

Original Article

Up-regulated long noncoding RNA AB073614 modulates the tumor cell proliferation, invasion and migration in human colorectal cancer

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Received September 20, 2018; Accepted October 25, 2018; Epub August 1, 2019; Published August 15, 2019

Abstract: Colorectal cancer (CRC) is a common cancer and the function of long noncoding RNA (lncRNA) AB073614 in CRC mainly unclear. Here, the expression of lncRNA AB073614 in CRC tissues was evaluated by quantitative real-time PCR (qRT-PCR). CCK-8 assays were conducted to explore the impact of AB073614 on cell proliferation. The effects of AB073614 on cell migration, invasion and apoptosis were evaluated by a Transwell *in vitro* assay. Apoptosis-related molecular marker expression levels were detected by Western blot analysis. In the present study, we confirmed that AB073614 was significantly upregulated in CRC tissues. A difference analysis in the lncRNA AB073614 expression in CRC patient group suggested that the expression of lncRNA AB073614 was independently associated with higher possibilities of high grade ($P = 0.0005$), tumor size (> 5 cm) ($P = 0.0001$), distant metastasis ($P = 0.0009$), and differentiation level ($P = 0.0037$). *In vitro* studies demonstrated that the knockdown of {"type": "entrez-nucleotide", "attrs":{"text": "AB073614", "term_id": "51555790", "term_text": "AB073614"}}AB073614 suppressed SW480 cell proliferation. Meanwhile, the overexpression of {"type": "entrez-nucleotide", "attrs":{"text": "AB073614", "term_id": "51555790", "term_text": "AB073614"}}AB073614 in SW480 cells accelerated cell growth and invasion, and suppressed cell apoptosis. In conclusion, our results suggest that AB073614 may function as a tumor promoter in CRC. Our findings may provide a therapeutic approach for the future treatment of CRC.

Keywords: Long noncoding RNA, AB073614, proliferation, migration, invasion, colorectal cancer

Introduction

Colorectal cancer (CRC) is the third most common malignant cancer worldwide and one of the leading causes of death. Previous research has proved that the incidence of CRC in China has rapidly increased [1]. Evidence has revealed numerous oncogenes which are responsible for the development of CRC [2-5]. The etiology of CRC is multifactorial and has remained poorly defined [6, 7].

Long noncoding RNAs (lncRNAs) exceed 200 nucleotides in length and are located in the nucleus or cytoplasm [8]. They widely participate in the regulation of gene expression and are involved in various diseases, including cancer [9]. Multiple studies have shown that lncRNAs regulate complex and diverse biological processes, including differentiation, tran-

scriptional regulation, tissue development, and tumorigenesis [10-16]. Also, lncRNAs are associated with various cellular processes, including the cell cycle, cell proliferation, apoptosis, and invasion [17]. However, we are only beginning to understand the nature and extent of the involvement of lncRNAs in tumorigenesis. Recently, the relationship between lncRNAs and tumors has drawn wide attention.

lncRNA AB073614 is a newly discovered lncRNA. Hu, et al. proved that AB073614 expression was significantly up-regulated in glioma tissues compared with normal brain tissues [18], indicating that AB073614 may function as a tumor promoter. In our study, we focused on the function of AB073614 in CRC. We demonstrated that AB073614 was markedly upregulated in CRC tissues compared with normal tissues ($P < 0.01$). Also, some of the clini-

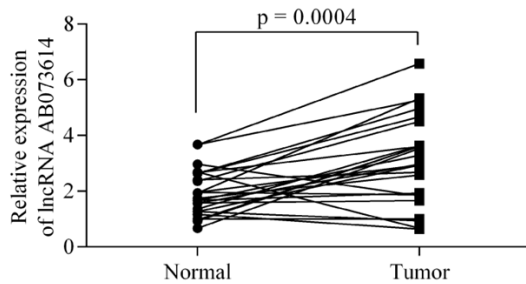


Figure 1. Expression of AB073614 in CRC tissues. AB073614 levels were upregulated in CRC tissues compared with non-cancerous tissues.

copathological characteristics of CRC patients, including tumor grade, tumor size, distant metastasis, and differentiation level, have a positive correlation with the level of AB073614. In vitro studies have shown that AB073614 can promote cell proliferation, migration and invasion, and can inhibit the cell apoptosis phenotype in CRC SW480 cells. That is to say, AB073614 plays a tumor promoter role in CRC progression.

Materials and methods

Clinical CRC samples

In this study, 64 pairs of tumor tissues and adjacent normal tissues of CRC patients, which were derived from surgical resections of primary tumors, were collected between January 2015 and December 2015 at the Fifth Affiliated Hospital, Sun Yat-sen University. Patients receiving preoperative adjuvant radiotherapy or adjuvant chemotherapy were not included in our study. The tissues were frozen in liquid nitrogen quickly and stored at -80°C until use. The clinicopathological data of the patients, such as age, gender, tumor grade, tumor size, lymphatic metastasis, distant metastasis, differentiation, and vascular invasion, were collected. The present study was approved by the Ethics Committee of the Fifth Affiliated Hospital, Sun Yat-sen University and signed, written consent forms were obtained from all the recruited patients.

Cell lines and antibody

The human CRC SW480 cell line used in this study was purchased from ATCC. The cells were cultured according to ATCC recommendations: RPMI 1640 medium with 10% FBS (fetal bovine serum, Gibco, USA) and penicillin/streptomycin

(Sigma, USA) supplemented in a 37°C incubator supplied with 5% CO_2 and 95% air. Commercially available antibodies were used for all immunoblotting and immunofluorescence studies. Anti-Bax, anti-Bcl-2, anti-MMP9 were obtained from the Abcam Company (UK). The anti-GAPDH was obtained from the Shanghai Kangchen Bio-tech Company (China). All secondary antibodies used were obtained from Boster (China).

Cell transfection

The siRNAs targeting human LncRNA AB073614 (si-AB073614) and the negative control were obtained from GenePharma (Shanghai, China). LncRNA AB073614 expression plasmids were constructed using pCDNA3.1 (+) basic vectors in our laboratory. These molecular products were transfected into SW480 cells when the cells reached 80-90% confluence, using Lipofectamine 2000 (Invitrogen, USA), according to the instructions provided by the manufacturer.

RNA extraction and qRT-PCR analyses

Total RNA from cells was extracted using the Trizol reagent (Takara, Japan). The RNA was reverse transcribed using a Bestar qPCR RT Kit (DBI Bioscience, Germany) according to the manufacturer's instructions. The RNA amplification and detection were performed using a Bestar qPCR RT Kit under the ABI 9700 PCR amplifier system (Applied Biosystems, USA). The primer sequences were as follows: The primers for AB073614 were 5'-TCTGCTCTGGGTCTTACAC-3' and 5'-TGCAACCACATGTAACACA-3'; the primers for GAPDH were 5'-CCCATCACCATCTTCCAGGAG-3' and 5'-GTTGTCATGGATGACCTTGGC-3'. qRT-PCR was performed in triplicate, and the relative expression of AB073614 was calculated using the comparative cycle threshold (CT) ($2^{-\Delta\Delta\text{CT}}$) method with GAPDH as the endogenous control to normalize the data.

Cell proliferation assay

Cell proliferation was detected using a cell counting kit-8 (CCK-8, Beyotime Biotechnology, China) according to the manufacturer's instructions. The infected cells were seeded at a density of 1×10^4 cells/well in a 96-well flat-bottom cell culture dish. 10 μl CCK-8 solution was added to each well, and incubated at 37°C for

Table 1. Difference in the lncRNA AB073614 expression in CRC patients grouped by clinicopathological characteristics

Clinicopathological characteristics	Number of patients	Expression of lncRNA AB073614*	P-value
Sex			
Male	14	3.051 ± 0.5218	0.5393
Female	11	3.511 ± 0.5020	
Age (year)			
< 58	13	3.328 ± 0.3818	0.8356
≥ 58	12	3.173 ± 0.6495	
Grade			
I/II	12	2.158 ± 0.3512	0.0005
III/IV	12	4.440 ± 0.4509	
Tumor size			
≤ 5 cm	13	2.082 ± 0.3320	0.0001
> 5 cm	12	4.523 ± 0.4282	
Lymphatic metastasis			
Yes	12	3.656 ± 0.5677	0.2947
No	13	2.882 ± 0.4550	
Distant metastasis			
Yes	11	4.500 ± 0.4776	0.0009
No	14	2.274 ± 0.3551	
Differentiation			
Poor	12	4.285 ± 0.4964	0.0037
High	13	2.301 ± 0.3711	
Vascular invasion			
Yes	11	3.438 ± 0.6193	0.6602
No	14	3.108 ± 0.4430	

(*The relative expression of lncRNA AB073614 was calculated using $2^{-\Delta\Delta Cq}$ method and was shown as mean ± SE).

100 min. The 450 nm absorbance was detected by using an automatic microplate reader at 24, 48 and 72 h (BioTeke, China). Additionally, a standard curve was established to calculate the number of cells. Each experiment was repeated 3 times.

Western blotting

Monolayer cells were grown to 80-90% confluence and then washed in ice-cold PBS. The cells were lysed in a universal protein extraction buffer (Beyotime, China) applied with the protease inhibitor PMSF (Genbase, China) 48 h after transfection. For western blotting, proteins were mixed with a 5×SDS loading buffer (250 mM Tris-HCl (pH = 6.8), 10% SDS, 0.5% bromophenol blue, 50% glycerol, 5% β-mercaptoethanol) and heated to 95°C for 8 min before separating with 4-12% SDS-PAGE. The proteins were then transferred to PVDF mem-

branes (Millipore, USA), and detected with proper primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies. Finally, the membranes were viewed using a chemiluminescent HRP substrate (Millipore, USA).

Trans-well invasion assay

8 μm pore size cell culture inserts coated with collagen (0.1%) were used to culture serum starved 2×10^4 SW480 cells. The inserts were incubated with a serum-free medium at 37°C for 10 hours with 50 ng/ml MCP-1 as chemoattractant. After we stained the inserts with a crystal violet solution, the cells on the upper side of the trans-well membrane were removed by cotton swab for microscopic analysis [19]. Subsequently, we mounted the membranes on glass slides, and then the migrated cells were viewed by light microscopy. Five different fields of cells were counted using a 20× magnification.

Cell migration assay

Cell migration was determined using a trans-well chamber assay. Briefly, serum-starved cells were trypsin-harvested in an FBS-free medium. Then, 500 μl of medium containing 1% FBS was added to the lower chambers, while the SW480 cells were plated in the upper chamber. After 12 h of incubation, the cells on the top surface of the membranes were removed by cotton swabs and the cells on the bottom surface were fixed with 4% paraformaldehyde at 37°C for 20 min and stained with a crystal violet solution. Then the migrated cells were viewed by light microscopy. All the groups of experiments were performed in triplicate.

Colony formation assay

After transfection, the SW480 cells were seeded in 6-well plates at the density of 1.5×10^3 /well and cultured for 2 weeks to allow colony formation. The culture medium was changed every 3 days. The colonies were then fixed in

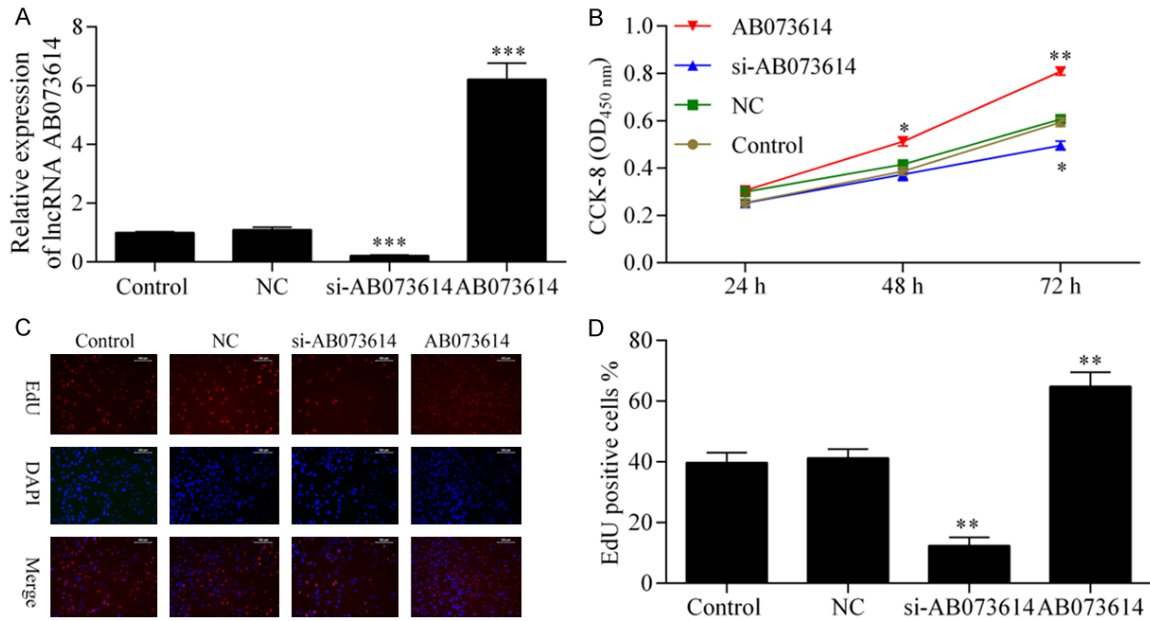


Figure 2. Effects of AB073614 expression on CRC cell proliferation. A. The relative levels of AB073614 were normalized to GAPDH in SW480 cell lines. SW480 was detected after the cells were transfected with si-AB073614, the AB073614 expression vector, or the control using real-time PCR. B. A CCK-8 assay was performed in SW480. Si-AB073614-transfected SW480 cells exhibited significantly lower growth rates than the control cells at day 3 after plating, and AB073614 vector transfected cells promoted cell growth. C, D. Cell proliferation in the si-AB073614, AB073614 expression vector or control transfected SW480 cells detected by an EdU staining assay.

100% methanol and stained with a crystal violet solution. Subsequently, the number of macroscopically observable colonies was recorded.

Cell cycle assay

The cell cycles were examined using a Cell Cycle Analysis Kit (MultiSciences, Netherlands). The cells were fixed with 70% ethanol at 4°C overnight and treated with RNaseA (0.02 mg/ml) in the dark at RT for 30 min, then stained with propidium iodide (PI) and analyzed by a FACSCalibur flow cytometer (BD, USA) according to the manufacturer's instructions.

Apoptosis assay

A Coulter Epics XL flow cytometer (Beckman Coulter, CA) was used to analyze the apoptotic rates using an annexin V-Alexa Fluor/PI apoptosis detection kit (Ebioe, China). The annexin V-Alexa Fluor/PI staining was executed according to the manufacturer's specifications.

5-ethynyl-2-deoxyuridine cell proliferation assay

The cells were seeded into 96-well plates. The cell proliferation ability was measured by a 5-ethynyl-2'-deoxyuridine (EdU) kit (Molecular

Probes, USA), following the instructions. A 100 µL culture medium and 5 mol/L EdU were added to each well, then they were incubated for 2 hours. After 30 min, a 4% paraformaldehyde fixation and 10 minutes treatment with 0.5% Triton, Hoechst 33342 (10 µg/ml) reaction solution (Sigma, USA) was added to each well, and then they were stained with 4,6-diamidino-2-phenylindole nuclear. After being washed with PBS three times, the cells were observed under fluorescence microscopy (Olympus, Japan) at 40× magnification, and condensed or fragmented nuclei were considered as apoptotic [20].

Statistical analysis

SPSS 20.0 software (Chicago, USA) was used to analyze the data with independent samples using a *T*-test or a Chi-square test. All values were represented as the mean ± standard deviation (SD). Statistical significance difference was defined as *P* < 0.05.

Results

LncRNA AB073614 is upregulated in CRC tissues

Based on the RT-qPCR, we found that the expression levels of AB073614 were signifi-

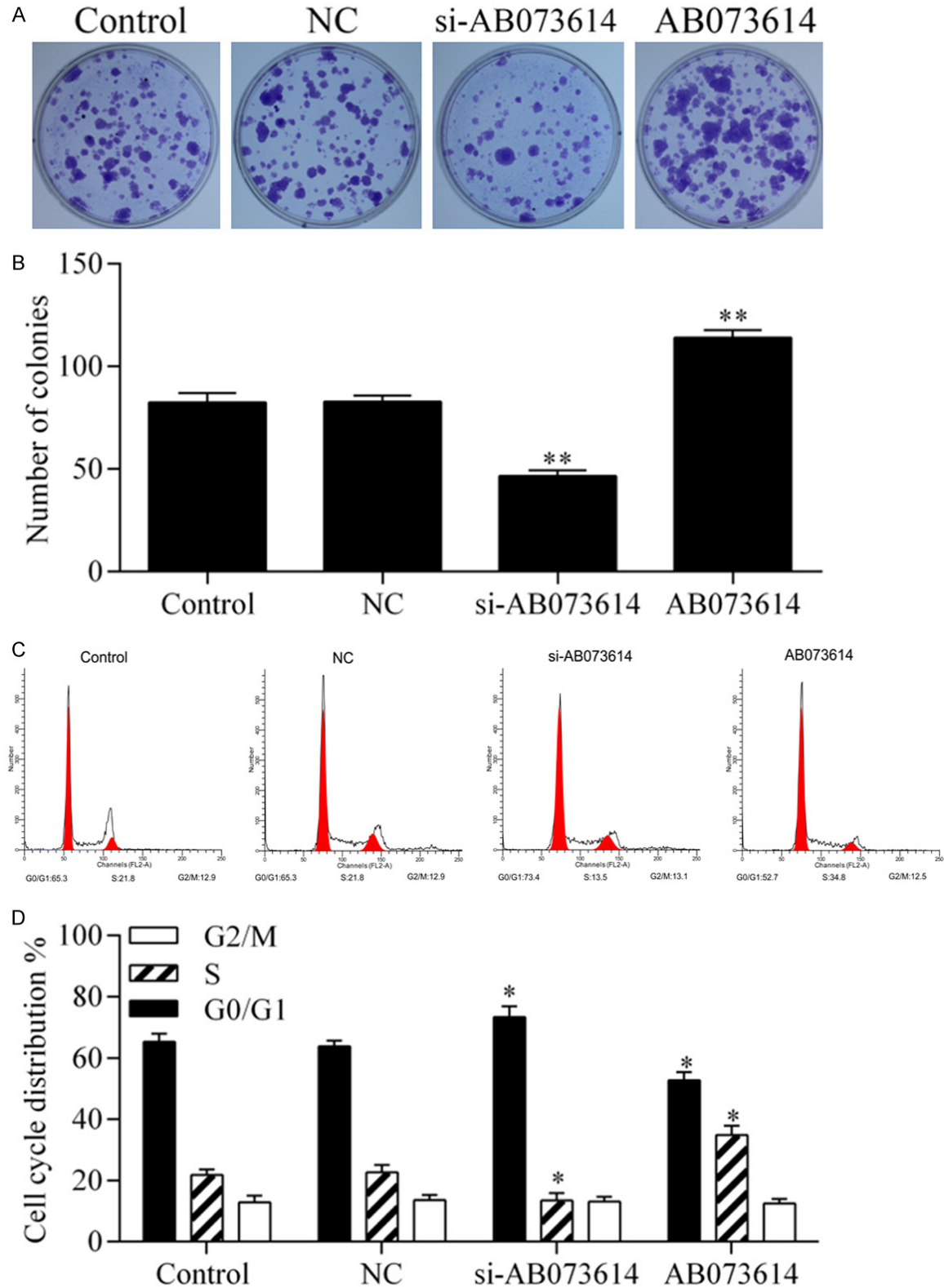


Figure 3. LncRNA AB073614 has a positive role on CRC cell proliferation and colony formation. A, B. Colony formation assay using si-AB073614, AB073614 expression vector or control transfected SW480 cells. C, D. Cell cycle assay in SW480 cells transfected with si-AB073614, AB073614 expression vector or control plasmids.

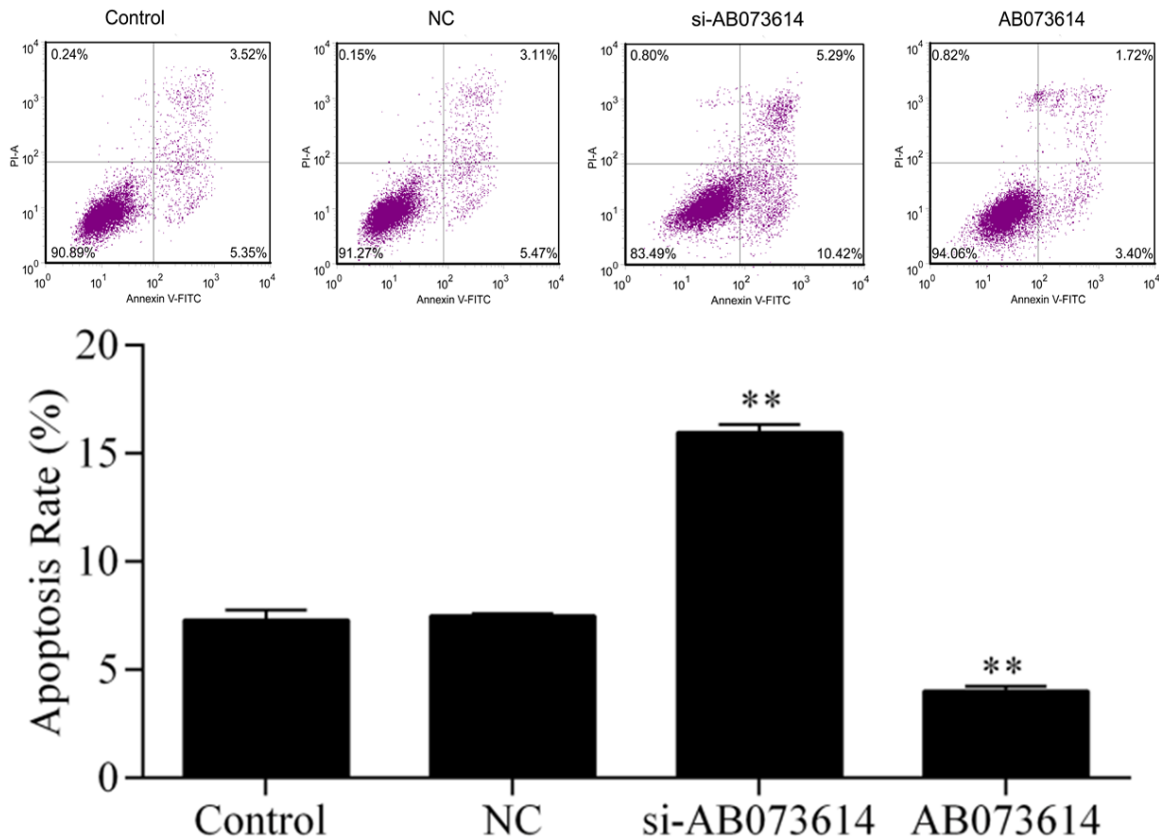


Figure 4. AB073614 inhibits SW480 cell apoptosis. Cell apoptosis rates were determined in the SW480 cells transfected with si-AB073614, the AB073614 expression vector, or the control plasmids.

cantly upregulated in CRC tissues compared with adjacent non-cancerous tissues ($P = 0.0004$, **Figure 1**).

LncRNA AB073614 has a positive correlation with tumor clinicopathological characteristics

AB073614 is overexpressed in CRC tissues, and we sequentially explored the correlation of AB073614 expression and the tumors' clinicopathological characteristics. As shown in **Table 1**, the level of AB073614 was markedly promoted in high grade CRC tumor tissues compared with the low grade tissues (I/II, 2.158 ± 0.3512 vs. III/IV, 4.440 ± 0.4509 , $P = 0.0005$). Tumor tissues with larger tumor sizes had higher expressions of AB073614 (≤ 5 cm, 2.082 ± 0.3320 vs. > 5 cm, 4.523 ± 0.4282 , $P = 0.0001$). Similarly, tumors with distant metastases (Yes, 4.500 ± 0.4776 vs. No 2.274 ± 0.3551 , $P = 0.0009$) and differentiation (Poor, 4.285 ± 0.4964 vs. High, 2.301 ± 0.3711 , $P = 0.0037$) characteristics had a significant difference in their AB073614 level (**Table 1**).

LncRNA AB073614 has a positive role on CRC cell proliferation and colony formation

First, we assessed the efficiency of the si-AB073614 and AB073614 overexpression vectors. The RT-qPCR showed that si-AB073614 can markedly knockdown the expression of AB073614, and the AB073614 overexpression vector can obviously increase the level of AB073614 (**Figure 2A**). To assess the biological role of AB073614 in CRC, we investigated the effect of AB073614 on cell proliferation. Next, a CCK-8 assay was conducted to analyze the proliferation of the transfected cells. The data showed that the knockdown of AB073614 significantly inhibited the proliferation of SW480 cells 72 hours after transfection, and the ectopic expression of AB073614 can promote the proliferation of SW480 cells (**Figure 2B**). Furthermore, the cell cycle assay showed that the knockdown of AB073614 can inhibit the G0/G1 phase of the cell cycle (**Figure 3C, 3D**). These results were further confirmed by an EdU staining assay (**Figure 2C, 2D**), suggesting that

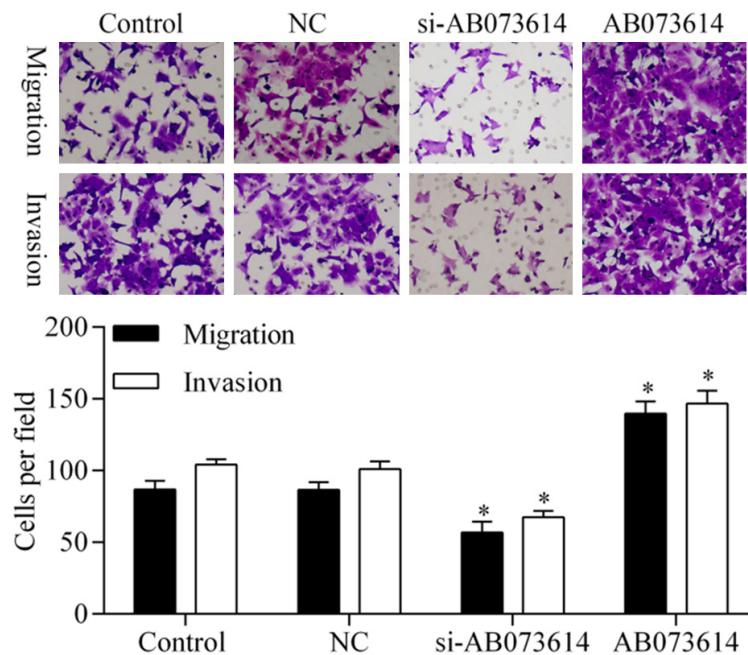


Figure 5. Migration and invasion assay using si-AB073614, the AB073614 expression vector, or the control transfected SW480 cells.

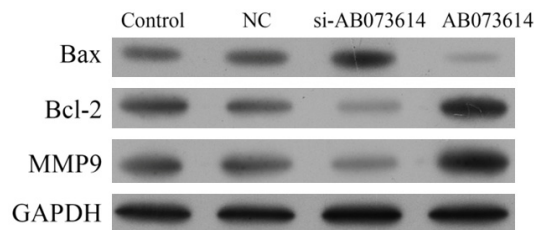


Figure 6. SW480 cells were transfected with si-AB073614, the AB073614 expression vector, or the control plasmids. After 24 h, the cells were lysed for the Western blot analysis, Bax, Bcl-2, MMP9 and GAPDH were detected.

AB073614 is involved in the promotion of cell proliferation. Furthermore, AB073614 overexpression markedly increased colony formation, and the knockdown of AB073614 markedly decreased colony formation in SW480 cells (Figure 3A, 3B).

LncRNA AB073614 has a positive role on CRC cell migration and invasion

To investigate the function of AB073614 in metastasis, the effects of AB073614 on the migrated ability of CRC cells were assessed by migration and invasion assays. As shown in Figure 5, the silencing of AB073614 in SW480 cells caused a significant reduction in cell mi-

gration and invasion. Similarly, the ectopic expression of AB073614 can promote the migration and invasion of SW480 cells.

The effects of LncRNA AB073614 expression on CRC cell apoptosis

To investigate the apoptosis functions of AB073614 in SW480 cells, a flow cytometer assay was performed to detect the rate of apoptosis. The results suggested that the AB073614 had a negative effect on SW480 cell apoptosis (Figure 4). To further measure the effect of AB073614 on the apoptosis of CRC cells, a Western blot was performed to examine the expression of apoptosis-related markers in SW480 cells after transfection

with si-AB073614, the AB073614 expression vector, or control plasmids. As shown in Figure 6, AB073614 in SW480 cells remarkably decreased the expression of Bax (which functions as an apoptotic activator) and meanwhile greatly increased the expression of Bcl-2 (promoting cellular survival), in comparison with the control groups. Also, AB073614 stimulates MMP9 expression, which is associated with the degradation of the extracellular matrix. These data suggest that AB073614 contributes to CRC cell proliferation partly by affecting the apoptosis process, and further experiments are needed to elucidate the potential mechanism.

Discussion

Recently, many lncRNAs have been identified, and the participation of lncRNAs in a wide repertoire of biological processes has been a topic of intense contemporary research [21]. So far, the clinical significance and biological functions of lncRNAs in CRC have been identified [22].

LncRNA AB073614 is a new lncRNA transcript which was first considered a *Homo sapiens* primary hepatoblastoma cDNA [23]. Dysregulated lncRNAs have been reported in many types of cancer and these lncRNAs have complex mo-

lecular mechanisms, which have been reviewed elsewhere [24, 25]. Cheng, et al. firstly showed that AB073614 expression was significantly up-regulated in ovarian cancer tissues compared with their normal counterparts [26]. Furthermore, they found that the knockdown of AB073614 expression significantly inhibited ovarian cancer cell proliferation and invasion. Hu, et al. reported that lncRNA AB073614 expression was significantly up-regulated in cancerous tissues compared with normal brain tissues [18]. Those results indicated that AB073614 might serve as a tumor promoter in glioma. However, the mechanism of AB073614 in regulating glioma was not reported.

Multiple studies have revealed that abnormal expressions of some lncRNAs are related to CRC tumorigenesis [27-30]. It remains unclear whether lncRNA AB073614 has important biological functions in CRC. In the present study, we confirmed that AB073614 was significantly upregulated in CRC tumor tissues, compared with normal tissues. Similarly, the level of AB073614 has a positive correlation with some clinicopathological characteristics of CRC patients, including high tumor grade, larger tumor size (> 5 cm), distant metastasis, and differentiation, which is further evidence that AB073614 acts as a tumor promoting regulator in the pathogenesis of CRC.

To further investigate the functions of lncRNA AB073614 in CRC, we explored the effects of lncRNA AB073614 on CRC cell biology. Furthermore, we found that AB073614 resulted in an obvious promotion in the cell proliferation rate and in the cells' migratory and invasion capacity. We examined the expression levels of the hallmarks of apoptosis in CRC cells with si-AB073614 and AB073614 expression vector transfection.

For now, the molecular mechanisms by which lncRNAs promote tumor development and metastasis are not fully understood. Many reports have shown that miRNAs lead to the oncogenic activity of some lncRNAs. Others have suggested that lncRNAs promote cancer progression by regulating the expressions of protein-coding genes. Our findings showed that AB073614 may regulate CRC progress by upregulating Bcl-2 and downregulating Bax in CRC cell lines. These findings remind us to confirm whether lncRNA AB073614 promotes CRC growth and metastasis by regulating the expression of genes encoding proteins.

Taken together, these findings indicate that lncRNA {"type": "entrez-nucleotide", "attrs": {"text": "AB073614", "term_id": "51555790", "term_text": "AB073614"}}AB073614 plays a direct role in the modulation of CRC progression and may be considered as a novel prognostic marker for CRC. Thus, lncRNA {"type": "entrez-nucleotide", "attrs": {"text": "AB073614", "term_id": "51555790", "term_text": "AB073614"}}AB073614 plays an oncogenic role in CRC and represents a potential target for CRC treatment.

Acknowledgements

This study was supported by the Science and Technology Foundation of Zhuhai (No. ZHW-2015-200).

Disclosure of conflict of interest

None.

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