Abstract. The long non-coding RNA colon cancer-associated transcript 2 (CCAT2) is abnormally expressed in various types of malignant tumor tissues and considered to be an oncogene, including for esophageal cancer (EC). Radiotherapy is an important and widely used cancer treatment. However, some patients with EC do not respond to radiotherapy. This study was designed to investigate effects of CCAT2 expression on radiotherapy dynamics for EC cells and to explore underlying molecular mechanisms. Reverse transcription-quantitative PCR was used to measure CCAT2 expression in EC tissues, normal esophageal mucosa, EC cells and normal human esophageal epithelial cells. TUNEL assays were used to assess the effect of CCAT2 on X-ray-induced apoptosis of EC cells. Protein expression was detected by western blot. CCAT2 was highly expressed in EC tissues and EC cells, and was negatively associated with radiotherapy efficacy in patients with EC. In vitro, knockdown of CCAT2 enhanced radiosensitivity of EC cells and promoted apoptosis by increasing Bax/Bcl2 and active-caspase 3/caspase 3 following X-ray treatment. In addition, CCAT2 negatively regulated miR-145 and P70 ribosomal protein S6 kinase 1 (p70S6K1) expression, and inhibited phosphorylation of Akt, ERK and p70S6K1 in EC cells. After X-ray treatment, CCAT2 negatively regulated protein levels of p53, P21 and c-Myc. These results showed that CCAT2 promoted the radiotherapy resistance of EC cells via negative regulation of the miR-145/p70S6K1 and the p53 signaling pathways and associated elements may be potential targets for improving the sensitivity of EC radiotherapy.

Introduction

Esophageal cancer (EC) is the eighth-ranked cancer of incidence worldwide and the sixth leading cause of cancer-related deaths in 2012 (1,2). China is the country with the highest incidence and mortality rates from EC globally, with a 5-year overall survival rate <30% (3). Most patients with EC are in advanced stages of the disease upon first diagnosis and outcomes for surgical treatment are correspondingly very poor. Therefore, concurrent chemoradiotherapy has become the standard treatment for advanced localized EC, but treatment outcomes still remain poor (4). In recent years, there has been a rapid development in diagnosis and treatment of EC, and although the therapeutic outcomes are better than before, certain EC patients still have local recurrences of tumors and metastasis after treatment (5,6). Such cases exemplify why radiotherapy resistance is considered one of the major predictive factors for poor prognosis and poor outcomes for EC patients (7). Therefore, it is necessary to find new markers that help to accurately predict radiotherapy resistance, so that novel detection targets and treatment methods for EC can be developed and tested.

Long non-coding RNAs (lncRNAs) play an important role in the development of various cancers, and have become a hot topic for tumor research due to tumor tissue specificity and the potential application in tumor diagnosis and treatment outcome prediction (8,9). lncRNAs are a type of RNA that is >200 bp in length, lacks or has only a minor portion of an open reading frame, which does not encode a protein, is usually composed of multiple exons spliced together, is transcribed by RNA polymerase II, and has a histone modifier similar to the encoded protein (10,11). An increasing number of lncRNAs have been found to be closely associated with the development of tumors in recent years (10,11), and studies have indicated that lncRNAs could be used as promoters of development and progression for certain types of tumors (12,13).

Colon cancer-associated transcript 2 (CCAT2), a lncRNA, received its name when it was first discovered in colorectal cancer (14). Thereafter, CCAT2 was confirmed to be abnormally expressed in various other tumor types, including breast cancer, ovarian cancer, gastric cancer, non-small cell lung cancer, cervical cancer and esophageal squamous cell...
carcinoma (15). Additional previous studies have indicated that CCAT2 is highly expressed in tumor tissues of esophageal squamous cell carcinoma and is associated with poor patient prognosis (16,17). Although few studies on the relationship between CCAT2 and radiotherapy resistance exist, previous research has confirmed that CCAT2 can inhibit the expression of miR-145 in cancer cells (18), and confirmed that the miR-145/p70S6K pathway is associated with radiotherapy resistance in cancer cells (19). In addition, previous studies have found that P70 ribosomal protein S6 kinase 1 (p70S6K1), an important effector protein downstream of the apoptosis-associates Akt/ERK signaling pathway, is a target gene of miR-145 (20). Therefore, our study was designed to investigate effects of CCAT2 expression on radiotherapy treatments for EC cells and to examine whether CCAT2 promoted radiotherapy resistance in EC cells via the miR-145/p70S6K1 and the p53 signaling pathways.

Materials and methods

**Tissue and ethics statement.** A total of 60 EC tissue and 21 healthy esophageal mucosa samples were collected for biopsy between June 2016 and June 2018 at Cangzhou Central Hospital. The EC tissues included 38 male and 22 female patients with a mean age at diagnosis of 60.46±9.24 years and the healthy esophageal mucosa samples included 12 male and 9 female patients with a mean age of 59.62±4.72 years. Inclusion criteria were based on the following: i) No anticancer treatment before tissue acquisition; ii) no other malignant tumors or chronic infectious diseases present; iii) normal tissue samples were confirmed by lack of any lesions in tissues; and iv) completion of clinical information, such as age, gender, medical history and a treatment plan. Exclusion criteria included: i) Failure to complete 3 years of follow-up; ii) death due to other diseases or accidents; iii) pregnancy, lactating and drug abusers; and iv) withdrawal from other types of medical treatments, such as participate in and withdraw from other clinical trials for drugs or biotherapies. All EC patients received radiation therapy after surgery. According to the patient's specific conditions, the prescription dose was 50–66 Gy. The divided dose was 2 Gy/day, once a day, 5 times a week, 6-7 weeks in total. Patients were treated with the Varian 23EX and 120-leaf MLC 600C linear accelerators. Patients were treated with 4 Gy irradiation for 24 h after transfection. All EC patients received radiation therapy after surgery. Patients were treated with the Varian 23EX and 120-leaf MLC 600C linear accelerators. Patients were treated with 4 Gy irradiation for 24 h after transfection.

**RT-qPCR.** Levels of CCAT2, miR-145 and p70S6K1 mRNA were detected by RT-qPCR as previously described (21). Briefly, TRIZol® (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from tissues or cells and RT was performed under the following conditions: 37°C for 15 min, 85°C for 5 sec and hold at 4°C. qPCR was performed using the following protocol: 95°C for 30 sec, followed by 40 cycles of 90°C for 5 sec and 65°C for 30 sec. PCR primer sets were as follows: CCAT2 forward (F), 5'-CCCTTGTCAA ATTGCTTAACCT-3' and reverse (R), 5'-TTATCGTGTCCCT CTGGTTTATGGAT-3' and GAPDH-F, 5'-GGGTGTTCA CACCCCGGGGAAGTG-3' and GAPDH-R, 5'-ACCCGTGACTGGT-3'. The 2^(-ΔΔCq) method (22) was used to calculate relative expression levels of genes. GAPDH as a reference gene and in addition we used U6 as a reference gene in miR quantification.

**Fluorescence in situ hybridization.** A fluorescent probe for binding to the human version of the CCAT2 gene was synthesized by Genomeditech Co., Ltd. Fluorescence levels of in situ hybridization of CCAT2 in different EC cells was determined as described previously (18). We analyzed samples using fluorescence microscopy (magnification, x20) and LAS AF Lite 4.0 image analysis software (Leica Microsystems, Inc.).

**Dual-luciferase reporter assay.** The Dual-Lucy Assay kit (cat. no. D00100; Beijing Solarbio Science & Technology Co.,
Ltd.) was used to detect luciferase activity following the manufacturer’s protocol. Briefly, cells were collected at 48 h after transfection and lysed for 5 min on ice before centrifugation (12,000 x g for 1 min at room temperature) to collect the cell supernatant. Five volumes of firefly luciferase reaction solution or Renilla luciferase reaction solution were added to the cell lysate and the enzyme activity was detected. Firefly luciferase activity was normalized to Renilla luciferase activity.

**TUNEL assays.** We seeded 0.6–0.8x10⁶ cells in Lab-Tek Chambered Coverglass and cultured for 24 h. Then, we added DAPI (5 µg/ml) to the culture medium for 5 min at 37°C, then removed the medium, washed samples twice with PBS and applied 4% paraformaldehyde to fix samples at room temperature for 20 min. Cell membranes were penetrated by applying 0.3% Triton X-100 in PBS for 1 h at room temperature. Next, TUNEL test solution (50 µl) was added and samples were incubated in the dark for 60 min at 37°C. Products were washed twice with PBS and analyzed using fluorescence microscopy (magnification, x20). We have observed ≥10 fields of view per sample and LAS AF Lite 4.0 image analysis software was used to evaluate the data.

**Western blotting.** The EpiQuik Whole Cell Extraction Kit (cat. no. OP-0003; Amyjet Scientific) to extract the total protein from cells. Levels of Bax (cat. no. ab32503; 1:1,000), Bcl2 (cat. no. ab32124; 1:2,000), caspase 3 (cat. no. ab13847; 1:2,000), active-caspase 3 (cat. no. ab2302; 1:500), p70S6K1 (cat. no. ab9366; 1:1,000), phosphorylated (P) -p70S6K1 (cat. no. ab60948; 1:500), P-ERK (cat. no. ab184699; 1:1,000), ERK (cat. no. ab201015; 1:2,000), P-Akt (cat. no. ab81283; 1:2,000), Akt (cat. no. ab64148; 1:2,000),
p53 (cat. no. ab131442; 1:1,000), P21 (cat. no. ab159520; 1:3,000), c-Myc (cat. no. ab32072; 1:1,000) and GAPDH (cat. no. ab181602; 1:5,000; all from Abcam) protein were detected by western blot as previously described (21). We used TBST diluted with 5% skim milk as a blocking solution at room temperature for 1 h. Primary and secondary antibodies (goat anti-rabbit HRP-conjugated IgG; cat. no. ab6721; 1:1,000 or goat anti-mouse HRP-conjugated IgG; cat. no. ab205719; 

Figure 2. CCAT2 expression affects radiosensitivity of esophageal cancer cells. (A) CCAT2 expression in TE-3 and ECA109 cells with and without X-ray irradiation. CCAT2 expression in TE-3 and ECA109 cells transfected with Si-CCAT2 or control detected by (B) reverse transcription-quantitative PCR and (C) fluorescence in situ hybridization; scale bar, 50 µm. (D) Quantification of TUNEL assay results and (E) fluorescence images for TE-3 and ECA109 cells transfected with Si-CCAT2 or control; scale bar, 50 µm. **P<0.001 vs. Control. CCAT2, long non-coding RNA colon cancer-associated transcript 2; Si, small interfering RNA.
1:2,000; from Abcam) were diluted with blocking solution and were incubated for 2 or 1 h at room temperature, respectively. ImageJ (version 1.37; National Institutes of Health) was used to analyze gray values of protein bands and GAPDH was used as the control.

Statistical analysis. Data were analyzed using SPSS 20.0 (IBM Corp.). Data are presented as the mean ± SD of at least 3 independent experiments. Two treatment groups were compared using unpaired Student's t-test or Chi-square test. Multiple group (>2) comparisons were made using one-way ANOVA followed by Duncan's test. Kaplan-Meier analysis was used to generate survival plots and significance was assessed by log-rank test. P<0.05 was considered to indicate a statistically significant difference.

Results

**CCAT2 is associated with radiotherapy efficacy in EC patients.** A total of 60 EC tissues and 21 normal esophageal mucosa were obtained and CCAT2 levels were determined. As seen in Fig. 1A, CCAT2 levels were significantly higher in EC tissues compared with normal esophageal mucosa (P<0.05). The same result was observed in vitro for various cell lines, where CCAT2 was significantly increased in EC cells, including TE-1, TE-3, ECA109, KYSE410 and KYSE520 compared with normal HEECs (P<0.05; Fig. 1B).

EC patients were assigned into two treatment groups based on CCAT2 expression level; namely a high and an low CCAT2 group. Upon conclusion of radiotherapy treatments, patients were evaluated for resultant effects according to Response Evaluation Criteria In Solid Tumor in 2000 (23) and we identified 22 cases of complete response (CR) + partial response (PR) and 12 cases of no response (NR) in the high CCAT2 group. In the low CCAT2 group, we identified 23 cases of CR+PR and 3 cases of NR (Fig. 1C). The 3-year overall survival of EC patients in the low CCAT2 group was significantly longer than for patients with EC in the high CCAT2 group (Fig. 1D).

**Knockdown of CCAT2 induces enhanced radiosensitivity of EC cells.** Firstly, we found that X-ray treatment significantly increased in CCAT2 expression in EC cells in vitro (Fig. 2A). We knocked down CCAT2 expression using si-CCAT2 (Fig. 2B and C) to study the effect of CCAT2 expression on...
radiosensitivity of EC cells and found that knockdown of CCAT2 significantly enhanced apoptosis of EC cells after X-ray treatment (Fig. 2D and E). Moreover, we found that knockdown of CCAT2 significantly increased Bax/Bcl2 protein expression and significantly increased the active-caspase 3/caspase 3 ratio in EC cells after X-ray treatment (Fig. 3).

**CCAT2 negatively regulates the miR-145/p70S6K1 and the Akt/ERK/p70S6K1 signaling pathways.** As presented in Fig. 4A, si-CCAT2 was significantly reduced CCAT2 expression and significantly increased miR-145 expression. Furthermore, we showed that transfection with Up-CCAT2 significantly increased CCAT2 expression and significantly decreased miR-145 expression. Additionally, miR-145-mimics significantly increased in miR-145 expression and significantly decreased CCAT2 and p70S6K1 mRNA expression (Fig. 4B). miR-145-inhibitor transfection resulted in a significant decrease in miR-145 levels and significant increases CCAT2 and p70S6K1 mRNA expression. Results from the dual luciferase reporter assay suggested that in the WT, miR-145-mimic transfection significantly decreased luciferase activity and miR-145-inhibitor transfection significantly increased luciferase activity compared with the miR-145-NC transfected cells (Fig. 4C). However, miR-145-mimic and miR-145-inhibitor did not affect the luciferase activity in the MUT group. As seen in Fig. 4D, transfection with miR-145-mimic significantly
decreased p70S6K1 protein expression and miR-145-inhibitor transfection significantly increased p70S6K1 protein expression compared with the miR-145-NC transfected cells.

In addition, in TE-3 cells exposed to X-ray irradiation, we found that knockdown of CCAT2 significantly decreased levels of p70S6K1, P-p70S6K1, P-ERK and P-Akt and CCAT2
overexpression significantly increased levels of p70S6K1, P-p70S6K1, P-ERK and P-Akt (Fig. 5). Results further indicated that knockdown of CCAT2 significantly decreased in phosphorylation of p70S6K1, ERK and Akt, and overexpression significantly increased activation of these proteins.

CCAT2 negatively regulates the p53 signaling pathway. Levels of CCAT2 expression did not significantly affect p53 expression in TE-3 cells without X-ray treatment; however, knockdown of CCAT2 significantly increased and overexpression significantly decreased p53 protein expression in TE-3 cells after X-ray treatment compared with the control (Fig. 6A). Next, we knocked down p53 expression by transfection with si-p53; a decrease in protein expression is only visible in the X-ray treated cells due to the absence of protein expression in the non-X-ray exposed cells (Fig. 6B). Knockdown of p53 significantly increased in CCAT2 expression in TE-3 cells with or without X-ray treatment compared with the control (Fig. 6C). In addition, knockdown of CCAT2 significantly increased and overexpression of CCAT2 significantly decreased levels of p21 and c-Myc protein expression in TE-3 cells after X-ray treatment compared with the control (Fig. 6D).

Discussion

In the present study, we found that CCAT2 was highly expressed in EC tissues and cells, which was consistent with available literature (16,17). On further analysis of the association between CCAT2 expression and radiotherapy efficacy in patients with EC, we found that EC patients with low levels of CCAT2 had better efficacy and outcomes from radiotherapy, and longer overall survival times. Tumor staging and other treatment options for EC patients may have additional effects on the efficacy of radiotherapy and overall survival times and require further analysis in the future. However, our results suggested that CCAT2 may be involved in the dynamics of sensitivity of EC patients to radiotherapy.

Using in vitro experiments, we found that X-ray treatment increased levels of CCAT2 in EC cells and knockdown of CCAT2 promoted X-ray-induced apoptosis of EC cells. Although no prior research has reported associations between CCAT2 expression and EC cell apoptosis, it was reported that overexpression of CCAT2 inhibits apoptosis of cervical cancer (24) and hepatocellular carcinoma cells (25). At present, neoadjuvant chemoradiotherapy or chemotherapy alone have become the standards for treatment of localized developing and advanced EC (26,27). Accordingly, radiotherapy has played a key role in the treatment of EC patients and has been rapidly developed as a course for treatment in recent decades (28). As radiotherapy is an important treatment for EC patients, radiotherapy resistance has recently attracted more attention. Previous studies indicated that radiotherapy can induce tumor cell apoptosis and that sensitivity of tumors to radiotherapy is consistent with sensitivities of tumor cells to apoptosis (29,30). Our results suggested that CCAT2 enhanced the resistance of EC cells to radiotherapy by inhibiting apoptosis.
Although studies that established a link between CCAT2 expression and tumor cell radiotherapy sensitivity are rare, potential molecular mechanisms can be hypothesized. Firstly, previous studies indicated CCAT2 inhibits expression of miR-145 in tumor cells (18) and miR-145 enhances radiosensitivity of cervical cancer (31), prostate cancer (32) and hepatocellular carcinoma cells (33). Our results also indicated that CCAT2 and miR-145 inhibited each other in EC cells. Moreover, our results indicated that miR-145 inhibited p70S6K1 protein expression. Previous studies indicated that the PI3K/Akt signaling pathway and downstream kinase p70S6K1 are important in mediating cell proliferation, differentiation and apoptosis (34,35). Activation of PI3K is a primary signal for inhibition of apoptosis, and PI3K induces Akt activation, which in turn activates p70S6K1 ultimately resulting in the inhibition of cell apoptosis (34,35). Our results indicated that CCAT2 decreased phosphorylation of Akt, ERK and p70S6K1 in EC cells. Therefore, indicating that CCAT2 enhanced the resistance of EC cells to radiotherapy by inhibiting apoptosis via inhibiting the miR-145/p70S6K1 signaling pathway and by activating the Akt/ERK/p70S6K1 signaling pathway.

Radiotherapy is based on the energy transmitted by radiation destroying the chromosome of the cell causing DNA damage in tumor cells, stopping cell growth by inducing apoptosis and halting proliferation of tumor cells (36,37). The p53 signaling pathway plays an important role in some of the aspects related to cellular DNA damage, repair, and apoptosis (38,39). We found that CCAT2 downregulated expression of p53, p21 and c-Myc proteins. When DNA damage occurs in cells, elevated levels of p53 induce a variety of downstream events, including cell cycle arrest, DNA repair or differentiation, and apoptosis (38,39). p53 activates the expression of p21, which induces cell cycle inhibition and repairs of damaged DNA. Further, if DNA damaged cannot be repaired smoothly, p53 regulates apoptosis (40,41). In the p53-dependent apoptotic signaling pathway, p21 is indirectly involved in apoptosis through cell cycle arrest, but is not essential for apoptosis (40,41). In addition, studies have indicated that c-Myc regulates the function and activity levels of p21 in association with apoptosis (42,43). Furthermore, it was found that c-Myc does not affect binding of p53 to p21, but that p53 selectively inhibits the activation of p21, thereby regulating p21 expression beneficial for p53-mediated apoptosis (42,43).

In conclusion, we found that CCAT2 was highly expressed in EC tissues and that it promotes the resistance of radiotherapy treatment in patients with EC and EC cells by decreasing apoptosis via inhibiting the miR-145/p70S6K1 signaling pathway. CCAT2 further inhibited the p53 signaling pathway and induced activation of the Akt/ERK/p70S6K1 signaling pathway. Our findings provided novel information to help further methodological approaches for the management of patients with EC.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors’ contributions

MW and LW conceived and designed the study. XH, JZ, ZZ, MZ and XL performed the experiments and analyzed the data. MW prepared and revised the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was performed with the approval of the Ethics Committee of Cangzhou Central Hospital. All aspects of the study complied with the Declaration of Helsinki. All participants provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

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