Understanding the mechanism underlying the acquisition of radioresistance in human prostate cancer cells

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Abstract. Acquisition of radioresistance (RR) has been reported during cancer treatment with fractionated irradiation. However, RR is poorly understood in the prognosis of radiotherapy. Although radiotherapy is important in the treatment of prostate cancer (PCa), acquisition of RR has been reported in PCa with an increased number of cancer stem cells (CSCs), neuroendocrine differentiation (NED) and epithelial-mesenchymal transition. However, to the best of our knowledge, the mechanism underlying RR acquisition during fractionated irradiation remains unclear. In the present study, human PCa cell lines were subjected to fractionated irradiation according to a fixed schedule as follows: Irradiation (IR)1, 2 Gy/day with a total of 20 Gy; IR2, 4 Gy/day with a total of 20 Gy; and IR3, 4 Gy/day with a total of 56 Gy. The expression of cluster of differentiation (CD)44, a CSC marker, was identified to be increased by fractionated irradiation, particularly in DU145 cells. The expression levels of CD133 and CD138 were increased compared with those in parental cells following a single irradiation or multiple irradiations; however, the expression levels decreased with subsequent irradiation. RR was evidently acquired by exposure to 56 Gy radiation, which resulted in increased expression of the NED markers CD133 and CD138, and increased mRNA expression levels of the pluripotency-associated genes octamer-binding transcription factor 4 and Nanog homeobox. These data indicate that radiation-induced CSCs emerge due to the exposure of cells to fractionated irradiation. In addition, the consequent increase in the expression of NED markers is possibly induced by the increased expression of pluripotency-associated genes. Therefore, it can be suggested that cancer cells acquire RR due to increased expression of pluripotency-associated genes following exposure to fractionated irradiation.

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Introduction

Prostate cancer (PCa) is the second most common cancer type in men worldwide (1). In Japan, the number of patients diagnosed with PCa has increased since the use of prostate-specific antigen screening (2). In 2017, the number of individuals diagnosed with PCa was 86,100, which ranked as the third most common type of disease in Japanese men. Radiotherapy is effective for treating localized PCa; however, 20-40% of patients with high-risk PCa experience tumor recurrence or distant metastases (3-6).

One of the causes of tumor recurrence and metastasis is that cancer cells acquire radioresistance (RR) during fractionated irradiation (7). It has been reported that RR is acquired in various cancer types, including head and neck cancer, non-small cell lung cancer, breast cancer and PCa (8-11). RR cells demonstrate resistance to the induction of apoptosis, decreased production of reactive oxygen species (ROS) (12), increased activation of DNA repair (13), and exhibit high migratory and invasive abilities (14). RR PCa cells exhibit a number of features, including an increase in the number of cancer stem cells (CSCs) (15-17), increased neuroendocrine differentiation (NED) (18-20) and increased epithelial-mesenchymal transition (15,16).

The prostatic epithelium is composed of basal cells, luminal cells and neuroendocrine (NE) cells, the majority of which are secretory cells. Although the physiological role of NE cells is unknown (21), NED has been reported to be caused by androgen deprivation or radiotherapy, which results in the emergence of NE-like cells (19,22). NE-like cells do not proliferate and this dormant phenotype renders them resistant to the cancer treatment (19,20). However, CSCs cause tumor recurrence and metastasis following treatment due to their ability to undergo self-renewal and their differentiation into various cell types to form the tumor bulk (23-27). PCa cells expressing surface antigens, including cluster of differentiation (CD)44 (also termed Hermes) (23,24), CD133 (also termed prominin-1) (16) and CD138 (also termed syndecan-1) (26),

exhibit CSC-like properties. Therefore, these surface antigens are used as CSC markers. Furthermore, it has been reported that pluripotency-associated genes serve important roles in maintaining CSC characteristics (28-30) and in regulating NED (31).

Cancer cells acquire RR by fractionated irradiation, and certain features of RR cells have been described (8-17). However, to the best of our knowledge, the mechanism underlying RR acquisition during fractionated irradiation is unclear. An improved understanding of the mechanism of RR acquisition is required to facilitate the development of novel and effective treatment strategies. The present study aimed to identify the factors associated with the acquisition of RR during fractionated irradiation and investigate the acquisition mechanism.

Materials and methods

Cell lines and reagents. The human PCa cell lines, PC3 (bone metastatic cell line), DU145 (brain metastatic cell line) and LNCaP (lymph node metastatic cell line), were purchased from RIKEN BioResource Center (Tsukuba, Japan). The cells were cultured at 37˚C in a 5% CO_2 environment in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Japan Bioserum Co. Ltd. Hiroshima, Japan) and 1% penicillin/streptomycin (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Phycoerythrin (PE)-conjugated monoclonal mouse anti-human CD44 (catalog no. 338808), PE mouse IgG1, κ isotype control (catalog no. 400114), peridinin chlorophyll protein complex/cyanine5.5 (PerCP/Cy5.5)-conjugated monoclonal mouse anti-human CD138 (catalog no. 356510) and PerCP/Cy5.5 mouse IgG1, κ isotype control (catalog no. 400150) were purchased from BioLegend, Inc. (Tokyo, Japan). Allophycocyanin (APC)-conjugated monoclonal mouse anti-human CD133/1 (catalog no. 130-090-826) and APC mouse IgG1, κ isotype control antibody (catalog no. 130-092-214) were purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). TB Green Premix Ex Taq and ROX Reference Dye (catalog no. RR820A) were purchased from Takara Bio Inc. (Otsu, Japan).

X-ray irradiation of PCa cell lines. DU145, PC3 and LNCaP cells were irradiated with X-ray (150 kV, 20 mA, 1.0 Gy/min) through a 0.5-mm aluminum and 0.3-mm copper filter using an X-ray generator (MBR-1520R-3; Hitachi Ltd., Tokyo, Japan), with a distance of 45 cm between the focus and target. The dose was monitored with a thimble ionization chamber placed next to the sample during irradiation. Cells were subjected to fractionated irradiation according to the following schedule: Irradiation (IR)1, 2 Gy/day with a total dose of 20 Gy; IR2, 4 Gy/day with a total dose of 20 Gy; and IR3, 4 Gy/day with a total dose of 56 Gy.

Colony formation assay. The clonogenic potency was estimated by a colony formation assay. DU145, PC3 and LNCaP cells were seeded in appropriate numbers as presented in Table I, subjected to X-ray irradiation, fixed with methanol (Wako Pure Chemical Industries, Ltd.) for 1 min at room temperature, 10-20 days after irradiation and stained with Giemsa for 2 h at room temperature (Wako Pure Chemical Industries, Ltd.). Colonies consisting of >50 cells were counted. The survival fraction for each cell line was calculated as the plating efficiency of the irradiated samples compared with that of the non-irradiated samples.

Flow cytometric analysis. To analyze the expression of the CSC markers, the cells were incubated in 100 μl PBS without calcium chloride and magnesium chloride [PBS(-); Takara Bio Inc.] containing 5% FBS used as blocking agent and PE anti-human CD44 (3 μl/10^6 cells), CD133/1-APC (3 μl:10^6 cells), PerCP/Cy5.5 anti-human CD138 (3 μl/10^6 cells) or respective mouse IgG1 isotype control antibodies (3 μl/10^6 cells) for 15 min at 4˚C in the dark. Following staining, the cells were collected by centrifugation at 300 x g for 5 min at 4˚C, resuspended in PBS and analyzed by direct immunofluorescence flow cytometry using a BD FACS Aria™ Cell Sorter (BD Biosciences, San Jose, CA, USA). The fluorescence values of the respective isotype controls were subtracted from the fluorescence data of CD44, CD133 and CD138. The results were analyzed using the Kaluza software (version 1.5a; Beckman Coulter, Inc., Brea, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from DU145, PC3 and LNCaP cells using the RNeasy® Mini kit (Qiagen GmbH, Hilden, Germany) and diluted to 100 ng/μl in nuclease-free water. Complementary DNA (cDNA) was synthesized from the isolated total RNA using a High-Capacity cDNA Reverse Transcriptase kit (Thermo Fisher Scientific Inc.). RT-qPCR was performed in a 20-μl reaction mixture containing 1X TB Green Premix Ex Taq II, primer pairs (sequences summarized in Table II) at a concentration of 0.5 μM and the cDNA template (100 ng/μl total RNA for cDNA synthesis). GAPDH mRNA was used as an endogenous control. The conditions for the Real-Time qPCR system (StepOne Plus; Thermo Fisher Scientific, Inc.) were set at 95˚C for 30 sec, followed by 40 cycles of incubation at 95˚C for 5 sec and 54˚C for 30 sec. The results were analyzed using the $2^{-\Delta\Delta Cq}$ method (32).

Statistical analysis. Statistically significant differences between the control group and the experimental group were determined using Student's t-test or Welch's t-test for comparisons between two groups. Multiple comparisons were performed with one-way analysis of variance followed by the Tukey-Kramer method or Scheffe's F test. Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA) with the add-in software Statcel (version 3; OMS Publishing, Inc., Saitama, Japan) was used to perform the statistical analyses. Data are presented as the mean ± standard deviation from three independent experiments. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Establishment of RR cells. Fractionated irradiation was performed according to a previously reported schedule to establish the RR cells. DU145-IR3 cells were obtained by subjecting DU145 cells to 4 Gy/day with a total irradiation dose of 56 Gy. Since PC3 and LNCaP cells failed to proliferate

Table I. Cell number seeded for the colony formation assay.

Absorbed dose [Gy]	Cell line								
	PC3-P	PC3-IR1	PC3-IR2	DU145-P	DU145-IR1	DU145-IR2	DU145-IR3	LNCaP-P	LNCaP-IR1
0	200	100	100	200	200	200	100	200	200
1		300			300			300	800
2	500	500	200	500	500	500	200	500	500
3	800								
4	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	2,000
5	2,000								10,000
6	3,000	2,000	5,000	3,000	2,000	3,000	3,000	5,000	20,000
8	5,000	3,000	30,000	5,000	3,000	10,000	10,000	20,000	20,000
10	8,000	5,000	60,000	20,000	5,000	20,000	20,000		40,000

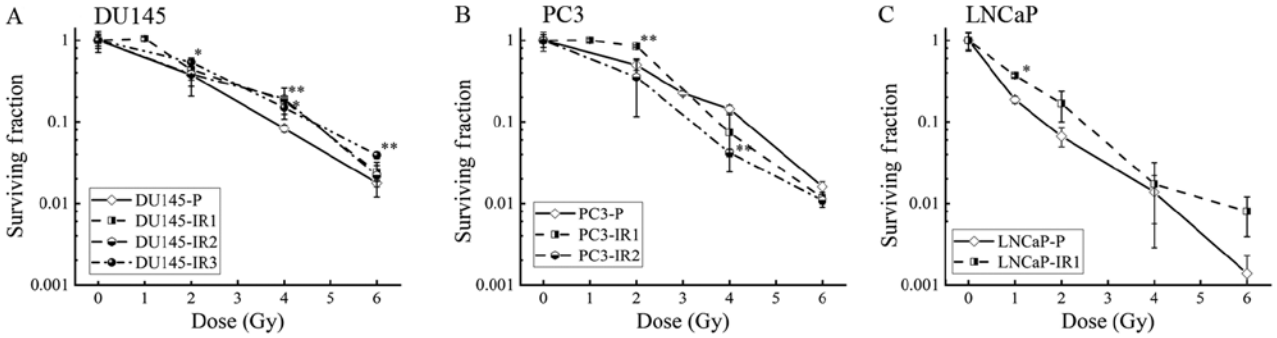

Figure 1. Evaluation of the viability of irradiated cells by colony formation assay. (A) Comparison of the viability of DU145-P and DU145-IR1-3 cells. (B) Comparison of the viability of PC3-P, PC3-IR1 and PC3-IR2 cells. (C) Comparison of the viability of LNCaP-P and LNCaP-IR1 cells. P-values were calculated by (A and B) one-way analysis of variance followed by the Tukey-Kramer method or (C) Welch's t test. *P<0.05 and **P<0.01 vs. P. P, parental; IR1, 2 Gy/day (total 20 Gy); IR2, 4 Gy/day (total 20 Gy); IR3, 4 Gy/day (total 56 Gy).

following irradiation treatment, IR3 in PC3 cells, and IR2 and IR3 in LNCaP, these RR cells were not established. Therefore, the changes in viability of DU145-IR3 cells compared with DU145-parental (P) cells were analyzed by colony forming assay. DU145-IR3 cells acquired significant RR between 2-6 Gy compared with DU145-P cells (Fig. 1A). Since PC3 and LNCaP cells did not proliferate following irradiation with 56 Gy, these cells were only subjected to fractionated irradiation with a total dose of 20 Gy. To investigate whether there was a change in the cell characteristics between 10 fractions and 5 fractions, 2 Gy for 10 fractions (IR1) and 4 Gy for 5 fractions (IR2) were used, respectively. In the DU145 and PC3 cells, IR1 and IR2 cells were established; however, in LNCaP cells only IR1 cells were established. In the PC3-IR1 and LNCaP-IR1 cells, the cell viability was increased following exposure to 1-2 Gy radiation compared with that in the PC3-P and LNCaP-P cells, respectively; however, no change was observed in the high dose range. Additionally, in DU145-IR2 and PC3-IR2 cells, no significant increase in cell viability was identified compared with that in the DU145-P and PC3-P cells, respectively (Fig. 1A-C).

Expression of CSC markers following fractionated irradiation. It has been reported that the expression of CSC markers increases upon X-ray irradiation and that the markers are highly expressed in RR cells compared with P cells (16). Therefore, the present study performed flow cytometry analysis to evaluate the variations in the expression of CD44, CD133 and CD138 in PC3, DU145, and LNCaP cell lines following exposure to fractionated irradiation (IR1, 2 Gy/day with a total dose of 20 Gy). Since the expression of CD133 in PC3 cells, and of CD44 in LNCaP cells were not detectable, the expression profiles of CD44 and CD138 in PC3 cells, CD44, CD133 and CD138 in DU145 cells, and CD133 and CD138 markers in LNCaP cells were confirmed (Fig. 2A and B). The variations in the ratio of CD44, CD133 and CD138 markers were analyzed during fractionated irradiation. In addition, the percentages of $CD44^{+}/CD138^{+}$ PC3 cells, $CD44^{+}/CD133^{+}/CD138^{+}$ DU145 cells and $CD133^{+}/CD138^{+}$ LNCaP cells were analyzed (Fig. 2C-E). CD44 was highly expressed in PC3-P cells (51.99±12.45%) and DU145-P cells (74.84±0.79%), and the expression was significantly increased in IR1 cells (PC3-IR1, 79.32±1.44%; DU145-IR1, 83.71±5.18%) compared with that in P cells (Fig. 2C and D). CD133 was increased by 2 Gy x 1 fraction in the DU145 and LNCaP cells (1.73±1.07 and 5.99±0.39%, respectively) compared with that in the P cells (0.43±0.44 and 0.120±0.19%, respectively). In LNCaP cells, CD133 expression decreased by 2 Gy x 2-8 fractions, but increased again following 2 Gy x 9 and 10 fractions (0.27±0.24 and 0.82±1.05%, respectively; Fig. 2E). The expression of CD138 could not be confirmed in PC3-P cells, but was increased by

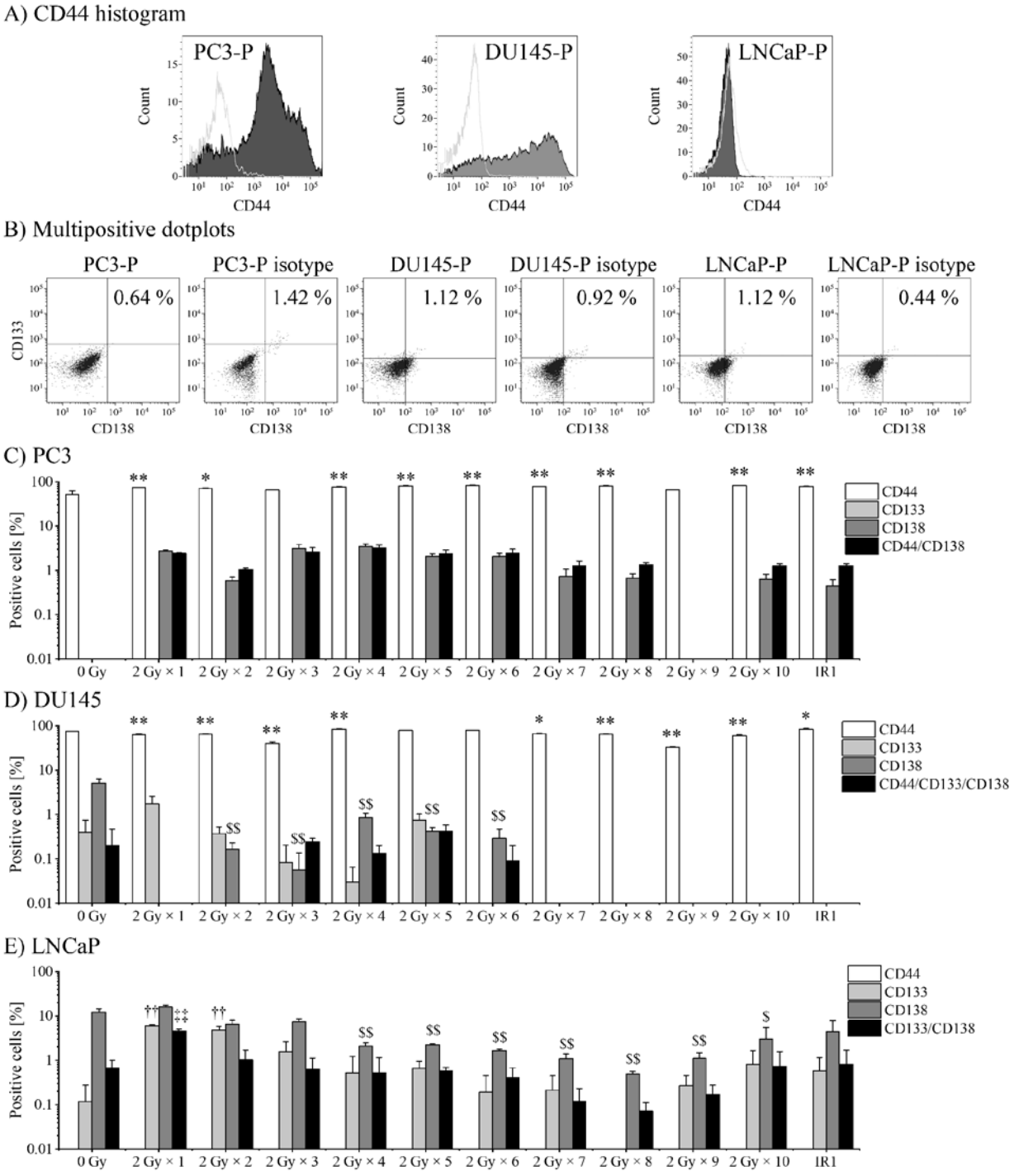

Figure 2. Expression of cancer stem cell markers following fractionated irradiation. The fraction of cells expressing CD44, CD133 and CD138 markers was analyzed by flow cytometry. Representative histograms and dot plots of PC3-P, DU145-P and LNCaP-P cells are presented to illustrate the identification of multipositive cells. (A) The $CD44^+$ cell population (grey area) was gated and the fluorescence value of the isotype control population (empty area) was subtracted. (B) $CD133^+$ and $CD138^+$ cells in the gated $CD44^+$ population of PC3-P cells and DU145-P cells, and the ungated LNCaP-P cells. $CD133^+$ and $CD138^+$ cells were subtracted from their respective isotype controls. The percentage of $CD44^+$, $CD133^+$, $CD138^+$ and multipositive cells of (C) PC3 cells, (D) DU145 cells and (E) LNCaP cells. The ratio of $CD44^+/CD138^+$ cells in the PC3 cell population, the ratio of $CD44^+/CD133^+/CD138^+$ cells in DU145 cell population and the ratio of $CD133^+/CD138^+$ cells in the LNCaP cell population have been depicted for the multipositive cells. P-values were calculated by the Tukey-Kramer method following one-way analysis of variance. $^*P<0.05$, $^{**}P<0.01$ vs. $CD44^+$ population of P cells; $^{\dagger\dagger}P<0.01$ vs. $CD133^+$ population of LNCaP-P cells; $^{\$}P<0.05$, $^{\$\$}P<0.01$ vs. $CD138^+$ population of P cells; and $^{\ddagger\ddagger}P<0.01$ vs. $CD133^+/CD138^+$ population of LNCaP-P cells. IR1, 2 Gy/day (total 20 Gy); P, parental; CD, cluster of differentiation.

2 Gy x 4 fractions (3.51±0.59%) and decreased by subsequent fractions. In DU145 cells, CD138 was highly expressed in P cells (5.15±1.30%), but decreased following fraction. In LNCaP cells, the expression of CD138 increased by 2 Gy x 1 fraction compared with that in P cells (15.93±1.72 vs. 12.16±3.05%), decreased with subsequent fraction and began to increase with 2 Gy x 9 and 10 fractions (1.10±0.47 and 2.99±3.05%, respectively; Fig. 2E). Expression of CD44/CD138 in PC3-P cells was increased by

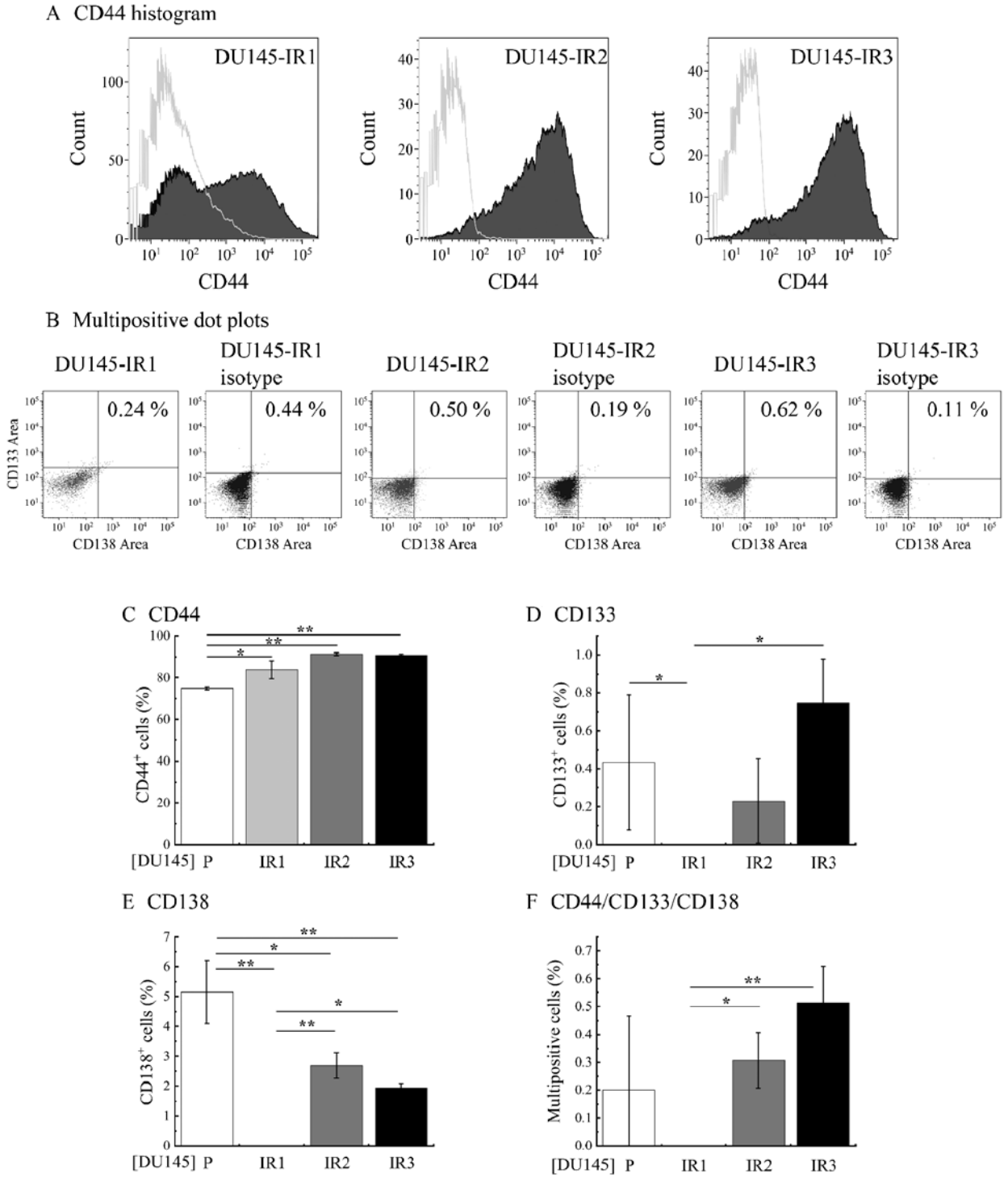

Figure 3. Changes in the ratio of CSC markers in cells subjected to fractionated irradiation. The ratio of cells expressing CD44, CD133 and CD138 was analyzed by flow cytometry. (A) The $CD44^+$ cell population (grey area) was gated and the fluorescence value of the isotype control population (empty area) was subtracted. (B) $CD133^+$ and $CD138^+$ cells in the gated $CD44^+$ population of DU145-P, -IR1, -IR2, and -IR3 cells. $CD133^+$ and $CD138^+$ cells were subtracted from their respective isotype controls. The ratio of (C) $CD44^+$, (D) $CD133^+$, (E) $CD138^+$ and (F) $CD44^+/CD133^+/CD138^+$ cells. P-values were calculated by the Tukey-Kramer method following one-way analysis of variance. $^*P<0.05$ and $^{**}P<0.01$. IR1, 2 Gy/day (total 20 Gy); IR2, 4 Gy/day (total 20 Gy); IR3, 4 Gy/day (total 56 Gy); P, parental; CD, cluster of differentiation.

2 Gy x 1 fraction (0.27±0.34%) and was enhanced the most by 2 Gy x 4 fractions (3.23±0.51%). In DU145 cells, no marked variation in the frequency of $CD44^+/CD133^+/CD138^+$ cells was identified following fractionated irradiation. In LNCaP cells, the expression of CD133/CD138 was significantly increased by 2 Gy x 1 fraction (4.62±0.40%) compared with that in P cells (0.68±0.31%), and the expression was decreased by subsequent fraction. Therefore, it is clear that the proportion of $CD44^+$ cells increases following fractionated irradiation. The fractions of $CD133^+$ and $CD138^+$ cells increased by 2 Gy x 1 fraction or subsequent irradiations; however, their expression was decreased by subsequent irradiation.

Table II. Primer sequences of the target genes.

Primer	Sequence (5'-3')
NSE	
Forward	AGCTGCCCCTGCCTTAC
Reverse	GAGACAAACAGCGTTACTTAG
CgA	
Forward	GCGGTGGAAGAGCCATCAT
Reverse	TCTGTGGCTTCACCACTTTTCTC
SOX2	
Forward	ATGCACAACTCGGAGATCAGC
Reverse	CCTTCTTCATGAGCGTCTTGG
c-Myc	
Forward	GCCACGTCTCCACACATCAG
Reverse	TCTTGGCAGCAGGATAGTCCTT
NANOG	
Forward	TAGCAATGGTGTGACGCAGAAG
Reverse	TCTGGTTGCTCCACATTGGAAGG
OCT4	
Forward	GAGGCAACCTGGAGAATTTGTTCC
Reverse	ATGTGGCTGATCTGCTGCAGTG
KLF4	
Forward	GCGAGTCTGACATGGCTGT
Reverse	GTCGCTTCATGTGGGAGAG
GAPDH	
Forward	GTGAAGGTCGGAGTCAACG
Reverse	TGAGGTCAATGAAGGGGTC

NSE, neuron-specific enolase; CgA, chromogranin A; OCT4, octamer-binding transcription factor 4; NANOG, Nanog homeobox; SOX2, sex determining region Y-box 2; KLF4, Kruppel-like factor 4.

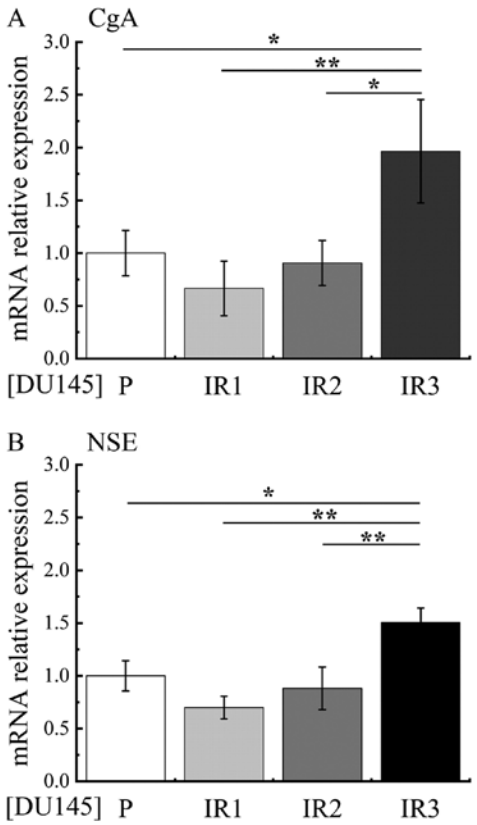

Figure 4. Differential expression of the NED markers CgA and NSE following fractionated irradiation. The relative mRNA expression levels of (A) CgA and (B) NSE were analyzed by reverse transcription-quantitative polymerase chain reaction. P cells were used as the control. P-values were calculated by the Tukey-Kramer method following one-way analysis of variance. *P<0.05 and **P<0.01. IR1, 2 Gy/day (total 20 Gy); IR2, 4 Gy/day (total 20 Gy); IR3, 4 Gy/day (total 56 Gy); P, parental; NED, neuroendocrine differentiation; CgA, chromogranin A; NSE, neuron-specific enolase.

Analysis of CSC markers, NED markers and pluripotency-associated genes in RR cells. To investigate how the levels of CSC markers, NED markers and pluripotency-associated genes change during RR acquisition by fractionated irradiation, these factors were analyzed in P cells, cells that did not acquire RR by fractionated irradiation and RR cells. PC3 cells and LNCaP cells did not exhibit RR with 4 Gy. Since DU145 cells did acquire RR with 2-6 Gy, CSC markers, NED markers and pluripotency-associated genes were examined in DU145-P, DU145-IR1, DU145-IR2 and DU145-IR3 cells.

Representative histograms and dot plots are presented in Fig. 3A and B. Expression of the CD44 marker was significantly increased in DU145-IR1 cells (Fig. 3C) compared with that in DU145-P cells; however, the expression of CD133 and CD138 markers was not identified in DU145-IR1 cells. In DU145-IR2 and DU145-IR3, the proportion of $CD44^{+}$ cells increased compared with that observed for DU145-P and DU145-IR1 cells (DU145-IR2, 91.27±0.79%; DU145-IR3, 90.75±0.58%). In addition, the proportion of $CD133^{+}$ (DU145-IR2, 0.23±0.27%; DU145-IR3, 0.75±0.28%) and $CD138^{+}$ cells (DU145-IR2, 2.70±0.53%; DU145-IR3, 1.93±0.20%) was significantly higher for DU145-IR2 and DU145-IR3 cells compared with that for DU145-P cells (Fig. 3D and E). Furthermore, the proportion of $CD44^{+}/CD133^{+}/CD138^{+}$ cells was low for the P cells (0.22±0.24%) and was not confirmed in IR1 cells, and was significantly increased in the IR2 (0.31±0.10%) and IR3 cells (0.51±0.13%) compared with that in the IR1 cells (Fig. 3F).

Secondly, to investigate NED, mRNA expression levels of the NED markers chromogranin A (CgA) and neuron-specific enolase (NSE) were measured by RT-qPCR. The expression levels of CgA and NSE were decreased in DU145-IR1 cells compared with those in DU145-P cells, whereas the expression levels were significantly increased in DU145-IR3 cells compared with those in DU145-P, DU145-IR1 and DU145-IR2 cells (Fig. 4).

Thirdly, the relative mRNA expression levels of the pluripotency-associated genes c-Myc, octamer-binding transcription factor 4 (OCT4), Nanog homeobox (NANOG), sex determining region Y-box 2 (SOX2) and Kruppel-like factor 4 (KLF4) were analyzed by RT-qPCR. The relative mRNA expression levels of OCT4 and NANOG were significantly increased in DU145-IR3 cells compared with those in DU145-IR2 cells (Fig. 5A and B). However, no significant increases in the relative mRNA expression levels of c-Myc, SOX and KLF4 were identified in DU145-IR3 cells (Fig. 5C-E).

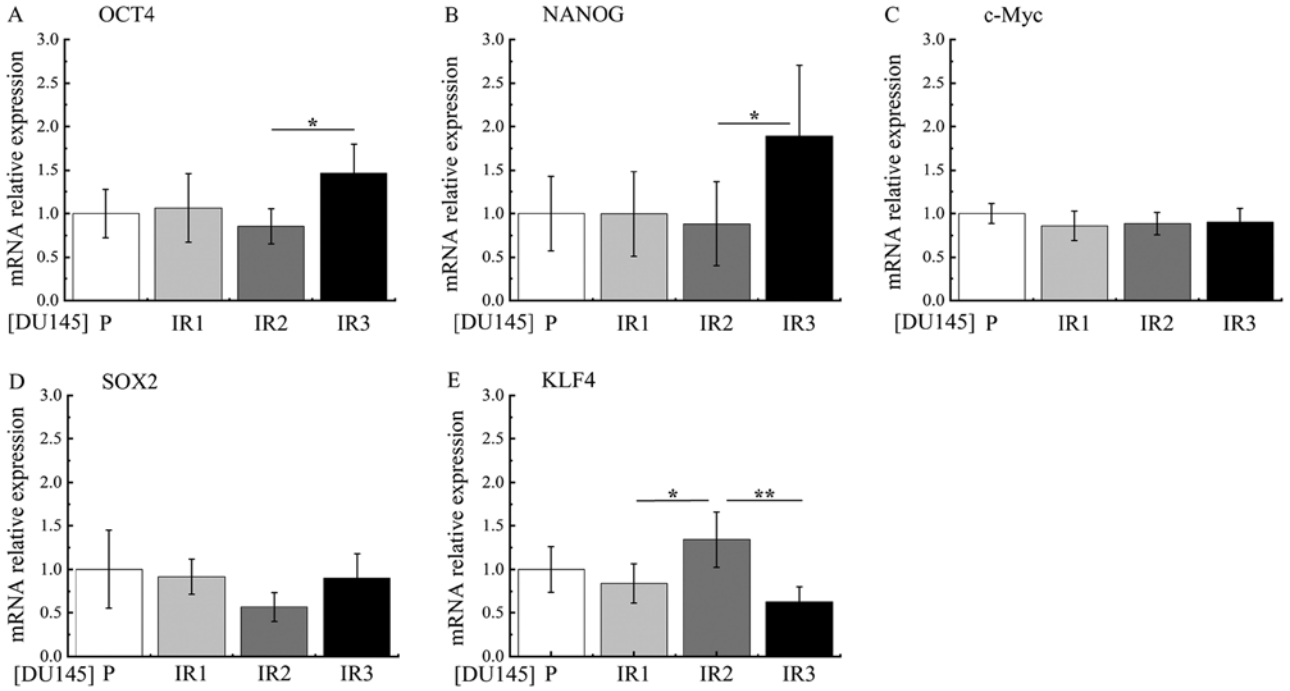

Figure 5. Expression of pluripotency-associated genes following fractionated irradiation. The relative mRNA expression levels of (A) OCT4, (B) NANOG, (C) c-Myc, (D) SOX2 and (E) KLF4 were analyzed by reverse transcription-quantitative polymerase chain reaction. P cells were used as the control. P-values were calculated by the Tukey-Kramer method following one-way analysis of variance. *P<0.05 and **P<0.01. IR1, 2 Gy/day (total 20 Gy); IR2, 4 Gy/day (total 20 Gy); IR3, 4 Gy/day (total 56 Gy); P, parental; OCT4, octamer-binding transcription factor 4; NANOG, Nanog homeobox; SOX2, sex determining region Y-box 2; KLF4, Kruppel-like factor 4.

Analysis of CSC markers, NED markers and pluripotency-associated genes in DU145-P, DU145-IR1, DU145-IR2 and DU145-IR3 cells revealed that the CSC markers were re-expressed, the levels of NED markers were increased and the mRNA expression levels of the pluripotency-associated genes OCT4 and NANOG were increased during fractionated irradiation.

Discussion

The present study examined factors associated with RR acquisition, including CSC markers, NED markers and pluripotency-associated genes, to understand the acquisition mechanism of RR, which remains a predictive factor for a poor prognosis following radiotherapy (16,19,31,33). The results of the current study revealed that the proportion of $CD44^{+}$ cells increased following fractionated irradiation. The percentages of $CD133^{+}$ and $CD138^{+}$ cells were identified to increase by single irradiation or multiple irradiations compared with the percentages of P cells; however, the expression levels of these markers decreased with subsequent irradiation. Furthermore, when RR was acquired by 56 Gy irradiation, the CSC markers CD133 and CD138 were re-expressed, the levels of NED markers increased and the mRNA expression levels of the pluripotency-associated genes OCT4 and NANOG increased.

Radiotherapy has been reported to eliminate the majority of non-CSCs (6). However, few CSCs are identified in cancer types that demonstrate relative RR by lowered ROS production and efficient DNA repair (34,35), which results in a poor prognosis. The present results revealed increased expression of CD44 triggered by fractionated irradiation and increased expression levels of CD133 and CD138 by single irradiation or multiple irradiations, which is consistent with the concept of CSCs (16,23). While the expression levels of CD133 and CD138 were decreased by continued fractionated irradiation, they subsequently increased in RR cells. Consistent with this result, Lagadec *et al* (36) reported that the proportion of $CD24^{-/low}/CD44^{high}$ cells was increased by multiple irradiations; however, with 2 Gy x 8 irradiations the proportion returned to the same level compared with that in non-irradiated cells, in breast cancer cell lines. These results suggest that the number of hypothetical CSCs decreases relative to the non-CSCs if the irradiation exceeds the tolerable dose by fractionated irradiation. To the best of our knowledge, the present study provides the first evidence that the frequency of CSCs decreases once during fractionated irradiation and then increases again.

Since NE-like cells do not proliferate and express survival genes, including survivin and B-cell lymphoma 2 (37,38), they may be a cause of treatment failure. In addition, NE-like cells secrete several peptide hormones and eutopic bioactive hormones, including serotonin, and promote the growth of surrounding tumor cells (18,29,40). Furthermore, NED is a reversible process and the dedifferentiated cells can resume proliferation (41). Therefore, NE-like cells exhibit a dormancy phenotype and are a predictive factor for a poor prognosis. Deng *et al* (19) established RR cells by fractionated irradiation and demonstrated that NED was induced by the increasing nuclear content of cyclic AMP response element binding protein of the basic leucine zipper family and cytoplasmic accumulation of activating transcription factor 2. The same

study reported that NE-like cells that emerge by fractionated irradiation maintain RR even if they are dedifferentiated and resume proliferation. The current study confirmed that the NED markers CgA and NSE did not increase by 20 Gy radiation exposure, but increased only in the RR cells.

The OCT4, SOX2 and NANOG proteins co-occupy the promoters of various target genes and contribute to pluripotency and the self-renewal of embryonic stem cells (42). In addition, c-Myc, OCT4, SOX2 and KLF4 are essential for the generation of the pluripotent phenotype from differentiated cells (43), and their expression is associated with tumor progression (28-31,44). OCT4 and NANOG modulate the expression of various CSC-associated molecules, including CD44 and CD133 (31,33), and the overexpression of NANOG upregulates CSC markers, including CD44 and CD133 (31). Furthermore, OCT4 is highly expressed in $CD133^+$ cells and OCT4-knockdown inhibits the expression of CD133 and sensitizes the cells to radiation and chemotherapy (44). In addition, OCT4-positive cells have been reported to co-express the NED markers CgA and synaptophysin in PCa samples (45).

In the present study, CD44 expression increased, while the CD133 and CD138 markers were re-expressed by fractionated irradiation, and OCT4 and NANOG mRNA expression levels increased only in RR cells. These results suggest that pluripotency-associated genes were upregulated by fractionated irradiation, resulting in the acquisition of CSC properties by the cancer cells. These findings strongly suggest that radiation-induced CSCs emerge by fractionated irradiation. Whether or not OCT4 can regulate the progression of NED remains unclear; however, these findings suggest that OCT4 may contribute to pluripotency and the maintenance of NED.

In summary, the current data demonstrate that the expression levels of CD133 and CD138 are increased by single irradiation or multiple irradiations but are decreased by fractionated irradiation. However, CD133 and CD138 are re-expressed by repeating fractionated irradiation. It can be suggested that the increased expression of pluripotency-associated genes caused by fractionated irradiation may result in the emergence of induced CSCs and the progression of NED, which triggers RR acquisition in PCa.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

KM, RS, SM, ET and YH conceived the study and participated in its design and coordination. KM, RS and KH drafted the manuscript. KM, RS and KH performed the experiments, and analyzed and interpreted the data. SM, ET and YH critically revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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