Translational Oncology 18 (2022) 101304

Contents lists available at ScienceDirect

Translational Oncology

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

Original Research

LncRNA DLGAP1-AS2 promotes the radioresistance of rectal cancer stem cells by upregulating CD151 expression *via* E2F1

Sheng-Ying Xiao [a], Zhi-Guang Yan [b], Xiao-Dong Zhu [c], Jun Qiu [a], Yi-Chen Lu [a], Fu-Ren Zeng [a,*]

[a] Department of Oncology, Hunan Provincial People's Hospital (The First Affiliated Hospital of Hunan Normal University), No.89, Guhan Road, Changsha, Hunan Province 410024, P R China
[b] Department of Orthopedics, Ningxiang Hospital Affiliated to Hunan University of Chinese Medicine, Ningxiang, Hunan Province 410600, P R China
[c] Department of Radiation Oncology, Guangxi Medical University Cancer Hospital, Nanning, Guangxi Province 530021, P R China

ARTICLE INFO

Keywords:
Radiation resistance
Rectal cancer stem cells
LncRNA DLGAP1-AS2
E2F1
CD151
AKT/mTOR/cyclinD1 pathway

ABSTRACT

Background: Radiotherapy resistance is one of the major causes of rectal cancer treatment failure. LncRNA DLGAP1-AS2 participates in the progression of several cancers. We explored the role and potential mechanism of DLGAP1-AS2 in the radioresistance of rectal cancer stem cells.

Methods: HR8348-R cells, radioresistant cells from HR8348 after irradiation, were isolated into CD133 negative ($CD133^-$) and positive ($CD133^+$) cells. Cell proliferation, apoptosis, migration and tumorsphere formation were determined by CCK-8, flow cytometry, wound healing assay and tumorsphere formation assay, respectively. CD133, tumor stem cell drug resistance gene (MDR1 and BCRP1), DNA repair marker (γ-H2AX) and AKT/mTOR/cyclinD1 signaling were measured by Western blot. The relationship between DLGAP1-AS2 and E2F1 was verified using RIP. The interaction between E2F1 and CD151 promoter was confirmed using dual-luciferase reporter gene assay and ChIP. AKT inhibitor API-2 was employed for validating the effect of AKT/mTOR/cyclinD1 signaling in the radioresistance of rectal cancer cells.

Results: The DLGAP1-AS2 level was increased in $CD133^+$ cells after irradiation. DLGAP1-AS2 knockdown inhibited the proliferation, migration and tumorsphere formation while stimulating apoptosis in $CD133^+$ cells. DLGAP1-AS2 inhibition downregulated the expression of CD133, MDR1, BCRP1 and γ-H2AX and suppressed AKT/mTOR/cyclinD1 activation. DLGAP1-AS2 upregulated the expression of CD151 by interacting with E2F1. API-2 neutralized the promotive effects of overexpressed CD151 on radioresistance.

Conclusion: DLGAP1-AS2 accelerates the radioresistance of rectal cancer cells through interactions with E2F1 to upregulate CD151 expression *via* the activation of the AKT/mTOR/cyclinD1 pathway.

Introduction

Rectal cancer is a common malignancy that continues to have a highly variable outcome [1, 2]. The clinical treatment of rectal cancer involves surgical removal and adjuvant therapies, including chemotherapy and radiotherapy [3]. Nevertheless, the prognosis is still not optimistic in rectal cancer patients [4], while radiotherapy resistance is one of major causes of the failure of rectal cancer management [5]. Cancer stem cells possess a highly effective DNA injury response system, contributing to the resistance of cancer cells to radiotherapeutic treatment [6]. Cancer stem cells are highly resistant to the current therapeutics and are the major cause of cancer recurrence [7]. A previous study has reported that cancer stem cells can perpetuate themselves *via* autorestoration and are highly resistant to the existing therapeutic methods [7]. The number of cancer stem cells and their inherent radioresistance characteristics are important parameters influencing the local control of cancer after radiotherapy [8]. The promoted stemness of colorectal cancer cells induced by cancer-associated fibroblasts increases radiation resistance [9]. CD133 is a five-transmembrane glycoprotein, which is considered to be a marker for distinguishing and separating colorectal cancer stem cells [10, 11]. Investigating the molecular mechanism of radiotherapy resistance in cancer stem cells is important for improving the clinical efficacy of radiotherapy in cancer patients. Currently, there is limited information on the role of cancer stem cells and their radiotherapy resistance mechanismin rectal cancer.

Long noncoding RNAs (lncRNAs), a category of recently identified

* Corresponding author.
E-mail address: 850694453@qq.com (F.-R. Zeng).

https://doi.org/10.1016/j.tranon.2021.101304
Received 2 September 2021; Received in revised form 28 November 2021; Accepted 29 November 2021

non-coding RNAs (ncRNAs) with a length of about 200 nucleotides, play important roles in various tumor behaviors [12]. LncRNA EGOT downregulation inhibits cell growth and improves the radiosensitivity of rectal cancer cells [13]. LncRNA-mediated regulation of cancer stem cells is significant in the radioresistance of cancers following radiotherapy [14, 15]. LncRNA DLGAP1-AS2 is discovered to be located at the human chromosome 18p11 [16]. Recently, it has been shown that DLGAP1-AS2 accelerates the progression of cholangiocarcinoma through miR-505 and GALNT10 [17]. In addition, DLGAP1-AS2 silencing represses cell migration and invasion by modulating the methylation of miR-154–5p in hepatocellular carcinoma [18]. However, the role of DLGAP1-AS2 in rectal cancer and radiotherapy resistance is still unclear.

As a well-characterized E2F family member, E2F1 is upregulated in colorectal cancer [19]. E2F1 is involved in promoting tumor growth in colorectal cancer [20]. It is also found that overexpression of E2F1 contributes to the oxaliplatin resistance of colorectal cancer cells [21]. E2F1 plays an important role in the progression of gastric cancer by maintaining gastric cancer stemness properties *via* the regulation of stemness-related genes [22]. E2F1 activation enhances the progression of neuroendocrine prostate cancer and self-renewal of prostate cancer stem cells [23]. CD151, a widely expressed transmembrane protein within the mammalian tetraspanin superfamily, is revealed to be a key player in the development of malignant diseases [24]. CD151 regulates CEACAM6, LGR5 and Wnt signaling to boost the progression of colorectal cancer [25]. In colorectal cancer, CD151 can be regulated by hypoxia to control cell adhesion and metastasis [26]. It has also been validated that increased CD151 expression is an important prognosis marker of poor outcome in colon cancer [27]. However, the relationship between E2F1 and CD151 in rectal cancer has not been reported.

Studies have demonstrated that activation of the AKT/mTOR/cyclinD1 pathway plays a role in the increased aggressiveness of oral cancer cells following low-level laser therapy [28]. The activated PI3K/AKT pathway after radiation facilitates the induction of cancer stem-like cells in colorectal cancer [29]. The downregulated cyclin D1 induced by MG132 pretreatment is associated with enhanced anti-growth and antimetastasis effects of radiation in human non-small-cell lung cancer cells [30]. CD151 knockdown could reduce AKT and mTOR phosphorylation in osteosarcoma [31]. However, the role of the AKT/mTOR/cyclinD1 pathway in the radioresistance of rectal cancer stem cells is still unclear.

In the present work, we mainly explored the effects of DLGAP1-AS2 knockdown, which decreased the radioresistance of rectal cancer stem cells *in vitro* by modulating CD151 expression *via* E2F1. Furthermore, we also investigated whether the AKT/mTOR/cyclinD1 signaling pathway was involved. Our results provided convincing evidence that knockdown of DLGAP1-AS2 could serve as a promising method for alleviating radiotherapy resistance in rectal cancer stem cells.

Materials and methods

Cell culture and treatment

HR8348 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI1640 medium (Thermo Fisher, Waltham, MA, USA) with 10% FBS, penicillin (100 U/mL, Sigma Aldrich, St. Louis, MO, USA) and streptomycin (100 μg/mL, Sigma). They were grown in an incubator with a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Radioresistant cells were obtained according to the previous description [29]. Briefly, cells were exposed to radiation (0.5 Gy) twice per day for 62 days using a Gammacell 3000 Elan irradiator (MDS Nordion, Ottawa, ON, Canada). After another 31 days in the absence of radiation, the acquisition of cell radioresistance was assessed by cell survival, recorded as HR8348-R cells.

Isolation of $CD133^+/CD133^-$ cells

Flow cytometry was carried out to evaluate the $CD133^+$ population. Prior to cell detachment and centrifugation, the cells were washed with sterile PBS. The pellet was blocked in filter-sterilized 2% BSA in PBS for 0.5 h. They were then treated with PE-labeled CD133 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) at 4 °C for 30 min. The samples incubated with the corresponding control IgG antibody were applied to set the gating level. After washing with PBS, $CD133^+/CD133^-$ cell sorting was performed with FACSAria (BD Biosciences, Mountain View, CA, USA). $CD133^-$ and $CD133^+$cells were cultured in serum-containing DMEM and DMEM F12 50/50 with added growth factors, respectively. These cells were exposed to radiation (5 Gy) prior to subsequent experiments.

Cell transfection

The sh-NC, sh-DLGAP1-AS2, overexpression vector (oe)-NC, oe-E2F1 and oe-CD151 were synthesized by GeneChem Company (Shanghai, China). For the knockdown of DLGAP1-AS2, specific short hairpin RNA (shRNA) targeting DLGAP1-AS2 was constructed using the pGPU6-GFP-Neo gene vector. The sequences for constructing the plasmids of shRNA were showed following: sh-NC: 5′-GTTCTCCGAACGTGTCACGT-3′, sh-DLGAP1-AS2: 5′-GCAAAATTAAAGATTTAGAAG-3′. The cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h, the cells were used for subsequent research. Moreover, 10 μmol/L API-2 (AKT inhibitor; Tocris Cookson Inc., Ellisville, MO, USA) was utilized to treat cells for 48 h.

CCK-8 assay

In the logarithmic growth phase, the cells were harvested and detached with 0.25% trypsin. The cells (2×10^3 cells/well) were seeded in 96-well plates. After culture for 0, 1, 2, 3, 4 and 5 days, sterile Cell Counting Kit-8 solution (10 μL) was added to each well prior to incubation at 37 °C for another 2 h. Finally, a Thermo Multiskan MK3 reader (Thermo Fisher) was used for optical density (OD) value analysis at 450 nm.

Cell apoptosis analysis

The Annexin V-FITC-PI apoptosis detection kit (Vazyme, Biotech Co., Ltd, Nanjing, China) was used for cell apoptosis detection according to the manufacturer's instructions. The cells (1×10^6 cells/well) were cultured in 6-well plates. Then, the cells were collected with 500 μL binding buffer and stained with Annexin V-FITC (5 μL) and PI (5 μL). Flow cytometry was used for apoptosis evaluation.

Wound healing assay

Cell migration was calculated by wound healing assay. Briefly, the cells were added into 6-well plates at 3×10^5 cells/well. A 10-μl sterile pipette tip was utilized to scrape the cell monolayer. Then, the cell migration path was tracked at 0 h and 24 h after wounding using a phase contrast microscope (Olympus Corporation, Tokyo, Japan). The wound healing area was analyzed using ImageJ software (National Institutes of Health, Bethesda, MA, USA).

Tumorsphere-formation assay

Tumorsphere-formation assay was carried out for evaluating self-renewal ability of the cancer cells [29]. After preparing a single-cell suspension, the cells were cultured in ultra-low-attachment 6-well or 96-well plates in the medium consisting of DMEM/F12 supplemented with 2% B27 supplements (Invitrogen), 10 ng/mL bFGF (Peprotech, Rocky Hill, NJ, USA) and 10 ng/mL EGF. They were cultured for 7 days;

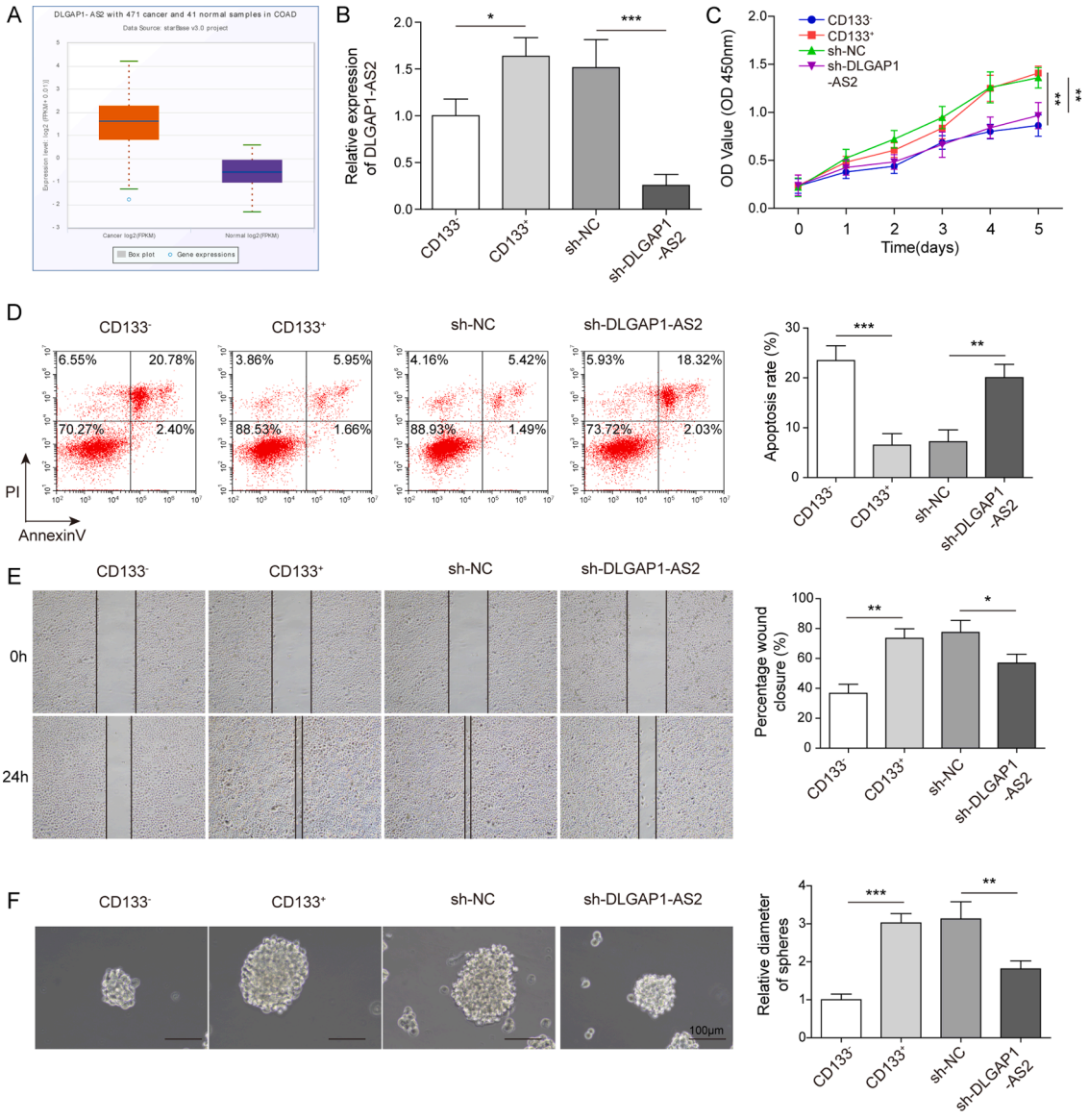

Fig. 1. LncRNA DLGAP1-AS2 knockdown inhibited the radiation resistance of rectal cancer stem cells.
(A) The starBase website predicted that DGAP1-AS2 was upregulated in colorectal cancer. HR8348-R cells, radioresistant cells obtained from HR8348 cells exposed to irradiation, were isolated into $CD133^{+}$ and $CD133^{-}$ cells, and $CD133^{+}$ cells were transfected with sh-DLGAP1-AS2 or sh-NC.(B) The DLGAP1-AS2 level analyzed by qRT-PCR. (C) CCK-8 assay showed cell proliferation. (D) Flow cytometry for cell apoptosis measurement. (E) Cell migration assessed using wound healing assay. (F) The images and size of tumoursphere cells assessed with tumoursphere-formation assay. *P<0.05, **P<0.01, ***P<0.001. The error bar represents the mean ± SD. Each experiment was repeated three times.

the sphere morphology was determined using a fluorescence microscope (Olympus), and the sphere number was counted.

RNA binding protein immunoprecipitation (RIP)

A EZMagna RIP kit (Shanghai Haoran Biological Technology Co., Ltd, Shanghai, China) was applied to analyze the interaction between DLGAP1-AS2 and E2F1 protein. Briefly, the cells resuspended in RIPA lysis (Beyotime, Shanghai, China) were centrifuged before supernatant removal. A 75 μL portion of beads was washed with RIP wash buffer and then resuspended. Each sample was treated with IgG antibody (1:300, Abcam, Cambridge, MA, USA) or E2F1 antibody (1:200, Abcam) for 1 h. The mixture was resuspended in RIP immunoprecipitation buffer (900 μL). Then, the samples placed on a magnetic base were washed with RIP wash buffer. For RNA extraction, 0.5 mg/mL proteinase K and 0.1% SDS were added into the complexes at 55 °C for 0.5 h.

Dual luciferase reporter gene assay

The JASPAR was used for predicting the putative E2F1 binding sites in the CD151 promoter. Wild-type (WT) and mutant (MUT) CD151 sequences containing the predicted E2F1 binding sites were cloned into psiCHECK-2 luciferase reporter plasmid (Promega, Madison, WI, USA) to produce luciferase reporter systems, including CD151-WT, CD151-S1 (MUT2&3), CD151-S2(MUT1&3) or CD151-S3(MUT1&2). The oe-NC or oe-E2F1 were co-transfected into cells with CD151-WT, CD151-S1, CD151-S2 or CD151-S3. After transfection for 48 h, luciferase reporter gene assay was carried out with the luciferase assay kit (Biovision, Milpitas, CA, USA). The internal reference gene was determined by renilla luciferase.

Chromatin immunoprecipitation (ChIP)

Cells were fixed for 10 min with formaldehyde, and a sonicator was

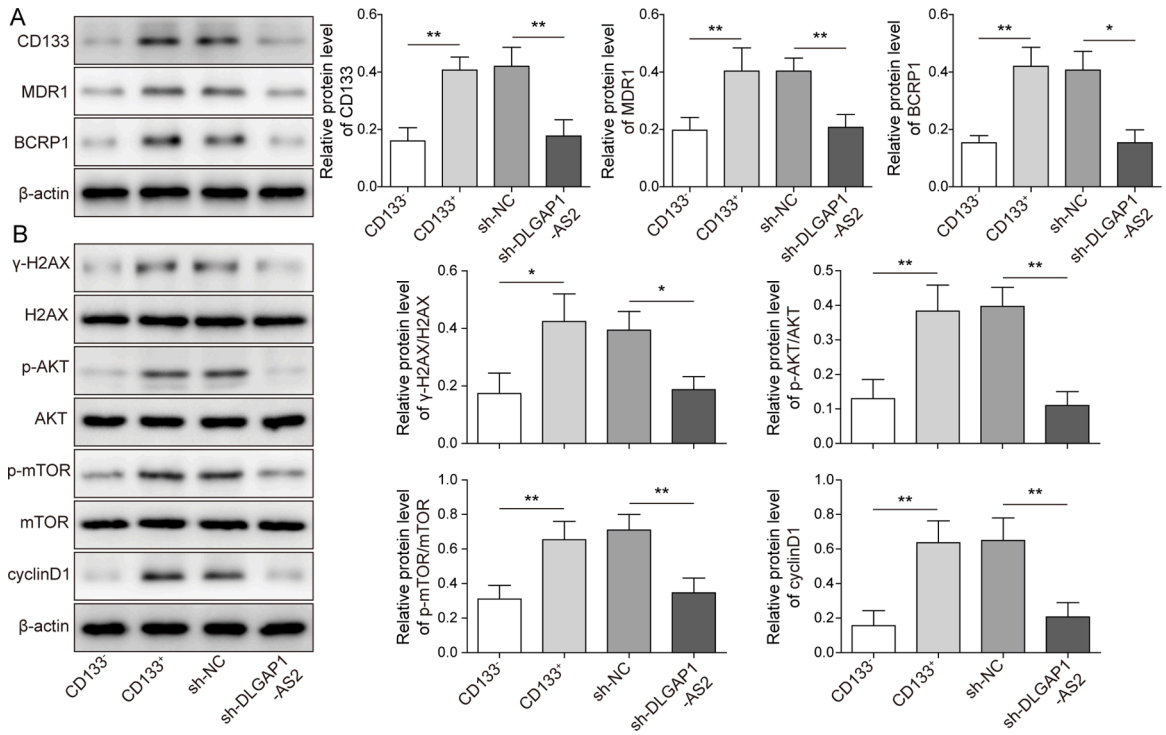

Fig. 2. LncRNA DLGAP1-AS2 knockdown inhibited the radiation resistance of rectal cancer stem cells.
(A–B) The protein levels of CD133, MDR1, BCRP1 and γ-H2AX and AKT/mTOR/cyclinD1 signaling detected by Western blotting. *$P<0.05$, **$P<0.01$, ***$P<0.001$. The error bar represents the mean ± SD. Each experiment was repeated three times.

used to fragment the chromatin. The cells were centrifuged before transferring into two tubes and separately incubated with E2F1 antibody (1: 200, Abcam) and IgG antibody (1: 300, Abcam) at 4 °C overnight. After precipitation with Protein Agarose/Sepharose, the complex was centrifuged. The nonspecific complex was eluted and de-crosslinked at 65 °C overnight. A purified DNA fragment extracted with phenol/chloroform was used as the amplification template and the fold enrichment of the CD151 promotor was validated by qRT-PCR. In addition, we also detected the interaction of E2F1and CD151 following DLGAP1-AS2 knockdown.

qRT-PCR

Total RNAs from the cells were isolated by the TRIzol kit (Thermo Fisher) and then quantitated with NanoDrop (Thermo Fisher). Single-stranded cDNA was synthesized with a SuperScript II Reverse Transcriptase (Thermo Fisher). qRT-PCR was performed using an SYBR Premix Ex Taq kit (TakaRa Biotechnology Co., Ltd., Dalian, China) in an ABI 7500 thermocycler (Applied Biosystems; Thermo Fisher). GAPDH was applied as an internal control. The relative gene expression was analyzed with the $2^{-\Delta\Delta Ct}$ method. The primer sequences were presented:

DLGAP1-AS2: F 5′-ACATCGTGGCTGAATGAACA-3′,
R 5′-ATCAGTGGGGAGGAAGGAGT-3′;
DLGAP1: F 5′- GCTAAATGCTCTCAGCTGCAC −3′,
R 5′- GCGCAGCAACTTTTGGAGAA −3′;
E2F1: F 5′-ATGTTTTCCTGTGCCCTGAG-3′,
R 5′-AGATGATGGTGGTGGTGAC-3′;
CD151: F 5′-ACAACTGCTGCTTCTGGCTG-3′,
R 5′-AGCAGGATGAAGTACAAGCG-3′;
GAPDH: F 5′-TAGATGACACCCGTCCCTGA-3′,
R 5′-ACCTCCACCTGTCCTTAGTG −3′.

Western blot

Protein was extracted from cells with a protein lysis buffer (Beyotime). Total proteins were quantitated with a BCA assay kit (Pierce, Rockford, IL, USA), followed by separation with 10% SDS-PAGE gels. Subsequently, the proteins were removed from gel to PVDF membrane and then blocked with 5% milk for 1 h. After incubation with primary antibody overnight at 4 °C, the membranes were incubated by HRP-conjugated secondary antibody for 2 h. Lastly, the blots were measured by ECL substrate. The primary antibodies included: anti-CD133 (130–092–395, 1:100, Miltenyi Biotec), anti-CD151 (SAB1402716, 1:50, Sigma), anti-E2F1 (ab4070, 1:500; Abcam), anti-MDR1 (ab170904, 1:100, Abcam), anti-BCRP1 (ab207732, 1:100, Abcam), anti-γ-H2AX (ab81299, 1:400; Abcam), anti-H2AX (ab11175, 1:200, Abcam), anti-AKT (#9272, 1:1000, Cell Signaling, Danvers, MA, USA), anti-p-AKT (#9271, 1:1000, Cell Signaling), anti-mTOR (ab134903, 1:100, Abcam), anti-p-mTOR (#2971, 1:100, Cell Signaling), anti-cyclinD1 (#2922, 1:1000, Cell Signaling) and anti-β-actin (A1978, 1:6000, Sigma).

Statistical analysis

Data were expressed as mean ± SD. Statistical analyses were carried out by SPSS 20.0. The data were analyzed by unpaired two-tailed Student's *t*-test (two groups) and one-way analysis of variance (multiple groups), followed by Tukey's post-test. All the samples were assayed in triplicate. $P<0.05$ was considered as statistically significant difference.

Results

LncRNA DLGAP1-AS2 knockdown inhibited the radioresistance of rectal cancer stem cells after irradiation

DLGAP1-AS2 has been revealed to accelerate cholangiocarcinoma progression [17]. Bioinformatics tool starBase website was used, which

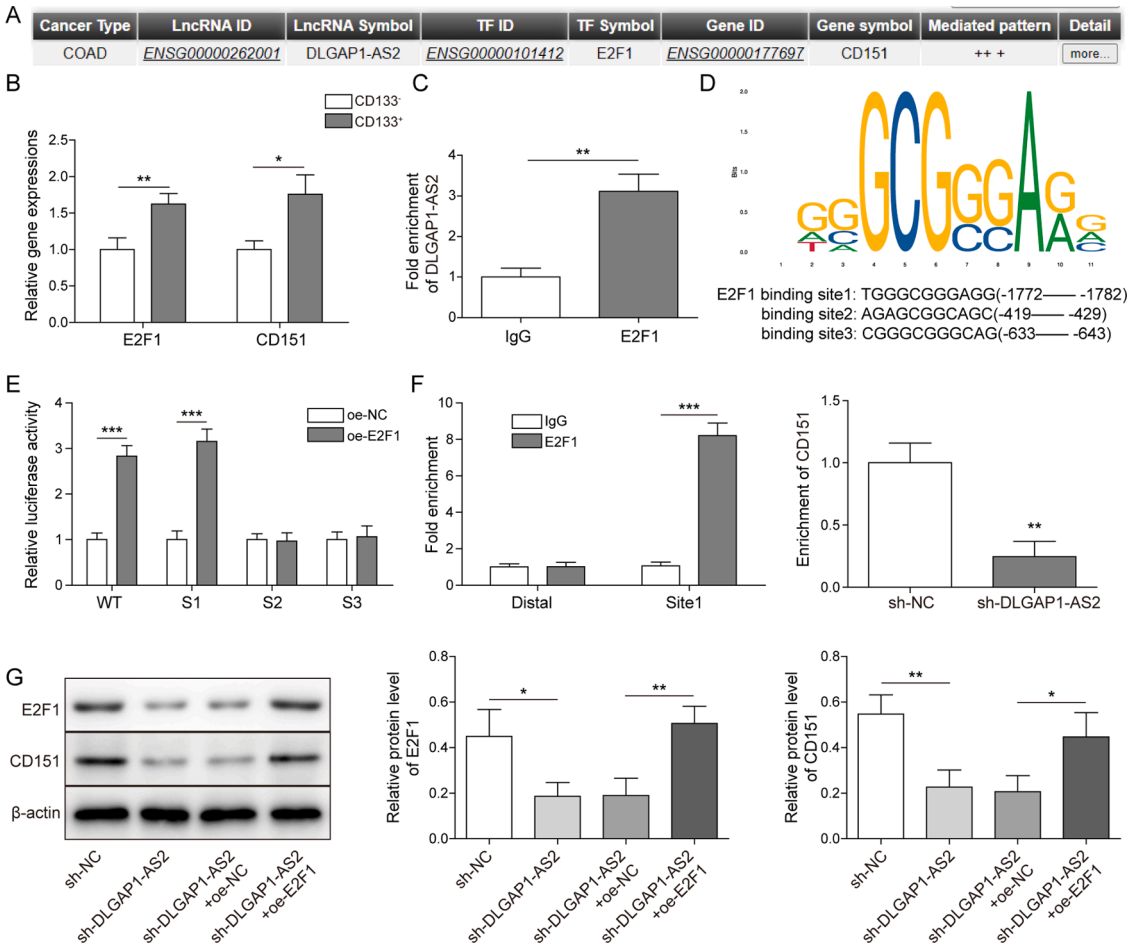

Fig. 3. DLGAP1-AS2 upregulated CD151 expression by modulating E2F1.
(A) The LncMAP website predicted that DLGAP1-AS2 regulated CD151 expression through E2F1 in colorectal cancer. (B) qRT-PCR showed the expressions of E2F1 and CD151 in $CD133^+$ and $CD133^-$ cells. (C) The interaction between E2F1 and DLGAP1-AS2 assessed by RIP assay. (D) The binding sites of E2F1 at the CD151 promoter predicted by JASPAR. (E) The relationship of E2F1 and CD151 promoter was examined by dual luciferase reporter gene assay. (F) ChIP assay for confirming the binding of E2F1 at the CD151 promoter region. (G) E2F1 and CD151 proteins were determined with Western blot. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. The error bar represents the mean ± SD. Each experiment was repeated three times.

predicted that DGAP1-AS2 was upregulated in colorectal cancer (Fig. 1A). To confirm the bioinformatics prediction, we then performed series of experiments to affirm the function of DLGAP1-AS2 in colorectal cancer. Radioresistance has been proposed to be related to the existence of cancer stem cells [7]. To explore the role and mechanism of DLGAP1-AS2 in the radioresistance of rectal cancer cells, HR8348-R cells with radioresistance were obtained from HR8348 cells exposed to radiation and then isolated into CD133 negative ($CD133^-$) and positive ($CD133^+$) cells. After radiation exposure, compared with $CD133^-$ cells, DLGAP1-AS2 expression was significantly increased in $CD133^+$ cells; therefore, we knocked down DLGAP1-AS2 in $CD133^+$ cells ($p<0.05$) (**Fig. 1B**). Moreover, the DLGAP1 mRNA level was downregulated in $C133^+$ cells following radiation ($p<0.05$), while it was slightly increased after the knockdown of DLGAP-AS2, but there was no statistically significant difference ($p>0.05$) (**Fig. S1**). In comparison with $CD133^-$ cells, the proliferation of $CD133^+$ cells was promoted after radiation, while DLGAP1-AS2 knockdown inhibited cell proliferation ($p<0.05$) (**Fig. 1C**). The cell apoptosis rate in $CD133^+$ cells was lower than that in $CD133^-$ cells, whereas the downregulation of DLGAP1-AS2 increased the cell apoptosis of $CD133^+$ cells exposed to radiation ($p<0.05$) (**Fig. 1D**). Compared with $CD133^-$ cells, cell migration was promoted in $CD133^+$ cells after radiation exposure, which was inhibited by sh-DLGAP1-AS2 ($p<0.05$) (**Fig. 1E**). In addition, the knockdown of DLGAP1-AS2 noticeably suppressed the tumorsphere formation of $CD133^+$ cells upon radiation exposure ($p<0.05$) (**Fig. 1F**). Compared with $CD133^-$ cells, CD133, MDR1, BCRP1 and γ-H2AX protein levels were elevated, and the AKT/mTOR/cyclinD1 pathway was activated, as indicated by the increased p-AKT, p-mTOR and cyclinD1 levels in $CD133^+$ cells after radiation exposure, while they were reversed by DLGAP1-AS2 silencing ($p<0.05$) (**Fig. 2A–2B**). Our data indicated that DLGAP1-AS2 knockdown suppressed the radiation resistance of rectal cancer stem cells.

DLGAP1-AS2 upregulated CD151 expression by interacting with E2F1

We next investigated the mechanism of DLGAP1-AS2 in the radiation resistance of rectal cancer stem cells. LncMAP website, a valuable tool for predicting the lncRNA-TF-gene regulatory network in cancers, predicted that DLGAP1-AS2 could regulate CD151 expression through transcription factor E2F1 in colorectal cancer (**Fig. 3A**). Subsequently, in our study, we examined the expressions of E2F1 and CD151 in $CD133^-$ and $CD133^+$ cells. In comparison with $CD133^-$ cells, E2F1 and CD151 levels were remarkably increased in $CD133^+$ cells after irradiation ($p<0.05$) (**Fig. 3B**). RIP assay showed an interaction between DLGAP1-AS2 and E2F1 ($p<0.05$) (**Fig. 3C**). JASPAR predicted there were three putative binding sites of E2F1 at the CD151 promoter region (**Fig. 3D**). Subsequently, dual luciferase reporter gene assay revealed that the overexpression of E2F1 significantly enhanced the activity of binding site 1 at CD151 promoter (**Fig. 3E**). The interaction between E2F1 and

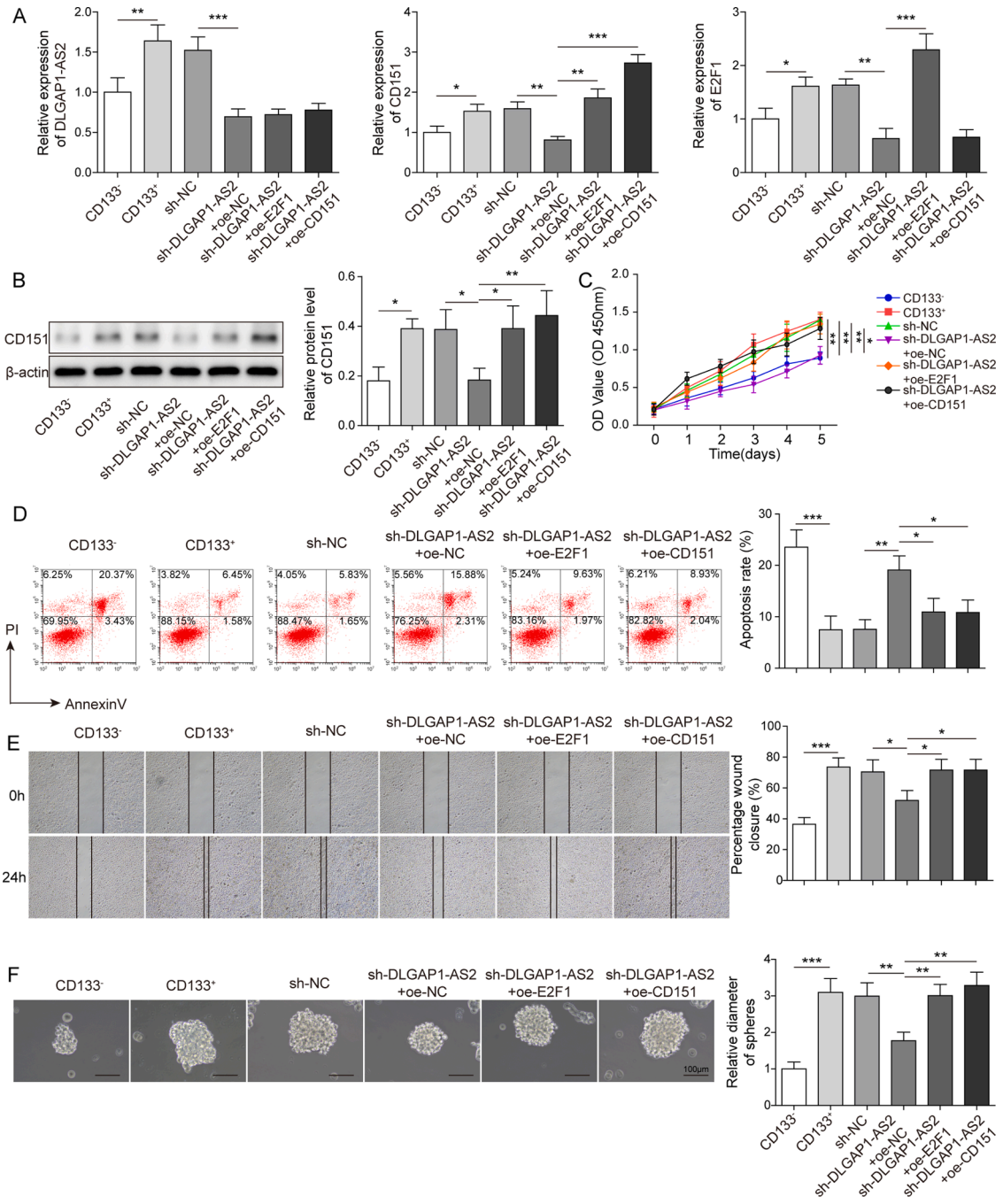

Fig. 4. DLGAP1-AS2 upregulated CD151 by interacting with E2F1 to regulate the radiation resistance of rectal cancer stem cells.
$CD133^+$ cells were transfected with sh-NC, sh-DLGAP1-AS2, oe-NC, oe-E2F1 or oe-CD151 after irradiation. (A) DLGAP1-AS2; the mRNA levels of CD151 and E2F1 measured by qRT-PCR. (B) CD151 protein levels analyzed with Western blot. (C) CCK-8 assay for cell proliferation detection. (D) Flow cytometry for the detection of cell apoptosis. (E) Cell migration evaluated using wound healing assay. (F) Tumorsphere-formation assay to measure the size of tumorsphere cells. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. The error bar represents the mean ± SD. Each experiment was repeated three times.

CD151 was further validated by ChIP assay, showing that the amplification product amount obtained by site 1 primer was higher in cells incubated with E2F1 antibody than in cells incubated with IgG ($p<0.05$), while no significant difference was measured in that from the distal primer (Fig. 3F). Moreover, RIP assay verified that DLGAP1-AS2 could regulate the binding of E2F1 at the CD151 promoter ($p<0.05$) (Fig. 3F). The expressions of E2F1 and CD151 were downregulated after DLGAP1-AS2 knockdown in $CD133^+$ cells exposed to radiation ($p<0.05$), which was reversed by E2F1 overexpression ($p<0.05$) (Fig. 3G). Our results revealed that DLGAP1-AS2 upregulated CD151 expression by modulating E2F1.

DLGAP1-AS2 promotes the radioresistance of rectal cancer stem cells by modulating E2F1 to upregulate CD151 expression

For exploring whether DLGAP1-AS2 regulated the radioresistance of rectal cancer stem cells by the E2F1/CD151 axis, sh-NC, sh-DLGAP1-AS2, oe-NC, oe-E2F1 or oe-CD151 were transfected to $CD133^+$cells after irradiation, alone or in combination. DLGAP1-AS2 silencing significantly downregulated the expressions of E2F1 and CD151 in $CD133^+$cells upon radiation exposure, while it was reversed by the overexpression of E2F1 or CD151 ($p<0.05$) (Fig. 4A–4B). Inhibition of DLGAP1-AS2 suppressed cell proliferation and promoted apoptosis,

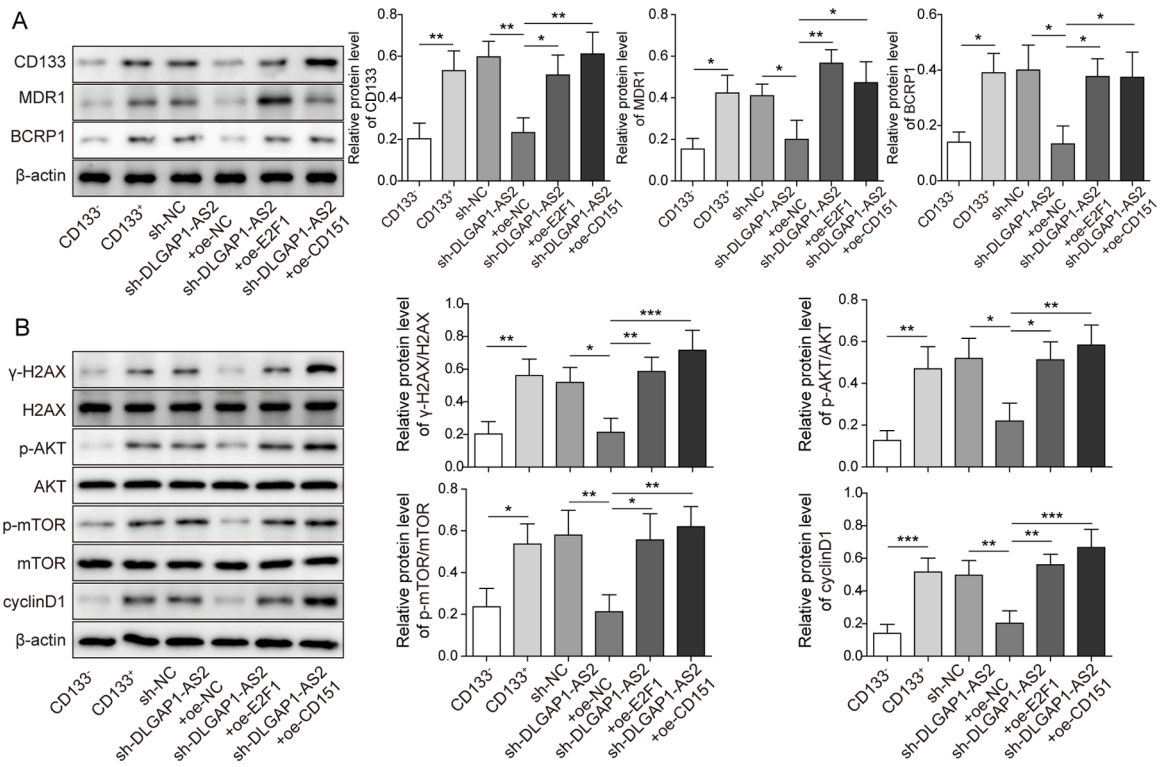

Fig. 5. DLGAP1-AS2 upregulated CD151 by interacting with E2F1 to regulate the radiation resistance of rectal cancer stem cells.
(A–B) Western blot for measuring the protein levels of CD133, MDR1, BCRP1 and γ-H2AX and AKT/mTOR/cyclinD1 signaling. *$P<0.05$, **$P<0.01$, ***$P<0.001$. The error bar represents the mean ± SD. Each experiment was repeated three times.

which was antagonized after the overexpression of E2F1 or CD151 in $CD133^+$ cells exposed to radiation ($p<0.05$) (Fig. 4C–4D). Besides, the knockdown of DLGAP1-AS2 inhibited cell migration and tumorsphere-formation, both of which were facilitated by the overexpression of E2F1 or CD151 ($p<0.05$) (Fig. 4E–4F). Subsequently, the upregulation of E2F1 or CD151 reversed the decreased CD133, MDR1, BCRP1, γ-H2AX and the inhibited AKT/mTOR/cyclinD1 pathway induced by DLGAP1-AS2 knockdown in $CD133^+$ cells after radiation exposure ($p<0.05$) (Fig. 5A–5B). We observed that DLGAP1-AS2 promotes the radiation resistance of rectal cancer stem cells by interacting with E2F1 to upregulate CD151.

Inhibition of AKT/mTOR/cyclinD1 signaling weakened the promotive effects induced by overexpressed CD151 on the radioresistance of rectal cancer stem cells following irradiation

To investigate the function of the AKT/mTOR/cyclinD1 pathway on the radioresistance of rectal cancer stem cells, 10 μmol/L AKT inhibitor API-2 was employed to treat cells for 48 h. CD151 overexpression apparently elevated p-AKT, p-mTOR and cyclinD1 expressions in $CD133^+$ cells after irradiation, which were inhibited by API-2 ($p<0.05$) (Fig. 6A). CD151 overexpression promoted cell proliferation, inhibited apoptosis and facilitated the migration of $CD133^+$ cells exposed to radiation, while these effects was reversed by API-2 ($p<0.05$) (Fig. 6B–6D). API-2 antagonized the promoted tumorsphere formation caused by the overexpression of CD151 ($p<0.05$) (Fig. 6E). Finally, the overexpression of CD151 increased CD133, MDR1, BCRP1 and γ-H2AX in $CD133^+$ cells exposed to radiation, which were neutralized by API-2 ($p<0.05$) (Fig. 7). We suggested that inhibition of the AKT/mTOR/cyclinD1 signaling pathway alleviated the facilitated impact of CD151 on the radioresistance of rectal cancer stem cells.

Discussion

Rectal cancer has been recognized as the most common malignant neoplasm of the gastrointestinal tract [32], while radiotherapy is one of the major treatment approaches for rectal cancer [33]. Studies have shown that radioresistance contributes to the failure of radiotherapy, which can be affected by lncRNAs [15]. Herein, we demonstrated that a high level of DLGAP1-AS2 was observed in $CD133^+$ rectal cancer stem cells. Knockdown of DLGAP1-AS2 regulated the radioresistance of rectal cancer stem cells following radiation. DLGAP1-AS2 interacted with E2F1 to elevate CD151 expression and then regulate the radioresistance of rectal cancer stem cells *via* the AKT/mTOR/cyclinD1 pathway, which indicated that DLGAP1-AS2 played a critical role in modulating the radiosensitivity of rectal cancer.

Radiotherapy is an effective treatment for many cancers; however, radioresistance is frequently encountered [34]. CD133, a marker for cancer stem cells, is associated with resistance to preoperative chemo-radiotherapy in low rectal cancer [35]. $CD133^+$ cells show reduced sensitivity to chemotherapy in colorectal cancer [36]. Radioresistance of cancer stem cells is related to the activation of anti-apoptosis genes and the increase in the self-renewal capability of cancer cells [37]. It has been investigated that lncRNAs play vital roles in the mediation of therapy resistance in a variety of cancers, including colorectal cancer [35]. LncRNA OIP5-AS1 regulates radioresistance in colorectal cancer cells [38]. Silencing of lincRNA-p21 results in enhanced stemness and radiation resistance in glioma stem cells [39]. LncRNA DLGAP1-AS2 inhibition represses cell migration and invasion in hepatocellular carcinoma [18], facilitates cholangiocarcinoma progression [17] and is significantly associated with the survival of patients with Wilms' tumor [40]. The role of lncRNA DLGAP1-AS2 in modulating the radiosensitivity of cancer cells, as well as its role in rectal cancer, is still unclear. In the current study, DLGAP1-AS2 knockdown restrained cell proliferation, migration and tumorsphere formation, whereas it promoted the apoptosis of $CD133^+$ HR8348-R cells after X-ray irradiation.

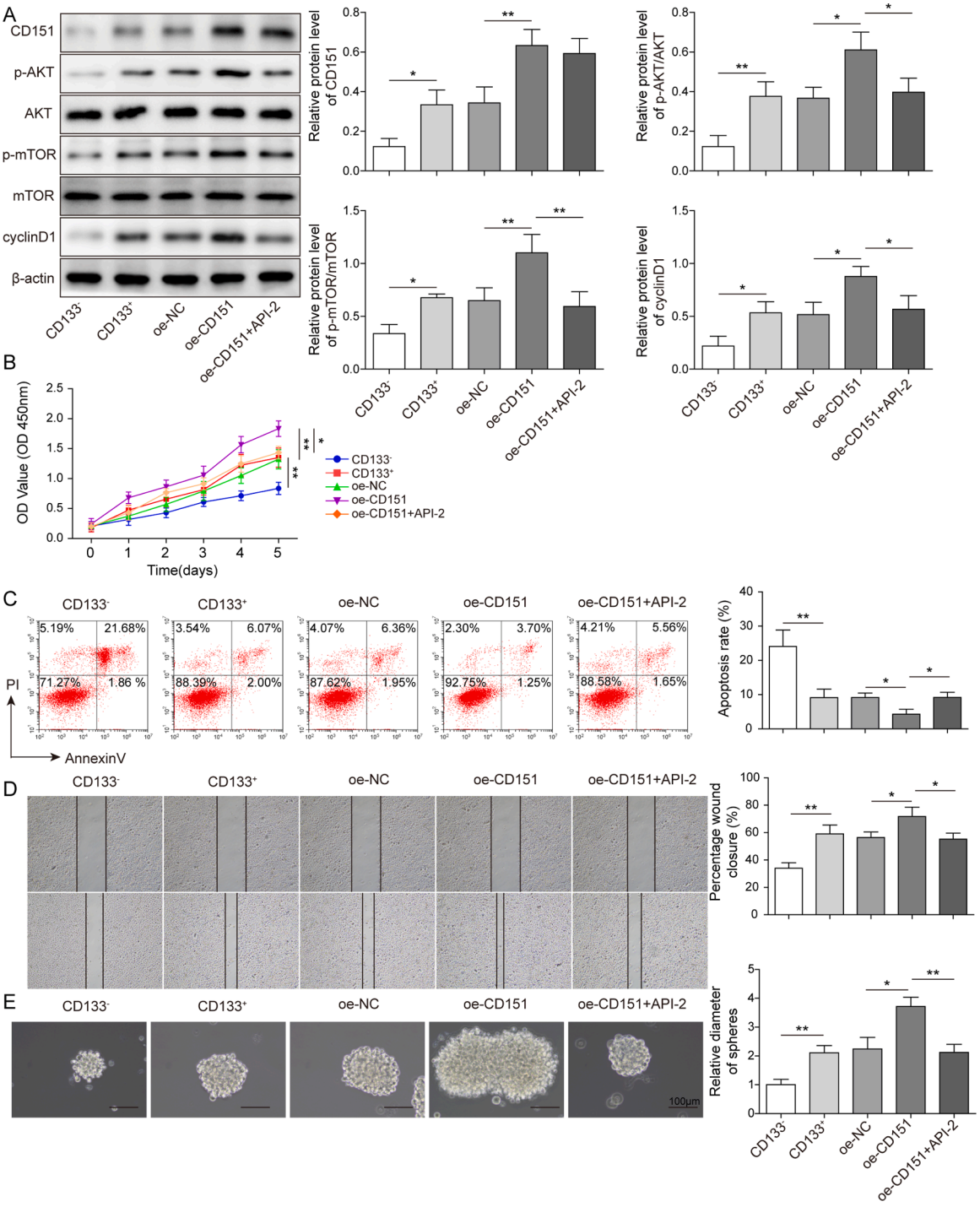

Fig. 6. Inhibiting AKT/mTOR/cyclinD1 signaling weakened the promotive effect of overexpressed CD151 on the radioresistance of rectal cancer stem cells. $CD133^+$ cells were transfected with oe-CD151, oe-NC or API-2, alone or in combination upon radiation exposure. (A) Western blot was used to determine the protein expressions. (B) Cell viability assessed with CCK-8 assay. (C) Flow cytometry for cell apoptosis evaluation. (D) Cell migration detected using wound healing assay. (E) The images and size of tumorsphere cells evaluated using tumorsphere-formation assay. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. The error bar represents the mean ± SD. Each experiment was repeated three times.

Furthermore, the cancer stem cell marker CD133, the drug-resistance genes MDR1 and BCRP1 and the DNA repair biomarker γ-H2AX were all reduced after DLGAP1-AS2 silencing in $CD133^+$ cells. Therefore, we proposed that the knockdown of lncRNA DLGAP1-AS2 diminished the radioresistance of rectal cancer stem cells. Our study explored, for the first time, the role of DLGAP1-AS2 in the radiation response of rectal cancer cells. Accordingly, targeting DLGAP1-AS2 may be an effective strategy to promote rectal cancer radiosensitivity.

A previous study indicated that lncRNA DLGAP1-AS2 regulates glioma progression by elevating the level of YAP1 [16]. The tumor promotive action of E2F1 has been proved in various cancers, including ovarian cancer [41] and colorectal cancer [42]. E2F1 is observed to play a critical role in the chemosensitivity regulation of colorectal cancer [20]. CD151 silencing can suppress cell viability, migration and invasion in colorectal cancer cells and inhibit mouse xenografts [25]. LncMAP and JASPAR websites show the association among lncRNA

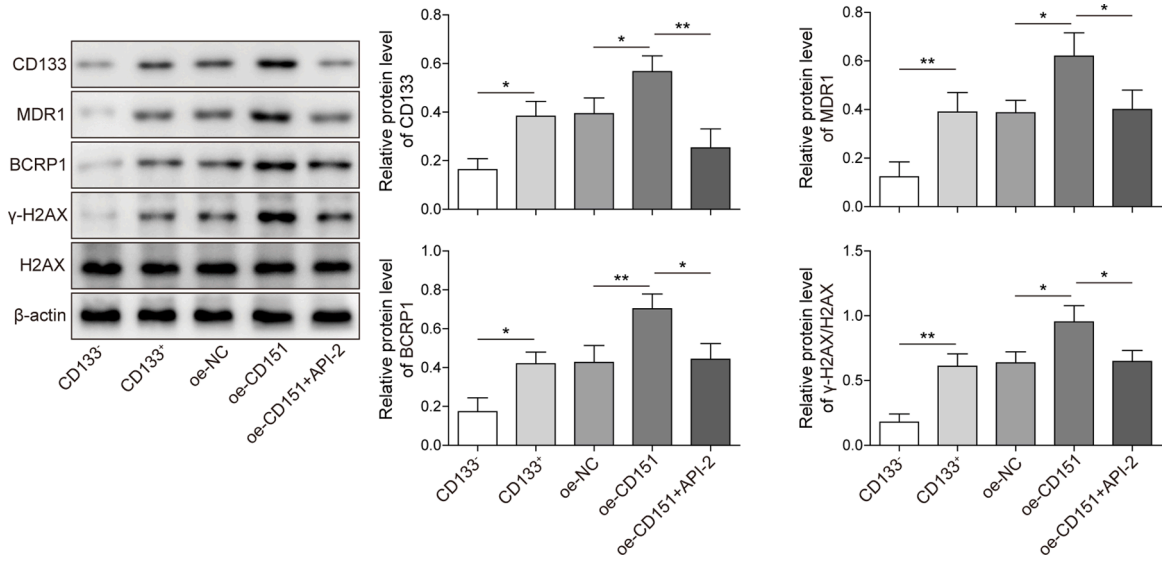

Fig. 7. Inhibiting AKT/mTOR/cyclinD1 signaling weakened the promotive effect of overexpressed CD151 on the radioresistance of rectal cancer stem cells. Protein levels of CD133, MDR1, BCRP1 and γ-H2AX were detected with Western blot. *$P<0.05$, **$P<0.01$, ***$P<0.001$. The error bar represents the mean ± SD. Each experiment was repeated three times.

DLGAP1-AS2, E2F1 and CD151. We confirmed that DLGAP1-AS2 bound to E2F1. Moreover, our data revealed the binding of E2F1 at the CD151 promoter region by ChIP and dual luciferase reporter gene assay. Importantly, DLGAP1-AS2 interacted with E2F1 to upregulate CD151 expression. In addition, overexpression of E2F1 or CD151 antagonized the impacts of DLGAP1-AS2 knockdown on cell proliferation, apoptosis, migration and tumorsphere formation of $CD133^+$ cells. Taken together, our findings indicated that lncRNA DLGAP1-AS2 modulated the radiation resistance of rectal cancer stem cells through the E2F1/CD151 axis. To the best of our knowledge, this is the first study to validate E2F1/CD151 as downstream factors modulated by DLGAP1-AS2 that participates in regulating the radiosensitivity of rectal cancer stem cells.

PI3K/AKT/mTOR signaling is commonly activated in most cancers, which is associated with resistance to radiotherapy [43]. Activation of the PI3K/AKT/mTOR pathway promotes radioresistance in hepatocellular carcinoma [44]. Activating the PI3K/AKT/mTOR signaling pathway is involved in the radiation resistance of colorectal cancer cells following radiation exposure [45]. The growth of oral cancer cells is modified through the regulation of the AKT/mTOR/cyclinD1 signaling pathway following low-level laser therapy [28]. Whether the AKT/mTOR/cyclinD1 signaling pathway is involved in the radioresistance of rectal cancer cells needs to be verified. We provided the first direct evidence showing that the activated AKT/mTOR/cyclinD1 pathway in $CD133^+$ cells was inhibited after DLGAP1-AS2 silencing. AKT inhibitor API-2 suppressed the activated AKT/mTOR/cyclinD1 pathway induced by the overexpression of CD151. We also confirmed that the overexpression of CD151 facilitated the radioresistance of rectal cancer stem cells, which was alleviated after the suppression of AKT/mTOR/cyclinD1 signaling. Our data demonstrated that the AKT/mTOR/cyclinD1 signaling pathway was implicated in the regulation of DLGAP1-AS2, influencing the radioresistance of rectal cancer stem cells.

Conclusions

In conclusion, we found that the knockdown of lncRNA DLGAP1-AS2 reduced the radiotherapy resistance of rectal cancer stem cells by regulating E2F1 leading to decreased CD151 *via* the inhibition of AKT/mTOR/cyclinD1 signaling, which revealed that lncRNA DLGAP1-AS2 silencing might be one of the useful strategies for enhancing the sensitivity of rectal cancer stem cells to radiotherapy.

Abbreviations

long non-coding RNAs (lncRNAs); long non-coding RNA (lncRNA); DLGAP1 antisense RNA 2 (DLGAP1-AS2); cluster of differentiation 151 (CD151); E2 promoter binding factor 1 (E2F1); protein kinase B (AKT); the mammalian target of rapamycin (mTOR); multidrug resistance protein 1 (MDR1); breast cancer resistance protein (BCRP1); Ser-139 phosphorylated histone H2AX (γ-H2AX); long noncoding RNA (lncRNA); eosinophil granule ontogeny transcript (EGOT); *N*-acetylgalactosaminyltransferase 10 (GALNT10); carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6); leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5); Yes-associated protein 1 (YAP1); 3-phosphoinositide-dependent protein kinase-1 (PDK1); long intergenic noncoding RNA (lincRNA); Y box binding protein 1 (YB1); interleukin 9 (IL-9); estrogen receptor beta (ERβ)

Authors' contribution

SYX: Conceptualization; Funding acquisition;
ZGY: Writing-original draft;
XDZ: Data curation; Resources;
JQ: Methodology; Formal analysis;
YCL: Investigation; Software; Visualization;
FRZ: Project administration; Supervision; Validation; Writing-review & editing.

All authors have read and approved the final version of this manuscript to be published.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to express our sincere gratitude to the reviewers for their constructive comments.

Funding

This work was supported by the Renshu Fund Project (RS201815).

Ethical approval

Not applicable. This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

Not Applicable. This article does not contain any studies with human participants performed by any of the authors.

Availability of data and material

All data generated or analyzed during this study are included in this article. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101304.

References

[1] F. Iafrate, et al., Preoperative staging of rectal cancer with MR Imaging: correlation with surgical and histopathologic findings, Radiographics 26 (2006) 701–714.
[2] F. Bray, et al., Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA Cancer J. Clin. 68 (2018) 394–424.
[3] S. Li, et al., Combined liposome-mediated cytosine deaminase gene therapy with radiation in killing rectal cancer cells and xenografts in athymic mice, Clin. Cancer Res. 11 (2005) 3574–3578.
[4] F. Lopez-Campos, et al., Watch and wait approach in rectal cancer: current controversies and future directions, World J. Gastroenterol. 26 (2020) 4218–4239.
[5] A.M. Buckley, N. Lynam-Lennon, H. O'Neill, J. O'Sullivan, Targeting hallmarks of cancer to enhance radiosensitivity in gastrointestinal cancers, Nat. Rev. Gastroenterol. Hepatol. 17 (2020) 298–313.
[6] E. Abad, D. Graifer, A. Lyakhovich, DNA damage response and resistance of cancer stem cells, Cancer Lett. 474 (2020) 106–117.
[7] M.R. Atashzar, et al., Cancer stem cells: a review from origin to therapeutic implications, J. Cell. Physiol. 235 (2020) 790–803.
[8] A. Schulz, F. Meyer, A. Dubrovska, K. Borgmann, Cancer Stem Cells and Radioresistance: DNA Repair and Beyond, Cancers (Basel) 11 (2019).
[9] L. Liu, et al., Cancer associated fibroblasts-derived exosomes contribute to radioresistance through promoting colorectal cancer stem cells phenotype, Exp. Cell Res. 391 (2020), 111956.
[10] F. Ren, W.Q. Sheng, X. Du, CD133: a cancer stem cells marker, is used in colorectal cancers, World J. Gastroenterol. 19 (2013) 2603–2611.
[11] S. Saigusa, et al., Correlation of CD133, OCT4, and SOX2 in rectal cancer and their association with distant recurrence after chemoradiotherapy, Ann. Surg. Oncol. 16 (2009) 3488–3498.
[12] Y. Chi, D. Wang, J. Wang, W. Yu, J. Yang, Long non-coding RNA in the pathogenesis of cancers, Cells 8 (2019).
[13] C. Li, et al., LncRNA EGOT/miR-211-5p affected radiosensitivity of rectal cancer by competitively regulating ErbB4, Onco Targets Ther 14 (2021) 2867–2878.
[14] H.C. Chi, C.Y. Tsai, M.M. Tsai, C.T. Yeh, K.H. Lin, Roles of long noncoding RNAs in recurrence and metastasis of radiotherapy-resistant cancer stem cells, Int. J. Mol. Sci. 18 (2017).
[15] J. Zhu, et al., Molecular mechanisms of lncRNAs in regulating cancer cell radiosensitivity, Biosci. Rep. 39 (2019).
[16] W. Miao, et al., LncRNA DLGAP1-AS2 modulates glioma development by up-regulating YAP1 expression, J. Biochem. 167 (2020) 411–418.
[17] Z. Liu, L. Pan, X. Yan, X. Duan, The long noncoding RNA DLGAP1-AS2 facilitates cholangiocarcinoma progression via miR-505 and GALNT10, FEBS Open Bio 11 (2021) 413–422.
[18] K. Chen, et al., lncRNA DLGAP1-AS2 knockdown inhibits hepatocellular carcinoma cell migration and invasion by regulating miR-154-5p methylation, Biomed. Res. Int. 2020 (2020), 6575724.
[19] Z. Fang, et al., YY1 promotes colorectal cancer proliferation through the miR-526b-3p/E2F1 axis, Am J Cancer Res 9 (2019) 2679–2692.
[20] C. Zhou, et al., circCAMSAP1 promotes tumor growth in colorectal cancer via the miR-328-5p/E2F1 axis, Mol. Ther. 28 (2020) 914–928.
[21] Z. Fang, et al., NFYB-induced high expression of E2F1 contributes to oxaliplatin resistance in colorectal cancer via the enhancement of CHK1 signaling, Cancer Lett. 415 (2018) 58–72.
[22] Y. Fu, C. Hu, P. Du, G. Huang, E2F1 maintains gastric cancer stemness properties by regulating stemness-associated genes, J. Oncol. 2021 (2021), 6611327.
[23] M. Hagiwara, et al., MUC1-C activates the BAF (mSWI/SNF) complex in prostate cancer stem cells, Cancer Res. 81 (2021) 1111–1122.
[24] M.E. Hemler, Targeting of tetraspanin proteins–potential benefits and strategies, Nat. Rev. Drug Discov. 7 (2008) 747–758.
[25] T. Yang, et al., CD151 promotes colorectal cancer progression by a crosstalk involving CEACAM6, LGR5 and Wnt signaling via TGFbeta1, Int J Biol Sci 17 (2021) 848–860.
[26] C.W. Chien, et al., Regulation of CD151 by hypoxia controls cell adhesion and metastasis in colorectal cancer, Clin. Cancer Res. 14 (2008) 8043–8051.
[27] H. Hashida, et al., Clinical significance of transmembrane 4 superfamily in colon cancer, Br. J. Cancer 89 (2003) 158–167.
[28] F.F. Sperandio, et al., Low-level laser therapy can produce increased aggressiveness of dysplastic and oral cancer cell lines by modulation of Akt/mTOR signaling pathway, J. Biophotonics 6 (2013) 839–847.
[29] J.H. Park, et al., Radiation-activated PI3K/AKT pathway promotes the induction of cancer stem-like cells via the upregulation of SOX2 in colorectal cancer, Cells 10 (2021).
[30] J. Liu, et al., Proteasome inhibitor MG132 enhances the antigrowth and antimetastasis effects of radiation in human nonsmall cell lung cancer cells, Tumour Biol. 35 (2014) 7531–7539.
[31] Z. Wang, et al., CD151-mediated adhesion is crucial to osteosarcoma pulmonary metastasis, Oncotarget 7 (2016) 60623–60638.
[32] E. Monastyrska, et al., Prospective assessment of the quality of life in patients treated surgically for rectal cancer with lower anterior resection and abdominoperineal resection, Eur. J. Surg. Oncol. 42 (2016) 1647–1653.
[33] A.S. Myint, J.P. Gerard, Role of radiotherapy in the treatment of rectal cancer in older patients, Eur. J. Surg. Oncol. 46 (2020) 349–357.
[34] Z. Bi, et al., Nanoparticles (NPs)-meditated LncRNA AFAP1-AS1 silencing to block Wnt/beta-catenin signaling pathway for synergistic reversal of radioresistance and effective cancer radiotherapy, Adv. Sci. (Weinh) 7 (2020), 2000915.
[35] D. Fanale, M. Castiglia, V. Bazan, A. Russo, Involvement of non-coding RNAs in chemo- and radioresistance of colorectal cancer, Adv. Exp. Med. Biol. 937 (2016) 207–228.
[36] S.H. Lim, et al., CD133-positive tumor cell content is a predictor of early recurrence in colorectal cancer, J. Gastrointest. Oncol. 5 (2014) 447–456.
[37] M. Signore, L. Ricci-Vitiani, R. De Maria, Targeting apoptosis pathways in cancer stem cells, Cancer Lett. 332 (2013) 374–382.
[38] Y. Zou, et al., LncRNA OIP5-AS1 regulates radioresistance by targeting DYRK1A through miR-369-3p in colorectal cancer cells, Eur. J. Cell Biol. 97 (2018) 369–378.
[39] W. Yang, et al., MiR-146b-5p overexpression attenuates stemness and radioresistance of glioma stem cells by targeting HuR/lincRNA-p21/beta-catenin pathway, Oncotarget 7 (2016) 41505–41526.
[40] P. Ren, M. Hu, A three long non-coding RNA signature to improve survival prediction in patients with Wilms' tumor, Oncol. Lett. 18 (2019) 6164–6170.
[41] R. Farra, B. Dapas, M. Grassi, F. Benedetti, G. Grassi, E2F1 as a molecular drug target in ovarian cancer, Expert Opin. Ther. Targets 23 (2019) 161–164.
[42] J. Chen, et al., E2F1/SP3/STAT6 axis is required for IL-4-induced epithelial-mesenchymal transition of colorectal cancer cells, Int. J. Oncol. 53 (2018) 567–578.
[43] M.W. Han, et al., Phosphorylation of PI3K regulatory subunit p85 contributes to resistance against PI3K inhibitors in radioresistant head and neck cancer, Oral Oncol. 78 (2018) 56–63.
[44] O.A. Bamodu, et al., Elevated PDK1 expression drives PI3K/AKT/MTOR signaling promotes radiation-resistant and dedifferentiated phenotype of hepatocellular carcinoma, Cells 9 (2020).
[45] A. Kim, et al., Inhibition of Y box binding protein 1 suppresses cell growth and motility in colorectal cancer, Mol. Cancer Ther. 19 (2020) 479–489.