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Promoted Instability in an X-ray Irradiated Chromosome Transferred into Werner Syndrome Cells

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Ionizing radiation is a potent genotoxic agent that can induce delayed biological effects referred to as genomic instability. Delayed chromosomal instability has been studied as a typical phenotype of genomic instability in the progeny of irradiated cells, but the mechanisms by which it arises remain obscure. The previous chromosome transfer study revealed that chromosomal instability could be transmitted to the progeny of unirradiated recipient cells by a chromosome exposed to ionizing radiation[1]. To determine whether the transmitted chromosomal instability is promoted in cells with compromised genomic integrity, we examined chromosome transfer to the cells which have defect of DNA repair genes, such as ataxia telangiectasia mutated gene (*ATM)*, Nijmegen breakage syndrome protein 1 gene (*NBS1*) and Werner syndrome (*WRN*) gene. Unfortunately, we could not get clone of *ATM* or *NBS1* deficient cells but we could get Werner syndrome cell line (WS780: WRN^{mut}) which carries transferred chromosome 9. The results indicated that both unirradiated and irradiated chromosomes 9 were stable after chromosome transfer in microcell hybrids derived from non-WS control cells (GM638: WRN^{wt}). In contrast, although all six WRN^{mut}-derived microcell hybrids had no rearrangements in the transferred-unirradiated chromosome 9, 11-28% of cells showed the rearranged chromosome 9 in three out of seven WRN^{mut}-derived microcell hybrids transferred with the 6 Gy-irradiated chromosome 9. Thus, the present study demonstrates the possibility that chromosome instability mediated by an irradiated chromosome is promoted in WS cells that harbor multiple defects of genomic integrity.

Key words: chromosomal instability, chromosome transfer, Werner Syndrome

1. Introduction

Radiation-induced genomic instability is described as abnormal phenotypes, such as reproductive cell death, delayed chromosomal instability and gene mutations, found in the progeny of cells survived exposure to ionizing radiation[2-5]. Among them, the chromosomal instability has been well studied as one of the non-targeted effects of ionizing radiation. However, the mechanisms underlying the perpetuation of chromosomal instability are still not clear. To investigate the transmissible nature of delayed chromosomal instability, we previously transferred an irradiated human chromosome 11 into unirradiated mouse

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recipient cells using microcell-mediated chromosome transfer and examined the integrity of the transferred human chromosome in microcell hybrid cells[1]. Although the unirradiated human chromosome was stable in the recipient cells, the irradiated human chromosome showed multiple aberrations including deletions, translocation with a recipient mouse chromosome, Robertsonian translocations, rings and fragments in microcell hybrid cells[1]. In other work, UV-irradiated human chromosome 21 also exhibited chromosomal instability in mouse recipient cells after chromosome transfer[6]. These results indicate that chromosomal instability is transmitted to the progeny of unirradiated cells by the chromosome exposed to ionizing radiation or UV.

Werner syndrome (WS) is a human progeroid syndromes caused by a mutation in the *WRN* gene, which encodes a member of RecQ helicase family implicated in the maintenance of genomic integrity[7]. The WS cells accumulate DNA damage, telomere abnormalities and exhibit genomic instability[8, 9] due to the defect in the WRN protein that participate in DNA repair, recombination, and replication[8]. In particular, several studies have demonstrated that WS cells show hypersensitivity to replication inhibitors and DNA damaging agents that cause replication folk stalling[10-13]. Consistent with those results, biochemical studies revealed that the WRN protein has a function in unwinding DNA structures associated with stalled replication forks[14]. In addition, the WRN protein has been shown to physically and/or functionally interact with proteins that have a role in DNA replication including proliferating cell nuclear antigen (PCNA)[15, 16], replication protein A (RPA)[17, 18], Flap endonuclease (FEN-1)[19, 20], and polymerase δ (Pol δ)[21, 22]. These interactions with replication proteins also imply that the WRN protein might have a function in responding to blocked replication. In addition, the WRN protein has multiple roles in DNA repair (base excision repair and DNA double strand break repair), transcription, and telomeres (replication, recombination and repair)[8].

To investigate how the level of inherent genomic integrity of recipient cells affects the transmitted instability of irradiated chromosomes, we transferred an irradiated human chromosome 9 into unirradiated WS cells by microcell-mediated chromosome transfer and examined the stability of the transferred chromosome

2. Materials and Method

2.1. Cells and cell culture

Mouse A9 cells containing a single copy of human chromosome 9, which was tagged with a neomycin resistance gene, were used as a chromosome donor. A WS patient cell line immortalized by introduction of SV40 DNA (WS780: WRN^{mut}) was used as a recipient, also SV40-transformed human fibroblast cell line was used as a non-WS control (GM638: WRN^{wt}). The WS780 has mutation in *WRN* gene (1336C>T) resulting in an amino acid change at codon 368 from arginine to a stop codon, and expression in WRN^{wt} and WRN^{mut} cells were confirmed previously[23]. A9 cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM; Nissui Pharmaceutical, Tokyo) supplemented with 10% fetal bovine serum (FBS; Trace Bioscience, Melbourne), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.8 mg/ml G418 (Life Technologies, Carlsbad, CA). WS780 cells and GM638 cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100μg/ml streptomycin. Microcell hybrid cells were cultured in the DMEM described above supplemented with 0.8 mg/ml G418. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

2.2. Irradiation

Exponentially growing A9 cells were irradiated with 6 Gy of X-rays using an X-ray generator (M-150 WE; Softex, Osaka) operating at 150 kVp and 5 mA with a 0.1mm Cu filter at a dose rate of 0.425 Gy/min. Twenty four hours after exposure, microcell fusion was performed.

2.3. Microcell fusion

Microcell fusion was performed as described previously.[13] Briefly, donor cells (1×10^6 cells) were inoculated in a 25 cm^2 flask, and microcells were induced by treatment with 50 ng/ml Colcemid in the DMEM containing 20% FBS and 0.8 mg/ml G418 for 48 h. The flasks were filled with serum free medium (SFM) containing 10 μg/ml cytochalasin B (Sigma Chemical Co., St. Louis, MO), and then microcells were isolated by centrifugation at 11,000 rpm for 30 min at 34°C. The crude microcells were purified by filtration through a series of polycarbonate filters with pore sizes of 8 μm, 5μm and 3 μm. The purified microcells were re-suspended in SFM containing 25 μg/ml phytohemagglutinin (PHA; Sigma Chemical Co., St. Louis, MO) and attached to the recipient cells by incubation at 37°C for 15 min. The cells were treated with 3 ml of polyethylene glycol (PEG; Sigma Chemical Co., St. Louis, MO) mixed with SFM (PEG; SFM, 1: 1.4) for 30 sec, overlaid with 3 ml of a low-concentration PEG (PEG : SFM, 1: 3) and treated for another 40 sec. After washing with SFM three times, the cells were filled with the DMEM containing 10% FBS. After 48 h of incubation at 37°C, the recipient cells were replated for selection in the DMEM containing 10 % FBS and 0.8 mg/ml G418 for 3 – 4 weeks. G418 resistant microcell hybrids were isolated and grown in the DMEM containing 10% FBS and 0.8 mg/ml G418.

2.4. Whole chromosome painting-Fluorescence in situ hybridization (WCP-FISH)

Exponentially growing cells were treated with colcemid (60 ng/ml) for 2 h. Chromosome samples were prepared as described previously[13]. For FISH analysis, the slide was air-dried overnight and immersed in pre-treatment solution (2 X SSC / 0.5% NP-40, pH 7.0) for 30 min at 37°C. Then, the slide was immersed in denaturing solution (70% formamide in 2 X SSC, pH 7.0) for 3 min at 72 °C. After dehydration by successive treatments with 70%, 80% and 100% ethanol for each 5 min, the slide was dried with an airjet. A DNA probe that was specific for chromosome 9 (Q-Biogene, Montreal) was denatured at 72°C for 10 min and applied to a chromosome slide. The slide was covered with the probe mixture and glass coverslip and sealed with rubber cement to avoid evaporation. The hybridization was performed at 37°C overnight in the humidified atmosphere. After hybridization, the coverslip was removed and the slide was incubated in wash buffer (0.5 X SSC/ 0.1% SDS) for 5 min at 65°C, rinsed in PBD buffer (Q-Biogene, Montreal) at room temperature for 5 min, and stained with 8 μ l of 20 ng/ml DAPI (Q-Biogene, Montreal) in antifade. The metaphase chromosomes were observed using a fluorescence microscope (Olympus, Tokyo) and digital images were recorded using a CCD camera (Olympus, Tokyo). P-arm contained in the transferred chromosome 9 was partially deleted, so we could distinguish transferred and intrinsic chromosomes 9 by its shape. One hundred cells of each micro cell hybrid were scored and determined the frequency of chromosome instability.

3. Results

3.1. Stability of an unirradiated human chromosome 9 in WS cells.

We first transferred an unirradiated chromosome 9 into WS cells (WRN^{mut}) and non-WS control cells (WRN^{wt}) by microcell fusion, and isolated micro cell hybrids that contained the transferred chromosome 9. The *WRN* gene expression in WRN^{wt} and WRN^{mut} cells were confirmed previously[24]. Because we used the A9 cells that carried a chromosome 9 of which p-arm was partially deleted, we could distinguish the transferred chromosome 9 from the chromosomes 9 of the recipient cells. As shown in Figure 1, WCP-FISH analysis indicated two copies of intact chromosome 9 in the WRN^{wt} cells (Fig. 1A, arrows), and the transferred chromosome 9 (Fig. 1B, shorter p-arm, arrowhead) could be distinguished from the recipient chromosomes 9 (Fig. 1B, arrows) in the microcell hybrid, WRN^{wt}-9-3. As shown in Figure 1C (arrows), all recipient chromosomes 9 in the WRN^{mut} were rearranged and, therefore, the transferred chromosome 9 (Fig. 1D, arrowhead) could be distinguished from the recipient chromosomes 9 (Fig. 1D, arrows) in the microcell hybrid, WRN^{mut}-9-3.

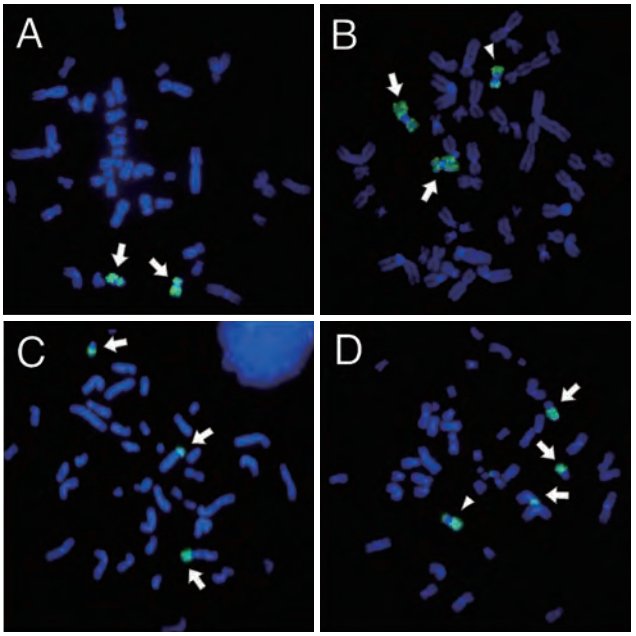

Fig. 1. Representative images of metaphase spreads of WRN^{wt} cells, WRN^{mut} cells and their microcell hybrid clones. A, A metaphase spread of the recipient WRN^{wt} cells. B, A metaphase spread of microcell hybrid WRN^{wt}-9-3 cells. C, A metaphase spread of the recipient WRN^{mut} cells. D, A metaphase spread of microcell hybrid WRN^{mut}-9-3 cells. Chromosomes were stained with DAPI and chromosomes 9 were visualized by WCP-FISH. Arrows and an arrowhead represent the recipient chromosomes 9 and a transferred chromosome 9 (shorter p-arm), respectively.

We isolated two microcell hybrids (WRN^{wt}-9-3 and WRN^{wt}-9-26) from WRN^{wt} cells and six microcell hybrids (WRN^{mut}-9-1, WRN^{mut}-9-3, WRN^{mut}-9-8, WRN^{mut}-9-9, WRN^{mut}-9-10, and WRN^{mut}-9-14) from the WRN^{mut} cells (Table 1). In those microcell hybrids isolated from the WRN^{wt} cells, only 1 % of the cells in one hybrid (WRN^{wt}-9-26) showed chromosomal aberrations after chromosome transfer (Table1). In those microcell hybrids isolated from the WRN^{mut} cells, although structural abnormalities were observed in two hybrids (WRN^{mut}-9-1 and WRN^{mut}-9-8), the aberrations were observed in all cells of each hybrid (Table 1).

3.2. Stability of an irradiated human chromosome 9 in WS cells.

We then transferred a 6 Gy-irradiated chromosome 9 into the WRN^{wt} cells and the WRN^{mut} cells, and investigated chromosome stability in the isolated (WRN^{wt}-I9-2, WRN^{wt}-I9-3, WRN^{wt}-I9-5, and WRN^{wt}-I9-8) and seven microcell hybrids (WRN^{mut}-I9-3, WRN^{mut}-I9-8, WRN^{mut}-I9-10, WRN^{mut}-I9-13, WRN^{mut}-I9-19, WRN^{mut}-I9-21 and WRN^{mut}-I9-22) from the WRN^{wt} and the WRN^{mut} cells, respectively (Table 1).

Table 1. Stability of transferred chromosome 9 in microcell hybrids

Cell	Dose (Gy)	Transferred Ch. 9	Percentage of cells with Ch. 9 aberration (%)	Aberration types (%)	
				Translocation	Dicentric chromosome
WRN^{wt}-9-3	0	Intact	0	0	0
WRN^{wt}-9-26	0	Intact	1	1	0
WRN^{mut}-9-1	0	Translocation	0	0	0
WRN^{mut}-9-3	0	Intact	0	0	0
WRN^{mut}-9-8	0	Translocation	0	0	0
WRN^{mut}-9-9	0	Intact	0	0	0
WRN^{mut}-9-10	0	Intact	0	0	0
WRN^{mut}-9-14	0	Intact	0	0	0
WRN^{wt}-I9-2	6	Translocation	0	0	0
WRN^{wt}-I9-3	6	Intact	0	0	0
WRN^{wt}-I9-5	6	Intact	0	0	0
WRN^{wt}-I9-8	6	Intact	0	0	0
WRN^{mut}-I9-3	6	Intact	13	10	3
WRN^{mut}-I9-8	6	Intact	11	9	2
WRN^{mut}-I9-10	6	Intact	2	2	0
WRN^{mut}-I9-13	6	Translocation	0	0	0
WRN^{mut}-I9-19	6	Intact	0	0	0
WRN^{mut}-I9-21	6	Intact	0	0	0
WRN^{mut}-I9-22	6	Intact	28	23	5

In those microcell hybrids isolated from the WRN^{wt} cells, three microcell hybrids (WRN^{wt}-I9-3, WRN^{wt}-I9-5 and WRN^{wt}-I9-8) showed no structural aberrations (Table 1) while one hybrid (WRN^{wt}-I9-2) showed the same chromosome aberration in all cells, and developed no further rearrangements (Table 1).

In those microcell hybrids isolated from the WRN^{mut} cells, two hybrids (WRN^{mut}-I9-19 and WRN^{mut}-I9-21) showed no aberrations, but the remaining five micro cell hybrids each showed chromosomal aberrations (Table 1). WRN^{mut}-I9-13 had only one type of aberration in all cells, however, WRN^{mut}-I9-3 showed multiple abnormalities including translocations with the chromosomes of recipient cells (Table 1, Fig. 2A; b, c, f, g) and a dicentric chromosome (Table 1, Fig. 2A; d, h). Similarly, WRN^{mut}-I9-22 showed a translocation with a chromosome of recipient cells (Table 1, Fig. 2B; b, e) and a dicentric chromosome (Table 1, Fig. 2B; c, f).

Although we compared the number of cells with the rearranged chromosome 9 after chromosome transfer between unirradiated (n=6) and irradiated (n=7) chromosome 9 containing WRN^{mut} microcell hybrids (Table 1), we failed to find the significant difference between them ($P = 0.0699$ by Mann-Whitney U test). However, in contrast to the fact that no rearrangements were occurred in six microcell hybrids that were transferred with unirradiated chromosome 9, 11-28% of cells showed the rearranged chromosome 9 after chromosome transfer in three out of seven microcell hybrids that were transferred with the 6 Gy-irradiated chromosome 9.

4. Discussion

In the present study, we first transferred a unirradiated chromosome 9 into WRN^{wt} cells and WRN^{mut} cells, and isolated microcell hybrids. The transferred chromosome 9 were almost stable in WRN^{wt} cells, also, four out of six microcell hybrids from WRN^{mut} cells (WRN^{mut}-9-3, WRN^{mut}-9-9, WRN^{mut}-9-10 and WRN^{mut}-9-14) showed no aberrations in transferred intact chromosome 9 (Table 1). We could not observe transferred intact chromosome 9 in two microcell hybrids (WRN^{mut}-9-1 and WRN^{mut}-9-8), indicating that aberrations occurred before or just after chromosome transfer into the recipient cells. Importantly, these chromosome aberrations were stable and no further rearrangements emerged in those hybrids (WRN^{mut}-9-1 and WRN^{mut}-9-8). These results suggest that the unirradiated chromosome 9 was stable in the recipient WRN^{wt} cells and WRN^{mut} cells after chromosome transfer (Table 1).

Next, we transferred a irradiated chromosome 9 into WRN^{wt} cells and WRN^{mut} cells, and isolated microcell hybrids. Three out of four microcell hybrids from WRN^{wt} cells (WRN^{wt}-I9-3, WRN^{wt}-I9-5 and WRN^{wt}-I9-8) showed no aberrations in transferred intact chromosome 9 (Table 1). Although we could not observe transferred intact chromosome 9 in WRN^{wt}-I9-2, no further aberrations

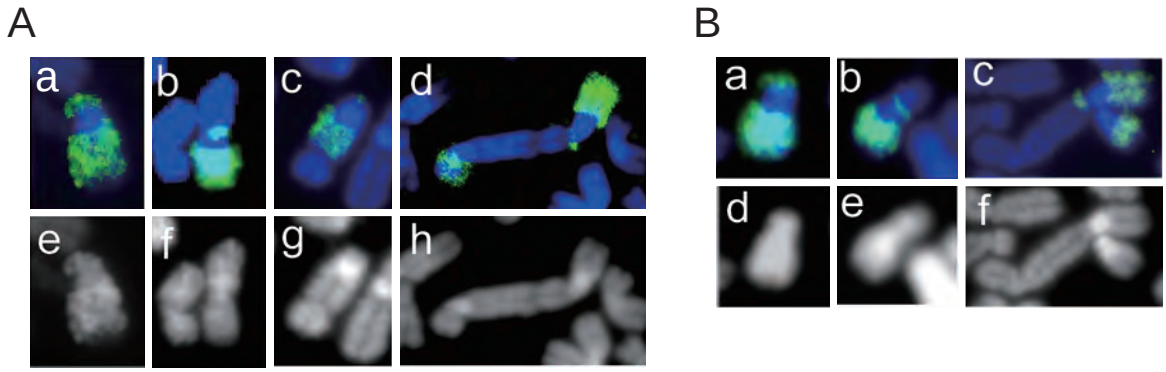

Fig. 1. Representative images of a rearranged chromosome 9 in microcell hybrid clones. A, Microcell hybrid WRN^{mut}-I9-3 cells; a and e, a transferred chromosome 9; b and f, a translocation involving a transferred chromosome 9; c and g, a translocation involving a transferred chromosome 9; d and h, a dicentric chromosome involving a transferred chromosome 9. B, Microcell hybrid WRN^{mut}-I9-22 cells; a and d; a transferred chromosome 9; b and e, a translocation involving a transferred chromosome 9; c and f, a dicentric chromosome involving a transferred chromosome 9. Chromosomes were stained with DAPI (A, e-h; B, d-f) and chromosomes 9 were visualized by WCP-FISH (A, a-d; B, a-c).

were observed (Table 1). These results suggest that the irradiated chromosome 9 was stable in the recipient WRN^{wt} cells. On the other hand, four out of seven microcell hybrids from WRN^{mut} cells (WRN^{mut}-I9-3, WRN^{mut}-I9-8, WRN^{mut}-I9-10 and WRN^{mut}-I9-22) showed chromosomal instability in transferred intact chromosome 9 (Table 1, Fig. 2). We failed to find the significant difference between unirradiated and irradiated chromosome 9 containing WRN^{mut} microcell hybrids, however, we could observe chromosomal instability in microcell hybrids from WRN^{mut} cells transferred a irradiated chromosome 9 (Table 1, Fig. 2). Our results suggest that the irradiated chromosome in WS cells has a higher probability to be unstable than that in non-WS cells, implying that the instability of chromosome exposed to radiation might be promoted in recipient cells inherently deficient in genomic integrity. Also, it is possible that delayed chromosomal instability is induced not by damaged chromosome but other factors. We previously reported that accumulation of DNA double-strand breaks and chromosome instability were observed in primary cells of WS patient (WS3RGB: WRN^{mut}) in a spontaneous condition[9]. But unirradiated chromosomes were relatively stable in both WRN^{wt} cells and WRN^{mut} cells (Table 1) suggesting that the frequency of spontaneous DSBs might not be evident in the SV40-transformed WS cells (WS780: WRN^{mut}) which we used in this experiment, also chromosome transfer itself did not affect the chromosomal stability. And the transferred irradiated chromosomes were unstable only in WRN^{mut} cells (Table 1). So we interpret these data as irradiated chromosome itself carries instable factor and the recipient cells inherently deficient in genomic integrity enhance the chromosomal instability.

Radiation-induced genomic instability is characterized by several endpoints including frequent non-clonal mutations, high frequency of chromosomal aberrations and cell death occurring within a clonal cell population at a delayed period after radiation exposure[2]. Although the mechanism underlying radiation-induced delayed genomic instability is poorly understood, we have previously found that the irradiated chromosome is itself unstable and potentially interacts with unirradiated chromosomes in later generations[1]. In addition, we examined the transfer of a human chromosome irradiated with UV into unirradiated mouse cells to determine whether genomic instability was induced by UV damage. The results revealed the induction of instability in both the transferred human chromosome and the recipient mouse chromosomes[6], suggesting that an irradiated chromosome retains potentially unstable sites that can recombine with other chromosomes. So far, we have no evidence that the active sites are consistent with the primary DNA damage sites induced by ionizing radiation or UV radiation. However, it is noteworthy that the typical DNA damage induced by UV is not DNA double-strand breaks but oxidative damage[24-26]. The fact that oxidative damage can induce delayed chromosomal aberrations is particularly relevant for the mechanism of the induction of genomic instability in WS cells. Blander *et al.* reported that WS cells exhibit delayed and attenuated accumulation of p53 after exposure to UV[27]. In addition, they also demonstrated that in response to UV exposure, the WRN protein translocates from the nucleolus to nucleoplasmic foci and the redistribution extent of the WRN protein is correlated with the UV dose[28]. Biochemical studies also demonstrated that the WRN protein binds to the catalytic domain of pol λ and specifically stimulates DNA gap filling by pol λ over 8-oxo-G followed by strand displacement synthesis[29]. These results suggest that the WRN protein may play a role in the oxidative damage response in signaling or

facilitating specific types of DNA repair.

Although we could not identify a specific pathway or type of DNA damage involved in inducing instability in the transferred chromosome, we speculate that the telomeres are the target candidate for mediating chromosomal instability since we observed that the transferred chromosomes were mostly translocated to the end of chromosomes (Fig. 2) and were also involved in forming end-to-end chromosome fusions (Fig. 2). These results suggest that telomere instability was involved in chromosome rearrangements observed in microcell hybrids of WS cells.

There are some possible reasons for involvement of telomeres in induction of chromosomal instability in WS cells. First, oxidative stress induces telomere abnormalities in WS cells. It has been suggested that telomere repeats (TTAGGG) are targets of oxidative stress[30], and oxidative stress induces abnormal telomere FISH signals especially in WS cells[9]. Hence, the radical scavenger APM (ascorbic acid phosphoric ester magnesium salt) extends the replicative life span of WS cells, as well as normal human cells. These reports suggest that telomeres of WS cells are more vulnerable than normal cells and more susceptible to oxidative damage.

Second, alteration of telomeric chromatin state in WS cells leads to telomere abnormality. Michishita *et al*. reported that deletion of SIRT6, an NAD^+-dependent histone H3 lysine 9 (H3K9) deacetylase, leaded to telomere abnormalities including abnormal telomere FISH signals and end-to-end chromosomal fusions and also that SIRT6 was required for the stable association of the WRN protein at telomeres and for preventing replication-associated telomere defects[31]. Their results suggest that deacetylation of lysine 9 of histone H3 at telomeric chromatin by SIRT6 leads to an altered chromatin state that is required for efficient WRN association to telomeres in S phase[31]. It is probable that ionizing radiation or UV causes the altered telomere chromatin state that leads to telomere instability and that this abnormal chromatin state may be retained as the memory of irradiation. Recent study reported that histone acetylation states dramatically changed during the radiation induced DNA damage response[32], so it is possible that the telomere chromatin states is affected by radiation. Also, it has been considered that fusions between dysfunctional telomeres or even between dysfunctional telomeres and DSBs can initiate breakage-fusion-bridge (BFB) cycles, which can lead chromosome instability[33].

Lastly, replication stress at telomeres in WS cells may induce telomere abnormalities. Accumulating evidence suggests that the WRN protein has a role in responding to blocked replication[10-22]. Especially, G-rich telomere sequence can fold into G-quaduplex structures (G4 DNA)[34], which can possibly block DNA synthesis, and are unwound by the WRN protein[35]. In addition, Damerla *et al*. demonstrated that the lack of the WRN protein induced telomere sequence-specific deletion, which was possibly caused by G4 DNA structure, by using telomere repeat containing shuttle vector[36]. It is probable that radiation-induced oxidative damage in telomeres may stimulate the formation of G4 DNA structure.

In summary, the present study demonstrates the possibility that genomic instability mediated by an irradiated chromosome is promoted in WS cells that harbor multiple defects of genomic integrity.

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