Gene 590 (2016) 244–249

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Downregulation of cell division cycle 25 homolog C reduces the radiosensitivity and proliferation activity of esophageal squamous cell carcinoma

Yachao Yin [a], Xiaoyan Dou [c], Shimiao Duan [b], Lei Zhang [b], Quanjing Xu [b], Hongwei Li [b], Duojie Li [b,*]

[a] Department of Radiotherapy, Anhui Cancer Hospital, Hefei, Anhui 230031, China
[b] Department of Radiotherapy, The First Affiliated Hospital of Bengbu Medical College, Bengbu 233004, Anhui, China
[c] Cyrus Tang Hematology Center, Jiangsu Instiute of Hematology, Soochow University, Suzhou 215123, Jiangsu, China

ARTICLE INFO

Article history:
Received 10 January 2016
Received in revised form 11 May 2016
Accepted 13 May 2016
Available online 14 May 2016

Keywords:
CDC25C
Esophageal cancer
Radiosensitivity
RNA interference

ABSTRACT

Radiation therapy is one of the most important methods of contemporary cancer treatment. Cells in the G2 and M phases are more sensitive to radiation therapy, and cell division cycle 25 homolog C (CDC25C) is essential in shifting the cell cycle between these two phases. In this study, the knockdown of CDC25C in human esophageal squamous carcinoma EC9706 cells was mediated by transfecting shRNA against human CDC25C-subcloning into pGV248. The levels of CDC25C mRNA and protein expression were assessed by reverse transcription–polymerase chain reaction (RT-PCR) and western blotting, respectively. Moreover, cell proliferation and radiosensitivity were measured. Stable CDC25C-knockdown EC9706 cell lines were successfully established. Furthermore, the proliferation of both control and CDC25C-shRNA-EC9706 cells was inhibited after the cells were treated with increasing X-ray doses, and the proliferation of the control cells was affected more significantly ($p < 0.05$). Moreover, cell colony formation assays allowed us to reach the same conclusion. Taken together, our experiments demonstrated that the knockdown of CDC25C can reduce both the radiotherapy sensitivity and the proliferation activity of EC9706 cells. Thus, CDC25C might be a potential biomarker for radiotherapy treatment.

1. Introduction

Esophageal squamous cell carcinoma (ESCC) is a prevalent malignancy worldwide, with the number of deaths increasing over time. Despite long-term efforts to develop novel cancer therapies, radiation therapy is still widely recognized as the current standard therapy for esophageal cancer. However, the radioresistance of ESCC decreases the efficacy of the treatment and knowledge of the associated molecular mechanisms may help to overcome this resistance (Zhao and Gu, 2014).

Recent evidence indicates that primary injury inhibiting damage repair and the cell cycle may increase the sensitivity of tumor cells to radiotherapy (Landsverk et al., 2011a). The cell division cycle protein 25 homolog C (CDC25C) gene encodes a 55-kDa protein including 14 introns and 14 exons, which is located in the chromosome 5q31 region, with a total length of approximately 2115 bp (Franckhauser et al., 2013). As a downstream molecule of ataxia telangiectasia mutated gene syndrome (ATM), CHK1 and CHK2, CDC25C is crucial for the cell cycle in the G2–M phase transition (Li et al., 2013). The G1–S and G2–M phase transitions are arrested in normal cells after exposure to ionizing radiation (Wang, 2014). It has been widely shown that the mutation of tumor suppressor gene P53 can induce tumorigenesis (Gaglia and Lahav, 2014). In fact, the P53 gene is mutated in the majority of tumor cells (Bieging et al., 2014). A previous study found that the cell cycle in G1–S phase blocking was inhibited when the P53 gene was mutated (Dimitrova et al., 2014), which makes tumor cells more dependent on the G2–M phase checkpoint for DNA repair (Landsverk et al., 2011b). Previous studies have shown that the over-expression of CDC25C is significantly associated with the better survival of locally advanced esophageal carcinoma patients treated with preoperative radiotherapy. He et al. (2013)) suggested that CDC25C might be used as an indicator for the response of esophageal squamous carcinoma patients in radiation therapy. However, the role of CDC25C in radiosensitization is still poorly understood.

In this study, human esophageal squamous carcinoma (EC9706) cell lines were selected as a cell model. Stable CDC25C-knockdown EC9706

Abbreviations: CDC25C, cell division cycle 25 homolog C; RT-PCR, reverse transcription-polymerase chain reaction; ESCC, Esophageal squamous cell carcinoma; CDK1-cyclinB, cyclin-dependent kinase1-cyclin B; shRNA, small hairpin RNAs; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; CE, cloning efficiency; SF, survival fraction; SER, sensitization enhancement ratio; SEM, standard error of the mean; ANOVA, assessed by one-way analysis of variance; NC, stable transfected with empty vector plasmid cell lines; ATM, ataxia telangiectasia mutated gene syndrome; HRS, low-dose radiation hypersensitivity; IRR, radiation resistance enhancement.

* Corresponding author.
E-mail addresses: 976920812@qq.com (Y. Yin), yycyyc1988@163.com (D. Li).

http://dx.doi.org/10.1016/j.gene.2016.05.025
0378-1119/

cell lines were established. Cell proliferation and colony formation were analyzed in CDC25C-knockdown EC9706 cells treated with various X-ray doses. The study aimed to explore the potential role of CDC25C in radiosensitization.

2. Materials and methods

2.1. Cell culture

The cell lines EC9706 and human kidney cell line HEK293T, purchased from ATCC, were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, USA) supplemented with 10% fetal bovine serum (FBS, BSZH, USA), 100 U/ml penicillin, and 100 U/ml streptomycin (Beyotime, China) under standard incubator conditions (37 °C, 5% CO_2).

2.2. Lentiviral shRNA cloning and transfection

Knockdown of CDC25C was induced by the transfection of cells with lentiviral vector-loaded CDC25C small hairpin RNAs (shRNAs) designed by Shanghai Genechem Co., Ltd. (Shanghai, China). Four shRNAs of CDC25C and a control were used, as follows:

shRNA1, 5′-CCGGGTCCCATTACTACTGTTCCAACTCGAGTTGGAACAG TAGTAATGGGACT3′; shRNA2, 5′-CCGGGAAGAGAATAATCATCGT GTTCTCGAGAACACGATGATTATTCTCTTCTTTTTG-3′; shRNA3, 5′-CCGG GCCTTGAGTTGCATAGAGATTCTCGAGAATCTCTATGCAACTC AAGGCTTTTTG-3′; shRNA4, 5′-CCGGGACAACACAATACCAGAT AAACTCGAGTTTATCTG GTATTGTGTTGTCTTTTTG-3′; shRNA-NC, 5′-CCGGTTCTCCGAACGTGT CAC GTCTCGAGACGTGACACGTTCGGAGAATTTTTG-3′.

EC9706 cells were individually transfected with vector pGV248 and the four different CDC25C-shRNAs. The element sequence of the vector hU6-MCS-Ubiquitin-EGFP-IRES-puromycin is shown in Fig. 1.

The cultured supernatant containing the viral particles was pooled at 24, 48 and 72 h after transfection. This supernatant was used as the shRNA viral stock solution. For lentiviral infection, the shRNA viral stock solution was added into EC9706 cell culture medium for 24 h in the presence of 4 μg/mL polybrene. After transfection, cells were selected using standard complete cultured medium and 1 μg/ml puromycin (the minimum concentration of puromycin (1 μg/ml) resulted in complete cell death after 3 days). At 72 h after transfection, the cell colonies resistant to puromycin were selected.

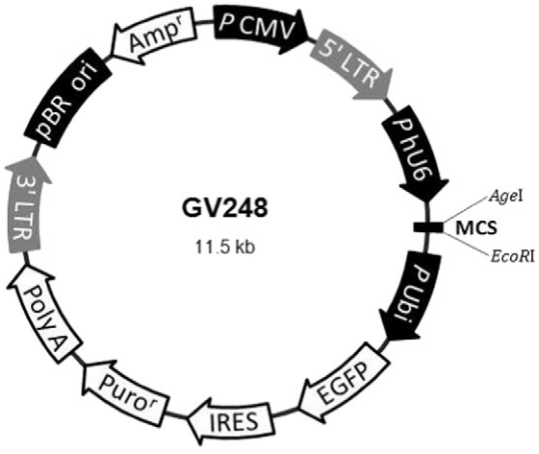

Fig. 1. Element sequence of vector hU6-MCS-Ubiquitin-EGFP-IRES-puromycin.

2.3. RT-PCR

Knockdown of CDC25C was further confirmed by RT-PCR. Briefly, total RNA was extracted from EC9706 cells using Trizol reagent (Sangon Biotech Co., Ltd., Shanghai, China) according to the protocol provided by the manufacturer. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control. Primer sequences for CDC25C detection were as follows: 5′-TGGTCACCTGGATTCTTC-3′ (forward) and 5′-ACCATTCGGAGTGCTA CA-3′ (reverse). Primer sequences for GAPDH detection were as follows: 5′-GTGAAGGTCGGAGTCAACG-3′ (forward) and 5′-GGTGAAGACGCCAGTGGACTC-3′ (reverse). The amplification conditions were as follows: 94 °C for 3 min, followed by 30 cycles for 30 s at 94 °C, 56 °C for 30 s, 72 °C for 1 min, ending with an additional extension step of 10 min at 72 °C. Finally, the PCR products were electrophoresed by 1.5% agarose gel electrophoresis and stained with ethidium bromide to analyze the products using UV light transilluminator visualization.

2.4. Western blot analysis

To calculate the protein expression levels of CDC25C, western blotting was performed as described previously (Bieging et al., 2014). Briefly, after transfection, EC9706 cells were washed with cold phosphate-buffered saline (PBS) three times and lyzed with radioimmunoprecipitation assay (RIPA) buffer solution (Beyotime, Shanghai, China). Supernatants were collected at 12,000 *g* for 20 min at 4 °C. The concentrations of samples were determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). Equal concentrations of proteins were mixed with sodium dodecyl sulfate (SDS) sample buffer, loaded onto 10% SDS–polyacrylamide gels for electrophoresis, then transferred onto a polyvinylidene fluoride (PVDF) membrane. β-actin was used as the loading control. Membranes were then blocked with nonfat milk for 1 h. Then the membranes were washed with tris-buffered saline and Tween 20 (TBST) three times, and incubated overnight at 4 °C with the following primary antibodies: anti-β-actin (1:1000, Millipore, USA) and anti-CDC25C (1:1000, Nanjing Enogene Biotech. Co., Ltd., China). After that, the membranes were washed with TBST three times, incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Biosharp, China) for 2 h at room temperature, and then detected by enhanced chemiluminescence (ECL-Plus kit, Beyotime, China). The fold changes of the protein levels were analyzed by the Image Lab software.

2.5. Irradiation

The EC9706 cells were first cultured in 96-well plates at a density of 5000 cells/well and incubated for 24 h at 37 °C in 5% CO_2. Then, the cells were irradiated at varying dose rates of 0, 1, 2, 4, and 6 Gy/min in appropriate conditions (GA 180°, CA 0°). Cell survival assays at each dose rate were repeated 5 times.

For the colony formation assay, EC9706 cell lines were plated in 6-well plates at a density of 500 cells/well in medium supplemented with 10% FBS. On the second day, the cells were treated at dose rates of 0, 1, 2, 4, and 6 Gy/min.

2.6. Assessment of proliferation by MTT assay

Cell proliferation was detected using the MTT assay. After incubation for 24, 48, and 72 h, the culture medium was replaced by 5% MTT solution with 200 μl per well. After 4 h, dimethyl sulfoxide (DMSO) was added to resolve the formazan generated from MTT. Finally, the absorbance value of each well was recorded on a microplate reader at a wavelength of 490 nm to calculate cell proliferation.