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Original Article

Long Non-Coding RNA CKMT2-AS1 Reduces the Viability of Colorectal Cancer Cells by Targeting AKT/mTOR Signaling Pathway

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Abstract

Background: Colorectal cancer (CRC) has not only seriously affected people's lives, but also burdened the government healthcare system. Long non-coding RNAs (lncRNA) have attracted more and more attention in the cancer study field.

Methods: Experiments were completed in the Medical Research and Innovation Center of Shanghai Pudong Hospital, China from 2019 to 2020. Cell cycle was detected by western blot analyzing and flow cytometry. Apoptosis analysis were determined using flow cytometry or western blot analysis. LncRNA *CKMT2-AS1* was knocked down by shRNA transfection.

Results: We found *CKMT2-AS1* was the most significant=0.0105 for SW480 and *P*=0.0071 for HCT116) difference lncRNA between colorectal cancer treated with autophagy inducer and colorectal cancer without any treatment. Effective shRNA-*CKMT2-AS1* was also designed. Following, we found the treatment of autophagy inducer and autophagy inducer + shRNA-NC were able to suppress the proliferation of both SW480 and HCT116 cells. In addition, the treatment of autophagy inducer + shRNA-*CKMT2-AS1* significantly reduced the apoptosis of SW480 and HCT116 cells induced by autophagy. Furthermore, we found the phosphorylation of mTOR, AKT was enhanced in SW480, and HCT116 cells treated with autophagy inducer + shRNA-*CKMT2-AS1* compared to the cells treated with autophagy inducer of autophagy inducer + shRNA-NC.

Conclusion: Enhancing the expression of *CKMT2-AS1* will become a promising strategy to prevent the progress of colorectal cancer.

Keywords: Colorectal cancer; Long non-coding RNAs; Gene

Introduction

Colorectal cancer (CRC) is cancer develops from the colon or rectum (part of the large intestine), which also known as colon cancer and bowel cancer (1). Symptoms of colorectal cancer include a change in bowel movements, blood in the stool; feeling tired all the time and weight loss (2). According to epidemiological statistics, colorectal cancer is the third most common cancer in the world, accounting for about 10% of all cases (3). There were 1.4 million new cases in 2012, and the disease caused 694,000 deaths (3). This situation is more common in developed countries, where more than 65% of cases are found. Besides, females are rarer than males (4). Even in the developed country, for example, in the United States, the five-year survival rate of CRC is around 65%. It already seriously affects people's life and government healthcare systems, thus some effective

strategies are under urgent need. However, the mechanism of the development of colorectal cancer is still not clear (5), it will help to develop an effective cure for the treatment of colorectal cancer.

Long non-coding RNA (lncRNA) is a type of RNA, defined as transcripts that are longer than 200 nucleotides and are not translated into protein (6). However, primary studies reported that lncRNAs were transcriptional "noise" without biological functions (7). In recent decades, LncRNA has become a necessary regulator in almost all aspects of biology. More and more evidence showed that lncRNAs play a significant role in cancer development (5, 8). For instance, prostate cancer non-coding RNA 1 (*PRNCR1*) was associated with the development of prostate cancer (9). *LncRNA-HOST2* played an important role in regulating the biological behavior of ovarian cancer cells (10). In colorectal cancer, the new lncRNA (*RP11-462C24.1*) was discovered that low expression of *RP11-462C24.1* was correlated with patients' bad prognosis (11). Moreover, Han et al found lncRNA was associated with lymph node metastasis through microarray analysis, and there were 545 differentially expressed lncRNAs in metastatic lymph nodes compared with normal lymph nodes (12). However, no potential lncRNA associated with colorectal cancer has been reported.

Autophagy is a natural, regulated mechanism of cells that can remove dysfunctional or unnecessary components from itself (13). It allows cells to orderly degrade and recycle some of the cellular components (14). The effects of autophagy in cancer have been highly reviewed and studied. Autophagy worked not only as a tumor suppressor but also as a cytokine for tumor cell survival. However, recent researches showed that autophagy was more likely to be used as a cancer suppressor, rather than cytokines for tumor cell survival (15).

Herein, we explore the differentially expressed lncRNAs, and we try to explore the relationship between lncRNAs and autophagy. This study may provide a promising target gene to prevent the development and progression of colorectal cancer.

Materials and Methods

Cell culture

SW480 and HCT116 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 % penicillin and 1 % streptomycin at 37 °C with 5% CO_2 atmosphere. The medium was changed every two days.

Cell cycle analysis

This experiment was completed in the Medical Research and Innovation Center of Shanghai Pudong Hospital, China in 2019. After digesting, SW480 and HCT116 cells were directly plated at a density of 6×10^5 cells/well in 96-well plates. Before incubation, the cells were cultured overnight. Cell cycle analysis was analyzed using Cell Cycle Analysis Kit according to the introduction of the manufacturer. In particular, SW480 and HCT116 cells were respectively treated with PBS or autophagy inducer, autophagy inducer + shRNA-NC, autophagy inducer + shRNA-*CKMT2-AS1*. Cells were collected, washed with PBS for two times, and then cell cycle was analyzed.

Apoptosis analysis

The apoptosis of SW480 and HCT116 cells with different treatments were detected using the Apoptosis Detection Kit, which followed the instructions of the manufacturer. Cells were cultured in a 6-well plate at the density of 1×10^5 cells/well, and then different treatment was performed. At indicated times, cells were collected then stained with 300 µL mixed staining solution containing 5 ul PI solution and 5 ul Annexin V-FITC for 20 min in the dark at room temperature. Finally, the apoptosis of SW480 and HCT116 cells with different treatments were performed by flow cytometry (FACScan, BD Biosciences).

Western blot

SW480 and HCT116 cells with different treatments were collected, then total proteins were extracted from cells lysis buffer, and the concentration of protein was measured using the Bradford method (Beyotime, Nantong, China) according to the instructions of the manufacturer. Twenty grams of proteins were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. Following, the membrane was blocked with 5% non-fat milk for 1 hour at room temperature, and then the membrane was treated with target primary antibodies at 4 °C overnight. After that, membranes were washed with 0.2% PBST for 3 times and treated with corresponding secondary antibodies for 2 h. Finally, the membrane was washed with 0.2% PBST for 3 times, the specific protein bands were visualized using the enhanced chemiluminescence reagents

shRNA transfection

SW480 and HCT116 cells were transiently transfected with negative control shRNA (200nM) and shRNA-*CKMT2-AS1* (200nM) respectively, which follows the instructions of the manufacturer. These shRNAs were purchased from Funengbio Co. (Shanghai, China)., The transfected cells were obtained for experiments after 24 h of culture.

Statistical analysis

All results were exhibited as the mean ± S.D. And the statistical analysis was performed using GraphPad Prism 8.0 software. Differences between two groups were evaluated using student *t*-test, and differences among the groups were assessed using one-way ANOVA. Significance levels were set at $*P < 0.05$ and $**P < 0.01$.

Results

Autophagy inducer reverses 5-FU induced cell apoptosis

As shown in Fig. 1A, we found that 5-FU was able to significantly inhibit the proliferation of SW480 cells, and promote its apoptosis (Fig. 1B). However, we found the autophagy inducer was capable of reducing the cytotoxicity of 5-FU. After the treatment of autophagy inducer, the proliferation of 5-FU pre-treated SW480 cells increased (Fig. 1C). On the contrary, the apoptosis of 5-FU pre-treated SW480 cells reduced (Fig. 1D). Besides, the autophagy inducer dramatically enhanced the expression of LC3II/LC3I in both SW480 and HCT116 cells compared to cells without any treatment (Fig. 1E).

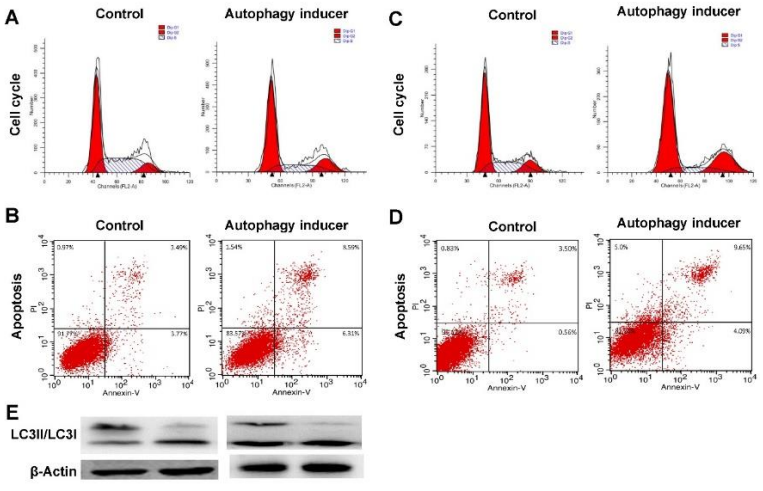

Fig. 1: Autophagy inducer reverses 5-FU induced cell apoptosis. (A) The cell cycle analysis of SW480 cells treated with 5-FU, cells without any treatment as control. (B) The apoptotic analysis of SW480 cells treated with 5-FU, cells without any treatment as control. (C) The cell cycle analysis of SW480 cells treated with 5-FU, 5-FU + autophagy inducer. (B) The apoptotic analysis of SW480 cells treated with 5-FU, 5-FU + autophagy inducer. (E) The expression of LC3II/LC3I in both SW480 and HCT116 cells treated with autophagy inducer, cells without any treatment as control

Differentially expressed lncRNA analysis

To screen out the differentially expressed lncRNA, the lncRNA microarray was performed. We found there were 16 upregulated lncRNAs and 3 downregulated lncRNAs in both SW480 and HCT116 cells. As shown in Fig. 2A and 2B, the upregulated lncRNAs including *HLA-J*, *CKMT2-AS1*, *MIR22HG*, *LOC105373456*, *LOC105370410*, *MIR34AHG*, *LOC105369748*, *LOC646626*, *LINC02535*, *GBAP1*, *FTO-IT1*, *SSTR5-AS1*, *LOC101929004*, *LOC103091866*, *OR7E91P*, *LOC105375119*. In addition, the downregulated lncRNAs including *LINC01389*, *LINC01003*, *LOC107985279* (Fig. 2C and 2D). Then we used RT-PCR to verify further these differentially expressed lncRNAs in both SW480 and HCT116 cells. As shown in Fig. 2E and 2F, most of the expression results of lncRNAs were consistent with lncRNA microarray results, and the expression of *CKMT2-AS1* showed the most significant difference.

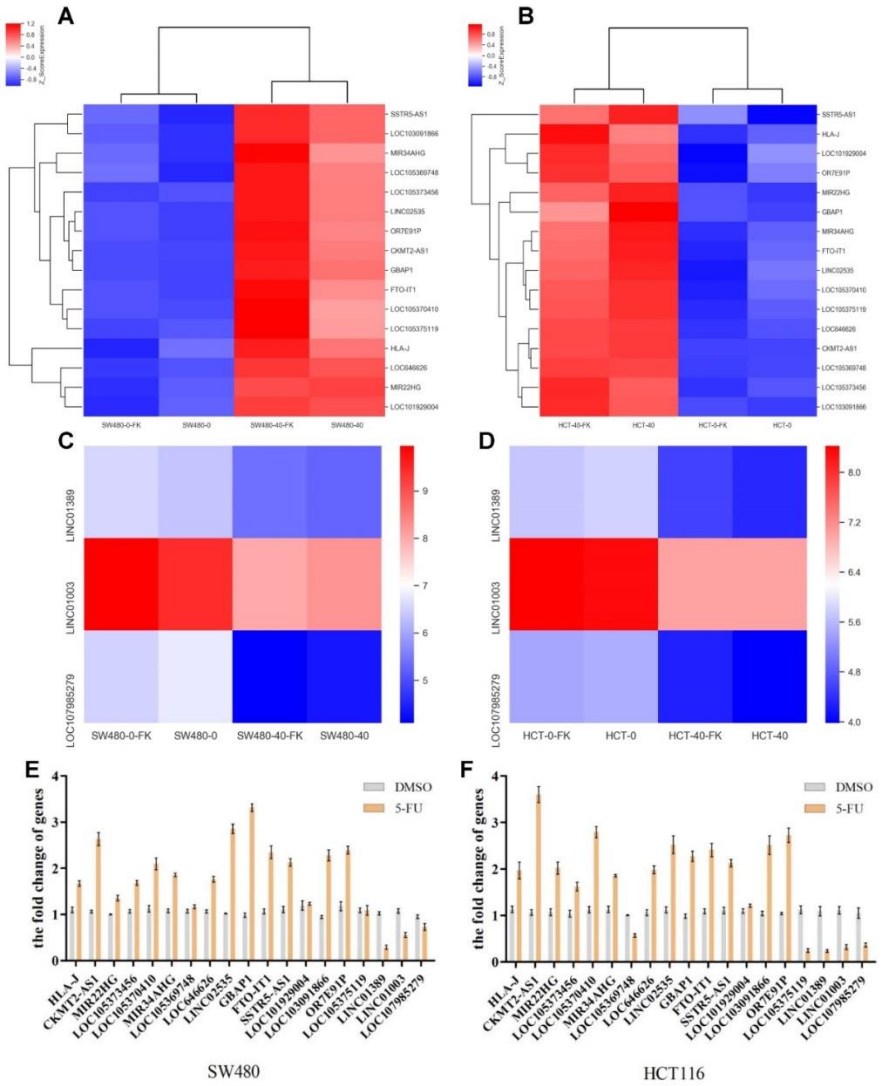

Fig. 2: The differentially expressed lncRNAs. (A,B) The upregulated 16 lncRNAs in both SW480 and HCT116 cells treated with 5-FU, cells without any treatment as control. (C,D) The downregulated 3 lncRNAs in both SW480 and HCT116 cells treated with 5-FU, cell without any treatment as control. (E,F) The relative expression of 16 upregulated and 3 downregulated lncRNAs in both SW480 and HCT116 cells

CKMT2-AS1 shRNA interference

To verify further the function of *CKMT2-AS1* in colorectal cancer cells, the shRNA interference was performed. We designed 3 different shRNA of *CKMT2-AS1*, and we found shRNA-*CKMT2-AS1* had the highest efficiency compared to the shRNA-NC, shRNA-*CKMT2-AS1-2* and shRNA-*CKMT2-AS1-3* (Fig. 3). Furthermore, the treatment of autophagy inducer and autophagy inducer + shRNA-NC were able to suppress the proliferation of both SW480 and HCT116 cells. However, the treatment of autophagy inducer + shRNA-*CKMT2-AS1* was able to reverse the inhibition of proliferation induced by autophagy (Fig. 4A). Moreover, the treatment of autophagy inducer and autophagy inducer + shRNA-NC also increased the apoptosis of both SW480 and HCT116 cells. On the contrary, the treatment of autophagy inducer + shRNA-*CKMT2-AS1* significantly reduced the apoptosis of SW480 and HCT116 cells induced by autophagy (Fig. 4B).

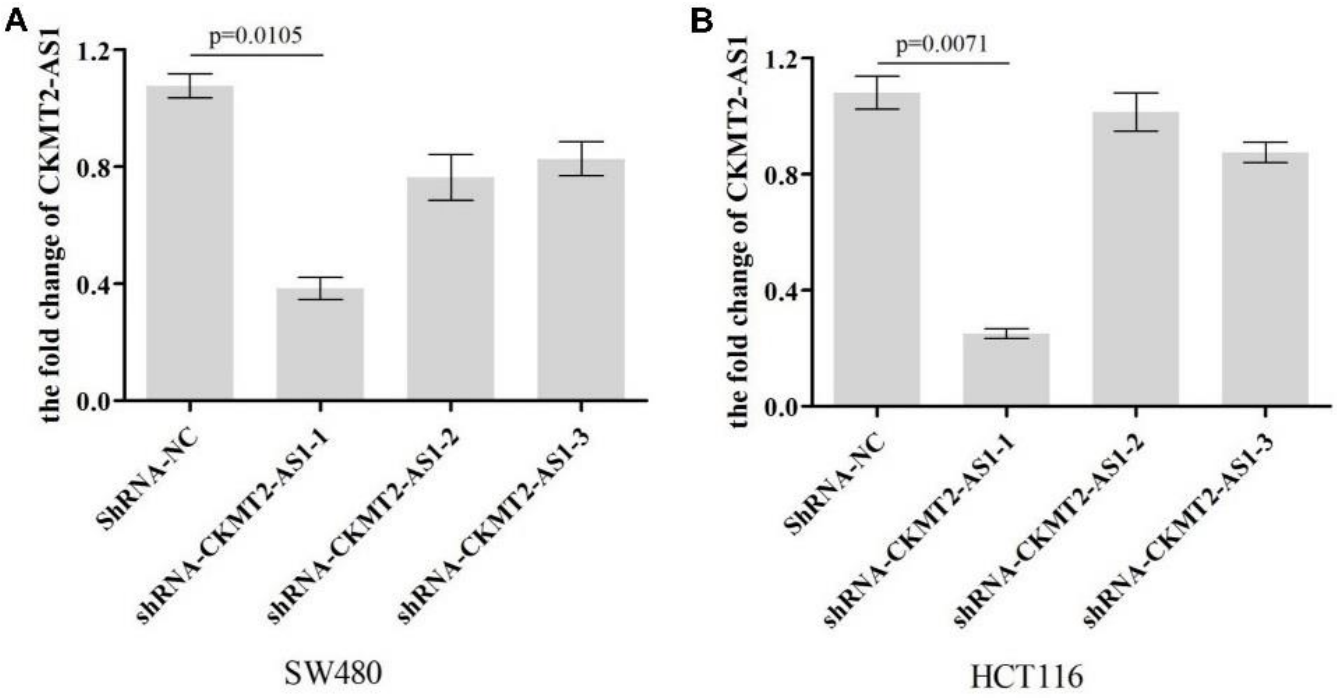

Fig. 3: The relative expression of CKMT2-AS1 in both SW480 and HCT116 cells treated with shRNA-NC, shRNA-CKMT2-AS1-1, shRNA-CKMT2-AS1-2, and shRNA-CKMT2-AS1-3

Silencing CKMT2-AS1 recovers the expression of p-mTOR, p-AKT

Following, we explored the potential mechanism of Silencing *CKMT2-AS1* reduced apoptosis of SW480 and HCT116 cells. As shown in Fig. 5A and 5B, the phosphorylation of mTOR and AKT reduced in SW480 and HCT116 cells treated with autophagy inducer of autophagy inducer + shRNA-NC compared to control cells. Moreover, we found the phosphorylation of mTOR, AKT was enhanced in SW480, and HCT116 cells treated with autophagy inducer + shRNA-*CKMT2-AS1-2* compared to the cells treated with autophagy inducer of autophagy inducer + shRNA-NC.

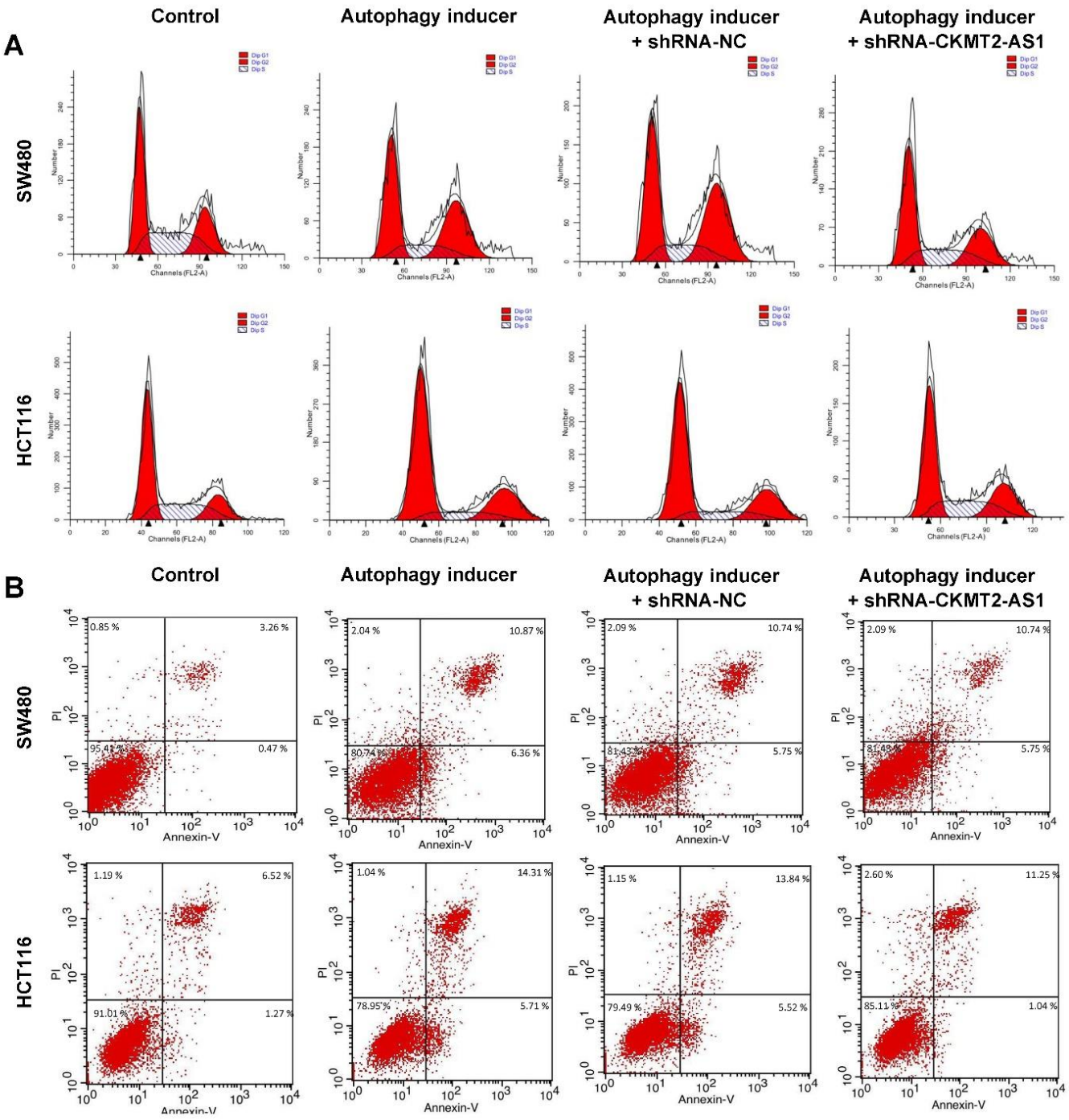

Fig. 4: CKMT2-AS1 shRNA interference. (A) The cell cycle analysis of both SW480 and HCT116 cells treated with autophagy inducer, autophagy inducer + shRNA-NC, autophagy inducer + shRNA-CKMT2-AS1, cells without any treatment as control. (B) The apoptotic analysis of both SW480 and HCT116 cells treated with autophagy inducer, autophagy inducer + shRNA-NC, autophagy inducer + shRNA-CKMT2-AS1, cells without any treatment as control

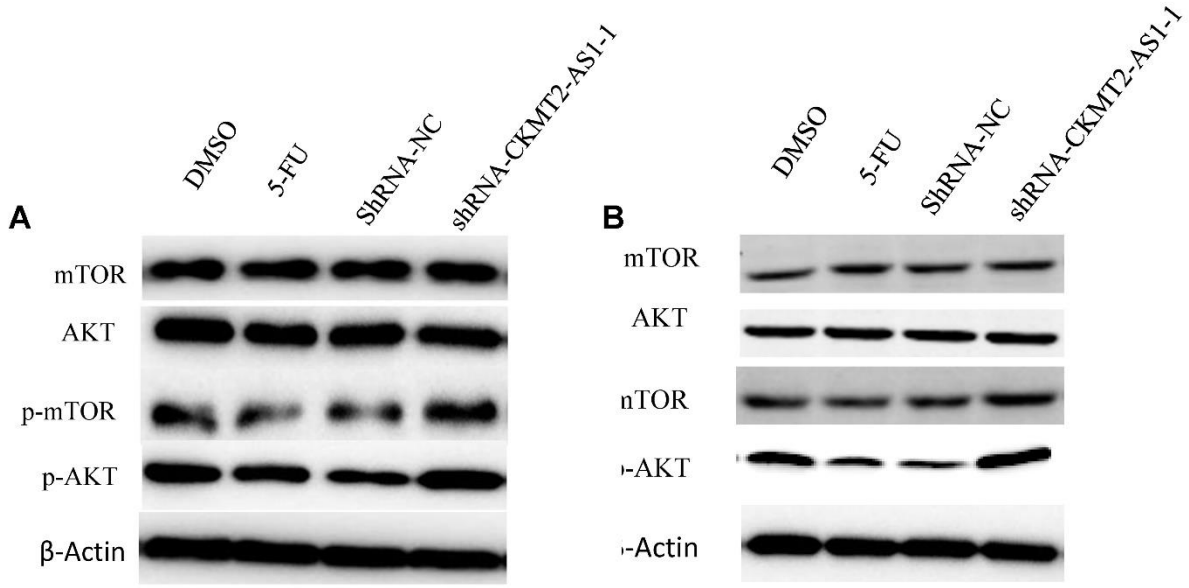

Fig. 5: The relative expression of mTOR, p-mTOR, AKT, p-AKT on both SW480 (A) and HCT116 (B) cells treated with DMSO, 5-FU, shRNA-NC and shRNA-CKMT2-AS1-1

Discussion

It is becoming increasingly apparent that many genomic mutations in cancer are located in regions that do not encode proteins. However, these regions are usually transcribed into lncRNA (5, 8). The latest application of next-generation sequencing technology in an increasing number of cancer transcriptomes has indeed revealed thousands of lncRNAs whose abnormal expression is associated with different types of cancer (5, 16). These lncRNAs play an important role in gene regulation, resulting in affecting different aspects of cellular homeostasis, including survival, proliferation, genomic stability and migration (17). In our study, we found the lncRNA, *CKMT2-AS1*, has relationships with the development of colorectal cancer. The expression of *CKMT2-AS1* was significantly increased in SW480 and HCT116 cells treated with 5-FU, and the inhibition of *CKMT2-AS1* expression will reduce the apoptosis of cancer cells. Then we explored its potential mechanism.

PI3K's downstream effector, Akt, is often over-activated in human cancer (18). The key downstream effector of Akt that promotes tumorigenesis is mTOR (19, 20). In the PI3K/Akt/mTOR pathway, there are two tumor suppressors on both sides of Akt. One is PTEN which acts as a brake upstream of Akt (21), and another is TSC1/TSC2 heterodimer that acts as a brake upstream of mTOR and downstream of Akt (22). In defect of the TSC1/TSC2 brake, mTOR activity is released through an inhibitory feedback mechanism to inhibit Akt. In our study, we found 5-FU was able to induce autophagy in SW480 and HCT116 cells, and it decreased the phosphorylation of Akt and mTOR. While the co-treatment of 5-FU and shRNA-*CKMT2-AS1* was capable of recovery the phosphorylation of Akt and mTOR. Thus, we speculated that *CKMT2-AS1* was able to suppress the viability of colorectal cancer cells.

There are few references on the role of *CKMT2-AS1* on cancers. *CKMT2-AS1* was down-regulated in colorectal cancer cell lines (23). Moreover, *CKMT2-AS1* was down-regulated in

colorectal cancer tissues (24). However, both of the two studies did not discuss the role of *CKMT2-AS1* in colorectal cancer. We found the *CKMT2-AS1* was associate with autophagy, and the inhibition of *CKMT2-AS1* will increase the viability of colorectal cancer cells. These suggest that enhancing the expression of *CKMT2-AS1* will become a promising strategy to prevent the progress of colorectal cancer.

Conclusion

Enhancing the expression of *CKMT2-AS1* will become a promising strategy to prevent the progress of colorectal cancer

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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