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Original Article

Overexpression of lncRNA AOC4P indicates a poor prognosis for colorectal cancer and regulates cell metastasis by epithelial mesenchymal transition

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Abstract: Background: The long non-coding RNA amine oxidase, copper containing 4, pseudogene (lncRNA AOC4P) has been recognized as a tumor suppressor in hepatocellular carcinoma. However, whether AOC4P is involved in colorectal cancer (CRC) progression remains unclear. Methods: LncRNA AOC4P expression in CRC tissues and cell lines was determined by quantitative real-time PCR (qRT-PCR). Kaplan-Meier analysis was used to explore the prognostic significance of AOC4P in CRC patients. MTT assay and migration assay were used to determine the function of AOC4P on CRC progression. Moreover, epithelial-mesenchymal transition (EMT) related molecular markers were detected by Western blotting. Results: In the current study, we found that lncRNA AOC4P was downregulated in CRC tissues and cell lines, decreased expression of AOC4P was closely correlated with advanced tumor stage and distant metastasis. Kaplan-Meier analysis showed that CRC patients with low AOC4P expression had poor overall survival than patients with high AOC4P expression. Furthermore, in vitro assays revealed that AOC4P overexpression significantly suppressed CRC cells proliferation, migration and invasion in vitro. In addition, we found that overexpression of AOC4P inhibited EMT phenotype in CRC cells. Conclusion: Our data revealed that lncRNA AOC4P plays an important role in the progression of CRC and mediates the EMT in CRC. This finding may provide a therapeutic approach for future treatment of CRC.

Keywords: Colorectal cancer, long non-coding RNA, AOC4P, progression, EMT

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed and lethal malignancy worldwide [1]. Despite the advancement of surgery, radiation and chemotherapy, the 5-year survival rate of CRC patients has not significantly improved during the past decades [2]. It is mainly due to regional recurrence and lymph node metastasis [3]. Therefore, it is critical to identify new biomarkers and therapeutic targets to improve clinical management of CRC.

Long non-coding RNAs (lncRNAs) are commonly considered as a kind of non-coding RNA longer than 200 nucleotides with no protein-coding capability [4]. Accumulation data revealed that lncRNAs play significant roles in a large range of biological processes, including cell development, differentiation, and many other biological processes [5-7]. Aberrant lncRNAs expression play important roles in tumor progression, including CRC. For example, Li et al showed that lncRNA FOXP4-AS1 was an unfavorable prognostic factor and regulated colorectal cancer cell proliferation and apoptosis [8]. Wu et al found that HOTAIR was a powerful predictor of metastasis and poor prognosis and associated with epithelial-mesenchymal transition in colon cancer [9]. Huang et al found that lncRNA CASC2 functioned as a competing endogenous RNA by sponging miR-18a in colorectal cancer [10]. However, the expression and function of lncRNA AOC4P in CRC remain unclear.

In the present study, we explore the expression and function of lncRNA AOC4P in CRC. Our data showed that AOC4P was downregulated in CRC tissues and cell lines, decreased expression of AOC4P was closely correlated with advanced

tumor stage, distant metastasis and poor overall survival of CRC patients. Function assays showed that AOC4P overexpression significantly suppressed CRC cell proliferation, migration and invasion in vitro. In addition, we found that overexpression of AOC4P inhibited EMT phenotype in CRC cells. Thus, these findings suggested that AOC4P might serve as a potential therapeutic target for the treatment of CRC.

Materials and methods

Clinical specimens

The study cohort consisted of 68 patients with CRC who underwent surgical resection at Shandong Provincial Hospital Affiliated to Shandong University between 2011 and 2012. Clinical stage was evaluated on the basis of the International Union Against Cancer (UICC) TNM classification system. Histological differentiation and distant metastasis were classified according to the World Health Organization (WHO) classification criteria. This study was approved by the Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University, and written informed consent was obtained from each patient.

Cell culture and transfection

The human normal intestinal mucous cell line CCC-HIE-2 and four CRC cell lines (SW480, HCT116, HT29 and SW620) were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. All cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) containing 10% fetal bovine serum (FBS; Invitrogen) and maintained in a humidified incubator at 37°C with 5% CO_2.

pCDNA3.1-AOC4P and the pCDNA3.1 plasmid were purchased from Invitrogen. Cells were then transfected with 1 μg of either the pCDNA3.1-AOC4P or the pCDNA3.1 plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

RNA isolation and quantitative real-time PCR

Total RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using a Reverse Transcription Kit (Takara). AOC4P expression levels were measured with qRT-PCR using an ABI7500 system and the SYBR Green PCR Master Mix (Takara). Each assay was performed in triplicate, and relative AOC4P expression was normalized to GAPDH. The fold change of AOC4P in CRC relative was determined by the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation assay

Cell proliferation was assayed using the Cell Counting Kit-8 (CCK-8, Promega) according to the manufacturer's protocol. The transfected cells were plated in 96-well plates (3000 cells/well). Cell proliferation was detected every 24 h according to the manufacturer's protocol. Briefly, 10 μl of CCK-8 solution was added to each well and incubated for 2 h at 37°C. The solution was then measured spectrophotometrically at 450 nm.

Cell migration and invasion assays

Cells were plated in the upper chamber of transwell assay inserts (Millipore) containing 200 μl of serum-free DMEM with a membrane to test migration. The lower chambers were filled with DMEM containing 10% FBS. The cells on the filter surface were fixed with methanol, stained with crystal violet, and photographed with a digital microscope after 24 h. The cell numbers were calculated in five random fields for each chamber. The transfected cells were plated in the top chamber containing a Matrigel-coated membrane (BD Biosciences) in 500 μl serum free DMEM to test cell invasion. There was 750 μl of 10% FBS-DMEM in the bottom chambers. The invasion function was determined after 48 h.

Western blotting

Cells were lysed in RIPA buffer with protease and phosphatase inhibitors (Roche). Equal amounts (30 μg) of the protein were electrophoresed by SDS-PAGE, transferred to NC membranes and incubated with the primary antibodies (Abcam). The primary antibody incubation for 10 h was followed by incubation with a HRP-conjugated secondary antibody for 2 h. The blots were visualized using an ECL kit (ComWin Biotech), and quantified using the Image J Software, normalized to GAPDH.

Statistical analysis

Statistical analyses were performed using SPSS version 18.0. The significance of differ-

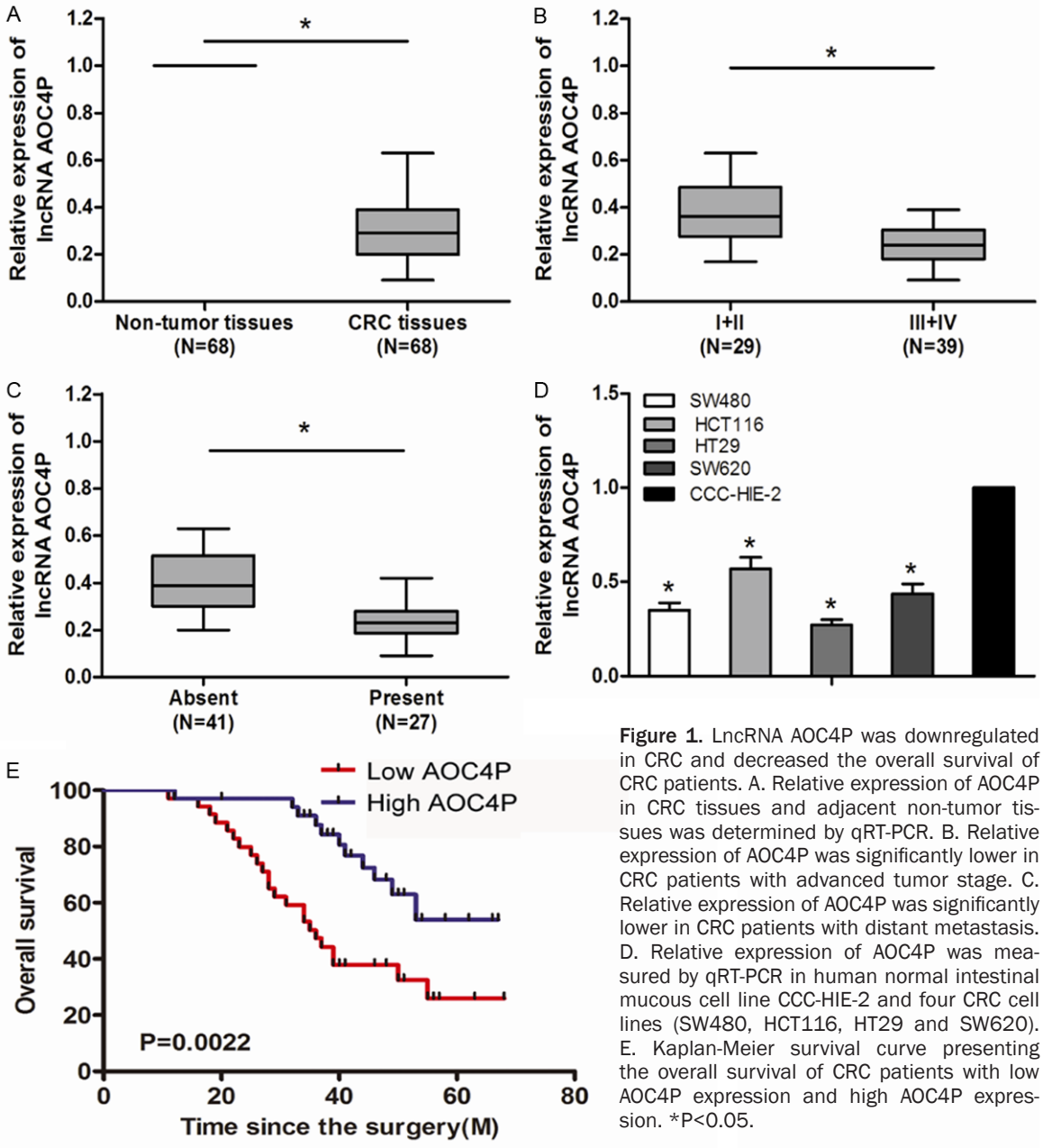

Figure 1. LncRNA AOC4P was downregulated in CRC and decreased the overall survival of CRC patients. A. Relative expression of AOC4P in CRC tissues and adjacent non-tumor tissues was determined by qRT-PCR. B. Relative expression of AOC4P was significantly lower in CRC patients with advanced tumor stage. C. Relative expression of AOC4P was significantly lower in CRC patients with distant metastasis. D. Relative expression of AOC4P was measured by qRT-PCR in human normal intestinal mucous cell line CCC-HIE-2 and four CRC cell lines (SW480, HCT116, HT29 and SW620). E. Kaplan-Meier survival curve presenting the overall survival of CRC patients with low AOC4P expression and high AOC4P expression. *P<0.05.

ences was assessed using Student's t-test or a one-way ANOVA. All data are expressed as the means ± SD. *P*-values of less than 0.05 were considered to be statistically significant.

Results

LncRNA AOC4P is downregulated in CRC

To explore potential biological functions of lncRNA AOC4P in CRC, we detected AOC4P expression by qRT-PCR in CRC tissues and adjacent non-tumor tissues from 68 patients with CRC. Our data showed that AOC4P expression was significantly decreased in CRC tissues compared to adjacent non-tumor tissues (**Figure 1A**, P<0.05). Furthermore, a correlation analysis of AOC4P expression with clinicopathological features showed that AOC4P expression was downregulated in advanced tumor stage (**Figure 1B**, P<0.05) and correlated with distant metastasis (**Figure 1C**, P<0.05). Moreover, we determined AOC4P expression in human normal intestinal mucous cell line CCC-HIE-2 and four CRC cell lines (SW480, HCT116, HT29 and SW620) by qRT-PCR, we found that AOC4P expression was downregulated in all

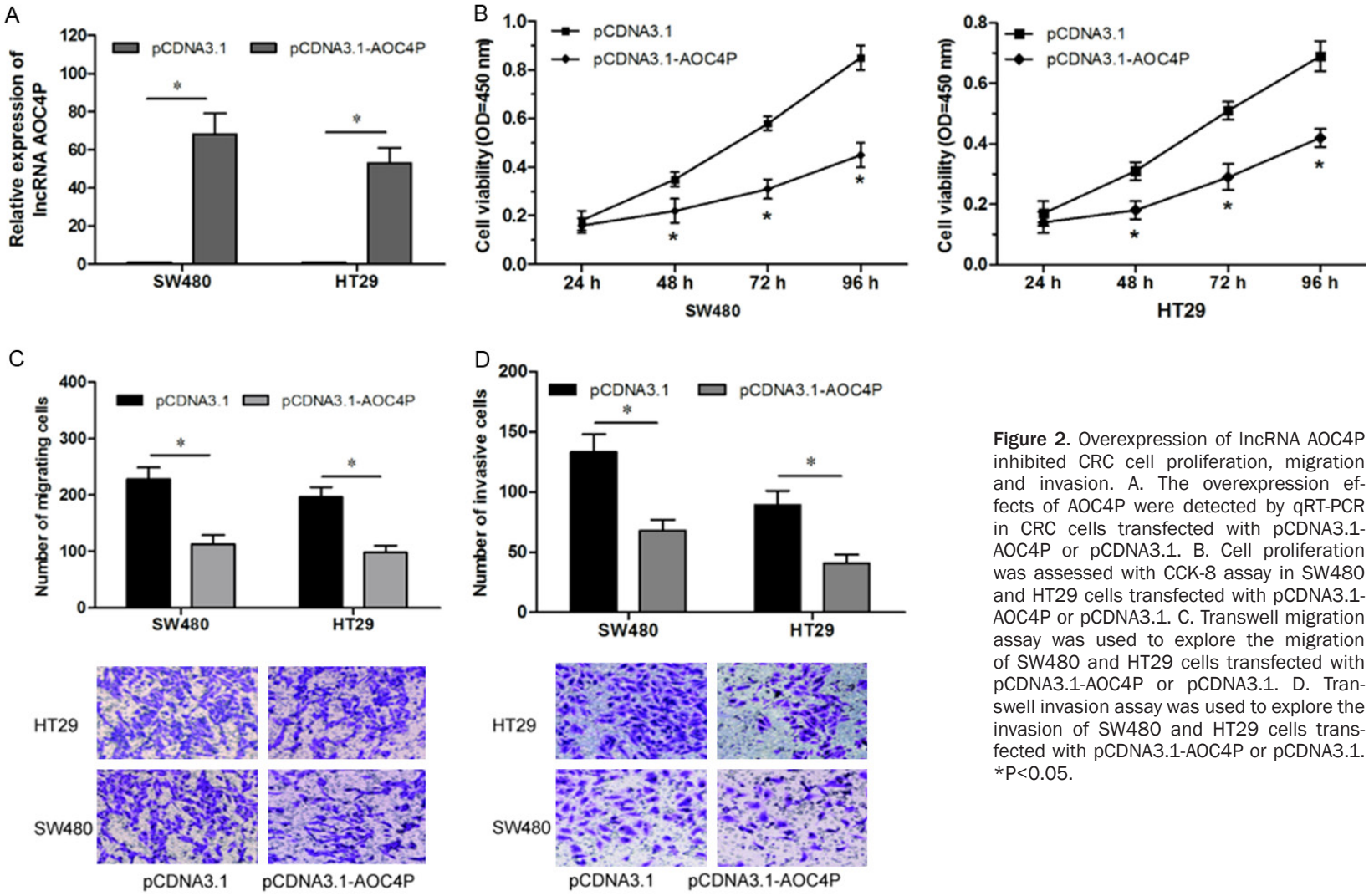

Figure 2. Overexpression of lncRNA AOC4P inhibited CRC cell proliferation, migration and invasion. A. The overexpression effects of AOC4P were detected by qRT-PCR in CRC cells transfected with pCDNA3.1-AOC4P or pCDNA3.1. B. Cell proliferation was assessed with CCK-8 assay in SW480 and HT29 cells transfected with pCDNA3.1-AOC4P or pCDNA3.1. C. Transwell migration assay was used to explore the migration of SW480 and HT29 cells transfected with pCDNA3.1-AOC4P or pCDNA3.1. D. Transwell invasion assay was used to explore the invasion of SW480 and HT29 cells transfected with pCDNA3.1-AOC4P or pCDNA3.1. *P<0.05.

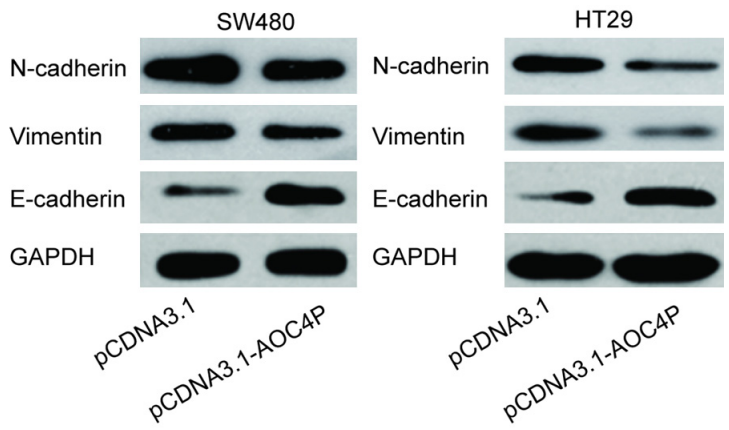

Figure 3. LncRNA AOC4P regulates EMT in CRC cell. Western blotting was used to explore the expression of EMT-related molecular markers (E-cadherin, N-cadherin and Vimentin) in SW480 and HT29 cells transfected with pCDNA3.1-AOC4P or pCDNA3.1.

four CRC cells when normalized to CCC-HIE-2 cells (**Figure 1D**, P<0.05). In addition, the prognosis of AOC4P expression in CRC patients was explored by Kaplan-Meier analysis followed by the log-rank test. Our results showed that patients with low AOC4P expression had a significantly poorer prognosis than those with high AOC4P expression (**Figure 1E**, P<0.05). These results suggested that AOC4P play important roles in CRC progression and development.

LncRNA AOC4P inhibits CRC cell proliferation, migration and invasion

To further explore the roles of AOC4P on regulating CRC cell phenotypes, SW480 and HT29 cells were transfected with pCDNA3.1-AOC4P or pCDNA3.1 plasmid, and the transfection efficiency was detected by qRT-PCR (**Figure 2A**, P<0.05). Forced overexpression of AOC4P significantly suppressed cell proliferation ability of SW480 and HT29 cells (**Figure 2B**, P<0.05). In addition, we also detected the effect of AOC4P on the motility of CRC cells. Transwell migration assay showed that the migration capacities of SW480 and HT29 cells transfected with pCDNA3.1-AOC4P were significantly decreased compared with cells transfected with pCDNA3.1 (**Figure 2C**, P<0.05). The invasion capacities of SW480 and HT29 cells transfected with pCDNA3.1-AOC4P were also significantly decreased compared with cells transfected with pCDNA3.1 (**Figure 2D**, P<0.05). These findings indicated that AOC4P could serve as a tumor suppressor in progression of CRC.

LncRNA AOC4P regulates epithelial-mesenchymal transition in CRC cell

Epithelial-mesenchymal transition (EMT) is the remarkable presentation for cell migration and invasion. To explore whether AOC4P expression was involved in the EMT process, the epithelial marker E-cadherin, and the mesenchymal markers N-cadherin and Vimentin were detected by western blotting. As showed in **Figure 3**, Vimentin and N-cadherin were downregulated after AOC4P overexpression while E-cadherin was increased after AOC4P overexpression in SW480 and HT29 cells. These data suggested that AOC4P contributed to CRC metastasis may be partly via affecting EMT process, and further experiments are needed to elucidate the potential mechanism.

Discussion

Colorectal cancer (CRC) is one of the most common malignant cancers worldwide. Despite of the recent rapid promotion in the diagnosis and therapy, the prognosis of CRC remains poor [11]. The improvement of CRC survival rate requires a clear understanding of pivotal molecular mechanisms from the initiation and progression of CRC. Thus, the exploration of novel diagnostic and therapeutic molecular targets for CRC is particularly crucial.

LncRNAs play critical regulatory roles in diverse cellular processes such as chromatin remodeling, transcription, post-transcriptional processing and intracellular trafficking [12]. Recent studies showed that lncRNAs could act as oncogenes or tumor suppressor genes to affect tumorigenesis [13]. For example, Zhang et al showed that upregulation of lncRNA MALAT1 correlated with tumor progression and poor prognosis in clear cell renal cell carcinoma [14]. Zhao et al showed that lncRNA ANRIL promoted the invasion and metastasis of thyroid cancer cells through TGF-β/Smad signaling pathway [15]. Sun et al found that decreased expression of lncRNA GAS5 indicated a poor prognosis and promoted cell proliferation in gastric cancer

[16]. However, the function of lncRNA in tumor progression is still unclear.

AOC4P (amine oxidase, copper containing 4, pseudogene) was originally detected in hepatocellular carcinoma, they showed that lncRNA AOC4P could act as a tumor suppressor by enhancing Vimentin degradation and suppressing the EMT [17]. However, the expression and function of AOC4P in CRC are still unclear. In the present study, we detected that AOC4P expression was downregulated in CRC tissues and cell lines, decreased expression of AOC4P was associated with advanced tumor stage and distant metastasis of CRC patients. Kaplan-Meier analysis revealed that patients with low AOC4P expression had poor overall survival than patients with high AOC4P expression group. Furthermore, function assay showed that AOC4P overexpression significantly suppressed CRC cell proliferation, migration and invasion in vitro. These findings suggested that AOC4P could act as a tumor suppressor in the progression of CRC.

Epithelial mesenchymal transition (EMT) is a key step toward cancer metastasis, a biological process where epithelial cells lose their polarity and undergo transition into a mesenchymal phenotype [18, 19]. The EMT process plays a critical role in cancer invasion and metastasis [20]. In the present study, we explored the expression of hallmarks of EMT in CRC cells with AOC4P overexpression. Our data revealed that AOC4P overexpression reduced Vimentin and N-cadherin expression and upregulated E-cadherin expression. These findings suggested that AOC4P was a critical regulator in preventing EMT in CRC.

Taken together, our findings suggested that lncRNA AOC4P might function as a tumor suppressor that was involved in CRC carcinogenesis and could be a potential therapeutic target for the treatment of CRC.

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Disclosure of conflict of interest

None.

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