Lawitz EJ, McCone J, Shiffman ML, Galler GW, Lee WM, Reindollar R, King JW, Kwo PY, Ghalib RH, Freilich B, Nyberg LM, Zeuzem S, Poynard T, Vock DM, Pieper KS, Patel K, Tillmann HL, Noviello S, Koury K, Pedicone LD, Brass CA, Albrecht JK, Goldstein DB, McHutchison JG. 2010. Interleukin-28B polymorphism improves viral kinetics and is the strongest pretreatment predictor of sustained virologic response in genotype 1 hepatitis C virus. Gastroenterology 139: 120–129.

Toyoda H, Kumada T, Kiriyama S, Tanikawa M, Hisanaga Y, Kanamori A, Tada T, Arakawa T, Fujimori M, Niinomi T, Ando N, Yasuda S, Sakai K, Kimura J. 2011. High ability to predict the treatment outcome of peginterferon and ribavirin combination therapy based on the reduction in HCV RNA levels at 4 weeks after starting therapy and amino acid substitutions in hepatitis C virus in patients infected with HCV genotype 1b J Gastroenterol 46:501–509.

Yu JW, Wang GQ, Sun LJ, Li XG, Li SC. 2007. Predictive value of rapid virological response and early virological response on sustained virological response in HCV patients treated with pegylated interferon α-2a and ribavirin. J Gastroenterol Hepatol 22:832–836.

Zeuzem S, Herrmann E, Lee JH, Fricke J, Neumann AU, Modi M, Colucci G, Roth WK. 2001. Viral kinetics in patients with chronic hepatitis C treated with standard or peginterferon alpha2a. Gastroenterology 120:1438–1447.

nature genetics

Meta-analysis identifies nine new loci associated with rheumatoid arthritis in the Japanese population

Yukinori Okada[1–3,40], Chikashi Terao[4,5,40], Katsunori Ikari[6,40], Yuta Kochi[1,2,40], Koichiro Ohmura[5], Akari Suzuki[1], Takahisa Kawaguchi[4], Eli A Stahl[7,8], Fina A S Kurreeman[7–9], Nao Nishida[10], Hiroko Ohmiya[3], Keiko Myouzen[1], Meiko Takahashi[4], Tetsuji Sawada[11], Yuichi Nishioka[12], Masao Yukioka[13], Tsukasa Matsubara[14], Shigeyuki Wakitani[15], Ryota Teshima[16], Shigeto Tohma[17], Kiyoshi Takasugi[18], Kota Shimada[17], Akira Murasawa[19], Shigeru Honjo[20], Keitaro Matsuo[21], Hideo Tanaka[21], Kazuo Tajima[22], Taku Suzuki[6,23], Takuji Iwamoto[6,23], Yoshiya Kawamura[24], Hisashi Tanii[25], Yuji Okazaki[26], Tsukasa Sasaki[27], Peter K Gregersen[28], Leonid Padyukov[29], Jane Worthington[30], Katherine A Siminovitch[31], Mark Lathrop[32,33], Atsuo Taniguchi[6], Atsushi Takahashi[3], Katsushi Tokunaga[10], Michiaki Kubo[34], Yusuke Nakamura[35], Naoyuki Kamatani[36], Tsuneyo Mimori[5], Robert M Plenge[7,8], Hisashi Yamanaka[6], Shigeki Momohara[6,41], Ryo Yamada[37,41], Fumihiko Matsuda[4,38,39,41] & Kazuhiko Yamamoto[1,2,41]

Rheumatoid arthritis is a common autoimmune disease characterized by chronic inflammation. We report a meta-analysis of genome-wide association studies (GWAS) in a Japanese population including 4,074 individuals with rheumatoid arthritis (cases) and 16,891 controls, followed by a replication in 5,277 rheumatoid arthritis cases and 21,684 controls. Our study identified nine loci newly associated with rheumatoid arthritis at a threshold of $P < 5.0 \times 10^{-8}$, including *B3GNT2*, *ANXA3*, *CSF2*, *CD83*, *NFKBIE*, *ARID5B*, *PDE2A-ARAP1*, *PLD4* and *PTPN2*. *ANXA3* was also associated with susceptibility to systemic lupus erythematosus ($P = 0.0040$), and *B3GNT2* and *ARID5B* were associated with Graves' disease ($P = 3.5 \times 10^{-4}$ and 2.9×10^{-4}, respectively). We conducted a multi-ancestry comparative analysis with a previous meta-analysis in individuals of European descent (5,539 rheumatoid arthritis cases and 20,169 controls). This provided evidence of shared genetic risks of rheumatoid arthritis between the populations.

Rheumatoid arthritis is a complex autoimmune disease characterized by inflammation and the destruction of synovial joints and affects up to 1% of the population worldwide. To date, more than 35 rheumatoid arthritis susceptibility loci, including *HLA-DRB1*, *PTPN22*, *PADI4*, *STAT4*, *TNFAIP3* and *CCR6*, among others, have been identified by GWAS in multiple populations[1–12] and by several meta-analyses of the original GWAS[13–16]. In particular, each meta-analysis of these GWAS uncovered a number of loci that were not identified in the single GWAS, leading to recognition of the enormous power of the meta-analysis approach for detecting causal genes in disease. However, these previous meta-analyses have been performed solely in European populations[13–16] and not in Asian ones. As multi-ancestry studies on validated rheumatoid arthritis susceptibility loci showed the existence of both population-specific and shared genetic components of rheumatoid arthritis[10,17], additional studies in Asian populations might provide useful insight into the underlying genetic architecture of rheumatoid arthritis, which would otherwise be difficult to capture using the studies in a single population. Here, we report a meta-analysis of GWAS and a replication study for rheumatoid arthritis in a Japanese population that was conducted by the Genetics and Allied research in Rheumatic diseases NETworking (GARNET) consortium[10,12]. We subsequently performed a multi-ancestry comparative analysis that incorporated results from a previously conducted meta-analysis of individuals of European ancestry[15].

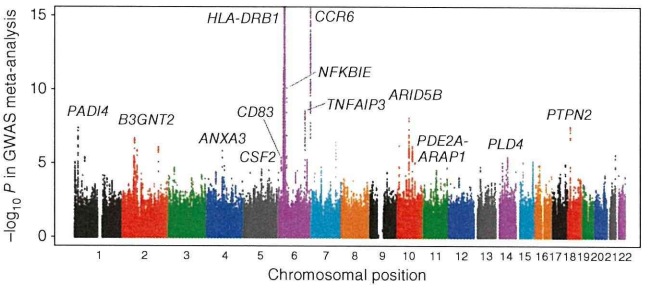

Figure 1 Manhattan plots of the GWAS meta-analysis for rheumatoid arthritis in the Japanese population. The genetic loci that satisfied the genome-wide significance threshold of $P < 5.0 \times 10^{-8}$ (gray line) in the meta-analysis or in the combined study of the meta-analysis and the replication study are presented. The *y* axis shows the $-\log_{10} P$ values of the SNPs in the meta-analysis. The SNPs for which the *P* values were smaller than 1.0×10^{-15} are indicated at the upper limit of the plot.

A full list of author affiliations appears at the end of the paper.

Received 24 October 2011; accepted 1 March 2012; published online 25 March 2012; doi:10.1038/ng.2231

Table 1 Results of the GWAS meta-analysis and the replication studies for rheumatoid arthritis

rsID[a]	Chr.	Position (bp)	Cytoband	Gene(s)	Associations in Japanese										Associations in Europeans[c]			
					GWAS meta-analysis					Replication study		Combined study			GWAS meta-analysis			
					Allele	Allele 1 freq.									Allele 1 Freq.			
					1/2	RA	Control	OR (95% CI)[b]	*P*	OR (95% CI)[b]	*P*	OR (95% CI)[b]	*P*		RA	Control	OR (95% CI)[b]	*P*
SNPs with significant associations ($P < 5.0 \times 10^{-8}$ in the combined study)																		
rs11900673	2	62306165	2p15	*B3GNT2*	T/C	0.31	0.28	1.15 (1.08–1.21)	3.5×10^{-6}	1.09 (1.04–1.14)	6.0×10^{-4}	1.11 (1.07–1.15)	1.1×10^{-8}		0.13	0.13	1.05 (0.98–1.13)	0.17
rs2867461	4	79732239	4q21	*ANXA3*	A/G	0.46	0.44	1.13 (1.08–1.19)	4.7×10^{-6}	1.12 (1.08–1.17)	1.2×10^{-7}	1.13 (1.09–1.17)	1.2×10^{-12}		0.37	0.37	0.98 (0.92–1.04)	0.52
rs657075	5	131458017	5q31	*CSF2*	A/G	0.38	0.36	1.12 (1.06–1.18)	3.2×10^{-5}	1.11 (1.06–1.16)	3.8×10^{-6}	1.12 (1.08–1.15)	2.8×10^{-10}		0.10	0.10	1.04 (0.95–1.13)	0.37
rs12529514	6	14204637	6p23	*CD83*	C/T	0.16	0.14	1.19 (1.10–1.27)	6.8×10^{-6}	1.11 (1.05–1.18)	6.0×10^{-4}	1.14 (1.09–1.19)	2.0×10^{-8}		0.055	0.053	1.11 (0.99–1.24)	0.074
rs2233434	6	44340898	6p21.1	*NFKBIE*	G/A	0.24	0.21	1.23 (1.16–1.31)	9.2×10^{-11}	1.17 (1.11–1.23)	2.2×10^{-9}	1.19 (1.15–1.24)	5.8×10^{-19}		0.059	0.040	1.57 (1.11–2.21)	0.0099
rs10821944	10	63455095	10q21	*ARID5B*	G/T	0.39	0.36	1.17 (1.11–1.23)	1.0×10^{-8}	1.15 (1.10–1.20)	3.0×10^{-10}	1.16 (1.12–1.20)	5.5×10^{-18}		0.29	0.26	1.11 (1.05–1.17)	1.9×10^{-4}
rs3781913	11	72051144	11q13	*PDE2A-ARAP1*	T/G	0.71	0.69	1.11 (1.05–1.17)	3.2×10^{-4}	1.13 (1.08–1.18)	6.7×10^{-7}	1.12 (1.08–1.16)	5.8×10^{-10}		0.45	0.43	1.04 (0.99–1.09)	0.13
rs2841277	14	104462050	14q32	*PLD4*	T/C	0.72	0.69	1.11 (1.05–1.18)	2.8×10^{-4}	1.18 (1.13–1.24)	7.0×10^{-12}	1.15 (1.11–1.19)	1.9×10^{-14}		0.47	0.46	1.02 (0.96–1.09)	0.54
rs2847297	18	12787694	18p11	*PTPN2*	G/A	0.37	0.33	1.16 (1.11–1.23)	3.5×10^{-8}	1.06 (1.01–1.11)	0.013	1.10 (1.07–1.14)	2.2×10^{-8}		0.36	0.34	1.10 (1.05–1.15)	9.2×10^{-5}
SNPs with suggestive associations ($5.0 \times 10^{-8} \leq P < 5.0 \times 10^{-6}$ in the combined study)																		
rs4937362	11	127997949	11q24	*ETS1-FLI1*	T/C	0.71	0.68	1.13 (1.07–1.19)	2.0×10^{-5}	1.07 (1.02–1.12)	0.0061	1.09 (1.06–1.13)	7.5×10^{-7}		0.46	0.44	1.06 (1.01–1.11)	0.015
rs3783637	14	54417868	14q22	*GCH1*	C/T	0.76	0.74	1.13 (1.07–1.20)	6.5×10^{-5}	1.07 (1.02–1.13)	0.0062	1.10 (1.06–1.14)	2.0×10^{-6}		0.88	0.88	0.99 (0.88–1.11)	0.87
rs1957895	14	60978085	14q23	*PRKCH*	G/T	0.40	0.39	1.12 (1.06–1.18)	4.1×10^{-5}	1.07 (1.02–1.12)	0.0022	1.09 (1.05–1.13)	3.6×10^{-7}		0.093	0.089	1.01 (0.95–1.07)	0.73
rs6496667	15	88694672	15q26	*ZNF774*	A/C	0.38	0.35	1.13 (1.07–1.19)	4.7×10^{-5}	1.07 (1.02–1.11)	0.0050	1.09 (1.05–1.13)	1.4×10^{-6}		0.21	0.20	1.07 (1.01–1.13)	0.031
rs7404928	16	23796341	16p12	*PRKCB1*	T/C	0.65	0.62	1.13 (1.07–1.19)	1.5×10^{-5}	1.05 (1.01–1.10)	0.026	1.08 (1.05–1.12)	4.0×10^{-6}		0.75	0.75	1.01 (0.94–1.09)	0.79
rs2280381	16	84576134	16q24	*IRF8*	T/C	0.86	0.84	1.16 (1.08–1.25)	1.0×10^{-4}	1.09 (1.03–1.15)	0.0049	1.12 (1.07–1.17)	2.4×10^{-6}		0.62	0.60	1.05 (0.99–1.11)	0.081
SNPs in previously reported rheumatoid arthritis susceptibility loci ($P < 5.0 \times 10^{-8}$ in the GWAS)																		
rs766449	1	17547439	1p36	*PADI4*	T/C	0.44	0.40	1.17 (1.11–1.24)	4.6×10^{-8}	–	–	–	–		0.38	0.37	1.09 (1.03–1.05)	0.0022
rs2157337	6	32609122	6p21.3	*HLA-DRB1*	C/T	0.59	0.44	1.99 (1.88–2.11)	2.6×10^{-118}	–	–	–	–		0.69	0.46	2.50 (2.39–2.62)	$< 1.0 \times 10^{-300}$
rs6932056	6	138284130	6q23	*TNFAIP3*	C/T	0.092	0.073	1.35 (1.23–1.49)	3.2×10^{-9}	–	–	–	–		0.044	0.034	1.41 (1.24–1.60)	1.3×10^{-7}
rs1571878	6	167460832	6q27	*CCR6*	C/T	0.54	0.48	1.31 (1.24–1.39)	3.2×10^{-19}	–	–	–	–		0.47	0.43	1.13 (1.08–1.19)	5.9×10^{-7}

Chr., chromosome; Freq., frequency; RA, rheumatoid arthritis; OR, odds ratio; CI, confidence interval.
[a]SNPs with $P < 5.0 \times 10^{-6}$ in the combined study of the GWAS meta-analysis and the replication study or SNPs with $P < 5.0 \times 10^{-8}$ in the GWAS meta-analysis are annotated according to forward strand and NCBI Build 36.3. Full results of the replication study are provided in **Supplementary Table 3**. [b]Odds ratio of allele 1. [c]Associations in the previous meta-analysis in European populations[15].

The meta-analysis included 4,074 rheumatoid arthritis cases (with 81.4% and 80.4% of the subjects being positive for antibody to cyclic citrullinated peptide (anti-CCP) and rheumatoid factor, respectively) and 16,891 controls from three GWAS of Japanese subjects (from the BioBank Japan Project[10,18], Kyoto University[12] and the Institute of Rheumatology Rheumatoid Arthritis (IORRA)[19]; **Supplementary Table 1**). After the application of stringent quality control criteria, including principal-component analysis (PCA; **Supplementary Fig. 1**) for each GWAS, the meta-analysis was conducted by evaluating ~2.0 million autosomal SNPs with minor allele frequencies (MAFs) ≥0.01, which were obtained through whole-genome imputation of genotypes on the basis of the HapMap Phase 2 East Asian panels (Japanese in Tokyo (JPT) and Han Chinese in Beijing (CHB)). The inflation factor of the test statistics in the meta-analysis λ_{GC} was as low as 1.036, suggesting no substantial effects of population structure (**Supplementary Table 2**). The quantile-quantile plot of *P* values showed a marked discrepancy in the values in its tail from those anticipated under the null hypothesis that there is no association—even after removal of the SNPs located in the human leukocyte antigen (HLA) region, the major rheumatoid arthritis susceptibility locus—thereby showing the presence of significant associations in the meta-analysis (**Supplementary Fig. 2**).

We identified seven loci in the current meta-analysis that satisfied the genome-wide significance threshold of $P < 5.0 \times 10^{-8}$. These included previously known rheumatoid arthritis susceptibility loci, such as *PADI4* at 1p36, *HLA-DRB1* at 6p21.3, *TNFAIP3* at 6q23 and *CCR6* at 6q27 (refs. 1,3,6,10,15) (the smallest $P = 2.6 \times 10^{-118}$ was found at the *HLA-DRB1* locus; **Fig. 1** and **Table 1**). To our knowledge, the other three loci identified, *NFKBIE* at 6p21.1, *ARID5B* at 10q21 and *PTPN2* at 18p11, are newly associated ($P = 9.2 \times 10^{-11}$, 1.0×10^{-8} and 3.5×10^{-8}, respectively).

To validate the associations identified in the meta-analysis, we conducted a replication study of two independent Japanese rheumatoid arthritis case-control cohorts (cohort 1: 3,830 rheumatoid arthritis cases and 17,920 controls, cohort 2: 1,447 rheumatoid arthritis cases and 3,764 controls; **Supplementary Table 1**). To increase the number of subjects and enhance statistical power, genotype data obtained from other GWAS projects conducted for non-autoimmune diseases in Japanese using Illumina platforms were used for the replication control panels. For each of the 46 loci that exhibited $P < 5.0 \times 10^{-4}$ in

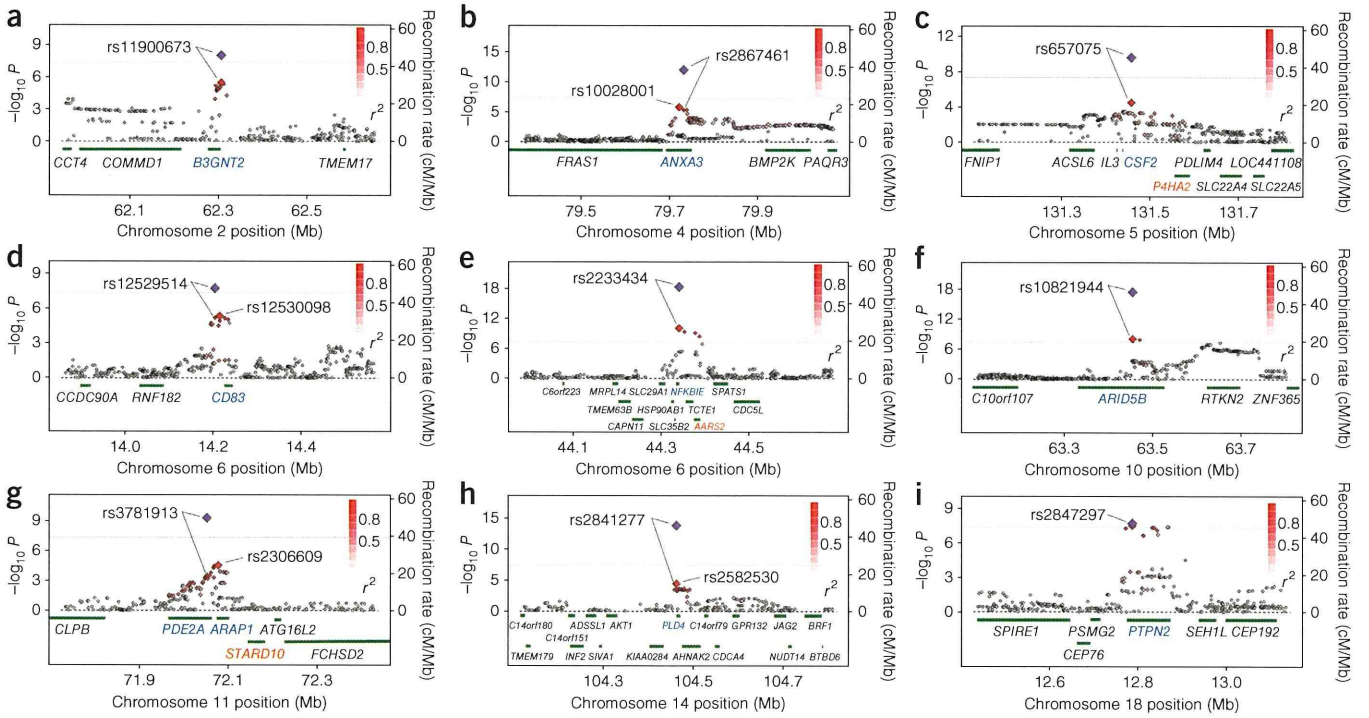

Figure 2 Regional plots of the loci newly associated with rheumatoid arthritis at the genome-wide significance threshold of $P < 5.0 \times 10^{-8}$ in the combined study of the meta-analysis and the replication study. (**a**–**i**) Regional plots are shown at *B3GNT2* (**a**), *ANXA3* (**b**), *CSF2* (**c**), *CD83* (**d**), *NFKBIE* (**e**), *ARID5B* (**f**), *PDE2A-ARAP1* (**g**), *PLD4* (**h**) and *PTPN2* (**i**). Diamonds represent the $-\log_{10} P$ values of the SNPs, and the red diamonds represent the $-\log_{10} P$ values of the SNPs in the meta-analysis. Red color for the smaller circles represents the r^2 value with the most significantly associated SNP (larger red circle). The purple circle represents the *P* value in the combined study. The blue line shows the recombination rates given by the HapMap Phase 2 east Asian populations (release 22). RefSeq genes at the loci are indicated below. Genes nearest to the marker SNPs at the loci are colored blue (**Supplementary Note**), and genes implicated in eQTL analysis are colored red (**Supplementary Table 4**). At 11q13, two genes (*PDE2A* and *ARAP1*) that are nearest to the SNP selected for the replication study and the most significant SNP in the meta-analysis are highlighted. The plots were drawn using SNP Annotation and Proxy Search (SNAP) version 2.2.

the meta-analysis and had not been reported as rheumatoid arthritis susceptibility loci[1–16], we selected a marker SNP for the replication study (Online Methods and **Supplementary Table 3**).

In the combined analyses of the meta-analysis and the replication study, including a total of 9,351 rheumatoid arthritis cases and 38,575 controls, we identified six newly associated loci, in addition to the *NFKBIE*, *ARID5B* and *PTPN2* loci, that satisfied the significance threshold of $P < 5.0 \times 10^{-8}$, including *B3GNT2* at 2p15, *ANXA3* at 4q21, *CSF2* at 5q31, *CD83* at 6p23, *PDE2A-ARAP1* at 11q13 and *PLD4* at 14q32 (**Figs. 1** and **2** and **Table 1**). Of these loci, *NFKBIE* had the smallest *P* value (5.8×10^{-19}). Although association with rheumatoid arthritis has been described for the *CSF2* and *PTPN2* loci[11,15,16,20,21], ours is the first report to our knowledge validating these associations with a threshold of $P < 5.0 \times 10^{-8}$. Suggestive associations were also observed in *ETS1-FLI1* at 11q24, *GCH1* at 14q22, *PRKCH* at 14q23, *ZNF774* at 15q26, *PRKCB1* at 16p12 and *IRF8* at 16q24 ($5.0 \times 10^{-8} \leq P < 5.0 \times 10^{-6}$). A summary of the genes in the newly associated loci and the results of *cis* expression quantitative trait locus (*cis* eQTL) analysis of the marker SNPs are provided (**Supplementary Table 4** and **Supplementary Note**).

Previous studies have reported associations of rheumatoid arthritis susceptibility loci with other autoimmune diseases[4,10,15,16]. Therefore, we assessed the association of these newly identified susceptibility loci with systemic lupus erythematosus (SLE) by examining the results of an SLE GWAS in the Japanese population (891 cases and 3,384 controls)[22] and in Graves' disease by genotyping 1,783 cases[10] (the controls from the SLE analysis were used for testing for Graves' disease). We observed significant associations of the *ANXA3* locus with SLE and of the *B3GNT2* and *ARID5B* loci with Graves' disease, which showed the same directional effects of the alleles as in rheumatoid arthritis ($P < 0.05/9 = 0.0056$, Bonferroni correction of the number of loci; **Supplementary Table 5**). It should be noted that relatively small sample sizes in the SLE and Graves' disease cohorts might yield limited statistical power, and further evaluations enrolling larger numbers of subjects would be desirable.

To highlight genetic backgrounds of rheumatoid arthritis that are common and divergent in different ancestry groups, we conducted a multi-ancestry comparative analysis of the present study in Japanese and a previous GWAS meta-analysis in Europeans that included 5,539 rheumatoid arthritis cases and 20,169 controls[15] (**Fig. 3a–c**). First, we compared associations in the reported[1–16] or newly identified rheumatoid arthritis susceptibility loci (**Fig. 3a** and **Supplementary Table 6**). Of the 46 rheumatoid arthritis risk variants evaluated, 6 were monomorphic in Japanese, and all were polymorphic in Europeans. We observed significant associations at 22 loci in Japanese and at 36 loci in Europeans (false discovery rate (FDR) < 0.05, $P < 0.0030$), with 14 loci being shared between the populations. Of the newly associated rheumatoid arthritis susceptibility loci identified in our Japanese meta-analysis, significant associations were also observed in the European meta-analysis at the *ARID5B* and *PTPN2* loci ($P = 1.9 \times 10^{-4}$ and 9.2×10^{-5}, respectively; **Table 1**). Significant positive correlation of odds ratios was observed between the studies ($r = 0.822$, $P = 8.1 \times 10^{-11}$; **Fig. 3b**), suggesting that a substantial proportion of genetic factors are shared between

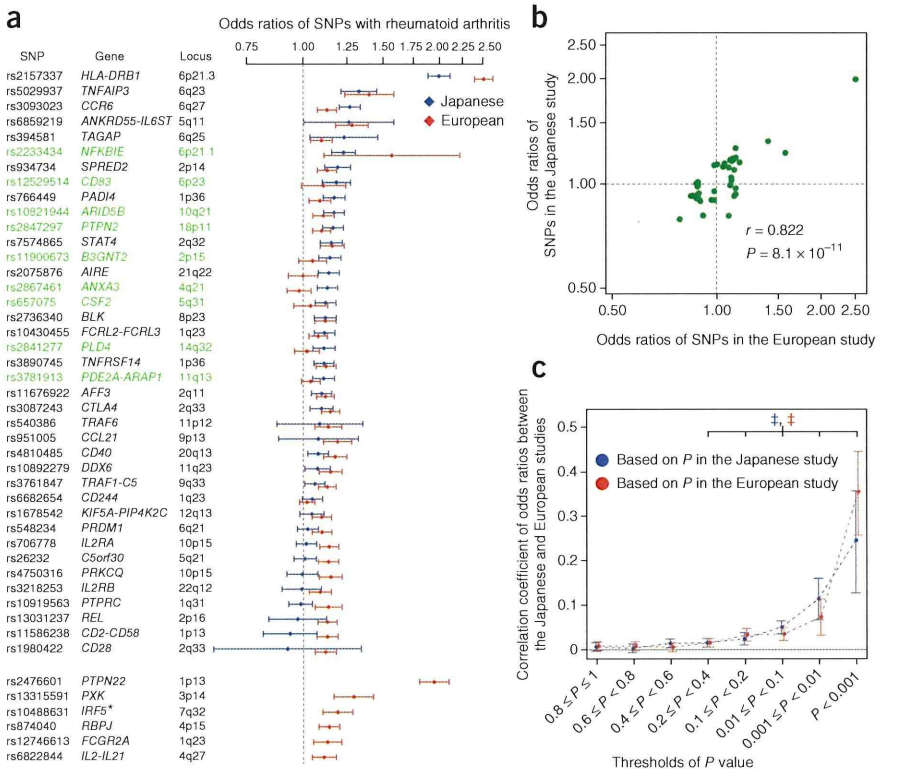

Figure 3 Overlap of the associations with rheumatoid arthritis between Japanese and European populations. (**a**) Forest plots of SNPs in the rheumatoid arthritis susceptibility loci (**Supplementary Table 6**). We selected the genetic loci that have been validated to be associated with rheumatoid arthritis susceptibility by showing associations in the reports of multiple cohorts or satisfying the genome-wide significant threshold ($P < 5.0 \times 10^{-8}$) in previous studies, including in the meta-analysis and replication phases[1–16]. For each of the loci, the most significant SNP among those reported in the previous or present study were selected[1–16]. SNPs in the newly identified rheumatoid arthritis susceptibility loci are colored green. Odds ratios and 95% confidence interval (CI) values are based on rheumatoid arthritis risk alleles, and the SNPs are ordered according to the odds ratios in the Japanese study. Several SNPs were monomorphic in the Japanese population. The odds ratios of these SNPs in the European study are presented below. The asterisk indicates that an association of another variant at the *IRF5* locus was reported in the Japanese population[24]. (**b**) Correlation of the odds ratios of the SNPs in the validated rheumatoid arthritis susceptibility loci between the two populations. SNPs that were polymorphic in both populations were used; odds ratios were based on the minor allele in the Japanese population. (**c**) Correlation of the odds ratios of the genome-wide SNPs, excluding the rheumatoid arthritis susceptibility loci. Correlations were evaluated for sets of SNPs stratified by the thresholds based on the meta-analysis *P* values in each population after pruning of the SNPs by LD ($r^2 < 0.3$). Correlation coefficient and 95% CI are indicated on the *y* axis. Significant correlation of the odds ratios was observed (‡, $P < 0.005$), even for the SNPs that showed moderate associations with rheumatoid arthritis (meta-analysis $P < 0.4$ in each population).

the two ancestry groups[17]. When the rheumatoid arthritis cases of the Japanese GWAS meta-analysis were stratified into anti-CCP–positive or rheumatoid factor–positive cases ($n = 3,209$) and controls ($n = 16,891$), similar results were observed (data not shown). Nevertheless, most of the SNPs assessed here are not necessarily causal variants, and further fine mapping of the loci is warranted to precisely evaluate the shared genetic predisposition between the populations.

Next, we compared regional associations within each of the loci and identified unique patterns in the *ARID5B* locus at 10q21 (**Supplementary Fig. 3**). In Japanese, three peaks of association were observed ($P = 1.0 \times 10^{-8}$ at rs10821944, $P = 5.7 \times 10^{-8}$ at rs10740069 and $P = 8.5 \times 10^{-6}$ at rs224311). These three variants were in weak linkage disequilibrium (LD) in Japanese ($r^2 < 0.10$), indicating independent associations with each of the other SNPs that satisfied a region-wide significance threshold of $P < 3.5 \times 10^{-5}$ (conditional $P = 4.3 \times 10^{-6}$, 1.7×10^{-5} and 1.8×10^{-5}, respectively) (**Supplementary Fig. 3**). In contrast, there was only one peak of association in Europeans ($P = 1.2 \times 10^{-6}$ at rs12764378; $r^2 = 0.59$ with rs10821944 in Europeans), and no additional association was observed in conditional analysis with rs12764378 (the smallest conditional $P = 2.2 \times 10^{-4}$), suggesting that the number of independent associations may be different at this locus in the two populations.

Finally, we conducted polygenic assessment for common variants showing modest associations to rheumatoid arthritis (those not meeting the genome-wide association threshold). This approach has been recognized to be a means to explain a substantial proportion of genetic risk[23]. For the SNPs that were shared between the two meta-analyses but not included in the validated rheumatoid arthritis susceptibility loci, we adopted LD pruning of the SNPs ($r^2 < 0.3$). We then evaluated the correlation of odds ratios of the SNPs between the two meta-analyses and observed a significant positive correlation ($r = 0.023$, $P < 1.0 \times 10^{-300}$). When the SNPs were stratified according to the *P* values in each meta-analysis, significant positive correlations of odds ratios were observed for the SNPs, even for those showing modest association ($P < 0.4$ in the meta-analysis of Japanese or Europeans; $r = 0.014$–0.36 for each *P* value range, $P < 0.005$ for each correlation test) (**Fig. 3c**). Correlations (r) of odds ratios observed herein suggest substantial overlap of the genetic risk of rheumatoid arthritis between the two populations, not only in the validated rheumatoid arthritis susceptibility loci but also at the loci showing nonsignificant associations. This suggests the usefulness of a meta-analysis approach involving multiple ancestry groups in identifying additional susceptibility loci.

In summary, we identified multiple new loci associated with rheumatoid arthritis through a large-scale meta-analysis of GWAS in Japanese. Multi-ancestry comparative analysis provided evidence of significant overlap in the genetic risks of rheumatoid arthritis between Japanese and Europeans. Thus, findings from the present study should contribute to the further understanding of the etiology of rheumatoid arthritis.

URLs. GARNET consortium, http://www.twmu.ac.jp/IOR/garnet/home.html; The BioBank Japan Project (in Japanese), http://biobankjp.org/; International HapMap Project, http://www.hapmap.org/; PLINK, http://pngu.mgh.harvard.edu/~purcell/plink/; EIGENSTRAT, http://genepath.med.harvard.edu/~reich/Software.htm; MACH and mach2dat, http://www.sph.umich.edu/csg/abecasis/MACH/index.

html; R statistical software, http://cran.r-project.org/; SNAP, http://www.broadinstitute.org/mpg/snap/index.php; NCBI GEO database, http://www.ncbi.nlm.nih.gov/geo/.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

The authors acknowledge the essential role of the GARNET consortium in developing the study. In this study, the following GARNET members are included: CGM of RIKEN, University of Tokyo, the BioBank Japan Project, Kyoto University and IORRA. We would like to thank all the doctors and staff who participated in sample collection for the RIKEN cohort and the BioBank Japan Project. We thank K. Kobayashi and M. Kitazato for their technical assistance. We thank T. Raj for calculation of composite of multiple signals (CMS). We thank M. Kokubo for DNA extraction, GWAS genotyping and secretarial assistance. We would also like to thank H. Yoshifuji, N. Yukawa, D. Kawabata, T. Nojima, T. Usui and T. Fujii for collecting DNA samples. We thank Y. Katagiri for her technical efforts. We also appreciate the contribution of E. Inoue and other members of the Institute of Rheumatology, Tokyo Women's Medical University, for their efforts on the IORRA cohort. This study was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) in Japan, the Ministry of Health, Labour and Welfare (MHLW) in Japan, the Japan Society for the Promotion of Science (JSPS), Core Research for Evolutional Science and Technology (CREST), Solution-Oriented Research for Science and Technology (SORST), INSERM and the Okawa Foundation for Information and Telecommunications.

AUTHOR CONTRIBUTIONS

Y. Okada, C.T., K.I., Y. Kochi and K.O. designed the study and drafted the manuscript. Y. Okada, C.T., K.I., T.K., H.O., N.N., M.T., M.L., K. Tokunaga and M.K. managed genotyping and manipulation of GWAS data. Y. Okada, Y. Kochi, C.T. and K.I. managed genotyping of replication cohorts. Y. Okada, T.K., H.O., E.A.S., A. Takahashi and R.Y. performed statistical analysis. Y. Kochi, A.S., K. Myouzen, T. Sawada, Y. Nishoka, M.Y., T. Matsubara, S.W., R.T. and S.T. collected samples and managed phenotype data for the rheumatoid arthritis cohorts from the BioBank Japan Project and CGM, RIKEN. C.T., K.O., T.K., M.T., K. Takasugi, K.S., A.M., S.H., K. Matsuo, H. Tanaka, K. Tajima and M.L. collected samples and managed phenotype data for the rheumatoid arthritis cohorts from Kyoto University. K.I., T. Suzuki, T.I., Y. Kawamura, H. Tanii, Y. Okazaki and T. Sakaki collected samples and managed phenotype data for the rheumatoid arthritis cohorts from IORRA. Y. Kochi managed the data for the SLE and Graves' disease cohorts. A.S., C.T. and K.I. analyzed the sera of subjects with rheumatoid arthritis. E.A.S., F.A.S.K., P.K.G., J.W., K.A.S., L.P. and R.M.P. managed the data for the rheumatoid arthritis cohorts in European populations. A. Taniguchi, A. Takahashi, K. Tokunaga, M.K., Y. Nakamura, N.K., T. Minori, R.M.P., H.Y., S.M., R.Y., F.M. and K.Y. supervised the overall study.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/naturegenetics/.
Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Suzuki, A. *et al.* Functional haplotypes of *PADI4*, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nat. Genet.* **34**, 395–402 (2003).
2. Kochi, Y. *et al.* A functional variant in *FCRL3*, encoding Fc receptor–like 3, is associated with rheumatoid arthritis and several autoimmunities. *Nat. Genet.* **37**, 478–485 (2005).
3. The Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* **447**, 661–678 (2007).
4. Remmers, E.F. *et al. STAT4* and the risk of rheumatoid arthritis and systemic lupus erythematosus. *N. Engl. J. Med.* **357**, 977–986 (2007).
5. Plenge, R.M. *et al. TRAF1–C5* as a risk locus for rheumatoid arthritis—a genomewide study. *N. Engl. J. Med.* **357**, 1199–1209 (2007).
6. Plenge, R.M. *et al.* Two independent alleles at 6q23 associated with risk of rheumatoid arthritis. *Nat. Genet.* **39**, 1477–1482 (2007).
7. Barton, A. *et al.* Rheumatoid arthritis susceptibility loci at chromosomes 10p15, 12q13 and 22q13. *Nat. Genet.* **40**, 1156–1159 (2008).
8. Suzuki, A. *et al.* Functional SNPs in *CD244* increase the risk of rheumatoid arthritis in a Japanese population. *Nat. Genet.* **40**, 1224–1229 (2008).
9. Gregersen, P.K. *et al. REL*, encoding a member of the NF-κB family of transcription factors, is a newly defined risk locus for rheumatoid arthritis. *Nat. Genet.* **41**, 820–823 (2009).
10. Kochi, Y. *et al.* A regulatory variant in *CCR6* is associated with rheumatoid arthritis susceptibility. *Nat. Genet.* **42**, 515–519 (2010).
11. Freudenberg, J. *et al.* Genome-wide association study of rheumatoid arthritis in Koreans: population-specific loci as well as overlap with European susceptibility loci. *Arthritis Rheum.* **63**, 884–893 (2011).
12. Terao, C. *et al.* The human *AIRE* gene at chromosome 21q22 is a genetic determinant for the predisposition to rheumatoid arthritis in Japanese population. *Hum. Mol. Genet.* **20**, 2680–2685 (2011).
13. Raychaudhuri, S. *et al.* Common variants at *CD40* and other loci confer risk of rheumatoid arthritis. *Nat. Genet.* **40**, 1216–1223 (2008).
14. Raychaudhuri, S. *et al.* Genetic variants at *CD28*, *PRDM1* and *CD2/CD58* are associated with rheumatoid arthritis risk. *Nat. Genet.* **41**, 1313–1318 (2009).
15. Stahl, E.A. *et al.* Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. *Nat. Genet.* **42**, 508–514 (2010).
16. Zhernakova, A. *et al.* Meta-analysis of genome-wide association studies in celiac disease and rheumatoid arthritis identifies fourteen non-HLA shared loci. *PLoS Genet.* **7**, e1002004 (2011).
17. Kurreeman, F. *et al.* Genetic basis of autoantibody positive and negative rheumatoid arthritis risk in a multi-ethnic cohort derived from electronic health records. *Am. J. Hum. Genet.* **88**, 57–69 (2011).
18. Nakamura, Y. The BioBank Japan Project. *Clin. Adv. Hematol. Oncol.* **5**, 696–697 (2007).
19. Yamanaka, H. *et al.* Influence of methotrexate dose on its efficacy and safety in rheumatoid arthritis patients: evidence based on the variety of prescribing approaches among practicing Japanese rheumatologists in a single institute-based large observational cohort (IORRA). *Mod. Rheumatol.* **17**, 98–105 (2007).
20. Yamada, R. *et al.* Association between a single-nucleotide polymorphism in the promoter of the human interleukin-3 gene and rheumatoid arthritis in Japanese patients, and maximum-likelihood estimation of combinatorial effect that two genetic loci have on susceptibility to the disease. *Am. J. Hum. Genet.* **68**, 674–685 (2001).
21. Tokuhiro, S. *et al.* An intronic SNP in a RUNX1 binding site of *SLC22A4*, encoding an organic cation transporter, is associated with rheumatoid arthritis. *Nat. Genet.* **35**, 341–348 (2003).
22. Okada, Y. *et al.* A genome-wide association study identified *AFF1* as a susceptibility locus for systemic lupus eyrthematosus in Japanese. *PLoS Genet.* **8**, e1002455 (2012).
23. Stranger, B.E., Stahl, E.A. & Raj, T. Progress and promise of genome-wide association studies for human complex trait genetics. *Genetics* **187**, 367–383 (2011).
24. Shimane, K. *et al.* A single nucleotide polymorphism in the *IRF5* promoter region is associated with susceptibility to rheumatoid arthritis in the Japanese patients. *Ann. Rheum. Dis.* **68**, 377–383 (2009).

[1]Laboratory for Autoimmune Diseases, Center for Genomic Medicine (CGM), RIKEN, Yokohama, Japan. [2]Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan. [3]Laboratory for Statistical Analysis, CGM, RIKEN, Yokohama, Japan. [4]Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan. [5]Department of Rheumatology and Clinical Immunology, Graduate School of Medicine, Kyoto University, Kyoto, Japan. [6]Institute of Rheumatology, Tokyo Women's Medical University, Tokyo, Japan. [7]Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA. [8]Broad Institute, Cambridge, Massachusetts, USA. [9]Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands. [10]Department of Human Genetics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan. [11]Department of Rheumatology, Tokyo Medical University Hospital, Tokyo, Japan. [12]Yamanashi Prefectural Central Hospital, Yamanashi, Japan. [13]Department of Orthopaedic Surgery, Yukioka Hospital, Osaka, Japan. [14]Matsubara Mayflower Hospital, Hyogo, Japan. [15]Osaka Minami National Hospital, Osaka, Japan. [16]Department of Orthopedic Surgery, Tottori University, Tottori, Japan. [17]Department of Rheumatology, National Hospital Organization, Sagamihara Hospital, Kanagawa, Japan. [18]Center for Rheumatic Diseases, Dohgo Spa Hospital, Ehime, Japan. [19]Department of Rheumatology, Niigata Rheumatic Center, Niigata, Japan. [20]Saiseikai Takaoka Hospital, Toyama, Japan. [21]Division of Epidemiology and Prevention, Aichi Cancer Center Research Institute, Aichi, Japan. [22]Aichi Cancer Center Hospital and Research Institute, Aichi, Japan. [23]Department of Orthopaedic Surgery, Keio University, Tokyo, Japan. [24]Yokohama Clinic, Warakukai Medical Corporation, Yokohama, Japan. [25]Department of Psychiatry, Mie University School of Medicine, Mie, Japan. [26]Metropolitan Matsuzawa Hospital, Tokyo, Japan. [27]Graduate School of Education, University of Tokyo, Tokyo, Japan. [28]The Feinstein Institute for Medical Research, North Shore–Long Island Jewish Health System, Manhasset, New York, USA. [29]Rheumatology Unit,

Department of Medicine in Solna, Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden. [30]Arthritis Research Campaign–Epidemiology Unit, The University of Manchester, Manchester Academic Health Science Centre, Manchester, UK. [31]Division of Medicine, University of Toronto, Mount Sinai Hospital and University Health Network, Toronto, Ontario, Canada. [32]Commisariat a l'Energie Atomique (CEA), Institut Genomique, Centre National de Genotypage, Evry, France. [33]Fondation Jean Dausset, Centre d'Etude du Polymorphisme Humain, Paris, France. [34]Laboratory for Genotyping Development, CGM, RIKEN, Yokohama, Japan. [35]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan. [36]Laboratory for International Alliance, CGM, RIKEN, Yokohama, Japan. [37]Unit of Statistical Genetics, Center for Genomic Medicine Graduate School of Medicine Kyoto University, Kyoto, Japan. [38]Core Research for Evolutional Science and Technology (CREST) Program, Japan Science and Technology Agency, Kawaguchi, Japan. [39]Institut National de la Santé et de la Recherche Médicale (INSERM), Unité U852, Kyoto University Graduate School of Medicine, Kyoto, Japan. [40]These authors contributed equally to this work. [41]These authors jointly directed this work. Correspondence should be addressed to Y.K. (ykochi@src.riken.jp) or K.O. (ohmurako@kuhp.kyoto-u.ac.jp).

ONLINE METHODS

Subjects. The Japanese participants in the meta-analysis (4,074 rheumatoid arthritis cases and 16,891 controls) and the replication study (5,277 rheumatoid arthritis cases and 21,684 controls) were obtained through the collaborations of the GARNET consortium (**Supplementary Table 1**)[10,12]. The meta-analysis was conducted on three independent GWAS (from the BioBank Japan Project[18] with 2,414 rheumatoid arthritis cases and 14,245 controls[10], Kyoto University with 1,237 rheumatoid arthritis cases and 2,087 controls[12] and IORRA[19] with 423 rheumatoid arthritis cases and 559 controls). The replication study consisted of two independent cohorts (cohort 1 included 3,830 rheumatoid arthritis cases and 17,920 controls, and cohort 2 included 1,447 rheumatoid arthritis cases and 3,764 controls). We employed a case-control cohort of SLE (891 cases and 3,384 controls)[22] and 1,783 cases with Graves' disease[10]. Details of 5,539 rheumatoid arthritis cases and 20,169 controls included in the meta-analysis in European populations were described elsewhere[15]. All participants provided written informed consent for participation in the study, as approved by the ethical committees of the institutional review boards. Detailed descriptions of the participating subjects are provided (**Supplementary Note**).

Genotyping and quality control in the GWAS. Genotyping platforms and quality control criteria for the GWAS, including cutoff values for sample call rates, SNP call rates, MAF and Hardy-Weinberg P values, are given (**Supplementary Table 2**). For the subjects enrolled in each of three GWAS, we excluded closely related subjects with first- or second-degree kinship, which was estimated using PLINK version 1.06 (see URLs). We also excluded the subjects determined to be ancestry outliers from East Asian populations using PCA performed by EIGENSTRAT version 2.0 (see URLs) along with HapMap Phase 2 panels (release 24; **Supplementary Fig. 1**). Genotype imputation was performed on the basis of the HapMap Phase 2 East Asian populations, using MACH version 1.0.16 (see URLs) in a two-step procedure as described elsewhere[25]. We excluded imputed SNPs with MAF < 0.01 or *Rsq* < 0.5 from each of the GWAS. Associations of the SNPs with rheumatoid arthritis were assessed by logistic regression models assuming additive effects of the allele dosages of the SNPs using mach2dat software (see URLs).

Meta-analysis. We included 1,948,139 autosomal SNPs that satisfied quality control criteria in all three GWAS (**Supplementary Table 2**). SNP information was based on a forward strand of the NCBI build 36.3 reference sequence. The meta-analysis was performed using an inverse variance method assuming a fixed-effects model from the study-specific effect sizes (logarithm of odds ratio) and the standard errors of the coded alleles of the SNPs determined with the Java source code implemented by the authors[25]. Genomic control corrections[26] were carried out on test statistics of the GWAS using the study-specific inflation factor (λ_{GC}) and was applied or reapplied to the results of our current meta-analysis (**Supplementary Fig. 2**).

Replication study. We selected a SNP for the replication study from each of the loci that exhibited $P < 5.0 \times 10^{-4}$ in the meta-analysis that had not previously been reported as rheumatoid arthritis susceptibility loci[1–16] (**Supplementary Table 3**). For control subjects, we used genotype data obtained from additional GWAS for non-autoimmune diseases or healthy controls, genotyped using Illumina HumanHap550 BeadChips or HumanHap610-Quad BeadChips, and the cases for rheumatoid arthritis and Graves' disease were genotyped with the TaqMan genotyping system (Applied Biosystems; **Supplementary Table 1**). Selection of the SNP was conducted according to the following criteria: if the SNP with the most significant association in the locus was genotyped in the replication control panel, then that SNP was selected; otherwise, a tag SNP in the replication control panel with the strongest LD was selected (mean $r^2 = 0.89$). For the three SNPs that yielded low call rates (<90%), we alternatively selected proxy SNPs with the second strongest LD. As a result, average genotyping call rates of the SNPs were 99.9% and 99.0% for the controls and cases, respectively. We then evaluated concordance rates between the assayed genotypes by applying these two different methods to samples from 376 subjects who were randomly selected. This procedure yielded high concordance rates of ≥99.9%. Associations of the SNPs were evaluated using logistic regression assuming an additive-effects model of genotypes in R statistical software version 2.11.0 (see URLs). The combined study of the meta-analysis and replication study was performed using an inverse variance method assuming a fixed-effects model[25].

***Cis* eQTL analysis.** For each marker SNP of the newly identified rheumatoid arthritis susceptibility locus, correlations between SNP genotypes and expression levels of genes located 300 kb upstream or downstream of the SNP measured in B-lymphoblastoid cell lines (GSE6536) were evaluated using data from the HapMap Phase 2 east Asian populations[27].

Multi-ancestry analysis of the meta-analyses in Japanese and Europeans. We evaluated the associations of the variants in the validated rheumatoid arthritis susceptibility loci by comparing the results from the current meta-analysis in Japanese with those from a previous meta-analysis in Europeans[15]. We assessed two variants in the *IRF5* locus, where different causal variants were identified in the two populations[24]. For the conditional analysis of the regional associations in the *ARID5B* locus (**Supplementary Fig. 3**), we repeated the meta-analysis at that locus by incorporating genotypes of the referenced SNP(s) as additional covariate(s). For comparison of the odds ratios of the SNPs, we first selected SNPs that were shared between the meta-analyses in Japanese and Europeans. Next, we removed the SNPs located more than 1 Mb away from each of the marker SNPs in the validated rheumatoid arthritis susceptibility loci, except for in the HLA region, where we removed the SNPs located between 24,000,000 bp to 36,000,000 bp on chromosome 6 because of the existence of long-range haplotypes with rheumatoid arthritis susceptibility in this region[28]. LD pruning of the SNPs was conducted for the SNP pairs that were in LD ($r^2 \geq 0.3$) in both HapMap Phase 2 East Asian and Utah residents of Northern and Western European ancestry (CEU) populations (release 24). Correlations of the odds ratios were evaluated using R statistical software version 2.11.0.

25. Okada, Y. *et al.* Identification of nine novel loci associated with white blood cell subtypes in a Japanese population. *PLoS Genet.* **7**, e1002067 (2011).
26. de Bakker, P.I. *et al.* Practical aspects of imputation-driven meta-analysis of genome-wide association studies. *Hum. Mol. Genet.* **17**, R122–R128 (2008).
27. Stranger, B.E. *et al.* Population genomics of human gene expression. *Nat. Genet.* **39**, 1217–1224 (2007).
28. Okada, Y. *et al.* Contribution of a haplotype in the HLA region to anti-cyclic citrullinated peptide antibody positivity in rheumatoid arthritis, independently of HLA-DRB1. *Arthritis Rheum.* **60**, 3582–3590 (2009).

研究成果の刊行に関する一覧表

研究分担者　琉球大学大学院医学研究科 准教授　齊藤峰輝

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Saito M, Matsuzaki T, Satou Y, Yasunaga J, Saito K, Arimura K, Matsuoka M, Ohara Y	In vivo expression of the HBZ gene of HTLV-1 correlates with proviral load, inflammatory markers and disease severity in HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP).	Retrovirology	6	19	2009
Saito M	Immunogenetics of human T-cell leukemia virus type 1 (HTLV-1)- associated myelopathy/ tropical spastic paraparesis (HAM/TSP).	Inflammation and Regeneration	29	310-316	2009
齊藤峰輝	HAM/TSP の病態に関する最近の考え方	血液・腫瘍科	58	600-606	2009
Taniura N, Saito M, Okuwa T, Saito K, Ohara Y	Different subcellular localization of Theiler's murine encephalomyelitis virus leader proteins of GDVII and DA strains in BHK-21 cells.	J Virol	83	6624-6630	2009
Saito M	Immunogenetics and the pathological mechanisms of human T-cell leukemia virus type 1- (HTLV-1) -associated myelopathy/ tropical spastic paraparesis (HAM/TSP).	Interdisciplinary Perspectives on Infectious Diseases		Article ID 478461	2010
齊藤峰輝	HAM/TSP 病態研究の最近の進歩	血液・腫瘍科	60	642-650	2010
Saito K, Saito M, Taniura N, Okuwa T, Ohara Y	Activation of the PI3K-Akt pathway by human T cell leukemia virus type 1 (HTLV-1) oncoprotein Tax increases Bcl3 expression, which is associated with enhanced growth of HTLV-1-infected T cells.	Virology	403	173-180	2010

Okuwa T, Taniura N, Saito M, Himeda T, Ohara Y	Opposite effects of two nonstructural proteins of Theiler's murine encephalomyelitis virus regulates apoptotic cell death in BHK-21 cells.	Microbiol Immunol	54	639-643.	2010
Tanaka R, Takahashi Y, Kodama A, Saito M, Ansari AA, Tanaka Y	Suppression of CCR5-tropic HIV type 1 infection by OX40 stimulation via enhanced production of β-chemokines.	AIDS Res Hum Retroviruses	26	1147-1154	2010
Kodama A, Tanaka R, Zhang LF, Adachi T, Saito M, Ansari AA, Tanaka Y	Impairment of in vitro generation of monocyte-derived human dendritic cells by inactivated human immunodeficiency virus-1: Involvement of type I interferon produced from plasmacytoid dendritc cells.	Hum Immunol	71	541-550	2010
Adachi T, Tanaka R, Kodama A, Saito M, Takahashi Y, Ansari AA, Tanaka Y.	Identification of an unique CXCR4 epitope whose ligation inhibits infection by both CXCR4 and CCR5 tropic human immunodeficiency type-I viruses.	Retrovirology	8	84	2012
Saito M, Bangham CR	Immunopathogenesis of Human T-cell leukemia virus type 1 (HTLV-1) -associated myelopathy/tropical spastic paraparesis (HAM/TSP): Recent perspectives.	Leukemia Research and Treatment		Article ID 259045	2012

Retrovirology

BioMed Central

Research

Open Access

In vivo expression of the HBZ gene of HTLV-I correlates with proviral load, inflammatory markers and disease severity in HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP)

Mineki Saito*[1,4], Toshio Matsuzaki[2], Yorifumi Satou[3], Jun-ichirou Yasunaga[3], Kousuke Saito[1], Kimiyoshi Arimura[2], Masao Matsuoka[3] and Yoshiro Ohara[1]

Address: [1]Department of Microbiology, Kanazawa Medical University, Ishikawa 920-0293, Japan, [2]Department of Neurology and Geriatrics, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8520, Japan, [3]Laboratory of Virus Immunology, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan and [4]Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Okinawa 903-0215, Japan

Email: Mineki Saito* - mineki@med.u-ryukyu.ac.jp; Toshio Matsuzaki - zaki7@mta.biglobe.ne.jp; Yorifumi Satou - ysatou@virus.kyoto-u.ac.jp; Jun-ichirou Yasunaga - jyasunag@virus1.virus.kyoto-u.ac.jp; Kousuke Saito - kousukes@kanazawa-med.ac.jp; Kimiyoshi Arimura - ari@m2.kufm.kagoshima-u.ac.jp; Masao Matsuoka - mmatsuok@virus.kyoto-u.ac.jp; Yoshiro Ohara - ohara@kanazawa-med.ac.jp

* Corresponding author

Published: 19 February 2009

Retrovirology 2009, **6**:19 doi:10.1186/1742-4690-6-19

Received: 27 November 2008
Accepted: 19 February 2009

This article is available from: http://www.retrovirology.com/content/6/1/19

Abstract

Background: Recently, human T-cell leukemia virus type I (HTLV-I) basic leucine zipper factor (HBZ), encoded from a minus strand mRNA was discovered and was suggested to play an important role in adult T cell leukemia (ATL) development. However, there have been no reports on the role of HBZ in patients with HTLV-I associated inflammatory diseases.

Results: We quantified the HBZ and tax mRNA expression levels in peripheral blood from 56 HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients, 10 ATL patients, 38 healthy asymptomatic carriers (HCs) and 20 normal uninfected controls, as well as human leukemic T-cell lines and HTLV-I-infected T-cell lines, and the data were correlated with clinical parameters. The spliced HBZ gene was transcribed in all HTLV-I-infected individuals examined, whereas tax mRNA was not transcribed in significant numbers of subjects in the same groups. Although the amount of HBZ mRNA expression was highest in ATL, medium in HAM/TSP, and lowest in HCs, with statistical significance, neither tax nor the HBZ mRNA expression per HTLV-I-infected cell differed significantly between each clinical group. The HTLV-I HBZ, but not tax mRNA load, positively correlated with disease severity and with neopterin concentration in the cerebrospinal fluid of HAM/TSP patients. Furthermore, HBZ mRNA expression per HTLV-I-infected cell was decreased after successful immunomodulatory treatment for HAM/TSP.

Conclusion: These findings suggest that *in vivo* expression of HBZ plays a role in HAM/TSP pathogenesis.

Background

Human T-cell lymphotropic virus type 1 (HTLV-1) is a replication-competent human retrovirus [1,2] which is associated with adult T-cell leukemia (ATL) [3,4] and with a slowly progressive neurological disorder HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [5,6]. In HTLV-1 infection, approximately 5% develop ATL [7] and another 2%-3% develop chronic inflammatory diseases involving the central nervous system (HAM/TSP), the eyes [8], the lungs [9], the joints [10], or the skeletal muscles [11]; most infected individuals, however, remain healthy in their lifetime (healthy asymptomatic carriers: HCs). Although the factors that cause these different manifestations of HTLV-1 infection are not fully understood, previous population association studies suggested that both viral and host genetic factors influence the outcome of infection [12].

Among several HTLV-1 genes, a transcriptional activator Tax encoded in the pX region is thought to play a central role in immortalization, oncogenesis and inflammation through its pleiotropic activity [13]. In HAM/TSP patients, it has been reported that several cytokines, chemokines and matrix metalloproteinases transactivated by Tax protein such as tumor necrosis factor-α (TNF-α) [14], monocyte chemoattractant protein-1 (MCP-1) [15] and matrix metalloproteinase (MMP)-9 [16] are overexpressed in the infiltrating mononuclear cells in the patients' spinal cords. In addition, a previous report from the United States suggested that the level of HTLV-1 tax mRNA expression in HTLV-1-infected cells (mRNA/DNA ratio) was significantly higher in HAM/TSP patients than HCs, and this finding correlated with the HTLV-1 proviral load, Tax-specific CD8+ T cell frequency and disease severity of the patients [17]. A report from Japan also indicated that HTLV-1 tax mRNA expression was higher in HAM/TSP than HCs, although the mRNA/DNA ratio was similar between both groups [18]. These results suggest an important role of Tax in the induction of HAM/TSP.

It has been reported that among fresh leukemic cells isolated from ATL patients, about 60% of cases do not express the tax transcript [19]. In tax transgenic mouse models, the mice develop a wide range of tumors such as neurofibrosarcomas, mesenchymal tumors, and mammary adenomas, or even skeletal abnormalities including osteolytic bone metastases [20-27]; however, no leukemias or lymphomas were identified except in three models, which used respectively the granzyme B promoter [28], Lck proximal promoter [29] and Lck distal promoter [30]. These findings suggest that Tax is required for malignant transformation but not essential for the maintenance of leukemic cells *in vivo*. Recently, a novel basic leucine zipper protein encoded by the complementary strand of the HTLV-1 genome, named HTLV-1 basic leucine zipper factor (HBZ), was characterized [31]. HBZ is expressed in all ATL cells [32], promotes proliferation of T-lymphocytes in its RNA form [32], suppresses Tax-mediated transactivation through the 5' LTR [31,33], promotes CD4+ T-lymphocyte proliferation in transgenic mice [32], and enhances infectivity and persistence in HTLV-1-inoculated rabbits [34].

In this study, we investigated whether HTLV-1 HBZ mRNA expression is associated with clinical and laboratory markers reported in HAM/TSP patients, including HTLV-1 proviral load, neopterin concentration in cerebrospinal fluid (CSF), and motor disability score. In addition, to confirm the previous observations [17,18], we have also investigated the tax mRNA expression in ATL patients, HAM/TSP patients, and HCs by using the same technology but in a larger number of subjects.

Methods

Patients and cells

Human leukemic T-cell lines (Jurkat, MOLT-4, and CEM) and HTLV-1-infected T-cell lines (C5/MJ, SLB1, HUT102, MT-1, MT-2, and MT-4) were cultured in RPMI 1640 medium supplemented with 10% FCS. The diagnosis of HAM/TSP was done in accordance with World Health Organization criteria [35]. The diagnosis of ATL was made on the basis of clinical features, hematological characteristics, serum antibodies against HTLV-1 antigens, and detection of the HTLV-1 viral genome inserted into leukemia cells by Southern blot hybridization. All the PBMC samples used in this study were collected prior to treatment by a Histopaque-1077 (Sigma) density gradient centrifugation, washed and stored in liquid nitrogen until use. This research was approved by the institutional review boards of the authors' institutions, and informed consent was obtained from all individuals.

Quantification of HTLV-I proviral load, tax and HBZ mRNA expression, anti-HTLV-I antibody titers and neopterin concentration in cerebrospinal fluid

RNA was extracted from PBMCs using RNeasy Mini Kit with on-column DNase digestion (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using TaqMan Gold RT-PCR Kit (Applied Biosystems, Tokyo, Japan). For cDNA synthesis from extracted mRNA, 2 μg total RNA, 10 μl 10×TaqMan RT buffer, 22 μl $MgCl_2$ (25 mM), 20 μl dNTPs mixture (at a final concentration of 500 μM each), 5 μl random hexamers (50 μM), 2 μl RNase inhibitor (20 U/μl), and 2.5 μl (50 U/μl) Moloney murine leukemia virus reverse transcriptase were added to a total volume of 100 μl. Samples were incubated at 25°C for 10 minutes and 48°C for 30 minutes, and reactions were stopped by heating to 95°C for 5 minutes. Genomic DNA was extracted from the frozen PBMCs by QIAamp blood kit

(QIAGEN, Tokyo, Japan). We, then, carried out a real time quantitative PCR using ABI Prism 7900 HT Fast Real-Time PCR System (Applied Biosystems) to examine the HTLV-1 proviral load [36] and tax mRNA expression [17] in PBMCs or HTLV-1 infected cell lines as reported previously. The amount of the HTLV-1 proviral load was calculated using β-actin as an internal control through the following formula: copy number of HTLV-1 tax per cell = [(copy number of tax)/(copy number of β-actin/2)]. The sequences of primers for HTLV-1 provirus were as follows: 5'-CAA ACC GTC AAG CAC AGC TT-3' and 5'-TCT CCA AAC ACG TAG ACT GGG T-3', and the probe was 5'-TTC CCA GGG TTT GGA CAG AGT CTT CT-3'. HBZ mRNA expression levels were also quantified by real time quantitative PCR using the same method for tax mRNA [17]. Namely, serially diluted cDNA from HTLV-1 infected MT-2 cells was used for generating standard curves for the value of HTLV-1 tax or HBZ mRNA and hypoxanthine ribosyl transferase (HPRT) mRNA, and the relative HTLV-1 tax or HBZ mRNA load was calculated by the following formula: HTLV-1 tax mRNA load = value of tax/value of HPRT. HTLV-1 HBZ mRNA load = value of HBZ/value of HPRT. We used aliquots of the same standard MT-2 cDNA preparation for all assays and the correlation values of standard curves were always more than 99%. The sequences of primers for tax mRNA detection were as follows: 5'-ATC CCG TGG AGA CTC CTC AA-3' and 5'-ATC CCG TGG AGA CTC CTC AA-3', and the probe was 5'-TCC AAC ACC ATG GCC CAC TTC CC-3'. The sequences of primers for HBZ mRNA detection were as follows: 5'-AGA ACG CGA CTC AAC CGG-3' and 5'-TGA CAC AGG CAA GCA TCG A-3', and the probe was 5'-TGG ATG GCG GCC TCA GGG CT-3'. As the probes for tax and HBZ mRNA surrounded the splice junction site of each mRNA, we detected HBZ splicing isoform, which is the most abundant HBZ transcript and contributed significantly to HBZ protein synthesis [37-39], but not unspliced form in this study. We used the HPRT primers and probe set (Applied Biosystems) for internal calibration. The tax and HBZ probes were labeled with fluorescent 6-carboxyfluorescein (FAM) (reporter) at the 5' end and fluorescent 6-carboxy tetramethyl rhodamine (TAMRA) (quencher) at the 3' end. All assays were performed in triplicate. The sensitivity of our real-time RT-PCR assay was determined using MT-2 cells diluted serially with PBMCs from a healthy uninfected donor. The HTLV-1 mRNA signal (both tax and HBZ) could be detected in a dose-dependent manner with a sensitivity limit as low as one MT-2 cell in 10^6 PBMCs. Neopterin levels were evaluated by HPLC with fluorometric detection methods as described previously [40]. Serum HTLV-1 antibody titers were determined by a particle agglutination method (Serodia-HTLV-1®, Fujirebio, Japan).

Clinical evaluation

Motor dysfunction seen in HAM/TSP patients was evaluated by clinical neurologists according to the Osame Motor Disability Score (OMDS) [41], which grades motor dysfunction from zero (normal walking and running) to 13 (complete bedridden) as follows: 1 = normal gait but runs slow; 2 = abnormal gait; 3 = abnormal gait and unable to run; 4 = need support while using stairs; 5 = need one hand support in walking; 6 = need two hands support in walking; 7 = need two hands support in walking but is limited to 10 m; 8 = need two hands support in walking but is limited to 5 m; 9 = unable to walk but able to crawl on hands and knees; 10 = crawls with hands; 11 = unable to crawl but can turn sideways in bed; 12 = unable to turn sideways but can move the toes. We have used OMDS throughout our previous studies [41-43] because this is a neurological measure of disability weighted toward ambulation and was specifically developed to evaluate motor dysfunction seen in HAM/TSP patients. It is therefore more suitable for evaluating HAM/TSP motor symptoms than the widely used EDSS [44]. The laboratory data were examined by an investigator who was not involved in the patients' clinical care, and the neurologists who made the clinical evaluation did not have access to the laboratory data.

Statistical analysis

The Mann-Whitney U test was used to compare data between two groups. Correlations between variables were examined by Spearman rank correlation analysis. Values of $p < 0.05$ were considered statistically significant.

Results

HTLV-I tax and HBZ mRNA load in HAM/TSP, ATL and HCs

A total of 56 HAM/TSP patients, 10 ATL patients and 38 HCs completed the evaluation. Twenty normal uninfected healthy controls (NCs) were used as negative controls. The HTLV-1 proviral load in this study represents the copy number of HTLV-1 tax per cell (for HTLV-1 infected cell lines) or PBMC (for HAM/TSP, ATL and HCs) (Table 1). Therefore, the HTLV-1 proviral load represents the population of infected cells in PBMCs when one cell harbors one provirus. However, since recent data by Kamihira et al. indicated that 43 out of 321 ATL specimens (17.8%) showed two or more bands by Southern blot analysis after *EcoRI* digestion [45], we reviewed the Southern blot data of our 10 ATL patients. As a result, two distinct bands of over 9 kb were observed in *EcoRI* digestion in samples from two ATL patients, indicating at least the biclonal integration of HTLV-1 proviral DNA. The incidence of multibands in our cases (two out of ten: 20%) was comparable with the data by Kamihira et al. (17.8%). The

Table 1: HTLV-1 mRNA load, proviral load and mRNA/DNA ratio in HTLV-1 – infected individuals and T-cell lines.

Cell line	HBZ mRNA[a]	tax mRNA[b]	Proviral load[c]	HBZ mRNA/DNA[d]	tax mRNA/DNA[e]
C5/MJ	13.3	0.062	8.1	1.64	0.0076
HUT102	1.2	26.35	19.3	0.063	1.37
MT1	25.2	0.011	7.1	3.56	0.0015
MT2	7.8	1.24	16.2	0.48	0.077
MT4	2.4	1.71	12.6	0.19	0.135
SLB1	25.8	87.4	115.5	0.22	0.756
HAM/TSP*	0.74 (0.023–33.50)	0 (0–0.041)	0.051 (0.0008–0.41)	19.10 (0.81–273.45)	0 (0–0.32)
HCs*	0.15 (0.0013–6.42)	0 (0–0.000078)	0.0089 (0.0001–0.10)	16.67 (0.21–7358.91)	0 (0–0.11)
ATL*	31.43 (5.93–225.64)	0.000018 (0–0.59)	1.14 (0.25–2.88)	24.04 (13.77–135.83)	0 (0–0.29)

*The results represent the median and range (n = 56 for HAM/TSP, n = 38 for HCs and n = 10 for ATL)
[a]HTLV-1 HBZ mRNA load = value of HBZ/value of HPRT
[b]HTLV-1 tax mRNA load = value of tax/value of HPRT
[c]Proviral load: HTLV-1 tax copy number per cell
[d]HBZ mRNA/DNA ratio = HTLV-1 HBZ mRNA load/Proviral load
[e]tax mRNA/DNA ratio = HTLV-1 tax mRNA load/Proviral load

number of HTLV-1 proviral load in MT-2 cells measured by our quantitative PCR method (16.2 copies/cell) was also comparable with the previous report (12.6 copies/cell) [46].

The HTLV-1 proviral load was significantly greater in HAM/TSP patients (median 0.051, range 0.0008–0.41) than HCs (median 0.0089, range 0.0001–0.10) (P = 0.000011, Mann Whitney U test, Table 1). The HTLV-1 HBZ mRNA level was highest in ATL, medium in HAM/TSP, and lowest in HCs with statistical significance (Table 1 and Figure 1A). It is noteworthy that we could detect HTLV-1 HBZ gene transcripts in all infected individuals tested. Interestingly, there were three cases with extremely high data of HBZ mRNA in HCs (Figure 1C). Since recent report by Shimizu et al. indicated that HTLV-1-specific T-cell responsiveness widely differed among HTLV-1 carriers [47], these extremely high data of HBZ mRNA might be explained by immunological diversity observed in HCs. In contrast, although the HTLV-1 tax mRNA levels in ATL patients was significantly higher than HCs (p = 0.014, Mann-Whitney U test), the HTLV-1 tax mRNA levels between HCs-HAM/TSP and HAM/TSP-ATL did not reach statistical difference (Figure 1B). We could not detect any HTLV-1 tax and HBZ mRNA expression in any of the 20 NCs and 3 uninfected human leukemic T-cell lines (Jurkat, MOLT-4, and CEM) tested (data not shown).

Comparison of HTLV-1 tax and HBZ mRNA load with HTLV-1 proviral load

To test whether higher HBZ mRNA levels reflect higher proviral load, we adjusted the tax or HBZ mRNA load (i.e. value of tax or HBZ/value of HPRT) by the HTLV-1 proviral load (i.e. HTLV-1 tax copy number per cell). As a result, neither tax nor the HBZ mRNA/DNA ratio differed significantly between each clinical group (i.e. HAM/TSP-HCs, HAM/TSP-ATL and HCs-ATL) (figure 1C, D). Interestingly, although both HTLV-1 proviral load and tax mRNA/DNA ratio were higher in HTLV-1-infected cell lines (C5/MJ, SLB1, HUT102, MT-1, MT-2, and MT-4) than PBMCs, HBZ mRNA/DNA ratio was even higher in PBMCs than HTLV-1-infected cell lines (Table 1). Consistent with the previous observations that HBZ suppresses Tax mediated transactivation through the 5' LTR [31,33,48], HBZ mRNA load tended to be higher in cell lines with lower tax mRNA load, and indeed HBZ mRNA/DNA ratio was inversely correlated with tax mRNA/DNA ratio in 6 HTLV-1-infected cell lines (Spearman's rank correlation coefficient r = -0.943, P = 0.035) (Table 1 and data not shown), although such correlation was not observed between HBZ and tax mRNA/DNA ratio in PBMCs from HAM/TSP patients, ATL patients, HCs and all groups combined (data not shown). As shown in Figure 2, the HTLV-1 HBZ mRNA load was significantly correlated with HTLV-1 proviral load in HAM/TSP patients (P = 0.0005, r = 0.470 by Spearman rank correlation analysis), HCs (P = 0.0013, r = 0.528) and all groups combined (P < 0.000001, r = 0.686), but not in ATL patients (P = 0.300, r = 0.345). The tax mRNA load was correlated with the HTLV-1 proviral load in HCs (P = 0.045, r = 0.444), ATL patients (P = 0.045, r = 0.673), and all groups combined (P < 0.01, r = 0.365), but not in HAM/TSP patients (P = 0.411, r = 0.210).

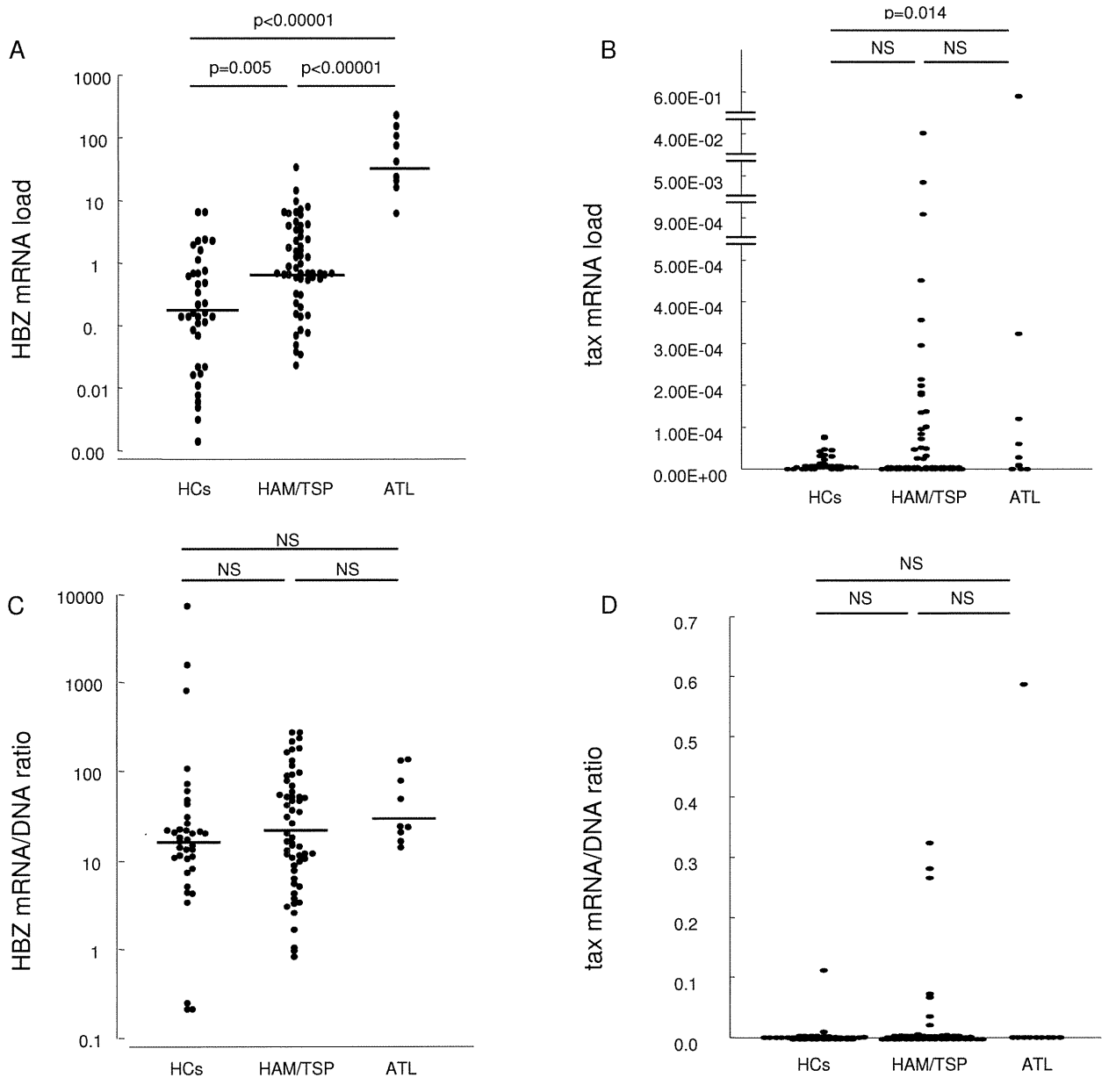

Figure 1

HTLV-I tax and HBZ mRNA load in patients with HAM/TSP, ATL and asymptomatic HTLV-I carriers. A. HTLV-I HBZ mRNA load was highest in ATL, medium in HAM/TSP, and lowest in HCs. B. The HTLV-I tax mRNA load between HCs and HAM/TSP, HAM/TSP and ATL did not reach statistical significance, although the HTLV-I tax mRNA load in ATL patients was significantly higher than HCs (p = 0.014, Mann Whitney U test). C and D. To normalize the HTLV-I tax or HBZ mRNA expression level per provirus, the mRNA/DNA ratio was calculated by dividing the HTLV-I tax or HBZ mRNA load by the HTLV-I proviral load. Neither the HBZ (C) nor the tax (D) mRNA/DNA ratio differed significantly between each clinical group (HAM/TSP – HCs, HAM/TSP – ATL, HCs – ATL). The zero value of tax gene transcripts was observed in 60.7% of HAM/TSP patients (34 out of 56), 71.1% of HCs (27 out of 38) and 30.0% of ATL patients (3 out of 10). The medians are represented by horizontal lines and the statistical differences between them were calculated with a Mann Whitney U test.

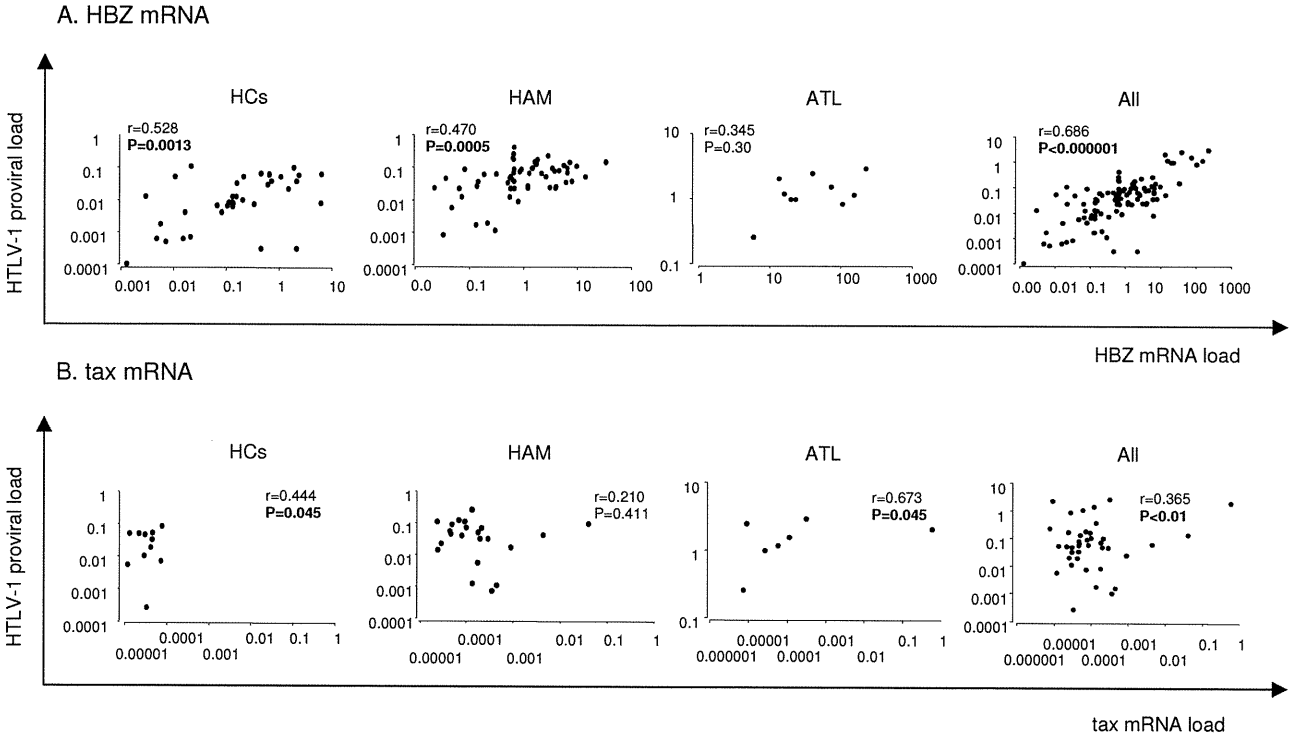

Figure 2

Correlation between HTLV-I proviral load and HTLV-I mRNA load in HTLV-I infected individuals. A. The HTLV-I HBZ mRNA load was significantly correlated with HTLV-I proviral load in HAM/TSP patients alone (P = 0.0005, r = 0.470 by Spearman rank correlation analysis), HCs alone (P = 0.0013, r = 0.528) and all groups combined (P < 0.000001, r = 0.686) but not in ATL patients (P = 0.300, r = 0.345). B. The tax mRNA load correlated with the HTLV-I proviral load in HCs (P = 0.045, r = 0.444), ATL patients (P = 0.045, r = 0.673) and both group combined (P < 0.01, r = 0.365) but not in HAM/TSP patients (P = 0.411, r = 0.210). The zero value of tax gene transcripts did not appear in the figures. Correlations were examined by Spearman rank correlation analysis.

Comparison of HBZ mRNA load with tax mRNA load among HTLV-I infected individuals in different clinical status

To investigate the mutual expression status of HBZ and tax mRNA in different clinical status, we calculated the ratio of HBZ mRNA/tax mRNA in 22 HAM/TSP patients, 11 HCs and 7 ATL patients, who express both tax and HBZ mRNA in PBMCs. HTLV-1 tax mRNA was not expressed in 60.7% (34 out of 56) of HAM/TSP patients, 71.1% (27 out of 38) of HCs and 30.0% (3 out of 10) of ATL patients, whereas HTLV-1 HBZ mRNA was expressed in all the infected individuals tested. As shown in figure 3, HBZ mRNA/tax mRNA ratio in PBMCs was significantly increased in ATL patients than HAM/TSP patients and HCs (P = 0.013 and 0.0051, Mann-Whitney U test, respectively), indicating very high HBZ transcript levels relative to tax, especially in ATL patients.

Correlation of HTLV-I HBZ mRNA load with CSF neopterin concentration and disease severity in HAM/TSP patients

To investigate the relationship between HTLV-1 mRNA load and various laboratory markers, HTLV-1 proviral load, CSF neopterin concentration and anti-HTLV-1 antibody titers were quantified and compared with motor dysfunction of HAM/TSP patients. Since neopterin is a low molecular weight pteridine compound released from macrophages upon stimulation with γ-interferon secreted by activated T cells, the measurement of neopterin concentrations in body fluids like blood serum, CSF or urine provides information about cellular immune activation in humans under the control of type 1 T helper cells [49]. As shown in table 2, we showed that the CSF neopterin level, which was positively correlated with proviral load, was also positively correlated with the HBZ mRNA load in HAM/TSP patients (Spearman's rank correlation coefficient P = 0.0052, r = 0.437). However, such a correlation was not observed between neopterin and HTLV-1 tax mRNA load (P = 0.544, r = 0.228). Motor dysfunction evaluated by OMDS significantly correlated with HTLV-1 HBZ mRNA load (P = 0.023, r = 0.328), but again not with HTLV-1 tax mRNA load (P = 0.401, r = 0.241).

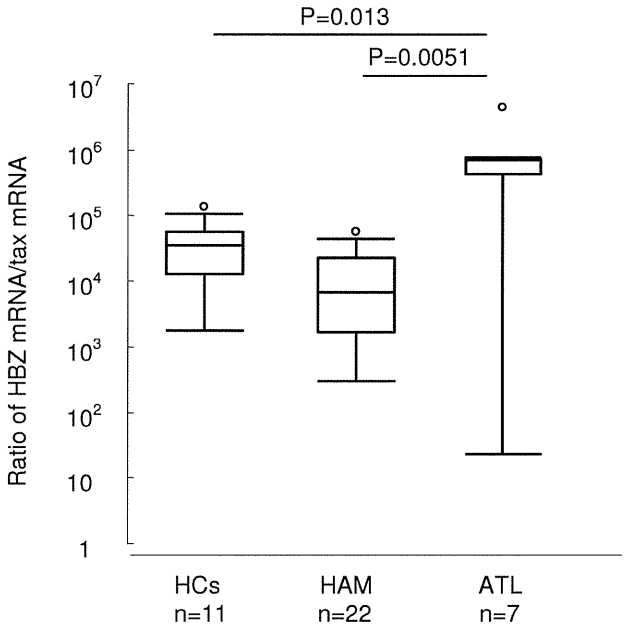

Figure 3
Comparison of HBZ mRNA load with tax mRNA load among HTLV-I infected individuals in different clinical status. The ratio of HBZ mRNA/tax mRNA was significantly increased in ATL patients (median 700,512.24, range 23.11 – 4,308,413.02) than HAM/TSP patients (median 4,932.41, range 295.63–56,082.14) or HCs (median 35,602.96, range 1,804.77–137,999.33). The statistical differences between groups were calculated with a Mann Whitney U test.

HBZ mRNA load and HBZ mRNA/DNA ratio in PBMCs was decreased in HAM/TSP patients after effective IFN-treatment

Finally, to determine whether HTLV-1 mRNA load and mRNA/DNA ratio are associated with clinical improvement, we measured the HTLV-1 (both tax and HBZ) mRNA load and mRNA/DNA ratio before, during, and after interferon-alpha (IFN-α) treatment in four HAM/TSP patients who received 4 weeks of daily administration. Three million international units (IU) of IFN-α (human lymphoblastoid interferon-HLBI, Sumiferon® by Sumitomo Pharmaceutical Co., Osaka, Japan) were administrated per intramuscular injection. Two patients (HAM1 and 2) showed marked clinical improvement with the changes of the OMDS, whereas two patients (HAM3 and 4) did not show clinical improvement (without the changes of the OMDS) (Additional file 1). The HBZ mRNA load and mRNA/DNA ratio was decreased after IFN-α treatment in two patients who showed clinical improvement, whereas the HBZ mRNA load and mRNA/DNA ratio was stable during the treatment in two patients without clinical improvement (Additional file 1 and Figure 4). In contrast, the tax mRNA load and mRNA/DNA ratio did not show such a clear correlation with clinical improvement.

Discussion

In this study, we demonstrated that there was a statistically significant difference in the HTLV-1 HBZ mRNA load, but not tax mRNA load, in PBMCs between HAM/TSP patients and HCs. This is probably because tax mRNA was not expressed in significant numbers of individuals tested (60.7% of HAM/TSP patients, 34 out of 56; 71.1% of HCs, 27 out of 38; 30.0% of ATL patients, 3 out of 10), whereas HTLV-1 HBZ mRNA was expressed in all the infected individuals tested. There was also a statistically significant correlation between HTLV-1 HBZ mRNA load and HTLV-1 proviral load both in HAM/TSP patients and HCs, whereas tax mRNA load correlated with the HTLV-1 proviral load only in HCs but not in HAM/TSP patients. Recently, Usui et al. reported a similar observation [37]. Namely, HBZ spliced isoform mRNA was detectable in samples from most HCs and ATL patients, and was significantly correlated with the HTLV-1 proviral load. These results indicate that the regulation of HBZ mRNA expression is different from that of tax mRNA. It seems likely that HBZ mRNA is near-equally expressed by all provirus-positive cells despite different clinical status, while tax

Table 2: Results of rank correlation test between clinical and virological parameters.

	Proviral load		HBZ mRNA[a]		tax mRNA[b]		HBZ mRNA/DNA[c]		tax mRNA/DNA[d]	
	r	p	r	p	r	p	r	p	r	p
OMDS	0.169	0.285	0.328	**0.023**	0.241	0.401	0.252	0.091	0.257	0.300
Neopterin in CSF	0.512	**0.001**	0.437	**0.0052**	0.228	0.544	0.121	0.442	0.211	0.608
Serum Ab	0.117	0.431	0.185	0.194	0.234	0.333	0.102	0.497	0.248	0.279
CSF Ab	0.071	0.639	0.042	0.801	-0.0029	0.322	-0.046	0.690	0.0025	0.345

OMDS: Osame Motor Disability Scale for HAM/TSP
[a]HTLV-I HBZ mRNA load = value of HBZ/value of HPRT
[b]HTLV-I tax mRNA load = value of tax/value of HPRT
[c]HBZ mRNA/DNA ratio = HTLV-I HBZ mRNA load/Proviral load
[d]tax mRNA/DNA ratio = HTLV-I tax mRNA load/Proviral load

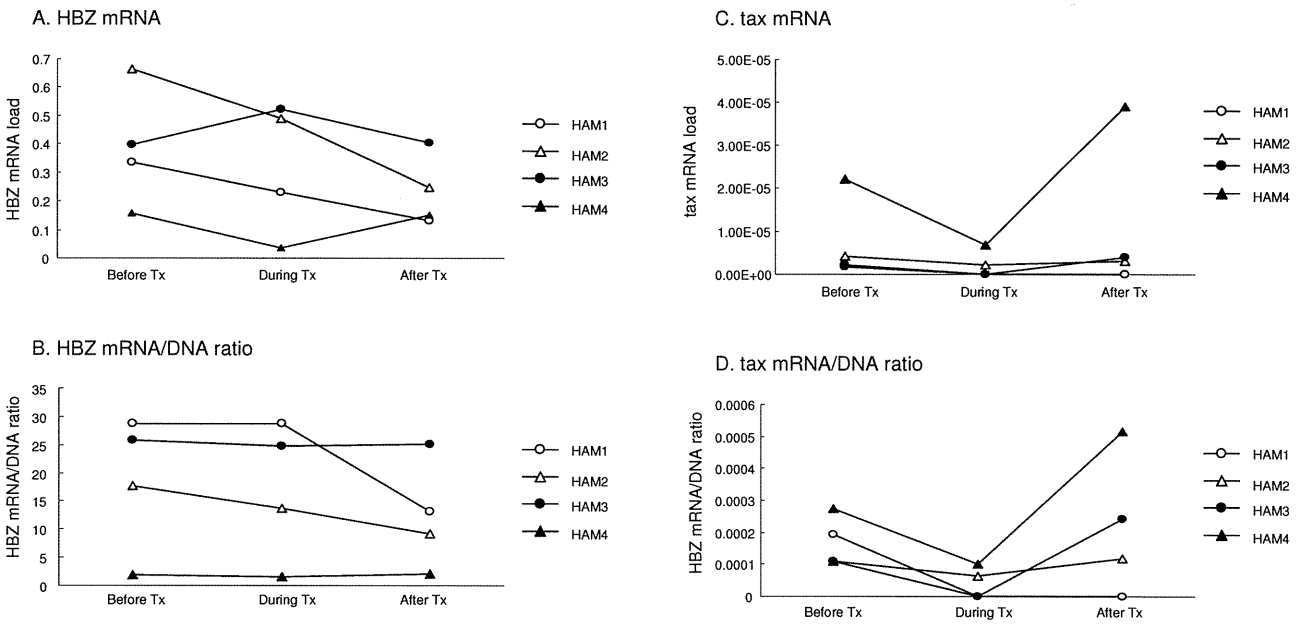

Figure 4

HBZ mRNA load and HBZ mRNA/DNA ratio in PBMCs were decreased in HAM/TSP patients after effective IFN-α treatment. To investigate whether HTLV-I mRNA load and mRNA/DNA ratio are associated with clinical improvement, we measured the HBZ mRNA/DNA ratio in four HAM/TSP patients who received 4 weeks of daily IFN-α administration (three million international units of IFN-α per one intramuscular injection). Two HAM/TSP patients with clinical improvement in Osame Motor Disability Score (OMDS) (HAM1 and 2) showed decreased HBZ mRNA load and HBZ mRNA/DNA ratio during the IFN-α treatment, whereas two HAM/TSP patients without clinical improvement in OMDS (HAM3 and 4) showed stable HBZ mRNA load and HBZ mRNA/DNA ratio during the IFN-α treatment. In contrast, the tax mRNA load and tax mRNA/DNA ratio did not show such a clear correlation with clinical improvement.

mRNA expression levels are variable in different clinical status.

When HTLV-1 tax or HBZ mRNA load was adjusted with HTLV-1 proviral DNA load (i.e. calculate mRNA/DNA ratio), the amount of tax and HBZ mRNA expressed per provirus was not significantly different between HAM/TSP patients and HCs, suggesting that the higher HTLV-1 proviral load seen in HAM/TSP patients caused higher HTLV-1 HBZ mRNA expression. This is consistent with our previous study using different methods for mRNA and DNA quantification [18], but differed from a previous American study using exactly the same methods, which showed significantly higher mRNA/DNA ratio in HAM/TSP patients than HCs [17]. In contrast to the previous study, which showed significant correlation between disease severity in HAM/TSP patients and both HTLV-1 tax mRNA load and mRNA/DNA ratio [17], we could not find such a correlation between clinical parameters of HAM/TSP patients including disease severity and both HTLV-1 tax mRNA load and mRNA/DNA ratio (Table 2). As we have already confirmed and reported the same levels of Tax protein expression in HTLV-1-infected PBMCs between HAM/TSP patients and HCs in the same cohort [50], the observed discrepancy may be due to the differences of a number of host genetic and virologic factors in HTLV-1 infected individuals, including differences in HLA haplotypes [51-53], differences in the amount of soluble suppressive factors and CD8+ T-cell responses, and differences in HTLV-1 tax genomic sequences [54]. As a recent report indicated that HTLV-I infection was associated with activated T-cell immunity in Jamaicans but with diminished T-cell immunity in Japanese persons [55], the interaction between different genes and/or environmental factors is also likely to contribute to the observed differences between the two populations. Namely, genetic resistance to infectious diseases that is formed by complex host genetic effects might be complicated further by pathogen diversity and environmental factors.

Another important observation is that the amount of HTLV-1 HBZ mRNA expression per provirus was more than a thousand times higher than tax mRNA expression both in HAM/TSP patients and HCs. Surprisingly, the amount of HTLV-1 HBZ mRNA expression per provirus was even higher in HTLV-1-infected PBMCs than in

infected cell lines, whereas tax mRNA expression was significantly higher in cell lines than infected PBMCs. Since HBZ suppresses Tax-mediated viral transcription [31], the abundant expression of HBZ mRNA in HTLV-1-infected PBMCs will be one of the molecular mechanisms involved in viral latency by suppressing HTLV-1 transcription and Tax expression, which may be a significant advantage to the virus in the infected cell by preventing its detection through a CTL response. Since we and others [37] found that down-regulation of tax mRNA (higher HBZ mRNA/tax mRNA ratio) was characteristic of primary ATL cells, imbalanced expression between HBZ and tax may induce the outgrowth of HTLV-1-transformed T cell and increase the risk of ATL, which is associated with a Tax-low or -negative phenotype.

We also found that the HTLV-1 HBZ mRNA load significantly correlated with the neopterin concentrations in CSF of HAM/TSP patients. Since neopterin levels in CSF have been used as an immunologic marker for monitoring disease activity and treatment efficacy of HAM/TSP [40,42,56], the quantitative analysis of HTLV-1 HBZ mRNA might also be used to monitor HAM/TSP disease activity. As expected, motor dysfunction of HAM/TSP patients evaluated by the OMDS score significantly correlated with HTLV-1 HBZ mRNA load ($P = 0.023$) but not with HTLV-1 tax mRNA load ($P = 0.401$). The correlation between HBZ mRNA load and two independent clinical parameters reflecting disease activities strongly suggest its stronger relevance than both tax mRNA and proviral load for HAM/TSP pathogenesis. This is further supported by the data that both HBZ mRNA load and HBZ mRNA/DNA ratio were decreased in HAM/TSP patients after effective IFN-α treatment. Collectively, our results suggest that higher HTLV-1 HBZ mRNA load may have relative prognostic value for the assessment of disease progression and could also be used as a surrogate marker to predict long-term outcome in HAM/TSP patients.

In summary, we showed that spliced HBZ gene was transcribed in all the HTLV-1 infected individuals examined, whereas tax mRNA was not transcribed in more than half in the same groups. Moreover, our data demonstrated a significant correlation between HTLV-1 HBZ mRNA load and HTLV-1 proviral load, neopterin concentrations in CSF and motor disability seen in HAM/TSP patients, indicating that HTLV-1 HBZ mRNA load may be a valid predictor of disease progression. Our present findings suggest that HTLV-1 HBZ mRNA expression plays a role not only in ATL, but also in the pathogenesis of the HTLV-1-associated inflammatory disease HAM/TSP.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MS designed and performed the experiments, analyzed the data, and wrote the paper; TM and KA provided clinical samples and assembled clinical database. YS and JY provided clinical samples and performed experiments. KS performed experiments, analyzed and interpreted data. MM made contribution to the conception and design of the study. YO contributed to obtaining funding and gave advice.

Additional material

Additional file 1

Changes in HBZ mRNA load and HBZ mRNA/DNA ratio in PBMCs of HAM/TSP patients after IFN- treatment.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1742-4690-6-19-S1.doc]

Acknowledgements

We are grateful to the staff and blood donors of Kagoshima University Hospital. We also thank Dr. Ryuji Kubota for providing the clinical samples, Prof. Masahiro Fujii of Niigata University for the gift of HTLV-I-infected T-cell lines (C5/MJ, SLB1, and MT-4), and Ms. Sumie Saito of Kanazawa Medical University for technical assistance. This work was supported by the Ministry of Health, Labor and Welfare, Japan (Neuroimmunological Disease Research Committee Grant to Y.O.); Takeda Science Foundation (to M.S.); Kanazawa Medical University (Grants H2007-11, H2008-11, C2008-2, and S2008-8 to M.S.).

References

1. Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC: **Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma.** *Proc Natl Acad Sci USA* 1980, **77:**7415-7419.
2. Yoshida M, Miyoshi I, Hinuma Y: **Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease.** *Proc Natl Acad Sci USA* 1982, **79:**2031-2035.
3. Hinuma Y, Nagata K, Hanaoka M, Nakai M, Matsumoto T, Kinoshita KI, Shirakawa S, Miyoshi I: **Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera.** *Proc Natl Acad Sci USA* 1981, **78:**6476-6480.
4. Yoshida M, Seiki M, Yamaguchi K, Takatsuki K: **Monoclonal integration of human T-cell leukemia provirus in all primary tumors of adult T-cell leukemia suggests causative role of human T-cell leukemia virus in the disease.** *Proc Natl Acad Sci USA* 1984, **81:**2534-2537.
5. Gessain A, Barin F, Vernant JC, Gout O, Maurs L, Calender A, de The G: **Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis.** *Lancet* 1985, **2:**407-410.
6. Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A, Matsumoto M, Tara M: **HTLV-I associated myelopathy, a new clinical entity.** *Lancet* 1986, **1:**1031-1032.
7. Arisawa K, Soda M, Endo S, Kurokawa K, Katamine S, Shimokawa I, Koba T, Takahashi T, Saito H, Doi H, Shirahama S: **Evaluation of adult T-cell leukemia/lymphoma incidence and its impact on non-Hodgkin lymphoma incidence in southwestern Japan.** *Int J Cancer* 2000, **85:**319-324.
8. Mochizuki M, Watanabe T, Yamaguchi K, Takatsuki K, Yoshimura K, Shirao M, Nakashima S, Mori S, Araki S, Miyata N: **HTLV-I uveitis: a distinct clinical entity caused by HTLV-I.** *Jpn J Cancer Res* 1992, **83:**236-239.

9. Sugimoto M, Nakashima H, Watanabe S, Uyama E, Tanaka F, Ando M, Araki S, Kawasaki S: **T-lymphocyte alveolitis in HTLV-I-associated myelopathy.** *Lancet* 1987, **2:**1220.
10. Nishioka K, Maruyama I, Sato K, Kitajima I, Nakajima Y, Osame M: **Chronic inflammatory arthropathy associated with HTLV-I.** *Lancet* 1989, **1:**441.
11. Higuchi I, Montemayor ES, Izumo S, Inose M, Osame M: **Immunohistochemical characteristics of polymyositis in patients with HTLV-I-associated myelopathy and HTLV-I carriers.** *Muscle Nerve* 1993, **16:**472-476.
12. Bangham CR, Osame M: **Cellular immune response to HTLV-1.** *Oncogene* 2005, **24:**6035-6046.
13. Yoshida M: **Multiple viral strategies of HTLV-1 for dysregulation of cell growth control.** *Annu Rev Immunol* 2001, **19:**475-496.
14. Umehara F, Izumo S, Ronquillo AT, Matsumuro K, Sato E, Osame M: **Cytokine expression in the spinal cord lesions in HTLV-I-associated myelopathy.** *J Neuropathol Exp Neurol* 1994, **53:**72-77.
15. Umehara F, Izumo S, Takeya M, Takahashi K, Sato E, Osame M: **Expression of adhesion molecules and monocyte chemoattractant protein -1 (MCP-1) in the spinal cord lesions in HTLV-I-associated myelopathy.** *Acta Neuropathol (Berl)* 1996, **91:**343-350.
16. Umehara F, Okada Y, Fujimoto N, Abe M, Izumo S, Osame M: **Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in HTLV-I-associated myelopathy.** *J Neuropathol Exp Neurol* 1998, **57:**839-849.
17. Yamano Y, Nagai M, Brennan M, Mora CA, Soldan SS, Tomaru U, Takenouchi N, Izumo S, Osame M, Jacobson S: **Correlation of human T-cell lymphotropic virus type 1 (HTLV-1) mRNA with proviral DNA load, virus-specific CD8(+) T cells, and disease severity in HTLV-1-associated myelopathy (HAM/TSP).** *Blood* 2002, **99:**88-94.
18. Furukawa Y, Osame M, Kubota R, Tara M, Yoshida M: **Human T-cell leukemia virus type-1 (HTLV-1) Tax is expressed at the same level in infected cells of HTLV-1-associated myelopathy or tropical spastic paraparesis patients as in asymptomatic carriers but at a lower level in adult T-cell leukemia cells.** *Blood* 1995, **85:**1865-1870.
19. Matsuoka M: **Human T-cell leukemia virus type I (HTLV-I) infection and the onset of adult T-cell leukemia (ATL).** *Retrovirology* 2005, **2:**27.
20. Hinrichs SH, Nerenberg M, Reynolds RK, Khoury G, Jay G: **A transgenic mouse model for human neurofibromatosis.** *Science* 1987, **237:**1340-1343.
21. Nerenberg M, Hinrichs SH, Reynolds RK, Khoury G, Jay G: **The tat gene of human T-lymphotropic virus type I induces mesenchymal tumors in transgenic mice.** *Science* 1987, **237:**1324-1329.
22. Green JE, Hinrichs SH, Vogel J, Jay G: **Exocrinopathy resembling Sjogren's syndrome in HTLV-1 tax transgenic mice.** *Nature* 1989, **341:**72-74.
23. Iwakura Y, Tosu M, Yoshida E, Takiguchi M, Sato K, Kitajima I, Nishioka K, Yamamoto K, Takeda T, Hatanaka M, *et al.*: **Induction of inflammatory arthropathy resembling rheumatoid arthritis in mice transgenic for HTLV-I.** *Science* 1991, **253:**1026-1028.
24. Ruddle NH, Li CB, Horne WC, Santiago P, Troiano N, Jay G, Horowitz M, Baron R: **Mice transgenic for HTLV-I LTR-tax exhibit tax expression in bone, skeletal alterations, and high bone turnover.** *Virology* 1993, **197:**196-204.
25. Hall AP, Irvine J, Blyth K, Cameron ER, Onions DE, Campbell ME: **Tumours derived from HTLV-I tax transgenic mice are characterized by enhanced levels of apoptosis and oncogene expression.** *J Pathol* 1998, **186:**209-214.
26. Gao L, Deng H, Zhao H, Hirbe A, Harding J, Ratner L, Weilbaecher K: **HTLV-I Tax transgenic mice develop spontaneous osteolytic bone metastases prevented by osteoclast inhibition.** *Blood* 2005, **106:**4294-4302.
27. Furuta Y, Aizawa S, Suda Y, Ikawa Y, Kishimoto H, Asano Y, Tada T, Hikikoshi A, Yoshida M, Seiki M: **Thymic atrophy characteristic in transgenic mice that harbor pX genes of human T-cell leukemia virus type I.** *J Virol* 1989, **63:**3185-3189.
28. Grossman WJ, Kimata JT, Wong FH, Zutter M, Ley TJ, Ratner L: **Development of leukemia in mice transgenic for the tax gene of human T-cell leukemia virus type I.** *Proc Natl Acad Sci USA* 1995, **92:**1057-1061.
29. Hasegawa H, Sawa H, Lewis MJ, Orba Y, Sheehy N, Yamamoto Y, Ichinohe T, Tsunetsugu-Yokota Y, Katano H, Takahashi H, Matsuda J, Sata T, Kurata T, Nagashima K, Hall WW: **Thymus-derived leukemia-lymphoma in mice transgenic for the Tax gene of human T-lymphotropic virus type I.** *Nat Med* 2006, **12:**466-472.
30. Ohsugi T, Kumasaka T, Okada S, Urano T: **The Tax protein of HTLV-I promotes oncogenesis in not only immature T cells but also mature T cells.** *Nat Med* 2007, **13:**527-528.
31. Gaudray G, Gachon F, Basbous J, Biard-Piechaczyk M, Devaux C, Mesnard JM: **The complementary strand of the human T-cell leukemia virus type I RNA genome encodes a bZIP transcription factor that down-regulates viral transcription.** *J Virol* 2002, **76:**12813-12822.
32. Satou Y, Yasunaga J, Yoshida M, Matsuoka M: **HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells.** *Proc Natl Acad Sci USA* 2006, **103:**720-725.
33. Basbous J, Arpin C, Gaudray G, Piechaczyk M, Devaux C, Mesnard JM: **The HBZ factor of human T-cell leukemia virus type I dimerizes with transcription factors JunB and c-Jun and modulates their transcriptional activity.** *J Biol Chem* 2003, **278:**43620-43627.
34. Arnold J, Yamamoto B, Li M, Phipps AJ, Younis I, Lairmore MD, Green PL: **Enhancement of infectivity and persistence in vivo by HBZ, a natural antisense coded protein of HTLV-1.** *Blood* 2006, **107:**3976-3982.
35. Osame M: *Review of WHO Kagoshima meeting and diagnostic guidelines for HAM/TSP* New York: Raven Press; 1990.
36. Nagai M, Usuku K, Matsumoto W, Kodama D, Takenouchi N, Moritoyo T, Hashiguchi S, Ichinose M, Bangham CR, Izumo S, Osame M: **Analysis of HTLV-I proviral load in 202 HAM/TSP patients and 243 asymptomatic HTLV-I carriers: high proviral load strongly predisposes to HAM/TSP.** *J Neurovirol* 1998, **4:**586-593.
37. Usui T, Yanagihara K, Tsukasaki K, Murata K, Hasegawa H, Yamada Y, Kamihira S: **Characteristic expression of HTLV-1 basic zipper factor (HBZ) transcripts in HTLV-1 provirus-positive cells.** *Retrovirology* 2008, **5:**34.
38. Murata K, Hayashibara T, Sugahara K, Uemura A, Yamaguchi T, Harasawa H, Hasegawa H, Tsuruda K, Okazaki T, Koji T, Miyanishi T, Yamada Y, Kamihira S: **A novel alternative splicing isoform of human T-cell leukemia virus type 1 bZIP factor (HBZ-SI) targets distinct subnuclear localization.** *J Virol* 2006, **80:**2495-2505.
39. Cavanagh MH, Landry S, Audet B, Arpin-Andre C, Hivin P, Pare ME, Thete J, Wattel E, Marriott SJ, Mesnard JM, Barbeau B: **HTLV-I antisense transcripts initiating in the 3'LTR are alternatively spliced and polyadenylated.** *Retrovirology* 2006, **3:**15.
40. Nomoto M, Utatsu Y, Soejima Y, Osame M: **Neopterin in cerebrospinal fluid: a useful marker for diagnosis of HTLV-I-associated myelopathy/tropical spastic paraparesis.** *Neurology* 1991, **41:**457.
41. Izumo S, Goto I, Itoyama Y, Okajima T, Watanabe S, Kuroda Y, Araki S, Mori M, Nagataki S, Matsukura S, Akamine T, Nakagawa M, Yamamoto I, Osame M: **Interferon-alpha is effective in HTLV-I-associated myelopathy: a multicenter, randomized, double-blind, controlled trial.** *Neurology* 1996, **46:**1016-1021.
42. Saito M, Nakagawa M, Kaseda S, Matsuzaki T, Jonosono M, Eiraku N, Kubota R, Takenouchi N, Nagai M, Furukawa Y, Usuku K, Izumo S, Osame M: **Decreased human T lymphotropic virus type I (HTLV-I) provirus load and alteration in T cell phenotype after interferon-alpha therapy for HTLV-I-associated myelopathy/tropical spastic paraparesis.** *J Infect Dis* 2004, **189:**29-40.
43. Matsuzaki T, Saito M, Usuku K, Nose H, Izumo S, Arimura K, Osame M: **A prospective uncontrolled trial of fermented milk drink containing viable Lactobacillus casei strain Shirota in the treatment of HTLV-I associated myelopathy/tropical spastic paraparesis.** *J Neurol Sci* 2005, **15:237(1–2):**1-2.
44. Kurtzke JF: **Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS).** *Neurology* 1983, **33:**1444-1452.
45. Kamihira S, Sugahara K, Tsuruda K, Minami S, Uemura A, Akamatsu N, Nagai H, Murata K, Hasegawa H, Hirakata Y, Takasaki Y, Tsukasaki K, Yamada Y: **Proviral status of HTLV-I integrated into the host genomic DNA of adult T-cell leukemia cells.** *Clin Lab Haematol* 2005, **27:**235-241.