Effects of Cerebrospinal Fluid From Patients With Parkinson Disease on Dopaminergic Cells

Wei-Dong Le, MD, PhD; Dominic B. Rowe, MBBS, FRACP; Joseph Jankovic, MD; Wenjie Xie, MD; Stanley H. Appel, MD

Background: The pathogenesis of substantia nigra pars compacta neuronal injury in Parkinson disease (PD) remains unknown. Cerebrospinal fluid (CSF) has been reported to contain factors toxic to dopaminergic neurons. Objectives: To determine whether the cytotoxic effects of CSF of PD patients are specific for dopaminergic neurons, dependent on prior levodopa therapy, and mediated by the cytokine tumor necrosis factor α (TNF-α).

Design: Specimens of CSF were evaluated in dopaminergic (MES 23.5) and nondopaminergic (N18TG2) cell lines for cytotoxicity by viability assay and by the inhibition of tyrosine hydroxylase. After specificity and time and dose response were established, CSF specimens were assayed in a blinded manner. The TNF-α levels in CSF were determined by enzyme-linked immunosorbent assay. The toxicity of TNF-α in MES 23.5 cells was determined.

Setting: A university-based research facility.

Subjects: There were 4 groups of subjects: normal control subjects (n = 10), control subjects with neurologic disease (n = 8), PD patients treated with levodopa (n = 10), and untreated subjects with PD (n = 20).

Results: Specimens of CSF from 15 (50%) of 30 PD patients and 2 (11%) of 18 control subjects were cytotoxic to dopaminergic MES 23.5 cells and were nontoxic to the parental cell line N18TG2. There was no correlation between the degree of PD CSF cytotoxicity, levodopa therapy, or the severity and duration of PD. Terminal deoxynucleotidyl transferase-mediated biotin-deoxyuridine triphosphate nick-end labeling (TUNEL) for DNA fragmentation suggested the involvement of apoptotic mechanisms. The inhibition of tyrosine hydroxylase was an early effect of cell injury by PD CSF and correlated with the viability assay. The mean TNF-α level was 2.6-fold higher in CSF specimens from PD patients than in those of controls. The addition of recombinant human TNF-α equivalent to the highest level determined in PD CSF was not cytotoxic to MES 23.5 cultures.

Conclusions: Blinded CSF specimens from PD patients, regardless of therapy, contain factors that cause specific dopaminergic neuronal cell injury. These factors are present in a substantial proportion of CSF specimens from patients with early PD, before the institution of medical therapy. Levels of TNF-α are elevated in the CSF of PD patients, but TNF-α is not responsible for the cytotoxicity.

Arch Neurol. 1999;56:194-200

Parkinson disease (PD) is a degenerative disorder of the central nervous system characterized pathologically by the loss of dopaminergic neurons in the substantia nigra (SN) pars compacta and other neuronal populations. The causes of sporadic PD remain unknown, and the reasons for the susceptibility of mesencephalic dopaminergic neurons also remain unknown. Following the description of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine–induced parkinsonism, several studies have examined the role of endogenous and exogenous cytotoxic factors in neuronal injury. Carvey et al and Klawans first demonstrated that CSF specimens from PD patients were cytotoxic to dopaminergic neurons in primary mesencephalic cell culture. Carvey et al published an abstract that suggested that prolonged stereotactic infusion of PD CSF into the SN area in rats produced controversial posturing and amphetamine-induced rotational behavior, indicative of severe nigral damage. The question remains as to what factors in PD CSF are responsible for dopaminergic neuronal cytotoxic effects. Antibodies to dopaminergic neurons were found in the CSF of some PD patients and were postulated to be involved in the cytotoxicity to dopaminergic neurons. Cytokine levels, including those of tumor necrosis factor α (TNF-α) and interleukin 1β, are elevated in PD CSF, and cytokines might be involved in neuronal damage.
SUBJECTS AND METHODS

PATIENT DATA AND CSF COLLECTION

A total of 48 CSF specimens from patients with PD and age-matched control subjects were obtained through the Neurology Clinic, Baylor College of Medicine, Houston, Tex, and the CSF Bank of the Parkinson Study Group, Rochester, NY. Patients with PD (n = 30) included 20 untreated patients and 10 being treated with levodopa. The untreated PD patients either never received levodopa or dopamine-receptor agonists or had not received therapy for at least 3 months before a lumbar puncture. Age-matched control subjects (n = 18) included 10 normal subjects and 8 patients with neurologic disease (ND): amyotrophic lateral sclerosis (n = 2), Alzheimer disease (n = 2), peripheral neuropathy (n = 1), Guillain-Barré syndrome (n = 1), stroke (n = 1), and compressive cervical myelopathy (n = 1). The clinical diagnosis of each patient was established on the basis of history, physical examination findings, and appropriate investigations. Neuropathological confirmation of the clinical diagnosis was not available for any patient. For each subject, 4- to 5-mL specimens of CSF were obtained by lumbar puncture after informed consent and approval from the appropriate institutional ethics committee. The specimens were aliquoted, coded, and stored at −80°C until assayed. The ages (±SD) were similar between the groups (untreated PD patients, 63.6 ± 9.8 years; levodopa-treated PD patients, 67.5 ± 6.9 years; normal subjects, 56.9 ± 11.0 years; and ND controls, 58.9 ± 11.0 years; P < .05). Clinical severity, as assessed by the UPDRS (total score of subscales I, II, and III: range, 0-134),13 was similar between the PD groups (untreated patients, 32.4 ± 9.6; levodopa-treated patients, 37.6 ± 9.2; P = .65), although the average disease duration was shorter in the untreated group (1.6 ± 0.8 years vs 3.1 ± 1.3 years; P = .006). The 10 levodopa-treated PD patients all received a levodopa-carbidopa combination product with daily dose ranges of 300/75 mg to 600/150 mg, and 3 of these patients also received selegeline hydrochloride (5-10 mg/d).

CELL CULTURE AND ASSAYS

OF CSF CYTOTOXICITY

The hybrid dopaminergic cell line MES 23.5 was previously developed from the nondopaminergic cell line N18TG2 in our laboratory.13,14 Both cell lines were grown on polyornithine-coated T-75 flasks (Corning Inc, Corn ing, NY) in the Dulbecco modified Eagle medium (Life Technologies Inc, Gaithersburg, Md), which contains Sato components (Sigma Immunochemicals, St Louis, Mo), and 2% heat-inactivated newborn calf serum (Hyclone Laboratories Inc, Logan, Utah) at 37°C in a 95% air–5% carbon dioxide humidified incubator. When 50% confluent, the cells were either transferred to new T-75 flasks or plated (10⁴ cells/cm²) onto polyornithine-coated 96-well plates for cell counting or 24-well plates with glass coverslips for fluorescence viability assays. Cells were differentiated in serum-free defined Dulbecco modified Eagle medium with 1 mmol/L cyclic adenosine monophosphate (Sigma Immunochemicals) for 2 to 3 days before CSF treatment. All CSF specimens were incubated (1-5:1-50 dilutions) in a blinded manner with the differentiated MES 23.5 cells for 3 days (cell viability assay) or 2 days (TH-inhibition assay) in triplicate. Cytotoxicity was assessed by cell structure and cell viability staining.13,16 The MES 23.5 and N18TG2 cells have smooth round cell bodies with multiple processes on phase-contrast microscopy. After PD CSF treatment, some of the MES 23.5 cells appeared to have round or irregularly shaped cell bodies with retracted and fragmented processes, with a few cells notably shrunken. Cell viability staining was performed with fluorescein diacetate (Molecular Probes, Inc, Eugene, Ore), 37 µmol/L, and propidium iodide (Sigma Immunochemicals), 10 µmol/L. Cell survival was calculated as a percentage of the ratio of viable cells to total cell number, with results expressed as the percentage of viable cells. Nuclear DNA fragmentation in cells was visualized by the terminal deoxynucleotidyl transferase–mediated biotin-doxuryridine triphosphate nick-end labeling (TUNEL) method of in situ end labeling.17 Briefly, the treated MES 23.5 cells were fixed in 2% paraformaldehyde for 15 minutes, washed in phosphate-buffered sodium and distilled water, and then incubated at 37°C for 60 minutes with biotinylated deoxyuridine triphosphate (Oncoz Inc, Gaithersburg). After the addition of avidin-peroxidase, 3-3′ diaminobenzidine staining was assessed by light microscopy.

Tyrosine hydroxylase was measured by the coupled nonenzymatic dopa-decarboxylation method of Bostwick and Le.18 Briefly, 25-µL aliquots of buffer containing tyrosine labeled with radioactive carbon (¹⁴C) (NEN Life Science Products, Boston, Mass; 48.6 mCi/mmol) and cofactors were incubated with microwell-cultured cells for 20 minutes at 37°C. The newly synthesized [¹⁴C]-dopa was decarboxylated by the addition of potassium ferrocyanide (Sigma Immunochemicals), 30 mmol/L, and heated for 30 minutes at 55°C. The released radioactive carbon dioxide was absorbed on filter paper impregnated with hyamine hydroxide (Sigma Immunochemicals) and quantified by scintillation counting (RackBeta 1214; LKB Wallac, Turku, Finland).

TNF-α ELISA AND TNF-α CYTOTOXICITY

The TNF-α levels in CSF were measured by ELISA (R&D Systems Inc, Minneapolis, Minn) as described previously.19 The coefficients of variation for intra-assays and interassays were 5% and 9%, respectively. Each specimen of CSF was measured in duplicate in 3 separate experiments. Recombinant human TNF-α (Pharmingen, San Diego, Calif) was incubated (range, 10 000-20 000 pg/mL) with MES 23.5 cell cultures plated at a density of 10⁴ per well. Three days after the incubation with TNF-α, the MES 23.5 cells were assessed for cytotoxicity as described above.

STATISTICAL ANALYSIS

All analyses were performed with commercially available software (Statview, version 4.5, Abacus Concepts, Berkeley, Calif). The Mann-Whitney U test was used in the analysis of clinical data. The Student t test was used for the analysis of experimental data. For further nonparametric data analysis, we used the Kruskall-Wallis test, Mann-Whitney U test, and the calculation of Spearman rank correlation coefficients. Statistical significance was regarded as P < .05.
In addition, Hao et al\textsuperscript{12} reported that unidentified low-molecular-weight components in PD CSF inhibit the development of mesencephalic neurons in vitro. Whether the cytotoxic effects of PD CSF are specific for dopaminergic neurons, these factors are related to levodopa therapy, or cytokines play a role in CSF cytotoxicity is not yet known.

In this study, we assessed the cytotoxicity of blinded CSF specimens from PD patients (both untreated and levodopa treated) and from control subjects in the hybrid dopaminergic mesencephalic cell line MES 23.5\textsuperscript{13} and the nondopaminergic parental cell line N18TG2 using 2 independent methods. Fifteen (50\%) of 30 CSF specimens from PD patients have moderate but significant cytotoxic effects on MES 23.5 cells compared with 2 (11\%) of 18 control CSF specimens. Cytotoxicity was selective for the dopaminergic cell line, having no effect on the nondopaminergic parental cell line N18TG2. Cytotoxicity was demonstrated in 7 (70\%) of 10 levodopa-treated and 8 (40\%) of 20 untreated PD patients. Although there was a trend for the degree of cytotoxicity to be greater in specimens from patients on levodopa therapy, this was not statistically significant. Furthermore, cytotoxicity was not related to either duration or severity of PD as determined by the Unified Parkinson’s Disease Rating Scale (UPDRS).\textsuperscript{14} DNA fragmentation was a prominent feature, which possibly implicates apoptotic mechanisms of cell injury. The inhibition of MES 23.5 cell tyrosine hydroxylase (TH) by CSF produced concordant results to the cell viability assay. The levels of TNF-\(\alpha\) were significantly higher in CSF specimens from PD patients than from control subjects in an enzyme-linked immunosorbent assay (ELISA). In toxicity studies with MES 23.5 cells, however, TNF-\(\alpha\) alone did not account for the observed cytotoxic effect of PD CSF.

**RESULTS**

**PD CSF CYTOTOXICITY**

To determine the time- and dose-dependent cytotoxic effects of PD CSF, 5 CSF specimens (3 from untreated PD patients, and 2 from levodopa-treated PD patients) were incubated with MES 23.5 cells at dilutions of 1:5 to 1:50 for 1 to 3 days (Figure 1). Cytotoxicity was assessed blindly by determining cell morphologic changes and cell viability. The cytotoxicity of PD CSF was time and dose dependent (Figure 2). Specimens of CSF (dilution range, 1:5-1:50) showed little cytotoxic effect by 1 day of incubation, but an effect was noticeable in 2 days’ incubation and was substantial after 3 days. Typical morphologic changes on phase-contrast microscopy were the retraction of neurites and shrinkage of the cell body (Figure 1, B and C). Higher concentrations of PD CSF increased the amount of cell loss (Figure 1, C). Ten CSF specimens (1:5 dilution) from levodopa-treated PD patients and 8 CSF specimens from ND control subjects demonstrated no notable cytotoxicity in N18TG2 cell cultures after 3 days (data not shown).
To examine the specificity of CSF, 48 specimens were subsequently examined in a blinded manner. Following incubation with differentiated MES 23.5 cells at a dilution of 1:5 for 3 days, cell viability was estimated using fluorescein diacetate–propidium iodide staining. Of the 30 PD CSF specimens, 8 (40%) of 20 from untreated PD patients and 7 (70%) of 10 from levodopa-treated PD patients produced significant cytotoxicity, defined as cell viability below 70% of control (Figure 3). Of the control specimens, 1 (10%) of 10 from normal subjects and 1 (a patient with Guillain-Barré syndrome) (13%) of 8 from ND controls also had a significant reduction of cell viability. There was a significant difference between PD patients (treated plus untreated) and control subjects (normal subjects plus those with ND) (P = .006; Kruskal-Wallis test). Separate comparisons of the untreated PD patients (P = .02) and levodopa-treated PD patients (P = .01) with control subjects were also statistically significant. Specimens of CSF from levodopa-treated PD patients demonstrated a trend for greater cytotoxic effect than those from untreated PD patients, although the difference between these groups was not significant (P = .08).

To correlate the PD CSF cytotoxicity in MES 23.5 cell cultures with the patient’s clinical profile, a Spearman correlation analysis was performed, and no significant correlation was found between the cytotoxicity of CSF and either UPDRS scores (P = .11) or disease duration (P = .35).

To explore the possible involvement of apoptotic mechanisms in the CSF-induced cell injury, MES 23.5 cells were incubated with 3 PD CSF specimens known to be cytotoxic and then stained for DNA fragmentation with the TUNEL technique. A typical pattern that included nuclear chromatin condensation and nuclear fragmentation was apparent in 21% to 40% of MES 23.5 cells after 2 days’ incubation with PD CSF at a 1:5 dilution, suggesting the involvement of apoptotic mechanisms (Figure 4).

**PD CSF INHIBITS TH ACTIVITY**

To determine whether a decrease in TH activity is an early indicator of cell injury following treatment with PD CSF, specimens were incubated with differentiated MES 23.5 cells for 2 days and then assayed for TH activity. Ten (30%) of 20 specimens from untreated PD patients and 6 (60%) of 10 specimens from levodopa-treated PD patients significantly inhibited TH activity (Figure 5). Of the control specimens, those from 1 (10%) of 10 normal subjects and 1 (13%) of 8 ND control subjects inhibited TH activity (PD patients vs controls, P = .001). Subgroup analysis identified significant differences for both untreated PD patients vs controls (P = .008) and levodopa-treated PD patients vs controls (P = .003). Although there was a trend for greater inhibition of TH from CSF specimens of levodopa-treated PD patients compared with untreated PD patients, the difference failed to achieve significance (P = .12). When the 2 assay systems were directly compared, the same CSF specimens (except 1 from a PD patient) that inhibited TH were also cytotoxic for MES 23.5 cells (Spearman ρ = 0.771; P < .001).

**TNF-α LEVELS ARE ELEVATED IN THE CSF OF PD PATIENTS**

To measure TNF-α levels, the CSF specimens were examined by ELISA (Table). The mean (±SD) level of TNF-α in PD CSF specimens (102 ± 26 pg/mL) was 3.4-fold higher than that of normal subjects (30.5 ± 12.2 pg/mL) (P < .001) and 2.1-fold higher than that of ND controls (49.0 ± 25.0 pg/mL) (P < .01). Again, there was a trend for TNF-α levels to be higher in levodopa-treated PD patients than in untreated PD patients, although this was not significant (P = .10). The TNF-α levels did not correlate with either cell viability or TH inhibition. To explore whether TNF-α is involved in cytotoxicity, MES 23.5 cells were treated with recombinant human TNF-α for 3 days and viability assays performed. Only concentrations greater than 1 ng/mL, 4-fold greater than the highest level of TNF-α measured in this series, produced identifiable cytotoxic effects in MES 23.5 cells (Figure 6).

**COMMENT**

The hybrid MES 23.5 cell line, developed by fusing rat embryonic mesencephalon cells with murine N18TG2 neuroblastoma cells, has been used to study the molecular mechanisms potentially involved in the degeneration of dopaminergic neurons in PD.34 This cell line displays many properties of developing neurons of the SN13 and offers several advantages, including greater homogeneity than primary cultures and susceptibility to both free radical–mediated cytotoxicity and calcium-dependent cell death.35,16 For these reasons, MES 23.5 cells were used in the assay systems.
Cerebrospinal fluid from PD patients was cytotoxic to dopaminergic cells in a time- and dose-dependent manner. The CSF-mediated cytotoxicity was selective because the nondopaminergic parental cell line N18TG2 did not exhibit cytotoxicity. Although the cytotoxic effect varied among specimens, 50% of the CSF specimens from PD patients showed significant toxicity on MES 23.5 cells, with only 1 CSF specimen from each control group demonstrating such an effect. Follow-up examination of these 2 control subjects will be performed to determine whether they represent preclinical PD. Alternatively, several toxic factors (eg, 4-hydroxynonenal) have been identified in CSF from patients with other neurologic diseases that might be responsible for this observation.19,20 Cerebrospinal fluid specimens from PD patients also produced significant inhibition of TH activity in the culture systems with a shorter incubation time, suggesting that TH inhibition may be an earlier indicator of cell injury. The degree of cytotoxicity or TH inhibition did not correlate with UPDRS scores or the duration of PD. However, CSF specimens from untreated PD patients significantly impaired cell viability (40%) and TH (50%). Although the molecular mechanisms by which dopaminergic cells were damaged are unclear from this study, TUNEL staining demonstrated nuclear chromatin condensation and nuclear fragmentation in some cells treated with CSF from 3 PD patients at a dilution of 1:5 for 2 days, suggesting that apoptotic mechanisms may be involved (Figure 4). This is the first blinded study suggesting that factors that could contribute to cell injury are present in the CSF of untreated PD patients.

Cerebrospinal fluid from PD patients was cytotoxic to dopaminergic cells in a time- and dose-dependent manner. The CSF-mediated cytotoxicity was selective because the nondopaminergic parental cell line N18TG2 did not exhibit cytotoxicity. Although the cytotoxic effect varied among specimens, 50% of the CSF specimens from PD patients showed significant toxicity on MES 23.5 cells, with only 1 CSF specimen from each control group demonstrating such an effect. Follow-up examination of these 2 control subjects will be performed to determine whether they represent preclinical PD. Alternatively, several toxic factors (eg, 4-hydroxynonenal) have been identified in CSF from patients with other neurologic diseases that might be responsible for this observation.19,20 Cerebrospinal fluid specimens from PD patients also produced significant inhibition of TH activity in the culture systems with a shorter incubation time, suggesting that TH inhibition may be an earlier indicator of cell injury. The degree of cytotoxicity or TH inhibition did not correlate with UPDRS scores or the duration of PD. However, CSF specimens from untreated PD patients significantly impaired cell viability (40%) and TH (50%). Although the molecular mechanisms by which dopaminergic cells were damaged are unclear from this study, TUNEL staining demonstrated nuclear chromatin condensation and nuclear fragmentation in some cells treated with CSF from 3 PD patients at a dilution of 1:5 for 2 days, suggesting that apoptotic mechanisms may be involved (Figure 4). This is the first blinded study suggesting that factors that could contribute to cell injury are present in the CSF of untreated PD patients.

Cerebrospinal Fluid Tumor Necrosis Factor α (TNF-α) Levels

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Subjects</th>
<th>TNF-α Level, pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated patients with PD</td>
<td>20</td>
<td>82.0 ± 28.8†‡§</td>
</tr>
<tr>
<td>Levodopa-treated patients</td>
<td>10</td>
<td>121.0 ± 62.0¶#</td>
</tr>
<tr>
<td>with PD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal subjects</td>
<td>10</td>
<td>30.5 ± 12.2</td>
</tr>
<tr>
<td>ND controls</td>
<td>8</td>
<td>49.0 ± 25.0</td>
</tr>
</tbody>
</table>

*Values expressed as mean ± SD, determined in duplicate in 3 experiments using TNF-α enzyme-linked immunosorbent assay. PD indicates Parkinson disease; ND, neurologic disease.
†P < .001 compared with normal subjects.
‡P < .005 compared with ND controls.
§P < .005 compared with normal subjects plus ND controls.
¶P < .01 compared with normal subjects plus ND controls.
#P < .01 compared with ND controls.
The nature of the cytotoxic factors in the CSF of PD patients is unclear, and these factors are not solely the result of levodopa therapy. Although there was a trend for greater cytotoxic effects of CSF from levodopatreated PD patients than from untreated patients, the difference between the 2 groups was not significant. Therefore, levodopa alone is unlikely to be responsible for cytotoxicity. Also, the concentrations of levodopa and dopamine in CSF specimens were too low—usually less than 100 pmol/L—to produce cytotoxicity in this cell line (data not shown). Low-molecular-weight metabolites of levodopa and dopamine, however, could produce additional cell injury. A study by Hao et al suggested that levodopa metabolites might contribute to CSF cytotoxicity. N-methyl-(R)salsolinol, a dopamine metabolite found in CSF, is cytotoxic in vitro and in vivo. The lipid peroxidation product, 4-hydroxynonenal, was demonstrated in the CSF of patients with Alzheimer disease and amyotrophic lateral sclerosis. This highly toxic compound forms adducts with protein, which were immunohistochemically demonstrated in the SN of patients with PD. Therefore, 4-hydroxynonenal is likely also in the CSF of a proportion of patients with PD.

Cytokines may play a role in CSF-mediated cytotoxicity. In the present study, TNF-α levels in PD CSF specimens were markedly elevated compared with those of age-matched normal subjects and ND controls (Table), consistent with other reports. Tumor necrosis factor α has been implicated in the apoptosis of neurons. Furthermore, TNF-α-positive glia have been detected in the SN of PD patients but not in that of control subjects. It is unlikely that TNF-α alone is responsible for dopaminergic cell injury because the concentrations of recombinant human TNF-α required to induce cytotoxicity of cultured MES 23.5 cells were 4-fold above the highest level documented in PD CSF in this series (Figure 6). Because TNF-α levels are probably the result of a concentration gradient, the key questions are whether the concentration of TNF-α at the surface of SN pars compacta neurons is much higher and whether this is sufficient to induce neuronal injury. In addition, no correlation was found between TNF-α levels and either cytotoxicity or TH inhibition.

Other factors in PD CSF such as immunoglobulins could also contribute to dopaminergic cell injury. Studies from our laboratory have documented that selective nigral damage can be induced by immunization in guinea pigs, either with bovine mesencephalic tissue or with differentiated MES 23.5 cells. Stereotactic microinjection of immunoglobulins from guinea pigs immunized with MES 23.5 cells or from PD patients can produce substantial and specific nigral damage in rats.

This study has extended previous observations that cytotoxic factors are present in the CSF of PD patients. Unlike previous studies, this study was performed blinded and included specimens from a large number of untreated PD patients. We conclude that factors that may injure dopaminergic neuronal cells are present early in the clinical course of PD and, therefore, may contribute to the pathogenesis of PD. Many factors are likely present in the CSF, including dopamine metabolites, toxic products of lipid peroxidation, immune components, cytokines, and other trophic factors. Additional studies will be needed to define the nature of these cytotoxic factors and their relevance to the pathogenesis of neuronal loss in PD.

Accepted for publication June 29, 1998.

This study was supported by a grant from the Research Foundation of the Methodist Hospital, by the Claude and Marie Hammill Foundation, and by a fellowship from the Huffington Center on Aging, Baylor College of Medicine (Dr Rowe), Houston, Tex.

The Parkinson Study Group, Rochester, NY, provided cerebrospinal fluid specimens and clinical data, and Charles F. Contant, MD, Baylor College of Medicine, assisted with statistical analysis.

Corresponding author: Wei-Dong Le, MD, PhD, Department of Neurology, Baylor College of Medicine, 6501 Fannin, Suite NB 302, Houston, TX 77030.

REFERENCES