**NBS1 I171V variant underlies individual differences in chromosomal radiosensitivity within human populations**

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Genetic information is protected against a variety of genotoxins including ionizing radiation (IR) through the DNA double-strand break (DSB) repair machinery. Genome-wide association studies and clinical sequencing of cancer patients have suggested that a number of variants in the DNA DSB repair genes might underlie individual differences in chromosomal radiosensitivity within human populations. However, the number of established variants that directly affect radiosensitivity is still limited. In this study, we performed whole-exome sequencing of 29 Japanese ovarian cancer patients and detected the NBS1 I171V variant, which is estimated to exist at a rate of approximately 0.15% in healthy human populations, in one patient. To clarify whether this variant indeed contributes to chromosomal radiosensitivity, we generated NBS1 I171V variant homozygous knock-in HCT116 cells and mice using the CRISPR/Cas9 system. Radiation-induced micronucleus formation and chromosomal aberration frequency were significantly increased in both HCT116 cells and mouse embryonic fibroblasts (MEFs) with knock-in of the NBS1 I171V variant compared with the levels in wild-type cells. These results suggested that the NBS1 I171V variant might be a genetic factor underlying individual differences in chromosomal radiosensitivity.

Ionizing radiation (IR) induces DNA double-strand breaks (DSBs). When DNA DSBs are left unrepaird, such damage results in the loss or rearrangement of genomic information, leading to cell death or carcinogenesis. To maintain genomic integrity, mammalian cells respond to DNA DSBs through a variety of pathways, including DNA repair, cell cycle checkpoint, and apoptosis1,2. It has been shown that, within human populations, there are individual differences in the capacity of cells to repair DNA DSBs, which we define as chromosomal radiosensitivity in this paper. The term "radiosensitivity" is used to describe different events in irradiated cells. In general, "cellular radiosensitivity" is defined as the cellular lethality post-irradiation underlying the occurrence of acute radiation-induced tissue damage, whereas "chromosomal radiosensitivity," which can be evaluated by several cytogenetic assays, is thought to be associated with the susceptibility to developing radiation-induced cancer3,4.

The cytokinesis-blocked micronucleus (CBMN) assay5, which is an elaborate procedure to evaluate chromosomal radiosensitivity by counting micronuclei formed by unrepaired DSB-derived chromosomal fragments, demonstrated the existence of mildly radiosensitive cases within a small population of healthy individuals6. It has been shown that DNA DSB repair gene variants might be the cause of individual differences in chromosomal radiosensitivity. To determine whether variants of DNA repair genes are involved in individual differences in radiosensitivity, it is informative to measure the chromosomal radiosensitivity of primary cells carrying the

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genetic variants of interest. However, chromosomal radiosensitivity might be influenced by many confounding factors, such as age, smoking, environment, and other genetic background. It is therefore necessary to evaluate the genetic variants underlying individual differences in chromosomal radiosensitivity in a cell line with a uniform genetic background.

We previously reported genome editing technology-mediated candidate variant knock-in in human cultured cells with a uniform genetic background to confirm that heterozygous ATM mutations, which cause a rare autosomal-recessive disease, ataxia-telangiectasia [A-T; Online Mendelian Inheritance in Man (OMIM): 607585], indeed underlie chromosomal radiosensitivity. Since these carriers of most hyper-radiosensitive recessive disorders such as A-T exist at a rate of ~1% in human populations, it was suggested that the heterozygous mutations might also determine individual differences in chromosomal radiosensitivity within human populations.

The association between increased chromosomal radiosensitivity and sporadic breast cancer risk has been reported, and a number of DNA DSB repair genes including BRCA1, BRCA2, ATM, CHEK2, PALB2, TP53, Rad51C, Rad51D, and NBS1 have been identified as being causative of hereditary breast and ovarian cancer (HBOC) 10. On the basis of this background, multi-gene panel testing of the DNA repair genes is clinically used to determine the genetic basis underlying individual differences in chromosomal radiosensitivity within human populations.

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Table 1. Nonsense or frameshift mutations in the HBOC core gene identified by whole-exome sequencing in 29 ovarian cancer patients.

| Gene | Variant ID | Nucleic acid change | Amino acid change | Allele frequency (gnomAD) | ClinVar | Mutation-taster | Ovarian cancer patient |
|------|------------|---------------------|------------------|--------------------------|---------|-----------------|----------------------|
| BRCA1 | rs80357692 | c.3329_3330insA     | p.Lys1110Gln      | n.d.                     | Disease causing | 3                |
|      | rs80357526 | c.1953_1956delGAAA  | p.Lys651fs        | 0.0000067                | Pathogenic       | Disease causing | 6                    |
|      | rs80357661 | c.2767_2770delGTTA  | p.Val923fs        | 0.0000067                | Pathogenic       | Disease causing | 28                   |
| BRCA2 | n.d.       | c.1314_1315delTTT   | p.Asp438fs        | n.d.                     | Disease causing | 8                |
|      | rs80358920 | c.6952C>T           | p.Arg2318*        | n.d.                     | Pathogenic       | Disease causing | 10                   |

Results

Identification of NBS1 I171V variant in a Japanese ovarian cancer patient and generation of knock-in HCT116 cell clones using genome editing technology. To screen genetic variants underlying chromosomal radiosensitivity, we performed whole-exome sequencing of genomic DNA from the peripheral blood cells of 29 Japanese ovarian cancer patients. Average coverage for the exons was more than 100 ×. Since most of the mutations detected in HBOC patients are located in the DNA repair genes, we first extracted genetic variants in the top 10 HBOC genes, namely, BRCA1, BRCA2, ATM, CDH1, CHEK2, PALB2, TP53, Rad51C, Rad51D, and NBS1 10. We also narrowed down the candidate variants on the basis of filtering criteria consisting of the ClinVar database evaluation, genomic position, function, and zygosity. As expected, of these patients, five cases harbored heterozygous mutations of either BRCA1 or BRCA2 (Table 1). In addition, heterozygous ATM missense variants (rs55141177 and rs587782298) in two ovarian cancer patients and a heterozygous TP53 missense variant (rs201382018) in two patients, which might be causative mutations of ataxia-telangiectasia (A-T, OMIM: 607585) and Li-Fraumeni syndrome (OMIM: 191170), respectively, were detected (Table 2). We also identified heterozygous variant c.511A>G, p.Ile171Val, in the NBS1 gene (NM: 002485.5, OMIM: 602667.0007), which encodes a component of the MRN1–RAD50–NBS1 (MRN) complex sensing DNA DSB sites for appropriate repair 17, in one patient (Table 2, Fig. 1a,b). The NBS1 c.511A>G, p.Ile171Val variant was predicted to be “disease-causing” and “probably damaging” by MutationTaster and Polyphen2, respectively. In addition, it was previously reported that a Japanese homozygote of this variant showed aplastic anemia rather than the typical features of Nijmegen breakage syndrome, such as severe microcephaly, immunodeficiency, and cancer predisposition 18. These findings suggested that the NBS1 c.511A>G, p.Ile171Val variant might be involved in individual differences in chromosomal radiosensitivity within human populations.

To demonstrate that the NBS1 c.511A>G, p.Ile171Val variant indeed underlies chromosomal radiosensitivity, we attempted to generate cultured human cells with knock-in of this variant along with a uniform genetic
| Gene | Variant ID | Nucleic acid change | Amino acid change | Allele frequency (gnomAD) | ClinVar | Mutation-taster | Polyphen-2 (score) | Ovarian cancer patients |
|------|------------|---------------------|------------------|---------------------------|--------|----------------|-------------------|------------------------|
| BRCA1 | rs1597830733 | c.4900A>G | p.Arg1634Gly | n.d. | Uncertain significance | Polymorphism | BENIGN (0.081) | 1, 2*, 3, 4*, 5, 6, 7, 8*, 9*, 11, 12*, 13, 15, 16*, 17, 18, 19*, 20, 25*, 27, 28 |
| ATM | rs16942 | c.3548A>G | p.Lys1183Arg | 0.3486 | Uncertain significance | Polymorphism | BENIGN (0) | 1, 2*, 3, 4*, 5, 6, 7, 8*, 9*, 11, 12*, 13, 15, 16*, 17, 18, 19*, 20, 22, 23*, 25*, 27, 28 |
| PALB2 | rs80357244 | c.811G>A | p.Val271Met | 0.00009232 | Conflicting interpretations of pathogenicity | Polymorphism | POSSIBLY DAMAGING (0.879) | 4 |
| ATM | rs551411717 | c.8288G>A | p.Arg2763Gln | 0.0001315 | Uncertain significance | Disease causing | PROBABLY DAMAGING (0.994) | 13 |
| PALB2 | rs587782298 | c.2771G>A | p.Arg924Gln | 0.00007556 | Uncertain significance | Disease causing | PROBABLEY DAMAGING (0.522) | 25 |
| TP53 | rs201382018 | c.31G>C | p.Glu11Gln | 0.0000709 | Conflicting interpretations of pathogenicity | Polymorphism | PROBABILITY DAMAGING (0.996) | 1, 26 |
| RAD51D | rs56026142 | c.176G>A | p.Gln559Arg | 0.1072 | Benign/Likely benign | Polymorphism | BENIGN (0) | 2, 3, 4, 7, 13, 14, 15, 17*, 18*, 19, 26, 29* |
| PALB2 | rs756778249 | c.1540G>A | p.Gly514Arg | 0.0001315 | Conflicting interpretations of pathogenicity | Polymorphism | BENIGN (0.145) | 10 |
| PALB2 | rs141749524 | c.2228A>G | p.Glu11Gln | 0.00007777 | Conflicting interpretations of pathogenicity | Polymorphism | BENIGN (0) | 26 |
| TP53 | rs192336678 | c.1809C>A | p.Tyr603Cys | 0.0001175 | Conflicting interpretations of pathogenicity | Polymorphism | BENIGN (0) | 7 |
| NBS1 | rs61754966 | c.511A>G | p.Ile171Val | 0.0015 | Conflicting interpretations of pathogenicity | Disease causing | PROBABILITY DAMAGING (1) | 18 |

Table 2. Missense variants in the HBOC core gene identified by whole-exome sequencing in 29 ovarian cancer patients. Asterisks indicate homozygosity of the variants. Variants with a “benign” status as evaluated in the ClinVar database are not included in this table.
Figure 1. Generation of NBS1 I171V knock-in HCT-116 cells. (a) Structure of human NBS1 protein. NBS1 contains FHA and BRCT1/2 domains at the N terminus and several DNA repair protein-binding regions at the C terminus. FHA and BRCT1/2 domains are involved in the IR-induced nuclear foci with phosphoproteins such as γ-H2AX. NBS1 I171V is located in the BRCT1 domain. (b) Sanger sequencing confirmed NBS1 I171V heterozygosity in ovarian cancer patient 18. (c) Targeting strategy for NBS1 I171V knock-in using the CRISPR/Cas9 system. Blue bases indicate silent mutations. (d–f) Sanger sequencing of NBS1+/+ parental HCT116 cell (d), NBS1+/+ edited HCT116 cell clone 1 (e), NBS1I171V/I171V-edited HCT116 cell clone 1 (f). A single base substitution of the NBS1 I171V variant and a silent Sca I site are indicated by yellow boxes.
Chromosomal radiosensitivity is more enhanced in NBS1<sup>I171V/I171V</sup>-edited HCT116 cells than in NBS1<sup>+/+</sup> HCT116 cells. To investigate whether the NBS1 c.511A>G, p.Ile171Val variant affects the cellular lethality after γ-ray irradiation, we performed the colony survival assay of the set of NBS1 genome-edited cell clones. Consistent with previous reports<sup>21,22</sup>, the NBS1<sup>+/−</sup> clone showed much higher lethal radiosensitivity than the others. The NBS1<sup>I171V/I171V</sup> cell clone did not show significant cellular lethality after less than 2 Gy of γ-ray irradiation in comparison with the NBS1<sup>+/+</sup> cell line (Fig. 2c, S3a–c, Table S1), while their lethal radiosensitivity was clearly segregated from those of the NBS1<sup>−/−</sup> cell line in the higher dose exposure. These results implied that the NBS1 c.511A>G, p.Ile171Val variant might contribute to cellular radiosensitivity.

Next, to quantify effect of the NBS1 c.511A>G, p.Ile171Val variant on radiosensitivity at the chromosomal level, radiation-induced micronuclei (MN) in the binucleated (BN) cells were measured (Fig. 2d–f). We evaluated the average ratio of MN to BN cells scored on more than 1000 BN images acquired from Metafer system as a semiautomatic approach<sup>4</sup>, in three independent conditions along with the standard error for each point and create representative dose–response calibration curves. The NBS1<sup>−/−</sup> cell clones showed extreme IR-induced micronucleus formation, while the NBS1<sup>I171V/I171V</sup> cell clones demonstrated a highly radiosensitive phenotype in comparison with the NBS1<sup>+/−</sup> parental HCT116 cell line and NBS1<sup>−/−</sup> cell clone with silent mutations (Fig. 2g, S3d–f, Table S2). To compare the chromosomal radiosensitivity among these cell clones in a more quantitative manner, we used a linear-quadratic model (MN frequency = c + βD + αD<sup>2</sup>, D: exposure dose) to analyze the dose–response curves of the ratio of MN/BN cells among the NBS1-edited HCT116 cell clones. α, β, and c coefficients were scored by the chromosomal aberration calculation (Cabas) software version 2.0 (http://www.pu.kielce.pl/ibiol/cabas)<sup>25</sup>. Since a linear-quadratic model is converted to (MN frequency - c)/D = αD + β, the sum of α and β coefficients at D = 1 Gy accurately represents the radiosensitivity of cells to γ-ray irradiation<sup>26</sup>. The mean data scores of the α and β coefficients from the NBS1-edited HCT116 cell clones were obtained (Table 3), indicating that biallelic NBS1 null mutations and the c.511A>G, p.Ile171Val variant contributed to approximately 2.0- and 1.3-fold increases of chromosomal radiosensitivity, respectively, in the HCT116 cell genetic background.

To confirm the radiosensitivity of the NBS1 c.511A>G, p.Ile171Val variant at the chromosomal level more directly, we stained the chromosomes with Giemsa dye in the γ-ray-irradiated NBS1 edited cell clones, and then measured the chromosomal abnormalities including chromatid and chromosome gaps and breaks<sup>23</sup>. Consistent with the results of the semiautomatic CBMN assay, the NBS1<sup>+/−</sup> cell clones showed the highest ratio of radiation-induced chromosomal abnormalities, while the NBS1<sup>I171V/I171V</sup> cell clones demonstrated an incidence of them intermediate between those of the NBS1<sup>−/−</sup> cells and the NBS1<sup>+/+</sup> cell clones (Fig. 2h–k, S3g–i, Table S3). These findings revealed that the NBS1 c.511A>G, p.Ile171Val variant has potency for enhancing chromosomal radiosensitivity.

Chromosomal radiosensitivity increases in a manner dependent on the copy number of Nbs1<sup>I171V</sup> variant. We attempted to generate heterozygous NBS1<sup>I171V/+</sup> HCT116 cell clones using the genome editing method mentioned above. However, the candidate cell clones obtained were all genetically mosaic consisting of NBS1<sup>+/+</sup> and NBS1<sup>I171V/I171V</sup> cells, and no heterozygous NBS1<sup>I171V/+</sup> single-cell clones were isolated.

Using genome editing technology in mouse fertilized embryos, it is possible to establish heterozygous cell clones for the ssODN knock-in allele. We thus attempted to establish Nbs1 I171V knock-in mice by co-electroporation of CRISPR/Cas9 RNP, which constitutes recombinant Cas9 protein, chemically synthesized crRNA and tracrRNA, and ssODN containing the variant, into mouse fertilized eggs (Fig S1a). Two mouse lines were generated from distinct electroporated embryos, in which the Nbs1<sup>+/+</sup>, Nbs1<sup>I171V/+</sup>, Nbs1<sup>I171V/I171V</sup> mice were introduced into the genome. Sanger sequencing confirmed correct knock-in of the variant in both lines. Since the two mouse lines did not exhibit different phenotypes, we mainly addressed data obtained from one line.

No obvious phenotypes were observed in heterozygous mutant mice up to 1 year of age. When they were inbred, wild-type (Nbs1<sup>+/+</sup>), heterozygous (Nbs1<sup>I171V/+</sup>), and homozygous (Nbs1<sup>I171V/I171V</sup>) mice were born at the expected Mendelian ratio. Nbs1<sup>I171V/I171V</sup> mice developed normal in the laboratory environment and showed normal reproductive ability, and did not show any hematological profile such as aplastic anemia, which was previously reported in a human homozygote of the NBS1 c.511A>G, p.Ile171Val variant<sup>11</sup> (Fig. S2).

To compare chromosomal radiosensitivity among the Nbs1<sup>+/+</sup>, Nbs1<sup>I171V/+</sup>, and Nbs1<sup>I171V/I171V</sup> mice, we intercrossed the Nbs1<sup>I171V/+</sup> mouse to generate MEFs with each Nbs1 genotype (Fig S1b–d). Western blotting analysis revealed that both Nbs1<sup>I171V/+</sup> MEFs and Nbs1<sup>I171V/I171V</sup> MEFs showed almost the same amount of Nbs1 protein as the Nbs1<sup>+/+</sup> MEFs (Fig. 3a). Next, to quantify the effect of the copy number of the Nbs1 I171V allele on chromosomal radiosensitivity, we measured the MN frequencies post-IR in these MEF lines using the semiautomatic CBMN assay (Fig. 3b–d). The Nbs1<sup>+/−</sup> MEFs showed the highest rate of IR-induced micronucleus formation, while the Nbs1<sup>−/−</sup> cell clones showed extreme IR-induced chromosomal aberrations, while the Nbs1<sup>I171V/I171V</sup> cell clones demonstrated an incidence of them intermediate between those of the Nbs1<sup>−/−</sup> cells and the Nbs1<sup>+/+</sup> cell clones (Fig. 2h–k, S3g–i, Table S3). These findings revealed that the NBS1 c.511A>G, p.Ile171Val variant has potency for enhancing chromosomal radiosensitivity.

Discussion

NBS1 encodes the NBS1 protein, which consists of 754 amino acids and plays a central role in DNA DSB repair as a member of the MRN protein complex, which recognizes DNA DSB sites and orchestrates the DNA damage response<sup>28</sup>. NBS1 protein interacts with multiple phosphorylated DNA repair proteins through the FHA/BRCT domain.
domains (a forkhead-associated domain and two BRCA1 C-terminal domains) and is recruited to DNA DSB sites. The NBS1 I171V variant, which was identified in this study, is located in the BRCT1 domain (Fig. 1a)\cite{26}. It was reported that the overexpression of the NBS1 I171V protein in HeLa cells interfered with phosphorylated MDC1 and inhibited IR-induced formation of foci of NBS1\cite{27}. Although overexpression analyses of the mutant protein are quick and informative, it is more precise to evaluate its effect on chromosomal radiosensitivity under the endogenous and physiological expression level. Consistent with previous overexpression studies, the endogenous promoter driven NBS1 I171V protein in HCT116 cells and MEFs also interfered chromosomal stability and cellular survival post IR irradiation. These results suggested that the NBS1 I171V variant might be involved in individual radiosensitivity within human populations. However, the epidemiological cancer risk of this variant remains controversial. Therefore, we attempted to evaluate the precise effect of this variant on chromosomal radiosensitivity in a uniform genetic background.

In the presence of conflicting forward genetics data, it is difficult to evaluate whether the variants of interest are indeed involved in chromosomal radiosensitivity. The number of similar situations is increasing with the recent clinical sequencing using deep-sequencers for genetic diagnosis\cite{16,19}. There are several criteria for evaluating the pathological significance of variants. We propose that a reverse genetics approach could be useful because this directly investigates the causality. In this study, we used the CRISPR/Cas9 system to introduce the NBS1 I171V variant into human cultured HCT116 cells and mouse fertilized eggs. The biallelic NBS1 I171V variant in human and mouse genomes significantly enhanced IR-induced micronucleus formation and chromosomal aberrations, clarifying that this variant underlies chromosomal instability after IR irradiation.

In general, simple knock-out using the CRISPR/Cas9 system can be applied in any proliferative human cultured cell lines. Previously, we generated ATM heterozygous knock-out hTERT-RPE1 cells derived from normal retinal tissues to demonstrate that the ATM heterozygous mutations affect cellular radiosensitivity\cite{25}. The activity of homologous directed recombination (HDR) repair in human cultured cells from normal tissues is generally insufficient for ssODN-mediated knock-in. Therefore, here we used HCT116 cells with sufficient HDR activity and a diploid karyotype from colon cancer tissue. As expected, we developed two independent HCT116 cell clones with the biallelic NBS1 I171V variant. However, NBS1\textsuperscript{I171V/+} cells were not obtained because the ssODN-non-targeted allele contained indel mutations in the CRISPR/Cas9 system-recognition site; however, mosaic colonies of NBS1\textsuperscript{I171V/+} cells and NBS1\textsuperscript{I171V/I171V} cells were formed. To control the copy number of the NBS1 I171V variant in the genome and to confirm that the biological effects of the variant on radiosensitivity are conserved among species, we generated mice heterozygous for the NBS1 I171V variant. Intercrossing of their heterozygous mice enabled MEFs with three copy numbers of the variant to be obtained. Notably, IR-induced chromosomal instabilities in the MEFs increased in a manner dependent on the copy number of the NBS1 I171V variant. Unlike a human NBS1 I171V homozygote, the homozygous mice did not show aplastic anemia. Although heterozygotes of NBS1 I171V are estimated to exist at a rate of approximately 0.15% in human populations (Table 2), a second homozygote has not been reported, implying that the existence of other gene mutations underlying aplastic anemia in the reported homozygote cannot be completely ruled out. Notably, NBS1 I171V did not increase the spontaneous frequencies of MN in HCT116 cells and MEFs in the spine of NBS1 I171V carrier mice. Further investigations are needed to confirm whether the heterozygous and homozygous NBS1 I171V exhibit a predisposition for cancer with or without IR irradiation, and to elucidate how NBS1 I171V protein interferes the DNA damage response after IR irradiations. Taking the above findings together, it is safe to conclude that the NBS1 I171V variant is a genetic factor underlying individual differences of radiation-induced chromosomal instability at the cellular level.

A series of radiation biological and epidemiological studies has suggested that many variants in the DNA repair genes might contribute to the existence of a portion of radiosensitive populations\cite{39}. Besides the NBS1 I171V variant, here we also extracted several suspicious variants in the ATM and TP53 genes from Japanese ovarian cancer patients. Whether they are merely polymorphisms or indeed determinants of IR-induced chromosomal instability and carcinogenesis is a key question for precision medicine in the fields of radiology and
convincing genetic markers for personal radiological protection and accurate genetic diagnosis. CO2. cultured in DMEM supplemented with 20% FBS and penicillin/streptomycin at 37 °C in humidified air with 5% leukemia38, the higher-throughput validation of single-nucleotide variants by reverse genetics might provide prime editing technologies are applied to the human haploid cell line HAP1 derived from chronic myeloid Whole-exome analysis. Materials and methods, BRCA1 oncology. Indeed, in breast cancer patients with variants such as in BRCA1, BRCA2, and ATM, it is recommended that the contralateral breast dose in mammography and radiotherapy be minimized in order to prevent secondary carcinogenesis33. Since chromothripsis, which is massive and clustered genomic rearrangements, occurs in micronuclei to drive cancer evolution44,45, the frequent radiation-induced micronucleus formation in the carriers with NBS1 I171V and other DNA repair gene variants might enhance the risk of secondary carcinogenesis. Thus, knowledge of the genetic basis underlying individual differences in chromosomal radiosensitivity might allow us to provide safer approaches in the context of CT imaging and radiotherapy.

Although the reverse genetics approach used in this study is useful for precision medicine, the throughput should be improved. Recent advances of CRISPR/Cas systems provide the base-editor technology based on several cytidine deaminases and their chemical evolved products fused to catalytically dead Cas9 (dCas9), thereby converting the base pair at the specific site directly36. Prime editing technology based on dCas9 fused with an engineered reverse transcriptase is another potential approach of base pair conversion37. If the base-editor and prime editing technologies are applied to the human haploid cell line HAP1 derived from chronic myeloid leukemia48, the higher-throughput validation of single-nucleotide variants by reverse genetics might provide convincing genetic markers for personal radiological protection and accurate genetic diagnosis.

Materials and methods

Whole-exome analysis. This study was reviewed and approved by the Ethics Committee of Hiroshima University Hospital, and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from the participants. Genomic DNA was extracted from the peripheral lymphocytes of ovarian cancer patients and exonic DNA was captured using Agilent SureSelect Human All Exon V5 kit. Sequencing was performed with 150-bp paired-end reads on a HiSeq2500 (Illumina). We used BWA (http://bio-bwa.sourceforge.net/) for alignment and mapping, Samtools (http://samtools.sourceforge.net/) and Picard (http://broadinstitute.github.io/picard/) for SAM/BAM handling, GATK (http://www.broadinstitute.org/gatk/) and Samtools for variant calls, and Annovar (http://annovar.openbioinformatics.org/) for annotation, as described previously. Functional predictions associated with amino acid changes were performed using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) and MutationTaster (http://www.mutationtaster.org/index.html). All reported genomic coordinates were in the Genome Reference Consortium Human build 37 (GRCh37/hg19) assembly. PCR amplification followed by Sanger sequencing with an Applied Biosystems 3130 sequencer (ThermoFisher) was used to validate mutations identified by whole-exome sequencing.

Cell cultures. HCT116 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and gentamycin at 37 °C in humidified air with 5% CO2. Primary MEFs were cultured in DMEM supplemented with 20% FBS and penicillin/streptomycin at 37 °C in humidified air with 5% CO2.

Animals and generation of MEFs. This study was reviewed and approved by the Ethics Committee for Experimental Animals of Hiroshima University, and all animals were treated in accordance with the guidelines of the Institutional Animal Care and Use Committee. The E13.5 embryos were thoroughly minced with razor or scalpel blades. The cells spun down at 500× g for 5 min were incubated in DMEM supplemented with 20% FBS and penicillin/streptomycin at 37 °C in humidified air with 5% CO2. After 24 h, a resuspension of the 2.5% trypsin/EDTA (Gibco)-treated cells was filtered through a 40 µm cell strainer and subsequently centrifuged at 500× g for 5 min. The cellular pellet was resuspended in DMEM supplemented with 20% FBS and penicillin/streptomycin and cultured in 60-mm culture dishes.

Generation of NBS1 I171V HCT116 knock-in genome-edited cells. A total of 200 pmol 100-mer ssODNs carrying the NBS1 c.511 A>G, p.I171V 100-mer variant and a silent Scal site (5′-CCTTTCAATTGTGAGGCTGCTTCTCCAGAGGAAATAATTATTAATCCAGCAATTTATATATATATATATATATATGAG-3′) or the silent mutations (5′-CCTTTCAATTGTGAGGCTGCTTCTCCAGAGGAAATAATTATTAATCCAGCAATTTATATATATATATATATATATATGAG-3′).
Figure 3. Radiation-induced chromosomal instability of Nbs1 I171V edited MEF clones. (a) Western blotting analysis data showing the expression levels of Nbs1 protein in Nbs1 I171V edited MEF clones. Full-length gel is presented in Supplementary Fig. 5. The GAPDH antibody was used as a loading control. The intensity of Nbs1 bands was normalized to that of GAPDH and shown as a percentage, regarding the score of Nbs1+/+ clone 1 as 100%. (b–d) Metafer MN Search images showing the cytokinesis-blocked Nbs1 I171V edited MEFs stained with DAPI. Arrowheads indicate MN. Nbs1+/+BN cell without MN (b), Nbs1I171V+/+BN cell with one MN (c), Nbs1I171V/I171V-BN cell with three MN (d). (e) Percentage of IR-induced MN formation in Nbs1 I171V edited MEF clones (mean ± SEM; t-test; n = 3; > 1000 BN cells). (f, g) Representative metaphase of Nbs1 I171V-edited MEFs after 4 Gy irradiation. Remarkable aberrations are enlarged. Arrows indicate chromosomal breakages. (i) Frequency of IR-induced chromosomal aberrations in Nbs1 I171V edited MEF clones (mean ± SEM; t-test; n = 3; > 50 cells).
AAACATGTTGAAATATATATCTACATGCTAGCA-3') and 1 μg of the pX459 plasmid vector [pSpCas9(BB)-2A-Puro; Addgene plasmid #62988] for editing of exon 5 of the NBS1 gene were cotransfected into 1 × 10^6 HCT116 cells with Kit V (Lonza) and program D-032 (HCT116) (Nucleaseor^2 2b device; Lonza), in accordance with the manufacturer’s protocol. Twenty-four hours after transfection, cells were treated with 1 μg/ml puromycin (Nacalai Tesque) for 48 h. After 2 weeks, the drug-resistant colonies were isolated and divided into two aliquots: one was transferred into a well of a 96-well plate for clonal expansion, while the other was lysed and used for PCR with a pair of primers for the amplification of NBS1 exon 5 (forward primer 5′-ATG CTT GAA TAA GCT GTT CTA CAG AT-3′, Fasmac), and ssODN carrying I171V and ScaI and then run on 2.0% agarose gel. The ScaI-sensitive products were analyzed by direct-sequence genotyping (ABI 3130 sequencer).

### Western blotting analysis.

Cells were resuspended in lysis buffer (20 mM Tris–HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% NP-40, 0.1% sodium deoxycholate, protease inhibitors, and 10 mM NaF) and incubated on ice for 20 min. Samples were centrifuged at 8000×g for 20 min and the supernatants were collected. Protein concentration in the supernatant was determined by Bradford protein assay. Cell lysates were then boiled with sample buffer (60 mM Tris–HCl pH 6.8, 1% SDS, 5% β-mercaptoethanol) for 5 min. Proteins were separated via SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% skim milk in TBS-T (0.05% Tween-20) for 30 min. Primary antibodies were diluted in blocking buffer and the membrane was incubated with diluted antibodies overnight at 4°C. HPR-conjugated anti-rabbit and anti-mouse IgG (#NA934V and #NA931V, respectively; GE Healthcare) were used as secondary antibodies. Images were acquired using ImageQuant LAS4000mini system (GE Healthcare). Quantitation of the levels of NBS1, MRE11 and GAPDH mouse monoclonal antibody (sc-23223, Santa Cruz Biotechnology).

### Generation of Nbs1 I171V knock-in genome-edited mice.

C57BL6 mouse fertilized eggs were electroporated with recombinant Cas9 protein (GeneArt Platinum Cas9 Nuclease, ThermoFisher), chemically synthesized NBS1-cRNA (5′-cauuauguccuuaagugaGAA TAC TTT TCT GAA TTT CT-3′, Fasmac), chemically synthesized tracrRNA (5′-UGG CAC CGA GUC GGU GCU-3′), and ssODN carrying Nbs1 I171V and EcorI enzyme digestion sequence (silent mutations) using NEPA21 (NEPAGENE). These eggs were transferred into pseudo-pregnant C57BL6 mouse fertilized eggs electroporated with recombinant Cas9 protein (GeneArt Platinum Cas9 Nuclease, ThermoFisher), chemically synthesized NBS1-cRNA (5′-cauuauguccuuaagugaGAA TAC TTT TCT GAA TTT CT-3′, Fasmac), chemically synthesized tracrRNA (5′-UGG CAC CGA GUC GGU GCU-3′), and ssODN carrying Nbs1 I171V and EcorI enzyme digestion sequence (silent mutations) using NEPA21 (NEPAGENE). These eggs were transferred into pseudo-pregnant female mice. Genomic DNA from the tail of F0 generation mice was analyzed by PCR with a pair of forward/reverse primers for the amplification of Nbs1 exon 5 (forward primer 5′-TGGACTAGGGTCTGTGCAGTTGAAATAGCTGTTTCTACAGAT-3′, and reverse primer 5′-CGCTCTTACGCTCACTGTTATGC-3′). PCR products were checked by EcoRI enzyme digestion (New England Biolabs) and DNA direct sequencing (ABI 3130 sequencer). The ssODN knock-in male mice were mated with C57BL6 females and the offspring were genotyped to confirm the germline transmission of the ssODN knock-in allele. Heterozygous knock-in mice were then intercrossed to produce homozygous mice. All animal methods were performed in accordance with the ARRIVE guidelines.

### Table 4. Radiosensitivity coefficients (α, β, c, and α + β) in Nbs1 I171V variant knock-in MEFs. α, β, and c coefficients were extracted from dose–response calibration curves in CBMN assay using Cabas software.

| Cell line ID/genotype | β ± SE × 10^–3 (Gy^2) | α ± SE × 10^–3 (Gy) | c ± SE × 10^–3 (Gy) | Radiosensitivity score [α + β] |
|-----------------------|------------------------|---------------------|---------------------|-----------------------------|
| Nbs1/I171V+/- clone 1 | 0.997 ± 0.77           | 14.48 ± 1.92        | 22.56 ± 2.61        | 15.48 ± 1.17                |
| Nbs1/I171V+/- clone 2 | 2.576 ± 0.70           | 8.033 ± 2.86        | 24.83 ± 0.94        | 10.60 ± 2.25                |
| Nbs1/I171V+/+ clone 1 | 3.960 ± 0.58           | 10.46 ± 1.70        | 27.34 ± 1.72        | 14.42 ± 1.30                |
| Nbs1/I171V+/+ clone 2 | 4.261 ± 0.47           | 9.662 ± 2.44        | 26.94 ± 2.46        | 13.92 ± 1.97                |
| Nbs1/I171V/I171V clone 1 | 5.840 ± 1.18          | 15.71 ± 4.20        | 31.75 ± 1.70        | 21.55 ± 3.09                |
| Nbs1/I171V/I171V clone 2 | 5.148 ± 1.63          | 17.97 ± 6.28        | 33.09 ± 1.00        | 23.12 ± 4.65                |

RT-PCR. RT-PCR analysis was carried out as described previously. Briefly, HCT116 genome editing cells in 96-well plates were cultured in DMEM supplemented with 10% FBS for 24 h. The cells were washed with ice-cold PBS, lysed, and then, cDNAs were synthesized with reverse transcriptase from their extracted total RNA using Cells to CT Kit (ThermoFisher), in accordance with the manufacturer’s protocol. Expression was measured by TaqMan PCR analysis using MRE11 and GAPDH TaqMan probes (ThermoFisher) on a CFX Connect™ Real-Time PCR Detection System (BioRad); the levels of MRE11 expression were normalized to those of GAPDH. The primers and TaqMan probes were MRE11 (Hs00967437_m1) and GAPDH (Hs99999905_g1).
**Gamma irradiation.** Cells were irradiated from 1 to 4 Gy ($^{137}$Cs gamma ray source, 148 TBq, Gammacell 40 Exactor; Best Theratronics). The dose rate used was around 1 Gy/min.

**Semiautomatic cytochalasin-block micronucleus (CBMN) assay.** Cells were seeded in six-well plates, incubated for 4 h, and then irradiated. After 44 h of incubation, 3 μg/ml cytochalasin B (WAKO) was added to block cytokinesis for 24 h. Cells were harvested and centrifuged. The pellet was treated with hypotonic solution (75 mM KCl) for 15 min at 37 °C and subsequently rinsed in Carnoy solution (methanol: acetic acid, 3:1) three times for fixation. The slides were prepared using HANABI Metaphase Spreader (ADSTEC, Japan) and stained with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI). These cells were scanned at 10× magnification with a Carl Zeiss Axioplan Imager Z1 connected to Metafer 4 software version 3.11.4 (MetaSystems GmbH, Alltussheim Germany). Captured images were analyzed with the Metafer 4 MN program (MetaSystems). Criteria for selecting BN cells and MN were as previously described. All scanned images of BN cells and MN were re-evaluated visually to exclude false-positive and -negative images of BN cells and for MN to be scored in at least 1000 BN cells from each condition.

**Survival assay.** An appropriate number of cells were irradiated and then seeded in three 100-mm dishes per condition. After 11 days of incubation, cell colonies were fixed with 100% methanol at room temperature for 2 min and stained with 10% Giemsa (Wako) for 2 min. Survival curves were obtained by counting the number of cell colonies.

**Chromosomal aberration analysis.** Cells were seeded in six-well plates, incubated for 4 h (HCT116 cells) or 6 h (MEFs), and then irradiated. After 24 h of incubation, colcemid (0.1 mg/ml, Gibco) was added to induce arrest in metaphase for 1 h (HCT116 cells) or 6 h (MEFs). The cells were then treated with a hypotonic solution (0.075 M KCl) for 15 min at 37 °C and subsequently fixed with Carnoy solution. Slides were prepared using HANABI Metaphase Spreader (ADSTEC, Japan) and then stained with 10% Giemsa solution (Wako) for 5 min. Chromosome images were scanned at 10× and 63× magnifications with Metafer 4 software (MetaSystems, Germany) connected to a motorized ZEISS AxioImager M1 (Zeiss, Germany). Chromosome gaps and breaks were scored visually by fewer than 25 metaphases per slide of well-spread metaphase chromosomes. Fifty metaphase chromosomes from each condition were examined for spontaneous chromosomal aberrations.

**Statistical analysis.** The experiments were performed independently three times, and the data are shown as mean ± s.e. Differences between groups were evaluated for statistical significance using Student’s t-test. Values of $p < 0.05$ were considered to be statistically significant.

Received: 2 April 2021; Accepted: 13 September 2021
Published online: 04 October 2021

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Acknowledgements
We thank Drs. Nori Nakamura, Junya Kobayashi, and Nobuyuki Hamada for helpful discussions. We also thank Dr. Kosuke Hosoba, Ms. Yukiji Tonouchi, Tomoko Watanabe, and Junko Murakami for technical assistance. This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to T.M.: 20K21845, 18H04979, and 18H02624; to S.M.: 19H04270); Grant-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare (to S.M.); the Center of World Intelligence Projects for Nuclear S&T and Human Resource Development from the Japan Science and Technology Agency (to S.M.); Grant-in-Aid for Network Joint/Usage Research Center for Radiation Disaster Medical Science (to S.M. and T.M.); AMED-PRIDE from the Japan Agency for Medical Research and Development, AMED (to T.M.: JP18gm5910011h0004); research grants from the Naito Foundation (to S.M. and T.M.); Ono Medical Research Foundation (to T.M.); Daiichi Sankyo Foundation of Life Science (to T.M.); and Takeda Science Foundation (to T.M.).

Author contributions
K.T., T.M., H.Y., S.A., K.F., and E.R. performed the experiments. I.K., E.H., and Y.K. collected the clinical samples. S.M. and T.M. conceived this study. S.M., T.Y., H.T., Y.K., M.K., S.O., and T.M. supervised this study. K.T., T.M., and S.M. wrote the paper. All authors interpreted the data.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-98673-7.

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