

# A frequent nonsense mutation in exon 1 across certain HLA-A and HLA-B alleles in leukocytes of patients with acquired aplastic anemia



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## ABSTRACT

Leukocytes that lack expression of HLA alleles are frequently detected in patients with acquired aplastic anemia (AA) who respond to immunosuppressive therapy, although the exact mechanisms underlying the HLA loss and HLA allele repertoire likely to acquire loss-of-function mutations are unknown. We identified a common nonsense mutation at codon 19 (c.19C>T, p.R7X) in exon 1 (*Exon1<sup>mut</sup>*) of different HLA-A and -B alleles in HLA-lacking granulocytes from AA patients. A droplet digital polymerase chain reaction assay capable of detecting as few as 0.07% *Exon1<sup>mut</sup>* HLA alleles in total DNA revealed that the mutation was present in 29% (101/353) of AA patients, with a median allele frequency of 0.42% (range, 0.071% to 21.3%). *Exon1<sup>mut</sup>* occurred in only 12 different HLA-A (n=4) and HLA-B (n=8) alleles, including *B\*40:02* (n=31) and *A\*02:06* (n=15), which correspond to four HLA class I supertypes (A02, A03, B07, and B44). The percentages of patients who possessed at least one of these 12 HLA alleles were significantly higher in the 353 AA patients (92%,  $P<0.001$ ) and in 83 AA patients with copy number neutral loss of heterozygosity in chromosome 6p (100%,  $P<0.001$ ) than the percentage (81%) in 18,604 Japanese healthy individuals. Eighty-two percent (37/45) of AA patients with *Exon1<sup>mut</sup>* responded to immunosuppressive therapy. Small populations of leukocytes that lack particular HLA-A or B alleles due to *Exon1<sup>mut</sup>* are common in AA patients. The detection of *Exon1<sup>mut</sup>* using a droplet digital polymerase chain reaction assay without the need for HLA typing may serve as a powerful tool for diagnosing the immune pathophysiology of patients with bone marrow failure.

## Introduction

Acquired aplastic anemia (AA) is a rare condition characterized by pancytopenia and bone marrow hypoplasia resulting from immune-mediated suppression of hematopoietic stem progenitor cells (HSPC).<sup>1</sup> Among several different immune mechanisms, cytotoxic T lymphocytes that recognize auto-antigens presented by HSPC are thought to play a critical role in the development of AA,<sup>2,7</sup> based on the finding that leukocytes that lack particular HLA-A or HLA-B alleles (HLA-allele-

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lacking leukocytes: HLA-LL) are often detected in the peripheral blood of AA patients.<sup>9,9</sup> The presence of HLA-LL represents compelling evidence to support the involvement of cytotoxic T lymphocytes specific to HSPC in the development of bone marrow failure, and the detection of these leukocytes would be useful for diagnosing immune pathophysiology in patients with AA and other types of bone marrow failure, including low-risk myelodysplastic syndrome. However, assays for detecting HLA-LL, such as flow cytometry using monoclonal antibodies specific to HLA-A or HLA-B alleles and single nucleotide polymorphism (SNP) arrays, have not been popularized because of the need for HLA typing and low frequencies of copy number neutral loss of heterozygosity of the short arm of chromosome 6 (6pLOH).<sup>10,11</sup>

6pLOH was considered the most common way for HSPC to lose HLA alleles.<sup>10-15</sup> We recently reported that, using targeted deep sequencing with a next-generation sequencer, somatic loss-of-function mutations of *HLA-B\*40:02* were frequently detected in granulocytes of AA patients possessing *HLA-B\*40:02*. These results strongly suggested that antigen presentation by HSPC via *HLA-B\*40:02* plays a critical role in the pathogenesis of AA.<sup>9</sup> Loss-of-function mutations in HLA class I alleles other than *HLA-B\*40:02* were also detected in patients with AA. Babushok *et al.* identified mutations in several HLA class I alleles in leukocytes of AA patients, including *HLA-A\*33:03*, *A\*68:01* and *HLA-B\*14:02*.<sup>14</sup> We recently analyzed leukocytes of AA patients with 6pLOH and detected somatic loss-of-function mutations in *HLA-A\*02:06* and *B\*54:01*.<sup>16,17</sup> However, HLA class I alleles responsible for autoantigen presentation in AA patients without *HLA-B\*40:02*, who account for approximately 80% of all AA patients, are largely unknown due to the limited number of AA patients who have been studied for loss-of-function mutations in HLA class I alleles.

To identify HLA class I alleles other than *HLA-B\*40:02* that are involved in the autoantigen presentation of AA, we performed targeted next-generation sequencing in AA patients with HLA-LL who had HLA class I alleles other than *HLA-B\*40:02*. During the course of the mutation analysis, we identified a nonsense mutation at codon 19 (c.19C>T, p.R7X) in exon 1 (*Exon1<sup>mut</sup>*) of some HLA-A or HLA-B alleles. Surprisingly, *Exon1<sup>mut</sup>* was shared by different HLA-A or HLA-B alleles and was prevalent in AA patients although their variant allelic frequencies (VAF) were very low. A sensitive assay that can detect *Exon1<sup>mut</sup>* could help to identify HLA-A or HLA-B alleles that are responsible for autoantigen presentation and provide insight into the immune pathophysiology of bone marrow failure.

Against this backdrop, we developed a highly sensitive droplet digital PCR (ddPCR) assay for detecting *Exon1<sup>mut</sup>*, and determined the prevalence of *Exon1<sup>mut</sup>* and HLA alleles likely to acquire this mutation in AA patients.

## Methods

Detailed information on the materials and methods are provided in the *Online Supplementary Data*.

## Patients

Twenty Japanese AA patients with HLA-LL who did not have an *HLA-B\*40:02* allele were analyzed for the presence of loss-of-function mutations in HLA alleles. We studied a total of 353

**Table 1. Baseline characteristics of the patients with aplastic anemia.**

Characteristics	N. of patients
Total	353
Age in years, median (range)	63 (11-93)
Sex, male/female	167/186
Severity	
Non-severe AA	202
Severe/very severe AA	151
Patients with increased GPI <sup>-</sup> granulocytes, n (%)	245 (69.4)
None (<0.003%)	108
0.003-1.0%	177
>1.0%	68
IST prior to sampling, n (%)	61 (17.2)
CsA±TPO-RA	31
rATG+CsA±TPO-RA	30

GPI<sup>-</sup>: glycosylphosphatidylinositol-anchored proteins deficient; AA: aplastic anemia; IST: immunosuppressive therapy; CsA: cyclosporine; TPO-RA: thrombopoietin receptor agonist; ATG: antithymocyte globulin.

Japanese AA patients, including the 20 patients who were further analyzed for the prevalence and clinical significance of *Exon1<sup>mut</sup>* in AA between 2010 and 2018 (Table 1). A schematic of the experiments is provided in *Online Supplementary Figure S1*. All patients were genotyped for HLA-A, HLA-B, HLA-C, and HLA-DRB1 alleles using the PCR sequence-specific oligonucleotide method. All patients provided consent to participation in this study, which was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Kanazawa University Institute of Medical, Pharmaceutical, and Health Sciences.

## Detection of glycosylphosphatidylinositol-deficient cells and cells with 6p loss of heterozygosity

Glycosylphosphatidylinositol-anchored protein-deficient (GPI<sup>-</sup>) cells were detected using high sensitivity flow cytometry, as previously described.<sup>18</sup> 6pLOH was assessed using a SNP array-based method with GeneChip 500K arrays (Affymetrix, Japan) or a ddPCR assay with a QX200 AutoDG Droplet Digital PCR System (Bio-Rad, CA, USA), as previously described.<sup>9,10</sup>

## Deep sequencing of HLA class I genes

From peripheral blood samples of the 20 patients with HLA-LL, which were stained with anti-HLA-allele-specific and lineage-specific monoclonal antibodies, paired fractions of granulocytes and CD3<sup>+</sup> T cells were sorted and were subjected to DNA extraction (*Online Supplementary Figure S2*). The monoclonal antibodies used in this study are summarized in *Online Supplementary Table S1*. Nucleotide sequences of HLA-A and HLA-B genes in sorted granulocytes of patients with HLA-LL were determined using a next-generation sequencer (Miseq; Illumina, CA, USA).

## Digital droplet polymerase chain reaction assay for detecting *Exon1<sup>mut</sup>*

We developed a sensitive ddPCR assay for precise detection of *Exon1<sup>mut</sup>* in the peripheral blood of AA patients using the QX200 ddPCR system. Briefly, we designed two different sets of primer pairs complementary to the consensus sequences of HLA-A and HLA-B alleles, and locked nucleic acid-based probes complementary to wild-type and mutant-specific sequences (*Online Supplementary Table S2*). Detailed protocols for ddPCR are provided in the *Online Supplementary Methods*.

## Determination of HLA alleles that acquired *Exon1<sup>mut</sup>*

HLA alleles that acquired *Exon1<sup>mut</sup>* were determined by deep sequencing with the next-generation sequencer, or deduced from

alleles contained in the lost haplotype due to 6pLOH that was accompanied by *Exon1<sup>mut</sup>* (Online Supplementary Figure S3). The low VAF of *Exon1<sup>mut</sup>* (VAF <1%) was confirmed by deep sequencing with unique molecular identifiers (xGen® Dual Index UMI Adapters: Integrated DNA Technologies, IA, USA).<sup>19</sup> The correlation between *Exon1<sup>mut</sup>* VAF determined by deep sequencing with unique molecular identifiers and those determined by the ddPCR assay was examined using 24 different samples (Online Supplementary Figure S4). HLA class I alleles acquiring *Exon1<sup>mut</sup>* were determined using the nearest allele-specific SNP. Details on deep

sequencing with unique molecular identifiers are provided in the Online Supplementary Methods.

**Statistical analysis**

Comparisons were performed using the Fisher exact test for categorical variables and Mann-Whitney U test for continuous variables with a two-tailed significance level of 0.05. Statistical analyses were performed using the EZR software program.<sup>20</sup> Graphs were generated using GraphPad PRISM7.0 (GraphPad Software Inc, CA, USA).

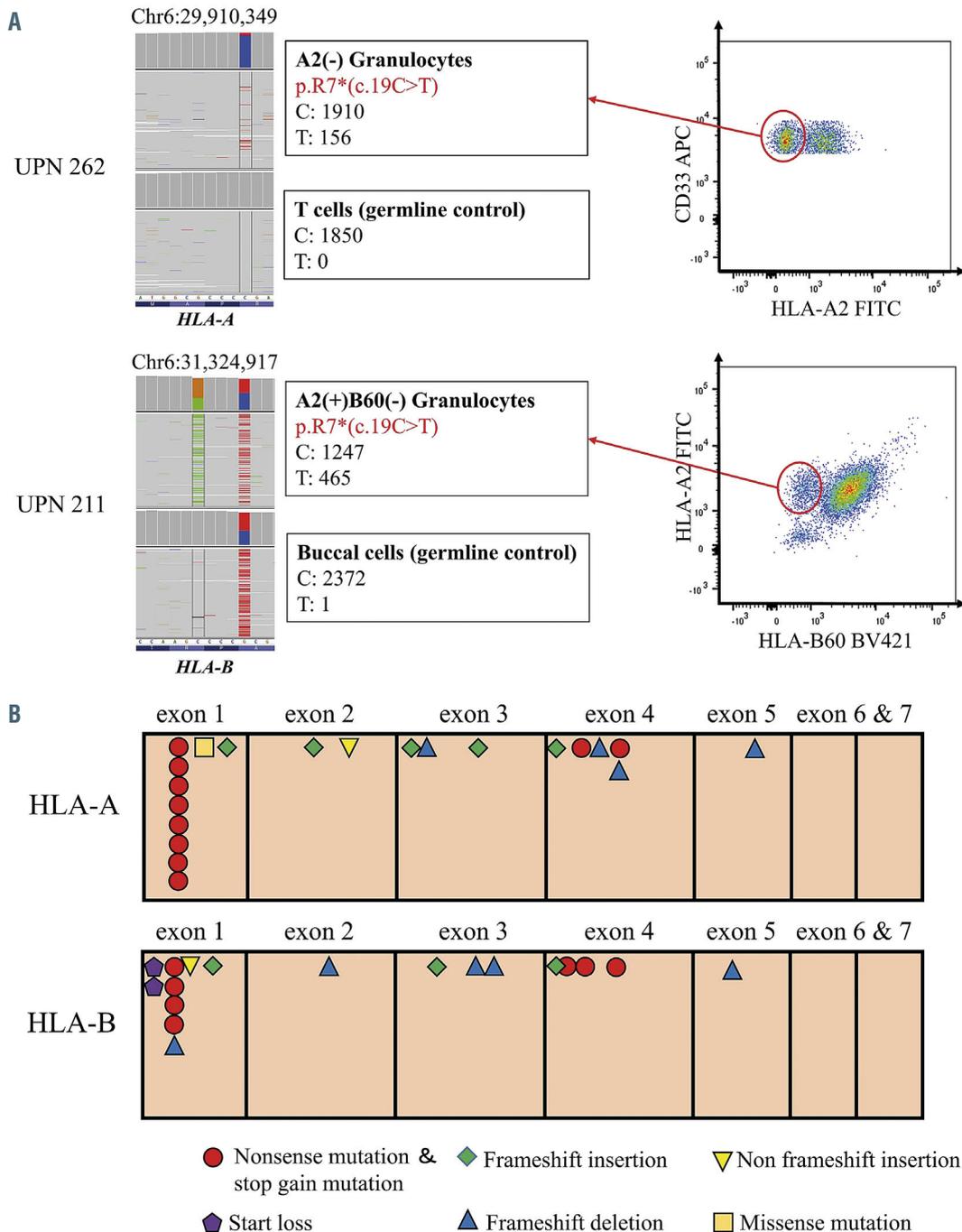


Figure 1. Identification of *Exon1<sup>mut</sup>* in patients with aplastic anemia. (A) *Exon1<sup>mut</sup>* [p.R7\*(c.19C>T)] was detected by targeted deep sequencing of sorted HLA-A2- granulocytes (UPN 262) and HLA-A2\*B60- granulocytes (UPN 211) in two patients with aplastic anemia. Sequencing results of sorted HLA-allele lacking leukocytes from these two patients and germline controls and flow cytometry results of granulocytes are shown. (B) Loss-of-function mutations detected in 14 patients by targeted deep sequencing. *Exon1<sup>mut</sup>* was detected in HLA-A alleles of eight patients and HLA-B alleles of four patients. UPN: unique patient number.

Results

Identification of *Exon1<sup>mut</sup>* in different HLA-A and HLA-B alleles in HLA-lacking leukocytes from patients with aplastic anemia

To identify HLA class I alleles other than *HLA-B\*40:02* that are critically involved in autoantigen presentation in AA, we sequenced HLA-A and HLA-B alleles of sorted granulocytes from 20 patients with HLA-LL not possessing *HLA-B\*40:02*. The clinical characteristics of these 20 patients are shown in *Online Supplementary Table S3*. HLA-A<sup>-</sup> granulocytes or HLA-A<sup>+</sup>B<sup>+</sup> granulocytes, accounting for 2.4-99.8% of all granulocytes, were detected in these patients (*Online Supplementary Figure S5, Online Supplementary Table S3*). Median read depths of the HLA-A and HLA-B alleles were 925 and 1,012 for targeted deep sequencing and 43,013 and 35,267 for amplicon sequencing, respectively. Of the 20 AA patients assessed, six had 6pLOH alone, ten had various loss-of-function mutations in addition to 6pLOH, and four had only somatic loss-of-function mutations in HLA-A. Three (UPN 210, 335, and 348) of the 14 patients with loss-of-function mutations had the mutations in HLA-B of sorted HLA-A<sup>+</sup> granulocytes.

Of note, 12 of 14 patients with loss-of-function mutations had *Exon1<sup>mut</sup>* in HLA-A (*A\*02:06*, n=7; *A\*31:01*, n=1) and HLA-B (*B\*13:01*, n=1; *B\*40:01*, n=2; and *B\*54:01*, n=1). The other two patients (UPN 335 and UPN 210) had different loss-of-function mutations from *Exon1<sup>mut</sup>* in *HLA-B\*40:03* and *HLA-B\*54:01*, respectively. Interestingly, a frameshift mutation of *HLA-B\*54:01* also occurred at codon 19 (c.19delC, p.R7Efs) in exon 1 (Figure 1A and B, *Online Supplementary Table S4*).

*Exon1<sup>mut</sup>* detection using a sensitive droplet digital polymerase chain reaction assay

To detect *Exon1<sup>mut</sup>* with high sensitivity and specificity, we established a ddPCR assay that allows for precise measurement of mutant allele frequency without the need for HLA typing. Tested samples containing a fixed amount of wild-type DNA and serial dilutions of *Exon1<sup>mut</sup>* template DNA revealed a detection limit of 0.07% for both HLA-A and HLA-B (*Online Supplementary Figure S6*). The ddPCR assay yielded 0% to 0.042% (median, 0.009%) positive dots in peripheral blood of 24 healthy individuals, validating the cut-off value of 0.07%. The ddPCR assay was able to detect *Exon1<sup>mut</sup>*, which had an allelic frequency of <1.0%

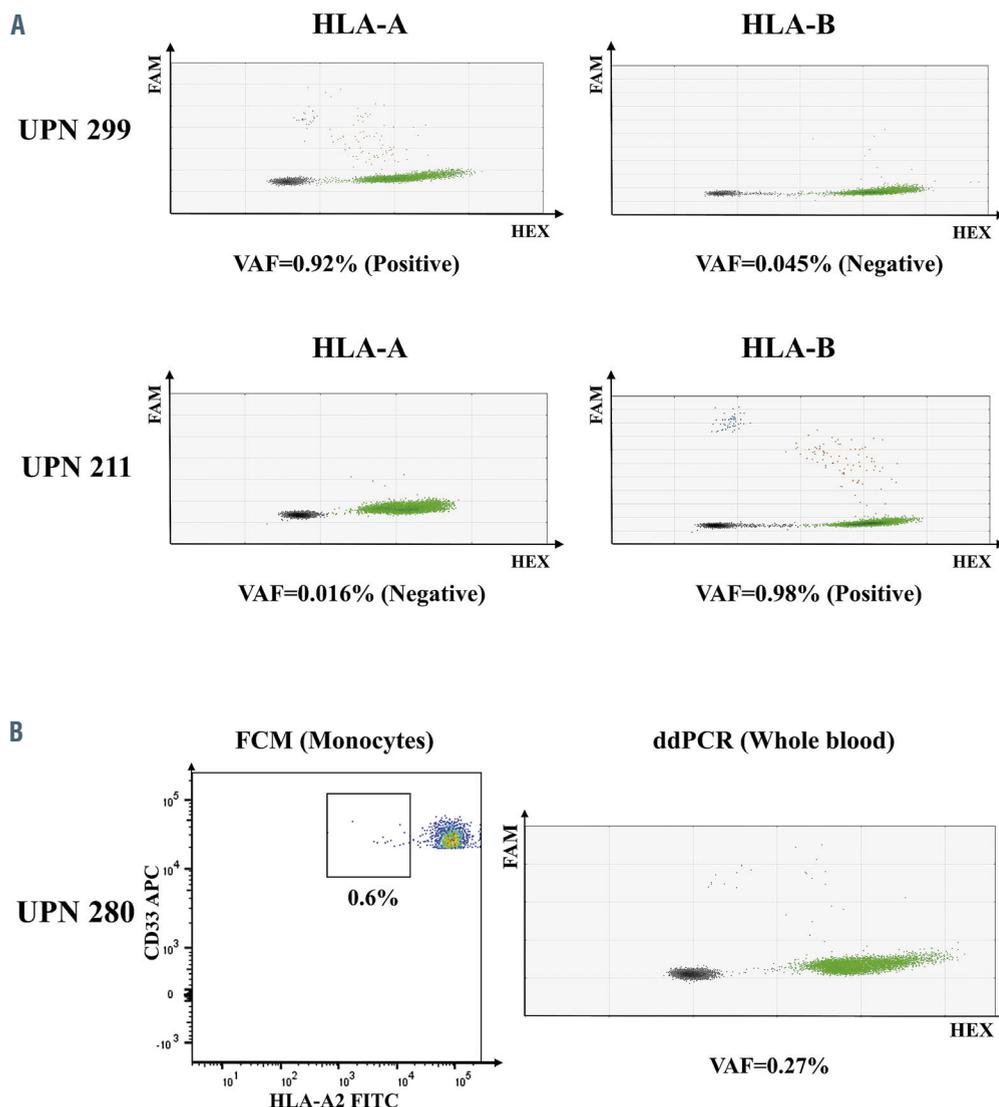


Figure 2. Detection of *Exon1<sup>mut</sup>* using the droplet digital polymerase chain reaction assay. (A) Representative droplet digital polymerase chain reaction (ddPCR) plots of *Exon1<sup>mut</sup>* DNA in HLA-A\*02:06 of UPN 299 and 0.98% *Exon1<sup>mut</sup>* DNA in HLA-B\*40:01 of UPN 211. (B) A minor population of HLA-allele-lacking leukocytes in UPN 280 detected by flow cytometry and the ddPCR assay. The ddPCR assay detected 0.27% *Exon1<sup>mut</sup>* in whole blood where granulocytes and monocyte with *Exon1<sup>mut</sup>* were diluted with lymphocytes without *Exon1<sup>mut</sup>*. The percentage of *Exon1<sup>mut</sup>*-positive cells was consistent with the percentage of HLA-A2<sup>-</sup> monocytes (0.6%) detected by flow cytometry. UPN: unique patient number; VAF: variant allele frequency; FCM: flow cytometry.

(Figure 2A), clearly showing the presence of HLA-LL in patients for whom flow cytometry analysis of monocytes using anti-HLA-A2 antibodies produced unequivocal results regarding the presence of HLA-A2-lacking monocytes (Figure 2B).

**Prevalence of *Exon1<sup>mut</sup>* in patients with aplastic anemia**

Using two different ddPCR mixtures for HLA-A and HLA-B, the presence of *Exon1<sup>mut</sup>* was evaluated in all of the 353 patients. *Exon1<sup>mut</sup>* was detected in 101 (28.6%) of the 353 patients, with the median frequency of 0.42% (range, 0.071% to 21.3%). The prevalence of *Exon1<sup>mut</sup>* was similar in HLA-A and HLA-B alleles in both treatment-naïve patients (n=291) and treated patients (n=62). Among the AA patients with *Exon1<sup>mut</sup>*, those who had been treated had a higher median frequency of *Exon1<sup>mut</sup>* than those who were untreated (0.96% vs. 0.33%,  $P=0.0079$ ) (Figure 3A). Figure 3B shows the relationships between the presence of cells with *Exon1<sup>mut</sup>*, 6pLOH and a paroxysmal nocturnal hemoglobinuria (PNH) phenotype in the patients. Fifty-six (55.4%) and 67 (66.3%) of the 101 patients with *Exon1<sup>mut</sup>* had 6pLOH and PNH phenotype cells, respectively. Ten (2.8%) of the 353 patients had *Exon1<sup>mut</sup>* alone. The frequency of *Exon1<sup>mut</sup>* was much lower than that of 6pLOH in 36 patients possessing both clones ( $P<0.001$ ) (Figure 3C).

**Long-term persistence of *Exon1<sup>mut</sup>*-positive cells**

Serial blood samples were available for longitudinal analyses of *Exon1<sup>mut</sup>* in 13 patients who responded to immunosuppressive therapy (cyclosporine [CsA] alone in 6 and rabbit antithymocyte globulin [rATG] + CsA in 5) or anabolic steroids (n=2). *Exon1<sup>mut</sup>* was persistently detected for 14-86 months in nine patients, including one patient (UPN 299) who had been off treatment for 7 years, suggesting that *Exon1<sup>mut</sup>*-positive leukocytes are derived from long-lasting HSPC (Figure 4A). The VAF of *Exon1<sup>mut</sup>* increased in two (15%, UPN 333 and UPN 339), remained stable in four (31%), and decreased in three (23%) patients. *Exon1<sup>mut</sup>* became undetectable at 7-33 months after the first detection of *Exon1<sup>mut</sup>* in the other four patients, all of whom were being treated with CsA. Figure 4B shows a gradual decline of the *Exon1<sup>mut</sup>* frequency over 3 years in one patient (UPN 213).

**HLA-A and HLA-B alleles that acquire *Exon1<sup>mut</sup>***

Among the 101 patients with *Exon1<sup>mut</sup>*, HLA alleles that acquired *Exon1<sup>mut</sup>* could be determined by targeted deep sequencing with (n=21) or without (n=37) unique molecular identifiers, or deduced from alleles contained in the lost haplotype due to 6pLOH that was accompanied by *Exon1<sup>mut</sup>* (n=10) (Online Supplementary Figure S2). In the

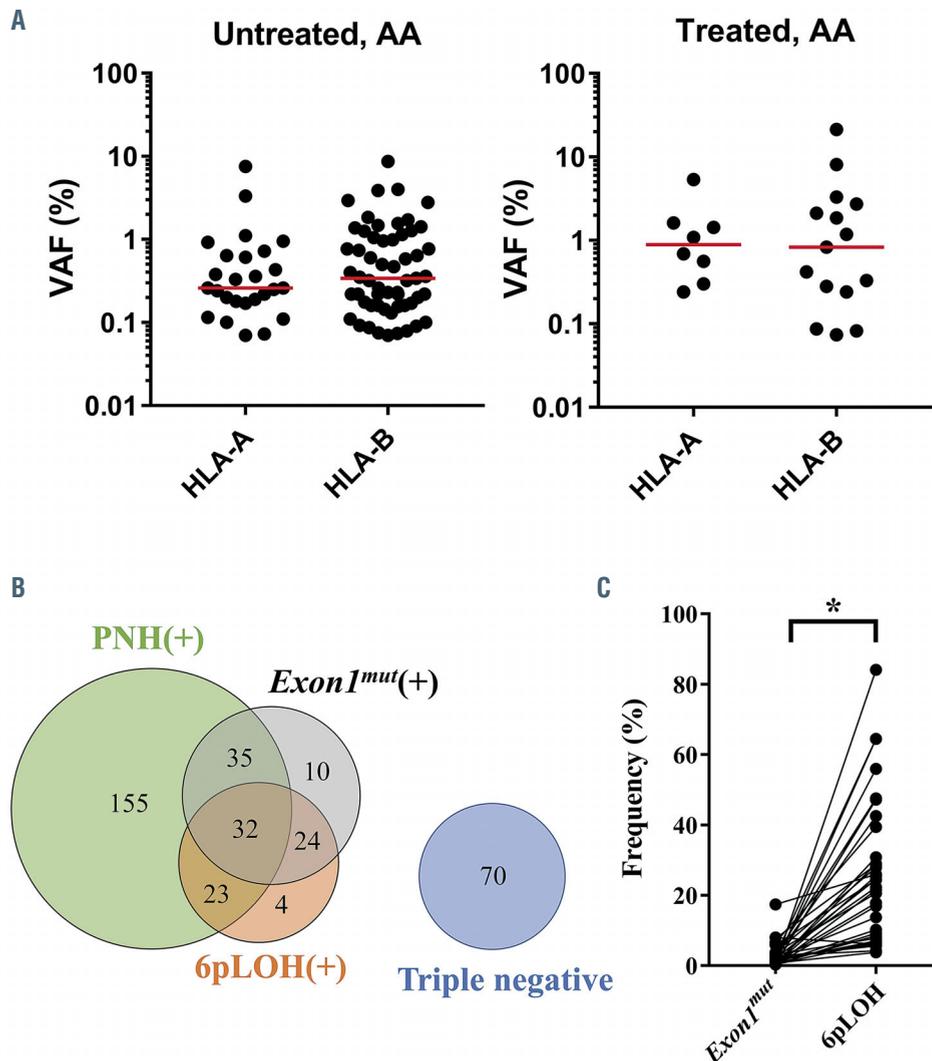


Figure 3. Prevalence and clone size of *Exon1<sup>mut</sup>* in patients with aplastic anemia. (A) Mutant allele frequency of *Exon1<sup>mut</sup>* in 291 and 62 *Exon1<sup>mut</sup>* positive untreated and treated patients with aplastic anemia, respectively. Red bars indicate median allele frequencies of *Exon1<sup>mut</sup>*. (B) The prevalence of *Exon1<sup>mut</sup>* and its relationship with paroxysmal nocturnal hemoglobinuria phenotype (PNH) and copy number neutral loss of heterozygosity of the short arm of chromosome 6 (6pLOH). *Exon1<sup>mut</sup>*-positive patients accounted for 58% of 6pLOH+PNH+ patients and for 67% of 6pLOH+ patients. (C) Frequency of *Exon1<sup>mut</sup>* and 6pLOH in individual patients who were positive for both mutant clones. The frequencies of both clones were determined by droplet digital polymerase chain reaction analysis. \* $P<0.001$ . VAF: variant allele frequency; AA: aplastic anemia; UPN: unique patient number.

other 33 patients with *Exon1<sup>mut</sup>*, HLA-A or HLA-B alleles that acquired *Exon1<sup>mut</sup>* could not be determined or deduced due to very low VAF (<0.2%), the absence of allele-specific SNP near *Exon1<sup>mut</sup>* in HLA-A or HLA-B alleles that are useful for identify missing HLA alleles, or the absence of coexisting 6pLOH. For the 68 patients in whom HLA alleles that acquired *Exon1<sup>mut</sup>* could be determined, the following 12 alleles were identified: *A\*02:01* (n=2), *A\*02:06* (n=15), *A\*02:07* (n=1), *A\*31:01* (n=3), *B\*13:01* (n=2), *B\*40:01* (n=3), *B\*40:02* (n=31), *B\*40:03* (n=1), *B\*44:03* (n=1), *B\*54:01* (n=6), *B\*55:02* (n=2), and *B\*56:01* (n=1)

(Figure 5A). HLA class I supertypes of these alleles, which are defined by similarities in the antigen-presenting amino-acid motif of HLA alleles, were confined to only four supertypes: A02, A03, B07, and B44, except for *HLA-B\*13:01* that does not belong to any of the 14 supertypes.<sup>21</sup>

When comparing the frequency of these 12 alleles between a healthy control population and our study cohort, 81% of 18,604 healthy Japanese individuals possessed at least one of the 12 alleles, while the prevalence was 92% in the 353 patients with AA ( $P<0.001$ ) and 100% in the 83 patients with 6pLOH ( $P<0.001$ ) (Figure 5B), sug-

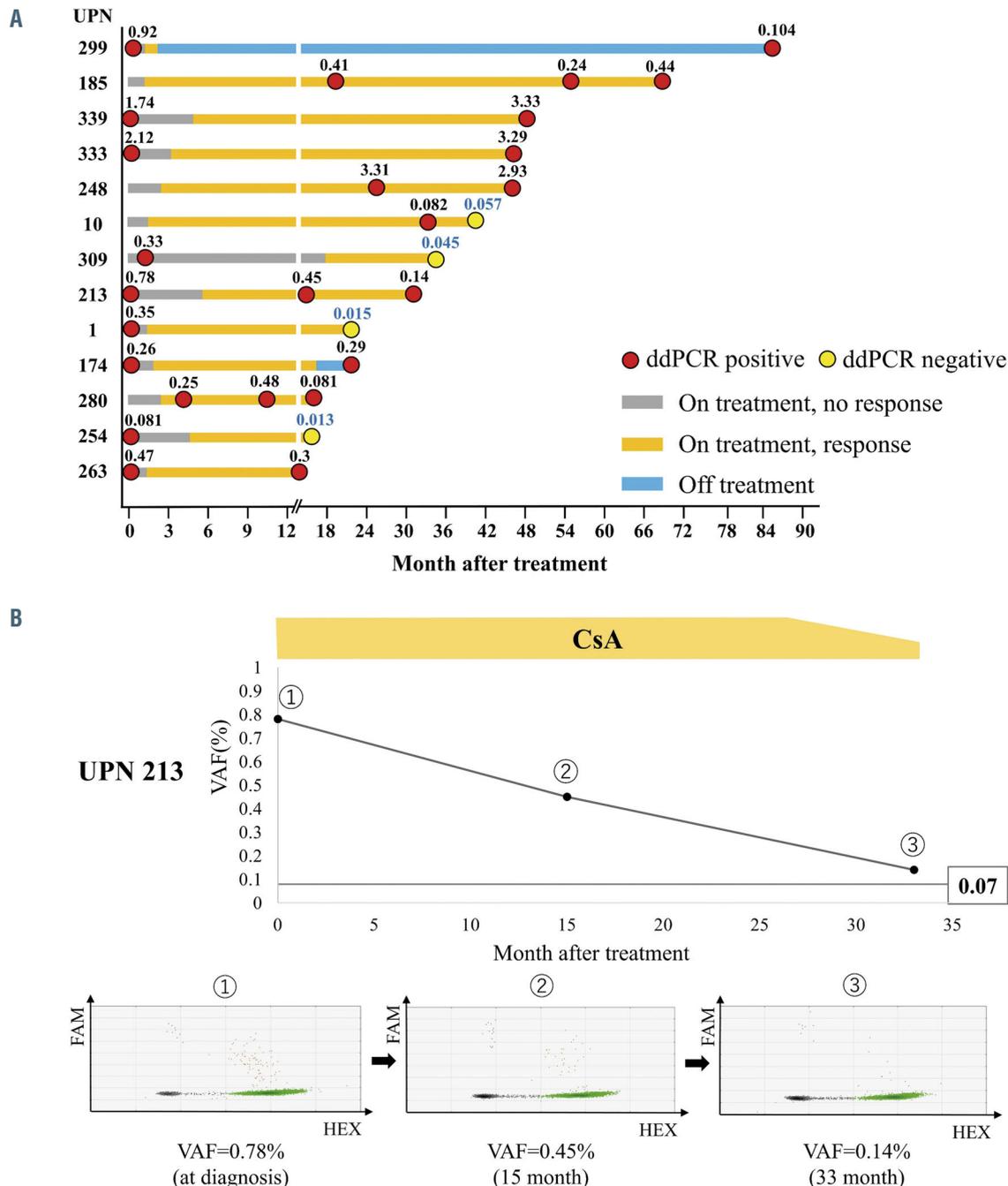


Figure 4. Temporal changes in allelic frequency of *Exon1<sup>mut</sup>*. (A) Allelic frequencies of *Exon1<sup>mut</sup>* determined at different time points in 13 patients and their disease status. (B) Representative scattergrams from UPN 213 showing a gradual decline in *Exon1<sup>mut</sup>* frequency over the course of 3 years. UPN: unique patient number; ddPCR: droplet digital polymerase chain reaction; CsA: cyclosporine A; VAF: variant allele frequency.

gesting the involvement of these alleles in the development of AA.

**Loss of HLA-B expression from *Exon1<sup>mut</sup>*-positive leukocytes**

Although *Exon1<sup>mut</sup>* in leukocytes is expected to result in lack of the corresponding HLA allele, the phenotype of these leukocytes is difficult to examine since the VAF of *Exon1<sup>mut</sup>* is very low. We previously established six induced pluripotent stem (iPS) cell clones from peripheral blood monocytes of an AA patient (UPN 333) whose monocytes included approximately 60% HLA-A24<sup>+</sup>Bw6<sup>-</sup> cells (Figure 6A).<sup>17</sup> Deep sequencing revealed the presence of *Exon1<sup>mut</sup>* in sorted HLA-A24<sup>+</sup>Bw6<sup>-</sup> cells and also in one (clone C1) of the six iPS cell clones. When a wild-type iPS clone (clone E1) and clone C1 were induced to differentiate into CD34<sup>+</sup> cells, all clone E1-derived CD34<sup>+</sup> cells expressed HLA-Bw6 (B5401), while all clone C1-derived CD34<sup>+</sup> cells lacked HLA-Bw6 (Figure 6B). The ddPCR assay using DNA from wild-type and *Exon1<sup>mut</sup>*-positive iPS cell-derived CD34<sup>+</sup> cells revealed that the VAF of *Exon1<sup>mut</sup>* were 0.041% and 49%, respectively, as expected (Figure 6C).

**Clinical characteristics of aplastic anemia patients with *Exon1<sup>mut</sup>***

Of the 291 patients whose peripheral blood samples were examined for *Exon1<sup>mut</sup>* and GPI<sup>-</sup> cells before treatment, 151 were evaluable for response to immunosuppressive therapy (CsA alone, n=68; CsA+rATG, n=83). The other 140 patients were excluded from the analysis of the relationship between the response to immunosuppressive therapy and the presence of *Exon1<sup>mut</sup>* or GPI<sup>-</sup> cells because no data on the response to immunosuppressive therapy were available in 84, and the remaining 56 received no treatment (n=25) or treatments other than immunosuppressive therapy (n=31), such as anabolic steroids and thrombopoietin receptor agonists, and allogeneic stem cell transplantation. An increase in GPI<sup>-</sup> cells was noted in 76% (34/45) of patients with *Exon1<sup>mut</sup>* and in 76% (81/106) without *Exon1<sup>mut</sup>* (P=1.0). In terms of response to immunosuppressive therapy, 82% (37/45) of patients with *Exon1<sup>mut</sup>* responded to CsA (n=14) or CsA+rATG (n=23), while 75% (79/106) of those without *Exon1<sup>mut</sup>* responded to CsA (n=35) or CsA+rATG (n=44) (P=0.40). The response rate to immunosuppressive thera-

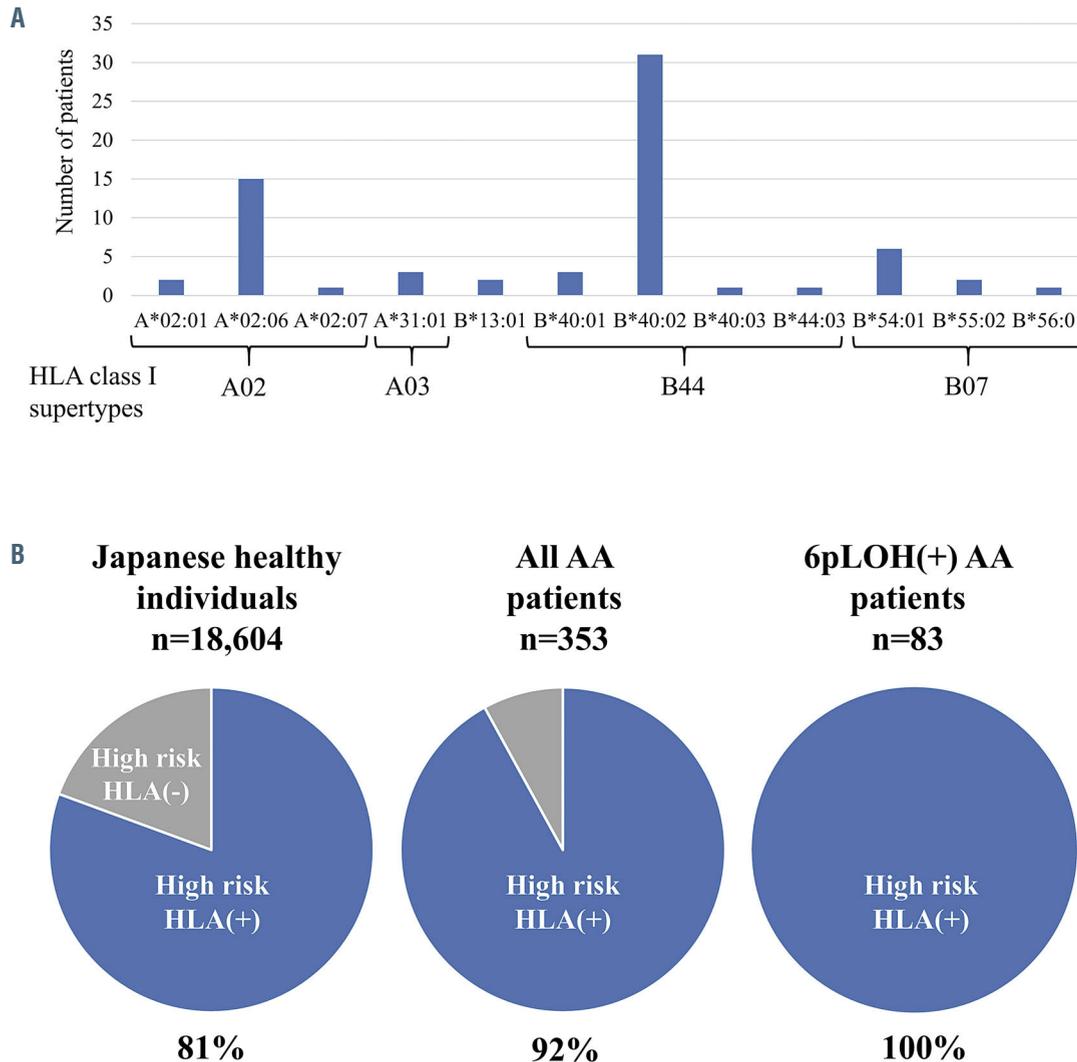


Figure 5. HLA alleles that acquired *Exon1<sup>mut</sup>*. (A) The number of cases that acquired *Exon1<sup>mut</sup>* in the corresponding HLA alleles are shown. HLA numbers shown below the bar graph denote the HLA class I supertypes to which each allele belongs. (B) Proportions that the 12 HLA alleles account for in the different populations. AA: aplastic anemia; 6pLOH: copy number neutral loss of heterozygosity of the short arm of chromosome 6.

py in patients with *Exon1<sup>mut</sup>* was significantly higher than that (54%, 13/24) in patients who were negative for all of *Exon1<sup>mut</sup>*, GPI<sup>-</sup> cells, and 6pLOH ( $P=0.023$ ).

**Discussion**

Targeted deep sequencing of HLA genes of leukocytes obtained from AA patients with HLA-LL revealed a unique nonsense mutation at codon 19 (c.19C>T, p.R7X) in exon 1 (*Exon1<sup>mut</sup>*) of different HLA-A and HLA-B alleles. This mutation has been previously reported in Japanese and American AA patients, but did not draw attention because the mutation was detectable in only a limited number of patients.<sup>9,14</sup> Our highly sensitive ddPCR assay enabled the detection of minor *Exon1<sup>mut</sup>* clones and detected the mutant DNA in nearly one third of Japanese AA patients regardless of the presence of 6pLOH. *Exon1<sup>mut</sup>* was also detected in two of eight Finnish AA patients we stud-

ied (*unpublished observation*). Interestingly, a frameshift mutation (c.19delC, p.R7Efs) was also identified at codon 19 of *HLA-B\*54:01* in a patient (UPN 210) without *Exon1<sup>mut</sup>*, suggesting that the codon 19 in exon 1 of *HLA-A* and *HLA-B* may be a specific position at which somatic mutations are likely to occur.

The loss of HLA from CD34<sup>+</sup> cells due to *Exon1<sup>mut</sup>* was verified by phenotypic analysis of *Exon1<sup>mut</sup>*-positive iPS cell-HSPC that were derived from monocytes of an AA patient who had approximately 14% *Exon1<sup>mut</sup>*-positive cells among the granulocyte population.<sup>17</sup> *Exon1<sup>mut</sup>* has also been detected in several squamous cell carcinomas, such as head and neck tumors, oral cancers, and anal cancers, in previous studies.<sup>22-25</sup> The solid tumors that lost HLA class I expression due to *Exon1<sup>mut</sup>* were thought to have escaped T-cell attack and acquired a proliferative advantage. Taken together, these findings suggest that *Exon1<sup>mut</sup>* is a common mechanism by which HSPC lose HLA, allowing them to escape from the effects of cytotoxic T lymphocytes in AA patients.

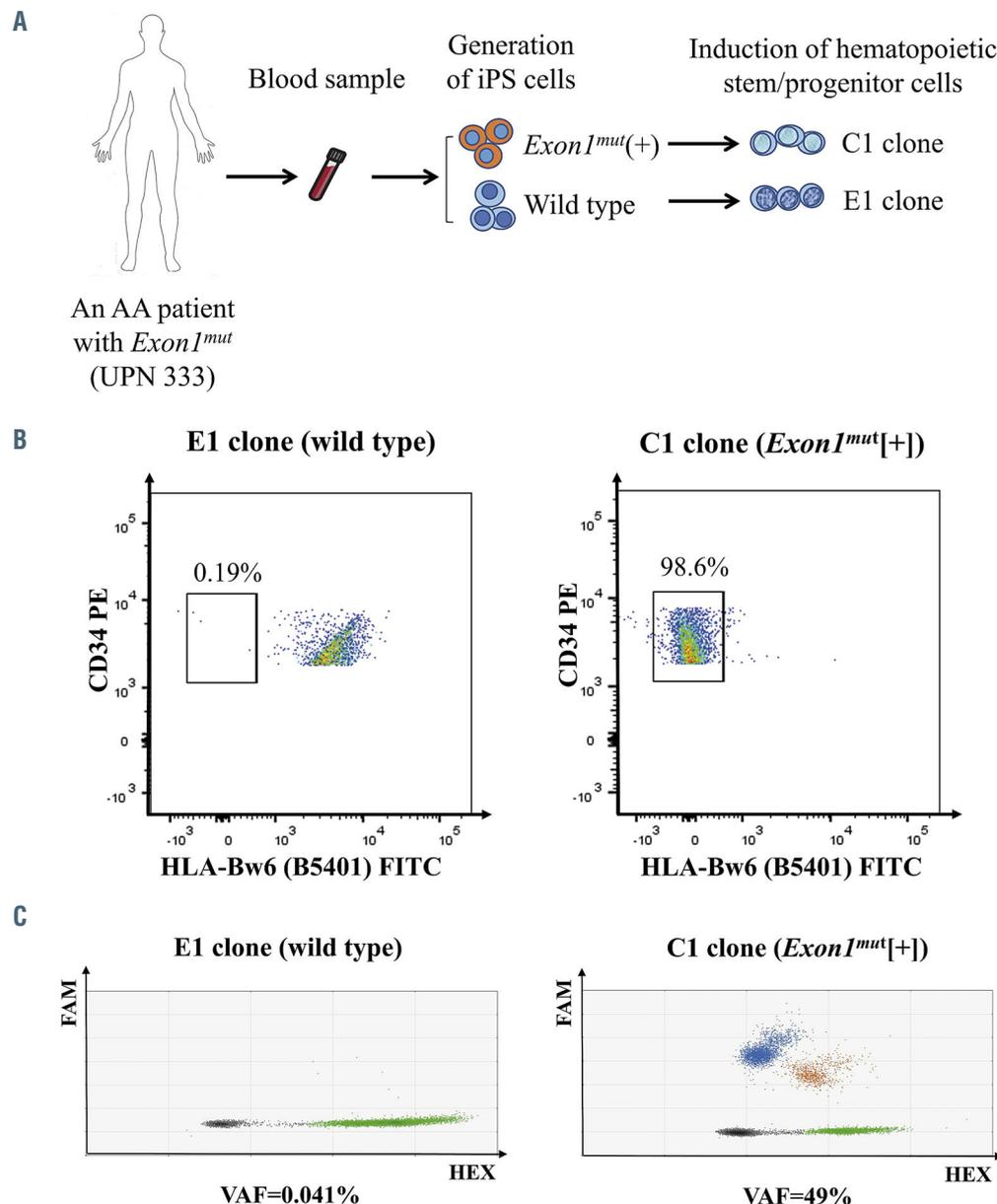


Figure 6. HLA allele expression by *Exon1<sup>mut</sup>*-positive hematopoietic stem and progenitor cells. (A) Establishment of induced pluripotent stem cell (iPSC)-derived hematopoietic stem cells from monocytes of an aplastic anemia patient with *Exon1<sup>mut</sup>* (UPN 333). (B) HLA-Bw6 (B5401) expression by CD34<sup>+</sup> cells derived from a wild-type iPSC clone (left) and an *Exon1<sup>mut</sup>*-positive iPSC clone (right). (C) *Exon1<sup>mut</sup>* detection in DNA from wild-type (left) and *Exon1<sup>mut</sup>*-positive (right) iPSC-derived CD34<sup>+</sup> cells. Numbers below the scattergram denote the variant allele frequency of *Exon1<sup>mut</sup>*. AA: aplastic anemia; UPN: unique patient number; iPSC cells: induced pluripotent stem cells; VAF: variant allele frequency.

We previously used targeted deep sequencing to identify frequent loss-of-function mutations in three HLA class I alleles, *B\*40:02*, *A\*02:06*, and *B\*54:01*.<sup>9,16,17</sup> The highly sensitive ddPCR assay described herein that was capable of detecting *Exon1<sup>mut</sup>* newly identified three HLA-A (*A\*02:01*, *A\*02:07*, *A\*31:01*) and six HLA-B alleles (*B\*13:01*, *B\*40:01*, *B\*40:03*, *B\*44:03*, *B\*55:02*, *B\*56:01*) as HLA alleles that are susceptible to allelic loss. Compared with their frequency in the general Japanese population, these HLA alleles were found to be highly enriched in AA patients. Among the 14 HLA class I supertypes that are defined based on similarities in the antigen-presenting peptide motif, the 12 alleles mentioned above belong to only four of the supertypes.<sup>21</sup> These findings suggest that autoantigens of AA may be presented to T cells by these specific HLA alleles on HSPC.

Like HSPC positive for 6pLOH, those positive for *Exon1<sup>mut</sup>* are thought to escape the attack of cytotoxic T lymphocytes specific to autoantigens presented by the missing HLA-A or HLA-B allele and contribute to hematopoiesis over the long-term. However, it is unclear why *Exon1<sup>mut</sup>* occurs more frequently in HSPC than loss-of-function mutations in other positions of HLA class I alleles. Shukla *et al.* reported different hotspots of mutations in class I HLA genes according to cancer type, and identified *Exon1<sup>mut</sup>* only in head and neck squamous cell cancers.<sup>24</sup> HSPC may thus share a common property in that *Exon1<sup>mut</sup>* is likely to occur in class I HLA genes in head and neck squamous cell cancers.

The median VAF of *Exon1<sup>mut</sup>* in patients with *Exon1<sup>mut</sup>* patients was only 0.42%, a level that cannot be detected by targeted deep sequencing. This low VAF was in sharp contrast to the high proportion of concomitant 6pLOH in individual patients (Figure 3C). We previously reported that 6pLOH<sup>+</sup> leukocytes were often polyclonal, consisting of leukocytes having different breakpoints of uniparental disomy in the short arm of chromosome 6.<sup>10</sup> This polyclonality may account for the high proportion of 6pLOH. Although the leukocytes with *Exon1<sup>mut</sup>* represent a minor leukocyte population, the long-term (1-7 years) persistence of these mutated leukocytes indicates that they are derived from HSPC with self-renewal capacity. Arends *et al.* showed that clone size of cells with somatic mutations of epigenetic regulation genes expanded from most immature hematopoietic stem cells to mature peripheral blood cells in patients with clonal hematopoiesis of indeterminate potential.<sup>26</sup> Leukocyte positive for *Exon1<sup>mut</sup>* may also be derived from most immature hematopoietic stem cells. The persistence of similarly minor clones in peripheral blood has been reported for GPI<sup>+</sup> granulocytes in AA, the median frequency of which was 0.25%.<sup>18,27</sup> In contrast to PIGA-mutated or 6pLOH<sup>+</sup> leukocytes, which can be oligoclonal and dysfunctional due to the lack of all GPI-anchored proteins or a large segment of 6p, *Exon1<sup>mut</sup>*-positive leukocytes are derived from a single HSPC that is phenotypically normal except for the lack of one HLA allele. According to Dingli's hypothesis, approximately 400 HSPC are actively involved in human hematopoiesis.<sup>28</sup> Thus, the small proportion of *Exon1<sup>mut</sup>*-positive leukocytes among the entire leukocyte population may reflect an average clone size of individual HSPC in the bone marrow.

HLA-LL are useful markers that indicate the presence of an immune pathophysiology in patients with bone mar-

row failure. Here we showed a high response rate to immunosuppressive therapy in patients with *Exon1<sup>mut</sup>*, although patients without *Exon1<sup>mut</sup>* also had a high response rate likely due to the high prevalence of GPI<sup>+</sup> cells.<sup>29-31</sup> Several methods can be used to detect HLA-LL, including flow cytometry assays with monoclonal antibodies specific to HLA-A or HLA-B alleles, ddPCR or SNP arrays for detecting 6pLOH, and targeted deep sequencing.<sup>8-10,12</sup> However, these methods require HLA typing of patients, take a long time to produce results, and are unable to detect HLA-LL that account for less than 1% of total leukocytes. The ddPCR assay used in the present study to detect *Exon1<sup>mut</sup>* enables the detection of HLA-LL accounting for as few as 0.07% of the total leukocyte population within 6 h of blood collection, highlighting the powerful nature of this assay for diagnosing immune pathophysiology in patients with bone marrow failure.

### Disclosures

No conflicts of interest to disclose.

### Contributions

HM, TI, KT, YZ and SN collected clinical data and blood samples. FA performed HLA genotyping. YF and SO conducted the SNP array analyses. YZ, HT, TO, HK and AM generated an original monoclonal antibody specific to HLA-B13, B60 and B61. HM and TY performed cell sorting. HM, KHosomichi, TI, YZ and AT performed deep sequencing. HM, YZ, NMA and TCD performed the droplet digital polymerase chain reaction. KC and YY generated the induced pluripotent stem cells. MIE performed the *in vitro* experiments. HM, KHosokawa and SN designed the research and wrote the manuscript. All authors critically reviewed the manuscript and checked the final version.

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