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## Brief Genetics Report

# Analysis of Parent-Offspring Trios Provides Evidence for Linkage and Association Between the Insulin Gene and Type 2 Diabetes Mediated Exclusively Through Paternally Transmitted Class III VNTR Variable Number Tandem Repeat Alleles

Stewart J. Huxtable, Philip J. Saker, Lema Haddad, Mark Walker, Timothy M. Frayling, Jonathan C. Levy, Graham A. Hitman, Stephen O'Rahilly, Andrew T. Hattersley, and Mark I. McCarthy

Variation at the variable number tandem repeat (VNTR) minisatellite 5' of the insulin gene (INS) is associated with several phenotypes, including type 1 diabetes, polycystic ovary syndrome, and birth weight. Case-control studies have suggested that class III VNTR alleles are also associated with type 2 diabetes, but results have been inconsistent and may reflect population stratification. To explore further the role of the INS-VNTR in type 2 diabetes susceptibility, we used family-based association methods in 155 parent-offspring trios from the British Diabetic Association-Warren Trios repository, each ascertained via a Europid proband with type 2 diabetes. Overall, there was no significant association between diabetes and the INS-VNTR genotype, with 65 of 119 heterozygous parents (55%) transmitting class III and 54 class I ( $P = 0.16$ , one-sided). However, whereas maternal transmissions followed Mendelian expectation, there was a marked excess of class III transmission from the 49 heterozygous fathers (34 [69%] vs. 15,  $P = 0.003$  vs. 50% expectation,  $P = 0.003$  vs. maternal transmission). These results confirm that variation within the TH-INS-IGF2 locus, most plausibly at the VNTR itself,

influences type 2 diabetes susceptibility. By demonstrating that this effect is mediated exclusively by the paternally derived allele, these findings implicate imprinted genes in the pathogenesis of type 2 diabetes. *Diabetes* 49:126-130, 2000

**T**he variable number tandem repeat (VNTR) regulatory polymorphism 5' to the insulin gene (INS) has been implicated in several important human phenotypes, including type 1 diabetes (1), polycystic ovary syndrome (PCOS) (2,3), and size at birth (4). However, the role of INS-VNTR variation with respect to type 2 diabetes susceptibility has been much less clearly defined (1,5,6). In Europid populations, INS-VNTR allele lengths fall into two broad classes: class I (26-63 repeats) and class III (141-209 repeats). Existing case-control data have suggested that class III VNTR alleles are associated with a modest, but inconsistent, increase in risk of type 2 diabetes (odds ratio ~1.4) (1,6), and recent analyses of the Hertfordshire birth cohort suggest that this relationship becomes stronger (odds ratio ~4.6) if adjustment is made for birth weight and postnatal growth (6).

We were motivated to use parent-offspring trios to explore the relationship between variation within INS and type 2 diabetes for two reasons. First, all previous data (1,6) have come from case-control studies, which raises the concern that they may represent false-positive results arising through population substructure (7). Family-based association methods, correctly applied, are not vulnerable to this source of error, because they detect only those associations due to linkage (7). Second, marked parent-of-origin effects have been observed at INS-VNTR in studies of type 1 diabetes (1) and PCOS (3), and this study design allowed us to determine whether differences between paternal and maternal patterns of transmission were evident in type 2 diabetes.

The British Diabetic Association-Warren Trios Collection comprises parent-offspring trios ascertained through European (97% British/Irish) type 2 diabetic probands with both parents alive (8). Approximately 2.5% of our original cohort

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From the Division of Medicine, Imperial College School of Medicine, St. Mary's Hospital, and MRC-Clinical Sciences Centre, Hammersmith Hospital, London (S.J.H., P.J.S., L.H., M.I.M.); the Department of Medicine (M.W.), School of Medicine, Newcastle-upon-Tyne; the Department of Diabetes and Vascular Medicine (T.M.F., A.T.H.), School of Postgraduate Medicine and Health Sciences, University of Exeter, Exeter; the Diabetes Research Laboratories (J.C.L.), Radcliffe Infirmary, Oxford; the Medical Unit (G.A.H.), St. Bartholomew's and the Royal School of Medicine and Dentistry, London; and the Departments of Medicine and Clinical Biochemistry (S.O.), Addenbrooke's Hospital, Cambridge, U.K..

Address correspondence and reprint requests to Dr. Mark I. McCarthy, Reader in Molecular Genetics, Department of Metabolic Medicine, Imperial College School of Medicine (St. Mary's Hospital), Norfolk Place, London W2 1PG, U.K. E-mail: m.mccarthy@ic.ac.uk.

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S.J.H. and P.J.S. contributed equally to this work.

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HNF-1 $\alpha$ , hepatocyte nuclear factor-1 $\alpha$ ; MODY, maturity-onset diabetes of the young; PCOS, polycystic ovary syndrome; PCR, polymerase chain reaction; TRD, transmission ratio distortion; VNTR, variable number tandem repeat.

TABLE 1  
Demographic and anthropometric details in the 155 probands from the families

	Male probands	Female probands
n	97	58
Age at diagnosis (years)	41 (25–58)	39 (25–55)
Treatment		
Diet only	17 (18)	15 (26)
Oral agents	65 (67)	34 (59)
Insulin	15 (15)	9 (16)
Have parents with previously diagnosed diabetes (%)	43	40
Height (m)	1.77 (1.70–1.81)	1.63 (1.58–1.66)
Weight (kg)	95.7 (81.3–106.7)	90.0 (72.0–105.3)
BMI (kg/m <sup>2</sup> )	30.1 (26.9–33.9)	33.1 (26.9–39.1)
Waist-to-hip ratio	0.96 (0.93–1.00)	0.90 (0.85–0.97)

Data for age at diagnosis are medians (range); the other continuous variables are medians (interquartile range); data for treatment are n (%).

of type 2 diabetic subjects had two living parents, and ~1.3% were eventually recruited to the study. Inclusion of other known subtypes of diabetes (maturity-onset diabetes of the young [MODY], type 1 diabetes) was minimized through clinical, immunological, and genetic tests (RESEARCH DESIGN AND METHODS; 8). Apart from a relatively early age of diagnosis (median 40 years) arising from the need to ascertain both parents, proband characteristics are in line with those seen in other European type 2 diabetic populations (Table 1). The high rates of previously diagnosed diabetes in the parents, together with the early diagnosis of diabetes and obesity seen in the probands, suggest that these trios are enriched for genetic determinants of type 2 diabetes and obesity.

The INS-VNTR was typed using the –23 HphI polymorphism (9) in 155 complete parent-offspring trios. This biallelic polymorphism has been repeatedly shown to be in extremely tight linkage disequilibrium (>99.7% concordance) with VNTR class in Caucasians (1), and we have therefore inferred VNTR class directly from the HphI genotyping results.

Of 310 parents, 119 were heterozygous (I/III) and hence informative. Of these, 65 (55%) transmitted the class III allele

TABLE 2  
Class III transmission rates from heterozygous parents in 155 pedigrees typed for the INS-VNTR

	n	Transmitted (class III)	Transmitted (class I)	% Transmitted (class III)	P
Overall	119	65	54	55	0.16
Fathers	49	34	15	69	0.003
Mothers	54	23	31	43	NS

The overall n exceeds that for mothers and fathers, because there were eight pedigrees in which parent of origin could not be unequivocally determined. P values represent uncorrected one-sided binomial tests assuming Mendelian expectation (50% transmission). NS, not significant for one-sided test (due to deviation in favor of class I transmission); by two-sided binomial test, P = 0.28.

to their diabetic offspring (65 vs. 54, P = 0.16, assuming expectation of 50% transmission) (Table 2). In eight of the trios, all three members were heterozygous, and parental origin could not be unambiguously assigned. Transmissions from 54 heterozygous mothers and 49 fathers (in 91 trios) were therefore available for the study of parent-of-origin effects. In mothers, there was no deviation from Mendelian expectation (23 [43%] class III vs. 31 class I, P = 0.28, two-sided), whereas in fathers, we found a marked excess of class III transmission (34 [69%] vs. 15, P = 0.003 vs. 50% expectation). These parental differences in transmission were highly significant (P = 0.003, Fisher's exact test). Excess transmission of class III from fathers was seen in diabetic offspring of both sexes (male offspring: 21 class III transmissions out of 30 [70%]; female: 13 of 19 [68%]).

Parental differences in transmission probabilities of this type have generally been interpreted as evidence for the action of imprinted genes. However, it is clear that unequal transmission may arise through other mechanisms, including differential survival of parents with different genotypes, non-genetically mediated maternal prenatal transmission of disease risk (10), and parent-of-origin differences in transmission ratio distortion (TRD) (11). None of these alternatives provides a likely explanation of our findings. First, there was no support for differential survival effects in our data set: VNTR genotype distributions were similar in fathers and mothers (P = 0.78), and there was no significant relationship between genotype and paternal (P = 0.67) or maternal (P = 0.77) age at the time of study (Table 3). Second, reevaluation of the evidence for a paternal parent-of-origin effect using a logistic model that explicitly incorporates maternal effects (10) (Table 4) produced no diminution of the evidence for excess transmission of paternal class III alleles (model 1: likelihood ratio test = 9.5, df = 1, P = 0.002; model 2: likelihood ratio test = 10.3, df = 1, P = 0.001). Finally, in the absence of DNA from unaffected siblings, we could not directly test for TRD in our pedigrees; however, a study of >1,400 Caucasian families ascertained without respect to disease status found no parent-of-origin differences in TRD at this locus (11). Notably, this last study reported evidence for background TRD favoring class I transmission (54% class I vs. 46% class III), suggesting that the full extent of the excess paternal class III transmission in our study may have been underestimated by assuming Mendelian expectation. Background TRD also provides an explanation for the nonsignificant excess of class I transmission seen in mothers. Given exclusion of these alternative explanations, we conclude that the excess transmission of class III alleles represents a genuine genetically mediated type 2 diabetes susceptibility effect that is restricted to paternally derived alleles and therefore points toward parental imprinting as the etiologic mechanism.

We observed no significant associations between INS-VNTR genotypes in probands and available quantitative phenotypes (age at diagnosis, weight, BMI, waist circumference, and waist-to-hip ratio). (Additional information about these probands can be found in an online appendix at [www.diabetes.org/diabetes/appendix.asp](http://www.diabetes.org/diabetes/appendix.asp).) Similarly, none of these variables appeared to be related to the parental origin of the inherited VNTR alleles. In the absence of birth weight or postnatal growth data on the offspring, we were unable to determine whether the class III association is confined to "nonchangers" (those who follow their genetic growth tra-

TABLE 3  
INS-VNTR genotype distribution and age at study by genotype among trios' parents

	INS-VNTR genotype		
	I/I	I/III	III/III
Fathers			
n (%)	82 (53)	57 (37)	16 (10)
Age at study (years)	73.6 (69.5–77.7)	74.2 (67.7–79.7)	75.1 (69.4–79.5)
Mothers			
n (%)	82 (53)	62 (40)	11 (7)
Age at study (years)	71.8 (67.2–78.4)	72.5 (69.4–77.2)	73.5 (68.0–79.2)

Data are n (%) or median (interquartile range). Evidence that the difference between paternal and maternal transmissions is not due to differential survival effects is shown. There was no difference between mothers and fathers for either genotype ( $P = 0.78$ , Kruskal-Wallis exact test) or allele frequencies ( $P = 0.66$ , likelihood ratio  $\chi^2$  exact test). Likewise, there was no relationship between age at the time of study and VNTR genotype in fathers ( $P = 0.67$ ) or mothers ( $P = 0.77$ ).

jectory during infancy), as suggested by the analysis of the Hertfordshire birth cohort (6).

We have previously shown that VNTR-associated susceptibility to PCOS is similarly restricted to paternally derived class III alleles. In these studies (2,3), 20 of 26 (77%) heterozygous fathers transmitted class III to PCOS offspring but only 14 of 30 (47%) mothers did so (fathers:  $P = 0.003$  vs. Mendelian expectation). PCOS is a common endocrinopathy associated with multiple metabolic abnormalities, including defects in insulin secretion and action and a sevenfold increase in the prevalence of type 2 diabetes (12). The similarity of the INS-associated parental effects in these two closely related conditions reinforces our findings in the type 2 diabetic trios and supports the existence of common pathogenetic mechanisms underlying PCOS and type 2 diabetes.

TABLE 4  
Parent-of-origin likelihood ratio test analysis of transmission data from the 155 Warren trios

	Parameter estimates (95% CI)	Likelihood ratio statistic (df)	P
Model 1			
$I_M$	0.24 (0.09–0.61)	9.5 (1)	0.002
$S_1$	2.31 (1.13–4.73)	5.7 (1)	0.017
$S_2$	2.99 (0.83–10.78)	2.8 (1)	0.092
$S_1$ and $S_2$	—	5.8 (2)	0.056
Model 2			
$I_M$	0.26 (0.11–0.62)	10.3 (1)	0.0013
S	2.25 (1.11–4.57)	5.5 (1)	0.019

Logistic regression analysis was implemented to estimate parameters and derive likelihood ratio test results for the model proposed by Weinberg (10). In the general model (model 1), evidence for a parent-of-origin effect favoring paternal class III transmission is supported by the estimate of  $I_M = 0.24$ , which the likelihood ratio test confirms as significantly  $<1$  ( $P = 0.002$ ). Model 1 also provides borderline evidence for a prenatal maternal effect ( $S_1 = S_2 = 1$ , likelihood ratio test = 5.75,  $df = 2$ ,  $P = 0.056$ ). Re-estimation of parameters under model 2, which includes only a single S parameter (see RESEARCH DESIGN AND METHODS), makes little difference to the evidence for a paternal genetic parent-of-origin effect ( $P = 0.0013$ ) but provides clearer support for an independent maternal class III effect mediated via nongenetic mechanisms (likelihood ratio test = 5.48,  $df = 1$ ,  $P = 0.019$ ).

In this study, we used *HphI* genotypes to examine variation at INS-VNTR, relying on the very tight linkage disequilibrium between these sites in Europeans. There is substantial evidence implicating the VNTR as the main functional variant within the TH-INS-IGF2 locus responsible for the type 1 diabetes susceptibility effect (1). While detailed study of the region will be required to confirm that the same applies to type 2 diabetes and PCOS, the VNTR represents the strongest candidate for the association we have observed. The INS-VNTR lies immediately ( $<5$  kb) upstream of IGF2, a known mediator of fetal growth and development (13). IGF2 is maternally imprinted, and INS-VNTR variation has been shown to influence IGF2 transcription in human placenta (14). In pigs, IGF2 is a major quantitative trait locus determining muscle mass and fat deposition (15,16), phenotypes of relevance to type 2 diabetes pathogenesis. Taken together with the data presented here, we speculate that the diabetogenic effect of paternally derived class III alleles is mediated through transcriptional regulation of maternally imprinted genes, with IGF2 a prime candidate. Given the role of IGF2 in fetal growth (13), variation at the TH-INS-IGF2 locus may provide a genetic explanation for the observed relationship between intrauterine development and adult disease (17).

As described above, the trios studied were ascertained through highly selected type 2 diabetic subjects. We estimate that  $\sim 1.3\%$  of all subjects with clinical type 2 diabetes approached were eventually recruited into the study (8). This raises questions about the generalizability of our findings. This is a difficult issue to address directly, since other data sets that allow estimation of parent-of-origin effects are likely to be subject to similar ascertainment restrictions. However, we note that class III associations have been detected in several case-control studies with more representative patient cohorts (1,6). Because our family-based analysis indicates that these associations are not due to population stratification, these previous studies can be taken as evidence that INS-encoded susceptibility effects extend to typical type 2 diabetic populations. The relative weakness of the associations in these case-control studies may reflect the inability to discriminate maternal and paternal meioses rather than the selected nature of the Warren trios.

This study emphasizes the value of the parent-offspring trios study design, even in late-onset diseases in which ascertainment of parental samples can be extremely difficult. Apart from the well-known advantages of family-based asso-

ciation methods in avoiding the confounding effects of population stratification (7), recent insights into the biology of the INS-VNTR, including parent-of-origin effects (1,3), TRD (11), and paramutagenic interactions (18), have each followed directly from the application of this study design and would not have been detectable in a case-control (or sibling-control) study. Indeed, we suggest that conventional approaches to genetic analysis—including case-control and linkage studies—have, through dilution of the paternal class III effect by noncontributory maternal meioses, consistently underestimated the role played by variation at the TH-INS-IGF2 locus in the pathogenesis of type 2 diabetes. Transmission ratio distortion and misclassification of late-onset type 1 diabetic subjects within type 2 diabetic cohorts (both of which will favor class I transmission) may have compounded difficulties in detecting class III-associated effects. If parent-of-origin effects at type 2 diabetes susceptibility genes are widespread (as would be consistent with the observed excess maternal transmission of this condition [19]), identification and characterization of these loci will require development and implementation of analytical approaches that are sensitive to parental origin.

In summary, analysis of the insulin gene VNTR minisatellite in 155 European type 2 diabetic parent-offspring trios has demonstrated that variation at this regulatory element is a significant determinant of type 2 diabetes susceptibility. By demonstrating that susceptibility is exclusively mediated by paternally derived alleles, these data highlight the potential role of imprinted genes in the pathogenesis of type 2 diabetes.

#### RESEARCH DESIGN AND METHODS

Family data set. Analyses were performed on 155 parent-offspring trios from the British Diabetic Association–Warren (Type 2 Diabetes) Trios collection, each ascertained through a proband with a clinical diagnosis of type 2 diabetes and two living parents. More complete details of the ascertainment and characterization methods are available (8), but the key criteria were as follows: Proband had 1) a diagnosis of diabetes after age 25 years, and at least 1 year between diagnosis and recruitment; 2) all four grandparents of Caucasian (European) origin; and 3) clinical exclusion of type 1 diabetes, MODY, and mitochondrial diabetes on the basis of personal and family history. Informed consent was obtained from all family members for extraction of genomic DNA and subsequent genetic analysis; only complete trios were included. Family relationships were confirmed with a panel of five microsatellite markers from chromosomes 7 and 20 with a cumulative heterozygosity >99.5%. A screen for mutations at hepatocyte nuclear factor-1 $\alpha$  (HNF-1 $\alpha$ ) position 291, the single most common site for MODY mutations in the U.K., was negative in all probands (20). All samples were tested for presence of antibodies to GAD in the laboratory of Prof. Franco Bottazzo, Royal London Hospital, by means of previously described methods (21). On the basis of control population data, probands with anti-GAD titers >10 U (range 13–318) were excluded. Clinical characteristics of probands from the 155 GAD<sup>+</sup> trios studied are provided in Table 1.

Genotyping. Analysis of the insulin gene was carried out with the –23 bp A/T allelic polymorphism, which alters an HphI restriction site (9). Polymerase chain reaction (PCR) amplification of a 441-bp region spanning the –23 HphI polymorphism was performed in a 30- $\mu$ l reaction containing 50 ng genomic DNA, 22 pmol each primer (INS04 5'-tccaggacagcgtcatcg-3' and INS05 5'-agcaatggcgggtgacctca-3'), 0.75 U Taq DNA polymerase, and 1.5 mmol/l Mg<sup>2+</sup>. PCR amplification was performed under the following conditions: denaturation at 94°C (4 min), followed by 38 cycles of 94°C (30 s), 64°C (30 s), and 72°C (40 s), with a final extension at 72°C (5 min). Then, 9  $\mu$ l PCR product was digested with 5 U HphI restriction endonuclease (New England Biolabs [U.K.], Hitchin, U.K.) in a 25- $\mu$ l reaction at 37°C for >2 h, followed by resolution of fragments on a 3% agarose gel in Tris-borate/EDTA (TBE) electrophoresis buffer and ethidium bromide staining. All analyses were performed in duplicate and discrepant results (<2%) retyped. VNTR classes were inferred on the basis of extremely tight linkage disequilibrium (>99.5% concordance) between the –23 HphI polymorphism and VNTR class in Caucasian populations, as repeatedly demonstrated in studies of type 1 diabetes (1). Statistical analyses. The transmission/disequilibrium test was used to assess evidence for cosegregation between affection status in the offspring and HphI alle-

les (7). Only a single affected offspring (the proband) was studied in each family. The significance levels of deviations from expectation were evaluated using binomial tests (midpoint) and are, unless otherwise indicated, one-sided given the prior evidence favoring a class III association. Given previous evidence of parent-of-origin effects in type 1 diabetes and PCOS (1,3), detection of such effects was one of our primary end points; however, we made no prior assumptions concerning the direction (paternal or maternal) of any parent-of-origin effects given conflicting data in type 1 diabetes (1).

The parent-of-origin likelihood ratio test formulated by Weinberg (10) was used to confirm that observed differences between paternal and maternal transmissions were not the result of prenatal (nongenetic) maternal transmission effects. The parameters in the general model (model 1) are  $I_M$  (representing the parent-of-origin effect, and parameterized such that  $I_M$  is <1 if a paternally derived copy of a class III allele is associated with a greater increase in risk than a maternally derived copy),  $S_1$ , and  $S_2$  (representing the effects of nongenetic transmission of disease risk associated with one and two maternal copies of a class III allele, respectively). Parameter estimates were obtained by logistic regression implemented within SPSS for Windows (version 8.0; SPSS, Chicago) (10). Evidence for a paternal imprinting effect is obtained by demonstrating that  $I_M$  is <1. Because the relatively small number of class III homozygotes made estimates of the  $S_2$  parameter imprecise, we also implemented a second model (model 2) that included only a single S parameter (i.e.,  $S_1 = S_2 = S$ , equivalent to the assumption of a dominant maternal prenatal effect).

Quantitative trait-association studies were performed using SPSS for Windows (version 8) after transformation of variables (where appropriate). Male and female probands were analyzed separately. Due to the multiple tests conducted, we adopted an a priori threshold for significance of  $P < 0.01$  for each analysis to control the type 1 error rate (see online appendix at [www.diabetes.org/diabetes/appendix.asp](http://www.diabetes.org/diabetes/appendix.asp)).

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