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Excess processing of oxidative damaged bases causes hypersensitivity to oxidative stress and low dose rate irradiation

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Abbreviations: *E.coli*, *Escherichia coli*; H_2O_2, hydrogen peroxide; 8-oxoG, 8-oxoguanine; AP-site. apurinic/apyrimidinic-site; DSB, double strand break; BER, base excision repair; ROS, reactive oxygen species

Abstract

Ionizing radiation such as X-ray and γ-ray can directly or indirectly produce clustered or multiple damages in DNA. Previous studies have reported that overexpression of DNA glycosylases in *Escherichia coli* (*E. coli*) and human lymphoblast cells caused increased sensitivity to γ-ray and X-ray irradiation. However, the effects and the mechanisms of other radiation, such as low dose rate radiation, heavy-ion beams or hydrogen peroxide (H_2O_2) are still poorly understood. In the present study, we constructed a stable HeLaS3 cell line overexpressing hOGG1 protein. We determined the survival of HeLaS3 and HeLaS3/hOGG1 cells exposed to UV, heavy-ion beams, γ-rays and H_2O_2. The results showed that HeLaS3 cells overexpressing hOGG1 were more sensitive to γ-rays, $OH^{\bullet}$ and H_2O_2, but not to UV or heavy-ion beams, than control HeLaS3. We further determined the levels of 8-oxoG foci and of chromosomal double-strand breaks by detecting γ-H2AX foci formation in DNA. The results demonstrated that both γ-rays and H_2O_2 induced 8-oxoG foci formation in HeLaS3 cells. hOGG1-overexpressing cells had increased amounts of γ-H2AX foci and decreased amounts of 8-oxoG foci compared with HeLaS3 control cells. These results suggest that excess hOGG1 removes the oxidatively damaged 8-oxoG in DNA more efficiently and therefore generates more double-strand breaks. Micronucleus formation also supported this conclusion. Low dose rate γ-ray effects were also investigated. We first found that over-expression of hOGG1 also caused increased sensitivity to low dose rate γ-ray irradiation. The rate of micronucleus formation supported the notion that low dose rate irradiation increased genome instability.

Introduction

Reactive oxygen species (ROS) are generated by exogenous stimuli such as ionizing radiation and various chemical oxidants. ROS are also generated in living cells by normal cellular metabolism [1-3]. ROS generate a wide variety of DNA lesions such as base damages, apurinic and apyrimidinic (AP) sites, and DNA strand breaks [4-8]. These damages may cause loss of genetic stability, and altered cellular regulation associated with aging and many diseases, including cancer [9-12]. Among the damages, 8-oxoguanine (8-oxoG) is an important mutagenic lesion, which can cause cellular mutations and genomic instability [13, 14]. To repair the damages and to maintain genome stability, oxidatively damaged bases are primarily repaired by base excision repair enzymes in most organisms [10, 11, 15-17]. It is well known, that fapy-DNA glycosylase (example: *E.coli* MutM, human hOGG1) plays an important role in protection against cellular damage by ROS. It removes 8-oxoG, which is paired with cytosine, from damaged DNA [18, 19]. AP sites arise after removal of 8-oxoG by hOGG1. AP sites inhibit the replication fork and consequently cause cell death and mutations or strand breaks [20].

Ionizing radiation such as X-rays, γ-rays and α-rays, can directly or indirectly produce clustered or multiple damages in DNA, including oxidatively damaged bases [21-23]. Previous studies showed that over expression of *E.coli* MutM protein in wild-type *E. coli* cells increased the cell susceptibility to γ-ray and α-ray irradiations [24]. These studies suggested that excessive DNA glycosylases present in living cells would remove damaged bases which were caused by ionizing radiation, and as a result clustered base damages would be converted into DSBs in bacterial cells [24 ,25]. Yang et al. reported that overexpression of human OGG1 in TK6 cells also caused increased sensitivity to γ-ray irradiation [26]. However, little is known about the effects of recognition and processing in eukaryotic cells of oxidatively

damaged bases caused by other types of radiation and other ROS such as hydrogen peroxide (H_2O_2).

Previous studies demonstrated that overexpression of human hOGG1 in *E.coli* cells could also increase the cell sensitivity under H_2O_2 stress conditions [24]. In the present study, HeLaS3 cells which stably overexpressed hOGG1 were established. The susceptibility to γ-rays and H_2O_2 was increased in these hOGG1-overexpressing cells. Moreover, the amounts of 8-oxoG and γ-H2AX, and the number of micronuclei, were analyzed. Increases in the amount of γ-H2AX and the number of micronuclei, and a decrease in the amount of 8-oxoG foci, were detected in the hOGG1-overexpressing cells when exposed to γ-rays and H_2O_2. We found for the first time that hOGG1-overexpressing cells were more sensitive to low dose rate γ-ray irradiation, and the genome-stability was also decreased.

Materials and methods

Cell culture

HeLaS3 cells were grown in Dulbecco's modified Eagle's medium (D-MEM) (Wako) containing L-glutamine supplemented with 10% fetal bovine serum or FBS at 37^{o}C in 5% CO_2 and 100% humidity [27].

Plasmid

A plasmid expressing hOGG1-FLAG was constructed as follows: The plasmid pBluescript bearing the human Ogg1-type1a gene was amplified by polymerase chain reaction (PCR) and subcloned into the pTargeT plasmid vector [28].

Transfection and stable cell line construction

Stable cell lines overexpressing human hOGG1 were produced using the Lipofectamine2000 (Invitrogen) according to the company's guidelines. HeLaS3 90-95% confluent cells grown in

24-well plates were transfected with 1-2 μg pTargeT-hOGG1-1a plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. After transfection, cells were incubated at 37^{o}C in a CO_2 incubator for 24~48 hours. Then the cells were passaged at 1:10~30 into fresh growth medium. The following day, 500 μg/ml Geneticin disulfate was added to the growth medium. The cells were selected in this growth medium containing Geneticin disulfate for 2 weeks at 37^{o}C, 5% CO_2. Several single colonies were then selected and screened by several methods. The clones ("HeLaS3/hOGG1") were maintained in growth medium with 200 μg/ml Geneticin (G418).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNAs were isolated from HeLaS3 or HeLaS3/hOGG1-1a cells that were about 90% confluent with an RNAqueous-4PCR kit (Ambion) according to the manufacturer's instructions. cDNA was prepared by reverse transcription using M-MuLV Reverse Transcriptase (MBI Ferrmentas). Gene transcripts were identified by reverse transcription-polymerase chain reaction. For the Ogg1 gene, 5'-ACACTGGAGTGGTGTACTAGCG-3'/5'-GCCGATGTT GTTGTTGGAGG-3', and for the ape1 gene 5'-ATAGGCGATGAGGATCATGA-3'/5'-CAACATTCTTGGATCGAGCA-3', and for the GAPDH, 5'-CCATGGAGAAGGCTGGGG-3'/5'-CAAAGTTGTCATGGATGACC-3' were used as primers. The amplification was performed with one denaturing cycle at 95^{o}C for 5 min, then 35 cycles at 94^{o}C for 50 s, at 60^{o}C for 50 s, at 72^{o}C for 1 min, and one final extension at 72^{o}C for 15 min. RT-PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. The products were quantitated with Fuji film BAS-2500 image Analyze (Fuji Photo Film).

Real-Time PCR

Total RNAs were isolated from HeLaS3 or HeLaS3/hOGG1-1a cells which were about 90% confluent with an RNAqueous-4PCR kit (Ambion) according to the manufacturer's instructions. cDNA was prepared by reverse transcription using M-MuLV Reverse Transcriptase (MBI Ferrmentas). Real Time PCR was performed using LightCycler96 (Roche) with THUNDERBIRD SYBR qPCR Mix (TOYOBO). The amplification was performed with one denaturing cycle at 95 ^{o}C for 60 s, then 45 cycles at 95 ^{o}C for 15 s, at 60 ^{o}C for 30 s, at 72 ^{o}C for 45 s. Gene amplification specificity was verified by melting curve analyses. For the Ogg1 gene, 5'-ACACTGGAGTGGTGTACTAGCG-3'/5'-TAGCTGGAAGTACTTGCGCA-3', and for the GAPDH 5'-CCATGGAGAAGGCTGGGG-3'/5'-CAAAGTTGT CATGGATGACC-3' were used as primers.

Survival assay

Cells were grown to about 80% confluence, and then suspended by trypsinization, and the number of cells was counted. The cell suspensions were appropriately diluted and plated in 100-mm dishes. After 16-24 h incubation in a CO_2 incubator, the cells were irradiated with γ-ray, UV or heavy-ion beams at the doses indicated in the text. Low dose rate γ irradiation (1mGy/min) was also carried out. After incubation for 10 days, cells were fixed and stained with crystal violet. For H_2O_2, H_2O_2 combined with Fe^{2+} treatment, the cells were treated with each agent at the indicated concentration for 30 min and then incubated for 10 days. For UV irradiation, UVC lamp (HITACHI-GL15, 254 nm, 1 J/m^{2}s) was used for irradiation. To prevent attenuation of UV, the cells were treated without medium. Colonies of > 50 cells were counted as survivors and the surviving fraction was calculated.

Irradiation of heavy-ion beams

The cells were irradiated with heavy-ion beams (290 MeV/nucleon) generated at the Heavy Ion Medical Accelerator in Chiba (HIMAC) at the National Institute of Radiological Science (NIRS) in Japan. The characteristics, irradiation procedures and dosimetry of heavy-ion beams have been described elsewhere [29]. Briefly, the dosimetry was characterized by three different methods: an ionization chamber method, a fluence measurement method using CR39 track detectors and measurement by silicon diode. The linear energy transfer (LET) value and dose rate were estimated to be 13.3 keV/μm and 1.0 Gy/min. The irradiation doses at the sample position were determined by multiplying the fluence by the LET using the following formula [30] :

$$\text{Dose (Gy)} = \text{Fluence (ions/cm}^2\text{)} \times \text{LET (keV/μm)} \times 1.602 \times 10^{-9}$$

All irradiation was carried out at room temperature.

Preparation of cell extracts

Extracts for western blotting: each type of cell was grown to > 90% confluence in 60-mm dishes. The cells were washed twice with ice-cold phosphate buffered saline (PBS) and collected using a rubber scraper. The cells were suspended in an equal volume of PBS. After adding an equal volume of 2X sample buffer (4% SDS, 100 mM Tris-HCl pH 6.8, 20% glycerol, 12% 2-mercaptoethanol, and BPB), the mixture was sonicated using a Bioruptor (Cosmo Bio) (30 sec ON, 1.5 min OFF, repeatedly for a total of 15 min).

Western blotting analysis

15-30 μg of each sample was loaded onto a 10% SDS-polyacrylamide gel and electrophoresed. The proteins were transferred to a nitrocellulose membrane and blocked with Tween-PBS (TBS) (PBS containing 0.1% Tween 20) and 5% dry milk. The membrane was

incubated with a 1:1000 dilution of anti-FLAG M2 antibody (Sigma) overnight at 4 ^{o}C. The membrane was washed three times in TBS and incubated with a 1:3000 dilution of HRP-conjugated anti-mouse second antibody overnight at 4 ^{o}C. The membrane was washed three times in TBS and analyzed using ECL luminescence reagent (GE Healthcare) and the band was detected using X-ray film (Fujifilm). For UV irradiation, UVC lamp (HITACHI-GL15, 254 nm, 1 J/m^{2}s) was used for irradiation. To prevent attenuation of UV, the cells were treated without medium. After irradiation, sample extraction and western blotting assay were performed.

Detection of cellular localization of hOGG1-FLAG

Cells were seeded in 3.5-cm glass bottom dishes. When the cell density reached about 80% confluency, the cells were washed with PBS, and fixed with 4% formaldehyde in PBS for 30 min. Then the cells were washed with PBS for 5 min, and treated with 0.2% Triton X-100 in PBS for 2 min to enhance antibody penetration. The cells were blocked at room temperature for 15 min with 10% goat serum in TN buffer (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl), and then incubated with FLAG M2 mouse antibody (Sigma) in TNT buffer (TN buffer containing 0.05% Tween 20) for 1 hour at room temperature. The cells were washed 3 times with PBS and treated with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Invitrogen) in TNT buffer at room temperature for 1 hour. Then fluorescence was observed using a fluorescence microscope (LX70, Olympus).

Observation of 8-oxoG foci formation

Cells were seeded in 3.5-cm glass bottom dishes (1x10^5 cells/dish) and incubated in D-MEM containing 10% serum for 24-48 h. After the cells were treated with H_2O_2, UV, γ-ray or heavy-ion beams, the cells were further cultured in D-MEM containing 10% serum for 3

hours. The cells were washed 3 times with 2 ml PBS and fixed with 2 ml ice-cold methanol for 20 min on ice. Then the fixed cells were washed twice with 2 ml PBS at room temperature, and treated with 100 μl RNase (10 μg/ml) at 37^{o}C for 1 hour. The cells were permeabilized by treatment 3 times for 3 min each with 2 ml PBD (PBS containing 0.1％ Tween 20, 0.1％ Triton X-100), and then treated for 30 min with 2 ml of 2 M HCl and finally with 2 ml of 50 mM Tris for 10 min. The cells were blocked at room temperature with 100 μl Block Ace for 30 min, then 100 μl of the diluted primary antibody (8-oxoG antibody(JaICA): Block Ace=1:100) was added, and the cells were incubated with the antibody for 2 hours at room temperature. Then the cells were washed 3 times with 2 ml PBS, and 100 μl Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Invitrogen) (secondary antibody: Block Ace=1:300) was added, and the cells were incubated with this antibody solution at 4^{o}C in the dark for 16 hours. The cells were washed 3 times with 2 ml PBS, and then observed under a microscope (LX70, Olympus). The conditions of exposure to various stresses were as follows: γ ray: 5 Gy; Low dose rate γ ray: 6 Gy; H_2O_2: 300 μM, 30 min; UV. 15 J; heavy-ion beams, 2.5 Gy [31].

Double-strand break detection

The cells were grown in 3.5-cm dishes until they reached a density of 2x10^{5} cells/dish. The cells were irradiated with 3 Gy of γ-rays (at 0.8 Gy/min). Then irradiated and unirradiated cells were incubated at 37^{o}C for the indicated times. The cells were fixed with 4% formaldehyde in PBS for 20 min. Then they were washed with PBS and treated with 0.2% Triton X-100 in PBS for 2 min, washed with PBS twice, and incubated with 10% normal goat serum in TN buffer. The cells were incubated with a 1:500 dilution of anti-γ-H2AX mouse antibody (Upstate Inc.) in TNT buffer for 1 hr at room temperature. The cells were washed three times with PBS and incubated with 1:500 dilution of Alexa Fluor 488-conjugated

anti-mouse antibody (Molecular Probes) in TNT buffer for 1 hr at room temperature. The cells were washed three times with PBS, stained with 1 μg/ml DAPI for 20 min at room temperature, and visualized by fluorescence microscopy. At least 200 cells were scored for each experiment.

Measurement of the rate of micronucleus formation

The cells were cultured until they were about 30 % confluent, and then the culture medium was supplemented with 2.5 mM thymidine (final concentration), and culturing was continued for 24 h. The cells were washed twice with PBS, then fresh culture medium was added to the dishes, and the cells were incubated for 10 h. Then 1 mM hydoroxyurea (final concentration) was added to the culture medium, and incubation was carried out for 14-16 h. After washing the dishes twice with PBS, fresh culture medium was added. The cells were irradiated with γ-ray at 2 Gy or exposed to 300 μM H_2O_2, and then cultured for 24 h. The cells were fixed with 4% formaldehyde in PBS for 10 min, and permeabilized with 0.5% Triton X-100 in PBS for 5 min. DNA was stained using DAPI, and the rate of micronucleus formation was detected by microscopic observation.

For low dose rate γ-rays, the cells were irradiated with γ-rays at 1.5 Gy, and then cultured for 24 h. The cells were fixed with 4% formaldehyde in PBS for 10 min, and permeabilized with 0.5% Triton X-100 in PBS for 5 min. DNA was stained using DAPI and the rate of micronucleus formation was detected by microscopic observation. At least 200 cells were scored for each experiment.

Data analysis

We calculated standard errors and carried out Student's t-test for statistical test.

Results

Construction of hOGG1 overexpression HeLaS3 cell lines

Our previous study showed that *E.coli* cells overexpressing hOGG1 were more susceptible to ionizing radiation than control *E.coli* cells [24]. To elucidate the mechanism of the radiation sensitivity and to investigate how the cells respond to H_2O_2, heavy-ion beams and low dose rate irradiation, we constructed a plasmid bearing the hOGG1 gene. HeLaS3 cells were cultured and transfected with hOGG1-1a plasmid containing the FLAG tag at the C-terminal using Lipofectamine 2000. Geneticin-resistant colonies were picked up and grown in D-MEM, 10% serum with 500~1000 μg/ml Geneticin. After three rounds of extensive screening, we established HeLaS3/hOGG1-1a cells, which stably overexpressed hOGG1-1a. Several clones of HeLaS3 cells overexpressing hOGG1-1a were obtained. The candidate clones were confirmed by several methods.

Overexpression of hOGG1-1a in HeLaS3 cells was checked by RT-PCR, Real Time PCR, western blotting and immunofluorescence staining. Total RNAs were isolated from control HeLaS3 cell and HeLaS3/hOGG1-1a cells. Reverse transcription of the RNA samples was performed using an RNAqueous-4 PCR kit (Ambion Inc.) according to the manufacturer's instructions. Then PCR was carried out using hOGG1 primers and control GAPDH primers. The PCR products were separated by electrophoresis on a 1.5% agarose gel and quantified by comparison with the GAPDH products. The results showed that the hOGG1 level was increased 2.5 fold in the transfected cell line compared with the control HeLaS3 cells (Figure.1a). Real Time PCR was also carried out using cDNA which used in reverse transcriptional PCR.

The results showed that the hOGG1 level was increased 1.6 fold in the transfected cell line (Figure.1b).

The transfected HeLaS3 cell line expressed hOGG1-FLAG fusion protein, which could be detected by western blotting using anti-FLAG antibody. The anti-FLAG-stainedband was detected in the extract of the HeLaS3/hOGG1 overexpression cells (Figure.1c).

hOGG1-type1a has a strong nuclear localization signal and a weak mitochondrial targeting sequence, and is mainly localized to nuclei [23, 32]. We therefore examined the subcellular localization of the transfected hOGG1-1a proteins by performing immunofluorescence staining using anti-FLAG antibody. As shown in Figure.1d, FLAG-tagged hOGG1 was localized mainly in the nucleus in HeLaS3/hOGG1 cells. The HeLaS3 cells overexpressing hOGG1-1a were therefore used for the following experiments.

Figure 1

Overexpression of hOGG1 increased the cell susceptibility to γ-ray irradiation and hydrogen peroxide treatment

Previous studies showed that overexpression of hOGG1 increased the cell sensitivity to γ-ray irradiation in *E. coli* cells and human TK6 cells [24, 26]. First, we investigated whether overexpression of hOGG1 caused radiosensitivity in HeLaS3 cells. γ-ray survival was measured by a colony-formation assay. The results in Figure. 2a show that hOGG1-overexpressing HeLaS3 had enhanced cell sensitivity to γ-irradiation. γ-rays generate reactive oxygen species and produce clustered damage to DNA.

We further investigated the effect of H_2O_2 on HeLaS3 cell lines and HeLaS3/hOGG1 cells. The results showed, when the cells were treated with 100 μM, 200 μM or 300 μM H_2O_2, overexpression of hOGG1 increased the cell sensitivity to H_2O_2 (Figure. 2b). On the other hand, cells exposed to UV showed similar sensitivity between HeLaS3 and HeLaS3/hOGG1

cells (Figure.2c). Subsequently, we investigated the effect of $OH^{\cdot}$ on cell survival after treatment of the cells with H_2O_2 and Fe^{2+}. The results showed, overexpression of hOGG1 increased the cell sensitivity to $OH^{\cdot}$ (Figure. 2d).

The above results suggested that processing of oxidative damage by hOGG1 contributes to sensitivity to oxidative stress.

Figure 2

8-oxoG was removed efficiently in HeLaS3/hOGG1-overexpressing cells after exposure to γ-rays and H_2O_2

γ-ray irradiation damages cellular DNA by directly attacking the DNA backbone and bases or by indirect effects such as generating reactive oxygen species (ROS) by ionizing cellular H_2O and O_2 [33]. Ionizing radiation produces clustered damages, which contain two or more lesions induced within one or two helical turns of the DNA [34-37]. Non-DSB clustered damages induced by ionizing radiation are less readily repaired than isolated lesions [38-42]. The above cell survival data showed that the sensitivity of the hOGG1-overexpressing cells was enhanced against γ-rays and H_2O_2, but not against UV. What are the differences between these stresses or exposures? Formation of 8-oxoG foci was detected by immunofluorescent staining in each type of cells, which were treated with various stresses. As shown in Figure.3b and 3c, the formation of 8-oxoG foci was significantly increased in γ-ray irradiated and H_2O_2-treated HeLaS3 cells. 8-oxoG foci were hardly observed in UV- and heavy-ion beams treated cells (Figure. 3d and 3e). On the other hand, in hOGG1-overexpressing cells, when exposed to γ-rays and H_2O_2, 8-oxoG foci formation was clearly lower than that in HeLaS3 cells exposed to these treatments (Figure. 3g and 3h). These results suggested that γ-ray

irradiation and H_2O_2 treatment generated clustered 8-oxoG, and the generation of such 8-oxoG foci was efficiently decreased in hOGG1-overexpressing cells.

Figure 3

hOGG1-overexpressing cells showed increased DSB formation after γ-ray irradiation and H_2O_2 exposure

HeLaS3 cells overexpressing hOGG1 were sensitive to γ-ray irradiation. We therefore measured the level of DSBs to examine the reason for this higher sensitivity. We checked the formation of γ-H2AX, the phosphorylated form of H2AX histone that is increased in response to DSBs (Figure. 4a). The cells were irradiated with 3 Gy γ rays, and then incubated for the indicated times, and the γH2AX foci were observed by microscopy and the percentage of foci-positive cells was determined. The results are shown in Figure.4a. The peak of the percentage of foci-positive cells in HeLaS3 was observed at 30 min after irradiation, whereas that in HeLaS3/hOGG1-1a cells was observed at 90 min. In HeLaS3/hOGG1-1a cells the amount of γ-H2AX foci at 90 min of incubation was higher than that in HeLaS3 cells. These results suggested that far more DSBs were generated post γ irradiation in hOGG1-overexpressing cells compared with the control cells. We carried out detection of phosphorylation of H2AX after UVC irradiation by western blotting assay. As a result, phosphorylation of H2AX after UVC irradiaton was detected in both HeLaS3 and HeLaS3/hOGG1 cells, no difference was detected obviously (data not shown).

To further support this suggestion, the rate of micronucleus formation was also measured. The cultured cells were synchronized with thymidine and then with hydoroxyurea, and the G1 phase cells were irradiated with 2 Gy γ-rays or exposed to 300 μM H_2O_2, and cultured for a

further 24 hours, then fixed with formaldehyde and stained with DAPI. As shown in Figure. 4c, in OGG1-overexpressing cells, the rate of micronucleus formation was significantly increased by 2 fold compared with that in HeLaS3 cells when exposed to 2 Gy γ-rays. In response to H_2O_2 treatment, the rate of micronucleus formation of the hOGG1-overexpressing cells was about 2.3-fold higher compared with that in HeLaS3 cells. Furthermore, compared with non-treated hOGG1-overexpressing cells, γ-ray-irradiated cells generated about 8-fold more micronuclei and H_2O_2 -treated cells generated about 7-fold more micronuclei. These results of γ-H2AX and micronucleus formation assays indicated that hOGG1 increases DSBs and genome instability through excess removal of 8-oxoG in oxidative stress conditions.

Figure 4

hOGG1-overexpressing cells are more sensitive to low dose rate γ-irradiation than HeLaS3 cells, but not more sensitive to heavy-ion beams

Previous studies showed that high LET radiation (for example: α-rays and heavy-ion beams) mainly affected cells by direct effects, and the effects of heavy-ion beams were less dependent on oxygen partial pressure [43].We investigated the contribution of oxidative damage induced by a heavy-ion beams. The cells were irradiated with heavy-ion beams at 0, 2, 4 or 6 Gy. The results showed that the hOGG1-overexpressing HeLaS3 cell survival was similar to that of the HeLaS3 cell line when treated with particular heavy-ion beams irradiation (Figure.5b). The micronucleus formation assay did not show a significant difference between hOGG1-overexpressing cells and HeLaS3 cells (Figure.5c).

Furthermore we investigated whether overexpression of hOGG1 confers radiosensitivity against low dose rate γ-irradiation in HeLaS3/hOGG1 cells. The cells were exposed to low

dose rate γ-ray (1 mGy/min) irradiation up to 6 Gy. The cell survival was measured by colony formation. The results in Figure.5a showed that overexpression of hOGG1 enhanced cellular sensitivity to low dose rate γ-irradiation. The micronucleus formation assay also showed that overexpression of hOGG1 increased genome instability (Figure. 5c).

Figure 5

Discussion

It is thought that ionizing radiation produces a unique form of DNA damage called non-DSB clustered damage, which contains two or more lesions induced within one or two helical turns of the DNA [32-34]. It has been shown that non-DSB clustered damages induced by ionizing radiation could be recognized and removed by DNA glycosylases, such as *E.coli* MutM and hOGG1, and then were converted to DSBs. Therefore, clustered damages might be biologically significant. However, the mechanisms of recognition of the clustered damaged DNA in human cultured adherent cells and the effects of ROS in cells overexpressing hOGG1 have not been fully studied. In addition, the effect of heavy-ion beams has no enough reports. The effects of low dose or low dose rate irradiation have also remained unknown. It has also remained unknown whether ROS-induced clustered DNA damages influence the cell survival. Thus, there are still many questions waiting to be answered.

Many studies using synthetic duplex oligonucleotides containing two closely opposed lesions and purified enzymes have demonstrated that processing of bi-stranded clustered damages by BER enzymes may actually convert non-lethal clustered damages into lethal DSBs [44-46]. The formation of DSBs depended on whether or not the DNA glycosylases were able to recognize and cleave at the sites of both lesions [47].

hOGG1-overexpressing lymphoblast TK6 cells were susceptible to γ-ray irradiation[22]. In the present study, we constructed a stable HeLaS3 cell line which overexpressed hOGG1 (Figure. 1), and examined the cell sensitivity to γ-ray irradiation and H_2O_2 treatment. The results showed that hOGG1-overexpressing HeLaS3 cells were more sensitive to γ-radiation, H_2O_2 and $OH^{\cdot}$ treatment compared with control HeLaS3 cells (Figure.2a, 2b, 2d). We also investigated the cell sensitivity to UVC irradiation, and the results demonstrated that hOGG1-overexpressing cells showed similar sensitivity to UV radiation compared with control cells. (Figure.2c). It is well known that pyrimidine dimers are the major products formed by UV irradiation [48] and hOGG1 does not remove these kinds of damage. The results shown in Figure.2a, Figure.2b and Figure.2d suggested that the HeLaS3/hOGG1-1a cell line established here can recognize oxidatively damaged DNA.

To explore the reason for this phenomenon, examination of 8-oxoG, which is a major substrate of OGG1, was important. 8-oxoG formation in HeLaS3 cells is markedly increased when the cells are exposed to γ-rays or H_2O_2. We treated the cells with H_2O_2 at 37 ^{o}C. At high temperature, H_2O_2 might attack DNA more actively than at low temperature. This may be the reason why we could detect 8-oxoG foci with H_2O_2 treatment. On the other hand, in hOGG1-overexpressing cells, the number of 8-oxoG foci was decreased (Figure.3). These results showed that 8-oxoG was efficiently removed in hOGG1-overexpressing HeLaS3 cells. γH2AX and micronuclei formation are indicators of DSBs, and both markers were increased in hOGG1-overexpressing cells (Figure.4). The 8-oxoG in the clustered damages caused by H_2O_2 and γ-ray radiation was effectively removed, and as a result DSBs were generated and consequently cell death was induced.

In the environment of outer space, prolonged exposure to various heavy ion species is predicted. In addition to the cosmic radiation that comes from outer space, organisms are

exposed to various kinds of radiation generated from the ground. In order to elucidate the effects of heavy particle beams, experiments were conducted here using the hOGG1-overexpressing cells. hOGG1-overexpressing cells did not show increased susceptibility to heavy-ion beams (Figure.5b), that is, they not show an increase of 8-oxoG foci (Figure.3e, j). Considering the fact that we detected a large amount of DNA strand breaks caused by heavy-ion beams (Figure.5c), the effect of heavy-ion beams on cells seemed to have a larger contribution via direct damage of DNA than via indirect effects (including generation of ROS).

The effect of low dose and low dose rate radiation is highly controversial. The biological effects of low dose rate ionizing radiation are difficult to measure. In the present study, the results showed that cells overexpressing hOGG1 are more sensitive to low dose rate γ-rays than HeLaS3 cells (Figure.5a). However, we could not detect 8-oxoG foci formation (data not shown). Why were the hOGG1-overexpressing cells more sensitive to low dose rate radiation than HeLaS3 notwithstanding the lack of 8-oxoG foci accumulation? We consider it likely that low dose rate irradiation generates ROS continuously by reacting with H_2O in cells. The ROS generate only small amounts of 8-oxoG. hOGG1 has a DNA glycosylase activity and hardly shows AP lyase activity [49]. hOGG1 removes 8-oxoG from damaged DNA, and then generates an AP site. hOGG1-overexpressing cells do not show increased APE1 expression (data not shown). It is possible that the activity of DNA glycosylase caused an accumulation of AP sites, which are would block DNA replication and RNA transcription. These hypotheses are currently under investigation in our laboratory.

This study showed that overexpression of OGG1 and excess removal of 8-oxoG led to genome instability and cell death(Figure6). In this study, hOGG-overexpressing cells were susceptible to γ-rays, H_2O_2, and low dose rate γ-rays. γ-rays produce clustered damages,

including 8-oxoG. H_2O_2 produces large amounts of 8-oxoG. Low dose rate γ-rays might produce ROS, which generate only small amounts of 8-oxoG. The phenotypes of hOGG1-overexpressing cells indicate the types of damage produced by each stress. hOGG1-overexpressing cells can thus be useful for analysis of damages which are induced by various stressors.

Figure 6

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Conflict of interest statement

The authors declare that there are no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

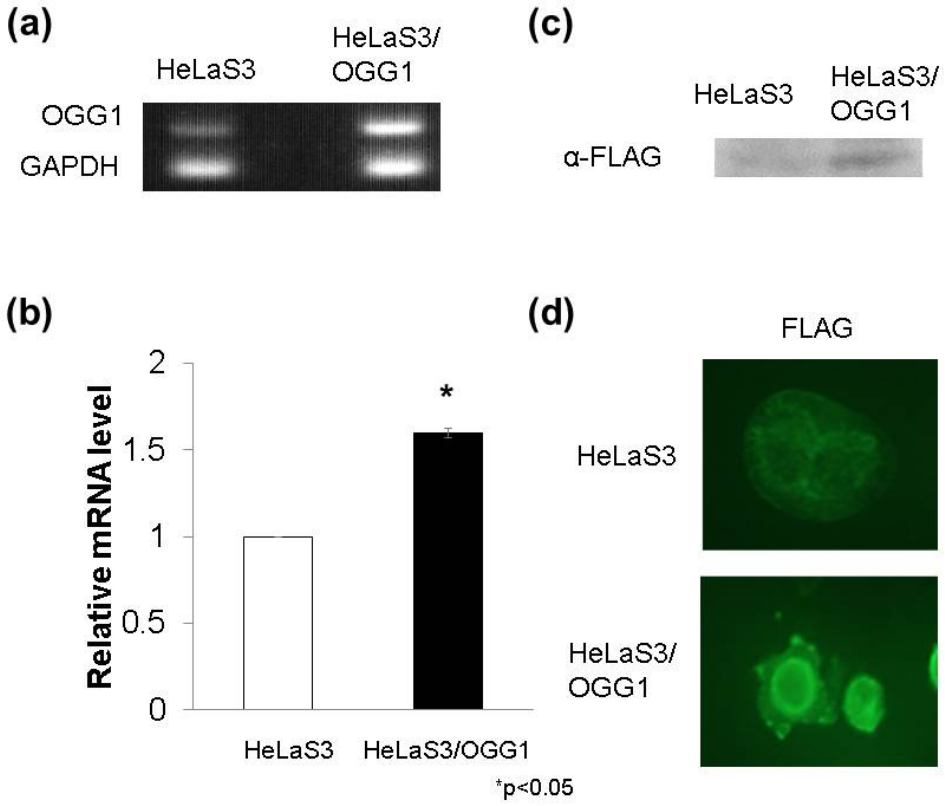

Figure. 1. Overexpression of hOGG1 in HeLaS3 cells (**a**) Total RNAs were isolated from control HeLaS3 and hOGG1-overexpressing cells. Gene transcripts were detected by RT-PCR. The products were separated by electrophoresis on a 1.5% agarose gel, and visualized by ethidium bromide staining. (**b**) Real Time PCR was carried out using cDNA from total RNA. The values represent the mean ± S.E. (n = 3). HeLaS3 cells versus HeLaS3/OGG1 cells by student's t test. (**c**) hOGG1 protein expression in HeLaS3 was analyzed with anti-FLAG antibody. (**d**) Localization of overexpressed hOGG1 protein in HeLaS3 cell was confirmed by an immunostaining assay. Immunostaining was performed with anti-FLAG antibody and Alexa Fluor 488-conjugated anti-mouse IgG. The green fluorescence of hOGG1 was observed using microscopy.

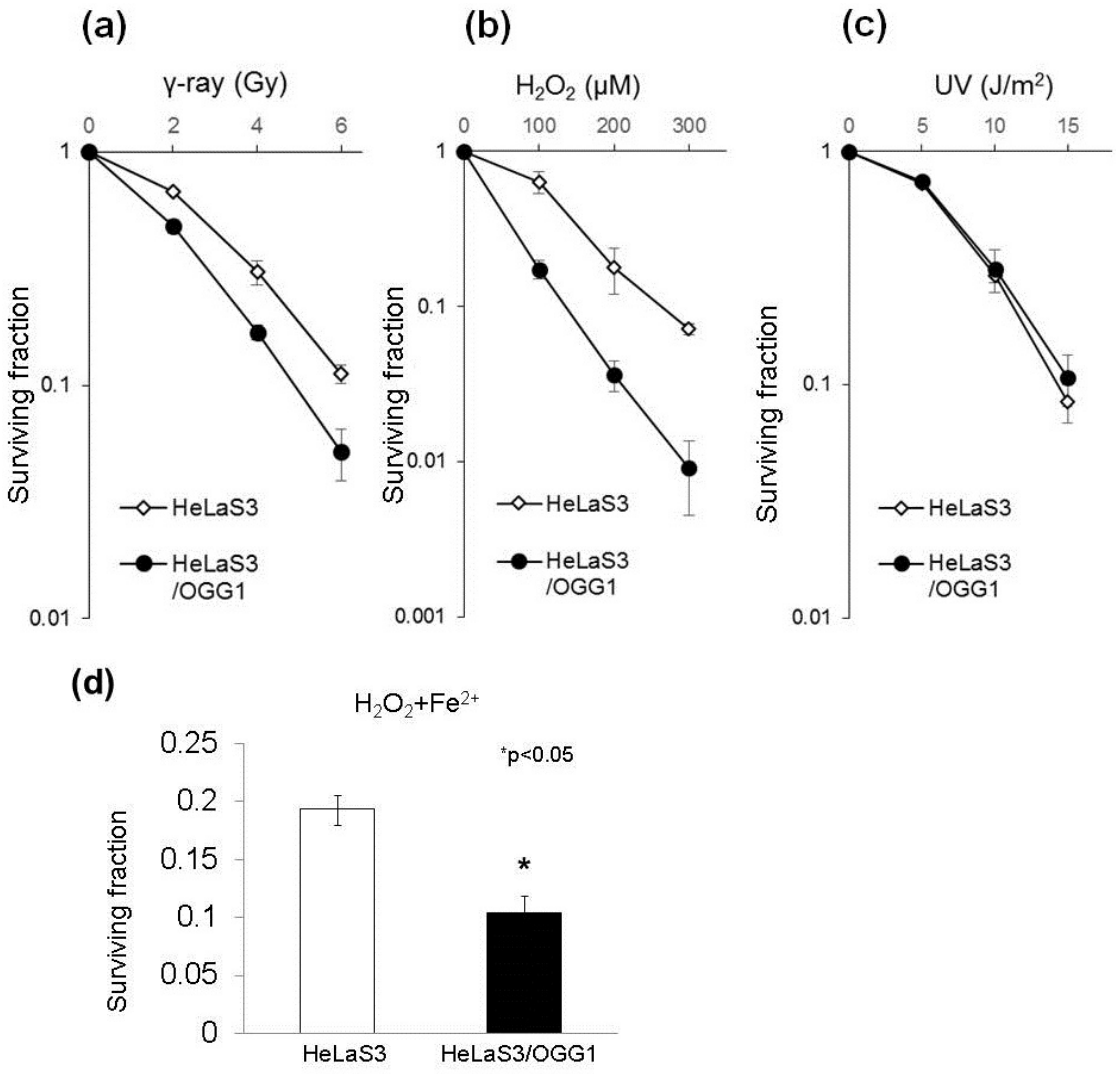

Figure. 2. Survival of HeLaS3 cells and hOGG1-overexpressing cells exposed to various stresses. Exponentially growing cells were appropriately diluted and reseeded in 10-cm culture dishes. Then, the cells were exposed to various stresses. After incubation for 14 days, colonies were counted. (**a**) Survival of HeLaS3 cells and hOGG1-overexpressing cells following exposure to γ-rays. (**b**) Survival of HeLaS3 cells and hOGG1-overexpressing cells following exposure to hydrogen peroxide. (**c**) Survival of HeLaS3 cells and hOGG1-overexpressing cells following exposure to UV. (**d**) Survival of HeLaS3 cells and hOGG1-overexpressing cells following exposure to $OH^{\bullet}$. H_2O_2 300 μM and $FeSO_4$ 50 μM. HeLaS3 cells versus HeLaS3/OGG1 cells by student's t test. The values represent the mean ±

S.E. (n = 3). -◇-: control HeLaS3 cells -●-: hOGG1-overexpressing cells.

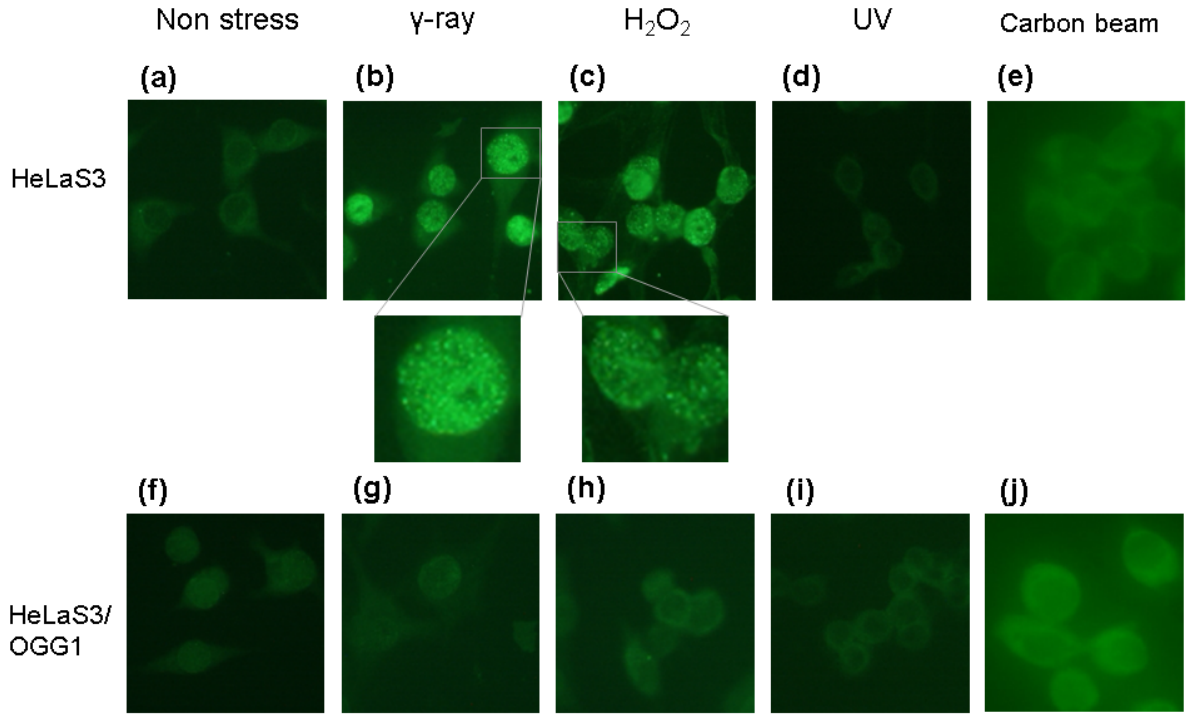

Figure. 3. Immunostaining detection of 8-oxoG foci following exposure to various stresses. Cells were exposed to 5 Gy γ-rays (**b**, **g**), 300 μM hydrogen peroxide(**c**, **h**), 15 J/m^2 UV (**d**, **i**), 2 Gy heavy-ion beams (**e**, **j**) or no treatment (**a, f**). Immunostaining was performed with anti-8-oxoG antibody and Alexa Fluor 488-conjugated anti-mouse IgG. The green fluorescence corresponding to 8-oxoG was observed using microscopy.

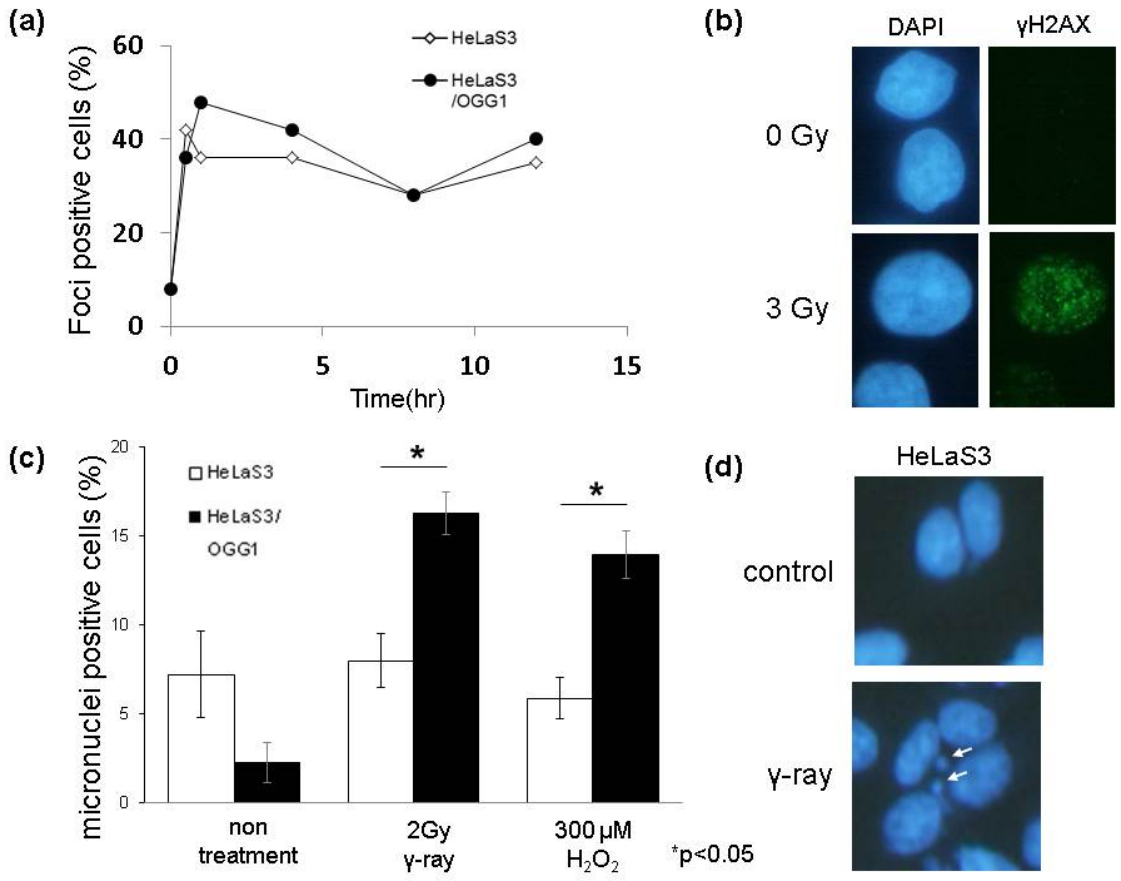

Figure. 4. Analysis of DNA strand break formation and genomic instability by γH2AX formation and micronuclei formation. (**a**) Immunostaining detection of γH2AX foci. Cells were exposed to γ-rays at 3 Gy. Immunostaining was performed with anti-γH2AX antibody and Alexa Fluor 488-conjugated anti-mouse IgG. The green fluorescence corresponding to γH2AX was observed using microscopy. The percentage of γH2AX foci-positive cells was determined. -◇-: control HeLaS3 cells -●-: hOGG1-overexpressing cells. (**b**) Immunostaining for γH2AX in hOGG1-overexpressing cells. (**c**) DAPI staining detection of micronuclei. Synchronized cells were exposed to γ-rays at 2 Gy, and hydrogen peroxide at 300 μM. After further incubation for 24 hours, DAPI staining was performed, and the blue fluorescence of micronuclei was observed using microscopy. HeLaS3 cells versus HeLaS3/OGG1 cells by

student's t test. (**d**) DAPI staining to detect micronuclei in HeLaS3 cells.

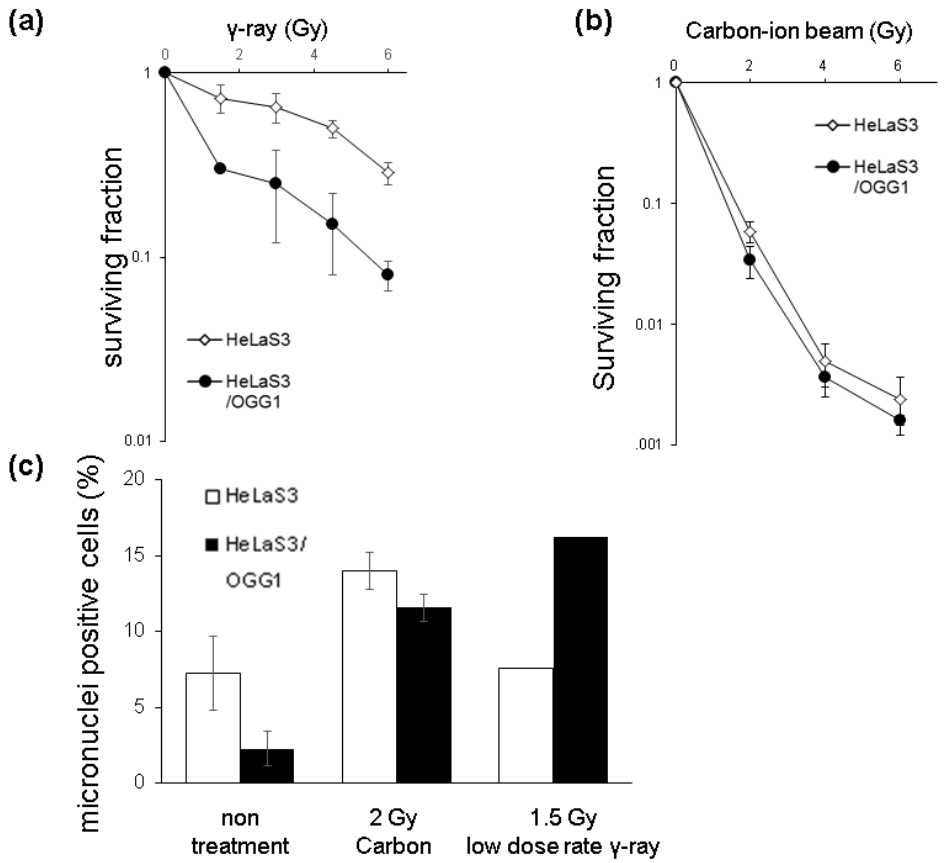

Figure. 5. (**a**) Survival of HeLaS3 cells and hOGG1-overexpressing cells following exposure to low dose rate γ-rays (1 mGy/min). Exponentially growing cells were appropriately diluted and reseeded in 6-cm culture dishes. After incubation for 10 hours, the cells were exposed to γ-rays at 37°C. After exposure to each dose, the cells continued to be incubated in a 37°C CO_2 incubator. The cells were incubated for a total of 12 days. The values shown represent the mean ± S.E. (n = 3). **(b)** Survival of HeLaS3 cells and hOGG1-overexpressing cells following exposure to heavy-ion beams. The values represent the mean ± S.E. (n = 3). -◇-: control HeLaS3 cells -●-: hOGG1-overexpressing cells. (**c**) DAPI staining detection of micronuclei. The cells were exposed to heavy-ion beams at 2 Gy, and low dose rate γ-rays at 1.5Gy. After further incubation for 24 hours, DAPI staining was performed, and the blue fluorescence of

micronuclei was observed using microscopy.

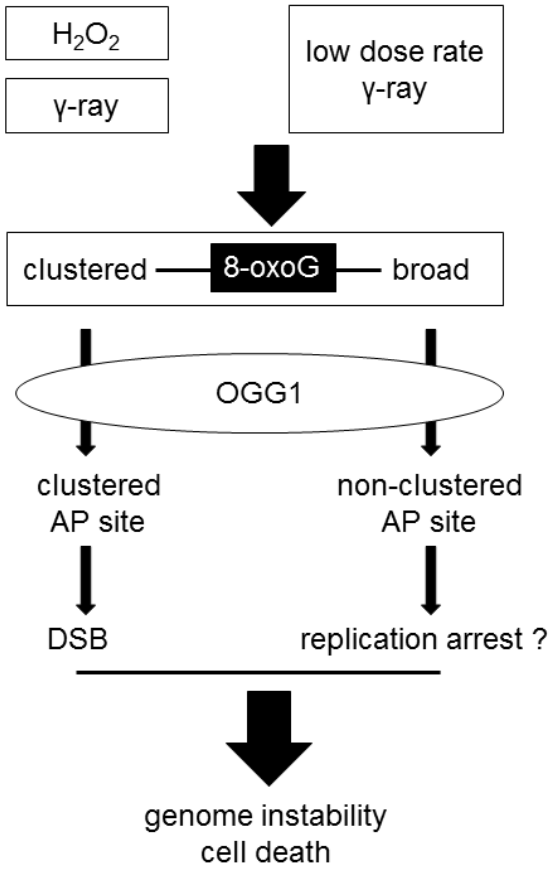

Figure.6. The mechanism of cell death in OGG1-overexpressing cells

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