follows: (1) Optic neuritis in NMO could be much more serious than in MS, and often leads to blindness, (2) MRI scan of NMO often reveals presence of an extensive lesion extending over three vertebral segments (Figure 1), referred to as 'Longitudinally extensive spinal cord lesion' (LESL), (3) Oligoclonal bands (OBs) commonly found in the cerebrospinal fluid of MS is only rarely seen in NMO, (4) NMO may show brain lesions, although they are different from characteristic MS lesions. However, the patients during an early stage of NMO or those who have been actively treated may not show the characteristic clinical profile of NMO, and could be misdiagnosed. In this regard, a recent discovery of the specific serological marker of NMO (NMO-IgG or anti-AQP4 antibody) [Lennon *et al.* 2004; Lennon *et al.* 2005] has opened a new gate for diagnosis of NMO. The NMO-specific autoantibody was first identified in the sera from NMO as 'NMO-IgG' based on the ability to stain mouse CNS tissue. The target antigen of NMO-IgG was subsequently identified to be AQP4 [Lennon *et al.* 2005], which has led to establishment of assays that are more feasible and more sensitive than the original NMO-IgG assay [Paul *et al.* 2007; Tanaka *et al.* 2007; Takahashi *et al.* 2006].

Recent studies have shown that anti-AQP4 antibody or NMO-IgG can be detected in a large majority of NMO/OSMS patients, whereas most patients with conventional MS are anti-AQP4 negative [Paul *et al.* 2007; Tanaka *et al.* 2007; Nakashima *et al.* 2006]. Although, it has been argued whether NMO and MS represent distinct entities or not [Weinshenker *et al.* 2006; Kikuchi and Fukazaw, 2005], discovery of anti-AQP4 antibody has obviously strengthened the idea that typical NMO cases are distinct from MS in the pathogenesis. Furthermore, pathological analysis has recently demonstrated a remarkable loss of AQP4 [Misu *et al.* 2007; Roemer *et al.* 2007] along with concomitant absence of glial fibrillary acidic protein, a marker of astrocytes [Misu *et al.* 2007] in the lesions of NMO but not of MS. Although primary targets in MS are thought to be myelin and myelin-forming oligodendrocytes, the results of pathological studies suggest that astrocytes could be attacked by antibodies against AQP4 in NMO, further highlighting the differences between NMO and MS.

As mentioned above, patients predominantly manifesting optic nerve and spinal cord signs have been traditionally diagnosed as OSMS in Japan. A recent analysis showed that a majority of the OSMS patients are anti-AQP4 antibody positive and accompany the LESL, implying that most cases of OSMS could be diagnosed as NMO. However, some of the patients exhibited neither aniti-AQP4 nor LESL [Tanaka *et al.* 2007]. It is possible that these patients may

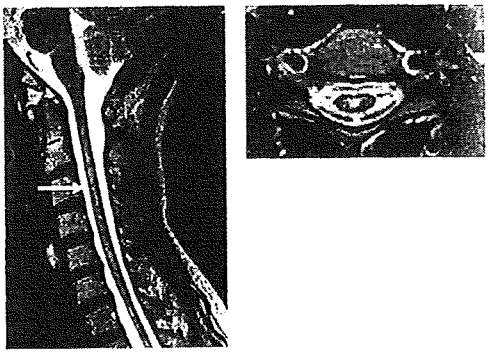

Figure 1. Longitudinally extensive spinal cord lesion (LESL) in a case of NMO T2-weighted cervical MRI demonstrates an extension of T2 high density involving central gray matter, which is characteristic of LESL associated with NMO.

Table 1. Brief history on NMO research.

Report	Year	Author
A report on a possible case with NMO	(1870)	Allbutto
A first case report on definitive NMO	(1894)	Devic
Proposal of a first diagnostic criteria	(1999)	Wingerchuk *et al.*
Discovery of NMO-IgG	(2004)	Lennon *et al.*
Identification of as a target of NMO-IgG	(2005)	Lennon *et al.*
Development of immunofluorescence assay for anti-AQP4 antibodies	(2006)	Takahashi *et al.*
Proposal of revised diagnostic criteria	(2006)	Wingerchuk *et al.*
Demonstration of AQP4 loss in NMO lesions	(2007)	Roemer *et al.*; Misu *et al.*

AQP4, aquaporin 4; NMO, neuromyelitis optica

belong to the category of MS, although the distribution of lesions resembles that of NMO.

Previously, presence of brain lesions and symptoms was an exclusion criterion for NMO. However, the revised diagnostic criteria allow diagnosis of NMO for patients who have brain lesions, provided that the MRI findings do not meet the diagnostic criteria for MS [Wingerchuk *et al.* 2006]. However, Matsuoka *et al.* reported on the presence of NMO patients, who have multiple juxtacortical or periventricular ovoid lesions in the brain, which is characteristic of MS, but not of NMO [Matsuoka *et al.* 2007]. Although this information may be used to argue against the distinction between MS and NMO, we would rather interpret that the patients might have both MS and NMO simultaneously. This possibility needs to be verified rigorously in future studies.

As such, discovery of anti-AQP4 antibody has greatly influenced on the understanding the pathogenesis of NMO. However, it remains unclear whether anti-AQP4 truly plays a role in the formation of destructive lesions in the optic nerve and spinal cord, although the selective loss of AQP4 in the NMO lesions indicate the pathogenic role of anti-AQP4 antibody. A number of investigators are trying to reproduce the pathology of NMO in rodents by passively transferring anti-AQP4 antibody. However, the results have not been published yet. Currently, it remains possible that pathogenic autoantibody in NMO may target CNS antigens other than AQP4.

Cerebrospinal fluid findings in NMO

Cerebrospinal fluid (CSF) examination could also be useful for distinguishing NMO from MS. For instance, presence of prominent CSF pleocytosis ($>50 \times 10^6$ WBC/L) during acute phase could be regarded as supporting diagnosis of NMO but not of MS [Wingerchuk *et al.* 1999]. It is also of note that OBs could be detected more frequently in MS than in NMO [Bergamaschi *et al.* 2004; Misu *et al.* 2002]. Misu *et al.* previously reported that OBs are negative in the Japanese OSMS patients who have no brain lesions on MRI [Misu *et al.* 2002]. However, Bergamaschi *et al.* have recently reported that presence of OBs could be demonstrated in 27% of NMO, when CSF samples were examined repeatedly [Bergamaschi *et al.* 2004]. Notably, the authors pointed out that OBs could be continuously detected during the course of MS, whereas appearance of OBs appears to be temporary in NMO, indicating the importance of repeated CSF examination to distinguish NMO from MS. Very recently, Jarius *et al.* have reported that a polyspecific humoral response against measles, rubella, and varicella zoster virus (MRZ) was positive in 37 out of 42 CSF samples from MS, but was detected only in one out of 20 samples from NMO. They suggest that assessment of the MRZ reaction in the CSF could also help in distinguishing MS and NMO [Jarius *et al.* 2008]. Taken together, these results indicate that a combination of CSF and serum studies may further improve diagnostic certainty.

Activation of IL-17/IL-8 axis in NMO

Besides an elevation of anti-AQP4, recent work has shown that IL-17 and IL-8 are specifically increased in the CSF from NMO [Ishizu *et al.* 2005]. IL-17 is a proinflammatory cytokine mainly produced by activated T cells, whose role in allergy and autoimmune inflammation has been highlighted lately. IL-8 is a chemokine whose major role is to recruit neutrophils. Of note, IL-8 production from macrophages and epithelial cells is promoted by IL-17. Because neutrophil infiltration is dominant in the necrotic lesions of NMO [Ishizu *et al.* 2005], the authors have argued that intrathecal activation of IL-17/IL-8 axis may uniquely contribute to the formation of destructive lesions found in NMO. If this is the case, an important question should be directed to the relationship between the IL-17/IL-8 axis and B cell immunity associated with an elevation of anti-AQP4 antibody. Though very little was known about the relationship between IL-17 and B cells, it has recently been reported that IL-17-producing T cells, namely Th17 cells [Bettelli *et al.* 2007; Steinman, 2007], would promote spontaneous formation of a germinal center and augment production of pathogenic autoantibodies in a model of systemic autoimmune disease [Hsu *et al.* 2008]. In the next section, we discuss on our hypothetical model in which the Th17 cell/B cell interaction plays a role in the pathogenesis of NMO.

Th17 cell biology and pathogenesis of NMO

Th17 cells are a novel helper T cell subset distinct from Th1 or Th2. Because it has been shown that Th17 cells play a decisive role in a variety of inflammatory processes, the biology

of Th17 cells is currently the subject of broad interest [Bettelli *et al.* 2007; Steinman 2007]. Before Th17 cells were identified, studies had emphasized the role of Th1 cells that produce interferon-γ in the pathogenesis of MS and its animal model experimental autoimmune encephalomyelitis (EAE). However, it now becomes clear that Th17 cells are crucial in the induction of EAE, and lymphocytes infiltrating the brain of MS would contain Th17 cells [Tzartos *et al.* 2008]. Although the pathogenic role of Th17 cells is sometimes being overemphasized, involvement of Th1 cells has been confirmed in various inflammatory pathologies. Interestingly, Th1 cells and Th17 cells express different sets of chemokine receptors [Sato *et al.* 2007], indicating that they might be recruited to different types of inflammatory lesions or to different anatomical sites.

Differentiation of rodent Th17 cells depends on IL-6 and transforming growth factor (TGF)-β [Bettelli *et al.* 2007] whereas human Th17 cells appear to be induced in the presence of IL-6 and IL-1β [Acosta-Rodriguez *et al.* 2007]. IL-23 is required for the expansion and maintenance of Th17 cells. As such IL-6 and IL-23 are now thought to be key cytokines in the generation of pathogenic Th17 cells.

The relation between Th17 cells and production of anti-AQP4 antibody is still not clear but could be speculated on the results of animal experiments. It is noteworthy that IL-17 produced by Th17 cells has recently been found to promote the germinal center formation in a spontaneous autoimmune disease model by altering the B cell chemotactic response, which leads to a massive production of pathogenic autoantibody [Hsu *et al.* 2008]. In contrast, blocking IL-17 signaling was inhibitory to the production of autoantibody and prevented the development of the autoimmune disease. These results indicate that Th17 cells would contribute to augmenting B cell autoimmunity through a mechanism distinct from its proinflammatory action. Notably, presence of a germinal center-like structure was demonstrated in the subarachnoid space of a rodent NMO model, which has been created by introducing genes for both T cell receptor (TCR) and B cell receptor for myelin oligodendrocytes glycoprotein (MOG) [Bettelli *et al.* 2006; Krishnamoorthy *et al.* 2006]. The mice spontaneously develop optic neuritis and myelitis. Furthermore, it is thought that collaboration of T cells (Th17) and B cells play a critical role in shaping the unique lesion distribution in this mouse model. If human NMO also involves a Th17 cell/B cell interaction, cytokines, chemokines and their receptors that play a role in Th17 cell-dependent production of pathogenic autoantibody could be potential therapeutic targets in NMO. The hypothetical model will be verified in a future study.

Interferon-β and NMO

Although a small preliminary report suggests the efficacy of interferon-β on OSMS [Saida *et al.* 2005], another study does not recommend its use for NMO in comparison with immunosuppressive agents [Papeix *et al.* 2007]. The most prominent and common side effects of interferon are a flu-like syndrome of fever, headache, myalgia, arthralgia, and general malaise. Furthermore, there are several case reports in Japan documenting a worsening of NMO [Warabi *et al.* 2007] or development of large brain lesions in NMO patients after starting interferon-β [Shimizu *et al.* 2008].

Although the clinical reports need to be carefully analyzed before making a conclusion, some cautions should be made upon the fact that type I interferon (including interferon-α and $-\beta$) would worsen or trigger the development of some antibody-mediated autoimmune diseases. For example, therapeutic use of type I interferon for cancer and hepatitis has been shown to cause exacerbation of SLE, thyroiditis, diabetes, psoriasis, rheumatoid arthritis, autoimmune hemolytic anemia, and myasthenia gravis [Baccala, *et al.* 2005; Theofilopoulos *et al.* 2005; Gota and Calabrise 2003; Stewart, 2003]. Among these, SLE and type I interferon has been causally linked following intensive analysis [Banchereau and Pascual, 2006; Pascual *et al.*' 2006]. Early studies reported increased serum levels of IFN-α in lupus patients, which correlate with disease activity [Kim *et al.* 1987l; Ytterberg and Schnitzer, 1982]. More recently, microarray studies have identified increased expression of interferon-α- and interferon-γ-induced genes in peripheral blood lymphocytes of SLE patients in correlation with disease severity [Bennett *et al.* 2003; Baechler *et al.* 2003; Crow *et al.* 2003; Han *et al.* 2003]. Consistently, interferon-α was recently identified as the serum factor in SLE that could induce differentiation of dendritic cells with efficacious

antigen-presenting ability [Blanco *et al.* 2001]. Type I interferon might also contribute to immune complex formation in SLE by directly activating B cells [Le bon *et al.* 2001]. These results highlight the augmenting effect of type I interferon on antibody-mediated autoimmunity, which differs greatly from that of MS.

It is also of note that interferon-β shows a potential to induce IL-6 *in vitro* [Satoh *et al.* 2006] and *in vivo* [Nakatsuji *et al.* 2006]. IL-6 is a key cytokine involved in the induction of Th17 cells as well as growth and differentiation of B cells. Satoh *et al.* examined the gene expression profile of peripheral blood lymphocytes after culture with interferon-β and found a number of inflammatory cytokines including IL-6 are up-regulated. Nakatsuji *et al.* has shown that the level of serum IL-6 after injection of interferon-β would correlate with side effects such as headache in the patients with MS, but ironically also predict the efficacy of interferon-β treatment in MS. Taken these together, injection of interferon-β could lead to induction of IL-6 at least transiently. From a theoretical point of view, one may argue that the IL-6-stimulatory property of interferon-β is not beneficial for treating NMO involving B cells and Th17 cells, both of which are responsive to IL-6. A systematic retrospective survey for interferon-β treated NMO patients will clarify if this concern is appropriate or not.

According to recent studies, abnormalities found in the brain MRI of NMO ranged from 10 to 50%. Asymptomatic brain lesions are now thought to be common in NMO, and symptomatic brain lesions do not exclude the diagnosis of NMO. Cabrera-Gómez *et al.* has reported that none of the brain MRI abnormalities in NMO were compatible with the criteria of MS brain lesions proposed by Barkhof *et al.* (1997) [Cabrera-Gómez *et al.* 2007]. As an extreme example, we show a patient with NMO, who developed a few large lesions in the brain white matter two months after starting interferon-β (Figure 2). A recent report by Shimizu *et al.* has also described the presence of similar NMO patients who developed large brain lesions after starting interferon-β [Shimizu *et al.* 2008]. The initial clinical and radiological features of our patient were consistent with NMO, and anti-AQP4 antibody was positive. This case suggests to us that a unique pattern of NMO lesion distribution could be transformed into another pattern of disease after undergoing

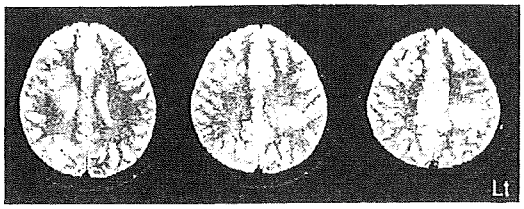

Figure 2. Development of large white matter lesions in a case of neuromyelitis optica (NMO) 2 months after starting interferon-β This young female patient was aquaporin 4 antibody-positive and showed a clinical and radiological picture characteristic of NMO. However, two months after starting interferon-β 1b treatment, she developed signs of brain hemispheres and MRI showed multiple large white matter lesions.

imunomodulation. We also speculate that interferon-β treatment might have triggered the unusual relapse in NMO.

Therapy of NMO in practice

At present, very little information is available that helps physicians and patients choose the best treatment for NMO. In general, treatment of acute exacerbation of NMO may start with intravenous corticosteroids (typically 1,000 mg of methylprednisolone for 3–5 consecutive days). Because the efficacy of plasma exchange was reported in NMO-IgG-positive patients with NMO [Watanabe *et al.* 2007a], plasmapheresis could be considered if clinical improvement is not satisfactory. However, effects of plasmapheresis are not consistent, and anti-AQP4 antibody could rise rapidly after plasmapheresis (Figure 3). To prevent the rebound of pathogenic antibody titers after plasma exchange, a combination therapy with immunosuppressive agents may be needed in some cases. Figure 3 demonstrates the clinical course of representative patients who were treated with plasmapheresis (plasma exchange or immunoadsorption (IA)). In the first case (Figure 3(a)), intravenous methylprednisolone (IVMP) treatment was found to reduce anti-AQP4 antibody titers in the serum, which was accompanied with some clinical improvement. However, as residual symptoms were not tolerable, plasma exchange was subsequently applied, which led to further recovery and disappearance of anti-AQP4 antibody. In the second case (Figure 3(b)), IVMP treatment was followed by plasmapheresis by using IA. We found that the first course of the IVMP plus IA tended to increase the titers of

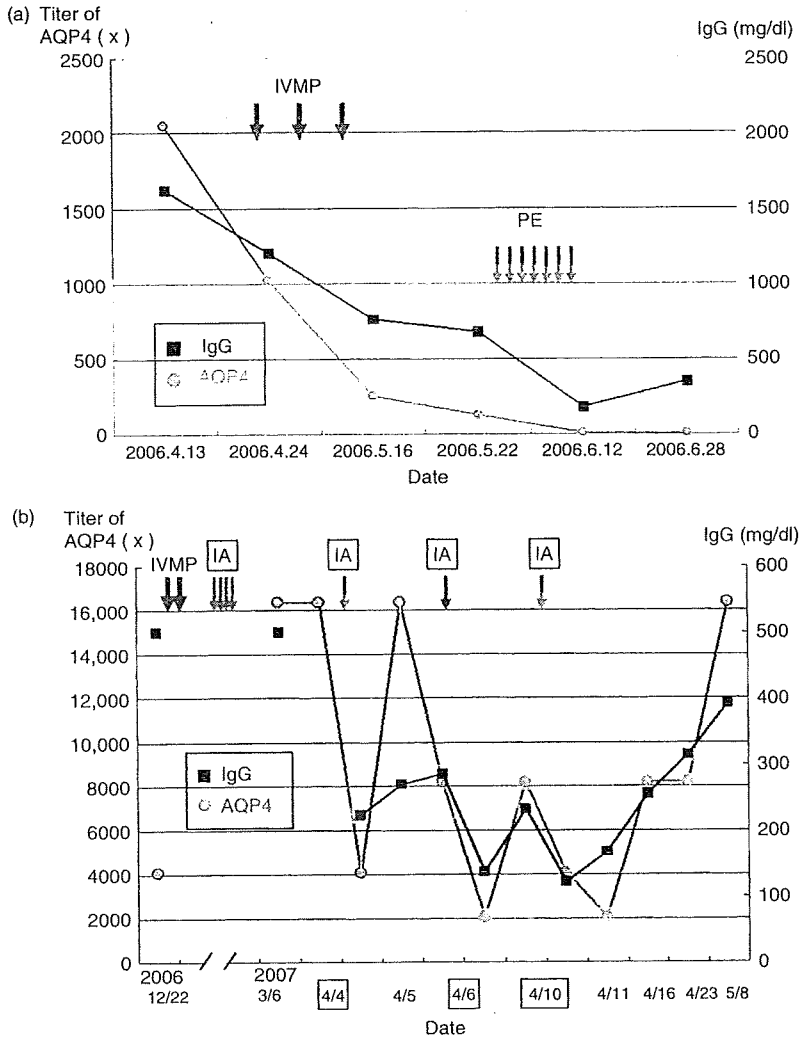

Figure 3. Treatment of NMO with plasmapheresis: representative cases (a) This 36-year-old female developed dysesthesia of the right leg and a constrictive band sensation in the chest region. A few days later, she experienced high fever, the loss of visual perception, progressive muscle weakness, and severe disturbance of sensation in all the limbs. She could not stand and suffered from neurogenic bladder. Treatment was initiated by the administration of 1000 mg/day of methylprednisolone (IVMP) for three consecutive days; this was followed by plasma exchange (PE) therapy which was conducted seven times over a two-week-period. The treatment was judged successful by clinical improvement as well as reduction of anti-aquaporin 4 (AQP4) antibody. (b) This 54-year-old female became completely paraplegic and was confined to bed after the development of thoracic transverse myelitis in December 2006. Although IVMP (1000 mg/day for five days followed by 500 mg/day for three days) and immunoadsorption (IA) therapies (four times) were applied, anti-AQP4 titers were somewhat elevated. So we checked the anti-AQP4 titer and total IgG before and after each of successive IA sessions. IA effectively removed the antibody and reduced the IgG amount after every IA session. But the titer and IgG returned rapidly. The anti-AQP4 antibody exhibits a higher rate of return to the basal level than that of the serum IgG. On evaluation on one month after the last IA, the patient's clinical improvement was very limited, and the anti-AQP4 antibody titer returned to the level of before starting the treatment.

anti-AQP4 antibody eleven weeks after starting the treatment. Subsequently, we measured the antibody titers and amount of serum IgG before and after each successive IA treatment. On each occasion, IA effectively removed the antibody and reduced the IgG amount. However, anti-AQP4 as well as total immunoglobulins recovered very quickly and returned to the pre-treatment level one month after the last IA. We attempted to add an immunosuppressive

drug, but the patient could not tolerate the side effects. The unsatisfactory result indicates that the primary target of therapy should be plasma cells producing pathogenic autoantibody.

To control the production of antibody, azathioprine could be used during the remission phase of NMO, often in combination with oral prednisone. Mandler *et al.* treated seven patients with newly diagnosed NMO with prednisone and azathioprine for 18 months. They found that relapses were prevented completely for more than 18 months and the patients improved significantly in the Expanded Disability Status Scale score [Mandler *et al.* 1998]. Figure 4 shows the clinical course of an anti-AQP4 antibody positive NMO patient being treated in our clinic. This NMO patient was in a state of remission for almost four years after two clinical attacks. However, she suddenly developed optic neuritis and myelitis at 57 years of age, and then interferon-β 1b therapy was introduced. The patient did not respond to the therapy, and clinical activity seemed to be even exacerbated. Because of frequent relapses, azathioprine (100 mg/day) was prescribed in addition. The patient then entered a state of remission, which was maintained even after stopping interferon-β. This interesting case indicates the efficacy of azathioprine in NMO.

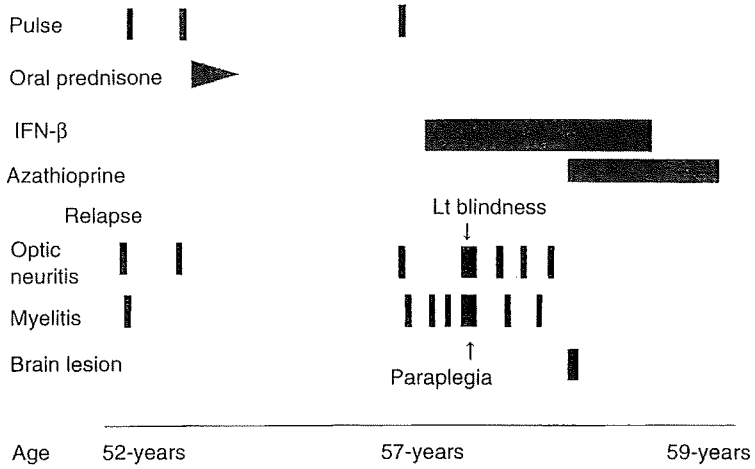

Figure 4. A patient with NMO who did not respond to interferon-β (IFN-β) but to azathioprine. Interferon-β was introduced to this female patient with NMO, as the patient's condition became active. However, there was no noticeable clinical benefit. After adding azathioprine, the patient entered a good remission state without any signs of relapses. Subsequently, we have withdrawn interferon-β, and the remission state is still continuing.

Recently, a retrospective investigation revealed that low-dose corticosteroids might reduce the rate of relapses in NMO [Watanabe *et al.* 2007b]. In some NMO patients, monthly intravenous infusion of immunoglobulin was reported to be effective [Bakker and Metz 2004]. Intravenous infusions of mitoxantrone hydrochloride (12 mg/m^2, monthly for six months followed by three additional treatments every three months) appeared to reduce relapses [Weinstock-Guttman *et al.* 2006]. As mitoxantrone would very potently suppress B-cell immunity directly or through a macrophage-mediated mechanism [Fidler *et al.* 1986], its efficacy in NMO is not unexpected. An open-label study of rituximab (a monoclonal antibody specific for CD20$^+$ B cells) showed an effective outcome for NMO [Cree *et al.* 2005]. Rituximab is an attractive treatment option for NMO because of its selective action against B cells. However, the potential risk and side effects should be taken into consideration. As an alternative therapeutic option, a single case report showed the efficacy of mycophenolate mofetil (2 g/day), which controls T cell-dependent antibody responses through purine synthesis inhibition [Falcini *et al.* 2006]. There is also a case report suggesting efficacy of glatiramer acetate on NMO [Bergamaschi *et al.* 2003].

Concluding remark

NMO is an autoimmune CNS disease characterized by the presence of anti-AQP4 antibody. According to the latest criteria for diagnosis, typical cases of NMO could be easily differentiated from MS by measuring anti-AQP4 antibody and examining the presence of LESL by spinal MRI. However, patients who have been treated with interferon-β or immunosuppressive drugs may show an atypical presentation, such as association of large brain lesions or clinical presentation of NMO without accompanying detectable anti-AQP4 antibody titers. Moreover, if the available anti-AQP4 assay is not sensitive enough, it might be hard to make a conclusive diagnosis of NMO. Interestingly, transgenic mice bearing MOG-specific T cell and B cell receptor are reported to exhibit NMO-like pathology, in which collaboration between T cells and B cells is critical [Bettelli *et al.* 2006; Krishnamoorthy *et al.* 2006]. By contrast, it remains unclear whether anti-AQP4 antibody may be truly pathogenic. It is rather promising to target B cells by a

monoclonal antibody like rituximab or block the T cell-B cell interaction by available drugs. An increase of IL-17 in the CSF also tempts us to consider therapy that modulates IL-6 or IL-23 signaling, which is involved in the generation and maintenance of Th17 cells. Because of recent advances in research, it may not take so long to establish a reasonable and more efficacious protocol for treatment of NMO.

Acknowledgment

We thank Dr Toshiyuki Takahashi at Tohoku University for measuring anti-AQP4 antibody levels.

Conflict of interest statement

None declared.

References

Acosta-Rodriguez, E., Napolitani, G., Lanzavecchia, A. and Sallusto, F. (2007) Interleukin 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 8: 942–949.

Allbutt, T. (1870) On the ophthalmoscopic signs of spinal disease. *Lancet* 1: 76–78.

Baccala, R., Kono, D.H. and Theofilopoulos, A.N. (2005) Interferons as pathogenic effectors in autoimmunity. *Immunol Rev* 204: 9–26.

Baechler, E.C., Batliwalla, F.M., Karypis, G., Gaffney, P.M., Ortmann, W.A., Espe, K.J. *et al.* (2003) Inerferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. *Proc Natl Acad Sci USA* 100: 2610–2615.

Bakker, J. and Metz, L. (2004) Devic's neuromyelitis optica treated with intravenous gamma globulin (IVIG). *Can J Neurol Sci* 31: 265–267.

Banchereau, J. and Pascual, V. (2006) Type I interferon in systemic lupus erythematosus and other autoimmune diseases. *Immunity* 25: 383–392.

Barkhof, F., Filippi, M., Miller, D.H., Sheltens, P., Campi, A., Polman, C.H. *et al.* (1997) Comparison of MRI criteria at first presentation to predict conversion to clinically definite multiple sclerosis. *Brain* 120: 2059–2069.

Bennett, L., Palucka, A.K., Arce, E., Cantrell, V., Borvak, J., Banchereau, J. and Pascual, V. (2003) Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. *J Exp Med* 197: 711–723.

Bergamaschi, R., Uggetti, C., Tonietti, S., Egitto, M.G. and Cosi, V. (2003) A case of relapsing neuromyelitis optica treated with glatiramer acetate. *J Neurol* 250: 359–361.

Bergamaschi, R., Tonietti, S., Franciotta, D., Candeloro, E., Tavazzi, E., Piccolo, G. *et al.* (2004) Oligoclonal bands in Devic's neuromyelitis optica and multiple sclerosis: differences in repeated cerebrospinal fluid examinations. *Mult Scler* 10: 2–4.

Bettelli, E., Baeten, D., Jäger, A., Sobel, R.A. and Kuchroo, V.K. (2006) Myelin oligodendrocyte glycoprotein-specific T and B cells cooperate to induce a Devic-like disease in mice. *J Clin Invest* 116: 2393–2402.

Bettelli, E., Korn, T. and Kuchroo, V.K. (2007) Th17: the third member of the effector T cell trilogy. *Curr Opin Immunol* 19: 652–657.

Blanco, P., Palucka, A.K., Gill, M., Pascual, V. and Banchereau, J. (2001) Induction of dendritic cell differentiation by IFN-α in systemic lupus erythematosus. *Science* 294: 1540–1543.

Cabrera-Gómez, J.A., Quevedo-Sotolongo, L., Gonzalez-Quevedo, A., Lima, S., Real-Gonzalez, Y., Cristofol-Corominas, M. *et al.* (2007) Brain magnetic resonance imaging findings in relapsing neuromyelitis optica. *Mult Scler* 13: 186–192.

Cree, B.A., Lamb, S., Morgan, K., Chen, A., Waubant, E. and Genain, C. (2005) An open label study of the effects of rituximab in neuromyelitis optica. *Neurology* 64: 1270–1272.

Crow, M.K., Kirou, K.A. and Wohlgemuth, J. (2003) Microarray analysis of interferon-regulated genes in SLE. *Autoimmunity* 36: 481–490.

Devic, E. (1894) Myelite subaiguë compliquée de névrite optique. *Bull Med* 8: 1033–1034.

Falcini, F., Trapani, S., Ricci, L., Resti, M., Simonini, G. and de Martino, M. (2006) Sustained improvement of a girl affected with Devic's disease over 2 years of mycophenolate mofetil treatment. *Rheumatology (Oxford)* 45: 913–915.

Fidler, J.M., DeJoy, S.Q. and Gibbons, J.J. (1986) Selective immunomodulation by the antineoplastic agent mitoxantrone. I. Suppression of B lymphocyte function. *J Immunol* 15;137: 727–732.

Gota, C. and Calabrese, L. (2003) Induction of clinincal autoimmune disease by therapeutic interferon-α. *Autoimmunity* 36: 511–518.

Han, G.M., Chen, S.L., Shen, N., Ye, S., Bao, C.K. and Gu, Y.Y. (2003) Analysis of gene expression profiles in human systemic lupus erythematosus using oligonucleotide microarray. *Genes Immun* 4: 177–186.

Hsu, H.-C., Yang, P., Wang, J., Wu, Q., Myers, R., Chen, J. *et al.* (2008) Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice. *Nature Immunol* 9: 166–175.

Ishizu, T., Osoegawa, M., Mei, F.J., Kikuchi, H., Tanaka, M., Takakura, Y. *et al.* (2005) Intrathecal

activation of the IL-17/IL-8 axis in opticospinal multiple sclerosis. *Brain* 128(Pt 5): 988–1002.

Jacob, A., Matiello, M., Wingerchuk, D.M., Lucchinetti, C.F., Pittock, S.J. and Weinshenker, B.G. (2007) Neuromyelitis optica: changing concepts. *J Neuroimmunol* 187: 126–138.

Jarius, S., Franciotta, D., Bergamaschi, R., Rauer, S., Wandinger, K.P., Petereit, H.F. *et al.* (2008) Polyspecific, antiviral immune response distinguishes multiple sclerosis and neuromyelitis optica. *J Neurol Neurosurg Psychiatry* epub February 12.

Kikuchi, S. and Fukazawa, T. (2005) OSMS is NMO, but not MS: confirmed by NMO-IgG? *Lancet Neurol* 4: 594–595.

Kim, T., Kanayama, Y., Negoro, N., Okamura, M., Takeda, T. and Inoue, T. (1987) Serum levels of interferons in patients with systemic lupus erythematosus. *Clin Exp Immunol* 70: 562–569.

Krishnamoorthy, G., Lassmann, H., Wekerle, H. and Holz, A. (2006) Spontaneous opticospinal encephalomyelitis in a double-transgenic mouse model of autoimmune T cell/B cell cooperation. *J Clin Invest* 116: 2385–2392.

Le bon, A., Schiavoni, G., D'Agostino, G., Gresser, I., Belardelli, F. and Tough, D.F. (2001) Type I interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. *Immunity* 14: 461–470.

Lennon, V.A., Wingerchuk, D.M., Kryzer, T.J., Pittock, S.J., Lucchinetti, C.F., Fujihara, K. *et al.* (2004) A serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis. *Lancet* 364: 2106–2012.

Lennon, V.A., Kryzer, T.J., Pittock, S.J., Verkman, A.S. and Hinson, S.R. (2005) IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 channel. *J Exp Med* 202: 473–477.

Mandler, R.N., Ahmed, W. and Dencoff, J.E. (1998) Devic's neuromyelitis optica: a prospective study of seven patients treated with prednisone and azathioprine. *Neurology* 51: 1219–1220.

Matiello, M., Jacob, A., Wingerchuk, D.M. and Weinshenker, B.G. (2007) Neuromyelitis optica. *Curr Opin Neurol* 20: 255–260.

Matsuoka, T., Matsushita, T., Kawano, Y., Osoegawa, M., Ochi, H., Ishizu, T. *et al.* (2007) Heterogeneity of aquaporin-4 autoimmunity and spinal cord lesions in multiple sclerosis in Japanese. *Brain* 130: 1206–1223.

Misu, T., Fujihara, K., Nakashima, I., Miyazawa, I., Okita, N., Takase, S. *et al.* (2002) Pure optic-spinal form of multiple sclerosis in Japan. *Brain* 125: 2460–2468.

Misu, T., Fujihara, K., Kakita, A., Konno, H., Nakamura, M., Watanabe, S. *et al.* (2007) Loss of aquaporin 4 in lesions of neuromyelitis optica: distinction from multiple sclerosis. *Brain* 130: 1224–1234.

Nakashima, I., Fujihara, K., Miyazawa, I., Misu, T., Narikawa, K., Nakamura, M. *et al.* (2006) Clinical and MRI features of Japanese patients with multiple sclerosis positive for NMO-IgG. *J Neurol Neurosurg Psychiatry* 77: 1073–1075.

Nakatsuji, Y., Nakano, M., Moriya, M., Kishigami, H., Tatsumi, C., Tada, S. *et al.* (2006) Beneficial effect of interferon-β treatment in patients with multiple sclerosis is associated with transient increase in serum IL-6 level in response to interferon-β injection. *Cytokine* 36: 69–74.

Papeix, C., Vidal, J.S., de Seze, J., Pierrot-Deseilligny, C., Tourbah, A., Stankoff, B. *et al.* (2007) Immunosuppressive therapy is more effective than interferon in neuromyelitis optica. *Mult Scler* 13: 256–259.

Paul, F., Jarius, S., Aktas, O., Bluthne, R.M., Baue, R.O., Appelhan, H. *et al.* (2007) Autoantibody to aquaporin 4 in the diagnosis of neuromyelitis optica. *PLoS Med* 4: e133.

Pascual, V., Farkas, L. and Banchereau, J. (2006) Systemic lupus erythematosus: all roads lead to type I interferons. *Curr Opin Immunol* 18: 676–682.

Roemer, S.F., Parisi, J.E., Lennon, V.A., Benarroch, E.E., Lassmann, H., Bruck, W. *et al.* (2007) Pattern-specific loss of aquaporin-4 immunoreactivity distinguishes neuromyelitis optica from multiple sclerosis. *Brain* 130: 1194–1205.

Saida, T., Tashiro, K., Itoyama, Y., Sato, T., Ohashi, Y., Zhao, Z. *et al.* (2005) Interferon beta-1b is effective in Japanese RRMS patients: a randomized multicenter study. *Neurology* 64: 621–630.

Sato, W., Aranami, T. and Yamamura, T. (2007) Cutting Edge. Human Th17 cells are identified as bearing $CCR2^{+}CCR5^{-}$ phenotype. *J Immunol* 178: 7525–7529.

Satoh, J., Nanri, Y., Tabunoki, H. and Yamamura, T. (2006) Microarray analysis identifies a set of CXCR3 and CCR2 ligand chemokines as early IFNβ -responsive genes in peripheral blood lymphocytes in vitro: an implication for IFNβ -related adverse effects in multiple sclerosis. *BMC Neurol* 6: 18.

Shimizu, Y., Yokoyama, K., Misu, T., Takahashi, T., Fujihara, K., Itoyama, Y., Iwata, M. *et al.* (2008) Development of extensive brain lesions following interferon beta therapy in relapsing Neuromyelitis optica and longitudinally extensive myelitis. *J Neurol* 255: 305–307.

Steinman, L. (2007) A brief history of T_H17, the first major revision in the T_H1/T_H2 hypothesis of T cell-mediated tissue damage. *Nat Med* 13: 139–145.

Stewart, T.A. (2003) Neutralizing interferon alpha as a therapeutic approach to autoimmune diseases. *Cytokine Growth Factor Rev* 14: 139–154.

Takahashi, T., Fujihara, K., Nakashima, I., Misu, T., Miyazawa, I., Nakamura, M. *et al.* (2006) Establishment of a new sensitive assay for anti-human aquaporin-4 antibody in neuromyelitis optica. *Tohoku J Exp Med* 210: 307–313.

Tanaka, K., Tani, T., Tanaka, M., Saida, T., Idezuka, J., Yamazaki, M. *et al.* (2007) Anti-aquaporin 4 antibody in selected Japanese multiple sclerosis patients with long spinal cord lesions. *Mult Scler* 13: 850–855.

Theofilopoulos, A.N., Baccala, R., Beutler, B. and Kono, D.H. (2005) Type I Interfesons (α/β) in immunity and autoimmunity. *Annu Rev Immunol* 23: 307–336.

Tsartos, J.S. *et al.* (2008) Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol* 172: 146–155.

Warabi, Y., Matsumoto, Y. and Hayashi, H. (2007) Interferon beta-1b exacerbates multiple sclerosis with severe optic nerve and spinal cord demyelination. *J Neurol Sci* 252: 57–61.

Watanabe, S., Nakashima, I., Misu, T., Miyazawa, I., Shiga, Y., Fujihara, K. *et al.* (2007a) Therapeutic efficacy of plasma exchange in NMO-IgG-positive patients with neuromyelitis optica. *Mult Scler* 13: 128–132.

Watanabe, S., Misu, T., Miyazawa, I., Nakashima, I., Shiga, Y., Fujihara, K. *et al.* (2007b) Low-dose corticosteroids reduce relapses in neuromyelitis optica: a retrospective analysis. *Mult Scler* 13: 968–974.

Weinshenker, B.G., Wingerchuk, D.M., Nakashima, I., Fujihara, K. and Lennon, V.A. (2006) OSMS is NMO, but not MS: proven clinically and pathologically. *Lancet Neurol* 5: 110–111.

Weinstock-Guttman, B., Ramanathan, M., Lincoff, N., Napoli, S.Q., Sharma, J., Feichter, J. *et al.* (2006) Study of mitoxantrone for the treatment of recurrent neuromyelitis optica (Devic disease). *Arch Neurol* 63: 957–963.

Wingerchuk, D.M., Ramanathan, M., Lincoff, N., Napoli, S.Q., Sharma, J., Feichter, J. *et al.* (1999) The clinical course of neuromyelitis optica (Devic's syndrome). *Neurology* 53: 1107–1114.

Wingerchuk, D.M., Lennon, V.A., Pittock, S.J., Lucchinetti, C.F., Weinshenker, B.G. *et al.* (2006) Revised diagnostic criteria for neuromyelitis optica. *Neurology* 66: 1485–1489.

Wingerchuk, D.M., Lennon, V.A., Lucchinetti, C.F., Pittock, S.J., Weinshenker, B.G. *et al.* (2007) The spectrum of neuromyelitis optica. *Lancet Neurol* 6: 805–815.

Yttenberg, S.R. and Schnitzer, T.J. (1982) Serum interferon levels in patients with systemic lupus erythematosus. *Arthritis Rheum* 25: 401–406.

J Hum Genet (2007) 52:643–649
DOI 10.1007/s10038-007-0154-1

ORIGINAL ARTICLE

Redefining the disease locus of 16q22.1-linked autosomal dominant cerebellar ataxia

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Received: 31 January 2007 / Accepted: 2 May 2007 / Published online: 5 July 2007

Abstract The 16q22.1-linked autosomal dominant cerebellar ataxia (16q-ADCA; Online Mendelian Inheritance in Man [OMIN] #117210) is one of the most common ADCAs in Japan. Previously, we had reported that the patients share a common haplotype by founder effect and that a C-to-T substitution (–16C>T) in the *puratrophin*-1 gene was strongly associated with the disease. However, recently, an exceptional patient without the substitution was reported, indicating that a true pathogenic mutation might be present elsewhere. In this study, we clarified the disease locus more definitely by the haplotype analysis of families showing pure cerebellar ataxia. In addition to microsatellite markers, the single nucleotide polymorphisms (SNPs) that we identified on the disease chromosome were examined to confirm the borders of the disease locus. The analysis of 64 families with the –16C>T substitution in the *puratrophin*-1 gene revealed one family showing an ancestral recombination event between SNP04 and SNP05 on the disease chromosome. The analysis of 22 families without identifiable genetic mutations revealed another family carrying the common haplotype centromeric to the *puratrophin*-1 gene, but lacking the –16C>T substitution in this gene. We concluded that the disease locus of 16q-ADCA was definitely confined to a 900-kb genomic region between the SNP04 and the –16C>T substitution in the *puratrophin*-1 gene in 16q22.1.

Keywords 16q-ADCA · Pure cerebellar ataxia · Haplotype · SNP · Founder effect · SCA4

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Introduction

Autosomal dominant cerebellar ataxia (ADCA) is a clinical entity of heterogeneous neurodegenerative diseases that show dominantly inherited, progressive cerebellar ataxia that can be variably associated with other neurological and systemic features (Harding 1982). ADCA is now classified by the responsible mutations or gene loci. Subtypes of ADCA of which causative genes or gene loci have been identified are known as spinocerebellar ataxia type (SCA) 1, 2, 3 (or Machado-Joseph disease), 4–8, 10–19, 21–23, 25, 26, 28, dentatorubral and pallidoluysian atrophy (DRPLA), and ADCA with mutation in the fibroblast growth factor (FGF) 14 gene (Schöls et al. 2004; Yu et al. 2005; Cagnoli et al. 2006).

Among these, mutations in SCA1, SCA2, SCA3/MJD, SCA6, SCA7, SCA17, and DRPLA have been identified as

the expansions of a trinucleotide (CAG) repeat that encodes the polyglutamine tract, uniformly causing the aggregation of polyglutamine-containing causative protein (Ross and Poirier 2004). The expansion of noncoding trinucleotide (CAG or CTG) or pentanucleotide (ATTCT) repeats are involved in SCA8, SCA10, and SCA12 (Holmes et al. 1999; Koob et al. 1999; Matsuura et al. 2000). Very few families are affected by missense mutations in beta-III spectrin (*SPTBN2*) (SCA5 (see Ikeda et al. 2006)), voltage-gated potassium channel KCNC3 (SCA13 (see Waters et al. 2006)), protein kinase C gamma (PKC gamma) (SCA14 (see Chen et al. 2003)), and *FGF14* genes (ADCA with FGF14 mutation (see van Swieten et al. 2003). However, genes or even loci remain unidentified for 20–40% of families with ADCA (Sasaki et al. 2003).

We had previously found that Japanese families with ADCA map to the human chromosome 16q22.1 (16q-ADCA), the gene locus of SCA4 (Flanigan et al. 1996; Hellenbroich et al. 2005; Nagaoka et al. 2000). However, our families show clinically pure cerebellar ataxia without other neurological signs, such as sensory neuropathy or pyramidal tract signs seen in SCA4. All 16q-ADCA patients shared a common haplotype, presumably due to inheritance from a disease chromosome of a founder (Takashima et al. 2001). Our haplotype analysis of 52 families with DNA polymorphic microsatellite markers revealed that they all share a common haplotype for the 400-kb genomic region in 16q22.1 (Ishikawa et al. 2005). Within this region, we found that a heterozygous single nucleotide C-to-T substitution (–16C>T) in the untranslated region of the *puratrophin*-1 gene was entirely segregated with all patients, suggesting a strong association with the disease. This substitution was also found in other cohorts of Japanese families with ataxia (Ouyang et al. 2006; Onodera et al. 2006), while it was not found in Caucasian patients in Europe (Wieczorek et al. 2006). The frequency of 16q-ADCA is considered to be relatively high in Japan, counted as the third or fourth major subtype of ADCA after MJD, SCA6, and DRPLA (Takano et al. 1998; Sasaki et al. 2003; Ohata et al. 2006).

However, one group recently reported an exceptional patient without the –16C>T substitution in the *puratrophin*-1 gene, in a family in which all of the other affected subjects carried the substitution (Ohata et al. 2006). This patient shared the common haplotype in a region centromeric to the substitution in the *puratrophin*-1 gene, suggesting that a true pathogenic mutation may be present in a different gene lying centromeric to the –16C>T substitution in the *puratrophin*-1 gene. Moreover, other patients sharing the common haplotype centromeric to the substitution in the *puratrophin*-1 gene without the substitution might exist.

In this study, we re-examined the haplotype of families showing ataxia in order to clarify a common genomic region shared in all 16q-ADCA patients. Because slippage mutation might cause minor deviations in repeat size for microsatellite markers (Ikeda et al. 2004), single nucleotide polymorphisms (SNPs) detected by ourselves on the disease chromosome were used in the analysis to confirm recombinant regions that are not conserved among families.

Materials and methods

Haplotype analysis

DNA samples from patients showing ataxia referred to our department were examined. After informed consent was obtained, genomic DNA was extracted from peripheral blood lymphocytes or lymphoblastoid cell lines by the use of methods described elsewhere (Ishikawa et al. 1997). All families were excluded for SCA1, SCA2, SCA3/ MJD, SCA6, SCA7, SCA8, SCA12, SCA14, SCA17, and DRPLA by testing for mutations in the disease genes.

Firstly, common haplotypes of the 16q-ADCA families with the –16C>T substitution in the *puratrophin*-1 gene were analyzed. Genotypes were determined for 19 microsatellite markers (D16S3043, D16S3031, D16S3019, CTATT01, TAGA02, GGAA05, D16S397, GGAA10, GATA01, D16S421, TA001, GA001, 17 msm, D16S3107, GGAA01, CTTT01, GT01, D16S3095, D16S512) in 16q22.1 by the use of methods described elsewhere (Ishikawa et al. 2005). Compared to our previous study (Ishikawa et al. 2005), several new markers with high specificity to the 16q-ADCA chromosome were added and the region analyzed was expanded to beyond the previous critical region spanning GATA01 and 17 msm (Ishikawa et al. 2005) in order to determine the maximum genomic region conserved in all of the affected individuals from all of the families. Although the phase of the markers were not confidently determined in families that have only a few examined members, the possibility that they carried the haplotype was indicated in those cases.

Secondly, haplotypes of families without the –16C>T substitution in the *puratrophin*-1 gene were also analyzed to see if they had the common haplotype centromeric to the substitution in the *puratrophin*-1 gene. Their genotypes were determined for 14 markers (D16S3043, D16S3019, CTATT01, TAGA02, GGAA05, D16S397, D16S3086, GATA01, GA001, 17 msm, CTTT01, GT01, D16S3095, D16S512), which are relatively highly specific to the common haplotype in 16q-ADCA.

Single nucleotide polymorphisms

We searched for single nucleotide polymorphisms (SNPs) on the disease chromosome by ourselves because most of

Table 1 The haplotype analysis of 16q22.1-linked autosomal dominant cerebellar ataxia (16q-ADCA) families with the –16CT substitution of the *puratrophin*-1 gene. The gray squares indicate that the alleles are one repeat-unit different fromthe common allele of 16q-ADCA and the black squares indicate alleles with two or more repeat-unitdifferences. One repeat-unit difference was seen for markers D16S397, GGAA10,GATA01, and TA001, close to the *puratrophin*-1 gene in several families, and greaterrepeat-units differences were observed for GGAA05 and other centromeric markers. Similarly, greater repeat-units differences were observed for 17msm and markers lyingtelomeric to 17msm. n.e.=not examined

Marker	most common haplotype	family No. frequency in control (%)	P2	P4	P14	T2	T3	T4	T5	T6	T7	T12	T15	T19	T21	T25	T26	T28	T30	T37	T42	T43	T44	T46
D16S3043	1	25.0	1	1/6	7	5	8	1	1/8	1	1/5	5	1/7	n.e.	1/8	n.e.	n.e.	1	n.e.	1	n.e.	1/5	1/5	1
D16S3031	9	68.1	9	9	9	9	9	9	10	1	10	9	9/10	9	9	9	9	1/9	9	9	9	9	9	9
D16S3019	4	41.4	4	4	4	4	4	4/5	3/4	3/4	3/4	1/4	4	n.e.	4	n.e.	n.e.	4/7	3/4	2/4	n.e.	3/4	3/4	1
CTATT01	1	32.4	1	2/4	1	1	1	1/4	1	1	1	1	1/3	n.e.	1/3	n.e.	n.e.	1/3	n.e.	1	n.e.	1/2	1	0/3
TAGA02	4	10.3	4	6	4	4	4	4/6	4/6	4/5	4	2/4	4/5	n.e.	4/5	n.e.	n.e.	4/5	4	3/4	n.e.	5/6	4/5	2/6
GGAA05	1	1.4	1	6	1	1	5	1	1	1	2	1	2/4	1/3	1/5	5	1/2	1/7	1/5	1/3	1/3	1/5	2/4	3/6
D16S397	1	47.1	n.e.	1/2	1	1	1	0/4	1/2	1/3	1/3	2/3	1	n.e.	1	n.e.	n.e.	1/4	-3/0	-3/1	n.e.	-3/1	1	-3/1
GGAA10	3	13.2	3	3	3	3	4	3/5	3	3	3	3	3	3/6	2/3	2/4	3/7	3/5	3/8	4/6	3	3/6	3/5	3/7
GATA01	2	44.1	2	2/3	2	2	2	3	3	3	2	2	1/2	2/3	1/3	3/4	1/3	1/3	1	2	3	1/3	2/3	2/3
D16S421	3	75.7	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3/4	3	3	3
puratrophin-1(C/T)	T	0.0	T	T	T	T	T	T	T	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C
TA001	1	23.8	1	1	1	1	1	1	1	1	1	1	1	1/9	1/7	2/8	1/6	1/10	1/9	1/9	1	1/9	1/9	1/5
GA001	4	0.1	4	4	4	4	4	4	4	4/7	4/7	4	4	1/4	4/8	4/5	4/7	4/7	4/6	4/11	4/5	4/7	4/5	4/7
17msm	2	8.3	2	2	2	2	2	2	2	2	2	5	2	2/5	2/5	2/4	2/4	2/4	2/4	2/5	2/4	2/4	4/6	2/4
D16S3107	7	13.9	7	7	7	7	7	7	6	7	7	7	5	6	5/6	3/7	6/10	6	7	5/7	3/7	6/7	4/7	6/7
GGAA01	6	18.8	6	6	6	6	6	6	6	7	6	6	1/6	3/6	2/6	2/6	4/6	6/7	6	6	6	6/7	6/7	5/6
CTTT01	8	28.2	8	8	8	8	9/10	8	5	9	8	10	8	9/10	3/9	9/10	8/10	8	8/11	6/9	8/15	9/10	1/7	3/8
GT01	6	15.8	6	6	6	6	6	6	7	6	6	4	6	2/6	1/6	4/6	4/6	2/6	5/6	4/6	6	3/6	3/4	4/6
D16S3095	1	9.7	2	3	1	0	1	1	2	3	2	1/2	1	1	1/2	1/2	1/2	1/3	1	1	1/2	1/3	2	1
D16S512	1	32.3	n.e.	2/4	1	4	1/5	4	2/4	1/5	1/5	1/5	4	n.e.	4/5	n.e.	n.e.	5	1	1/2	n.e.	2/4	4	4/5

the SNPs obtained from public databases were not present on the disease allele or did not have enough specificity to the disease chromosome. SNPs were revealed by direct sequencing of the genomic DNA from a homozygous patient who carries the common haplotypes between D16S3031 and GT01 in both of the chromosomes. Primers were designed to amplify about 800 bp from genomic DNA (primer sequences are available on request), and polymerase chain reaction (PCR) and sequencing were performed with the same methods as previously described (Ishikawa et al. 2005). Comparing the sequenced data and the annotated databases with use of DNASIS (Hitachi) software revealed many SNPs. With the sequenced data of the control genomic DNA, SNPs with high specificity to the 16q-ADCAs were chosen. With these SNPs, 16q-ADCA families were analyzed to reveal the borders of the maximally conserved genomic region.

Results

Haplotype analysis of 16q-ADCA with the –16C>T substitution in the *puratrophin*-1 gene

One hundred and twenty-five patients from 64 families were diagnosed as 16q-ADCA based on the clinical features and the presence of the –16C>T substitution in the *puratrophin*-1 gene. The families included 52 families that we had previously reported (Ishikawa et al. 2005) and 12 new families that had not been reported elsewhere. They all share similar haplotypes around the *puratrophin*-1 gene. The most common haplotype among these families are shown in the left column in Table 1. Twenty-two families out of the 64 families showed different alleles at least for one of the DNA markers as shown in Table 1. The remaining 42 families, which are not listed in Table 1, harbored or had the possibility to harbor the common haplotype.

There was one repeat-unit difference from the common alleles for D16S397, GGAA10, GATA01, and TA001 close to the *puratrophin*-1 gene in 13 out of 22 families. For centromeric DNA markers from the *puratrophin*-1 gene, such as GGAA05, TAGA02, D16S3031, and D16S3043, eight families (P4, P14, T2, T3, T6, T12, T25, T46) harbored alleles with greater differences in repeat number (more than two repeat-units). Furthermore, families P4 and T46 carried different alleles in three consecutive markers, GGAA05, TAGA02, and CTATT01.

Similarly, for telomeric DNA markers such as 17 msm, D16S3107, CTTT01, and GT01, greater differences were seen in three families (T12, T15, T44). Especially, families T12 and T44 harbored different alleles for markers 17 msm, CTTT01, and GT01, which were highly specific to the common haplotype.

The presence of large differences in repeat number and successively different alleles would indicate that the families were sharing the common chromosomal region,

inherited from a founder, between markers GGAA05 and 17 msm.

Haplotype analysis of families without identifiable genetic mutations

Twenty-three patients from 22 families presenting pure cerebellar ataxia did not carry identifiable genetic mutations. Nine families showed autosomal dominant inheritance, and the other families had no apparent family history. Their haplotypes are shown in Table 2. Although no family carried entirely identical alleles to the common haplotype consecutively for the markers telomeric to the *puratrophin*-1 gene, one family (U09) harbored the identical alleles for the markers between D16S3043 and GATA01 centromeric to the *puratrophin*-1 gene. It suggested the possibility that the U09 family have the common haplotype of 16q-ADCA in the region centromeric to the –16C>T substitution in the *puratrophin*-1 gene.

Haplotype analysis with SNPs

Four markers, GGAA05, D16S397, GGAA10, and GATA01 centromeric to the *puratrophin*-1 gene, showing different alleles in Table 1 suggested that ancestral chromosomal recombination might have occurred around the markers. Family U09 and the family reported by Ohata et al. 2006) also suggested ancestral chromosomal recombination around the substitution in the *puratrophin*-1 gene. Therefore, we searched the SNPs around these four markers and the *puratrophin*-1 gene. Five SNPs were identified around the marker GGAA05, one SNP around D16S397, four SNPs around GGAA10, one SNP around GATA01, and two SNPs around the *puratrophin*-1 gene (Table 3). SNP05 and SNP06 showed high specificity to the disease chromosome because they were absent in 200 control chromosomes.

Eighteen families showed different alleles for GGAA05, D16S397, GGAA10, or GATA01 (Table 1). Among them, sufficient amounts of DNA samples were not available in four families (T25, T26, T30, T42). The remaining 14 families were analyzed as shown in Table 4. While 13 out of the 14 families carried all of the same SNPs, family T46 did not carry SNP01, SNP02, SNP03, and SNP04. This confirmed that the genomic region between SNP01 and SNP04 of family T46 was a recombinant region, which was not conserved in all families.

These SNPs were also analyzed for the U09 family suspected of having the common haplotype of 16q-ADCA (Table 4). The family had all 13 SNPs, including SNP05 and SNP06, which are highly specific to the disease chromosome. This strongly suggested that family U09 shared the 16q-ADCA common haplotype centromeric to the –16C>T substitution in the *puratrophin*-1 gene.

Discussion

16q-ADCA is one of the most common ataxic diseases in Japan. We previously showed that 52 families shared the common haplotype in the genomic 400-kb region between the markers GATA01 and 17 msm by analysis with

Table 2 The haplotype analysis of families without identifiable genetic mutation. The black squares indicate that the families carry the identical alleles to the common alleles of 16q-ADCA, and the gray squares indicate alleles with one repeat-unit difference. Only family U09 harbored the identical alleles consecutively for the markers from D16S3043 to GATA01, suggesting that this family may harbor the common haplotype of 16q-ADCA. n.c.=notclear. A.D.=autosomal dominant inheritance was suspected

		Family No.	U01	U02	U03	U04	U05	U06	U07	U08	U09	U10	U11	U12	U13	U14	U15	U16	U17	U18	U19	U20	U21	U22
		Family history	n.c.	n.c.	n.c.	A.D.	n.c.	n.c.	n.c.	A.D.	n.c.	n.c.	A.D.	A.D.	A.D.	n.c.	A.D.	A.D.	A.D.	A.D.	n.c.	n.c.	n.c.	n.c.
Marker	most common haplotype	frequency in control (%)																						
D16S3043	1	25.0	1/6	4/8	1/2	1/7	1	4/6	5	5	1/7	1/7	4/5	4/5	1/6	1/5	5	1/6	5/7	1/6	1	1	1/5	1/8
D16S3019	4	41.4		1/4	4	1	3/4	5	3/4	4	4	3/4	1	2	4	4/5	4/5	3/4	1	2	1	1	1/3	1/2
CTATT01	1	32.4	3/4	1/4	1/3	3	2/4	1/2	3	3	1/4	1/3	1/3	1/3	4	1	1/3	2/5	1	2/3	1/3	1/2	1	1/3
TAGA02	4	10.3	3/5	6	2/4	4/5	5/6	4/6	5/6	5/6	4/5	5	5	6/7	5	2/6	3/5	4/5	6	4/5	4/6	3/6	2/6	5/6
GGAA05	1	1.4	3	4/5	4/5	4	5	3/4	4/6	4	1/3	3	2/4	4	4	5	4/5	2/6	4	4/5	4/6	5/6	4/6	4/5
D16S397	1	47.1	-1/1	1/3	2/3	4/6	4	1	3	4	1	3	1/4	1	1	1/2	1/4	3/5	1/4	2/6	1	-3/3	1/3	-3/6
D16S3086	2	65.7	2	2/3	3/4	3/4	3	2	3	3	2	3	2/4	2	2	2	2/3	2/3				3	2/3	3/4
GATA01	2	44.1	2	1/2	1/3	2/4	3	3	1/3	3	1/2	2/3	2	2/3	2	2/3	2/3	2	1	0/2	3/4	2	1	0/2
puratrophin-1 (C/T)	T	0.0	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
GA001	4	0.1	1/8	6/8	8	8/9	8	7/11	8/9	1	5/7	7/10	6/7	5/8	5	5/8	5/9	1/7	6/7	5/7	6/9	6/8	7/9	7/8
17msm	2	8.3	3/4	4	4/5	4/5	5	2/6	5	2	5	3/4	4	3/4	4	1/3	2/4	7	5	2/6	4	2/4	4/5	2/5
CTTT01	8	28.2	5/7	8/10	6/10	6/9	4/5	7/10	5/6	9	5/10	6/7	9	7/9	5/6	7/8	7/10	5/11	5/7	7/10	8/10	4/7	6/8	7/9
GT01	6	15.8	2/4	4/6	2/6	2/4	2/5	1/2	2/3	7	2/4	2/6	1/4	4/6	3/4	4	2/6	3/4	4/7	2/6	3/6	4/6	5/8	2
D16S3095	1	9.7	2	1/3	2/3	2/5	1/3	2/3	2/4	6	2	2/3	2/4	1/2	3	2/3	1/2	1/3	3/4	3/4	1/2	2/6	2/4	2/4
D16S512	1	32.3	2/4	1/5	4/5	2/4	2/4	4	2/4	1	2	2/4	1/4	4/5	4	2/4	4	2/3	1/4	2	4/5	4/5	4/5	4

Table 3 Single nucleotide polymorphisms (SNPs) on the disease chromosome of 16q22.1-linked autosomal dominant cerebellar ataxia (16q-ADCA). We identified thirteen SNPs by ourselves. SNP05 and SNP06 were absent in control chromosomes (n=200) and are thought to be highly specific to the disease chromosome

SNP/marker		Position on Chr 16	SNP change on 16q-ADCA	Frequency in control (%)
	GGAA05	64,938,933		
SNP01		64,972,150	A → G	27.8
SNP02		64,977,170	A → C	22.2
SNP03		64,977,733	T → C	30.0
SNP04		64,982,678	C → T	27.8
SNP05		65,049,292	G → A	0.0
	D16S397	65,295,770		
SNP06		65,337,827	A → G	0.0
SNP07		65,449,825	C → T	56.3
SNP08		65,451,833	T → A	45.5
	GGAA10	65,452,426		
SNP09		65,457,741	T → A	42.4
SNP10		65,458,302	T → C	45.5
SNP11		65,669,454	T → C	30.3
	GATA01	65,700,022		
SNP12		65,771,917	G → A	18.2
SNP13		65,793,152	C → T	8.7
puratrophin-1 (C/T)		65,871,434	C → T	0.0

microsatellite markers. Within this region, we had found that the single nucleotide –16C>T substitution in the *puratrophin*-1 gene was strongly associated with the disease (Ishikawa et al. 2005). Since then, a number of patients with the substitution and the common haplotype were reported in various areas of Japan. However, a report of the one exceptional patient without the substitution in the family in which all other affected subjects carried the substitution (Ohata et al. 2006) raised the possibility that a true pathogenic mutation may be present in a different gene. This exceptional patient indicated that the mutation might be lying centromeric to the substitution in the *puratrophin*-1 gene, where the patient shared the common haplotype with other affected individuals in the family.

Here, we re-examined the 16q-ADCA families with the –16C>T substitution in the *puratrophin*-1 gene with microsatellite markers and found four possible centromeric borders of the disease locus (GATA01, D16S397, GGAA10, GGAA05), based on the difference of alleles. We searched for informative SNPs around the markers capable of distinguishing the chromosomes derived from a founder and analyzed haplotypes with the SNPs. Because all of the examined families carried SNPs around the markers GATA01, D16S397, and GGAA10, ancestral chromosomal recombination around the markers was not confirmed. The differences in alleles for these markers was only one repeat-unit, suggesting that the allele differences

Table 4 The haplotype analysis with single nucleotide polymorphisms (SNPs). Fourteen familiesof 16q-ADCA with different alleles for microsatellite markers and family U09 are shown.The gray squares indicate that the family carried the SNPs common to 16q-ADCA. Family T46 did not carry the common SNPs from SNP01 to SNP04. This is consistent with the findingon microsatellite markers (Table 1), further suggesting that the centromeric border of thedisease locus is SNP04. Family U09 carried all of the 13 SNPs. This would also supportthe theory that family U09 shares the 16q-ADCA common haplotype centromeric to the substitution inthe *puratrophin*-1 gene

SNP	SNP change on 16q-ADCA	frequency in control (%)	family No.														
			P4	T3	T4	T5	T6	T7	T12	T15	T21	T28	T37	T43	T44	T46	U09
SNP01	A → G	27.8	G/A	G/A	G/A	G	G	G/A	G	G/A	G/A	G/A	G	G/A	G	A	G/A
SNP02	A → C	22.2	C/A	C/A	C/A	C	C	C/A	C	C/A	C/A	C/A	C	C/A	C/A	A	C/A
SNP03	T → C	30.0	C/T	C/T	C/T	C	C	C/T	C	C/T	C/T	C/T	C	C/T	C	T	C/T
SNP04	C → T	27.8	T/C	T	T/C	T	T	T/C	T	T/C	T/C	T	T	T/C	T	C	C/T
SNP05	G → A	0.0	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G
SNP06	A → G	0.0	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A
SNP07	C → T	56.3	T	T/C	T/C	T	T	T/C	T/C	T	T	T/C	T/C	T/C	T	T/C	T/C
SNP08	T → A	45.5	A	A/T	A/T	A	A	A/T	A/T	A	A	A/T	A/T	A/T	A	A/T	A
SNP09	T → A	42.4	A	A/T	A/T	A	A	A/T	A/T	A	A	A/T	A/T	A/T	A	A/T	A
SNP10	T → C	45.5	C	C/T	C/T	C	C	C/T	C/T	C	C	C/T	C/T	C/T	C/T	C/T	C
SNP11	T → C	30.3	C	C/T	C/T	C/T	C	C/T	C	C	C/T	C/T	C/T	C/T	C	C/T	C/T
SNP12	G → A	18.2	A	A/G	A/G	A/G	A/G	A/G	A/G	A	A/G	A/G	A/G	A/G	A	A/G	A/G
SNP13	C → T	8.7	T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	T	C/T	C/T
puratrophin-1(C/T)	C → T	0.0	T	T	T	T	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	C

in GATA01, D16S397, and GGAA10 might have resulted not from recombination events, but from the microsatellite slippage mutation (Ikeda et al. 2004). On the other hand, four families (P4, T3, T25, T46) showed great allele differences in GGAA05 and one family (T46) did not carry four SNPs, confirming that family T46 did not share the genomic region centromeric to GGAA05 with the other 16q-ADCA families. This strongly indicates that the centromeric border of the disease locus of 16q-ADCA could be placed at SNP04.

The U09 family had the identical alleles for all markers and SNPs in the region centromeric to the –16C>T substitution in the *puratrophin*-1 gene. It is impossible to conclude that the family has the common haplotype of 16q-ADCA because only one examined family member was available for the present genetic analysis. However, carrying the rare alleles for GGAA05 and infrequent SNPs, both highly specific to the disease chromosome, strongly suggests that the U09 family shares a part of the 16q-ADCA common haplotype. The patient in the U09 family developed pure cerebellar ataxia later in life without apparent family history. Because 16q-ADCA patients were found among sporadic cases (Ouyang et al. 2006), these clinical features of the U09 family are consistent with those of 16q-ADCA. Importantly, this family had not been reported previously and, therefore, would be the second case of 16q-ADCA without the substitution in the *puratrophin*-1 gene following the family reported by Ohata et al. (2006). These cases indicate that the telomeric end of the disease locus could be placed at the –16C>T substitution in the *puratrophin*-1 gene.

Haplotype analysis of a number of 16q-ADCA families with microsatellite markers and SNPs in this study suggests that the gene locus of 16q-ADCA could be re-assigned to a 900-kb genomic region between SNP04 and the substitution in the *puratrophin*-1 gene (Fig. 1). This region partly overlaps with, but is not the same as, the candidate region previously set by Ohata et al. (2006). They showed that three large 16q-ADCA families shared a common haplotype between D16S3086 and D16S412, and suggested the possibility that real pathogenic mutation would exist in the region between TTCC01 and the –16C>T substitution in the *puratrophin*-1 gene. However, the allele difference for TTCC01 in their families was only one repeat-unit, and all of their patients shared identical allele for TAGA02, lying centromeric to TTCC01. Since the possibility of slippage mutation remains as an explanation for the allele difference seen in TTCC01, as we observed for GATA01, D16S397, and GGAA10, it would be cautious to place the centromeric border at the marker TTCC01. Given that the allele differences in TTCC01 is due to slippage mutation, the centromeric border in their families would be alternatively set at D16S503, since an obligate recombination was seen between D16S503 and TAGA02. It would be, thus, important to analyze GGAA05 and specific SNPs in their families to see to what extent their patients harbor conserved haplotypes.

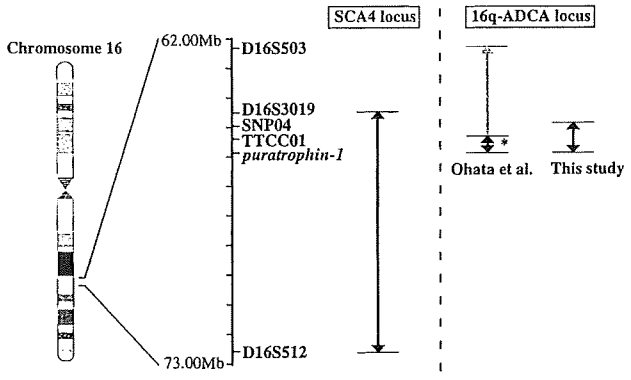

Fig. 1 A summary of critical intervals for 16q-ADCA and SCA4. Our study could define the disease locus of 16q-ADCA to a 900-kb genomic region between SNP04 and the –16C>T substitution in the *puratrophin*-1 gene. This region is completely inside the candidate locus of SCA4 (Flanigan et al. 1996).The haplotype region (*asterisk*) between TTCC01 and the *puratrophin*-1 gene shown by Ohata et al. (2006) is also shown, together with an alternative critical region between D16S503 and the *puratrophin*-1 gene (see text for details)

Although we found a patient without the –16C>T substitution in the *puratrophin*-1 gene, the substitution was present in all patients except the one in the U09 family (i.e., 125/126=99.2% sensitivity; 100% specificity) and, thus, the *puratrophin*-1 genetic change still remains to be a useful marker. Molecular diagnosis with multiple microsatellite markers and SNPs will help to identify 16q-ADCA patients more accurately. Through the present study, we showed that the truly pathogenic mutation would lie in a 900-kb genomic region between SNP04 and the –16C>T substitution in the *puratrophin*-1 gene. Further investigations for finding a genetic mutation within the critical region are needed to elucidate the molecular pathogenesis of 16q-ADCA.

References

Cagnoli C, Mariotti C, Taroni F, Seri M, Brussino A, Michielotto C, Grisoli M, Di Bella D, Migone N, Gellera C, Di Donato S, Brusco A (2006) SCA28, a novel form of autosomal dominant cerebellar ataxia on chromosome 18p11.22-q11.2. Brain 129:235–242

Chen DH, Brkanac Z, Verlinde CL, Tan XJ, Bylenok L, Nochlin D,Matsushita M, Lipe H, Wolff J, Fernandez M, Cimino PJ, Bird TD,Raskind WH (2003) Missense mutations in the regulatory domain of PKC gamma: a new mechanism for dominant nonepisodic cerebellar ataxia. AmJ Hum Genet 72:839–849

Flanigan K, Gardner K, Alderson K, Galster B, Otterud B, Leppert MF, Kaplan C, Ptacek LJ (1996) Autosomal dominant spinocerebellar ataxia with sensory axonal neuropathy (SCA4): clinical description and genetic localization to chromosome 16q22.1. Am J Hum Genet 59:392–399

Harding AE (1982) The clinical features and classification of the late onset autosomal dominant cerebellar ataxias. A study of 11 families, including descendants of the "Drew family of Walworth." Brain 105:1–28

Hellenbroich Y, Pawlack H, Rub U, Schwinger E, Zuhlke Ch (2005) Spinocerebellar ataxia type 4. Investigation of 34 candidate genes. J Neurol 252:1472–1475

Holmes SE, O'Hearn EE, McInnis MG, Gorelick-Feldman DA, Kleiderlein JJ, Callahan C, Kwak NG, Ingersoll-Ashworth RG, Sherr M, Sumner AJ, Sharp AH, Ananth U, Seltzer WK, Boss MA, Vieria-Saecker A-M, Epplen JT, Riess O, Ross CA, Margolis RL (1999) Expansion of a novel CAG trinucleotide repeat in the 5′ region of PPP2R2B is associated with SCA12. Nat Genet 23:391–392

Ikeda Y, Dalton JC, Moseley ML, Gardner KL, Bird TD, Ashizawa T, Seltzer WK, Pandolfo M, Milunsky A, Potter NT, Shoji M, Vincent JB, Day JW, Ranum LP (2004) Spinocerebellar ataxia type 8: molecular genetic comparisons and haplotype analysis of 37 families with ataxia. Am J Hum Genet 75:3–16

Ikeda Y, Dick KA, Weatherspoon MR, Gincel D, Armbrust KR, Dalton JC, Stevanin G, Durr A, Zuhlke C, Burk K, Clark HB, Brice A, Rothstein JD, Schut LJ, Day JW, Ranum LP (2006) Spectrin mutations cause spinocerebellar ataxia type 5. Nat Genet 38:184–190

Ishikawa K, Tanaka H, Saito M, Ohkoshi N, Fujita T, Yoshizawa K,Ikeuchi T, Watanabe M, Hayashi A, Takiyama Y, Nishizawa M, Nakano I,Matsubayashi K, Miwa M, Shoji S, Kanazawa I, Tsuji S, Mizusawa H(1997) Japanese families with autosomal dominant pure cerebellar ataxia map to chromosome 19p13.1-p13.2 and are strongly associated with mild CAG expansions in the spinocerebellar ataxia type 6 gene in chromosome 19p13.1. Am J Hum Genet 61:336–346

Ishikawa K, Toru S, Tsunemi T, Li M, Kobayashi K, Yokota T, Amino T, Owada K, Fujigasaki H, Sakamoto M, Tomimitsu H, Takashima M, Kumagai J, Noguchi Y, Kawashima Y, Ohkoshi N, Ishida G, Gomyoda M, Yoshida M, Hashizume Y, Saito Y, Murayama S, Yamanouchi H, Mizutani T, Kondo I, Toda T, Mizusawa H (2005) An autosomal dominant cerebellar ataxia linked to chromosome 16q22.1 is associated with a single-nucleotide substitution in the 5′ untranslated region of the gene encoding a protein with spectrin repeat and Rho guanine nucleotide exchange-factor domains. Am J Hum Genet 77:280–296

Koob MD, Moseley ML, Schut LJ, Benzow KA, Bird TD, Day JW, Ranum LP (1999) An untranslated CTG expansion causes a novel form of spinocerebellar ataxia (SCA8). Nat Genet 21:379–384

Matsuura T, Yamagata T, Burgess DL, Rasmussen A, Grewal RP, Watase K, Khajavi M, McCall AE, Davis CF, Zu L, Achari M, Pulst SM, Alonso E, Noebels JL, Nelson DL, Zoghbi HY, Ashizawa T (2000) Large expansion of the ATTCT pentanucleotide repeat in spinocerebellar ataxia type 10. Nat Genet 26:191–194

Nagaoka U, Takashima M, Ishikawa K, Yoshizawa K, Yoshizawa T, Ishikawa M, Yamawaki T, Shoji S, Mizusawa H (2000) A gene on SCA4 locus causes dominantly inherited pure cerebellar ataxia. Neurology 54:1971–1975

Ohata T, Yoshida K, Sakai H, Hamanoue H, Mizuguchi T, Shimizu Y, Okano T, Takada F, Ishikawa K, Mizusawa H, Yoshiura K, Fukushima Y, Ikeda S, Matsumoto N (2006) A −16C>T substitution in the 5′ UTR of the puratrophin-1 gene is prevalent in autosomal dominant cerebellar ataxia in Nagano. J Hum Genet 51:461–466

Onodera Y, Aoki M, Mizuno H, Warita H, Shiga Y, Itoyama Y (2006) Clinical features of chromosome 16q22.1 linked autosomal dominant cerebellar ataxia in Japanese. Neurology 67:1300–1302

Ouyang Y, Sakoe K, Shimazaki H, Namekawa M, Ogawa T, Ando Y, Kawakami T, Kaneko J, Hasegawa Y, Yoshizawa K, Amino T, Ishikawa K, Mizusawa H, Nakano I, Takiyama Y (2006) 16q-linked autosomal dominant cerebellar ataxia: a clinical and genetic study. J Neurol Sci 247:180–186

Ross CA, Poirier MA (2004) Protein aggregation and neurodegenerative disease. Nat Med 10 Suppl:S10–S17

Sasaki H, Yabe I, Tashiro K (2003) The hereditary spinocerebellar ataxias in Japan. Cytogenet Genome Res 100:198–205

Schöls L, Bauer P, Schmidt T, Schulte T, Riess O (2004) Autosomal dominant cerebellar ataxias: clinical features, genetics, and pathogenesis. Lancet Neurol 3:291–304

van Swieten JC, Brusse E, de Graaf BM, Krieger E, van de Graaf R, deKoning I, Maat-Kievit A, Leegwater P, Dooijes D, Oostra BA, Heutink P(2003) A mutation in the fibroblast growth factor 14 gene is associated with autosomal dominant cerebellar ataxia. Am J Hum Genet72:191–199

Takano H, Cancel G, Ikeuchi T, Lorenzetti D, Mawad R, Stevanin G, Didierjean O, Durr A, Oyake M, Shimohata T, Sasaki R, Koide R, Igarashi S, Hayashi S, Takiyama Y, Nishizawa M, Tanaka H, Zoghbi H, Brice A, Tsuji S (1998) Close associations between prevalences of dominantly inherited spinocerebellar ataxias with CAG-repeat expansions and frequencies of large normal CAG alleles in Japanese and Caucasian populations. Am J Hum Genet 63:1060–1066

Takashima M, Ishikawa K, Nagaoka U, Shoji S, Mizusawa H (2001) A linkage disequilibrium at the candidate gene locus for 16q-linked autosomal dominant cerebellar ataxia type III in Japan. J Hum Genet 46:167–171

Waters MF, Minassian NA, Stevanin G, Figueroa KP, Bannister JP, Nolte D, Mock AF, Evidente VG, Fee DB, Muller U, Durr A, Brice A, Papazian DM, Pulst SM (2006) Mutations in voltage-gated potassium channel KCNC3 cause degenerative and developmental central nervous system phenotypes. Nat Genet 38:447–451

Wieczorek S, Arning L, Alheite I, Epplen JT (2006) Mutations of the puratrophin-1 (PLEKHG4) gene on chromosome 16q22.1 are not a common genetic cause of cerebellar ataxia in a European population. J Hum Genet 51: 363–367

Yu GY, Howell MJ, Roller MJ, Xie TD, Gomez CM (2005) Spinocerebellar ataxia type 26 maps to chromosome 19p13.3 adjacent to SCA6. Ann Neurol 57:349–354

Fibroblast growth factor 20 gene and Parkinson's disease in the Japanese population

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Received 3 January 2007; accepted 20 February 2007

A genetic association between the fibroblast growth factor 20 (*FGF20*) gene and Parkinson's disease has been found by the pedigree disequilibrium test. This association, however, was not replicated by a case–control association study. In order to clarify the association between the *FGF20* gene and Parkinson's disease, we attempted to replicate this association by a case–control association study using a large number of Japanese samples (1388 patients and 1891 controls). rs1721100 exhibited a significant difference in allele C versus G (P=0.0089), and in genotype CC + CG versus GG (P=0.0053). Haplotype association analysis showed that haplotype 2 was the protective haplotype for Parkinson's disease (permutation-P=0.0075). These results suggest that the *FGF20* gene is a susceptibility gene for Parkinson's disease in the Japanese population. *NeuroReport* 18:937–940 © 2007 Lippincott Williams & Wilkins.

Keywords: association, case–control study, fibroblast growth factor 20 (*FGF20*), Parkinson's disease, single nucleotide polymorphism, susceptibility gene

Introduction

Parkinson's disease (OMIM #168600) is one of the most common neurodegenerative diseases, characterized by resting tremor, cogwheel rigidity, bradykinesia, and impaired postural reflexes. These clinical features result primarily from the loss of dopaminergic neurons in the substantia nigra. Various medical treatments improve Parkinson's disease symptoms, but do little to deter disease progression [1]. In Mendelian-inherited Parkinson's disease, eight causal genes have been identified (*SNCA*, *parkin*, *UCHL1*, *PINK1*, *DJ1*, *LRRK2/dardarin*, *ATP13A2*, and *NR4A2/Nurr1*). Sporadic Parkinson's disease is a complex disorder, with multiple genetic and environmental factors influencing disease risk [2]. Identifying genetic risk factors for Parkinson's disease will be helpful in elucidating the pathogenesis of Parkinson's disease.

Genome-wide, non-parametric linkage analyses of Parkinson's disease families have revealed significant linkage in multiple chromosomal regions [3–6]. One of these prominent regions of linkage was found on chromosome 8p (LOD score 2.2 at D8S520) [6]. Subsequently, van der Walt *et al.* chose to examine the *FGF20* gene in their investigation of biological candidate genes for Parkinson's disease susceptibility in this region. The *FGF20* gene is approximately 9.3 kb (*http://genome.ucsc.edu/*), and is located approximately 6.2 Mb from a peak marker D8S520 on chromosome 8p22–p21.3 [7]. FGF20 is a neurotrophic factor that exerts strong neurotrophic properties within brain tissue, and regulates central nervous development and function [8]. FGF20 is preferentially expressed in the substantia nigra [9], and it has been reported to be involved in dopaminergic neurons survival [10]. In order to assess the genetic association of the *FGF20* gene with Parkinson's disease, they genotyped five single nucleotide polymorphisms (SNPs) [ss20399076 (rs12718379), rs1989756, rs1989754, rs1721100, and ss20399075 (rs12720208)] lying within the *FGF20* gene in 644 families from the United States, performed the pedigree disequilibrium test (PDT), the genotype PDT, the multilocus-genotype PDT, and the family-based association test, and discovered a highly significant association of Parkinson's disease with one intronic SNP, rs1989754 (P=0.0006), and two SNPs, rs1721100 (P=0.02) and rs12720208 (P=0.0008), located in the 3′ regulatory region. Furthermore, they detected a haplotype that is positively associated with risk of Parkinson's disease (P=0.0003), whereas a second haplotype was

found to be negatively associated with risk of Parkinson's disease (P=0.0009). Consequently, they concluded that the *FGF20* gene was a susceptibility gene for Parkinson's disease [11].

Subsequently, Clarimon *et al.* sought to replicate the association of the *FGF20* gene with Parkinson's disease by performing a case–control association study with four SNPs [rs1989756, rs1989754, rs1721100, and ss20399075 (rs12720208)] using Finnish and Greek samples. They found a difference in allele frequency in only rs1989754, but the difference was not significant after the Bonferroni correction. They also found no significant difference in the distribution of haplotypes between patients and controls. They hence failed to replicate the association of the *FGF20* gene with Parkinson's disease [12]. Thus, it is still controversial as to whether the *FGF20* gene is a susceptibility gene for Parkinson's disease or not. We here conducted a case–control association study using a large number of Japanese samples in order to evaluate the association of the *FGF20* gene with risk of Parkinson's disease.

Materials and methods

We recruited 1388 unrelated Parkinson's disease patients (age, 65.7±9.8; male/female ratio, 0.84) and 1891 unrelated controls (age, 48.5±17.6; male/female ratio, 1.08). The diagnosis of Parkinson's disease was based on the presence of two or more of the cardinal features of Parkinson's disease (tremor, rigidity, bradykinesia, and postural instability), according to the criteria for Parkinson's disease [13]. Patients were evaluated by certified neurologists specializing in Parkinson's disease. The average age of onset was 57.7±11.1 years. All patients and controls were of Japanese ancestry. Informed consent was obtained from each individual, and approval for the study was obtained from the University Ethical Committees. Genomic DNA was extracted from venous blood using standard procedures.

The TaqMan SNP Genotyping Assay (Applied Biosystems, Foster, California, USA) was employed for five SNPs (rs12718379, rs1989756, rs1989754, rs1721100, and rs12720208). SNP information was obtained from the dbSNP database (*http://www.ncbi.nlm.nih.gov/SNP/*) and the International HapMap Project database (*http://hapmap.org*) [14].

All statistical analyses were performed by using the software SNPAlyze (Dynacom, Japan). Genotype deviation from Hardy–Weinberg equilibrium was assessed by the χ^2 test. The statistical significance of a case–control association was evaluated by the χ^2 test, and odds ratio and its 95% confidence intervals (CIs) were calculated by the Bootstrap method. Haplotype frequencies were estimated using an expectation-maximization algorithm [15]. We evaluated pair-wise linkage disequilibrium (LD) among SNPs by D' value, and r^2 as standards for LD. Case–control haplotype analyses were carried out by calculating the permutation P-value on the basis of 10 000 replications [16].

Results

Two SNPs (rs1989756 and rs12720208) of the five SNPs, examined by van der Walt *et al.*, showed a monomorphism in 95 individuals drawn from the Japanese population, and therefore these two SNPs were excluded from further analysis. Three SNPs (rs1989754, rs1721100, and

Table 1 Summary of the association of three SNPs between Parkinson's disease patients and controls

SNP ID	Alleles 1–2	Strand[a]	Genotype: Patient 1/1	1/2	2/2	Total	Frequency of allele 1	Genotype: Control 1/1	1/2	2/2	Total	Frequency of allele 1	Allele 1 versus Allele 2: Odds ratio (95% CI[b])	*P*-value	Genotype 11 versus 12+22: Odds ratio (95% CI[b])	*P*-value	Genotype 11+12 versus 22: Odds ratio (95% CI[b])	*P*-value
rs12718379	A-G	Reverse	249	641	481	1371	0.42	375	902	597	1874	0.44	1.11 (1.01–1.23)	0.041	1.13 (0.95–1.34)	0.19	1.16 (1.00–1.34)	0.054
rs1989754	G-C	Forward	261	628	477	1366	0.42	381	895	586	1862	0.44	1.10 (1.00–1.22)	0.055	1.09 (0.92–1.28)	0.34	1.17 (1.01–1.35)	0.040
rs1721100	C-G	Forward	270	639	458	1367	0.43	407	925	542	1874	0.46	1.14 (1.03–1.26)	0.0089	1.13 (0.94–1.36)	0.17	1.24 (1.06–1.43)	0.0053

[a]Relative to the transcriptional direction.
[b]CI, confidence intervals; SNP, single nucleotide polymorphism.

rs12720208) are included in HapMap. rs12720208 also shows no polymorphism in JPT (Japanese in Tokyo) HapMap, consistent with the genotyping results of our samples. In the *FGF20* gene region, 171 SNPs were observed in dbSNP. According to JPT HapMap, the Tagger method showed that two SNPs (rs1989754 and rs1721100) can represent the remainder of the HapMap SNPs of the *FGF20* gene region, as tag SNPs with a criteria of $r^2 > 0.8$ and a minor allele frequency > 0.1, although the number of tag SNPs differed between JPT and CEU [CEPH (Utah residents with ancestry from northern and western Europe)] [17]. Thus, we considered that a case–control association study using three SNPs [two tag SNPs (rs1989754 and rs1721100) plus rs12718379] was appropriate for assessing the association of the *FGF20* gene with Parkinson's disease.

Table 1 shows the results of the SNP genotyping in the Parkinson's disease patients and controls. The association of rs1721100 was significant in allele 1 versus allele 2 [frequency of allele 1; 43% in patients and 46% in controls, P=0.0089, odds ratio 1.14 (95% CI, 1.03–1.26)] and in genotype 11 + 12 versus 22 [P=0.0053, odds ratio 1.24 (95% CI, 1.06–1.43)]. The association with rs1721100 was significant even after the Bonferroni correction (tests for three SNPs). As for rs12718379, a decrease in frequency of allele 1 was found in patients compared with controls [frequency of allele 1; 42% in patients and 44% in controls, P=0.041, odds ratio 1.11 (95% CI, 1.01–1.23)]. As for rs1989754, there was a difference in frequency of genotype 11 + 12 versus 22 between patients and controls [P=0.040, odds ratio 1.17 (95% CI, 1.01–1.35)]. Neither rs12718379 nor rs1989754, however, showed a significant association with Parkinson's disease after the Bonferroni correction. The genotype frequencies of all three SNPs were not significantly different from the values expected from the Hardy–Weinberg equilibrium.

We calculated the LD among the three SNPs in patients and controls. D' values (absolute value) and r^2 for pair-wise LD of controls are shown in Table 2. A high LD was detected between each pair of SNPs, and the same trend was observed in patients and in the JPT samples of the HapMap database (data not shown). These findings suggested that the three SNPs were in single LD, and we therefore performed haplotype association analysis. Haplotype frequencies of the three SNPs were estimated in patients and controls (Table 3). Two common haplotypes (haplotypes 1 and 2) covered >90% of the population haplotypes in both patients and controls. The frequency of haplotype 2 (A-G-C) was significantly less in patients than controls (38% in patients and 41% in controls, permutation-P=0.0075). This indicates that haplotype 2 is a protective haplotype for Parkinson's disease in the Japanese population. Taken together, our genetic analyses support the *FGF20* gene being a susceptibility gene for Parkinson's disease in the Japanese population.

Table 2 Linkage disequilibrium between SNPs in the *FGF20* gene

SNP ID	rsl2718379	rsl989754	rsl721100
rsl2718379	—		
rsl989754	0.94 (0.98)	—	
rsl721100	0.68 (0.86)	0.72 (0.88)	—

r^2 (D') values of controls are shown for each pair of single nucleotide polymorphisms (SNPs).

Discussion

Our results are consistent with the report by van der Walt *et al.* [11], which showed an association of the *FGF20* gene with risk of Parkinson's disease. The significance of the *FGF20* gene for Parkinson's disease susceptibility in our study, however, was not so strong as that shown by van der Walt *et al.* This discrepancy may result from: (i) the ethnic differences between the Japanese samples and samples from the United States; the association in the Japanese population might be smaller than in the United States, or (ii) the difference in epidemiological approaches; we performed a case–control association study by the χ^2 test in unrelated samples, while they analysed family-based samples by the PDT. rs12720208, the strongly associated SNP in the report by van der Walt *et al.*, was excluded from our study because we were not able to find polymorphism of this SNP in the Japanese samples. It is interesting that rs1721100, the most strongly associated SNP in our study, and rs12720208, however, are both located in the 3′ UTR region of the *FGF20* gene. LD indices between rs12720208 and rs1721100 showed that these two SNPs are in a single LD block (D'=1) and that the correlation was not strong (r^2=0.28) (on the basis of CEU HapMap).

On the other hand, the case–control association study by Clarimon *et al.* [12] failed to replicate the association of the *FGF20* gene with risk of Parkinson's disease, although the rs1989754 G allele frequency was higher in patients than controls in the Finnish samples (52% in patients and 42% in controls, P=0.03 before Bonferroni correction). However, as their sample size was not large enough, their study does not disprove the association of the *FGF20* gene with Parkinson's disease convincingly if the influence for Parkinson's disease in the Greek and Finnish population is to the same extent as in our Japanese sample. The sample size of their study was considerably smaller than ours (Finnish series, 144 patients and 135 controls; Greek series, 151 patients and 186 controls in their study, compared with 1388 patients and 1891 controls in our study). As mentioned in their report, their experiment had 80% power to detect

Table 3 Haplotype association analysis using three SNPs in the *FGF20* gene

	Base at SNP			Haplotype frequency		
Haplotype ID	rsl2718379	rsl989754	rsl721100	Patient	Control	*P*-value
Haplotype 1	G	C	G	0.53	0.50	0.054
Haplotype 2	A	G	C	0.38	0.41	0.0075
Haplotype 3	G	C	C	0.045	0.047	0.75
Haplotype 4	A	G	G	0.035	0.028	0.11

risks from 1.7 to 3.6 in the Finnish samples and from 1.6 to 2.1 in the Greek samples, whereas the odds ratio of the *FGF20* gene in our data was 1.14. The possibility of type 2 errors in their study could not be excluded as an explanation for this negative finding. Another explanation for lack of replication could be genetic heterogeneity; there might not be an association between the *FGF20* gene and Parkinson's disease in the Greek and Finnish populations, whereas there might be in the Japanese and the United States-based population.

In this study, the three SNPs (rs12718379, rs1989754, and rs1721100) showed a difference between patients and controls to some degree. After Bonferroni correction, however, a significant association was detected only in rs1721100. The correlations between rs1721100 and the other two SNPs were not strong (r^2=0.68 with rs12718379 and 0.72 with rs1989754), which might explain the different extents of significance among the three SNPs.

The *EFHA2* gene is located 25 kb upstream of the 5′ UTR, and the *MSR1* gene is located 800 kb downstream of the *FGF20* gene. The entire region of the *FGF20* gene is within a single LD block of 20.8 kb (on the basis of JPT HapMap). Moreover, no other known genes reside within this LD block. Therefore, we concluded that our positive finding results from the association between the *FGF20* gene and Parkinson's disease.

Conclusion

We performed a case–control association study using a large number of samples (1388 Parkinson's disease patients and 1891 controls) in the Japanese population, and found a significant association of Parkinson's disease with rs1721100 and haplotype 2 (A-G-C) in the *FGF20* gene. Our results, together with those of van der Walt *et al.*, demonstrate an association of the *FGF20* gene with Parkinson's disease in two different ethnic groups. This evidence suggests the involvement of the *FGF20* gene in the pathogenesis of Parkinson's disease.

Acknowledgements

The authors are grateful to the Parkinson's disease patients who participated in this study. The authors thank Drs Akira Oka, Hidetoshi Inoko, and Katsushi Tokunaga for control samples. They also thank Dr Helena A. Popiel for editing the manuscript. This work was supported by the 21st century COE program and Research Grants (17019044 and 17590874), both from the Ministry of Education, Culture, Sports, Science and Technology of Japan; by the Grant for Research on Measures for Intractable Diseases (H17-Q-15-1) from the Ministry of Health, Labor and Welfare of Japan; and by a grant from Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Agency (JST).

References

1. Rascol O, Payoux P, Ory F, Ferreira JJ, Brefel-Courbon C, Montastruc JL. Limitations of current Parkinson's disease therapy. *Ann Neurol* 2003; **53**:S3–S12.
2. Warner TT, Schapira AH. Genetic and environmental factors in the cause of Parkinson's disease. *Ann Neurol* 2003; **53**:S16–S23.
3. DeStefano AL, Golbe LI, Mark MH, Lazzarini AM, Maher NE, Saint-Hilaire M, *et al.* Genome-wide scan for Parkinson's disease: the GenePD study. *Neurology* 2001; **57**:1124–1126.
4. Hicks AA, Petursson H, Jonsson T, Stefansson H, Johannsdottir HS, Sainz J, *et al.* A susceptibility gene for late-onset idiopathic Parkinson's disease. *Ann Neurol* 2002; **52**:549–555.
5. Pankratz N, Nichols WC, Uniacke SK, Halter C, Rudolph A, Shults C, *et al.* Genome screen to identify susceptibility genes for Parkinson disease in a sample without parkin mutations. *Am J Hum Genet* 2002; **71**:124–135.
6. Scott WK, Nance MA, Watts RL, Hubble JP, Koller WC, Lyons K, *et al.* Complete genomic screen in Parkinson disease: evidence for multiple genes. *JAMA* 2001; **286**:2239–2244.
7. Kirikoshi H, Sagara N, Saitoh T, Tanaka K, Sekihara H, Shiokawa K, *et al.* Molecular cloning and characterization of human FGF-20 on chromosome 8 p21.3-p22. *Biochem Biophys Res Commun* 2000; **274**:337–343.
8. Jeffers M, Shimkets R, Prayaga S, Boldog F, Yang M, Burgess C, *et al.* Identification of a novel human fibroblast growth factor and characterization of its role in oncogenesis. *Cancer Res* 2001; **61**:3131–3138.
9. Ohmachi S, Watanabe Y, Mikami T, Kusu N, Ibi T, Akaike A, *et al.* FGF-20, a novel neurotrophic factor, preferentially expressed in the substantia nigra pars compacta of rat brain. *Biochem Biophys Res Commun* 2000; **277**:355–360.
10. Ohmachi S, Mikami T, Konishi M, Miyake A, Itoh N. Preferential neurotrophic activity of fibroblast growth factor-20 for dopaminergic neurons through fibroblast growth factor receptor-1c. *J Neurosci Res* 2003; **72**:436–443.
11. Van der Walt JM, Noureddine MA, Kittappa R, Hauser MA, Scott WK, McKay R, *et al.* Fibroblast growth factor 20 polymorphisms and haplotypes strongly influence risk of Parkinson disease. *Am J Hum Genet* 2004; **74**:1121–1127.
12. Clarimon J, Xiromerisiou G, Eerola J, Gourbali V, Hellstrom O, Dardiotis E, *et al.* Lack of evidence for a genetic association between FGF20 and Parkinson's disease in Finnish and Greek patients. *BMC Neurol* 2005; **5**:11.
13. Bower JH, Maraganore DM, McDonnell SK, Rocca WA. Incidence and distribution of parkinsonism in Olmsted County, Minnesota, 1976–1990. *Neurology* 1999; **52**:1214–1220.
14. The International HapMap Consortium. The International HapMap Project. *Nature* 2003; **426**:789–796.
15. Excoffier L, Slatkin M. Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. *Mol Biol Evol* 1995; **12**: 921–927.
16. Fallin D, Cohen A, Essioux L, Chumakov I, Blumenfeld M, Cohen D, *et al.* Genetic analysis of case/control data using estimated haplotype frequencies: application to APOE locus variation and Alzheimer's disease. *Genome Res* 2001; **11**:143–151.
17. De-Bakker PI, Yelensky R, Pe'er I, Gabriel SB, Daly MJ, Altshuler D. Efficiency and power in genetic association studies. *Nat Genet* 2005; **37**:1217–1223.