Frequent loss of HLA alleles associated with copy number-neutral 6pLOH in acquired aplastic anemia

Supplementary Information

Frequent loss of HLA alleles associated with copy number-neutral 6pLOH in acquired aplastic anemia

Short title: HLA allelic loss due to 6pLOH in aplastic anemia

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Supplementary Tables

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Supplementary Tables

Supplementary Table 1. 6pLOH(+) AA cases.

UID	Age (year)	Sex	Disease Status	Response to IST (type of IST)¶	Months from diagnosis
19	69	M	Before Therapies	PR (CyA)	0.1
12	45	M	During Remission	CR (CyA+AS)	200 (8†)
17	18	M	Before BMT	NA	8
304	54	M	Immediate after teatment	NE# (ATG+CyA)	1 (1†)
11	75	M	Before Therapies	PR (ATG+CyA)	0.2
21	42	M	During Remission	PR (CyA)	26 (5†)
24	42	M	Before Therapies	PR (CyA+AS)	0.3
26	93	F	During Remission	PR (CyA)	204 (6†)
27	26	F	Before BMT	NA	8
10	53	F	Before BMT	NA	15
8	53	M	During Remission	CR (ATG+CyA)	53 (9†)
23	3	M	Before BMT	NA	9
25	32	F	During Remission	CR (CyA)	163 (6†)
9	39	M	During Remission	CR (AS)	50 (8†)
20	60	M	Before Therapies	PR (CyA)	0.3
14	59	M	During Remission	PR (CyA)	96 (6†)
22	12	F	Before BMT	NA	105
18	63	M	During Remission	PR (ATG+CyA)	48
15	12	F	Before BMT	NA	14
41	50	F	During Remission	PR (AS)	180 (6†)
28	81	M	Before Therapies	NA	0.3
29	23	M	Before BMT	NA	16
305	33	M	Before Therapies	PR (ATG+CyA)	2 (2†)
13	22	M	During Remission	CR (ATG+CyA)	98 (16†)
306	25	M	Before Therapies	NE# (ATG+CyA)	2 (2†)
16	6	F	Before BMT	NA	8
30	8	M	Before BMT	NA	9
72	29	M	Before Therapies	CR (ATG+CyA)	0.2
36	42	M	During Remission	CR (CyA)	41 (4†)
124	69	M	Before Therapies	CR (ATG+CyA)	0.3
223	74	F	During Remission	PR (CyA)	74 (6†)
215	62	M	Before Therapies	PR (ATG+CyA)	0.2
181	78	M	Before Therapies	NA	0.4
97	71	M	Before Therapies	PR (CyA)	0.2
252	34	M	During Remission	CR (ATG+CyA)	124
118	64	F	Before Therapies	PR (ATG+CyA)	0.2
298	60	F	Before Therapies	CR (ATG+CyA)	0.4
188	64	F	Before Therapies	NA	0.1
291	20	F	Before Therapies	NA	0.1
196	27	M	During Remission	PR (ATG+CyA)	49

Too early to evaluate.

† The HLA-A missing cells in peripheral blood were confirmed to persist over indicated periods in flow cytometry.

‡ The 6pUPD component was not detected 4 years before.

¶ NA: data not available, NE: not evaluable, CR: complete response, PR: partial response, ATG: anti-thymocyte globulin, CyA: cyclosporine A, AS: anabolic steroid.

Supplementary Table 2. Antibodies used in flow cytometry.

Surface Antigen	Isotype	Fluorescein	Source
CD3	IgG1	PerCP-Cy5.5	BD Biosciences
CD11b	IgG1	PE	BD Biosciences
CD11b	IgG1	APC	Beckman Coulter
CD19	IgG1	APC-Cy7	Beckman Coulter
CD33	IgG1	APC	Beckman Coulter
CD34	IgG1	FITC	BD Biosciences
CD34	IgG1	PE	BD Biosciences
HLA-ABC	IgG1	FITC	BD Biosciences
HLA-ABC	IgG1	PE	BD Biosciences
HLA-A2/28	IgG2a	FITC	ONE LAMBDA
HLA-A3	IgM	Biotine	ONE LAMBDA
HLA-A9/24	IgG2b	FITC	ONE LAMBDA
HLA-A11	IgM	Biotine	ONE LAMBDA
HLA-A25/26	IgM	Biotine	ONE LAMBDA
HLA-A30/31	IgM	Biotine	ONE LAMBDA
HLA-A33	IgM	Unconjugated	ONE LAMBDA
Streptavidin	-	PE	BD Biosciences
Goat anti-Mouse Ig	-	PE	BD Biosciences

Abbreviations: PerCP-Cy5.5, PerCP-Cy5.5 Tandem; PE, phycoerthrin; APC, Allophycocyanin; APC-Cy7, Allophycocyanin-Cy7 Tandem; FITC, fluorescein isothiocyanate

Supplementary Table 3. Demographic information of the 6,613 JMDP case series.

Diseases		Number of Pts	M / F	Age at Dx (Median) (y)
AA		407	229 / 178	0 - 64 (15)
ALL		1606	923 / 683	0 - 68 (21)
AML		1827	1075 / 752	0 - 72 (34)
CML		1014	657/357	1 - 60 (33)
MDS		825	511/314	0 - 106 (41)
NHL		566	359/207	2 - 68 (44)
Others		368	247/121	0 - 67 (27.5)
	MM	73	51/22	34 - 63 (52)
	HD	69	53/16	0 - 43 (4)
	othL	68	40/28	5 - 67 (46)
	ID	51	39/12	0 - 20 (4)
	O	42	24/18	1 - 62 (25.5)
	HOD	18	10/8	1 - 52 (29)
	ML	14	9/5	4 - 59 (49.5)
	SOT	7	5/2	8 - 57 (37)
	AUL	5	4/1	5 - 27 (10)
	MF	5	2/3	15 - 54 (37)
	CLL	4	2/2	31 - 58 (52)
	MPD	4	4/0	38 - 54 (48.5)
	FSCC	1	0/1	36
	LPD	1	0/1	7
	NB	1	0/1	3
	PMF	1	0/1	60
	PNH	1	1/0	38
	TLBL	1	1/0	32
	TPLL	1	1/0	27
	WAS	1	1/0	4

Abbreviations:

AA: aplastic anemia, ALL: acute lymphoid leukemia, AML: acute myeloid leukemia, CML: chronic myeloid leukemia, MDS: myelodysplastic syndrome, NHL: non-Hodgkin’s lymphoma, MM: multiple myeloma, HD: hereditary disease, othL: other lymphoid neoplasm, ID: immunodeficiency syndrome, O: others, HOD: Hodgkin lymphoma, ML: malignant lymphoma, SOT: solid tumor, AUL: acute unclassified leukemia, MF: primary myelofibrosis, CLL: chronic lymphocytic leukemia, MPD: myeloproliferative disorder, FSCC: follicular small-cell cleaved lymphoma, LPD: lymphoproliferative disorder, NB: neuroblastoma, PMF: primary myelofibrosis, PNH: paroxysmal nocturnal hemoglobinuria, TLBL: T-lymphoblastic leukemia, TPLL: T-prolymphocytic leukemia, WAS: Wiscott-Aldrich syndrome.

Supplementary Table 4. Univariate and multivariate logistic regression analyses.

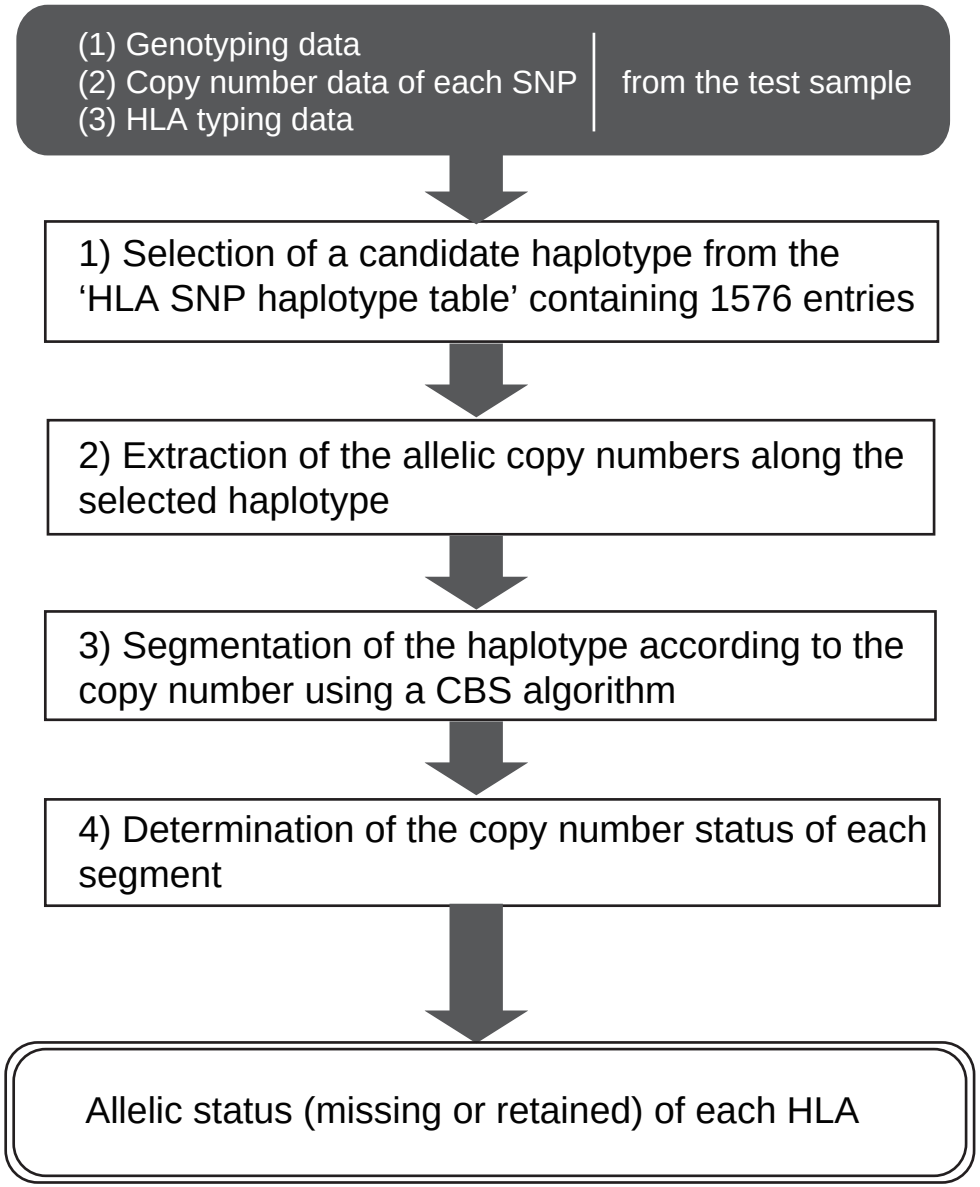
	Univariate		Multivariate		
Covariate	Odds Ratio	*P*-value	Rank‡	Odds Ratio	*P*-value
A*02:01	2.2	0.28	–	8.8	0.032
A*02:06	1.3E+01	0.002	–	1.6E+06	<0.0001
A*24:02	0.17	0.0002	3	0.88	0.93
A*31:01	1.0E+05	<.0001	–	1.6E+16	<0.0001
B*40:02	3.6E+05	<.0001	–	4.0E+16	<0.0001
B*52:01 / Cw*12:02§	0.053	0.0001	6	7.6E+03	0.67
Cw*01:02	0.30	0.13	5	0.63	0.75
Cw*03:04	3.2E+01	<.0001	14	6.8	0.22
Cw*07:02	0.52	0.33	4	0.50	0.71
Cw*08:01	0.57	0.45	9	3.7	0.41
DRB1*09:01 / DQB1*03:03§	1.6	0.39	12	9.7E-06	0.072
DRB1*15:01 / DQB1*06:02§	2.6	0.17	–	5.3E-11	0.0069
DRB1*15:02	0.17	0.0039	1	3.4E-07	1.0
DQB1*0301	2.6	0.17	13	2.6E-06	0.11
DQB1*06:01	0.21	0.0044	11	0.19	0.12
DPB1*02:01	2.0	0.19	7	2.8E-06	0.54
DPB1*04:02	0.57	0.45	8	2.7	0.42
DPB1*05:01	1.4	0.46	2	1.1	0.97
DPB1*09:01	0.14	0.0045	10	3.4E+04	0.40

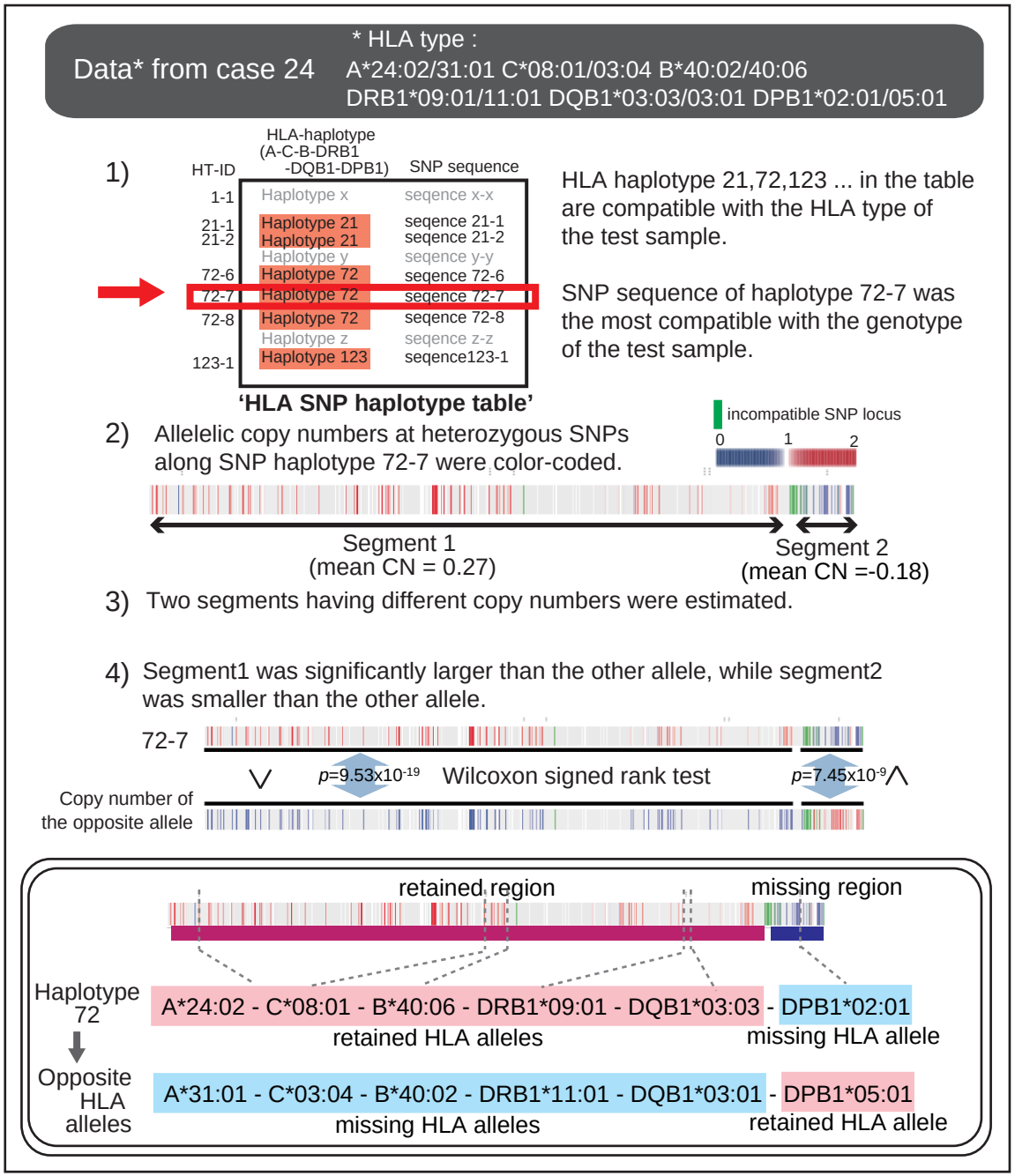
‡ The rank of elimination for backward stepwise selection

§ Complete association

Supplementary Figures

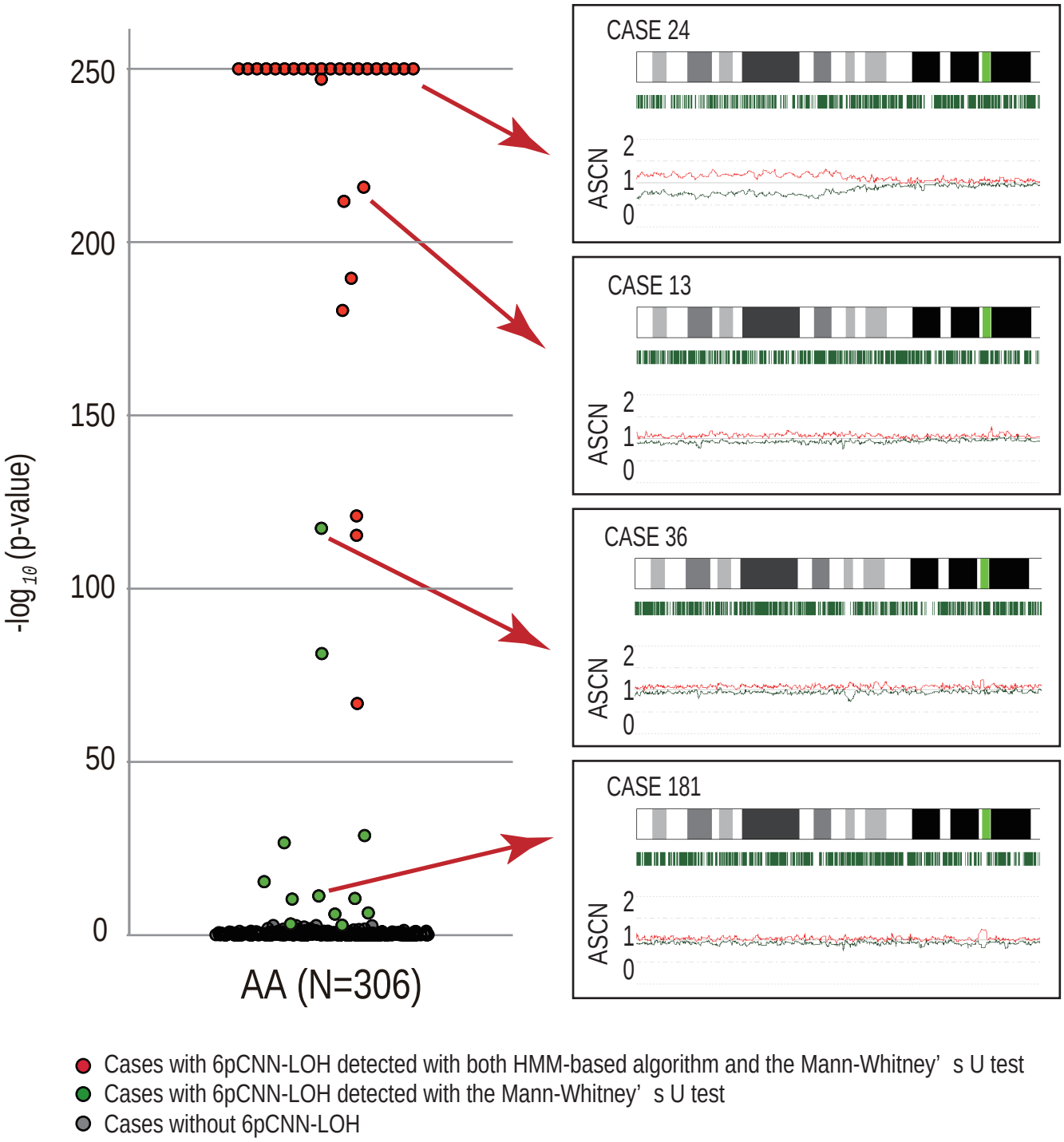
Supplementary Figure 1





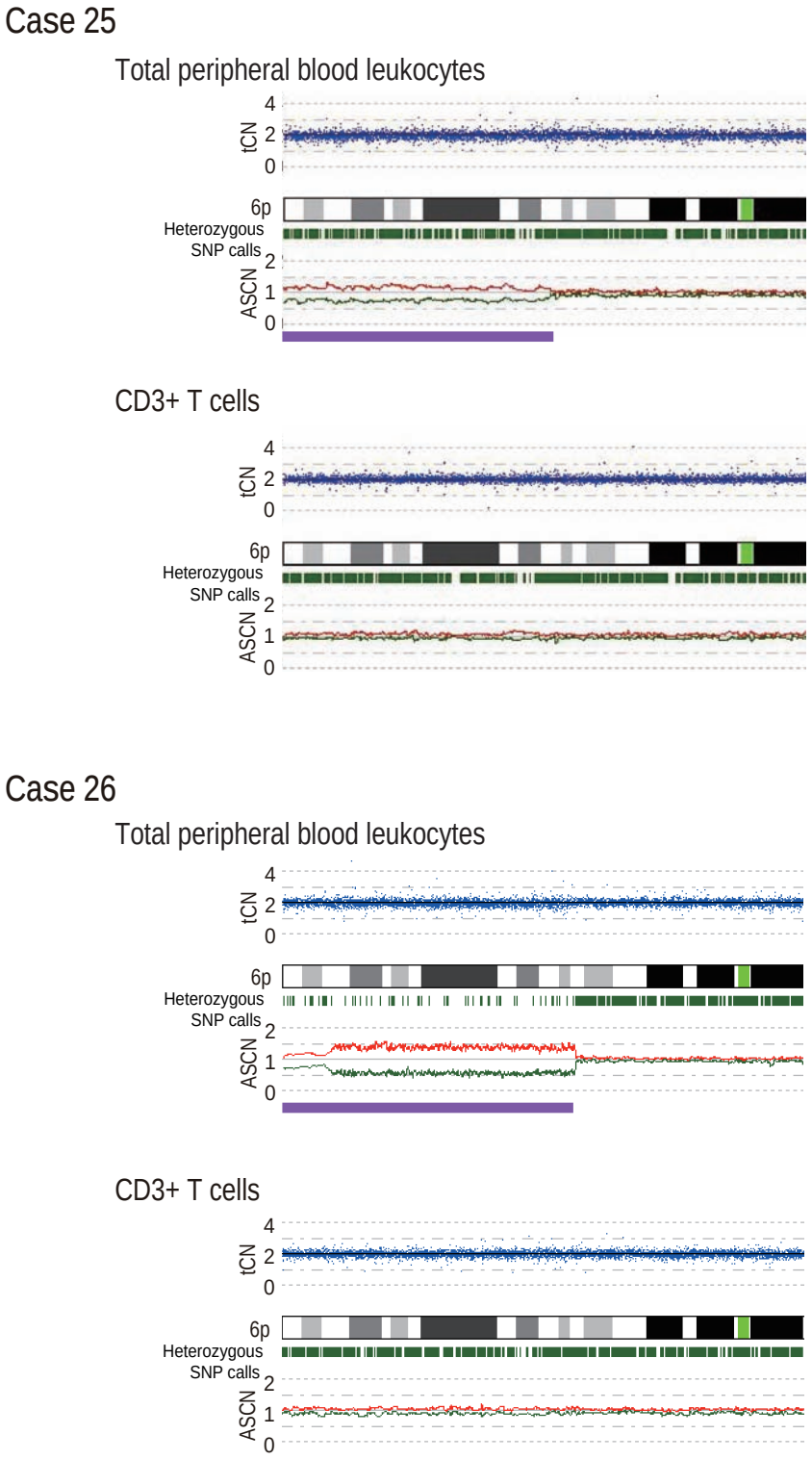
Supplementary Figure 1. Imputation of missing HLA alleles.
Procedure of imputation of the allelic status of HLA is shown in the left panel. In the right panel, this analysis was applied to case 24, in which haplotype of A*31:01 - C*03:04 - B*40:02 - DRB1*11:01 - DQB1*03:01 - DPB1*02:01 was estimated to be missed.

Supplementary Figure 2



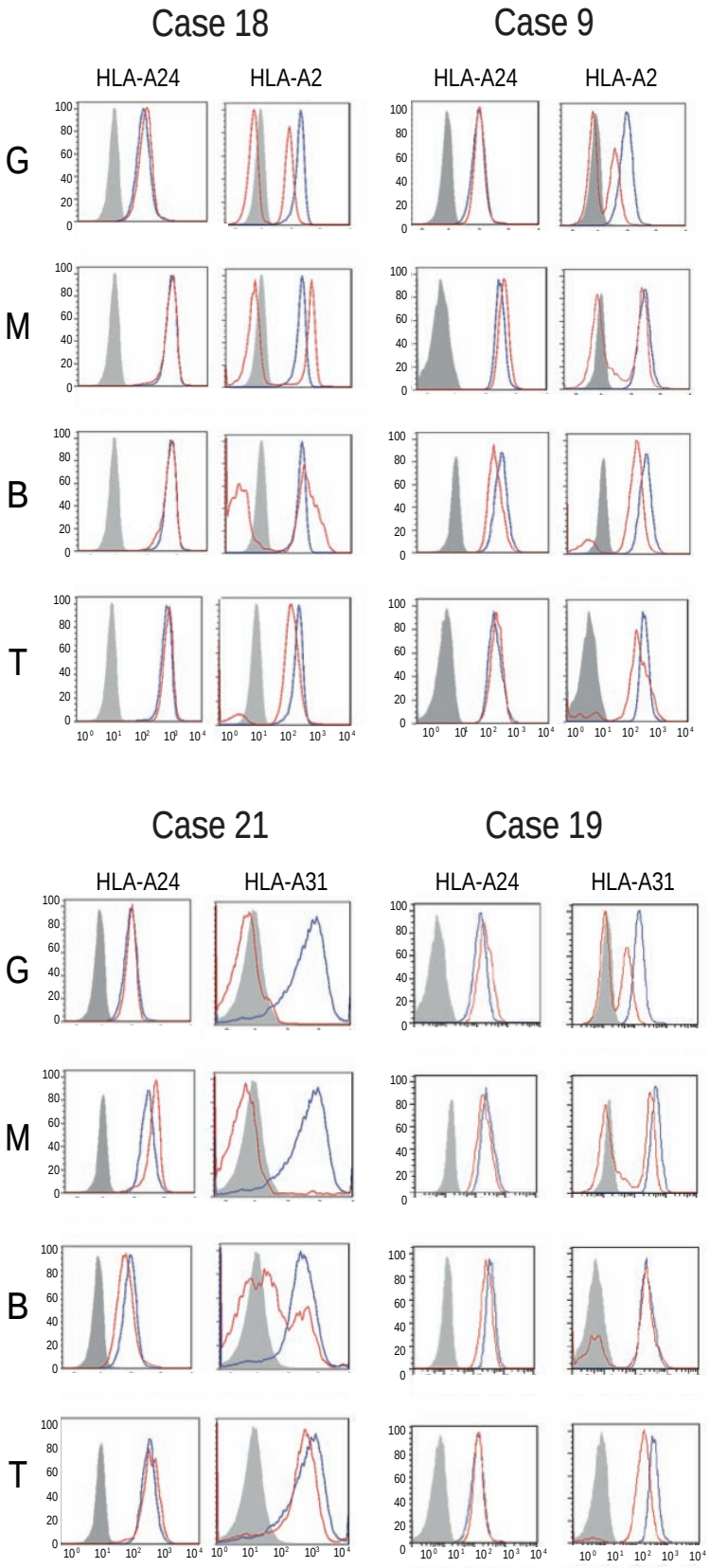
Supplementary Figure 2. Sensitive detection of 6pLOH in AA cases.
CNN-LOH in 6p was detected as dissociation of allele-specific copy number graphs in SNP array analysis using a HMM-based algorithm. However, CNN-LOH in 6p was detected with greater sensitivity by statistically comparing the mean difference in allele-specific copy numbers in 6p with that in non-6p regions (left panel). To avoid too many false positive findings, the threshold P-value was set to control FDR to 0.01. Because allele-specific copy numbers were measured at a large number of SNP sites, the power of detecting allelic imbalance outperforms that of HMM-based detection. In cases 24 and 13, dissociation of allele-specific copy numbers in 6p was detected by a HMM-based algorithm in CNAG. On the other hand, in cases 36 and 181, the allelic imbalance in 6p was too small to be detected by HMM, but the difference in allele-specific copy numbers in 6p was highly significant, indicating the presence of an allelic imbalance in 6p.

Supplementary Figure 3



Supplementary Figure 3. CNN-LOHs in 6p: Acquired genetic events.
CNN-LOH in 6p was not detectable in the DNA of $CD3^+$ T cells isolated from two patients (case 25 and 26), but was clearly detected in that of total peripheral blood leukocytes, indicating that the CNN-LOH detected in the 6pLOH(+) patients is not constitutional.

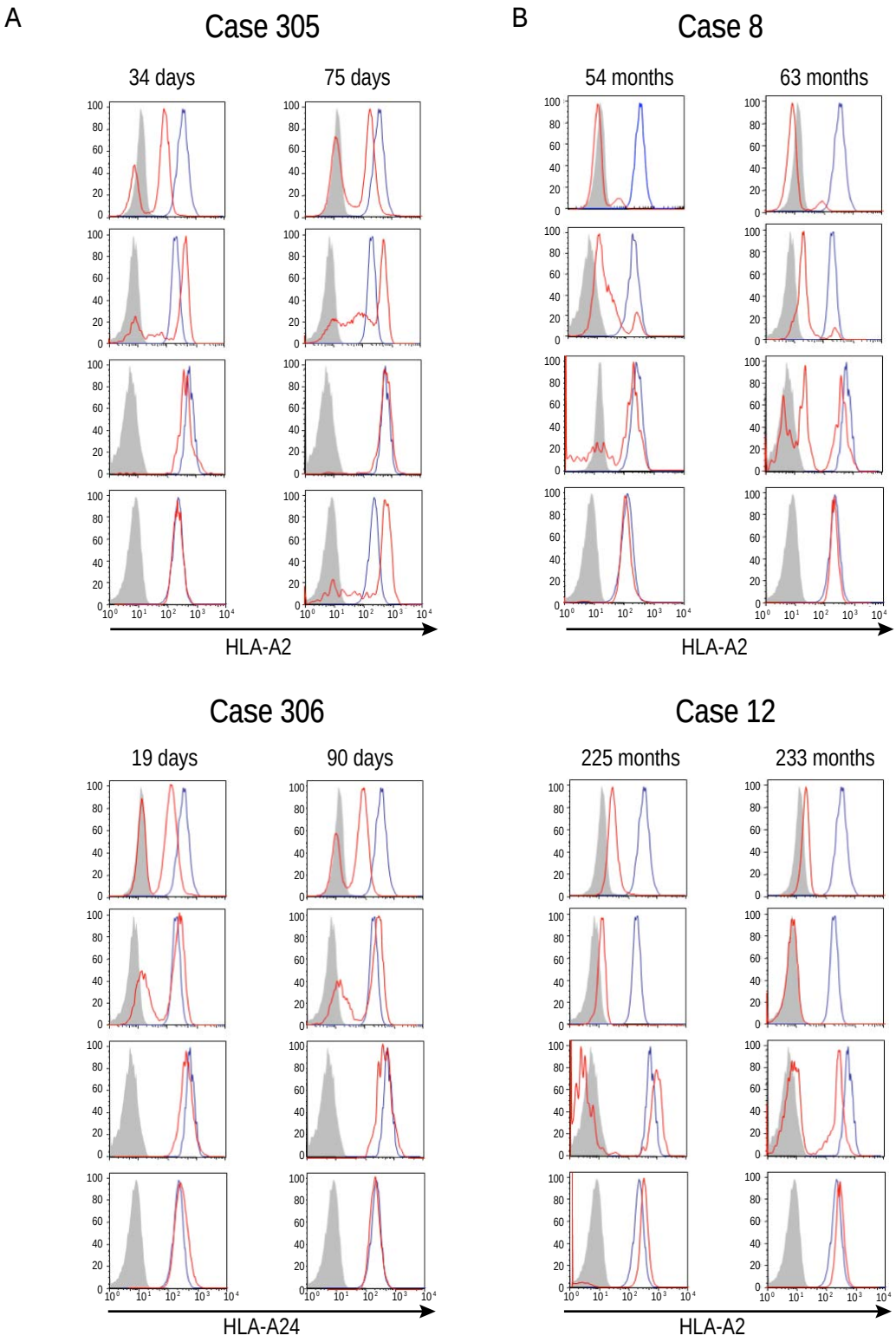
Supplementary Figure 4



Supplementary Figure 4. Uniparental expression of HLA-A antigens in additional cases with CNN-LOH in 6p.

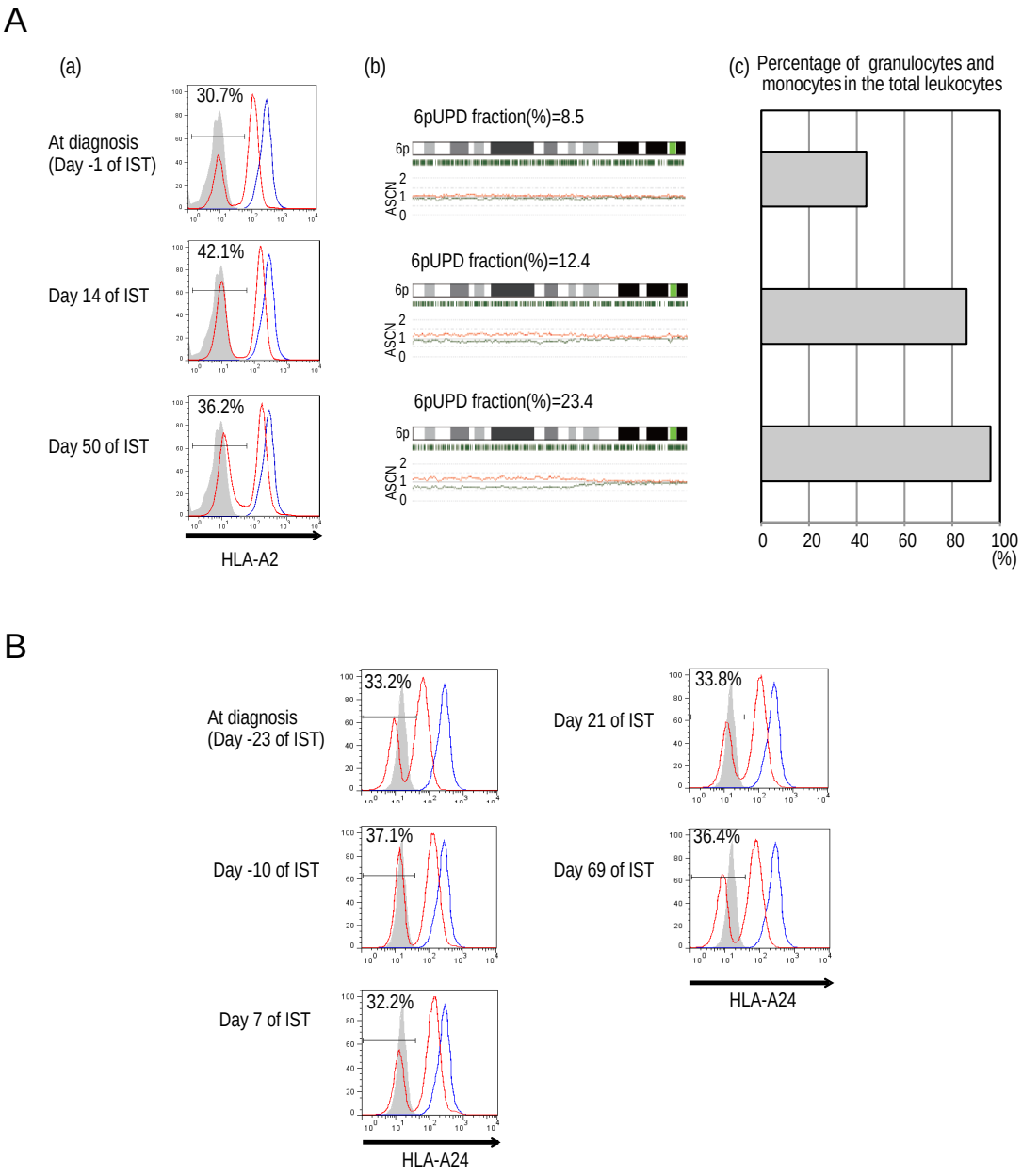
Uniparental HLA-A expression in different peripheral blood compartments in additional 4 cases (cases 18, 9, 21 and 19) are shown, where the analyses for missing HLA-A antigens are demonstrated in the right panels for each case. Red and blue lines indicate the results for the patients and healthy controls, respectively. G, granulocytes; M, monocytes; B, B lymphocytes; T, T lymphocytes.

Supplementary Figure 5



Supplementary Figure 5. Persistence of 6pLOH(+) cells in patients with AA.
Blood samples obtained at different time points from two newly-diagnosed AA patients (A, cases 305 and 306) and two AA patients in remission after immunosuppressive therapy (B, cases 8 and 12) were examined for the presence of leukocytes lacking HLA-A antigen The numbers on the histograms represent the time after the diagnosis of AA. Case 305 received ATG plus cyclosporine (CsA) therapy 34 days after the diagnosis of AA and was recovering from AA when the second sample was obtained 75 days after the diagnosis. Case 306 had been treated with CsA plus G-CSF from day 23 after the diagnosis and only the neutrophil count was on the rise at the time of second sampling (day 67 after the treatment). Case 8 had been in CR for 60 months after ATG plus CsA therapy while case 12 had been in PR for 124 months with CsA plus anabolic steroids.

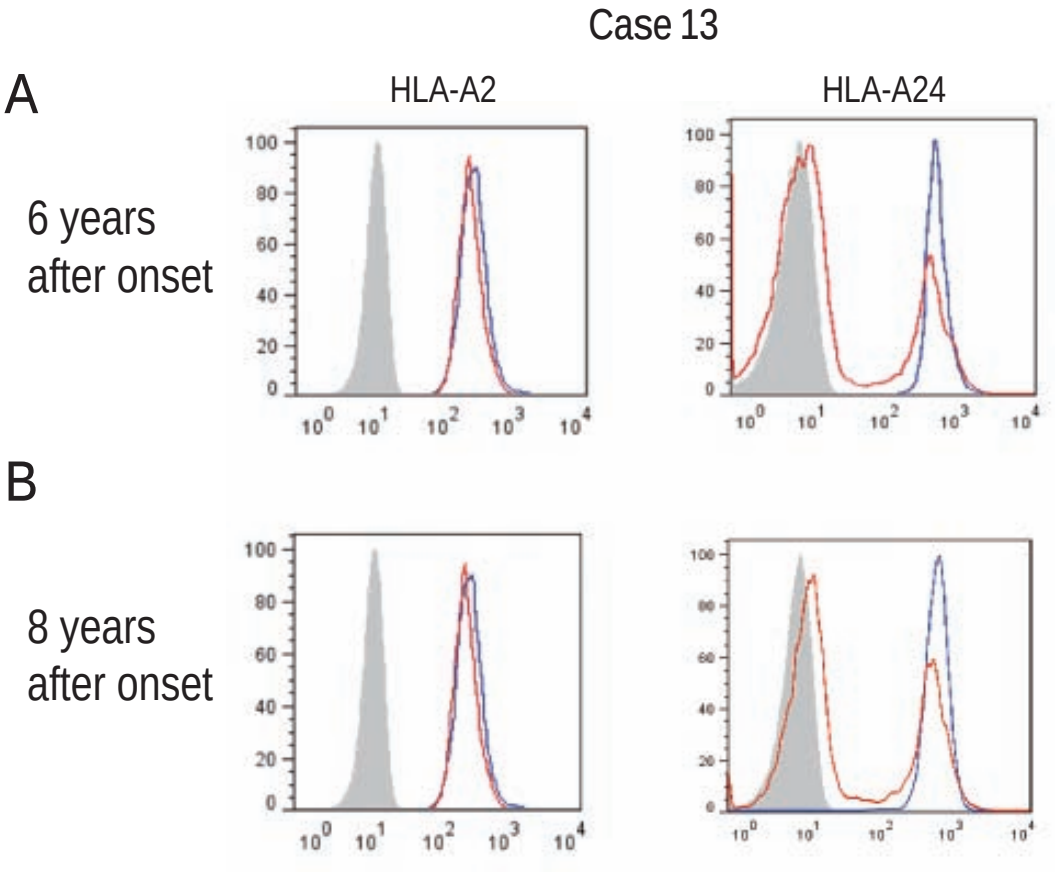
Supplementary Figure 6



Supplementary Figure 6. Changes in the percentage of granulocytes lacking HLA-A antigens in convalescent patients after IST.

The results of chronological flow cytometry of the HLA-A antigen expression by granulocytes in case 305 (A) and 306 (B) are shown. (A) The percentages of the HLA-A2 missing cells determined by flow cytometry remained unchanged during the convalescent phase of 2-3 months in case 305 (a), but the percentage estimated from the width between the dissociated lines in the SNP array analysis increased with time after IST (b). This was due to the increase in myeloid elements (granulocytes and monocytes) in the peripheral blood associated with the response to IST (c). (B) The percentage of HLA-A24 missing granulocytes remained stable for 3 weeks before therapy and also for 10 weeks after IST.

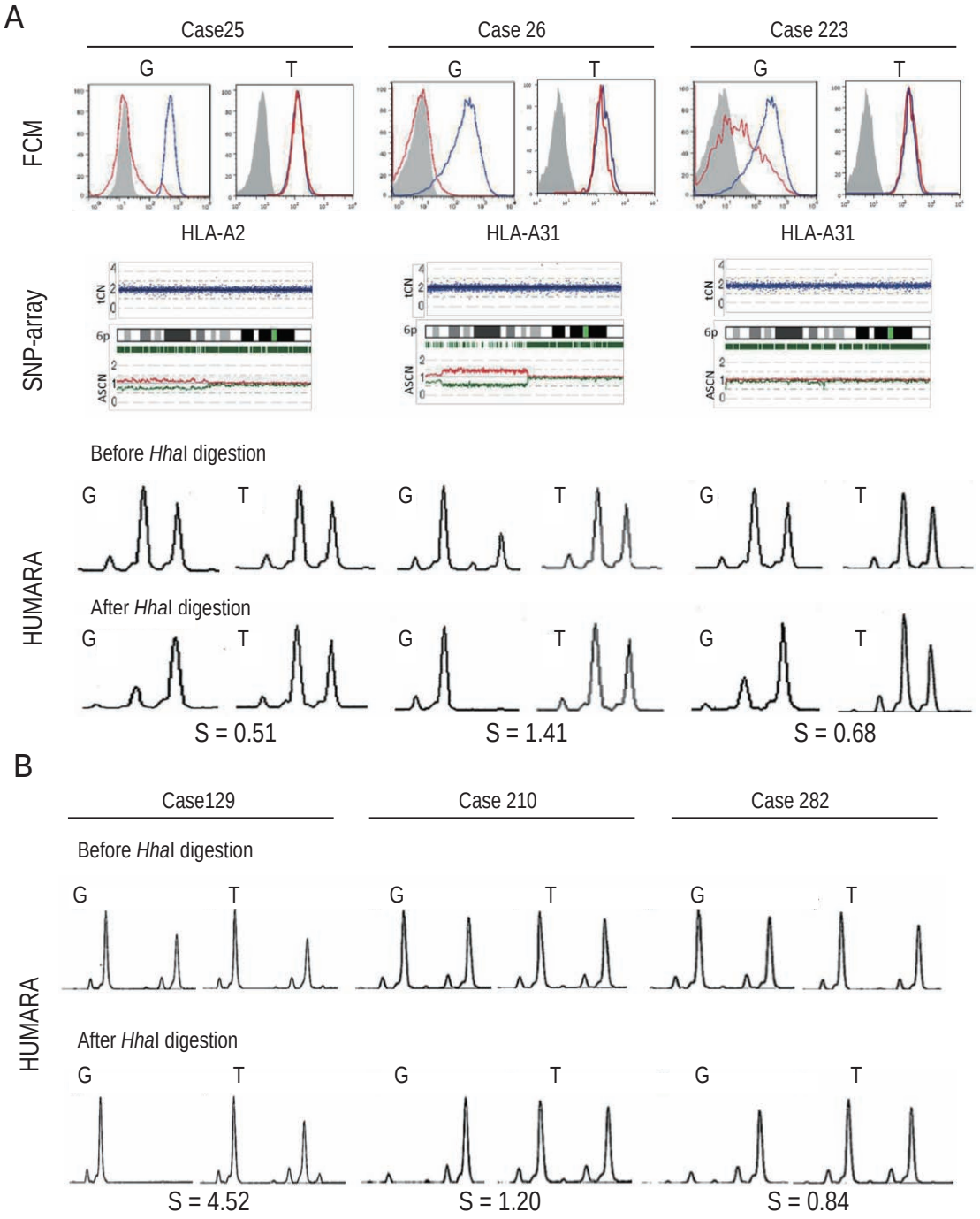
Supplementary Figure 7



Supplementary Figure 7. Chronological assessment of uniparental HLA-A expression in BM CD34⁺ fractions in case 13.

BM CD34⁺ cells obtained from case 13 in July of 2008 (A) and those obtained in July of 2010 (B) were analyzed for uniparental expression of HLA-A antigens by flow cytometry. HLA-A24 missing cells constituted 71.3% (A) and 65.2% (B) of the total CD34⁺ cells. The red and blue lines represent the expression of the indicated HLA-A antigens in the patient and a healthy control, respectively.

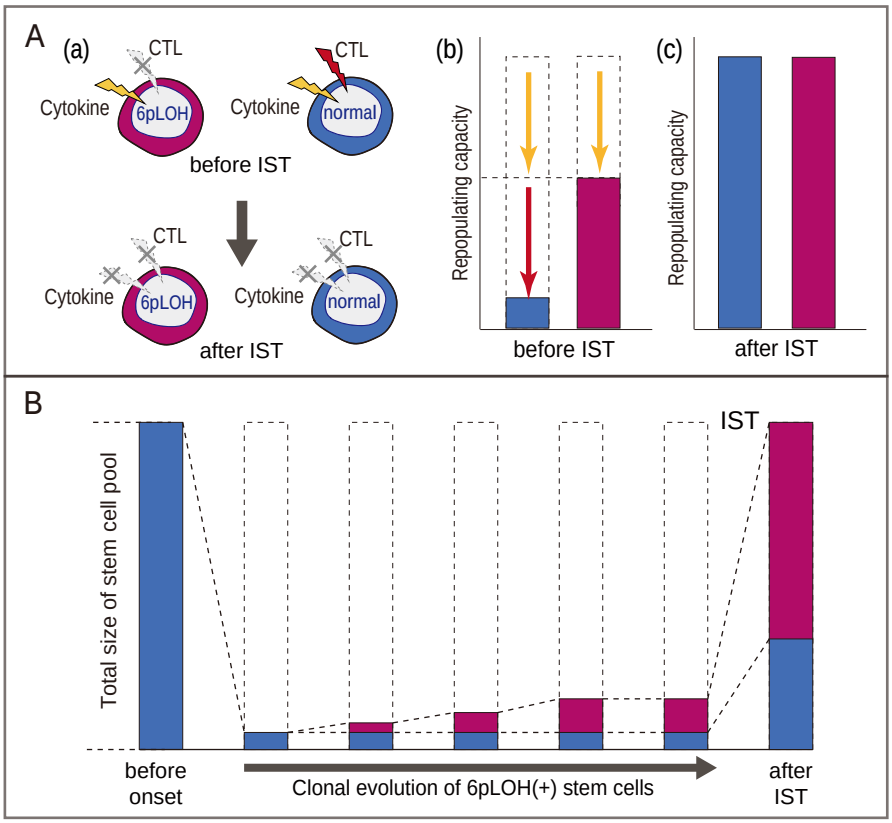
Supplementary Figure 8



Supplementary Figure 8. HUMARA-based clonality analysis of peripheral blood granulocytes in female cases with or without CNN-LOH in 6p.

A. The clonality in peripheral blood granulocytes detected by HUMARA in 3 6pLOH(+) cases (bottom panels). After digestion with a methylation sensitive restriction enzyme (*Hha*I), the genomic DNA from granulocytes (G), but not from T cells (T), showed skewed patterns of PCR amplification of the human androgen receptor gene, indicating the presence of clonal granulocytes. The uniparental expression of the indicated HLA-A antigens in granulocytes (red lines in the upper panels) in flow cytometry and the presence of CNN-LOH in 6p in whole blood (middle panels) in SNP array analysis are also presented. The expression of corresponding HLA-A antigens in healthy controls is shown by blue lines (upper panels). B. Representative results of HUMARA showing the presence of clonal granulocytes in 6pLOH negative females. To diagnose clonality in granulocytes, the ratios of both allele areas (C values) before (lower allele/higher allele; A/B) and after (lower allele/higher allele; A'/B') *Hha*I digestion were determined for granulocytes (CG) and T lymphocytes (CL) as a marker of skewing in each cell population. The absolute values of log(CG/CL) (S values) were then determined and used as a marker of clonality of granulopoiesis. The clonal granulopoiesis was judged to be present when the S value was >0.50.

Supplementary Figure 9



Supplementary Figure 9. The influence of IST on the proportion of normal and 6pLOH(+) leukocytes in patients with AA.

A. The normal (6pLOH(-)) hematopoietic stem cells (HSCs) in a patient with AA are being inhibited by both cytotoxic lymphocytes (CTLs, yellow arrow) and inflammatory cytokines (red arrow), while the 6pLOH(+) HSCs are being inhibited by only cytokines due to the absence of HLA antigens required for presentation of target auto-antigens to CTLs (a). As a result, the repopulating capacity of normal HSCs would be more severely impaired than that of 6pLOH(+) HSCs (b). IST capable of eliminating the inhibitory effect of both CTLs and cytokines allows normal HSCs and 6pLOH(+) HSCs to repopulate the patient's BM to the same degree (c). B. The CTL attack against HSCs reduces the HSC pool, and allows 6pLOH(+) HSCs to grow, although both normal and 6pLOH(+) HSCs remain suppressed due to the presence of inflammatory cytokines. The relative numbers of 6pLOH(+) to (-) stem cells will become a fixed value that is determined by the severity of the autoimmune insults in the steady state before IST. Once the autoimmune insults disappear, nothing could discriminate a 6pLOH(+) stem cell from a 6pLOH(-) stem cell, and therefore, both 6pLOH(+) and 6pLOH(-) HSCs will produce the same number of progeny on average, and feed the same number of mature blood cells. As a consequence, the relative number of 6pLOH(+) stem cells to 6pLOH(-) stem cells should be maintained, both after and while the severely reduced hematopoietic stem cell pool is re-expanded by removal of the inciting autoimmunity, including inhibitory cytokines.