·Cancer Stem Cell Column·

Effects of heterochromatin in colorectal cancer stem cells on radiosensitivity

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[Abstract] Background and Objective: Radiotherapy (RT) is a major non-surgical modality in the comprehensive treatment for colorectal adenocarcinoma. The radioresistance of cancer stem cells (CSCs) is a key factor that influences therapeutic effectiveness. This study was to investigate the effects of specific chromosome structure and histone modification in CSCs in colorectal adenocarcinoma radioresistance. **Methods:** Samples were collected from resected human colorectal adenocarcinomas. Subcutaneous colorectal cancer model was established in nude mice. Immunohistochemistry showed that xenografts generated from bulk colorectal cancer cells resembled the original tumor specimen. Flow cytometry was performed to sort CSCs ($CD133^+$) and non-CSCs ($CD133^-$) from both resected samples of colorectal adenocarcinoma and xenograft before and after high single-dose radiation. The markers labeling heterochromatin (H3K9me3, HP1-α and H3K4me1) and euchromatin (H3K4me3) in $CD133^+$ and $CD133^-$ nucleus were detected by immunoflurescence. **Results:** There was distinct difference in chromatin structure between colorectal CSCs ($CD133^+$) and non-CSCs ($CD133^-$). The chromatin displayed compact patches in $CD133^+$ nucleus, but loosely latticed structure in $CD133^-$ nucleus; immunoflurescence verified that the compact patches existing in CSCs was generated from heterochromatin construction. In addition, the vacuole-like defect in heterochromatin regions of CSCs was observed within 24 h after exposure to 10 gray (Gy) single-dose RT. Interestingly, this phenomenon was repaired from 96 h, and recovered to dense plaque structure in heterochromatin regions of CSCs after 144 h. However, no significant difference in non-CSCs was observed after RT exception for a loose chromatin structure. **Conclusions:** CSCs play a role in radiosensitivity in colorectal cancer. The mechanism may be related to heterchromatin formation and histone methylation.

Key words: Colorectal neoplasm, cancer stem cell, high-single-dose radiation, radioresistance, heterochromatin

Colorectal adenocarcinoma is a common type of malignant tumor, but it has become one of malignant tumors with a high incidence and mortality[1]. Standardized treatment for colorectal cancer includes surgery, radiotherapy and chemotherapy. Radiotherapy has been widely recognized as an effective non-surgical treatment. However, the resistance of radiotherapy remains a problem for clinicians. Once the radiation resistance was overcome, the efficacy of radiotherapy and prognosis would be improved.

Solid malignant tumor is a complex system controlled by multi-factors and departed from normal body. Its growth and maintenance depend on the regulation and interaction among different types of cells in the microenvironment. With the growth of tumor, different types of tumor cells demonstrate cellular heterogeneity and differences in differentiating abilities. A limited number of cells had similar biological behavior and characteristics to normal stem cells. This group of cells, with the capacities of self-renewal and multi-differentiation and maintenance of the proliferative potential of tumors, are called as cancer stem cells (CSCs)[2-5]. With in-depth study of CSCs, Michael Clarke *et al*. have isolated a group of cancer cells with CD133 expression from human colorectal adenocarcinoma. These cells have the same characteristics with the other CSCs, and revert to tumor heterogeneity in vivo and in vitro[6].

The ideal cancer treatment is to control 100% of the tumor cells effectively. Unfortunately, a small number of cancer cells are resistant to the treatment, which causes disease progression and recurrence. Recent studies have shown that CSCs are more resistant to chemotherapy than non-CSCs[7,8]. Therefore,

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recurrence of the treated cancer may be related to resistance of CSCs[9]. Radiosensitivity of glioblastoma CSCs was significantly lower than that of non-CSCs[10]. The similar results were confirmed in breast cancer[11]. The mechanisms of high radiation resistance of CSCs mainly focus on the following points: a high degree of radiation resistance of CSCs may be related to DNA repair capacity, which promotes angiogenesis by enhancing vascular endothelial growth factor (VEGF) expression[12], or to hypoxic niche, and so on[13].

With the finding of CSCs in humor colorectal adenocarinoma, more attention has been paid to the studies of characteristics of CSCs and therapeutic sensitivity. However, the mechanism of CSCs radiosensitivity has not been completely clarified. Previous studies about radiation resistance of CSCs mainly focused on the effects of tumor microenvironment or DNA repair ability. It is well known that in eukaryotic cells, the chromosome DNA and histones are regulated each other. Histone packs DNA and the post-transcriptional modification of histone regulates DNA[14]. In this study, we compared the chromatin structure of colorectal cancer CSCs ($CD133^+$) and non-CSC ($CD133^-$) before and after radiation, and analyzed the characteristics and changes of histone modification so as to find out whether there is a relationship between characteristic chromosome structure and radiation resistance of tumor cells.

Materials and Methods

Materials

Human colorectal adenocarcinoma samples were collected from 16 patients (N1–N16) who underwent surgery of anorectum in West China Hospital of Sichuan University. There were 10 men and 6 women, aged 34–80 years. Among them, there were seven cases of ascending colon adenocarcinoma, three cases of descending colon adenocarcinoma, four cases of sigmoid colon cancer, and two cases of rectal cancer. Five cases were in clinical stage IIIb, seven in stage IIIc and four in stage IV. All patients were confirmed by histopathology. The experiments were approved by the Ethics Committee of West China Hospital of Sichuan University and informed consent was obtained from all the patients. The tumor tissues were washed by sterile phosphate buffer solution (PBS) and doused overnight in DMEM with 25 u/mL penicillin, 25 μg/mL streptomycin and 10 μg/mL amphotericin B. After removing the necrotic tissues and adipose tissues, we kept the samples under sterile and cold conditions until treatment.

Methods

Establishment of subcutaneous human colorectal cancer mouse model Forty of 6–8 weeks old nude mice, 20 males and 20 females, were purchased from Experimental Animal Center, Chinese Academy of Medical Sciences (animal qualified code: SCSK Beijing, 2009-0004). Animals were housed in SPF-grade breeding room and operations were performed in the light of principles of asepsis.

Cancer cells of 1×10^6 were inoculated to the right and left flanks of nude mice. All nude mice were exposed to 350 cGy systemic radiation to enhance the rate of tumor formation. The tumor growth was monitored regularly. The volume of tumor was calculated by the following formula: $V\,(mm^3) = A\,(mm) \times B\,(mm)^2 / 2$. A and B stand for the longest and shortest diameters of the tumor, respectively. Eleven subcutaneous colorectal cancer models were successfully established from surgical specimens of 16 patients, and the rate of tumor formation was 68%. All animal experiments complied with the relevant laws and regulations of Sichuan University Animal Ethics Committee.

Immunohistochemistry After stripping, the tumor tissues were fixed in 10% formalin and continuous resection of 5 μm in thickness was made. Tissues were dewaxed by xylene and rehydrated by distilled water. Slices were treated with 10 mmol/L citrate buffer (pH6) and antigen was repaired by microwave heating. Endogenous peroxidase was blocked by 3% hydrogen peroxide methanol solution. Then antibodies were added to the slices, and incubated for 1 h at room temperature. DAB (DakoCytomation) was used to colorize. The antibodies and dilutions were: CDX-2 (BioGenex, 1:100), CK20 (Dako, 1:50), and CK7 (Dako, 1:50) CEA (Fujian Maxim Biotechnological Co. Ltd., 1:50).

Flow cytometry After shearing, tumor tissues were digested by IV collagenase (Sigma) for 1.5 h. Tumor tissues were prepared into single cell suspensions by filtrating and gradient centrifugation. In order to improve the purification of sorted CSCs, $Lineage^+$ cells, murine cells and dead cells were removed. The markers of Lineage included CD2, CD31, CD45 and CD64 antibodies (BD, Biosciences). Dead cells were labeled with 7-ADD (BD, Biosciences). The cell suspensions were divided into two tubes, one was a blank control and the other was added phycoerythrin-coupled anti-CD133 (CD133-phycoerythrin) monoclonal antibody (BD, Biosciences), and incubated at 4℃ overnight. $CD133^+$ and $CD133^-$ cells were separated by florescence excitated flow cytometry (FACS, Becton Dickinson, Franklin Lakes, NJ).

Radiation on subcutaneous xenografts in human colorectal cancer Once the tumor volume reached 0.2–0.5 mm^3, the mice without anesthesia were fixed on a dedicated device in order to fully expose the tumor area. The radiation device used was 6 MV linear accelerator (ELEKTA 1232 Medical Linear Accelerator), and the dose rate was 2 Gy/min. One side of the flank in nude mice was exposed to 10 Gy single dose of radiation. And the other side without treatment served as control. To observe dynamic changes in chromatin structure of $CD133^+$ and $CD133^-$ cells which were exposed to single high-dose radiation, nude mice were killed after 1 h, 24 h, 96 h and 144 h of radiation, respectively. Both sides of the xenografts were stripped, labeled and cryopreserved until use.

Indirect immunofluorescence staining $CD133^+$ and $CD133^-$ cells sorted by flow cytometry were dripped on the slides, labeled and dried at room temperature. After the slides were completely dried, an equal volume of 4% paraformaldehyde and 1% Triton-100 was added to fix at room temperature for 20 min. PBS containing 1% BSA was used to wash away the fixative, and PBS with 3% BSA was added to block at room temperature for 30 min. Slides were washed by the same method. Antibodies were diluted by PBS

with 1% BSA and added to the slides, which were incubated at 4℃ overnight. PBS was used to wash the slides the next day, and slides were incubated with secondary antibody at room temperature for 1 h in dark. After the secondary antibody was washed away by PBS, slides were incubated with 1:2000 diluted DAPI for 3 min, and mounted after washing by PBS for three times. The blue florescence signal emitted by DAPI was used to show the chromosome structure. The eukaryotic cell specific markers of heterochromatin H3K9me3 (Trimethyl-Histone H3 Lys9, Rabbit, 1:500, Upstate), HP1α (Histone protein-1, Mouse, 1:500, Upstate), H3K4me1 (Monomethyl-Histone H3Lys4, Rabbit, 1:500, Upstate) and euchromatin specific marker H3K4me3 (Trimethyl-Histone H3Lys4, Rabbit, 1:500, Upstate) were used for immunofluorescence staining in CSCs and non-CSCs, respectively.

Image collection Indirect immunofluorescence stained cells were observed under Zeiss axis plane fluorescence microscope. The images were collected and analyzed by Zeiss AxioCam MR Camera and Axiovision 3.1 software. The nuclear structures were clearly shown by strongly amplified 100× oil lens.

Results

Phenotype and CD133 expression are consistent in human colorectal cancer surgical specimens and animal models

Histological analysis clearly displayed the morphology of human colorectal cancer surgical specimens and xenografts in animal models and the matched phenotype of tumor cells (Figure 1). In addition, flow cytometry showed that CD133 expression rates were 0.1%–10% (average 1.7%) and 1.4%–11.4% (average 2.3%) in surgical specimens and xenografts, respectively (Figure 2). An average of 5×10^4 $CD133^+$ cells were isolated from each sample, and the number of $CD133^-$ cells reached 1×10^5.

Nuclear morphology of human colorectal CSCs and non-CSCs is different

The chromosome structure of $CD133^+$ and $CD133^-$ cells was significantly different as shown by DAPI staining. The nuclei of $CD133^+$ cells demonstrated clearly a compacted patch structure of chromatin and the patches were scattered throughout the nucleus, or arranged orderly in the inner nuclear membrane. The nuclear structure of $CD133^-$ cells was sheet or mesh, and several unstained cavities or clearances were scattered (Figure 3). The differences of chromosome structure between CSCs and non-CSCs were consistent in both surgical specimens and xenografts.

Compacted patch structure of chromatin in CSCs is a component of heterochromatin

In $CD133^+$ cells, both anti-H3K9me3 antibody and anti-HP1 antibody were enriched in the dense chromatin area as shown by immunofluorenscence, and the fluorescent region was almost identical to DAPI stained region. With overlaying analysis of various fluorescent signals collected by fluorescence microscopy, we found that anti-H3K9me3 antibody, anti-HP1 antibody and DAPI were co-localized in the dense chromatin region. In contrast, anti-H3K9me3 antibody and anti-HP1 antibody were weakly stained and loosely latticed in $CD133^-$ cells, and only stained in the interspace of chromatin without DAPI stained (Figure 4A). Similarly, anti-H3K4me1 antibody and DAPI were co-stained in the dense nuclear chromatin of $CD133^+$ cells, while in $CD133^-$ cells the staining of anti-H3K4me1 antibody was similar to anti-H3K9me3 antibody (Figure 4B).

In order to prove that the condensation of colorectal CSCs chromatin was induced by heterochromatin, we labeled euchromatin in two groups of cells simultaneously. Specific

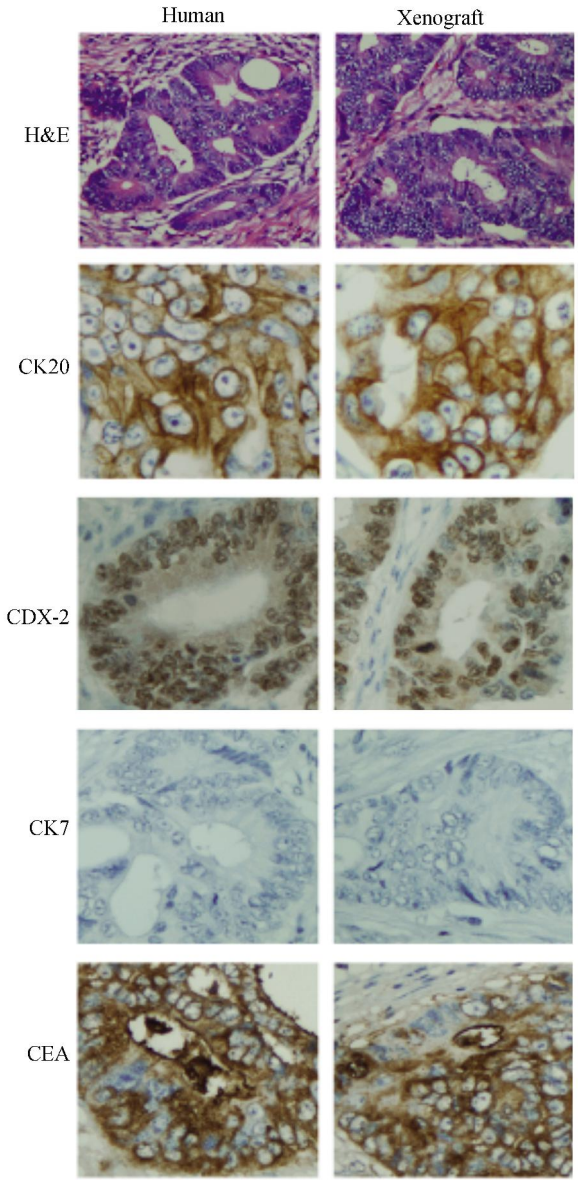

Figure 1 According to HE staining (×20) and immunohistochemical analyses (×40) of CK20, CK-7, CEA and CDX-2, xenografts resemble the original tumor of the patient

CK20 is expressed on cell membrane; CK7 shows no expression; CEA is expressed in cytoplasm; CDX-2 is expressed in nucleus.

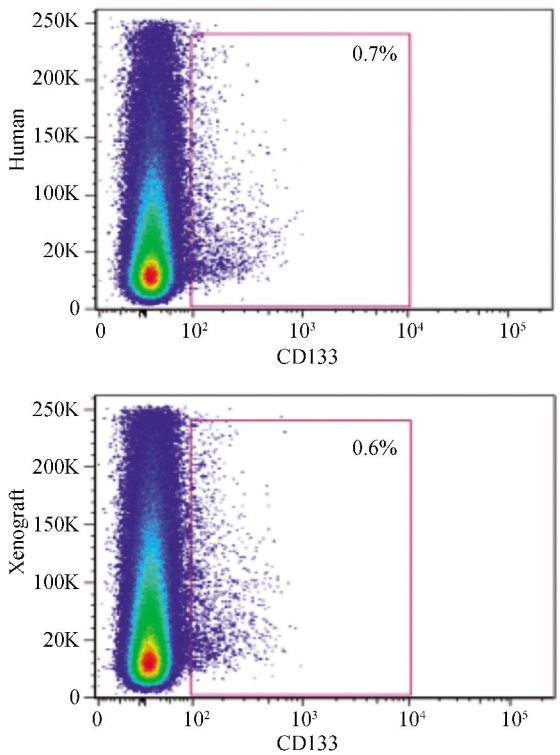

Figure 2 Flow cytometric analysis of CD133 expression in xenografts and tumors

CD133 expression in xenografts (X7) is similar to tumors (N7). Data are representative of X1–X11 tumors.

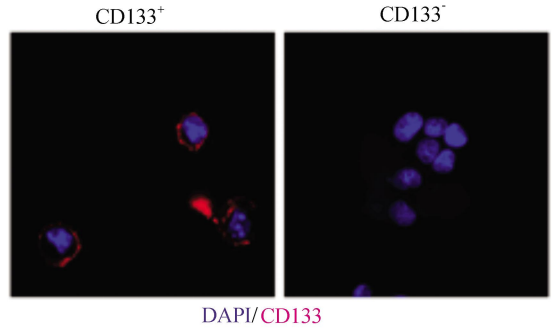

Figure 3 Significant differences between CSCs ($CD133^+$) and non-CSCs ($CD133^-$) in nuclei (×100)

Obvious chromatin condensation formed in $CD133^+$ cells, and loose and frothy structure in $CD133^-$ cells.

euchromatin markers were selected, and anti-H3K4me3 antibody was used to stain $CD133^+$ and $CD133^-$ cells. The results showed that except for the dense chromatin region, other regions of nucleus were stained by anti-H3K4me3 antibody in $CD133^+$ cells, indicating that except compact patches, other regions of nucleus were euchromatin. In the nucleus of $CD133^-$ cells, the chromatin was all stained by anti-H3K4me3 antibody, indicating the distribution of euchromatin (Figure 4C).

Single high-dose radiation induces short-term vacuolar-like defects in colorectal CSCs chromatin

As shown in immunofluorescence staining, after 1–24 h of radiation, DAPI staining was consistent with pre-radiation in $CD133^+$ cells, and displayed dense blue fluorescent patches. However, the staining of heterochromatin markers was changed obviously as compared with pre-radiation. The fluorescent signals of anti-H3K9me3, anti-HP1 and anti-H3K4me1 antibodies were detected only in the peripheral patches with the overlaying analysis, while the staining of heterochromatin markers in the inner patches were shown as vacuolar-like defects. The

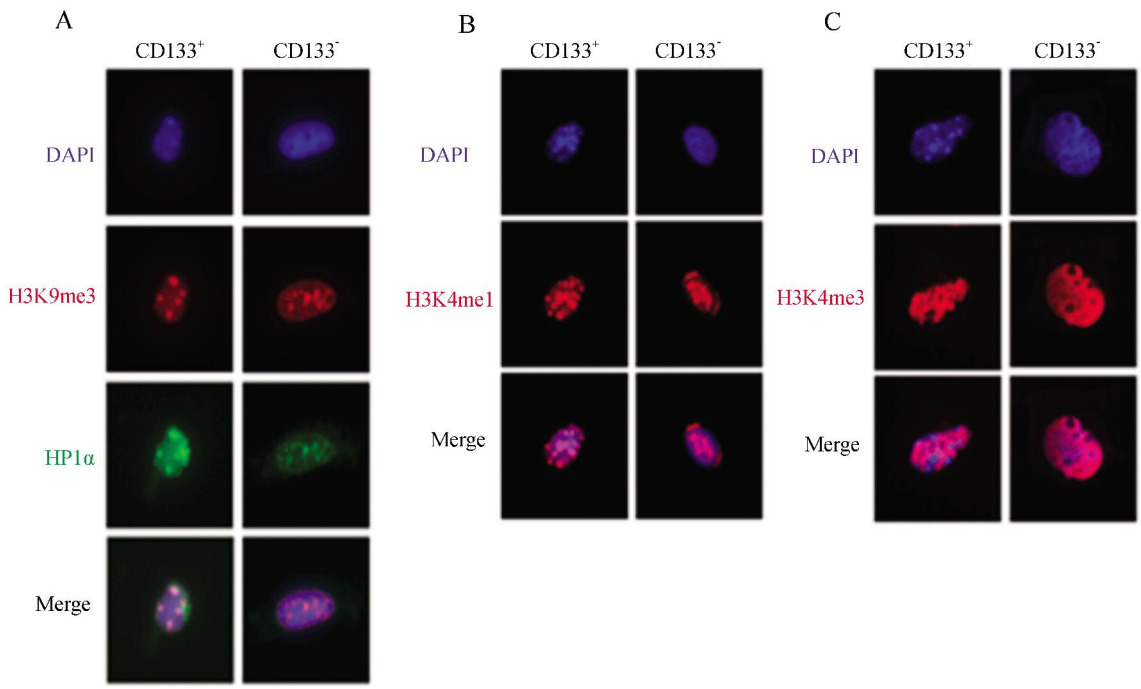

Figure 4 Heterochromatin formation in human colorectal CSCs ($CD133^+$) (×100)

euchromatin marker H3K4me3 had no significant differences as compared with pre-radiation. After irradiation, $CD133^-$ cells did not show any significant changes. The staining of anti-H3K4me3 antibody and DAPI displayed slightly looser chromatin structure than pre-radiation, which may be induced by chromatin damage. No change occurred in the non-radiated tumor cells as shown in Figure 5.

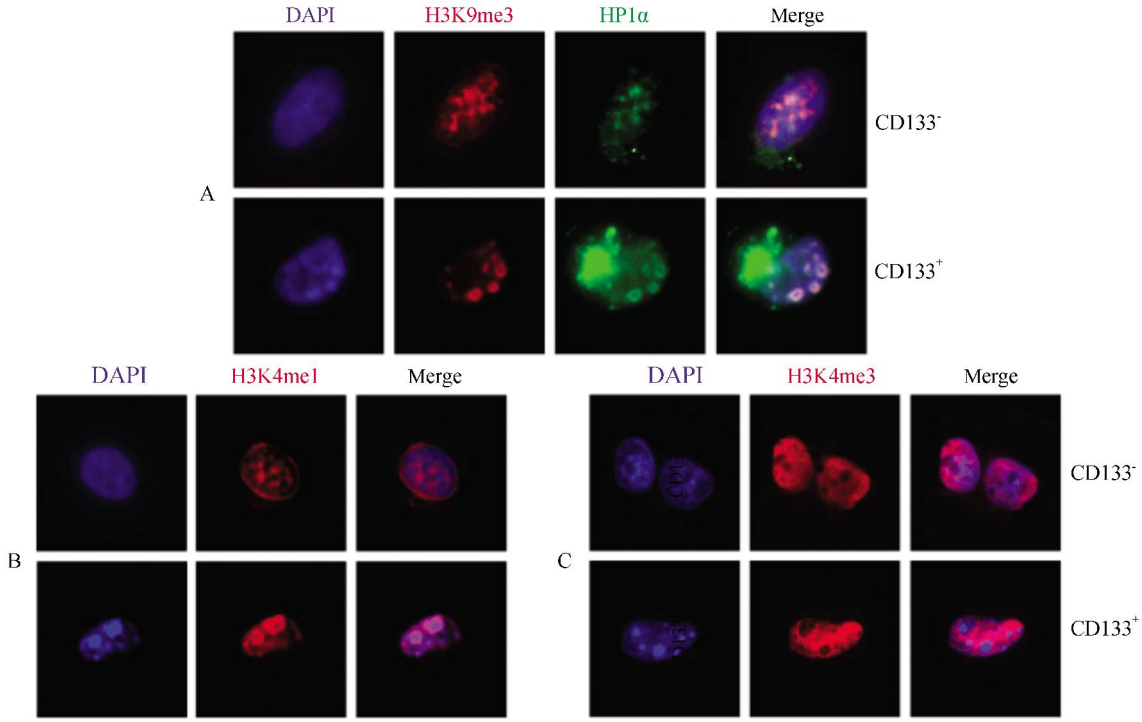

Figure 5 Vacuole-like defects in heterochromatin regions of CSCs ($CD133^+$) observed within 24 h after 10 Gy single-dose radiotherapy (×100)

Repair of colorectal CSCs vacuolar-like defect and reconversion of heterochromatin

In order to observe the dynamic process of nuclear chromatin in CSCs and non-CSCs cells, the changes were observed after 1 h, 24 h, 96 h and 144 h of radiation. Heterochromatin markers H3K9me3, HP1 and H3K4me1, and euchromatin marker H3K4me3 were stained by immunofluorescence. After 1 h of radiation, heterochromatin of CSCs showed obvious vacuolar-like defects, and the result was similar to that after 24 h of radiation. However, after 96 h, the nuclear chromatin structure was changed, and the vacuolar-like defects induced by 1 h and 24 h radiation were repaired and recovered to a dense structure as pre-radiation. Nuclear heterochromatin in $CD133^+$ cells was generally reverted to a compacted patch structure (Figure 6) at 144 h.

Discussion

Human colorectal adenocarcinoma is one of malignant tumors with a high morbidity and mortality. Although radiotherapy is an important non-surgical treatment, radiation resistance and relapse still occur after treatment, which affect the efficacy of radiotherapy. More and more studies have shown that CSCs are more resistant to radiotherapy than non-CSCs. As CSCs are the most important factor of tumor proliferation, tumor growth maintenance and cellular heterogeneity, the resistance of CSCs to radiotherapy did affect the overall efficacy of radiotherapy and prognosis[10,12,15]. Currently, in tumor cell chromosome, except for the DNA repair capacity which can affect radiosensitivity, histone modification such as methylation, acetylation or phosphorylation, can also increase the resistance to radiation in tumor cells[16].

In this study, we observed the changes of histone in two groups of cells after single high-dose radiation and studied the modification of chromatin histone in human colorectal CSCs and non-CSCs in an attempt to explore the causes of radiation resistance in human colorectal cancer.

This study has indicated that the chromatin structure was different in human colorectal CSCs ($CD133^+$) and non-CSCs ($CD133^-$). In $CD133^+$ cells, the chromatin displayed a compacted patch structure, while in $CD133^-$ cell, the chromatin showed a loose sheet or a latticed structure. The compacted patch structure in CSC nucleus was proved to be heterochromatin by immuofluorescence staining. Single high-dose radiation of 10 Gy was exposed to the cells, and after 1 h and 24 h of radiation, vacuole-like defects appeared in the heterochromatin region of colorectal CSCs. Compared with non-radiated group, except for a slightly loose chromatin structure, no other obvious changes were found in non-CSCs cells. After 96 h of radiation, the vacuole-like

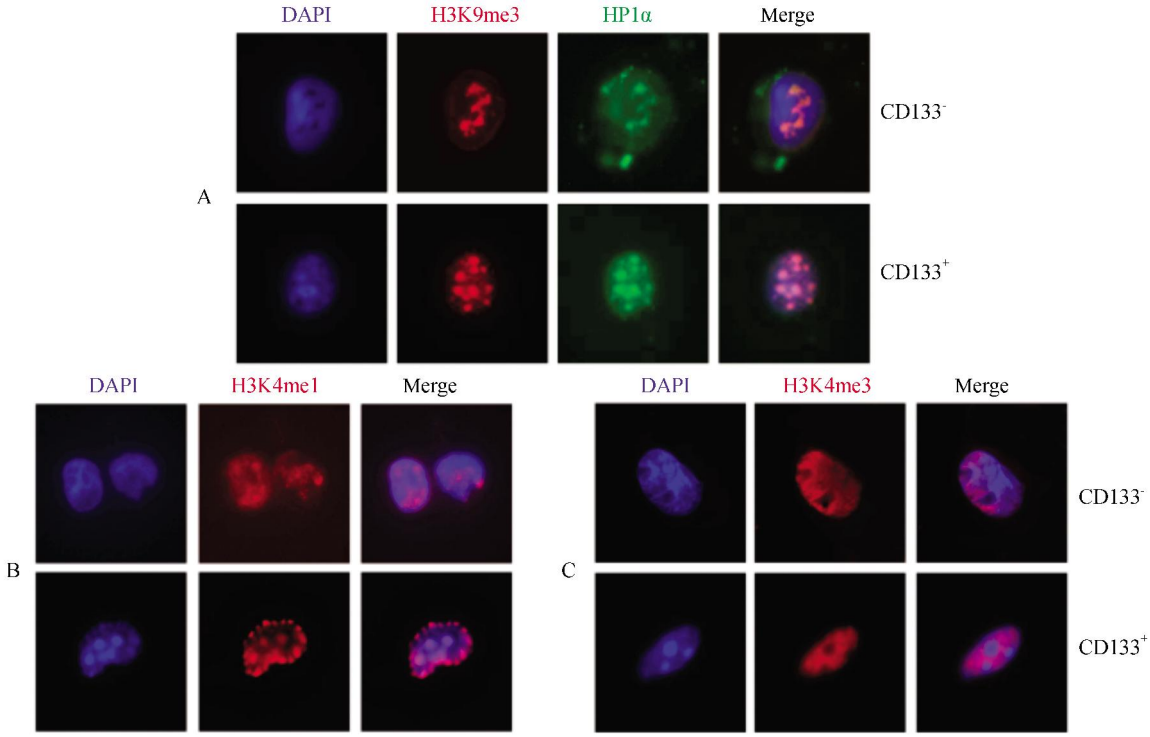

Figure 6 Vacuole-like defects at heterochromatin in CSCs (CD133+) became compacted patches 144 h after radiation (×100)

defects in heterochromatin of CSCs were repaired, and reverted to a compacted patch structure similar to pre-radiation.

Heterochromatin of eukaryotic cells affects the gene expression and chromosome segregation through histone modification. Current data have shown that methylation of histone H3-lys9 (H3K9) created a site of high affinity of HP1 binding, and the binding of HP1 and methylated histone H3K9 was essential for heterochromatin formation[17-19]. Furthermore, studies also found that from fission yeast to human, the methylation of H3K4 and H3K9 were conversed in heterochromatin and euchromatin, which meant the methylation of H3K9 was high and the methylation of H3K4 was low in heterochromatin, conversely, H3K4 was highly methylated and H3K9 was lowly methylated in euchromatin[20]. Histone lysine residue was methylated by methyltransferase (HMTs), which was the most important means for regulating epigenetic expression. The formation and maintenance of heterochromatin were regulated by the interaction of HP1, histone lysine residues and HMTs. In short, HMT-histone lysine methylation-HP1 axis is the core of heterochromatin[18,19].

Histone maintains the morphology and function of heterochromatin through HMTs mediated methylation modification. High-dose radiation may change the heterochromatin structure through interfering with the binding of methyl and histone lysine residue or enhancing the defects of methyl, which causes different degrees of defects. However, HMT-histone lysine metylation-HP1 axis is not affected irreversibly, the defects of heterochromatin caused by radiation could be finally reversed by this pathway. On the other hand, it is indicated that this axis is related to tumor radiotherapy resistance. Therefore, finding an ideal HMT inhibitor to effectively block HMTs mediated histone methylation and further interfering with the heterochromatin repair would improve and reverse the tumor resistance to radiotherapy.

Recently, a large number of studies have reported that interference of histone modification and combination of histone modification related enzyme inhibitors could promote tumor cell apoptosis and enhance the sensitivity of radiotherapy and chemotherapy[21]. In the study of histone deacetylase (HDAC), trichostatin A or other HDAC inhibitors increased the radiosensitivity of squamous cell carcinoma[22]. Suberoylanilide hydroxamic acid (SAHA) was shown to increase the anti-proliferative effect caused by radiotherapy in human prostate cancer[23]. The same results also found in lung cancer, pelvic cancer and brain gliomas[24]. However, the studies about HMTs inhibitors were rarely reported.

In summary, compared with non-CSCs, CSCs showed more significant resistance to radiation in malignant tumors. This founding suggested that if CSCs were not controlled effectively by radiotherapy, the efficacy and prognosis would be affected directly, even leading to treatment failure due to tumor recurrence. In the present study, we found the compacted patch structure in human colorectal CSCs was dense heterochromatins. After exposure to 10 Gy radiation, the vacuolar-like defects in CSCs heterochromatin were observed in short time, but the defect can be repaired and recovered to the same compacted patch structure as before radiation at last. No obvious changes

were found in non-CSCs before and after radiation.

The existence of heterochromatin in human colorectal CSCs and dynamic changes of heterochromatin after high-dose radiation suggested that the radioresistance of CSCs may be closely related to histone modification and heterochromatin formation. Further studies of the mechanisms would help understand the causes of radiation resistance in human colorectal caner and find effective means to solve the existing problems.

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