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Long non-coding RNA CCAT1 promotes metastasis and poor prognosis in epithelial ovarian cancer

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ABSTRACT

In this study, we reported that long non-coding RNA (lncRNA) CCAT1 was upregulated in epithelial ovarian cancer (EOC) tissues, and was associated with FIGO stage, histological grade, lymph node metastasis and poor survival of EOC patients. Multivariate Cox regression analysis showed that CCAT1 was an independent prognostic indicator. While CCAT1 downregulation inhibited EOC cell epithelial-mesenchymal transition (EMT), migration and invasion, CCAT1 upregulation promoted EOC cell EMT, migration and invasion. We further identified and confirmed that miR-152 and miR-130b were the targets of CCAT1, and CCAT1 functioned by targeting miR-152 and miR-130b. Subsequently, ADAM17 and WNT1, and STAT3 and ZEB1 were confirmed to be the targets of miR-152 and miR-130b, respectively, and could be regulated by CCAT1 in EOC cells. Knockdown of anyone of these four proteins inhibited EOC cell EMT, migration and invasion. Taken together, our study first revealed a critical role of CCAT1-miR-152/miR-130b-ADAM17/WNT1/STAT3/ZEB1 regulatory network in EOC cell metastasis. These findings provide great insights into EOC initiation and progression, and novel potential therapeutic targets and biomarkers for diagnosis and prognosis for EOC.

1. Introduction

Epithelial ovarian cancer (EOC), which accounts for ~ 90% of all ovarian cancers, is the deadliest gynecological cancer and the fifth-leading cause of cancer-related death in women worldwide [1,2]. It is estimated that each year over 240,000 women are diagnosed with EOC, resulting in at least 100,000 deaths per year in the world [3]. EOC is characterized by late clinical manifestation, subtle symptomatology, and fast disease progression. As a consequence, up to 75% of patients with EOC have already developed metastases at first diagnosis. Despite the great advances achieved in surgery and chemotherapy over the last few decades, the prognosis of EOC patients remains poor, with a 5-year survival rate of only 30% [4]. Tumor metastasis and recurrence are the major causes of the poor prognosis of EOC patients. Therefore, there exists a crucial need for understanding of the molecular mechanisms involved in EOC metastasis, and identification of reliable markers that can predict the clinical results of EOC patients. There is an increasing body of evidence to suggest that epithelial-mesenchymal transition (EMT) is one of the major molecular mechanisms inducing cancer metastasis [5–9]. EMT occurs at the initial stage of cancer metastasis, and involves a disassembly of cell-cell junctions, which makes cells lose cell-cell adhesion, and gain migratory and invasive traits, resulting in tumor aggressiveness.

Long non-coding RNAs (lncRNAs) with more than 200 nucleotides in length, initially considered as the transcriptional noise, are emerging as crucial regulators in various physiologic and pathophysiologic processes, including cancer. For example in ovarian cancer, lncRNA LSINCT5 is over expressed and affects cellular proliferation [10]. LncRNA HOST2 regulates cell biological behaviors through a mechanism involving microRNA let-7b [11]. LncRNA HOTAIR, AB073614 and ANRIL can predict poor prognosis and promote tumor metastasis [12–14]. Recently, lncRNA CCAT1 is widely reported to be increased in many cancers and involved in various cellular processes related to carcinogenesis. For example, CCAT1 promotes gallbladder cancer development via negative modulation of miRNA-218-5p; promotes hepatocellular carcinoma progression by functioning as let-7 sponge; promotes cell proliferation and invasion in pancreatic cancer, laryngeal squamous cell carcinoma and colon cancer; and is a novel biomarker of poor prognosis in patients with breast cancer, cholangiocarcinoma and oral squamous cell carcinoma [15–22]. One recent study has described that in EOC, CCAT1 expression is increased in the metastatic cell line SKOV3.ip1 compared with its parental cell line SKOV3 [23]. However, there are few reports about the clinical significance and biological function of CCAT1 in EOC.

In this study, we determined the expression of CCAT1 in 72 paired EOC tissues and adjacent normal tissues, and found that CCAT1 was

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upregulated in EOC tissues. We further analyzed the clinical significance and found that CCAT1 was correlated with FIGO stage, histological grade, lymph node metastasis and shorter survival, and was an independent prognostic factor. Via loss- and gain-of-function experiments, we demonstrated that CCAT1 promoted EOC cell EMT, migration and invasion. We further investigated the molecular mechanism, and found that CCAT1 promoted EOC cell metastasis by interacting with miR-152 and miR-130b, and upregulating ADAM17, WNT1, STAT3 and ZEB1 expression.

2. Materials and methods

2.1. Human samples

A total of 72 paired EOC tissue and corresponding adjacent non-tumor tissue specimens were collected from patients who underwent surgery at the First Affiliated Hospital of Zhengzhou University between 2007 and 2011. All diagnoses of EOC were confirmed by histology. None of EOC cases received radiotherapy, chemotherapy, or hormonal therapy before operation. These tissue specimens were frozen immediately in liquid nitrogen and stored at − 80 °C until use. This study was approved by the First Affiliated Hospital ethics committee, and all patients provided informed consent before participation of this study. Overall survival (OS) was defined as the interval between primary surgery and death or the last follow-up visit.

2.2. Cell lines and cell culture

Normal human ovarian surface epithelium (HOSE) cells were obtained from Pricells (Wuhan, China). The human EOC cell lines HO8910 and HO8910PM were purchased from American Type Culture Collections (ATCC, Rockville, Md, USA), and OVCAR3, SKOV3 and Caov3 were obtained from Chinese Type Culture Collection (CTCC, Shanghai, China). These cells were cultured under the conditions as suggested by the manufacturers.

2.3. Quantitative real-time PCR (qRT-PCR)

Total RNA samples were extracted by using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and was reverse transcribed to cDNA by using the Prime-Script™ one step RT-PCR kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). qRT-PCR analyses for CCAT1, ADAM17, WNT1, STAT3, and ZEB1 were performed using the SYBR Green qPCR (Toyobo, Osaka, Japan) according to the manufacturer's protocol, and GAPDH level was measured as an internal control. miR-152 and miR-130b expression were quantified by TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA), and U6 was applied as an internal control.

2.4. Cell transfection

The siRNA targeting CCAT1, ADAM17, WNT1, STAT3 and ZEB1 and the plasmid of pCDNA3.1-CCAT1 were purchased from AuraGene (Changsha, China). The mimics for miR-152 and miR-130b, and their respective controls were obtained from Fulen Gen (Guangzhou, China). Cells were transfected with siRNAs, plasmids or mimics by Lipofectamine 2000 reagent (Invitrogen). At 48 h post-transfection, cells were collected.

2.5. Western blot assay

Western blot was performed as described previously [24]. Briefly, total proteins were extracted using RIPA lysis buffer (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The lysates were separated via 10% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (PVDF, Millipore, Bedford, MA, USA). The membranes were incubated with primary antibodies against E-cadherin, vimentin, N-cadherin, CSF1, ADAM17, KLF4, WNT1, ERBB3, STAT3, IGF1, ZEB1 or β-actin, followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody. The blots were detected by using an ECL kit (Pierce, Rockford, IL, USA).

2.6. Migration assay

Cells with 1×10^5 cells/well were seeded into 12-well plates, and were cultured at 37 °C until cell confluence reached approximately 90%. A scratch was created by using a 10-μl pipette tip. The cells were rinsed with PBS, cultured in fetal bovine serum (FBS; Invitrogen)-free medium, and then photographed at 0 and 48 h with an inverted microscope (Motic, Xiamen, China). Migration distance was defined as the difference between the gap of 0 and 48 h.

2.7. Invasion assay

Invasion assay was performed using Transwell chambers with 8.0-μm pore membranes (Millipore). Cells (1×10^5 cells) were resuspended in 200 μl of FBS-free medium, and seeded into the top chamber with Matrigel-coated membrane. In addition, 500 μl medium with 10% FBS was added into the bottom chamber as a chemo-attractant. After 48 h of incubation, invaded cells were fixed, stained with 0.1% crystal violet, and counted via an inverted microscope (Motic).

2.8. Luciferase reporter assay

HO8910PM cells were co-transfected with miRNA mimics or mimic control and pMIR-report luciferase vector containing the gene fragments with wild-type (WT) or mutant (MUT) binding sites of miR-152 or miR-130b by Lipofectamine 2000. Two days after transfection, cells were collected and luciferase activity was determined by dual luciferase reporter gene assay kit (BioVision, Milpitas, CA, USA).

2.9. Statistical analysis

All experiments were repeated at least three times. Data were presented as the means ± standard deviation (SD). The comparisons between groups were performed via Student's two-tailed *t*-test, Fisher's exact tests, chi-square test or one-way variance analysis (OVA) by using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Kaplan-Meier method and log-rank test were performed for patients' survival analyses. Multiple Cox proportional hazards regression was performed to identify the independent factors with a major impact on the survival of the patients. Values of $P < 0.05$ were considered significant.

3. Results

3.1. CCAT1 is upregulated in EOC and associated with the prognosis of EOC patients

The expression level of CCAT1 was determined by qRT-PCR in 72 EOC patients. The results showed that CCAT1 expression was significantly up-regulated in 90.3% (65/72) EOC tissues compared with adjacent non-tumor tissues (Fig. 1A and B). For the clinicopathological correlation analysis, 72 EOC patients were divided into two groups: high CCAT1 group (n = 36) and low CCAT1 group (n = 36) by adopting the median of CCAT1 expression in EOC tissues as a cut-off value. As shown in Table 1, high CCAT1 expression was closely associated with FIGO stage, histological grade and lymph node metastasis, but no association was identified between CCAT1 expression and age, histological type or the occurrence of ascites (Table 1). Furthermore, Kaplan-Meier and log-rank test analyses showed that EOC patients with high CCAT1 expression had shorter OS than those with low CCAT1 expression (Fig. 1C). Multivariate Cox regression analysis

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