Table 1. Association of rs2275294 in *ZNF512B* with ALS

	No. of subjects		Risk allele frequency			Odds ratio (95% CI)
	Case	Control	Case	Control	*P*-value	
Discovery series	454	958	0.491	0.422	6.3×10^{-4}	1.32 (1.13–1.55)
Sample set 1	249	1030	0.512	0.434	1.8×10^{-3}	1.37 (1.12–1.66)
Sample set 2	602	2256	0.481	0.416	5.6×10^{-5}	1.30 (1.14–1.48)
Combined	1305	4244				
Meta-analysis[a]					9.3×10^{-10}	1.32 (1.21–1.44)
Joint analysis					6.7×10^{-10}	1.32 (1.21–1.44)

[a]By the Mantel–Haenszel method.

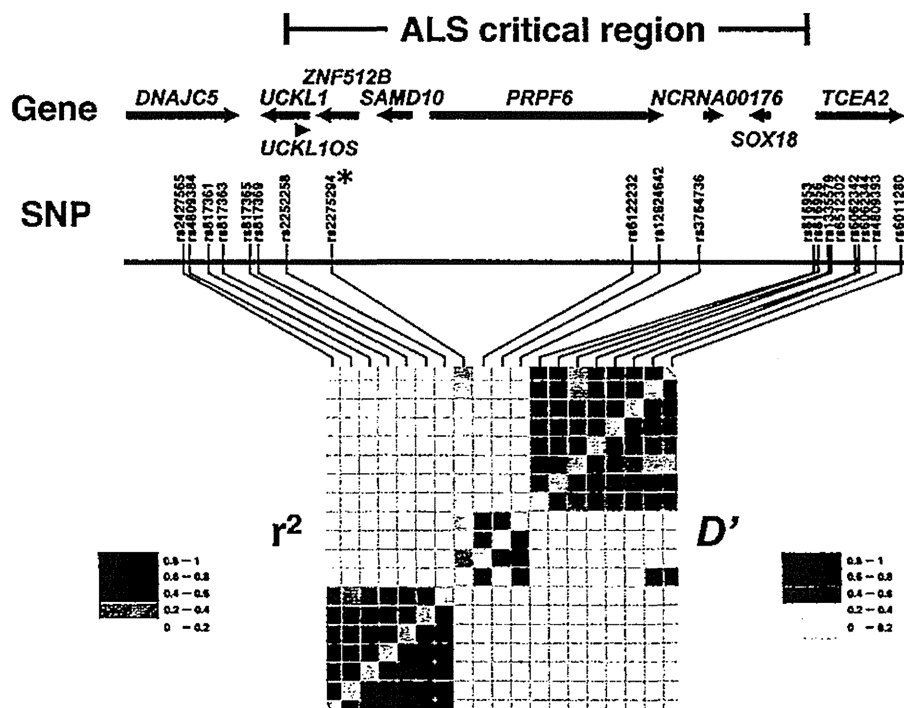

Figure 1. Genomic structure and linkage disequilibrium (LD) map in the ALS critical region. Top, an SNP map of a 111 kb genomic region containing *ZNF512B*. The orientation of each gene is indicated by a green arrow. An asterisk shows the landmark SNP. Bottom, an LD map as measured by D' (lower right triangle) and r^2 (upper left triangle).

examined and no significant differences in age and gender distribution were found among rs2275294 genotyped. The associations with rs2275294 were significant in two sample sets ($P = 4.1 \times 10^{-3}$ and 1.4×10^{-4}), even after adjusting for age and gender in a logistic regression analysis.

Genome analysis of the ALS critical region containing rs2275294

We constructed a linkage disequilibrium (LD) map around rs2275294 on the basis of the genotyping data for Japanese subjects used in HapMap (HapMap JPT). Because rs2275294 was unmapped in the HapMap data, we genotyped the SNP for the HapMap JPT samples and integrated the data with the HapMap JPT data. We found that rs2275294 was in strong LD with the two SNPs rs6122232 and rs3764736 ($D' > 0.85$). Subsequently, the critical region could be confined to a 111 kb interval flanked by rs2252258 and rs816953 on chromosome 20q13.33 (Fig. 1). This region included four genes (*ZNF512B, SAMD10, PRPF6* and *SOX18*) and a part of *UCKL1*, as well as two non-protein-coding RNAs (*UCKL1OS* and *NCRNA00176*). In order to identify a more significantly associated SNP, we searched for SNPs in each gene by re-sequencing genomic DNA of 48 ALS subjects. A total of 24 SNPs were identified and their level of association was examined using 455 cases and 452 controls, but rs2275294 remained the most significantly associated (Supplementary Material, Table S3).

Functional analysis of rs2275294

To gain insight into the biological significance of rs2275294, luciferase reporter plasmids corresponding to a genomic

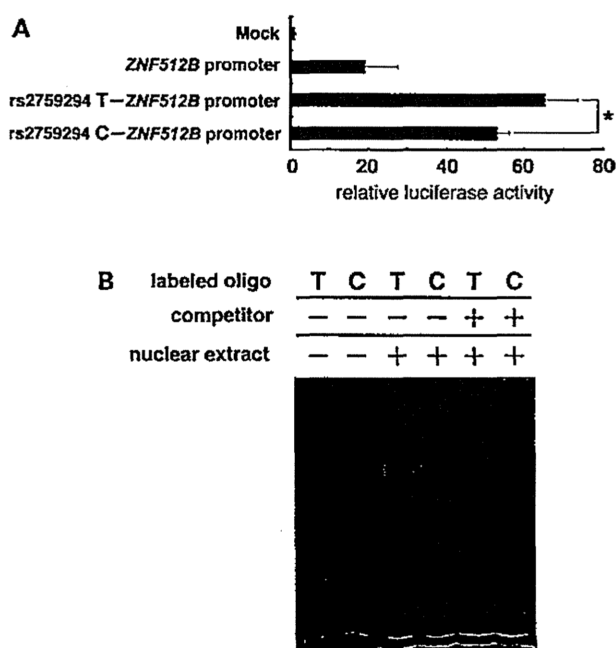

Figure 2. Functional analysis for rs2275294 in *ZNF512B*. (**A**) Difference in the enhancer activity of genomic DNA segments containing rs2275294. Luciferase assay in SK_N_Be(2)C cells. Enhancer activity was lower in the ALS-susceptibility allele (C allele). *ZNF512B* promoter:native promoter (nts −820 to −74) of *ZNF512B*. Data represent the mean ± SEM ($n = 6$). $^{*}P < 0.01$ (Student's t-test). (**B**) Difference in binding of nuclear proteins to a cis-element containing rs2275294. An EMSA using nuclear extracts from SK_N_AS cells. The specific band was weaker in the ALS-susceptibility allele (C allele).

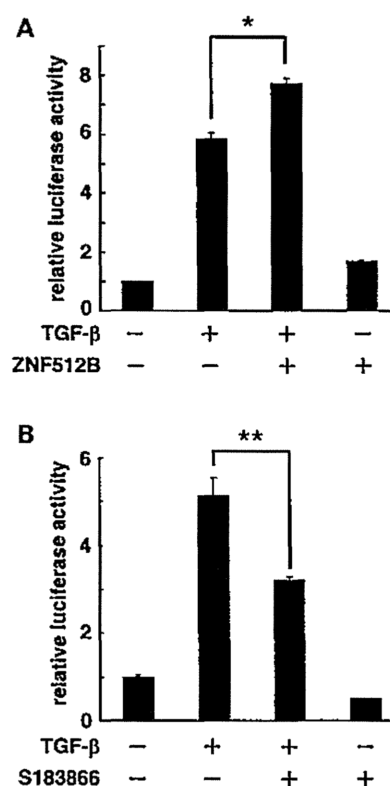

Figure 3. ZNF512B is a positive regulator of the TGF-β signal. (**A**) Luciferase assay using SBE4-luciferase. ZNF512B trans-activated the TGF-β-induced SMAD transcriptional activity in the SK_N_AS cell line ($^{*}P < 0.0005$). (**B**) S183866, a *ZNF512B*-targeting siRNA oligonucleotide repressed the TGF-β-dependent SBE4-luciferase activity ($^{**}P < 0.005$).

DNA fragment containing rs2275294 were constructed and a luciferase assay using the human neuroblastoma cell line SK_N_Be(2)C was performed. Constructs containing the ALS-susceptibility allele (C allele) of rs2275294 showed lower enhancer activity than those containing the non-susceptibility allele, indicating that the SNP affects the *ZNF512B* transcription level (Fig. 2A). We then examined the allelic difference in the binding of genomic DNA containing rs2275294 to nuclear proteins by the electrophoretic mobility shift assay (EMSA). The DNA–protein complex from the C allele showed weaker binding (Fig. 2B). Thus, it is feasible that the presence of the susceptibility allele leads to lower *ZNF512B* levels as a consequence of decreased enhancer activity.

ZNF512B is a positive regulator in the TGF-β signaling pathway

Proteomics analysis has suggested that ZNF512B functions as a regulator of the TGF-β signaling pathway (54). We examined the effect of ZNF512B on TGF-β signaling using the TGF-β-dependent SMAD2/3-specific luciferase assay (55) in a HepG2 cell (data not shown). SMAD2/3-mediated reporter activity after TGF-β stimulation was enhanced by ZNF512B over-expression. The TGF-β-dependent reporter activity was activated by ZNF512B over-expression in a neuroblastoma cell line SK_N_AS (Fig. 3A) and a glioblastoma cell line U87MG (Supplementary Material, Fig. S4). Next, we knocked down expression of the endogenous *ZNF512B* in SK_N_AS by using the short-interfering RNA (siRNA) technique. Real time polymerase chain reaction (PCR) showed that *ZNF512B* siRNA significantly reduced *ZNF512B* transcription, and TGF-β-dependent reporter activity was repressed by the siRNA (Fig. 3B).

ZNF512B expression in the spinal cord of ALS

The localization of ZNF512B in the spinal cord of ALS patients was investigated by immuno-histochemical studies. The immuno-reactivity for an anti-ZNF512B polyclonal antibody was intense in motor neuron cells in the anterior horn of the spinal cords of ALS patients, while it was barely detectable in those of controls (Fig. 4A–D). Glial cells in the anterior horn did not show ZNF512B immuno-reactivity.

DISCUSSION

By a large-scale case–control association study using gene-based SNPs and enrolling a total of more than 5500 subjects, we identified *ZNF512B* at chromosome 20q13.33 as a new susceptibility gene for ALS. rs2275294 in *ZNF512B* had

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Figure 4. Immuno-histochemical localization of ZNF512B in the spinal cord of ALS patients (A and B) and controls (C and D). The ZNF512B immuno-reactivity was intense in motor neuron cells in the anterior horn of ALS patients, while it was hardly detectable in those of controls. Glial cells in the anterior horn did not show ZNF512B immuno-reactivity. Scale bar, 100 μm.

significant association that satisfied a genome-wide significance level ($P = 9.3 \times 10^{-10}$). rs2275294 affected *ZNF512B* transcription *in vitro*, and the ALS-susceptibility allele (C allele) showed lower enhancer activity for the *ZNF512B* promoter. Therefore, *ZNF512B* is presumably lower in those who have the susceptibility allele than in those who have the non-susceptibility allele. *ZNF512B* over-expression enhanced TGF-β signaling, while its knockdown decreased the signal. Our findings suggest that ZNF512B is an important positive regulator of TGF-β and that lowered ZNF512B expression is implicated in the pathogenesis of ALS susceptibility via decreased TGF-β signal.

In this study, we screened the genic regions using >52 000 gene-based SNPs from the JSNP database. The number of SNPs and their coverage are not sufficient to screen the entire genome. Our study must have many false negatives. Current commercial GWAS platforms are considered superior to ours in terms of the study power and the coverage of SNPs in the human genome. In contrast, the false-positive association of rs2275294 is unlikely. The inflation factor was low and principal component analysis showed no evidence of population stratification. We validated the association in independent Japanese panels. The statistical significance of the association for the combined *P*-values by two different methods fulfilled criteria of the genome-wide significance level. The results of the two analyses were very similar, which further shows that a hidden confounder in our population is unlikely. In addition, there was no difference in the population structures among the case–control sets by Wright's F statistics (53) throughout the study. The MAF of rs2275294 in 744 Japanese samples deposited in dbSNP is similar to that of our controls (0.414).

In spite of its very significant association in our study, rs2275294 in *ZNF512B* has not been found in the previous GWASs. Several explanations can be considered. The main reason is that rs2275294 was not included in the platforms of the previous GWASs. Only 15 SNPs in Illumina 610K SNP Array were mapped to the 111 kb genomic region (1 SNP/7.4 kb) corresponding to the ALS critical region we determined. Also, only 10 SNPs in Affymetrix SNP Array 6.0 were mapped to the genomic region (1 SNP/11.1 kb). In addition, rs2275294 is not even mapped in the HapMap JPT database, nor included in the CEU and YRI HapMap data sets. In the Illumina and Affymetrix SNP arrays, the numbers of SNPs in the *ZNF512B* locus are only two and one, respectively. Their coverages of *ZNF512B*-SNPs in the ALS critical region were very low, 2/15 (13%) and 1/10 (10%), respectively. The low coverage of the region might have led to the false-negative association in the previous GWASs. No SNP was in strong LD ($r^2 > 0.8$) with rs2275294 in CHB-JPT, CEU and YRI in the 1000 Genomes data (Supplementary Material, Table S4). Hence, we speculate that rs2275294 has been identified by virtue of our platform. Still another explanation is the ethnic difference of ALS susceptibility.

A number of GWASs in ALS have been performed recently. They report the identification of five candidate genes and two candidate loci (30–35). Among them, only five gene loci (*DPP6, ITPR2, FLJ10986, KIFAP3* and *UNC13A*) were included in our platform. We checked 16 SNPs in *DPP6*, 23 in *ITPR2*, 2 in *FLJ10986*, 9 in *KIFAP3* and 4 in *UNC13A*; however, their associations were not replicated in our study (Supplementary Material, Table S5). The small number of samples and the low coverage of SNPs in our platform may have resulted in false-negative association. Ethnic differences may be another reason for no replication. The 9p21.2 SNP that has been reported in the previous study (42) was not included in the present study. The tested SNPs for previous associations were negative, but no evidence can be provided for the chromosome 9p21.2 locus. Because the powers of Japanese and Chinese were only 0.37 and 0.11, respectively (42), the negative association may be due to a lack of power in the study. More extensive association studies using larger panels of Japanese samples will be required to conclude the associations between previous candidate genes and ALS.

ZNF512B was originally identified as a *KIAA1196* in the course of the Kazusa Human cDNA project (56). The *ZNF512B* cDNA is 5919 bp long and encodes an 893 amino-acid protein that is ubiquitously expressed in various tissues, including the brain and spinal cord (56). Our immunohistochemical studies confirmed its localization in the spinal cord. The ZNF512B protein showed no significant homology with any proteins in the public database. It contains six C2H2-type zinc finger domains and is predicted to act as a transcription factor. The ALS-susceptibility SNP rs2275294 was localized to intron 12 of *ZNF512B*. We have demonstrated that the genomic region containing rs2275294 can act as an enhancer of the *ZNF512B* promoter and that the susceptibility allele of rs2275294 had reduced transcriptional activity, which was likely due to its decreased binding capacity to trans-factors. Further studies for the upstream factors of *ZNF512B* are necessary to clarify the molecular pathogenesis of ALS related to *ZNF512B*.

We showed that ZNF512B is a positive regulator of the canonical TGF-β signaling pathway through SMAD2/3. TGF-β signal is essential for the survival of neurons (44–46). Upregulation of PAI-1 by SMAD3-dependent induction in astrocytes mediates the neuroprotective activity of TGF-β against NMDA receptor-mediated excitotoxicity (57). TGF-β signal has been implicated in the pathogenesis of ALS. Plasma TGF-β1 level is significantly increased in ALS patients compared with healthy controls, and there is a significant positive correlation between TGF-β1 concentration in ALS patients and duration of their disease (58). A microarray analysis showed a 4.8-fold increased expression of *SMAD4* in sALS compared with neurologically normal controls (59).

Also, phosphorylated SMAD2/3 immuno-reactivity is increased in the remaining spinal motor neurons and glial cells in sporadic and familial ALSs, as well as in *Sod1* transgenic mice (60). These findings suggest that the TGF-β signal is increased in ALS.

Several studies have shown an association between duration of ALS and TGF-β levels. Houi *et al.* (58) found a positive correlation between the plasma concentration of TGF-β1 in ALS patients and the duration of disease. Another group reported that TGF-β1 concentrations in serum and cerebrospinal fluid did not differ between ALS patients and controls, but were higher in ALS patients with a terminal clinical status than in controls (61). These data suggest that TGF-β is increased in the motor neuron cells of ALS patients during the disease process. As ZNF512B is a critical enhancer of TGF-β signaling, its genetic association may be related to the progression of the disease rather than its onset.

We have demonstrated the localization of ZNF512B in the spinal cord of ALS patients, and that ZNF512B expression in the motor neurons of ALS patients was significantly increased compared with that of controls (Fig. 4). It is biologically plausible that ZNF512B is a positive regulator (co-activator) of neuroprotective TGF-β signaling (Fig. 3) and may act as a protector against ALS. Taken together with the results of luciferase assay and EMSA that showed allelic differences in *ZNF512B* expression level (Fig. 2), a patient harboring the susceptibility allele would have decreased *ZNF512B* expression level compared with a patient harboring non-susceptibility alleles. The decreased *ZNF512B* enhancer activity by the susceptibility allele leads to insufficient increase in ZNF512B, which leads to insufficient increase in the TGF-β signal that results in decreased potential for survival and/or recovery of motor neurons. The discovery of this ALS-susceptibility gene and its pathway should shed light on ALS pathogenesis and facilitate development of targeted therapies.

MATERIALS AND METHODS

Subjects

A total of 1305 ALS patients diagnosed as having probable, probable and laboratory-supported, or definite ALS according to the El Escorial revised criteria (62) were included in the study. All subjects were unrelated Japanese individuals. We obtained a total of 703 DNA samples from the Biobank Japan project (63). All patients were screened for mutations in *SOD1*, *TARDBP* and *ANG* and none was detected. The mean age of cases was 60.8 years (range: 28–82 years), and 66.1% were male. 74.4% had a spinal onset, 19.6% a bulbar one and 6% a multiple and the others. We obtained a total of 602 DNA samples from the Japanese Consortium for Amyotrophic Lateral Sclerosis Research (JaCALS), Jichi Medical University and The University of Tokyo. The mean age was 61.5 years (range: 27–89 years), and 62.0% were male. 70.4% of the patients had a spinal onset and the remaining had a bulbar one. We recruited 4244 controls through several medical institutes in Japan. Their mean age was 66.8 years (range: 18–98 years), and 48.0% were male. All controls had negative medical and family histories for neurodegenerative disorders. Written informed consent was obtained from all the subjects. The ethical committees at the participating institutions approved this project.

SNP genotyping

Using standard protocols, genomic DNA was extracted from the peripheral blood leukocytes. SNPs were genotyped using the multiplex PCR-based invader assay (Third Wave Technologies) as described previously (64). A total of 52 608 gene-based SNPs were selected from the JSNP database on the basis of the haplotype block structure reported previously (43,65). We calculated the total number of independent SNPs in this study to be 43 052 (the SNPs in LD: $r^2 > 0.80$ were considered as one SNP). We checked the cryptic relatedness for each pair of samples by identity-by-state by estimating the average number of shared alleles between two individuals (V_I) using 48 884 autosomal SNPs. Six individuals in controls were related ($V_I > 1.65$). They were excluded from the analysis. A stepwise screening method was adopted to increase the statistical power (66). In stage 1, 92 ALS and 233 control subjects were analyzed. We applied the SNP quality control filters of call rate of ≥ 0.95 in both cases and controls and *P*-value of Hardy–Weinberg equilibrium (HWE) test of $\geq 1.0 \times 10^{-2}$ in controls. A total of 48 939 SNPs on autosomal chromosomes passed the quality control filters and were analyzed for the association. The data of this study are available at the JSNP database (http://snp.ims.u-tokyo.ac.jp/). Among the SNPs analyzed in stage 1, 893 SNPs showing the smallest *P*-values (0.01 or smaller) were selected for stage 2. Three models (i.e. allelic, dominant and recessive) were tested for the association. Since these three models are not independent, 893 SNPs were isolated. In stage 2, we genotyped an additional 1087 subjects consisting of 362 ALS cases and 725 controls. Stage 1 and stage 2 were defined as the discovery series of this research and the following sample sets were defined as sample set 1 and sample set 2.

SNP discovery

Appropriate genome sequences were extracted from the UCSC Genome Bioinformatics website. The critical region contained five genes (*ZNF512B, SAMD10, PRPF6, SOX18* and part of *UCKL1*) and two non-protein-coding RNAs (*UCKL1OS* and *NCRNA00176*). We defined the exon–intron boundaries of each gene and designed PCR primer sets for the critical region except for repetitive sequence regions. Each PCR was performed with 5 ng of mixed genomic DNA derived from three ALS subjects; 16 mixed samples were amplified in the GeneAmp PCR system 9700 (PE Applied Biosystems) under the following conditions: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 96°C for 30 s, annealing at 60–65°C for 30 s, extension at 72°C for 2 min and post-extension at 72°C for 7 min. PCR products served as templates for direct sequencing by the fluorescent dye-terminator cycle sequencing method.

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Statistical analysis

For general statistical analyses, we used R statistical environment version 2.6.1 and programs created by our group. The Chi-square test or Fisher's exact test was applied to a two-by-two contingency table in three genetic models: an allele frequency model, a dominant-effect model and a recessive-effect model. Principal component analysis was performed using the smartpca program (52). We calculated the association in case–control status of stage 1 by using a twstats of EIGENSOFT (52). The top six principal components were associated with case–control status. Genotype data from the HapMap project were used (67) to estimate the population structure. The significance of stratification was determined using the Wright method (53). The Mantel–Haenszel method was used for meta-analysis. An automated laboratory system and bar-coding were employed to reduce clerical errors. The accuracy of our system has been guaranteed in data of the HapMap project (67). We checked HWE and personally retyped some SNPs from genome screening in duplicated samples. We also obtained age- and gender-adjusted odds ratios by logistic regression analysis by program R. Haploview 4.1 was used to infer the LD structure of the ALS critical region. An LD pattern was created based on the JPT HapMap data. Luciferase assay data were analyzed by Student's *t*-test.

Luciferase assay

We cloned DNA fragments containing rs2275294, nucleotides (nts) 190–208 of intron 12 of *ZNF512B*. The fragments for both alleles as three tandem copies were inserted into pGL3-Basic vector (Promega) upstream of its luciferase gene in 5′–>3′ orientation together with the *ZNF512B* core promoter of nts −820 to −74 of its 5′ flanking region. We transfected SK_N_Be(2)C cells with 400 ng of each reporter construct using FuGene 6 transfection reagent (Roche) together with 8 ng of pRL-TK vector (Promega) as a control. After 24 h, the cells were lysed in a passive lysis buffer and luciferase activities were measured using Dual-Luciferase Reporter Assay System (Toyo Ink). The entire coding sequence of ZNF512B was cloned into pcDNA3.1, which had a Myc-tag sequence. We also co-transfected with SBE4 (four copies of Smad Binding Element) luciferase reporter vector (55)/Myc-tagged ZNF512B or SBE_4-luciferase reporter vector/Myc-tagged pcDNA3.1, and pRL-TK vector using Trans-IT LT reagent (TAKARA Bio). After 24 h, we treated the cells with 10 ng/ml of TGF-β for 24 h. The cells were lysed in a passive lysis buffer and luciferase activities were measured using Dual-Luciferase Reporter Assay System (Toyo Ink).

Electrophoretic mobility shift assay

A nuclear extract from SK_N_AS cells was prepared as previously described (68) and incubated with oligonucleotides (nts 184–203 of intron 12 of *ZNF512B*) that were labeled with digoxigenin-11-ddUTP using the Dig Gel Shift Kit (Roche). The reaction was carried out at a room temperature with an additional 1 mg/ml of poly[d(I-C)]. For the competition assay, the nuclear extract was pre-incubated with unlabeled oligonucleotides (200-fold molar excess) before adding digoxigenin-labeled oligonucleotide. The protein–DNA complex was separated on a non-denaturing 6% polyacrylamide gel in 0.25× Tris-borate-EDTA buffer. We transferred the gel to membrane and detected the signal with a chemiluminescent detection system (Roche) according to the manufacturer's instructions.

RNAi experiment

Double-strand stealth RNAi oligonucleotides (ZNF512B-S183866 for ZNF512B and negative universal control medium GC duplex for negative control) were purchased from Invitrogen. The RNAi oligonucleotides were transfected into a cell line using Lipofectamine RNAiMAX reagent according to the manufacturer's instructions (Invitrogen). After 24 h, we also transfected with SBE_4-luciferase reporter vector and pRL-TK vector. We treated the cells with TGF-β (10 ng/ml) for 24 h, collected the cells and measured luciferase activity using the Dual-Luciferase Reporter Assay System (Toyo Ink).

Immuno-histochemistry

Autopsy specimens of lumbar spinal cord were obtained from clinically and histopathologically diagnosed ALS patients (13 males and 9 females, age 41–79 years) and from neurologically normal patients (4 males and 3 females, age 42–76 years). The autopsy times in relation to death for the cases and controls (average ± SD) were 4.0 ± 2.8 h and 4.5 ± 5.2 h, respectively. 6-μm-thick sections were prepared from paraffin-embedded tissues. The sections were microwaved for 20 min in 50 mM citrate buffer (pH 6.0) and then treated with a TNB blocking buffer (PerkinElmer) before incubation with an anti-ZNF512B antibody (Santa Cruz Biotechnology, 1:200). The immuno-reactivity was detected using EnVision+ System-HRP (Dako). The sections were photographed with an optical microscope (BX51, Olympus).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGMENTS

We thank all ALS patients who participated in the study. We also thank all members of Japanese ALS Association and all participating doctors and staff from collaborating institutes. The DNA samples used for this research were provided from the Leading Project for Personalized Medicine in the Ministry of Education, Culture, Sports, Science and Technology, Japan, and from JaCALS. JaCALS members included Drs M. Ito, J. Senda (Nagoya University), H. Takano (Niigata University), A. Kawata, H. Hayashi (Tokyo Metropolitan Neurological Hospital), I. Aiba (Higashi Nagoya National Hospital), A. Taniguchi (Mie University), Y. Izumi (University of Tokushima), M. Sakai, M. Konagaya (Suzuka National Hospital), H. Mizusawa (Tokyo Medical and Dental University), T. Yuasa (Kamagaya General Hospital), T. Fujita (Takumikai

Neurology Clinic), M. Ikeda, K. Okamoto (Gunma University), S. Akimoto, H. Sasaki (Hokkaido University), T. Imai (Miyagi National Hospital) and S. Kuzuhara (National Center Hospital of Neurology and Psychiatry).

Conflict of Interest statement. None declared.

FUNDING

This work was supported by grants from the Leading Project of Ministry of Education, Culture, Sports, Science and Technology Japan; Health and Labour, Sciences Research Grants for Research on Measures for Intractable Diseases and Comprehensive Research on Aging and Health from the Ministry of Health, Labour and Welfare, Japan; and by a Grant-in-Aid for Scientific Research (C) (19500314) from the Ministry of Education, Culture, Sports, Science and Technology Japan (A.I.).

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Familial Clusters of HTLV-1-Associated Myelopathy/ Tropical Spastic Paraparesis

Satoshi Nozuma[1], Eiji Matsuura[1]*, Toshio Matsuzaki[2], Osamu Watanabe[1], Ryuji Kubota[2], Shuji Izumo[2], Hiroshi Takashima[1]

1 Department of Neurology and Geriatrics, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima city, Japan, 2 Department of Molecular Pathology, Center for Chronic Viral Diseases, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima city, Japan

Abstract

Objective: HTLV-1 proviral loads (PVLs) and some genetic factors are reported to be associated with the development of HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). However, there are very few reports on HAM/TSP having family history. We aimed to define the clinical features and laboratory indications associated with HAM/TSP having family history.

Methods: Records of 784 HAM/TSP patients who were hospitalized in Kagoshima University Hospital and related hospitals from 1987 to 2012 were reviewed. Using an unmatched case-control design, 40 patients of HAM/TSP having family history (f-HAM/TSP) were compared with 124 patients suffering from sporadic HAM/TSP, who were admitted in series over the last 10 years for associated clinical features.

Results: Of the 784 patients, 40 (5.1%) were f-HAM/TSP cases. Compared with sporadic cases, the age of onset was earlier (41.3 vs. 51.6 years, $p<0.001$), motor disability grades were lower (4.0 vs. 4.9, $p=0.043$) despite longer duration of illness (14.3 vs. 10.2 years, $p=0.026$), time elapsed between onset and wheelchair use in daily life was longer (18.3 vs. 10.0 years, $p=0.025$), cases with rapid disease progression were fewer (10.0% vs. 28.2%, $p=0.019$), and protein levels in cerebrospinal fluid (CSF) were significantly lower in f-HAM/TSP cases (29.9 vs. 42.5 mg, $p<0.001$). There was no difference in HTLV-1 PVLs, anti-HTLV-1 antibody titers in serum and CSF, or cell number and neopterin levels in CSF. Furthermore, HTLV-1 PVLs were lower in cases with rapid disease progression than in those with slow progression in both f-HAM/TSP and sporadic cases.

Conclusions: We demonstrated that HAM/TSP aggregates in the family, with a younger age of onset and a slow rate of progression in f-HAM/TSP cases compared with sporadic cases. These data also suggested that factors other than HTLV-1 PVLs contribute to the disease course of HAM/TSP.

Citation: Nozuma S, Matsuura E, Matsuzaki T, Watanabe O, Kubota R, et al. (2014) Familial Clusters of HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis. PLoS ONE 9(5): e86144. doi:10.1371/journal.pone.0086144

Editor: Steven Jacobson, National Institutes of Health, United States of America

Received June 6, 2013; **Accepted** December 5, 2013; **Published** May 6, 2014

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Funding: This study was supported by Health and Labour Sciences Research Grants from the Ministry of Health Labour and Welfare and JSPS KAKENHI Grant Numbers 25293205 and 24133701. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: pine@m.kufm.kagoshima-u.ac.jp

Introduction

HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is characterized by slow progressive spastic paraparesis and positivity for anti-HTLV-1 antibodies in both serum and cerebrospinal fluid (CSF) [1,2]. Worldwide, at least 10–20 million people are infected with HTLV-1 [3]. However, although the majority of infected individuals remain lifelong asymptomatic carriers, approximately 2%–5% develop adult T-cell lymphomas [4,5] and another 0.25%–3.8% develop HAM/TSP [1,2]. Although the mechanisms underlying the development of HAM/TSP are not fully understood, several risk factors are closely associated with HAM/TSP. In particular, HTLV-1 proviral loads (PVLs) are significantly higher in HAM/TSP patients than in asymptomatic carriers and are also higher in genetic relatives of HAM/TSP patients than in non-HAM-related asymptomatic carriers [6]. Host genetic factors, including human leukocyte antigen (HLA) and non-HLA gene polymorphisms affect the occurrence of HAM/TSP [7], indicating that HTLV-1 PVLs and genetic backgrounds may influence individual susceptibility to HAM/TSP. Although several reports of familial adult T-cell lymphoma have been published [8,9], to our knowledge, there is only one case report of patient with HAM/TSP having family history (f-HAM/TSP) [10]. Hence, little is known about the prevalence and character of f-HAM/TSP cases. In this study, the characteristic clinical and laboratory features of f-HAM/TSP cases are defined and compared with those of sporadic cases.

Methods

Ethics Statement

This study was approved by the Institutional Review Boards of Kagoshima University. All participants provided written informed consent.

Design

We used an unmatched case-control design to identify the phenotypic features of f-HAM/TSP. f-HAM/TSP cases were identified as patients with multiple family members suffering from HAM/TSP. Controls were defined as HAM/TSP patients who were not genetically related to other HAM/TSP patients.

Subjects

f-HAM/TSP cases were extracted from our database of individuals diagnosed with HAM/TSP in Kagoshima University Hospital and related hospitals from 1987 to 2012. Controls included consecutive patients with sporadic HAM/TSP who were evaluated in our department between January 2002 and June 2012. HAM/TSP was diagnosed according to the World Health Organization diagnostic criteria, and the updated criteria of Castro-costa Belem [11]. Clinical information was obtained from the medical records of patient attendance at our hospital. In other cases, clinical data were obtained from the clinical records of patients or directly from the referring clinicians. Clinical variables included sex, age, age of onset, and initial symptoms. Neurological disabilities were assessed using Motor Disability Grading (MDG), modified from the Osame Motor Disability Scale of 0 to 10, as reported previously [12]. Motor disability grades were defined as follows: 5, needs one-hand support while walking; 6, needs two-hand support while walking; and 7, unable to walk but can crawl. We used a different assessment for the subgroup of more than grade 6 because their disease state significantly interfered with their lifestyle and necessitated the use of wheelchairs in daily life. The subgroup of patients with rapid progression was defined by deterioration of motor disability by more than three grades within two years. Anti-HTLV-1 antibody titers in serum and CSF were detected using enzyme-linked immunosorbent assays and particle agglutination methods (Fijirebio Inc, Tokyo, Japan). HTLV-1 PVLs in peripheral blood mononuclear cells (PBMCs) were assayed using quantitative PCR with the ABI PRISM 7700TM sequence detection system as reported previously [6].

Statistical Analysis

Data were analyzed using SPSS-20 (SPSS, Chicago, Illinois). Statistical analyses were performed using parametric (t-test) and non-parametric tests (Mann–Whitney test) for continuous variables and χ^2 (Pearsonχ^2 test/Fisher exact test) for categorical variables. Significant differences were then adjusted for potential confounders (age and sex) using multiple linear regression analysis. Survival was estimated according to the Kaplan–Meier method. The final endpoint was defined by a MDG score of 6. Patients with MDG scores of 6 almost wheelchair bound in daily life. The log rank test was used in Kaplan–Meier analyses. Differences were considered significant when $p<0.05$.

Results

Clinical characteristics of f-HAM/TSP

Of the 784 patients diagnosed with HAM/TSP between January 1987 and June 2012, 40 (5.1%) were f-HAM/TSP. The sex ratio was 33 males : 7 females. Of these 40 cases, 10 had parents or children (25.0%), 27 had siblings (67.5%), and three had other relatives (7.5%) diagnosed with HAM/TSP. Three individuals from one family were diagnosed with HAM/TSP, whereas only two individuals were diagnosed with HAM/TSP in all other families. In f-HAM/TSP cases, the age of onset was earlier (41.3 vs. 51.6 years, $p<0.001$), cases with rapid progression were fewer (10.0% vs. 28.2%, $p=0.019$), motor disability grades were lower (4.0 vs. 4.9, $p=0.043$) despite longer duration of illness (14.3 vs. 10.2 years, $p=0.026$), and time elapsed between onset and wheelchair use in daily life was longer (18.3 vs. 10.0 years, $p=0.025$) compared with sporadic cases. Sex and initial symptoms did not differ significantly between f-HAM/TSP and sporadic cases (Table 1). Twelve patients of f-HAM/TSP, and 38 of the 128 sporadic cases reached endpoint MDG scores of 6. Significant differences were then adjusted for potential confounders (age and sex) using multivariate analysis. Age of onset, duration of illness, MDG scores, and time elapsed between onset and wheelchair use in daily life remained significantly different after multivariate analysis (Table 1). The proportion of patients with rapid progression did not differ significantly between the groups, although there was a trend toward a higher proportion in sporadic cases. Kaplan–Meier analyses revealed that approximately 30% of both f-HAM/TSP and sporadic cases needed a wheelchair in daily life in 15 years after onset, and approximately 50% of patients from both groups needed it in 20 years after onset (Figure 1). Although sporadic patients needed wheelchairs earlier in most cases, the difference in the ratio of the patients with MDG score above six was not statistically significant between the groups. Finally, we compared differences in the age of onset between parent–child and sibling cases in f-HAM/TSP cases. Age of onset in parent–child f-HAM/TSP cases was significantly younger than that in sibling f-HAM/TSP cases (29.9±10.0 vs. 45.1±13.0 years, $p=0.002$).

Laboratory parameters and PVLs in f-HAM/TSP cases

Protein levels in CSF were significantly lower in f-HAM/TSP cases than in sporadic cases (29.9 vs. 42.5 mg/dl, $p<0.001$). This difference in CSF protein level remained significant after multivariate analysis. Anti-HTLV-1 antibody titers in serum and CSF, and cell numbers and neopterin levels in CSF were not significantly different between two groups. Moreover, HTLV-1 PVLs did not differ significantly. (Table 2).

Clinical and laboratory findings in patients with rapid disease progression

Previous studies suggest that an older age of onset is associated with rapid disease progression. Similar findings are found in the present study. The percentage of rapid progression tended to increase with older age of onset in both f-HAM/TSP and sporadic groups (Figure 2). We compared the characteristics of 124 sporadic HAM/TSP patients with rapid and slow progression who were admitted to Kagoshima University Hospital in series during the last 10 years (Table 3). Patients with rapid progression were significantly older at onset than those with slow progression (62.3 vs. 47.4 years, $p<0.001$), although sex and initial symptoms did not differ significantly between rapid and slow progression groups. However, the time elapsed between onset and wheelchair use in daily life was markedly shorter among patients with rapid progression (1.5 vs. 14.4 years, $p<0.001$). Cell numbers, protein levels, and anti-HTLV-1 antibody titers in CSF were significantly higher in patients with rapid progression than in those with slow progression (11.6 vs. 3.2, $p<0.001$; 55.3 vs. 36.7 mg/dl, $p<0.001$; 1,251 vs. 416, $p<0.014$, respectively). Interestingly, HTLV-1 PVLs were significantly lower in patients with rapid progression than in those with slow progression (370 vs. 1,245 copies, $p<0.001$). Furthermore, we compared the differences between women and men in patients with rapid progression because the reason remains unknown why HAM/TSP is common in female

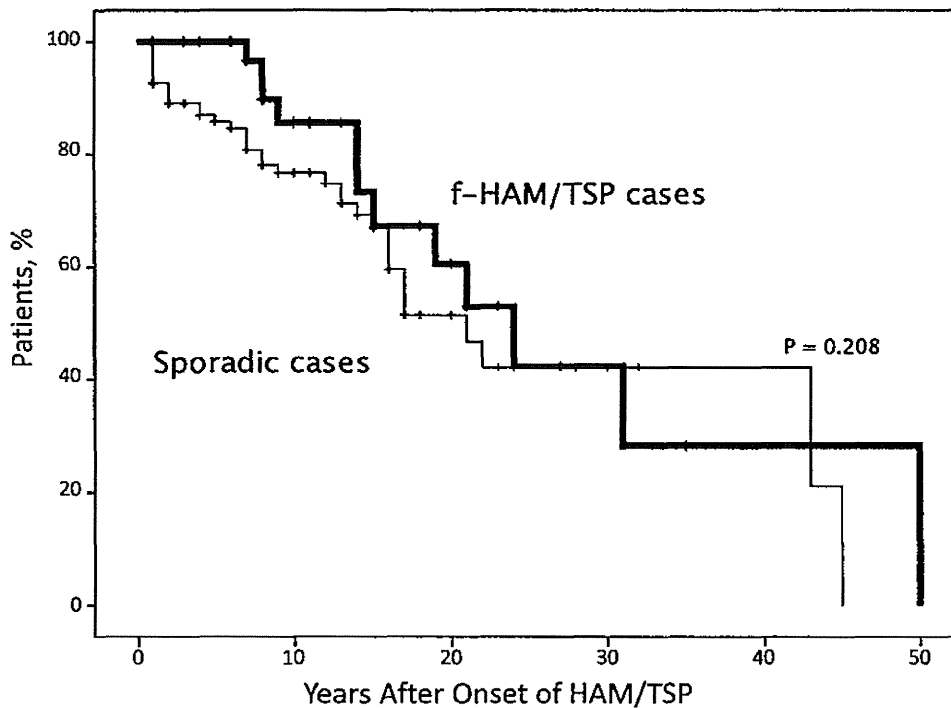

Figure 1. Kaplan-Meier estimates of the time from disease onset to assignment of motor disability scores of 6. In sporadic cases, more patients reached the score of six at an early stage; however, the difference was not significant. Approximately 30% of both f-HAM/TSP cases and sporadic cases needed a wheelchair in daily life in 15 years after onset and approximately 50% of patients from both groups needed a wheelchair in 20 years after onset.
doi:10.1371/journal.pone.0086144.g001

than in male. There was no significant difference between women and men in the age of onset (61.5 y.o.±12.6 vs. 62.7 y.o.±12.5), in the incidence of rapid progression (26.3% vs. 32.3%) and in MDG score (5.4 vs. 5.0; mean).

Discussion

We demonstrated that among 784 HAM/TSP patients, 40 (5.1%) had family members with the disease. The lifetime risk of developing HAM/TSP is 0.25% of HTLV-1 carriers in Japan

Table 1. Clinical features of f-HAM/TSP cases or sporadic cases of HAM/TSP.

	f-HAM/TSP cases (40 cases)	Sporadic cases (124 cases)	p value	p value†
Female ratio (%)	78.8% (7 males : 33 females)	66.4% (31 males : 93 females)	NS	
Age	55.6±13.0 (23–79)	61.8±12.5 (15–83)	**0.008**	
Age of onset	41.3±13.9 (14–65)	51.6±15.9 (13–78)	**<0.001**	**0.017**
Duration of illness (years)	14.3±11.4 (1–49)	10.2±9.6 (0–45)	**0.026**	**0.017**
Initial symptoms				
Gait disturbance	50.0%	52.4%	NS	
Urinary disturbance	32.5%	26.6%	NS	
Sensory disturbance	12.5%	14.5%	NS	
Others	5%	6.5%	NS	
Rapid disease progression	4 cases (10.0%)	35 cases (28.2%)	**0.019**	0.069
Motor disability score	4.0±2.0 (0–7)	4.9±1.5 (0–8)	**0.043**	**0.036**
Score more than 6	12 cases (30.0%)	38 cases (30.7%)	NS	
Time elapsed between onset and wheelchair use in daily life (years)	18.3±12.4 (7–50)	10.0±10.4 (1–45)	**0.025**	**0.020**

Data are presented as mean values ± s.d., (range),
†Adjusted for age and sex.
doi:10.1371/journal.pone.0086144.t001

Table 2. Laboratory findings of familial clusters or sporadic cases of HAM/TSP.

	f-HAM/TSP cases (40cases)	Sporadic cases (124 cases)	p value	p value[†]
Anti-HTLV-1 antibodies*				
Titer in Serum	20,787±31,004, N=37	31,009±36,075, N=109	NS	
Titer in CSF	2,310±11,741, N=31	672±1,274, N=111	NS	
Cerebrospinal fluid				
Cell number (/mm^3)	3.0±2.5, N=25	5.7±10.0, N=109	NS	
Protein (mg/dl)	29.9±9.4, N=22	42.5±19.3, N=109	<0.001	0.007
Neopterin (pmol/ml)	83.2±118.1, N=18	38.3±56.8, N=35	NS	
HTLV-1 proviral loads (Copies/10^4 PBMCs)	930±781, N=32	968±1,746, N=101	NS	

* Particle Aggregation Method.
Data are presented as mean values ± s.d., N=sample number,
[†]Adjusted for age and sex.
doi:10.1371/journal.pone.0086144.t002

[13]. Although clustering of familial adult T-cell lymphomas has been reported [8,9], to our knowledge the prevalence of familial clusters of HAM/TSP has not been described. A study in Peru showed that 30% of HAM/TSP patients have family members with paralytic neurological disorders, but the cause of paralysis was not evaluated [14]. In the present study, we included f-HAM/TSP diagnosed in medical institutions and excluded cases with a family history of neurological disorders. Thus, the actual incidence rates of f-HAM/TSP may be higher than those reported here. Interestingly, although HTLV-1 PVL has been associated with the development and clinical progression of HAM/TSP [15–17], there was no significant difference between f-HAM/TSP and sporadic cases in the present study. Because previous studies reported that HTLV-1 PVLs of asymptomatic carriers in relatives of HAM/TSP patients were higher than those in non-HAM-related asymptomatic carriers [6], relatives of HAM/TSP are believed to be at a higher risk of developing HAM/TSP. Interestingly, our data suggest that HAM/TSP patients aggregate in families and factors other than HTLV-1 PVLs may contribute to HAM/TSP.

Compared with sporadic HAM/TSP, the clinical characteristics of f-HAM/TSP have a younger age of onset and longer time elapsed between onset and wheelchair use in daily life. Although we were unable to identify the reason for earlier onset among f-HAM/TSP cases, one can speculate that mild symptoms, such as urinary and sensory disturbances, may be identified earlier by family members who are familiar with HAM/TSP symptoms. However, the present data show no difference in initial symptoms

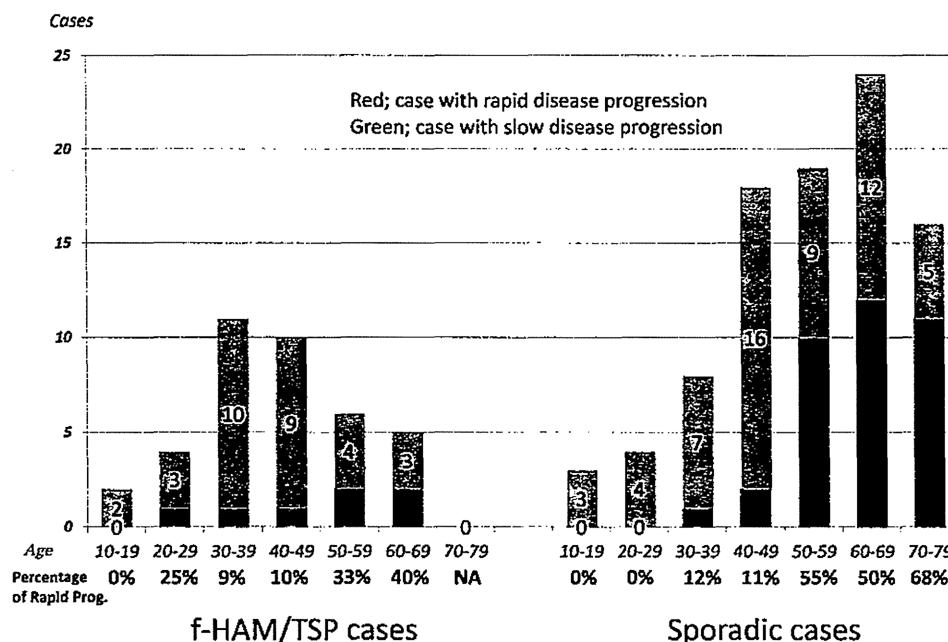

Figure 2. Age-specific proportions of rapid disease progression. The proportion of cases with rapid disease progression tended to increase with the older age of onset.
doi:10.1371/journal.pone.0086144.g002

Table 3. Clinical and laboratory findings of sporadic HAM/TSP with rapid/slow disease progression.

Type of disease progression	Rapid progression	Slow progression	p value
Female ratio (%)	71.4% (10 males : 25 females)	76.4% (21 males : 68 females)	NS
Age of onset	62.3±9.6, N=35	47.4±15.9, N=89	**<0.001**
Age of onset of f-HAM/TSP cases	60.5±3.7, N=4	39.2±12.9, N=36	**0.002**
Duration between onset and inability to walk alone (years)	1.5±0.9, N=13	14.4±10.4, N=25	**<0.001**
Anti-HTLV-1 antibodies*			
Titer in Serum	31,894±36,845, N=34	30,608±35,965, N=75	NS
Titer in CSF	1,251±1,800, N=34	416±852, N=77	**0.014**
Cerebrospinal fluid			
Cell number (/mm^3)	11.6±16.6, N=34	3.2±3.5, N=75	**<0.001**
Protein (mg/dl)	55.3±24.3, N=34	36.7±13.0, N=75	**<0.001**
Neopterin (pmol/ml)	74.9±107.9, N=8	27.4±23.4, N=27	0.255
HTLV-1 proviral loads (Copies/10^4 PBMCs)	370±327, N=32	1,245±2,046, N=69	**<0.001**

* Particle Aggregation Method.
Data are presented as mean values ± s.d., N=sample number.
doi:10.1371/journal.pone.0086144.t003

between f-HAM/TSP and sporadic cases. In all cases, the age of onset and initial symptoms of HAM/TSP were evaluated by the neurologists during hospitalization. Because inflammatory processes are less marked in f-HAM/TSP cases, as indicated by significantly lower protein levels in CSF, f-HAM/TSP cases may show slow progression of disease.

We need to discuss the possibility that the two groups compared represent different mode of HTLV transmission, i.e. vertical vs. sexual transmission. To clarify genetic backgrounds, sporadic HAM/TSP with seropositive carrier family members may be a more appropriate control, but are not available at present. The incidence of female cases showing no significant differences between f-HAM/TSP and sporadic cases, and between rapid and slow disease progression, might suggest less possibility of sporadic cases due to sexual transmission.

Although the subgroup of patients with rapid progression has not been clearly defined, previous studies suggest that rapid progression occurs in 10%–30% of all patients with HAM/TSP [12,14,16], and is associated with an older age of onset [14–16]. In the present study, the age of onset in patients with rapid progression was significantly older than that in patients with slow progression between f-HAM/TSP and sporadic cases, and the proportion of patients with rapid progression increased with the older age of onset (Figure 2). Among sporadic cases, cell numbers and protein levels in CSF were significantly higher in patients with rapid progression, suggesting that inflammation is more active in the spinal cords of patients with rapid progression and that cytotoxic T-lymphocyte (CTL) immune responses may be more intensive. Therefore, lower PVLs in PBMCs of patients with rapid disease progression may be attributed to the strong killing ability of the CTL. However, PVLs were higher in PBMCs of patients with HAM/TSP than in asymptomatic carriers [6]. In addition, the killing ability of CTLs in patients with HAM/TSP does not differ from that in asymptomatic carriers [18]. Hence, strong immune responses may be associated with the disease course. The onset of disease may require other factors that lead to strong immune responses. A late onset may also be associated with alterations of the immune function in HTLV-1-infected patients. Indeed, an increased age has been associated with autoimmune disorders, such as myasthenia gravis and rheumatoid arthritis, and may be partly explained by immune intolerance and accumulation of autoantibodies in older individuals [19,20].

In conclusion, we demonstrated that patients with HAM/TSP aggregate in some families. Compared with sporadic cases, the age of onset was younger and rates of disease progression were slower among familial cases, whereas HTLV-1 PVLs did not differ between f-HAM/TSP and sporadic groups. The present data suggest that factors other than HTLV-1 PVLs contribute to the disease course of HAM/TSP. Our data also suggested strong immune responses in the spinal cord of HAM/TSP patients with rapid progression. Further studies on HTLV-1, immune response to HTLV-1 and genetic factor in patients with rapid progression might provide new insights into HAM/TSP pathogenesis.

Acknowledgments

We would like to express the deepest appreciation to Yuka Komai for collecting information from patients and Dr. Moe Moe Aye for reading our manuscript.

Author Contributions

Conceived and designed the experiments: HT SI OW. Performed the experiments: SN EM. Analyzed the data: SN EM. Contributed reagents/materials/analysis tools: SN EM TM RK. Wrote the paper: SN EM.

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Saito et al. Retrovirology 2013, 10:51
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Increased expression of OX40 is associated with progressive disease in patients with HTLV-1-associated myelopathy/tropical spastic paraparesis

Mineki Saito[1,6*], Reiko Tanaka[1], Shiho Arishima[2], Toshio Matsuzaki[3], Satoshi Ishihara[4], Takashi Tokashiki[4], Yusuke Ohya[4], Hiroshi Takashima[3], Fujio Umehara[5], Shuji Izumo[2] and Yuetsu Tanaka[1]

Abstract

Background: OX40 is a member of the tumor necrosis factor receptor family that is expressed primarily on activated $CD4^+$ T cells and promotes the development of effector and memory T cells. Although OX40 has been reported to be a target gene of human T-cell leukemia virus type-1 (HTLV-1) viral transactivator Tax and is overexpressed *in vivo* in adult T-cell leukemia (ATL) cells, an association between OX40 and HTLV-1-associated inflammatory disorders, such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), has not yet been established. Moreover, because abrogation of OX40 signals ameliorates chronic inflammation in animal models of autoimmune disease, novel monoclonal antibodies against OX40 may offer a potential treatment for HTLV-1-associated diseases such as ATL and HAM/TSP.

Results: In this study, we showed that OX40 was specifically expressed in $CD4^+$ T cells naturally infected with HTLV-1 that have the potential to produce pro-inflammatory cytokines along with Tax expression. We also showed that OX40 was overexpressed in spinal cord infiltrating mononuclear cells in a clinically progressive HAM/TSP patient with a short duration of illness. The levels of the soluble form of OX40 (sOX40) in the cerebrospinal fluid (CSF) from chronic progressive HAM/TSP patients or from patients with other inflammatory neurological diseases (OINDs) were not different. In contrast, sOX40 levels in the CSF of rapidly progressing HAM/TSP patients were higher than those in the CSF from patients with OINDs, and these patients showed higher sOX40 levels in the CSF than in the plasma. When our newly produced monoclonal antibody against OX40 was added to peripheral blood mononuclear cells in culture, HTLV-1-infected T cells were specifically removed by a mechanism that depends on antibody-dependent cellular cytotoxicity.

Conclusions: Our study identified OX40 as a key molecule and biomarker for rapid progression of HAM/TSP. Furthermore, blocking OX40 may have potential in therapeutic intervention for HAM/TSP.

Keywords: HTLV-1, OX40, HAM/TSP, ADCC, Immunotherapy

* Correspondence: mineki@med.kawasaki-m.ac.jp
[1]Department of Immunology, Graduate School of Medicine, University of the Ryukyus, 207 Uehara, Okinawa 903-0215, Japan
[6]Present Address: Department of Microbiology, Kawasaki Medical School, 577 Matsushima, Kurashiki 701-0192, Japan
Full list of author information is available at the end of the article

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Background

Human T-cell leukemia virus type 1 (HTLV-1) was the first human oncogenic retrovirus to be identified and associated with distinct human diseases such as adult T-cell leukemia (ATL) [1,2] and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [3,4]. HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities [5]. In addition to neurological symptoms, some HAM/TSP patients also exhibit autoimmune-like disorders such as uveitis, arthritis, T-lymphocyte alveolitis, polymyositis, and Sjögren syndrome [6]. Major pathological features of HAM/TSP are chronic inflammation of the spinal cord, characterized by perivascular lymphocytic cuffing and parenchymal lymphocytic infiltration that includes HTLV-1-infected $CD4^+$ T cells [7]. In HAM/TSP patients, the median HTLV-1 proviral load (PVL), which reflects the *in vivo* number of HTLV-1-infected lymphocytes, is more than 10 times higher than that in asymptomatic carriers (ACs) [8]. An increase in PVL typically coincides with worsening of clinical symptoms [9]. Increased concentrations of inflammatory markers such as neopterin [10], tumor necrosis factor (TNF)-α, interleukin (IL)-6, and interferon (IFN)-γ [11], and increase in HTLV-1 antigen-specific intrathecal antibody synthesis [12] have been observed in the cerebrospinal fluid (CSF) of HAM/TSP patients. More recently, it has been reported that IFN-stimulated genes were overexpressed in circulating leukocytes and the expression correlated with the clinical severity of HAM/TSP [13]. These findings indicate that a pro-inflammatory environment, associated with increased numbers of HTLV-1-infected cells, is a characteristic immunologic profile of HAM/TSP.

OX40, also known as CD134 or TNFRSF4, is a member of the TNF co-stimulatory receptor family and is expressed on activated T cells [14]. OX40 is specifically up-regulated by the HTLV-1 viral transactivator Tax [15,16]. The ligand of OX40 (OX40L), which belongs to the TNF superfamily, was first identified as glycoprotein 34 (gp34) on HTLV-1-transformed cells [17], and it was later found to bind OX40 [18]. OX40-OX40L interactions alter the activity and differentiation of many kinds of immune cells, including regulatory T cells (Tregs), T cells, antigen-presenting cells (APCs), natural killer (NK) cells, and natural killer T (NKT) cells [14]. Previous studies have reported that OX40 is constitutively expressed in ATL cells and participate in cell adhesion [19]. Specifically, OX40 and OX40L directly mediate the adhesion of activated normal $CD4^+$ T cells, as well as HTLV-1-transformed T cells, to vascular endothelial cells [20]. Immunohistochemical staining of skin biopsy specimens from ATL patients also showed constitutive expression of OX40, suggesting its role in leukemic cell infiltration, in addition to *in vivo* cell adhesion [19].

Recent research has also shown the importance of OX40-OX40L interactions in the development of immune-mediated diseases. In particular, a strong reduction in disease severity or a complete lack of disease has been reported when OX40 or OX40L is absent or neutralized in animal models of multiple sclerosis (MS) [21], allergic asthma [22], colitis [23], diabetes [24], arthritis [25], atherosclerosis [26], graft versus host disease [27], and allograft rejection [28]. Although HTLV-1 causes an aggressive T cell malignancy (i.e., ATL) and chronic inflammatory diseases such as HAM/TSP, an association of OX40 with the inflammatory diseases observed in HTLV-1-infected individuals has not yet been established.

In this study, we investigated the expression of OX40 in HAM/TSP patients and found that the increased expression of OX40 is associated with the rapidly progressive disease. We also used an in-house monoclonal antibody (mAb) against human OX40 to test the potential of OX40 as a target molecule for immunotherapy.

Results

Tax-dependent constitutive expression of OX40 in HTLV-1-infected T cells

OX40 and OX40L have been reported to be overexpressed in HTLV-1-infected human T-cells lines [15,19,20]. These findings were obtained using northern blot or western blot analysis using whole cells; hence, our first aim was to confirm and extend these findings at the single-cell level using flow cytometry. Therefore, we used mAbs against human OX40 (clone B-7B5) and human OX40L (clone 5A8) produced in our laboratory. We analyzed six HTLV-1-infected human T-cell lines (HUT-102, MT-1, MT-2, MT-4, SLB-1, and C5/MJ). C5/MJ, SLB-1, and MT-4 cells have not been previously tested for OX40/OX40L expression. As shown in Figure 1A, expression levels were different in each cell line: OX40 was overexpressed on the surface of the Tax positive (Tax+) T-cell lines (HUT-102, MT-2, MT-4, SLB-1, and C5/MJ), but OX40 was not expressed on the surface of the Tax negative (Tax-) MT-1 cell line or the uninfected T cell line (CEM-OX40L). Consistent with previous studies, these findings suggested that OX40 expression is Tax dependent. In contrast, OX40L was not always expressed on the surface of HTLV-1-infected human T-cell lines or on the uninfected T cell line (CEM-OX40), irrespective of Tax expression (Figure 1B).

Next, we confirmed whether OX40 and OX40L protein expression on the cell surface is induced by Tax at the single-cell level by flow cytometry. We used JPX-9 cells [29], a Jurkat (HTLV-1 negative human T cell leukemia cell line) subclone generated by stable transfection of a functional Tax expression-plasmid vector, and induced Tax expression by adding $CdCl_2$ into the culture medium (final concentration: 10 μM). As shown in Figure 1C, treatment of JPX-9 cells with $CdCl_2$ induced expression of

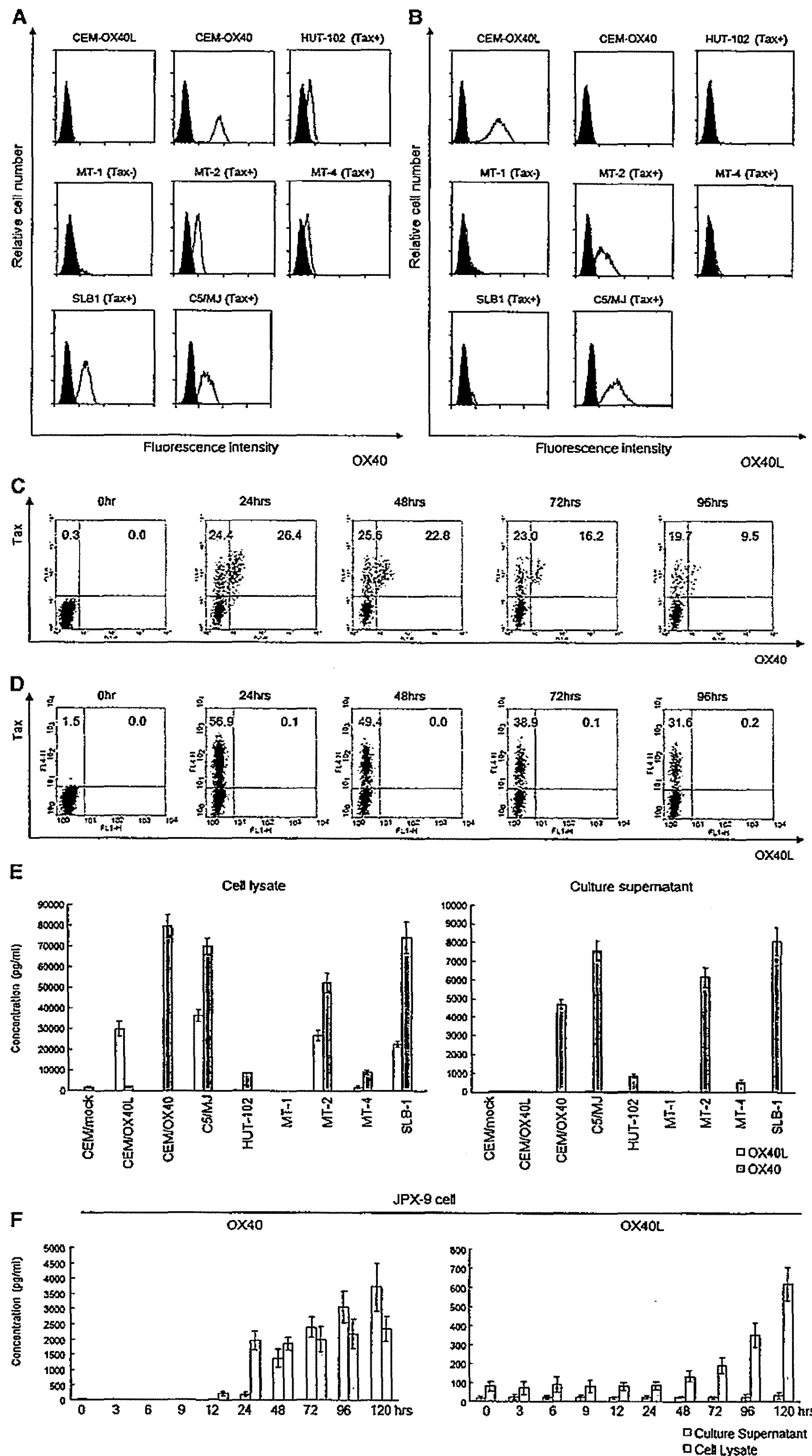

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Figure 1 Tax-dependent constitutive expression of OX40 in HTLV-1-infected T-cell lines and Tax-inducible JPX-9 cell line. **A.** Representative histograms of OX40 expression in 6 HTLV-1 infected T-cell lines (HUT-102, MT-1, MT-2, MT-4, C5/MJ, SLB-1) and two HTLV-1-uninfected T-cell lines (CEM-OX40L and CEM-OX40). Shaded histograms represent the isotype control. Tax+ or Tax- means whether these cells express Tax (Tax+) or not (Tax-). **B.** Representative histograms of OX40L expression in 6 HTLV-1 infected T-cell lines (HUT-102, MT-1, MT-2, MT-4, C5/MJ, SLB-1) and two HTLV-1-uninfected T-cell lines (CEM-OX40L and CEM-OX40). Shaded histograms are isotype controls. **C.** Flow cytometric analysis of expression of OX40 after induction of Tax in JPX-9 cells. **D.** Flow cytometric analysis of expression of OX40L after induction of Tax in JPX-9 cells. **E.** Soluble OX40 and OX40L levels in cell culture supernatant and cell lysate from 6 HTLV-1 infected T-cell lines (HUT-102, MT-1, MT-2, MT-4, C5/MJ, SLB-1) and three HTLV-1-uninfected T-cell lines (CEM-mock, CEM-OX40L and CEM-OX40). **F.** Soluble OX40 and OX40L levels in cell culture supernatant and cell lysate from JPX-9 cell line treated with $CdCl_2$ along with the induction of viral transactivator Tax.

Tax, and OX40 was expressed exclusively in cells that also expressed Tax. In contrast, OX40L was not expressed in JPX-9 cells even after 96 hours post Tax-induction (Figure 1D).

Previous reports indicated that the soluble forms of OX40 (sOX40) and OX40L (sOX40L) were detectable in serum of patients with autoimmune disease and cancer [30,31]. We therefore examined whether sOX40 and sOX40L levels were elevated in culture supernatants from HTLV-1 infected T-cell lines and JPX-9 cells before and after induction of Tax. In agreement with our flow cytometry data (Figure 1A), sOX40 was detected in both culture supernatants and cell lysates of Tax positive C5/MJ, HUT102, MT-2, MT-4, and SLB-1 cells (Figure 1E, gray bar). However, sOX40L was not detected in culture supernatants of any of the samples tested, but it was readily detectable in cell lysates of Tax positive C5/MJ, MT-2, MT-4 and SLB1 cells (Figure 1E, light gray bar). We next examined whether soluble OX40 and OX40L are induced by Tax in JPX-9 cells. Addition of $CdCl_2$ to the culture medium of JPX-9 cells resulted in a concomitant increase in sOX40 expression within 24 hours, indicating a strong correlation and functional link between Tax and sOX40 expression (Figure 1F, left panel). Interestingly, although OX40L was already present before induction of Tax, OX40L expression was increased after 24 hours but was never released into the culture supernatant as sOX40L within 120 hours after induction of Tax (Figure 1F, right panel).

Functional OX40 is specifically expressed on the surface of T cells naturally infected with HTLV-1 that have the potential to produce pro-inflammatory cytokines

Next, we tested whether OX40 or OX40L expression is also activated in naturally infected T cells isolated directly from HTLV-1-infected individuals. PBMCs were collected from three non-infected controls (NCs), three ACs, and four HAM/TSP patients. PBMCs were isolated from blood samples and harvested directly, or after a 16-hour in vitro cultivation in the absence of any growth factors or mitogens. After harvesting, cell samples were fixed and processed for concomitant detection of Tax, OX40, or OX40L, and CD4 expression by flow cytometry. Similar to the findings for JPX-9 cells, OX40 was detected with an anti-OX40 mAb (clones B-7B5) after 16 hours of in vitro cultivation (Figure 2A), but OX40L was not detected in cultured PBMCs from a HAM/TSP patient (HAM/TSP1) (Figure 2B). Figure 2C shows that the Tax protein was detected in $CD4^+$ T cells after cultivation. Similar to the JPX-9 cell experiments, OX40 was expressed almost exclusively in the naturally infected $CD4^+$ T cells that also expressed Tax (Figure 2D). Similar findings were observed in all samples tested, irrespective of disease status (i.e., HAM/TSP or ACs) (Additional file 1: Figure S1 and Additional file 2: Table S1). The cells from NCs did not express either OX40 or Tax in $CD4^+$ T cells, before or after cultivation (data not shown). Real time RT-PCR also showed that mRNA expression of HTLV-1 tax and OX40 in $CD4^+$ T cells was increased after cultivation, both in HAM/TSP patients and ACs (Figure 2E).

It has recently been reported [32], that the expression of another co-stimulatory member of the TNFR family, 4-1BB, is also up-regulated ex vivo in $CD4^+$ T cells from HTLV-1-infected individuals, and it was found to be correlated with Tax expression (Additional file 1: Figure S2A and B). However, the expression of OX40 is more specific for Tax^+CD4^+ cells than 4-1BB (Figure 2D and Additional file 1: Figure S2C).

Next, we sought to determine if OX40, expressed on the surface of Tax^+CD4^+ T cells from HTLV-1-infected individuals, is functional. We incubated aliquots of Fc-blocked PBMCs with biotinylated recombinant soluble OX40L at a concentration of 2.5 mg/ml for 30 min on ice. Cells were then fixed and processed for concomitant detection of Tax, CD4, and PE-streptavidin by flow cytometry. As shown in Additional file 1: Figure S3, the frequency of $CD4^+$ T cells that were positively stained with biotinylated recombinant soluble OX40L and PE-streptavidin was similar to the percentage of $CD4^+$ T cells stained by anti-OX40 mAb, indicating that these cells expressed functional OX40.

We further analyzed if $CD4^+OX40^+$ T cells in HAM/TSP patients were capable of producing the inflammatory and neurotoxic cytokines, IFN-γ and TNF-α, which, according to the bystander damage hypothesis, could cause central nervous system (CNS) inflammation and demyelination seen in HAM/TSP patients [33,34]. The frequency of pro-inflammatory cytokine positive cells within the $OX40^+CD4^+$ and Tax^+CD4^+ populations from

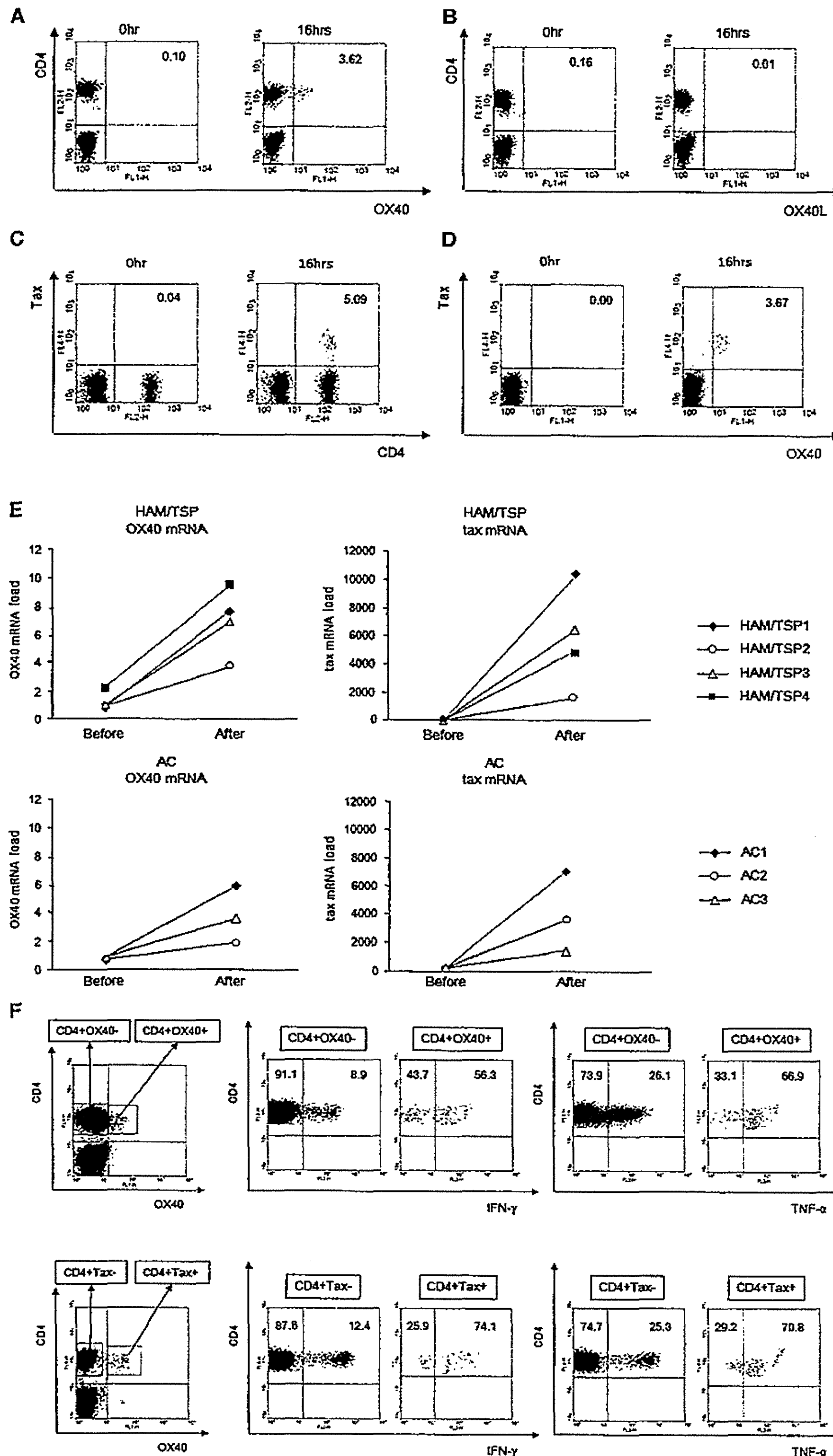

Figure 2 (See legend on next page.)

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Figure 2 OX40 is specifically expressed on the surface of T cells naturally infected with HTLV-1 that have the potential to produce pro-inflammatory cytokines. A. OX40 was detected on $CD4^+$ T cells of HAM/TSP patient with anti-OX40 mAb (clones B-7B5) after 16 hours *in vitro* cultivation in the absence of any growth factors or mitogen. **B.** OX40L was not detected on $CD4^+$ T cells of HAM/TSP patient with anti-OX40L mAb (clones 5A8) after 16 hours *in vitro* cultivation in the absence of any growth factors or mitogen. **C.** Tax protein was detected in $CD4^+$ T cells of HAM/TSP patient after 16 hours *in vitro* cultivation. **D.** OX40 was expressed almost exclusively in naturally infected $CD4^+$ T cells that also expressed Tax in HAM/TSP patient. **E.** Both HTLV-1 tax and OX40 mRNA expression in $CD4^+$ T cells was increased after 16 hours *in vitro* cultivation. **F.** The frequency of pro-inflammatory cytokine positive cells within the $OX40^+CD4^+$ and Tax^+CD4^+ populations from HTLV-1 infected individuals are significantly higher than $OX40^-CD4^+$ and Tax^-CD4^+ T cells, respectively (p<0.001, Student's t- test). One representative experiment of HAM/TSP patient (HAM/TSP1) is shown.

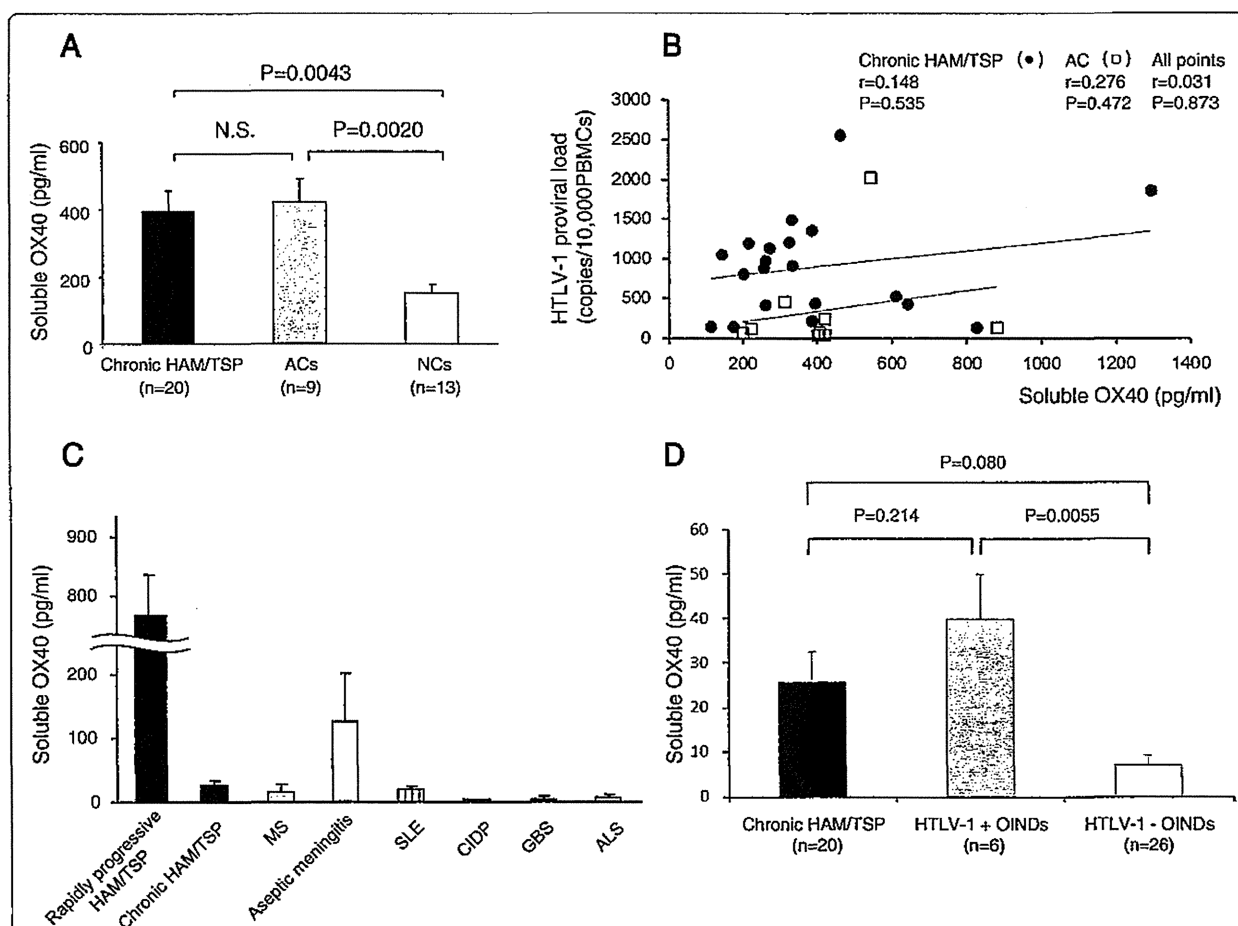

Figure 3 Increased expression of OX40 in vivo in rapidly progressive HAM/TSP patients. A. The plasma levels of soluble OX40 (sOX40) measured by ELISA. The plasma levels of sOX40 in typical HAM/TSP patients (chronic HAM: n=20), asymptomatic carriers (ACs: n=9) and normal uninfected healthy controls (NCs: n=13). **B.** No correlation between the plasma levels of sOX40 and HTLV-1 proviral load (tax copies/ 10,000PBMCs) from 29 HTLV-1 infected individuals (20 chronic HAM/TSP patients and 9 ACs). Data were analyzed by Spearman rank correlation. **C.** The cerebrospinal fluid (CSF) levels of sOX40 in rapidly progressive HAM/TSP patients (n=3), chronic HAM/TSP patients (n=22) and other neurological diseases including multiple sclerosis (MS) (n=12), aseptic meningitis (n=8), systemic lupus erythematosus (SLE) with neurological manifestations (n=5), chronic inflammatory demyelinating polyneuropathy (CIDP) (n=9), Guillain-Barré syndrome (GBS) (n=6), and amyotrophic lateral sclerosis (ALS) (n=9). Chronic HAM/TSP means typical cases fulfilling diagnostic criteria and rapidly progressive HAM/TSP is defined by patients' incapacity to walk unaided within three months after symptoms' onset. **D.** The levels of sOX40 in the CSF from HTLV-1 infected other inflammatory neurological diseases (HTLV-1+ OINDs), i.e. any inflammatory neurological disorders except for HAM/TSP which occurred in HTLV-1 infected individuals, was not significantly different from that of chronic HAM/TSP, whereas the levels of sOX40 from HTLV-1+ OINDs was significantly increased than that of non-infected OINDs (HTLV-1- OINDs). HTLV-1+ OINDs: 1 multiple sclerosis (MS), 1 SLE with neurological manifestations, 4 aseptic meningitis. HTLV-1- OINDs: 9 MS, 5 SLE with neurological manifestations, 7 CIDP, 5 GBS.

HAM/TSP patients are significantly higher than $OX40^-$ $CD4^+$ and Tax^-CD4^+ T cells, respectively ($p < 0.001$, Student's *t*- test) (Figure 2F and Table 1).

Increased expression of OX40 in vivo in rapidly progressive HAM/TSP patients

To investigate if OX40 expression is associated with in vivo pathogenesis of HAM/TSP, we first measured the plasma concentration of sOX40 and sOX40L in 20 chronic HAM/TSP patients, 9 ACs, and 13 NCs by ELISA by using monoclonal antibodies generated in our laboratory (Figure 3A). None of the samples had detectable levels of sOX40L (data not shown), but we could readily detect sOX40. The median level of sOX40 in NCs was 149.5 pg/ml (range 13–328 pg/ml). Significantly higher sOX40 levels were found in chronic HAM/TSP patients (median 395.2 pg/ml, range 113–1295 pg/ml) and ACs (median 423.8 pg/ml, range 201–881 pg/ml) than in NCs (p=0.0043 for differences between HAM/TSP and NCs, p=0.0020 for differences between ACs and NCs). The difference between chronic HAM/TSP patients and ACs was not statistically significant. No positive correlation was found between sOX40 in the plasma and HTLV-1 PVL in infected individuals (i.e., chronic HAM/TSP patients and ACs) (Spearman's rank correlation coefficient n=29, r=0.031, P=0.873; Figure 3B). We then tested disease specificity by measuring the levels of sOX40 in the CSF from both rapidly progressive and chronic HAM/TSP patients, and in patients with other neurological disorders, with and without inflammation (e.g., 12 MS, 8 aseptic meningitis, 5 systemic lupus erythematosus with neurological manifestations, 9 chronic inflammatory demyelinating polyneuropathy, 6 Guillain-Barré syndrome, and 9 amyotrophic lateral sclerosis patients). As shown in Figure 3C, CSF sOX40 levels were markedly increased in patients with rapidly progressive HAM/TSP (n=3) and aseptic meningitis (n=8). The CSF sOX40 levels in other HTLV-1-infected inflammatory neurological diseases, i.e. any inflammatory neurological disorders except for HAM/TSP that occurred in HTLV-1 infected individuals, (HTLV-1+ OINDs, n=6) was not significantly different from chronic HAM/TSP (n=20), whereas the sOX40 level of HTLV-1+ OINDs was significantly increased compared to non-infected OINDs (HTLV-1- OINDs, n=26; Figure 3D).

Of the HAM/TSP patients studied, paired CSF and plasma samples, i.e., blood and CSF were collected on the same day, were available for six patients. HAM/TSP patients No.10-12 had a lower concentration of sOX40 in the CSF than in the plasma (Table 2), and the patients showed a typical clinical course of HAM/TSP (i.e. slowly progressive symmetrical myelopathy) and had no history of rapid exacerbation. In contrast, HAM/TSP patients No.13-15, who had higher concentrations of sOX40 in the CSF than in the plasma, showed a rapidly progressive clinical course (i.e. patients became unable to walk within three months after onset of initial symptoms).

Expression of OX40 in inflammatory mononuclear cells in spinal cord lesions of HAM/TSP patient with short disease duration and progressive symptoms

We also examined autopsy specimens from HAM/TSP patients by immunohistochemical staining. Although there was reduced or no OX40 protein expression in HAM/TSP patients who had a long duration of illness and who no longer had active inflammation (a representative example is shown in Figure 4A), we observed marked OX40 expression in inflammatory round-shaped mononuclear cells around the blood vessels in spinal cord lesions from one HAM/TSP patient (Figure 4B). This patient (patient 1 in refs [35-38], who had a shorter disease duration of up to 2.5 years after the onset of neurological symptoms) showed predominant infiltration of $CD4^+$ T cells [36] that also expressed tax mRNA [38], pro-inflammatory cytokines [37], and matrix metalloproteinases [39]. In contrast, we observed only low background staining for OX40L in spinal cord tissues of all the HAM/TSP patients examined (a representative example is shown in Figure 4C) compared to positive control (Figure 4D).

Anti-OX40 monoclonal antibody specifically eliminated naturally infected $CD4^+$ T cells via antibody-dependent cell-mediated cytotoxicity (ADCC) in cultured PBMCs

We investigated the role of OX40 in HTLV-1 naturally infected $CD4^+$ T cells, by testing the effects of an anti-human OX40 mAb on Tax expression. As shown in Figure 5, anti-OX40 mAb (clone B-7B5) reduced the percentage of Tax-positive cells, whereas the isotype control mAb (clone 2C2: anti-HIV-1 gp21, mouse IgG1) had no effect on Tax expression (Figure 5, 1st, 2nd, and 3rd panels from left). Culture of PBMCs with anti-CD16/CD32 (Fc receptor) antibody to block Fc receptors abolished Tax suppression by anti-OX40 mAb (Figure 5, 4th panels from left), suggesting that the effect of the anti-OX40 mAb (B-7B5) is mainly mediated by ADCC. We further tested the effects of the $F(ab')_2$ fragment of anti-OX40 mAb (B-7B5) and found that the $F(ab')_2$ fragment did not suppress Tax expression; this finding supports an ADCC mechanism of action of the anti-OX40 mAb (Figure 5, right panels).

Anti-OX40 monoclonal antibody specifically eliminated OX40-positive HTLV-1 infected cells in cultured PBMCs

We examined whether suppression of OX40 expression either reduced the frequency of Tax-positive cells or selectively eliminated HTLV-1-infected cells by isolating $CD4^+$ T cells from PBMCs before and after culture, extracting genomic DNA, and measuring HTLV-1 PVL. HTLV-1 PVL in $CD4^+$ T cells was significantly reduced