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The V81M variant of tyrosine hydroxylase is associated with more severe freezing of gait in Parkinson's disease

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Introduction: Many of the symptoms and signs of Parkinson's disease (PD) arise from the death of midbrain dopamine neurons that utilize tyrosine hydroxylase (TH) as the rate-limiting enzyme in catecholamine biosynthesis.
Methods: We investigated whether the presence of a common TH polymorphism affects the clinical outcomes in 101 PD subjects. We further examined the effect of this polymorphism on the purified recombinant enzyme.
Results: PD subjects homozygous for the common V81M polymorphism, have higher overall freezing of gait scores after controlling for disease duration, although this polymorphism does not associate with the occurrence of PD or FOG. *In vitro* functional assays on pure recombinant wild type TH and V81M TH revealed that the K_m of the mutant enzyme for tyrosine was twice that of the wild-type. This polymorphism, however, did not change the stability of the enzyme, nor did it affect the V_{max} or K_m for the co-substrate BH_4.
Conclusion: The data suggest that presence of a homozygous V81M polymorphism is associated with more severe FOG, possibly due to lower catecholamine synthetic capacity. Further studies are warranted to investigate the role of subtle changes in catecholamine availability in the development of FOG.
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1. Introduction

Parkinson's disease (PD), the second most common neurodegenerative disorder, presents initially with motor disabilities of resting tremor, bradykinesia, and rigidity [1]. As the disease progresses, gait and balance difficulties also occur. Freezing-of-gait (FOG) is one of common gait disorders in PD, and contributes to significant clinical disability such as falls. The pathological hallmark of PD is the loss of dopamine neurons in the substantia nigra pars compacta, including a reduction in tyrosine hydroxylase (TH), the rate limiting enzyme in catecholamine synthesis [2,3]. This protein loss is most significant at the nerve terminals.

TH is a homotetramer in which each of the subunits contains regulatory, catalytic, and tetramerization domains [2]. The enzyme utilizes tyrosine, BH_4 and O_2 as co-substrates, and Fe^{2+} as a cofactor. TH protein levels and activity can be regulated at several points, including mRNA expression, regulation of RNA stability, and translation including post-translational phosphorylation and binding of effector proteins. TH knock-out mice are embryonically lethal, but one intact copy is sufficient to maintain dopamine synthesis, as indicated by the fact that heterozygote animals are viable and express normal levels of catecholamines [4,5]. There are genetic polymorphisms in several regions of the TH gene, and some of these have been postulated to be involved in the pathology of PD and other forms of parkinsonism [6,7].

A non-synonymous coding region polymorphism in the regulatory domain of TH, V81M (rs6356), has been described by independent studies with an average reported allelic frequency of 0.33 [6,7], but neither the specific effect of this polymorphism on the enzyme, nor the potential functional consequences for PD patients, has been reported. This report is the first such comprehensive analysis on the effect of V81M on clinical presentations of PD and TH function.

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2. Methods

2.1. Subjects

Blood samples were obtained from 101 PD patients and 68 controls for DNA analysis. Patients were selected from a large cohort currently followed at the Hershey Medical Center Neurology Outpatient Clinic for an ongoing longitudinal study. PD diagnosis [8] was performed by a movement disorder specialist. All PD subjects involved in this study are managed by a movement disorder specialist and on optimized medication regimens. Disease duration was obtained from subject history. Unified Parkinson's Disease Rating Scale part-3 (UPDRS-III) scores were used as a measure of general motor capability [9] and assessed twice: once 12 h after the last dose of PD medication (UPDRS-OFF) and then shortly after administration of the subject's regular PD medication (UPDRS-ON). The levodopa-equivalent daily dose (LEDD) was calculated to estimate the effect of combined dopaminergic medications on each subject [10]. FOG was assessed using the Freezing of Gait Questionnaire (FOG-Q) [11,12]. Written informed consent was obtained from all subjects and the study was approved by the Penn State Hershey Institutional Review Board (IRB# 40726) and conducted in accordance with the principles of the Declaration of Helsinki.

2.2. Genetic analyses

Venous blood was collected from all participants and DNA extraction performed using the DNeasy Blood & Tissue Kit (QIAGEN; Valencia, CA). We utilized a TaqMan SNP Genotyping assay designed against the rs6356 SNP (Life Technologies, Thermo Fisher Scientific, Grand Island, NY). Follow-up and confirmation sequencing were performed with previously reported primers for exon 3 of the human tyrosine hydroxylase gene using Polymerase Chain Reaction (Hot Start Taq DNA Polymerase, QIAGEN) and Sanger amplicon sequencing to determine the presence of the V81M polymorphism [13]. Agarose gel electrophoresis (1.5% Agarose gel, Sigma–Aldrich, St. Louis, MO, in Tris/Acetic Acid/EDTA Buffer, Bio-Rad, Hercules, CA) of the PCR products (performed at 150 V for 1 h) was followed by gel extraction (NucleoSpin Gel and PCR Clean-up Kit, Macherey–Nagel, Bethlehem, PA). Sanger sequencing was performed to determine the DNA sequence of exon 3 (Eurofins MWG Operon, Huntsville, AL). The results were compared against the genomic sequence of native human TH using ClustalW2 by EBI (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and the complementary strand sequence was obtained using Reverse Complement (http://www.bioinformatics.org/sms/rev_comp.html).

2.3. Site-directed mutagenesis and expression of mutant recombinant TH construct

The full length cDNA for hTH1 was purchased from OriGene in a custom vector (Rockville, MD), and the hTH1 coding sequence was cloned (as an N-terminal hexa-histidine fusion protein) into the pET28-tev expression vector as described previously for the human tryptophan hydroxylase 2 gene [14]. Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) with the following primers: 5′-GGGAAGGCCATGCTAAACCTG-3’ (forward) and 5′-GAAGAGCAGGTTTAGCATGGC-3’ (reverse) purchased from Integrated DNA Technologies (Coralville, IA). The following parameters were used in the thermal cycler: 30 s denaturation at 95 °C, 1 min annealing at 55 °C, 8 min extension at 68 °C, performed for 18 cycles. Following *DpnI* digestion, the plasmids were transformed into XL-1 Blue supercompetent cells, and positive colonies were selected using kanamycin (25 mg/mL) LB Agar plates (Sigma, St. Louis, MO). Selected colonies were grown overnight in liquid LB cultures at 37 °C, and the plasmids isolated using the NucleoSpin Plasmid Isolation Kit (Macherey–Nagel, Bethlehem, PA). The sequencing of the plasmids was performed by Eurofins Operon (Huntsville, AL) with the following primers: 5′-ATGCCCACCCCCGACGCCACC-3’ (forward),5′-CTAGCCAATGGCACTCAGCGCATGG-3’ (reverse), as well as T7 promoter (forward) and T7 terminator (reverse) primers. Positive colonies then were transformed into the BL21-CodonPlus(DE3)-RIL *E.coli* strain for protein expression.

2.4. Bacterial expression of wild-type human TH

The expression of the recombinant protein in *E. coli* was established using ZYP-5052 medium as previously described [14,15]. The expression was performed by shaking the cultures at 215 rpm and 15 °C until the saturation density (measured at 600 nm) reached 9, after which the cultures were harvested and stored at −80 °C.

2.5. Purification of recombinant hTH1

Harvested bacterial cultures were lysed with BugBuster, Amine Free (EMD Chemicals Inc., San Diego, CA) containing benzonase and r-lysozyme with EDTA-free protease inhibitor cocktail. The recombinant hexa-histidine fusion TH was purified on a 5 mL HT Nickel column (GE Healthcare, Pittsburgh, PA, USA), as described previously [14]. 50%-saturated ammonium sulfate was used to precipitate the Ni-column-purified proteins. The pellet then was dissolved in size exclusion chromatography buffer (25 mM HEPES, 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA), and size exclusion chromatography was performed using a 25 mL Superdex 200 10/300 GL column (GE Healthcare). Protein standards were purchased from Sigma (MW-GF-1000 Protein Standard Kit).

2.6. SDS-PAGE analysis

SDS-PAGE analysis was utilized to document the expression and purity of the recombinant proteins. Pre-cast 4–12% NuPAGE BisTris gels (Invitrogen) were used to resolve denatured proteins, as described previously [14]. Coomassie Blue dye was used to visualize the resolved proteins (Pierce).

2.7. Enzyme activity assay

Tyrosine hydroxylase activities of the pure recombinant proteins were assessed using a radioenzymatic assay that monitors the release of $^{3}H_2O$, as described previously by Reinhard et al. [16]. Bradford protein assay (Bio-Rad) was used to determine protein concentration. Chemicals used for the activity assay were obtained from Sigma, except for the activated charcoal (Darco G-60, Fisher Scientific). In order to assay for the kinetic parameters, varying concentrations for either one of the substrates were employed in the presence of a fixed concentration of the counterpart (100 μM tyrosine or 100 μM BH_4) with ambient oxygen. Resulting activity values were normalized to the amount of protein present to obtain specific activities. Prism Software V.5 (GraphPad, San Diego, CA) was used to calculate kinetic constants (K_m, V_{max}). The stability assay was performed in a similar manner, with the incubation of the protein at 37 °C for varying periods of time up to 90 min. The activity values at different time points were plotted against time on a semi-log plot, and the enzyme activity half-life was calculated.

2.8. Statistical analysis

The differences in V_{max}, K_m, and $t_{1/2}$ values were analyzed using

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