Haplotype analysis of the IGF2-INS-TH gene cluster in Parkinson's disease

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Short title: IGF2-INS-TH haplotypes in Parkinson's disease

Abstract: 184 words **Text word count:** 2422

Number of: text pages: 8 figures: 1 tables: 3 **Supplementary tables:** 1

Abstract

Idiopathic Parkinson's disease is a common movement disorder characterised by a loss of dopaminergic neurons in the substantia nigra. Its pathogenesis is postulated to involve complex interactions between genetic susceptibility and environmental exposures. The *IGF2-INS-TH* gene cluster on the telomeric end of human chromosome 11 is a gene rich region expressing several proteins important for dopamine neuron homeostasis. We used a haplotyping approach to determine whether common genetic variation in the *IGF2-INS-TH* cluster influences the risk of idiopathic Parkinson's disease in a Caucasian case-control group recruited from Brisbane, Australia. Three tagging polymorphisms, the SNPs, rs680 and rs689 and the microsatellite, HUMTH01, were genotyped in 215 cases and 215 age- and gender-matched controls. Eight common haplotypes accounted for 91% of the genetic variation in our control group and one haplotype, *IGF2-INS-TH**6, was significantly under-represented among the cases with idiopathic Parkinson's disease (OR = 0.42, 95% C.I. = 0.25-0.72, p-value = 0.001). Analysis of the individual polymorphisms showed that the *IGF2*-rs680 alternate 'A' allele accounted for the majority of the protective effect. Our findings suggest that common genetic variants in the *IGF2-INS-TH* cluster modify susceptibility to idiopathic Parkinson's disease.

Key words: association, chromosome 11, insulin-like growth factor 2, Parkinson's disease, tyrosine hydroxylase

INTRODUCTION

Idiopathic Parkinson's disease (PD) is characterised by the selective loss of dopamine neurons in the substantia nigra and is responsive to the exogenous administration of levodopa (L-DOPA) (Litvan *et al.* 2007). The neurons that degenerate contain the rate-limiting enzyme for dopamine synthesis, tyrosine hydroxylase (TH). Growth factors, including insulin-like growth factors (IGF) can protect against environmental toxins used to produce animal models of PD (Quesada and Micevych 2004; Chung *et al.* 2005). While the cause/s of PD remain unknown, there is evidence that complex interactions between genetic susceptibility and environmental exposures are involved (Le Couteur *et al.* 2002). In particular, the genetic component of PD is postulated to be a combination of small incremental risks from common variants of multiple genes. Linkage disequilibrium-based mapping approaches, such as haplotype analysis, allow this common disease - common variant hypothesis to be investigated using a minimal amount of markers per loci (Doris 2002).

The telomeric end of the short arm of chromosome 11 (11p15.5) is a gene-rich region containing the *IGF2*, proinsulin precursor (*INS*) and *TH* genes (Figure 1); all of which are important for dopamine neuron homeostasis. A previous haplotype analysis of the *IGF2-INS-TH* region identified three 'tagging' polymorphisms (Figure 1): the single nucleotide polymorphisms (SNP), *IGF2*-rs680 and *INS*-rs689; and HUMTH01, a tetranucleotide repeat marker in the *TH* gene (Rodriguez *et al.* 2004). Associations were subsequently found between particular *IGF2-INS-TH* haplotypes and cardiovascular risk factors, such as high body fat percentage, plasma triglycerides and blood pressure (Rodriguez *et al.* 2004) and initiation of regular smoking (Rodriguez *et al.* 2006). This latter study supported other groups who found associations between the HUMTH01 marker *per se* and smoking behaviour (Anney *et al.* 2004; Olsson *et al.* 2004). Interestingly, these same risk factors occur less

frequently in PD patients compared with controls (Haugarvoll et al. 2005; Scigliano et al. 2006), suggesting that common variants in the *IGF2-INS-TH* gene cluster may also affect susceptibility for PD. The *TH* gene has been previously discounted as a PD susceptibility gene, although only single polymorphic markers were investigated in these studies (Plante-Bordeneuve et al. 1994; Kunugi et al. 1998; Mizuta et al. 2006). Furthermore the potential confounding influence of smoking behaviour was not considered in these studies. We therefore investigated the potential role of *IGF2*, *INS* and *TH* genes, along with the known protective factor smoking, in PD susceptibility.

***** Figure 1 about here*****

MATERIALS AND METHODS

Case recruitment and phenotyping

Caucasian PD cases were recruited by an experienced neurologist (PS) at both private and public movement disorders clinics in Brisbane, Australia. Informed consent for participation in the study was obtained following approval by the human research ethics committees at each of the participating institutions, in accordance with the Declaration of Helsinki. Standard criteria for the diagnosis of probable PD was used (Gelb et al. 1999). Patients were excluded if they had no response to levodopa, or they had cognitive decline. A standard questionnaire was completed to obtain detailed epidemiological information including smoking behaviour. Subjects were considered smokers if they had ever smoked at least one cigarette per day for a period of a year or more (Deng et al. 2004). Controls of Caucasian ancestry were a combination of spouses (37%) and electoral roll volunteers who underwent the same data collection procedures and were excluded if screening responses suggested any possibility of movement or cognitive disorders. Blood was collected for subsequent DNA extraction from

215 PD cases (mean age = $71 \pm$ S.D. of 9 years, M/F = 129/86) and 215 age and gender-matched controls (mean age = $71 \pm$ 9 years, M/F = 129/86).

Genotyping

Design and implementation of genotyping assays for SNPs, rs680 and rs689, was carried out by the Australian Genome Research Facility in Brisbane, using the Sequenom Platform.

HUMTH01 PCR amplification (Sharma et al. 1998) was performed using the primers:

forward 5' (HEX)-TCCAAAAAATCCAAGATGGC-3' and reverse 5'-

ACAGGGAACACAGACTCCATG-3'. Briefly 50 ng genomic DNA was incubated with the TH primers (both 0.2 μ M) in a solution of 1.5 mM MgCl₂, 0.1 mM dNTPs, 0.5 U native Taq polymerase in a final volume of 20 μ l (Invitrogen, Carlsbad, California, USA). Cycling conditions were 94°C for 3 minutes, then 35 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 35 seconds, followed by 72°C for 7 minutes. PCR products were resolved by electrophoresis on a 6% 7M urea denaturing polyacrylamide gel, using the Gelscan 2000 real-time gel system (Corbett Research, Mortlake, NSW, Australia). Identical results were obtained on duplicates of 10% of the samples. Genotyping efficiency was 98% for both the rs680 and rs689 assays and 100% for HUMTH01; 209 controls (mean age = $71 \pm$ S.D. of 9 years, M/F = 126/83, smokers = 53%) and 210 PD cases (mean age = $71 \pm$ S.D. of 9 years, mean age at onset = $58 \pm$ S.D. of 10 years, M/F = 125/85, smokers = 47%) were included in the final haplotype analysis (see below). *IGF2-INS-TH* haplotype nomenclature has been previously described (Rodriguez et al. 2004).

Statistics

A nominal statistical significance level of < 0.05 was used for all tests unless modified for multiple comparisons using Bonferroni's correction. Hardy-Weinberg equilibrium analyses

were performed (web-based χ^2 analyses:

http://www.kursus.kvl.dk/shares/vetgen/_Popgen/genetik/applets/kitest.htm) to assess

genotyping reliability. Haplotype differences between PD cases and controls were assessed

with χ^2 analysis using the 'EH' program (version 15; <http://www.genemapping.cn/eh.htm>).

The program utilises an expectation-maximisation algorithm to handle unphased data.

Haplotype crude odds ratios (OR) were calculated using a web-based calculator

(<http://statpages.org/ctab2x2.html>). Linkage disequilibrium analysis was calculated using the

'2LD' program (<http://www.mrc-epid.cam.ac.uk/Personal/jinghua.zhao/software.htm>)

incorporating 'EH' generated haplotype data. Uncommon haplotypes were combined to

reduce cells and all other haplotypes used as the reference. Further explorative statistics to

determine any underlying individual allelic or genotypic polymorphisms associated with PD

were assessed using χ^2 analyses (SPSS Version 13.0). Binomial logistic regression modelling

was used to adjust the ORs for age, gender, and smoking status. Variables that were

significantly difference in these univariate regression analyses were included in a final

multivariate regression model.

RESULTS

The rs680 and HUMTH01 markers were in Hardy-Weinberg equilibrium for both cases and control populations ($p > 0.6$). The rs689 marker for cases was in Hardy-Weinberg equilibrium ($p = 0.6$), but the controls were outside Hardy-Weinberg equilibrium for this marker (0.04).

However, there were no ambiguities between replicates for these samples. Eight common

IGF2-INS-TH haplotypes accounted for 91% of all haplotypes among the controls (Table 1).

A χ^2 analysis of all haplotype frequencies showed a significant difference between PD cases and controls ($\chi^2 = 20.4$, degrees of freedom (df) = 8, $p \leq 0.01$). Further analysis of individual

haplotypes showed that *IGF2-INS-TH**6 (*6) was significantly under-represented in PD (OR = 0.42 95% C.I. = 0.25-0.72, $p \leq 0.001$) whereas *8 was over-represented (OR = 2.31 (1.17-4.56), $p \leq 0.02$) (Table 1). However, the *8 effect would not survive a strict adjustment for multiple comparisons (level of significance ≤ 0.006). The *6 haplotype consisted of the alternate alleles for both SNPs combined with the longest HUMTH01 allele 'e', whereas *8 had the major *IGF2* allele (G) and HUMTH01 'c' allele (Table 1).

***** Table 1 about here *****

To determine whether the haplotype effects above could be attributed to any particular individual polymorphisms, further exploratory statistics were performed. There were no allele or genotype associations observed for rs689, however both the minor allele 'A' of rs680 (OR = 0.73, 95% C.I. = 0.54-0.99, p -value = 0.04) (Supplementary Table S1) and 'AA' homozygotes cases (OR = 0.37, 95% C.I. = 0.17-0.83, p -value = 0.02) (Table 2) were significantly reduced among PD. As previously reported (Sharma et al. 1998), five different HUMTH01 alleles (a, b, c, d and e) relating to 6, 7, 8, 9 and 9.3 TCAT repeats were observed in our group. A χ^2 analysis of all HUMTH01 alleles showed no significant differences ($\chi^2 = 7.45$, $df = 4$, p -value ≥ 0.11) between cases and controls. However genotype analysis showed that 'e' allele carriers were under-represented among PD cases (non-corrected OR = 0.66, 95% C.I. = 0.45-0.97, p -value = 0.04) (Table 2 and Supplementary Table S1).

***** Table 2 about here *****

Finally a logistic multivariate regression analysis including the rs680 'AA' genotype and HUMTH01 'e' carrier genotypes was carried out. This suggested that the majority of the *6 protective effect was associated with rs680 (OR = 0.72, 95% C.I. = 0.53-0.98, p -value = 0.03) (Table 3). The protective effect seen with HUMTH01 'e' allele carriers was reduced to a strong trend (OR = 0.68, 95% C.I. = 0.46-1.01, $p = 0.06$) (Table 3) suggesting that the e allele also contributes to the outcome, albeit to a lesser extent.

*** Table 3 about here ***

DISCUSSION

This is the first case-control analysis of *IGF2-INS-TH* haplotype in PD, although polymorphic variability in the *TH* gene has been previously investigated (Plante-Bordeneuve et al. 1994; Kunugi et al. 1998; Mizuta et al. 2006). Our results indicate that the expression of the common haplotype *6 reduces the risk for PD. Multivariate logistic regression modelling suggested that the rs680 'A' allele probably accounts for the majority of this effect as the significance of the HUMTH01 'e' carrier genotype was lost in this analysis (Table 3). There was modest linkage disequilibrium between the HUMTH01 and rs680 markers ($D' = 0.48$; $p = 0$) so it is possible that both may be proxies for the same functional entity with an influence on disease outcome. However, given that HUMTH01 'e' carrier status, adjusted for the influence of the rs680 genotype, continued to make a modest contribution to the outcome it seems more likely that there are at least two protective entities on the *6 background.

We did not find significant differences in the overall allele frequencies of the HUMTH01 microsatellite, consistent with previous negative case-control reports (Plante-Bordeneuve et al. 1994) (Supplementary Table S1). However HUMTH01 may act as a marker for causative variants rather than being a functional variant *per se*. In that case, the detection of subtle differences between common haplotypes in our study suggests that analysis of HUMTH01 alone gives an incomplete picture of the common genetic variation in and around the *TH* gene. While the 'e' allele of HUMTH01 features in the *6 haplotype, it is also seen on the *3 and *4 backgrounds, whose frequencies were similar between cases and controls in our study, thus diluting any independent association effect. This diluting effect could also explain the

variation seen in previous *in vitro* and *in vivo* studies assessing HUMTH01's effect on TH expression. One *in vitro* study revealed a length-dependent reduction in transcription up to eight repeats (c allele) but no further reduction or enhancement of transcription with larger repeats (Albanese et al. 2001). Their result suggests that the 'a' allele should be associated with the highest TH activity *in vivo*. However *in vivo* studies actually suggest that the longer 'e' allele has the highest enzyme activity (Sharma et al. 1998; Zhang et al. 2004), although this finding also lacks consistency (Wei et al. 1997).

TH gene variability has been linked to cardiovascular disease. The study of Sharma *et al.* (1998) showed that the 'e' allele was over represented in hypertensive individuals, consistent with *4 and *8 containing this allele being associated with elevated cardiovascular risk factors (Rodriguez et al. 2004). Cardiovascular risk factors such as increased blood sugar, triglycerides, total lipids and systolic blood pressure occur significantly less frequently in PD compared with controls (Haugarvoll et al. 2005; Scigliano et al. 2006), consistent with the observed *6 protective effect for PD (present study). Rather than a significant *TH* effect, Rodriguez *et al.* (2004) suggest that the long repeat insertion in the *INS* promoter contributes most to cardiovascular association with the *IGF2-INS-TH* haplotypes. In the present study we did not find any underlying association between the *INS* gene and PD.

Variability in the *TH* gene has also been linked to smoking behaviour. Smoking behaviour is negatively associated with PD (De Reuck et al. 2005; Wirdefeldt et al. 2005). In non-PD Caucasian cohorts the HUMTH01 'b' allele confers protection against tobacco dependence (Anney et al. 2004; Olsson et al. 2004). However we failed to see any over-representation of this allele in PD and were unable to find any differences in *IGF2-INS-TH* frequencies when smoking status and life-long cigarette smoking exposure in terms of pack-years were

investigated as dependent variables in our cohort (data not shown). This may be due to our cohort being selected for PD, although we included smoking status as a covariant in our association studies.

The *IGF2* gene or gene product has not been previously investigated in PD. The *IGF2* gene is imprinted in prenatal development but both alleles are expressed in the adult human brain (Pham et al. 1998). Our results suggest that a polymorphism in this gene or a causative entity in tight linkage disequilibrium with the rs680 'A' allele is protective for PD. The rs680 'A' allele has been associated with higher plasma IGF2 protein levels and lower body weights (O'Dell et al. 1997; Gaunt et al. 2001; Sandhu et al. 2003). Interestingly, low body mass index, potentially reflecting a wider metabolic disturbance, is observed in PD (Bachmann and Trenkwalder 2006), although plasma IGF2 levels have not been assessed in PD patients to our knowledge. The *IGF2* gene has also been linked to the neurodegeneration in Alzheimer's disease where reduced levels of insulin, IGF1, and IGF2 and their receptors transcripts are seen with advancing stages of the disease (Rivera et al. 2005).

As with all genetic association studies, there are many potential biases that can influence outcome and lead to false-positive findings, so replication of these findings in an independent sample is necessary for confirmation. The advantages of our study include the consistency of the genetic data from our tightly age and gender matched cases and controls, and the use of a validated questionnaire to assess non-genetic factors. In particular our genotyping data was comparable to other Caucasian cohorts for HUMTH01 (Anney et al. 2004; Olsson et al. 2004; Rodriguez et al. 2006; Sharma et al. 1998) and both SNPs (Rodriguez et al. 2006). Despite this, the rs689 SNP remained outside Hardy-Weinberg equilibrium in our control population even after replication. Admittedly our controls were a convenient sample of both electoral roll

volunteers and spouses, but the potential overmatching of the latter for environmental factors seems unlikely to affect our results.

Overall our results suggest that there is at least one common variant in the *IGF2-INS-TH* gene cluster that affects or is associated with susceptibility to PD. It remains unclear which gene in the cluster harbours this variant(s) and whether the effect is brain-specific or part of a more systemic syndrome.

ACKNOWLEDGEMENTS

We wish to thank Madelaine Ranola and Coral Gartner for assistance with case ascertainment, Tonia Russell and Gerhard Siebert for assistance with DNA processing, and Heather McCann, Francine Griffiths and Nadeeka Dissanayaka for laboratory assistance. The Australian Brain Foundation provided core funding for this project (LL, KD) with other research funds from NHMRC project grants #222727 (GH, DR) and #401537 (GM, GH, PS, DR), and the Geriatric Medical Foundation of Queensland (GM).

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Tables

Table 1. Common *IGF2-INS-TH* haplotypes for cases and controls

Label ^a	rs680-rs689-TH	%Freq. Ca (N)	%Freq. Co (N)
*1	G-A-a	23.4 (97.9)	21.9 (91.9)
*5	A-A-d	11.7 (49.0)	14.1 (59.3)
*2	G-A-b	16.4 (68.5)	13.4 (56.4)
*3	G-A-e	9.9 (41.4)	12.3 (51.6)
*4	G-T-e	12.1 (50.4)	11.4 (47.7)
*6^{bd}	A-T-e	5.1 (21.1)	11.0 (46.2)
*7	G-A-c	3.2 (13.6)	3.7 (15.6)
*8 ^{cd}	G-T-c	6.3 (26.4)	2.9 (12.0)
Others (*9-20)		11.9 (50.2)	9.3 (38.8)
PD-case control χ^2 analysis = 20.4, df =8, p-value $\leq$ 0.01			

^a haplotype nomenclature from Rodriguez *et al.*, 2004^b unadjusted OR = 0.42, 95% C.I. = 0.25-0.72, p = 0.001; Ref. = all other haplotypes^c unadjusted OR = 2.31, 95% C.I. = 1.17-4.56, p = 0.02; Ref. = all other haplotypes^d significant p-value = 0.006 corrected for 9 comparisons

Table 2. Genotype frequencies and analysis of individual polymorphisms

polymorphism		cases (%)	controls (%)	OR ^a	95% C.I.	p-value
<i>IGF2</i> -rs680	no 'A' allele	114 (54)	101 (48)	1.00		
	1 'A' allele	86 (41)	85 (40)	0.89	0.59-1.34	0.58
	2 'A' alleles	11 (5)	25 (12)	0.35	0.16-0.77	0.01
<i>INS</i> -rs689	no 'T' allele	117(55)	103 (49)	1		
	1 'T' allele	79 (37)	97 (46)	0.71	(0.48 – 1.07)	0.10
	2 'T' alleles	16 (8)	10 (5)	1.35	0.59 – 3.13	0.48
HUMTH01	no 'e' allele	104 (48)	83 (39)	1.00		
	1 'e' allele	90 (42)	105 (49)	0.67	0.45-1.01	0.06
	2 'e' alleles	21 (10)	27 (13)	0.59	0.31-1.14	0.12

^a adjusted for age, gender and smoking status by logistic regression modelling

Table 3. Results of multivariate logistic regression analysis

Risk factor	OR	95% C.I	p-value
age	1.00	0.98-1.03	0.82
gender	1.06	0.71-1.60	0.76
smoking status	0.78	0.52-1.17	0.22
HUMTH01 'e' allele carriers	0.68	0.47-1.03	0.07
<i>IGF2</i> -rs680 'AA' genotype	0.37	0.17-0.80	0.01

Figure Legend

Figure 1. Schematic of the IGF2-INS-TH gene cluster at Chr:11p15.5.

Insulin-like growth factor (*IGF*) 2, insulin precursor (*INS*) and tyrosine hydroxylase (*TH*) genes are found together at the telomeric end of the short arm of chromosome 11 (11p15.5) along with the *IGF2* anti-sense gene (*IGF2AS*) transcribed from the negative strand. Three polymorphisms tag the majority of common genetic variation in this region: *IGF2*-rs680 (SNP in 3'UTR of *IGF2*), *INS*-rs689 (SNP in intron 1 of *INS*) and HUMTH01 (tetranucleotide repeat in intron1 of *TH*).

Figure 1

Table S1: Allele and genotype frequencies and analyse

Genotypes					95% confidence intervals		
	Genotype	cases (%)	controls (%)	OR ^a	lower	upper	p-value ^b
<i>IGF2</i> -rs680	GG	114 (54)	101 (48)	1			
	AG	86 (41)	85 (40)	0.89	0.59	1.34	0.58
	AA	11 (5)	25 (12)	0.35	0.16	0.77	0.01
<i>INS</i> -rs689	AA	117 (55)	103 (49)	1			
	AT	79 (37)	97 (46)	0.71	0.48	1.07	0.10
	TT	16 (8)	10 (5)	1.35	0.59	3.13	0.48
	Genotype	cases (%)	controls (%)	OR ^a	lower	upper	p-value ^c
HUMTH01	others			1			
	e carriers	111 (52)	132 (61)	0.66	0.45	0.97	0.04
	ee	21 (10)	27 (13)	0.59	0.31	1.14	0.12
	c carriers	42 (20)	34 (16)	1.32	0.8	2.18	0.28
	cc	3 (1)	1(0.5)	n.d			
Alleles					95% confidence intervals		
	Allele	cases (%)	controls (%)	OR ^d	upper	lower	p-value
<i>IGF2</i> -rs680	G	314 (74)	287 (68)				
	A	108 (24)	135 (32)	0.73	0.54	0.99	0.04
<i>INS</i> -rs689	A	313 (74)	303 (72)				
	T	111 (26)	117 (28)	0.92	0.68	1.24	0.58
	Allele	cases (%)	controls (%)	OR ^e	upper	lower	p-value ^f
HUMTH01	a	114 (27)	104 (24)	1.16	0.76	1.77	
	b	79 (18)	63 (15)	1.24	0.74	2.06	0.54
	c	48 (11)	36 (8)	0.78	0.50	1.22	0.45
	d	57 (13)	68 (13)	0.76	0.53	1.08	0.23
	e	132 (31)	159 (31)	1.16	0.76	1.77	0.12
c2 = 7.45, df = 4, p ≤ 0.11							

^a odds ratios adjusted for age, gender and smoking status by logistic regression modelling^b significance level at p = 0.025^c significance level at p = 0.005 following correction for eight comparisons^d unadjusted odds ratios^e unadjusted odds ratios; ref = other alleles^f significance level at p = 0.01