Supplemental information

Sustained clonal hematopoiesis by HLA-lacking hematopoietic stem cells without driver mutations in aplastic anemia

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Running Title: Clonal hematopoiesis by HLA(-) HSPCs in AA

SUPPLEMENTAL METHODS

SUPPLEMENTAL FIGURES

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Next-generation sequencing data processing

Paired-end 100-bp reads were mapped to the reference genome (GRCh37) using Burrows-Wheeler Aligner (bwa) v.0.7.12.[1] bwa-generated SAM files were converted to the BAM format, then sorted and indexed using SAM tools v.1.2.[2] Duplicated reads were marked with Picard v.1.52 (https://github.com/broadinstitute/picard).

Mutation calling

The heuristic somatic mutation caller, VarScan 2,[3] was used for somatic mutation and LOH calling. The mutations were reviewed using Unified Genotyper in the Genome Analysis Toolkit (GATK) v3.4[4] and the alignment data were visually compared among granulocytes, T cells and buccal mucosa cells using IGV.[5] The functional information on somatic mutations was annotated using ANNOVAR.[6]

Reference

1. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2009;25(14):1754-60.

2. Li H, Handsaker B, Wysoker A, Fennell T, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. 2009;25(16):2078-9.

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4. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res.* 2010;20(9):1297-303.

5. Robinson JT, Thorvaldsdóttir H, Winckler W, et al. Integrative genomics viewer. *Nat Biotechnol.* 2011;29(1):24-6.

6. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res.* 2010;38(16):e164.

SUPPLEMENTAL DISCUSSION

Escape hematopoiesis by a 6pLOH(+) clone that was forced to proliferate by a thrombopoietin receptor agonist

Romiplostim was given to one patient (Case 12) who was refractory to platelet transfusions from random donors due to anti-HLA antibodies as an emergency measure after approval by an ethical committee of a local hospital, when the patient developed cerebral bleeding. Romiplostim miraculously restored the patient's hematopoiesis by a 6pLOH(+) HSPC without a help of immunosuppressive therapy (IST). Romiplostim is a thrombopoietin receptor agonist (TPO-RA) that is reported to be as efficacious in the treatment of acquired aplastic anemia (AA) as eltrombopag.[1-3] Case 12 achieved complete hematologic recovery with romiplostim alone despite the fact that no concomitant IST was given. The high number of somatic mutations with high valiant allele frequencies (VAFs) detected in this patient might have developed in the process of the extensive proliferation of a 6pLOH(+) HSPC that was forced to proliferate due to the administration of romiplostim. This hypothesis needs to be studied by examining other AA patients with 6pLOH(+) HSPCs who are treated with TPO-RA alone using whole-exome sequencing (WES). The efficacy of romiplostim in the treatment of AA must be evaluated in a clinical trial setting.

Reference

1. Lee JW, Jang JH, Lee SE, Jung CW, Park S. and Oh IH. Efficacy and safety of romiplostim in patients with aplastic anemia refractory to immunosuppressive therapy: 1-year interim analysis of phase 2 clinical trial [abstract]. *Blood.* 2016 128:3910

2. Townsley DM, Scheinberg P, Winkler T, et al. Eltrombopag added to standard immunosuppression for aplastic anemia. *N Engl J Med.* 2017;376(16):1540-1550.

3. Townsley DM, Cooper JN, Winkler T, et al. Myeloid neoplasm gene somatic mutations in patients with severe aplastic anemia treated with eltrombopag and standard immunosuppression [abstract]. *Blood.* 2016 128:727

SUPPLEMENTAL TABLES

Supplemental Table 1. The monoclonal antibodies used for flow cytometry

Antigen	Isotype	Conjugate	Source
CD3	Mouse IgG1	PerCP-Cy5.5	BD Biosciences
CD3	Mouse IgG1	PE	BD Biosciences
CD19	Mouse IgG1	APC-Cy7	Beckman Coulter
CD33	Mouse IgG1	APC	Beckman Coulter
HLA-A2/28	Mouse IgG2a	FITC	One Lambda
HLA-A2/28	Mouse IgG2a	PE	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

Abbreviations: Ig, immunoglobulin; PerCP-Cy5.5, peridinin-chlorophyll proteins-Cy5.5 tandem; PE, phycoerythrin; APC, allophycocyanin; APC-Cy7, allophycocyanin-Cy7 tandem; FITC, fluorescein isothiocyanate.

Supplemental Table 2. The genes analyzed by targeted sequencing

Gene	Function	Reported disease	Reference
ASXL1	Epigenetic regulation	MDS	Leukemia. 2014 Feb;28(2):241-7.
ATRX	Epigenetic regulation	MDS	Leukemia. 2014 Feb;28(2):241-7.
DNMT3A	Epigenetic regulation	AML	Leukemia. 2014 Feb;28(2):241-7.
EED	Epigenetic regulation	MDS	Blood. 2012 Feb 2;119(5):1208-13.
EZH2	Epigenetic regulation	MDS	Leukemia. 2014 Feb;28(2):241-7.
TET2	Epigenetic regulation	MDS	Leukemia. 2014 Feb;28(2):241-7.
ATM	Transcription	MDS	Leukemia. 2014 Feb;28(2):241-7.
BCOR	Transcription	AML	Leukemia. 2014 Feb;28(2):241-7.
BCORL1	Transcription	AML	Leukemia. 2014 Feb;28(2):241-7.
PHF6	Transcription	MDS	Leukemia. 2014 Feb;28(2):241-7.
RUNX1	Transcription	AML	Leukemia. 2014 Feb;28(2):241-7.
TP53	Transcription	MDS	Leukemia. 2014 Feb;28(2):241-7.
WT1	Transcription	AML	Leukemia. 2014 Feb;28(2):241-7.
GNAS	Signal transduction	MDS	Leukemia. 2014 Feb;28(2):241-7.
KRAS	Signal transduction	MDS	Leukemia. 2014 Feb;28(2):241-7.
NF1	Signal transduction	MDS	Leukemia. 2014 Feb;28(2):241-7.
STAT3	Signal transduction	PRCA/AA	Blood. 2013 Oct 3;122(14):2453-9.
MPL	Receptor	MDS	Leukemia. 2014 Feb;28(2):241-7.
POT1	Telomere related gene		Genes Chromosomes Cancer. 2006 Mar;45(3):247-56.
RAP1	Telomere related gene		Genes Chromosomes Cancer. 2006 Mar;45(3):247-56.
TERT	Telomere related gene	Dyskeratosis congenita	Curr Opin Pediatr. 2012 Feb;24(1):23-32.
SRSF2	RNA splicing	MDS	Nature. 2011 Sep 11;478(7367):64-9.
U2AF1	RNA splicing	MDS	Nature. 2011 Sep 11;478(7367):64-9.

ZRSR2	RNA splicing	MDS	Nature. 2011 Sep 11;478(7367):64-9.
RAD21	Cohesin	AML	Nat Genet. 2013 Oct;45(10):1232-7.
STAG2	Cohesin	MDS	Nat Genet. 2013 Oct;45(10):1232-7.
LAMB4	Cell adhesion	MDS	Leukemia. 2014 Feb;28(2):241-7.
PEG3	Apoptosis	AA	N Engl J Med. 2015 Jul 2;373(1):35-47.
PIGA	GPI anchor	AA	N Engl J Med. 2015 Jul 2;373(1):35-47.
SETBP1	Bind to SET nuclear oncogene	MDS	Nat Genet. 2013 Aug;45(8):942-6.
IDH2	Epigenetic regulation	AML	Leukemia. 2014 Feb;28(2):241-7.
RBBP4	Epigenetic regulation		Cancer Genet Cytogenet. 2003 Oct 15;146(2):89-101.
SUZ12	Epigenetic regulation	MDS/MPD	Blood. 2012 Feb 2;119(5):1208-13.
CUX1	Transcription	MDS	Am J Hematol. 2012 Nov;87(11):1010-6.
CSMD1	Signal transduction	CMML	Leukemia. 2016 Apr;30(4):906-13.
JAK1	Signal transduction		
JAK2	Signal transduction	MPD	Leukemia. 2014 Feb;28(2):241-7.
JAK3	Signal transduction		
SH2B3	Signal transduction		
SMC1A	Cohesin	AML	Nat Genet. 2013 Oct;45(10):1232-7.
SMC3	Cohesin	MDS	Nat Genet. 2013 Oct;45(10):1232-7.
BRCC3	Ubiquitination	MDS	Leukemia. 2014 Feb;28(2):241-7.
CBL	Ubiquitination	MDS	Leukemia. 2014 Feb;28(2):241-7.
DIS3	RNA processing and degradation	AML	Nature. 2012 Jan 11;481(7382):506-10.
RIT1	Signal transduction	CMML	Leukemia. 2013 Sep;27(9):1943-6.
PRPF8	RNA splicing	MDS	Leukemia. 2014 Feb;28(2):241-7.
DHX29	ATP-Dependent RNA Helicase	AML	Blood Cancer J. 2016 Dec 16;6(12):e510.
MECOM	transcriptional regulator and oncoprotein	MDS	
KDM6A	Histone Demethylase	APL	J Clin Invest. 2011 Apr;121(4):1445-55.

ETV6	Transcription factor	AL	Leukemia. 2014 Feb;28(2):241-7.
BRPF1	Histone acetyltransferase activity	AML	J Cell Physiol. 2014 Nov;229(11):1571-4.
CCR9	beta chemokine receptor family		
KDM3B	Histone demethylase	AML	Leuk Lymphoma. 2018 Jan;59(1):204-213.
C11orf34 (PLET1)	keratinocyte migration and cellular adhesion		
IKZF1	chromatin remodeling	ALL	
ERBB2	viral oncogene homolog 2	breast cancer, brain tumor	
SLIT1	molecular guidance in cellular migration		
LRCH1	Structure protein	knee osteoarthritis	
DPP4	adenosine deaminase-binding protein		
PRR5L	Associates with the mTORC2 complex		
RFX1	HLA class II expression		

Abbreviations: MDS, myelodysplastic syndrome; AML, acute myelogenous leukemia; PRCA, pure red cell aplasia; AA, acquired aplastic anemia; DKC, dyskeratosis congenital; MPD, myeloproliferative disease; CMML, chronic myelomonocytic leukemia; AL, acute leukemia; ALL, acute lymphoblastic leukemia.

Supplemental Table 3. The somatic mutations of genes identified by targeted sequencing

Case number	Gene	Chromosome	Exonic function	Position	Genome change	Amino acid change	VAF	dbSNP138	COSMIC ID
1	*DNMT3A*	2	nonsynonymous SNV	NM_153759	C2077T	R693C	0.44	rs377577594	COSM1166704, COSM53042
1	*ZRSR2*	X	stopgain	NM_005089	C505T	R169X	0.36		COSM1716885
1	*TET2*	4	frameshift deletion	NM_001127208	452_458 del	V151fs	0.04		Novel
1	*TET2*	4	frameshift deletion	NM_001127208	2045_2051del	H682fs	0.17		Novel
2	*DNMT3A*	2	nonsynonymous SNV	NM_153759	A1637G	Y546C	0.19	rs147828672	COSM133126
2	*RFX1*	19	synonymous SNV	NM_002918	G918C	T306T	0.15		Novel
3	*PRR5L*	11	nonsynonymous SNV	NM_001160168	G211A	E71K	0.12		Novel
3	*LRCH1*	13	frameshift deletion	NM_001164213	1734_1737del	E578fs	0.12		Novel
3	*DPP4*	2	synonymous SNV	NM_001935	G375A	R125R	0.2		Novel
4	*CBL*	11	nonsynonymous SNV	NM_005188	C1624A	L542I	0.33		Novel

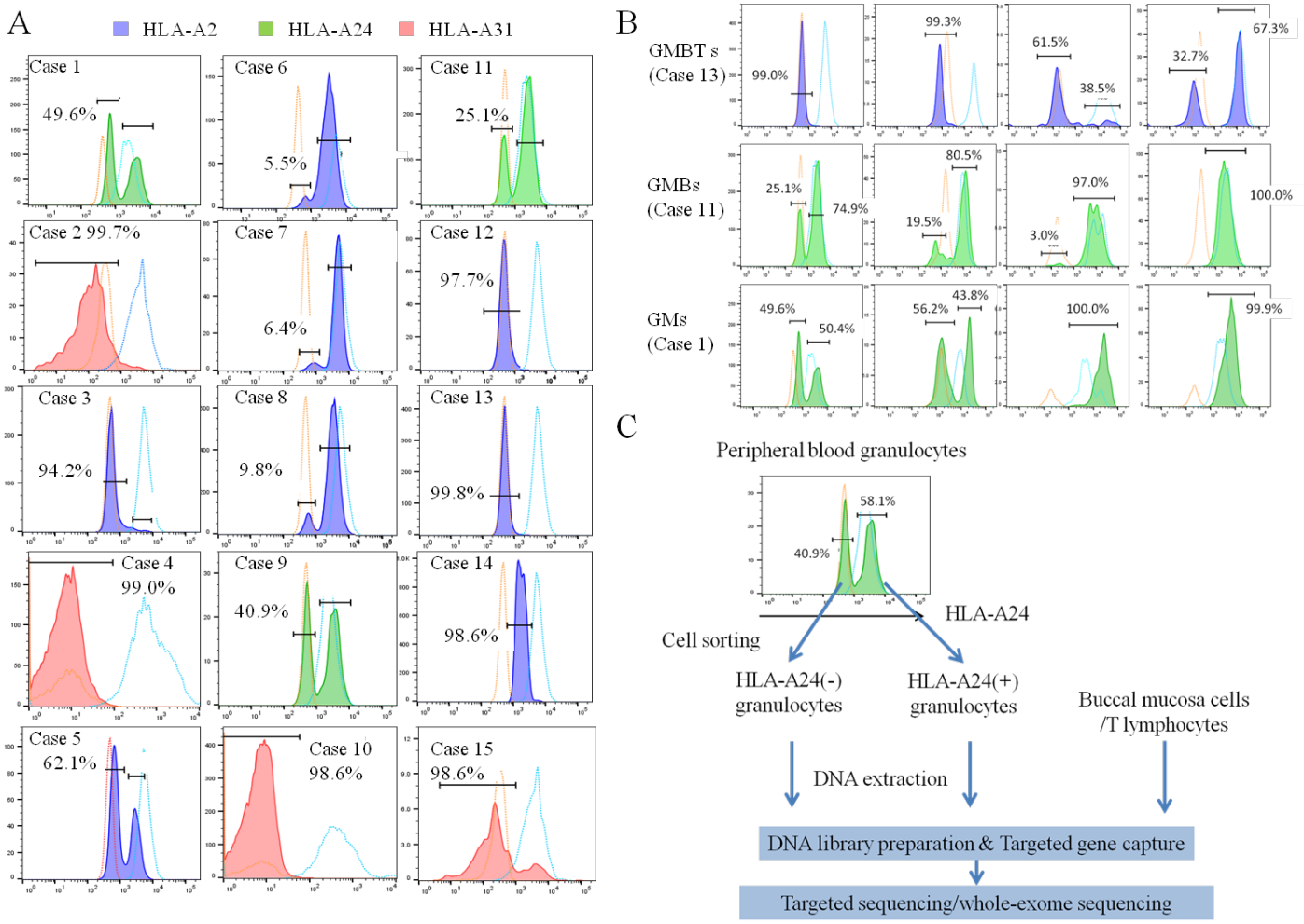
Supplemental Table 4. The somatic mutations of genes identified by whole-exome sequencing

Case number	Gene	Chromosome	Exonic function	Position	Genome change	Amino acid change	VAF	dbSNP138	COSMIC ID
12	*SPEG*	2	synonymous SNV	NM_005876	C2367A	A789A	0.22		Novel
12	*DCAF4*	14	synonymous SNV	NM_001163508	G276A	T92T	0.24		Novel
12	*MYSM1*	1	stopgain	NM_001085487	T2427G	Y809X	0.36		Novel
12	*MYSM1*	1	nonsynonymous SNV	NM_001085487	T2425C	Y809H	0.36		Novel
12	*COX4I2*	20	nonsynonymous SNV	NM_032609	C415T	R139W	0.39	rs200392726	Novel
12	*OR2AT4*	11	nonsynonymous SNV	NM_001005285	G913T	A305S	0.41		Novel
12	*PPAP2B*	1	nonsynonymous SNV	NM_003713	T284C	I95T	0.44		Novel
12	*FAM154B*	15	nonsynonymous SNV	NM_001008226	C689T	P230L	0.44		Novel
12	*PEX5*	12	nonsynonymous SNV	NM_000319	G1319A	G440D	0.45		Novel
12	*GPR56*	16	nonsynonymous SNV	NM_001145773	T694G	S232A	0.46		Novel
12	*SRRM2*	16	nonsynonymous SNV	NM_016333	C1975T	R659C	0.47		Novel
12	*NLRP4*	19	nonsynonymous SNV	NM_134444	G863A	R288Q	0.47		Novel
12	*SP5*	2	nonsynonymous SNV	NM_001003845	T826C	C276R	0.51		Novel
12	*HSPG2*	1	synonymous SNV	NM_005529	C6849T	G2283G	0.51		Novel
12	*FER1L5*	2	nonsynonymous SNV	NM_001113382	T4855C	Y1619H	0.51		Novel
13	*OLFM4*	13	nonsynonymous SNV	NM_006418	G419A	R140Q	0.21		Novel
13	*CRTAC1*	10	nonsynonymous SNV	NM_001206528	T2C	M1T	0.22		Novel
13	*SCG2*	2	synonymous SNV	NM_003469	G306A	R102R	0.22		COSM1017067
13	*GPT*	8	nonsynonymous SNV	NM_005309	A929T	H310L	0.23		Novel
13	*ZNF623*	8	synonymous SNV	NM_014789	T42A	T14T	0.24		Novel

13	*SERPINF1*	17	nonsynonymous SNV	NM_002615	T20C	L7P	0.26		COSM1317826, COSM1317825, COSM3401548
13	*ZNF462*	9	nonsynonymous SNV	NM_021224	G4982A	R1661Q	0.47		Novel
14	*NPY2R*	4	synonymous SNV	NM_000910	C547T	L183L	0.47		Novel
14	*ZNF502*	3	nonsynonymous SNV	NM_001134441	A1099T	I367F	0.47		Novel
14	*GRID2IP*	7	synonymous SNV	NM_001145118	G1464A	T488T	0.51		Novel
14	*DHX35*	20	nonsynonymous SNV	NM_001190809	C20T	P7L	0.53		Novel
15	*AKAP10*	17	nonsynonymous SNV	NM_007202	A449C	H150P	0.28		Novel
15	*SERPINA9*	14	synonymous SNV	NM_001284275	C636T	G212G	0.42		Novel
15	*ATXN1L*	16	nonsynonymous SNV	NM_001137675	G298A	V100M	0.45		Novel
15	*MSH3*	5	synonymous SNV	NM_002439	A444G	Q148Q	0.48		Novel
15	*EDEM3*	1	nonsynonymous SNV	NM_025191	A2713G	K905E	0.51		Novel

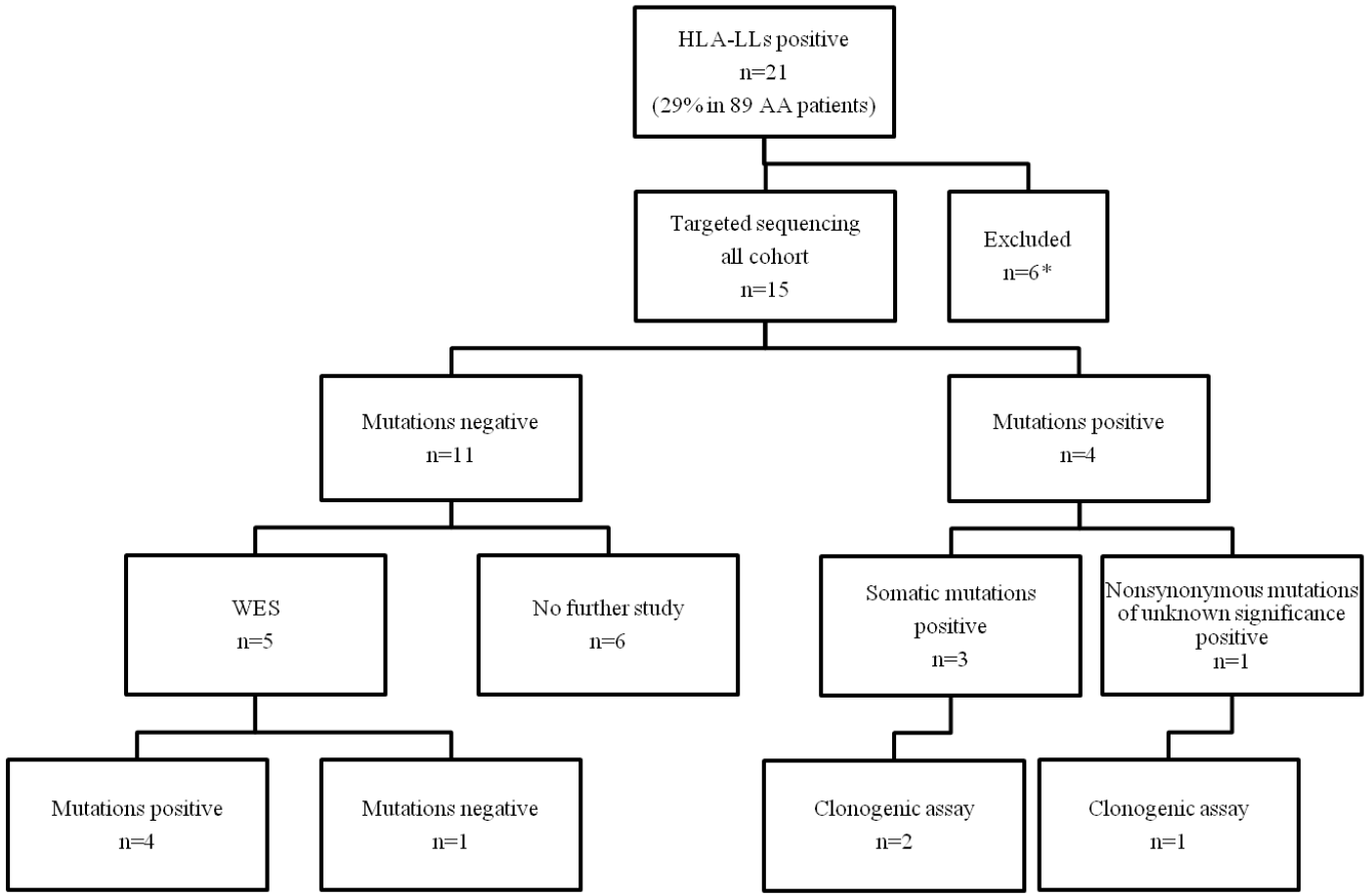
SUPPLEMENTAL FIGURES

Supplemental Figure 1. HLA-A allele-lacking leukocytes and the gene analysis procedure



A: HLA(-) granulocytes and their percentages in each case. The open histogram represents the HLA-A allele expression of normal controls. B: The lineage patterns of HLA-A allele-lacking leukocytes of the representative cases. G, granulocytes; M, monocytes; B, B lymphocytes, T, T lymphocytes. C: The procedure for sorting and sequencing of HLA-A24(-) and HLA-A24(+) granulocytes.

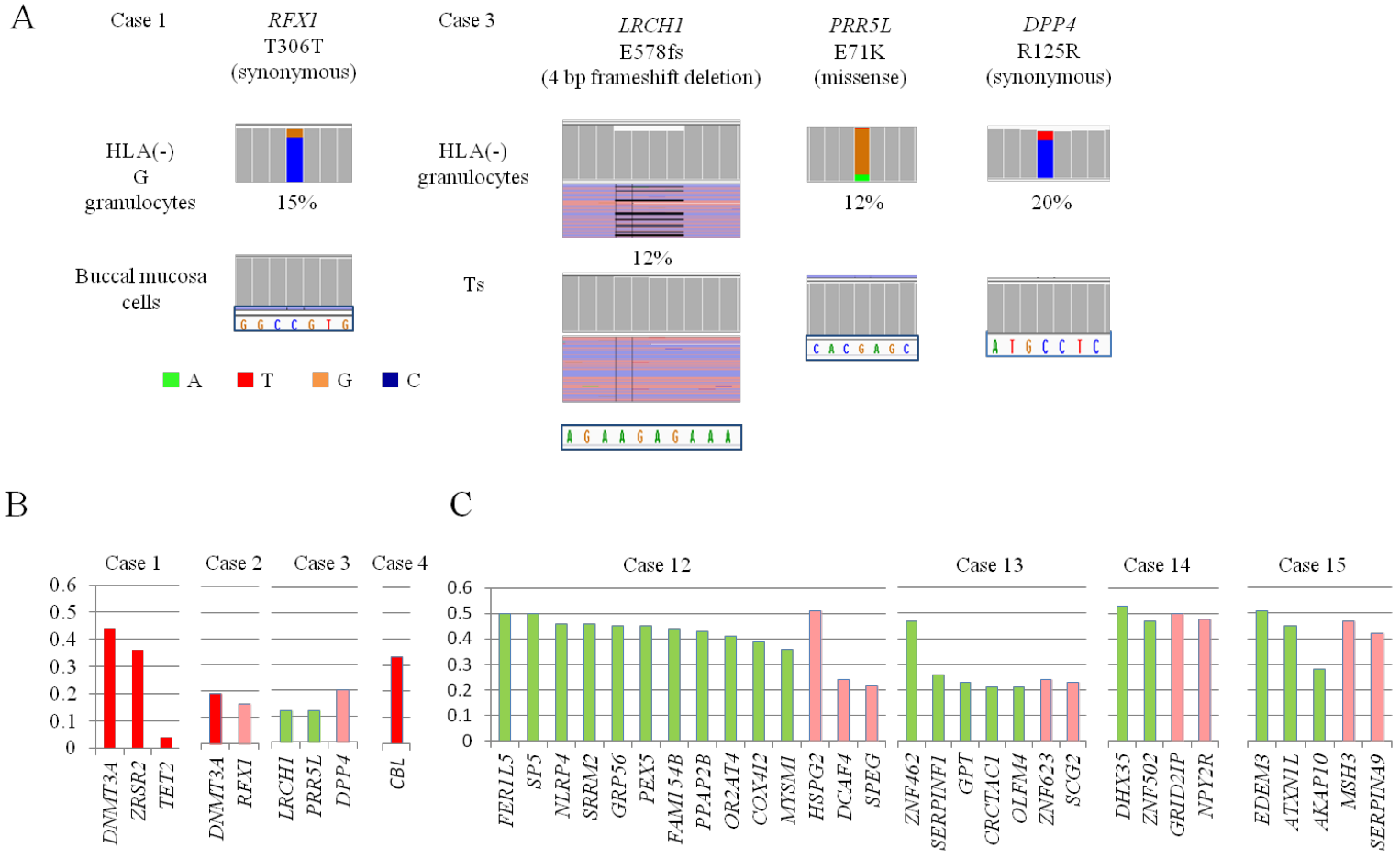
Supplemental Figure 2. A flow chart of the gene analyses



* Six patients were excluded from this study for the following reasons: three had HLA(-) cells only in B cells, one died of infection before sampling for this study, one had a disease history of <1 year, and the percentage of HLA(-) granulocyte was too low (<1%) to obtain a sufficient amount of DNA for the

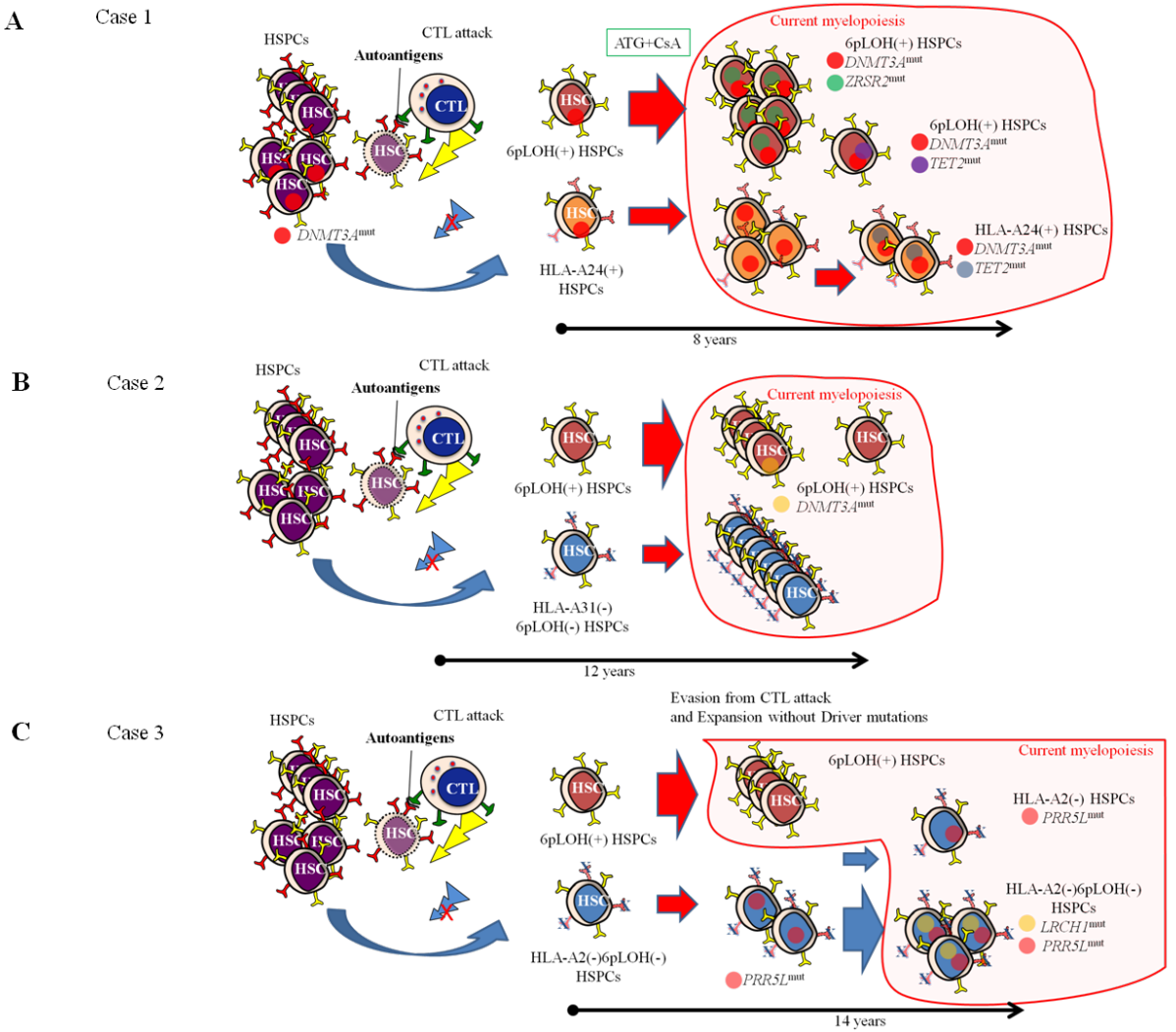
analysis in one.

Supplemental Figure 3. Charts of the synonymous and nonsynonymous mutations of unknown significance detected by targeted sequencing and the VAFs of each mutation detected by targeted and whole-exome sequencing.



A: Four synonymous and nonsynonymous mutations of unknown significance were detected by targeted sequencing. The synonymous mutation in *RFX1* was only detected in the HLA(-) granulocytes of Case 1. An *LRCH1* frameshift mutation, *PRR5L* missense mutation and *DPP4* synonymous mutation were also only detected in the HLA(-) granulocytes of Case 3. B: The variant allele frequencies (VAFs) of each somatic mutation in targeted sequencing. C: The VAFs of each gene mutation in four cases were detected by whole-exome sequencing. Somatic mutations (red), nonsynonymous mutations of unknown significance (green) and synonymous mutations (pink) are shown.

Supplemental Figure 4. The clonal architecture of HLA(-) granulocytes deduced from the genetic analyses.

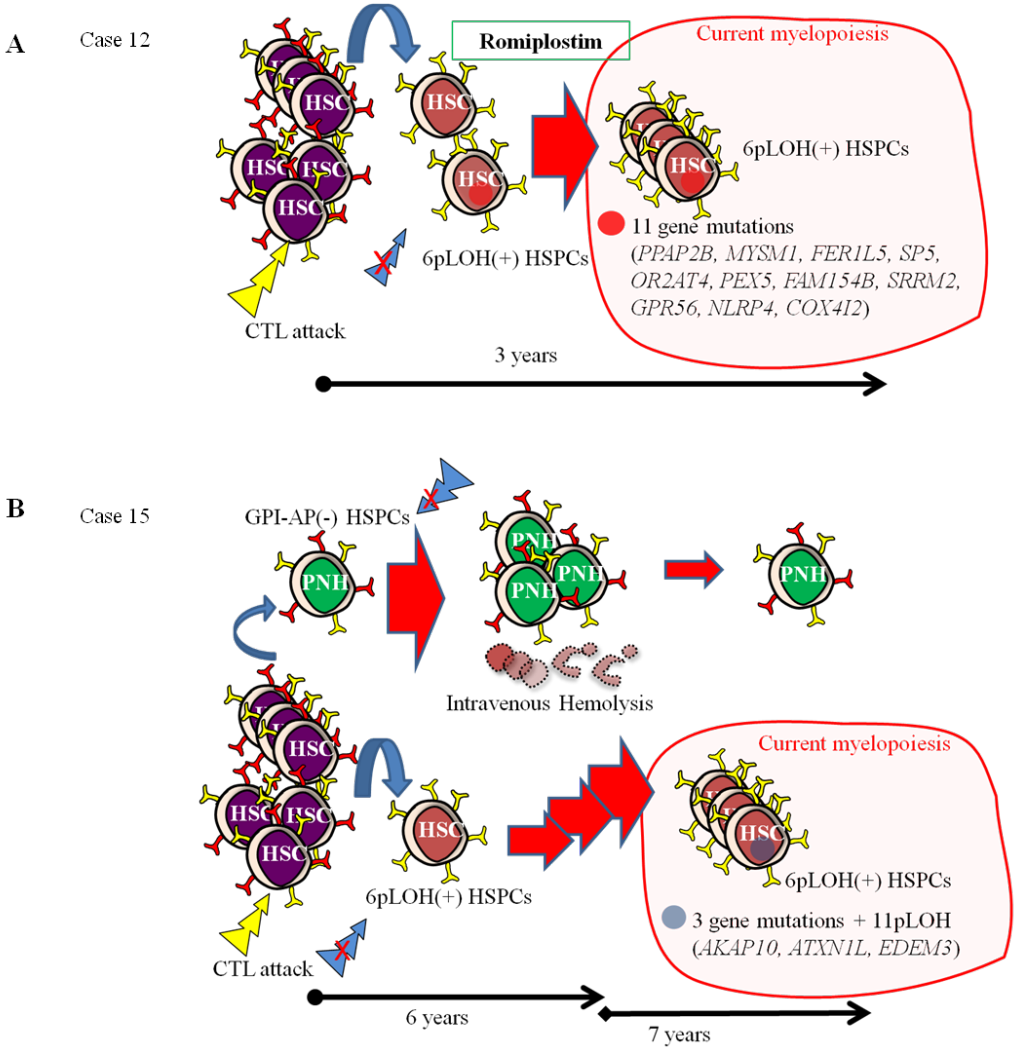


A: 6pLOH occurred in a *DNMT3A*^{mut} HSPC, and the 6pLOH(+)*DNMT3A*^{mut} HSPC survived the CTL attack when the patient developed AA and proliferated to support Case 1's hematopoiesis. In the process of clonal proliferation of the 6pLOH(+)*DNMT3A*^{mut} HSPC, a *ZRSR2* or *TET2* mutation occurred in the 6pLOH(+)*DNMT3A*^{mut} HSPC, leading to acceleration of the clonal proliferation of 6pLOH(+)*DNMT3A*^{mut}*ZRSR2*^{mut} and 6pLOH(+)*DNMT3A*^{mut}*TET2*^{mut} HSPCs. A 6pLOH(-)*DNMT3A*^{mut} HSPC also underwent a *TET2* mutation and clonal proliferation independently after escaping from the CTL attack.

B: 6pLOH occurred in an HSPC, and the 6pLOH(+) HSPC survived the CTL attack when the patient developed AA and proliferated to support Case 2's hematopoiesis. In the process of clonal proliferation of the 6pLOH(+) HSPC, *DNMT3A* mutation occurred in the 6pLOH(+) HSPC, and the 6pLOH(+)*DNMT3A*^{mut} HSPC gradually proliferated. An HLA-A31(-)6pLOH(-) HSPC that also survived the CTL attack partly supported Case 2's hematopoiesis for a long time.

C: 6pLOH occurred in an HSPC, and the 6pLOH(+) HSPC survived the CTL attack when the patient developed AA and proliferated to support Case 3's hematopoiesis without undergoing secondary mutations. An HLA-A2(-)6pLOH(-) HSPC underwent a *PRR5L* mutation, and a subclone of the HLA-A2(-)6pLOH(-)*PRR5L*^{mut} HSPC underwent an *LRCH1* mutation.

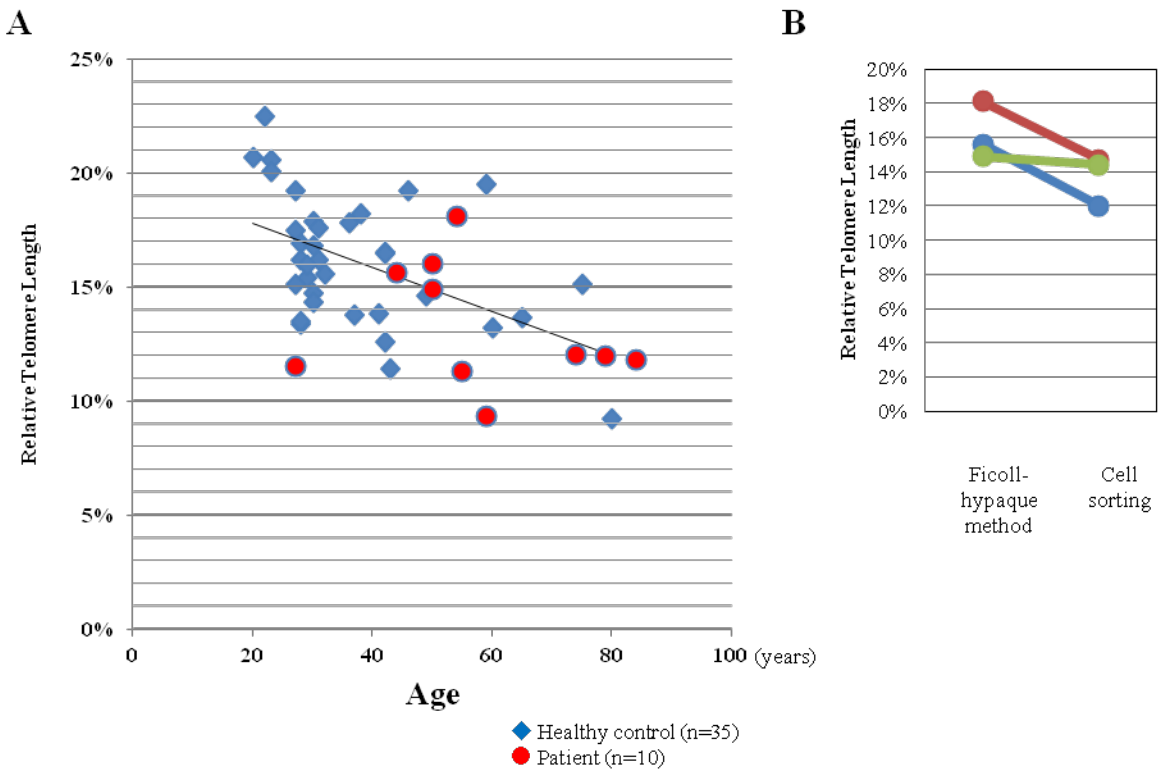
Supplemental Figure 5. The clonal architecture of HLA(-) granulocytes deduced from the whole-exome sequencing.



A: A few 6pLOH(+) HSPCs survived the CTL attack when Case 12 developed severe AA, and one of the mutated HSPCs that carried multiple passenger gene mutations was stimulated to grow by romiplostim, which had been administered to support the patient's hematopoiesis.

B: Both GPI-AP(-) and 6pLOH(+) HSPCs survived when the patient developed AA, and only GPI-AP(-) HSPCs supported Case 15's hematopoiesis for the initial six years. When the PNH clone(s) spontaneously regressed, 6pLOH(+) HSPCs that carried three passenger mutations started to grow for some reason to support the patient's hematopoiesis.

Supplemental Figure 6. Measuring the telomere length in AA patients by flow-FISH



A. The relative telomere length of granulocytes from healthy individuals of different ages (n=35) and AA patients possessing HLA(-) granulocytes (n=10).

B. A comparison of the telomere length between granulocytes collected by the Ficoll-Hypaque method and those collected by cell sorting.