Knockdown of Long Non-Coding RNA (lncRNA) Colon Cancer-Associated Transcript-1 (CCAT1) Suppresses Oral Squamous Cell Carcinoma Proliferation, Invasion, and Migration by Inhibiting the Discoidin Domain Receptor 2 (DDR2)/ERK/AKT Axis

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Background: Emerging evidence shows that lncRNAs are involved in carcinogenesis or suppression in diverse cancers. This study assessed the biological role of lncRNA CCAT1 in OSCC and explored the underlying molecule mechanism.

Material/Methods: CCAT1 and DDR2 expression was measured by qRT-PCR. Colony formation assay and CCK-8 assay were performed to evaluate cell proliferation. Cell cycle was determined by flow cytometric analysis and Western blot analysis. In addition, wound healing and Transwell assay were used to assess cell migration and invasion, respectively. RNA immunoprecipitation (RIP) assay were employed to identify the interaction between DDR2 and CCAT1. Protein levels involved in DDR2/ERK/AKT pathway were estimated by Western blot assay.

Results: The findings revealed that CCAT1 expression was upregulated in OSCC cell lines. Knockdown of CCAT1 repressed cell proliferation, blocked the cell cycle, and suppressed the invasion and migration of TCA-8113 cells. Moreover, DDR2 expression in OSCC cell lines was downregulated and CCAT1 silencing repressed the expression of DDR2. RIP assays validated the binding of CCAT1 and DDR2 protein. Moreover, CCAT1 silencing suppressed the ERK/AKT signaling through DDR2 in TCA-8113 cells.

Conclusions: Downregulation of CCAT1 suppressed TCA-8113 cell proliferation, invasion, and migration by inactivation of the ERK/AKT pathway via inhibition of DDR2, suggesting the value of CCAT1 in diagnosis and treatment of patients with OSCC.

MeSH Keywords: MAP Kinase Signaling System • Mouth Neoplasms • RNA, Long Noncoding
Background

Oral squamous cell carcinoma (OSCC) is a subset of head and neck squamous cell carcinoma (HNSCC), characterized by high lymph node metastasis and invasiveness [1,2]. Due to the high percentage of patients at advanced stages and the characteristic metastasis, OSCC usually has a poor prognosis, with a 5-year survival rate of less than 50% [3]. Although advances have been made in surgery, radiotherapy, chemotherapy, and combined treatments, difficulties in early diagnoses still limit the effectiveness of these therapies in patients with OSCC [4]. Therefore, it is urgent to better understand pathogenesis of OSCC and to develop more biomarkers for early diagnosis and prognostic prediction of OSCC.

Long noncoding RNAs (lncRNAs) are transcripts that do not contain a functional open reading frame (ORF), but play extensive roles in many biological processes, including gene expression, epigenetic regulation, and post-transcription regulation [5,6]. Moreover, lncRNAs exert functional roles in progression of various cancers as tumor suppressors or oncogenic factors [7,8]. Colon cancer-associated transcript-1 (CCAT1) was found to be dysregulated in colon cancer [9]. Previous studies demonstrated that CCAT1 is abnormally expressed and acts as a tumor promotor in several cancers, including gallbladder cancer [10], esophageal carcinoma [11], and gastric cancer [12]. However, whether lncRNA CCAT1 serves as a tumor promotor in OSCC is still unclear.

Discoidin domain receptor (DDR) 2, a receptor tyrosine kinase, can be activated by binding with collagen, followed by activation of a series of intracellular pathways, including p38, JNK, ERK1/2, Notch-1, and NF-κB [13, 14]. DDR2 is associated with several cellular responses during cell proliferation, differentiation, metastasis, and ECM mediated by matrix metalloproteinase (MMP) [15-17]. Additionally, it is well established that DDR2 functions as a regulatory factor in many human cancers, such as lung cancer [18], hepatocellular carcinoma [19], and breast carcinoma [20]. DDR2 has also been showed to be closely connected with cell invasion and metastasis in HNSCC [21]. Bioinformatics analysis revealed that CCAT1 and DDR2 might have the binding sites. In view of this, we speculated that CCAT1 can interact with DDR2. In the present study, we assessed CCAT1 expression in OSCC cell lines and the effects of CCAT1 silencing on OSCC cell proliferation, invasion, and migration. Additionally, our mechanistic investigation of the regulatory effects of CCAT1 revealed the molecular targets and signaling repress proliferation and metastasis of OSCC.

Material and Methods

Cell culture

OSCC cell lines CAL-27, SCC-4, SCC-9, and SCC-15 were obtained from the American Tissue Culture Collection (ATCC, USA). The OSCC cell line TCA-8113 and oral epithelial cell line HIOE were obtained from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C.

Cell transfection

Vectors used in the study including shRNA-CCAT1-1/2, pcDNA-DDR2, shRNA-NC, and pcDNA-NC were constructed by GeneChem (Shanghai, China). TCA-8113 cells were cultured in six-well plates for 24 h and transfected with the vectors respectively with Lipofectamine 2000 according to manufacturer’s protocol. The cells were then harvested at 48 h after transfection for further experiments. The sequences of shRNA-NC-CCAT1, pcDNA-DDR2 and negative controls were summarized in Supplementary Table 1.

Quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted from cultured cells by TRIzol reagent (Life technology, USA). The purity of total RNA was assessed with A NanoDrop 3000 spectrophotometer (Thermo Scientific, Waltham, MA) at the wavelength of 260 and 280 nm. Then, the RNAs were reversely transcribed into cDNA using PrimeScript RT Master Mix (Takara, Japan) and PCR amplification was performed with the SYBR Premix ExTaq kit (Takara, Japan) in an ABI PRISM 7900 Real-Time system (Applied Biosystems, USA). The PCR amplification program was as follow: 94°C for 60 sec, followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 60 sec. Primer sequences were as follows: CCAT1, forward primer: 5’-TCACTGACAACATCGACTTTGAAG-3’, and reverse primer: 5’-GGAGAAAGGGGCTTACACATAGCTG-A-3’; DDR2, forward primer: 5’-GTCCAGGCGACGACACATCAT-3’, and reverse primer: 5’-GGAGCACGAGGCTGAGG-3’; GAPDH, forward primer: 5’-GGAGAAGGGCTGAGGCTGAGG-3’; and reverse primer: 5’-GGAGCACGAGGCTGAGGCTGAGG-3’. All expression data were normalized to the expression of GAPDH.

Cell counting kit-8 assay

Cell proliferation was evaluated by Cell Counting Kit-8 (CCK-8) assay. Briefly, after transfection for 48 h, TCA-8113 cells were seeded in a 96-well plate (2×10³/well) and cultured in DMEM
for 24, 48 and 72 h. CCK-8 reagent was added into each well and incubated the cells for 3 hours. Following that, the absorbance of each well was measured with a microplate reader at a wavelength of 450 nm. Each group was replicated for 6 times.

**Colony formation assay**

Transfected cells were seeded into six-well plates at a density of 4×10⁴/well and cultured in regular culture medium at 5% CO₂ and 37°C for 2 weeks. After washing trice with PBS, the cells were fixed with 4% paraformaldehyde for 15 min, followed by staining with 0.1% crystal violet for 15 min. The colony number was then observed and counted (>50 cells/culture) with a light microscope (Olympus Corp., Tokyo, Japan). The assays were independently repeated 3 times.

**Flow cytometric analysis**

Flow cytometric analysis was carried out to detect the effect of CCAT1 silence on the cell cycle distribution. TCA-8113 cells were harvested by trypsinization at 48 h after transfection. After centrifuged for 5 min at 300 g, cells were stained with the Cell Cycle Analysis Kit (GenScript, USA). The data were analyzed with a FACScan flow cytometer (BD Bioscience, USA) and BD CellQuest software (BD Bioscience, USA).

**Western blot analysis**

After 48 h transfection, cells were collected and lysed with RIPA lysis buffer. The protein concentration was measured by a Bradford reagent (Bio-Rad). Next, equal amounts of cell lysates were subjected to 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane, followed by the blockage of 5% of non-fat dried milk for 2 h. The membrane was incubated with primary antibodies anti-CDK2, anti-cyclinD1, anti-P27, anti-MMP2, anti-MMP9, anti-DDR2, anti-p-ERK, anti-p-AKT, anti-ERK, anti-AKT and anti-GAPDH (Abcam) at 4°C overnight and incubated with horseradish peroxidase-conjugated secondary antibodies at 37°C for 2 h. The protein-antibody complexes were detected and visualized by ECL Detection System (Life technologies, Gaithersburg, MD, USA). GAPDH served as an internal control.

**Wound healing scratch assay**

Cell migration was evaluated by wound healing scratch assay. Transfected cells were seeded into six-well plates (4×10⁴/well) and cultured until the confluence. The cell monolayer was scratched by a white pipette tips and washed by PBS 3 times for removing the cell debris. After incubation for 24 h, migrated cells onto the wound surface were counted under an inverted microscopy. Five fields were chosen randomly to analyze in each well.

**Cell invasion assay**

Cell invasive capacity was assessed by using the Transwell chamber (Corning Costar, Cambridge, MA). Transfected cells were harvested and suspended in serum-free DMEM. Then, the cell suspensions were placed into the upper chamber and to the lower chamber we added medium containing 10% FBS. After 24 h, cells on the upper surface were removed with a cotton swab, while the invasive cells in the lower chamber were fixed with 4% paraformaldehyde and stained with 1% crystal violet. The stained cells were counted in 5 randomly selected visual fields per well under a light microscope (Nikon, Japan).

**RNA immunoprecipitation (RIP) assay**

A Magna RIP Kit (Millipore, USA) was used to confirm the interaction between DDR2 and CCAT1 according to the manufacturer’s instructions. TCA-8113 cells were fixed with formaldehyde, lysed by RIP lysis buffer, and then incubated with magnetic beads conjugated with human anti-Ago2 antibody or normal mouse IgG control (Millipore, USA). Subsequently, the precipitated RNAs were extracted and analyzed by qRT-PCR.

**Statistical analysis**

IBM SPSS Statistics18.0 and GraphPad Prism 6 were used to perform the data analysis. The data represent the mean±SD. Differences between 2 groups were compared using the t test. One-way analysis of variance (ANOVA) was performed for multiple comparisons. A value of P<0.05 was considered to be statistically significant.

**Results**

**CCAT1 expression was highly expressed in OSCC cell lines**

To identify whether CCAT1 might be a promising gene for diagnosis and treatment of OSCC, we detected the mRNA expression of CCAT1 in several OSCC cell lines by qRT-PCR. As shown in Figure 1, CCAT1 expression was dramatically elevated in OSCC cells when compared with normal oral epithelial cells.

**CCAT1 silencing suppressed cell proliferation of TCA-8113 cells**

To study the role of CCAT1 in OSCC cells, we transfected interfering CCAT1 (shRNA- CCAT1-1/2) or empty vectors into TCA-8113 cells for silencing of CCAT1. The effect of transfection was investigated by qRT-PCR (Figure 2A). Based on these results, shRNA- CCAT1-1 was used in the following experiments. CCK-8 assay was employed to evaluate the proliferation of TCA-8113 cells. As shown in Figure 2B, cell proliferation
was remarkably inhibited by knockdown of CCAT1 compared with the shRNA-NC group. In addition, colony formation assay also showed a decreased number of colonies after transfection with shRNA-CCAT1-1 (Figure 2C). The results suggest that downregulation of CCAT1 represses cell proliferation of TCA-8113 cells.

**Knockdown of CCAT1 inhibited TCA-8113 cell cycle**

To identify the influence of CCAT1 silencing on cell cycle of TCA-8113 cells, cycle distribution was explored by flow cytometry. As presented in Figure 3A, downregulation of CCAT1 enhanced the proportion of cells in G0/G1 phase and decreased the proportion of cells in S phase. Moreover, results from Western blot assay showed that transfection with shRNA-CCAT1-1 attenuated the levels of CDK2 and cyclinD1 but elevated the p27 protein level in TCA-8113 cells in comparison with the control or shRNA-NC group (Figure 3B). These data demonstrate that inhibition of CCAT1 blocks cell cycle progression in TCA-8113 cells.

**Figure 1.** CCAT1 is upregulated in several OSCC cell lines. The mRNA expressions of CCAT1 in CAL-27, TCA-8113, SCC-4, SCC-9, and SCC-15 cells and oral epithelial cell line HIOEC were detected by qRT-PCR. Each bar represents the mean±SD calculated from 3 independent experiments. *** P<0.001 versus control.

**Figure 2.** CCAT1 silencing inhibits TCA-8113 cell proliferation. (A) CCAT1 mRNA expression was detected after transfection with shRNA-CCAT1-1/2. (B) Cell proliferation was evaluated by CCK-8 assay. (C) colony formation assay was employed to assess the cloning capacity. Each bar represents the mean±SD calculated from 3 independent experiments. ** P<0.01, *** P<0.001 versus control; ** P<0.01, *** P<0.001 versus shRNA-NC groups.
Silencing of CCAT1 repressed migration and invasion of TCA-8113 cells

Next, we investigated the effects of CCAT1 knockdown on OSCC cell migration and invasion. As shown in Figure 4A, after 24-h incubation, cells without treatment quickly migrated onto the wound area, while few cells with CCAT1 silencing migrated. The number of invasive cells was notably reduced in TCA-8113 cells transfected with shRNA-CCAT1-1 compared with the control (Figure 4B). Western blot assay results revealed that the activity of MMP2 and MMP9 was obviously decreased when cells were transfected with shRNA-CCAT1-1 (Figure 4C). These results indicate that the migratory and invasive capacity of TCA-8113 cells can be inhibited by down-regulation of CCAT1.

CCAT1 regulated DDR2

Previous studies have demonstrated that DDR2 regulates activity of MMP2/9 and ERK pathway, and serves as a tumor regulator in several types of squamous cell carcinoma [18]. Thus, we speculated that DDR2 contributes to the inhibitory effect of CCAT1 on OSCC cells. As shown in Figure 5A, DDR2 expression was markedly upregulated in OSCC cell lines in contrast to the control. Moreover, a reduction in protein and mRNA expression of DDR2 was observed in TCA-8113 cells upon shRNA-CCAT1-1 transfection (Figure 5B, 5C). To further verify the relationship between CCAT1 and DDR2, RIP assay was carried out. As presented in Figure 5D, the combination complex of CCAT1-1 and DDR2 was enriched in Ago2 immunoprecipitates in comparison with the control IgG immunoprecipitates. The data suggest that CCAT1 can bind to DDR2 and modulate DDR2.

CCAT1 inhibited ERK/AKT signaling pathway via DDR2

To achieve the upregulation of DDR2, DDR2 overexpression vectors were transfected into TCA-8113 cells. The mRNA and protein expression of DDR2 were assessed at 48 h after transfection (Figure 6A, 6B). Western blot analysis results showed that the protein level of phosphorylated ERK and AKT was reduced by shRNA-CCAT1-1, while DDR2 overexpression resulted in upregulation of p-ERK and p-AKT in shRNA-CCAT1-1-transfected TCA-8113 cells. At the same time, the level of total ERK and AKT did not altered in each group (Figure 6C). These results indicate that CCAT1 regulates TCA-8113 cells through mediating the ERK/AKT axis via DDR2.

Discussion

OSCC is a type of head and neck squamous cell carcinoma that has a poor 5-year survival rate. There are few obviously...
effective treatments for OSCC. In the present study, we explored the functions of CCAT1 in TCA-8113 proliferation, invasion, and migration. Furthermore, the underlying mechanism involved in DDR2/ERK/AKT axis was also revealed.

lncRNAs have been proved to exert numerous biological effects in various cancers. The IncRNA CCAT was first reported to be upregulated in colon cancer and has been shown to be abnormally expressed in some carcinomas, including OSCC [22]. Zhang et al. showed that CCAT is upregulated in esophageal squamous cell carcinoma (ESCC) tissues and is related to poor prognosis. CCAT knockdown inhibited ESCC proliferation and migration by regulation expression of SPRY4 and HOXB13 in vitro and in vivo [11]. Zhu et al. reported that CCAT accelerated proliferation

Figure 4. Effects of CCAT1 silencing on the migration and invasion of TCA-8113 cells. (A) Cell migration was investigated by wound healing scratch assay in CCAT1-silenced cells. (B) Transwell assay was applied for exploring the invasive capacity in CCAT1-silenced cells. (C) Levels of MMP2 and MMP9 were assessed by Western blot analysis after transfection with shRNA-CCAT1-1. Each bar represents the mean±SD calculated from 3 independent experiments. ** P<0.01, *** P<0.001 versus control; ## P<0.01, ### P<0.001 versus shRNA-NC groups.
and metastasis in hepatocellular carcinoma cells [23]. In addition, CCAT1 was found to be strongly expressed in lung SCC (LSCC), head and neck SCC (HNSCC), and ESCC and was modulated by TP63 or SOX2, which both promote cell proliferation and metastasis [24–6]. CCAT1 silencing suppressed proliferation and clonogenic capacity, while CCAT1 overexpression increased both the cell viability and colony number of SCC cells [27]. In our study, CCAT1 was overexpressed in OSCC cell lines compared with the control cells. The silencing of CCAT1 repressed cell proliferation by inhibiting cell viability, reducing colony numbers, and obstructing the cell cycle. Additionally, CCAT1 silencing attenuated migration and invasion of TCA-8113 cells, which is in agreement with previous reports.

Tyrosine kinase discoidin domain receptors (DDRs) are intimately linked with intracellular activities and functions. It has been reported that DDR2 takes part in various cellular processes involved in tumor progression, including cell proliferation, migration, invasion, and adhesion [28,29]. Previous studies have shown that DDR2 is upregulated in human malignant tumors, including oral squamous cell carcinoma [30]. Thus, DDR2 has been used as a diagnostic marker in some special cancer types. In addition, studies also found that inhibition of DDR2 diminished migration and invasion in metastatic melanoma cells by repression of MMP2 and MMP9 expression [31]. Based on these previous studies, we speculated that CCAT1 knockdown alleviates invasion and migration of TCA-8113 cells by mediating DDR2 expression. In the present study, we focused on the interaction of CCAT1 and DDR2. The data showed that DDR2 expression was upregulated in several OSCC cell lines. Downregulation of CCAT1 in TCA-8113 cells resulted in the decreased level of DDR2 mRNA and protein. Also, the RIP assay results validated the combination of DDR2 and CCAT1, suggesting that CCAT1 exerts regulatory effects on the development of TCA-8113 cells by targeting DDR2.

ERK/AKT signaling is a primary determinant in cell proliferation, migration, differentiation, and apoptosis [32]. This pathway is often activated in human cancers, which makes it a good anticancer target [33]. It has been demonstrated that ERK signaling is dysregulated in OSCC [34]. Wang et al. reported that FAP silencing inhibited cell proliferation, metastasis, and invasion by blockade of the PTEN/P13K/AKT and Ras-ERK signaling in OSCC cells [35]. Yu et al. found that CXCR4 facilitated OSCC...
Figure 6. CCAT1 suppresses activation of the ERK/AKT signaling pathway through targeting DDR2. The mRNA (A) and protein expression (B) of DDR2 after transfection of pcDNA-DDR2 were measured by qRT-PCR and Western blot assay, respectively. (C) Phosphorylated levels of ERK and AKT were detected in CCAT1-silenced TCA-8113 cells with or without transfection of pcDNA-DDR2. Each bar represents the mean±SD calculated from 3 independent experiments. *** P<0.001 versus control; * P<0.05, ### P<0.001 versus shRNA-NC groups; @ P<0.05, @@ P<0.01 versus shRNA-CCAT1+pcDNA-NC group.

cell migration and invasion by enhancing MMP 9/13 expression by activating the ERK signaling [36]. Moreover, the modulation of ERK in cancer is associated with the regulative effect of DDR2. Xie et al. revealed that DDR2 induced invasion and metastasis in hepatocellular carcinoma cells via activation of the ERK pathway [19]. In addition, Zhang et al. discovered that Nrp1 promoted DDR2-ERK-Runx2 cascade by inhibition of DDR2 degradation in osteoblast differentiation [37]. In the study, the expression of ERK signaling was detected. Transfection with shRNA-CCAT1-1 inhibited the phosphorylation of ERK and AKT, while DDR2 overexpression increased the phosphorylated expression of ERK and AKT after CCAT1 silencing, indicating knockdown of CCAT in TCA-8113 cells suppressed activation of the ERK pathway by regulating DDR2.

This study has several limitations. We chose several OSCC cell lines to detect the CCAT1 expression, but these cell lines cannot represent all OSCC cell lines. In addition, cell experiments were performed; thus, we could only understand the role of CCAT1 in OSCC at the cellular level. Further research is needed to assess the effects of CCAT1 on other OSCC cell lines and in animal experiments.

Conclusions

In summary, our study confirmed the crucial role of CCAT1 in regulation of OSCC progression. These results revealed that CCAT1 silencing inhibits cell proliferation, invasion, and migration in TCA-8113 cells through inactivation of the ERK/AKT signaling pathway via repressing DDR2. Therefore, CCAT may be a potential biological marker and therapeutic target for tumorigenesis of OSCC.
Conflicts of interest

None.

Supplementary Data

**Supplementary Table 1.** The list of shRNA and pcDNA sequence.

| Gene          | Primer                                                                 |
|---------------|------------------------------------------------------------------------|
| shRNA-CCAT1-1 | CACCCCAT TCCATTCTTTCTCTCTTCATCAAGAGATGAAAGAAGATGAAATGGA ATGTTTTTGT    |
| shRNA-CCAT1-2 | CACCGAAGCAGGCAGAAAGCCGT ATCTTAATTCAAGAGATTAAGATACGGCTTTCTGCCTGCTTTTTTTG |
| shRNA-NC      | CGCCGCCCCACCTTGGGACCTCTCCCT TCAAGA GGGGAG AGTGCCGAAAGGTGTTTTTGGAAAT  |
| pcDNA-DDR2    | CGGGGTACCCCTCCTGCCCCTAACTTACTATG                                      |
| pcDNA-NC      | CTAGCTAG CACAACATCGACTTTGAAGTT                                         |

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