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Molecular Analysis of Carbon Ion-Induced Mutations in DNA Repair-Deficient Strains of *Saccharomyces cerevisiae*

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Abstract: Mutations caused by ion beams have been well-studied in plants, including ornamental flowers, rice, and algae. It has been shown that ion beams have several significantly interesting features, such as a high biological effect and unique mutation spectrum, which is in contrast to low linear energy transfer (LET) radiation such as gamma rays. In this study, the effects of double strand breaks and 8-oxo-2′-deoxyguanosine (8-oxodG) caused by ion-beam irradiation were examined. We irradiated repair-gene-inactive strains *rad52*, *ogg1*, and *msh2* using carbon ion beams, analyzed the lethality and mutagenicity, and characterized the mutations. High-LET carbon ion-beam radiation was found to cause oxidative base damage, such as 8-oxodG, which can lead to mutations. The present observations suggested that nucleotide incorporation of oxidative damage gave only a limited effect on cell viability and genome fidelity. The ion-beam mutations occurred predominantly in 5′-ACA-3′ sequences, and we detected a hotspot at around +79 to +98 in *URA3* in wild-type and mutant lines, suggesting the presence of a mutation-susceptible region.

Keywords: carbon ion beam; mutation; *S. cerevisiae*; 8-oxodG; high LET

1. Introduction

The effects of ion beam irradiation on biological samples have been investigated using a wide range of ion species produced by accelerators. Ion beams have been thought to induce a broad mutation spectrum due to their high linear energy transfer (LET), and mutation breeding using ion beams has seen significant developments. The biological effects of ionizing radiation depend on LET [1]. It has been shown in mammalian cells, plants, and microorganisms that the relative biological effectiveness (RBE) depends on LET and generally reaches a peak in a LET range of 100–200 keV/μm [2–4]. The analysis of mutations in *Arabidopsis* demonstrated that ion beams induce deletions of large regions and inversions at the molecular level [5,6] and also some mutants with novel phenotypes [7,8]. Mutations caused by ion beams have also been well-studied in plants, such as ornamental flowers [9], rice [10], and algae [11].

Our research group has been studying ion-beam induced mutations in budding yeast, as a model of eukaryote cells [12]. Yeasts grow faster than plants, and a series of repair-deficient strains are

available for study, allowing mutational analysis with ease. The mutagenic effects of ion beams were examined using *URA3* as a marker gene, and showed that high-LET (107 keV/μm) carbon ion beams are more mutagenic than low-LET gamma rays [12]. A remarkable feature of carbon ion-induced yeast mutation was that the mutations were located at a hot-spot near the linker regions of nucleosomes on the *URA3* gene, whereas mutations induced by gamma-rays were located uniformly throughout the marker gene. However, the underlying mechanisms remain to be elucidated.

8-Oxo-2′-deoxyguanosine (8-oxodG), a major oxidative damage product produced by free radicals, is important because of its mutagenicity. DNA polymerase incorporates 8-oxodG opposite to deoxycytidine (dC) in template DNA. The incorporated 8-oxodG can pair with dA in next round of replication and lead to G:C to T:A transversion [13]. Here, we focused on the repair of double strand breaks (DSBs) and 8-oxodG. Carbon ion beams were used to irradiate repair-gene-inactive strains *rad52*, *ogg1*, and *msh2*, and the lethality and mutagenicity were analyzed. RAD52 is essential for all types of homologous recombination (HR) to repair DSBs in *S. cerevisiae* [14] and also eukaryotes [15]. The *ogg1* strain is deficient in DNA glycosylase activity and is not capable of removing 8-oxodG. On the other hand, MSH2 is a critical factor for mismatch repair pathways and binds to 8-oxodG:A mispairing as a MSH2-MSH6 complex to remove the incorporated 8-oxodG [16].

Here, we analyzed the effect of HR, base excision repair (BER), and mismatch repair on ion beam-induced mutations. The distribution of mutations in these repair-deficient lines was also analyzed to investigate the possible roles of nucleosome structure in the ion beam-induced mutations.

2. Materials and Methods

2.1. Strains and Growth Conditions

Wild-type and repair-deficient haploid strains of *S. cerevisiae* were used to investigate the repair pathway of DNA damage induced by the carbon ion beam. The relevant genotype was as follows: S288c (*MATα SUC2 gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6*), *G160/2b* (*MATα, rad52*), *ogg1* (*MATα, ogg1*), and *msh2* (*MATα, msh2*). G160/2b (MATα, *rad52*) was obtained from Yeast Genetic Stock Center (UC Berkeley). The *ogg1* and *msh2* strains were generated using one-step gene disruption by homologous recombination in this study. Briefly, the *kanMX*-containing plasmid pUG6 [17] was Polymerase Chain Reaction (PCR)-amplified with the primers OGG-F1 and OGG-R1 (Table S1) and introduced into S288c to disrupt the *OGG1* gene. To create *msh2*, yeast chromosomal DNA was PCR-amplified with MSH2-F1 and MSH2-R1 (Table S1) and cloned into pAUR101. The plasmid was digested by *Sna*BI and then introduced into S288c.

Yeast extract-Peptone-Dextrose (YPD) medium was used to grow yeast cultures at 30 °C. For the selection of *ura3* mutants, we employed YPD medium containing 0.1% 5-fluorortic acid (5-FOA). The yeast *URA3* gene encodes an orotidine-5-phosphate decarboxylase, an enzyme required for the biosynthesis of uracil. 5-FOA is converted by the action of this decarboxylase into a toxic product, 5-fluorouracil, which kills *URA3* cells but leaves *ura3* mutant cells alive. This positive selection has been proven to be highly efficient and selective [18].

2.2. Irradiation

The carbon ion ($^{12}C^{5+}$, 18.3 MeV/u) beam used in this study was generated by the cyclotron at the Takasaki Ion Accelerator for Advanced Radiation Application (TIARA), Takasaki Advanced Radiation Research Institute, National Institute for Quantum and Radiological Science and Technology, Japan. The LET of the carbon ion beam was 107 keV/μm. Yeast cells in the logarithmic growth phase were harvested by centrifugation, re-suspended in a fresh YPD medium, and the cell density was measured using a Neubauer improved cell counting chamber. Then, the cells were collected by filtering with a 0.45 μm nitrocellulose membrane filters (EMD Millipore, Billerica, MA, USA). The membrane filters, each containing $2 \times 10^{2-3}$ cells for determining survival rate or $2 \times 10^{7-8}$ cells for determining mutation

frequency, were put on Φ 60-mm plastic petri dishes and covered with a 5-μm thick sterilized Kapton film. The samples of yeast cells were kept at 4 °C and irradiated on the following day after preparation.

2.3. Survival Rate and Mutation Frequency

To measure cell survival, the irradiated membrane filters were incubated on YPD plates at 30 °C for 2 days. Survival rate was determined by counting the numbers of colonies on YPD plates. To measure the mutation frequency, the membranes were transferred to 5-FOA selection plates and cultured for 5 days at 30 °C, after which the numbers of mutants (*ura3*) were counted. The colonies that grew on the 5-FOA plate were scored as *ura3* mutants. The mutation frequency was calculated according to the numbers of colonies on the 5-FOA plates.

2.4. Sequence Analysis

The *ura3* mutants isolated from the 5-FOA plates were cultured and DNA was isolated using a DNeasy Tissue Kit (Qiagen KK, Tokyo, Japan) [19]. The *URA3* gene region of the mutants was amplified by PCR using Ex Taq polymerase (Takara Bio, Otsu, Japan). The PCR protocol was as described previously [20]. The nucleotide sequences were determined using an ABI2300 system (Applied Biosystems, Foster City, CA, USA). The sequence of the entire *URA3* structural gene region (804 bp) was examined for base alterations.

3. Results

3.1. Survival Rate

The survival rate of the haploid *rad52*, *ogg1*, and *msh2* cells irradiated with a carbon ion beam was measured. Figure 1 shows that *rad52* cells were extremely sensitive to carbon ion irradiation; approximately 26 times more sensitive than wild-type cells at 100 Gy. The survival ratios of *msh2* and *ogg1* cells showed similar trends to wild-type cells. These results suggested that the DNA strand breaks are the major determinant of survival reduction by ion beam irradiation and the increase in mismatched base pairs and oxidized guanines in the genome only partially affected cell survival.

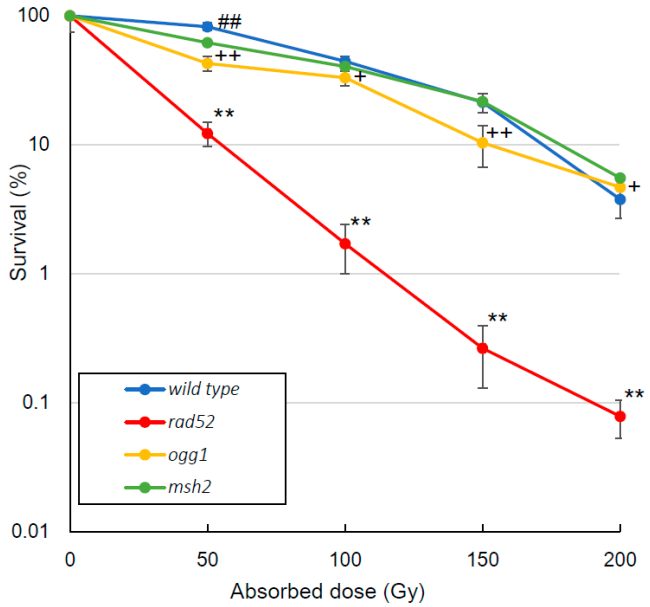

Figure 1. Survival rates for yeast strains S288c (wild-type), *rad52*, *ogg1*, and *msh2* after irradiation by carbon ion beams. Data are the means of five replicates. Data for yeast strain S288c (wild-type) were taken from [12]. Bars indicate SD. *rad52* vs. wild-type; ** $P < 0.01$, *ogg1* vs. wild-type; + $P < 0.05$, ++ $P < 0.01$, *msh2* vs. wild-type; ## $P < 0.01$.

3.2. Mutation Frequency

Next, the spontaneous mutation frequency in the *URA3* gene in the haploid wild-type, *rad52*, *ogg1*, and *msh2* cells was measured using the *ura3* positive selection system. The mutation frequency in *rad52* cells was 4.2×10^{-6}, which was 24-fold higher than that in the wild-type (1.8×10^{-7}) (Figure 2). The *ogg1* and *msh2* cells showed a much higher spontaneous mutation frequency (5.0×10^{-6}). These findings indicated the mutator phenotype of the DNA-repair-deficient strains used here.

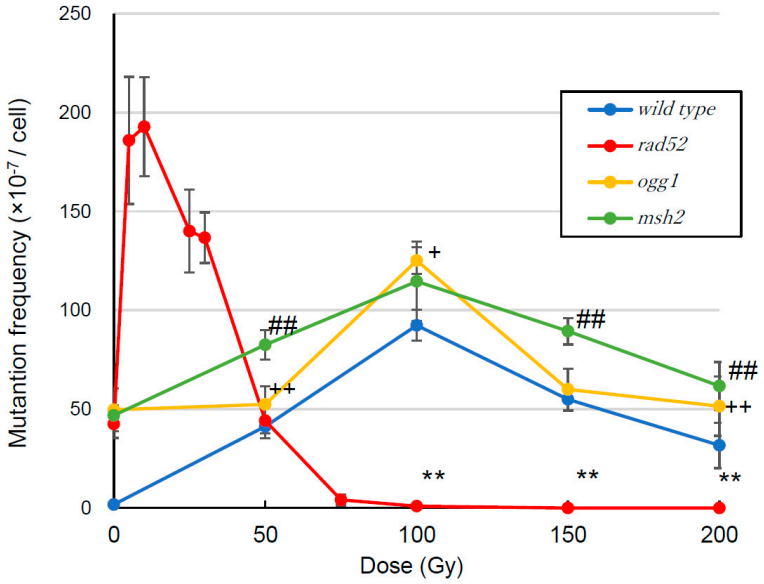

Figure 2. Mutation frequency for yeast strains S288c (wild-type), *rad52*, *ogg1*, and *msh2* after irradiation with carbon ion beams. Data are the means of five replicates. Data for yeast strain S288c (wild-type) were taken from [12]. Bars indicate the SD. *rad52* vs. wild-type; ** $P < 0.01$, *ogg1* vs. wild-type; + $P < 0.05$, ++ $P < 0.01$, *msh2* vs. wild-type; ## $P < 0.01$, at 50 Gy, 100 Gy, 150 Gy, and 200 Gy, respectively.

When haploid cells of wild-type, *rad52*, *ogg1*, and *msh2* were irradiated with a carbon ion beam, the mutation frequency of *rad52* showed a maximum at 10 Gy (2.0×10^5). The mutation frequencies of *ogg1* and *msh2* were highest at 100 Gy (1.3×10^5 and 1.1×10^5, respectively; Figure 2).

3.3. Mutation Spectrum

Table 1 shows the types of mutations in *ura3* mutants caused by carbon ion irradiation of *rad52*, *ogg1*, and *msh2* strains. In the case of the *rad52* strain, GC to TA transversion was observed at a frequency of 61.9%. In the *ogg1* strain, GC to TA transversion was observed in 72.0%, but no deletion or insertion mutations were seen. In the *msh2* strain, single base deletions were often observed (46%). Frame-shift mutations mainly occurred at the A or T base (40%) (Table 2).

Table 1. Types of mutations induced by carbon ion beam irradiation (linear energy transfer [LET] 107 keV/um). Data for the wild-type (S288c strain) was taken from our previous report [12].

Type of Mutation	*rad52*		*ogg1*		*msh2*		Wild-type	
	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
Base substitution								
Transversions	31	73.8	47	94.0	16	32.0	35	68.6
G:C to T:A	26	61.9	36	72.0	13	26.0	21	41.2
G:C to C:G	3	7.1	0	0.0	2	4.0	12	23.5
A:T to C:G	1	2.4	7	14.0	0	0.0	1	2.0
A:T to T:A	1	2.4	4	8.0	1	2.0	1	2.0
Transitions	7	16.6	3	6.0	10	20.0	7	13.7
G:C to A:T	7	16.6	3	6.0	9	18.0	7	13.7
A:T to G:C	0	0.0	0	0.0	1	2.0	0	0.0
Deletions	2	4.8	0	0.0	23	46.0	8	15.7
Insertions	2	4.8	0	0.0	1	2.1	1	2.0
Total	42	100	50	100.0	50	100.0	51	100.0

Table 2. Spectrum of deletion and insertion mutations in *ura3* mutants of wild-type and *msh2* caused by carbon ion beam irradiation.

Genotype	Mutation	Occurrence
Wild-type	$A_2 \rightarrow A_1$	1/51
	$C_2 \rightarrow C_1$	1/51
	$C_1 \rightarrow C_0$	5/51
	$T_4 \rightarrow T_3$	1/51
	$T_5 \rightarrow T_6$	1/51
rad52	$T_1 \rightarrow T_0$	1/42
	$T_1 \rightarrow T_2$	2/42
	$A_2 \rightarrow A_1$	1/42
msh2	$A_5 \rightarrow A_4$	6/50
	$A_4 \rightarrow A_3$	6/50
	$C_3 \rightarrow C_2$	3/50
	$C_1 \rightarrow C_0$	1/50
	$T_6 \rightarrow T_5$	6/50
	$T_5 \rightarrow T_4$	1/50
	$T_6 \rightarrow T_7$	1/50

Notably, in all strains, G (or C) residues in the 5′-ACA-3′ (or 5′-TGT-3′) sequence tended to change. If the base substitution occurred randomly in any sequence, the ratio of 5′-ACA-3′ (or 5′-TGT-3′) would be 2/64 ≈ 3.1%. However, these mutations were at least more than three times as frequent as mutations with other combinations of three bases (*rad52* strain, 10-fold [30.9%]; *ogg1* strain, 9-fold [28.0%]; *msh2* strain, 3-fold [8.0%]) (Tables S2–S4). This sequence may be a hot-spot for base substitution mutation, which is at least unrelated to the function of *RAD52*, *OGG1*, and *MSH2*.

Figure 3 shows the distribution of mutation sites in the *URA3* gene. It is likely that the mutation sites are non-uniformly distributed, particularly in *rad52*, *msh2*, and wild-type.

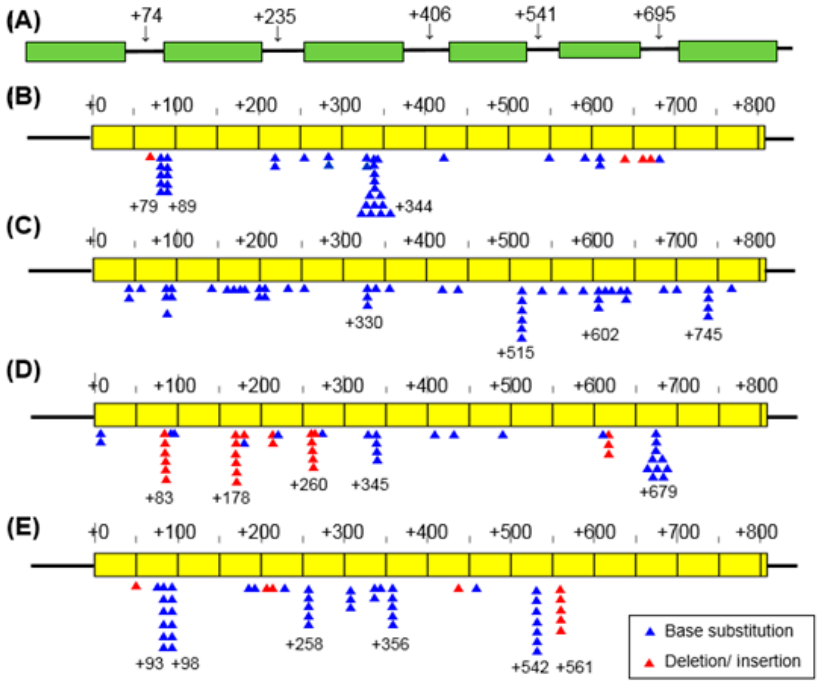

Figure 3. Distribution of mutation sites induced by carbon ion beams (LET 107 keV/μm) in wild-type and repair-deficient yeast strains. (**A**) Deduced nucleosome structure described in Tanaka et al. [21]. Arrows indicate the approximate position of linker DNA. (**B**–**E**) Mutation sites observed in *rad52* (**B**), *ogg1* (**C**), *msh2* (**D**), and wild-type (**E**). Blue triangles: substitution mutations. Red triangles: deletion/insertions. Numbers represent the nucleotide positions of the *URA3* gene, in which +1 indicates the position of A in the initiation codon ATG. The gray boxes in (**A**) indicates histone proteins. The white boxes in (**B**) to (**E**) indicate the PCR amplified region. Data of yeast strain S288c (wild-type) was taken from [12].

4. Discussion

4.1. Survival Rate

The *rad52* strain was hypersensitive to the high-LET carbon ion beam (107 keV/μm) (Figure 1). In our previous study, the *rad52* strain also showed hypersensitivity to a carbon ion beam with a relatively low LET (13 keV/μm) [20]. It has been shown that DSBs are mainly repaired by HR or the non-homologous end joining (NHEJ) pathway. NHEJ functions in the G_1/S/G_2 phases, whereas HR becomes active only in the S/G_2 phase after DNA replication [22]. The widely accepted role of *RAD52* is to repair DSBs by HR. The high sensitivity of the *rad52* strain indicated that HR is essential for the repair of DSBs induced by ion beams with the wide range of LET in yeast. In contrast, no significant difference was observed in radiation sensitivity between *ogg1*, *msh2*, and wild-type. This observation suggested that the incorporation of the oxidative damage into the genome only slightly affected cell survival after ion beam irradiation in yeast.

4.2. Mutation Frequency

rad52 cells showed the highest mutation frequency at 10 Gy (Figure 2). However, the mutation frequency of the *rad52* strain decreased steeply at doses higher than 10 Gy. In contrast, the *ogg1* and *msh2* strains showed peak mutation frequencies at 100 Gy. This suggested that DNA strand breaks are highly mutagenic and also sources of critical damage for cell survival.

The mutation frequency caused by ion beam irradiation of the *ogg1* strain was higher than that observed in wild-type ($P < 0.01$ at 100 Gy). 8-oxodG is used as a substrate by DNA polymerases and is inserted opposite C and G [23]. The present results suggested that ion beam mutations such as GC to

TA transversion were mainly caused by the production of 8-oxodG in the nucleotide pool or in the DNA of irradiated cells. In general, low-LET radiation mainly produces oxidative damage [24]. However, our results suggested that oxidative damage also occurred following exposure to high-LET radiation.

4.3. Mutation Spectrum

Spontaneously generated mutations are usually transitions, whereas mutations generated by ion beams are predominantly transversions [25]. The predominance of G:C to T:A transversion in all strains suggested that oxidative damage such as 8-oxodG is the major DNA damage resulting in base substitution (Table 1) [26]. The transversion/transition ratio induced by carbon ion beam irradiation was 5.0 in the wild-type, whereas it was 4.4 in *rad52*, 15.7 in *ogg1*, and 1.6 in *msh2*. The high transversion/transition ratio in *ogg1* was reasonable, because OGG1 is the primary enzyme responsible for the excision of 8-oxodG. A high transversion/transition ratio was also reported in spontaneous mutation in the *ogg1* strain, in which 95% of natural mutations were base substitutions, with transversion mutations accounting for 70% [27]. Oxidative DNA damage is thought to be generated through three pathways. First, direct oxidation; second, oxidatively damaged DNA precursors are incorporated into new DNA strands by the action of DNA polymerase; and thirdly incorporation of oxidized DNA precursor into DNA during DNA repair processes. Although it is difficult to determine which of these processes is dominant, the results from the *msh2* strain suggested a clue. *msh2*, which is a mismatched repair inactive strain, also showed a slightly higher mutation rate than wild-type ($P < 0.01$ at 50 Gy, $P < 0.05$ at 100 Gy). Because the mismatch repair process works after replication, this result may suggest that 8-oxodG is incorporated into DNA through DNA replication. When DNA carrying a damaged base is replicated, an incorrect base may be inserted contralateral to the damaged base in the complementary strand, leading to a mutation in the next round of DNA replication [28]. Moreover, in the *rad52* strain, the percentages of G:C to A:T mutations, which were caused by 8-oxo-G were higher than in wild-type [12]. These results suggested that 8-oxo-G is taken in during normal replication and NHEJ pathway.

In *rad52* and *ogg1* cells, about 31% and 28% of C substitute mutations occurred within 5′-ACA-3′ sequences, respectively. This ratio was notably higher than the ratio expected from random occurrence. The same tendency was observed in wild-type cells irradiated with a carbon ion beam [12]; however, no such tendency was reported in spontaneous mutations in yeast [29]. Although the mechanism is unclear, there may be sequence specificity in mis-incorporation by DNA polymerase.

MSH2, which forms a heterodimer with MSH6 to make the mismatch repair complex, is the major determinant by which misincorporation of A opposite to 8-oxodG is corrected [28]. In *msh2* cells, the percentage of deletion mutations and G:C to T:A transversion was the highest (Table 1). It is reasonable to interpret that the remaining 8-oxodG:A pairs induce G:C to T:A transversion. In the *msh2* strain, 83% of deletions occurred at poly (A/T) sequences (Table 2). Spontaneous mutation observed in the yeast *msh2* strain also showed a similar tendency [28]. Therefore, the mutation spectrum shown here is not specific to ion beam irradiation, but rather similar to those of spontaneous mutation.

Our previous results showed that yeast mutations induced by carbon ion beams were located at a hot-spot near the linking regions of nucleosomes on the *URA3* gene [12]. In contrast, gamma ray-induced mutations were located uniformly throughout the marker gene. The damage induced by high-LET radiation opens the chromatin structure, making the DNA more susceptible to attack by Reactive Oxygen Ppecies (ROS) generated by low-LET radiation [30]. It was hypothesized that the non-uniform distribution of carbon-ion-induced mutations in the *URA3* gene might be related to the nucleosome structure. This notion was supported by the studies on nucleotide excision repair and photoreactivation in yeast, which suggested that DNA damage was repaired slowly on the nucleosomes but quickly on the linkers [31].

Comparison of mutation sites in wild-type, *rad52*, *ogg1*, and *msh2* showed the presence of unique hotspots. In *ogg1*, the mutations distributed uniformly, except for weak hotspots at +515 and +745, as previously seen in gamma-irradiated wild-type cells [12]. In *msh2*, the mutations were distributed

at around +83, +178, +260, +345, and +679, which were not consistent with the linker region in the nucleosome structure. Nevertheless, all the lines had mutations at around +79 to +98. Therefore, these positions are especially susceptible to mutation, which might be independent of HR, BER, and mismatch repair process.

In order to examine the above hypothesis, a yeast strain with a *URA3* gene with an altered nucleosome structure was prepared. In a preliminary experiment, the distribution of mutations in *URA3* with an altered nucleosome structure were inconsistent with those in the wild-type, suggesting that the distribution of mutation is dependent on the nucleosome structure. We will accumulate further data to examine the above hypothesis.

Supplementary Materials: The following are available online at http://www.mdpi.com/2412-382X/3/3/14/s1, Table S1. Primers used in this study, Table S2. DNA sequence context of mutations among carbon ion beam irradiation mutations in *ura3* of *rad52*-type. Box indicates that the sequence "3′-ACA-5" or "3′-TGT-5" changed. Underline indicates the position of mutation, Table S3. DNA sequence context of mutations among carbon ion beam irradiation mutations in *ura3* of *ogg1*-type. Open box indicates that the sequence "3′-ACA-5" or "3′-TGT-5" changed. Underline indicates the position of mutation, Table S4. DNA sequence context of mutations among carbon ion beam irradiation mutations in *ura3* of *msh2*- type. Open box indicates that the sequence "3′-ACA-5" or "3′-TGT-5" changed. Underline indicates the position of mutation.

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