ANP32A modulates cell growth by regulating p38 and Akt activity in colorectal cancer

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Abstract. Acidic leucine-rich nuclear phosphoprotein-32A (ANP32A) possesses multiple biochemical activities, has been found to be decreased or absent in malignant tumors. However, new findings have shown that it is expressed in greater amounts in advanced cancers than in early-stage tumors. The role and clinical significance of ANP32A in colorectal cancer (CRC) is still unknown. In the present study, the expression of ANP32A was assessed in 68 CRC patients by IHC, and then the correlation of its expression with clinicopathological factors was investigated using the Allred, Klein and immune response scoring system analysis. Western blot and real-time PCR analyses were used to assess ANP32A expression and the activity of Akt and p38 in cancer and normal tissues. These data indicated a significant association between ANP32A expression and the activity of Akt and p38, besides the tumor differentiation status in CRC patients. IHC and western blotting data revealed that ANP32A was overexpressed in CRC patients, and ANP32A levels were higher in poorly differentiated tumors. Protein and mRNA analysis revealed that with a high expression of ANP32A, the activation of Akt was enhanced, while the p-38 phosphorylation level was decreased in CRC tissues. MTT assay and functional studies revealed that knockdown of ANP32A inhibited cell growth and induced p38 phosphorylation and Akt dephosphorylation. The present study indicated that ANP32A promoted CRC proliferation by inhibition of p38 and activation of Akt signaling pathways and suggested that ANP32A may play a potential role in CRC diagnosis and therapy.

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Introduction

Colorectal cancer (CRC) has one of the greatest incidences and mortality rates of any type of cancer in Europe and the United States. In China, the mortality of CRC ranks fifth in malignant tumors (1), but a rapid rise has been observed in recent years (2). Nearly 70% of CRC patients survive no more than 5 years after diagnosis. Surgical excision combined with chemotherapy is the preferred treatment for early-stage CRC, and thus can lead to good prognosis and higher overall survival rates. However, in advanced cases of CRC with multi-organ metastasis, only one-tenth of CRC patients survive to the 5-year mark (3). Fluorouracil and capecitabine are widely used chemotherapeutic drugs for CRC (4). However, their use is usually limited due to the drug resistance of cancer. Although targeted agents anti-VEGF and anti-EGFR have been introduced for treatment of CRC, the overall death rate in advanced CRC remains high since EGFR therapeutic antibodies are only effective in ~25% of advanced CRC patients (5). EGFR-targeted therapy fails mainly due to mutations in KRAS, NRAS and BRAF. Furthermore, amplification of ERBB2 and MET caused primary resistance (5,6). Cells that acquired resistance to cetuximab remained sensitive to the combination of anti-EGFR and anti-MEK (6). Thus, early identification of molecular heterogeneity is crucial for the treatment of metastatic CRC.

ANP32A, known as PP32, I-1^{PP2A}, Lanp and PHAPI, is an acidic phosphate protein consisting of a leucine-rich repeat (LRR) amino terminal, and stretched acid-rich amino acids at the carboxy terminus (7), expressed in normal tissues as well as in pancreatic, breast and prostate cancer and other malignant tumors (8-10). ANP32A plays an important role in cell proliferation, apoptosis, transcriptional regulation, signal transduction and other processes (11). Most previous studies had shown that ANP32A is a tumor suppressor (9,12-14), while recent studies found that ANP32A is highly expressed in hepatocellular carcinoma (15), and oral squamous cell carcinoma (OSCC) (16). Velmurugan *et al* reported that ANP32A is a potential prognostic factor and closely related to the survival rate of OSCC patients (16). In CRC, however, the roles and mechanisms of ANP32A are still not clear.

Acidic leucine-rich nuclear phosphoprotein-32A (ANP32A) has been identified as an inhibitor of protein

phosphatase 2A (PP2A) (17), which is a serine/threonine protein phosphatase expressed in most mammalian tissues. PP2A has been shown to be a critical cellular regulator of cell metabolism, transcription, translation, RNA splicing, DNA replication, cell cycle progression, transformation and apoptosis (18). Studies have shown that PP2A activation induces apoptosis via the p38 MAPK-mediated pathway (18-22). The p38 MAP kinase is thought to be a promising cancer drug target, but its real therapeutic effect is unsatisfactory in CRC treatment (23). Zhang *et al* reported opposite responses in two subgroups of CRC cells to p38 inhibitors, which can explain why p38 inhibitors showed no positive effects on CRC in clinical settings (23). These findings are consistent with those reporting that p38 signaling has a dual function in colorectal tumorigenesis (24), and that the role and function of p38 in tumorigenesis may be affected by other unpredictable factors (25) including PP2A (23). Akt signaling has also been shown to be upregulated to promote colon cell growth required for the activity of PP2A (26). Numerous studies have revealed the relationship between ANP32A and PP2A, but there are few studies that have focused on the correlation between p38, Akt and ANP32A, which possesses the opposite effect of PP2A.

In the present study, we collected clinical specimens, analyzed the expression of ANP32A in CRC patients and its relationship with the differentiation of CRC. Meanwhile, due to the important role of p38 and Akt in CRCs, the relationship between ANP32A and these two proteins was investigated. The clarification of these results may be helpful in the treatment of CRC.

Material and methods

Clinical specimens. A total of 68 cases of CRC patients and matched tumor adjacent tissues were obtained from the Affiliated Hospital of Guilin Medical College to evaluate the relationship between the expression levels of ANP32A and the clinical/pathological factors. Tumor tissues were classified according to cancer tumor-node-metastasis (TNM) classification system issued by the Union for International Cancer Control. In order to avoid any factors that may affect the results of the experiment, all the selected specimens were examined without any treatment. All samples were studied with the consent of the patient and approval of the Guilin Medical College Ethics Committee.

CRC cell line. The CRC cell lines LoVo and SW480 were obtained from Xiangya Medical College of Central South University and stored in our laboratory. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Shanghai, China) containing 10% fetal bovine serum (FBS) (Gemini Biological Products, Calabasas, CA, USA) and 100 U/ml penicillin and streptomycin (Solarbio, Beijing, China) at 37˚C in humidified atmosphere with 5% CO_2.

Immunohistochemistry (IHC) analysis. Tissue sections were fixed by formalin and embedded by paraffin in this experiment. The cancer tissues were cut into 4-μm sections, and then hematoxylin/eosin staining was performed to confirm the presence of the original cancer as shown by its morphology. Tissue sections were dewaxed using xylene, and then tissue blocks were dehydrated in an ascending series of ethanol solutions. A 0.3% solution of hydrogen peroxide in methanol was added for 10 min to eliminate the effects of endogenous peroxidase and incubated with 10 mmol/l citrate buffer (pH 6.0). The tissues were blocked at 37˚C with 5% goat serum (Solarbio). The sections were then incubated with ANP32A rabbit polyclonal antibody (Abcam, Cambridge, UK) in room temperature for 1 h. The secondary antibody used was a horseradish peroxidase-conjugated antibody (MXB, Fuzhou, China). Each tumor was scored based on the ratio of the intensity of the nucleus and cytoplasm: negative staining and weak positive staining, ±; moderate staining, 1+; and strong staining, 2+. Staining intensity was confirmed by two pathologists. For the Allred scoring system, cut-off scores ≤4 and >4 were defined as low and high expression levels, respectively.

Cell transfection. Double-stranded small interfering RNA (siRNA) oligonucleotides were obtained from Bioligo, (Shanghai, China). The siRNA sequence targeting ANP32A (si-ANP32A) was 5-CAAUCGCAAACUUACCAAAGT-3; a fluorescently-labeled non-specific pri-miR sequence was used as a negative control (sequence targeting was 5-UUCUCCGAA CGUGUCACGUTT-3).

Total RNA isolation and quantitative real-time PCR. Total RNA was isolated from SW480 cells transfected with si-ANP32A and CRC tissue using TRIzol reagent (Tiangen Biotech Co., Ltd., Beijing, China). The isolated RNA was used as a template for the reverse transcription reaction. The primers were designed according to the corresponding cDNA sequences in GenBank. These primers listed in Table I were designed to detect ANP32A, AKT, p53 and β-actin (positive control).

Western blotting. Total protein was extracted from SW480 and LoVo cells, and CRC tissues. The primary antibodies of ANP32A (Abcam), p-Akt, p38, and p-p38 (Wanleibio, Shenyang, China) were used to detect the protein expression. β-actin (ZSGB-BIO, Beijing, China) was used as a loading control. Proteins of different molecular weights were isolated using 12% SDS-PAGE. A nitrocellulose membrane was then used to transfer the target protein. The membrane was incubated in 5% skim milk overnight at 4˚C, and then treated with horseradish peroxidase-labeled anti-mouse or anti-rabbit secondary antibodies for 1 h at 37˚C and detected with an enhanced chemiluminescence (ECL) reagent (Bio-Rad, Hercules, CA, USA).

MTT analysis. SW480 cells were divided into a si-ANP32A, negative and blank control group. Each group had quadruplicate wells for treatment, respectively. Cells at $2x10^4$ cells/ml were seeded into 96-well plates at a final volume of 100 μl and incubated at 37˚C and 5% CO_2 in saturated humidity. Cells were incubated with 20 μl of MTT for 4 h at 37˚C and 5% CO_2 in saturated humidity and treated with 150 μl of dimethyl sulfoxide (DMSO) after removal of the supernatant, and then shaken for 10 min on a plate shaker. Dissolved the optical density (OD) value of each well was measured at 490 nm wavelength by TECAN M200 (Tecan Group Ltd.,

Table I. Sequences of primers used in quantitative real-time PCR.

RNA species		Primer pairs
ANP32A	Sense	5'-CACCTCAATCGCAAACTTACCA-3'
	Antisense	5'-AACACATTTTCTCGGTAGTCGTT-3'
β-actin	Sense	5'-AAAGACCTGTACGCCAACAC-3'
	Antisense	5'-GTCATACTCCTGCTTGCTGAT-3'
Akt	Sense	5'-AGAACCTCATRCTGGACAA-3'
	Antisense	5'-CTCATGGTCCTGGTTGTAGA-3'
p53	Sense	5'-TCAACAAGATGTTTTGCCAACTG-3'
	Antisense	5-ATGTGCTGTGACTGCTTGTAGATG-3

ANP32A, acidic leucine-rich nuclear phosphoprotein-32A.

Männedorf, Switzerland). All experiments were performed at least three times. Growth inhibition rate = (control group OD value - OD value of experimental group) / control group OD values x 100%.

Statistical analysis. All data in the present study were evaluated using SPSS version 18.0 software (SPSS, Inc., Chicago, IL, USA). The statistical significance of the data was evaluated using the Student's t-test. All data are expressed as mean ± SD. The relationship between the expression of ANP32A and the clinicopathological parameters of CRC was assessed by the χ^2 test. For all tests, the level of significance was set at $P<0.05$.

Results

ANP32A expression in tumor and normal tissues of CRC patients. The clinical data of 68 patients with CRC are summarized in Table II. According to the histological grade of CRC, 23 patients well-differentiated tumors, whereas the remaining 45 patients had moderately or poorly differentiated tumors. In order to investigate the expression of ANP32A in CRC, we performed IHC. As shown in Fig. 1, positive IHC staining was mainly localized in the cytoplasm and nucleus of CRC cells, and ANP32A was highly expressed in poorly differentiated CRC. Total protein was extracted from CRC and matched tumor adjacent tissues, then subjected to western blot analysis was used to detect ANP32A expression. The results revealed that the expression of ANP32A was higher in CRC, which was consistent with the aforementioned IHC data in which a high level of ANP32A expression was detected. In addition, high ANP32A protein expression was found to have a statistically significant relationship to tumor differentiation (P=0.021) (Table III).

Cell proliferation is detected by MTT assay. In order to investigate the effect of ANP32A on cell proliferation, the cell viability was assessed in the present study via silencing of ANP32A in SW480 cells. The MTT assay revealed that the growth rate of SW480 cells transfected with siRNA targeting ANP32A was lower than that of the negative or blank control groups ($P<0.05$). The results revealed that the growth inhibition rates of the siRNA treatment group were 8.4, 15.6 and 14.1% at 24, 48 and 72 h, respectively (Fig. 2B). There was no significant difference between the negative or blank control groups. Collectively, these results indicated that knockdown of ANP32A can prevent proliferation of SW480 cells effectively.

Silencing of ANP32A affects the expression of genes associated with cell proliferation in SW480 cells. The expression of

Table II. Demographics and characteristics among CRC patients.

Factors	No.	%
Sex		
Female	43	63.24
Male	25	36.76
Age, years		
≤49	12	17.65
50-59	22	32.35
60-69	23	33.82
≥70	11	16.18
T (tumor size)		
TIS	3	4.41
I	9	13.24
II	19	27.94
III	36	52.94
IV	1	1.47
N (lymph node)		
N0	48	70.59
N1	14	20.59
N2	6	8.82
M (metastasis)		
M0	62	91.18
M1	6	8.82
AJCC cancer stage		
0	3	4.41
I	25	36.76
II		
IIA	18	26.47
III		
IIIA	1	1.47
IIIB	12	17.65
IIIC	3	4.41
IV		
IVA	4	5.88
IVB	2	2.94
Histological grade		
Well	23	33.82
Moderate	42	61.76
Poor	3	4.41

CRC, colorectal cancer; AJCC, American Joint Committee on Cancer; TIS, tumor *in situ*.

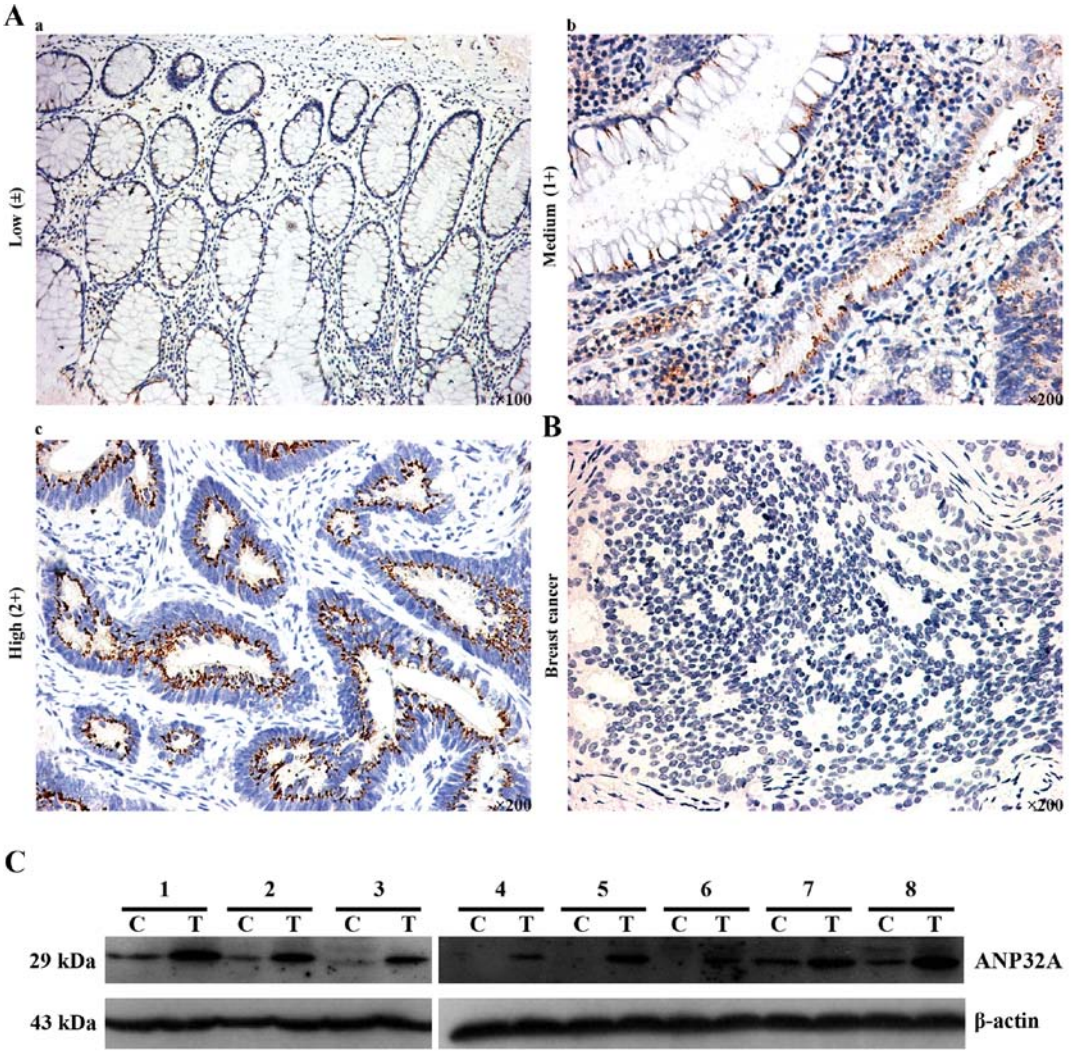

Figure 1. Higher expression of ANP32A in colorectal cancer (CRC) patients. (A) The staining intensities of ANP32A were identified in CRC tissues. Cells were stained using a scale (±, 1+, 2+), classified as follows: representative of low ANP32A, medium ANP32A and high ANP32A staining. (B) Breast cancer tissues were used as a negative control. (C) The protein level of ANP32A was analyzed by immunoblotting in CRC patients, and β-actin served as an internal control. There were 8 CRC patients randomly selected, of which group C were tumor-adjacent tissues of CRC patients and group T were cancer tissues of CRC patients.

Table III. Correlation of ANP32A expression with clinical-pathological characteristics using the Allred scoring system among CRC patients.

	Allred			
Factors	Low no. (n=37)	High no. (n=31)	χ^2	P-value
Histological grade			5.328	0.021
Well	17	6		
Moderate/poor	20	25		

CRC, colorectal cancer; ANP32A, acidic leucine-rich nuclear phosphoprotein-32A.

ANP32A was detected by western blotting. Higher levels of p-Akt expression were observed in CRC tissues than in normal tumor-adjacent tissues. Unlike the expression patterns of p-Akt, p38 and p-p38 were overexpressed in normal tumor-adjacent tissues (Fig. 3). As shown in Fig. 3A, the mRNA expression of total Akt was higher in the CRC tissues, as was the expression of ANP32A in the CRC tissues. The protein expression levels of ANP32A and p-Akt were higher in the CRC tissues and the level of phosphorylated p-38 was lower (Fig. 3B).

To elucidate the details underlying the molecular mechanism of ANP32A in CRC, SW480 cells with higher ANP32A expression were selected for the current investigation of the effects of ANP32A knockdown (Fig. 4A). For this reason, pre-treatment with siRNA targeting ANP32A for 48 h, and then quantitative real-time PCR experiments were performed to determine whether knockdown of ANP32A subsequently regulates Akt transcription. The data revealed that Akt mRNA expression was decreased by the inhibition of the expression of ANP32A, whereas the levels of p53 were increased (Fig. 4B). When normalizing to β-actin, ANP32A mRNA expression in the CRC samples was nearly 8.09-fold higher than that in the matched non-cancerous tissues, and the data were consistent with the previous western blotting test in which high expression of ANP32A was detected (Fig. 4B). The results indicated

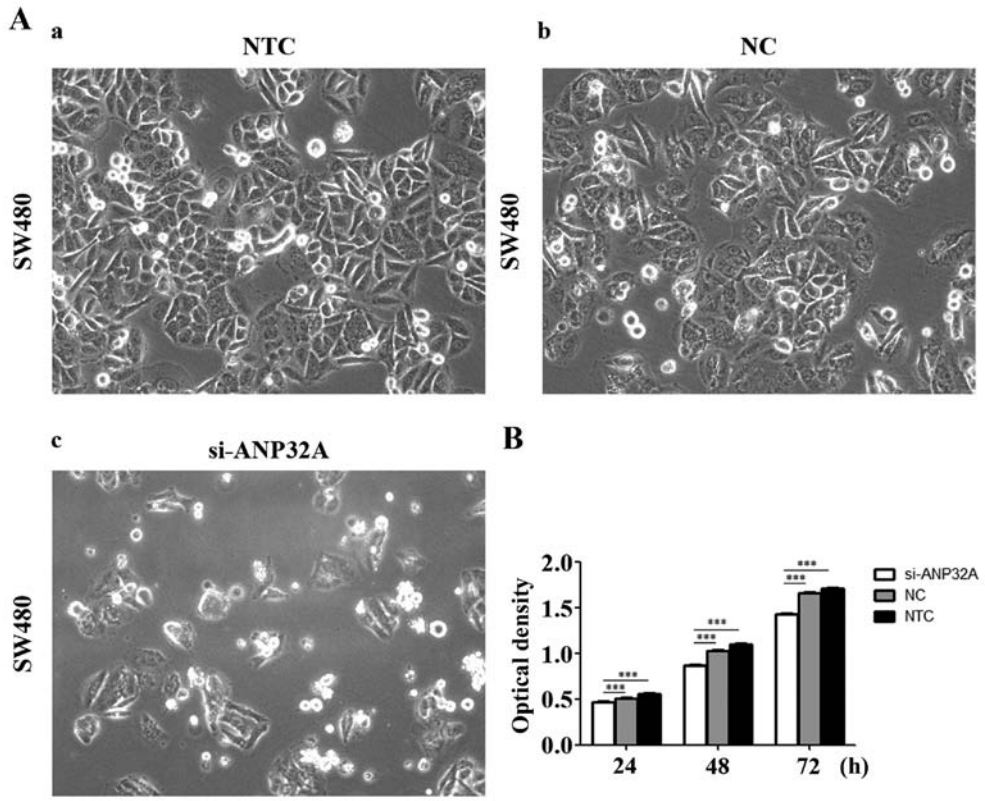

Figure 2. Knockdown of ANP32A by siRNA decreases the cell viability in SW480 cells. (A) SW480 cells were transfected with si-ANP32A or siControl for 24 h and microscopically photographed by inverted fluorescence microscope. (B) An MTT assay was used to detect the proliferation of SW480 cells after transfection at 24, 48 and 72 h, respectively. 'NC' refers to the negative control group, and 'NTC' to the blank control group. Densitometry values are represented in a bar chart, and the data is expressed as the mean values ± SD. The asterisks indicate significant differences among the siControl and si-ANP32A and blank control group (***P<0.001).

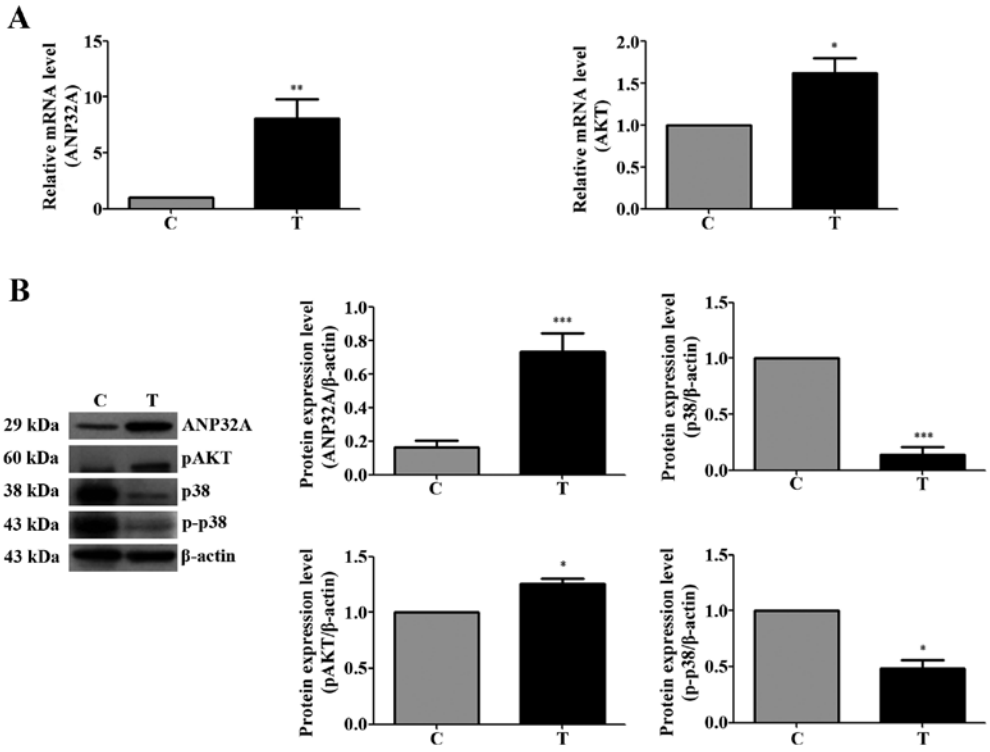

Figure 3. Expression of ANP32A, pAKT and p38 in cancer and tumor-adjacent tissues. (A) The expression of ANP32A or Akt mRNA was analyzed using quantitative RT-PCR and the results were calculated using $2^{-\Delta\Delta Ct}$. (B) The expression of ANP32A, pAKT, p38 and p-p38 were detected using western blot analysis. β-actin was used as an internal control for each protein. 'C' represents tumor-adjacent tissues of CRC patients, and 'T' represents cancer tissues of CRC patients. Densitometry values are represented in bar charts. Data is expressed as the mean values ± SD. The asterisks indicate significant differences between the 'C' control and the 'T' tumor group (*P<0.05, ***P<0.001).

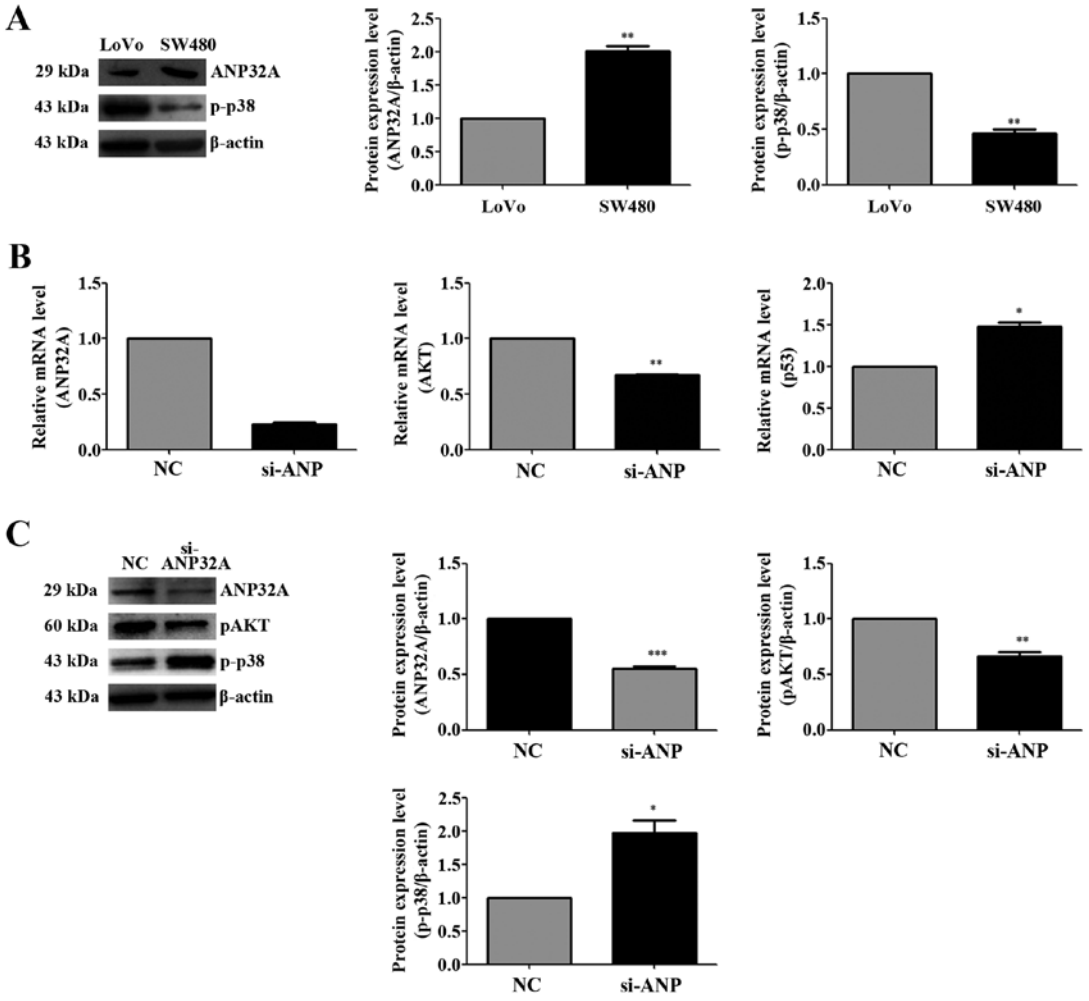

Figure 4. Effect of ANP32A on the expression of genes associated with proliferation. (A) The expression of ANP32A and p-p38 were detected using western blotting in SW480 and LoVo cells. (B) After SW480 cells were transfected with siControl (siCtrl) or si-ANP32A, the expression of ANP32A, Akt and p53 were analyzed by quantitative RT-PCR. (C) The expression of p-Akt and p-p38 were analyzed using western blotting after ANP32A expression knockdown. β-actin was used as a loading control. Densitometry values are represented in bar charts, the data is expressed as the mean values ± SD. The asterisks indicate the significant differences between LoVo and SW480 cells and the differences between the siControl and si-ANP32A groups (*P<0.05, **P<0.01, ***P<0.001).

that knockdown of ANP32A resulted in the decrease of p-Akt expression, but the increase of the expression of p-p38 (Fig. 4C). All of these data strongly demonstrated that silencing of ANP32A inhibited the Akt activation and increased the expression of p-p38 in SW480 cells. These results demonstrated that the expression of ANP32A is associated with the level of Akt and that it may promote the proliferation of CRC cells.

Discussion

Colorectal cancer (CRC) is a high-incidence and high-mortality form of cancer (27). The findings of numerous different studies have shown that several factors induce the formation of CRC, included genetic and epigenetic alterations (28,29). Some of these gene alterations can be applied as biomarkers for cancer diagnosis and provide strategies for cancer therapy (30). The human ANP32A gene is expressed differently in different tumor tissues. Low expression has been reported in prostate (31), pancreatic (9,32) and breast cancer (33,34), and B cell lymphoma (13). Early studies have shown that ANP32A is expressed as a modulator of cancer cell apoptosis *in vitro* and *in vivo* (35,36), including human non-small cell lung cancer (NSCLC) (37). In pancreatic cancer, Brody *et al* demonstrated that considerable expression of ANP32A was found in well-differentiated tumors, however decreased or absent expression of ANP32A was found to be related to poor differentiation (38). Notably, Velmurugan *et al* found ANP32A levels to be higher in tumor tissues and that high levels of ANP32A expression were associated with poor tumor differentiation in OSCC (16). Shi *et al* found that ANP32A was overexpressed in CRC using laser capture microdissection (LCM) and two-dimensional difference gel electrophoresis (2D-DIGE) (39), but no further information is available concerning ANP32A expression with tumor differentiation or its significance to the development of CRC. In the present study, the expression of ANP32A was evaluated in CRC and adjacent tumor-free tissue samples. IHC, western blotting, and real-time PCR analysis demonstrated that CRC specimens exhibited significantly higher ANP32A protein and mRNA levels than normal tissues nearby. There is a statistically positive correlation between the ANP32A protein expression levels and the degrees of tumor differentiation in cancer tissues. Overexpression of ANP32A has been shown to

suppress tumor cell growth and induce apoptosis in breast and non-small-cell lung cancer (33,37). It has been reported that artificially introducing ANP32A expression into pancreatic cell lines in which it has been absent can increase the rate of G1 arrest relative to control cells (38). The present study demonstrated that ANP32A knockdown by RNA interference suppresses the proliferation of CRC cells. These results revealed that ANP32A promotes tumor development in CRC cells by accelerating cell proliferation and that overexpression of ANP32A has an important role in tumorigenesis and progression. These results were inconsistent with results associated with other tumors. The results indicated that the expression of ANP32A varies in different types of cancer, and may have the opposite effects in different tumor tissues.

To identify the possible mechanisms by which ANP32A enhances the survival of colon cancer cells, the effects of ANP32A on molecules involved in the proliferation of CRC were investigated. Numerous studies have shown that p38 has a promotive effect in CRC, but newer studies have shown that p38 MAPK can act as a tumor suppressor in CRC (40), and that it constitutes a potential molecular target in the inhibition of colorectal carcinogenesis (41,42). A previous study revealed that the activation of p38 can lead to an increase in apoptosis in the LoVo CRC cell line (43). The p38 inhibitor SB203580 can reverse the effect of apoptosis upon curcumol treatment in LoVo cells (43). PP2A, an inhibitor of ANP32A, is a negative regulator of p38 activation (22,44-46). It can be easily hypothesized that ANP32A may have a positive effect in p38 regulation. Habrukowich *et al* revealed that knockdown of ANP32A expression induced p38 phosphorylation in human umbilical vein endothelial cells (HUVEC) (47). To confirm the activity of p38 in colon cancer tissues, the expression of phosphorylated p38 was assessed in CRC specimens and ANP32A silenced SW480 cells. Protein analysis data revealed the p38 phosphorylation levels to be lower in CRC than in normal cells, and that silencing of ANP32A upregulated p38 activation. Akt, according to previous studies, plays a key role in protein synthesis, cell metabolism and survival, and it is a key regulator of proliferation and metastasis in CRC cells (48-50). Phosphorylated Akt (p-Akt) can be used as a tissue biomarker to identify patients with favorable prognosis and to identify suitable therapeutic targets (41,51). It has been demonstrated that in addition to activation of p38, the activity of PP2A was also significantly increased by triptolide and hydroxycamptothecin, and the Akt survival pathway was also inhibited in A549 cells (46). Van Kanegan *et al* found that PP2A inhibition potentiates Akt phosphorylation in PC12 cells (52). To further confirm the effect of ANP32A in CRC and determine the correlation between ANP32A and p-Akt, function analysis of ANP32A was performed in SW480 cells. These protein and mRNA results revealed that, as the expression of ANP32A increased in colorectal tissues, phosphorylated Akt was upregulated. The results also revealed there to be less expression of Akt in the ANP32A-silenced cells than in other cells at both the mRNA and protein levels.

In summary, ANP32A was overexpressed in CRC patients. Knockdown of ANP32A was found to inhibit CRC SW480 cell growth and the net effect of ANP32A silencing was hyperphosphorylation of p38 and dephosphorylation of Akt. These results demonstrated that ANP32A may be a suitable molecule for the development of CRC, and the mechanism by which ANP32A promotes colon cancer growth may involve p38 inactivation and Akt activation. Further studies are required to improve our understanding of the specific effect of ANP32A in cancer. The involvement of other factors and signaling pathways in ANP32A interaction remains to be explored.

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