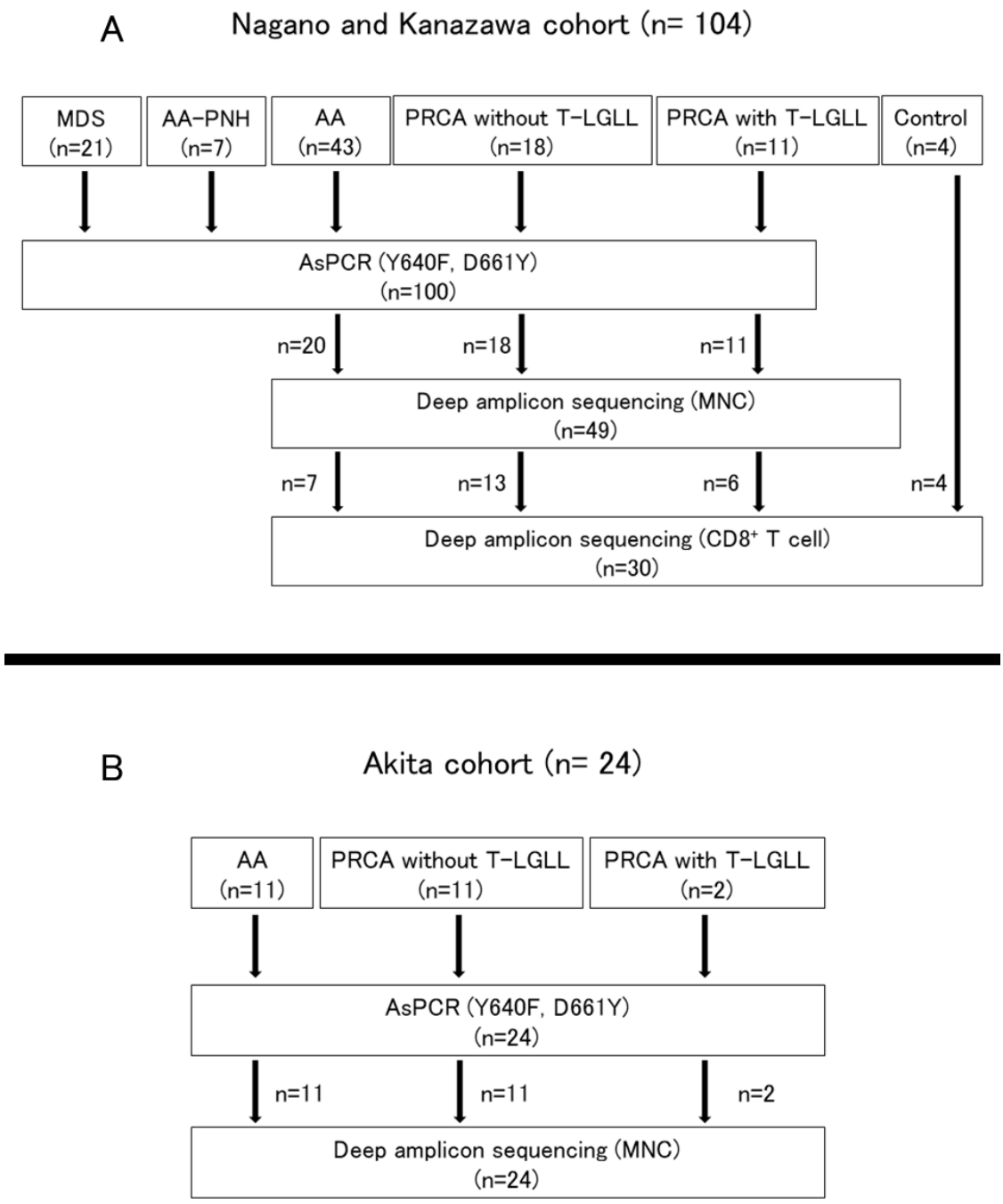
Supplemental Data

Supplement to: Kawakami T, et al. Frequent *STAT3* mutations in $CD8^+$ T cells from patients with pure red cell aplasia

Contents:

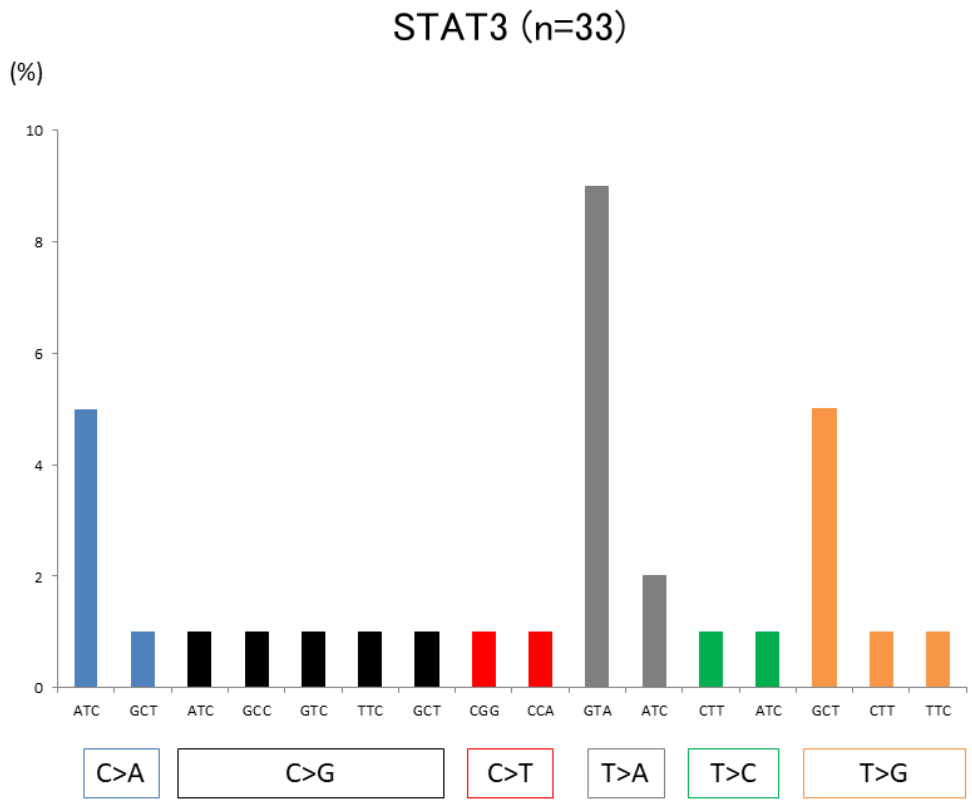
Supplemental Figure S1.



Design of this study. A: Nagano and Kanazawa cohort in which clinical data of the patients were available. B: Akita cohort in which DNA samples and the diagnosis of the patients available.

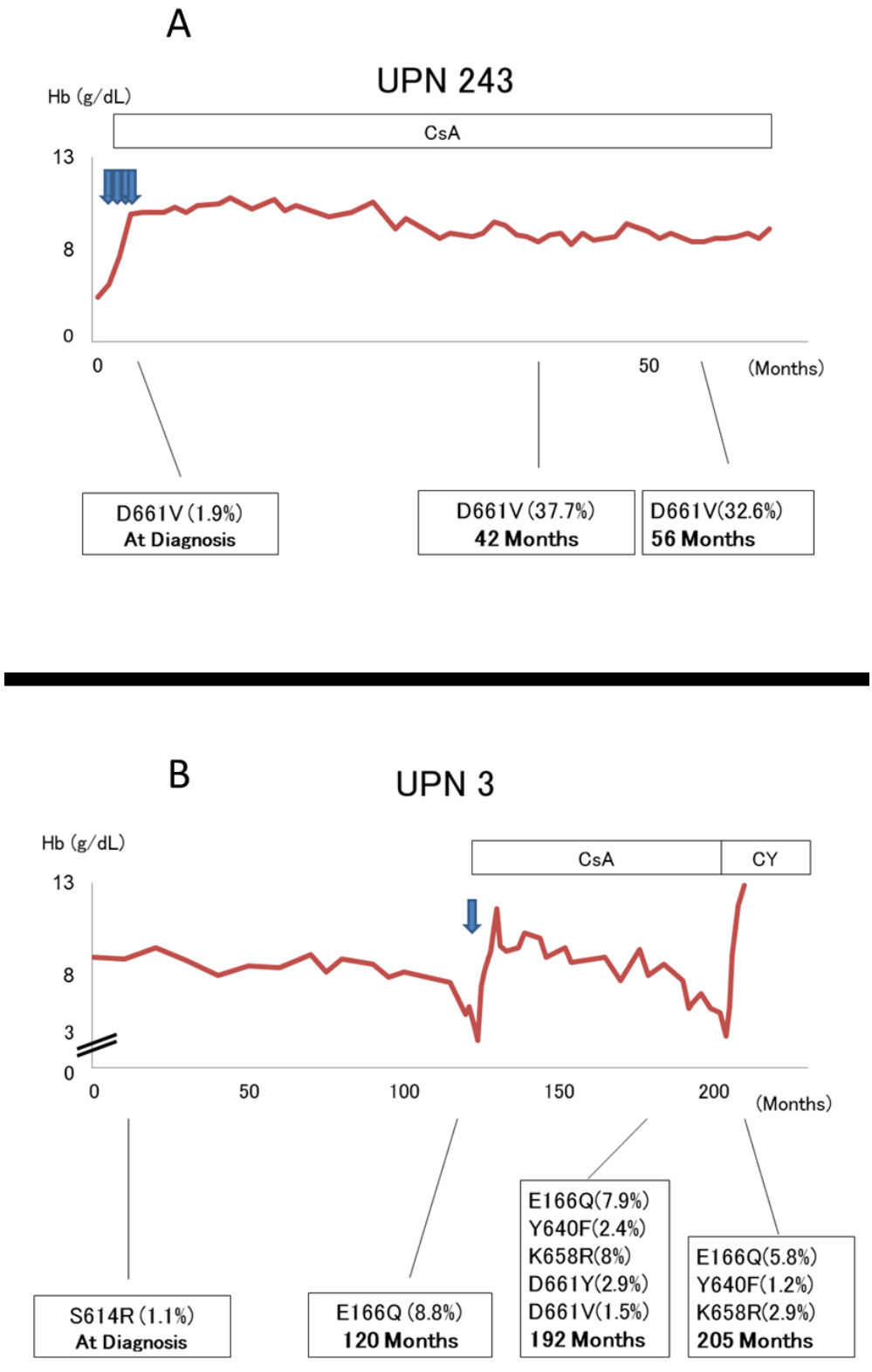
AsPCR: allele-specific PCR, MNC: mononuclear cell

Supplemental Figure S2.



The nucleotide substitution patterns of *STAT3* mutations with targeted sequencing. The types of nucleotide substitution were tallied manually and classified with different colors.

Supplemental Figure S3.

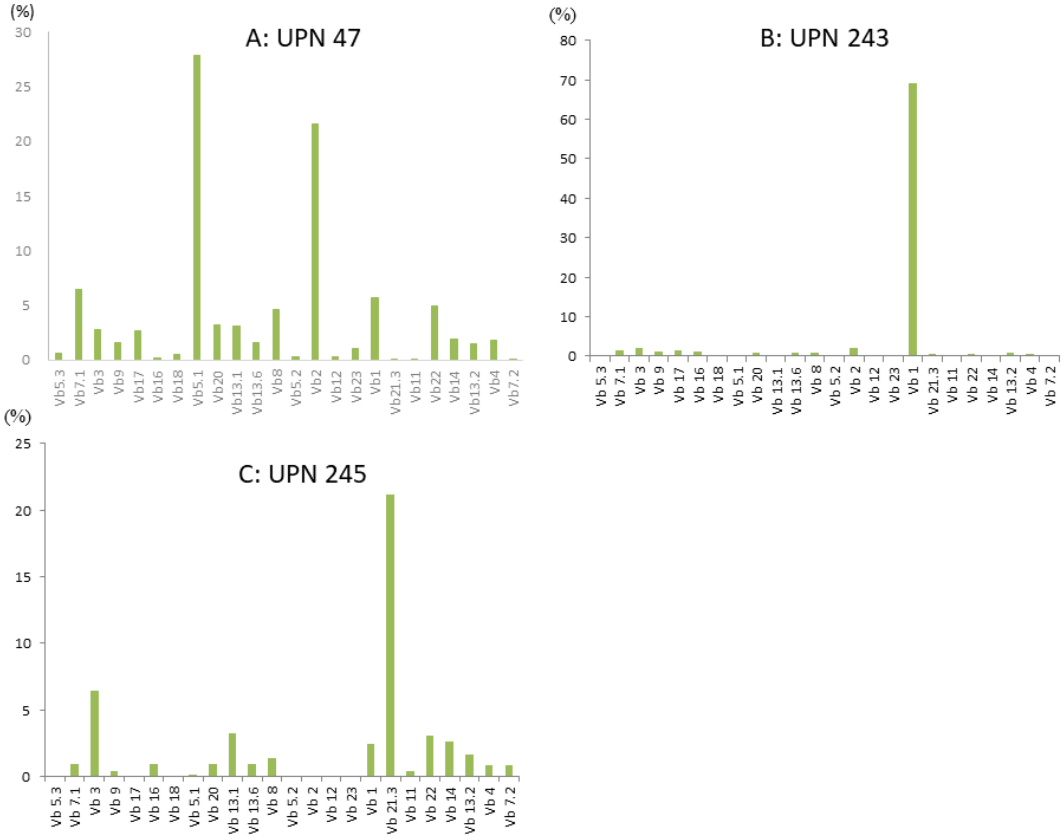


The time course analysis of the *STAT3* gene in two patients with PRCA. A: Unique patient number (UPN) 243, PRCA without T cell large granular lymphocytic leukemia (T-LGLL), received cyclosporine A (CsA) treatment under the diagnosis of idiopathic PRCA. She temporally responded but gradually became resistant to CsA. She had the D661V mutation at the time of her diagnosis and the variant allele frequency (VAF) of D661V increased with time.

B: UPN 3, PRCA with T-LGLL, began taking oral CsA 10 years after the diagnosis of T-LGLL to treat progressive anemia. Her anemia improved and was stable for 6 years with CsA, but she subsequently became resistant to CsA. Her anemia responded after the replacement of CsA with cyclophosphamide (CY). The *STAT3* mutation S614R (variant allele frequency; VAF 1.1%) was detected at the time of the diagnosis of T-LGLL and the E166Q (8.8%) mutation appeared when CsA therapy was started. E166Q (7.9%), Y640F (2.4%), K658R (8%), D661Y (2.9%), and D661V (1.5%) were detected at the time of CsA failure. After the administration of CY, the mutation profile became less complex.

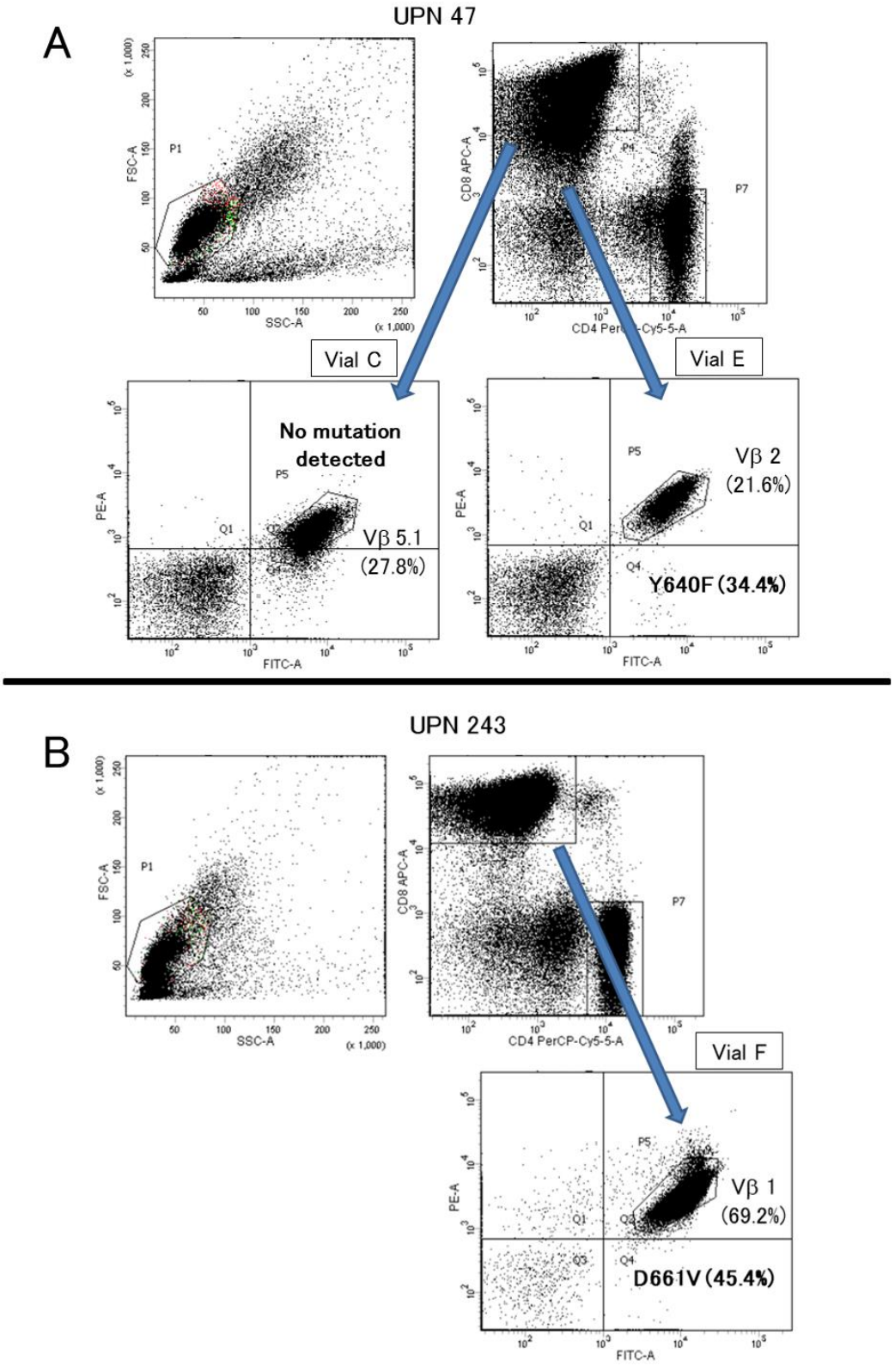
Blue arrows indicate red blood cell transfusion.

Supplemental Figure S4.



The distributions of the TCRVβ repertoire in $CD3^{+}CD8^{+}$ lymphocyte populations. A: UPN 47 (idiopathic PRCA, positive for Y640F and D661Y mutations). B: UPN 243 (idiopathic PRCA, with D661V). C: UPN 245 (thymoma-associated PRCA, with Y640F).

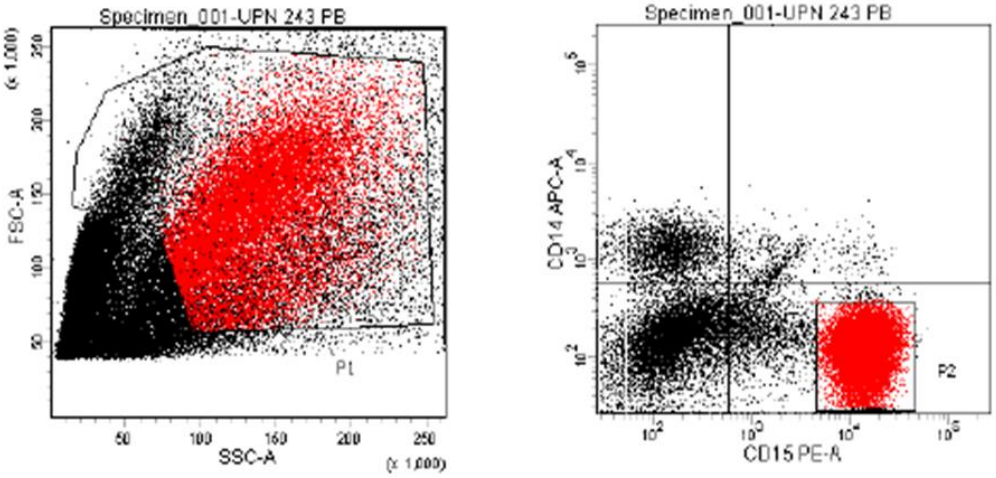
Supplemental Figure S5.



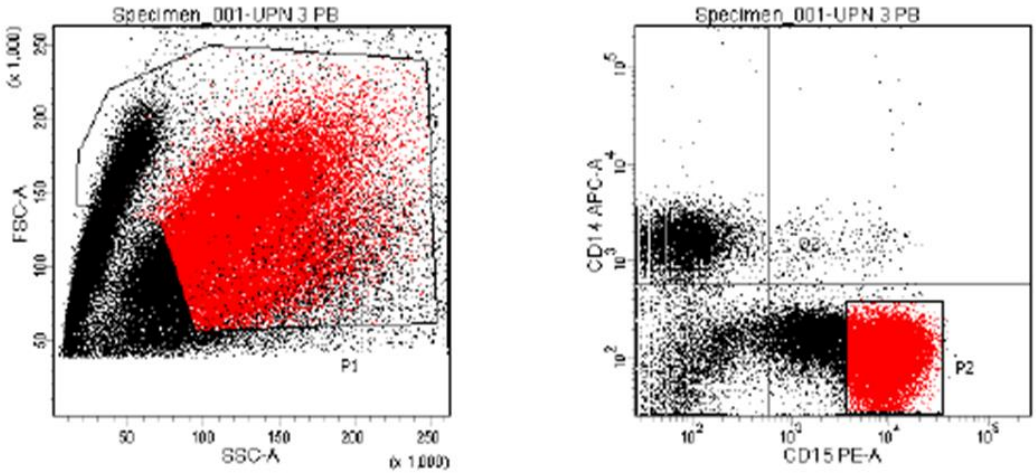
Scatterplots of sorted T cells and the results of amplicon sequencing for the *STAT3* genes in sorted fractions. UPN 47 represents idiopathic pure red cell aplasia (PRCA). The $CD8^+$ T cells were restricted to 27.8% of Vβ5.1 and 21.6% of Vβ2. Vβ5.1 was negative for *STAT3* mutations; however, a Y640F mutation was found in the Vβ2 population (A). UPN 243 represents idiopathic PRCA; most of the $CD8^+$ T cells expressed Vβ1 (69.2%). This abnormal Vβ fraction was positive for the *STAT3* D661V mutation (B).

Supplemental Figure S6.

A. UPN 243



B. UPN 3



FACS sorting of granulocytes and subsequent amplicon sequencing for the *STAT3* gene from patients with PRCA. Granulocytes were sorted from 2 patients: UPN243 with idiopathic PRCA positive for the D661V mutation (A); and UPN 3 with PRCA associated with T-LGLL positive for the E166Q, Y640F and K658R mutations. No mutations were detected in the granulocytes. The sorted granulocyte fractions are shown in the red area.

Supplemental Table S1: Diagnostic criteria

aplastic anemia (AA)
1 clinical manifestations of anemia, bleeding or fever
2 meet at least 2 of the following 3 criteria
1)Hemoglobin concentration: <100 g/L
2)Neutrophil count: $<1.5 \times 10^9$ /L
3)Platelet count: $<100 \times 10^9$ /L
3 rule out other diseases causing pancytopenia
4 auxiliary findings
1)without increased reticulocytes
2)reduction of nuclear cells, megakaryocytes, or increasing ratio of lymphocytes in BM
3)reduced hematopoietic cells in BM biopsy
4)elevated serum iron levels and lowered unsaturated iron binding capacity
5)MRI findings of reduced hematopoietic tissue and increased fat tissue
5 consider the possibility of AA with 1 and 2, rule out other disorders by 3 and increase certainty by 4

AA-paroxysmal nocturnal hemoglobinuria (PNH)
1 meet the diagnostic criteria for AA
2 existence of PNH-type blood cells
3 with hemolysis

pure red cell aplasia (PRCA)
1 chronic normocytic normochromic or macrocytic normochromic anemia with decreased or absent reticulocytes
2 cellular marrow with markedly decreased to absent nucleated red blood cells but normal granulocytic and megakaryocytic cell series
3 no apparent leukocytopenia or thrombocytopenia

T-cell large granular lymphocytic leukemia (T-LGLL)
1 persistent (>6 months) increase in the number of peripheral blood T-cell type large granular lymphocytes (LGL) ($> 2 \times 10^9$ /L)
2 evidence of TCRγ rearrangement is necessary when LGL count is 0.5-2 $\times 10^9$ /L

BM: bone marrow, MRI: magnetic resonance imaging

Supplemental Table S2: List of primers used for amplicon sequencing

primer name	sequence	primer name	sequence
STAT3_1_F	GTCCCATTCCCAGGGATAACTG	STAT3_1_R	CCTCCAGCTCTGCTTACTGAAT
STAT3_2_F	CAAGTAGCCGGAGGATGAAGTT	STAT3_2_R	GTGGTCAAAGTAGGCTTTTTGAAGAAA
STAT3_3_F	AAGATCAGAATTCAATCTAGCTTTCGAGA	STAT3_3_R	GGAGTACGTGCAGAAAACTCTCAC
STAT3_4_F	GGGACCAACTTCCCTTATAGGGA	STAT3_4_R	CCTGCAGCAATACCATTGACCT
STAT3_5_F	ATGTCTCTTGACTCAAACTGAAACCT	STAT3_5_R	AATCAGCTACAGCAGCTTGACA
STAT3_6_F	TGCCCGAGGCTTGTAACTTG	STAT3_6_R	TCACATGTGCATTGACCTCCTTTT
STAT3_7_F	TCTGCCACAAGACGCTGAAAT	STAT3_7_R	TGAATGGAAACAACCAGTCAGTGA
STAT3_8_F	TGAGAAAAGAGATGCTTCCAGGAAAG	STAT3_8_R	GGAGAGATTGACCAGCAGTATAGC
STAT3_9_F	GCTCCCTCAGGGTCTGTAAGAA	STAT3_9_R	GATGAACATGGAAGAATCCAACAACG
STAT3_10_F	TGAAATGCGGACCCAAGAGTTT	STAT3_10_R	CCCAATTGGAACCTGGGATCAAG
STAT3_11_F	GTGCAGCTCCTCAGTCACAAT	STAT3_11_R	AGTTTTGCTGTGCTGCTTAGACT
STAT3_12_F	GTCAGCATGTTGTACCACAGGAT	STAT3_12_R	GAAGAGATTTCCAAGGCTGTGAGA
STAT3_13_F	CCGCCTTAAGATCTAAACAGAGTTAAGA	STAT3_13_R	GAATCTCCAGGATGACTTTGATTTCAACTA
STAT3_14_F	CTAAGATAGGAGTACTTACTTGTCAATGC	STAT3_14_R	AAAAGGTTATGGGAGAGTTACTGACTTTTT
STAT3_15_F	TTAGATGAGGGAAAGGGACAAGGA	STAT3_15_R	ACTTCAGACCCGTCAACAAATTAAGAA
STAT3_16_F	GCATTCCCATTCCCACGAGAAT	STAT3_16_R	CCCAAGCTGAAAATGTACTACTTGACT
STAT3_17_F	GTCCTTCTCCACCCAAGTGAAA	STAT3_17_R	GCAATAACAACATTGTTCCTCCTCCTT
STAT3_18_F	GGAATGTCAGGATAGAGATAGACCAGT	STAT3_18_R	GATCTTTCCTTCCCATGTCCTGT
STAT3_19_F	GGGACTTGGTTACATCTGTGCA	STAT3_19_R	GCAGTAGACTTGGCTTTCCCAT
STAT3_20_F	CCTAACAGTGTCCCTCAGTAAAATCT	STAT3_20_R	GCGTGAGCCCATCTTCTCTTTC
STAT3_21_F	CCCAGACCAGGGATTTGTTTTGT	STAT3_21_R	GTCTGCTGCTGATTTTTATTCTCTTTCTC
STAT3_22_F	CCAAACTGCATCAATGAATCTAAAGTGC	STAT3_22_R	AGAGTTTGATGGCTTGTGTGTGT
STAT3_23_F	TCCGACCTATGCCCTTACTCTC	STAT3_23_R	GTATTCCCTCAGGTCAAGGAGTTTTT
STAT3_24_F	GCAGTAGGTGCTTGCAACTAGA	STAT3_24_R	CTAAGATTCAGTGAAAGCAGCAAAGAA
STAT3_25_F	AAGCTGTCACTGTAGAGCTGATG	STAT3_25_R	AACATATGCACACTTTGGTTTACAGTTG
STAT3_26_F	CGCCTTGCTCAGGAAAGAAAC	STAT3_26_R	GAGCATCGAGCAGCTGACTA
STAT3_27_F	TCCCAGTGGAAGTTTTTGTCCTG	STAT3_27_R	TGTCCTTCTCGTTACTGTAGGCT
STAT3_28_F	CCACATACCAAGTGTTTGAATTCTGC	STAT3_28_R	TTGGTCACCTACATAGTTGATTGTTCC
STAT3_29_F	ACACATTTGCTTGTTTAGATGAGGGAT	STAT3_29_R	CCAACATCTGTCAGATGCCAAATG
STAT3_30_F	TCTAATATTCACTTGCCTCCTTGACTCT	STAT3_30_R	TCCCAAGGAAATCTTTCTTTACTTCTGTTT
STAT3_31_F	CCCTCTCTCCCTCAAGGAAAAC	STAT3_31_R	ACAGGACACCTGCCTTTTTCTT
STAT3_32_F	ACTTTTGTCCACAAAATGAAGATCTCTGA	STAT3_32_R	TCAAATTCCCTGAGTTGAATTATCAGCTTA
STAT3_33_F	CCTTTGTAGGAAACTTTTTGCTGCA	STAT3_33_R	ACCTGTATACATTCACTTTGGTAATTAGCA
STAT3_34_F	GCGCCTCAGTCGTATCTTTCTG	STAT3_34_R	AGAGGGTGGACAACTGAACTAGTT
STAT3_35_F	CTCCAATGCAGGCAATCTGTTG	STAT3_35_R	ATGTTTTCTGACTTTGTTTTGGTTTCCA
STAT3_36_F	CCAAAAATTAAATGCCAGGAACATGGA	STAT3_36_R	GGCTATAAGATCATGGATGCTACCAATATC
STAT3_37_F	GTGCTGATAGAGAACATTCGACTCT	STAT3_37_R	TCAGAGCCATTCTTTATCATTCTTCCTTTT

Supplemental Table S3: List of primers used for additional amplicon sequencing

primer name	sequence	primer name	sequence
STAT3_add_1_F	AGAGTTTCTCTGCCAGTGTAGTCA	STAT3_add_2_R	ACCATCCCTCATCTAAACAAGCAAAT
STAT3_add_2_F	TTTCCAAACTGCATCAATGAATCTAAAGTG	STAT3_add_2_R	AGAGTTTGATGGCTTGTGTGTGT

Supplemental Table S4: List of primers used for Sanger sequencing

primer name	sequence	primer name	sequence
STAT3_ex3_F	GACTCTGCGGGTCCTGTTTC	STAT3_ex3_R	GTGTGTATGCGTCGGCTTCAGAG
STAT3_ex5_F	TGAGCTGTGATCATGCCACTGCAGCCC	STAT3_ex5_R	GCAGCGTCTCAAGCCAAACAAGAAG
STAT3_ex6_F	CAACTCAACAACACAAACTCAC	STAT3_ex6_R	TTTGAGGTTTTGGCTTCATTTG
STAT3_ex9_10_F	CCTAACAGTGTCCCTCAGTAAAATC	STAT3_ex9_10_R	TGTCTTCAGTATTTCCTTCCCCTTC
STAT3_ex12_13_14_F	TCCCTCAAGGAAAACACCCCAGTTG	STAT3_ex12_13_14_R	GTAGGCTTTTTGAAGAAACACAGAG
STAT3_ex15_F	CAAACAGTAATCATTCCACCTTCTC	STAT3_ex15_R	GCAGAGATGGAGTTTTGCTGTGCTGC
STAT3_ex18_F	ATCCTCAGGCCCGTCTACCTTCA	STAT3_ex18_R	AGCTGATTATTTTGTGGCCCATTGT
STAT3_ex19_20_F	ATCTCCACCCACCAGGGGGC	STAT3_ex19_20_R	AGGGAAGGGCTGGGATGGCA
STAT3_ex21_F	TCCCATCGGTCACCCCAACA	STAT3_ex21_R	GCCAGGCCACTGAACAGGGTG
STAT3_ex22_F	TCCCATTCCCAGGGATAACTGAGGA	STAT3_ex22_R	TCCTGCCGAGGCAGATGGCT
STAT3_ex23_F	AGAGCATCACACAAAGGGGACCA	STAT3_ex23_R	TCCTGCCGAGGCAGATGGCT
STAT3_ex24_F	GCAGGTAGGCGCCTCAGTCG	STAT3_ex24_R	TGCAGAGGGTGGACAACTGAACT

Supplemental Table S5: Subtypes of myelodysplastic syndrome

cohort	UPN	Subtypes
Nagano	56	RA
Nagano	61	RAEB-2
Nagano	79	RAEB-1
Nagano	80	RCMD
Nagano	82	MDS-U
Nagano	86	RAEB-2
Nagano	88	RAEB
Nagano	89	RA
Nagano	122	RT
Nagano	127	RA
Nagano	133	RCMD
Nagano	241	RAEB-1
Nagano	248	RCMD
Nagano	249	RCMD
Nagano	296	RA
Nagano	320	RA
Nagano	447	RCMD
Kanazawa	158	RCMD
Kanazawa	159	RA
Kanazawa	165	RAEB-1
Kanazawa	179	RA

RA: refractory anemia

RAEB: RA with excess blasts

RCMD: refractory cytopenia with multilineage dysplasia

MDS-U: MDS, unclassifiable

RT: refractory thrombocytopenia

Supplemental Table S6: Clinical characteristics of PRCA patients

cohort	UPN	gender	cause of PRCA	age, y	*STAT3* mutation	WBC, $\times 10^9$/L	lymphocyte, %	neutrophil, %	lymphocyte, $\times 10^9$/L	neutrophil, $\times 10^9$/L	Hemoglobin, g/L	Platelet, $\times 10^9$/L	Reticulocyte, %	Reticulocyte, $\times 10^9$/L	CD4, $\times 10^9$/L	CD8 $\times 10^9$/L	CD4/CD8	erythroid cells in BM, %	karyotype	TCR Vβ repertoire	TCRγ rearrangement	therapy	outcome	follow up duration, mo
Nagano and Kanazawa	45	F	idiopathic	59	negative	4.65	23	71	1.07	3.30	42	382	1.4	16.7	0.57	0.34	1.68	2.0	normal	N.T	negative	CsA	alive	20
	47	F	idiopathic	39	positive	4.31	50	38	2.16	1.64	71	405	1.5	27.8	0.95	0.80	1.19	1.6	normal	*Vβ 5.1 (27.8%) Vβ 2 (21.6%)	positive	mPSL,PSL, CY	dead	251
	53	F	drug	65	negative	2.88	42	52	1.22	1.50	57	243	0.5	9.2	0.80	0.22	3.64	0.8	normal	N.T	negative	watch	alive	1
	70	M	idiopathic	82	negative	5.10	30	58	1.54	2.97	48	168	0.6	7.9	1.11	0.25	4.44	4.4	normal	N.T	negative	PSL	alive	1
	90	M	parvovirus B19	49	negative	10.93	14	80	1.53	8.74	69	319	0.5	10.9	0.77	0.34	2.26	0.0	normal	N.T	positive	CsA	alive	134
	150	M	AIH	63	negative	3.96	29	55	1.15	2.18	67	208	0.4	7.0	*0.67	*0.67	*1.00	0.0	normal	*poly	negative	CsA	alive	30
	166	F	idiopathic	62	positive	3.90	N.T	N.T	N.T	N.T	42	179	0.0	0.0	N.T	N.T	N.T	1.2	normal	N.T	*negative	CsA	alive	300
	171	F	idiopathic	77	negative	3.84	11	75	0.42	2.88	65	303	0.8	16.0	0.16	0.22	0.73	4.6	normal	N.T	negative	CsA	alive	25
	182	F	CD, SjS	58	positive	2.25	22	67	0.50	1.50	66	113	0.7	2.0	0.23	0.22	1.05	3.6	normal	N.T	*negative	CsA	dead	204
	237	M	AIP	67	negative	5.68	27	59	1.53	3.35	72	362	0.2	4.1	0.56	0.66	0.85	0.8	normal	N.T	negative	PSL	dead	38
	243	F	idiopathic	70	positive	2.31	44	46	1.01	1.06	70	295	0.3	6.2	0.54	0.43	1.26	0.2	normal	*Vβ 1 (69.2%)	negative	CsA	alive	59
	245	M	thymoma	43	positive	2.96	42	47	1.24	1.39	66	249	0.9	15.9	*0.19	*0.39	*0.49	1.2	normal	*Vβ 21.3 (21.2%)	*positive	CsA, mPSL, PSL	dead	85
	278	F	thymic cancer	78	negative	5.68	35	58	1.99	3.29	52	213	0.2	3.4	1.15	0.40	2.88	0.0	normal	poly	negative	watch	alive	2
	281	F	idiopathic	81	negative	3.87	34	53	1.32	2.05	73	205	0.1	2.3	0.59	0.48	1.23	0.2	normal	poly	negative	CsA	alive	13
	397	F	thymoma	48	negative	4.76	31	66	1.48	3.14	40	417	0.5	7.2	0.68	0.72	0.94	0.4	normal	poly	negative	thymectomy	alive	6
	398	F	idiopathic	72	negative	3.83	8	85	0.31	3.26	53	296	0.3	4.3	*0.21	*0.22	*0.95	5.2	normal	*poly	*negative	CsA	alive	114
	399	F	idiopathic	67	positive	3.50	65	26	2.27	0.90	77	364	N.T	23.0	N.T	N.T	N.T	N.T	normal	N.T	*negative	CsA, PSL	alive	50
	411	F	idiopathic	81	negative	4.70	35	57	1.62	2.66	58	315	0.2	3.9	0.68	0.34	2.00	0.4	normal	poly	negative	watch	alive	1
	3	F	T-LGLL	20	positive	6.70	79	10	5.29	0.67	92	306	0.9	23.9	0.69	4.45	0.16	4.2	normal	*Vβ 23 (92%)	positive	CsA	alive	232
	7	F	T-LGLL	73	negative	5.53	70	13	3.87	0.72	91	426	0.3	9.0	0.61	3.33	0.18	0.4	normal	N.T	positive	CsA	alive	95
	8	F	T-LGLL	59	positive	5.87	66	20	3.87	1.17	99	276	0.8	22.0	0.82	2.31	0.35	NA	normal	Vβ 14 (76%) Vβ 22 (51%)	positive	watch	alive	141
	13	M	T-LGLL	58	positive	9.72	53	44	5.15	4.28	48	497	0.4	5.0	0.90	4.31	0.21	0.6	normal	poly	positive	PSL, CY, CsA	alive	133
	37	F	T-LGLL	45	positive	19.13	30	69	5.74	13.20	100	318	0.6	18.1	3.15	2.30	1.37	1.6	normal	N.T	positive	mPSL, ATG, CsA, CY	alive	261
	101	M	T-LGLL	78	negative	7.17	62	31	4.45	2.22	92	262	0.3	7.5	0.46	1.17	0.39	4.4	normal	N.T	positive	CsA	alive	44
	170	M	T-LGLL	72	positive	3.50	62	24	2.17	0.84	90	286	0.6	16.8	0.43	1.54	0.28	1.2	normal	N.T	positive	CsA, CY	alive	24
	192	F	T-LGLL	74	positive	3.28	29	60	0.95	1.97	75	254	0.2	4.8	0.07	0.87	0.08	0.4	normal	Vβ 16 (89%)	positive	CsA	alive	11
	194	F	T-LGLL	73	positive	8.47	64	23	5.42	1.95	86	584	0.5	13.0	0.49	4.93	0.10	3.4	normal	Vβ 3 (39.9%)	positive	CsA, CY	alive	9
	291	F	T-LGLL	71	positive	8.75	45	52	3.94	4.55	79	223	0.2	5.1	0.51	3.33	0.15	0.2	normal	Vβ 1 (93%)	positive	CsA	alive	13
	293	F	T-LGLL	65	positive	11.50	65	31	7.48	3.57	71	519	0.8	16.0	0.26	5.51	0.05	4.3	normal	*Vβ 7.1 (48%) Vβ 3 (43%)	positive	CsA	alive	16
Akita	452		thymoma		positive																negative			
	453		idiopathic		positive																negative			
	454		idiopathic		negative																negative			
	456		idiopathic		negative																negative			
	457		thymoma		negative																negative			
	458		thymoma		negative																positive			
	459		idiopathic		negative																positive			
	460		thymoma		negative																negative			
	461		SLE		negative																positive			
	462		idiopathic		negative																negative			
	463		SLE		negative																positive			
	451		T-LGLL		negative																negative			
	455		T-LGLL		positive																positive			

*Data obtained during therapy

AIH: autoimmune hepatitis, CD: Crohn's disease, SjS: Sjögren's syndrome, AIP: autoimmune pancreatitis, SLE: systemic lupus erythematosus. Data on points other than the diagnosis were not available from the Akita cohort.

Supplemental Table S7: CADD scores for *STAT3* mutations found in this study

AA change	base substitution	CADD score
p.R152W	c.C454T	35.0
p.D170G	c.A509G	29.7
p.F174V	c.T520G	28.9
p.S614R	c.A1840C	26.0
p.S614R	c.C1842A	25.8
p.S614R	c.C1842G	25.5
p.G618R	c.G1852C	34.0
p.Y640F	c.A1919T	24.2
p.D661H	c.G1981C	26.2
p.D661Y	c.G1981T	34.0
p.D661V	c.A1982T	25.1
p.D698H	c.G2092C	31.0

Combined Annotation Dependent Depletion (CADD) scores were calculated according to reference 33.