effective as oral pramipexole in patients with advanced PD,[22,23] no studies to date have assessed the efficacy and safety of rotigotine at doses > 8 mg/24 hours in patients with early stage PD. Therefore, we investigated the efficacy and safety of rotigotine at doses up to 16 mg/24 hours in Japanese patients with early stage PD.

Patients and Methods

The present trial, which was performed between September 2007 and April 2009, was a randomized, double-blind, placebo-controlled, 2-arm, parallel-group trial and was approved by the institutional review boards of the 41 centers in Japan at which the trial was performed. Informed consent was obtained from all patients who participated in the trial. One-hundred ninety-eight patients with early stage PD who had no concomitant treatment with L-dopa (age range, 30–79 years), Hoehn & Yahr scale scores from I to III, and Unified Parkinson's Disease Rating Scale (UPDRS) part II (activities of daily living) and III (motor function) scores ≥10 were enrolled. The clinical diagnosis of PD was made according to UK Brain Bank criteria.[24]

Patients were excluded if they had any of the following: psychiatric symptoms, including confusion, hallucination, delusion, excitation, delirium, and abnormal behavior at entry; symptomatic orthostatic hypotension; a history of epilepsy and/or convulsion; complications or history of serious cardiac disease and/or arrhythmia; severe renal or hepatic impairments; history of deep brain stimulation; and dementia; or of they had received L-dopa for > 6 months by the time of acquisition of informed consent or other drugs that could possibly affect PD symptoms from at least 4 weeks before the date of first treatment. Patients who had received L-dopa before study entry had to discontinue L-dopa at least 2 weeks before the date of the first treatment administration.

Treatments, outcome measures, and statistical analyses are described in detail in the Supplementary Methods. In brief, the trial consisted of a 4-week screening period followed by an 8-week titration period, a 4-week maintenance period, a 2-week taper period, and a 1-week safety follow-up period. Patients were treated with either rotigotine transdermal patches that delivered 2 mg/24 hours or corresponding placebo patches with a weekly increment of 2 mg/24 hours, up to a maximum dose of 16 mg/24 hours during the 8-week titration period. The primary endpoint was the change in UPDRS part II and III scores from baseline to the end of treatment (EOT).

TABLE 1. Patient demographics at baseline[a]

Characteristic	Rotigotine group, n = 88	Placebo group, n = 88	P
Sex			
Men	33 (37.5)	37 (42.0)	0.538[b]
Women	55 (62.5)	51 (58.0)	
Age, y			
<65	36 (40.9)	35 (39.8)	0.878[b]
≥65	52 (59.1)	53 (60.2)	
Duration of disease, y	2.0 ± 1.8	1.8 ± 1.9	0.275[c]
UPDRS score			
Part I: Activities of daily living	6.8 ± 3.9	7.4 ± 3.8	0.183[c]
Part III: Motor function	20.2 ± 9.2	20.8 ± 9.5	0.643[c]
Parts II + III	27.0 ± 11.8	28.2 ± 12.2	0.480[c]
Hoehn & Yahr score			
Average	2.1 ± 0.7	2.2 ± 0.6	0.630[c]
I	20 (22.7)	17 (19.3)	
II	45 (51.1)	48 (54.5)	
III	23 (26.1)	23 (26.1)	

[a]Values shown are the means ± standard deviation or no. of patients (%).
[b]χ^2 test.
[c]Wilcoxon test.
UPDRS, Unified Parkinson's Disease Rating Scale.

Results

Patient demographics are listed in Table 1, while the flow of enrolled patients into groups and their disposition are shown in Supplementary Figure 1. Eighteen patients were excluded; therefore, 180 patients were assigned randomly to receive either rotigotine (90 patients) or placebo (90 patients). Four patients were excluded from the full analysis set (FAS) because of a lack of efficacy assessments in 3 patients and Gerstmann-Straussler-Scheinker disease in 1 patient, leaving 176 patients in the FAS (88 in each group). Treatment was completed by 75 patients in the rotigotine group and by 80 patients in the placebo group; the other 25 patients discontinued after treatments had commenced.

Eighty-two of 88 patients in the rotigotine group entered the maintenance period, and individual optimal doses were determined in these patients. Doses received in the maintenance period were 4 mg/24 hours (2 patients), 6 mg/24 hours (9 patients), 8 mg/24 hours (6 patients), 10 mg/24 hours (10 patients), 12 mg/24 hours (8 patients), 14 mg/24 hours (3 patients), and 16 mg/24 hours (44 patients; 53.7%). The mean (± standard deviation) changes in UPDRS part II and III scores from baseline to EOT were −8.4 ± 9.7 and −4.1 ± 8.2 in the rotigotine and placebo groups, respectively (Fig. 1A), with a statistically significant difference between the 2 groups (95% confidence interval [CI], −7.0 to −1.7; $P = 0.002$). Changes in UPDRS part III scores from baseline to EOT also differed significantly (95% CI, −5.6 to −1.6; $P < 0.001$) between groups, but changes in UPDRS part II scores did not (95% CI, −1.6 to 0.2;

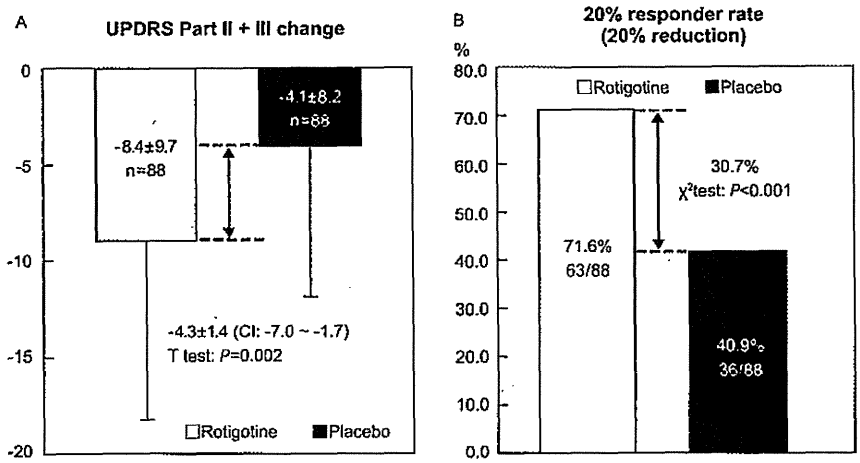

FIG. 1. Changes in United Parkinson's Disease Rating Scale part II (activities of daily living) and part III (motor function) scores are illustrated (A) from baseline to the end of treatment along with (B) 20% responder rates. CI, confidence interval.

$P = 0.125$). The 20% responder rate (defined as a patient with at least a 20% reduction in UPDRS part II and III scores from baseline to EOT) was 71.6% (63 of 88 patients) in the rotigotine group and 40.9% (36 of 88 patients) in the placebo group (Fig. 1B), and the difference between groups, again, was significant ($P < 0.001$).

A subgroup analysis revealed differences in the change in UPDRS part II and III sum scores from baseline to EOT between the rotigotine and placebo groups of −3.1, −3.8, and −6.5 in those who had baseline Hoehn & Yahr scores of I, II, and III, respectively, revealing that, with greater severity, there was a greater difference in the change in UPDRS part II and III sum scores.

Supporting Table 1 lists treatment-emergent adverse events (TEAEs) that had an incidence ≥5%. Seventy-eight patients (86.7%) in the rotigotine group and 65 patients (72.2%) in the placebo group experienced at least 1 TEAE, and most were mild or moderate in intensity. Two serious adverse events, namely, cerebral infarction and myelopathy, were reported in 2 rotigotine-treated patients who were hospitalized because of these events; it was later concluded that these events were not related to the trial drug. Supporting Table 2 lists the same events according to subgroup.

Discussion

The purpose of this trial was to assess the efficacy and safety of rotigotine transdermal patches at doses up to 16 mg/24 hours compared with placebo in patients with early stage PD who were receiving treatment with L-dopa. Rotigotine, when delivered at doses up to 16 mg/24 hours, reduced UPDRS part II and III scores from baseline to EOT (primary endpoint) by 8.4 ± 9.7, significantly exceeding the reduction caused by placebo ($P = 0.002$). This change in the UPDRS part II and III score was greater than that observed in previous randomized controlled trials of rotigotine in patients with early stage PD,[19–21] was similar to the change induced by extended-release pramipexole (a dopamine agonist) in such PD patients,[25] and was greater than the changes induced by the dopamine agonists sumanirole and ropinirole.[26] In addition, the 20% responder rate was 71.6% (63 of 88 patients) in the rotigotine group and 40.9% (36 of 88 patients) in the placebo, with a statistically significant difference between groups ($P < 0.001$). Similar to the change in UPDRS part II and III scores, the responder rate in the rotigotine group also was greater than that described in earlier studies.[20,21]

In an earlier trial of rotigotine in patients with early stage PD,[19] 91% of the patients treated with rotigotine (maximum, 8 mg/24 hours) reported at least 1 adverse event. Similarly, in the present trial, 86.7% of patients treated with rotigotine (maximum, 16 mg/24 hours) reported at least 1 adverse event. With the exception of application site reactions, the profiles of TEAEs in rotigotine-treated patients were similar among the present trial, previous trials of rotigotine in patients with early stage PD,[−22] and trials of other dopamine agonists.[5,7,27] Serious adverse events were observed in only 2 of the 90 patients (2.2%) who received rotigotine in our study. Thus, overall, rotigotine delivered at up to 16 mg/24 hours was well tolerated in this trial.

Conclusion

This is the first randomized, double-blind, placebo-controlled trial assessing the efficacy of

rotigotine transdermal patches at doses up to 16 mg/24 hours compared with placebo in patients with early stage PD who were not receiving treatment with L-dopa. Rotigotine delivered at doses up to 16 mg/24 hours is efficacious in patients with early stage PD. Doses greater than 8 mg/24 hours appear to result in higher responder rates. The responder rate of 71.6% in this trial is, to our knowledge, the highest among trials of rotigotine in patients with early stage PD. ■

Acknowledgments: We acknowledge the editorial support of Daniel McGowan, PhD, in the preparation of this article. We thank the following additional members of the Rotigotine Trial Group, who participated in this study as investigators: Takashi Kimura, Mikio Shoji, Satoshi Takahashi, Takashi Abe, Atsushi Takeda, Itaru Toyoshima, Kazuo Yoshizawa, Kimihito Arai, Hideki Shimura, Sadako Kuno, Hiroshi Kurisaki, Masayuki Yokochi, Kouichi Mizoguchi, Toshihiko Ohashi, Tetsushi Atsumi, Akira Inukai, Hideyuki Sawada, Harutoshi Fujimura, Yoshiyuki Mitsui, Toshio Inui, Mitsutoshi Yamamoto, Masahiro Nagai, Yoshio Tsuboi, Naokazu Sasagasako, Takayuki Kondo, Souichi Katayama, Fusako Yokochi, Hidetomo Murakami, Fumihito Yoshii, Ryousuke Takahashi, Shigenobu Nakamura, Yoshihisa Tatsuoka, Nobuo Kouhara, Koichi Hirata, Asako Takei, Yusuke Hazama, Youichi Hokezu, and Hiroshi Sugiyama.

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Please cite this article in press as: Takahashi et al., *ERBB4* Mutations that Disrupt the Neuregulin-ErbB4 Pathway Cause Amyotrophic Lateral Sclerosis Type 19, The American Journal of Human Genetics (2013), http://dx.doi.org/10.1016/j.ajhg.2013.09.008

REPORT

ERBB4 Mutations that Disrupt the Neuregulin-ErbB4 Pathway Cause Amyotrophic Lateral Sclerosis Type 19

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Amyotrophic lateral sclerosis (ALS) is a devastating neurological disorder characterized by the degeneration of motor neurons and typically results in death within 3–5 years from onset. Familial ALS (FALS) comprises 5%–10% of ALS cases, and the identification of genes associated with FALS is indispensable to elucidating the molecular pathogenesis. We identified a Japanese family affected by late-onset, autosomal-dominant ALS in which mutations in genes known to be associated with FALS were excluded. A whole- genome sequencing and parametric linkage analysis under the assumption of an autosomal-dominant mode of inheritance with incomplete penetrance revealed the mutation c.2780G>A (p. Arg927Gln) in *ERBB4*. An extensive mutational analysis revealed the same mutation in a Canadian individual with familial ALS and a de novo mutation, c.3823C>T (p. Arg1275Trp), in a Japanese simplex case. These amino acid substitutions involve amino acids highly conserved among species, are predicted as probably damaging, and are located within a tyrosine kinase domain (p. Arg927Gln) or a C-terminal domain (p. Arg1275Trp), both of which mediate essential functions of ErbB4 as a receptor tyrosine kinase. Functional analysis revealed that these mutations led to a reduced autophosphorylation of ErbB4 upon neuregulin-1 (NRG-1) stimulation. Clinical presentations of the individuals with mutations were characterized by the involvement of both upper and lower motor neurons, a lack of obvious cognitive dysfunction, and relatively slow progression. This study indicates that disruption of the neuregulin-ErbB4 pathway is involved in the pathogenesis of ALS and potentially paves the way for the development of innovative therapeutic strategies such using NRGs or their agonists to upregulate ErbB4 functions.

Amyotrophic lateral sclerosis (ALS) is a devastating neurological disorder in which the degeneration of motor neurons leads to progressive weakness and wasting of limb, bulbar, and respiratory muscles. Familial ALS (FALS) comprises 5%–10% of ALS cases, and the remaining cases are simplex cases of ALS (SALS). To date, more than 20 genes have been shown to be associated with ALS,[1] and these account for 75% of FALS and 14% of SALS cases.[2] Mutations that are found in FALS-associated genes but that are also identified in individuals with SALS are considered mutations with reduced penetrance or de novo mutations. Further discovery of genes associated with FALS is indispensable to elucidating the molecular backgrounds of both FALS and SALS.

Identification of genes associated with familial diseases has been accomplished through identification of the disease loci on the chromosomes by linkage analysis of large pedigrees and subsequent positional cloning of the genes. The majority of the FALS pedigrees, however, are not large and do not have multiple affected members as a result of the poor prognosis of the disease and the late age of onset, which makes it difficult to sufficiently narrow the candidate regions by linkage analyses and means that it takes a tremendous effort to identify the genes associated with FALS. The recent development of massively parallel

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http://dx.doi.org/10.1016/j.ajhg.2013.09.008.

Please cite this article in press as: Takahashi et al., *ERBB4* Mutations that Disrupt the Neuregulin-ErbB4 Pathway Cause Amyotrophic Lateral Sclerosis Type 19, The American Journal of Human Genetics (2013), http://dx.doi.org/10.1016/j.ajhg.2013.09.008

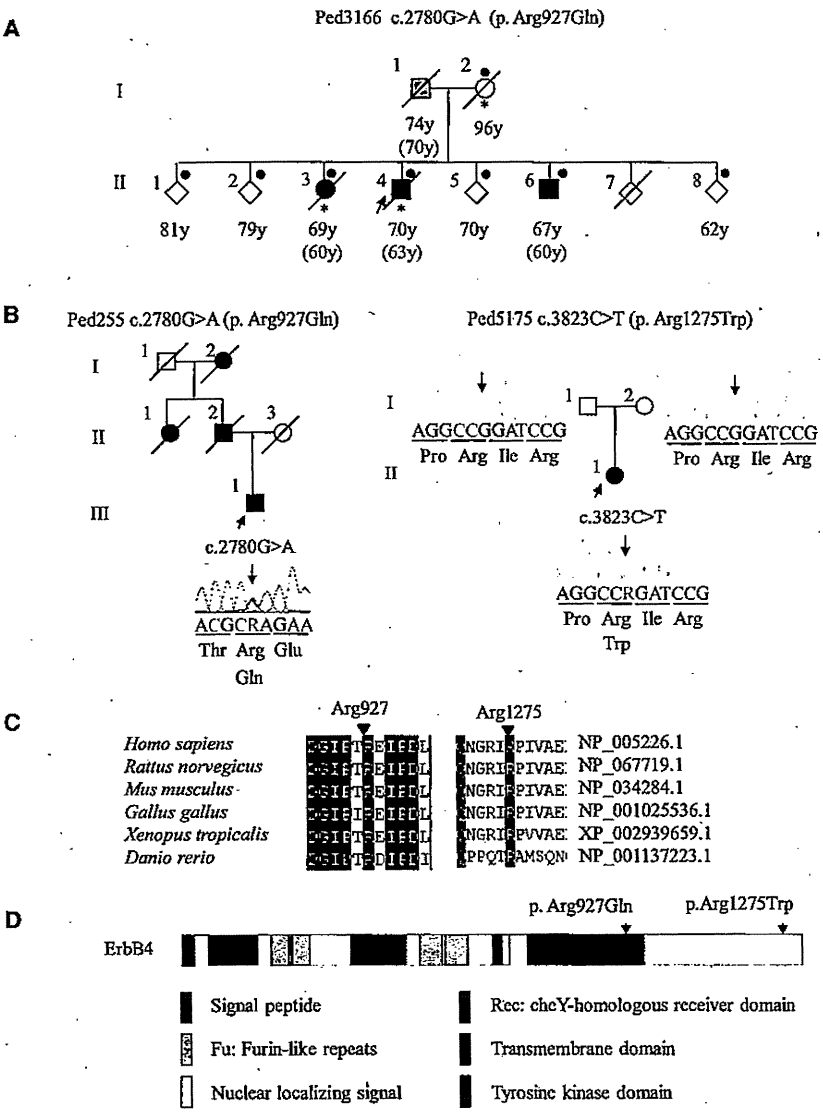

Figure 1. Pedigrees of ALS and Characterization of Mutations

(A) Pedigree charts of the index family. Filled symbols indicate affected individuals. The arrow indicates the proband. For confidentiality purposes, all unaffected siblings are indicated by diamonds. Dots or asterisks indicate individuals included in the linkage study or WGS, respectively. Age at present or age at death is shown under each individual, and ages at onset are shown in parentheses. The box with gray shading indicates that the individual's clinical information obtained from the family members strongly supports the diagnosis of ALS, although detailed neurological evaluations have not been conducted for this individual.

(B) Additional Canadian (Ped255) and Japanese (Ped5175) pedigrees with *ERBB4* mutations. The electropherograms of mutational data are shown beside each member. Nucleotide colors correspond to the colors in the electropherograms. The amino acids are designated below the nucleotide sequences. The blue arrows indicate the nucleotide positions of the mutations. In the electropherograms (Ped5175), nucleotide sequences of the reverse complementary strand are shown.

(C) Amino acid conservation. The amino acids Arg927 and Arg1275 are highly conserved among species.

(D) The protein structure along with the locations of amino acid substitutions are shown; amino acid substitutions are indicated by arrows. The amino acid substitution p. Arg927Gln resides in the tyrosine kinase domain, which mediates the key functions of ErbB4. The amino acid substitution p. Arg1275Trp resides in the C-terminal domain in the vicinity of multiple phosphorylation sites, which mediate downstream signaling pathways.

sequencing technologies has allowed us to overcome the difficulty by means of whole-genome sequencing (WGS) or exome analysis.

We identified a Japanese family with three affected siblings presenting with late-onset ALS (Figure 1A and Table 1). The familial history indicated that the mode of inheritance is probably an autosomal-dominant one. Mutational analysis of the proband (II-4) employing direct nucleotide sequence analysis, a microarray-based resequencing, or a repeat-primed PCR analysis excluded *SOD1*[MIM 147450], *ALS2*[MIM 606352], *DCTN1*[MIM 601143], *CHMP2B*[MIM 609512], *ANG*[MIM 105850], *TARDBP*[MIM 605078], *FUS*[MIM 137070] and *C9ORF72* [MIM 1614260] as the genes associated with FALS.[3,4] To identify a gene associated with FALS, we applied WGS in combination with a linkage analysis to the pedigree. Written informed consent was obtained from all the participants. This study was approved by the institutional review board at the University of Tokyo.

WGS was performed on three individuals (I-2, II-3 and II-4, as shown in Figure 1A) in the index pedigree. Paired-end DNA libraries were generated and subjected to massively parallel sequencing with a GAII Illumina Genome Analyzer in accordance with the manufacturer's instructions. The short read sequences obtained were aligned to the reference genome (NCBI37/hg19 assembly) via the Burrows-Wheeler Aligner.[5] Downstream analyses in which potential PCR duplicates were removed were processed with SAMtools.[6] Aligned reads were viewed on an Integrative Genomics Viewer.[7] Genomic sequence variations were identified with the SAMtools pileup command and annotated with Refseq, dbSNP135, 1000 Genomes, personal genome databases, the NHLBI GO Exome Sequencing Project (NHLBI-ESP) database, and an in-house variant database containing 41 whole genomes and 1,408 exomes in the Japanese population. The numbers of nonsynonymous variants that were identified in individuals I-2, II-3, and II-4 but that were not present in any of the

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Table 1. Clinical Characteristics of Affected Individuals

Pedigree Number	Pedigree 3166				Pedigree 255	Pedigree 5175
Ethnicity	Japanese				Canadian	Japanese
Inheritance	familial (autosomal dominant)				familial (autosomal dominant)	simplex
Mutation	c.2780G>A				c.2780G>A	c.3823C>T
Amino acid substitution	p. Arg927Gln				p. Arg927Gln	p. Arg1275Trp
Members	I-1	II-3	II-4 (proband)	II-6	III-3	II-1
Age at onset	70	60	63	60	67	45
Initial symptoms	bulbar	N.D.	upper limbs	respiration	upper limbs	upper limbs
Diagnostic criteria[a]	N.D.	N.D.	definite	definite	probable	probable
Progression	unable to walk after 3 years	ventilator -dependent after 5 years, locked-in state after 8 years	locked-in state after 5 years	ventilator- dependent after 1 year, locked-in state after 5 years	slow progression that significantly decelerated and finally stopped after 8 years	wheelchair- bound, MRS 1-2/5 in upper extremities after 5 years
Cognitive function	N.D.	N.D.	normal	normal	N.D.	normal
Age at death	74	69	70	66	N/A	N/A

Abbreviations are as follows: N.D., not described; MRS, manual muscle testing rating scale; and N/A, not applicable.
[a]El Escorial and Airlie House revised criteria.

databases (hereafter, variants not found in the databases are referred to as "novel") were 411, 404, and 382, respectively (Table S1). No novel nonsynonymous variants in genes known to be associated with FALS were included. Among the identified variants, 57 were identified both in the proband and in the affected sibling, but not in the mother, and were subjected to further analysis.

The individuals indicated by dots in Figure 1A were genotyped with Genome-Wide Human SNP Array 6.0 (Affymetrix). Linkage analysis and haplotype reconstruction were conducted with the pipeline software SNP-HiTLink[8] and Allegro version 2[9] under the assumption of an autosomal-dominant mode of inheritance and a disease-allele frequency of 0.000001. Parametric multipoint linkage analysis under the assumption of complete penetrance revealed three loci spanning 23.6 Mb on chromosomes 1, 6, and 13, having a maximum LOD score of 1.8 (Figure S1; penetrance = 1.0), and containing 88 annotated genes. However, no novel nonsynonymous variants were identified in the candidate regions. We then considered the possibility of reduced penetrance. When penetrance was reduced to 0.8 (Figure S1), seven additional loci had LOD scores > 0.7 and were thus shown to support linkage; these loci contained 809 annotated genes. Three heterozygous novel nonsynonymous variants were identified in these regions; among these variants, only c.2780G>A (p. Arg927Gln; dbSNP SubSNP ID ss831884245) substituting glutamine for arginine at codon 927 (p. Arg927Gln) in v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian) (*ERBB4* [MIM 600543; RefSeq accession number NM_005235.2]) was not present in 477 controls (Table S2). When we allowed further reduced penetrance, we identified 19 additional loci with LOD > 0; these loci contained 1,265 annotated genes. In these regions, we identified seven heterozygous novel nonsynonymous variants, among which three variants in *OR2D3* (RefSeq NM_001004684.1), *FTCD* (MIM 606806; RefSeq NM_206965.1), and *TJP2* (MIM 607709; RefSeq NM_001170414.2) were not present in 477 controls (Table S2). *OR2D3* is an olfactory receptor gene; the substituted amino acid in *OR2D3* is not conserved, and the substitution is predicted as benign by PolyPhen-2 analysis. *FTCD* and *TJP2* are associated with autosomal-recessive glutamate formiminotransferase deficiency (MIM 229100) and familial hypercholanemia (MIM 607748), respectively, and heterozygous carriers have not been described as exhibiting ALS. Taken together, the results pointed to c.2780G>A in *ERBB4* as the most likely pathogenic mutation.

We used a direct nucleotide sequence analysis method to conduct mutational analysis of *ERBB4* in 364 FALS and 818 SALS individuals by using an ABI 3100 sequencer and BigDye Terminator ver3.1 (Applied Biosystems). We used the ExonPrimer website to design oligonucleotide primers (Table S3). The mutation c.2780G>A was also identified in one Canadian FALS individual (Figure 1B). Unfortunately, DNA from other family members was not available to confirm segregation. To investigate a possibility that the c.2780G>A mutation identified in the Japanese and Canadian families is a common founder mutation, we compared the haplotypes with the c.2780G>A mutation in *ERBB4* of the Japanese and Canadian families (Figure S2). Different SNPs were observed 14 kbp and 5 kbp centromeric and telomeric to the mutation, respectively, indicating that disease haplotypes of the Japanese and Canadian families are different and that

Please cite this article in press as: Takahashi et al., *ERBB4* Mutations that Disrupt the Neuregulin-ErbB4 Pathway Cause Amyotrophic Lateral Sclerosis Type 19, The American Journal of Human Genetics (2013), http://dx.doi.org/10.1016/j.ajhg.2013.09.008

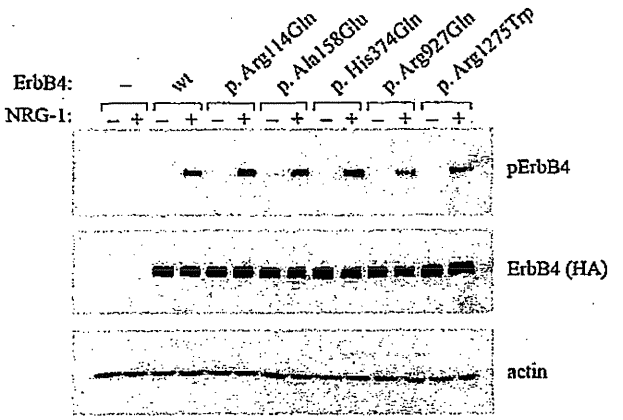

Figure 2. Functional Analysis of Wild-Type and Mutant ErbB4 upon Neuregulin-1 Stimulation

COS-7 cells transfected with an empty-vector control or plasmids encoding either wild-type (wt) or mutant HA-tagged ErbB4 (p. Arg114Gln, p. Ala158Glu, p. His374Gln, p. Arg927Gln, or p. Arg1275Trp) were stimulated with or without NRG-1, and the autophosphorylation activity of ErbB4 was analyzed by immunoblot analysis with antibodies against phospho-ErbB4 (Tyr1284) (Cell Signaling) and HA tag (Abcam), respectively. For loading controls, immunoblotting was performed with an anti-actin antibody (Santa Cruz Biotechnology). Three amino acid substitutions, including p.Arg114Gln, p.Ala158Glu, and p.His374Gln (rs760369), identified through mutational analysis of FALS and SALS individuals, were included in autophosphorylation assay. The substitutions p.Arg114Gln and p.Ala158Glu were not considered to be relevant to ALS because neither recurrence nor cosegregation was confirmed.

mutation occurred independently. We identified a de novo mutation of c.3823C>T (dbSNP SubSNP ID ss831884246), substituting tryptophan for arginine at codon 1275 (p. Arg1275Trp), in a Japanese SALS individual (Figure 1B) in whom a biological parent-descendant relationship was confirmed (Table S4) by the PLINK[10] algorithm. These mutations were neither present in the 477 Japanese controls nor registered in the in-house database containing 41 whole genomes and 1408 exomes, the 1000 Genomes database, or the NHLBI-ESP database, containing 6503 exomes. Furthermore, c.2780G>A was not present in 190 Canadian controls. The identification of c.2780G>A in two independent families of different ethnic backgrounds strongly supported c.2780G>A as the causative mutation for ALS. Given that de novo mutation rates have been estimated to be 1.20×10^{-8} per nucleotide per generation[11] and less than one nonsynonymous single-nucleotide variant (SNV)/generation,[12] the observation of the de novo mutation further supports the idea that c.3823C>T is likely to be the causative mutation for ALS in this individual. The mutation's substituted arginine residues, Arg927 and Arg1275, are highly conserved among species (Figure 1C), and the substitutions are predicted to be probably damaging by PolyPhen-2 analysis. The amino acid residue Arg927 resides in a tyrosine kinase domain, which is essential for the receptor tyrosine kinase activity, and Arg1275 is located in a C-terminal domain in the vicinity of multiple phosphorylation sites, which mediate downstream signaling pathways (Figure 1D). The clinical presentations of these ALS individuals with the *ERBB4* mutations are summarized in Table 1. The common clinical characteristics of the individuals included both upper and lower motor-neuron involvement diagnosed as definite or probable ALS according to El Escorial and Airlie House revised criteria, relatively slow disease progression, and no obvious cognitive impairment. The individuals with the c.2780G>A mutation were characterized by relatively late onset (the ages at onset ranged from 60–70 years) and a slightly reduced penetrance. In contrast, the individual with the c.3823C>T mutation was characterized by early onset (45 years of age).

ErbB4 is a member of the epidermal growth factor (EGF) subfamily of receptor tyrosine kinases (RTKs). It forms a homodimer or a heterodimer with ErbB2 or ErbB3 and is activated upon binding of neuregulins (NRGs) to the extracellular ligand-binding domain of ErbB4.[13] Activation of ErbB4 is mediated by increased tyrosine kinase activity upon NRG binding, resulting in autophosphorylation of the C-terminal tail.[14] To determine how the two mutations identified in the ALS individuals affect ErbB4 functions, we investigated the autophosphorylation of ErbB4 in cells expressing either wild-type or mutant (c.2780G>A or c.3823C>T) *ERBB4* in the presence of NRG-1. The *ERBB4* mutations were introduced into the pBABE-puro*ERBB4JM-aCYT-2HA* plasmid encoding HA-tagged ErbB4 JM-a CYT-2[15] by site-directed mutagenesis according to the protocol described in the Phusion Site-Directed Mutagenesis Kit (Thermo Fisher Scientific). After mutagenesis, all the constructs were verified by sequencing. The plasmids were transiently transfected into COS-7 cells via FuGENE 6 transfection reagent (Roche) in accordance with the manufacturer's instructions. Transfected cells were starved of serum overnight and stimulated with 0 or 50 ng/ml NRG-1 (R&D Systems) for 10 min at 37°C. After stimulation, the cells were lysed, and samples equivalent to 50 μg of total protein were separated through 8% SDS-PAGE gels. For detectiin of ErbB4 phosphorylation and total ErbB4 protein levels, immunoblotting was performed antibodies against phospho-ErbB4 (Tyr1284) (Cell Signaling) and HA-tag (Abcam), respectively. The two amino acid substitutions, p. Arg927Gln and p. Arg1275Trp, showed a clearly reduced autophosphorylation of ErbB4 (Figure 2). On the basis of these genetic and functional data, we concluded that the two mutations are causative mutations for ALS (ALS19).

This study revealed that a reduced autophosphorylation of ErbB4 upon NRG-1 stimulation is involved in the pathogenesis of ALS. *Erbb4* is specifically expressed in the soma of large motor neurons of the rat spinal cord.[16] The lack of *Erbb4* is embryonically lethal in mice, which displayed the derangement of motor-neuron axon guidance and pathfinding during embryogenesis.[17] Heterozygous-null mice showed a reduced body weight and delayed motor development, and brain-specific conditional knock-out mice

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demonstrated reduced spontaneous motor activity and grip strength of the hindlimbs.[18] Mice lacking cysteine-rich domain (CRD) isoforms of *Nrg-1* (*CRD-NRG-1*$^{-/-}$) die perinatally as a result of respiratory failure, lack detectable limb movement, and exhibit a loss of ~60% of spinal motor neurons.[19] Similarly, motor and sensory neuron-specific conditional *Nrg-1* knockout mice die at birth and showed marked retraction of motor-neuron axons.[20] Furthermore, a decrease in the amount of CRD-NRG-1 has been detected in the spinal motor neurons in FALS and SALS individuals and *Sod1* mutant mice at disease onset,[21] raising the possibility that disruption of the NRG-ErbB pathway is commonly involved in the motor-neuron degeneration underlying ALS. This study provides insight into ALS pathogenesis and is expected to pave the way for the development of innovative therapeutic strategies such as using NRGs or their agonists to upregulate ErbB4 functions.

Supplemental Data

Supplemental Data include two figures and four tables and can be found with this article online at http://www.cell.com/AJHG/.

Consortia

Consortium members of JaCALS include Ryoichi Nakamura, Hazuki Watanabe, Yuishin Izumi, Ryuji Kaji, Mitsuya Morita, Kotaro Ogaki, Akira Taniguchi, Ikuko Aiba, Koichi Mizoguchi, Koichi Okamoto, Kazuko Hasegawa, Masashi Aoki, Akihiro Kawata, Imaharu Nakano, Koji Abe, Masaya Oda, Masaaki Konagaya, Takashi Imai, Masanori Nakagawa, Takuji Fujita, Hidenao Sasaki, and Masatoyo Nishizawa.

Acknowledgments

We thank all the family members for participating in this study. This study was supported in part by KAKENHI (Grants-in-Aid for Scientific Research on Innovative Areas [22129001 and 22129002]) to S.T.; the Global COE Program from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and a grant-in-aid (H23-Jitsuyoka [Nanbyo]-Ippan-004) from the Ministry of Health, Labour, and Welfare, Japan to S.T. We acknowledge support to R.H.B. from ALS Therapy Alliance, Project ALS, P2ALS, the Angel Fund, the Pierre L. de Bourgknecht ALS Research Foundation, the Al-Athel ALS Research Foundation, the ALS Family Charitable Foundation, and grant 1R01NS050557 from the National Institute of Neurological Disorders and Stroke of the National Institutes of Health and support to G.A.N. from the MND Research Institute of Australia. P.G.-P. was supported by the Alfonso Martin Escudero Foundation (Madrid).

Received: May 12, 2013
Revised: August 26, 2013
Accepted: September 13, 2013
Published: October 10, 2013

Web Resources

The URLs for data presented herein are as follows:

1000 Genomes Project Database, http://www.1000genomes.org/
dbSNP135, http://www.ncbi.nlm.nih.gov/projects/SNP/
ExonPrimer, http://ihg.gsf.de/ihg/ExonPrimer.html
NCBI37/hg19 assembly, http://genome.ucsc.edu/
NHLBI GO Exome Sequencing Project (NHLBI-ESP), https://esp.gs.washington.edu/drupal
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
Personal genome databases, http://www.sequenceontology.org/resources/10Gen.html
PLINK algorithm, http://pngu.mgh.harvard.edu/purcell/plink/
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
Refseq, http://www.ncbi.nlm.nih.gov/projects/RefSeq/
UCSC Human Genome Browser, http://genome.ucsc.edu/

Accession Numbers

The dbSNP accession numbers for the c. 2780G>A and c. 3823C>T mutations reported for *ERBB4* in this paper are ss831884245 and ss831884246, respectively.

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INTERNAL MEDICINE

ORIGINAL ARTICLE

Cerebrospinal Fluid Neopterin, but not Osteopontin, is a Valuable Biomarker for the Treatment Response in Patients with HTLV-I-associated Myelopathy

Masahiro Nagai, Tomoaki Tsujii, Hirotaka Iwaki, Noriko Nishikawa and Masahiro Nomoto

Abstract

Objective The concentrations of neopterin and osteopontin in the cerebrospinal fluid (CSF) were measured in patients with HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) in order to evaluate their utility as biomarkers for the treatment response.

Methods Seven HAM/TSP patients were treated intravenously with high-dose methylprednisolone (1,000 mg/day) for 3 days. CSF samples were collected before and after the treatment. The neopterin and osteopontin concentrations were determined using high-performance liquid chromatography (HPLC) and an enzyme immunoassay, respectively. The clinical symptoms were evaluated using the Osame Motor Disability Score and the Urinary Disturbance Score.

Results Four out of the seven patients showed an improvement in motor function with the treatment, and were therefore classed as responders. The pre-treatment CSF neopterin concentration exceeded the upper limit of normal in all seven of the patients, and tended to be higher in treatment responders as compared to non-responders. The CSF neopterin concentration was reduced following treatment in all patients. The mean CSF neopterin concentration significantly ($p<0.01$) decreased following treatment by almost 60% (from 124.1±79.9 nmol/L to 49.2±29.8 nmol/L). The mean CSF osteopontin concentration was significantly ($p<0.01$) higher in the HAM/TSP patients in comparison to the 18 HTLV-1-seronegative patients who were designated as controls (9.54±4.53 mg/L vs. 3.72±3.04 mg/L). No significant ($p=0.47$) reduction of the CSF osteopontin concentration was observed following the intravenous administration of high-dose methylprednisolone.

Conclusion These results indicate that the CSF neopterin concentration, but not the osteopontin concentration, is a potentially valuable biomarker for monitoring the treatment response in HAM/TSP patients. Furthermore, high pre-treatment CSF neopterin concentrations may be a predictive biomarker for a response to intravenous high-dose methylprednisolone therapy.

Key words: HTLV-I, HAM/TSP, cerebrospinal fluid, neopterin, osteopontin, methylprednisolone

(Intern Med 52: 2203-2208, 2013)
(DOI: 10.2169/internalmedicine.52.0869)

Introduction

Human T-cell lymphotropic virus type I (HTLV-I) is an exogenous human retrovirus that has been demonstrated to be the etiological agent in adult T-cell leukemia as well as in a progressive neurological disease called HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP). The vast majority of HTLV-I-infected individuals are clinically asymptomatic, with <5% of infected individuals ever developing HAM/TSP. Clinically, HAM/TSP is characterized by muscle weakness, hyperreflexia, spasticity in the lower extremities and urinary disturbance associated with the preferential damage of the thoracic spinal cord (1). Although it is not yet completely understood how HTLV-I causes HAM/TSP, it is believed that increased HTLV-I

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Received for publication April 26, 2013; Accepted for publication June 2, 2013
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Table 1. Patient Characteristics

Patient no.	Age (years)	Sex	Disease duration (years)	CSF anti-HTLV-I antibody titer
1	70	M	13	1:512
2	81	F	2	1:800
3	48	F	2	1:256
4	48	F	4	1:800
5	77	F	5	1:512
6	60	F	2	1:512
7	56	F	10	1:128

F: female, M: male

Table 2. Rating Scale for the Osame Motor Disability Score

0	Normal walking and running
1	Normal gait but runs slowly
2	Abnormal gait
3	Abnormal gait and unable to run
4	Needs support while using stairs
5.	Needs 1-hand support in walking
6	Needs 2-hand support in walking
7	Needs 2-hand support in walking and is limited to 10 m
8	Needs 2-hand support in walking and is limited to 5 m
9	Unable to walk but able to crawl on hands and knees
10	Crawls with hands
11	Unable to crawl but can turn sideways in bed
12	Unable to turn sideways but can move toes
13	Completely bedridden

proviral loads and immune responses to HTLV-I infected cells play a pivotal role in the pathogenesis of this disorder (2).

Consequently, therapeutic strategies for HAM/TSP patients are directed towards these pathogenic phenomena. The first therapeutic strategy is to reduce HTLV-I loads, although anti-retroviral reagents such as reverse transcriptase inhibitors seem to be less effective for HTLV-I infections than for human immunodeficiency virus (HIV) infections. The second therapeutic strategy is to modulate the abnormal immune responses in HAM/TSP patients. Several immunosuppressive or immunomodulating therapies have been tried, including corticosteroids, interferon-α (IFN-α), azathioprine and plasmapheresis (3). Chronic oral prednisolone therapy was empirically the most effective for the improvement the neurological impairment that is associated with HAM/TSP. However, adverse events such as osteoporosis, glucose intolerance and gastroduodenal ulceration have limited its use for HAM/TSP patients, especially elderly postmenopausal women. We have chosen IV high-dose methylprednisolone as a first-line therapy because this treatment has a better tolerability profile than chronic oral prednisolone therapy. Another reason is because IV high-dose methylprednisolone therapy has been extensively and successfully used for patients with relapses of multiple sclerosis or other immune-mediated neurological disorders.

Although the anti-HTLV-I antibody titer in the cerebrospinal fluid (CSF) is used as a diagnostic biomarker of HAM/TSP, it is inadequate as a biomarker for monitoring the treatment response. However, increased CSF neopterin concentrations have been previously reported in patients with HAM/TSP (4, 5). Neopterin is a pyrazino-pyrimidine compound that is produced by macrophages after stimulation by IFN-γ from activated T cells. The concentration of neopterin in the CSF has been used as a biomarker for cellular immune response in the central nervous system (CNS). Osteopontin has multiple functions and is involved in the recruitment of macrophage and T cells in inflammatory lesions. Osteopontin enhances IFN-γ and interleukin (IL)-12 production and depresses the release of IL-10 from immune cells (6). It is up-regulated in the brains of patients with multiple sclerosis and in the spinal cords of mice with experimental autoimmune encephalomyelitis (7, 8). It is therefore believed that both neopterin and osteopontin could be valuable biomarkers for indicating the severity of inflammation and the degree of T cell activation in the CNS.

Chronic oral prednisolone therapy decreases CSF neopterin concentrations in HAM/TSP patients (3). However, it is not yet known if CSF neopterin concentrations change rapidly after treatment and might, therefore, be useful in predicting the response to corticosteroid therapy. The CSF osteopontin concentrations in HAM/TSP patients have not yet been investigated. We herein present the results of our study in which we monitored the CSF neopterin and osteopontin concentrations before and after IV high-dose methylprednisolone therapy in patients with HAM/TSP in order to evaluate their utility as biomarkers for treatment response.

Materials and Methods

The diagnosis of HAM/TSP was made according to the current WHO diagnosis guidelines. The anti-HTLV-I antibody titers were measured using a particle-agglutination (PA) test. The characteristics of the seven patients with HAM/TSP who were recruited are summarized in Table 1. They had not been previously treated with either immunosuppressive or immunomodulating agents. They were treated with IV infusions of high-dose methylprednisolone (1,000 mg/day) for 3 days following their hospital admission. None of the patients received any additional oral corticosteroid therapy following the IV administration of high-dose methylprednisolone. The Osame Motor Disability Score (OMDS) and the Urinary Disturbance Score (UDS) were used for the clinical evaluation. The OMDS rating scale is shown in Table 2. The UDS was calculated from the sum of the scores (0=normal, 1=slight, 2=moderate, 3=severe) for three symptoms (increased frequency of urination, feeling of residual urine, incontinence). CSF was obtained from each patient with written informed consent. Lumbar punctures were performed before and within 7 days of treatment completion. The collected CSF was stored at -80℃ until analyzed. The study was approved by the hospital ethics committee.

The CSF neopterin concentrations were measured using high-performance liquid chromatography (HPLC) with fluorometric detection. Thawed CSF aliquots (100 μL) were acidified with ice-cold 0.1 M HCl (100 μL) and kept on ice.

A mixture of 1% I_2 and 2% KI in 0.1 M HCl (50 μL) was then added, and the samples were incubated at room temperature under dark conditions. An aqueous solution of 1.5% ascorbic acid (50 μL) was then added to the mixture, which was then centrifuged at 10,000 rpm for 1 min. The supernatant (100 μL) was injected into a C18 column (150× 2.1 mm) with 3.5% methanol in water as the mobile phase. The quantification of osteopontin in the CSF samples was performed using a commercially available enzyme immunoassay kit (human osteopontin assay kit, Immuno-Biological Laboratories, Japan) according to the manufacturer's protocol.

Statistical analysis was performed using the JMP10 software program (SAS). The results are expressed as mean±SD and median values. The paired data from before and after treatment were analyzed by Wilcoxon's signed-rank test. The Mann-Whitney U test was used to compare groups. p values of <0.05 were considered to be statistically significant.

Results

Four out of the seven patients showed improvement in their motor function after treatment with IV high-dose methylprednisolone. One patient improved by two grades and the others improved by one grade on the OMDS rating scale (Table 3). An improvement in the urinary disturbance score was observed in four patients (Table 3). Three of the patients showed improvements on both scales, while one patient showed an improvement in the urinary disturbance score without an associated improvement in motor function and a second patient showed improved motor function without an improvement in the urinary disturbance score. Five patients had sensory disturbance (pain and numbness of lower limbs and back pain) before the treatment. The treatment alleviated pain in all five patients.

The CSF neopterin concentrations exceeded the upper limit of normal (30 nmol/L) in all of the patients before treatment. The mean CSF neopterin concentration was 124.1±79.9 nmol/L (median 89.9 nmol/L) for all seven patients prior to treatment. The CSF neopterin concentrations decreased in all of the patients after IV high-dose methylprednisolone therapy. The mean CSF neopterin concentration was significantly ($p<0.01$) reduced by almost 60% after treatment to 49.2±29.8 nmol/L (median: 43.4 nmol/L) (Table 4). The changes in the CSF neopterin concentrations according to the treatment parameters for each patient are shown in Fig. 1.

Table 3. Changes in the Disability Scores after IV High-dose Methylprednisolone Therapy

Patient no.	OMDS		UDS	
	Before	After	Before	After
1	5	4	6	5
2	10	8	9	7
3	4	4	5	3
4	3	3	3	3
5	5	4	3	3
6	10	9	7	6
7	5	5	3	3

OMDS: Osame Motor Disability Score, UDS: Urinary Disturbance Score

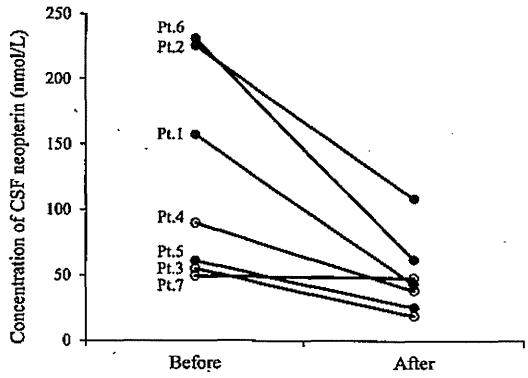

Figure 1. The CSF neopterin concentrations before and after IV high-dose methylprednisolone therapy. The closed and open symbols indicate clinical responders and non-responders, respectively.

Table 4. Changes in the CSF Parameters after IV High-dose Methylprednisolone Therapy

Patient no.	Neopterin (nmol/L)		Osteopontin (mg/L)		anti-HTLV-I antibody titer		Cells (/μL)		Protein (mg/dL)	
	Before	After	Before	After	Before	After	Before	After	Before	After
1	157.4	43.4	10.69	13.46	1:512	1:512	6	2	25	27
2	225.5	108.7	7.22	9.36	1:800	1:256	10	8	54	50
3	54.9	18.9	3.67	5.05	1:256	1:128	6	7	45	29
4	89.9	38.5	13.15	12.33	1:800	1:800	7	6	50	44
5	60.9	25.4	4.66	4.81	1:512	1:128	3	3	39	35
6	230.8	61.7	16.02	11.50	1:512	1:512	12	6	52	29
7	49.5	47.8	11.36	3.70	1:128	1:256	5	7	20	23

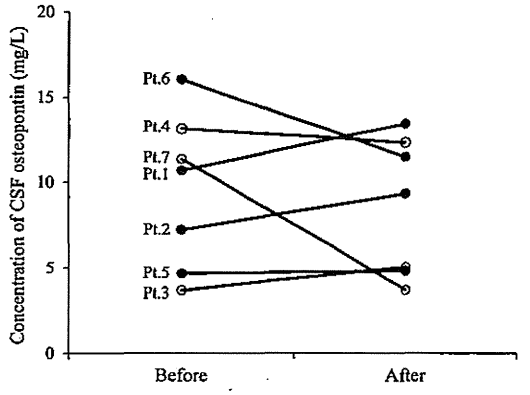

Figure 2. The CSF osteopontin concentrations before and after IV high-dose methylprednisolone therapy. The closed and open symbols indicate clinical responders and non-responders, respectively.

The mean CSF osteopontin concentration was 9.54±4.53 mg/L (median 10.69 mg/L) prior to treatment. Unlike for neopterin, our laboratory had not yet set an upper limit of normal for CSF osteopontin. We therefore compared CSF osteopontin concentrations for our seven HAM/TSP patients to those for 18 HTLV-I-seronegative patients with spondylosis as a control group. The mean CSF osteopontin concentration was 3.72±3.04 mg/L (median 3.52 mg/L) in the control group. The pre-treatment CSF osteopontin concentration in our seven HAM/TSP patients was significantly ($p<0.01$) higher than that of the control group. The mean CSF osteopontin concentration was 8.6±4.03 mg/L (median: 9.36 mg/L) after treatment in the seven HAM/TSP patients, which was not significantly ($p=0.47$) different than the pre-treatment values (Table 4). The pre-treatment and post-treatment CSF osteopontin concentrations for each HAM/TSP patient are shown in Fig. 2.

We defined HAM/TSP patients who showed an OMDS improvement as responders. The mean pre-treatment CSF neopterin concentration in responders (n=4) was 168.7±79.2 nmol/L (median 191.5 nmol/L), but only 64.8±22.0 nmol/L (median 54.9 nmol/L) in non-responders (n=3). While the pre-treatment CSF neopterin concentrations tended to be higher among responders compared to non-responders, the difference was not statistically significant ($p=0.056$). The mean pre-treatment CSF osteopontin concentration was 9.64±4.91 mg/L (median 8.95 mg/L) in responders and 9.34±5.04 mg/L (median 11.36 mg/L) in non-responders with no significant difference between the groups ($p=0.43$).

The HTLV-I antibody titer, and the number of cells and the amount of protein in the CSF were not significantly altered by the treatment (Table 4).

The IV high-dose methylprednisolone therapy was well tolerated by all of the patients. Although insomnia was observed as an adverse effect of treatment, it was transient. No serious adverse events were observed.

Discussion

We herein demonstrate that the CSF neopterin concentration significantly decreases following IV high-dose methylprednisolone therapy in patients with HAM/TSP. While the pre-treatment CSF osteopontin concentrations were significantly higher in HAM/TSP patients as compared to the controls, there were no statistically significant changes in the CSF osteopontin concentrations after treatment.

The coexistence of a high HTLV-I proviral load and HTLV-I-specific T cells is an important feature of HAM/TSP (9). This distinguishing feature is observed in both peripheral blood and CSF of patients with HAM/TSP (9, 10). Histopathological studies indicate the existence of HTLV-I-infected cells as well as a local inflammatory response in the spinal cord lesions of HAM/TSP patients (11, 12). It is therefore believed that the immune response to HTLV-I likely contributes to the inflammatory process of the CNS lesions in HAM/TSP patients and causes the clinical symptoms of HAM/TSP. Activated lymphocytes and macrophages up-regulate the production of pro-inflammatory cytokines such as IL-1, IL-6 and IFN-γ (13). The significant elevation of the levels of these cytokines has been described in the CSF of HAM/TSP patients (14, 15). High values of CSF neopterin have also been reported in HAM/TSP patients (4, 5). Neopterin is released by stimulated macrophages, and the concentration of CSF neopterin reflects the degree of the inflammatory response in the CNS. The concentration of CSF neopterin is significantly correlated with the HTLV-I proviral load, which is an important risk factor for the development of HAM/TSP (10). The CSF neopterin concentration is therefore useful as an adjunct to the diagnosis of HAM/TSP. In our study, we confirmed the elevation of CSF neopterin concentrations in HAM/TSP patients.

Moreover, we also demonstrated that CSF osteopontin concentrations are increased in HAM/TSP patients. To the best of our knowledge, this is the first report concerning osteopontin concentrations in HAM/TSP patients. Osteopontin is a secreted phosphoprotein that is produced by many kinds of cells including osteoblasts, activated lymphocytes, macrophages, vascular smooth muscle cells and kidney cells (16). It has a multifunctional capacity, and is involved in bone remodeling, tumor progression, atherosclerosis, inflammation and immunity (16). Osteopontin promotes the production of pro-inflammatory cytokines such as IL-12 and IFN-γ (6). Several studies have reported that osteopontin concentrations are significantly elevated in the CSF of patients with multiple sclerosis (17-19). Our finding suggests that a chronic inflammatory response in the CNS lesions of HAM/TSP is reflected by the CSF osteopontin concentrations as well as the CSF neopterin concentrations. If so, which is the better diagnostic marker for HAM/TSP? The CSF neopterin concentrations exceeded the upper limit of normal in all HAM/TSP patients. However, the CSF osteopontin concentrations in three of the seven HAM/TSP patients overlapped the range

measured for the control group. Thus, the CSF neopterin concentration would appear to be more suitable for discriminating HAM/TSP and non-HAM/TSP patients than CSF osteopontin concentration.

Various treatments have been tried for HAM/TSP patients (3). Almost all of the studies have been open-label trials or case series with the exception of a multicenter, randomized placebo-controlled, double-blind study of an IFN-α trial in Japan (20). However, no study has conclusively demonstrated a long-term clinical benefit. Well-designed clinical trials are therefore necessary in order to develop effective therapies which may improve the long-term prognoses for HAM/TSP patients (21). In addition, validated surrogate biomarkers are required for the determination of the effectiveness of investigational treatments.

It has been previously reported that approximately 70% of HAM/TSP patients who are treated with a chronic oral administration of prednisolone (n=131) improved by at least one OMDS grade (3). Furthermore, the treatment significantly decreased the reported CSF neopterin concentrations in 16 patients. The mean CSF neopterin concentration was also reduced from 155.4 nmol/L to 79.5 nmol/L by IV high-dose methylprednisolone therapy in eight of the previously reported patients, but the change was not statistically significant (3). Unfortunately, the timing of the CSF sampling after IV high-dose methylprednisolone therapy was not specified in that publication. The timing of CSF sampling is likely critical with such short-term therapies as IV high-dose methylprednisolone, since the CSF neopterin concentration seems to increase after the discontinuation of treatment. In our study, the CSF samples were collected within 7 days of the completion of the IV high-dose methylprednisolone therapy in order to attenuate the impact of the change on the CSF neopterin concentration. The CSF neopterin concentration changed rapidly after IV high-dose methylprednisolone therapy. This finding suggests that CSF neopterin is a sensitive biomarker for the evaluation of the early-phase response to treatments in HAM/TSP patients. This feature may be partially due to the short half-life of neopterin which has been estimated to be 90 minutes in the circulation (22). In contrast, there was no significant change in the CSF osteopontin concentrations that were was observed after IV high-dose methylprednisolone therapy in the HAM/TSP patients. Even though there is a possibility that a change in the CSF osteopontin concentration may arise several days after the treatment, it is clear that the CSF osteopontin concentration is unreliable as a biomarker for the assessment of an early-phase response to treatment. Although the influence of IV high-dose methylprednisolone therapy on HTLV-I proviral loads is still unclear, the therapy doesn't seem to reduce HTLV-I proviral loads. It has been reported that the osteopontin gene was transactivated by HTLV-I Tax protein (23). If the elevation of the CSF osteopontin levels in HAM/TSP patients is due to an HTLV-I infection of the osteopontin producing cells, but not the inflammatory response, then the unchanged osteopontin levels by IV high-dose methylprednisolone therapy are thus thought to be understandable.

Although it was not a statistically significant difference, and it is most likely due to the small number of patients, the pre-treatment CSF neopterin concentrations of those patients who responded to the IV high-dose methylprednisolone therapy tended to be higher than those of non-responders. This suggests that patients with relatively high values of CSF neopterin may have a more favorable response to IV high-dose methylprednisolone therapy. Additional patients need to be examined in order to confirm the belief that CSF neopterin is useful as a predictive biomarker for responders to IV high-dose methylprednisolone therapy.

The mechanism of action of for the corticosteroids on HAM/TSP remains to be elucidated. The anti-inflammatory properties of the corticosteroids may attenuate the degree of the inflammatory response in spinal cord lesions. This non-specific anti-inflammatory effect may result in clinical improvements in the patients, especially when the inflammation is very intense. Moreover, corticosteroids may also affect the HTLV-I infected cells or the immune response to HTLV-I. It has been demonstrated that betamethasone therapy decreased $CD4^+Tax^+$ T cells and increased $CD4^+Foxp3^+$ T cells (regulatory T cells) in the peripheral blood samples of patients with HAM/TSP (24). Corticosteroids therapy may reduce the erratic IFN-γ production by T-cells in patients with HAM/TSP, which is then followed by a reduction of neopterin release by the stimulated macrophages.

Our study did not address how long the clinical effect and the reduction of CSF neopterin concentration lasted after the IV high-dose methylprednisolone therapy had been discontinued. An open-label clinical trial of IV high-dose methylprednisolone has been reported from Brazil, in which 39 patients with HAM/TSP received IV high-dose methylprednisolone every 3-4 months (25). The Incapacity Status Scale showed a significant neurological improvement of 24.5% after a mean follow-up of 2.2 years. However, the CSF biomarkers were not reported in that trial. Further study will therefore be needed to clarify the long-term changes in the CSF neopterin concentration following the treatment of HAM/TSP patients.

In conclusion, our results indicate that the concentration of CSF neopterin, but not that of osteopontin, is a potentially valuable biomarker for monitoring treatment response in HAM/TSP patients. In addition, high pre-treatment CSF neopterin concentrations may be a predictive biomarker for response to IV high-dose methylprednisolone therapy.

The authors state that they have no Conflict of Interest (COI).

Acknowledgement

This study was supported by Grant-in-Aids for Orphan and Neurodegenerative Disorders from the Ministry of Health, Labour and Welfare, GJTS, and a Grant of Ehime University. The authors wish to thank Ms. Madoka Kubo for her excellent technical assistance.

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International Journal of General Medicine

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ORIGINAL RESEARCH

Evaluation of the effect of pregabalin on simulated driving ability using a driving simulator in healthy male volunteers

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Abstract: Pregabalin, a novel agent for treating partial epilepsy and peripheral neuropathic and central pain, was studied for its effect on driving performance in healthy volunteers. Sixteen healthy male volunteers who drove regularly were enrolled in a double-blind, parallel-group, placebo-controlled study assessing the effect of pregabalin on driving performance. Subjects received an oral dose of pregabalin 75 mg or placebo, and a second dose 12 hours later. A driving simulator was used to test simple and complicated braking reaction time, and simple and complicated steering-wheel techniques before the first dose, and 1 hour and 3 hours after the second dose of pregabalin or placebo. The effect of training during the driving test on the driving performance of each group was also evaluated. There were no statistically significant differences in driving performance between the pregabalin and the placebo groups. However, the pregabalin group showed no significant improvement in steering-wheel skills with training, whereas the placebo group showed a significant ($P<0.05$) improvement with training. In this study using a driving simulator, pregabalin did not impair driving performance but mildly reduced the training effects of driving experiments. Although pregabalin caused sleepiness, it had no severe effect on driving ability after a second dose of 75 mg after the initial introduction of pregabalin.

Keywords: pregabalin, driving, volunteers

Introduction

Pregabalin, an analog of GABA (γ-aminobutyric acid), is an anticonvulsant drug used in the treatment of partial epilepsy. Similar to its parent drug, gabapentin, pregabalin binds to the $\alpha_2\delta$ subunit of Ca^{2+} channels of presynaptic neurons.[1] This binding reduces the release of neurotransmitters including glutamate, noradrenaline, and substance P, which results in anticonvulsant, anxiolytic, and analgesic effects.[2]

Pregabalin was effective as an add-on therapy for seizure reduction in patients with partial epilepsy with or without secondary generalization in a randomized, double-blind, placebo-controlled trial.[3] It was well tolerated, with adverse effects generally reported to be mild-to-moderate somnolence, dizziness, ataxia, diplopia, and weight gain.[3] Pregabalin has been shown to ameliorate the symptoms of diabetic peripheral neuropathic pain and postherpetic neuralgia.[4] The sleep quality of these patients was also improved.[4] A clinical trial of pregabalin has shown a significant improvement in anxiety scores in patients with generalized anxiety disorder.[5] Pregabalin has been studied in patients with neuropathic pain due to spinal cord injury in a randomized, controlled trial.[6] There was a significant improvement in endpoint mean pain scores and assessments of sleep. However, dizziness and somnolence were reported as the most frequent adverse effects.

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Although pregabalin is well applied as an effective medication in various indications, the common side effects of drowsiness and dizziness have given rise to questions with respect to safety during driving and handling machinery while the patient is on medication. In this study, we evaluated the effect of pregabalin on the driving performance of healthy volunteers by using a driving simulator to provide more-detailed prescribing information for patients.

Materials and methods

The study was approved by the Ethical Committee of Ehime University Hospital. Written informed consent was obtained from the volunteers prior to participation in the study, which was performed in accordance with the principles of the Declaration of Helsinki.

Sixteen healthy male volunteers licensed to drive a motor vehicle and with regular driving experience and normal vision were enrolled in this randomized, double-blind, parallel-group, placebo-controlled study. A crossover design was not employed to exclude learning effects. Exclusion criteria consisted of abnormal complete blood count, infectious diseases, abnormalities on electrocardiogram and thoracic radiography, and use of any medication 72 hours prior to the study. The volunteers received a tablet of either pregabalin 75 mg (Lyrica®, Pfizer, Inc, New York, NY, USA) or matching placebo (lactose) on two occasions separated by a 12 hour interval while fasting, ie, a total pregabalin dose of 150 mg. This is because the study simulated normal clinical conditions for patients first starting to take pregabalin. Typically, a patient who is prescribed pregabalin by a physician will take a tablet of pregabalin after dinner and will drive his car the next morning after taking a second tablet of pregabalin after breakfast. Driving performance was evaluated using a driving simulator before taking the first dose, and 1 hour and 3 hours after taking the second dose. The rationale for driving assessment at these times was to study correlation with maximum plasma concentrations of pregabalin. Plasma pregabalin concentration, serum creatinine, and estimated glomerular filtration rate (eGFR) were also measured during the study. Physical examination, including vital signs and electrocardiogram tests, were performed before and after the study to detect any side effects of treatment.

Driving simulator

The volunteers underwent a driving test using a driving simulator (Safety Master NT-932; Niigata Tsushinki Co, Ltd, Niigata, Japan) (Figure 1), which has been adopted officially at drivers' license centers in Japan. They were examined on their coping responses to a complex of motor ability and perception tasks, using simple reaction time responding to red traffic signals, complicated reaction time responding to variable signals, simple steering-wheel technique responding to variable signals, and steering-wheel technique complicated by braking responding to variable signals. They were required to handle a steering-wheel to avoid collision with a wall and to respond to the change in color of signals on the display by pressing and releasing the accelerator or brake pedal as necessary. The total duration of the test is about 20 minutes and the total scores of the safety driving test are rated on a 5-point scale (1= very poor and unsafe, 2= moderately poor and unsafe, 3= marginal, 4= moderately safe, and 5= safe driving status) based on the mean result of test items in the driving performance test.

Figure 1 Driving simulator (Safety Master-NT 932).

Simple steering-wheel test

In the simple steering-wheel test, the screen displays horizontal bars on the left and right sides of the road. The subjects are required to avoid collision with the bars on both sides of the road. The mean numbers of steering-wheel errors on both sides was evaluated.

Complicated steering-wheel test

This test assesses the combination of complicated reaction times and simple steering-wheel tests. The screen of the driving simulator displays bars on both sides of the road, and red, yellow, or green traffic lights on either side of the road. The subjects respond to changes in variable signals by pressing or releasing the pedals, as in the test of complicated reaction time, while trying to not collide with the bars. The mean number of steering-wheel errors on both sides of the road was also evaluated.

Simple reaction time

This test evaluates the mean reaction time taken to release the accelerator when the red traffic light is displayed seven times on the screen of the driving simulator. A black traffic signal is displayed throughout the test as an indicator to continue pressing the accelerator. When the black signal is changed to red, the participants are required to release the accelerator and press the brake pedal.

Complicated reaction time

Individual reaction time taken in responding to variable signals of red, yellow, and green lights was tested in the driving simulator. The participants need to press the accelerator in response to a black light throughout the test and respond to a red light as in the test of simple reaction time. The accelerator needs to be released when the black light changes to yellow and pressed in response to the light's changing to green. The mean of ten traffic light changes is calculated by software in the driving simulator and displayed on screen.

Statistical evaluation

We evaluated the effect of the pregabalin treatment on driving performance compared to the placebo control group. The effect of training on the driving performance was also assessed in relation to baseline results recorded before taking the first dose. The Mann–Whitney *U*-test was applied to study the level of significance, with *P*-values <0.05 considered as statistically significant. Results are expressed as mean values ± standard deviation. Bonferroni's correction for multiplicity was applied.

Results

Baseline characteristics were similar for the pregabalin and placebo groups, respectively: age (26.0±2.9 years versus 28.6±3.5 years), body weight (69.0±4.7 kg versus 62.2±4.6 kg), serum creatinine (0.8±0.04 mg/dL versus 0.8±0.05 mg/dL), and eGFR (94.1±5.7 mL/minute versus 96.7±6.5 mL/minute). Maximum plasma pregabalin concentration was reached at 1 hour in six subjects and at 2 hours in two subjects (Table 1).

Six subjects in the pregabalin group (100%) and two in the placebo group (33.3%) reported feeling sleepy. Two subjects of the pregabalin group presented somnolence. None of the subjects in either group had severe adverse effects.

There was no significant difference in either simple or complicated braking reaction time between the pregabalin and placebo groups (Figure 2A and B). Simple and

Table 1 Individual plasma pregabalin concentrations at baseline (prior to first dose) and after the second dose

Subject number	Plasma pregabalin concentration (µg/mL)				
	Baseline	1 hour	2 hours	3 hours	4 hours
1	1.05	1.83	4.32	3.39	3.96
2	0.89	4.54	3.52	3.39	3.10
3	0.99	3.85	4.06	3.21	3.06
4	1.30	4.78	4.37	3.10	3.70
5	1.22	5.47	3.53	3.79	3.77
6	1.32	4.60	3.94	3.77	3.23
7	1.11	5.78	3.71	3.19	3.01
8	0.86	4.38	3.87	3.20	3.39

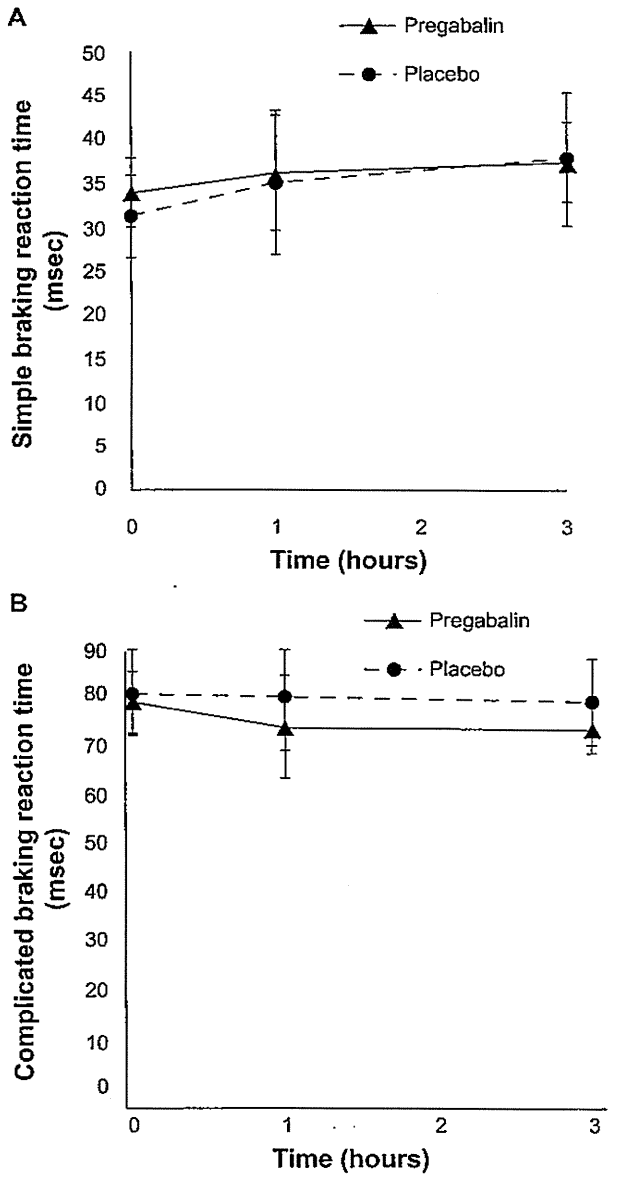

Figure 2 Effect of pregabalin on braking reaction time.
Notes: (A) Simple braking test: reaction time in response to a red light. **(B)** Complicated braking test: reaction time in response to variable signals. Results from both tests were unaffected by either pregabalin or placebo compared to baseline. There were also no significant changes in both simple and complicated braking reaction time between the pregabalin and placebo groups.

complicated steering-wheel techniques also showed no significant differences between the pregabalin and placebo groups (Figure 3A and B). When comparing the effect of training on driving performance, the placebo group showed an improvement in simple steering-wheel technique (Table 2; Figure 3A): the number of errors in steering-wheel handling was significantly reduced at 1 hour and 3 hours compared to baseline. However, the pregabalin group showed no improvement in steering-wheel handling with training. There were no differences between the groups or changes compared with baseline in simple and complicated braking reaction times, complicated steering-wheel handling tests, or total safety score comparing pregabalin and placebo (Figure 4).

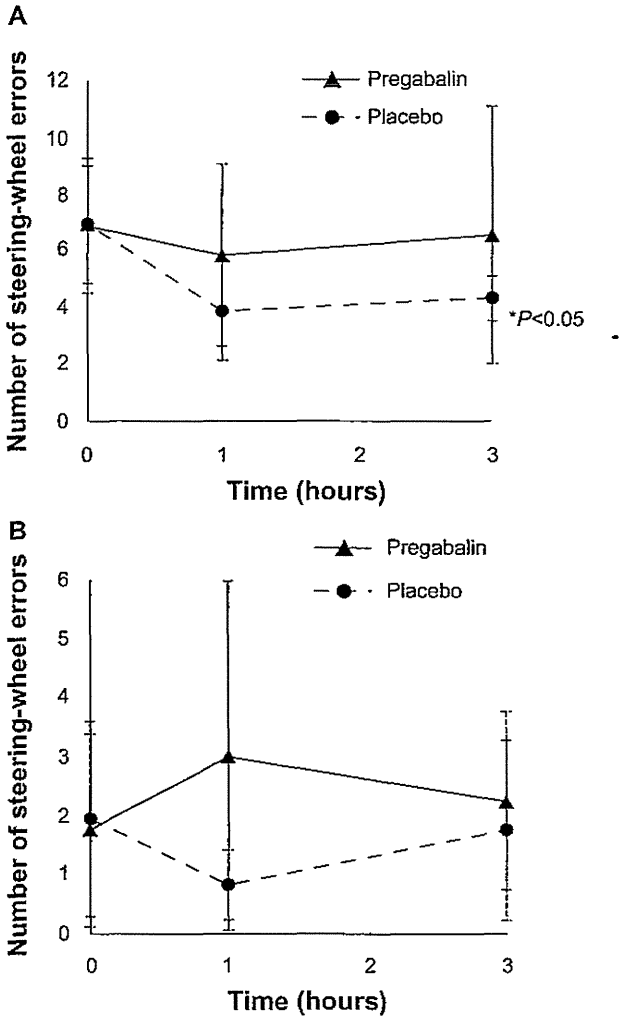

Figure 3 Effect of pregabalin on steering-wheel technique.
Notes: (**A**) Simple steering-wheel test: The number of simple steering-wheel errors evaluated at 1 and 3 hours was significantly ($P<0.05$) decreased compared to baseline in the placebo group, whereas the pregabalin group showed no significant change in the number of simple steering-wheel errors. (**B**) Complicated steering-wheel test: Both the pregabalin and the placebo groups exhibited no changes in complicated steering-wheel technique under variable signals.

Table 2 Effect of training on driving performance

Driving simulator test	*P*-value*	
	Baseline versus 1 hour	Baseline versus 3 hours
Pregabalin group (n=8)		
Simple braking reaction time	0.79	0.49
Complicated braking reaction time	0.67	0.71
Number of errors in simple steering-wheel test	0.22	0.42
Number of errors in complicated steering-wheel test	0.42	0.34
Placebo group (n=8)		
Simple braking reaction time	0.23	0.42
Complicated braking reaction time	0.56	0.40
Number of errors in simple steering-wheel test	0.01†	0.01†
Number of errors in complicated steering-wheel test	0.10	0.91

Notes: *Comparing baseline prior to first dose versus 1 hour or 3 hours after the second dose; †according to Bonferroni's correction, $P=0.01$ was estimated to be $P=0.04$.
Abbreviation: n, number.

Discussion

Central nervous system-depressant drugs increase the risk of traffic accidents[7] and are assumed to trigger acute impairment during driving.[8] Benzodiazepines, which are commonly prescribed antidepressant, anticonvulsant, and anesthetic drugs, have been particularly studied for their effect on collisions and driving performance. Benzodiazepines act at the level of the benzodiazepine receptor and enhance the effect of GABA, which causes somnolence in some individuals.[7]

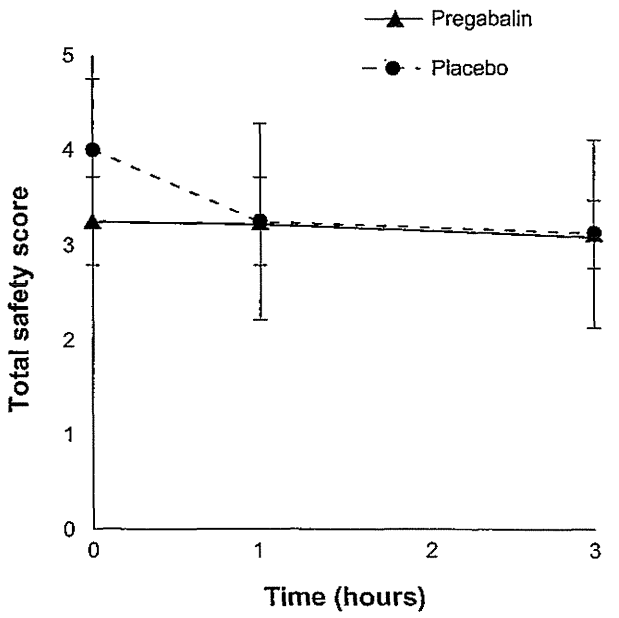

Figure 4 Effect of pregabalin on total safety test scores.
Note: Neither the pregabalin group nor the placebo group showed significant correlation with total safety test scores.

An epidemiological study has reported that collision risk is associated with long-acting but not short-acting benzodiazepines.[9] On-road crossover studies assessing the impact of benzodiazepines on driving among healthy subjects[10] or anxious patients[11] have also reported no impact on braking reaction time. A review study of real-life driving presented four cases with a positive relationship between plasma gabapentin concentration (2.0–15.5 mg/L) and impaired driving ability.[12] These subjects were reported for improper use of turn signal, slow speeds, and deviation from regular driving and braking.

Epidemiological studies of driving in the elderly receiving H_1-antihistamines have shown no significant association with car crashes.[13,14] In contrast, a study conducted among professional drivers reported an increase risk of car crashes with the prescription of antihistamines.[15] Drinking coffee is assumed to counteract the effect of sleepiness during driving. A simulated highway driving study proved that caffeinated coffee had a positive effect on driving performance and subjective sleepiness, compared to decaffeinated coffee.[16]

Patients with Parkinson's disease (PD) have been reported to have impaired driving performance.[17] Compared to healthy controls, PD patients made more safety errors both under baseline conditions and during distraction.[18] Motor assessments alone may not be enough to predict driving performance in PD patients. In a previous study of driving in PD patients using a driving simulator,[19] we found that patients with more impairment made more steering errors but showed no impairment of braking reaction time.[19]

In the present study, the use of pregabalin was not associated with braking reaction time, although some participants complained of sleepiness during the test. The sample size in this study was limited because of the number of available driving simulators and the time needed to perform the driving test, so further studies are needed. This finding is, however, compatible with other findings on the effect of pregabalin on braking reaction time. In a double-blind study among healthy volunteers,[20] pregabalin did not significantly impair braking reaction time compared to placebo, whereas a positive control with alprazolam caused significant impairment of breaking reaction time. However, pregabalin showed an influence on driving performance in our study, as steering-wheel handling skills were not improved with training whereas the placebo group showed a significant improvement of handling skills with training. Compared to braking reaction time, steering-wheel handling skills seem to be more sensitive to the effect of drugs. In our study, a dose of pregabalin 75 mg was used because we wanted to study the actual outpatient clinical condition – a dose of 75 mg is the recommended dose at the start of treatment. Our study might have some limitations due to the involvement of only young male participants and not including the highest approved dose of pregabalin. The driving simulator provides a comprehensive evaluation of driving, including perception or motor performance. Another recent study of pregabalin did not reveal any interference with psychomotor testing.[21] Further studies are needed to clarify the effects of pregabalin on driving.

In conclusion, pregabalin showed no severe central nervous system effects related to cognitive measures during simulated driving testing in healthy subjects. Because pregabalin may cause somnolence, on first prescription of the drug we advise patients to be careful when driving, rather than advising them to avoid driving a car totally.

Acknowledgments

The work was supported by Grants-in-Aid from the Research Committee for Parkinson's Disease, the Ministry of Health, Labour and Welfare of Japan, and SRFJ; and a Grant from Ehime University. The authors would like to express their thanks to all the clinical research coordinators of the Clinical Research Trial Centre of Ehime University Hospital.

Disclosure

The authors report no conflicts of interest in this work.

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