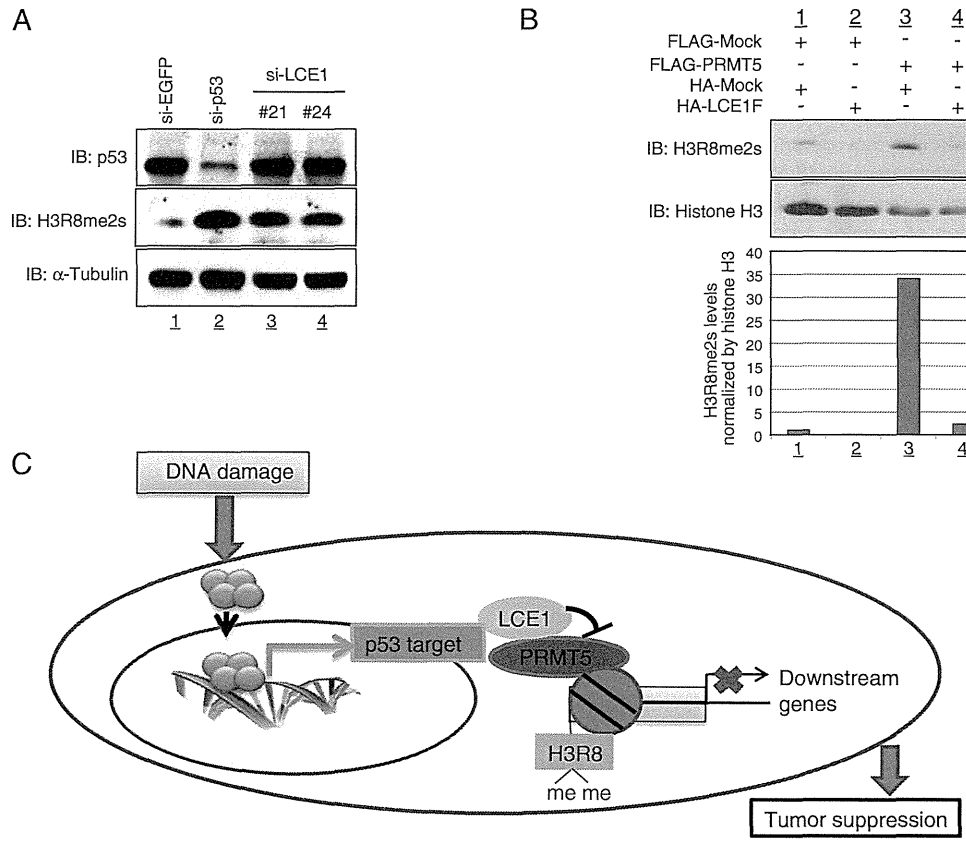

Figure 5. Possible mechanism of LCE1F on regulation of PRMT5 activity. (A) HCT116 $p53^{+/+}$ cells were transfected with siRNAs targeting EGFP, p53, or LCE1 for 24 hours and then treated with 1 μg/ml adriamycin. Proteins were extracted from the cells 36 hours after adriamycin treatment and immunoblotted with anti-p53, anti-H3R8 SDMA (H3R8me2s), or anti–α-tubulin (control) antibodies. (B) HEK293T cells were co-transfected with either FLAG-Mock or FLAG-PRMT5 and either HA-Mock or HA-LCE1F. Cells were harvested 48 hours after transfection and immunoblotted with anti-H3R8me2s or histone H3 (control) antibodies after histone purification. (C) A possible model of the network among p53, LCE1F, and PRMT5. p53 induced by DNA damage activates transcription of LCE1F, which suppresses the PRMT5 methyltransferase activity through their interaction.

targeting Enhanced green fluorescent protein (EGFP), p53, or LCE1 under treatment with adriamycin (Figure 5*A*) and found that knockdown of p53 and LCE1 significantly enhanced the methylation levels of H3R8 in HCT116 $p53^{+/+}$ cells, compared with the control cells, suggesting that LCE1 may modulate the PRMT5-dependent H3R8 methylation. To further validate this result, we co-transfected either FLAG-PRMT5 or FLAG-Mock and HA-LCE1F or HA-Mock into HEK293T cells and purified histone proteins from cell lysates. As shown in Figure 5*B*, in the presence of LCE1F, H3R8 methylation by PRMT5 protein was significantly suppressed. Taken together, our data indicate that LCE1F may negatively regulate PRMT5-dependent H3R8 methylation through the direct association to PRMT5.

Discussion

In this study, we showed that *LCE1* is a novel p53 target gene and that the LCE1F protein interacts with the arginine methyltransferase PRMT5. Through the interaction with PRMT5, LCE1F may suppress the PRMT5 methylation activity on arginine 8 of histone H3 (Figure 5*C*). Since PRMT5 is overexpressed in a wide range of human cancer and plays a critical role in tumorigenesis through the regulation of histone methylation, we unveiled a novel mechanism of tumor suppression mediated by p53.

The *LCE* gene cluster contains multiple conserved genes encoding stratum-corneum proteins [9,10,12,29]. Our study demonstrated that most of the members in the LCE1 group are transcriptionally regulated by the tumor suppressor p53 although the induction levels varied. Concordantly, *LCE1* genes were reported to be significantly upregulated in response to UVB irradiation of the skin cells [9]. UVR causes DNA damage, photoperoxidation of lipids, protein cross-linking, and isomerization of urocanic acid that lead to immunosuppression, photo-induced aging and cancer. p53 protein acts as a molecular sensor for the damages generated by UVR through mediating cell cycle arrest and apoptosis in damaged keratinocytes [30–34]. Taken together, cells may possess the function to express *LCE1* family genes through p53 activation to eliminate dangerous cells with DNA damages. Importantly, LCE1 family proteins show a high level of sequence similarity and LCE1F has more than 90% homology with other members (LCE1A-E; Table W6). In fact, as mentioned above, we confirmed the interaction between all of LCE1 proteins examined and PRMT5 (Figure W6). Since the expression of *LCE1* cluster genes is regulated by p53 as a whole (may not be all of the members), this protein family

members might play important roles to complementally or redundantly function as a tumor suppressor.

We identified PRMT5, a histone methyltransferase, as a key binding partner of the LCE1 proteins. Current progress of molecular medicine revealed that the enzymes relevant to histone methylation play critical roles in human carcinogenesis [28,35–45]. PRMT5 is one of the type II arginine methyltransferases, which catalyze the formation of symmetric dimethylation of arginine residues (SDMA) and regulates various cellular pathways [16,17,21]. A number of reports described the importance of this arginine methyltransferase in tumorigenesis [16,18,21,46–49]. Intriguingly, PRMT5 was reported to interact with BRG1- and hBRM-based hSWI/SNF chromatin remodelers and methylate arginine 8 on histone H3 [20]. The H3R8 methylation mediated by PRMT5-containing BRG1 and hBRM complexes directly repressed the expression of *suppressor of tumorigenicity 7* and *nonmetastatic 23*, tumor suppressor genes [20]. This line of pathway seems to be a key mechanism in the PRMT5-dependent tumorigenesis. Our data presented here have implied that LCE1, which is regulated by the tumor suppressor p53, negatively regulates H3R8 methylation mediated by PRMT5 (Figure 5, *A* and *B*), suggesting a new role of p53 in the regulation of histone modification. Since PRMT5 was reported to methylate p53 through the direct interaction and this methylation prevents p53-dependent apoptosis in cancer cells [50], we suggest an interesting feedback mechanism among p53, LCE1, and PRMT5.

Overall, our study elucidates a novel function of p53 as a tumor suppressor through the transcriptional regulation of the *LCE1* cluster genes. Further functional analysis may explore the importance of the LCE1 group proteins as tumor suppressors and the physiological relevance among p53 downstream genes.

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Appendix A. Supplementary Materials

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2014.07.008.

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Molecular and Cellular Pathobiology

Cancer Research

A Rare Polymorphic Variant of *NBS1* Reduces DNA Repair Activity and Elevates Chromosomal Instability

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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 of structural chromosomal aberrations in a patient with pediatric aplastic anemia 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. Furthermore, we found that heterozygosity in this polymorphic variant was associated with breast cancer risk. Finally, 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. *Cancer Res; 74(14); 3707–15. ©2014 AACR.*

Introduction

Nijmegen breakage syndrome, an autosomal recessive human disease, is because of 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 double-strand break (DSB) in DNA (2). NBS1 consists of 2 functional regions (Fig. 1A). Its C-terminal region contains binding motifs of MRE11 meiotic recombination 11 homolog A (*Saccharomyces 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; ref. 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 a patient with aplastic anemia (AA) 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

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

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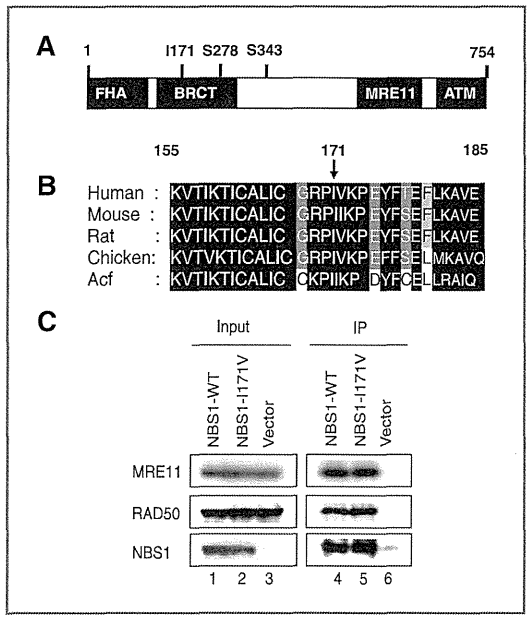

Figure 1. The polymorphic variant of *NBS1*-I171V is located in the BRCT domain. The structure of human NBS1 and the sequence alignment of the *NBS1*-I171V polymorphic variant region of various NBS1. A, NBS1 consists of functional regions: FHA and BRCT (BRCA1 C-terminus) domains at the N-terminus, MRE11, 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.

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.), mouse anti-phospho histone H2AX (Ser139) antibody (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*-wild-type (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 [150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.9), 20% glycerol, and 1 mmol/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 three 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 ×40 water immersion objective.

DR-GFP assay

Homologous recombination (HR) repair frequency in the cell lines using DR-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

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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 1,600 well-spread metaphase chromosomes were screened for structural chromosomal aberrations.

Sample collection, genotyping, and statistical analysis

We obtained DNA samples of 1,524 breast cancer cases and 1,462 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. 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′-TGAAACAAGCATTAAAGAGGGAA-3′. The odds ratios (OR) were calculated in a dominant mode. *P* values were calculated by using the χ^2 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 IR or the cross-linking agent 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 HR repair frequency in the NBS1-expressing cell lines with DR-GFP system (11, 12). When I-*Sce*I expression was induced, the cell line expressing NBS1-I171V showed a 3-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 PARP1 inhibitor (AZD2281) reduced growth of HR repair–deficient cells such as *BRCA1* and *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

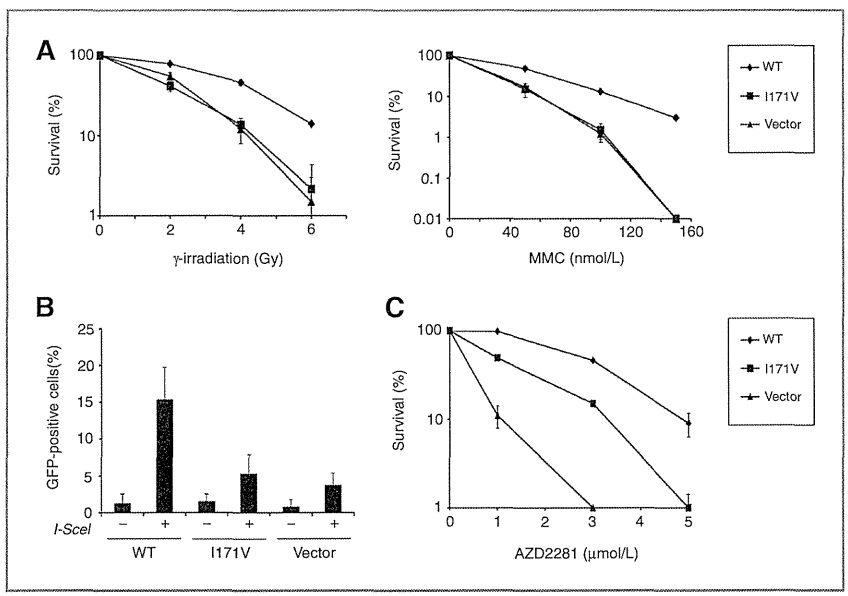

Figure 2. Reduced DSB repair activity in cells expressing an *NBS1-I171V* polymorphic variant. A, the survival of the GM07166VA7 cell lines expressing NBS1-WT (WT), NBS1-I171V (I171V), or the vector (vector) were analyzed by using a colony formation assay after exposure to 0, 2, 4, or 6 Gy of IR or 0, 50, 100, or 150 nmol/L of MMC. Data, 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. Data, mean ± SEM ($n = 3$). C, the survival of the GM07166VA7 cell lines expressing NBS1-WT (WT), NBS1-I171V (I171V), or the vector were analyzed by using a colony formation assay after exposure to 0, 1, 3, or 5 μmol/L of AZD2281. Data, mean ± SEM ($n = 3$).

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by the PARP inhibitor were different from those by IR or MMC in Fig. 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 Fig. 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 research 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 G_1–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 with 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 B). MRE11 foci were also identified in cells expressing NBS1-WT, but not in those expressing NBS1-I171V (Fig. 3C and D). Phosphorylated H2A histone family member X (γH2AX) foci were identified in both the cells expressing

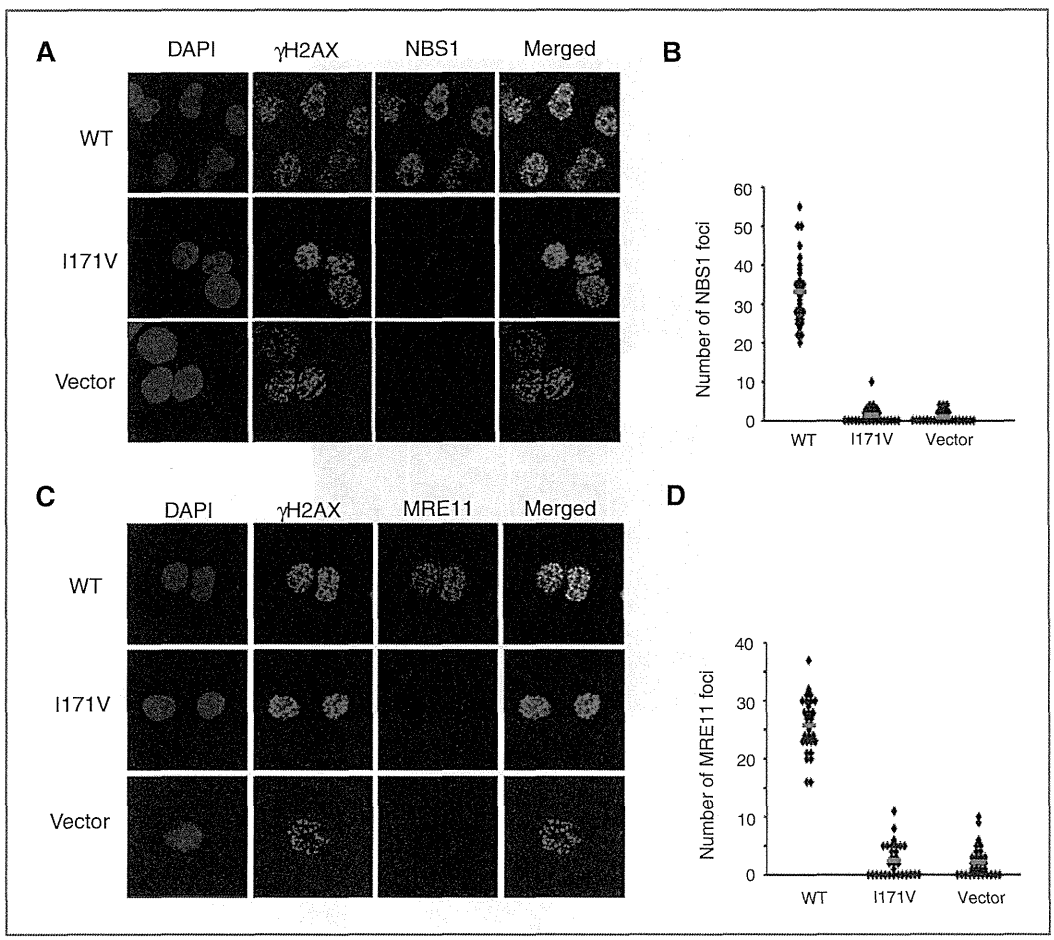

Figure 3. Reduced localization of NBS1 and MRE11 at the DSB sites in cells expressing an *NBS1-I171V* polymorphic variant. A and C, focus formation of NBS1, MRE11, and γH2AX. The GM07166VA7 cells expressing NBS1-WT (WT), NBS1-I171V (I171V), and the vector were irradiated with 10 Gy of IR. After 4 hours, the cells were incubated with antibodies directed against NBS1, γH2AX, or MRE11 and then stained with DAPI. B and D, scatter plots of NBS1 or MRE11 focus counts per cell in Fig. 3A or C are shown ($n = 30$). Red bars, median.

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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 B). 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 D). 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 that 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 Fig. 4 suggest that the decreased localization of NBS1-I171V to sites of IR-induced DSB results

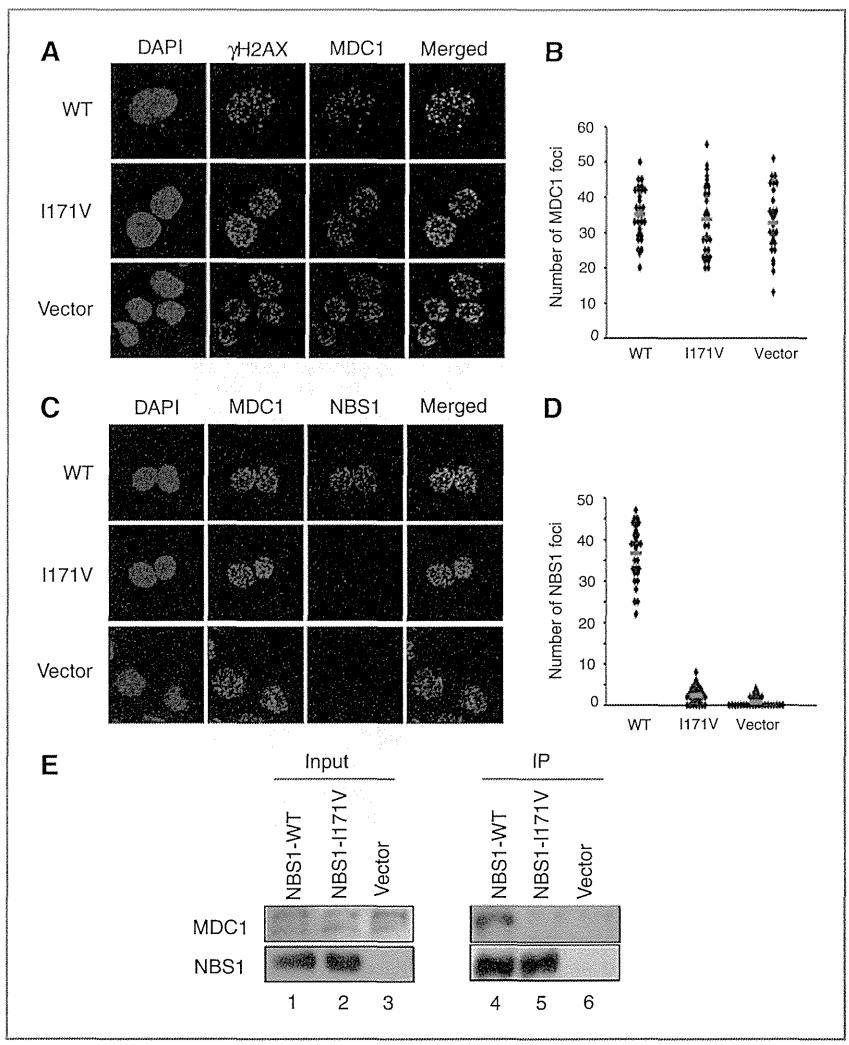

Figure 4. Reduced association of the NBS1-I171V polymorphic variant with MDC1. A and C, focus formation of γH2AX, MDC1, and NBS1. The GM07166VA7 cells expressing NBS1-WT (WT), NBS1-I171V (I171V), and the vector were irradiated with 10 Gy of IR. After 4 hours, the cells were incubated with antibodies directed against γH2AX, MDC1, or NBS1 and then stained with DAPI. B and D, scatter plots of MDC1 or NBS1 focus counts per cell in Fig. 4A or C are shown ($n = 30$). Red bars, 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 with anti-Flag antibody and then incubated with antibodies directed against MDC1 or NBS1.

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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 32 kDa 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 B). 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 D). 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 B), 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 *NBS1* gene is associated with cancer. It was reported that although null mutations in the mouse *NBS1* gene resulted in embryonic lethality at the

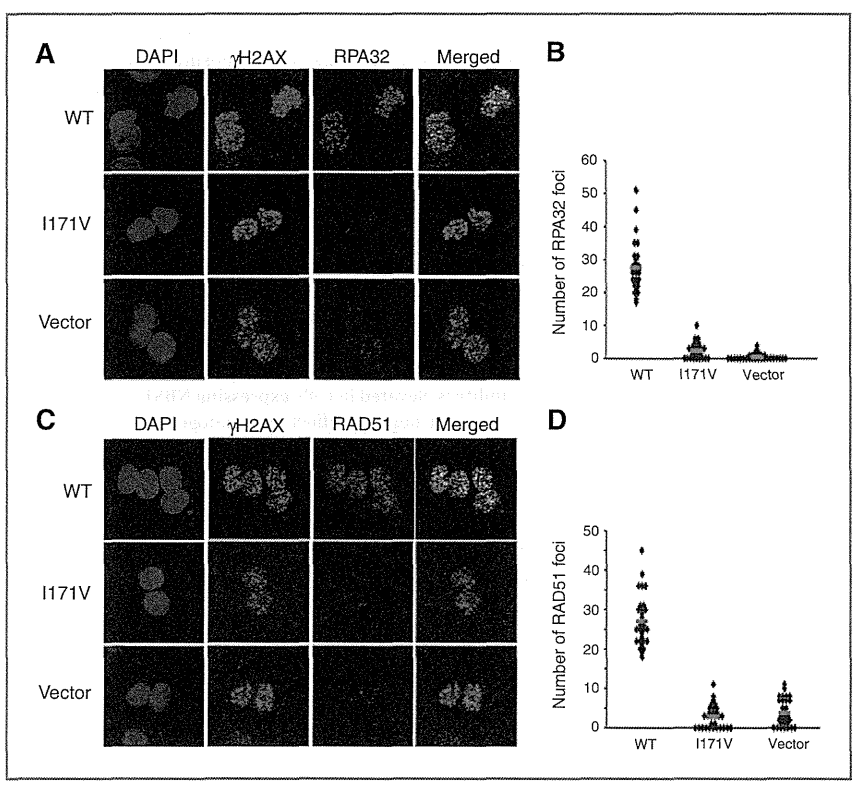

Figure 5. Reduced association of RPA32 and RAD51 at the DSB sites in cells expressing an *NBS1-I171V* polymorphic variant. A and C, focus formation of γH2AX, phospho-RPA32, and RAD51. The GM07166VA7 cells expressing NBS1-WT (WT), NBS1-I171V (I171V), and the vector were irradiated with 10 Gy of IR. After 4 hours, the cells were incubated with antibodies directed against γH2AX, phospho-RPA32, or RAD51 and then stained with DAPI. B and D, scatter plots of phospho-RPA32 or RAD51 focus counts per cell in Fig. 5A or C are shown ($n = 30$). Red bars, median.

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Table 1. Association of *NBS1* variation with breast cancer in Japanese

SNP Gene	Allele 1/2[a]	Groups	Case 11	Case 12	Case 22	RAF	P^b	OR[c] (95% CI)
rs61754966	G/A	Breast	0	23	1,501	0.0075	0.0048	3.19 (1.36–7.44)
NBS1		Control	0	7	1,455	0.0024		

NOTE: We analyzed 1,524 breast cancers and 1,462 controls.

[a]Allele 1, risk allele; Allele 2, nonrisk allele.

[b]*P* value and [c]OR were calculated in a dominant model (11 + 12 vs. 22).

blastocyst stage, heterozygous knockout ($NBS1^{+/-}$) 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_2 phases of the cell cycle (33). These reports strongly suggest that the *NBS1*-I171V polymorphic variant may increase breast cancer risk. Therefore, we analyzed the association of the *NBS1*-I171V variant with breast cancer in a Japanese population; patient characteristics are presented in Supplementary Table S1. Of the 1,524 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 ($n = 1{,}462$). None of the patients in the breast cancer group or in the control group carried the homozygous polymorphic variant of the *NBS1*-I171V substitution. The frequency of patients with the heterozygous *NBS1* polymorphic variant in the Japanese breast cancer group [OR, 3.19; 95% confidence interval (CI), 1.36–7.44; $P = 0.0048$] was significantly higher than that in the control group (Table 1). This result suggests that the *NBS1*-I171V variant increases the risk of breast cancer in Japanese women.

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 2- to 3-fold lower focus counts of NBS1, MRE11, phosphorylated RPA32, RAD51, or BRCA1 compared with the cell line expressing NBS1-WT or the vector (Supplementary Figs. 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 (DM), 7 chromatid gaps (CTG), and 8 chromatid breaks (CTB)/~1,600 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/~1,600 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

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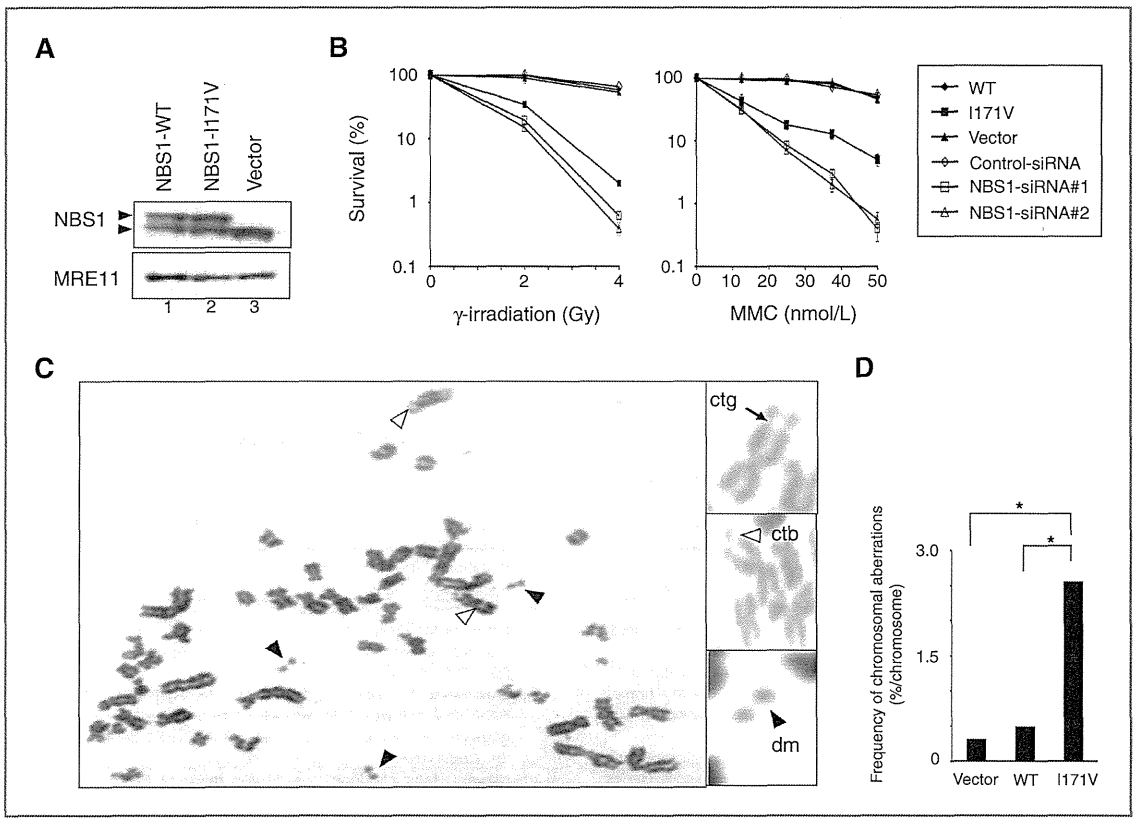

Figure 6. The DSB repair activity of endogenous NBS1 was repressed by the NBS1-I171V polymorphic variant in a dominant-negative manner. Expression vectors containing 3xFlag-His-HA-NBS1-WT cDNA, -NBS1-I171V cDNA, or no cDNA were stably transfected into HeLa cells. A, the expression levels of NBS1 in the 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, 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 nmol/L of MMC. Data, mean ± SEM ($n = 3$). C, a representative metaphase spread of the HeLa cells expressing NBS1-I171V after exposure to 6 Gy of IR. CTBs (white arrowheads), DMs (black arrowheads), and CTGs (arrows) are indicated. Noteworthy, structural chromosomal aberrations are shown at higher magnification in the right panels: CTG (top), CTB (middle), and DM (bottom). D, comparison of the frequencies of aberrations found in the cells used in A. *, $P < 0.005$.

Japanese population. Recently, it was reported that Mre11-mediated DDR restrains mammary hyperplasia by effecting an oncogene-induced G_2 arrest (34). Therefore, further research of NBS1-I171V function in the development of breast cancer could be necessary.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Yamamoto, T. Ohta
Development of methodology: Y. Yamamoto
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Yamamoto, M. Miyamoto, D. Tatsuda, M. Kubo, K. Matsuda, T. Watanabe
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Yamamoto, Y. Nakamura
Writing, review, and/or revision of the manuscript: Y. Yamamoto, H. Satoh, T. Watanabe, T. Ohta
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Kubo, H. Satoh, T. Watanabe
Study supervision: H. Nakagama, Y. Nakamura

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New Susceptibility and Resistance HLA-DP Alleles to HBV-Related Diseases Identified by a Trans-Ethnic Association Study in Asia

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Abstract

Previous studies have revealed the association between SNPs located on human leukocyte antigen (*HLA*) class II genes, including *HLA-DP* and *HLA-DQ*, and chronic hepatitis B virus (HBV) infection, mainly in Asian populations. *HLA-DP* alleles or haplotypes associated with chronic HBV infection or disease progression have not been fully identified in Asian populations. We performed trans-ethnic association analyses of *HLA-DPA1*, *HLA-DPB1* alleles and haplotypes with hepatitis B virus infection and disease progression among Asian populations comprising Japanese, Korean, Hong Kong, and Thai subjects. To assess the association between *HLA-DP* and chronic HBV infection and disease progression, we conducted high-resolution (4-digit) *HLA-DPA1* and *HLA-DPB1* genotyping in a total of 3,167 samples, including HBV patients, HBV-resolved individuals and healthy controls. Trans-ethnic association analyses among Asian populations identified a new risk allele *HLA-DPB1*09:01* ($P = 1.36 \times 10^{-6}$; OR = 1.97; 95% CI, 1.50–2.59) and a new protective allele *DPB1*02:01* ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) to chronic HBV infection, in addition to the previously reported alleles. Moreover, *DPB1*02:01* was also associated with a decreased risk of disease progression in chronic HBV patients among Asian populations ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65). Trans-ethnic association analyses identified Asian-specific associations of *HLA-DP* alleles and haplotypes with HBV infection or disease progression. The present findings will serve as a base for future functional studies of HLA-DP molecules in order to understand the pathogenesis of HBV infection and the development of hepatocellular carcinoma.

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Introduction

Hepatitis B virus (HBV) infection is a major global health problem, resulting in 0.5–1.0 million deaths per year [1]. The prevalence of chronic HBV infection varies. About 75% of the chronic carriers in the world live in Southeast Asia and East Pacific [2]. Due to the introduction of vaccination programs, the prevalence of HBV infection in many countries has gradually been decreasing with consequent decreases in HBV-related hepatocellular carcinoma (HCC) [3]. Although some HBV carriers spontaneously eliminate the virus, about 10–15% of carriers develop liver cirrhosis (LC), liver failure and HCC [4]. Moreover, the progression of liver disease was revealed to be associated with the presence of several distinct mutations in HBV infections [5]. Genetic variations in *STAT4* and *HLA-DQ* genes were recently identified as host genetic factors in a large-scale genome-wide association study (GWAS) for HBV-related HCC in China [6].

With regard to the genes associated with susceptibility to chronic HBV infection, *HLA-DP* and *HLA-DQ* genes were identified by GWAS in Japanese and Thai populations in 2009 [7] and 2011 [8], respectively. In addition, our previous GWAS confirmed and identified the association of SNP markers located on *HLA-DPA1* (rs3077) and *HLA-DPB1* (rs9277535) genes with susceptibility to chronic hepatitis B (CHB) and HBV clearance in Japanese and Korean subjects[9]. The significant associations of *HLA-DP* with CHB and HBV clearance have mainly been detected in Asian populations, such as Japanese [8,9], Thai [7], Chinese [10–12], and Korean [9]. In 2012, the association between *HLA-DPA1* gene SNPs and persistent HBV infection was replicated in a Germany non-Asian population for the first time; however, this showed no association with HBV infection [13]. These results seem to be explained by the fact that allele frequencies of both rs3077 (0.155, 0.587 and 0.743 for C allele, on HapMap CEU, JPT, and YRI) and rs9277535 (0.261, 0.558 and 0.103 for G allele, on HapMap CEU, JPT, and YRI) are markedly different between populations. Moreover, the previous study showed that HBsAg seropositivity rates were higher in Thailand and China (5–12%) than in North America and Europe (0.2–0.5%) [2]. These results suggest that comparative analyses of *HLA-DP* alleles and haplotypes in Asian populations would clarify key host factors of the susceptible and protective *HLA-DP* alleles and haplotypes for CHB and HBV clearance. Here, we performed trans-ethnic analyses of *HLA-DP* alleles and haplotypes in Asian populations comprising Japanese, Korean, Hong Kong and Thai individuals. The findings from this study will serve as a base for future functional studies of HLA-DP molecules.

Results

Characteristics of studied subjects

The characteristics of a total of 3,167 samples, including Japanese, Korean, Hong Kong and Thai subjects, are shown in Table 1. Each population included three groups of HBV patients, resolved individuals and healthy controls. The clinical definitions of HBV patients and resolved individuals are summarized in Materials and Methods. Some of the Japanese and all of the Korean samples overlapped with the subjects in our previous study [9,14].

We performed genotyping for *HLA-DPA1* and *HLA-DPB1* in all 3,167 samples, and a total of 2,895 samples were successfully genotyped. The characteristics of successfully genotyped samples are shown in Table S1.

Association of *HLA-DPA1* and *HLA-DPB1* alleles in Asian populations

As for a general Asian population, including 464 Japanese, 140 Korean, 156 Hong Kong, and 122 Thai subjects, five *HLA-DPA1* alleles and twenty-four *HLA-DPB1* alleles were observed (Table S2). The frequencies of *HLA-DPA1* and *HLA-DPB1* alleles were similar between Japanese and Korean subjects. On the other hand, the number of alleles with frequencies of 1–2% was larger in Hong Kong and Thai populations, despite the small sample size. Although the frequencies of *HLA-DP* alleles varied in Asian populations, *HLA-DPB1*05:01* was the most prevalent with over 30% in all populations.

The associations of *HLA-DPA1* and *HLA-DPB1* alleles with chronic HBV infection (i.e., comparison between HBV patients and healthy controls) are shown in Table S2. To avoid false positives caused by multiple testing, the significance levels were corrected based on the numbers of *HLA-DPA1* and *HLA-DPB1*

Table 1. Number of individuals in this study.

Population	Japanese	Korean	Hong Kong	Thai
Total number of samples	1,291	586	661	629
HBV patients	489	340	281	390
IC	114	-	-	-
CH	147	175	187	198
AE	21	-	-	-
LC	38	-	-	-
HCC	169	165	94	192
Mean age (y)	57.1	44.7	57.9	52.0
(min-max)	(20–84)	(18–74)	(32–86)	(21–84)
Gender (M/F)	338/151	265/75	239/42	289/101
Resolved individuals*	335	106	190	113
HCV (−)	249	106	190	113
HCV (+)	86	-	-	-
Mean age (y)	59.7	43.1	40.0	48.2
(min-max)	(18–87)	(12–66)	(18–60)	(39–66)
Gender (M/F)	173/162	61/45	113/77	83/30
Healthy controls	467	140	190	126
Mean age (y)	39.0**	33.7	26.2	46.6
(min-max)	(23–64)	(1–59)	(16–60)	(38–79)
Gender (M/F)	370/97	67/73	87/103	73/53

Abbreviation: IC, Inactive Carrier; CH, Chronic Hepatitis; AE, Acute Exacerbation; LC, Liver Cirrhosis; HCC, Hepatocellular Carcinoma.
* Resolved individuals were HBsAg negative and HBcAb positive.
** 419 of 467 healthy controls were de-identified, without information on age.
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alleles in the focal population. Briefly, the significance level was set at 0.05/(# of observed alleles at each locus) in each population (see Materials and Methods). With regard to high-risk alleles of *HLA-DPA1*, the most prevalent allele *HLA-DPA1*02:02* was significantly associated with susceptibility to HBV infection in Japanese ($P = 3.45 \times 10^{-4}$; OR = 1.39; 95% CI, 1.16–1.68) and Korean subjects ($P = 2.66 \times 10^{-5}$; OR = 1.89; 95% CI, 1.39–2.58), whereas this association was not observed in Hong Kong or Thai subjects. The association of *HLA-DPA1*02:01* with susceptibility to HBV infection was significant only in Japanese ($P = 2.61 \times 10^{-7}$; OR = 1.88; 95% CI, 1.46–2.41). The significant association of *HLA-DPA1*01:03* with protection against HBV infection was commonly observed among four Asian populations (Table S2). The pooled OR and 95% CI were 0.51 and 0.41–0.63, respectively in a meta-analysis ($P = 3.15 \times 10^{-10}$) (Fig. S1A).

As shown in Table S2, *HLA-DPB1* shows higher degree of polymorphism than *HLA-DPA1*. The most common allele in Asian populations, *HLA-DPB1*05:01*, was significantly associated with HBV susceptibility in both Japanese and Korean subjects. Although *HLA-DPB1*05:01* showed no significant association in the Hong Kong and Thai populations, the same direction of association (i.e., HBV susceptibility) was observed. Meta-analysis of the four populations revealed a significant association between *HLA-DPB1*05:01* and susceptibility to HBV infection ($P = 1.51 \times 10^{-4}$; OR = 1.45; 95% CI, 1.19–1.75) (Fig. S1B). The frequency of *HLA-DPB1*09:01* was significantly elevated in Japanese HBV patients (15.7%) as compared with healthy controls (8.7%) ($P = 3.70 \times 10^{-6}$; OR = 1.94; 95% CI, 1.45–2.62), and this association was most significant (i.e., the smallest P value) in the Japanese population. Because of lower allele frequencies of *HLA-DPB1*09:01* or lack of statistical power in the other populations, no significant associations were observed. A common allele in Thai subjects, *HLA-DPB1*13:01*, was significantly associated with susceptibility to HBV infection ($P = 2.49 \times 10^{-4}$; OR = 2.17; 95% CI, 1.40–3.47) with the same direction of associations in Japanese and Hong Kong (OR = 1.52 and 1.40, respectively).

*HLA-DPB1*04:02* was identified as the most protective allele for HBV infection in Japanese ($P = 1.59 \times 10^{-7}$; OR = 0.37; 95% CI, 0.24–0.55) and Korean subjects ($P = 1.27 \times 10^{-7}$; OR = 0.19; 95% CI, 0.10–0.38). Both *HLA-DPB1*02:01* and *HLA-DPB1*04:01* were also significantly associated with protection in the Japanese population, and the former was significantly associated with protection in Hong Kong subjects ($P = 9.17 \times 10^{-4}$; OR = 0.49; 95% CI, 0.32–0.76). This common allele among four Asian populations, *HLA-DPB1*02:01*, showed a significant association with protection against HBV infection ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) in a meta-analysis (Fig. S1B).

The frequencies of associated *HLA-DP* alleles in a comparison of HBV patients with healthy controls (Table S2) or with HBV-resolved individuals (Table S3) were similar in all four Asian populations. In the Japanese population, the associations of susceptible and protective *HLA-DPB1* alleles to chronic HBV infection seem weaker in the comparison of HBV patients with HBV-resolved individuals than in the comparison of HBV patients with healthy controls. Moreover, the results of association analyses showed no difference in the comparison of HBV patients with HBV-resolved individuals, including or excluding HCV positive individuals (Table S3). In contrast, the association became stronger in the comparison of HBV patients with HBV-resolved individuals among the Korean subjects. The protective allele *HLA-DPB1*04:01* was also identified to have a strong association with HBV clearance in Hong Kong subjects (Table S3). Moreover, in Hong Kong subjects, the *HLA-DPB1*05:01* associated with the risk for HBV infection showed lower frequency in HBV-resolved individuals (42.9%) than in the healthy controls (48.1%), which accounts for a strong association in the comparison of HBV patients with HBV-resolved individuals ($P = 6.24 \times 10^{-3}$; OR = 1.64; 95% CI, 1.14–2.36). Although the number of samples was insufficient, *HLA-DP*100:01* showed a significant association with protection against HBV infection in the Hong Kong population ($P = 3.05 \times 10^{-6}$; OR = 0.03; 95% CI, 0.0007–0.20).

Table 2. Association of number of *DPB1*02:01* alleles (i.e., 0, 1 or 2) with disease progression in CHB patients assessed by multivariate logistic regression analysis adjusted for age and sex.

Population	P value	OR (95% CI)
Japanese	0.000177	0.47 (0.32–0.70)
Korean	0.025358	0.55 (0.33–0.93)
Hong Kong	0.040842	0.46 (0.22–0.97)
Thai	0.087782	0.58 (0.31–1.08)
All*	1.55×10^{-7}	0.50 (0.39–0.65)

*Population was adjusted using dummy variables.

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As for disease progression in CHB patients among Asian populations, a protective effect of *HLA-DPB1*02:01* on disease progression was observed in the Japanese ($P = 4.26 \times 10^{-5}$; OR = 0.45; 95% CI, 0.30–0.67) and Korean populations ($P = 8.74 \times 10^{-4}$; OR = 0.47; 95% CI, 0.29–0.75) (Table S4). Multivariate logistic regression analysis adjusted for age and sex revealed that the number of *DPB1*02:01* alleles (i.e., 0, 1, or 2) was significantly associated with disease progression in CHB patients in Japanese ($P = 1.77 \times 10^{-4}$; OR = 0.47; 95% CI, 0.32–0.70) (Table 2). Moreover, protective effects of *DPB1*02:01* on disease progression in Asian populations ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65) were detected in a multivariate logistic regression analysis adjusted for age, gender, and population (Table 2).

Associations of *DPA1-DPB1* haplotypes in Asian populations

The estimated frequencies of *HLA DPA1-DPB1* haplotypes are shown in Table S5. The most frequent haplotype among the four Asian populations was *DPA1*02:02-DPB1*05:01*. The number of haplotypes with low frequencies of 1–2% was 10 in both Japanese and Korean subjects, whereas more haplotypes appeared with frequencies of 1–2% in Hong Kong and Thai subjects. The associations of *DPA1-DPB1* haplotypes with HBV infection are shown in Table S5. In the Japanese population, *DPA1*02:01-DPB1*09:01* showed the most significant association with susceptibility to HBV infection ($P = 3.38 \times 10^{-6}$; OR = 1.95; 95% CI, 1.46–2.64). The most common haplotype in the four Asian populations, *DPA1*02:02-DPB1*05:01*, was found to be significantly associated with susceptibility to HBV infection in the Japanese and Korean subjects ($P = 7.40 \times 10^{-4}$; OR = 1.37; 95% CI, 1.14–1.66 for Japanese, and $P = 4.50 \times 10^{-6}$; OR = 2.02; 95% CI, 1.48–2.78 for Korean). In the Thai subjects, *HLA-DPB1*13:01* was the most significant risk allele for HBV infection (Table S2); however, no significant associations were found for the three different haplotypes bearing *HLA-DPB1*13:01*: *DPA1*02:01-DPB1*13:01*, *DPA1*02:02-DPB1*13:01*, and *DPA1*04:01-DPB1*13:01*,indicating that the association of *HLA-DPB1*13:01* with susceptibility to HBV infection did not result from a specific *DPA1-DPB1* haplotype or combination with a specific *DPA1* allele.

In the Japanese population, both haplotypes *DPA1*01:03-DPB1*04:01* and *DPA1*01:03-DPB1*04:02* showed significant associations with protection against HBV infection ($P = 1.17 \times 10^{-5}$; OR = 0.32; 95% CI, 0.18–0.56 for *DPA1*01:03-DPB1*04:01* and $P = 1.95 \times 10^{-7}$; OR = 0.37; 95% CI, 0.24–0.55 for *DPA1*01:03-DPB1*04:02*). In the Korean subjects, a significant association of *DPA1*01:03-DPB1*04:02* was also demonstrated; however, no association was observed for *DPA1*01:03-DPB1*04:01*. Because the observed number of each haplotype was small, none of the other haplotypes showed a significant association with protection against HBV infection.

In order to identify trans-ethnic DPA1-DPB1 haplotypes associated with HBV infection, a meta-analysis was performed. A meta-analysis further revealed that the *DPA1*01:03-DPB1*02:01* haplotype was significantly associated with protection against HBV infection ($P = 1.45 \times 10^{-5}$; OR = 0.69; 95% CI, 0.58–0.82) (Fig. S1C).

Discussion

Among 2.2 billion individuals worldwide who are infected with HBV, 15% of these are chronic carriers. Of chronic carriers, 10–15% develops LC, liver failure and HCC, and the remaining individuals eventually achieve a state of nonreplicative infection, resulting in HBsAg negative and anti-HBc positive, i.e. HBV-resolved individuals. To identify host genetic factors associated with HBV-related disease progression may lead HBV patients to discriminate individuals who need treatment.

The *HLA-DPA1* and *HLA-DPB1* genes were identified as host genetic factors significantly associated with CHB infection, mainly in Asian populations [7–12], and not in European populations [13]. In the previous association analyses of *HLA-DPB1* alleles with HBV infection, one risk allele *HLA-DPB1*05:01* (OR = 1.52; 95% CI, 1.31–1.76), and two protective alleles, *HLA-DPB1*04:01* (OR = 0.53; 95% CI, 0.34–0.80) and *HLA-DPB1*04:02* (OR = 0.47; 95% CI, 0.34–.64), were identified in the Japanese population [7]. In this study, we further identified a new risk allele *HLA-DPB1*09:01* (OR = 1.94; 95% CI, 1.45–2.62) for HBV infection and a new protective allele *HLA-DPB1*02:01* (OR = 0.71; 95% CI, 0.56–0.89) in the Japanese population, in addition to the previously reported alleles (Table S2) [7]. The discrepancy in the association of *HLA-DPB1*09:01* allele with risk for HBV infection in a previous study [7] results from the elevated frequency of *HLA-DPB1*09:01* in the controls (12.2%), which is higher than our controls (8.7%). In this study, healthy subjects were recruited as controls. In contrast, individuals that were registered in BioBank Japan as subjects with diseases other than CHB were recruited as controls in the previous study [7], which may have included patients with diseases with which *HLA-DPB1*09:01* is associated. Although no significant association of *HLA-DPB1*09:01* with risk for HBV infection was observed in the Korean subjects, *HLA-DPB1*09:01* appears to have a susceptible effect on HBV infection, as it showed the same direction of association. When the association analyses in Japanese and Korean subjects were combined in meta-analysis, the association was statistically significant ($P = 1.36 \times 10^{-6}$; OR = 1.97; 95% CI, 1.50–2.59). Thus, *HLA-DPB1*09:01* may be a Northeast Asian-specific allele associated with risk for HBV infection.

Moreover, a significant association of *HLA-DPB1*13:01* with risk of HBV infection (OR = 2.17; 95% CI, 1.40–3.47) was identified in the Thai subjects. However, the frequency of *HLA-DPB1*13:01* in Thai healthy controls (11.5% in the present study) reportedly varies, ranging from 15.4% to 29.5%, due to the population diversity [15–17]. Therefore, a replication analysis is required to confirm the association of *HLA-DPB1*13:01* with HBV infection in the Thai subjects. There were four other marginally associated *HLA-DPB1* alleles with low allele frequencies below 5% in HBV patients and healthy controls, including *HLA-DPB1*28:01*, *-DPB1*31:01*, *-DPB1*100:01*, and *-DPB1*105:01*, in the Hong Kong and Thai subjects. Because these infrequent alleles may have resulted from false positive associations, the association needs to be validated in a large number of subjects.

*HLA-DPB1*02:01* showed a significant association with protection against HBV infection in both Japanese and Hong Kong populations (Table S2); however, the *HLA-DPB1*02:01* allele was not associated with HBV infection in the previous study [7]. Although *HLA-DPB1*02:01* showed no association in either Korean or Thai populations, a significant association of *HLA-DPB1*02:01* with protection against HBV infection among four Asian populations was detected in meta-analysis ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) (Fig. S1B). We therefore conclude that the present finding is not a false positive.

A recent report showed that *HLA-DPB1*02:01:02*, **02:02*, **03:01:01*, **04:01:01*, **05:01*, **09:01*, and **14:01* were significantly associated with response to booster HB vaccination in Taiwan neonatally vaccinated adolescents [18]. The *HLA-DPB1*02:01:02*, **02:02*, **03:01:01*, **04:01:01*, and **14:01* were significantly more frequent in recipients whose post-booster titers of antibodies against HBV surface antigen (anti-HBs) were detectable, on the other hand, *HLA-DPB1*05:01* and **09:01* were significantly more frequent in recipients who were undetectable. Moreover, the *HLA-DPB1*05:01* and **09:01* significantly increase the likelihoods of undetectable pre-booster anti-HBs titers. These results seem consistent with our findings, in which *HLA-DPB1*05:01* and **09:01* are associated with susceptibility to chronic hepatitis B infection.

We also identified a protective effect of *HLA-DPB1*02:01* allele on disease progression in Asian populations. Previous studies identified the association of HLA class II genes including *HLA-DQ* and *HLA-DR* with development of HBV related hepatocellular carcinoma in the Chinese population [6,19,20]. In this study using Japanese and Korean samples, we identified significant associations between *HLA-DPB1*02:01* and disease progression in CHB patients ($P = 4.26 \times 10^{-5}$; OR = 0.45; 95% CI, 0.30–0.67, for Japanese and $P = 8.74 \times 10^{-4}$; OR = 0.47; 95% CI, 0.29–0.75 for Korean) (Table S4). Although the association of *HLA-DPB1*02:01* with disease progression was weaker after adjustment for age and gender in Korean subjects ($P = 2.54 \times 10^{-2}$; OR = 0.55; 95% CI, 0.33–0.93), the same direction of association was observed (i.e. protective effect on disease progression) (Table 2). The protective effects of *HLA-DPB1*02:01* on disease progression showed a significant association after adjustment for age and gender in the Japanese population ($P = 1.77 \times 10^{-4}$; OR = 0.47; 95% CI, 0.32–0.70); moreover, a significant association between *HLA-DPB1*02:01* was observed among four Asian populations, under which population was adjusted by using dummy variables in a multivariate logistic regression analysis ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65) (Table 2).

The *HLA-DPA1* and *HLA-DPB1* belong to the HLA class II alpha and beta chain paralogues, which make a heterodimer consisting of an alpha and a beta chain on the surface of antigen presenting cells. This HLA class II molecule plays a central role in the immune system by presenting peptides derived from extracellular proteins. We identified two susceptible haplotypes (*DPA1*02:02-DPB1*05:01* and *DPA1*02:01-DPB1*09:01*) and three protective haplotypes (*DPA1*01:03-DPB1*04:01*, *DPA1*01:03-DPB1*04:02*, and *HLA-DPA1*01:03-DPB1*02:01*) to chronic hepatitis B infection, which may result in different binding

affinities between HLA-DP subtypes and extracellular antigens. Although functional analyses of HLA-DP subtypes to identify HBV-related peptides are not fully completed, identification of susceptible and protective haplotypes as host genetic factors would lead us to understand the pathogenesis of HBV infection including viral factors.

In summary, we identified a new risk allele *HLA-DPB1*09:01*, which was specifically observed in Northeast Asian populations, Japanese and Korean. Moreover, a new protective allele *HLA-DPB1*02:01* was identified among four Asian populations: Japanese, Korean, Hong Kong and Thai. The protective allele *HLA-DPB1*02:01* was associated with both chronic HBV infection and disease progression in chronic HBV patients. Identification of a total of five alleles, including two risk alleles (*DPB1*09:01* and *DPB1*05:01*) and three protective alleles (*DPB1*04:01*, *DPB1*04:02* and *DPB1*02:01*), would enable HBV-infected individuals to be classified into groups according to the treatment requirements. Moreover, the risk and protective alleles for HBV infection and disease progression, identified in this study by means of trans-ethnic association analyses, would be key host factors to recognize HBV-derived antigen peptides. The present results may lead to subsequent functional studies into HLA-DP molecules and viral factors in order to understand the pathogenesis of HBV infection and development of hepatocellular carcinoma.

Materials and Methods

Ethics Statement

All study protocols conform to the relevant ethical guidelines, as reflected in the *a priori* approval by the ethics committee of National Center for Global Health and Medicine, and by the ethics committees of all participating universities and hospitals, including The University of Tokyo, Japanese Red Cross Kanto-Koshinetsu Block Blood Center, The University of Hong Kong, Chulalongkorn University, Yonsei University College of Medicine, Nagoya City University Graduate School of Medical Sciences, Musashino Red Cross Hospital, Tokyo Medical and Dental University, Teine Keijinkai Hospital, Hokkaido University Graduate School of Medicine, Kurume University School of Medicine, Okayama University Graduate School of Medicine, Yamaguchi University Graduate School of Medicine, Tottori University, Kyoto Prefectural University of Medicine, Osaka City University Graduate School of Medicine, Nagoya Daini Red Cross Hospital, Ehime University Graduate School of Medicine, Kanazawa University Graduate School of Medicine, National Hospital Organization Osaka National Hospital, Iwate Medical University, Kawasaki Medical College, Shinshu University School of Medicine, Saitama Medical University, Kitasato University School of Medicine, Saga Medical School, and University of Tsukuba.

Written informed consent was obtained from each patient who participated in this study and all samples were anonymized. For Japanese healthy controls, 419 individuals were de-identified with information about gender, and all were recruited after obtaining verbal informed consent in Tokyo prior to 1990. For the 419 Japanese healthy individuals, written informed consent was not obtained because the blood sampling was conducted before the "Ethical Guidelines for Human Genome and Genetic Sequencing Research" were established in Japan. Under the condition that DNA sample is permanently de-linked from the individual, this study was approved by the Research Ethics Committee of National Center for Global Health and Medicine.

Characteristics of studied subjects

All of the 3,167 genomic DNA samples were collected from individuals with HBV, HBV-resolved individuals (HBsAg-negative and anti-HBc-positive) and healthy controls at 26 multi-center hospitals throughout Japan, Korea, Hong Kong, and Thailand (Table 1). In a total of 1,291 Japanese and 586 Korean samples, 1,191 Japanese individuals and all 586 Korean individuals were included in our previous study [9]. With regard to additional Japanese individuals, we collected samples from 48 healthy controls at Kohnodai Hospital, and 52 HBV patients at Okayama University Hospital and Ehime University Hospital, including 26 individuals with LC and 26 individuals with HCC. A total of 661 Hong Kong samples and 629 Thai samples were collected at Queen Mary Hospital and Chulalongkorn University, respectively.

HBV status was measured based on serological results for HBsAg and anti-HBc with a fully automated chemiluminescent enzyme immunoassay system (Abbott ARCHITECT; Abbott Japan, Tokyo, Japan, or LUMIPULSE f or G1200; Fujirebio, Inc., Tokyo, Japan). For clinical staging, inactive carrier (IC) state was defined by the presence of HBsAg with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of liver cirrhosis. Chronic hepatitis (CH) was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/L]) persisting over 6 months (by at least 3 bimonthly tests). Acute exacerbation (AE) of chronic hepatitis B was defined as an elevation of ALT to more than 10 times the upper limit of normal (ULN, 58 IU/L) and bilirubin to at least three times ULN (15 μmol/L). LC was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges and hypersplenism), platelet counts<100,000/cm^3, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. HCC was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy or a combination thereof.

The Japanese control samples from HBV-resolved subjects (HBsAg-negative and anti-HBc-positive) at Nagoya City University-affiliated healthcare center were used by comprehensive agreement (anonymization in a de-identified manner) in this study. Some of the unrelated and anonymized Japanese healthy controls were purchased from the Japan Health Science Research Resources Bank (Osaka, Japan). One microgram of purified genomic DNA was dissolved in 100 μl of TE buffer (pH 8.0) (Wako, Osaka, Japan), followed by storage at −20°C until use.

Genotyping of *HLA-DPA1* and *HLA-DPB1* alleles

High resolution (4-digit) genotyping of *HLA-DPA1* and *-DPB1* alleles was performed for HBV patients, resolved individuals, and healthy controls in Japan, Korea, Hong Kong, and Thailand. LABType SSO HLA DPA1/DPB1 kit (One Lambda, CA) and a Luminex Multi-Analyte Profiling system (xMAP; Luminex, Austin, TX) were used for genotyping, in according with the manufacturer's protocol. Because of the small quantity of genomic DNA in some Korean samples, we performed whole genome amplification for a total of 486 samples using GenomiPhi v2 DNA Amplification kit (GE Healthcare Life Sciences, UK), in accordance with the manufacturer's instruction.

A total of 2,895 samples were successfully genotyped and characteristics of these samples are summarized in Table S1.

Statistical analysis

Fisher's exact test in two-by-two cross tables was used to examine the associations between *HLA-DP* allele and chronic HBV infection or disease progression in chronic HBV patients,

using statistical software R2.9. To avoid false-positive results due to multiple testing, significance levels were adjusted based on the number of observed alleles at each locus in each population. For *HLA-DPA1* alleles, the number of observed alleles was 3 in Japanese, 4 in Korean, 5 in Hong Kong, and 5 in Thai subjects. Therefore, the significant levels for α were set at $\alpha = 0.05/3$ in Japanese, $\alpha = 0.05/4$ in Korean, $\alpha = 0.05/5$ in Hong Kong, and $\alpha = 0.05/5$ in Thai subjects. In the same way, significant levels for *HLA-DPB1* alleles were $\alpha = 0.05/10$, $0.05/11$, $0.05/12$, and $0.05/16$, respectively. Multivariate logistic regression analysis adjusted for age and sex (used as independent variables) was applied to assess associations between the number of *DPB1*02:01* alleles (i.e., 0, 1, or 2) and disease progression in CHB patients. To examine the effect of *DPB1*02:01* allele on disease progression in all populations, population was further adjusted by using three dummy variables (i.e., (c1, c2, c3) = (0, 0, 0) for Japanese, (1, 0, 0) for Korean, (0, 1, 0) for Hong Kong, and (0, 0, 1) for Thai) in a multivariate logistic regression analysis. We obtained the following regression equation: logit(p) = −3.905+0.083*age+(−0.929)*sex+(−0.684)**DPB1*02:01*+1.814*c1+(−0.478)*c2+0.782*c3. Significance levels in the analysis of disease progression in CHB patients were set as $\alpha = 0.05/10$ in Japanese, $\alpha = 0.05/11$ in Korean, $\alpha = 0.05/15$ in Hong Kong, and $\alpha = 0.05/15$ in Thai subjects. The phase of each individual (i.e., a combination of two *DPA1-DPB1* haplotypes) was estimated using PHASE software [21], assuming samples are selected randomly from a general population. In comparison of the estimated *DPA1-DPB1* haplotype frequencies, significant levels were set as $\alpha = 0.05/14$ in Japanese, $\alpha = 0.05/17$ in Korean, $\alpha = 0.05/17$ in Hong Kong, and $\alpha = 0.05/18$ in Thai subjects. Meta-analysis was performed using the DerSimonian-Laird method (random-effects model) in order to calculate pooled OR and its 95% confidence interval (95% CI). We applied meta-analysis for alleles with frequency>1% in all four Asian populations. The significance levels in meta-analysis were adjusted by the total number of statistical tests; $\alpha = 0.05/20$ for *DPA1* alleles, $\alpha = 0.05/57$ for *DPB1* alleles, and $\alpha = 0.05/74$ for *DPA1-DPB1* haplotypes.

Supporting Information

Figure S1 Comparison of odds ratios in association analyses for *HLA-DP* with chronic HBV infection among four Asian populations: (A) *HLA-DPA1* alleles; (B) *HLA-DPB1* alleles; and (C) *HLA DPA1-DPB1* haplotypes. Meta-analysis was performed using the DerSimonian-Laird method (random-effects model) to calculate pooled OR and its 95% confidence interval (95% CI). Bold depicts a statistically significant association after correction of significance level.
(DOCX)

Table S1 Individuals with successfully genotyped for *HLA-DPA1* and *HLA-DPB1*.
(DOCX)

Table S2 Frequencies of HLA-DP alleles in HBV patients and healthy controls among Asian populations.
(XLSX)

Table S3 Frequencies of HLA-DP alleles in HBV patients and resolved individuals among Asian populations.
(XLSX)

Table S4 Associations of HLA-DPB1 alleles with disease progression in CHB patients among Asian populations.
(XLSX)

Table S5 Estimated frequencies of HLA DPA1-DPB1 haplotypes in HBV patients and healthy controls among Asian populations.
(XLSX)

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Author Contributions

Conceived and designed the experiments: NN HS MS KT M. Mizokami. Performed the experiments: NN HS KK Y. Mawatari M. Kawashima M. Minami. Analyzed the data: NN HS M. Kawashima JO. Contributed reagents/materials/analysis tools: W-KS M-FY NP YP SHA K-HH K. Matsuura YT M. Kurosaki YA NI J-HK SH TI KY IS Y. Murawaki YI AT EO YH MH SK EM KS KH ET SM MW YE NM K. Murata M. Korenaga KT M. Mizokami. Wrote the paper: NN HS JO KT M. Mizokami.

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Risk Factors for Long-Term Persistence of Serum Hepatitis B Surface Antigen Following Acute Hepatitis B Virus Infection in Japanese Adults

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The proportion of patients who progress to chronicity following acute hepatitis B (AHB) varies widely worldwide. Moreover, the association between viral persistence after AHB and hepatitis B virus (HBV) genotypes in adults remains unclear. A nationwide multicenter study was conducted throughout Japan to evaluate the influence of clinical and virological factors on chronic outcomes in patients with AHB. For comparing factors between AHB patients with viral persistence and those with self-limited infection, 212 AHB patients without human immunodeficiency virus (HIV) coinfection were observed in 38 liver centers until serum hepatitis B surface antigen (HBsAg) disappeared or a minimum of 6 months in cases where HBsAg persisted. The time to disappearance of HBsAg was significantly longer for genotype A patients than that of patients infected with non-A genotypes. When chronicity was defined as the persistence of HBsAg positivity for more than 6 or 12 months, the rate of progression to chronicity was higher in patients with genotype A, although many cases caused by genotype A were prolonged cases of AHB, rather than chronic infection. Multivariate logistic regression analysis revealed only genotype A was independently associated with viral persistence following AHB. A higher peak level of HBV DNA and a lower peak of alanine aminotransferase (ALT) levels were characteristics of AHB caused by genotype A. Treatment with nucleotide analogs (NAs) did not prevent progression to chronic infection following AHB overall. Subanalysis suggested early NA initiation may enhance the viral clearance. *Conclusion*: Genotype A was an independent risk factor for progression to chronic infection following AHB. Our data will be useful in elucidating the association between viral persistence after AHB, host genetic factors, and treatment with NAs in future studies. (HEPATOLOGY 2014;59:89-97)

Abbreviations: AHB, acute hepatitis B; ALT, alanine aminotransferase; anti-HBc, antibody to hepatitis B core antigen; anti-HBs, antibody to HBsAg; HBeAg, hepatitis B e-antigen; CLIA, chemiluminescent enzyme immunoassay; EIA, enzyme immunoassay; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HIV, human immunodeficiency virus; IgM, immunoglobulin M; anti-HBe, antibody to HBeAg; NAs, nucleotide analogs; RPHA, reverse passive hemagglutination.

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