Long non-coding RNA CCAT1 regulates the biological behavior of osteosarcoma cells through the miR-454-3p/ZEB2 axis

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Abstract. – OBJECTIVE: To investigate the clinical value and mechanism of action of IncRNA CCAT1 in OS.

PATIENTS AND METHODS: Serum, tumor tissue and corresponding adjacent tissue of 84 cases of patients receiving treatment in our hospital from February 2014 to June 2015 were obtained. Healthy volunteers were recruited during the same period for serum collection. CCAT1 expression in serum and tissue samples were detected, and the value of its expression in osteosarcoma (OS) diagnosis and prognosis was analyzed. Stable and transient over-expression and inhibition vectors were established and transfected into OS cells. CCK-8, transwell, and flow cytometry were applied to determine the proliferation, invasion, and apoptosis of transfected cells, and the Dual-Luciferase reporter was utilized to determine the correlation of CCAT1 with miR-454-3p, miR-454-3p, and ZEB2 (zinc-finger E-box-binding homeobox-2).

RESULTS: In OS, CCAT1 was upregulated, and serum CCAT1 could be used as a marker for OS diagnosis, with an AUC value of 0.930. High CCAT1 expression predicted poor survival rate in patients. Inhibition of CCAT1 could suppress the proliferation and invasion of OS cells, and increase the apoptosis rate. Over-expression of miR-454-3p and inhibition of ZEB2 could also achieve the above effects. Dual-Luciferase reporter indicated that CCAT1 could target miR-454-3p, and miR-454-3p could target ZEB2. The rescue experiment proved that CCAT1 could regulate OS progression through the miR-454-3p/ZEB2 axis.

CONCLUSIONS: CCAT1 can be used as a diagnostic and prognostic marker for OS, promote OS cell proliferation and invasion, and inhibit apoptosis through the miR-454-3p/ZEB2 axis, which makes it a potential therapeutic target for OS.

Key Words: Osteosarcoma, CCAT1, MiR-454-3p, ZEB2, Biological function.
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However, there are relatively few studies on the clinical value and possible molecular mechanisms of CCAT1 in OS. Thus, potential targeting sites of CCAT1 and miR-454-3p were found through online bioinformatics analysis, but the relationship between the two is still unknown.

In this research, the expression of CCAT1 in OS tissues, serum and cells was first observed. Secondly, the correlation of CCAT1 with pathological features and survival rate of patients was analyzed. Finally, the role and molecular mechanism of CCAT1 in OS were investigated through in vitro experiments.

**Patients and Methods**

**Source of Serum and Tissue Samples**

This investigation was approved by the Institute of Medical Ethics of our Institute and followed the Declaration of Helsinki. The subjects involved in this study were 84 cases of OS patients (patient group) undergoing surgery in our hospital from February 2014 to June 2015. Inclusion criteria: patients were diagnosed as OS histologically. Patients and their family had signed the informed consent. Patients had complete clinical data. Patients did not receive chemotherapy or radiotherapy before. Exclusion criteria: patients were complicated with other malignant tumors. Patients were infected before enrollment. Patients whose expected survival was less than 3 months. Patients were suffered severe disease (diabetes, etc.). During the surgery, blood, cancer tissues and corresponding adjacent normal tissues of the patients were collected. The tissues were frozen in liquid nitrogen, and the serum was centrifuged from the blood. Then, all samples were stored in the refrigerator at -80°C for future use. Meanwhile, healthy subjects (normal group) were recruited and their serum was obtained. The patient group was followed up for 3 years, and the overall survival rate was followed up by telephone and outpatient review. Follow-up was conducted every one month.

**Cell Processing**

OS cell lines (SOSP-9607, KHOS, U2OS, MG63) and normal osteoblast cell lines (hFOB1.19) were bought from Xin Yu Biotech Co., Ltd., Shanghai, China. Cells were placed in Medico’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 UI/ml penicillin/100 µg/ml streptomycin (100X, Gibco, Carlsbad, CA, USA), and placed in an incubator at 37°C and 5% CO2. Cell transfection: CCAT1 inhibitory plasmid (si-CCAT1), miR-454-3p over-expression plasmid (miR-454-3p-mimics), zinc-finger E-box-binding homeobox-2 (ZEB2) inhibitory plasmid and corresponding negative controls (CCAT1-NC, ZEB2-NC) were established using pcDNA 3.1 as plasmid. They were transfected by the aid of Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA). Six hours later, the transfected cells were transferred into medium comprising 10% phosphate-buffered saline (PBS).

**qRT-PCR**

qRT-PCR was used to detect CCAT1 and miR-454-3p expression. The total RNA was extracted from the samples in the light of the manufacturer’s instructions of Trizol kit (Invitrogen, Carlsbad, CA, USA). A spectrophotometer was used for determination of purity, concentration and integrity of the extracted RNA. 2 µg total RNA was taken and reverse-transcribed into cDNA using reverse transcription kit (Invitrogen, Carlsbad, CA, USA). SYBR Premix Ex Taq II (TaKaRa, Dalian, China) was used for amplification, and qRT-PCR quantitative analysis was carried out on ABI 7500 PCR (Applied Biosystems, Foster City, CA, USA). Amplification system: 10 µL of SYBR Premix Ex Taq II (2X), 2 µL of cDNA, 0.8 µL of upstream primers, 0.8 µL of downstream primers, sterile purified water was supplemented to 20 µL. Amplification conditions: pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, and annealing and extension at 60°C for 30 s. A total of 40 cycles were performed. CCAT1 and miR-454-3p expression was calculated using $2^{-\Delta\Delta Ct}$, which was normalized to GADPH and U6. Primer sequence was shown in Table I.

**Western Blot**

Western blot was applied to detect ZEB2 protein expression. Total protein was extracted by the aid of radio immunoprecipitation assay (RIPA, Thermo Fisher Scientific, Waltham, MA, USA), and protein concentration was measured with the help of bicinchoninic acid assay (BCA) kit (Thermo Fisher Scientific, Waltham, MA, USA). The protein concentration was adjusted to 4 µg/µL and separated by 12% polyacrylamide gel electrophoresis. After that, it was transferred to polyvinylidene difluoride membrane, soaked with...
phosphate-buffered saline and tween (PBST) for 5 min and washed, blocked with 5% skimmed milk powder for 2 h. Then, ZEB2 (1:1000), β-catenin (1:1000) (Abcam, Cambridge, MA, USA) primary antibody was added to seal overnight at 4°C. The primary antibody was removed by washing membrane, and horseradish peroxidase (HRP)-labeled goat anti-rabbit (Abcam, Cambridge, MA, USA) secondary antibody (1:2000) was added, incubated at 37°C for 1 h, then rinsed with phosphate-buffered saline (PBS) for 3 times, with 5 min each time. Excess liquid was absorbed from the membrane with filter paper. Enhanced chemiluminescence (ECL) was used to illuminate and develop in a dark room. The protein bands were scanned to calculate the relative expression level of the target protein.

Cell Apoptosis Detection

Target cells after 24 h of transfection were collected and digested with 0.25% trypsin. After digestion, they were rinsed with PBS and added with 100 μL of 0.5% trypsinization buffer. They were centrifuged and resuspended in 0.5 mL suspension. Annexin-V-FITC (10 μL), propidium iodide (PI, 5 μL) was successively added, incubating at room temperature in the dark for 10 min. Determination of cell apoptosis was conducted with the help of a flow cytometry (Thermo Fisher Scientific, Waltham, MA, USA) and the apoptosis rate was calculated.

Dual-Luciferase Reporter

The lncRNA and miR target genes were predicted using starBase 3.0 and Targetscan 7.2. CCAT1 or ZEB2 was cloned into the pmirGLO Dual-Luciferase target expression vector using Lipofectamine™ 2000 kit. CCAT1-3'UTR wild-type (Wt), CCAT1-3'UTR mutant (Mut), ZEB2-3'UTR Wt and ZEB2-3'UTR Wt were transferred to the downstream of the Luciferase reporter gene for sequencing and identification of the construct-ed plasmids. The correctly sequenced plasmids were co-transfected with miR-424-3p-mimics or miR-NC into target cells, respectively. Dual-Luciferase reporter assay kit (Thermo Fisher Scientific, Waltham, MA, USA) was utilized when determining Luciferase activity.

Statistical Analysis

Statistical analysis of the data collected in this study was conducted using SPSS 20.0 (IBM, Armonk, NY, USA) software package, and GraphPad 7 software package was used to draw the required images. Comparison of enumeration data between the two groups adopted chi-square test. Measurement data comparison between the two groups adopted independent sample t-test. One-way analysis of variance was utilized for comparison among groups, represented with F. LSD-t test was used for afterwards pairwise comparison. Comparison of expression among multiple time points was analyzed using repeated mea-
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Bonferroni was used for back testing. ROC was used to draw the diagnostic curve of serum CCAT1 for OS. The overall survival of patients was plotted using K-M survival curve, and Log-rank test was used for analysis. When $p<0.05$, there were statistical differences.

Results

Clinical Value of CCAT1 in OS

According to qRT-PCR, CCAT1 expression was up-regulated in OS tissue, serum and cells. ROC curve exhibited that serum could be used as a marker for OS diagnosis, with an AUC of 0.930, a sensitivity of 88.10%, and a specificity of 85.71%. Patients were divided into low-expression group and high-expression group according to the median value of CCAT1 expression, and the correlation of CCAT1 with patients’ clinicopathological data and survival rate was analyzed. The results revealed that high CCAT1 expression was associated with patients’ high TNM staging (stage III+IV), lymph node metastasis, low differentiation and poor survival rate (Table II, and Figure 1).

CCAT1 can Promote OS Development

This study revealed that CCAT1 was up-regulated in OS and was related to high TNM staging, lymph node metastasis, differentiation, and survival of patients, suggesting that CCAT1 plays an important role in OS development. Therefore, we inhibited CCAT1 in SOSP-9067 (CCAT1 expression in this cell showed the greatest difference). After the transfection, transwell and flow cytometry were used to observe the proliferation, invasion and apoptosis of SOSP-9067 after CCAT1 inhibition. The results revealed that after SOSP-9067 inhibition, CCAT1, CCAT1 expression was down-regulated, the proliferation and invasion abilities were inhibited, and apoptosis was increased. This indicated that CCAT1 had a certain effect on OS (Figure 2).

MiR-454-3p can Be Targeting Regulated by CCAT1

According to the second paragraph of the Results section, it could be known that CCAT1 could inhibit the progression of OS, but the specific mechanism is not clear. To further explore the mechanism of CCAT1 in OS, targeted binding sites between CCAT1 and miR-454-3p were found according to online software starBase 3.0. The relationship between them was proved through Dual-Luciferase activity, and it was found that transfection of miR-454-3p-mimics could inhibit the activity of CCAT1-3’UTR Wt Luciferase but not 3’UTR Mut Luciferase activity. Furthermore, we found through qRT-PCR that miR-454-3p

Table II. CCAT1 and clinicopathological data.

<table>
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<tr>
<th>Factors</th>
<th>CCAT1 expression</th>
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<tr>
<td></td>
<td>High expression</td>
<td>Low expression</td>
<td>$\chi^2$</td>
<td>$p$-value</td>
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<tr>
<td></td>
<td>(n = 42)</td>
<td>(n = 42)</td>
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<tr>
<td>Gender</td>
<td>Male (n = 58)</td>
<td>28 (66.67)</td>
<td>30 (71.43)</td>
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<tr>
<td></td>
<td>Female (n = 26)</td>
<td>14 (33.33)</td>
<td>12 (28.57)</td>
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<td></td>
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<tr>
<td>Age (years)</td>
<td>&lt; 18 years old</td>
<td>24 (57.14)</td>
<td>22 (52.38)</td>
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<td>0.661</td>
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<tr>
<td></td>
<td>≥ 18 years old</td>
<td>18 (42.86)</td>
<td>20 (47.62)</td>
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<tr>
<td>Tumor size</td>
<td>≥ 5 cm (n = 49)</td>
<td>23 (58.97)</td>
<td>26 (71.79)</td>
<td>0.441</td>
<td>0.664</td>
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<tr>
<td></td>
<td>&lt; 5 cm (n = 35)</td>
<td>19 (41.03)</td>
<td>16 (28.21)</td>
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<td>TNM staging</td>
<td>Grade I+II (n = 47)</td>
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<td>29 (69.05)</td>
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<td></td>
<td>Grade III+IV (n = 37)</td>
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<td>14 (30.95)</td>
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<td>27 (64.29)</td>
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<tr>
<td>Differentiation</td>
<td>Low differentiation (n = 42)</td>
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<td>16 (38.10)</td>
<td>4.762</td>
<td>0.029</td>
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<tr>
<td></td>
<td>Middle+high differentiation (n = 42)</td>
<td>16 (38.10)</td>
<td>26 (61.90)</td>
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</table>
was increased when si-CCAT1 was transfected with SOSP-9607. This suggested that miR-454-3p could be targeting regulated by CCAT1 (Figure 3).

**Figure 1.** A, CCAT1 expression in the cancer tissues of OS patients was higher than that in the adjacent normal tissues. B, CCAT1 expression in serum of the patient group was higher than that of the normal group. C, CCAT1 expression increased in OS cells. D, ROC curve of serum CCAT1 in diagnosing OS, the AUC value was 0.934, the sensitivity was 88.10%, and the specificity was 85.71%. E, Patients with high expression had a lower overall survival rate than those with low expression (Log-rank: p=0.012). Note: * denotes p<0.05.

**Figure 2.** Effects of CCAT1 on cell biological function of OS. A, CCAT1 expression was down-regulated when SOSP-9607 was transfected with si-CCAT1. B, The proliferation ability was decreased when SOSP-9607 was transfected with si-CCAT1. C, The invasion ability was decreased when SOSP-9607 was transfected with si-CCAT1. D, The apoptosis rate was increased when SOSP-9607 was transfected with si-CCAT1. E, Flow cytometry image. Note: * denotes p<0.05.

CCAT1 can Promote OS Development Through MiR-454-3p
Results section, while whether CCAT1 could promote the development of OS through miR-454-3p remained unknown. To determine whether CCAT1 could inhibit OS progression through miR-454-3p, we over-expressed miR-454-3p in SOSP-9607 and observed the effects of such treatment on proliferation, invasion and apoptosis of SOSP-9607. The results showed that after transfection of SOSP-9607 with miR-454-3p-mimics, miR-454-3p expression was increased, the proliferation and invasion ability of SOSP-9607 was inhibited, and the apoptosis rate was increased. Subsequently, CCAT1 and miR-454-3p in SOSP-9607 were over-expressed at the same time. By observing the biological functions of cells after treatment, it was found that there was no difference in the proliferation and apoptosis after transfection with sh-CCAT1+miR-454-3p-mimics. Compared with transfection with miR-454-3p alone, however, the proliferation and invasion ability increased, and the apoptosis rate decreased. It suggested that at least part of CCAT1 could play a carcinogenic role in OS through miR-454-3p (Figure 4).

**miR-454-3p can Inhibit OS Progression through ZEB2**

In the above results, the CCAT1/miR-454-3p axis has been found to produce a marked effect on OS progression. To further improve the understanding of the mechanism of this axis, Targetscan 7.2 online software was applied for miR-454-3p target gene prediction, and binding sites between ZEB2 and miR-454-3p were found. The activity of Dual-Luciferase was detected, and it was found that transfection of miR-454-3p-mimics could inhibit the activity of ZEB2-3' UTR Wt without affecting the Luciferase activity of ZEB-3' UTR Mut. Through qRT-PCR, ZEB2 was found to be down-regulated after SOSP-9607 was transfected with si-ZEB2. Subsequently, we investigated the role of ZEB2 in OS, and inhibited ZEB2 in SOSP-9607. The results showed that after transfection of SOSP-9607 with si-ZEB2, ZEB2 was down-regulated, proliferation and invasion of SOSP-9607 were inhibited, and the apoptosis rate was increased. This indicated that miR-454-3p could play a role through ZEB2. Subsequently, SOSP-9607 was treated with ZEB2 and miR-454-3p inhibition simultaneously. After observing the biological functions of the cells after treatment, it was found that there was no difference in the proliferation and apoptosis after transfection with sh-CCAT1+miR-454-3p-mimics or miR-NC. Compared with transfection of si-ZEB2, however, the proliferation and invasion ability increased, and the apoptosis
According to this study, CCAT1 was upregulated in OS tissues, serum, and cells, and serum CCAT1 could be used as a marker for OS diagnosis, with an AUC value of 0.930. High CCAT1 expression was related to high TNM staging (stage III+IV), lymph node metastasis, low differentiation, and poor survival rate. In addition, cell experiments revealed that CCAT1 could promote OS cell proliferation and invasion and inhibit apoptosis through miR-454-3p/ZEB2 axis. In recent years, with the further understanding of IncRNA, its dysregulation has been considered as one of the important factors in the development of multiple tumors including OS12-14. CCAT1 is a member of IncRNA, which is generally highly expressed in tumors and associated with poor prognosis. In cholangiocarcinoma, CCAT1 is up-regulated and predicts a poor prognosis15. In oral squamous cell carcinoma, CCAT1 is also up-regulated and associated with poor therapeutic outcomes16. CCAT1 is elevated in ovarian cancer and is related to poor prognosis of patients. In addition, CCAT1 can promote cancer cell migration, invasion and epithelial mesenchymal transformation (EMT)17. However, the mechanism of CCAT1 in OS is still largely unknown. Here, we measured the expression of CCAT1 in OS tissues, serum and cells. By drawing the ROC curve, it was found that serum CCAT1 could be used as the marker for OS diagnosis, with an AUC value of 0.930. High CCAT1 expression was related to high TNM staging (stage III+IV), lymph node metastasis, low differentiation, and poor survival rate. In addition, cell experiments revealed that CCAT1 could promote OS cell proliferation and invasion and inhibit apoptosis through miR-454-3p/ZEB2 axis.

Discussion

According to this study, CCAT1 was upregulated in OS tissues, serum, and cells, and serum CCAT1 could be used as a marker for OS diagnosis, with an AUC value of 0.930. High CCAT1 expression was related to high TNM staging (stage III+IV), lymph node metastasis, low differentiation, and poor survival rate. In addition, cell experiments revealed that CCAT1 could promote OS cell proliferation and invasion and inhibit apoptosis through miR-454-3p/ZEB2 axis.

In recent years, with the further understanding of IncRNA, its dysregulation has been considered as one of the important factors in the development of multiple tumors including OS12-14. CCAT1 is a member of IncRNA, which is generally highly expressed in tumors and associated with poor prognosis. In cholangiocarcinoma, CCAT1 is up-regulated and predicts a poor prognosis15. In oral squamous cell carcinoma, CCAT1 is also up-regulated and associated with poor therapeutic outcomes16. CCAT1 is elevated in ovarian cancer and is related to poor prognosis of patients. In addition, CCAT1 can promote cancer cell migration, invasion and epithelial mesenchymal transformation (EMT)17. However, the mechanism of CCAT1 in OS is still largely unknown. Here, we measured the expression of CCAT1 in OS tissues, serum and cells. By drawing the ROC curve, it was found that serum CCAT1 could be used as the marker for OS diagnosis, with an
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AUC value of 0.930, sensitivity of 88.10%, and specificity of 85.71%. Moreover, high CCAT1 expression was related to high TNM staging (stage III+IV), lymph node metastasis, low differentiation and poor survival rate, indicating that CCAT1 could be used as a marker for OS diagnosis and prognosis, and may be involved in the pathogenesis of the disease. Then, in vitro experiments revealed that knocking down CCAT1 could suppress OS cell proliferation and invasion, and promote apoptosis, which was similar to previous studies suggesting that CCAT1 plays a carcinogenic role.

To further understand the function of CCAT1 in promoting OS progression, the online starBase 3.0 software was used to analyze the presence of targeted binding sites between CCAT1 and miR-454-3p, and Dual-Luciferase activity test showed that miR-454-3p could be regulated by CCAT1 in a targeted manner. MiR-454-3p has been previously reported to be a tumor suppressor, which can block the progression of glioblastoma, gastric cancer and cervical cancer. MiR-454-3p also acts as a tumor suppressor in OS. According to Niu et al., miR-454-3p is reduced in OS and inhibits the growth and invasion of cancer cells by regulating c-Met. Through cell experiments, it could be seen that miR-454-3p over-expression could inhibit the proliferation and invasion of OS cells and promote apoptosis, suggesting that miR-454-3p could suppress OS progression.

CCAT1 contributes to the progression of a variety of tumors through miR. In laryngeal squamous cell carcinoma, CCAT1 can promote cancer through miR-218/ZFX axis. In intrahepatic cholangiocarcinoma, CCAT1 can promote the migration, invasion and EMT of cancer cells via inhibiting miR-152. Subsequently, this study found that the proliferation and apoptosis

![Image](https://example.com/figure5.png)
after transfection with sh-CCAT1+miR-454-3p-mimics showed no statistical difference when compared with those transfected with miR-NC, while the proliferation and invasion capacity increased and the apoptosis rate decreased compared with those transfected with miR-454-3p-mimics. These results demonstrated that at least part of CCAT1 could play a carcinogenic role in OS through miR-454-3p.

To further improve the mechanism of CCAT1/miR-454-3p in OS, Targetscan 7.2 online software was utilized miR-454-3p target gene prediction, and it was found that there were binding sites between ZEB2 and miR-454-3p. After Dual-Luciferase activity detection, it was observed that ZEB2 could be targeted by miR-454-3p. As a member of the ZEB family, ZEB2 is considered to be a transcription factor involved in proliferation, apoptosis, invasion and other cellular functions. It is thought to be a carcinogenic gene in tumor. ZEB2 is up-regulated in OS and promotes the development of the disease; in addition, it can be regulated by miR-187 to play a role in cancer inhibition. In this paper, ZEB2 in OS cells was inhibited, and it was found that this treatment could suppress the proliferation and invasion of cancer cells and increase the apoptosis rate. Subsequently, both ZEB2 and miR-454-3p in OS cells were inhibited at the same time. By observing the biological functions of the cells, it was found that there was no difference in proliferation and apoptosis after transfection with si-ZEB2+miR-454-3p-inhibitor or with si-NC. Compared with cells transfected with si-ZEB2, the proliferation and invasion capacity increased, and the apoptosis rate decreased. Combined with the above research results, it could be known that CCAT1 can promote OS cell proliferation and invasion and inhibit apoptosis through the miR-454-3p/ZEB2 axis.

We showed that CCAT1 could be used as a diagnostic and prognostic marker for OS, and found for the first time that CCAT1 could promote OS cell proliferation and invasion and inhibit apoptosis through the miR-454-3p/ZEB2 axis. It is well known that the incidence of tumor cells to chemotherapy and radiotherapy is one of the main causes of treatment failure, and chemotherapy resistance can lead to tumor recurrence and metastasis. It is important to explore the molecular mechanism of chemotherapy resistance in different stages of cancer. Therefore, our research shows that CCAT1 may exert a certain influence on chemotherapy resistance of OS cells through miR-454-3p/ZEB2 axis, which can be explored in future studies. There are also some shortcomings in this study. For example, the relationship between CCAT1 and other targets has not been explored. Moreover, tumor formation in nude mice experiments have not been conducted to prove the effect of CCAT1 inhibitors in vivo. We hope that more research can be carried out in future researches to supplement and improve our results.

Conclusions

To sum up, CCAT1 can be used as a diagnostic and prognostic marker for OS and can promote OS cell proliferation and invasion and inhibit apoptosis through the miR-454-3p/ZEB2 axis, which can be a therapeutic target for OS therapy. The result indicates that CCAT1 is an important regulator of the development of OS and a potential therapeutic target for OS therapy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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8) Li Y, Zhu G, Ma Y, Qi H. LncRNA CCAT1 contributes to the growth and invasion of gastric cancer.
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