Long non-coding RNA CCAT2 regulates proliferation, drug sensitivity and metastasis of ovarian cancer

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Abstract

Introduction: Reports suggest that lncRNAs have implications in the development of several diseases including cancer. It is therefore believed that lncRNAs may act as therapeutic targets for cancer treatment. The treatment of ovarian cancer is mainly obstructed by lack of biomarkers and efficient drug targets. Against this backdrop, this study was undertaken to reveal the role and therapeutic implications of lncRNA CCAT2 in ovarian cancer.

Material and methods: Expression analysis was carried out with quantitative real-time polymerase chain reaction (qRT-PCR). Transfections were carried out using Lipofectamine 2000 reagent. Cell counting kit 8 (CCK-8) assays were used to determine the cell viability. AO/EB and annexin V/propidium iodide (PI) were used for detection of apoptosis. Wound healing and transwell assays were used to determine cell migration and invasion. The expression of the proteins was estimated by western blotting.

Results: The results showed that the expression of CCAT2 was significantly overexpressed in ovarian cancer tissues and cell lines. Si-RNA mediated silencing of CCAT2 resulted in the decrease of proliferation rate and colony formation potential of the OVACAR-3 cells via induction of apoptotic cell death, which was also accompanied by cleavage of PARP, downregulation of Bcl-2 and upregulation of Bax, caspase-3 and caspase-9. Suppression of CCAT2 enhanced the chemosensitivity of the OVACAR-3 cells to cisplatin and also decreased their migration and invasion.

Conclusions: The findings of this study revealed that lncRNA CCAT2 suppression inhibits the proliferation, drug sensitivity, and metastasis of ovarian cancer and may prove beneficial in ovarian cancer management.

Key words: ovarian cancer, long non-coding RNAs, apoptosis, metastasis.
ent diseases conditions such as cancer, lncRNAs have been shown to exhibit aberrant expression [4]. LncRNA CCAT2 has been shown to be dysregulated in cancerous tissues and has been shown to play vital roles in cancer-linked processes [5]. In gastric cancer, lncRNA CCAT2 has been shown to be overexpressed and has been shown to be associated with poor prognosis [6]. In lung adenocarcinomas, CCAT2 has been shown to control the invasion of the cancer cells [7]. The poor prognosis of esophageal carcinoma is also linked with upregulation of lncRNA CCAT2 [8]. Herein, we examined the expression and role of lncRNA CCAT2 in human ovarian cancer cells. Ovarian cancer is one of the common gynecological malignancies in women across the globe and accounts for 2.5% of all malignancies in women. It is responsible for 5% of all cancer-related deaths in women [9]. Although the incidence of ovarian cancer has declined over the last few decades, the clinical outcome is still far from decent [10]. It has been reported that in the United States alone more than 22,000 new ovarian cancer cases and 14,000 ovarian cancer deaths are recorded annually [11]. The late diagnosis and dearth of therapeutic targets impose hurdles in the treatment of ovarian cancer. Improvement of prevention through early detection and identification of the therapeutic targets may prove beneficial to curb ovarian cancer related mortality [12].

Herein we report that the lncRNA CCAT2 is significantly overexpressed in human ovarian cancer cells. Silencing of CCAT2 in OVACAR-3 cells results in the inhibition of cell proliferation and metastasis via blocking of the wnt/β-catenin signaling pathway. Overall, lncRNA CCAT2 may prove beneficial in the management of ovarian cancer and warrant further investigations.

Material and methods

Tissue samples and cell lines

Human ovarian cell lines (PA-1, SK-OV-3, Caov-3 and SW626) and a non-cancerous cell line (SV40) were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) containing penicillin (100 U/ml), streptomycin (100 U/ml) (Sigma-Aldrich, St. Louis, MO, USA), and 10% fetal bovine serum (FBS; Gibco) at 37°C in 5% CO₂.

Expression analysis

The RNA was extracted from the ovarian cancer cell lines using TRizol reagent and then subsequently purified by RNeasy Mini Kit (Qiagen). The miScript Reverse Transcription Kit (Qiagen) was then used to synthesize cDNA from the total RNA. Thereafter, cDNA was amplified using SYBR Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan). The cycling conditions were as follows: 94°C for 25 s, followed by 38 cycles of 95°C for 20 s, and 57°C for 30 s. The expression was estimated by the 2−ΔΔCt method and actin was used as an internal control.

Cell transfection

The si-CCAT2 and NC were synthesized by Ribobio (Guangzhou, China). The transfection was then carried out using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer’s instructions. As the OVACAR-3 cells reached 80%, the appropriate concentrations of si-CCAT2 or NC was transfected into OVACAR-3 cells.

Cell viability assay

For the determination of cell viability, the transfected OVACAR-3 cells were subjected to culturing in 96 well plates at 37°C for 24 h. The cells were then treated with CCK8 reagent and again incubated at 37°C. After 2 h, the OD was taken at different time intervals using a microplate reader. The colony formation of the OVACAR-3 cells was assessed as described previously [13].

Apoptosis assay

The transfected OVACAR-3 cells were cultured for 24 h at 37°C and then fixed with ethanol (70%) for 20 min. The cells were then subjected to PBS washing and subsequently stained with a solution of AO/EB. Finally, the cells were examined under a microscope to detect the induction of apoptosis. For determination of the percentage of apoptosis, the transfected cells were stained with annexin V/PI and subsequently examined by a flow cytometer.

Transwell assay

Transwell chambers with Matrigel were employed for the determination of cell invasion. The OVACAR-3 cells were transfected si-CCAT2 or NC and cultured for 24 h at 37°C. Afterwards, on the upper chamber around 250 ml of the cell culture suspension was added while empty fresh media were placed in the lower chamber to act as a chemotactrant. After around 24 h, the cells on the upper chamber were removed by swabbing while the cells that moved to the lower chamber were fixed and subsequently stained with crystal violet. Cell invasion was determined using an inverted microscope.

Wound-healing assay

The OVACAR-3 cells were transfected with si-NC or si-CCAT2 and cultured for 48 h at 37°C. This was
followed by removal of the media and subsequent washing of the cells with PBS. A scratch was made with a pipette tip and a photograph was taken. The plates were then kept at 37°C for 24 h and a photograph was taken again under a microscope.

**Western blotting**

The OVACAR-3 cells were transfected with si-CCAT2 or NC and cultured for 24 h at 37°C. Next the cultures were transferred to 50 ml centrifuge tubes and centrifuged at 12 000 rpm for 10 min. The pellets were washed with PBS and then resuspended in lysis buffer. BCA assay was used to determine the concentration of the proteins in each sample. Equal concentrations of the proteins from each sample were loaded and separated on the SDS-PAGE and then transferred to polyvinylidene fluoride membrane. These membranes were then treated with TBS and then exposed to primary antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C for 24 h. Afterwards, the cells were treated with HRP-conjugated anti-rabbit secondary antibody and subsequently chemiluminescence reagent was used for visualization of the proteins.

**Statistical analysis**

Data are shown as mean ± SD. Statistical analysis was done using Student’s t-test with GraphPad prism 7 software. Values of p < 0.05 were taken as indicating a significant difference.

**Results**

CCAT2 is upregulated in ovarian cancer tissues and cell lines

The expression of CCAT2 was examined in four different ovarian cancer cells lines and one...
normal SV40 cell line by qRT-PCR analysis. It was found that expression of CCAT2 was significantly upregulated in all the ovarian cancer cell lines with fold upregulation ranging between 3.2 to 4.5 relative to normal cell lines (Figure 1 A). It was further found that the OVACAR-3 cell line showed the highest expression of CCAT2 and was therefore selected for further investigation.

**Suppression of CCAT2 inhibits the proliferation of OVACAR-3 cells via apoptosis induction**

Next to ascertain the CCAT2 function in ovarian cancer, its expression was suppressed in the OVACAR-3 cells (Figure 1 B). The results revealed that inhibition of CCAT2 expression resulted in a reduction of the proliferation rate of the OVACAR-3 cells and also inhibited their colony formation potential (Figures 1 C, D). AO/EB staining analysis revealed that suppression of the CCAT2 expression caused the induction of apoptosis in the OVACAR-3 cells (Figure 2 A). Annexin V/PI staining also showed that the percentage of the apoptotic OVACAR-3 cells increased significantly upon CCAT2 silencing, which was also accompanied by cleavage of PARP downregulation of Bcl-2 and upregulation of Bax, caspase-3 and caspase-9 (Figures 2 B, C).

**Silencing of CCAT2 enhances drug sensitivity of OVACAR-3 cells**

The effects of CCAT2 inhibition on the chemosensitivity of the OVACAR-3 ovarian cancer cells were evaluated. The CCAT2 transfected OVACAR-3 cells were treated with 2 µM concentration of cisplatin and the cell viability was monitored at different time intervals by CCK-8 assay. The results showed that suppression of CCAT2 caused enhancement of the chemosensitivity of OVACAR-3 cells to cisplatin (Figure 3).

**Silencing of CCAT2 inhibits migration and invasion of OVACAR-3 cells**

Next the effects of CCAT2 were examined on the migration and invasion of the OVACAR-3 cells by wound healing and transwell assays respectively. The results showed that CCAT2 suppression significantly reduced the migration and invasion of the ovarian cancer cells (Figures 4 and 5).

**Silencing of CCAT2 inhibits the wnt/β-catenin signaling pathway**

The effects of CCAT2 silencing were also examined on the wnt/β-catenin signaling pathway in the OVACAR-3 cancer cells by western blot analysis. The results showed that CCAT2 silencing inhibited the expression of Aixin 1, β-catenin, c-myc, cyclin D1 and E-cadherin, indicating that CCAT2 silencing results in blocking the wnt/β-catenin signaling pathway in OVACAR-3 cells (Figure 6).

**Discussion**

The treatment of ovarian cancer is mainly obstructed by a dearth of therapeutic targets and biomarkers for early detection [14]. It has been reported that IncRNAs modulate the expression of many human genes that are involved in a wide ar-
Long non-coding RNA CCAT2 regulates proliferation, drug sensitivity and metastasis of ovarian cancer

Figure 3. LncRNA CCAT2 enhances chemosensitivity of OVACAR-3 cells to the anticancer drug cisplatin. The experiments were performed in three replicates and expressed as mean ± SD (*p < 0.05)

Figure 4. Wound heal assay showing cell migration in the NC and si-CCAT2 transfected OVACAR-3 ovarian cancer cells. The experiments were performed in triplicate

Figure 5. Transwell assay showing cell invasion in the NC and si-CCAT2 transfected OVACAR-3 ovarian cancer cells. The experiments were performed in triplicate

Figure 6. Western blots showing the effect of NC or si-CCAT2 transfection in OVACAR-3 cells on the expression of Aixin 1, β-catenin, c-myc, cyclin D1, E-cadherin and β-actin. The experiments were performed in triplicate

Ray of biological processes [3]. Because of the implications of IncRNAs for cellular and physiological processes, previous studies have revealed the potential of IncRNAs as therapeutic targets [4]. In this study the therapeutic implications of IncRNA CCAT2 were examined in the human ovarian cancer cells. The expression of CCAT2 was found to be significantly and aberrantly elevated in human ovarian cancer cells. These observations are also in agreement with the previous research findings wherein CCAT2 expression has been found to be elevated in cancerous tissues; for example, the expression of CCAT2 was reported to be overexpressed in esophageal carcinoma [8]. Similarly, CCAT2 was reported to be elevated in non-melanoma skin cancer [15]. In yet another study, the CCAT2 expression was shown to be upregulated in uterine cervical cancer [16]. Studies have also shown CCAT2 to be upregulated in ovarian cancer tissues, further confirming our findings [17]. To investigate the role of CCAT2 in ovarian cancer, its expression was suppressed in
OVACAR-3 ovarian cancer cells. It was found that Si-RNA mediated silencing of CCAT2 inhibited the proliferation and the colony formation ability of OVACAR-3 cells. A previously carried out study also showed that CCAT2 regulates proliferation of osteosarcoma cells [18]. AO/EB staining showed that CCAT2 silencing caused apoptosis in OVACAR-3 cells. Annexin V/PI staining showed that CCAT2 inhibition increased the percentage of apoptotic OVACAR-3 cells. The induction of apoptosis was also accompanied by cleavage of PARR downregulation of Bcl-2 and upregulation of Bax, caspase-3 and caspase-9. A previously carried out study also showed that inhibition of CCAT2 expression triggers apoptosis in gastric cancer cells [13]. The effects of CCAT2 silencing on the in vitro metastasis of OVACAR-3 cells were also examined by transwell and wound heal assay. The results showed that CCAT2 inhibition suppressed both migration and invasion of OVACAR-3 cancer cells. These results confirm a previous investigation wherein CCAT2 was shown to regulate the migration and invasion of cholangiocarcinoma cells [19]. In another study IncRNA was reported to exert its effects via regulation of the wnt/β-catenin signaling pathway in epithelial ovarian cancer cells [20]. The results showed that CCAT2 suppression also inhibits the wnt/β-catenin pathway in OVACAR-3 cells. Although the present study explored the therapeutic potential of CCAT2 in ovarian cancer, a pharmaceutical tool that may be used to modulate the expression of CCAT2 needs to be developed.

In conclusion, the findings of the present study show that CCAT2 is significantly upregulated in human ovarian cancer cells. Si-RNA mediated inhibition of CCAT2 in ovarian cancer cells inhibits proliferation of ovarian cancer cells via induction of apoptosis. CCAT2 suppression also causes inhibition of in vitro metastasis of ovarian cancer cells, suggesting the therapeutic potential of CCAT2 cells in ovarian cancer.

Conflict of interest
The authors declare no conflict of interest.

References