A rare polymorphic variant of NBS1 reduces DNA repair activity and elevates chromosomal instability

Yuki Yamamoto¹², Mamiko Miyamoto¹, Daisuke Tatsuda¹³, Michiaki Kubo⁴, Hitoshi Nakagama¹, Yusuke Nakamura⁵, Hitoshi Satoh², Koichi Matsuda⁵, Toshiki Watanabe² and Tsutomu Ohta¹,*

¹Division of Integrative Omics and Bioinformatics, National Cancer Center Research Institute, 5-1-1 Tsukiji Chuo-ku, Tokyo 104-0045, Japan.
²Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Japan.
³Institute of Microbial Chemistry (BIKAKEN), Japan.
⁴Center for Genomic Medicine, The Institute of Physical and Chemical Research (RIKEN), Japan.
⁵Human Genome Center Institute of Medical Science, The University of Tokyo, Japan.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Running title: DNA repair activity of a rare polymorphic variant of NBS1
Keywords: breast cancer / cancer risk / double-strand breaks (DSB) repair / NBS1 /
polymorphic variant

*Correspondence to: Tsutomu Ohta.
Division of Integrative Omics and Bioinformatics, National Cancer Center Research
Institute, 5-1-1 Tsukiji Chuo-ku, Tokyo 104-0045, Japan.
Phone: +81-3-3542-2511; Fax: +81-3-3248-1631; E-mail: cota@ncc.go.jp

The word count (excluding references): 4358 words
The total number of figures: 6 figures
The total number of table: 1 table

The word count of supplementary figure legends: 1039 words
The total number of supplementary figures: 8 figures
The total number of supplementary table: 1 table
Abstract

Failure to expeditiously repair DNA at sites of double-strand breaks (DSB) ultimately is an important etiologic factor in cancer development. NBS1 plays an important role in the cellular response to DSB damage. A rare polymorphic variant of NBS1 that resulted in an isoleucine to valine substitution at amino acid position 171 (I171V) was first identified in childhood acute lymphoblastic leukemia. This polymorphic variant is located in the N-terminal region that interacts with other DNA repair factors. In earlier work, we had identified a remarkable number structural chromosomal aberrations in a pediatric aplastic anemia (AA) patient with a homozygous polymorphic variant of NBS1-I171V, however, it was unclear whether this variant affected DSB repair activity or chromosomal instability. In this report, we demonstrate that NBS1-I171V reduces DSB repair activity through a loss of association with the DNA repair factor MDC1. Further, we found that heterozygosity in this polymorphic variant was associated with breast cancer risk. Lastly, we showed that this variant exerted a dominant-negative effect on wild-type NBS1, attenuating DSB repair efficiency and elevating chromosomal instability. Our findings offer evidence that the failure of DNA repair leading to chromosomal instability has a causal impact on the risk of breast cancer development.
Introduction

Nijmegen breakage syndrome, an autosomal recessive human disease, is due to a mutation in the NBS1 gene (1). The clinical features of this syndrome include growth retardation, immunodeficiency, and increased susceptibility to malignancies (1). A gene product of the nibrin gene [NBN, also known as the Nijmegen breakage syndrome 1 gene (NBS1)] is a member of the MRE11/RAD50/NBS1 (MRN) protein complex, which is involved in the repair of DSB in DNA (2). NBS1 consists of two functional regions (Fig. 1A). Its C-terminal region contains binding motifs of MRE11 meiotic recombination 11 homolog A (S. cerevisiae) (MRE11A, also known as MRE11) and ataxia telangiectasia-mutated (ATM) kinase (3-5), whereas its N-terminal region contains forkhead-associated (FHA) and breast cancer C-terminal (BRCT) domain that mediates phospho-dependent protein-protein interactions (6-8). A rare polymorphic variant of NBS1 that resulted in an isoleucine to valine substitution at amino acid position 171 (I171V) was first identified in childhood acute lymphoblastic leukemia (ALL) (9). The NBS1-I171V polymorphic variant is located in the BRCT domain, which is highly conserved in human, mouse, rat, chicken and African clawed frog (Fig. 1B). We have previously described an aplastic anemia (AA) patient in a Japanese child with a homozygous polymorphic variant of NBS1-I171V (10). We also determined that the chromosomes of lymphoblastic cell lines derived from this patient contained a remarkable number of structural chromosomal aberrations (10). However, it is unclear whether the NBS1-I171V polymorphic variant affects DSB repair activity and genomic instability.
In this study, we showed that NBS1-I171V decreased the localization of the MRN complex to sites of plural DSBs through its loss of association with MDC1. This aberrant localization resulted in decreased production of repairable single-stranded DNA and reduced DSB repair activity. We also showed that the heterozygous NBS1-I171V variant increased the risk of breast cancer in Japanese women. The NBS1-I171V reduced the DSB repair activity of endogenous NBS1 in a dominant-negative manner, and increased chromosomal instability.
Materials and Methods

Cell lines and antibodies

HeLa, HCC1937, GM07166VA7, GM07166VA7 transfected with DR-GFP reporter and HS-SY-II cells were obtained from the American Type Culture Collection (ATCC), Dr. K. Komatsu (Kyoto University, Japan) and Dr. S. Sonobe (Kouchi Medical School, Japan) respectively. The following commercially available antibodies were used: mouse anti-FLAG M2 monoclonal antibody (Sigma-Aldrich), rabbit anti-MDC1 antibody, mouse anti-MDC1 antibody, rabbit anti-p95 NBS1 antibody, rabbit anti-Mre11 antibody (Abcam), rabbit anti-phospho RPA32 (S4/S8) antibody (Bethyl Laboratories, Inc., NC), mouse anti-phospho histone H2AX (Ser139) (Upstate), rabbit anti-RAD50 antibody, rabbit anti-RAD51 antibody (Santa Cruz Biotechnology, Inc.) and rabbit anti-BRCA1 antibody (Merck Millipore).

Plasmids and stable cell lines

The plasmids of pDRGFP and pCBASceI were obtained from addgene. The full-length human NBS1 cDNA was a gift from Dr. Komatsu. The cDNA of NBS1-I171V or NBS1-WT with a 3xFlag-His6-HA-tag at C-terminus was generated by using PCR and then ligated into the pEB-Multi-Neo mammalian expression vector (Wako). These vectors were transfected into GM07166VA7 cells, GM07166VA7 cells containing DR-GFP reporter and HeLa cells. The cells were cultured in the presence of 800 or 600 μg/ml G418 (Calbiochem) for 2 weeks, after which clones were isolated and selected on the basis of their NBS1 expression, with the selected clones expressing equivalent levels of protein.
Immunoprecipitation and immunofluorescence analyses

For immunoprecipitation analysis, the cells were washed with phosphate-buffered saline (PBS) and sonicated in lysis buffer (150mmol/L NaCl, 20mmol/L Tris-HCl [pH 7.9], 20% glycerol, and 1mmol/L Pefabloc [a protease inhibitor; Roche]). The lysate (1 mg) was mixed with anti-Flag M2 Affinity Gel (Sigma-Aldrich) and incubated for 4 hours at 4°C. The gel was washed 3 times with lysis buffer. The immunoprecipitated proteins were separated by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then incubated with the indicated antibodies. For immunofluorescence analysis, the cells were cultured on glass coverslips, exposed to 10 Gy of ionizing radiation (IR). After 4 hours, the cells were fixed in ice-cold 4% paraformaldehyde for 1 hour, permeabilized with 0.1% TritonX-100 at room temperature, immersed in blocking reagent (2% Normal Swine Serum [Funakoshi Inc.], 0.05% TritonX-100) for 30 minutes at room temperature, and then incubated with the indicated primary antibodies overnight at 4°C. The cells were incubated with secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen) for 1 hour at room temperature and stained with 2 μg/ml of 4',6- diamidino-2-phenylindole dihydrochloride (DAPI) for 10 minutes at room temperature. Images were captured with a confocal laser microscope (Carl Zeiss) with a 40x water immersion objective.

DR-GFP assay
Homologous recombination (HR) repair frequency in the cell lines using DF-GFP system was performed as described previously (11, 12).

**Cell cycle assay**

An appropriate number of cells was plated and then exposed to 10 Gy of IR. After 6 hours, the cell cycle phase in the cells was analyzed by the Cell-Clock Mammalian Cell Cycle Assay kit (biocolor life science assays).

**Cell survival assay**

An appropriate number of cells was plated and then exposed to IR, mitomycin C (MMC) or a poly-(ADP-ribose) polymerase 1 (PARP1) inhibitor (AZD2281). After 10 days of incubation, the surviving fractions were calculated by counting the number of colonies.

**Small interfering RNA analysis**

For the small interfering RNA (siRNA) experiments, the siRNA for control (4390844; life technologies), MDC1 (s18578; life technologies) and NBS1 (s9291 and s9292; life technologies) were used. Transfection was performed as described previously (13).

**Cytogenetic analysis**

After exposure to IR, the cells were cultured for 3 days and then treated with colcemid (0.02 μg/ml) for 2 hours before being harvested. Chromosome slides were prepared by using standard protocols and then stained with a 5% Giemsa solution (Wako) for 30
minutes. For each cell line, about 1600 well-spread metaphase chromosomes were screened for structural chromosomal aberrations.

**Sample collection, genotyping, and statistical analysis**

We obtained DNA samples of 1524 breast cancer cases and 1462 controls from the Biobank Japan Project as described previously (PMID: 22951594). As part of this project, patients’ DNA samples were collected through a collaborative network of 66 hospitals throughout Japan. A list of participating hospitals can be found at the following website: [http://biobankjp.org/plan/member_hospital.html](http://biobankjp.org/plan/member_hospital.html). Genotyping of the *NBS1* variations at amino acid position 171 was performed by direct sequencing. The primers used for amplification were as follows: forward, 5’-TGGATGTAAACAGCCTCTTTGT-3’; reverse, 5’-TGAAACAAGCATTAAAGAGGAA-3’. The odds ratios (OR) were calculated in a dominant mode. *P* values were calculated by using the chi-square test.
Results and Discussions

NBS1-I171V polymorphic variant reduced DSB repair activity

To explore the biological consequences of the NBS1-I171V substitution, we generate cell lines that express the polymorphic variants of the NBS1 gene. First, we constructed the NBS1 cDNA to construct expression vectors that encoded a protein with either isoleucine (NBS1-WT) or valine (NBS1-I171V) at amino acid position 171. The vectors were stably transfected into human GM07166VA7 cells, which contain homozygous for the 657del5 mutation in exon 6. The mutation of NBS1 determines the synthesis of two truncated proteins of 26 kDa (p26) and 70 kDa (p70) (14). We isolated and selected clones with equivalent levels of NBS1 expression for further analysis (Fig. 1C lanes 1 and 2, and Supplementary Fig. S1A). Immunoprecipitation analysis in these cloned cell lines indicated that both NBS1-I171V and NBS1-WT bound to MRE11 and RAD50 homolog (S. cerevisiae) (RAD50) (Fig. 1C, lanes 4 and 5), suggesting that NBS1-I171V can incorporate into the MRN protein complex. We also used these NBS1-expressing cell lines to assess cell survival in response to DNA damage caused by ionizing radiation (IR) or the cross-linking agent mitomycin C (MMC). An analysis of cell survival revealed that the cell line expressing NBS1-I171V was more sensitive to IR and MMC than was the cell line expressing NBS1-WT (Fig. 2A). We also found that other cell line expressing NBS1-I171V was more sensitive to IR and MMC than was other cell line expressing NBS1-WT (Supplementary Fig. S1B). Next, we analyzed homologous recombination (HR) repair frequency in the NBS1-expressing cell lines with DR-GFP system (11, 12). When I-SceI expression was induced, the cell line expressing NBS1-I171V showed a three-fold lower
frequency in HR repair compared with the cell line expressing NBS1-WT (Fig. 2B). We also found that other cell line expressing NBS1-I171V showed lower frequency in HR repair compared with other cell line expressing NBS1-WT (Supplementary Fig. S1C). It was reported that a poly-(ADP-ribose) polymerase 1 (PARP1) inhibitor (AZD2281) reduced growth of HR repair-deficient cells such as \textit{BRCA1} and \textit{BRCA2} mutated cells (15, 16). Therefore, we examined cell survival in response to AZD2281. We found that the cell line expressing NBS1-I171V was more sensitive to AZD2281 than was the cell line expressing NBS1-WT, but was more resistant to AZD2281 than was the cell line expressing a vector (Fig. 2C and Supplementary Fig. S2A). This result indicated that damages by the PARP inhibitor were different from those by IR or MMC in Figure 2A. We showed that HR repair activity of GM07166VA7 cells expressing NBS1-I171V was almost similar to that of GM07166VA7 cells expressing a vector in Figure 2B. These results suggested that damages by the PARP inhibitor were repaired by HR repair-dependent and -independent manners of NBS1. We speculate that NBS1-I171V has the HR repair-independent activity to repair damages by the PARP inhibitor. However, the activity of NBS1-I171V is still unclear. Thus, further researches of NBS1-I171V function could be necessary.

It was reported that NBS cells exposed to IR showed an abnormal cell cycle (17). Therefore, we examined the cell-cycle status of the cells that had been exposed to IR. We found that the ratio of S phase in the cell expressing the NBS1-WT was reduced by IR treatment, but not in the cell expressing the NBS1-I171V or the vector (Supplementary Fig. S2B). This result suggests that G1/S phase arrest is failed in cells expressing NBS1-I171V.
These results suggest that the DSB repair activity of cells expressing NBS1-I171V is reduced compared to that of cells expressing NBS1-WT and prompted a more in-depth analysis of these cell lines.

**NBS1-I171V polymorphic variant decreased the localization of the MRN complex to sites of DSB**

NBS1 is known to be involved in an early step of DSB repair (2). Thus, we used immunofluorescence to examine whether NBS1-I171V localizes to sites of DSB in cells exposed to IR. We observed NBS1 foci in cells expressing NBS1-WT, but not in those expressing NBS1-I171V (Fig. 3A and 3B). MRE11 foci were also identified in cells expressing NBS1-WT, but not in those expressing NBS1-I171V (Fig. 3C and 3D). Phosphorylated H2A histone family member X (γH2AX) foci were identified in both the cells expressing NBS1-I171V and those expressing NBS1-WT, where they colocalized with the NBS1-WT foci or MRE11 foci (Fig. 3). These results suggest that after exposure to IR, the localization of NBS1-I171V to sites of DSB is less prominent than that of NBS1-WT.

**NBS1-I171V polymorphic variant reduced DSB repair activity through loss of association with MDC1**

Because mediator of DNA-damage checkpoint 1 (MDC1) recruits NBS1 to sites of DSB after IR treatment (18,19), we examined MDC1 localization in cells that had been exposed to IR. MDC1 foci were identified at sites of DSB in cells expressing either NBS1-I171V or
NBS1-WT (Fig. 4A and 4B). These results suggest that MDC1’s ability to localize to sites of DSB after IR treatment is unaffected by the NBS1-I171V substitution. We also determined that MDC1 foci localized at sites of DSB with NBS1-WT foci but not with NBS1-I171V foci after IR treatment (Fig. 4C and 4D). Next, we characterized the interaction between NBS1-I171V and MDC1 by conducting an immunoprecipitation analysis. After exposure to IR, a large amount of MDC1 coprecipitated with NBS1-WT, but not with NBS1-I171V (Fig. 4E, lanes 4 and 5). This result is consistent with the reports that both of FHA and BRCT domains in NBS1 are important for its association with MDC1 (8, 20), and NBS1-K160M mutant which resulted in an lysine to methionine substitution at amino acid position 160 within the BRCT domain reduces its binding activity to MDC1 (8). Therefore, we speculate that NBS1-I171V mutant within the BRCT domain affects a structure of the BRCT domain and abolishes the interaction between NBS1 and MDC1. The results in Figure 4 suggest that the decreased localization of NBS1-I171V to sites of IR-induced DSB results from its inability to bind to MDC1. We also found that the knockdown of MDC1 expression using MDC1-specific siRNA did not affect the sensitivity to IR in cell line expressing NBS1-I171V or the vector (Supplementary Fig. S3A). However, the knockdown of MDC1 expression increased the sensitivity to IR in cell line expressing NBS1-WT (Supplementary Fig. S3A). This result suggests that NBS1-I171V impairs the MDC1-NBS1-pathway.

The MRN complex initiates resection of DSB ends to create single-stranded 3’-overhangs that can be repaired by HR system (21). In addition, the complex recruits replication protein A (RPA), containing phosphorylated 32kDa replication protein A2
subunit (RPA2, also known as RPA32), to single-stranded DNA at sites of DSB (22). We examined the production of single-stranded DNA at the ends of DSB by using immunofluorescence to analyze the localization of phosphorylated RPA32 in cells exposed to IR. Phosphorylated RPA32 foci were identified at sites of DSB in cells expressing NBS1-WT, but not in those expressing NBS1-I171V or the vector control (Fig. 5A and 5B). Because RAD51 homolog (*S. cerevisiae*) (RAD51) also was recruited to the single-stranded 3’-overhangs after IR treatment (23), we examined RAD51 localization in cells that had been exposed to IR. RAD51 foci were identified at sites of DSB in cells expressing NBS1-WT, but not in those expressing NBS1-I171V or the vector (Fig. 5C and 5D). It was reported that BRCA1 (familial breast cancer susceptibility protein) also promoted induction of the single-stranded DNAs at sites of DSB (24). Therefore, we examined BRCA1 localization in cells that had been exposed to IR. BRCA1 foci were identified at sites of DSB in cells expressing NBS1-WT, but not in cells expressing NBS1-I171V or the vector (Supplementary Fig. S3C and S3D). These results are consistent with our finding that the localization of NBS1-I171V to sites of IR-induced DSB was less pronounced than that of NBS1-WT (Fig. 3A and 3B), and suggest that NBS1-I171V decreases the localizations of the MRN complex and BRCA1 to sites of DSB. These aberrant localizations result in decreased production of repairable single-stranded DNA and reduced DSB repair activity.

NBS1-I171V polymorphic variant increases the risk of breast cancer

To date, the *NBS1*-I171V polymorphic variant was detected frequently only in Polish patients with breast cancer, head and neck cancer, and colorectal cancer (25-28). However,
other groups did not find a similar association in European patients with breast cancer, leukemia, or lymphoma (29-31). It remains unclear whether this particular polymorphic variant of the \textit{NBS1} gene is associated with cancer. It was reported that while null mutations in the mouse \textit{NBS1} gene resulted in embryonic lethality at the blastocyst stage, heterozygous knockout (\textit{NBS1}\textsuperscript{+/-}) mice developed a wide array of tumors (32). Moreover, cell cycle-dependent association of BRCA1 with the MRN protein complex contributes to the activation of HR-mediated DSB repair in S and G\textsubscript{2} phases of the cell cycle (33). These reports strongly suggest that the \textit{NBS1}-I171V polymorphic variant may increase breast cancer risk. Therefore, we analyzed the association of the \textit{NBS1}-I171V variant with breast cancer in a Japanese population; patient characteristics are presented in Supplementary Table S1. Of the 1524 Japanese women with cancer, 23 (1.6\%) carried the heterozygous polymorphic variant. Only 7 women (0.48\%) with the heterozygous polymorphic variant were found in the control group (\textit{n} = 1462). None of the patients in the breast cancer group or in the control group carried the homozygous polymorphic variant of the \textit{NBS1}-I171V substitution. The frequency of patients with the heterozygous \textit{NBS1} polymorphic variant in the Japanese breast cancer group [odds ratio (OR) = 3.19, 95\% confidence interval (CI) = 1.36–7.44, \textit{P} = 0.0048] was significantly higher than that in the control group (Table 1). This result suggests that the \textit{NBS1}-I171V variant increases the risk of breast cancer in Japanese women.

\textit{NBS1}-I171V polymorphic variant elevated chromosomal instability
The above finding may suggest that NBS1-I171V exerts a dominant-negative effect on the function of NBS1-WT. To test this hypothesis, we expressed the codon 171 polymorphic variants of NBS1 in HeLa cells, which also express endogenous NBS1. Clones were isolated and selected on the basis of their exogenous and endogenous NBS1 expression, with the selected clones expressing equivalent levels of protein (Fig. 6A, lanes 1 and 2). Using these cell lines, we assessed cell survival in response to DNA damage caused by exposure to IR or MMC. The cell line expressing NBS1-I171V was more sensitive to IR and MMC than was the line expressing NBS1-WT or the vector (Fig. 6B). We also found that other cell line expressing NBS1-I171V was more sensitive to IR and MMC than was other cell line expressing NBS1-WT (Supplementary Fig. S4B). Next, we analyzed the localization of NBS1, MRE11, phosphorylated RPA32, RAD51 or BRCA1 in cells exposed to IR. The cell line expressing NBS1-I171V showed a two- to threefold lower focus counts of NBS1, MRE11, phosphorylated RPA32, RAD51 or BRCA1 compared with the cell line expressing NBS1-WT or the vector (Supplementary Fig. S4D, S4E, S5, S6 and S7). These results suggest that the DSB repair activity of endogenous NBS1 was reduced by NBS1-I171V in a dominant-negative manner. Figure 6A showed that total amount of NBS1 protein in HeLa cell line expressing NBS1-I171V or NBS1-WT was almost same as that in HeLa cell line expressing a vector. Because NBS1-I171V incorporates into the MRN protein complex (Fig. 1C), the half of the MRN protein complex contains NBS1-I171V in HeLa cell line expressing NBS1-I171V. Therefore, we think that decreased the amount of MRN protein complex containing wild-type NBS1 in HeLa cell line expressing
NBS1-I171V shows the dominant negative effect in response to DNA damage caused by exposure to IR or MMC.

We also assessed the structural aberrations of the chromosomes in each cell line after exposure to IR and discovered that the number of aberrations in the cell line expressing NBS1-I171V [28 double minutes (DMs), 7 chromatid gaps (CTGs), and 8 chromatid breaks (CTBs) / ~1600 chromosomes; a representative metaphase spread is shown in Fig. 6C] was significantly higher than that of cell line expressing either NBS1-WT or the vector control (NBS1-WT: 4 DMs and 4 CTGs, vector control: 4 DMs, 1 CTG, and 1 CTB; both / ~1600 chromosomes; Fig. 6D). We also found that the number of aberrations in other cell line expressing NBS1-I171V was significantly higher than that of other cell line expressing either NBS1-WT or the vector control (Supplementary Fig. S8). These results suggest that chromosomal instability is elevated in cells expressing NBS1-I171V because its dominant-negative effects on endogenous NBS1 reduce DSB repair. This result is consistent with our previous finding that the chromosomes of lymphoblastic cell lines derived from the patient’s father, who carried the heterozygous polymorphic variant of the NBS1-I171V substitution, contained a remarkable number of structural chromosomal aberrations (10).

Conclusion

We have demonstrated that the NBS1-I171V variant reduces DSB repair activity through loss of association with MDC1. Moreover, the reduced activity of NBS1 in cells expressing the NBS1-I171V variant elevated chromosomal instability in these cells and increased the
risk of breast cancer in a Japanese population. Recently, it was reported that Mre11-mediated DDR restrains mammary hyperplasia by effecting an oncogene-induced G2 arrest (34). Therefore, further researches of NBS1-I171V function in the development of breast cancer could be necessary.
References


24. Schlegel BP, Jodelka FM, Nunez R. BRCA1 Promotes Induction of ssDNA by Ionizing


Acknowledgments

We thank Dr. K. Komatsu (Kyoto University, Japan) for the NBS1 cDNA, GM07166VA7 cells and GM07166VA7 cells transfected with DR-GFP reporter, and Dr. M. Jasin (Sloan Kettering Cancer Center, USA) for the DR-GFP construct. This work was supported in part by the National Cancer Center Research and Development Fund (23-A-4), and Grants-in-Aid for Cancer Research from the Ministry of Health, Labor, and Welfare of Japan. This work was also conducted as part of the BioBank Japan Project, which was supported by the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Author Contributions

Figure Legends

Figure 1. The polymorphic variant of NBS1-I171V is located in the BRCT domain.

The structure of human NBS1 and the sequence alignment of the NBS1-I171V polymorphic variant region of various NBS1. A, NBS1 consists of functional regions: forkhead-associated (FHA) and BRCT (BRCA1 C-terminus) domains at the N-terminus, MRE11 and ATM interacting motifs at the C-terminus, two ATM/ATR-phosphorylated serine residues (S278, S343). B, Modified sequence alignment of the NBS1-I171V polymorphic variant region of various NBS1 from human, mouse, rat, chicken and ACF (African clawed frog, Xenopus laevis). C, Expression vectors containing 3xFlag-His-HA-NBS1-WT cDNA, -NBS1-I171V cDNA, or no cDNA (vector) were stably transfected into GM07166VA7 cells. Extracts from the cell lines were immunoprecipitated (IP) with anti-Flag antibody and then incubated with antibodies directed against MRE11, RAD50 or NBS1.

Figure 2. Reduced DSB repair activity in cells expressing an NBS1-I171V polymorphic variant.

A, The survival of the GM07166VA7 cell lines expressing NBS1-WT (WT), NBS1-I171V (I171V) or the vector (vector) were analyzed by using a colony formation assay after exposure to 0, 2, 4 or 6 Gy of IR or 0, 50, 100 or 150 n mol/L of MMC. The data shown are the mean ± SEM (n = 3). B, HR repair activity in the GM07166VA7 cell lines expressing NBS1-WT (WT), NBS1-I171V (I171V), or the vector (vector) was measured with the DR-GFP assay. The data shown are the mean ± SEM (n = 3). C, The survival of the
GM07166VA7 cell lines expressing NBS1-WT (WT), NBS1-I171V (I171V) or the vector (vector) were analyzed by using a colony formation assay after exposure to 0, 1, 3 or 5 μmol/L of AZD2281. The data shown are the mean ± SEM (n = 3).

**Figure 3. Reduced localization of NBS1 and MRE11 at the DSB sites in cells expressing an NBS1-I171V polymorphic variant.**

A, C, Focus formation of NBS1, MRE11 and γH2AX. The GM07166VA7 cells expressing NBS1-WT (WT), NBS1-I171V (I171V) and the vector (vector) were irradiated with 10 Gy of IR. After 4 hours, the cells were incubated with antibodies directed against NBS1, γH2AX or MRE11, and then stained with DAPI. B, D, Scatter plots of NBS1 or MRE11 focus counts per cell in Fig. 3A or Fig. 3C were shown (n = 30). The read bars represent median.

**Figure 4. Reduced association of the NBS1-I171V polymorphic variant with MDC1.**

A, C, Focus formation of γH2AX, MDC1 and NBS1. The GM07166VA7 cells expressing NBS1-WT (WT), NBS1-I171V (I171V) and the vector (vector) were irradiated with 10 Gy of IR. After 4 hours, the cells were incubated with antibodies directed against γH2AX, MDC1 or NBS1, and then stained with DAPI. B, D, Scatter plots of MDC1 or NBS1 focus counts per cell in Fig. 4A or Fig. 4C were shown (n = 30). The read bars represent median.

E, The GM07166VA7 cells expressing NBS1-WT, NBS1-I171V and the vector were irradiated with 10 Gy of IR. After 4 hours, whole cell lysates were prepared. The cell lysates were immunoprecipitated (IP) with anti-Flag antibody and then incubated with
antibodies directed against MDC1 or NBS1.

**Figure 5. Reduced association of RPA32 and RAD51 at the DSB sites in cells expressing an NBS1-I171V polymorphic variant.**

A, C, Focus formation of γH2AX, phospho RPA32 and RAD51. The GM07166VA7 cells expressing NBS1-WT (WT), NBS1-I171V (I171V) and the vector (vector) were irradiated with 10 Gy of IR. After 4 hours, the cells were incubated with antibodies directed against γH2AX, phospho RPA32 or RAD51, and then stained with DAPI. B, D, Scatter plots of phospho RPA32 or RAD51 focus counts per cell in Fig. 5A or Fig. 5C were shown (n = 30). The read bars represent median.

**Figure 6. The DSB repair activity of endogenous NBS1 was repressed by the NBS1-I171V polymorphic variant in a dominant-negative manner.**

Expression vectors containing 3xFlag-His-HA-NBS1-WT cDNA, -NBS1-I171V cDNA, or no cDNA were stably transfected into HeLa cells. A, The expression levels of NBS1 in the cell extracts were analyzed with antibodies directed against NBS1. The upper *arrowhead* indicates exogenous NBS1 and the lower *arrowhead* indicates endogenous NBS1. B, The survival of the cell lines expressing NBS1-WT (WT), NBS1-I171V (I171V) or the vector (vector), or the survival of HeLa cells transfected with NBS1 siRNA (NBS1-siRNA#1 and siRNA#2) or control siRNA (control-siRNA) was analyzed by using colony formation assays after exposure to 0, 2 or 4 Gy of IR or 0, 12.5, 25, 37.5 or 50 n mol/L of MMC. The data shown are the mean ± SEM (n = 3). C, A representative metaphase spread of the HeLa...
cells expressing NBS1-I171V after exposure to 6 Gy of IR. Chromatid breaks (CTB, white arrowheads), double minutes (DM, black arrowheads), and chromatid gaps (CTG, arrows) are indicated. Noteworthy structural chromosomal aberrations are shown at higher magnification in the right panels: chromatid gap (top panel), chromatid break (middle panel), and double minute (bottom panel). D, Comparison of the frequencies of aberrations found in the cells used in Fig. 6A. *, $P < 0.005$. 
Table 1. Association of NBS1 variation with breast cancer in Japanese

<table>
<thead>
<tr>
<th>SNP Gene</th>
<th>Allele (G/A)</th>
<th>Groups</th>
<th>Case</th>
<th>RAF</th>
<th>P</th>
<th>OR (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBS1</td>
<td>G/A</td>
<td>Breast</td>
<td>0 23 1501</td>
<td>0.0075</td>
<td>0.0048</td>
<td>3.19 (1.36-7.44)</td>
</tr>
<tr>
<td>rs61754966</td>
<td>G/A</td>
<td>Control</td>
<td>0 7 1455</td>
<td>0.0024</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

We analyzed 1,524 breast cancers and 1,462 controls. aAllele 1; risk allele, Allele 2; non risk allele. P value and Odd ratio (OR) were calculated in a dominant model (11+12 vs 22).
Fig. 1. Y. Yamamoto et al.
Fig. 2. Y. Yamamoto et al.
Fig. 3. Y. Yamamoto et al.
**Fig. 4. Y. Yamamoto et al.**

**A**

<table>
<thead>
<tr>
<th></th>
<th>DAPI</th>
<th>γH2AX</th>
<th>MDC1</th>
<th>Merged</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I171V</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vector</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**

Number of MDC1 foci

- WT
- I171V
- Vector

**C**

<table>
<thead>
<tr>
<th></th>
<th>DAPI</th>
<th>MDC1</th>
<th>NBS1</th>
<th>Merged</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I171V</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vector</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**D**

Number of NBS1 foci

- WT
- I171V
- Vector

**E**

<table>
<thead>
<tr>
<th>INPUT</th>
<th>NBS1-WT</th>
<th>NBS1-I171V</th>
<th>Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDC1</td>
<td><img src="MDC1_INPUT.png" alt="Image" /></td>
<td><img src="MDC1_INPUT.png" alt="Image" /></td>
<td><img src="MDC1_INPUT.png" alt="Image" /></td>
</tr>
<tr>
<td>NBS1</td>
<td><img src="NBS1_INPUT.png" alt="Image" /></td>
<td><img src="NBS1_INPUT.png" alt="Image" /></td>
<td><img src="NBS1_INPUT.png" alt="Image" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IP</th>
<th>NBS1-WT</th>
<th>NBS1-I171V</th>
<th>Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDC1</td>
<td><img src="MDC1_IP.png" alt="Image" /></td>
<td><img src="MDC1_IP.png" alt="Image" /></td>
<td><img src="MDC1_IP.png" alt="Image" /></td>
</tr>
<tr>
<td>NBS1</td>
<td><img src="NBS1_IP.png" alt="Image" /></td>
<td><img src="NBS1_IP.png" alt="Image" /></td>
<td><img src="NBS1_IP.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Fig. 5. Y. Yamamoto et al.
Fig. 6. Y. Yamamoto et al.
A rare polymorphic variant of NBS1 reduces DNA repair activity and elevates chromosomal instability

Yuki Yamamoto, Mamiko Miyamoto, Daisuke Tatsuda, et al.

Cancer Res  Published OnlineFirst May 15, 2014.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-13-3037

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2014/05/15/0008-5472.CAN-13-3037.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.