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LncRNA CRNDE is involved in radiation resistance in hepatocellular carcinoma via modulating the SP1/PDK1 axis

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Hepatocellular carcinoma (HCC) is defined as a universal malignancy while radiation therapy is the effective treatment for it. This study validated the mechanism of long non-coding RNA (lncRNA) colorectal neoplasia differentially expressed gene (CRNDE) in radiation resistance in HCC. LncRNA CRNDE upregulation was detected in HCC cells. The radiation-resistant cell strains Huh7R and SNU-387R were established. After silencing lncRNA CRNDE, the cell colony formation ability, cell activity, apoptosis, cell cycles, and γ-H2AX positive rate in Huh7R and SNU-387R were detected. Silencing lncRNA CRNDE decreased the cell activity, colony formation ability, and cell number in the G2 phase and facilitated DNA damage and apoptosis. The binding relations of specificity protein 1 (SP1) with lncRNA CRNDE and 3-phosphoinositide dependent protein kinase 1 (PDK1) were verified. LncRNA CRNDE regulated PDK1 transcription by binding to transcription factor SP1. PDK1 overexpression partially reversed the inhibition of silencing lncRNA CRNDE on radiation resistance in HCC cells. The transplanted tumor mouse model was established and showed that silencing lncRNA CRNDE decreased tumor volume and weight and Ki67-positive cells in HCC mice in vivo. Collectively, lncRNA CRNDE was upregulated in HCC cells and promoted PDK1 transcription by binding to SP1, thus enhancing radiation resistance in HCC cells.

Key words: hepatocellular carcinoma, radiation resistance, lncRNA CRNDE, transcription factor, SP1

Hepatocellular carcinoma (HCC), as the major common category of primary carcinoma of the liver, mainly exists in patients who have liver-related diseases [1]. The incidence of HCC has been ranking 6th globally with a high mortality rate [2]. Unfortunately, the number of patients with HCC has been increasing and the prognosis of it is still unsatisfactory [3]. Radiation therapy has been considered a viable way for HCC treatment via utilizing high-dose radiation to control HCC, maximize and, to some extent, reduce the toxicity caused by radiation [4]. Nonetheless, radiation resistance that could be triggered by various factors is a primary obstacle in HCC treatment [5]. The above contents suggested that it is urgent and necessary to explore the responsive mechanism and feasible treatment of HCC.

Long non-coding RNAs (lncRNAs), >200 nucleotides long, are defined as transcripts of RNA [6]. LncRNAs could exert various functions to modulate HCC, such as lncRNA uc.134 and lncRNA CDKN2BAS [7, 8]. The prior study elucidated that lncRNA colorectal neoplasia differentially expressed gene (CRNDE) has an impact on the progression of HCC, including proliferation as well as invasion [9], and silencing lncRNA CRNDE could attenuate the resistance of radiation therapy in ovarian carcinoma [10]. Therefore, lncRNA CRNDE was introduced to study its mechanism in radiation resistance in HCC.

On a separate note, lncRNA that is retained in the nucleus could be combined with and recruit transcription factors thus modulating the transcription of downstream genes [11]. Specificity protein 1 (SP1) has been defined as an exemplary and basic transcription factor since it was widely correlated to cell processes including differentiation, senescence, and proliferation [12], and SP1 was also important in carcinogenesis because SP1 could activate or inhibit tumor genes [13], and SP1 expression was associated with tumorigenesis of HCC [14]. Hence, lncRNA CRNDE may modulate SP1 and thus affecting radiation resistance in HCC.

The 3-phosphoinositide dependent protein kinase 1 (PDK1 or PDPK1), as a type of phosphorylation-regulated kinase, could activate and modulate cell processes as well as certain signaling pathways [15]. PDK1 was previously

reported to be highly expressed in HCC [16]. In addition, PDK1 suppression was associated with an increase in sensitivity of radiation resistance [17]. Therefore, further exploration of PDK1 on radiation resistance in HCC is advisable for treating this debilitating carcinoma.

From the above associations, we speculated that lncRNA CRNDE may have an impact on radiation resistance in HCC with the help of SP1/PDK1 and hoped to provide a new theoretical perspective for increasing the efficiency of radiation sensitivity in cancer cells.

Materials and methods

Ethics approval. This study was performed with the approval of the Clinical Ethical Committee of Guangdong Second Provincial General Hospital. All animal experiments follow the ARRIVE guidelines and the Guide for the Care and Use of Laboratory Animals.

Bioinformatics. The visualization website StarBase of The Cancer Genome Atlas (TCGA) website (starbase.sysu.edu.cn/) was adopted to predict the relation between lncRNA CRNDE expression and survival prognosis in HCC patients. The database (http://lncatlas.crg.eu/) was used to predict the subcellular localization of lncRNA CRNDE, and the database (http://jaspar.genereg.net/) was adopted to explore the binding sites of SP1 to PDK1.

Cell culture and transfection. Human normal hepatic epithelial cells THLE-3 and HCC cell line SNU-387 were all provided by the American Type Culture Collection (ATCC; Manassas, VA, USA). BEL-7405 was purchased from the National Infrastructure of Cell Line Resource (Beijing, China); MHCC-97H and Huh7 from the Cell Bank of the Type Culture Collection (Wuhan, China); HCCLM3 from the Cell Bank of the Chinese Academy of Sciences (Beijing, China). All the obtained cells were cultured in the Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) in a constant temperature incubator at 37 °C with 5% CO_2.

The cells in the logarithmic phase were respectively seeded in 6-well plates at 2×10^5 cells/well for 24 h, and then si-NC, si-CRNDE-1, si-CRNDE-2, si-SP1, pcDNA3.1-NC, and pcDNA3.1-PDK1 (Shanghai GeneChem Co., Ltd., Shanghai, China; siRNA 40–100 nM, pcDNA3.1 30 nM) were transfected into cells respectively using Lipofectamine 2000 (11668-019, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. After 48 h of transfection, the following experiments were conducted.

Establishment of radiation-resistant cell strains. To establish radiation-resistant cell strains, X-ray Linear Accelerator (RS2000, RadSource, Suwanee, USA) was used to expose Huh7 and SNU-387 cells to 2 Gy (130 kV, 5 mA) every 48 h, which continued for 30 cycles, namely, the cumulative dose was 60 Gy within 2 months. After 2 months, the viable cells were the radiation-resistant HCC strains (Huh7R and SNU-387R). The medium was changed every 72 h or every cell passage after cell fusion.

Colony formation assay. HCC cells were seeded in 6-well plates and allowed to adhere to the wall for 24 h, and singly irradiated by different doses of X-rays (0, 2, 4, 6, and 8 Gy) with the dose rate of 1 Gy/min. After 2 weeks of irradiation, the cells were subjected to fixation of 4% paraformaldehyde and staining of 0.5% crystal violet at normal temperature for 10 min. The colonies greater than 50 cells per dish were counted and photographed using a digital camera (Olympus, Tokyo, Japan). The ability for cell survival was quantified using the survival fraction (SF); SF = the number of formed colonies/the number of inoculated colonies × inoculation efficiency; when non-irradiated, the inoculation efficiency = the actual number of formed colonies : the number of original inoculated cells.

Cell counting kit-8 (CCK-8) assay. CCK-8 kit (Dojindo, Kumamoto, Japan) was adopted to detect cell activity. The cell concentration was adjusted to 2×10^3 cells/well, and cells were seeded in 96-well plates. The cells were irradiated for 0, 24, 48, and 72 h, and then each well was added with CCK-8 solution. The plates were continuously cultured at 37 °C for 4 h. The absorbance was measured at 450 nm via a microplate reader.

Flow cytometry. Annexin V APC-PI apoptosis kit (50474; BD Biosciences, San Jose, CA, USA) was adopted to verify apoptosis. The cells of different treatment groups were seeded in 24-well plates and resuspended with a binding buffer (500 μl), followed by the addition of 5 μl Annexin V-FITC (BD Biosciences) and 5 μl propidium iodide. Next, the cells were mixed and incubated in dark conditions at normal temperature for 15 min. Flow cytometry (NovoCyte, ACEA Biosciences, San Diego, CA, USA) was conducted to analyze the apoptosis of the cells.

A cell cycle staining kit (BD Pharmingen™, Franklin Lakes, New Jersey, USA) was adopted to evaluate cell cycle distribution. The cells were fixed with 70% ethanol at 4 °C for 2 h and treated with DNA staining solution and permeating solution at room temperature for 15 min in the dark, after which the cells were examined via flow cytometry.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA of HCC cells was collected using TRIzol kit (Invitrogen, Carlsbad, CA, USA). The total RNA sample (5 μl) was diluted 20 times using RNA-free ultrapure water, and the absorbance at 260 nm and 280 nm of the ultraviolet spectrophotometer was observed to determine the concentration and purity of RNA. The ratio of OD260/OD280 between 1.7–2.1 meant that the purity was high and it could be used for the following experiments. The collected RNA underwent reverse-transcription into cDNA via a Transcriptor First Strand cDNA Synthesis kit (TaKaRa Biotechnology, Tokyo, Japan). qRT-PCR was conducted using SYBR Green II (TaKaRa Biotechnology). The cells were pre-denaturated at 95 °C for 10 min, denaturated for 40 cycles at 95 °C for 10 s, annealed at 60 °C for 20 s, and extended at 72 °C for 34 s. The threshold value was designated manually

at the lowest point where each logarithmic amplification curve rose in parallel to obtain the Ct value (threshold cycle) of each reaction tube; the data were presented using the $2^{-\Delta\Delta Ct}$ method [18]; the cell experiment was conducted 3 times repeatedly. U6 served as an internal reference of lncRNA CRNDE and GAPDH as an internal reference of genes. The primer sequences synthesized by Sangon Biotech (Shanghai, China) are presented in Table 1.

Immunofluorescence for DNA damage. The re-suspended cells were seeded in 96-well plates and subjected to fixation with 4% buffered paraformaldehyde and permeabilization of 1% Triton X-100 when cell fusion reached 80–90%. After being blocked with 5% skim milk solution for 30 min, the cells were incubated with γ-H2AX antibody (ab81299, Abcam, Cambridge, UK) at 37 °C for 2 h, and with secondary antibody goat anti-rabbit IgG (1:5000, ab6721, Abcam) at 37 °C for 1 h, and then incubated with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) at 37 °C for 5 min. At last, a fluorescence microscope (Olympus) was adopted to examine the cells. Cells containing more than 10 γ-H2AX foci cells were considered as positive for DNA damage.

Nuclear/cytosol fractionation assay. The cell nucleus and cytoplasm extraction kit (Beyotime, Shanghai, China) was used to separate the nuclear and cytoplasmic portions of the cells following the manufacturer's instructions. Briefly, after being washed with phosphate buffer saline (PBS), the cells were placed in 200 μl cytoplasmic protein extract A/protease inhibitor buffer on ice for 15–20 min. Then, the cells were treated with 10 μl cytoplasmic protein extract B and centrifuged at 12,000×g at 4 °C for 10 min to separate the nucleus precipitates from the cytoplasm. After being re-suspended in 50 μl nucleoprotein extraction buffer, the nucleus was stirred on ice for 30 min. The nucleus was centrifuged at 12,000×g at 4 °C for 10 min, and then the supernatant was harvested as the nuclear extract, and analyzed by qRT-PCR.

RNA immunoprecipitation. Magna RIP RNA-Binding Protein Immunoprecipitation kit (Millipore, Darmstadt, Germany) was used for RNA immunoprecipitation. Briefly, the cells were lysed and mixed with anti-SP1 (1:1000, ab231778, Abcam) or IgG (1:2500, ab150077, Abcam). The RNA binding to antibodies was pulled down by protein A/G magnetic bead and quantified via qRT-PCR.

Table 1. qPCR primer sequences.

Name of primer	Sequences (5'–3')
CRNDE-F	5'-ATGCTTCCATAATACATTTGG-3'
CRNDE-R	5'-CAAACTGTGTGCAGACACTCA-3'
SP1-F	5'-ATGAGAACAGCAACAACTCCC-3'
SP1-R	5'-AGTTGTGTGGCTGTGAGGTCA-3'
PDK1-F	5'-ATGAGTGACCGAGGAGGTGGC-3'
PDK1-R	5'-ATGTAGTTGAAAAGTCTGTCA-3'
U6-F	5'-CGCTTCGGCAGCACATATAC-3'
U6-R	5'-AATATGGAACGCTTCACGA-3'
GAPDH-F	5'-GGCACAGTCAAGGCTGAGAATG-3'
GAPDH-R	5'-ATGGTGGTGAAGACGCCAGTA-3'

Dual-luciferase report gene experiment. The database (http://jaspar.genereg.net/) was used to predict the binding sites of SP1 to PDK1. To evaluate luciferase activity, Huh7R and SNU-387R cells were transfected into 12-well plates when the cells reached 70% confluence and 1-h of serum starvation. The artificially synthesized Vector-PDK1 3'UTR gene segment was introduced into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (E1330, Promega, USA). A mutation site at the binding site was designed on the wild-type PDK1, and after being digested with a restriction endonuclease, T4 DNA ligase was used to insert the target fragment into the pmirGLO vector. All the above operations were entrusted to Shanghai GeneChem Co., Ltd. The cells were transfected with PDK1-WT or PDK1-MUT plasmids and pcDNA3.1-SP1 or pcDNA3.1 NC (GenePharma, Shanghai, China) using Lipofectamine 2000. After 48 h, the cells were harvested and analyzed using a dual-luciferase assay kit (Promega) following the manufacturer's instructions.

Chromatin immunoprecipitation (Ch-IP). Ch-IP analysis was performed using Ch-IP assay kits (Abcam) to determine the binding sites of SP1 to PDK1, and DNA fragments of 200–1000 bp were obtained by ultrasonic fragmentation. HCC cells were cross-linked with formaldehyde and incubated overnight at 4 °C. Non-specific IgG antibodies (ab172730, Abcam) and SP1 (ab231778, 1:1000, Abcam) were precipitated by chromatin, and the cross-link of DNA was reversed by NaCl. PDK1 expression was verified using qRT-PCR; PDK1 forward primer: 5'-GAGCCTG-GTCCCCTCTGA-3' and PDK1 reverse primer: 5'-GATTG-GTTCGCGCGAGGT-3'; the data were analyzed using the $2^{-\Delta\Delta CT}$ method.

Western blot. RIPA lysis buffer (Beyotime) containing protease inhibitor cocktail (Sigma, St. Louis, MO, USA) was added to the cells and mixed. Then the cells were lysed on ice for 30 min and centrifuged at 18,800×g for 10 min to collect the supernatant. The concentration of protein was examined by bicinchoninic acid (BCA) protein assay kits (Rockford, IL, USA). The protein samples were subjected to 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was shaken and blocked with 5% skim milk prepared in Tris-buffered saline with Tween 20 (TBST) for 1 h, and incubated overnight at 4 °C with primary rabbit polyclonal antibody SP1 (ab231778, 1:1000, Abcam). After being washed with TBST for 5 min, the cells were incubated with goat anti-rabbit IgG (ab205718, 1:10000, Abcam) labeled by horseradish peroxidase (HRP) for 1 h. At last, the enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) was used to display the protein bands, and ImageJ (version 1.48; National Institutes of Health, Bethesda, MD, USA) was adopted to examine the protein bands with GAPDH as an internal reference.

Subcutaneously implanted tumor model in nude mouse. The nude mice (aged 6 weeks and weighing 18–22 g) used in this study were all provided by Southern Medical

University, Guangzhou, China [License No.: SCXK (Guangdong) 2016-0041]. Recombinant lentivirus sh-CRNDE was used to decrease CRNDE expression in Huh7R cells, and plasmid construction and lentivirus packaging were entrusted to GenePharma Co., Ltd. (Shanghai, China), with sh-NC serving as a control. The cells were infected with the lentivirus for 48 h based on the total viral load of 40 multiplicity of infection (MOI)/cell, and then the cells stably transfected sh-CRNDE were screened by puromycin (Sigma) for subsequent experiments. Huh7R cells (5×10^6 cells/0.1 ml) were re-suspended in PBS solution and subcutaneously implanted into the right hind leg of male nude mice, with Huh7 radiation-resistant cell strains infected with sh-NC as a control. The tumor volume (mm^3) = (length × $width^2$)/2 was calculated, and when the palpable tumor volume extended 100 mm^3, the mice were anesthetized with 40 mg/kg pentobarbital sodium and treated with radiotherapy. The tumor size was measured every 2 days following the initiation of radiotherapy, and after 15 days, the mice were subjected to an intraperitoneal injection of 200 mg/kg pentobarbital sodium for euthanasia to collect tumor tissues. Each group consisted of 12 mice, six of which were subjected to qRT-PCR detection, and six were subjected to histological staining.

Radiotherapy. The mice, under anesthesia, were placed prone on the membrane fixation plate with the right hind leg carrying the tumor exposed to the radiation field, and other parts of the mice were covered with a lead plate for protection. The linear accelerator 12 MeV electron beam was used for irradiation. The irradiation field with a diameter of 3 cm plus 1 cm compensation glue was used for irradiation, and the source-skin distance was 100 cm. The day when the palpable tumor volume had reached 100 mm^3 was regarded as the 1st day, and the mice were given 6 Gy of radiotherapy on the 1st, 3rd, and 5th day [19].

Immunohistochemistry (IHC). IHC was conducted in sections that were formalin-fixed and paraffin-embedded. The tumor sections with a thickness of 3 μm were dewaxed using xylene and rehydrated using gradient alcohol and distilled water. The endogenous peroxidase activity was blocked using distilled water with 3% H_2O_2 for 5 min. Normal horse serum was adopted to block the nonspecific binding at 37 °C for 30 min. Then, the sections were cultured with Ki-67 (1:500; ab16667, Abcam). Based on the manufacturer's instructions, the VetastainABC kit (Vector Laboratories, Burlingame, CA, USA) was used for the determination.

Statistical analysis. SPSS21.0 statistical software (IBM SPSS Statistics, Chicago, IL, USA) and GraphPad Prism 8.01 statistical software (GraphPad Software Inc., San Diego, CA, USA) were used for statistical analysis and mapping of data. Measurement data in this study were indicated as mean ± standard deviation. The t-test was used for analysis comparisons between two groups; one-way ANOVA or two-way ANOVA was performed for comparisons among multiple groups; and the Tukey test was used for the post-hoc test; $p<0.05$ indicated that the difference was statistically meaningful; $p<0.01$ indicated that the difference was highly statistically significant; $p<0.001$ indicated that the difference was extremely statistical significance.

Results

LncRNA CRNDE elevation in HCC cells. To investigate the impact of lncRNA CRNDE on HCC, we observed the relation between lncRNA CRNDE expression and the survival prognosis in HCC patients via TCGA's visualization website (starbase.sysu.edu.cn/), which elucidated that lncRNA CRNDE was elevated in HCC patients and the survival prognosis of patients with highly expressed lncRNA CRNDE was poor (Figure 1A). LncRNA CRNDE expression in human HCC cell lines BEL-7405, SNU-387, MHCC-97H, HCCLM3, and Huh7 was verified through qRT-PCR, with human normal hepatic epithelial cell line THLE-3 as a control. The results identified that lncRNA CRNDE expression in different HCC cell lines was higher than that of lncRNA CRNDE in THLE-3, and lncRNA CRNDE expression was the highest in Huh7 and the lowest in SNU-387 ($p<0.001$, Figure 1B), suggesting that lncRNA CRNDE elevation was verified in HCC cells.

LncRNA CRNDE expression was related to radiation resistance in HCC cells. As radiation therapy has been considered an effective method for malignant tumor treatment, we speculated that lncRNA CRNDE may be related to the radiation sensitivity in HCC cells. To verify our speculation, we selected Huh7 and SNU-387 cells to establish the radiation-resistant cell strains Huh7R and SNU-387R. Then, we used different doses (0, 2, 4, 6, and 8 Gy) of X-ray to irradiate the parent cell strains and the radiation-resistant cell strains of Huh7 and SNU-387, and noticed that the radiation-resistant cell strains had a better survival rate ($p<0.001$, Figure 2A), and we selected the dose of 4 Gy with nearly 50% survival rate of parent cells for following experiments. qRT-PCR results revealed higher lncRNA CRNDE expression in radiation-resistant cell strains ($p<0.001$, Figure 2B). And under 4 Gy radiation, the cell activity and colony formation ability of radiation-resistant cell strains were better than that of parent cell strains and the cell apoptosis rate was reduced ($p<0.001$, Figures 2C-2E). And the radiation-resistant cells were blocked in the G2 phase ($p<0.001$, Figure 2F). Meanwhile, the positive expression of γ-H2AX in radiation-resistant cell strains was lower than that of parent cell strains ($p<0.001$, Figure 2G). The above results suggested that lncRNA CRNDE expression was related to radiation resistance in HCC cells.

Silencing lncRNA CRNDE alleviated radiation resistance in HCC cells. To explore the functions of lncRNA CRNDE in radiation resistance in HCC cells, siRNAs (si-CRNDE-1 and si-CRNDE-2) were transfected into Huh7R and SNU-387R cells to decline lncRNA CRNDE expression ($p<0.001$, Figure 3A), and si-SMYD3-1 with a better silencing effect was chosen for subsequent experiments. After trans-

fection with si-CRNDE-1, under 4 Gy radiation, cell activity and colony formation ability of the Huh7R and SNU-387R cells were limited, apoptosis was promoted ($p<0.001$, Figures 3B–3D), cell arrest in the G2 phase was attenuated (Figure 3E), and the positive expression of γ-H2AX in the cells was increased, indicating that DNA damage was aggravated ($p<0.001$, Figure 3F). The above results suggested that silencing lncRNA CRNDE could suppress radiation resistance in HCC cells.

LncRNA CRNDE regulated PDK1 transcription by binding to transcription factor SP1. To verify the downstream regulatory functions of lncRNA CRNDE to radiation resistance in HCC cells, the database (http://lncatlas.crg.eu/) was used to analyze the subcellular localization of lncRNA CRNDE, which showed that lncRNA CRNDE was mostly expressed in the nucleus of various cells (Figure 4A). The nuclear/cytosol fractionation assay further revealed that lncRNA CRNDE was mainly expressed in the nucleus of Huh7R and SNU-387R cells (Figure 4B). A previous study reported that lncRNAs located in the nucleus could regulate the transcription of downstream genes via binding to transcription factors [11], and lncRNA CRNDE could bind to transcription factor SP1 [20]. We then predicted the transcription factor SP1 and its downstream genes through the database (http://jaspar.genereg.net/) and found that there were binding sites between SP1 and the promoter region of its downstream gene PDK1 (Figure 4C), and PDK1 was associated with radiation resistance in HCC cells [17]. In Huh7R and SNU-387R cells, the binding relation between lncRNA CRNDE and SP1 was verified via the RIP assay ($p<0.001$, Figure 4D). The binding of SP1 and DNA in the PDK1 promoter region was proved by the Ch-IP assay: when lncRNA CRNDE was decreased, the DNA level of the PDK1 promoter region binding to SP1 was decreased ($p<0.001$, Figure 4E). Then, the binding of SP1 and PDK1 in the promoter region was further verified via a dual-luciferase report gene assay: after transfection with pcDNA3.1-SP1, the dual-luciferase activity in PDK1-WT was facilitated while there was no distinct difference in PDK1-MUT (Figure 4F). To verify the regulatory roles of SP1 in PDK1 in Huh7R and SNU-387R cells, si-SP1 was transfected into radiation-resistant HCC cells, with si-NC as a control. The decrease of SP1 protein in radiation-resistant HCC cells was detected via western blot ($p<0.001$, Figure 4G). qRT-PCR showed that the transcription level of PDK1 was downregulated with the decrease in SP1 protein ($p<0.001$, Figure 4H). The level of PDK1 transcription in radiation-resistant cells was upregulated compared with that of their parent cells ($p<0.001$, Figure 4I), and was suppressed after silencing lncRNA CRNDE ($p<0.001$, Figure 4J). Overall, these results demonstrated that lncRNA CRNDE bound to SP1 to promote PDK1 transcription.

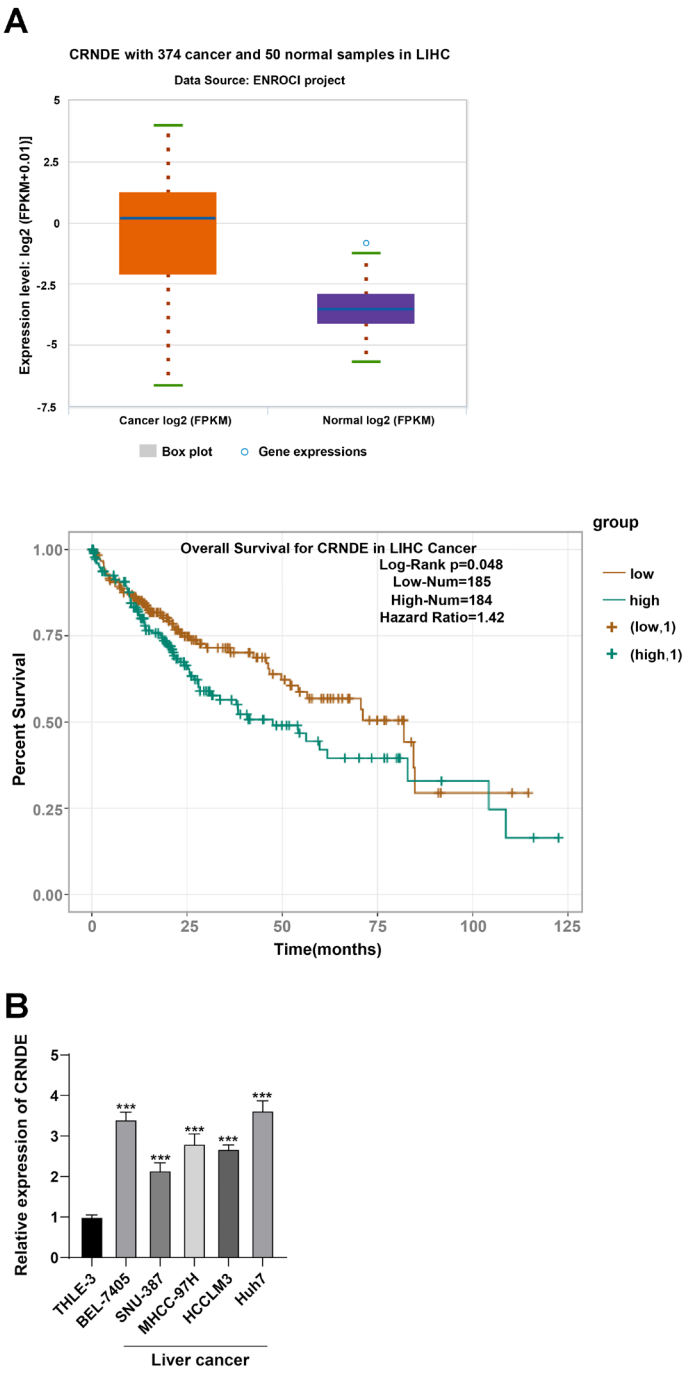

Figure 1. LncRNA CRNDE was upregulated in HCC cells. A) TCGA's visualization website starBase (starbase.sysu.edu.cn/) was performed to analyze lncRNA CRNDE expression in HCC patients as well as the relation between lncRNA CRNDE expression and the survival prognosis in HCC patients (HHC patients were divided into two groups: high CRNDE expression and low CRNDE expression, using the median of lncRNA CRNDE expression levels in the cancer tissues of HCC patients as the critical threshold); B) qRT-PCR was used to detect the expression of lncRNA CRNDE in BEL-7405, SNU-387, MHCC-97H, HCCLM3, and Huh7 cells. The cell experiment was conducted 3 times independently. The data in Figure B were measurement data, and data were expressed as mean ± standard deviation. Data in panels were analyzed using one-way ANOVA, followed by Tukey's post-hoc test; ***in Figure B represents p<0. 001, compared with the THLE-3 group.

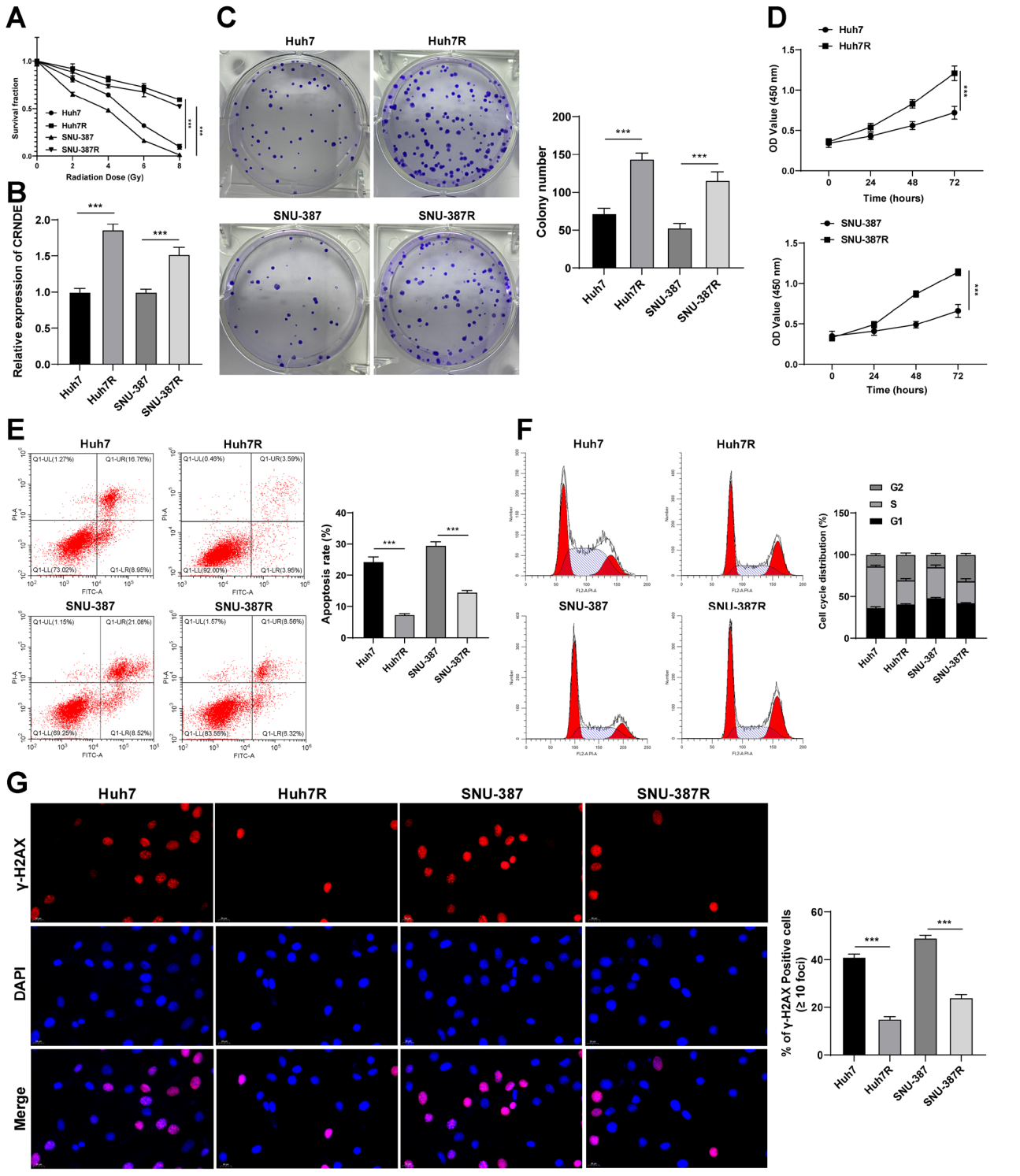

Figure 2. LncRNA CRNDE expression was connected with radiation resistance in HCC cells. The radiation-resistant cell strains of Huh7 and SNU-387 cells (Huh7R and SNU-387R) were established. A) Colony formation assay was conducted to calculate the SF of the parent cell strains and the radiation-resistant cell strains, under the different doses of X rays (0, 2, 4, 6, and 8 Gy); B) qRT-PCR was adopted to examine the expression of lncRNA CRNDE in the parent cell strains and the radiation-resistant cell strains; C) Colony formation assay was used to examine the clone formation ability; D) CCK-8 assay was performed to detect cell activity; E, F) Flow cytometry was conducted to examine cell apoptosis rate and cell cycle; G) Immunofluorescence staining was performed to verify the positive expression of γ-H2AX in the cells. The cell experiment was repeated 3 times independently. The data in the figures were all measurement data and data were expressed as mean ± standard deviation. Data in panels B, C, E, and F were analyzed using one-way ANOVA; data in panels A and D were analyzed using two-way ANOVA, followed by Tukey's post-hoc test; ***p<0.001.

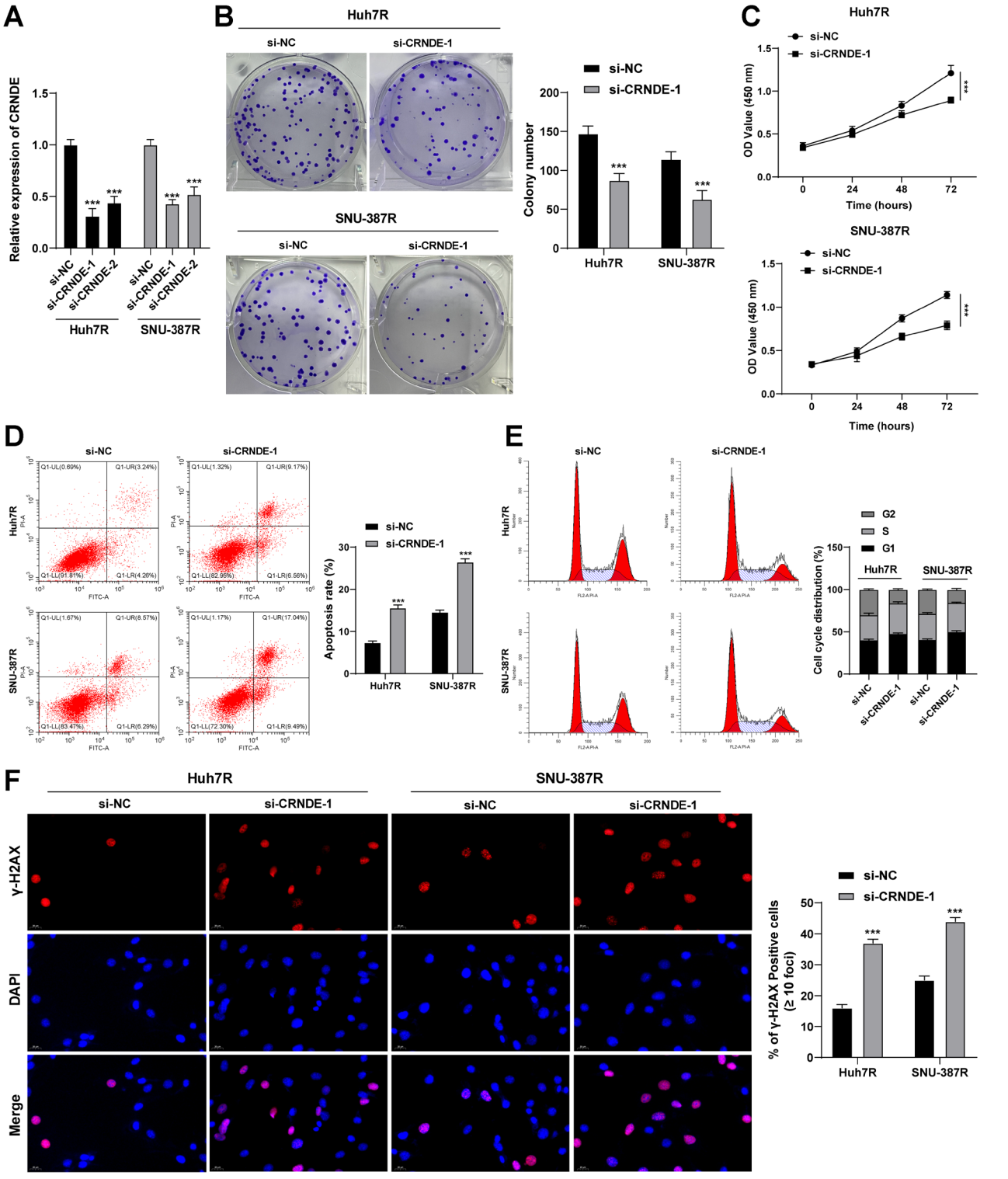

Figure 3. Silencing lncRNA CRNDE attenuated radiation resistance in HCC cells. siRNAs (si-CRNDE-1 and si-CRNDE-2) were transfected into Huh7R and SNU-387R cells, with si-NC as a control. A) qRT-PCR was adopted to detect the transfection efficiency. si-CRNDE-1 was used to decrease the expression of lncRNA CRNDE, and Huh7R and SNU-387R cells were subjected to 4 Gy radiation. B. C) Colony formation assay and CCK-8 assay were conducted to evaluate clone formation ability and cell activity; D, E) Flow cytometry was used to examine cell apoptosis and cell cycle; F) Immunofluorescence staining of γ-H2AX was performed to evaluate the DNA damage. The cell experiment was repeated 3 times independently. The data in the figures were all measurement data and data were expressed as mean ± standard deviation. Data in panels were analyzed using two-way ANOVA, followed by Tukey's post-hoc test; ***p<0.001.

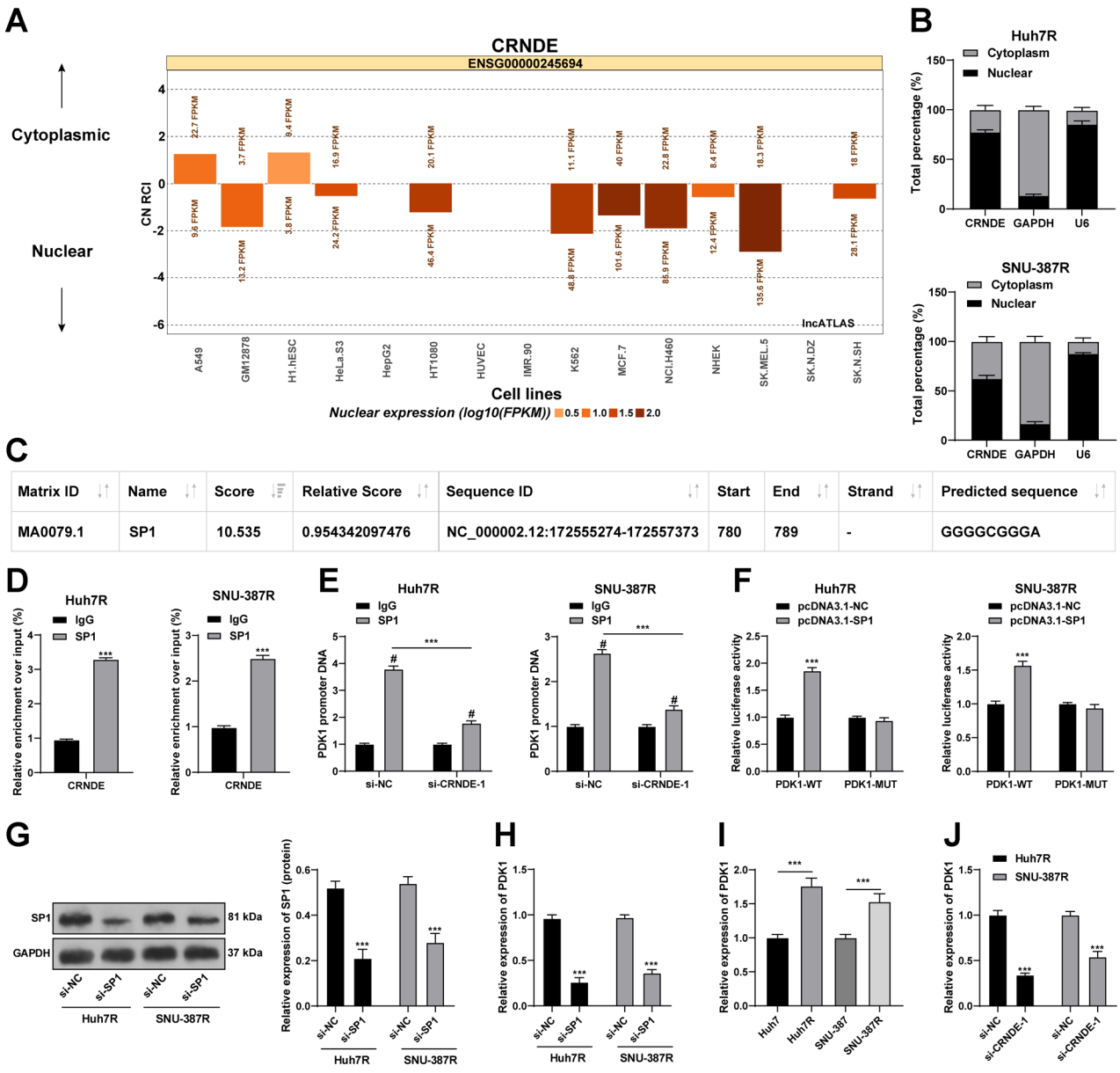

Figure 4. LncRNA CRNDE regulated PDK1 transcription by binding to transcription factor SP1. A) The database (http://lncatlas.crg.eu/) was used to predict the subcellular localization of lncRNA CRNDE; B) The nuclear/cytosol fractionation assay was conducted to verify the subcellular localization in Huh7R and SNU-387R cells; C) The database (http://jaspar.genereg.net/) was used to predict the binding sites between SP1 and PDK1; D) RIP assay was performed to testify the binding of lncRNA CRNDE and SP1; E, F) Ch-IP assay and dual-luciferase report gene experiment were adopted to verify the binding of SP1 and PDK1. si-SP1 was transfected into the radiation-resistant cell strains of Huh7 or SNU-387 cells, with si-NC as a control. G) Western blot was conducted to detect the expression of SP1 protein; H) qRT-PCR was used to examine the transcription level of PDK1; I) qRT-PCR was used to verify the expression of PDK1 in the parent cell strains and radiation-resistant cell strains of Huh7 or SNU-387 cells; J) qRT-PCR was used to detect the expression of PDK1 in the radiation-resistant cell strains transfected with si-NC and si-CRNDE-1. The cell experiment was repeated 3 times independently. The data in the figures were all measurement data and data were expressed as mean ± standard deviation. Data in panel D were verified using t-test; data in panels B, E, F, G, H, and J were analyzed using two-way ANOVA; data in panel I were analyzed using one-way ANOVA, followed by Tukey's post-hoc test; ***p<0.001; #p<0.05.

PDK1 overexpression partially reversed the inhibition of silencing lncRNA CRNDE on radiation resistance. To further verify the molecular regulatory mechanisms of PDK1 on radiation resistance in HCC cells, pcDNA3.1-PDK1 was transfected into HCC cells to upregulate the PDK1 expression in the cells, with pcDNA3.1-NC as a control (p<0.001, Figure 5A), and then combined with si-CRNDE-1 for a joint experiment. The experimental results showed that compared with the si-CRNDE-1 group, the cell activity, and colony formation ability in the pcDNA3.1-PDK1+si-

CRNDE-1 group were facilitated ($p<0.001$, Figures 5B, 5C), and the apoptosis rate was declined ($p<0.001$, Figure 5D), cell proportion in the G2 phase was increased (Figure 5E), and the positive rate of γ-H2AX were decreased ($p<0.001$, Figure 5F). The above results indicated that PDK1 upregulation could partially reverse the inhibition of silencing lncRNA CRNDE on radiation resistance in HCC cells.

Silencing lncRNA CRNDE attenuated radiation resistance in HCC mice *in vivo*. Huh7R cells were infected with sh-CRNDE to obtain the cells stably lowly-expressed lncRNA CRNDE ($p<0.001$, Figure 6A), and then the cells were subcutaneously injected into the mice. The functions of lncRNA CRNDE to radiation resistance of HCC cells were observed via radiotherapy. Compared with the sh-NC group, the tumor volume and weight of the sh-CRNDE group were reduced ($p<0.001$, Figures 6B, 6C), and the positive rate of Ki-67 was decreased ($p<0.001$, Figure 6D). And, the expressions of lncRNA CRNDE and PDK1 in tumors were downregulated ($p<0.001$, Figure 6E). The above results proved that silencing lncRNA CRNDE attenuated radiation resistance in HCC via inhibiting the promotion of SP1 on PDK1 transcription.

Discussion

HCC is defined as the major type of primary liver carcinoma, accounting for over 90% of primary liver carcinoma cases, and meanwhile, the survival rate of HCC is typically poor [21]. Radiation therapy is recognized as the viable option for HCC patients [22], whereas radiation resistance in HCC has impeded the clinical effect of HCC treatment [5]. Meanwhile, lncRNAs functioned as major molecular regulators to the progression of HCC [23]. In the current study, we investigated the mechanism of lncRNA CRNDE in radiation resistance in HCC via modulating the SP1/PDK1 axis.

Previous studies identified that lncRNA CRNDE had impacts on cell processes or progression of HCC via downregulating miR-384 or increasing SIX1 [24, 25]. To further investigate the effects of lncRNA CRNDE on HCC, we first confirmed the inverse correlation of lncRNA CRNDE expression and prognosis of HCC patients and verified the high lncRNA CRNDE expression in HCC cells via TCGA's visualization website starBase and qRT-PCR assay. The result was the same as the tendency of prior studies [25, 26]. Elevation of lncRNA is closely correlated to the poor prognosis of patients with cancer and resistance to radio-/chemo-therapy [27]. We speculated that lncRNA CRNDE may be correlated with the radiosensitivity in HCC. Therefore, we established radiation-resistant cell strains Huh7R and SNU-387R and used X-rays with different doses to irradiate the parent cell strains and radiation-resistant cell strains. We examined whether the survival ability of Huh7R and SNU-387R was better. Under the irradiation of 4 Gy, in the Huh7R and SNU-387R, lncRNA CRNDE was elevated, cell activity and colony formation ability were enhanced, and cell strains were blocked in the G2 phase, while apoptosis rate and the positive expression of γ-H2AX were decreased, indicating lncRNA CRNDE expression was concerned with radiation resistance in HCC cells. A preceding study elucidated that lncRNAs have been considered effective factors and biomarkers of chemotherapy and radiotherapy of HCC [28]. For example, lncRNA H19 exerted a modulating effect on radio-/chemo-resistances in HCC cells [29]. To further assess the possible mechanism of lncRNA CRNDE in radiation resistance in HCC, siRNAs were transfected into Huh7R and SNU-387R cells to suppress the lncRNA CRNDE expression. After silencing lncRNA CRNDE expression, the cell proliferation and colony formation abilities were limited, and cell arrest in the G2 phase was attenuated, whereby apoptosis rate and the positive expression of γ-H2AX were elevated, indicating DNA damage of the cells was aggravated. Previous studies have elucidated that silencing lncRNA CRNDE exerted a favorable role in reducing radiation resistance in ovarian carcinoma and lung adenocarcinoma [10, 30]. Overall, the above results suggested that silencing lncRNA CRNDE suppressed radiation resistance in HCC.

To explore the downstream regulatory functions of lncRNA CRNDE to HCC, we identified the subcellular localization of lncRNA CRNDE. The testing result of the nuclear/cytosol fractionation assay proved that lncRNA CRNDE was mainly retained in the nucleus of Huh7R and SNU-387R cells. Furthermore, lncRNAs located in the nucleus could downregulate the transcription of its downstream genes via interacting with transcription factors [11, 31]. Prior studies showed that lncRNA CTBP1-AS2 elevation induced by SP1 facilitated tumorigenesis of HCC [32], and lncRNA CRNDE bound to SP1 accelerated the progression of osteosarcoma through the Wnt/β-catenin network [33]. Then, the binding sites between SP1 to the promoter region of the downstream gene PDK1 were predicted. A prior study proved that SP1 protein was suppressed by ATL-1, and the interaction between SP1 and Stat3 inhibited by ATL-1 downregulated PDK1 expression, affecting lung cancer progression [34], and PDK1-targeted miR-155-5p had an impact on the sensitivity of cisplatin resistance in HCC [35]. Subsequently, the binding relations between SP1 to lncRNA CRNDE and PDK1 were identified, respectively. Afterward, we noticed that after lncRNA CRNDE downregulation, the DNA level of the PDK1 promoter region binding to SP1 was declined; PDK1 expression was decreased after silencing SP1. PDK1 expression was elevated in Huh7R and SNU-387R strains but declined after silencing lncRNA CRNDE. The increase in PDK1 was detected in HCC cells [36]. Previous studies have demonstrated that SP1 was elevated in HCC cells and silencing SP1 could limit cell proliferation and facilitate apoptosis [37], and the increases in SP1 and lncRNA HOTAIR elevated PDK1 expression [20]. The above results demonstrated that lncRNA CRNDE modulated PDK1 transcription via binding to SP1.

PDK1 expression was considered to be correlated with the radiosensitivity of cancers, such as melanoma and lung cancer [38, 39]. To further explore the mechanism of PDK1

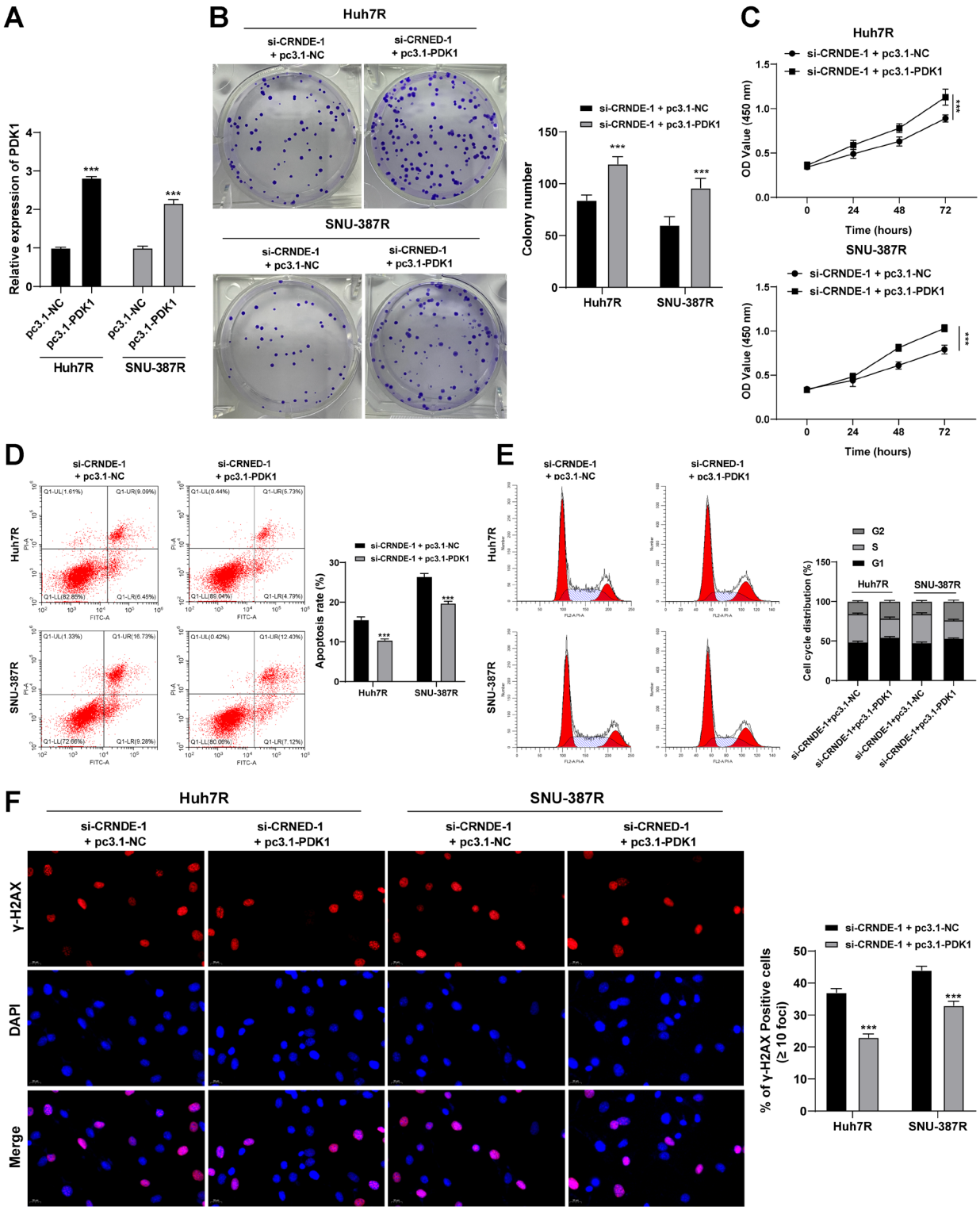

Figure 5. PDK1 overexpression partially reversed the inhibition of silencing lncRNA CRNDE on radiation resistance. Overexpression plasmid pcDNA3.1-PDK1 was transfected into HCC cell lines, with pcDNA3.1-NC as a control. A) qRT-PCR was performed to verify the transfection efficiency. Under 4 Gy radiation, HCC cell lines with lowly expressed lncRNA CRNDE were simultaneously transfected with pcDNA3.1-PDK1. B, C) Colony formation assay and CCK-8 assay were performed to detect the clone formation ability and cell activity; D, E) Flow cytometry was used to examine cell apoptosis and cell cycle; F) Immunofluorescence staining of γ-H2AX was conducted to examine the DNA damage. The cell experiment was repeated 3 times independently. The data in the figures were all measurement data and data were expressed as mean ± standard deviation. Data in panels were analyzed using two-way ANOVA, followed by Tukey's post-hoc test; *** p<0.001.

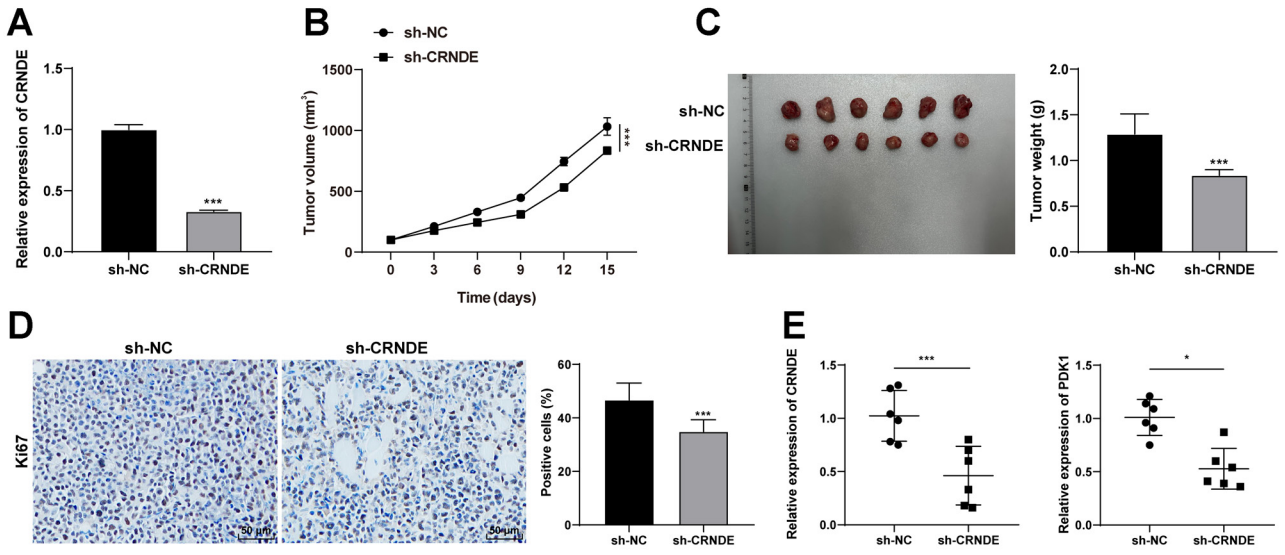

Figure 6. Silencing lncRNA CRNDE attenuated radiation resistance in HCC *in vivo*. sh-CRNDE was used to infect Huh7R cells, with sh-NC as a control. A) qRT-PCR was performed to examine the expression of lncRNA CRNDE in Huh7R cells. Huh7R cells with lowly expressed lncRNA CRNDE were subcutaneously injected into the right hind leg of the mice. B, C) The weight and volume of the tumors were measured; D) Immunofluorescence staining of Ki-67 was used to analyze the positive rate of Ki-67; E) qRT-PCR was used to detect the expressions of lncRNA CRNDE and PDK1 in the tumors. N=6; the data in the figures were all measurement data; data were expressed as mean ± standard deviation; Data in panels A, C, D, and E were analyzed using the t-test; data in panel B were analyzed using two-way ANOVA, followed by Tukey's post-hoc test; *p<0.05; ***p<0.001.

in radiation resistance in HCC cells, PDK1 in HCC cells was overexpressed and was combined with si-CRNDE-1, which then showed that, in the pcDNA3.1-PDK1+si-CRNDE-1 group, the cell activity and colony formation ability were facilitated, and cell number in the G2 phase was increased, while the apoptosis rate and positive rate of γ-H2AX were declined. Bamodu et al. concluded that PDK1 overexpression enhanced the radiosensitivity as well as a dedifferentiated phenotype in HCC [17], further supporting that PDK1 overexpression partially antagonized the inhibition of silencing lncRNA CRNDE on radiation resistance in HCC cells.

Finally, the nude mice were subjected to subcutaneous injection of Huh7R cells with silencing lncRNA CRNDE. The tumor weight and volume of the sh-CRNDE group were reduced, the positive rate of Ki-67 was decreased, and the expressions of lncRNA CRND and PDK1 in tumors were suppressed. Briefly, the above findings indicated that silencing lncRNA CRNDE attenuated radiation resistance in HCC *in vivo*.

Together, the above-described results revealed that lncRNA CRNDE was elevated in HCC cells and related to HCC cells radiation resistance, meanwhile, lncRNA CRNDE in the nucleus enhanced PDK1 transcription through binding to SP1 and thus accelerating radiation resistance in HCC cells. Nonetheless, we only studied the mechanism of the lncRNA CRNDE/SP1/PDK1 axis in radiation resistance in HCC cells and failed to study the roles of the interaction between lncRNA CRNDE and other transcription factors and downstream genes in radiation resistance. Going forward, we shall explore other transcription factors and downstream genes that bind to lncRNA CRNDE so as to further decode the molecular mechanisms of lncRNA CRNDE in radiation resistance in HCC cells.

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