Septin 3 Gene Polymorphism in Alzheimer's Disease

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Septin 3 is a novel member of the septin subfamily of GTPase domain proteins that was recently identified in human neuronal cells. These proteins are involved in vesicle trafficking, neurite outgrowth, and neurofibrillary tangle formation; however, the expression and functional role of septin 3 in normal neuronal tissues and as an etiological agent in neurological disorders is currently unclear. To further characterize these parameters, the present study analyzed the expression of three isoforms of septin 3 (A, B, and C) in fetal and adult human brains and polymorphism of the *septin 3* exon 11 microsatellite in control, pure Alzheimer's disease (AD), Lewy body variant (LBV) of AD, and Parkinson's disease. Septin 3 mRNAs for isoforms A and B, but not C, were detected in the frontal cortex of fetus and adult human samples, as measured by reverse transcription-coupled polymerase chain reaction. Genotype analyses indicated that polymorphic *septin 3* alleles were distributed in two peaks of frequency in both control and disease groups. Categorization of the alleles into short (S) and long (L) types revealed a significant difference between AD patients and controls ($p = 0.034$ by chi-square test). Furthermore, the S-allele homozygosity was significantly underrepresented in AD compared with control ($p = 0.015$ by chi-square test). These results suggest that polymorphism in exon 11 of *septin 3* may have a determinative role in the pathogenesis of AD.

Key words: Septin 3; Isoforms; Polymorphism; Alzheimer's disease;
Lewy body variant of Alzheimer's disease; Parkinson's disease; CYP2D6

SEPTINS are a group of proteins that were first identified in a genetic screen of budding yeast *Saccharomyces cerevisiae* for mutants defective in cytokinesis (9). Further studies led to the characterization of septins in eurkaryotes (8). Ten homologues have since been identified in mammals (12,15), many of which are present in the brain (14,22). Recently, involvement of neuronal septins in vesicle trafficking (2,10), neurite outgrowth (23), and neurofibrillary tangle for-

mation (13) was reported, and one of the neuronal septins, CDCrel-1, was identified as a substrate of parkin, a ubiquitin-protein ligase that is present as the *PARK2* mutant in individuals with autosomal recessive juvenile parkinsonism (26). These findings suggest functional roles of human septins in neuronal networking, survival, and, possibly, degeneration.

Septin 3 was originally cloned as a gene upregulated upon neuronal differentiation of a human terato-

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carcinoma cell line (16). Its encoding protein, septin 3, belongs to a family of GTPases (40–50 kDa) that are distinct from small Ras-like GTPases or other known GTPases (12). Human *septin 3* is highly homologous to mouse *Sep3* (24) and rat *G-septin* (25). The *septin 3* gene has been mapped to chromosome 22q13.2 in the vicinity of *CYP2D6* gene and contains a microsatellite with dinucleotide repeats in exon 11 (Fig. 1). Although chromosome 22 was not identified as a linkage region in recent genomic screenings for Alzheimer's disease (AD), previous studies have shown an association between the *CYP2D6* polymorphism and Parkinson's disease (PD) or Lewy body variant (LBV) of AD (1,18,19,21). To further clarify the role of septin 3 in normal and diseased tissues, the present study aimed to assess the expression of septin 3 isoforms in human fetal and adult cerebral cortices and analyze whether microsatellite polymorphism of *septin 3* is associated with pure AD, LBV, or PD. In addition, a possible linkage between *CYP2D6* polymorphism and *septin 3* polymorphism was investigated.

MATERIALS AND METHODS

Subjects

Seventy-four control subjects (mean age ± SD, 74.8 ± 8.8 years), 99 patients with AD (78.7 ± 8.8

years), 58 patients with LBV (78.5 ± 8.0 years), and 45 patients with PD (74.2 ± 6.1 years) were studied. The diagnosis of the diseases was pathologically confirmed at autopsy. The presence of Lewy bodies in neocortical and subcortical regions served as a criterion for differentiating LBV from AD. The control group contained 28 subjects whose brains exhibited no neuropathological changes characteristic of AD, LBV, or PD, and 46 subjects who had been clinically diagnosed not to have dementia or parkinsonism. The latter subjects were included in the control group to supplement a limited number of control brains available for the allelic association study. All subjects were Caucasian. Brain samples were obtained from the University of California (UCSD; San Diego, CA) Alzheimer's Disease Research Center (ADRC), the University of Pennsylvania Medical Center (Philadelphia, PA), and the National Neurological Research Specimen Bank in Los Angeles (CA). Blood samples of the control group were obtained from UCSD-ADRC. Informed consent was obtained for all samples.

Reverse Transcription-Coupled Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the frontal cortex of a control subject by the guanidinium/CsCl method (6). First-strand cDNA was synthesized from 3 μg total RNA with oligo(dT)$_{20}$ primer (Toyobo Co., Ltd.,
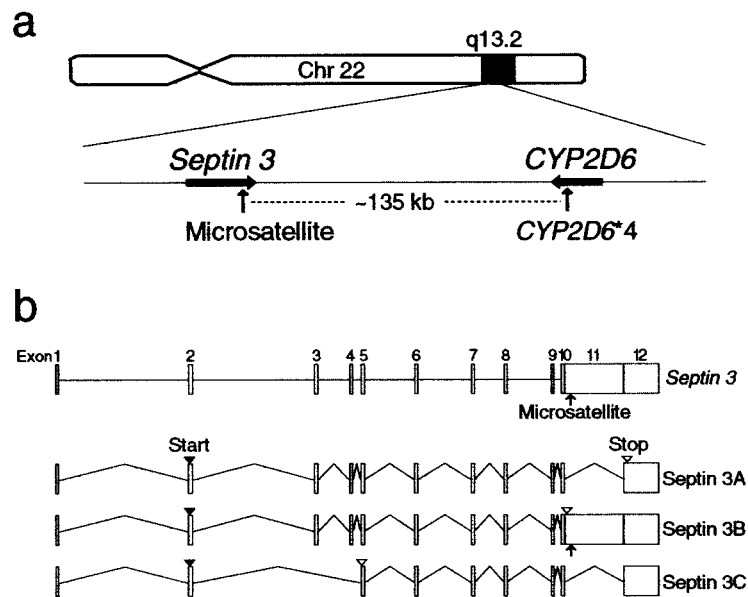

Figure 1. Chromosomal locus and genomic structure of *septin 3*. (a) *Septin 3* gene is mapped to chromosome 22q13.2 in the vicinity of *CYP2D6* gene. The distance between the two polymorphic sites, *septin 3* exon 11 microsatellite and *CYP2D6*4, is approximately 135 kb. (b) *Septin 3* gene consists of 12 exons (boxes) and contains a microsatellite in exon 11 (arrow). Septin 3 has three isoforms, A, B, and C, that are produced by alternative splicing of a single transcript. Start and stop codons are indicated by closed and open arrowheads, respectively. Based on Ensembl's Human Genome Browser (http://www.ensembl.org/Homo_sapiens/) and Methner et al. (16).

Osaka, Japan) using 100 units of Moloney murine leukemia virus reverse transcriptase ReverTra Ace (Toyobo Co., Ltd.) in a total volume of 20 µl. The cDNA thus formed served as a template for polymerase chain reaction (PCR). The following primers were used to detect mRNA of septin 3 isoforms A (GenBank, Bethesda, MD; septin 3A, Accession No. AF285107), B (septin 3B, AF285109), and C (septin 3C, AF285108): 5′-GAT TCA TGT CCA AAG GGC TC-3′ (septin 3A, 3B, and 3C; forward), 5′-ACA GCA CTA AAA AGG GCT TGG-3′(3A, reverse), 5′-CTT ACA AGG GAC TCT CCA GG-3′ (3B, reverse), and 5′-TTC AAG CTC CTT GCT GAG GT-3′ (3C, reverse). PCR was performed with 0.65 unit of Pyrobest DNA polymerase (Takara Bio Inc., Otsu, Japan) in 20 µl of 1× PCR buffer containing 0.4 µl cDNA prepared as above, 0.2 mM each dNTP mixture, 1.0 mM MgCl$_2$, and 0.2 µM each of the primers. The mixture was subjected to 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1.5 min, followed by a final extension at 72°C for 2 min. The Human Fetal Brain Large-Insert cDNA library (BD Biosciences Clontech, Palo Alto, CA) was also subjected to PCR. The PCR products were electrophoresed on 1.5% agarose gel containing ethidium bromide.

Analysis of Septin 3 Polymorphism

Genomic DNA was extracted from frozen brain samples or leukocyte preparations by the phenol/chloroform method. Polymorphism of *septin 3* was analyzed by PCR with primers flanking the polymorphic region. The sequence of the forward primer was 5′-GTC TGG TAT TTG TGG AGC ATC-3′, corresponding to the sequence from nt 1170 to 1190 in exon 11 of septin 3B mRNA (GenBank, Accession No. AF285109), and that of the reverse primer was 5′-CTT ACA AGG GAC TCT CCA GG-3′, complementary to the sequence from nt 1318 to 1299 in exon 11. PCR was performed with 1.0 unit of rTaq DNA polymerase (Toyobo Co., Ltd.) in 20 µl of 1× PCR buffer containing 0.1 µg genomic DNA, 0.2 mM each dNTP mixture, 1.5 mM MgCl$_2$, and 0.2 µM each of the primers. [α-^{32}P]dCTP 1.5 µCi was added to this mixture. The mixture was subjected to 32 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 75°C for 30 s, followed by a final extension at 75°C for 5 min. The PCR products were denatured and separated on a 6% polyacrylamide gel containing 8.0 M urea. The alleles were identified by autoradiography. An M13mp18 DNA sequencing ladder was used as a size marker.

Identification of CYP2D6*4

Analysis of the *CYP2D6*4 allele was carried out by the PCR-*Bst*NI restriction fragment length polymorphism technique (19). The sequence of the forward primer was 5′-GCC TTC GCC AAC CAC TCC G-3′, corresponding to the sequence from nt 3359 to 3377 in exon 3 of *CYP2D6* gene (GenBank, Accession No. M33388), and that of the reverse primer was 5′-AAA TCC TGC TCT TCC GAG GC-3′, complementary to the sequence from nt 3713 to 3694 in intron 4. PCR amplification was performed as described above for *septin 3* polymorphism, except for omission of [α-^{32}P]dCTP and annealing at 60°C. The PCR product of 355 bp was digested with *Bst*NI restriction endonuclease. The *CYP2D6*4 allele missing the recognition site gave a 355-bp band, whereas the wild-type allele was digested to give 105- and 250-bp bands.

Statistical Analysis

Genetic association was examined using the contingency tables and incorporated the use of Kruskal-Wallis and Wilcoxon rank sum statistics. Odds ratio (OD) and 95% confidence interval (CI) were generated from subset 2 × 2 tables, with simple tests for association calculated using the chi-square test or Fisher's exact test. The latter test was applied when at least one expected value was smaller than five. These statistical analyses were carried out by using InStat, version 2.03 (GraphPad Software, San Diego, CA) and SAS, version 8.2 (SAS Statistical, Software Inc., Cary, NC).

RESULTS

Detection of Septin 3 mRNAs by RT-PCR

Expression of three isoforms of septin 3 was analyzed in fetal and adult human brains by RT-PCR. Septin 3A and 3B mRNAs were clearly detected as the bands of expected sizes in both fetal and adult human brains, whereas septin 3C mRNA was not detectable in either tissue (Fig. 2).

Septin 3 *Polymorphism in AD, LBV, and PD*

Microsatellite polymorphism in the *septin 3* gene was investigated for a possible association with AD, LBV, or PD. The size of PCR products carrying *septin 3* polymorphism ranged from 135 to 157 bp. *Septin 3* alleles were distinguished based on the size of their PCR products. Due to the low allele frequency on either extreme of the distribution, the tails were
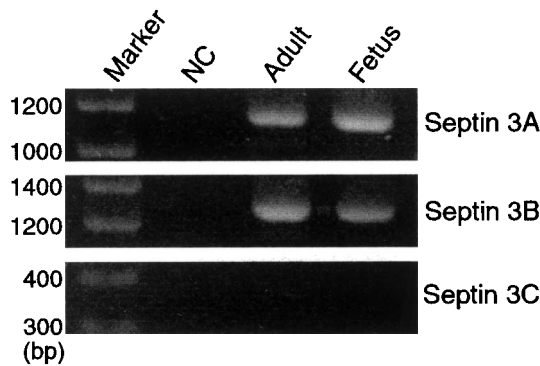
Figure 2. Detection of septin 3 mRNAs in human brains. cDNA synthesized from mRNA isolated from fetal and adult human brains was used as templates for PCR. Three sets of primers were used to detect alternatively spliced forms of septin 3 mRNA. The expected sizes of septin 3A, 3B, and 3C mRNAs were 1160, 1308, and 397 bp, respectively. Septin 3A and 3B, but not 3C, mRNAs were detected in both fetal and adult brains. Sterilized water was used as a negative control (NC) for PCR.

recategorized as ≤145 and ≥155 for analysis. Heterozygosity of this microsatellite polymorphism among control subjects was 0.757 (56 out of 74), and the polymorphic information content (3) was 0.688. Apparently, in both control and disease groups, alleles were distributed in two peak frequencies: a major peak at allele 147 and a minor peak at allele 153 (Table 1). The statistical difference was observed upon comparison of AD distribution to that of controls using the Wilcoxon rank sum test (test statistic $= 5.504$, $p = 0.019$). Analysis of the frequency of each allele indicated that *septin 3* allele ≤145 was underrepresented in AD and LBV, compared with controls (AD vs. control, OD = 0.32, 95% CI = 0.11–0.95, $p = 0.039$ by Fisher's exact test; LBV vs. control, OD = 0.22, 95% CI = 0.05–1.01, $p = 0.044$ by Fisher's exact test). *Septin 3* allele 153 was overrepresented in AD (AD vs. control, OD = 1.63, 95% CI = 1.01–2.62, $p = 0.044$ by chi-square test). How-

ever, these differences were not statistically different after Bonferroni's correction (critical p value for 6 alleles = 0.0083).

Based on the allele distribution in two high frequency peaks, *septin 3* polymorphic alleles were dichotomized. The alleles of 149 and shorter were termed short-type (S) allele, and those of 151 and longer were termed long-type (L) allele in this study. After this categorization, a significant difference was found between control and AD (AD vs. control, $p = 0.034$ by chi-square test) (Table 2). In LBV and PD, the allelic distribution of *septin 3* polymorphism did not differ significantly from control. The proportions of three *septin 3* genotypes, S/S, S/L, and L/L, in control and disease groups are summarized in Table 3. The progression from S/S to L/L was assumed to be ordinal for statistical analysis. There was no significant difference in overall genotypic distribution between the control and disease groups (Kruskal-Wallis statistic = 5.087, $p = 0.167$); however, a statistically significant difference in genotype distribution was noted between AD patients and controls (Wilcoxon rank sum statistic = 4.706, $p = 0.030$). Genotype S/S was found to be underrepresented in AD compared with controls (AD vs. control, OD = 0.46, 95% CI = 0.25–0.87, $p = 0.015$ by chi-square test) (Fig. 3). Conversely, AD patients carrying at least one L allele (S/L, L/L) were significantly more abundant than controls. This difference was significant even after Bonferroni's correction (critical p value for 3 genotypes = 0.017). The frequency of genotypes S/L or L/L did not differ significantly in any disease groups from control.

Septin 3 *Polymorphism and* CYP2D6 *Polymorphism*

A possible linkage between *CYP2D6* polymorphism and *septin 3* polymorphism due to their proximity was investigated. *CYP2D6*4* (formerly designated as *CYP2D6* B mutation) allele was used as a

TABLE 1
DISTRIBUTION OF *SEPTIN 3* ALLELES IN CONTROL AND DISEASE GROUPS

Group	n	≤145	147	149	151	153	≥155
Control	148	11 (7.4)	58 (39.2)	32 (21.6)	5 (3.4)	36 (24.3)	6 (4.1)
AD	198	5 (2.5)	68 (34.3)	40 (20.2)	8 (4.0)	68 (34.3)	9 (4.5)
LBV	116	2 (1.7)	54 (46.6)	20 (17.2)	3 (2.6)	32 (27.6)	5 (4.3)
PD	90	2 (2.2)	37 (41.1)	19 (21.1)	1 (1.1)	28 (31.1)	3 (3.3)

n: number of chromosomes analyzed. Number in parentheses indicates percentage.
Overall statistic (Kruskal-Wallis test) = 6.062, $df = 3$, $p = 0.109$.
Wilcoxon rank sum statistic:
AD vs. controls: test statistic = 5.504, $df = 1$, $p = 0.019$.
LBV vs. controls: test statistic = 0.355, $df = 1$, $p = 0.551$.
PD vs. controls: test statistic = 0.854, $df = 1$, $p = 0.355$.

TABLE 2
FREQUENCY OF *SEPTIN 3* ALLELES IN CONTROL
AND DISEASE GROUPS

Group	n	S	L	χ^2	p Value
Control	148	101 (68.2)	47 (31.8)		
AD	198	113 (57.1)	85 (42.9)	4.480	0.034
LBV	116	76 (65.5)	40 (34.5)	0.219	0.640
PD	90	58 (64.4)	32 (35.6)	0.364	0.546

n: number of chromosomes analyzed. S: short-type allele (≤149). L: long-type allele (≥151). Number in parentheses indicates percentage.
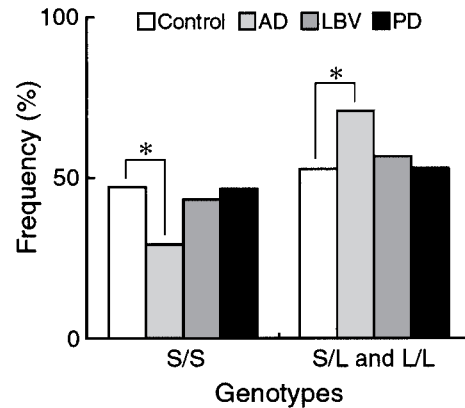Overall statistic (Kruskal-Wallis test) = 5.120, df = 3, p = 0.163.

Figure 3. Frequency of *septin 3* genotypes with L allele (S/L, L/L) or without L allele (S/S) in control and disease groups. *χ^2(1) = 5.142, p = 0.015 by chi-square test for AD versus control.

marker of *CYP2D6* polymorphism. Analysis of the allele frequency showed a strong linkage between *septin 3* allele 147 and the *CYP2D6*4 allele (W/*4 vs. W/W, OD = 2.88, 95% CI = 1.95–4.27, $p < 0.0001$ by chi-square test; *4/*4 vs. W/W, OD = 18.9, 95% CI = 5.56–64.5, $p < 0.0001$ by Fisher's exact test) (Table 4). In addition, a significant difference was observed in the overall allelic distribution of the *septin 3* locus between W/W genotype and W/*4 genotype (Wilcoxon rank sum statistic = 10.647, p = 0.001).

CYP2D6 *Polymorphism in AD, LBV, and PD*

Genotypic distribution and allelic frequency of *CYP2D6*4 in control and disease groups are summarized in Table 5. Overall genotype distribution by groups was not statistically significant (Kruskal-Wallis statistic = 7.352, p = 0.061); however, a statistically significant difference was found between LBV patients and controls (Wilcoxon rank sum statistic = 5.238, p = 0.022). Analysis of the frequency of indi-

TABLE 3
GENOTYPIC DISTRIBUTION OF *SEPTIN 3* POLYMORPHISM
IN CONTROL AND DISEASE GROUPS

Group	N	S/S	S/L	L/L
Control	74	35 (47.3)	31 (41.9)	8 (10.8)
AD	99	29 (29.3)	55 (55.6)	15 (15.2)
LBV	58	25 (43.1)	26 (44.8)	7 (12.1)
PD	45	21 (46.7)	16 (35.6)	8 (17.8)

N: number of subjects analyzed. S: short-type allele (≤149). L: long-type allele (≥151). Number in parentheses indicates percentage.
Overall statistic (Kruskal-Wallis test) = 5.087, df = 3, p = 0.165.
Wilcoxon rank sum statistic:
AD vs. controls: test statistic = 4.706, df = 1, p = 0.030.
LBV vs. controls: test statistic = 0.212, df = 1, p = 0.645.
PD vs. controls: test statistic = 0.325, df = 1, p = 0.568.

vidual genotypes indicated that genotype W/W was underrepresented and W/*4 was overrepresented in LBV compared with controls (W/W, OD = 0.37, 95% CI = 0.17–0.80, p = 0.011 by chi-square test; W/*4, OD = 2.64, 95% CI = 1.18–5.92, p = 0.017 by chi-square test). These differences were statistically significant after Bonferroni's correction (critical p value for 3 genotypes = 0.017). The *CYP2D6*4 allele was significantly overrepresented in LBV (28.3%) compared with controls (15.5%) (OD = 2.15, 95% CI = 1.13–4.09, p = 0.018 by chi-square test).

DISCUSSION

Septin 3 has been shown to occur in three alternatively spliced forms, septin 3A, 3B, and 3C [Fig. 1b and (16)]. Septin 3A and 3B differ in the absence or presence of exon 11, respectively. The omission of exon 11 in septin 3A leads to translation of C-terminal amino acids from exon 12, whereas the presence of exon 11 in septin 3B leads to translation of 13 amino acids followed by a stop codon and an additional 3′ untranslated region (3′ UTR). Septin 3C is an atypical member lacking a highly conserved central domain, which is produced by omission of exons 3 and 4 leading to a frameshift and early stop. In a previous study, only septin 3B was reported as detectable in adult human brain by Northern blotting (16). In the present study, the expression of both septin 3A and 3B mRNAs was demonstrated in fetal and adult human brains, a difference that may be due to primer selection, RNA purity, etc.

Human *septin 3* gene is highly homologous to mouse *Sep3* and rat *G-septin* genes. Mouse *Sep3* is

TABLE 4
ASSOCIATION OF *SEPTIN 3* POLYMORPHISM WITH *CYP2D6* POLYMORPHISM

CYP2D6 Genotype	n	≤145	147	149	151	153	≥155
				Septin 3 Allele			
W/W	340	14 (4.1)	98 (28.8)	82 (24.1)	12 (3.5)	118 (34.7)	16 (4.7)
W/*4	156	4 (2.6)	84 (53.8)*	19 (12.2)	5 (3.2)	38 (24.4)	6 (3.8)
*4/*4	26	0 (0.0)	23 (88.5)†	3 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

n: number of chromosomes analyzed. W: wild-type allele of *CYP2D6*. *4: *CYP2D6*4 allele. Number in parentheses indicates percentage.

Wilcoxon rank sum statistic (W/W vs. W/*4) = 10.647, *df* = 1, *p* = 0.001.

*$\chi^2(1)$ = 28.82, *p* < 0.0001 (chi-square test for W/*4 vs. W/W), critical *p* value = 0.0083.

†$\chi^2(1)$ = 38.82, *p* < 0.0001 (Fisher's exact test for *4/*4 vs. W/W), critical *p* value = 0.0083.

upregulated in neuronal tissues during development (24), while rat G-septin was identified as a specific substrate for type I cGMP-dependent protein kinase (PKG) and detected as a phosphoprotein in nerve termini (25). Therefore, septin 3 and its modification by PKG may play an important role in brain functions.

Identification of three septins (Nedd5, H5, and Diff6) in neurofibrillary tangles, neuropil threads, and dystrophic neurites in AD brains suggests their involvement in neurological disorders (13). To test whether septin 3 interacts with any of these septins, we prepared and analyzed HeLa cells in which septin 3 and Nedd5 were transiently coexpressed. Our preliminary analysis revealed that septin 3 was coimmunoprecipitated with Nedd5, suggesting their interaction and possible involvement of septin 3 in

neurodegenerative processes (unpublished data). In this context, noteworthy is our present result that homozygous *septin 3* S allele was underrepresented among AD patients, which suggests a recessively protective effect of S allele on AD pathogenesis or a dominantly causative effect of L allele.

The *septin 3* microsatellite is located within the 3′ UTR in exon 11, which is retained only in septin 3B mRNA. Elements of 3′ UTR, such as the poly(A) tail and AU-rich sequences, modulate reportedly mRNA translation and stability (4,5,11). In other genes, dinucleotide or trinucleotide repeats within the 3′ UTR have been identified as binding sites for RNA binding proteins that regulate localization, stability, and translation of mRNA (7,17,20). It seems possible that the *septin 3* polymorphism act as *cis*-acting regula-

TABLE 5
GENOTYPIC DISTRIBUTION AND ALLELIC FREQUENCY OF *CYP2D6*4 BY GROUP

Group	N	W/W	W/*4	*4/*4	n	W	*4
			Genotype			Allele	
Control	71	52 (73.2)	16 (22.5)	3 (4.2)	142	120 (84.5)	22 (15.5)
AD	99	68 (68.7)	28 (28.3)	3 (3.0)	198	164 (82.8)	34 (17.2)
LBV	46	23 (50.0)	20 (43.5)	3 (6.5)	92	66 (71.7)	26 (28.3)
PD	45	27 (60.0)	14 (31.34)	4 (8.9)	90	68 (75.6)	22 (24.4)

N: number of subjects analyzed. *n*: number of chromosomes analyzed. W: wild-type allele. *CYP2D6*4: *CYP2D6*4 allele. Number in parentheses indicates percentage.

Genotype Distribution:

Overall statistic (Kruskal-Wallis test) = 7.352, *df* = 3, *p* = 0.061.

Wilcoxon rank sum statistic:

AD vs. controls: test statistic = 0.159, *df* = 1, *p* = 0.690.

LBV vs. controls: test statistic = 5.238, *df* = 1, *p* = 0.022.

PD vs. controls: test statistic = 2.457, *df* = 1, *p* = 0.117.

Allele Distribution:

Overall statistic (Kruskal-Wallis test) = 7.833, *df* = 3, *p* = 0.050.

Wilcoxon rank sum statistic:

AD vs. controls: test statistic = 0.169, *df* = 1, *p* = 0.681.

LBV vs. controls: test statistic = 5.582, *df* = 1, *p* = 0.018.

PD vs. controls: test statistic = 2.872, *df* = 1, *p* = 0.090.

tory element(s), directly affecting posttranscriptional regulation of septin 3B mRNA by action of some RNA-binding protein(s).

The *septin 3* gene is located in close proximity to the *CYP2D6* gene (Fig. 1). A strong linkage was found between *septin 3* allele 147 and *CYP2D6**4 allele. Overrepresentation of *CYP2D6**4 allele in LBV, as revealed in the present study, confirmed our previous observation (18,21). *Septin 3* allele 147 was also overrepresented in LBV, although the difference did not reach a significant level. This trend is probably due to the linkage between *septin 3* allele 147 and pathogenic *CYP2D6**4 allele.

In summary, our present findings suggest that the polymorphism of *septin 3* gene is associated with the susceptibility of the brain to neurodegenerative changes specific for AD, but not LBV or PD. Further investigation of *septin 3* polymorphism in different ethnic populations and their relevance to changes in gene expression are required to confirm this notion.

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