PPP2R2B CAG Repeat Length in the Han Chinese in Taiwan: Association Analyses in Neurological and Psychiatric Disorders and Potential Functional Implications

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PPP2R2B, a protein widely expressed in neurons throughout the brain, regulates the protein phosphatase 2A (PP2A) activity for the microtubule-associated protein tau and other substrates. Altered PP2A activity has been implicated in spinocerebellar ataxia 12, Alzheimer’s disease (AD), and other tauopathies. Through a case-control study and a reporter assay, we investigated the association of PPP2R2B CAG repeat polymorphism with Taiwanese AD, essential tremor (ET), Parkinson’s disease (PD), and schizophrenia and clarified the functional implication of this polymorphism. The distribution of the alleles was not significantly different between patients and controls, with 68.6–76.1% alleles at lengths of 10, 13, and 16 triplets. No expanded alleles were detected in either group. However, the frequency of the individuals carrying the short 5-, 6-, and 7-triplet alleles was notably higher in patients with AD (5/180 [2.8%], Fisher’s exact test, P = 0.003; including 2 homozygotes) and ET (4/132 [3.0%], Fisher’s exact test, P < 0.001) than in the controls (1/625 [0.2%]). The PPP2R2B transcriptional activity was significantly lower in the luciferase reporter constructs containing the (CAG)5–7 allele than in those containing the common 10-, 13-, and 16-triplet alleles in both neuroblastoma and embryonic kidney cells. Therefore, our preliminary results suggest that the PPP2R2B gene CAG repeat polymorphism may be functional and may, in part, play a role in conferring susceptibility to AD and ET in Taiwan.

Key words: PPP2R2B; CAG repeat polymorphism; promoter assay; Alzheimer’s disease; essential tremor

INTRODUCTION

PPP2R2B (also known as Bβ), a regulatory B subunit of the heterotrimeric protein phosphatase 2A (PP2A) [Kamibayashi et al., 1994], is widely expressed in neurons throughout the brain [Mayer et al., 1991]. The regulatory B subunits regulate PP2A activity and cell specificity [Strack et al., 1998]. PP2A has been implicated in multiple cellular functions, including cell cycle regulation, tau dephosphorylation, and apoptosis [Virshup, 2000]. Recently, Holmes and colleagues reported a novel form of autosomal dominant ataxia—spinocerebellar ataxia 12 (SCA12)—caused by mutations in the PPP2R2B gene [Holmes et al., 2006]. The PPP2R2B gene is located on chromosome 18p11.21 and consists of 11 exons [Itoh et al., 1996]. The protein encoded by the PPP2R2B gene is composed of 440 amino acids and contains multiple protein phosphatase domains [Kamibayashi et al., 1994]. The PPP2R2B protein is found in neurons throughout the brain and has been implicated in various neurological disorders, including Alzheimer’s disease (AD), spinocerebellar ataxia 12 (SCA12), and essential tremor (ET) [Mayer et al., 1991; Strack et al., 1998].

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by expanded CAG repeats in the *PPP2R2B* gene [Holmes et al., 1999]. Repeat sizes ranged from 55 to 78 triplets in the mutant allele of the affected individuals and from 9 to 28 triplets in normal alleles [Holmes et al., 2001]. Subsequently, expanded repeats with 55–69 triplets were detected in six Indian families [Fujigasaki et al., 2001; Srivastava et al., 2001]. Moreover, SCA12 accounts for 16% (20/124) of all the autosomal dominant ataxia cases diagnosed in North India [Bahl et al., 2005]. The clinical presentations of SCA12 are variable. In addition to progressive cerebellar ataxia, it can manifest as upper limb tremor, dementia, psychiatric symptoms, and extrapyramidal features [Holmes et al., 1999, 2001; Fujigasaki et al., 2001; Srivastava et al., 2001]. The CAG repeats associated with SCA12 may lie upstream or within the 5′UTR region of the *PPP2R2B* transcript [Holmes et al., 1999, 2003]. Thus, it was postulated that the length of the CAG repeats alters the expression of the *PPP2R2B* gene and consequently affects the PP2A activity [Holmes et al., 2003].

Tau phosphorylation is regulated by PP2A and has been implicated to varying degrees in the pathogenesis of AD [Wang et al., 2001; Gong et al., 2003; Iqbal et al., 2005], PD [Frasier et al., 2005; Rodriguez-Navarro et al., 2007], and schizophrenia [Deutsch et al., 2006]. Therefore, given the proposal that repeat length may modulate *PPP2R2B* expression, it is worthwhile examining the *PPP2R2B* repeat length in these various diseases. The analysis is also important because of the overlap of the SCA12 phenotype with certain aspects of each AD, PD, schizophrenia, and essential tremor (ET). In this study, we examined the length of the CAG repeats in the *PPP2R2B* gene and its association with ethnic Chinese AD, ET, PD, and schizophrenia in Taiwan. Furthermore, the functional role of the CAG repeats was investigated using a dual luciferase reporter assay.

**MATERIALS AND METHODS**

**Subjects**

The study group comprised 180 sporadic AD patients (47.8% females, aged 73.1 ± 8.1 years), 132 ET patients (48.6% females, aged 63.4 ± 14.4 years), 681 PD patients (46.3% females, aged 69.5 ± 10.6 years), and 100 schizophrenia patients (44.0% females, aged 34.6 ± 9.9 years). Patients with AD, ET, and PD were recruited from the outpatient clinic of Chang Gung Memorial Hospital. Probable AD was diagnosed by consensus based on the criteria for probable AD of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) [McKhann et al., 1984]. All PD patients exhibited at least 2 of the 4 cardinal signs of PD: resting tremor, cogwheel rigidity, bradykinesia, and postural reflex impairment. Probable idiopathic PD was diagnosed according to the published criteria [Gelb et al., 1999]. ET was diagnosed based on the Tremor Investigational Group Criteria for definite or probable ET [Findley and Koller, 1995]. To the extent possible, we excluded other types of tremor with a recognizable cause, such as tremor caused by hyperthyroidism, medical intoxication, drug withdrawal, and chronic alcoholism. Schizophrenia patients recruited from the psychiatry clinics of China Medical University Hospital were diagnosed as described previously [Chen et al., 2005]. A group of 625 subjects (47.0% females, aged 57.2 ± 15.1 years) was enrolled as the normal control group. All the cases and controls in the study were Han Chinese in Taiwan and not related to each other. Each control was interviewed by one of the neurologists (C-M Chen and Y-R Wu) to rule out the possibility of dementia, tremor, parkinsonism, ataxia, and other neurological diseases. We believe that our approaches and use of an ethnically homogeneous population increased the power of the study to detect putative association. Each subject was informed of the study goals, and all examinations were performed after obtaining informed consent.

**Genetic Analysis**

DNA was extracted from leukocytes, and the CAG repeats in the *PPP2R2B* gene were determined by polymerase chain reaction (PCR) amplification [Holmes et al., 1999] and resolved on a linear polyacrylamide gel using an automated MegaBACE Analyzer. Allele sizes were determined by comparing the migration relative to the standard molecular weights. DNA sequencing was performed to assess the repeat size. Nonparametric Kruskal–Wallis test was used to compare the repeat sizes among different groups. Allele frequencies at each locus were estimated using the gene count method. A chi-square goodness of fit test was used to examine whether the controls used in this study were in the Hardy–Weinberg equilibrium. For this test, we combined all the rare genotypes (expected probability, <1%) into 1 group so that the expected frequencies of all genotype groups would be larger than 5 [Zar, 1999]; ultimately, there were 22 groups. The repeat size of the shorter allele, the longer allele, and the additive length of the 2 alleles were compared among the different groups by using ANCOVA to control for the age of the subjects. Fisher’s exact test was used to compare the probability of rare short alleles in the genotypes of the AD, ET, PD, and schizophrenia patients with that of the controls. Bonferroni adjusted α levels were used for these multiple comparisons (α = 0.05/4 [0.0125]). Using the current sample size (180 AD patients, 132 ET patients, and 625 controls) in our study, we evaluated the ability to detect an association between the short (CAG)$_{5-7}$ allele and AD or ET by power calculation implemented in PAWE version 1.2 [Gordon et al., 2002]. The genetic model-free method conferred a power of 0.90 to identify the association of the short (CAG)$_{5-7}$ allele frequency of 0.028 with AD and power of 0.88 to identify the association of the short (CAG)$_{5-7}$ allele frequency of 0.030 with ET.

**PPP2R2B Promoter Cloning and Functional Assay**

The amplified *PPP2R2B* promoter fragments (−870 to +23, where +1 represents the first nucleotide to be transcribed) containing 5, 6, 7, 10, 13, 16, and 26 CAG repeats were cloned into the pGEM-T Easy vector and sequenced. The cloned promoter fragments were inserted upstream of the firefly luciferase reporter gene in a dual luciferase reporter plasmid [Wang et al., 2006] containing the *Renilla* luciferase gene driven by the HSV-TK promoter. Human neuroblastoma cell lines IMR-32 and SK-N-SH and embryonic kidney cell line HEK-293 were plated on 12-well dishes (2 × 10$^4$/well), grown for 20 hr, and transfected by the lipofection method (Invitrogen, Carlsbad, CA) with the test dual luciferase reporter
plasmid (1 μg). The cells were grown for 48 hr. Cell lysates were prepared and the luciferase activity was measured using a luminometer by employing a dual luciferase assay system (Promega, Madison, WI). The activity of each promoter was directly measured as the ratio of the firefly luciferase level to the Renilla luciferase level. Three independent transfection experiments were conducted for each reporter construct. An allele-specific difference in luciferase activity was tested using the two-tailed Student’s t-test.

FIG. 1. Distributions of the SCA12 PPP2R2B CAG repeat lengths in controls and patients with AD, ET, PD, or schizophrenia. The percentages of the short 5- to 7-triplet alleles (A) or genotypes (B) in each group are shown in parentheses. Among the patients carrying the short (CAG)5–7 alleles, two AD patients were found to carry (CAG)7/(CAG)7, and three PD patients were found to carry (CAG)7/(CAG)10. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
RESULTS

Frequency Distributions of CAG Repeat Lengths

PCR amplification of the PPP2R2B locus yielded a pattern of 2 distinct alleles in 80% of the cases analyzed. Figure 1 shows the distributions of the CAG repeat lengths in both alleles in the 180 AD patients, 132 ET patients, 681 PD patients, 100 schizophrenia patients, and 625 normal control subjects. The controls were found to be in the Hardy–Weinberg equilibrium ($\chi^2 = 19.55$, df = 21, $P = 0.550$). Pathological expansions in the CAG repeats were not detected in any of the disease populations studied. The repeat size of the shorter allele (ANCOVA, $F_{4,1712} = 0.25$, $P = 0.911$), the longer allele (ANCOVA, $F_{4,1712} = 0.19$, $P = 0.946$), and the additive length (ANCOVA, $F_{4,1712} = 0.28$, $P = 0.890$) did not differ significantly between the controls and each of the AD, ET, PD, and schizophrenia patient groups after controlling for the age of the subjects. Table I compares the ranges of CAG repeats and frequencies of the most common 10 triplets in the Han Chinese in Taiwan and other populations [Fujigasaki et al., 2001; Srivastava et al., 2001; Brusco et al., 2002; Sulek et al., 2004]. As shown in Figure 1A, in the control group, the repeat length was 7–27 triplets and the most common lengths were 10 (28%), 13 (25%), and 16 (19%) triplets. In the patient groups, the repeat length exhibited a similar range (5–27 triplets) and the most common lengths were 10 (27–29%), 13 (25–30%), and 16 (16–21%) triplets. In both control and patients groups, the frequency of large alleles (>16 triplets) was 15%. Notably, As shown in Figure 1B, Fisher’s exact test revealed that the proportion of subjects carrying rare short (CAG)$_{5-7}$ alleles was higher in the AD patients (5/180 [2.8%], $P = 0.003$; including 2 homozygotes) and ET patients (4/132 [3.0%], $P < 0.001$) but not in the PD (6/681 [0.8%], $P = 0.075$) and schizophrenia (0/100 [0.0%], $P = 0.689$) patients as compared to the proportion in the control subjects (1/625 [0.2%]). The clinical features of the AD patients who carried the short (CAG)$_{5-7}$ alleles are displayed in Table II.

Promoter Functional Assay

To investigate the effect of the CAG repeat length on PPP2R2B expression, the promoter activity was assessed by measuring the ratio of the firefly luciferase activity to Renilla luciferase activity (internal control). For this purpose, transient transfection experiments were carried out using a dual luciferase reporter plasmid (Fig. 2A) in neuroblastoma and embryonic kidney cells. When the expressed luciferase level of the common 10-triplet allele was set as 100%, the rare 5-, 6-, and 7-triplet alleles were found to reduce the firefly luciferase synthesis to a level of 48–54% in the IMR-32 cells (open bars), 59–64% in the SK-N-SH cells (dotted bars), and 60–73% in the HEK-293 cells (filled bars), respectively ($P = 0.000–0.018$; Fig. 2B). While the common 13- and 16-triplet alleles displayed a similar promoter activity, the larger 26-triplet allele increased the gene expression level in the SK-N-SH cells (119%, $P = 0.036$) and the HEK-293 cells (131%, $P = 0.026$).

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**TABLE I. The Comparisons of Repeat Length Distribution in Unrelated Healthy in Chinese Han in Taiwan Versus Other Populations**

<table>
<thead>
<tr>
<th>Repeat range</th>
<th>Han Chinese in Taiwan (Present study)</th>
<th>Indian Srivastava et al. [2001]</th>
<th>Indian Fujigasaki et al. [2001]</th>
<th>French Fujigasaki et al. [2001]</th>
<th>Italian Brusco et al. [2002]</th>
<th>Polish Sulek et al. [2004]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rough frequency of the most common (CAG)$_{10}$ allele (%)</td>
<td>28</td>
<td>48</td>
<td>43</td>
<td>60</td>
<td>55</td>
<td>66</td>
</tr>
<tr>
<td>No. of chromosomes studied</td>
<td>1,250</td>
<td>270</td>
<td>200</td>
<td>314</td>
<td>254</td>
<td>200</td>
</tr>
</tbody>
</table>

**TABLE II. Clinical Features of AD Patients Carrying Short SCA12 PPP2R2B CAG Repeat Allele**

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex, age at onset (years)</th>
<th>Length of CAG repeat</th>
<th>Disease duration (years)</th>
<th>Clinical features at examination</th>
<th>MMSE (score)</th>
<th>Brain CT or MRI</th>
<th>EEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>M, 72</td>
<td>7/7</td>
<td>2</td>
<td>Visual hallucination, delusion, disorientation to time and place, memory impairment</td>
<td>12</td>
<td>Moderate diffuse cortical atrophy</td>
<td>Normal</td>
</tr>
<tr>
<td>Case 2</td>
<td>F, 76</td>
<td>7/7</td>
<td>2</td>
<td>Paranoid, aggressive behavior, disorientation to time and place, memory impairment</td>
<td>9</td>
<td>Moderate diffuse cortical atrophy</td>
<td>Diffuse intermittent theta waves</td>
</tr>
<tr>
<td>Case 3</td>
<td>M, 81</td>
<td>7/10</td>
<td>1</td>
<td>Recent memory impairment</td>
<td>25</td>
<td>Not available</td>
<td>Normal</td>
</tr>
<tr>
<td>Case 4</td>
<td>M, 77</td>
<td>7/13</td>
<td>1</td>
<td>Recent memory impairment</td>
<td>24</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Case 5</td>
<td>F, 69</td>
<td>7/19</td>
<td>2</td>
<td>Recent memory impairment</td>
<td>23</td>
<td>Mild diffuse cortical atrophy</td>
<td>Normal</td>
</tr>
</tbody>
</table>

MMSE, Mini-Mental Status Examination; CT, computed tomography; MRI, magnetic resonance imaging; EEG, electroencephalography.
**DISCUSSION**

This study examined the length of the CAG repeats of the SCA12 PPP2R2B gene in Taiwanese AD, ET, PD, and schizophrenia patients and in normal control subjects. No expanded alleles were detected in any of the disease populations studied. The repeat lengths of up to 31 [Srivastava et al., 2001] or 45 triplets [Fujigasaki et al., 2001] observed in the Indian controls in previous studies may explain the high prevalence of SCA12 in the Indian population. The low allele range (7–27 triplets) in Han Chinese controls in Taiwan is consistent with the absence of SCA12 cases in our previous [Wu et al., 2004] and the present studies.

Notably, the proportion of subjects carrying the short 5-, 6-, and 7-triplet alleles was higher in the AD patients (2.8%, including 2 homozygotes) and ET patients (3.0%) than in the controls (0.2%). The short 5- and 6-triplet alleles have not been reported previously. The 7-triplet allele is also extremely rare. It has been reported only twice: in a study of 2,986 alleles in controls and neurological patients (subjects with Huntington’s disease, PD, or sporadic or familial ataxias) of European ancestry [Holmes et al., 1999] and in a study of 270 normal alleles in an Indian population [Srivastava et al., 2001]. In the present study, the functional implications of the short 5-, 6-, and 7-triplet alleles were examined in neuroblastoma and embryonic kidney cells (Fig. 2B). While the promoter activity of the common 10-, 13-, and 16-triplet alleles was similar to that of the common 10-triplet allele (100–105% in IMR-32 and SK-N-SH cells and 100–108% in HEK-293 cells), the activity of the short 5-, 6-, and 7-triplet alleles was 48–69% that of the common 10-triplet allele. The expression levels of the 26-triplet allele were 112% (IMR-32), 119% (SK-N-SH), and 131% (HEK-293) that of the 10-triplet allele. The variations in the number of CAG repeats may alter the spacing of the flanking promoter elements and influence the gene expression level, similar to the expansion of dodecamer in the cystatin B promoter, which causes the EPM1 disease [Lalioti et al., 1999]. While the functional effects of a polymorphism on gene expression may be more complicated and context dependent than is often assumed [Cirulli and Goldstein, 2007], our in vitro assay may not faithfully reflect the in vivo effects of the PPP2R2B CAG repeat polymorphism. The expression level of PPP2R2B in patients carrying the short 5-, 6-, and 7-triplet alleles was further examined to support the role of PPP2R2B CAG repeat polymorphism in disease etiology. However, the expression of the PPP2R2B mRNA in peripheral leucocytes was too low to be efficiently detected (data not shown). Therefore, the comparison of PPP2R2B expression between patients who carried a short triplet and those with normal-length alleles was not performed.

It is believed that PP2A function is modulated mainly by the B subunits [Virshup, 2000]. Although the exact role of PPP2R2B in regulating PP2A activity remains to be clarified, the CAG repeat variation in PPP2R2B is assumed to influence PP2A activity. Increased PPP2R2B expression may abnormally enhance PP2A activity, thereby leading to hypophosphorylation of many PP2A substrates and finally, to SCA12. On the other hand, the shorter repeats associated with a significantly reduced expression level of PPP2R2B may reduce the PP2A activity and consequently induce tau hyperphosphorylation with a loss of tau-induced microtubule stability, and ultimately lead to increased susceptibility to AD or ET. These speculative relationships clearly necessitate further in vitro and in vivo investigations.

In summary, the results of the present study may suggest a functional role of the CAG repeats in PPP2R2B gene expression. Further studies are required to confirm whether the presence of the short 5–7-triplet alleles confers an increased susceptibility to AD or ET.
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