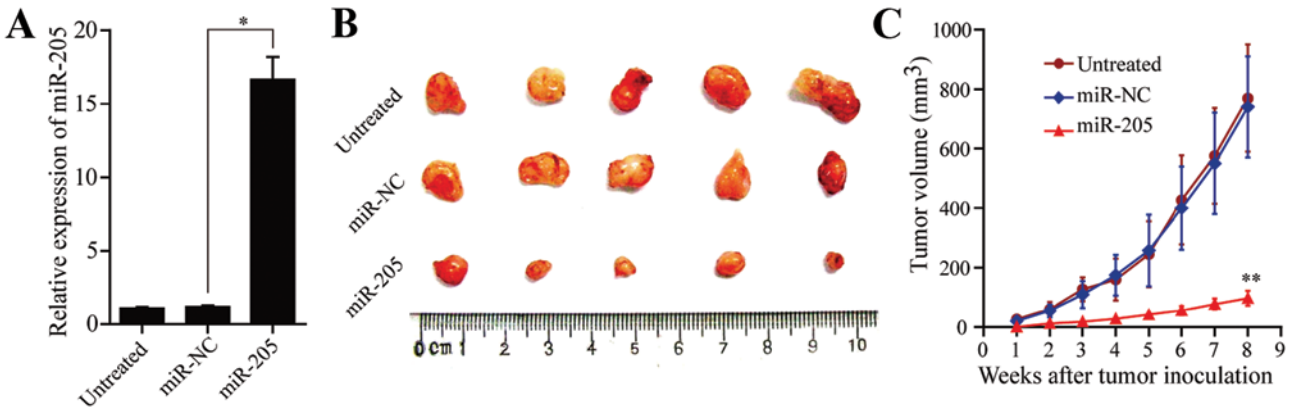

Figure 3. miR-205 inhibits SW-13 cell tumor growth. (A) Relative expression of miR-205 in stably transfected SW-13 cells. (B) Images of tumor xenograft in nude mice. (C) Effects of miR-205 on the growth of xenograft tumors, tumor sizes are shown as means ± SEM. (**P<0.01).

Bcl-2 expression functionally, qRT-PCR analysis was used to confirm that stably overexpressing miR-205 reduced mRNA expression of Bcl-2 compared to the miR-NC group (P<0.05, Fig. 4B). Western blotting was used to quantify expression of Bcl-2 protein due to miR-205 overexpression (Fig. 4C). IHC staining confirmed that Bcl-2 expression was negatively correlated with that of the miR-205 in xenograft tumor tissues (Fig. 4D). Therefore, miR-205 targets Bcl-2 through 3'UTR and regulates mRNA and protein expression.

miR-205 inhibits Bcl-2 expression via the intrinsic apoptotic pathway. Bcl-2 is reported to be a vital anti-apoptotic gene. Activation of Bcl-2 inhibits apoptosis by cleaving Bax, releasing cytochrome *c*, caspase-9 and -3 which are involved in the intrinsic apoptotic pathway (17). To determine whether the intrinsic apoptotic pathway is also activated through miR-205-mediated suppression of Bcl-2 in SW-13 cells, we measured Bax, caspase-9 and -3 expression via western blotting and noted that these genes were upregulated at the protein

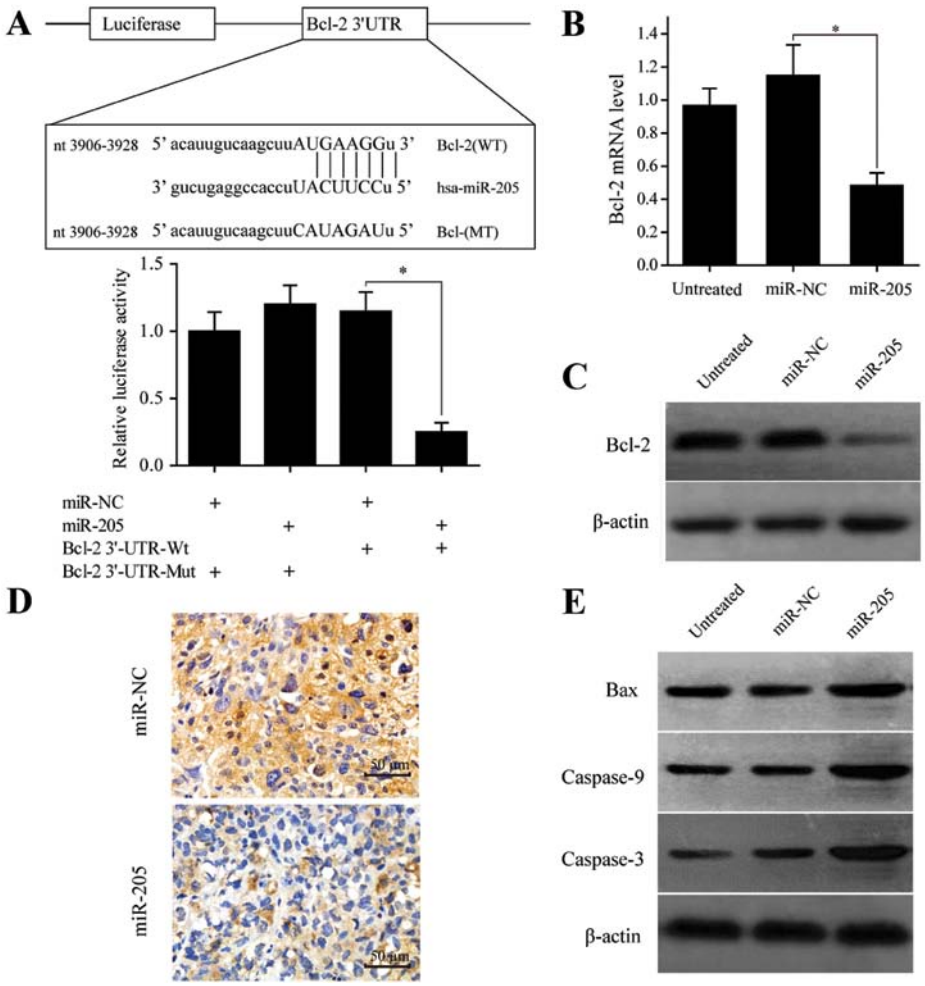

Figure 4. miR-205 induces SW-13 cell apoptosis via targeting the Bcl-2 gene. (A) The predicted miR-205 sequences contain the binding sites complementary to Bcl-2 3'UTR, including wild-type and mutant. miR-205 significantly repressed luciferase activity of wild-type Bcl-2. (B and C) Expression of Bcl-2 was verified by qRT-PCR and western blotting following stable overexpression of miR-205 in SW-13 cells. (D) Bcl-2 expression was confirmed by IHC in xenograft tumor tissues. Original magnification, x400. (E) Western blotting revealed upregulation of Bax, caspase-9 and -3 at the protein level in SW-13 cells stably overexpressing miR-205 (*P<0.05).

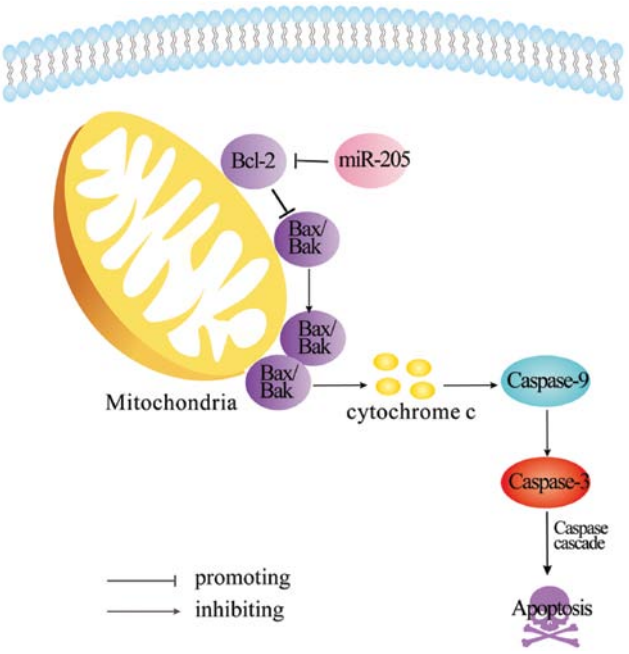

Figure 5. Suppression of Bcl-2 expression resulted in cleaving Bax, releasing cytochrome *c*, caspase-9 and -3 involved in the intrinsic apoptosis pathway due to miR-205, which induced SW-13 cell apoptosis.

level in miR-205-overexpressing SW-13 cells (Fig. 4E). Therefore, the Bcl-2-mediated intrinsic apoptotic pathway plays an important role in inducing SW-13 cell apoptosis due to miR-205.

Discussion

Research shows that miRNAs are critical regulators in various biological pathways and pathologic processes, including initiation and progression of cancer (9). miRNAs in tumors are thought also to modulate oncogenic or tumor suppressive pathways (18). Recently, the expression of miRNAs was confirmed in adrenocortical tumors and the goal was to identify miRNAs to differentiate adenomas from carcinomas. For instance, miR-483 was upregulated and miR-195 was downregulated in ACC compared with ACA (4,19), indicating a potential value of miRNAs in diagnosing ACC. Despite these advances, little is known about the composition and characteristic of miRNAs in ACC, and few targets of miRNAs in ACC have been identified.

We reported that miR-205 is downregulated in ACC compared to ACA, and overexpression of miR-205 not only induced apoptosis and impaired proliferation of SW-13 cells, but also repressed tumor growth *in vivo*. Thus, miR-205 functions as an anti-oncogene in ACC. The role of miRNAs in tumors depends on the tumor origin and tissue specificity. The known functions of miR-205 include inhibition of epithelial to mesenchymal transition (EMT) via significant suppression of E-cadherin, which is related to metastasis (20,21). In contrast, miR-205 regulated target genes by direct cleavage of mRNA or inhibition of protein synthesis through complementarity with 3'UTR. At this time, few genes have been identified as targets of miR-205, including anti-oncogenes PTEN (22) and SHIP2 (23), the oncogenes HER3 (24) and PKCε (25), and the angiogenic factor VEGFA (26), and finally the pro-metastatic genes Zeb1 and Zeb2 (27). In the present study, we propose that the Bcl-2 gene is a target of miR-205 through bioinformatics prediction software, and these data were verified by luciferase assay. mRNA and protein expression of Bcl-2 were negatively related to miR-205 in SW-13 cells and xenograft tumor tissues, suggesting that miR-205 inhibits tumor progression through targeting the 3'UTR of Bcl-2 in ACC. In addition, miR-205 was a tumor-suppressing gene by targeting Bcl-2 which has been documented in prostate cancer (28).

In the embryo stage, the fetal cortex accounts for 85% of the adrenal cortex but disappears quickly due to apoptosis after birth (29,31). Thus, it was speculated that similarities may exist between normal cortex and ACC which originated from defective apoptosis of the fetal cortex (31-33). Consequently, the research on apoptosis-related genes could be of great significance not only to provide new views to the biology of ACC, yet also to search potential prognostic and therapeutic markers. Bcl-2 predominantly localizes to the mitochondria which is the central coordinator of the apoptotic pathway (34). Bcl-2 is reported to vary from ACC to ACA at the mRNA expression level (35), yet the mechanism of Bcl-2 action is unclear. In the present study, we found that miR-205 is an upstream regulator inhibiting Bcl-2 expression. Also, the intrinsic apoptotic pathway was activated and this promoted caspase-dependent apoptosis in SW-13 cells (Fig. 5). Notably, other miRNAs target Bcl-2 to induce cancer-cell apoptosis, among them, miR-15/miR-16 and miR-148a negatively regulated Bcl-2 expression to induce leukemic and colorectal cancer cell apoptosis, respectively (36,37). In addition, miR-181b modulates multidrug resistance by targeting Bcl-2 in lung cancer cell lines (38). Thus, miR-205 with other miRNAs, targets Bcl-2 to create a network to regulate tumor cell death.

In conclusion, miRNA-205 serves as a tumor-suppressor in ACC by targeting anti-apoptotic Bcl-2, which induces SW-13 cell apoptosis via the intrinsic apoptotic pathway. Our results may provide a foundation for considering miR-205 as a biomarker to aid in the diagnosis and treatment of ACC. Extensive studies are required to confirm our findings and to validate the diagnostic accuracy of these miRNAs.

Acknowledgements

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