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

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and -B was frequently detected in acquired aplastic anemia. HLA alleles likely to acquire the common mutation were limited to 4 HLA-A and 8 HLA-B alleles in Japanese patients.

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Abstract

Leukocytes that lack HLA allelic expression are frequently detected in patients with acquired aplastic anemia (AA) who respond to immunosuppressive therapy (IST), 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 position 19 (c.19C>T, p.R7X) in exon 1 (*Exon1*^{mut}) of different HLA-A and -B alleles in HLA-lacking granulocytes from AA patients. A droplet digital PCR (ddPCR) assay capable of detecting as few as 0.07% *Exon1*^{mut} HLA alleles in total DNA revealed 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*^{mut} 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 4 HLA 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 (6pLOH) (100%, $P<0.001$) than that (81%) in 18,604 Japanese healthy individuals. 82% (37/45) of AA patients with *Exon1*^{mut} responded to IST. Small populations of leukocytes that lack particular HLA-A or B alleles due to *Exon1*^{mut} are common in AA patients. The detection of *Exon1*^{mut} using the ddPCR

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 (BM) hypoplasia resulting from immune-mediated suppression of hematopoietic stem progenitor cells (HSPCs) (1). Among several different immune mechanisms, cytotoxic T lymphocytes (CTLs) that recognize auto-antigens presented by HSPCs are thought to play a critical role in the development of AA (2-7), based on the finding that leukocytes that lack particular HLA-A or HLA-B alleles (HLA-allele-lacking leukocytes: HLA-LLs) are often detected in the peripheral blood (PB) of AA patients (8, 9). The presence of HLA-LLs represents compelling evidence to support the involvement of CTLs specific to HSPCs in the development of BM failure, and the detection of these leukocytes would be useful for diagnosing immune pathophysiology in patients with AA and other types of BM failure, including low-risk myelodysplastic syndrome. However, assays for detecting HLA-LLs, such as flow cytometry using monoclonal antibodies (mAbs) specific to HLA-A or HLA-B alleles and single nucleotide polymorphism (SNP) arrays, have not been popularized due to the need for HLA typing and low frequencies of copy number neutral loss of heterozygosity of the short arm of chromosome 6 (6pLOH) (10, 11).

6pLOH was considered the most common way for HSPCs to lose HLA alleles (10-15). We recently reported that, using targeted deep sequencing with a next

generation sequencer (NGS), 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 HSPCs via HLA-B4002 plays a critical role in the pathogenesis of AA (9). 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* (14). 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* (16, 17). However, HLA class I alleles responsible for autoantigen presentation in AA patients without *HLA-B*40:02*, who account for approximately 80% of the total AA patients, are largely unknown due to the limited number of AA patients that 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-LLs 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 position 19 (c.19C>T, p.R7X) in exon 1 ($Exon1^{mut}$) of some HLA-A or HLA-B alleles.

Surprisingly, *Exon1*^{mut} 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. If there was a sensitive assay that can detect *Exon1*^{mut}, this could help identify HLA-A or HLA-B alleles that are responsible for autoantigen presentation and provide insight into the immune pathophysiology of BM failure.

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

Methods

Detailed of materials and methods are provided in supplemental data.

Patients

Twenty Japanese AA patients with HLA-LLs who did not have *HLA-B*40:02* allele were analyzed for the presence of loss-of-function mutations in HLA alleles. A total of 353 Japanese AA patients including the 20 patients were further analyzed for the prevalence and clinical significance of *Exon1*^{mut} in AA between 2010 and 2018 (Table 1). A schematic of the experiments is provided in Supplemental Figure 1. 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 participate in this study, which was conducted in accordance with the Declaration of Helsinki. This study was approved by the ethics committee of the Kanazawa University Institute of Medical, Pharmaceutical, and Health Sciences.

Detection of GPI(-) and 6pLOH(+) cells

Glycosylphosphatidylinositol-anchored protein-deficient (GPI[-]) cells were detected using high sensitivity flow cytometry (FCM), as previously described (18). 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 (9, 10).

Deep sequencing of HLA class I genes

From PB samples of the 20 patients with HLA-LLs, which were stained with anti-HLA-allele-specific and lineage-specific mAbs, paired fractions of granulocytes and $CD3^+$ T cells were sorted and were subjected to DNA extraction (Supplemental Figure 2). The mAbs used in this study are summarized in Supplemental Table 1.

Nucleotide sequences of HLA-A and HLA-B genes in sorted granulocytes of HLA-LL(+) patients were determined using an NGS (Miseq; Illumina, CA, USA).

ddPCR assay for detecting Exon1^{mut}

We developed a sensitive ddPCR assay for precisely detecting *Exon1*^{mut} in the PB 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 (WT) and mutant-specific (MT) sequences (Supplemental Table 2). Detailed protocols for ddPCR are provided in the Supplemental Methods.

Determination of HLA alleles that acquired Exon1^{mut}

HLA alleles that acquired *Exon1*^{mut} were determined by deep sequencing with the NGS, or deduced from alleles contained in the lost haplotype due to 6pLOH that was accompanied by *Exon1*^{mut} (Supplemental Figure 3). The low VAF of *Exon1*^{mut} (VAF<1%) was confirmed by deep sequencing with unique molecular identifiers (UMI) (xGen® Dual Index UMI Adapters: Integrated DNA Technologies, IA, USA) (19). The correlation between *Exon1*^{mut}, VAF determined by deep sequencing with UMI and

those determined by the ddPCR assay was examined using 24 different samples (Supplemental Figure 4). HLA class I alleles acquiring *Exon1*^{mut} were determined using the nearest allele-specific SNPs. Details on deep sequencing with UMI are provided in the Supplemental Methods.

Statistical analysis

Comparisons were performed using Fisher's exact test for categorical variables and Mann-Whitney U test for continuous variables with a 2-tailed significance level of 0.05. Statistical analyses were performed using the EZR software program (20). Graphs were generated using GraphPad PRISM7.0 (GraphPad Software Inc, CA, USA).

Results

Identification of Exon1^{mut} *in different HLA-A and HLA-B alleles in HLA-LL(+) AA patients*

To identify HLA class I alleles other than *HLA-B*40:02* that are critically involved in autoantigen presentation in AA, HLA-A and HLA-B alleles of sorted granulocytes from 20 HLA-LL(+) AA patients not possessing *HLA-B*40:02* were sequenced. Clinical characteristics of the 20 patients are shown in Supplemental Table 3. HLA-A(-)

granulocytes or HLA-A(+)B(-) granulocytes, accounting for 2.4-99.8% of all granulocytes, were detected in these patients (Supplemental Figure 5 and Supplemental Table 3). Median read depths of the HLA-A and HLA-B alleles were 925, 1,012 for target deep sequencing and 43,013, 35,267 for amplicon sequencing, respectively. Of the 20 assessed AA patients, 6 had 6pLOH alone, 10 had various loss-of-function mutations in addition to 6pLOH, and 4 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(+) granulocytes. Of note, 12 of the 14 patients with loss-of-function mutations had *Exon1*^{mut} 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 2 patients (UPN 335 and UPN 210) had different loss-of-function mutations from *Exon1*^{mut} in *HLA-B*40:03* and *HLA-B*54:01*, respectively. Interestingly, a frameshift mutation of *HLA-B*54:01* also occurred at position 19 (c.19delC, p. R7Efs) in exon 1 (Figures 1A and 1B, Supplemental Table 4).

Exon1^{mut} *detection using a sensitive ddPCR assay*

To detect *Exon1*^{mut} 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 WT DNA and serial dilutions of *Exon1*^{mut} template DNA revealed a detection limit of 0.07% for both HLA-A and HLA-B (Supplemental Figure 6). The ddPCR assay yielded 0% to 0.042% (median, 0.009%) positive dots in PB of 24 healthy individuals, validating the cut-off value of 0.07%. The ddPCR assay was able to detect *Exon1*^{mut}, which had an allelic frequency of <1.0% (Figure 2A), clearly showing the presence of HLA-LLs in patients whose FCM analysis of monocytes using anti-HLA-A2 Abs produced unequivocal results regarding the presence of HLA-A2-lacking monocytes (Figure 2B).

Prevalence of Exon1^{mut} in AA patients

Using two different ddPCR mixtures for HLA-A and HLA-B, the presence of *Exon1*^{mut} was evaluated in all of the 353 patients. *Exon1*^{mut} 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*^{mut} was similar between HLA-A and HLA-B alleles in both treatment-naive patients (n=291) and treated patients (n=62), respectively. Treated *Exon1*^{mut}(+) AA patients had the higher median frequency of *Exon1*^{mut} than untreated *Exon1*^{mut}(+) AA patients (0.96% vs 0.33%, *P=0.0079*) (Figure 3A). Figure 3B shows the relationships between *Exon1*^{mut}(+), 6pLOH(+), and paroxysmal nocturnal

hemoglobinuria (PNH) phenotype cells (+) patients. Fifty-six (55.4%) and 67 (66.3%) of the 101 *Exon1*^{mut}(+) patients had 6pLOH and PNH phenotype cells, respectively. Ten (2.8%) of the 353 patients had *Exon1*^{mut} alone. The frequency of *Exon1*^{mut} were much smaller than those of 6pLOH in 36 patients possessing both clones (P<0.001) (Figure 3C).

Long-term persistence of Exon1*^{mut}*(+) cells

Serial blood samples were available for longitudinal analyses of *Exon1*^{mut} in 13 patients who responded to IST (cyclosporine [CsA] alone in 6 and rabbit antithymocyte globulin [rATG] + CsA in 5) or anabolic steroids (n=2). *Exon1*^{mut} was persistently detected for 14-86 months in 9 patients including 1 patient (UPN 299) who had been off treatment for 7 years, suggesting that *Exon1*^{mut}(+) leukocytes are derived from long-lasting HSPCs (Figure 4A). The VAF of *Exon1*^{mut}(+) increased in 2 (15%, UPN 333 and UPN 339), remained stable in 4 (31%), and decreased in 3 (23%). *Exon1*^{mut} became undetectable at 7-33 months after the first detection of *Exon1*^{mut} in the other 4 patients, all of whom were being treated with CsA. Figure 4B shows a gradual decline of the *Exon1*^{mut} frequency over 3 years in one patient (UPN 213).

HLA-A and HLA-B alleles that acquire Exon1^{mut}

Among the 101 *Exon1*^{mut}(+) patients, HLA alleles that acquired *Exon1*^{mut} could be determined by targeted deep sequencing with (n=21) or without UMI (n=37), or deduced from alleles contained in the lost haplotype due to 6pLOH that was accompanied by *Exon1*^{mut} (n=10) (Supplemental Figure 2). In the other 33 *Exon1*^{mut}(+) patients, HLA-A or HLA-B alleles that acquired *Exon1*^{mut} could not be determined or deduced due to very low VAFs (<0.2%), the absence of allele-specific SNPs near *Exon1*^{mut} 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*^{mut} 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 supertypes of these alleles, which are defined by similarities in the antigen-presenting amino-acid motif of HLA alleles, were confined to only 4 supertypes: A02, A03, B07, and B44, except for *HLA-B*13:01* that does not belong to any of the 14 supertypes (21).

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 1 of the 12 alleles, while the prevalence was 92% in the 353 patients with AA (P<0.001) and 100% in the 83 6pLOH(+) patients (P<0.001) (Figure 5B), suggesting the involvement of these alleles in AA development.

Loss of HLA-B expression from Exon1^{mut}(+) leukocytes

Although *Exon1^{mut}* 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^{mut}* is very low. We previously established 6 iPS cell clones from PB monocytes of an AA patient (UPN 333) whose monocytes included approximately 60% HLA-A24(+)Bw6(-) cells (Figures 6A) (17). Deep sequencing revealed the presence of *Exon1^{mut}* in sorted HLA-A24(+)Bw6(-) cells and also in 1 (clone C1) of the 6 iPS cell clones. When a WT iPS clone (clone E1) and clone C1 were induced to differentiate into CD34^{+} cells, all clone E1-derived CD34^{+} cells expressed HLA-Bw6 (B5401), while all clone C1-derived CD34^{+} cells lacked HLA-Bw6 (Figure 6B). The ddPCR assay using DNA from WT and *Exon1^{mut}*(+) iPS cell-derived CD34^{+} cells revealed the VAF of *Exon1^{mut}* to be 0.041% and 49%, respectively, as expected (Figure 6C).

Clinical characteristics of Exon1^{mut}(+) AA patients

Of the 291 patients whose PB samples were examined for *Exon1*^{mut} and GPI(-) cells before treatment, 151 were evaluable for response to IST (CsA alone, n=68; CsA+rATG, n=83). The other 140 patients were excluded from the analysis of the relationship between the IST response and the presence of *Exon1*^{mut} or GPI(-) cells because no data on the IST response were available in 84, and the remaining 56 received no treatment (n=25) or treatments other than IST (n=31), such as anabolic steroids and thrombopoietin receptor agonists, and allogeneic stem cell transplantation. Increased GPI(-) cells were noted in 76% (34/45) of *Exon1*^{mut}(+) and 76% (81/106) of *Exon1*^{mut}(-) patients (P=1.0). In terms of response to IST, 82% (37/45) of *Exon1*^{mut}(+) patients responded to CsA (n=14) or CsA+rATG (n=23), while 75% (79/106) of *Exon1*^{mut}(-) patients responded to CsA (n=35) or CsA+rATG (n=44) (P=0.40). The response rate to IST in *Exon1*^{mut}(+) patients was significantly higher than that (54%, 13/24) in patients who were negative for all of *Exon1*^{mut}, GPI(-) cells, and 6pLOH (P=0.023).

Discussion

Targeted deep sequencing of HLA genes of leukocytes obtained from HLA-LL(+) AA patients revealed a unique nonsense mutation at position 19 (c.19C>T, p.R7X) in exon 1 (*Exon1*^{mut}) 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 (9, 14). Our highly sensitive ddPCR assay allowed for the detection of minor *Exon1*^{mut} clones and detected the mutant DNA in nearly one third of Japanese AA patients regardless of the presence of 6pLOH. *Exon1*^{mut} was also detected in 2 of 8 Finnish AA patients we studied (Unpublished observation). Interestingly, a frameshift mutation (c.19delC, p.R7Efs) was also identified at position 19 of *HLA-B*54:01* in a patients (UPN 210) without *Exon1*^{mut}, suggesting that the position 19 in exon 1 of *HLA-A* and *HLA-B* may be a specific position where somatic mutations are likely to occur.

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

We previously used targeted deep sequencing to identify frequent loss-of-function mutations in 3 HLA class I alleles, including *B*40:02*, *A*02:06*, and *B*54:01* (9, 16, 17). The highly sensitive ddPCR assay described herein that was capable of detecting *Exon1*^{mut} newly identified 3 HLA-A (*A*02:01, A*02:07, A*31:01*) and 6 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 the frequency of these alleles in the general Japanese population, these HLA alleles were found to be highly enriched in AA patients. Among the 14 HLA supertypes that are defined based on similarities in the antigen-presenting peptide motif, the 12 alleles mentioned above belonged only to 4 of the supertypes (21). These findings suggest that autoantigens of AA may be presented to T cells by these specific HLA alleles on HSPCs.

Similar to 6pLOH(+) HSPCs, *Exon1*^{mut}(+) HSPCs are thought to escape CTL attack 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*^{mut} occurs more frequently in HSPCs 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*^{mut} only in head and neck squamous

cell cancers (HNSCs) (24). HSPCs thus may share a common property in that *Exon1*^{mut} is likely to occur in class I HLA genes in HNSCs.

The median VAF of *Exon1*^{mut} in *Exon1*^{mut} (+) 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(+) leukocytes were often polyclonal, consisting of leukocytes having different breakpoints of uniparental disomy in the short arm of chromosome 6 (10). This polyclonality may account for the high proportion of 6pLOH. Although *Exon1*^{mut} (+) leukocytes represent a minor leukocyte population, the long-term (1-7 years) persistence of these mutated leukocytes indicates that they are derived from HSPCs 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 (HSCs) to mature PB cells in patients with clonal hematopoiesis of indeterminate potential (26). *Exon1*^{mut} (+) leukocytes may also be derived from most immature HSCs. The persistence of similarly minor clones in PB has been reported for GPI(-) granulocytes in AA, the median frequency of which was 0.25% (18, 27). In contrast to *PIGA*-mutated or 6pLOH(+) leukocytes that can be oligoclonal and dysfunctional due to the lack of all GPI-APs or a large segment of 6p, *Exon1*^{mut}(+)

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 HSPCs are actively involved in human hematopoiesis (28). Thus, the small proportion of *Exon1*^{mut}(+) leukocytes among the entire leukocyte population may reflect an average clone size of individual HSPCs in the BM.

HLA-LLs are useful markers that indicate the presence of immune pathophysiology in patients with BM failure. Here we showed a high response rate to IST in *Exon1*^{mut}(+) patients, although *Exon1*^{mut}(-) patients also had a high response rate likely due to the high prevalence of increased GPI(-) cells (29-31). Several methods are used to detect HLA-LLs, including FCM assays with mAbs specific to HLA-A or HLA-B alleles, ddPCR or SNP arrays for detecting 6pLOH, and targeted deep sequencing (8-10, 12). However, these methods required HLA typing of patients, take a long time to produce results, and are unable to detect HLA-LLs that account for less than 1% of total leukocytes. The ddPCR assay used in the present study to detect *Exon1*^{mut} enables the detection of HLA-LLs accounting for as low as 0.07% of the total leukocyte population within 6 hours of blood collection, highlighting the powerful nature of this assay for diagnosing immune pathophysiology in patients with BM failure.

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H.M. is a PhD candidate at Kanazawa University and this work is submitted in partial fulfillment of the requirements for the PhD.

Authorship

Contribution: H.M., T.I., K.T., Y.Z., and S.N. collected clinical data and blood samples. F.A. performed HLA genotyping. Y.F., S.O. conducted the SNP array analyses. Y.Z., H.T., T.O., H.K., and A.M. generated an original mAb specific to HLA-B13, B60 and B61. H.M., T.Y. performed cell sorting. H.M., K.Hosomichi., T.I., Y.Z., and A.T. performed deep sequencing. H.M., Y.Z., N.M.A., and T.C.D. performed the droplet digital PCR. K.C and Y.Y. generated iPSCs. M.I.E. performed the *in vitro* experiments. H.M., K.Hosokawa., and S.N. designed the research and wrote the manuscript. All authors critically reviewed the manuscript and checked the final version.

Conflict of interest disclosure

The authors declare no competing financial interests.

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Table 1. Baseline characteristics of AA patients

Characteristics	No. of patients
Total	353
Median age, y (range)	63 (11-93)
Sex, M/F	167/186
Severity	
NSAA	202
SAA / VSAA	151
Patients with increased GPI(-) 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

Abbreviations: M, male; F, female; GPI(-), glycosylphosphatidylinositol-anchored proteins deficient; NSAA, non-severe aplastic anemia; SAA, severe aplastic anemia; VSAA, very severe aplastic anemia; IST, immunosuppressive therapy; CsA, cyclosporine; TPO-RA, thrombopoietin receptor agonist; ATG, antithymocyte globulin.

Figure legends

Figure 1. Identification of *Exon1*^{mut} in AA patients

A. *Exon1*^{mut} [p.R7*(C>T)] was detected by targeted deep sequencing of sorted HLA-A2(-) granulocytes (UPN 262) and HLA-A2(+)B60(-) granulocytes (UPN 211) in 2 AA patients. Sequencing results of sorted HLA-LLs from these 2 patients and germline controls and FCM results of granulocytes are shown. B. Loss-of-function mutations detected in 14 patients by targeted deep sequencing. *Exon1*^{mut} was detected in HLA-A alleles of 8 patients and HLA-B alleles of 4 patients.

Figure 2. Detection of *Exon1*^{mut} using the ddPCR assay.

A. Representative ddPCR plots of *Exon1*^{mut} in 2 AA patients. The ddPCR assay detected 0.92% *Exon1*^{mut} DNA in *HLA-A*02:06* of UPN 299 and 0.98% *Exon1*^{mut} DNA in *HLA-B*40:01* of UPN 211. B. A minor HLA-LLs population in UPN 280 detected by FCM and the ddPCR assay. The ddPCR assay detected 0.27% *Exon1*^{mut} in whole blood where *Exon1*^{mut}(+) granulocytes and monocytes were diluted with *Exon1*^{mut}(-) lymphocytes. The *Exon1*^{mut}(+) percentage was consistent with the percentage of HLA-A2(-) monocytes (0.6%) detected by FCM.

Figure 3. Prevalence and clone size of *Exon1*^{mut} in AA patients

A. Mutant allele frequency of *Exon1*^{mut} in 291 and 62 *Exon1*^{mut}(+) untreated and treated AA patients, respectively. Red bars indicate median allele frequencies of *Exon1*^{mut}. B. The prevalence of *Exon1*^{mut} and its relationship with PNH(+) and 6pLOH(+) patients. *Exon1*^{mut}(+) patients accounted for 58% of 6pLOH(+)PNH(+) patients and for 67% of 6pLOH(+) patients. C. Frequency of *Exon1*^{mut} and 6pLOH in individual patients who were positive for both mutant clones. Frequencies of both clones were determined by ddPCR. The asterisk indicates $P<0.001$.

Figure 4. Temporal changes in allelic frequency of *Exon1*^{mut}(+)

A. Allelic frequencies of *Exon1*^{mut} determined at different time points in 13 patients and their disease status are shown. B. Representative scattergrams from UPN 213 showing a gradual decline in *Exon1*^{mut} frequency over the course of 3 years.

Figure 5. HLA alleles that acquired *Exon1*^{mut}

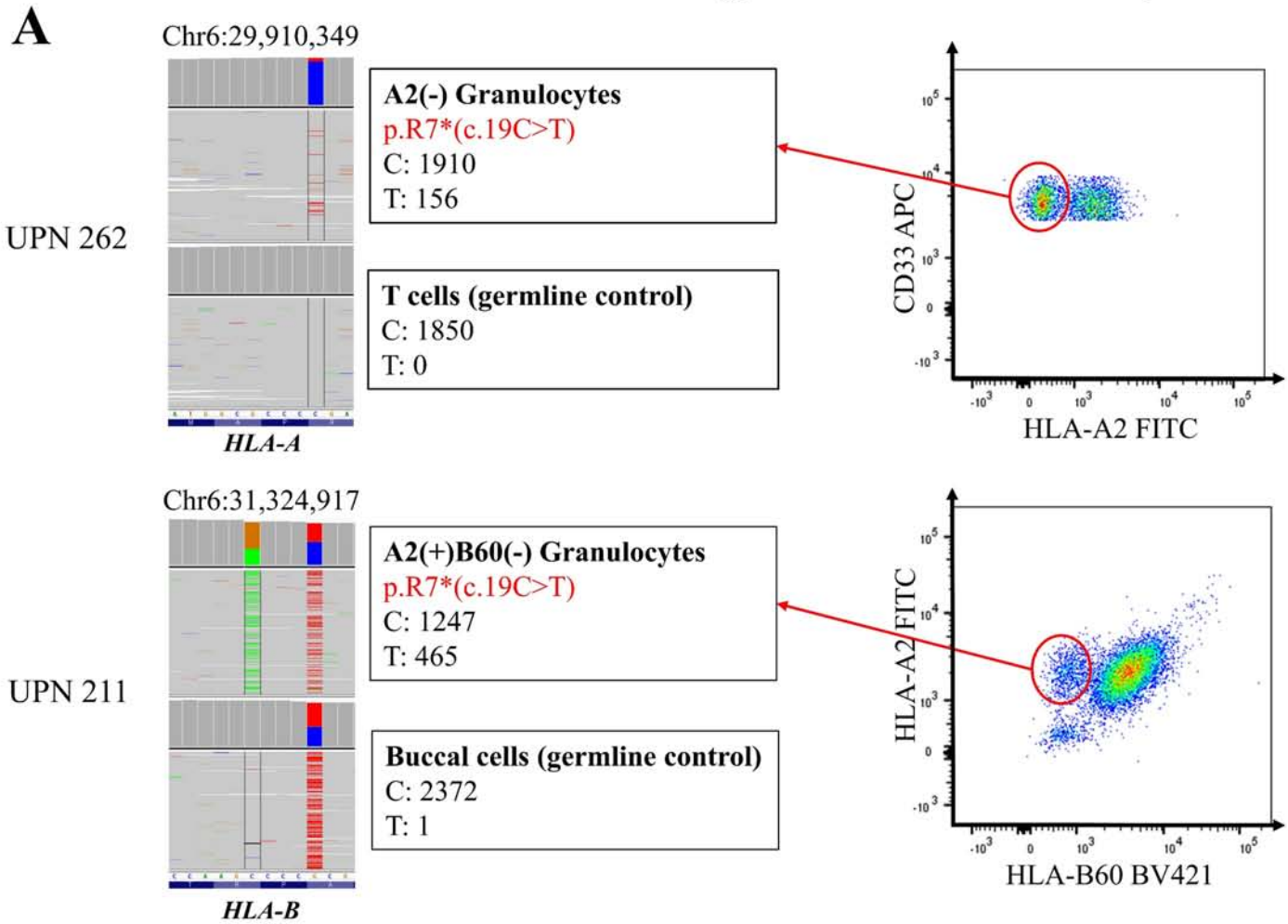
A. The number of cases that acquired *Exon1*^{mut} in the corresponding HLA alleles are shown. HLA numbers shown below the bar graph denote HLA supertypes that each allele belongs to. B. Proportions that the 12 HLA alleles account for in the different

populations.

Figure 6. HLA allele expression by *Exon1*^{mut}(+) HSPCs

A. Establishment of iPSC-derived hematopoietic stem cells from monocytes of an AA patients with *Exon1*^{mut} (UPN 333). B. HLA-Bw6 (B5401) expression by CD34^{+} cells derived from a wild-type iPS clone (left) and an *Exon1*^{mut}(+) iPS clone (right). C. *Exon1*^{mut} detection in DNA from wild-type (left) and *Exon1*^{mut}(+) (right) iPS cell-derived CD34^{+} cells. Numbers below the scattergram denote the variant allele frequency (VAF) of *Exon1*^{mut}.

Figure 1. Mizumaki, et al.



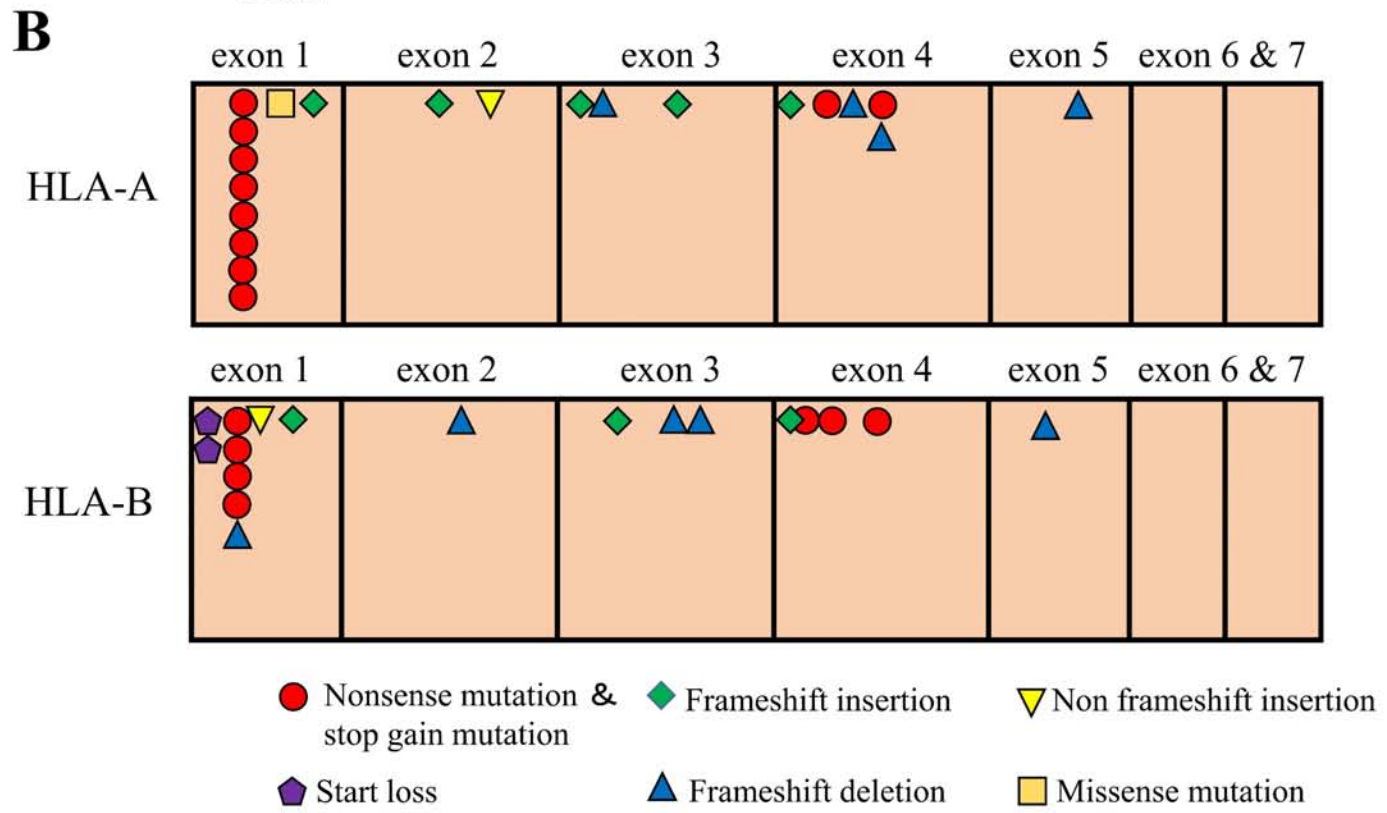


Figure 2. Mizumaki, et al.

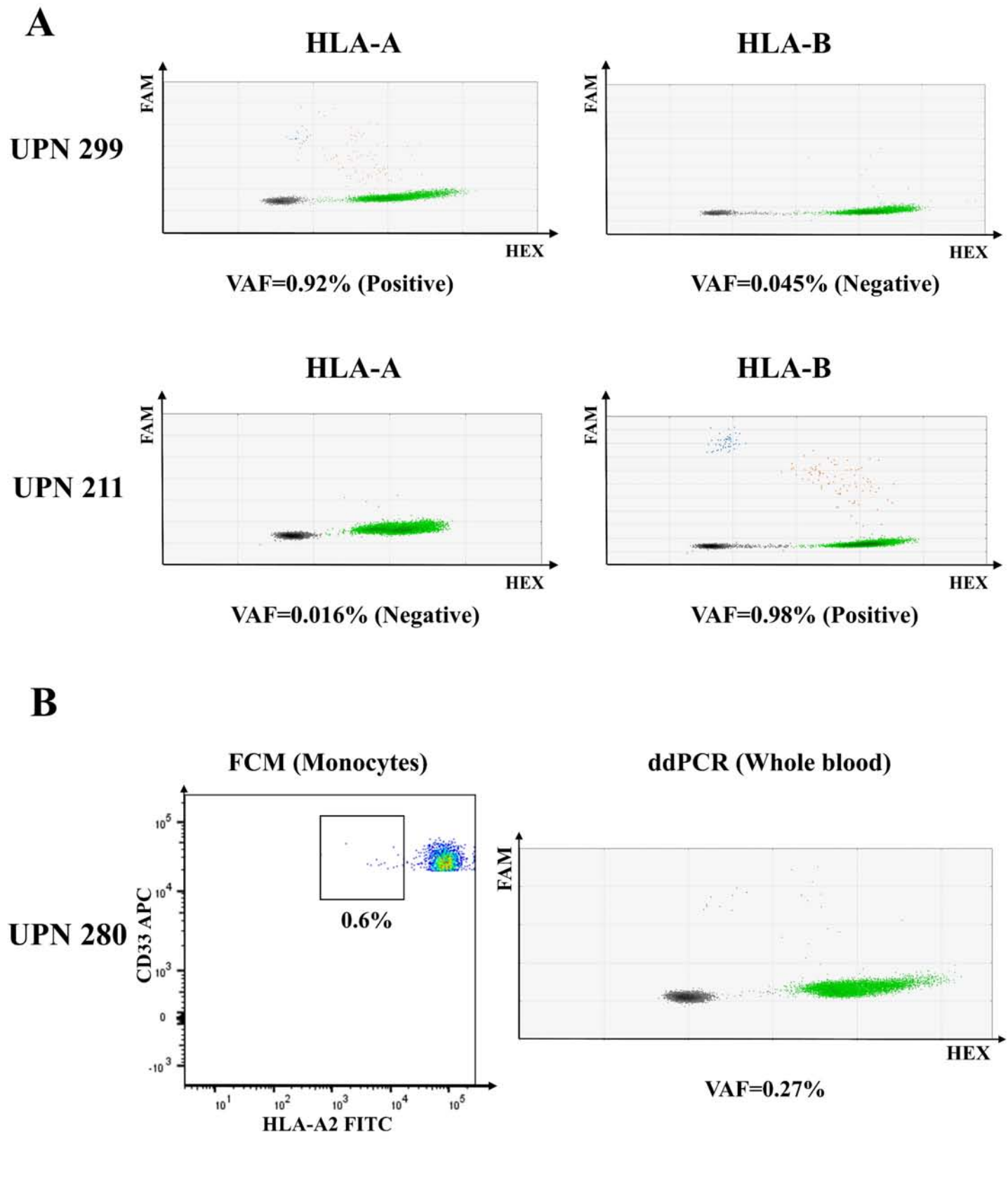


Figure 3. Mizumaki, et al.

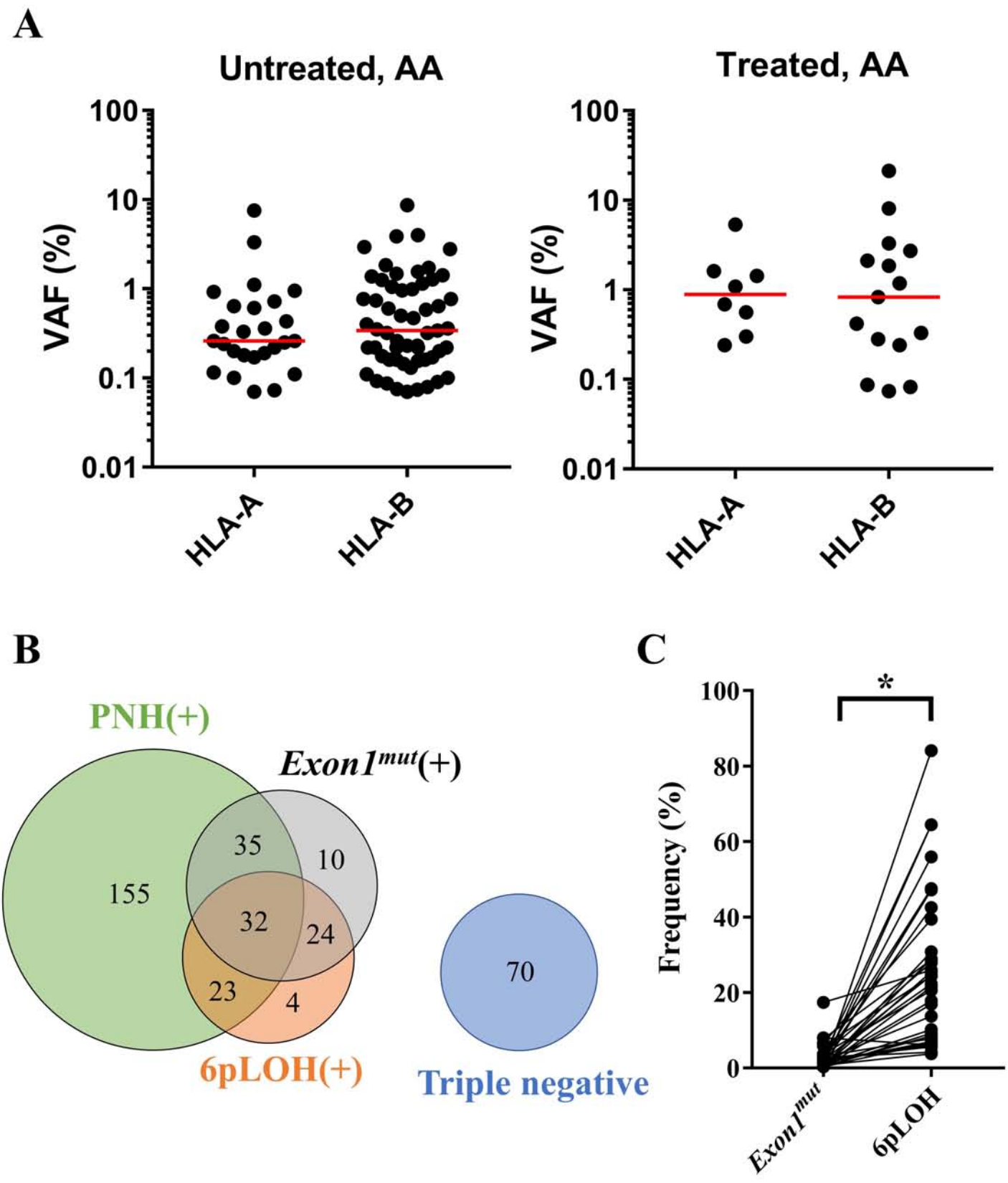
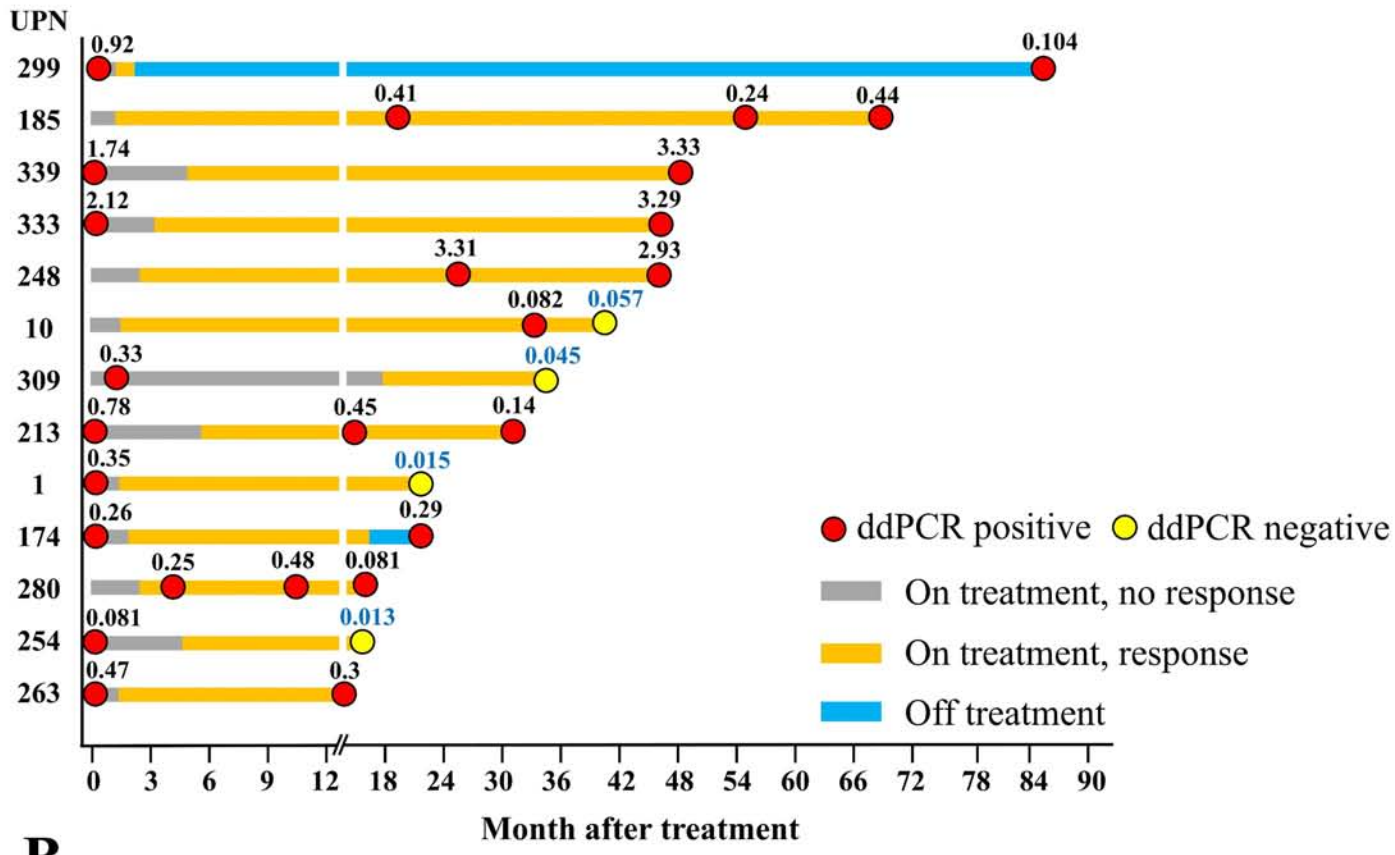


Figure 4. Mizumaki, et al.



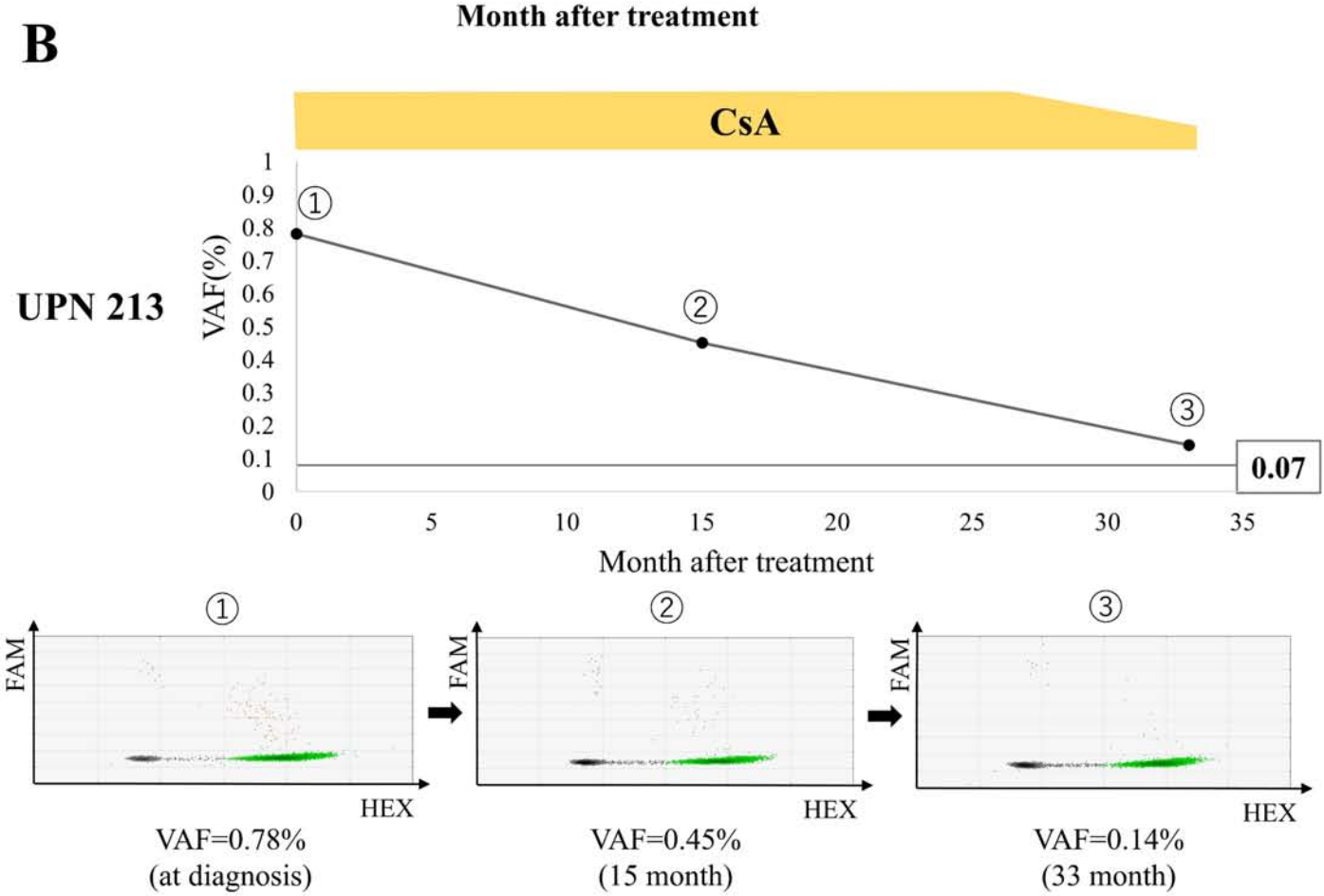
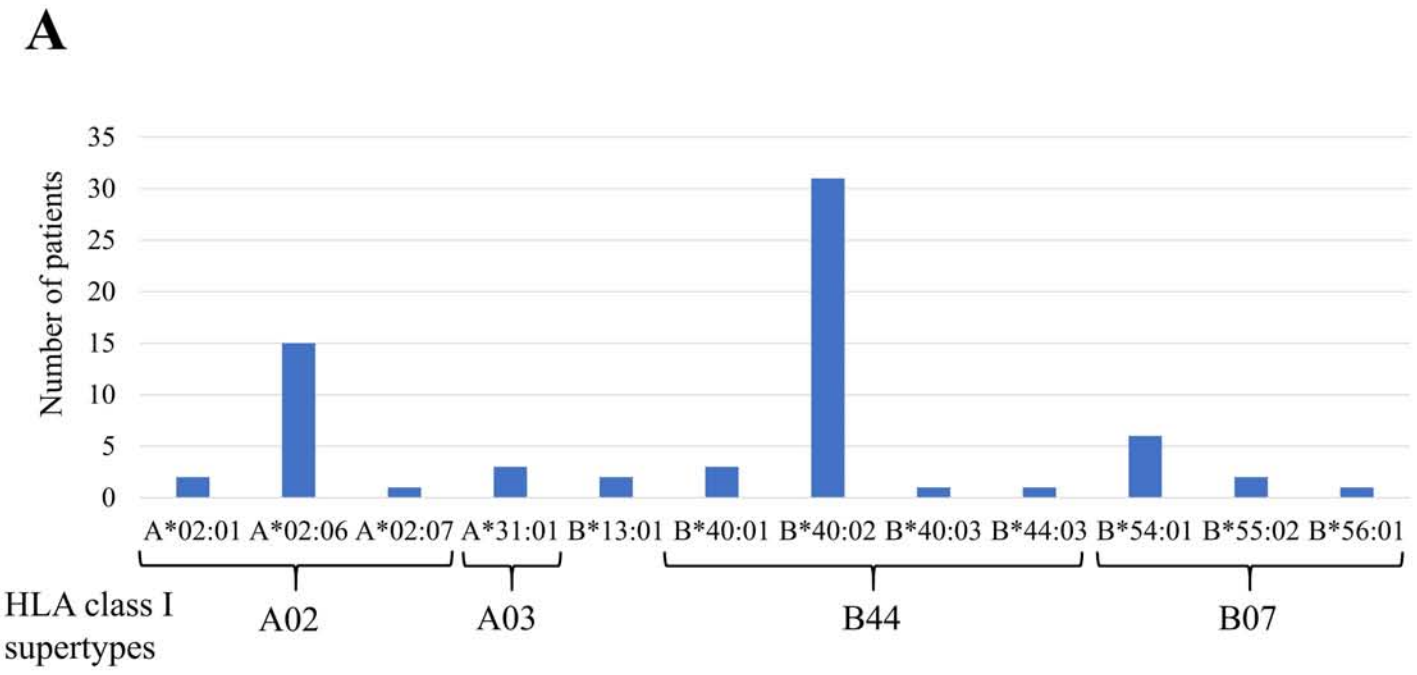


Figure 5. Mizumaki, et al.



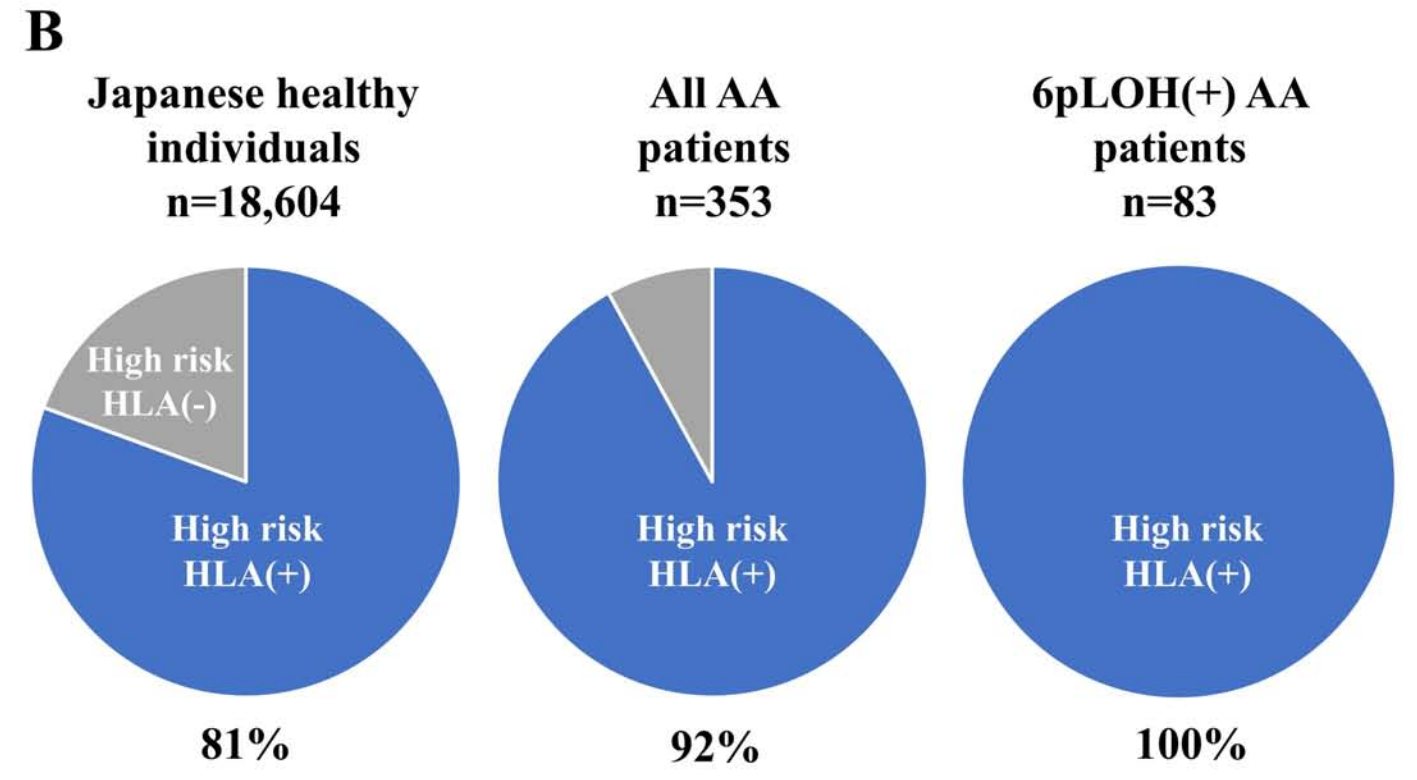
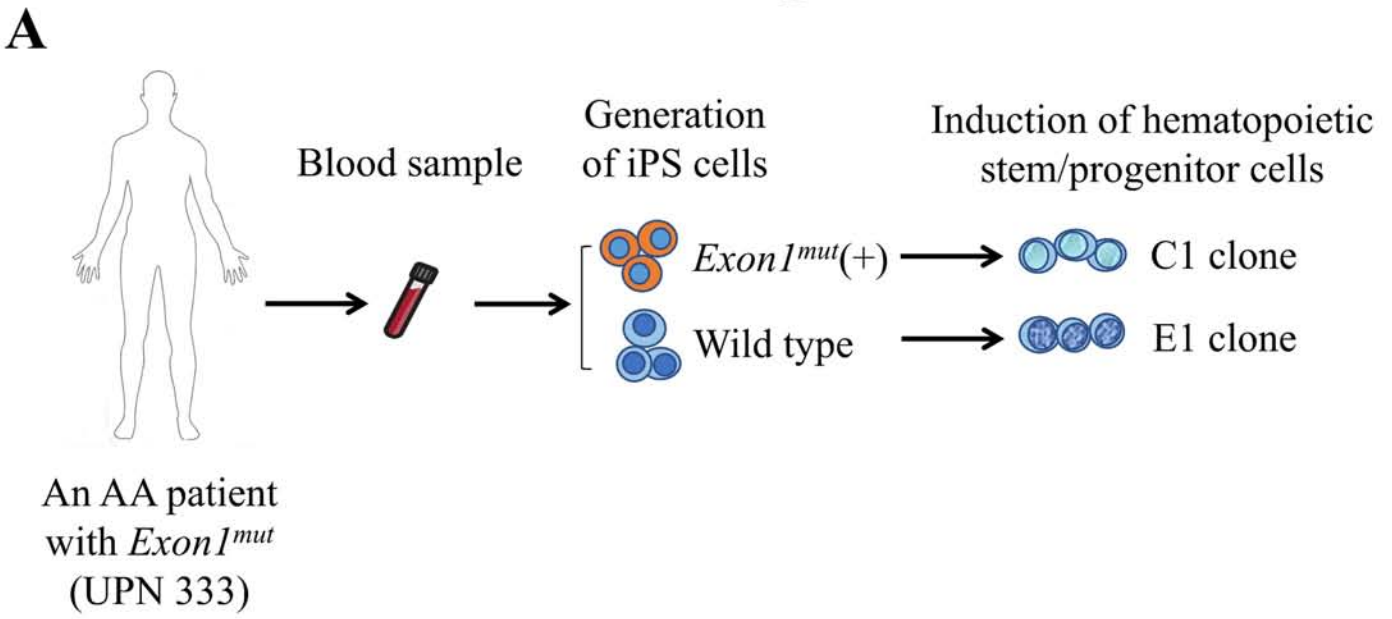
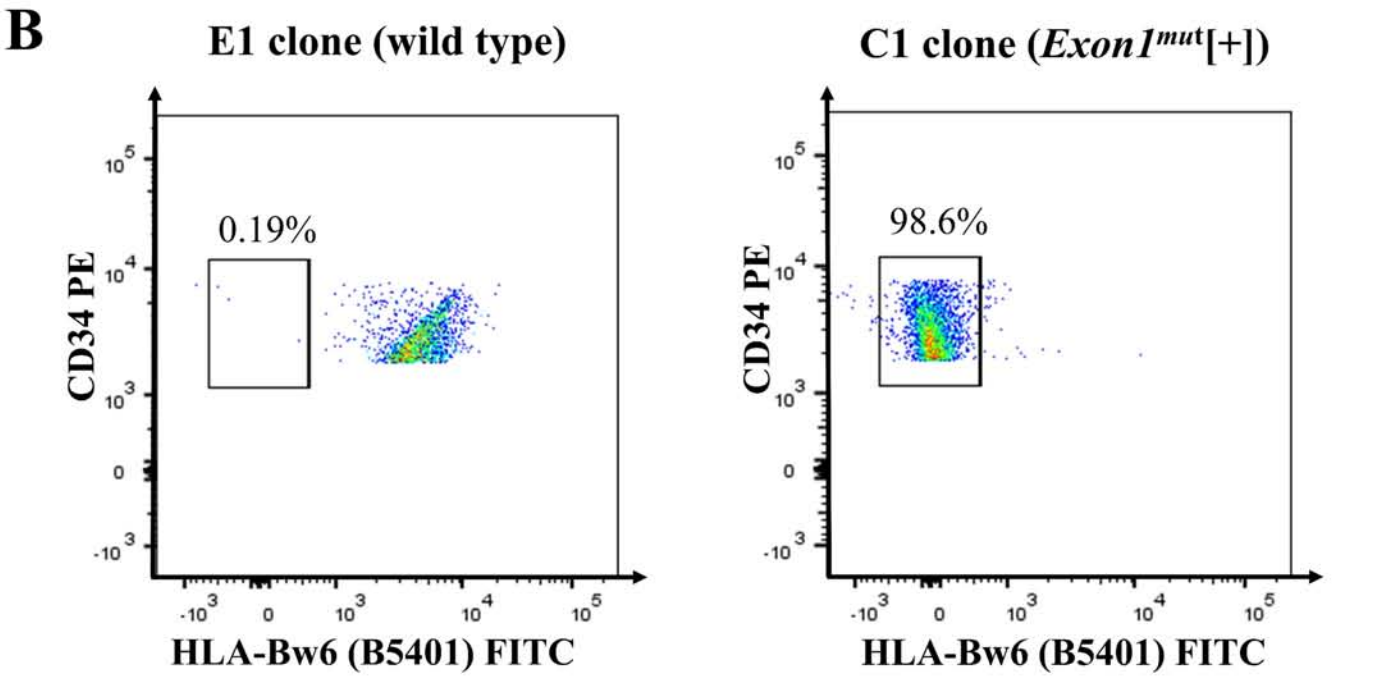
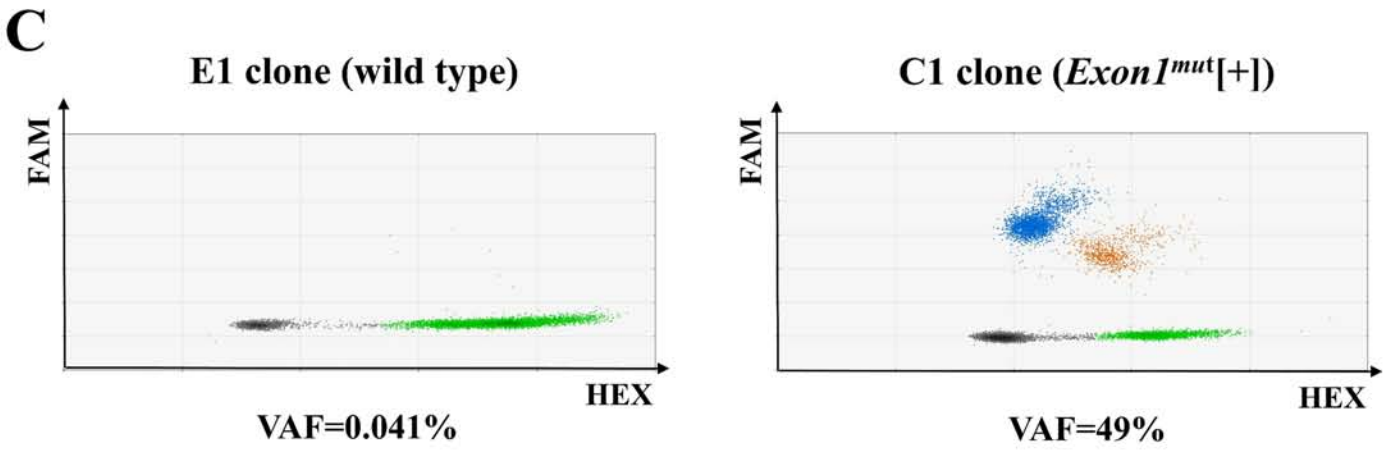


Figure 6. Mizumaki, et al.







Supplemental information

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

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SUPPLEMENTAL METHODS

SUPPLEMENTAL TABLES

SUPPLEMENTAL FIGURES

with 6pLOH.

Supplemental Figure 4. Correlation between the variant allele frequencies (VAF) determined by the ddPCR and those determined by target sequencing with the next generation sequencer.

Supplemental Figure 5. HLA-class I allele lacking granulocytes detected in 20 patients of the cohort 1.

Supplemental Figure 6. Determination of the cut-off allele frequency that can be detected by the ddPCR.

SUPPLEMENTAL METHODS

Patients

Twenty Japanese AA patients with HLA-LLs who did not have *HLA-B*40:02* allele were analyzed for the presence of loss-of-function mutations in HLA alleles. A total of 353 Japanese AA patients including the 20 patients (severe and very severe, n=151; non-severe, n=202; male, n=167; female, n=186; median age, 63 [range, 11–93] years) were further analyzed for the prevalence and clinical significance of $Exon1^{mut}$ in AA between 2010 and 2018. Of the 353 patients, 265 had been included in our previous studies (1, 2).

HLA typing and definition of AA severity

All patients were genotyped for HLA-A, HLA-B, HLA-C, and HLA-DRB1 alleles using the polymerase chain reaction (PCR) sequence-specific oligonucleotide method and 6pLOH was detected by GeneChip 500K arrays (Affymetrix) or droplet digital polymerase chain reaction (ddPCR) using a QX200 AutoDG Droplet Digital PCR System (Bio-Rad, Hercules, USA), as previously described (1, 2). HLA haplotypes of patients were estimated from the database of the Japanese population, which includes 18,604 healthy individuals, as previously described (3). Informed consent was obtained from all patients for the HLA-genotyping and genetic analyses. The study protocols

were approved by the ethical committee of Kanazawa University Institute of Medical, Pharmaceutical, and Health Sciences. Severe AA was diagnosed when at least two of the following criteria were met; the neutrophil count was $< 0.5 \times 10^9$/L, the platelet count was $< 20 \times 10^9$/L and the reticulocyte count was $< 20 \times 10^9$/L. Very severe AA was defined as a neutrophil count $< 0.2 \times 10^9$/L in addition to the criteria for severe AA (4). The response criteria have been described previously (5).

Detection and FACS sorting of HLA-LLs

Peripheral blood (PB) samples were stained with anti-HLA-A allele-specific and lineage-specific mAbs to detect leukocytes that lack particular HLA-A or HLA-B alleles (HLA-allele-lacking leukocytes: HLA-LLs). We used mAbs specific for each of HLA-B13, HLA-B60, and HLA-B61 that we developed (2) and HLA-Bw6, as well as mAbs specific for HLA-A, in order to detect HLA-A-positive ($HLA\text{-}A^+$) and HLA-B negative ($HLA\text{-}B^-$) granulocytes, when patients possessed these HLA-B alleles. The mAbs used in this study are summarized in Supplemental Table 1. HLA-LLs were detected using BD FACSCantoII (BD Biosciences, San Jose, CA, USA) and analyzed with the Flowjo (version 10.1) software program (Tree Star, Ashland, OR, USA).

From the PB samples of patients with HLA-LLs, paired fractions, including

granulocytes which lack the HLA class I allele (HLA[-] granulocytes) and granulocytes which retain the HLA class I allele (HLA[+] granulocytes), and $CD3^+$ T cells were sorted using a fluorescence-activated cell sorter (BD FACSAria™ Fusion, BD Biosciences, Franklin Lakes, NJ, USA). Sorted granulocyte populations and control cells ($CD3^+$ T cells or buccal mucosa cells) were subjected to DNA extraction (Supplemental Figure 2). All PB samples from the patients with HLA-LLs were collected during treatment.

Next generation sequencing of HLA class I genes

Nucleotide sequences of HLA-A and HLA-B in sorted granulocytes of the 20 HLA-LL(+) AA patients were determined by using the sequence capture method with a next generation sequencer (NGS). The sequence capture method based on hybridization between DNA of an adapter-ligated library (KAPA Hyper Prep Kit, Roche Diagnostics, Westfield, IN, USA) and a biotinylated DNA probe (SeqCap EZ choice kit, Roche Diagnostics, Westfield, IN, USA) custom-designed based on target sequences of HLA genes.(6) Paired-end sequence reads (350 bp read 1 and 250 bp read 2 in length) were obtained by using the MiSeq sequencer (illumina, San Diego, CA, USA). Somatic mutations were detected as difference from reference sequence with low variant allele

frequency (VAF <1%), and covering with at least 1000 reads. All of the mutations were validated by ultra-deep sequencing of HLA-A and HLA-B locus-specific long-range PCR amplicons (2, 7). The median read depths for HLA-A and HLA-B amplicons sequencing in the 20 patients were 43,031 (23,689-95,332) and 35,267 (19,378-59,846), respectively. HLA alleles were assigned using the Omixon Target software program (version 2.0.0, Omixon, Budapest, Hungary) for read alignment and genotype calling (reference sequence, IMGT/HLA Database 3.26.0).

Novel ddPCR assay for detecting *Exon1^{mut}*

We designed ddPCR assays to precisely detecting *Exon1^{mut}* in the PB using a QX200 AutoDG Droplet Digital PCR System (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer`s instructions. 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 (LNA)-based probes with a non-fluorescent quencher (Iowa Black®FQ[IBFQ]**)** complementary to wild-type (WT) and mutant-specific (MT) sequences, which were labeled with different fluorochromes (hexachlorofluorescein (HEX) for WT and carboxyfluoresein (FAM) for MT) using PrimerQuest and

BioPhysics (Integrated DNA Technologies, Coralville, IA, USA) (Supplemental Table 2).

The reaction mixtures for ddPCR consisted of the 2 LNA probes, 1 set of the primer pairs and 100 ng of genomic DNA, 5 μl of 4×ddPCR Multiplex Supermix (Bio-Rad), and deionized water in a final volume of 20 μl. Cycling conditions were as follows; 95°C/10 min for enzyme activation, followed by 40 cycles of 94°C/30 sec and 59.5°C/1 min, and 98°C/10 min for enzyme deactivation. Droplets were measured with the QX200 droplet reader and were analyzed with Quantasoft software ver 1.7.4 (Bio-Rad), according to the manufacturer's instructions. The fractional abundance of the mutant allele was obtained by dividing the number of copies of the mutant allele (FAM) by the total number of copies of the WT allele (HEX) plus the mutant allele (FAM). Results were considered evaluable when the number of accepted droplets per well was at least 10,000.

Deep sequencing using unique molecular identifiers

In order to confirm the presence of HLA allelic mutations at very low frequencies (VAF<1%), we performed targeted deep sequencing for HLA class I genes by using

sequence capture method with biotin-labelled oligo probes designed for all entire HLA class I genes and a unique molecular identifiers (UMI) for adapters.

To reduce the sequence read errors and increase the sensitivity of rare mutation detection, we used xGen® Dual Index UMI Adapters (Integrated DNA Technologies, Coralville, IA, USA), which reduces amplification errors and enables the accurate detection of true variants at as low as 0.2% VAF, in place of standard Illumina dual index adaptors in this assay (8). Enriched DNA fragments for entire HLA genes were sequenced by paired-end sequencing (2×150 bp) using an illumine HiSeq 4000 (Illumina, San Diego, CA, USA). To ensure accurate variant calling, we used three thresholds, at least 10,000× raw sequencing depth, at least 1,000× collapsed sequencing depth after UMI error correction, and at least 6 reads with alternative allele. The sequence reads carrying $Exon1^{mut}$ were used for HLA typing as HLA allele-specific tagSNP detection.

Next-generation sequencing data processing

Paired-end illumine reads were mapped to the reference genome (GRCh37) using Burrows-Wheeler Aligner (bwa) v.0.7.12.(9) bwa-generated SAM files were converted to the BAM format, then sorted and indexed using SAM tools v.1.2 (10). Duplicated

reads were marked with Picard v.1.52 (https://github.com/broadinstitute/picard). After alignment of reads, the heuristic somatic mutation caller, VarScan 2 (11), was used to detect somatic mutation. The mutations were reviewed using Unified Genotyper in the Genome Analysis Toolkit (GATK) v3.4 (12) and the alignment data from granulocytes, T cells and buccal mucosa cells were visually compared via IGV (13). The somatic mutations were given functional annotation and COSMIC (https://cancer.sanger.ac.uk/cosmic) mutation ID meaning by ANNOVAR (14).

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SUPPLEMENTAL TABLES

Supplemental Table 1. The monoclonal antibodies used for flow cytometry

Antigen	Isotype	Conjugate	Source
CD3	Mouse IgG1	PerCP-Cy5.5	BD Biosciences
CD19	Mouse IgG1	APC-Cy7	Beckman Coulter
CD33	Mouse IgG1	APC	Beckman Coulter
HLA-A2/28	Mouse IgG2a	FITC	One Lambda
HLA-A9/24	Mouse IgG2b	FITC	One Lambda
HLA-A9/24	Mouse IgG2b	Biotin	One Lambda
HLA-A30/31	Mouse IgM	Biotin	One Lambda
Streptavidin	NA	PE	BD Biosciences
HLA-Bw6	Mouse IgG2a	FITC	One Lambda
Mouse Ig	Goat polyclonal Ig	BV421	BD Horizon
HLA-B13/B60/B61	Human/mouse chimeric IgG with constant domein of mouse IgG1	None	Original

Abbreviations: Ig, immunoglobulin; PerCP-Cy5.5, peridinin-chlorophyll proteins-Cy5.5 tandem; PE, phycoerythrin; APC, allophycocyanin; APC-Cy7, allophycocyanin-Cy7 tandem; FITC, fluorescein isothiocyanate; BV421, BD Horizon Brilliant Violet 421

Supplemental Table 2. Sequence of primers and probes

Name	Description	Sequence（5’→3’）
primer set of HLA-A	Forward Primer	CGAGGATGGCCGTCATG
	Reverse Primer1	GGACACGGAGGTGTAGAAATAC
	Reverse Primer2	GGGACACGGATGTGAAGAAATA
	Reverse Primer3	GGACACGGATGTGGAGAAATA
	Reverse Primer4	GGACACGGATGTGGTGAAATA
primer set of HLA-B	Forward Primer1	GTCTCCTCAGACACCGAGAT
	Forward Primer2	TCTCCTCAGACGCCAAGAT
	Forward Primer3	TCCTCAGACGCCGAGAT
	Reverse Primer1	CTCCCTACAGAGGCCATTTC
	Reverse Primer2	CCGGCAGAGGCCATTTC
	Reverse Primer3	CCCACAGAGGCCATTTCC
Probe set	LNA probe for WT (HEX)	CC+C+C+GAA+CC
	LNA probe for MT (FAM)	C+C+C+T+GAA+CC

Abbreviations: LNA, locked nucleic acid; FAM, carboxyfluoresein; HEX, hexachlorofluorescein; MT, mutant-specific sequences; WT, wild-type specific sequences

+C, +G, and +T indicates bases which connect to LNA.

Supplemental Table 3. Patient characteristics

UPN	Age at diagnosis	Sex	Disease duration, year	Severity	Treatment	Time after IST, year	Response to IST	% of GPI(-) cells at diagnosis	
								Granulocytes	RBCs
211	73	F	1.7	NSAA	CSA	1.6	CR	0.061	0.036
335	18	M	1.7	VSAA	ATG+CSA+EPAG	1.7	NR	0.024	0.009
338	54	M	0.8	SAA	ATG+CSA+EPAG	0.7	NR	0.079	0.050
248	61	F	2.8	SAA	ATG+CSA	2.8	PR	0.012	0.000
262	75	M	0.3	SAA	CSA	0.3	PR	0.103	0.047
349	68	F	14.3	NSAA	CSA	14.3	CR	0.001	0.001
344	25	F	15.8	NSAA	CSA	15.7	CR	0.405	0.207
339	47	M	11.8	NSAA	AS	11.6	CR	0..000	0.000
333	53	M	3.9	NSAA	CSA	3.9	PR	3.499	0.407
309	51	F	3.1	VSAA	Romi+AS	NE	CR	0.000	0.000
334	81	M	10.3	SAA	ATG+CSA	10.3	CR	0.001	0.000
336	20	M	0.2	SAA	ATG+CSA	0.1	CR	1.833	0.085
210	80	M	2.1	NSAA	CSA＋EPAG	0.9	NR	7.33	0.700
340	42	M	12.6	SAA	CSA	12.6	CR	0.180	0.340
353	52	M	6.8	SAA	CSA	NE	CR	12.324	0.524
348	70	M	1.3	NSAA	CSA+EPAG+AS	1.3	PR	0.706	0.069
235	35	M	0.4	NSAA	ATG+CSA	0.3	CR	0.101	0.092
346	55	F	24.8	NSAA	None	NE	NE	0.172	1.039
242	68	M	0.5	NSAA	ATG+CSA	0.3	CR	0.058	0.035
285	69	M	0.2	SAA	ATG+CSA+EPAG	0.2	NR	0.078	0.008

Supplemental Table 3. Patient characteristics (continued)

UPN	% of HLA (-) granulocytes in total granulocytes		A	B	C	DRB1	A	B	C	DRB1
	Type of granulocytes	%	haplotype (1)				haplotype (2)			
211	A2(+)B4001(-)G	9.3	02:01	40:01	03:03	15:02	31:01	51:01	14:02	08:02
335	A2(-)G	90.0	02:01	40:03	03:04	14:05	03:01	44:02	05:01	13:01
338	A2(-)G	31.9	02:07	54:01	07:04	12:01	24:02	15:18	01:02	04:05
248	A2(+)B1301(-)G	29.8	02:01	13:01	03:04	12:02	24:02	52:01	12:02	15:02
262	A2(-)G	47.5	02:06	39:01	07:02	15:01	11:01	39:01	01:02	15:01
349	A31(-)G	99.4	31:01	48:01	03:04	09:01	02:06	39:01	07:02	15:01
344	A2(-)G	95.4	02:06	07:02	07:02	01:01	01:01	37:01	06:02	15:01
339	A2(-)G	80.1	02:06	39:01	07:02	08:05	24:02	15:18	07:04	04:01
333	A24(+)Bw6(-)G	74.4	24:02	54:01	01:02	04:01	01:01	37:01	06:02	10:01
309	A2(-)G	99.8	02:06	54:01	01:02	15:01	24:02	46:01	01:03	09:01
334	A2(+)B4001(-)G	22.0	24:02	40:01	07:02	12:01	11:01	67:01	07:02	15:01
336	A2(-)G	6.4	02:06	39:01	08:03	15:01	24:02	54:01	07:02	04:05
210	A24(-)G	30.1	24:02	54:01	01:02	04:05	02:06	35:01	03:03	15:02
340	A31(-)G	99.2	31:01	51:01	14:02	14:05	24:02	07:02	07:02	01:01
353	A2(-)G	2.4	02:06	48:01	08:03	16:02	26:02	15:01	03:03	15:01
348	A24(-)G	38.3	24:02	52:01	12:02	15:02	02:06	39:01	07:02	15:01
235	A2(-)G	2.9	02:06	59:01	01:02	04:05	24:02	07:02	07:02	01:01
346	A31(-)G	99.6	31:01	35:01	03:03	09:01	26:01	39:01	07:02	08:03
242	A2(-)G	20.5	02:06	07:02	07:02	15:02	33:03	44:03	14:03	13:02
285	A2(-)G	19.3	02:06	39:01	07:02	15:01	24:02	52:01	12:02	15:02

Abbreviations: UPN, unique patient number; F, female; M, male; GPI-AP(-), glycosylphosphatidylinositol-anchored proteins deficient;

NSAA, non-severe aplastic anemia; SAA, severe aplastic anemia; VSAA, very severe aplastic anemia; PNH, paroxysmal nocturnal hemoglobinuria; CsA, cyclosporine; ATG, antithymocyte globulin; AS, anabolic steroids; EPAG, eltrombopag; Romi, romiplastim; IST, immunosuppressive therapy; CR, complete response; PR, partial response; NR, no response; NE, not evaluated; G, granulocytes.

Supplemental Table 4. Loss-of-function mutations identified in HLA class I genes

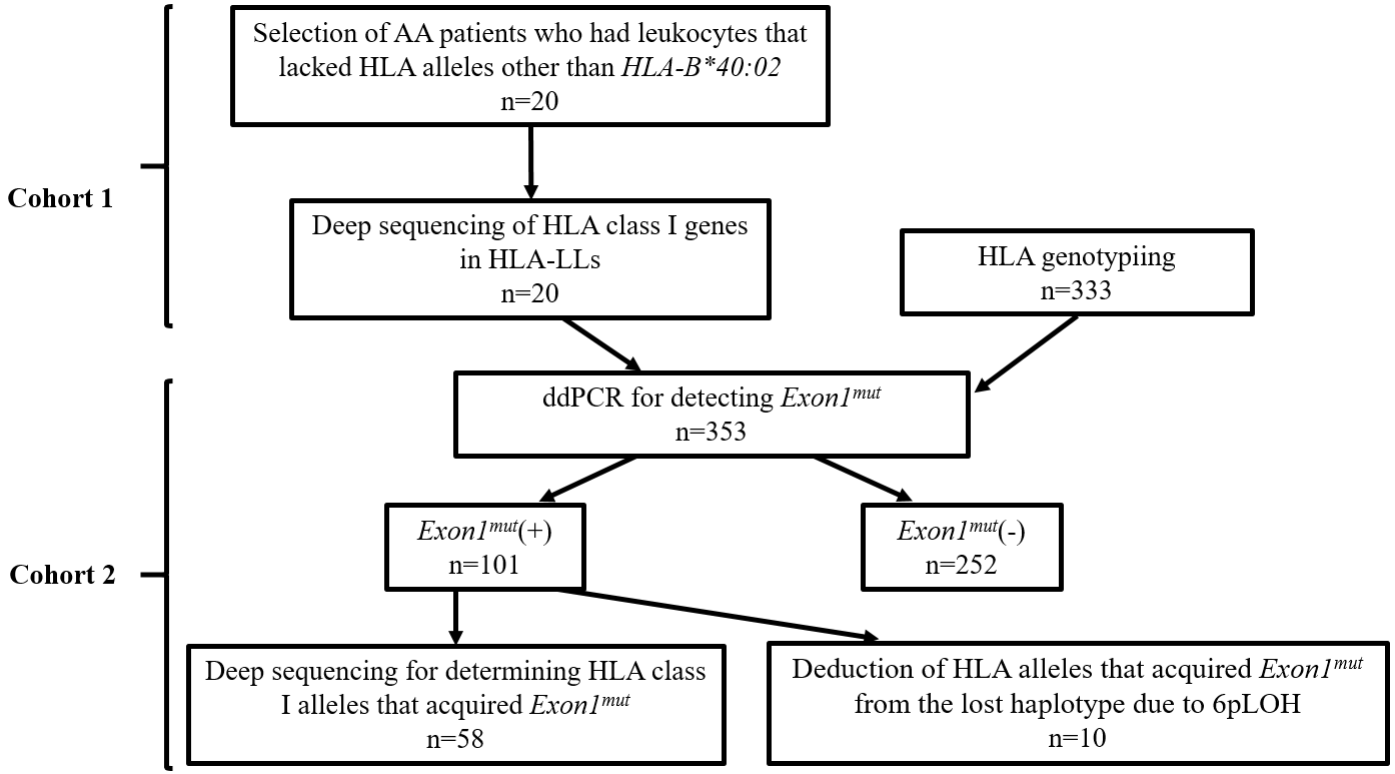
UPN	Mutation (n)	Mutated allele	Type of Mutation	Exon	Mutation (coding)	Mutation (protein)	Variant Allele Frequency (%)	COSMIC ID
211	1	B*40:01	Nonsense	1	c.19C>T	p.R7*	37.3	COSM3253080
334	3	B*40:01	Nonsense	1	c.19C>T	p.R7*	8.6	COSM3253080
			Frameshift insertion	3	c.438dupT	p.Y147Lfs	3.8	Novel
			Frameshift insertion	4	c.625dupC	p.T211Dfs	25.2	COSM5494349
262	3	A*02:06	Nonsense	1	c.19C>T	p.R7*	6.1	COSM3722056
			Missense	1	c.47C>A	p.A16D	19.6	Novel
			Nonsense	4	c.724C>T	p.Q242*	2.0	Novel
335	1	B*40:03	Frameshift deletion	5	c.907delC	p.Q303Sfs	25.4	Novel
242	3	A*02:06	Nonsense	1	c.19C>T	p.R7*	2.3	COSM3722056
			Frameshift deletion	3	c.402delC	p.L135Afs	17.8	Novel
			Frameshift insertion	3	c.556dupG	p.T187Hfs	2.9	Novel
336	3	A*02:06	Nonsense	1	c.19C>T	p.R7*	8.1	COSM3722056
			Frameshift deletion	4	c.831delG	p.E278Sfs	1.2	Novel
			Frameshift deletion	5	c.969delT	p.G324Efs	18.5	Novel
333	4	B*54:01	Nonsense	1	c.19C>T	p.R7*	7.1	COSM3253080
			start loss	1	c.1A>G	p.L2_M4del	3.3	Novel
			start loss	1	c.3G>A	p.L2_M4del	2.0	Novel
			Frameshift deletion	3	c.559_568del	p.E187Sfs	8.9	Novel
248	6	B*13:01	Nonsense	1	c.19C>T	p.R7*	15.1	COSM3253080
			Non frameshift insertion	1	c.27_28insCG	p.L10Gfs	1.6	Novel
			Frameshift deletion	2	c.209_220del	p.E70_P74delinsA	1.3	Novel
			stop gain	4	c.657dupT	p.D220fs	1.4	Novel
			Nonsense	4	c.748C>T	p.Q250*	1.9	COSM4833544
			stop gain	4	c.842dupA	p.Y280fs	1.3	Novel

339	1	A*02:06	Nonsense	1	c.19C>T	p.R7*	3.5	COSM3722056
349	3	A*31:01	Nonsense	1	c.19C>T	p.R7*	19.1	COSM3722056
			Frameshift insertion	3	c.417_418insGGAC	p.D140Gfs	1.2	Novel
			Frameshift insertion	4	c.621dupC	p.K207Qfs	1.8	COSM4765125
344	5	A*02:06	Nonsense	1	c.19C>T	p.R7*	9.3	COSM3722056
			Frameshift insertion	1	c.65dupC	p.W23Lfs	2.4	Novel
			Frameshift insertion	2	c.164_165insT	p.T55fs	1.5	Novel
			Non frameshift insertion	2	c.245_246insAGT	p.E82_Y83insV	2.0	Novel
			Nonsense	4	c.843C>A	p.Y281*	2.1	Novel
210	2	B*54:01	Frameshift deletion	1	c.19delC	p.R7Efs	1.4	Novel
			Frameshift deletion	3	c.577delC	p.R193Afs	2.7	Novel
235	3	A*02:06	Nonsense	1	c.19C>T	p.R7*	11.8	COSM3722056
			Frameshift deletion	3	c.474_475delCG	p.A159Gfs	4.5	Novel
			Frameshift deletion	4	c.772delA	p.R258Gfs	2.3	Novel
353	1	A*02:06	Nonsense	1	c.19C>T	p.R7*	31.2	COSM3722056

Red letters indicate *Exon1*^{mut} in HLA-A and –B genes.

SUPPLEMENTAL Figures

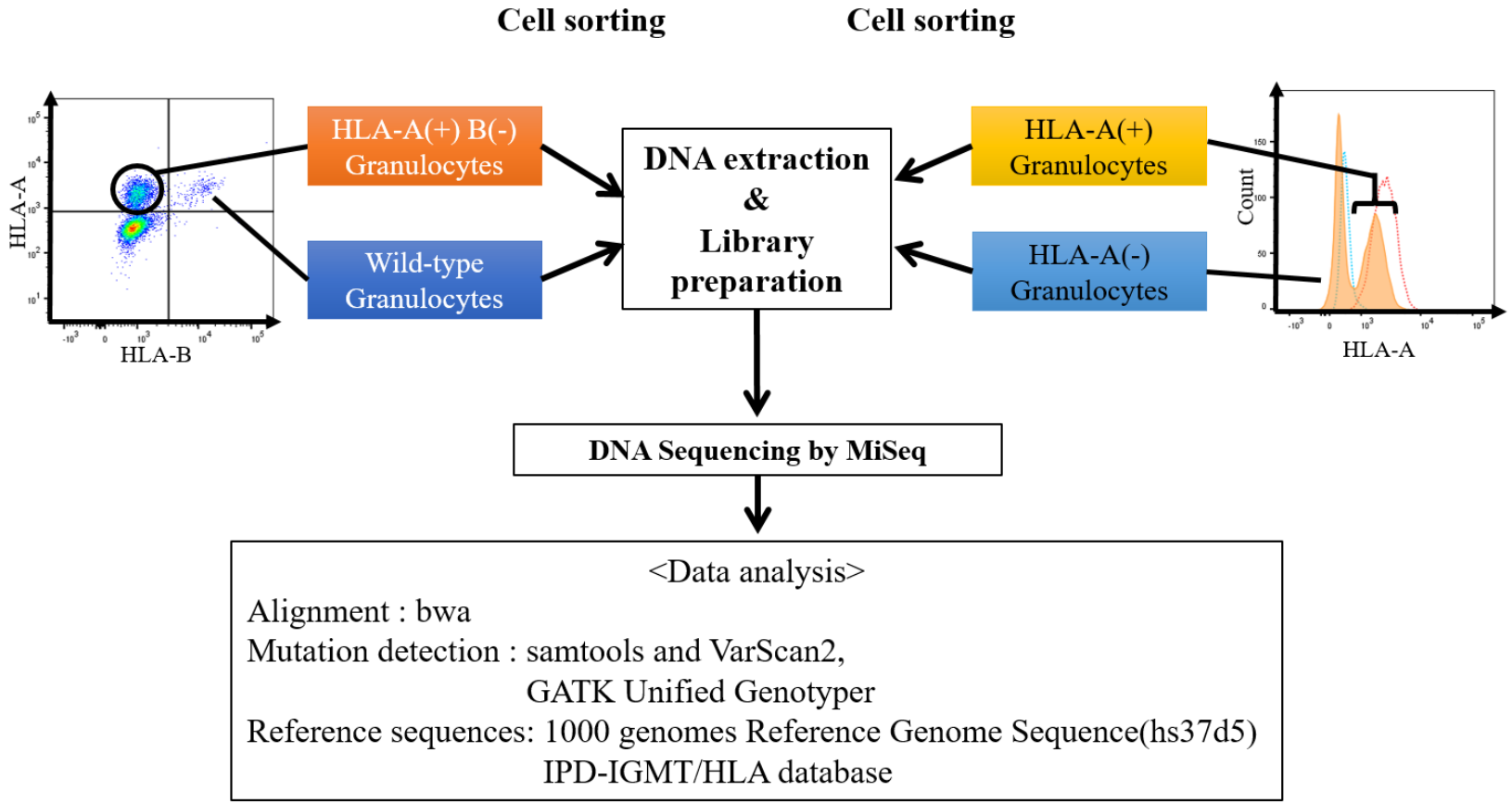
Supplemental Figure 1. Work flow diagram



Abbreviations: AA, aplastic anemia; HLA-LLs, HLA-allele-lacking leukocytes; 6pLOH, copy number neutral loss of heterozygosity of the short arm of chromosome 6.

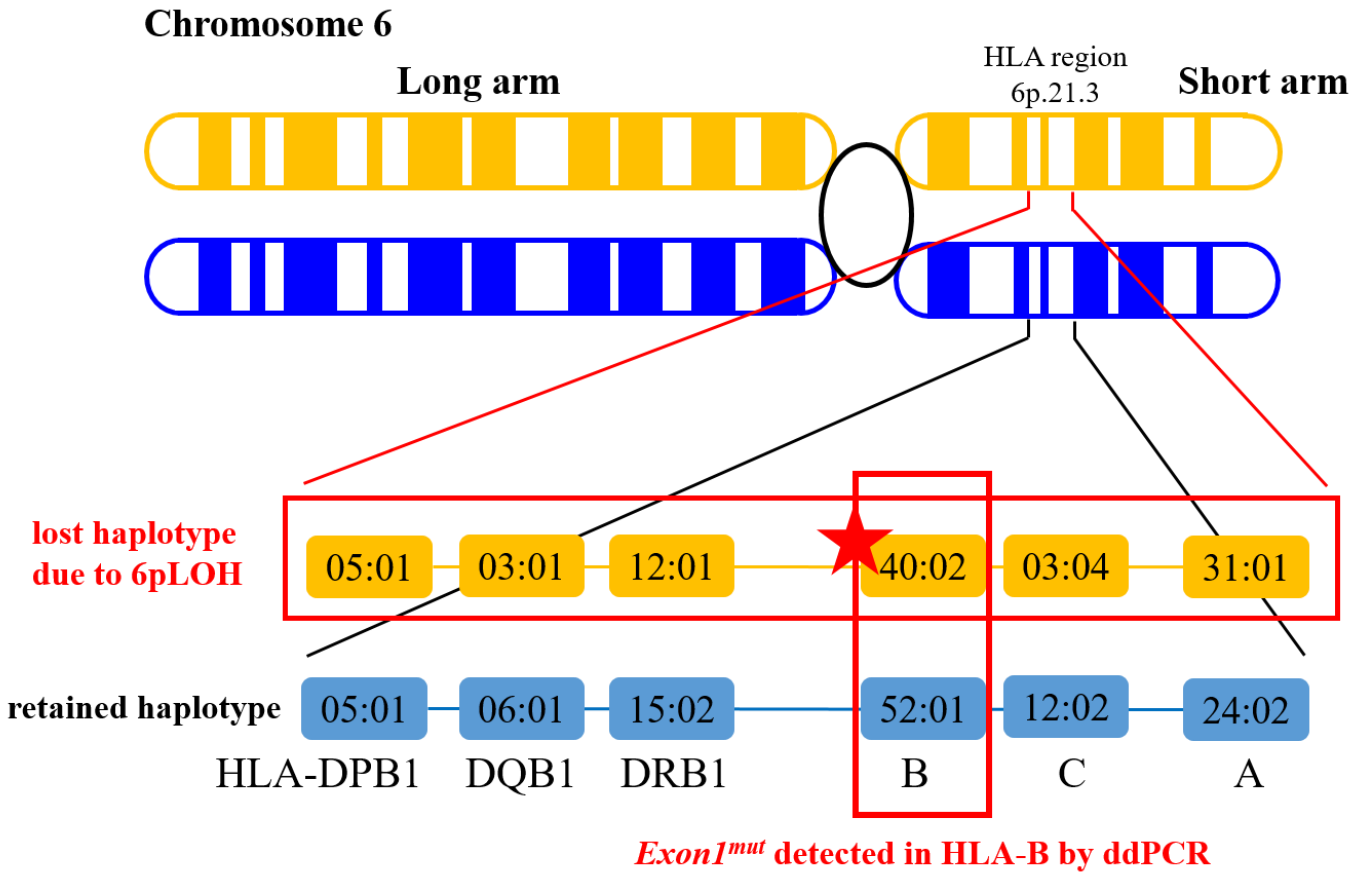
Each number denotes the number of patients studied.

Supplemental Figure 2. Deep sequencing of HLA class I genes



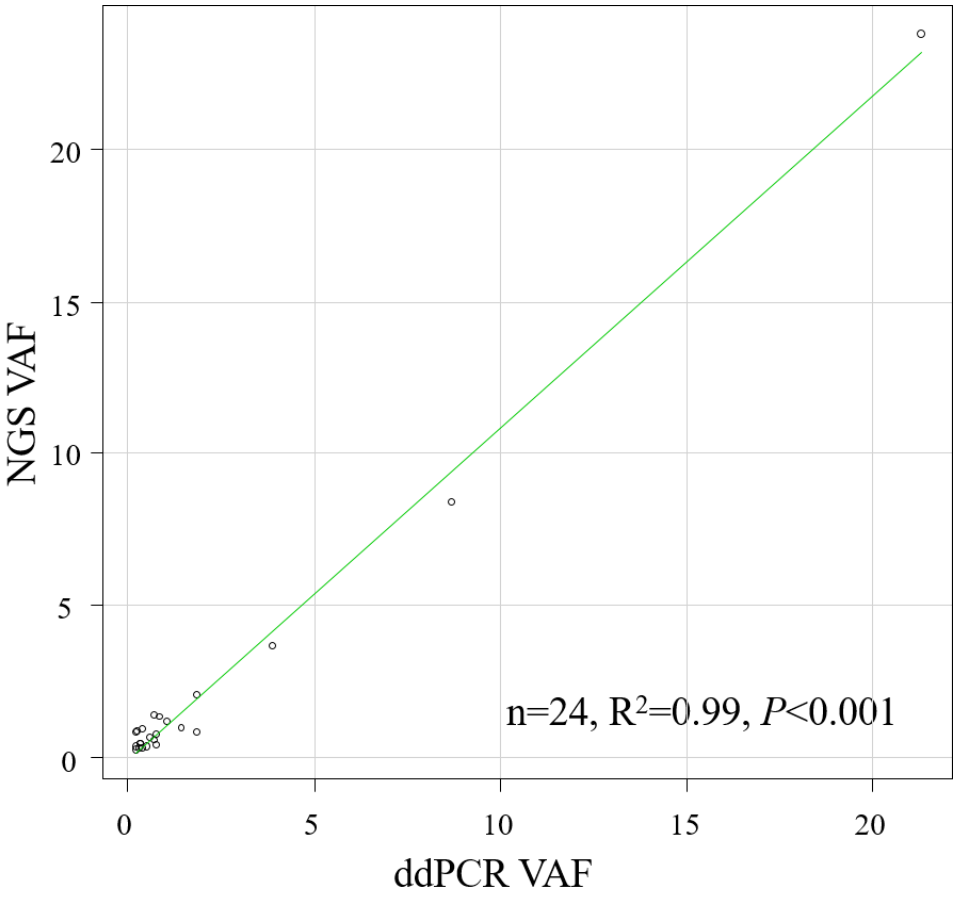
HLA(+) and HLA(-) granulocytes were sorted and subjected to targeted sequencing using a next generation sequencer (MiSeq, illumina). A representative scattergram of a patient (UPN 334) who had HLA-B4001-lacking granulocytes is shown.

Supplemental Figure 3. Estimation of the allele that acquiring *Exon1*^{mut} in a patient with 6pLOH



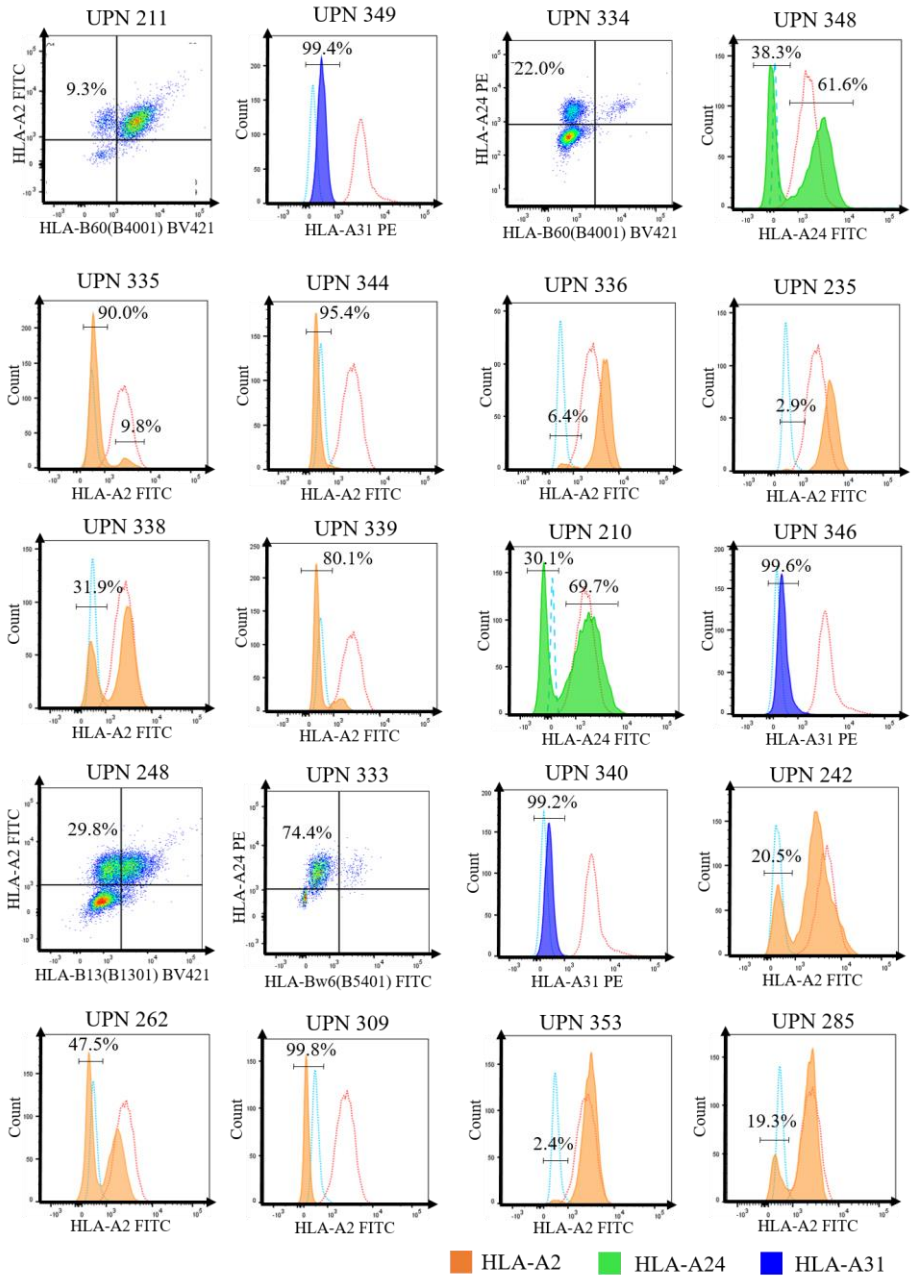
In this patient (UPN 341) whose HLA-B allele acquired *Exon1*^{mut} but could not be determined due to its very low VAF. The affected allele was estimated from an allele contained in the lost haplotype due to 6pLOH that was accompanied by *Exon1*^{mut}.

Supplemental Figure 4. Correlation between the variant allele frequencies (VAFs) determined by the ddPCR assay and those determined by deep sequencing with a next generation sequencer



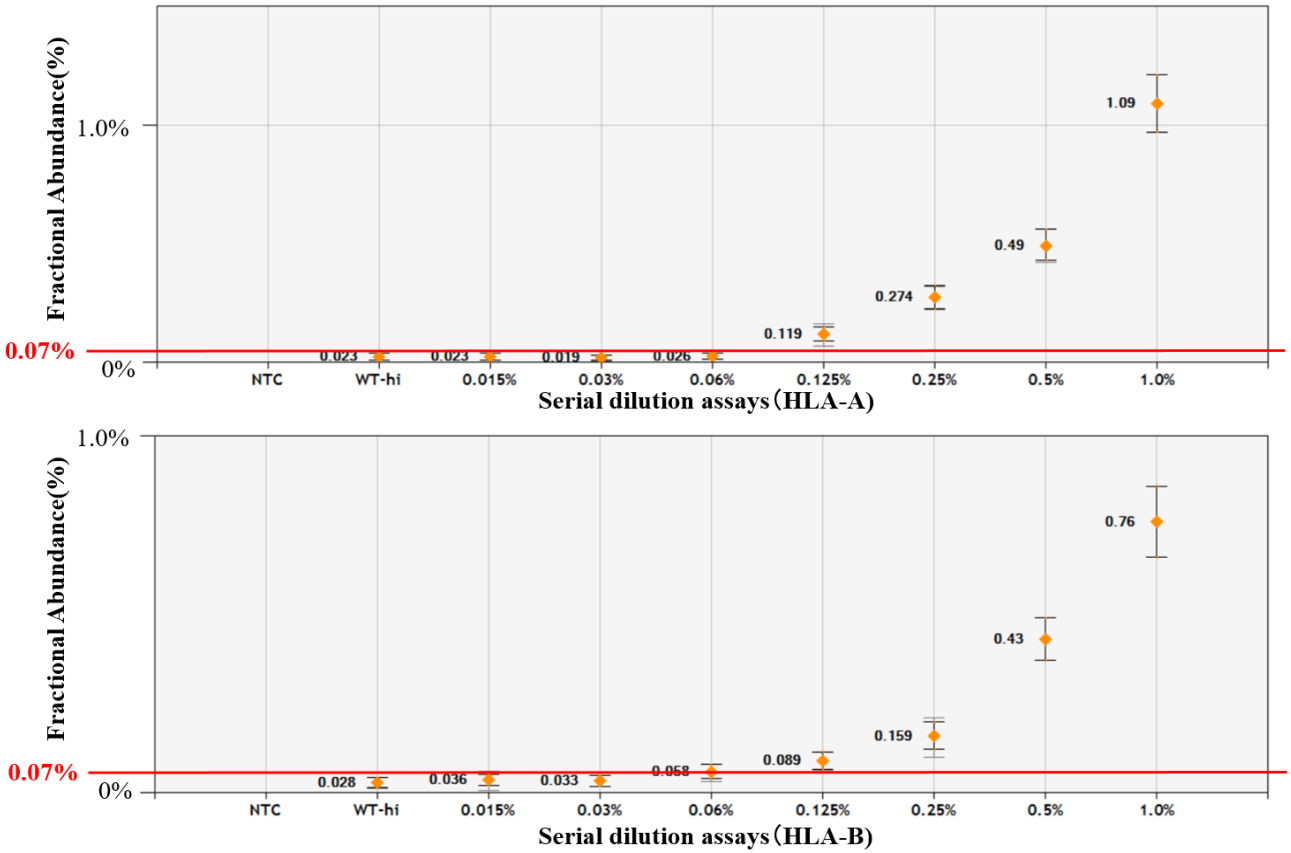
The correlation between *Exon1*^{mut}, VAFs determined by NGS with UMI and those determined by the ddPCR assay was examined using 24 different samples. The results of variant allele frequency (VAF) of the 24 samples are shown. The results of the deep sequencing with UMI were positively correlated with those of the ddPCR assay in the 24 samples (P<0.001, R^2=0.99).

Supplemental Figure 5. HLA-class I allele lacking granulocytes detected in 20 patients of the cohort 1



HLA-class I allele-lacking granulocytes and their percentages in the 20 patients are shown. The open histogram represents HLA-A expression of normal controls, including negative (blue) and positive controls (orange).

Supplemental Figure 6. Determination of the cut-off allele frequency detectable by the ddPCR



Tested samples containing a fixed amount of wild-type DNA and serial dilutions of *Exon1*^{mut} template DNA revealed a detection limit of 0.07% for both HLA-A and HLA-B. None of 24 healthy samples showed >0.042% *Exon1*^{mut} DNA (data not shown).