Novel NBS1 Heterozygous Germ Line Mutation Causing MRE11-Binding Domain Loss Predisposes to Common Types of Cancer

Hiromichi Ebi,1,2 Keitaro Matsuuo,3 Nobuyoshi Sugito,1 Motoshi Suzuki,1 Hirotaka Osada,4 Kazuo Tajima,3 Ryuzo Ueda,3 and Takashi Takahashi1

Abstract

DNA damage response (DDR) pathways maintain genomic stability. A 657del5 mutation of NBS1, a key DDR component, causing the rare cancer-predisposing Nijmegen breakage syndrome has been reported nearly exclusively in Slavic populations. In this study, we describe the first identification in a Japanese population of an unprecedented type of heterozygous NBS1 mutant, termed IVS11+2insT, lacking the MRE11- and ATM-binding site at the COOH terminus. Profoundly defective in crucial binding to MRE11, MDC1, BRCA1, and wild-type NBS1, the mutant caused impaired ATM phosphorylation in response to low-dose irradiation in a heterozygous state. Importantly, whereas IVS11+2insT was found in only 2 (0.09%) of 2,348 control subjects, it was identified in 2% (2 of 96) of heterozygotes with gastric cancer, 0.8% (3 of 376) of those with colorectal cancer, and 0.4% (2 of 532) of those with lung cancer, which were comparable to frequencies reported for other DDR-related genes known to confer cancer susceptibility. The presence of the heterozygous IVS11+2insT mutation seemed to be associated with an increased risk for gastrointestinal cancers, with an odds ratio of 12.6 and 95% confidence interval (95% CI) of 2.05 to 132.1 (P = 0.0001). The odds ratios separately calculated for gastric and colorectal cancers were 25.0 (95% CI, 1.78–346.0) and 9.43 (95% CI, 1.08–113.1), respectively. These findings suggest that Japanese heterozygotes with the IVS11+2insT mutation seem to be associated with an increased risk for the development of certain types of common cancers, warranting future investigation including detailed phenotypic characterization of age of onset and penetrance in heterozygotes, as well as screening in other ethnic groups. [Cancer Res 2007;67(23):11158–65]

Introduction

Accumulating evidence indicates that a DNA damage checkpoint is activated even in preneoplastic lesions, leading to cell cycle blockade or apoptosis, thereby constraining tumor progression (1, 2). Its abrogation is thought to be critical during multistep transformation processes and fully malignant cells of overt cancers frequently carry various defects in checkpoint mechanisms. Consistent with this notion, we have established that frequent impairment of various types of DNA damage and mitotic checkpoints occur in lung cancer (3–5), suggesting their involvement in lung carcinogenesis.

NBS1 plays important roles in the cellular response to DNA damage and is critical for maintaining genomic stability. This gene forms a multimeric complex with MRE11 and RAD50 (MRN complex), which have various functions such as DNA repair by homologous recombination or nonhomologous end-joining, mitotic recombination, DNA damage response (DDR), and telomere maintenance (6). Furthermore, homozygous germ line mutations in the NBS1 gene residing at 8q21.3 are responsible for Nijmegen breakage syndrome (NBS), a rare autosomal recessive genetic disease belonging to a group of disorders often referred to as chromosomal instability syndromes that displays increased cancer risk, especially for lymphoma development (7). The vast majority of patients with NBS are of Slavic origin and share the founder NBS1 mutation, 657del5, which has been shown to be hypomorphic due to the production of both NH2-terminal 26 kDa and COOH-terminal 70 kDa species (8).

In the present study, originally initiated for pursuing molecular dissection of DNA damage checkpoint impairment in lung cancers, we identified for the first time in Japanese subjects an unprecedented type of heterozygous germ line NBS1 mutation, termed NBS1 IVS11+2insT. In addition to its initial characterization, we describe the results of our extensive search for this novel germ line mutation in a total of 1,743 nonselected cases with various types of adult common cancers as well as in 2,348 control subjects. Our findings suggest that Japanese heterozygotes with the IVS11+2insT mutation may be predisposed to the development of certain common types of adult cancer, including stomach and colon cancers.

Materials and Methods

Cell lines. Six small cell lung cancer and three non–small cell lung cancer cell lines with the prefix ACC-LC were established in our laboratories (small cell lung cancer: ACC-LC-48, -49, -76, -80, -97, and -172; non–small cell lung cancer: ACC-LC-94, -176, and -319). The NCI-H460 and A549 cell lines were purchased from the American Type Culture Collection, whereas PC-10 was generously provided by Y. Hayata (Tokyo Medical University, Tokyo, Japan), and Calu-1 and Calu-6 was provided by L.J. Old (Memorial Sloan-Kettering Cancer Center, New York, NY). Using a TaKaRa PCR Mycoplasma Detection Kit (Takara Bio, Inc.), all cell lines were shown to be free from Mycoplasma contamination.

Antibodies. The antibodies used in this study were rabbit polyclonal NBS1 against the mid- to COOH-terminal portion (amino acids 399–751) of NBS1 (Oncogene Research); rabbit polyclonal NBS1 against the synthetic peptide corresponding to 24 amino acids in the COOH-terminal NBS1 (Calbiochem); MRE11 and RAD50 (Genetex); H2AX and γ-H2AX (Upstate); SMC1 and SMC1 Ser1396 (Bethyl Laboratories); ATM (Ab-3, Oncogene Research); ATM Ser1981 (Rockland Immunocchemicals); ATR, CDC25A, and

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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BRC1 (Santa Cruz Biotechnology); Chk2 (MBL); and Chk2 Thr68 (Cell Signaling Technology). Rabbit polyclonal anti-MDC1 was a kind gift from Junjie Chen (Yale University, New Haven, CT).

**Immunofluorescence.** Cells were grown on coverslips and irradiated at various doses. After 10 min, they were fixed in 1% paraformaldehyde for 15 min, followed by treatment with 0.2% Triton X-100 on ice for 20 min. The coverslips were then incubated with mouse monoclonal anti–γ-H2AX antibody and rabbit polyclonal anti–ATM Ser1981 antibody overnight at 4°C, followed by incubation with Alexa 488–conjugated goat anti-rabbit and Alexa 568–conjugated antimouse secondary antibodies (Molecular Probes). Confocal microscopy analysis was performed using an Olympus fluorescence microscope (Olympus, Japan) equipped with a ×100 objective lens and a Bio-Rad confocal imaging system (Radiance2100; Bio-Rad). The acquisition software used was a LaserSharp 2000 from Bio-Rad. Images at 488 and 568 nm were stacked and merged.

**Immunoprecipitation.** Cells were placed in NP40 lysis buffer [20 mmol/L HEPES (pH 7.8), 300 mmol/L NaCl, 1 mmol/L EDTA, and 1% NP40] supplemented with a protease inhibitor cocktail (Roche), then centrifuged and fractionated into supernatant and pellet fractions. For immunoprecipitation of MDC1, the pellet fraction was resuspended in buffer containing 20 mmol/L of Tris-Cl (pH 8.0), 1 mmol/L of MgCl2, and 20 mmol/L of NaCl, supplemented with Benzonase (Novagen) and the protease inhibitor cocktail for 30 min. The clarified extract was mixed with the supernatant fraction. Equal amounts of the mixture were then incubated overnight at 4°C with 1 to 2 μg of antibodies, followed by a 1-h incubation with 30 μL of protein G Sepharose (Amersham Biosciences). Immunocomplexes were washed four times with NP40 lysis buffer, boiled in SDS sample buffer, and loaded onto an SDS-polyacrylamide gel.

**Vectors and gene expression.** An NBS1-encoding pLXIN retroviral vector was kindly provided by P. Concannon (Benaroya Research Institute, Seattle, WA). Retroviral supernatants were generated by a 48-h transient cotransfection of the indicated retroviral and VSV-G expression plasmids in Plat-E cells (T. Kitamura, University of Tokyo, Tokyo, Japan). Stable cell lines were generated by transduction with the retroviral supernatants in the presence of 8 μg/mL of polybrene, after which neomycin-resistant cells (400 μg/mL) were selected.

**Radioreistant DNA synthesis assay.** One day after seeding in multiple 35-mm dishes (5–10 × 10^4 per dish), cells were incubated with [2-¹⁴C]thymidine (1.85 kBq/mL) for 16 h, then subjected to irradiation at 0, 2, 4, 6, 10, or 20 Gy at room temperature, and subsequently labeled with [methyl-3H]thymidine (74 kBq/mL) for 4 h. The cells were then rinsed with PBS, incubated with unlabeled medium for 30 min, rinsed with PBS again, and lysed in 0.5 mL of 0.25 mol/L of NaOH. Each lysate was transferred into 7.5 mL of scintillation cocktail (Hionic Fluor, Packard) and measured for radioactivity. DNA synthesis was estimated by ³H/¹⁴C dpm ratios, with the resulting value expressed as that compared with 100% of the nonirradiated controls.

**mRNA isolation and rapid amplification of cDNA ends analysis.** Total RNA was prepared using a RNeasy Mini Kit (QIAGEN) and mRNA was isolated with oligo(dT) latex beads (Oligotex dT30 Super, Nippon Roche Co.). We performed ³′-rapid amplification of cDNA ends (RACE) analysis using a SMART RACE cDNA amplification kit (Clontech), according to the manufacturer’s instructions. NBS1-specific sense primers (5′-ctgtgatcctcagggccatc-3′ and 5′-cagattaggaagttatg-3′) were used with a universal primer mix to generate PCR products, which were gel-separated and directly sequenced using an ABI3100 (Perkin-Elmer) DNA sequencer.

**PCR-based detection of IVS11+2insT.** To detect the one-base insertion at the ag-gt canonical splice junction between exon 11 and intron 11, a reverse primer was designed at the exon 11/intron 11 junction (5′-ctataccctctatatatgtgacctac-3′, inserted nucleotide indicated as a capital letter) and used for PCR with a forward primer (5′-aacttgtaggccttgtagggtaa-3′). This insertion prevented the amplification of the wild-type NBS1 (wt-NBS1), but not IVS11+2insT. For quality control, a region of wt-NBS1 was amplified with a forward primer, 5′-aacttgtaggccttgtagggtaa-3′, and a reverse primer, 5′-atctctatatatatatgtgacctac-3′. Genomic DNA from ACC-LC-80 served as a positive control for the case-control study. Direct sequencing analysis of positive samples from PCR screening was done to ensure the presence of the germ line NBS1 IVS11+2insT mutation.

**Clinical samples.** Tumor tissues from 200 lung carcinoma cases as well as a total of 1,743 peripheral-blood samples from patients with various malignancies, including 434 breast cancers, 532 lung cancers, 376 colorectal cancers, 109 malignant lymphomas, 196 esophageal cancers, and 96 gastric cancers, were collected at Aichi Cancer Center after obtaining written informed consent. Clinical samples were exposed to the indicated doses of irradiation, DNA synthesis was measured and plotted. Points, the averages of 12 lung cancer cell lines (details in Supplementary Fig. S1). T1G-112 (●), ACC-LC-80 (▲), and ACC-LC-172 (□).

**Figure 1.** Aberrant NBS1 protein in an S phase checkpoint–defective cell line. A, a proportion of the tested lung cancer cell lines had a defective S phase checkpoint. After a normal human fibroblast line (TIG-112) and various lung cancer cell lines were exposed to the indicated doses of irradiation, DNA synthesis was measured and plotted. Points, the averages of 12 lung cancer cell lines; details in Supplementary Fig. S1. TIG-112 (●), ACC-LC-80 (▲), and ACC-LC-172 (□). B, Western blot analysis of ACC-LC-80 cells. Cell extracts were used for Western blotting of ATM, ATR, RAD50, NBS1, MRE11, and α-tubulin, as indicated. Decreased protein levels of MRE11 and RAD50 were also observed in ACC-LC-80 cells.
informed consent. We used non–cancer patients at Aichi Cancer Center Hospital as controls, given the likelihood that our cases arose within this population base. Individuals selected randomly from our control population were earlier shown to be similar to the general population in terms of common lifestyle, such as smoking and drinking (9). Equivalence in the genotype distributions for the several types of polymorphism between our controls and the general population in Japan has been reported (10–12); therefore, our controls were considered to be representative. Furthermore, among non–cancer outpatients, 45% have been reported to be without abnormal findings in clinical examinations and 35% with benign nonspecific diseases (13). Therefore, we concluded that it was reasonable to assume that the internal and external validity for causal inference from the cases–control comparison was valid. All patients and control subjects were of Japanese ethnicity. This study was approved by the institutional review boards of Aichi Cancer Center and Nagoya University Graduate School of Medicine.

Statistical analysis. Fisher’s exact test was used to compare the frequencies of carriers with the total number of control subjects. All analyses were performed using Stata software (version 7; Stata Corp), with the two-sided significance level set at \( P < 0.05 \).

Results

Identification of NBS1 IVS11+2insT mutation in S phase checkpoint–defective cell line. First, we evaluated the S phase checkpoint status by examining radiosensitive DNA synthesis (RDS) in 14 lung cancer cell lines and normal human fibroblasts, and observed significantly decreased responses in 2 of the lung cancer cell lines, ACC- LC-80 and -172 (Fig. 1A; Supplementary Fig. S1). It has been reported that the S phase checkpoint is activated through at least two parallel branches in response to irradiation (14). Overexpression of CDC25A, which is a downstream molecule in the ATM-Chk2-CDC25A branch, was observed in a few cell lines, in accordance with a previous report (15); however, the expression status did not correlate with the checkpoint impairment (Supplementary Fig. S2A). Destruction of CDC25A and phosphorylation of Chk2 at Thr68 also seemed to be intact in the S phase checkpoint-impaired cell lines (Supplementary Fig. S2B and C). In contrast, Western blot analysis of the ATM-NBS1-SMC1 branch revealed the expression of an aberrantly-sized NBS1 (80 kDa), termed NBS1p80, as well as considerable decreases in NBS1 expression levels in the cell lines (Fig. 1B).

Requirement of COOH terminus of NBS1 for interaction with MDC1 and optimal ATM activation. The MRN complex plays roles in both the initial recognition of unrepaired double-strand breaks and subsequent DDR amplification, for which NBS1 with MDC1 and optimal ATM activation.

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Requirement of COOH terminus of NBS1 for interaction with MDC1 and optimal ATM activation. The MRN complex plays roles in both the initial recognition of unrepaired double-strand breaks and subsequent DDR amplification, for which interactions of NBS1 with MRE11, ATM, and MDC1 are known to be crucial (16–18). NBS1p80 lacking the MRE11 binding domain was absent from MRN complexes, as expected (Fig. 3A). To examine whether NBS1p80 interacts with wt-NBS1 through oligomerization mediated by the forkhead-associated (FHA) domain, we performed immunoprecipitation of cell lysates with an antibody against the COOH-terminal end of NBS1 that does not react with NBS1p80. The resulting immunoprecipitates were probed with the other anti-NBS1 antibody, which reacts with the mid- to COOH-terminal portion of NBS1, and the results clearly showed that NBS1p80 also failed to interact with wt-NBS1 regardless of irradiation (Fig. 3B). In addition, we observed no association with BRCA1, which is known to interact with RAD50 (ref. 19; Fig. 3C). Unexpectedly, we found that NBS1p80 could not be coimmunoprecipitated with MDC1, even though it retained the FHA domain, through which NBS1 is thought to associate with MDC1 (refs. 20, 21; Fig. 3D).

During our experiments, we also detected multiple foci of phosphorylated ATM and γ-H2AX in ACC-LC-80, even when the
cells were not exposed to irradiation (Fig. 4A), suggesting the constitutive presence of DNA damage. Western blot analysis confirmed the constitutive phosphorylation of ATM (Fig. 4B) and γ-H2AX (Supplementary Fig. S3) in ACC-LC-80 in the absence of irradiation. We also noted reduced phosphorylation of ATM at submaximal irradiation in ACC-LC-80, whereas NCI-H460 showed a normal response to irradiation (ref. 22; Fig. 4B). In addition, modest but reproducible elevations of ATM and SMC1 phosphorylation were observed in wt-NBS1–reconstituted ACC-LC-80 cells (Fig. 4C), which also suggested a possible enhanced detection of ongoing DNA damage. There were no differences in the RDS values with >2 Gy of irradiation between NBS1p80 and empty vector–transfected Calu-1 cells with wt-NBS1 (Supplementary Fig. S4), or between wt-NBS1 and empty retrovirus–transduced ACC-LC-80 cells with the heterozygous IVS11+2insT mutation (Supplementary Fig. S5). However, it was not possible to reliably evaluate the differences in RDS at submaximal activating doses. Taken together, these findings indicate that NBS1p80 is a nonfunctional mutant in terms of DDR propagation due to its inability to directly or indirectly bind with key DDR components, including MRE11, ATM, wt-NBS1, BRCA1, and MDC1. In addition, they also suggest the haploinsufficient nature of NBS1 in terms of proficient detection of low levels of DNA damage.

Identification of heterozygous germ line mutation of NBS1 IVS11+2insT in nonselected cancer patients. Next, we investigated whether this previously unreported NBS1 mutation could be detected in lung cancer tissues in vivo, using a PCR-based method that specifically amplified a genomic DNA fragment only if the one-base insertion mutation existed (Fig. 5). In our initial screening, the IVS11+2insT mutation was in fact identified in one tumor among 200 primary lung cancer cases. To further determine whether IVS11+2insT is present in the germ line and predisposes individuals carrying NBS1 IVS11+2insT to cancer development, we screened nonselective peripheral blood samples from 1,743 patients affected with various adult cancers, including breast carcinomas, lung carcinomas, colorectal adenocarcinomas, malignant lymphomas, esophageal carcinomas, and stomach carcinomas, as well as in 2,348 non–cancer control subjects (Table 1). Apparent PCR amplification was observed in 2 (2%) of 96 patients with gastric cancer, 3 (0.8%) of 376 patients with colorectal cancer, and 2 (0.4%) of 532 patients with lung cancer, whereas a positive amplification of the mutant allele was observed in only 2 (0.09%) of the control subjects. None of the 434 patients with breast cancer, 196 with esophageal cancer, and 109 with malignant lymphomas exhibited positive PCR amplifications. Subsequent sequencing analyses confirmed the identity of the IVS11+2insT mutation and all the cases were found to be heterozygous for IVS11+2insT.

Figure 3. Lack of interactions of NBS1p80 with components of the DDR pathway. A, lack of interactions of NBS1p80 with MRE11 and RAD50. Immunoprecipitates from either anti-NBS1 (left) or anti-MRE11 (right) antibodies were immunoblotted with anti-NBS1 (top), anti-MRE11 (middle), and anti-RAD50 (bottom) antibodies. B, lack of interaction between wt-NBS1 and NBS1p80. Immunoprecipitates from an antibody against the COOH-terminal end of NBS1 were immunoblotted with an antibody against the mid-to COOH-terminal portion of NBS1. Note that NBS1p80 was not coimmunoprecipitated with wt-NBS1 due to lack of the COOH-terminal portion of NBS1. C, lack of interactions of NBS1p80 with BRCA1. Immunoprecipitation was performed with anti-BRCA1 or control IgG antibodies, and the precipitated proteins were immunoblotted with anti-BRCA1 (top) and anti-NBS1 (bottom) antibodies. D, lack of interaction of NBS1p80 with MDC1. After 60 min of irradiation at 0 or 10 Gy, immunoprecipitation was performed with either anti-MDC1 or control IgG antibodies, and Western blotting analyses were performed using the anti-MDC1 (top) and anti-NBS1 (bottom) antibodies. Protein positions are indicated (left).
consistent with the finding that homozygous deletion of the MRE11-binding domain is lethal in embryonic mice (23). Analysis using a two-sided Fisher’s exact test revealed that the IVS11+2insT mutation resulting in the expression of NBS1p80 increases the risk for cancer occurrence in the gastrointestinal tract, with an odds ratio of 12.6 and 95% confidence interval [(95% CI), 2.05–132.1; P = 0.0001]. The presence of the heterozygous IVS11+2insT mutation seemed to confer an increased risk for the development of gastrointestinal tract cancers that originate in the stomach, with an odds ratio of 25.0 (95% CI, 1.78–346.0; P < 0.0001), and those with a colorectal origin with an odds ratio of 9.43 (95% CI, 1.08–113.1; P = 0.02). Although it was noted that patients with lung cancer harboring the mutation were at a stage of relatively early onset, the observed risk for lung cancer development did not reach statistical significance (odds ratio, 4.43; 95% CI, 0.32–61.1; P = 0.16).

**Discussion**

The NBS cases identified thus far have been found to be homozygous mostly for the 657del5 mutation and are nearly exclusively of Slavic ethnicity, with a carrier frequency approaching as high as 1 in 154 individuals in the Czech Republic (6). Individuals with NBS are known to have a high risk of developing predominantly hematologic malignancies. In addition, there are somewhat
controversial epidemiologic findings suggesting that even heterozygous carriers of the hypomorphic 657del5 mutation might be at an increased risk of developing malignant tumors, such as those associated with breast and prostate cancer, and melanomas (6, 24, 25). There is no known previous report of a germ line NBS1 mutation similar to IVS11+2insT identified in this study, which results in elimination of the COOH terminus of the NBS1 protein. It should also be noted that no germ line NBS1 mutation has been reported in Japanese subjects, except for a functionally uncertain I171V substitution in a single patient with aplastic anemia (26).

The 657del5 mutation, which accounts for >90% of all previously identified mutant alleles in NBS, is known to be hypomorphic and partially preserves NBS1 functions due to the expression of the COOH-terminal portion of 70 kDa (NBS1p70) containing the MRE11- and ATM-binding sites (Fig. 2C; ref. 8). In contrast to complete abrogation of irradiation-induced phosphorylation of ATM in NBS1-null cells, the phosphorylation is only attenuated in homozygous 657del5 NBS cells, indicating dependence on the presence of MRE11- and ATM-binding domains (18, 23). In this context, NBS1p70 has been shown to associate directly with MRE11 (8), whereas the present findings indicate that NBS1p80 lacks any association with MRE11, BRCA1, ATM, or wt-NBS1 itself, or unexpectedly, MDC1, which are all known to be crucial for DDR.

The present findings also suggest that formation of the MRN complex may be a prerequisite for a stabilizing interaction of NBS1 with MDC1. Alternatively, NBS1 may be associated with MDC1 not only at FHA domains, but also through the carboxyl terminal region. Using knockout mouse models, the haploinsufficiency of DDR proteins, including p53, BubR1, H2AX, and ATM have been shown in spontaneous and/or carcinogen-induced tumorigenesis (27–29). In the case of NBS1, the heterozygosity of experimentally complete knockout mice resulted in a modest elevation of chromosomal aberrations, shown by cytogenetic analysis (30, 31), with the development of a wide array of tumors including lung and gastric adenomas (31).

It is critical for cells to be able to cope with DNA single- and double-strand breaks arising from the process of daily DNA metabolism and environmental sources of damage (32). IVS11+2insT

Table 1. Germ line IVS11+2insT mutations in NBS1 gene in 1,743 patients with various types of cancers

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>No. analyzed</th>
<th>Age, mean ± SD</th>
<th>IVS11+2insT heterozygotes</th>
<th>OR (95% CI)*</th>
<th>P</th>
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<tr>
<td>Lung cancer</td>
<td>532</td>
<td>61.8 ± 10.0</td>
<td>2</td>
<td>4.43 (0.32–61.1)</td>
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<tr>
<td>Esophageal cancer</td>
<td>196</td>
<td>61.2 ± 8.4</td>
<td>0</td>
<td>12.6 (2.05–132.1)</td>
<td>0.0001</td>
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<tr>
<td>Gastric cancer</td>
<td>472</td>
<td>58.5 ± 10.6</td>
<td>5</td>
<td>25.0 (1.78–346.0)</td>
<td>&lt;0.0001</td>
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<tr>
<td>Colorectal cancer</td>
<td>376</td>
<td>58.7 ± 10.6</td>
<td>3</td>
<td>9.43 (1.08–113.1)</td>
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<td>Breast cancer</td>
<td>434</td>
<td>52.4 ± 10.8</td>
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<tr>
<td>Malignant lymphoma</td>
<td>109</td>
<td>54.1 ± 12.0</td>
<td>0</td>
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<td>Control subjects</td>
<td>2,348</td>
<td>56.7 ± 12.0</td>
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*OR, odds ratio; CI, confidence intervals.
†M, male; F, female.
‡AD, adenocarcinoma; LA, large cell carcinoma.
does not seem to augment RDS or have chemoradiosensitivity (data not
taken), at least at relatively high doses that cause extensive
DNA damage. However, it is possible that the probable haploin-
sufficiency of IVS11+2insT may compromise DRR at lower doses,
which are probably more relevant in terms of carcinogenic
involvement, although that cannot be revealed by a standard RDS
assay. Thus, it is interesting to note that multiple foci of
phosphorylated ATM and γ-H2AX were detected in nonirradiated
ACC-LC-80 cells. The modestly elevated levels of constitutive
phosphorylation of ATM and SMC1 seen in wt-NBS1–reconstituted
ACC-LC-80 cells may also support the notion that expression of
nonfunctional IVS11+2insT and wt-NBS1 at half the normal level is
insufficient for full detection of constitutive DNA damage, for which
the underlying mechanism remains to be elucidated.

The checkpoint and repair pathways facilitate cellular responses
to DNA damage, and a proportion of human cancers seems to be
attributable to the inheritance of genetic defects in these pathways.
In the present study, we observed the heterozygous germ line
IVS11+2insT mutation in 1.1% of gastrointestinal cancers, which
was associated with an increased risk for their development with
an odds ratio of 12.6 (95% CI, 2.05–132.1; P = 0.0001). Furthermore,
the presence of the heterozygous germ line IVS11+2insT mutation
was observed in 0.8% of nonselected Japanese colorectal cancer
patients, who showed a 9.4-fold increased risk for developing
colorectal cancer (95% CI, 1.08–113.1; P = 0.02). A number of studies
have been conducted to investigate whether DDR gene
mutations confer colorectal cancer susceptibility. An Ashkenazi
Jewish population with colorectal cancer was shown to be more
than twice as likely to carry a heterozygous mutation of BLM (odds
ratio, 2.45; 95% CI, 1.3–4.8; P = 0.0065; ref. 33). Furthermore, two
other studies reported a possible association between ATM and
colorectal cancer (relative risk, 2.54; 95% CI, 1.06–6.09; refs. 34, 35).
Although the association of colorectal cancer risk with CHEK2
variants remains controversial, among Dutch families with breast
upper, the 1100delC variant was found more frequently in
individuals with hereditary breast and colorectal cancer pheno-
types, as compared with those with non–hereditary breast and
colorectal cancers (odds ratio, 5.41; 95% CI, 2.29–12.8; P < 0.001;
ref. 36). Taken together, this seems to be considerable evidence for
the involvement of germ line mutations of DDR genes in the
pathogenesis of colorectal cancer.

Although the vast majority of adenocarcinomas of the stomach
occur sporadically without inherent predisposition, a small
portion of gastric cancers arise in inherited syndromes, including
hereditary nonpolyposis colon cancer syndrome, familial adeno-
matus polyposis, and hereditary diffuse gastric carcinoma. The
latter is an autosomal dominantly inherited gastric cancer
susceptibility syndrome caused by a germline mutation of the
CDH1 gene, however, there are no known reports of a germline
mutation of E-cadherin in gastric cancer among Japanese familial
cases (37, 38). Thus, NBS1 IVS11+2insT seems to be the first
reported example in Japan of a germline mutation that predisposes
individuals to gastric cancer development. The observed frequency (2%)
of IVS11+2insT in nonselected gastric cancer patients has a sizable effect when considering the enormous
number of gastric cancer patients in Japan, which is the most
prevalent form of cancer and adds 100,000 new cases each year
(39). Based on the present information, it is conceivable that
IVS11+2insT heterozygotes carrying this apparently defective allele
are susceptible to the development of certain types of cancers, such
as gastric and colon carcinomas. In addition, although it was
beyond the scope of this study and requires further investigation
using a much larger cohort, it is of note that lung cancer cases
with IVS11+2insT tend to have a younger onset and that previous
epidemiologic studies have suggested the existence of a rare auto-
somal gene contributing to early onset lung cancer in nonsmokers
(40, 41). In addition, familial aggregation of both lung and colon
cancers is of particular interest (41).

In conclusion, the present study is the first to identify a naturally
occurring NBS1 mutation that leads to the loss of the MRE11-
and ATM-binding sites at the COOH terminus with several functional
abrogations in a Japanese population. Our results suggest that
NBS1 IVS11+2insT confers significant susceptibility to cancers of
the gastrointestinal tract, including the stomach and colorectum.
Future detailed studies of the relationships of the heterozygous
germ line IVS11+2insT mutation with penetrance and other cli-
nicopathologic characteristics in various cancer types are war-
ranted. Finally, although we cannot rule out the possibility of a
founder effect, it would be of interest to investigate whether the
IVS11+2insT germ line mutation is present in other ethnic groups.

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