Brief Genetics Report

Association Studies of Genetic Variation in the *WFS1* Gene and Type 2 Diabetes in U.K. Populations

Jayne A.L. Minton,[1] Andrew T. Hattersley,[2] Katharine Owen,[2] Mark I. McCarthy,[3] Mark Walker,[4] Farida Latif,[1] Timothy Barrett,[1] and Timothy M. Frayling[2]

Mutations in the *WFS1* gene cause β-cell death, resulting in a monogenic form of diabetes known as Wolfram syndrome. The role of variation in *WFS1* in type 2 diabetes susceptibility is not known. We sequenced the *WFS1* gene in 29 type 2 diabetic probands and identified 12 coding variants. We used 152 parent-offspring trios to look for familial association; the R allele at residue 456 ($P = 0.04$) and the H allele at residue 611 ($P = 0.05$) as well as the R456-H611 haplotype ($P = 0.032$) were overtransmitted to affected offspring from heterozygous parents. In a further cohort of 327 type 2 diabetic subjects and 357 normoglycemic control subjects, the H611 allele and the R456-H611 haplotype were present in more type 2 diabetic subjects than control subjects (one-tailed $P = 0.06$ and $P = 0.023$, respectively). In a combined analysis, the H611 allele was present in 60% of all diabetes chromosomes and 55% of all control chromosomes (odds ratio [OR] 1.24 [95% CI 1.03–1.48], $P = 0.02$), and the R456-H611 haplotype was significantly more frequent in type 2 diabetic subjects than in control subjects (60 vs. 54%, OR 1.29 [95% CI 1.08–1.54], $P = 0.0053$). Our results provide the first evidence that variation in the *WFS1* gene may influence susceptibility to type 2 diabetes. *Diabetes* 51:1287–1290, 2002

Wolfram syndrome (MIM 222300) (1) is a monogenic form of diabetes and neurodegeneration characterized by childhood-onset diabetes and optic atrophy. Diabetes and progressive optic atrophy present in the first decade, sensorineural deafness and cranial diabetes insipidus present in the second decade (2), and neuropathic bladder and neurological complications, including ataxia and psychiatric symptoms, present in the third and fourth decades (3). This form of diabetes is insulin deficient and nonautoimmune; postmortem studies have shown loss of β-cells (4). Obligate carriers have an increased prevalence of type 2 diabetes and deafness in some but not all studies (4–6).

The *WFS1* gene maps to chromosome 4p16.3 (7) and consists of eight exons (8). WFS1 is widely expressed in tissues, including brain and pancreas, and has been localized to the endoplasmic reticulum (9). A wide spectrum of loss of function mutations have been reported in affected patients, with no obvious relation between mutation and phenotype (10). The function of WFS1 is unknown, but it is thought to be involved in the survival of islet β-cells and neurons. A previous study of three Japanese cohorts of type 1 diabetic patients and control subjects identified three coding variants in strong linkage disequilibrium (LD), R456H, H611R, and I720V, in which the rare allele was present in more type 1 diabetic patients than control subjects (odds ratios [ORs] 1.80–2.04, $P = 0.0005$–0.0093) (11). Carriers of the H456 allele showed decreased frequencies of autoimmune characteristics (islet cell antibody or GAD autoantibody positivity and decreased frequencies of HLA-DRB susceptibility alleles) (11). This raised the suggestion that these variants (or further variants in LD with them) are associated with a nonautoimmune process of β-cell dysfunction.

There have been no studies of the role of the *WFS1* gene in type 2 diabetes. We hypothesized that variation in the *WFS1* gene may contribute to β-cell dysfunction and hence account for some of the genetic susceptibility of type 2 diabetes.

We screened the coding region of *WFS1* for variants in 29 patients with type 2 diabetes. These subjects were randomly selected from the Diabetes U.K./Warren parent-offspring trios collection (Table 1). Sequencing of the coding region and intron/exon boundaries revealed 12 coding variants and 2 noncoding variants (Table 2). We selected five of these variants for association studies: F341F, R456H, R611H, K774K, and S855S. The I720V variant found in Japanese subjects was not observed. These five variants were chosen to ensure alleles associated with diabetes in previous studies were represented and to represent all common haplotypes across the gene—we could distinguish all haplotypes with a frequency >0.05.

To determine the role of these variants and their haplotypes in type 2 diabetes susceptibility, we used family-based association methods in 152 parent-offspring trios.

From the [1]Section of Medical and Molecular Genetics, Department of Pediatrics and Child Health, The Medical School, University of Birmingham, Edgbaston, U.K.; the [2]Department of Diabetes and Vascular Medicine, School of Postgraduate Medicine and Health Sciences, University of Exeter, Exeter, U.K.; the [3]Imperial College Genetics and Genomics Research Institute and Division of Medicine, Imperial College, London, U.K.; and the [4]Department of Medicine, Medical School, University of Newcastle, Newcastle, U.K.

Address correspondence and reprint requests to Tim Barrett, Section of Medical and Molecular Genetics, Department of Pediatrics and Child Health, The Medical School, University of Birmingham, Edgbaston, Birmingham, B15 2TT U.K. E-mail: t.g.barrett@bham.ac.uk.

Received for publication 20 September 2001 and accepted in revised form 4 January 2002.

EH, Estimated Haplotype; LD, linkage disequilibrium; OR, odds ratio; TDT, transmission disequilibrium test.

TABLE 1
Clinical details of subjects studied

	Case subjects			
	Trios probands	YT2D	Type 2 diabetes	Control subjects
n	152	170	157	357
Male (%)	63	54	57	50
Age (years)*	40 (35–45)	40.6 (36.5–44.8)	56 (50–63)	32 (29–35)
BMI	30.8 (26.9–36.4)	30.4 (26.5–33.6)	27.1 (24.5–30.3)	26.7 (24.2–29.8)
Treatment				
(% diet/oral hypoglycemic agent/insulin)	21/64/15	12/36/53	15/65/20	—

Continuous data are given as median (interquartile range). *Age at diagnosis for case subjects, age at study for control subjects. YT2D, young onset type 2 diabetes.

Inclusion of other known types of diabetes in the probands was previously minimized through clinical, immunological (all subjects GAD autoantibody negative), and genetic tests (12).

Using the transmission disequilibrium test (TDT) (13), both the R456 and H611 alleles and the R456-H611 haplotype showed borderline significant overtransmission to affected offspring from heterozygous parents ($P = 0.04$, $P = 0.05$, and $P = 0.032$, respectively) (Table 3). No significant deviations from the expected 50% transmission rates were observed for the three synonymous variants. However, the haplotype formed by the common alleles at all five positions showed borderline-significant overtransmission ($P = 0.057$) (Table 3). These observations were not independent, as we observed strong LD among the five variants investigated ($P = 0.002$ for LD between H611*R* and R456H and $P < 0.000001$ for LD among all five variants).

To test these results further, we undertook a case-control study using an additional 323 patients with type 2 diabetes from two cohorts (170 young-onset subjects diagnosed at <45 years of age and 157 subjects with at least one affected sibling) and 357 unrelated control subjects. All subjects were of white U.K. origin living in the county of Devon, U.K. (Table 1). We did not replicate the association with the R456H variant: the R456 allele was present in 94% of type 2 diabetic subjects and 96% of control subjects. For the H611*R* variant, the H allele was present in 387/654 alleles (59%) from diabetic subjects compared with 392/714 alleles (55%) from control subjects (one-sided *P* value in the direction of TDT result, $P = 0.06$, OR 1.19 [95% CI 0.96–1.48]). The R456-H611 haplotype also occurred more frequently in diabetic subjects than in control subjects (one-sided $P = 0.023$). When combining all results, the H611 allele is present at a higher frequency on type 2 diabetes chromosomes compared with control chromosomes (60 vs. 55%, OR 1.24 [1.03–1.48], $P = 0.02$) (Table 4). The R456-H611 haplotype is also significantly more frequent in diabetic sujects than control subjects (60 vs. 54%, OR 1.29 [1.08–1.54], $P = 0.0053$). All cohorts, individually and combined, were consistent with Hardy-Weinberg equilibrium ($P = 0.41$ for all diabetic subjects and $P = 0.59$ for all control subjects).

TABLE 2
Variants found in screening 29 young-onset type 2 diabetic subjects

Exon	Codon no.	Nucleotide change	Frequency of rare allele
	Noncoding	A71184G	37/58 (63)
	Noncoding	C71190T	37/58 (63)
5	K193Q	A617C	1/58 (1.7)
6	R228R	G684C	17/58 (29.3)
8	I333V	A997G	4/58 (6.9)
8	F341F	C1023T	5/58 (8.6)
8	V395V	T1183C	24/58 (41.4)
8	R456H	G1367A	3/58 (5.2)
8	N500N	T1500C	6/58 (10.3)
8	H611R	A1832G	23/58 (39.6)
8	W648R	T1942C	1/58 (1.7)
8	K774K	G2322A	5/58 (8.6)
8	K811K	A2433G	20/58 (34.4)
8	S855S	G2564A	17/58 (29.3)

Frequency data are *n* (%).

We sought further evidence for a role of the *WFS1* H611 allele in diabetes susceptibility by assessing β-cell dysfunction in control subjects. Fasting specific insulin and glucose were available for 351 normoglycemic subjects (median age 32 years). Using log-transformed estimates of β-cell function from the HOMA program (version 2.0) (14) H611H611 homozygotes had reduced β-cell function compared with other subjects (130% [122.6–137.9] vs. 136.8% [131.6–142.4], $P = 0.148$), although this did not reach statistical significance, and the effect was weaker when all three genotypes were compared ($P = 0.23$).

Our results show evidence for a possible association between *WFS1* gene variants and type 2 diabetes in the U.K. This is the first evidence for a role of WFS1 in susceptibility to type 2 diabetes. Association studies of gene variants with complex diseases are fraught with difficulties, including potential population stratification, low a priori odds of finding a genuine association (15), and lack of replication. In this study, we tried to avoid these potential pitfalls in a number of ways, including use of family-based association tests to avoid population stratification, selection of variants previously associated with a similar disease process in a gene in which rare mutations are known to cause β-cell dysfunction, and assessment of nominally significant results in secondary cohorts. Despite this, our results only reached significance (at $P = 0.05$) when all data were compared; therefore, further replication is required to confirm or refute our findings.

There are a number of differences between our study and a recent Japanese study (11). In the Japanese study, the R611 allele is associated with type 1 diabetes, and in our study, the H611 allele is associated with type 2 diabetes. In addition, the H611 allele frequency differs

TABLE 3
TDT results from 152 parent-offspring trios

Codon	Allele/haplotype* (frequency)	Transmitted	Untransmitted	Nominal *P* value	Transmitted rate† (95% CI)
341	T (0.08)	24	17	0.27	0.59 (0.43–0.74)
456	R (0.94)	23	11	0.04	0.68 (0.52–0.83)
611	H (0.58)	87	63	0.05	0.58 (0.50–0.66)
774	A (0.07)	22	19	0.64	0.54 (0.38–0.69)
855	A (0.28)	52	66	0.20	0.44 (0.35–0.53)
	1-R-H-1-1 (0.54)			0.057	
	2-R-R-2-1 (0.06)			0.83	
	1-R-R-1-2 (0.27)			0.265	
	X-R-H-X-X (0.56)			0.032	
	X-R-R-X-X (0.38)			0.08	

*Amino acid symbol given if variant nonsynonymous. Analysis of haplotypes was limited to those consisting of all five variants that occur with a frequency >0.05, and those consisting of the two nonsynonymous variants that individually were nominally significant (and had a frequency >0.05). Allele 1, common allele; allele 2, rare allele. Haplotypes in sequence 341-456-611-774-855. †significance at 0.05 level if 95% Cls do not cross 0.5 (expected transmission rate).

greatly between the two populations (88.7% in Japanese vs. 55% in white U.K. population). Given the borderline-significant TDT result with the 1-R-H-1-1 haplotype (Table 3), the H611 allele may only be a marker of a disease susceptibility haplotype.

Currently, there are no functional studies to support our findings because the function of WFS1 is unknown. To increase our knowledge of β-cell metabolism, we need to establish the function of WFS1 and determine how variants alter β-cell function. Our findings have to be tested in other populations and if replicated, we need to quantify the risk of diabetes conferred by these variants.

RESEARCH DESIGN AND METHODS

Table 1 gives details of the subjects from whom DNA was used. Type 2 diabetic subjects were from three sources: the previously described Diabetes U.K./Warren (type 2 diabetes) parent-offspring trios collection (12), a collection of young-onset (defined as >25 and ≤45 years of age at diagnosis) type 2 diabetic subjects, and unrelated type 2 diabetic subjects who were found to have at least one affected sibling. All affected subjects were GAD autoantibody negative. Control subjects consisted of parents from a consecutive birth cohort with normal (<6.0 mmol/l) fasting glucose and/or normal HbA_{1c} levels (<6.0%; Diabetes Control and Complications Trial–corrected). Fasting specific insulin and glucose measurements were available for 98.3% of these control subjects, including 174 pregnant women at 28 weeks' gestation. All subjects were of white U.K. origin and lived in the county of Devon, U.K., with the exception of the trios probands, which consisted of white U.K. probands from throughout the U.K. and was collected at three U.K. centers (Devon, London, and Newcastle).

PCR amplification of coding region of WFS1. In the present study, exon 8 was divided into nine overlapping fragments, and the primers used were the same as those previously described (8), except for the following: 5′-TGGAGATGAAG GACAGGTAG-3′, 5′-TTCGCCTTCTTCATCCCGCT-3′, 5′-AGCAAGGACTGCATC CCCT-3′, 5′AACTGCACGCCCACCA-3′, 5′-TGGTGGGCGTGCTGCAGTT-3′, 5′-AGCAAGGACTGCATCCCCT-3′, 5-CTCCAGGATGGTGCTGAACT-3′, 5′-CAG CGAGTTCAAAA-3′, and 5′-GGGTGGAGATGGCATGCAAT-3′. PCRs were performed using *Taq* DNA polymerase (Gibco), and cycling conditions included an initial denaturation at 96°C for 5 min followed by 35 cycles at 95°C for 30 s. The annealing temperature was 60°C for exons 2–7, 54°C for exon 8 fragment 1, 51°C for exon 8 fragment 2, 58°C for exon 8 fragment 3, and 56°C for exon 8 fragments 4–9. Extension was at 72°C for 45 s and a final extension for 15 min at 72°C. $MgCl_2$ was used in PCR amplification at a final concentration of 1.5 mmol/l.

Direct sequence analysis. PCR products amplified as previously described from each exon were directly sequenced on both strands, if necessary, using a BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems).

Genotyping. PCR amplification was performed as previously described. Ten microliters PCR product was digested with 10 units restriction endonuclease (F341F alters a BcgI restriction site, R456H alters a BstUI restriction site, R611H alters a *Hha*I restriction site, K774K alters a ApoI restriction site, and S855S alters a HpHI restriction site) (New England Biolabs U.K., Hitchin, U.K.) in a 20-μl reaction at optimal temperature for >2 h, followed by resolution of fragments on a 2% agarose gel in Tris-borate/EDTA (TBE) electrophoresis buffer and ethidium bromide staining. Results are shown in Table 3.

Statistics. All *P* values quoted are two-tailed unless stated otherwise. The TDT in the parent-offspring trios for both individual *WFS1* variants and haplotypes was performed using TRANSMIT (16). Transmisson rates were

TABLE 4
Summary of pooled analysis of H611R

	Allele frequency		Genotype *n* (frequency)		
	n	H	HH	HR	RR
Case subjects					
Trios probands	152	0.62	61 (0.42)	65 (0.43)	26 (0.17)
YT2D	170	0.59	57 (0.34)	85 (0.50)	28 (0.16)
Type 2 diabetes	157	0.60	61 (0.39)	66 (0.42)	30 (0.19)
Total case subjects	479	0.60	179 (0.38)	216 (0.45)	84 (0.18)
Control subjects					
Untransmitted trios chromosomes	152	0.54	43 (0.28)	79 (0.52)	30 (0.20)
Population control subjects	357	0.55	115 (0.32)	162 (0.45)	80 (0.22)
Total control subjects	509	0.55	158 (0.31)	241 (0.48)	110 (0.22)
OR (95% CI)		1.24 (1.03–1.48)*			
P		0.020	0.07†		

*Based on allele frequency differences between case and control subjects; †based on a χ^2 test of all three genotypes between case and control subjects.

calculated using standard statistics for proportions. The significance of LD among variants was calculated using the maximum likelihood outputs from TRANSMIT.

For the follow-up case-control study, the significance of allele and genotype frequency differences was calculated using χ^2 analyses. Haplotype frequency differences were calculated using the maximum likelihood output from the Estimated Haplotype (EH) program (17,18).

For the pooled case-control study, the trios probands were used as case subjects, and the two untransmitted alleles in each trio were put together as a control, as with the haplotype-based haplotype relative risk statistic of Terwilliger and Ott (17). Allele and genotype frequency differences were then calculated using χ^2 analyses, with overall allele numbers used to calculate ORs and 95% CIs using 2×2 contingency tables. To assess the overall significance of the R456-H611 haplotype, estimated frequencies from the EH and TRANSMIT outputs were used to calculate actual numbers of haplotypes (R456-H611 versus the other three haplotypes formed by these two variants) in all case and control chromosomes. Although we tested five variants, we did not correct for multiple testing because alleles at two of the variants tested had previously been associated with diabetes and because strong LD existed across the gene (D' values between 0.36 and 0.95 for H611R versus the other four variants in TDT analysis), meaning allele associations were not independent of each other.

To examine possible associations with β-cell function in the control subjects, we used log-transformed percent of β-cell function values, calculated by the HOMA 2.0 program. ANOVA and *t* tests using SPSS (version 9.0) were used to compare log-transformed percent of β-cell function across genotypes while correcting for sex and age. The percentage of β-cell function values quoted are back-transformed.

ACKNOWLEDGMENTS

We gratefully acknowledge funding for this study from Diabetes U.K. as well as from a Children Nationwide and Royal Society Research grant (to J.A.L.M. and T.B.). T.M.F. is a career scientist of the South and West National Health Service Research Directorate.

We are very grateful for the assistance of our colleagues Diane Jarvis (DNA extraction), Susan Ayres (collection of samples from type 2 diabetic subjects), Beatrice Knight, and Tina Turner (collection of samples from control subjects) as well as Penny Clark (Birmingham; measurement of insulin) and Sian Ellard (laboratory organization).

REFERENCES

1. Wolfram DJ: Diabetes mellitus and simple optic atrophy among siblings: report of four cases. *Mayo Clin Proc* 9:715–718, 1938
2. Barrett TG, Bundey SE, Macleod AF: Neurodegeneration and diabetes: UK nationwide study of Wolfram (DIDMOAD) syndrome. *Lancet* 346:1458–1463, 1995
3. Kinsley BT, Swift M, Dumont RH, Swift RG: Morbidity and mortality in the Wolfram syndrome. *Diabetes Care* 18:1566–1570, 1995
4. Karasik A, O'Hara C, Srikanta S, Swift M, Soeldner JS, Kahn CR, Herskowitz RD: Genetically programmed selective islet beta-cell loss in diabetic subjects with Wolfram's syndrome. *Diabetes Care* 12:135–138, 1989
5. Fraser FC, Gunn T: Diabetes mellitus, diabetes insipidus, and optic atrophy. An autosomal recessive syndrome? *J Med Genet* 14:190–193, 1977
6. Ohata T, Koizumi A, Kayo T, Watanabe A, Monoh., Higashi K, Ito S, Ogawa O, Wada Y, Takada G: Evidence of an increased risk of hearing loss in heterozygous carriers in a Wolfram syndrome family. *Hum Genet* 103:470–474, 1998
7. Collier DA, Barrett TG, Curtis D, Macleod A, Arranz MJ, Maassen JA, Bundey S: Linkage of Wolfram syndrome to chromosome 4p16:1 and evidence for heterogeneity. *Am J Hum Genet* 59:855–863, 1996
8. Inoue H, Tanizawa Y, Wasson J, Behn P, Kalidas K, Bernal-Mizrachi E, Mueckler M, Marshall H, Donis-Keller H, Crock P, Rogers D, Mikuni M, Kumashiro H, Higashi K, Sobue G, Oka Y, Permutt A: A gene encoding a transmembrane protein is mutated in patients with diabetes mellitus and optic atrophy (Wolfram syndrome). *Nat Genet* 20:143–148, 1998
9. Takeda K, Inoue H, Tanizawa Y, Matsuzaki Y, Oba J, Watanabe Y, Shinoda K, Oka Y: WFS1 (Wolfram syndrome 1) gene product: predominant subcellular localization to endoplasmic reticulum in cultured cells and neuronal expression in rat brain. *Hum Mol Genet* 10:477–484, 2001
10. Hardy C, Khanim F, Torres R, Scott-Brown M, Seller A, Poulton J, Collier D, Kirk J, Polymeropoulos M, Latif F, Barrett T: Clinical and molecular genetic analysis of 19 Wolfram syndrome kindreds demonstrating a wide spectrum of mutations in WFS1. *Am J Hum Genet* 65:1279–1290, 1999
11. Awata T, Inoue K, Kurihara S, Ohkubo T, Inoue I, Abe T, Takino H, Kanazawa Y, Katayama S: Missense variations of the gene responsible for Wolfram syndrome (WFS1/wolframin) in Japanese: possible contribution of the Arg456His mutation to type 1 diabetes as a nonautoimmune genetic basis. *Biochem Biophys Res Comm* 268:612–616, 2000
12. Frayling TM, Walker M, McCarthy MI, Evans JC, Allen LI, Lynn S, Ayres S, Millauer B, Turner C, Turner RC, Sampson MJ, Hitman GA, Ellard S, Hattersley AT: Parent-offspring trios: a resource to facilitate the identification of type 2 diabetes genes. *Diabetes* 48:2475–2479, 1999
13. Spielman RS, McGinnis RE, Ewens WJ: Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am J Hum Genet* 52:506–516, 1993
14. Levy JC, Matthews DR, Hermans MP: Correct homeostasis model assessment (HOMA) evaluation uses the computer program (Letter). *Diabetes Care* 21:2191–2192, 1998
15. Risch NJ: Searching for genetic determinants in the new millennium (Review). *Nature Jun* 15:847–856, 2000
16. Clayton D: A generalization of the transmission/disequilibrium test for uncertain-haplotype transmission. *Am J Hum Gene* 65:1170–1177, 1999
17. Terwilliger JD, Ott J: *Handbook of Human Genetic Linkage*. Baltimore, MD, Johns Hopkins, 1994
18. Xie X, Ott J: Testing linkage disequilibrium between a disease gene and marker loci (Abstract). *Am J Hum Genet* 53:1107, 1993