CDCA2 promotes tumorigenesis and induces radioresistance in oesophageal squamous cell carcinoma cells

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Abstract. Cell division cycle-associated 2 (CDCA2) overexpression has been demonstrated to serve a significant role in tumorigenesis in certain types of cancer. Nevertheless, its role in tumour proliferation and radioresistance in oesophageal squamous cell carcinoma (ESCC) remains to be elucidated. Thus, the present study aimed to elucidate these roles. Data were downloaded from The Cancer Genome Atlas (TCGA) to compare the gene expression profiles. The expression of CDCA2 was higher in ESCC tissues compared with normal tissues. Gene set enrichment analysis was performed based on the ESCC cohorts in TCGA database. This demonstrated that higher expression of CDCA2 was significantly associated with the expression of related components of the cell cycle phase transition and G2/M phase transition pathways. Collectively, the results revealed that CDCA2 could serve as an underlying target to regulate tumour growth and radioresistance among patients with ESCC.

Introduction

Oesophageal cancer (EC) is one of the commonest types of cancer worldwide with high morbidity, especially in Eastern Asia (1-3). It is also the sixth leading cause of cancer-related death globally, with 5.3% of all cancer deaths (age-standardized rates, 5.5 per 100,000) (2). EC has two main histological subtypes: Oesophageal adenocarcinoma (EA) and oesophageal squamous cell carcinoma (ESCC) (4). ESCC is one of the most aggressive squamous cell carcinomas and has a high prevalence in Asia, especially in China (5-7). Patients with advanced oesophageal squamous cell carcinoma who cannot tolerate or refuse to undergo surgery can choose radiotherapy (RT) (4,7,8). As a number of patients may suffer radioresistance, the outcomes of clinical treatment are unsatisfactory (9). Hence, it is necessary to improve the clinical outcomes to explore the related molecular mechanisms of the proliferation and radioresistance of ESCC.

Cell division cycle-associated protein 2 (CDCA2; also known as RepoMan), regulates the phosphatase of the core substrates throughout the cell cycle (10). It has been reported that CDCA2 can promote cell proliferation in colorectal cancer by activating the AKT/cyclin D1 pathway (11). CDCA2, which is highly expressed in oral squamous cell carcinoma and lung adenocarcinoma, respectively, can promote the growth of certain types of tumour (12,13). Previous studies have also reported that CDCA2 can modulate chromatin remodelling and DNA damage checkpoint activation (14,15).

At present, the biological functions of CDCA2 in ESCC have rarely been reported and hence the purpose of the present study was to examine the expression and biological behaviours of CDCA2 and evaluate its role in the process of tumour growth and radioresistance in ESCC. Targeting CDCA2 may be a novel therapeutic option to heighten the radiosensitivity of ESCC.

Materials and methods

The Cancer Genome Atlas (TCGA) database. The expression data of all genes in ESCC were downloaded from TCGA, comprising 93 samples (11 normal tissues and 82 tumour tissues).

Cell culture. A total of five types of human ESCC cell lines (ECA109, KYSE150, KYSE450, TE10 and TE13) and an oesophageal epithelial cell line (SHEE) were maintained at the Jiangsu Province Hospital Core Facility Center. All cell lines involved in the present study were purchased from the Shanghai Institute of Biochemistry and Cell Biology. The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific).
Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). All cells were cultured in a humidified chamber with 5% CO₂ at 37°C.

**RNA extraction and reverse transcription-quantitative (RT-q) PCR.** In accordance with the manufacturer’s instructions, total RNA was extracted from cells using TRIzol® (Thermo Fisher Scientific, Inc.). A NanoDrop spectrophotometer (ND-100, Thermo Fisher Scientific, Inc.) was used to detect the quality and concentration of RNA. RNA reverse transcription was conducted with a New Poly (A) Tailing kit (Thermo Fisher Scientific, Inc.) and a PrimeScript RT Master Mix kit (cat. no. RR036A; Takara Bio, Inc.). The temperature and duration of RT were as follows: 37°C for 15 min, followed by 85°C for 5 sec and 4°C for 10 min. RT-PCR was performed utilizing Universal SYBR Green Master Mix (cat. no. 4913914001; Roche Diagnostics) with a 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were: 95°C for 10 min, followed by 40 cycles at 95°C for 5 sec, 55°C for 30 sec and 72°C for 30 sec. Expression levels were calculated using the 2^ΔΔCq method (16). The relative mRNA expression was normalized to β-actin. All-in-one™ qPCR primers for CDC2A2 were purchased from GeneCopoeia, Inc. All experiments were repeated three times. Primer sequences for CDC2A2 and β-actin are as follows: CDC2A2 forward, 5’-TGCCTCAGGTATGTGGAAGA-3’ and reverse, 5’-CCCATCTGGCTCGCTG-3’ and reverse, 5’-GCTGTACCTCCAAGCTCC-3’.

**Lentivirus transfection.** Human CDC2A2-targeting short hairpin (sh)RNA sequences (CCGGCTGTTGCAAGAGGGAAAGTAACCTCATTCTTCTTCTTGCCACA GTTTTTT) were cloned into hU6-MCS-CMW-puromycin (Shanghai GenePharma Co., Ltd.). The titre of lentivirus was 2.37x10^8 TU/ml, and the best transduction efficiency in the current study was at multiplicity of infection=5. Lentivirus transduction was conducted according to the manufacturer’s instructions. Lentivirus was added into cells at room temperature. Cells were placed in an incubator at 37°C for 12 h, and then the medium was changed. As a negative control (NC), a shRNA with a scrambled sequence was used. The transfected cells were selected by puromycin until stably transfected cells were obtained. The concentration of puromycin in medium was 3 µg/ml. Transfection efficiency was assessed by western blot analysis and RT-qPCR. Subsequent experiments were performed at least 72 h after transfection.

**Cell Counting Kit-8 (CCK-8) assay.** Cells were seeded into a 96-well plate at a density of 3,000 cells per well. Following cell adherence, 100 µl of mixed solution (CCK-8 solution: RPMI-1640 medium=1:10) was added to each well according to the manufacturer’s instructions at the indicated time points (days 1, 2, 3 and 4). Following a 2-h incubation period at 37°C, absorbance was determined with a microplate reader (cat. no. ELx800; BioTek Instruments, Inc.).

**5-Ethynyl-2′-deoxyuridine (EdU) incorporation assay.** An EdU assay kit (Guangzhou RiboBio Co., Ltd.) was used to assess cellular proliferation. Cells were cultured in RPMI-1640 medium containing 10% FBS at 37°C in 24-well plates in triplicate at a density of 5x10^4 cells per well overnight. Cells were then maintained for 2 h at 37°C in medium containing 50 µM EdU and treated according to the manufacturer’s instructions. Typical images were captured under a fluorescence microscope (magnification, x40; Nikon Corporation). The proportion of EdU-positive cells among the cells from three random fields was analysed using ImageJ software (Java 1.6.0_20; National Institutes of Health).

**Clonogenic survival assays.** Unequal numbers of exponentially growing cells (0 Gy: 300 cells, 2 Gy: 600 cells, 4 Gy: 1,200 cells, 6 Gy: 3,000 cells and 8 Gy: 6,000 cells) were plated into 6-well plates. Following cell adherence, they were subjected to X-ray radiation. Immediately after radiation, the culture medium (RPMI-1640 medium containing 10% FBS) was renewed and the cells were cultured at 37°C for ≤12 days. The cells were fixed and then stained with crystal violet at room temperature for 30 min. The number of colonies (>50 cells/colony) were counted under a light microscope (magnification, x10). Plating efficiencies (PEs) were calculated as the number of colonies divided by the number of cells seeded. The surviving fraction (SF) of each radiation group was corrected by the PE of the nonirradiated control. Dose-response clonogenic survival curves were plotted on a log-linear scale. Cell survival curves based on the mean survival fractions of the cell line were fitted to a multitarget single-hit model: S=1-(1-e^{-D/D0})^N (17). The experiment was repeated three times.

**Immunofluorescence.** Immunofluorescence detection of γH2AX foci was utilized to evaluate DNA double-strand breaks (DSBs) in ESCC cells. The cells were seeded on a glass-bottomed dish and then irradiated with a single 8 Gy dose. Then, 2, 6 and 24 h after radiation, the cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. Cells were stained with a rabbit anti-γH2AX monoclonal antibody (1:200; cat. no. ab229914; Abcam) overnight at 4°C and then with Alexa 555 Fluor secondary antibody (1:500; cat. no. A0453; Beyotime Institute of Biotechnology) for 1.5 h at room temperature. The nuclei were counterstained with 2 µg/ml DAPI (cat. no. C1005; Beyotime Institute of Biotechnology) for confocal microscopy (Zeiss AG; magnification, x40) for 30 min at room temperature.

**Cell cycle detection.** First, cells were seeded into a 6-well plate at a density of 1x10^5 cells per well and treated with 6-MV X-ray radiation at doses of 0 or 8 Gy. Then, the cells were collected and stained with PI/RNase Staining Buffer (BD Biosciences) for 20 min at room temperature, according to the manufacturer’s protocol. The cell cycle was detected utilizing flow cytometry (FACSCalibur; BD Biosciences) and interpreted using FlowJo software (V10; FlowJo LLC). The experiment was repeated three times.

**Western blotting.** Total protein was separated from cell lysates on ice using RIPA buffer (Beyotime Institute of Biotechnology). An equal amount of protein (40 µg), whose concentration was quantified by a BCA kit (Beyotime Institute of Biotechnology), was separated on a 10% SDS-polyacrylamide gel and then
transferred to PVDF membranes. Afterwards, the membranes were blocked with 5% skimmed milk powder for 2 h at room temperature, followed by incubation with primary antibodies in dilution buffer at 4˚C overnight. The next day, HRP-linked anti-rabbit secondary antibodies (1:3,000; cat. no. 7074; Cell Signaling Technology, Inc.) were incubated at room temperature for 2 h. Western blotting analysis was conducted using a rabbit anti-CDCA2 monoclonal antibody (1:1,000; cat. no. 14976; Cell Signaling Technology, Inc.). An anti-GAPDH polyclonal antibody (1:5,000; cat. no. 10494-1-AP; ProteinTech Group, Inc.) was used as a loading control. The signals were visualized via an enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Inc.) and a chemiluminescence detection system.

Gene Set Enrichment Analysis (GSEA). GSEA was performed using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway gene sets in the Molecular Signatures Database against two probe-level expression matrices via the GSEA v3.0 software (www.broadinstitute.org/gsea).

Xenograft tumours in nude mice. The present study was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (approval no. 2103063). In total, 24 male BALB/c nude mice (17-20 g; 4-6 weeks old) were obtained from the Nanjing Medical University Animal Center and raised in a specific pathogen-free environment under a 12-h light-dark cycle at 23±1˚C and 50±5% humidity atmosphere. The mice were divided into four groups (n=6): i) shNC; ii) shCDCA2; iii) shNC and irradiation; and iv) shCDCA2 and irradiation. A total of 2x10⁶ KYSE450 cells were subcutaneously implanted into the flanks of mice. At ~20 days post injection, mice were exposed to irradiation (8 Gy) once in an RS 2000 Pro XRay Bioirradiator (Radsource) and then sacrificed at day 35. The mice were sacrificed with an intraperitoneal injection of 1% pentobarbital sodium at 100 mg/kg. The criteria for death were sustained non-spontaneous breathing for 2-3 min and no blink reflex.

Statistical analysis. The data are expressed as the mean ± standard deviation. Differences between groups were analysed using unpaired Student's t-test or one-way and mixed design ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference. Statistical analyses were performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc.). All experiments were repeated at least three times.

Results

CDCA2 is upregulated in human ESCC tissues and cell lines. First, ESCC-related gene expression profiles of tumours and normal tissues were analysed using information extracted from TCGA database (Fig. 1A and B). The results showed...
that, compared with the expression in normal tissues, the expression of CDCA2 in tumour samples was significantly upregulated (Fig. 1C). Afterwards, the CDCA2 expression levels were detected by RT-qPCR and western blot analysis in ESCC cell lines (Fig. 2A and D). As the results showed that CDCA2 was highly expressed in ESCC cells, especially in KYSE450 and TE13 cell lines, KYSE450 and TE13 cells were ultimately selected for further experiments. According to these results, it was hypothesized that CDCA2 might act as a regulator of tumour biological behaviour in ESCC. Since the function and mechanism of CDCA2 in the proliferation and radioresistance of ESCC cells remain unclear, it was intended to conduct related experiments.

To determine the important roles of CDCA2 in contributing to ESCC radiosensitivity, stable CDCA2 knockdown cell lines of both KYSE450 and TE13 cells were successfully generated by utilizing a specific shRNA against CDCA2. Then, RT-qPCR was performed to check the downregulation of CDCA2 in the two cell lines (Fig. 2B and C). Western blot analysis was also conducted to confirm the results (Fig. 2E and F).

**CDCA2 promotes the proliferation of ESCC cells.** CCK-8 assays were conducted to explore the influence of CDCA2 knockdown on ESCC proliferation. The proliferation rates of the cells in which the expression of CDCA2 was downregulated were significantly lower than those of the control groups (Fig. 3A and B). The results indicated that CDCA2 could regulate tumour growth and it was hypothesized that it might induce radioresistance in ESCC. The role of CDCA2 in cell proliferation was also verified via a more sensitive and specific EdU assay. As demonstrated in Fig. 3C and D, CDCA2 depletion in KYSE450 cells reduced the number of EdU-positive cells. Similar results were observed in TE13 cells (Fig. 3E and F). According to these results, the downregulation of CDCA2 could effectively inhibit ESCC cell proliferation.

**CDCA2 regulates the radiosensitivity of ESCC cells.** The role of CDCA2 in the radioresistance of ESCC was further determine. First, clonogenic survival assays were performed to assess the influence of CDCA2 on radiosensitivity. As demonstrated in Fig. 4A, the surviving cell fraction of KYSE450 cells with CDCA2 downregulation was distinctly lower compared with the control group, which was exposed to X-ray radiation. TE13 cells with reduced CDCA2 expression showed a similar outcome (Fig. 4B).

Immunofluorescence detection of γ-H2AX foci in KYSE450 and TE13 cell lines following X-ray radiation was used to explore whether CDCA2 was able to regulate radiation-induced DNA DSBs. As demonstrated in Fig. 4C-F, there were increased γ-H2AX signals at different time points (2 and 24 h) after irradiation in CDCA2-depleted cells compared with the control group. This suggested that downregulation of CDCA2 in ESCC cells could induce more DNA DSBs during radiation exposure. Collectively, downregulation of CDCA2 expression in oesophageal cancer cells enhanced cell radiation sensitivity.

**CDCA2 influences the cell cycle distribution of ESCC cells.** Previous studies have demonstrated that G2/M arrest is a pivotal mechanism for regulating radioresistance in ESCC (18-21). To explore whether CDCA2 could influence radiosensitivity in ESCC cells by regulating cell cycle distribution, every phase...
percentage with CDCA2 knockdown after radiation was quan-
tified (Fig. 5A-D). When exposed to X-ray radiation (8 Gy),
the CDCA2-downregulated cells had a higher G2/M phase
percentage than the controls in KYSE450 and TE13 cell lines
(Fig. 5E and F).

To study the possible link between CDCA2 and the cell
cycle phase transition pathway, GSEA was performed using
the KEGG pathway gene sets in the Molecular Signatures
Database against two probe-level expression matrices via the
GSEA v3.0 software (www.broadinstitute.org/gsea). It was
found that higher expression of CDCA2 was significantly
associated with the expression of related components of the
cell cycle phase transition and G2/M phase transition path-
ways (Fig. 6A and B). Collectively, the results revealed that
CDCA2 could regulate G2/M phase arrest to further influence
radioresistance in ESCC cells.

CDCA2 influences ESCC cell proliferation and radiosensi-
tivity in vivo. To confirm the effects of CDCA2 on ESCC cell
proliferation in vivo, BALB/c nude mice were injected with
TE13 cells transfected with shNC and shCDCA2. The tumour
weight of shCDCA2 group was significantly lower compared
with control group. This effect was evident after exposure to radiation (Fig. 6C and D) and markedly evident following exposure to radiation. The in vivo results were consistent with the in vitro results. This further confirmed that downregulation of CDCA in ESCC cells can inhibit cell proliferation and improve tumour radiation sensitivity.

**Discussion**

Numerous studies have demonstrated that the differences in the expression of various genes between cancer and adjacent normal tissues led to innovations in diagnostic techniques and chemotherapy treatment strategies for cancer (22,23). However, RT is regarded as the standard treatment modality in patients with ESCC who cannot be radically resected or those who refuse surgery (i.e., not fit for surgery) (4,7,8). RT serves a vital role in the local control of ESCC4 (1). However, distant metastasis and local recurrence frequently occur, thus causing RT resistance (1,9). Therefore, it is necessary to carry out genetic studies of oesophageal cancer. The expression levels of all known genes in oesophageal cancer and adjacent tissues were analysed in TCGA database. The present study confirmed that the expression of CDCA2 was higher in ESCC tissues compared with normal tissues. Several studies have demonstrated that CDCA2, as a cell division cycle-associated protein, is associated with tumour occurrence, progression and proliferation in several types of cancer, including melanoma, colorectal cancer, neuroblastoma, squamous cell carcinoma.
Figure 5. Inhibition of CDCA2 changes the cell cycle distribution in X ray-exposed ESCC cells. Cell cycle distribution of (A) KYSE450/shCDCA2 and (B) TE13/shCDCA2 cells. (C and D) Phase percentage with CDCA2 knockdown following radiation. (E and F) Downregulation of CDCA2 had a higher G2/M phase percentage. Mean ± standard deviation, n=3, **P<0.01, ***P<0.001, ****P<0.0001. CDCA2, cell division cycle-associated 2; ESCC, oesophageal squamous cell carcinoma; sh, short hairpin; NC, negative control; IR, ionising radiation; ns, not significant.
and others (11-13,24,25). Ionizing radiation affects cell proliferation through the cell cycle and cells at different stages of the cycle exhibit different radiation sensitivities (26,27). Hence, the present study explored the association between CDCA2 and radioresistance. To date, no reports, to the best of the authors' knowledge have examined the role of CDCA2 in ESCC radioresistance to induce radiosensitivity.

The in vitro experiments validated the abovementioned hypothesis. CDCA2 knockdown decreased ESCC cell proliferation. Dose-dependent clone formation was used to explore whether CDCA2 can affect radiosensitivity and the downregulation of CDCA2 inhibited the formation of cloning. These results revealed the action of CDCA2 in the regulation of ESCC radiosensitivity to induce radiosensitivity.

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![Image](image_url)

**Figure 6. GSEA analysis of CDCA2.** The knockdown of CDCA2 suppresses cell proliferation in vivo. (A and B) GSEA analysis showed that CDCA2 was associated with cell cycle phase transition pathways. (C) Representative xenografts each group were shown (n=3). (D) Tumour weight was recorded and presented as mean ± standard deviation. ***P<0.001, ****P<0.0001. GSEA, gene set enrichment analysis; CDCA2, cell division cycle-associated 2; sh, short hairpin; NC, negative control; IR, ionising radiation.

Histone H2AX is phosphorylated near DNA double-strand breaks and γH2AX can be used as a marker for DNA DSBs in chromatin and can be used to assess radiosensitivity (32). γH2AX phosphorylation foci were significantly higher in shCDCA2 cells at 2 and 24 h after radiation compared with the control groups. The persistence of γH2AX lesions has previously been demonstrated to be associated with radiosensitivity and the loss of lesions depends on effective DNA repair. As DNA is the main target of ionizing radiation and that DNA DSBs are a key lesion that causes cell death, CDCA2 knockdown can increase ESCC sensitivity to X-ray radiation (31,33).

Generally, cells are sensitive to radiation-induced DNA damage during G₂/M and G₁/S, while cells in the late S phase are most resistant to ionizing radiation (34). G₂/M phase arrest is the most sensitive stage of cell damage. After cells were exposed to X-ray irradiation, CDCA2 knockdown increased radiation-induced G₂/M phase arrest, as demonstrated by flow cytometry analysis.

Previous reports have demonstrated that downregulation of CDCA2 can induce G₁ arrest of lung adenocarcinoma cells and oral squamous cell carcinoma cells (12,13). In the
present study, CDCA2 showed a regulatory effect on the cycle distribution of oesophageal squamous cell carcinoma cells, however, it was the G1/M phase rather than the G2 phase. Radiation can increase the distribution in the G1/M phase and the present study showed that radiation combined with downregulation of CDCA2 can aggravate this effect. The reason may be that CDCA2 did not serve the G1-arresting function in these two cell lines, or the sample size was too small. The results of the present study did not contradict previous studies. To explore the relationship between CDCA2 and the cell cycle, GSEA was also conducted using the data of the ESCC cohorts from the TCGA database. Please provide a reference for this statement, remembering to update the reference list and in-text citations accordingly. The results of the present study revealed that CDCA2 could regulate the cell cycle distribution to further influence radioresistance in ESCC cells. However, the findings and mechanisms in the present study should be further tested in animal models and patient samples.

In conclusion, the present study found that silencing CDCA2 could suppress the growth and enhance the radiosensitivity of ESCC cells. CDCA2 is a potential molecular target of radioresensitization.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
BX, HuC, HoC and XS conceived the study, BX and HuC participated in data collection, analysis and interpretation, and drafted the manuscript. ZX and XY contributed to collecting samples and materials, and analyzing data. BX, HoC, ZX and XY participated in the analysis and interpretation of the results. All authors read and approved the final manuscript, and consented to publish this manuscript. XS and HYC confirmed the authenticity of all the raw data.

Ethics approval and consent to participate
The present study was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (approval no. 2103063) and was in accordance with China’s National Code of the Animal Care for Scientific Experimentation.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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