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ORIGINAL ARTICLE

LncRNA-PCAT1 controls the growth, metastasis and drug resistance of human colon cancer cells

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Summary

Purpose: *The long non-coding (lnc) RNAs have been shown to exhibit profound regulatory roles in maintaining the growth and proliferation of human cancer cells. Taking this fact into consideration, the current research work was scheduled to explore the regulatory control of lncRNA-PCAT1 in maintaining the growth and progression of human colon cancer cell.*

Methods: *The expression of lncRNA-PCAT1 was assessed through qRT-PCR method. DAPI and acridine orange (AO)/ethidium bromide (EB) staining protocols along with the colony formation protocols were performed to evaluate the viability of cancer cells. The migratory and invasion properties of cancer cells were examined by the wound-healing and transwell assays, respectively. Western blotting was used to assess the expression of proteins of interest. MTT assay was used for the assessment of cell proliferation.*

Results: *lncRNA-PCAT1 was highly up-regulated in the colon cancer tissues and cancer cell lines. The repression of lncRNA-PCAT1 in colon cancer cells reduced their viability through induction of Bax/Bcl-2 mediated apoptosis. The inhibition of lncRNA-PCAT1 expression further declined the migration and invasion of colon cancer cells along with the decline of cell proliferation and enhanced the chemosensitivity of colon cancer cells.*

Conclusion: *lncRNA-PCAT1 expression may be utilized as a vital prognostic tool in colon cancer and highlighted its regulatory effects in maintaining the colon cancer growth and proliferation.*

Key words: *long non-coding RNA, colon cancer, metastasis, proliferation, chemosensitivity, apoptosis*

Introduction

Colon cancer is one of the leading lethal malignancies. The current figures make it the 3rd most prevalent malignancy among the human cancers worldwide [1]. Another alarming fact regarding colon cancer is that about 20-30% of patients detected with this cancer are seen with metastasis at the time of diagnosis [2]. The currently used drugs can be administered in combination with anti-cancer molecules to possibly combat cancer more efficiently [3]. However, from such anti-cancer approaches no satisfactory results have been reported. The use of chemotherapeutic drugs is also limited by the development of multidrug resistance [4]. Considering all these, there is a need of exploring alternative approaches against the human colon cancer. Long non-coding RNAs (lncRNAs) have evolved as important regulatory molecules during recent years for their role in affecting the characteristics of various human cancers [5-7]. LncRNAs are usually longer than 200 nucleotides in length and their expression levels are relatively lower than the protein coding RNA molecules [8]. The lncRNA PCAT1 (lncRNA-PCAT1) has been shown to regulate the growth and metastasis of osteosar-

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coma cells [9] and it was also shown to affect the proliferation of human prostate cancer cells [10]. The up-regulation of lncRNA-PCAT1 was seen to be associated with poor prognosis in colorectal cancer [11]. This study was designed to examine the expression of lncRN.

Methods

Clinical specimens, cell lines and transfection of cancer cells

The clinical specimens pertaining to colon cancer and surrounding normal tissues were obtained from the colon cancer patients being treated at the First Hospital of China Medical University, under proper ethical guidelines. The tissue samples were immediately frozen in liquid nitrogen and stored at -80°C for future usage. Colon cancer cell lines DLD-1, HT-29 and HCT-116 and the normal colon epithelial cell line HCEC-1CT were purchased from the ATCC, USA. The cell lines were cultured using DMEM medium (Thermo Scientific Waltham, Massachusetts, United States) supplemented with 10% fetal bovine serum at 37°C and 5% CO_2 in a humidified incubator. The transfection of HCT-116 cancer cells with siRNA-negative control (si-NC) and si-PCAT1 was done using Lipofectamine 2000 (Thermo Scientific Waltham, Massachusetts, United States), following the manufacturer's protocol.

qRT-PCR analysis

Total RNA from tissues and cell lines was extracted using TRIzol reagent method following the manufacturer's guidelines (Thermo Scientific, Waltham, Massachusetts, United States). The DNA contamination was removed by treating the extracted RNA samples with DNAse I (Thermo Scientific [Waltham, Massachusetts, United States). The RNA was then used to synthesize the complementary DNA (cDNA) by Prime-Script™ one step kit (Takara). cDNA was used to assess the expression of lncRNA-PCAT1 in clinical samples and cell lines by performing quantitative real time PCR on QuantStudio 5.0 Real Time system (Thermo Scientific, Waltham, Massachusetts, United States). The quantitative comparison of lncRNA-PCAT1 expression was analyzed through 2^{-ddCt} method. Human actin gene was used as internal control in the expression analysis.

DAPI and AO/EB staining assays

The HCT-116 colon cancer cells were placed into 12-well plates at a density of 10^6 cells/well following their transfection with either si-NC or si-PCAT1. The cancer cells were allowed to grow for a period of 48h at 37°C in DMEM medium. Then, the cells were harvested and fixed with 70% ethanol after which they were stained with DAPI or AO/EB solution. The cells were then examined by fluorescent microscope.

Colony formation assay

The viability of HCT-116 cancer cells transfected with si-NC or si-PCAT1 was determined by colony formation assay. Briefly, about $6x10^3$ cancer cells were added per well of 6-well plates and cultured for a week. Post harvesting and phosphate buffered saline washing, the cells were fixed with methanol for 20 min. 0.1% crystal violet stain was used for staining the cells and finally their colony forming potential was examined.

Migration and invasion assays

The migratory potential of HCT-116 cancer cells transfected with si-NC or si-PCAT1 was estimated by wound-healing assay. The transfected cells were cultured in 6-well plates for 24 h till 80% confluence. A wound was made on the surface using pipette tip whose initial width was noted. After 24 h of incubation at 37°C, the width was again recorded, visualized under 100x light microscope and photographed.

The invasiveness of HCT-116 cancer cells transfected with si-NC or si-PCAT1 was determined by Transwell chamber coated with matrigel. The upper chamber was inoculated with 6000 transfected cells while the lower chamber was inoculated with DMEM medium only. Following incubation at 37°C for 72h, the cancer cells invading the lower chamber were harvested, fixed with 4% formaldehyde and stained with 0.1% crystal violet solution following which they were visualized using light microscope (100x).

Cell lysis and western blotting

The cell lysates were obtained by fractionation of cancer cells (transfected with si-NC or si-PCAT1) with RIPA lysis buffer. The total protein count of each lysate was calculated with Bradford reagent. Cell lysates with equal protein concentrations were run on 8% SDS-PAGE gel. The gel was then blotted to nylon membranes which were exposed to primary and secondary antibodies designed against the proteins of interest and finally protein expressions were examined. The expressions were standardized using human β-actin protein.

Cell proliferation assay

The MTT assay was performed for estimating the proliferative rates of HCT-116 cancer cells transfected with si-NC or si-PCAT1. In brief, the transfected cancer cells were administered or not 2.5μM 5-flourouracil and grown for 24, 48, 72 or 120h in 96-well plates. Afterwards, 0.5% MTT reagent was added to each well and incubated for 4h at 37°C. A spectrophotometer was used to record the absorbance at 570nm to determine the proliferation rate of cell culture samples. The absorbance values were plotted to compare the proliferation rates of cancer cells.

Statistics

For statistical validation of experimental data, the experiments were performed in triplicate and the values are shown as mean ± standard deviation (SD). Graphpad Prism 7.0 software was used to determine differences performing the Student's t- test and one-way ANOVA. P values ≤ 0.05 were considered as statistically significant.

Results

lncRNA-PCAT1 is up-regulated in colon cancer

qRT-PCR method was used to determine the expression of lncRNA-PCAT1 in the tissue samples and colon cell lines (normal and cancerous). The results indicated that the cancer tissues and cell lines (DLD-1, HT-29 and HCT-116) exhibited significantly higher expression levels of lnc-PCAT1 than the normal tissues and epithelial cell line (Figure 1A and 1B). Among the cancer cell lines, HCT-116 showed highest expression of lncRNA-PCAT1 and was thus chosen for further experimentations.

Inhibition of lncRNA-PCAT1 expression declines the viability of colon cancer cells through modulation of Bax/Bcl-2 ratio

To assess whether downregulation of lncRNA-PCAT1 had any effect on the viability of colon cancer cells, the HCT-116 cancer cells transfected with si-NC or si-PCAT1 were cultured for 48h and processed for DAPI and AO/EB staining assays. The results showed that the repression of lncRNA-PCAT1 expression was associated with reduction of viability of cancer cells (Figure 2A and 2B). The reduction of the viability was seen as decrease in the colony forming potential of colon cancer cells under si-PCAT1 transfection (Figure 3A). The decline in cell viability was shown to be due to cell apoptosis owing to the modification of Bax/Bcl-2 ratio. The protein concentration of Bax was seen to be increasing while that of Bcl-2 decreased in cancer cells transfected with lncRNA-PCAT1 (Figure 3B).

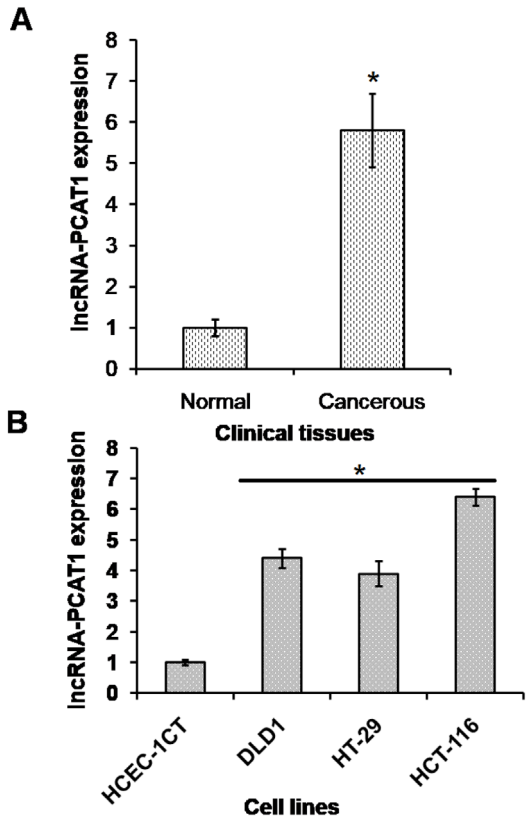

Figure 1. lncNRA-PCAT1 is over-expressed in colon cancer. **A:** qRT-PCR analysis of lncRNA-PCAT1 in normal and cancerous colon tissue samples showing upregulation of lncRNA-PCAT1 in colon cancer tissues. **B:** qRT-PCR analysis of lncRNA-PCAT1 in normal and cancer colon cell lines showing upregulation of lncRNA-PCAT1 in colon cancer cells. The experiments were performed in triplicate and shown as mean ± SD (*p<0.05).

HCT-116 cancer cell migration and invasion is reduced under lncRNA-PCAT1 transcript repression

The effects of downregulation of lncRNA-PCAT1 in HCT-116 cancer cells were also evident in the reduction of their migration rates (Figure 4A). Similar observation was drawn for the invasion of the colon cancer cells transfected with the silencing constructs of lncRNA-PCAT1 (Figure 4B). Together, these findings highlight the potential of lncRNA-PCAT1 in controlling the metastasis of human colon cancer.

Downregulation of lncRNA-PCAT1 in colon cancer cells decreases the cell proliferation and increases their chemosensitivity

The colon cancer cells transfected with si-NC or si-PCAT1 were administered with or without 2.5μM 5-flourouracil and their proliferation rates

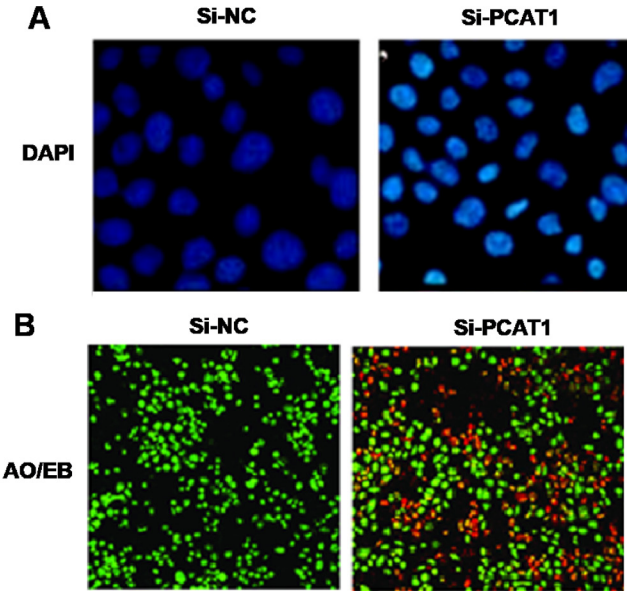

Figure 2. Repression of lncRNA-PCAT1 decreases the viability of colon cancer cells. **A:** DAPI staining of HCT-116 cancer cells transfected with si-NC or si-PCAT1. **B:** AO/EB dual-staining of HCT-116 cancer cells transfected with si-NC or si-PCAT. The figures show that silencing of lncRNA-PCAT1 induces apoptosis in HCR-116 colon cancer cells. The experiments were performed in triplicate.

were determined at 24, 48, 72, 96 and 120h time intervals of growth by the MTT assay. The values suggested that repression of lncRNA-PCAT1 expression led to significant decline in the proliferation rates of colon cancer cells (Figure 5). Moreover, the downregulation of lncRNA-PCAT1 in colon cancer cells enhanced their chemosensitivity to 5-flourouracil, implicating the molecular anticancer role of lncRNA-PCAT1 against the growth and proliferation of human colon cancer.

Discussion

Cancer was and still is a most deadly malignancy. The severity of cancer is evident from the morbidity and mortality it causes per year worldwide [12]. The currently employed anticancer approaches against the cancer progression are accompanied with a number of hurdles including the development of drug resistance contributing to fairly low success [13]. Considering all these, clinicians and researchers search for more robust and successful ways of cancer management. Colon cancer ranks 3rd globally in terms of incidence rates. The early prognosis of colon cancer is very difficult and a lot of colon cancer patients are metastatic when first diagnosed. The mortality of colon cancer is further increased owing to the development of drug resistant colon cancer cells, as highlighted by a multitude of research studies. The emergence of lncRNAs as vital anticancer molecules, as implicated by the recent cancer research, is seen with lot of promise to be utilized as an alternative approach

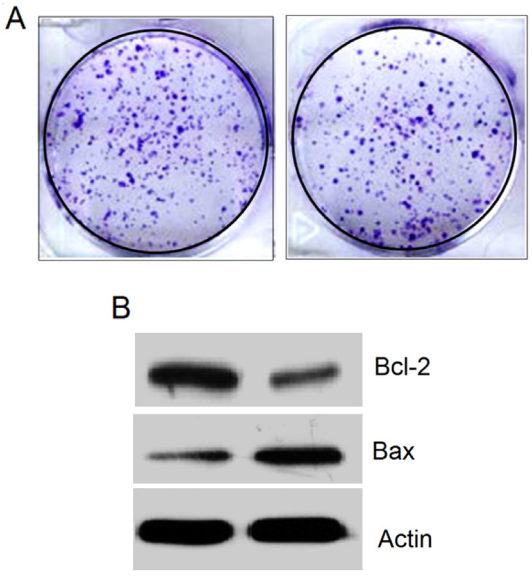

Figure 3. lncRNA-PCAT1 suppression induces the apoptotic cell death in colon cancer cells. **A:** Colony forming potential of HCT-116 cancer cells transfected with si-NC or si-PCAT1 showing PCAT1 silencing inhibits colony formation of the HCT-116 colon cancer cells. **B:** Western blotting of Bax and Bcl-2 proteins from HCT-116 cancer cells transfected with si-NC or si-PCAT1 showing inhibition of Bcl-2 and increase of Bax expression upon PCAT silencing. The experiments were performed in triplicate.

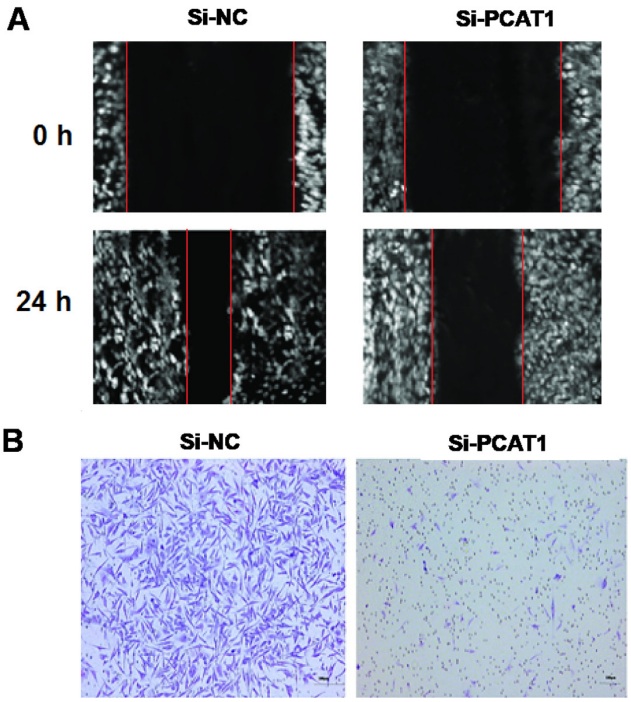

Figure 4. Inhibition of lncRNA-PCAT1 expression suppresses the colon cancer cell metastasis. **A:** Wound-healing assay showing inhibition of cell migration upon PCAT1 silencing in colon cancer cells. **B:** Transwell chamber assay showing inhibition of cell invasion upon PCAT1 silencing. The experiments were performed in triplicate.

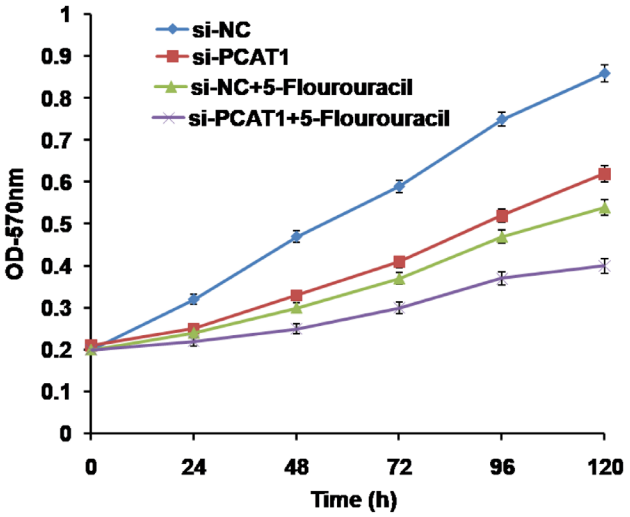

Figure 5. lncRNA-PCAT1 down-regulation leads to decline in the proliferation of colon cancer cells. MTT assay for the determination of proliferation rates of HCT-116 transfected with si-NC or si-PCAT1 and treated with or without 5-flourouracils showing that PCAT1 silencing increases the chemosensitivity of the colon cancer cells. The experiments were performed in triplicate and expressed as mean ± SD.

against the cancer growth and proliferation [14]. The lncRNAs are greater than 200 nucleotides long and have significant regulatory roles. The human cancers are shown to be associated with anomalous expression of lncRNAs and studies have shown that these regulatory molecules are important to cancer growth and development including its spread to neighbouring tissues [15]. In this regard, the molecular role of lncRNA-PCAT1 in maintaining the mechanics of human colon cancer was explored in the present research work. The results showed that the colon cancer is associated with upregulation of lncRNA-PCAT1. Such results were also obtained in previous studies on lncRNA-PCAT1 [16]. Our results further showed that silencing lncRNA-PCAT1 decreased the viability of colon cancer cells, which is in accordance with a previous report [17]. The decline in viability of cancer cells was due to induction of apoptotic cell death mediated by Bax/Bcl-2 pathway. Moreover, the metastatic potential of cancer cells was observed to diminish with the downregulation of lncRNA-PCAT1 [18] and our results also suggest similar effects. The proliferation of colon cancer cells was also seen to be reducing coupled with enhancement of their chemosensitiviy to 5-flourouracil. This observation is in conformity with the previous research work [19-22]. Taken together, the present study explored the regulatory potential of lncRNA-PCAT1 in human colon cancer which can be utilized in combination with the traditional chemotherapeutic anticancer approaches for achieving better results in the fight against this deadly disease.

Conclusion

The present study figured out the role of lncRNA-PCAT1 in the growth and progression of human colon cancer. The results revealed the utility of lncRNAs in general and lncRNA-PCAT1 in particular, as vital tools against colon cancer.

Acknowledgements

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Conflict of interests

The authors declare no conflict of interests.

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