Table 2
Percentage PD-1 expression on tetramer$^+$ cells within the CD8$^+$ lymphocyte populations from ACs, HAM/TSP patients, and CAIDs

	AC	HAM/TSP	CAIDs
HTLV-1 Tax/HLA-A*0201	56.2 ± 20.7%† (N = 8)	43.1 ± 28.2% (N = 5)	42.8 ± 11.3% (N = 5)
HTLV-1 Tax/HLA-A*2402	80.7 ± 22.4% (N = 20)	59.1 ± 24.9%* (N = 29)	68.3 ± 26.7% (N = 15)

Results represent the mean ± SD.
$^\dagger P < 0.05$ versus HTLV-1 Tax/HLA-A *2402.
$^* P < 0.05$ versus AC.

Table 3
Characteristics of SS, SLE, and RA in 15 patients with CAIDs*

	SS	SLE	RA
Rheumatoid factor (positive rate, %)	50	NE	66.7
Antinuclear Ab (%)	80	83.3	50
Anti-SS-A Ab (%)	80	0	0
Anti-SS-B Ab (%)	75	0	0
Anticentromere Ab (positive number, N)	1	NE	NE
Antiribonucleoprotein Ab (N)	1	2	NE
Anti-smooth muscle Ab (N)	1	2	NE
Anticardiolipin Ab (N)	NE	1	NE
Anti-ds DNA Ab (N)	NE	3	NE
Anti-CLβ2GP1 AB (N)	NE	1	NE
Anti-CCP Ab (N)	NE	NE	1
PD-1 on HTLV-1/HLA-A*2402 tetramer$^+$ CD8$^+$ cells (% ± SD)	80.9 ± 23.6	68.1 ± 25.5	57.5 ± 41.0

Ab = antibodies; NE = not examined; CCP = cyclic citrullinated peptide.
*Number was reduced because not all the participants agreed to take full examinations.

tetramer$^+$CD8$^+$ cells (Table 2). The proviral load in Tax11–19 tetramer$^+$ ACs was significantly lower than that in Tax301–309 tetramer$^+$ ACs [27]. These findings suggest that the Tax11–19-specific tetramer CD8$^+$ T cells act to strongly downregulate the proviral load [33]. The frequency of HTLV-1 Tax11–19/HLA-A*0201-specific CTLs may also be related to the HTLV-1 proviral load and clinical course in HAM/TSP patients [34] and the fact that the HTLV-1 mRNA load correlates with the proviral DNA load and the frequency of HTLV-1-specific CD8$^+$ T cells [35]. In the present study, the proviral load in CAIDs was significantly reduced compared with that in ACs (25.8 vs 61.3 copies/10^3 PBMCs; $p < 0.05$; data not shown). Our present results, therefore, support prior findings indicating that a high HTLV-1 proviral DNA load may be associated with higher mRNA expression, resulting in heightened virus-specific immune responses [35]. As the levels of viral antigen expression increase during HTLV-1 infection, HTLV-1-specific CD8$^+$ T cells may be generated that are unable to eliminate infected cells. Therefore, HTLV-1 can survive/replicate in the presence of an activated immune system that is trying to control the infection.

3.2. PD-1 expression by virus-specific/HLA-A*2402 tetramer$^+$CD8$^+$ cells in ACS, HAM/TSP patients, CAIDs, and healthy donors (HDs)

The percentage of CD8$^+$ lymphocytes expressing PD-1 was significantly higher in ACs, HAM/TSP, and CAIDs than in HDs. However, PD-1 expression on HTLV-1-specific CD8$^+$ cells from ACs and CAIDs was significantly higher than that on CMV- or EBV-specific CD8$^+$ cells, whereas no significant difference was observed in HAM/TSP. Interestingly, PD-1 expression was significantly downregulated on HTLV-1/HLA-A*2402-specific CD8$^+$ cells in HAM/TSP compared with ACs (Fig. 1, $p < 0.01$). PD-1 expression in CAIDs was also downregulated on HTLV-1/HLA-A*2402-specific CD8$^+$ cells compared with ACs (Fig. 1, $p = 0.07$). In a recent study, Abdelbary et al. reported a significantly higher frequency of PD-1-expressing HTLV-1 Tax/HLA-A*0201-specific CTLs in ACs compared with that in HAM/TSP patients [36].

In the present study, there was some evidence of autoantibodies, such as rheumatoid factor, in CAID patients (Table 3). However, there were no significant differences in PD-1 expression by HTLV-1/HLA-A*2402 tetramer$^+$CD8$^+$ cells from patients with SS, SLE, or RA (SS, 80.9 ± 23.6%; SLE, 68.1 ± 25.5%; RA, 57.5 ± 41.0%).

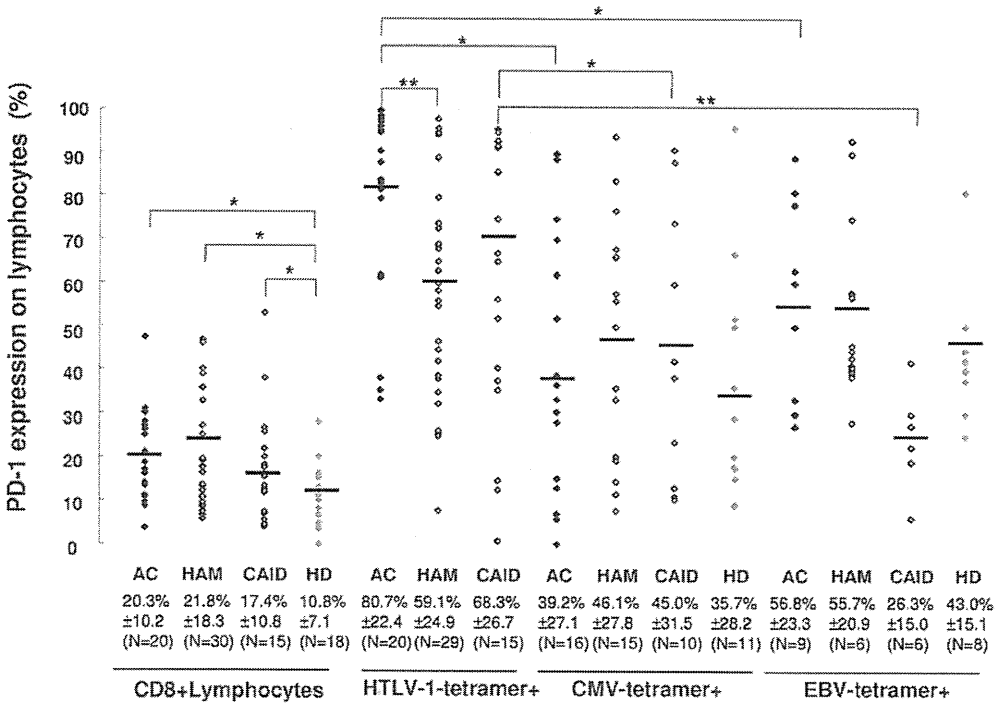

Fig. 1. PD-1 expression on virus-specific CD8$^+$ T lymphocytes from ACs, CAIDs, and HAM/TSP patients. The percentage of CD8$^+$ lymphocytes expressing PD-1 in HTLV-1-, CMV-, and EBV/HLA-A*2402 CD8$^+$ lymphocytes from ACs, HAM/TSP patients, CAIDs, and HDs. Horizontal bars indicate the mean percentage of PD-1-positive cells. The number below each subject type represents the mean ± SD. $^* p < 0.01$; $^{**} p < 0.05$ (Mann–Whitney U test).

3.3. PD-1/PD-L1 pathway in HTLV-1-specific CD8+ cells from ACS, HAM/TSP patients, and CAIDs

Generally, ACs had few lymphocytes positive for PD-L1 *ex vivo*; however, PD-L1 expression was upregulated on Tax-expressing lymphocytes from ACs cultured for 12 hours (5/6), but to a lesser extent in those from HD, HAM/TSP, and CAIDs (0/6, 1/6, and 1/6, respectively; Fig. 2). It is possible that the increased PD-L1 expression on HTLV-1-infected cells from ACs may be reflective of the *in vivo* lymphoid tissue microenvironment because cell-to-cell interactions in short-term culture mimic cellular interactions in lymphoid tissues *in vivo* [37,38]. Furthermore, the increased PD-1 expression seen in HTLV-1-specific $CD8^+$ T cells from ACs suggests that interactions with PD-L1 occur in ACs, but are less pronounced and, therefore, perhaps disrupted, in CAIDs and HAM/TSP.

To assess whether PD-1 expression on $CD8^+$ lymphocytes from ACs, HAM/TSP, and CAIDs has any functional significance, we examined cytolytic function using flow cytometric analysis of CD107a expression, a marker of degranulation. We incubated PBMCs from these subjects with culture medium alone or with medium containing an anti-PD-L1 antibody in the presence of an HTLV-1 Tax peptide for 6 hours. After culture in the presence of Tax peptide without PD-L1 blockade, the number of CD107a expressing te-

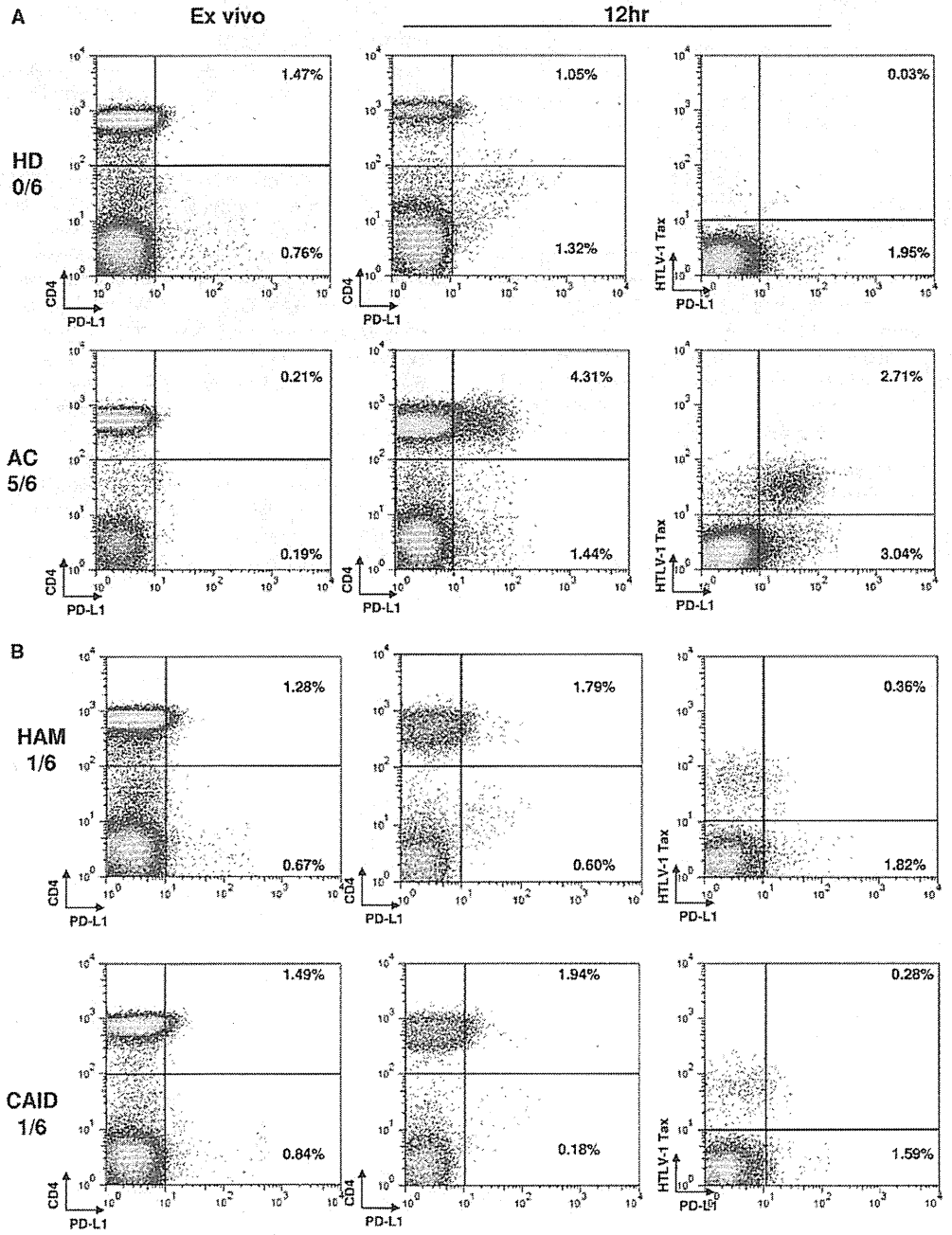

Fig. 2. HTLV-1-infected cells induce PD-L1 expression. Representative flow cytometry plots illustrating PD-L1 expression gated on lymphocytes from HD, AC, HAM/TSP, and CAIDs. (Left) Results obtained *ex vivo*; (right) results obtained after 12 hours in culture (gated on lymphocytes). Increases in PD-L1 expression were observed after culture of cells from ACs; these increases were less pronounced in HDs, HAM/TSP patients, and CAIDs. The numbers indicate the percentage of lymphocytes. There was no *ex vivo* expression of HTLV-1 Tax. All HTLV-1 $Tax^+PD\text{-}L1^+$ lymphocytes were $CD4^+CD25^+$. The numbers under the each subject group indicate the proportion of PD-L1 positive subjects. One representative result of PD-L1 expression from each group of 6 subjects is presented.

tramer$^+$ cells within the CD8$^+$ lymphocyte subset was significantly higher in HAM/TSP than in ACs or CAIDs ($p < 0.01$ and $p < 0.05$, respectively); however, PD-L1 blockade had no significant effect on PBMCs from ACs, HAM/TSP, and CAIDs subjects (Fig. 3A). The percentage of CD107a expressing tetramer$^+$ cells within the CD8$^+$ lymphocyte population from ACs, HAM/TSP, and CAIDs was 0.92, 1.5, and 0.61%, respectively (Fig. 3A). Furthermore, the percentage of CD8$^+$ cells within the lymphocyte population from ACs, HAM/TSP, and CAIDs was 28.8, 21.4, and 27.3%, respectively (data not shown). Therefore, the frequency of tetramer$^+$/CD107a$^+$ cells from

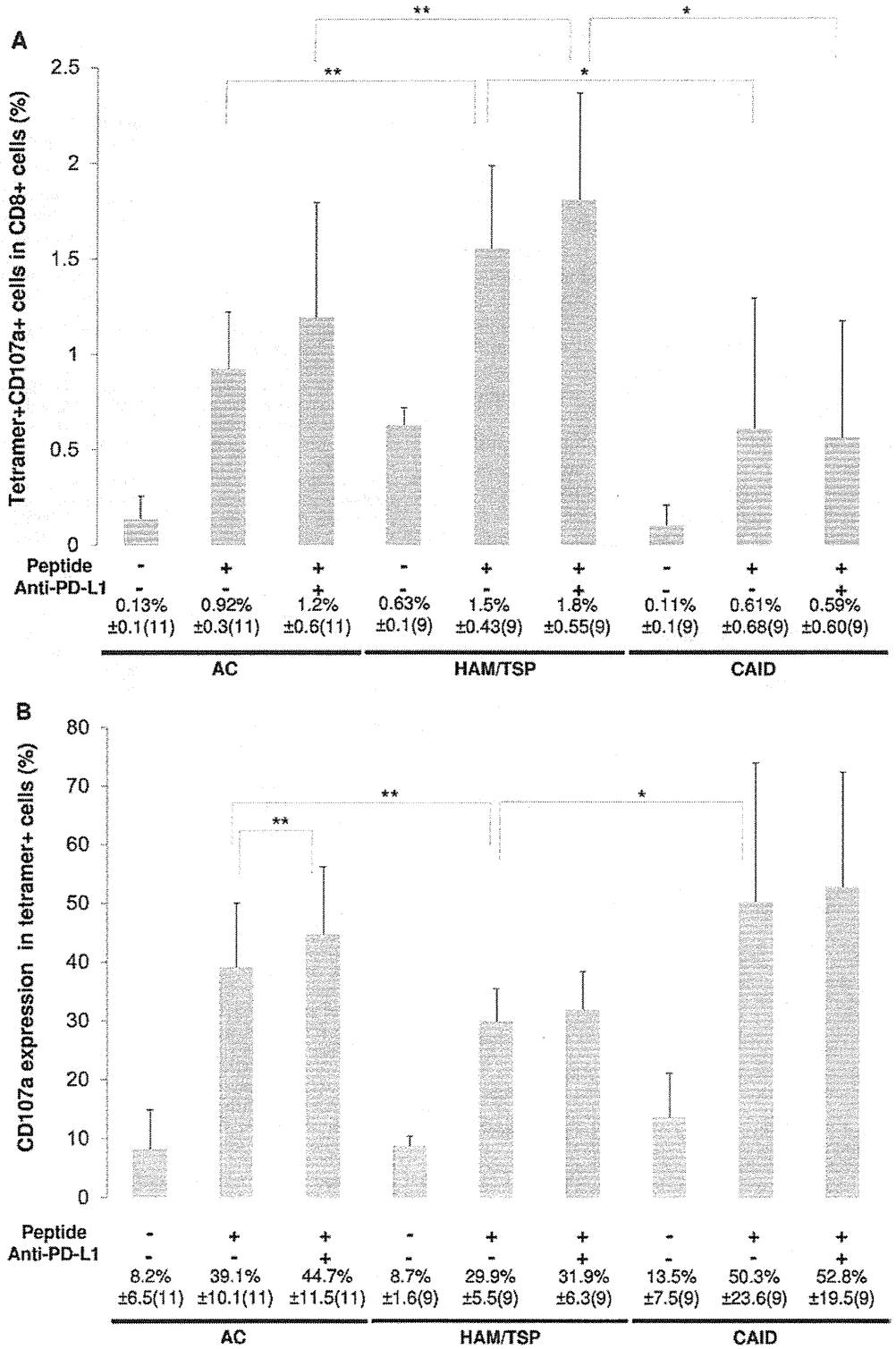

Fig. 3. The PD-1/PD-L1 pathway in HTLV-1-specific CD8$^+$ cells from ACs, HAM/TSP patients, and CAIDs. (A) Tetramer$^+$ CD107a expression in CD8$^+$ T lymphocytes treated with peptide and anti-PD-L1 blocking antibody. (B) Tetramer$^+$ CD107a expression in HTLV-1-specific CD8$^+$ T lymphocytes treated with peptide and anti-PD-L1 blocking antibody. The numbers represent the mean ± SD. *$p < 0.01$; **$p < 0.05$ (Wilcoxon matched pairs test).

ACs and CAIDs was 86.4 and 54.6%, respectively, of that in HAM/TSP. These results suggest that HTLV-1-specific $CD8^+$ cells maintain a state of high cytolytic function in HAM/TSP compared with ACs and CAIDs. However, despite the higher frequency of activated Tax301–309/HLA-A*2402 tetramer-binding $CD8^+$ T cells, HAM/TSP patients had significantly more infected T cells than ACs. Therefore, to clarify the function of HTLV-1-specific $CD8^+$ T cells in each of the subjects, we examined CD107a expression in HTLV-1-specific CTLs. In contrast to patterns of CD107a expression within the total $CD8^+$ lymphocyte subset, CD107a expression in HTLV-1-specific CTLs was significantly lower in HAM/TSP than in ACs and CAIDs (Fig. 3B; $p < 0.05$ and $p < 0.01$, respectively). This indicates that CAIDs mount the strongest HTLV-1-specific immune responses, even stronger than those of ACs. These results may explain, at least in part, the enhanced IL-15 expression observed in $CD14^+$ cells that mediate the immune dysregulation characterized by spontaneous degranulation of $CD8^+$ T cells in HAM/TSP patients [39]. PD-1 expression on HTLV-1-specific CTLs in HAM/TSP and CAIDs was also lower than on ACs. Moreover, the anti-PD-L1 blocking antibody significantly increased CD107a expression from 39.2 ± 10.1% to 44.7 ± 11.5% in ACs ($p < 0.05$). Because the effect of PD-L1 blockade is directly related to the baseline inducibility of PD-L1 (Fig. 2), and in light of the results suggesting that degranulation of $CD8^+$ T cells is only partially mediated by the PD-1/PD-L1 pathway, we conclude that other immune mediators are at play.

The downregulation of PD-1 on HTLV-1 Tax-specific CTLs and the loss of PD-L1 expression in CAIDs and HAM/TSP may underlie the apparently dysfunctional immune response against HTLV-1, but with a skewed impaired response by HTLV-1-specific CTLs, facilitating immune evasion by HTLV-1 infected cells and, thus, persistent infection.

Acknowledgments

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References

[1] Uchiyama T. Human T cell leukemia virus type I (HTLV-I) and human diseases. Annu Rev Immunol 1997;15:15–37.
[2] Popovic M, Reitz MS Jr, Sarngadharan MG, Robert-Guroff M, Kalyanaraman VS, Nakao Y, et al. The virus of Japanese adult T-cell leukaemia is a member of the human T-cell leukaemia virus group. Nature 1982;300:63–6.
[3] Gessain A, Barin F, Vernant JC, Gout O, Maurs L, Calender A, et al. Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. Lancet 1985;2:407–10.
[4] Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A, et al. HTLV-I associated myelopathy, a new clinical entity. Lancet 1986;1:1031–2.
[5] Matsuoka M, Jeang KT. Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. Nat Rev Cancer 2007;7:270–80.
[6] Verdonck K, González E, Van Dooren S, Vandamme AM, Vanham G, Gotuzzo E. Human T-lymphotropic virus 1: recent knowledge about an ancient infection. Lancet Infect Dis 2007;7:266–81.
[7] Bangham CR. HTLV-1 infection: role of CTL efficiency. Blood 2008;112:2176–7.
[8] Matano T. Retroviruses and autoimmune diseases. Jpn J Infect Dis 1999;52:106–9.
[9] Nishioka K, Maruyama I, Sato K, Kitajima I, Nakajima Y, Osame M. Chronic inflammatory arthropathy associated with HTLV-I. Lancet 1989;1:441.
[10] Terada K, Katamine S, Eguchi K, Moriuchi R, Kita M, Shimada H, et al. Prevalence of serum and salivary antibodies to HTLV-1 in Sjögren's syndrome. Lancet 1994;344:1116–9.
[11] Vernant JC, Buisson G, Magdeleine J, De Thore J, Jouannelle A, Neisson-Vernant C, et al. T-lymphocyte alveolitis, tropical spastic paresis, and Sjogren syndrome. Lancet 1988;1:177.
[12] Crawford TB, Adams DS, Cheevers WP, Cork LC. Chronic arthritis in goats caused by a retrovirus. Science 1980;207:997–9.
[13] Motokawa S, Hasunuma T, Tajima K, Krieg AM, Ito S, Iwasaki K, et al. High prevalence of arthropathy in HTLV-I carriers on a Japanese island. Ann Rheum Dis 1996;55:193–5.
[14] Helyer BJ, Howie JB. Renal disease associated with positive lupus erythematosus tests in a cross-bred strain of mice. Nature 1963;197:197.
[15] Akimoto M, Matsushita K, Suruga Y, Aoki N, Ozaki A, Uozumi K, et al. Clinical manifestations of human T lymphotropic virus type I-infected patients with systemic lupus erythematosus. J Rheumatol 2007;34:1841–8.
[16] Iwakura Y, Tosu M, Yoshida E, Takiguchi M, Sato K, Kitajima I, et al. Induction of inflammatory arthropathy resembling rheumatoid arthritis in mice transgenic for HTLV-I. Science 1991;253:1026–8.
[17] Green JE, Hinrichs SH, Vogel J, Jay G. Exocrinopathy resembling Sjögren's syndrome in HTLV-1 tax transgenic mice. Nature 1989;341:72–4.
[18] Kitajima I, Yamamoto K, Sato K, Nakajima Y, Nakajima T, Maruyama I, et al. Detection of human T cell lymphotropic virus type I proviral DNA and its gene expression in synovial cells in chronic inflammatory arthropathy. J Clin Invest 1991;88:1315–22.
[19] Nishioka K, Nakajima T, Hasunuma T, Sato K. Rheumatic manifestation of human leukemia virus infection. Rheum Dis Clin North Am 1993;19:489–503.
[20] Eguchi K, Matsuoka N, Ida H, Nakashima M, Sakai M, Sakito S, et al. Primary Sjögren's syndrome with antibodies to HTLV-I: clinical and laboratory features. Ann Rheum Dis 1992;51:769–76.
[21] Nakamura H, Kawakami A, Tominaga M, Hida A, Yamasaki S, Migita K, et al. Relationship between Sjögren's syndrome and human T-lymphotropic virus type I infection: follow-up study of 83 patients. J Lab Clin Med 2000;135:139–44.
[22] Nakamura H, Eguchi K, Nakamura T, Mizokami A, Shirabe S, Kawakami A, et al. High prevalence of Sjögren's syndrome in patients with HTLV-I associated myelopathy. Ann Rheum Dis 1997;56:167–72.
[23] Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. Annu Rev Immunol 2005;23:515–48.
[24] Blank C, Mackensen A. Contribution of the PD-L1/PD-1 pathway to T-cell exhaustion: an update on implications for chronic infections and tumor evasion. Cancer Immunol Immunother 2007;56:739–45.
[25] Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. Nat Immunol 2007;8:239–45.
[26] Sheppard KA, Fitz LJ, Lee JM, Benander C, George JA, Wooters J, et al. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. FEBS Lett 2004;574:37–41.
[27] Kozako T, Arima N, Toji S, Masamoto I, Akimoto M, Hamada H, et al. Reduced frequency, diversity, and function of human T cell leukemia virus type 1-specific CD8+ T cell in adult T cell leukemia patients. J Immunol 2006;177:5718–26.
[28] Kozako T, Yoshimitsu M, Fujiwara H, Masamoto I, Horai S, White Y, et al. PD-1/PD-L1 expression in human T-cell leukemia virus type 1 carriers and adult T-cell leukemia/lymphoma patients. Leukemia 2009;23:375–82.
[29] Kozako T. New strategy of adult T-cell leukemia treatment targeted for antitumor immunity and a longevity gene-encoded protein. Yakugaku Zasshi 2011;131:1061–72.
[30] Kozako T, Hirata S, Shimizu Y, Satoh Y, Yoshimitsu M, White Y, et al. Oligomannose-coated liposomes efficiently induce human T-cell leukemia virus-1-specific cytotoxic T lymphocytes without adjuvant. FEBS J 2011;278:1358–66.
[31] Akimoto M, Kozako T, Sawada T, Matsushita K, Ozaki A, Hamada H, et al. Anti-HTLV-1 tax antibody and tax-specific cytotoxic T lymphocyte are associated with a reduction in HTLV-1 proviral load in asymptomatic carriers. J Med Virol 2007;79:977–86.
[32] Kozako T, Akimoto M, Toji S, White Y, Suzuki S, Arima T, et al. Target epitopes of HTLV-1 recognized by class I MHC-restricted cytotoxic T lymphocytes in patients with myelopathy and spastic paraparesis and infected patients with autoimmune disorders. J Med Virol 2011;83:501–9.
[33] Bangham CR. The immune control and cell-to-cell spread of human T-lymphotropic virus type 1. J Gen Virol 2003;84:3177–89.
[34] Kubota R, Furukawa Y, Izumo S, Usuku K, Osame M. Degenerate specificity of HTLV-1-specific CD8+ T cells during viral replication in patients with HTLV-1-associated myelopathy (HAM/TSP). Blood 2003;101:3074–81.
[35] Yamano Y, Nagai M, Brennan M, Mora CA, Soldan SS, Tomaru U, et al. 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.
[36] Abdelbary NH, Abdullah HM, Matsuzaki T, Hayashi D, Tanaka Y, Takashima H, et al. Reduced Tim-3 expression on human T-lymphotropic virus type I (HTLV-I) Tax-specific cytotoxic T lymphocytes in HTLV-I infection. J Infect Dis 2011;203:948–59.
[37] Arima N, Arimura K, Tokito Y, Sakaki Y, Matsushita K, Orihara K, et al. HTLV-I Tax protein inhibits apoptosis induction but not G1 arrest by pyrrolidinedithiocarbamate, an anti-oxidant, in adult T cell leukemia cells. Exp Hematol 2004;32:195–201.
[38] Yoshida M. Multiple viral strategies of HTLV-1 for dysregulation of cell growth control. Annu Rev Immunol 2001;19:475–96.
[39] Enose-Akahata Y, Oh U, Grant C, Jacobson S. Retrovirally induced CTL degranulation mediated by IL-15 expression and infection of mononuclear phagocytes in patients with HTLV-I-associated neurologic disease. Blood 2008;112:2400–10.

＜シンポジウム 20—2＞難治性神経感染症 update

HAM スペクトラム

久保田龍二

(臨床神経 2011;51:1044-1046)
Key words：HTLV-I，HAM，プロウイルス量，細胞傷害性Tリンパ球，bystander damage

はじめに

HTLV-I 関連脊髄症（HAM）は，HTLV-I 感染者の約 300 人に 1 人発症する慢性の脊髄炎である．臨床的に両下肢痙性麻痺，排尿排便障害，両下肢の感覚障害を主徴とし，血清および髄液中の抗 HTLV-I 抗体が陽性である[1]．HTLV-I 感染経路は，感染細胞をふくむ母乳を介した母児感染が主であり，一部性交渉によるものもある．今まで IFN-α やステロイドなどの炎症をおさえる種々の治療法が試され一定の効果をみとめるものの，根治療法は未だ確立していない．本稿では，HAM の地理的広がり，臨床像の広がり，および発症機序とそれに基づく治療戦略につき述べる．

1. 全国 HTLV-I 感染疫学調査と全国 HAM 疫学調査

HTLV-I 感染症は，1990 年の厚生省疫学グループの報告で，日本の感染者は約 120 万人いるが今後感染者は減り続け，いずれは日本から消えてなくなるだろう，したがって全国一律の検査や対策は必要ないと報告され，全国的な対策はおこなわれなかった．しかし，2007 年の全国疫学調査では 108 万人の感染者がおり，ほとんど減っていないこと，また関東や中部地方などの都市部では増加していることが報告された．2008 年第 3 次 HAM 全国疫学調査では，アンケート結果より推定される 2008 年の全国 HAM 患者数は約 3,600 人であり，九州に次いで関東，近畿地方で患者が多かった．また，1995 年以降は全国で年間平均約 140 人の新規 HAM 患者が発症していると推定された．このように HTLV-I 感染者および HAM 患者数は減ってはおらず，都市部ではむしろ増大傾向を示した．これらの報告を受け，2011 年より，HTLV-I 母児感染の予防と，新規治療開発に重点を置いた厚生労働省による HTLV-I 総合対策が始まった．長崎県で先行している HTLV-I 陽性母親からの新生児の人工栄養哺育の介入試験では，新生児 HTLV-I 感染率は 20.3% から 2.5% に激減している．

2. HAM の臨床像の広がり

HAM の病巣の特徴は，血管周囲の単核球浸潤による炎症である．病巣分布は下部胸髄が中心であるが，頸髄や腰髄にも炎症像が広がり，全脊髄に病巣が広がる例も存在する．Aye らは，HAM 剖検例の脳脊髄の炎症細胞の分布を組織学的に検討し，頸髄，胸髄，腰髄だけでなく，大脳の皮質および白質にわたり，広範囲に炎症が広がっていることを報告している[2]．MRI 所見では，急性期の胸腰髄の腫大および T_2 高信号が，慢性期の胸腰髄の萎縮が報告されていたが，梅原らは頸髄で脊髄腫大や T_2 高信号などの MRI 所見をともなった HAM の一群を報告している[3]．HAM の臨床経過は，ほとんどの症例は緩徐進行性であるが，数週間～1，2 カ月で比較的急速に進行する例も存在する．われわれは 2 回の増悪および寛解を示し頸髄に T_2 高信号を示した多発性硬化症との鑑別が困難であった症例を報告している[4]．この症例では，増悪前後で末梢血中の HTLV-I プロウイルス量は 6.1 倍増加しているのに加え，髄液中では 11.0 倍増加し，増悪時には末梢血中プロウイルス量の約 10.5 倍におよぶ髄液中プロウイルス量を示した．さらに，増悪は多発性硬化症の数日間での悪化にくらべ，1～2 週間と長いことから，HAM の診断にいたった．HTLV-I キャリアとくらべ，HAM では末梢血プロウイルス量が約 10 倍高く，HAM 発症の最大のリスクと考えられており[5]，HAM の症状の増悪期には，髄液中および末梢血中でウイルス量が増加している傾向がある[6]．一方，HTLV-I 関連疾患の広がりとしては，HTLV-I は ATL（白血病），HAM だけでなく，多臓器にわたり炎症性疾患を発症する．HTLV-I 関連ぶどう膜炎（HAU），HTLV-I 関連関節症（HAAP），HTLV-I 関連気管枝肺胞炎（HAB）などに加え，Sjögren 症候群，筋炎，皮膚炎などとの関連も報告されている．HAM における他臓器の HTLV-I 関連疾患の合併を検討すると，HAB 80%，Sjögren 症候群 25%，HAAP 17%，HAU 8% と高率に他臓器の HTLV-I 関連炎症性疾患の合併が観察される[7]．

3. HAM の発症機序と治療戦略

HTLV-I は，成体内では主に CD4 陽性細胞に感染し，血流に乗って全身を循環している．通常末梢血中ではウイルス蛋白はほとんど検出されないが，HAM の脊髄の血管周囲では，CD4 陽性細胞の一部にウイルス mRNA が発現しており，感染細胞の浸潤がみとめられた[8]．われわれは，脊髄内に多数の

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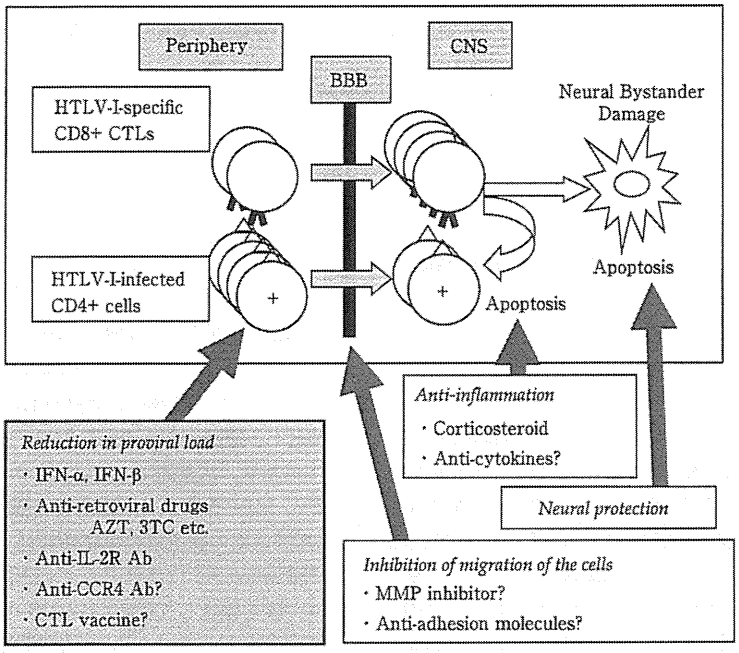

Fig. 1　HAM の発症機序と治療戦略.
HAM では，HTLV-I プロウイルス量の増大が最大の発症リスクである．HAM の脊髄では，末梢血中から HTLV-I 感染細胞と HTLV-I 特異的 CTL が中枢神経系へ浸潤し，炎症の結果として周囲の神経系細胞の障害（neural bystander damage）がおこっていると考えられる．HTLV-I 感染細胞を有効に排除する新規治療法が望まれる．

HTLV-I 特異的細胞傷害性 T リンパ球（CTL）の浸潤をみとめ，その周囲の CD4 陽性細胞，マクロファージならびにオリゴデンドロサイトにアポトーシスをみとめた．HTLV-I ウイルス蛋白は神経系細胞では検出されず，浸潤 CD4 陽性細胞にのみ検出された．以上より，HAM の脊髄では，末梢血より HTLV-I 感染リンパ球と HTLV-I 特異的 CTL が組織へ浸潤して炎症をおこし，周囲の神経系細胞が障害を受ける bystander damage がおこっていると考えられた．さらに，われわれは HTLV-I 関連肺疾患においても，感染細胞と HTLV-I 特異的 CTL の肺への集積を観察している．このように臓器特異的細胞に感染することなしに，末梢血中の HTLV-I 感染細胞および HTLV-I 特異的 CTL の浸潤により臓器に炎症をおこし，周囲の組織破壊をきたす bystander damage モデルは，HTLV-I が同一個体の中で，多数の臓器に炎症性疾患をおこしうることを説明できると考えている．この発症モデルにしたがえば，炎症のイニシエーターである HTLV-I 感染細胞の生体内からの除去が HAM の治療にもっとも重要な根本治療となりえる．また，臓器へ感染細胞の浸潤を抑制できれば HAM および HTLV-I 関連炎症性疾患の治療となりえる（Fig. 1）．

おわりに

HTLV-I 感染者および HAM 患者は減少していない．HAM の炎症は，全脊髄および脳にも広がっており，HAM は他の HTLV-I 関連炎症性疾患の合併が多い．新規 HAM の発症予防には，HTLV-I 感染の主な経路である母子感染を遮断することがもっとも重要である．また，HAM の治療には HTLV-I 感染細胞を減少させることが第一であり，感染細胞に対する標的治療または薬物療法の開発が急務である．

文　献

1) Osame M, Matsumoto M, Usuku K, et al. Chronic progressive myelopathy associated with elevated antibodies to human T-lymphotropic virus type I and adult T-cell leukemialike cells. Ann Neurol 1987;21:117-122.
2) Aye MM, Matsuoka E, Moritoyo T, et al. Histopathological analysis of four autopsy cases of HTLV-I-associated myelopathy/tropical spastic paraparesis: inflammatory changes occur simultaneously in the entire central nervous system. Acta Neuropathol 2000;100:245-252.
3) Umehara F, Nagatomo S, Yoshishige K, et al. Chronic progressive cervical myelopathy with HTLV-I infection: Variant form of HAM/TSP? Neurology 2004;63:1276-1280.
4) Hayashi D, Kubota R, Takenouchi N, et al. Accumulation of human T-lymphotropic virus type I (HTLV-I)-infected cells in the cerebrospinal fluid during the exacerbation of HTLV-I-associated myelopathy. J Neurovirol 2008;14:459-463.
5) Nagai M, Usuku K, Matsumoto W, et al. 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.
6) Takenouchi N, Yamano Y, Usuku K, et al. Usefulness of proviral load measurement for monitoring of disease activity in individual patients with human T-lymphotropic virus type I-associated myelopathy/tropical spastic paraparesis. J Neurovirol 2003;9:29-35.
7) Nakagawa M, Izumo S, Ijichi S, et al. HTLV-I-associated myelopathy: analysis of 213 patients based on clinical features and laboratory findings. J Neurovirol 1995;1:50-61.
8) Moritoyo T, Reinhart TA, Moritoyo H, et al. Human T-lymphotropic virus type I-associated myelopathy and tax gene expression in CD4 + T lymphocytes. Ann Neurol 1996;40:84-90.

Abstract

Clinical diversity of HAM/TSP

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HTLV-I is a human retrovirus and causes adult T cell leukemia and several inflammatory diseases such as HAM/TSP. The infection occurs via HTLV-I-infected cells and the main transmission rout is mother-to-child infection via breast-feeding. In Japan, total numbers of HTLV-I carriers and HAM/TSP patients are recently estimated to be 1.08 million and 3,600, respectively, which exhibit no reduction in numbers. Although the main lesion is in the thoracic cord of patients with HAM/TSP, the inflammatory regions characterized by mononuclear cells infiltration are disseminated throughout the central nervous system (CNS). The patients show higher proviral load compared to the carries and are frequently complicated with HTLV-I-associated inflammatory diseases in other organs, including uveitis, bronchoalveolitis, arthritis, and Sjögren syndrome. Pathologically, HTLV-I-infected lymphocytes and HTLV-I-specific cytotoxic T lymphocytes infiltrate the CNS from the peripheral blood and induce an inflammation without HTLV-I infection of CNS resident cells, leading to bystander damage in the resident cells. Inhibition of mother-to-child infection via breast-feeding is most important to prevent HTLV-I spread and a treatment to eliminate HTLV-I-infected cells should urgently be established.

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Key words: HTLV-I, HAM/TSP, proviral load, cytotoxic T lymphocyte, bystander damage

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Case report

A novel *MPZ* mutation in Charcot-Marie-Tooth disease type 1B with focally folded myelin and multiple entrapment neuropathies

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Abstract

Charcot-Marie-Tooth type 1B (CMT1B) is a demyelinating neuropathy caused by mutations in the myelin protein zero (*MPZ*) gene. Here, we describe a patient with CMT1B with focally folded myelin, a rarely reported phenotype of CMT1B, who initially presented with multiple entrapment neuropathies. She complained of palmar dysesthesia on both sides and on both soles of her feet in her 30's. She underwent bilateral carpal and tarsal tunnel release at age 44, which provided transient relief from the symptoms. A sural nerve biopsy performed at age 49 revealed focally folded myelin. Molecular genetic analysis revealed a novel Asn131Ser mutation in *MPZ*.

Keywords: Charcot-Marie-Tooth disease type 1B; Myelin protein zero; Focally folded myelin; Carpal tunnel syndrome

1. Introduction

Charcot-Marie-Tooth (CMT) disease is the most common inherited peripheral neuropathy and is both clinically and genetically heterogeneous. CMT type 1B (CMT1B) disease is an autosomal dominant demyelinating neuropathy caused by point mutations in the myelin protein zero (*MPZ*) gene that encodes the major structural component of peripheral myelin [1]. Most individuals with CMT1B develop one of two phenotypes: an early (childhood) onset neuropathy with very slow nerve conduction velocities (NCV) and predominantly demyelination on nerve biopsy, or a late (adult) onset neuropathy with minimal to moderately slowed NCV and predominant axonal neuropathy on nerve biopsy [1,2]. Among the demyelination types, some mutations in *MPZ* were associated with myelin uncompaction, and a few were associated with focal foldings of the myelin sheath without any obvious relation to the molecular lesion [1,3–9]. So far, more than 120 distinct mutations have been identified in *MPZ*, comprising missense and frameshift mutations or small deletions (see http://www.molgen.ua.ac.be/CMTMutations/default.cfm). Here we describe a CMT1B patient with an AAC→AGC transition in *MPZ*, encoding a novel Asn131Ser mutation, who exhibited focally folded myelin sheaths in a sural nerve biopsy. She underwent bilateral carpal tunnel and tarsal tunnel release 5 years before being diagnosed with CMT, which provided transient relief from the symptoms and improvements in nerve conduction parameters.

2. Case report

The patient is a 49-year-old female with a body mass index of 19.6. Her developmental motor milestones were almost normal. When she was an elementary school student, she could not run as fast as other classmates. At

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the age of 38, she noticed progressive weakness, dysesthesias, and wasting of upper extremities. At the age of 39 she presented with dysesthesias of the soles and consulted an orthopedic department. Nerve conduction velocities (NCV) were markedly reduced when she was 44. In the right median nerve, the motor NCV was 16.3 m/s, the distal motor latency (DML) was 13.6 ms, and the compound motor action potential (CMAP) amplitude was 112 μV. In the left median nerve, the motor NCV was 21.6 m/s, DML was 12.4 ms, and CMAP was 208 μV. A sensory nerve impulse could not be evoked in the right median nerve. In the left median nerve, the sensory NCV was 19.5 m/s, and the sensory nerve action potential (SNAP) amplitude was 1 μV. None of the nerve conduction parameters could be measured in lower extremities. The results of nerve conduction study were reconfirmed. She had obvious thenar atrophy and Phalen's signs were positive bilaterally. She suffered from constant uncomfortable palmar dysesthesias on both sides, which was stronger in the median nerve region than in the ulnar nerve region. Tinel's signs and nocturnal pain were not present.

Therefore, she underwent bilateral carpal tunnel release. The dysesthesias improved slightly, and the parameters of nerve conduction studies were improved two months after the operation (Fig. 1). In the right median nerve, the motor NCV was 14.5 m/s, DML was 8.28 ms, and CMAP was 724 μV. In the left median nerve, the motor NCV was 15.2 m/s, DML was 5.62 ms, and CMAP was 1.28 mV. In the right median nerve, the sensory NCV was 18.7 m/s and SNAP was 1 μV. In the left median nerve, the sensory NCV was 18.5 m/s and SNAP was 2 μV. At the age of 45, she underwent bilateral tarsal tunnel release because there were dysesthesias in soles and Tinel's signs were positive bilaterally. Nerve conduction parameters of tibial nerves could not be evoked on either sides before the operation. The dysesthesias improved slightly after the operation.

However, partial dysesthesias remained and nerve conduction parameters of NCS gradually worsened again. By the age of 47 none of the nerve conduction parameters could be measured, including those of the sural nerves; whereupon, she was referred to the Neurology department of our hospital. A neurological examination of the patient at 49 showed symmetrical muscle atrophy, hand weakness, absent deep tendon reflexes, and impairment of all sensory modalities in the upper extremities. Mild atrophy of the distal muscles and high arch was observed in the lower extremities, patellar and Achilles tendon reflexes were absent, and all sensory modalities were impaired up to knee level. Romberg sign was present. Cranial nerves were normal. Results of laboratory studies were all within normal ranges, including HbA1c, vitamin B_1, vitamin B_{12} and folate.

At this time, a sural nerve biopsy was taken (Fig. 2). Semithin sections (stained with toluidine blue) and teased fibers were examined. Severe loss of myelinated fibers and fibers with abnormally thickened myelin were visible on semithin epon cross sections. The density of myelinated fibers was 1291 fibers/mm^2. All teased fibers showed hypomyelinated and demyelinated segments and focal thickenings of myelin.

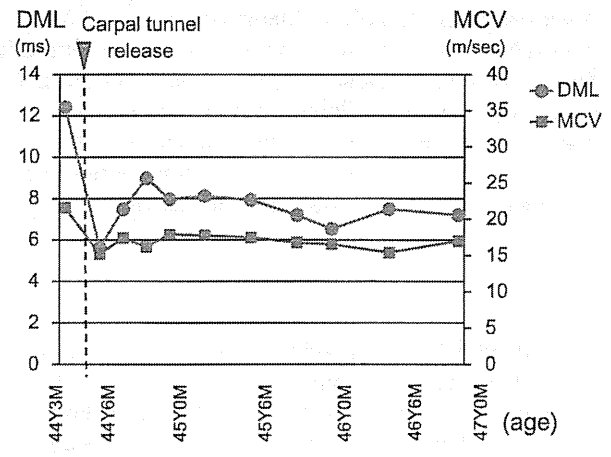

Fig. 1. Results of follow up analysis of distal motor latency (DML) and motor conduction velocity (MCV) on the left hand. After the carpal tunnel release, DML was improved although MCV remained at the same level.

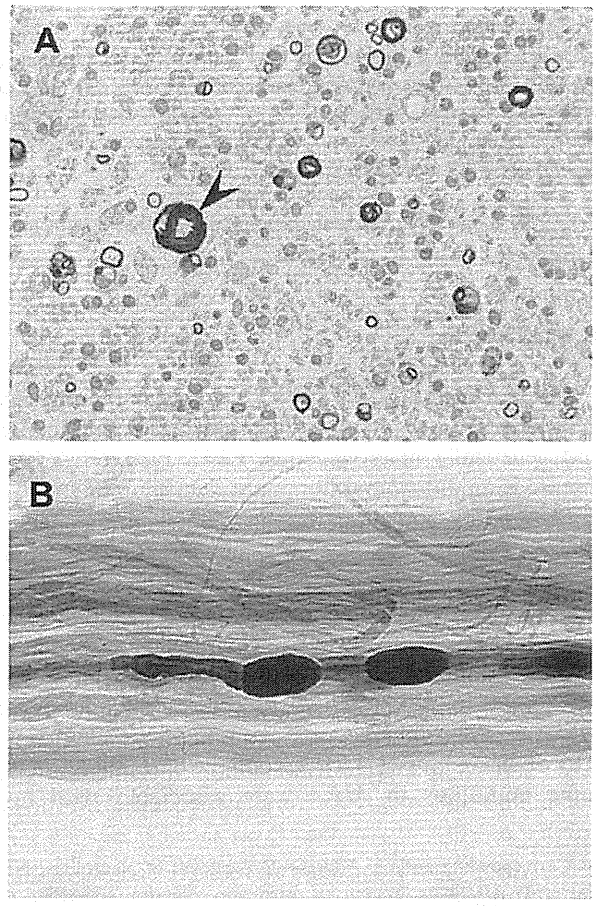

Fig. 2. Sural nerve biopsy specimen from the patient. (A) On cross section, marked loss of myelinated fibers was observed. A fiber with abnormal thickening of the myelin was indicated by an arrowhead. (B) On teased-fiber preparations, focal thickenings of the myelin sheath were evident.

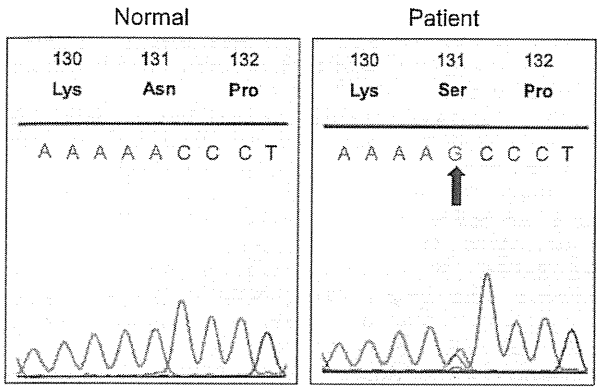

Fig. 3. Direct DNA sequencing of the *MPZ* exon 3 revealed an A→G transition (sense strand) at codon131. The transition resulted in an amino acid change from Asn to Ser in the *MPZ* protein.

Molecular analysis of the *PMP22* gene ruled out the presence of a duplication or deletion. Direct DNA sequencing of the *MPZ* exon 3 revealed an AAC→AGC transition (sense strand) and a TTG→TCG transition (antisense strand) at codon131. The transition resulted in an amino acid change from Asn to Ser in the *MPZ* protein (Fig. 3).

We also examined the proband's sister who was reported to present similar symptoms. Although she was reported to have fatigability in walking and an inability to run as fast as others in her childhood, neurological and electrophysiological findings were unremarkable. DNA sequencing of the *MPZ* exon 3 did not show Asn131-Ser mutation, either.

3. Discussion

We present a 49-year-old woman suffering from motor and sensory polyneuropathy with the onset of symptoms during the first decade of life. She underwent carpal and tarsal tunnel decompression of both hands and feet, yielding temporary relief from the dysesthesias and improvements in nerve conduction parameters. At the time of her referral to the Neurology department, she exhibited moderate weakness and atrophy of the hand muscles, as well as an impairment of all sensory modalities in the upper and lower extremities. A sural nerve biopsy revealed focally folded myelin sheaths. Molecular genetic analysis revealed a novel mutation in *MPZ* leading to an Asn131Ser substitution, indicative of CMT1B.

MPZ accounts for 50–60% of the protein in the peripheral myelin expressed exclusively by myelinating Schwann cells [1]. *MPZ* mutations usually cause a demyelinating variant of CMT1B, but there is a wide spectrum of phenotypic manifestations [1,2]. Gabreels-Festen et al. distinguished at least two morphological phenotypes in CMT1B patients from a pathological view point: (i) mutations resulting in disturbed compaction of myelin and (ii) mutations resulting in focally folding of myelin [5]. So far more than 120 distinct *MPZ* mutations have been identified, but only a few of them were found in patients with the focally folded myelin phenotype [3–9]. The reason why some *MPZ* mutations produce the focally folded myelin phenotype remains unclear.

The mutation, found in the present case, is located within the extracellular domain, which is important for homophilic interaction of *MPZ* protein. Most mutations so far identified in *MPZ* are actually located within the extracellular domain [10]. We searched across the JSNP (Japanese Single Nucleotide Polymorphism) database (http://snp.ims.u-tokyo.ac.jp) and the dbSNP (Single Nucleotide Polymorphism Database) (http://www.ncbi.nlm.nih.gov/projects/SNP/) that showed single nucleotide polymorphism in normal population, and confirmed that the AAC→AGC transition had not been reported as a single nucleotide polymorphism. A different Asn to Lys change was reported twice at the same codon [9,11]. A heterozygous AAC→AAA transition in the third exon of *MPZ*, resulting in an Asn to Lys substitution at codon131. Morphologically, a chronic demyelinating neuropathy with the remarkable aspects of a focally hypertrophic myelin sheath and major loss of myelinated fibers was observed in both cases, suggesting an important role of Asn131 in myelin maintenance.

There is a report that patients with CMT1B had recurrent lesions at compression sites that mimicked hereditary neuropathy with liability to pressure palsies [12]. They describe three patients from one family and one additional patient who had a heterozygous nonsense mutation, Tyr145Stop, corresponding to a T→A transition at nucleotide position 435 in exon 3 of *MPZ*. A family with the missense mutation Asp234Tyr within the intracellular domain of *MPZ*, who showed carpal tunnel syndrome, distal sensorimotor symptoms, cramps, restless legs syndrome, and neuropathic pain has also been described [10]. One of the patients responded to intravenous immunoglobulin and immunosuppression, suggesting a possible secondary inflammatory and autoimmune process, probably triggered by altered antigen presentation due to mutated *MPZ* [10]. There is also a report that the patients with Val102/fs null mutation have segmental conduction abnormalities mainly in ulnar nerves at the elbow, and excessive myelin folding and thickenings. The authors hypothesize that myelin thickenings at the paranodal region, in concurrence with compression at usual entrapment sites or minor repetitive trauma, may induce segmental conduction abnormalities [13].

Nerve conduction studies exhibit high sensitivity and specificity for the diagnosis of carpal tunnel syndrome and are the only objective measure for confirmation of the diagnosis [14]. Postoperative nerve conduction studies are especially useful for evaluating the effectiveness of surgery, as subjective symptoms of physical findings are sometimes difficult to quantify [15]. We followed the transition of nerve conduction parameters in this patient before and after the operation (Fig. 1); distal motor latency improved after the carpal tunnel release, which paralleled the improvement in dysesthesia symptoms.

As in previous reports, some mutations in *MPZ* express the symptoms of entrapment neuropathy [10,12]. *MPZ*

mutations should be considered in patients initially presenting with entrapment neuropathy, especially in patients with multiple lesions. To clarify this relationship, further studies of the clinical features of CMT1B patients are needed.

Acknowledgement

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References

[1] Shy ME, Jáni A, Krajewski K, et al. Phenotypic clustering in MPZ mutations. Brain 2004;127:371–84.
[2] Hattori N, Yamamoto M, Yoshihara T, et al. Study group for hereditary neuropathy in Japan. Demyelinating and axonal features of Charcot–Marie–Tooth disease with mutations of myelin-related proteins (PMP22, MPZ and Cx32): a clinicopathological study of 205 Japanese patients. Brain 2003;126:134–51.
[3] Tachi N, Kozuka N, Ohya K, Chiba S, Sasaki K. Tomaculous neuropathy in Charcot–Marie–Tooth disease with myelin protein zero gene mutation. J Neurol Sci 1997;153:106–9.
[4] Thomas FP, Lebo RV, Rosoklija G, et al. Tomaculous neuropathy in chromosome 1 Charcot–Marie–Tooth syndrome. Acta Neuropathol 1994;87:91–7.
[5] Gabreëls-Festen AA, Hoogendijk JE, Meijerink PH, et al. Two divergent types of nerve pathology in patients with different P0 mutations in Charcot–Marie–Tooth disease. Neurology 1996;47: 761–5.
[6] Kochanski A, Drac H, Kabzińska D, Hausmanowa-Petrusewicz I. A novel mutation, Thr65Ala, in the MPZ gene in a patient with Charcot–Marie–Tooth type 1B disease with focally folded myelin. Neuromuscul Disord 2004;14:229–32.
[7] Fabrizi GM, Taioli F, Cavallaro T, et al. Focally folded myelin in Charcot–Marie–Tooth neuropathy type 1B with Ser49Leu in the myelin protein zero. Acta Neuropathol 2000;100:299–304.
[8] Nakagawa M, Suehara M, Saito A, et al. A novel MPZ gene mutation in dominantly inherited neuropathy with focally folded myelin sheaths. Neurology 1999;52:1271–5.
[9] Kochański A, Drac H, Jedrzejowska H, Hausmanowa-Petrusewicz I. Focally folded myelin in Charcot–Marie–Tooth type 1B disease is associated with Asn131Lys mutation in myelin protein zero gene: short report. Eur J Neurol 2003;10:547–9.
[10] Schneider-Gold C, Kötting J, Epplen JT, Gold R, Gerding WM. Unusual Charcot–Marie–Tooth phenotype due to a mutation within the intracellular domain of myelin protein zero. Muscle Nerve 2010;41:550–4.
[11] Planté-Bordeneuve V, Guiochon-Mantel A, Lacroix C, Lapresle J, Said G. The Roussy-Lévy family: from the original description to the gene. Ann Neurol 1999;46:770–3.
[12] Magot A, Latour P, Mussini JM, Mourtada R, Guiheneuc P, Pereon Y. A new MPZ mutation associated with a mild CMT1 phenotype presenting with recurrent nerve compression. Muscle Nerve 2008;38:1055–9.
[13] De Angelis MV, Di Muzio A, Capasso M, Angiari C, Cavallaro T, et al. Segmental conduction abnormalities and myelin thickenings in Val102/fs null mutation of MPZ gene. Neurology 2004;63:2180–3.
[14] Jablecki CK, Andary MT, So YT, Wilkins DE, Williams FH. Literature review of the usefulness of nerve conduction studies and electromyography for the evaluation of patients with carpal tunnel syndrome. Muscle Nerve 1993;16:1392–414.
[15] Shurr DG, Blair WF, Bassett G. Electromyographic changes after carpal tunnel release. J Hand Surg Am 1986;11:876–80.

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A functional variant in *ZNF512B* is associated with susceptibility to amyotrophic lateral sclerosis in Japanese

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the selective loss of motor neurons. Several susceptibility genes for ALS have been reported; however, ALS etiology and pathogenesis remain largely unknown. To identify further ALS-susceptibility genes, we conducted a large-scale case–control association study using gene-based tag single-nucleotide polymorphisms (SNPs). A functional SNP (rs2275294) was found to be significantly associated with ALS through a stepwise screening approach (combined $P = 9.3 \times 10^{-10}$, odds ratio = 1.32). The SNP was located in an enhancer region of *ZNF512B*, a transcription factor of unknown biological function, and the susceptibility allele showed decreased activity and decreased binding to nuclear proteins. ZNF512B over-expression increased transforming growth factor-β (TGF-β) signaling, while knockdown had the opposite effect. ZNF512B expression was increased in the anterior horn motor neurons of the spinal cord of ALS patients when compared with controls. Our results strongly suggest that ZNF512B is an important positive regulator of TGF-β signaling and that decreased *ZNF512B* expression increases susceptibility to ALS.

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a heterogeneous motor neuron disease that results from selective death of motor neurons in the brain and spinal cord (1). The predominant clinical feature of ALS is progressive wasting and weakness of limb, bulbar and respiratory muscles. The mean survival of patients after onset of symptoms is 3–5 years. Its worldwide incidence and prevalence are 0.3–2.4 and 0.7–7.0 per 100 000 each year (2). The heritability of ALS is high, with twin studies estimating it at 0.61 and the unshared environment component at 0.39 (3).

Approximately 10% of ALS cases are familial (fALS), and the remaining 90% are sporadic (sALS). Genetic factors have been reported in ALS. Detailed information regarding ALS-related genes is available via amyotrophic lateral sclerosis online genetics database and the ALS mutation database (4,5). Most fALS is monogenic in origin. At least 15 fALS loci, under various modes of inheritance, have been identified by linkage studies, and pathogenic mutations have been described in 11 genes, *SOD1*, *NEFH*, *ALS2*, *DCTN1*, *VAPB*, *SETX*, *ANG*, *TARDBP*, *FUS*, *OPTN* and *DAO*, in fALS (6–19). Despite the abundance of genes and loci identified in fALS, mutations in these genes explain only a small minority of sALS (20).

Regarding susceptibility genes for sALS, >30 association studies based on the candidate-gene approach have been reported (21,22). Among them, *NEFH*, *APEX* and *ANG* have the most evidence; associations of these genes have been found in Caucasians (23–25) and replicated in several studies (7,22,26). However, many of the reported genes are still controversial. For example, the association of non-synonymous substitution (P413L) in the chromogranin B gene (*CHGB*) is reported in French, French-Canadian and Scandinavian ALS populations (27), but has not been found in a Dutch and another French population (28,29).

The genome-wide association study (GWAS) has identified five ALS-susceptibility genes (*FGGY*, *ITPR2*, *DPP6*, *KIFAP3* and *UNC13A*) and two loci (9p21.2 and 10q26.3) in Caucasian (30–35). These results are promising, but remain slightly controversial (36–39). The association of the 9p21.2 locus has been independently replicated in three studies (34,40,41), but is not found in all populations, including those from Japan and China (42). More studies are necessary to evaluate and confirm these previously reported ALS-susceptibility genes.

To identify novel susceptibility genes for ALS, we conducted a large-scale genetic association study in Japanese ALS patients using gene-based single-nucleotide polymorphisms (SNPs) (43). We identified a functional SNP that was significantly associated with ALS. The SNP was located in an enhancer region of *ZNF512B*, a previously uncharacterized transcription factor, and the susceptibility allele of the SNP had decreased enhancer activity for the *ZNF512B* promoter and decreased binding capacity to nuclear proteins. We found that in neuron cells, ZNF512B acts as a positive regulator of transforming growth factor-β (TGF-β) signaling, which is known to be neuroprotective and critical for maintenance and/or survival of neurons (44–46). We demonstrated the localization of ZNF512B in the spinal cord of ALS patients and it showed enhanced expression in motor neuron cells of the anterior horn when compared with controls.

RESULTS

Genome screening

We carried out a stepwise case–control association study (Supplementary Material, Fig. S1) as previously described (47–51). In stage 1 of the discovery series, 92 ALS and 233 control subjects were analyzed at 52 608 gene-based SNP loci selected from the JSNP database (43). Genotype information was successfully obtained for 48 939 SNPs on autosomal chromosomes passed after the quality control. Either the Chi-square test or Fisher's exact test was performed for three genetic models: dominant, recessive and allelic. Comparison of observed and expected distributions showed no evidence for inflation of the trend test statistics (inflation factor, lambda = 1.04; Supplementary Material, Fig. S2). Also, principal component analysis (52) in stage 1 and HapMap samples showed no evidence of population stratification between the case and control groups (Supplementary Material, Fig. S3). In stage 2 of the discovery series, 893 SNPs that showed *P*-values of ≤ 0.01 in stage 1 were genotyped for an additional 1087 subjects (362 ALS cases and 725 controls). Subsequently, 10 SNPs with *P*-values <0.001 were identified by the Chi-square test for the three models (Supplementary Material, Table S1).

Identification of genetic association between rs2275294 and ALS

We validated the association of these SNPs using independent subjects from Biobank Japan (sample set 1). In all, 249 ALS cases and 1030 controls were genotyped and validated the association in rs2275294 (allele model, $P = 1.8 \times 10^{-3}$). The SNP was then genotyped in an independent Japanese population consisting of 602 ALS cases and 2256 controls (sample set 2). Significant association was replicated in this population (allele model $P = 5.6 \times 10^{-5}$). The combined *P*-values for the stepwise association study calculated by the Mantel–Haenszel method and the joint analysis were 9.3×10^{-10} and 6.7×10^{-10}, respectively (Table 1). The combined *P*-values remained significant after Bonferroni correction ($9.3 \times 10^{-10} \times 52\,608 \times 3 = 1.47 \times 10^{-4}$). The *P*-values from the Mantel–Haenszel method and the joint analysis were very similar, supporting the fact that there is no hidden confounder in our population. The minor allele frequency (MAF) of rs2275294 in 744 samples of the Japan Biological Informatics Consortium (JBIC)-genotyping data deposited in the dbSNP database was similar to that of our controls (0.414).

Evaluation of rs2275294

We assessed the stratification using principal component analysis (52). The top six principal components were associated with case–control status. The association of rs2275294 with the top six principal components included as covariates (trend model $P = 0.00287$) was similar to that in stage 1 (trend model $P = 0.00246$), suggesting no stratification. Population stratification was also assessed by evaluating differences in population structure among all case and control sample sets using Wright's F statistics (53). There was no difference in the population structure among these groups (Supplementary Material, Table S2). Potential confounding factors were also

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.

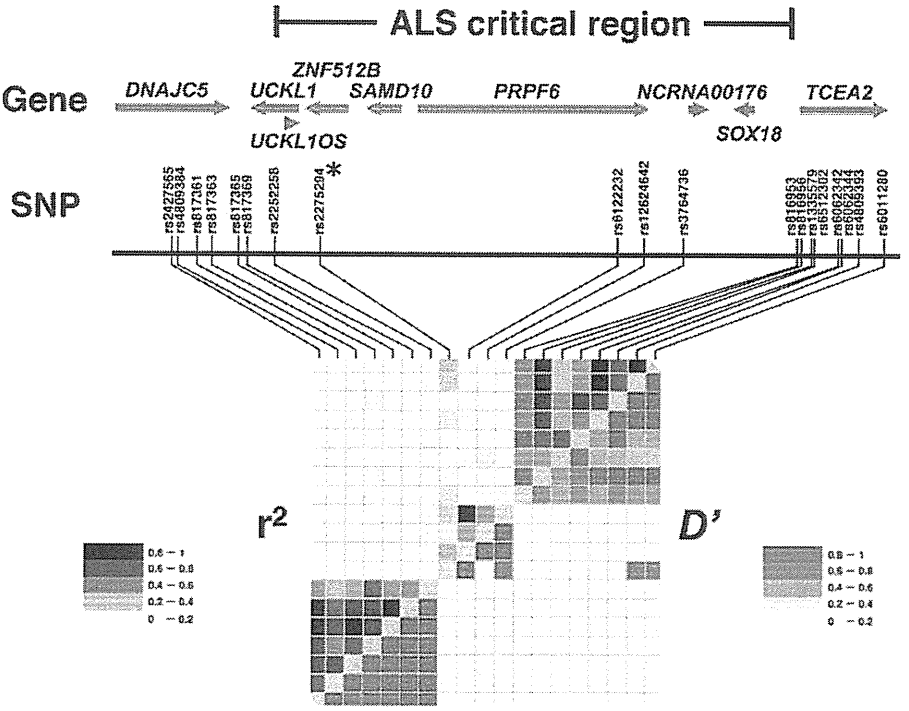

Figure 1. Genomic structure and linkage disequilibrium (LD) map in the ALS critical region. Top, an SNP map of a 111 kb genomic region containing *ZNF512B*. The orientation of each gene is indicated by a green arrow. An asterisk shows the landmark SNP. Bottom, an LD map as measured by D' (lower right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

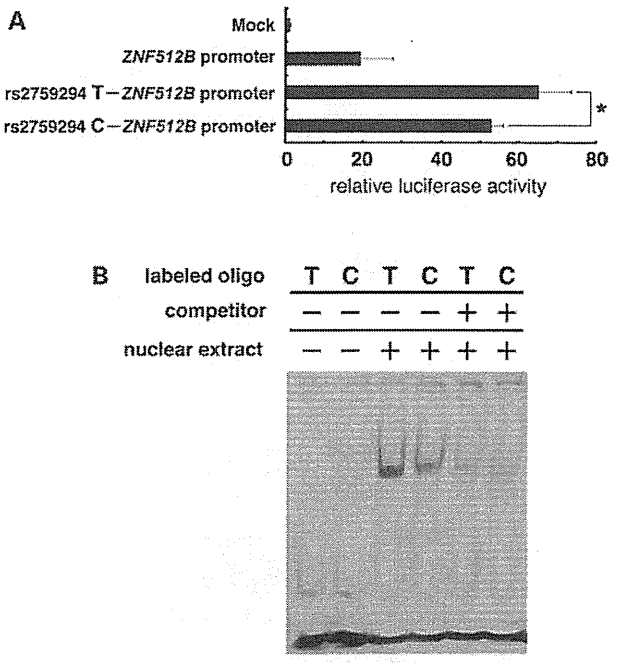

Figure 2. Functional analysis for rs2275294 in *ZNF512B*. (**A**) Difference in the enhancer activity of genomic DNA segments containing rs2275294. Luciferase assay in SK_N_Be(2)C cells. Enhancer activity was lower in the ALS-susceptibility allele (C allele). *ZNF512B* promoter:native promoter (nts −820 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).

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

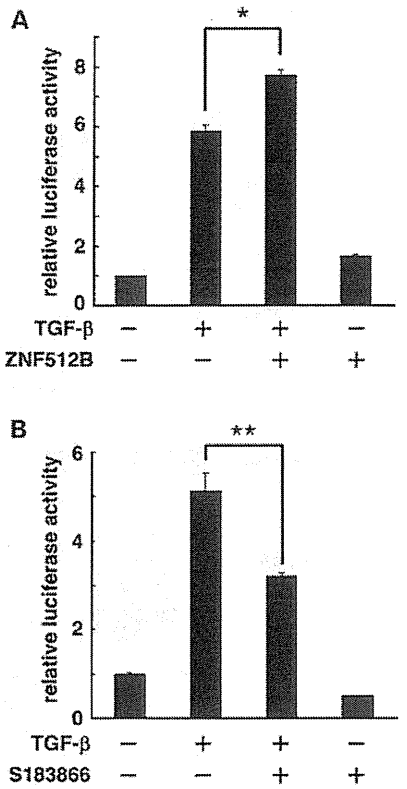

Figure 3. ZNF512B is a positive regulator of the TGF-β signal. (**A**) Luciferase assay using SBE4-luciferase. ZNF512B trans-activated the TGF-β-induced SMAD transcriptional activity in the SK_N_AS cell line ($^{*}P < 0.0005$). (**B**) S183866, a *ZNF512B*-targeting siRNA oligonucleotide repressed the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

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

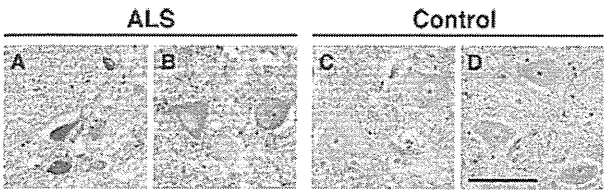

Figure 4. Immuno-histochemical localization of ZNF512B in the spinal cord of ALS patients (**A** and **B**) and controls (**C** and **D**). The ZNF512B immuno-reactivity was intense in motor neuron cells in the anterior horn of ALS patients, while it was hardly detectable in those of controls. Glial 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.

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.

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Conflict of Interest statement. None declared.

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REFERENCES

1. Wijesekera, L.C. and Leigh, P.N. (2009) Amyotrophic lateral sclerosis. *Orphanet J. Rare Dis.*, **4**, 3.
2. Cronin, S., Hardiman, O. and Traynor, B.J. (2007) Ethnic variation in the incidence of ALS: a systematic review. *Neurology*, **68**, 1002–1007.
3. Al-Chalabi, A., Fang, F., Hanby, M.F., Leigh, P.N., Shaw, C.E., Ye, W. and Rijsdijk, F. (2010) An estimate of amyotrophic lateral sclerosis heritability using twin data. *J. Neurol. Neurosurg. Psychiatry*, **81**, 1324–1326.
4. Wroe, R., Wai-Ling Butler, A., Andersen, P.M., Powell, J.F. and Al-Chalabi, A. (2008) ALSOD: the Amyotrophic Lateral Sclerosis Online Database. *Amyotroph. Lateral Scler.*, **9**, 249–250.
5. Yoshida, M., Takahashi, Y., Koike, A., Fukuda, Y., Goto, J. and Tsuji, S. (2010) A mutation database for amyotrophic lateral sclerosis. *Hum. Mutat.*, **31**, 1003–1010.
6. Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., Deng, H.X. *et al.* (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature*, **362**, 59–62.
7. Al-Chalabi, A., Andersen, P.M., Nilsson, P., Chioza, B., Andersson, J.L., Russ, C., Shaw, C.E., Powell, J.F. and Leigh, P.N. (1999) Deletions of the heavy neurofilament subunit tail in amyotrophic lateral sclerosis. *Hum. Mol. Genet.*, **8**, 157–164.
8. Yang, Y., Hentati, A., Deng, H.X., Dabbagh, O., Sasaki, T., Hirano, M., Hung, W.Y., Ouahchi, K., Yan, J., Azim, A.C. *et al.* (2001) The gene encoding alsin, a protein with three guanine-nucleotide exchange factor domains, is mutated in a form of recessive amyotrophic lateral sclerosis. *Nat. Genet.*, **29**, 160–165.
9. Hadano, S., Hand, C.K., Osuga, H., Yanagisawa, Y., Otomo, A., Devon, R.S., Miyamoto, N., Showguchi-Miyata, J., Okada, Y., Singaraja, R. *et al.* (2001) A gene encoding a putative GTPase regulator is mutated in familial amyotrophic lateral sclerosis 2. *Nat. Genet.*, **29**, 166–173.
10. Puls, I., Jonnakuty, C., LaMonte, B.H., Holzbaur, E.L., Tokito, M., Mann, E., Floeter, M.K., Bidus, K., Drayna, D., Oh, S.J. *et al.* (2003) Mutant dynactin in motor neuron disease. *Nat. Genet.*, **33**, 455–456.
11. Nishimura, A.L., Mitne-Neto, M., Silva, H.C., Richieri-Costa, A., Middleton, S., Cascio, D., Kok, F., Oliveira, J.R., Gillingwater, T., Webb, J. *et al.* (2004) A mutation in the vesicle-trafficking protein VAPB causes late-onset spinal muscular atrophy and amyotrophic lateral sclerosis. *Am. J. Hum. Genet.*, **75**, 822–831.
12. Chen, Y.Z., Bennett, C.L., Huynh, H.M., Blair, I.P., Puls, I., Irobi, J., Dierick, I., Abel, A., Kennerson, M.L., Rabin, B.A. *et al.* (2004) DNA/RNA helicase gene mutations in a form of juvenile amyotrophic lateral sclerosis (ALS4). *Am. J. Hum. Genet.*, **74**, 1128–1135.
13. Greenway, M.J., Andersen, P.M., Russ, C., Ennis, S., Cashman, S., Donaghy, C., Patterson, V., Swingler, R., Kieran, D., Prehn, J. *et al.* (2006) ANG mutations segregate with familial and 'sporadic' amyotrophic lateral sclerosis. *Nat. Genet.*, **38**, 411–413.
14. Kabashi, E., Valdmanis, P.N., Dion, P., Spiegelman, D., McConkey, B.J., Vande Velde, C., Bouchard, J.P., Lacomblez, L., Pochigaeva, K., Salachas, F. *et al.* (2008) TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. *Nat. Genet.*, **40**, 572–574.
15. Sreedharan, J., Blair, I.P., Tripathi, V.B., Hu, X., Vance, C., Rogelj, B., Ackerley, S., Durnall, J.C., Williams, K.L., Buratti, E. *et al.* (2008) TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science*, **319**, 1668–1672.
16. Kwiatkowski, T.J. Jr, Bosco, D.A., Leclerc, A.L., Tamrazian, E., Vanderburg, C.R., Russ, C., Davis, A., Gilchrist, J., Kasarskis, E.J., Munsat, T. *et al.* (2009) Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science*, **323**, 1205–1208.
17. Vance, C., Rogelj, B., Hortobagyi, T., De Vos, K.J., Nishimura, A.L., Sreedharan, J., Hu, X., Smith, B., Ruddy, D., Wright, P. *et al.* (2009) Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science*, **323**, 1208–1211.
18. Maruyama, H., Morino, H., Ito, H., Izumi, Y., Kato, H., Watanabe, Y., Kinoshita, Y., Kamada, M., Nodera, H., Suzuki, H. *et al.* (2010) Mutations of optineurin in amyotrophic lateral sclerosis. *Nature*, **465**, 223–226.
19. Mitchell, J., Paul, P., Chen, H.J., Morris, A., Payling, M., Falchi, M., Habgood, J., Panoutsou, S., Winkler, S., Tisato, V. *et al.* (2010) Familial amyotrophic lateral sclerosis is associated with a mutation in D-amino acid oxidase. *Proc. Natl Acad. Sci. USA*, **107**, 7556–7561.
20. Beleza-Meireles, A. and Al-Chalabi, A. (2009) Genetic studies of amyotrophic lateral sclerosis: controversies and perspectives. *Amyotroph. Lateral Scler.*, **10**, 1–14.
21. Simpson, C.L. and Al-Chalabi, A. (2006) Amyotrophic lateral sclerosis as a complex genetic disease. *Biochim. Biophys. Acta*, **1762**, 973–985.
22. Schymick, J.C., Talbot, K. and Traynor, B.J. (2007) Genetics of sporadic amyotrophic lateral sclerosis. *Hum. Mol. Genet.*, **16**, R233–R242.
23. Figlewicz, D.A., Krizus, A., Martinoli, M.G., Meininger, V., Dib, M., Rouleau, G.A. and Julien, J.P. (1994) Variants of the heavy neurofilament subunit are associated with the development of amyotrophic lateral sclerosis. *Hum. Mol. Genet.*, **3**, 1757–1761.
24. Hayward, C., Colville, S., Swingler, R.J. and Brock, D.J. (1999) Molecular genetic analysis of the APEX nuclease gene in amyotrophic lateral sclerosis. *Neurology*, **52**, 1899–1901.
25. Greenway, M.J., Alexander, M.D., Ennis, S., Traynor, B.J., Corr, B., Frost, E., Green, A. and Hardiman, O. (2004) A novel candidate region for ALS on chromosome 14q11.2. *Neurology*, **63**, 1936–1938.
26. Skvortsova, V., Shadrina, M., Slominsky, P., Levitsky, G., Kondratieva, E., Zherebtsova, A., Levitskaya, N., Alekhin, A., Serdyuk, A. and Limborska, S. (2004) Analysis of heavy neurofilament subunit gene polymorphism in Russian patients with sporadic motor neuron disease (MND). *Eur. J. Hum. Genet.*, **12**, 241–244.
27. Gros-Louis, F., Andersen, P.M., Dupre, N., Urushitani, M., Dion, P., Souchon, F., D'Amour, M., Camu, W., Meininger, V., Bouchard, J.P. *et al.* (2009) Chromogranin B P413L variant as risk factor and modifier of disease onset for amyotrophic lateral sclerosis. *Proc. Natl Acad. Sci. USA*, **106**, 21777–21782.
28. Blasco, H., Corcia, P., Veyrat-Durebex, C., Coutadeur, C., Fournier, C., Camu, W., Gordon, P., Praline, J., Andres, C.R. and Vourc'h, P. (2010) The P413L chromogranin B variation in French patients with sporadic amyotrophic lateral sclerosis. *Amyotroph. Lateral Scler.*, doi:10.3109/17482968.2011.522587.
29. van Vught, P.W., Veldink, J.H. and van den Berg, L.H. (2010) P413L CHGB is not associated with ALS susceptibility or age at onset in a Dutch population. *Proc. Natl Acad. Sci. USA*, **107**, E77; author reply E78.
30. Schymick, J.C., Scholz, S.W., Fung, H.C., Britton, A., Arepalli, S., Gibbs, J.R., Lombardo, F., Matarin, M., Kasperaviciute, D., Hernandez, D.G. *et al.* (2007) Genome-wide genotyping in amyotrophic lateral sclerosis and neurologically normal controls: first stage analysis and public release of data. *Lancet Neurol.*, **6**, 322–328.
31. Dunckley, T., Huentelman, M.J., Craig, D.W., Pearson, J.V., Szelinger, S., Joshipura, K., Halperin, R.F., Stamper, C., Jensen, K.R., Letizia, D. *et al.* (2007) Whole-genome analysis of sporadic amyotrophic lateral sclerosis. *N. Engl. J. Med.*, **357**, 775–788.
32. van Es, M.A., Van Vught, P.W., Blauw, H.M., Franke, L., Saris, C.G., Andersen, P.M., Van Den Bosch, L., de Jong, S.W., van 't Slot, R., Birve, A. *et al.* (2007) ITPR2 as a susceptibility gene in sporadic amyotrophic lateral sclerosis: a genome-wide association study. *Lancet Neurol.*, **6**, 869–877.

33. van Es, M.A., van Vught, P.W., Blauw, H.M., Franke, L., Saris, C.G., Van den Bosch, L., de Jong, S.W., de Jong, V., Baas, F., van't Slot, R. *et al.* (2008) Genetic variation in DPP6 is associated with susceptibility to amyotrophic lateral sclerosis. *Nat. Genet.*, **40**, 29–31.
34. van Es, M.A., Veldink, J.H., Saris, C.G., Blauw, H.M., van Vught, P.W., Birve, A., Lemmens, R., Schelhaas, H.J., Groen, E.J., Huisman, M.H. *et al.* (2009) Genome-wide association study identifies 19p13.3 (UNC13A) and 9p21.2 as susceptibility loci for sporadic amyotrophic lateral sclerosis. *Nat. Genet.*, **41**, 1083–1087.
35. Landers, J.E., Melki, J., Meininger, V., Glass, J.D., van den Berg, L.H., van Es, M.A., Sapp, P.C., van Vught, P.W., McKenna-Yasek, D.M., Blauw, H.M. *et al.* (2009) Reduced expression of the Kinesin-Associated Protein 3 (KIFAP3) gene increases survival in sporadic amyotrophic lateral sclerosis. *Proc. Natl Acad. Sci. USA*, **106**, 9004–9009.
36. Fernandez-Santiago, R., Sharma, M., Berg, D., Illig, T., Anneser, J., Meyer, T., Ludolph, A. and Gasser, T. (2009) No evidence of association of FLJ10986 and ITPR2 with ALS in a large German cohort. *Neurobiol. Aging*, **32**, 551e1–551e4.
37. Fogh, I., D'Alfonso, S., Gellera, C., Ratti, A., Cereda, C., Penco, S., Corrado, L., Soraru, G., Castellotti, B., Tiloca, C. *et al.* (2009) No association of DPP6 with amyotrophic lateral sclerosis in an Italian population. *Neurobiol. Aging*, **32**, 966–967.
38. Chiò, A., Schymick, J.C., Restagno, G., Scholz, S.W., Lombardo, F., Lai, S.L., Mora, G., Fung, H.C., Britton, A., Arepalli, S. *et al.* (2009) A two-stage genome-wide association study of sporadic amyotrophic lateral sclerosis. *Hum. Mol. Genet.*, **18**, 1524–1532.
39. Daoud, H., Belzil, V., Desjarlais, A., Camu, W., Dion, P.A. and Rouleau, G.A. (2010) Analysis of the UNC13A gene as a risk factor for sporadic amyotrophic lateral sclerosis. *Arch. Neurol.*, **67**, 516–517.
40. Laaksovirta, H., Peuralinna, T., Schymick, J.C., Scholz, S.W., Lai, S.L., Myllykangas, L., Sulkava, R., Jansson, L., Hernandez, D.G., Gibbs, J.R. *et al.* (2010) Chromosome 9p21 in amyotrophic lateral sclerosis in Finland: a genome-wide association study. *Lancet Neurol.*, **9**, 978–985.
41. Shatunov, A., Mok, K., Newhouse, S., Weale, M.E., Smith, B., Vance, C., Johnson, L., Veldink, J.H., van Es, M.A., van den Berg, L.H. *et al.* (2010) Chromosome 9p21 in sporadic amyotrophic lateral sclerosis in the UK and seven other countries: a genome-wide association study. *Lancet Neurol.*, **9**, 986–994.
42. Iida, A., Takahashi, A., Deng, M., Zhang, Y., Wang, J., Atsuta, N., Tanaka, F., Kamei, T., Sano, M., Oshima, S. *et al.* (2011) Replication analysis of SNPs on 9p21.2 and 19p13.3 with amyotrophic lateral sclerosis in East Asians. *Neurobiol. Aging*, **32**, 757e13–757e14.
43. Haga, H., Yamada, R., Ohnishi, Y., Nakamura, Y. and Tanaka, T. (2002) Gene-based SNP discovery as part of the Japanese Millennium Genome Project: identification of 190,562 genetic variations in the human genome. Single-nucleotide polymorphism. *J. Hum. Genet.*, **47**, 605–610.
44. Henrich-Noack, P., Prehn, J.H. and Krieglstein, J. (1994) Neuroprotective effects of TGF-beta 1. *J. Neural. Transm. Suppl.*, **43**, 33–45.
45. Iwasaki, Y., Shiojima, T., Tagaya, N., Kobayashi, T. and Kinoshita, M. (1997) Effect of transforming growth factor beta 1 on spinal motor neurons after axotomy. *J. Neurol. Sci.*, **147**, 9–12.
46. Krieglstein, K., Strelau, J., Schober, A., Sullivan, A. and Unsicker, K. (2002) TGF-beta and the regulation of neuron survival and death. *J. Physiol. Paris*, **96**, 25–30.
47. Ozaki, K., Ohnishi, Y., Iida, A., Sekine, A., Yamada, R., Tsunoda, T., Sato, H., Hori, M., Nakamura, Y. and Tanaka, T. (2002) Functional SNPs in the lymphotoxin-alpha gene that are associated with susceptibility to myocardial infarction. *Nat. Genet.*, **32**, 650–654.
48. Kubo, M., Hata, J., Ninomiya, T., Matsuda, K., Yonemoto, K., Nakano, T., Matsushita, T., Yamazaki, K., Ohnishi, Y., Saito, S. *et al.* (2007) A nonsynonymous SNP in PRKCH (protein kinase C eta) increases the risk of cerebral infarction. *Nat. Genet.*, **39**, 212–217.
49. Tomlinson, I.P., Webb, E., Carvajal-Carmona, L., Broderick, P., Howarth, K., Pittman, A.M., Spain, S., Lubbe, S., Walther, A., Sullivan, K. *et al.* (2008) A genome-wide association study identifies colorectal cancer susceptibility loci on chromosomes 10p14 and 8q23.3. *Nat. Genet.*, **40**, 623–630.
50. Miyamoto, Y., Shi, D., Nakajima, M., Ozaki, K., Sudo, A., Kotani, A., Uchida, A., Tanaka, T., Fukui, N., Tsunoda, T. *et al.* (2008) Common variants in DVWA on chromosome 3p24.3 are associated with susceptibility to knee osteoarthritis. *Nat. Genet.*, **40**, 994–998.
51. Suppiah, V., Moldovan, M., Ahlenstiel, G., Berg, T., Weltman, M., Abate, M.L., Bassendine, M., Spengler, U., Dore, G.J., Powell, E. *et al.* (2009) IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat. Genet.*, **41**, 1100–1104.
52. Price, A.L., Patterson, N.J., Plenge, R.M., Weinblatt, M.E., Shadick, N.A. and Reich, D. (2006) Principal components analysis corrects for stratification in genome-wide association studies. *Nat. Genet.*, **38**, 904–909.
53. Weir, B.S. (1996) *Genetic Data Analysis II.* Sinauer Associates, Sunderland.
54. Colland, F., Jacq, X., Trouplin, V., Mougin, C., Groizeleau, C., Hamburger, A., Meil, A., Wojcik, J., Legrain, P. and Gauthier, J.M. (2004) Functional proteomics mapping of a human signaling pathway. *Genome Res.*, **14**, 1324–1332.
55. Zawel, L., Dai, J.L., Buckhaults, P., Zhou, S., Kinzler, K.W., Vogelstein, B. and Kern, S.E. (1998) Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol. Cell*, **1**, 611–617.
56. Nagase, T., Ishikawa, K., Kikuno, R., Hirosawa, M., Nomura, N. and Ohara, O. (1999) Prediction of the coding sequences of unidentified human genes. XV. The complete sequences of 100 new cDNA clones from brain which code for large proteins in vitro. *DNA Res.*, **6**, 337–345.
57. Docagne, F., Nicole, O., Gabriel, C., Fernandez-Monreal, M., Lesne, S., Ali, C., Plawinski, L., Carmeliet, P., MacKenzie, E.T., Buisson, A. *et al.* (2002) Smad3-dependent induction of plasminogen activator inhibitor-1 in astrocytes mediates neuroprotective activity of transforming growth factor-beta 1 against NMDA-induced necrosis. *Mol. Cell Neurosci.*, **21**, 634–644.
58. Houi, K., Kobayashi, T., Kato, S., Mochio, S. and Inoue, K. (2002) Increased plasma TGF-beta1 in patients with amyotrophic lateral sclerosis. *Acta Neurol. Scand.*, **106**, 299–301.
59. Jiang, Y.M., Yamamoto, M., Kobayashi, Y., Yoshihara, T., Liang, Y., Terao, S., Takeuchi, H., Ishigaki, S., Katsuno, M., Adachi, H. *et al.* (2005) Gene expression profile of spinal motor neurons in sporadic amyotrophic lateral sclerosis. *Ann. Neurol.*, **57**, 236–251.
60. Nakamura, M., Ito, H., Wate, R., Nakano, S., Hirano, A. and Kusaka, H. (2008) Phosphorylated Smad2/3 immunoreactivity in sporadic and familial amyotrophic lateral sclerosis and its mouse model. *Acta Neuropathol.*, **115**, 327–334.
61. Ilzecka, J., Stelmasiak, Z. and Dobosz, B. (2002) Transforming growth factor-Beta 1 (tgf-Beta 1) in patients with amyotrophic lateral sclerosis. *Cytokine*, **20**, 239–243.
62. Brooks, B.R., Miller, R.G., Swash, M. and Munsat, T.L. (2000) El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. *Amyotroph. Lateral Scler. Other Motor Neuron Disord.*, **1**, 293–299.
63. Nakamura, Y. (2007) The BioBank Japan Project. *Clin. Adv. Hematol. Oncol.*, **5**, 696–697.
64. Ohnishi, Y., Tanaka, T., Ozaki, K., Yamada, R., Suzuki, H. and Nakamura, Y. (2001) A high-throughput SNP typing system for genome-wide association studies. *J. Hum. Genet.*, **46**, 471–477.
65. Tsunoda, T., Lathrop, G.M., Sekine, A., Yamada, R., Takahashi, A., Ohnishi, Y., Tanaka, T. and Nakamura, Y. (2004) Variation of gene-based SNPs and linkage disequilibrium patterns in the human genome. *Hum. Mol. Genet.*, **13**, 1623–1632.
66. Saito, A. and Kamatani, N. (2002) Strategies for genome-wide association studies: optimization of study designs by the stepwise focusing method. *J. Hum. Genet.*, **47**, 360–365.
67. International HapMap Consortium. (2005) A haplotype map of the human genome. *Nature*, **437**, 1299–1320.
68. Andrews, N.C. and Faller, D.V. (1991) A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res.*, **19**, 2499.