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A common mitochondrial DNA variant is associated with insulin resistance in adult life

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Summary Mitochondrial DNA is maternally inherited. Mitochondrial DNA mutations could contribute to the excess of maternal over paternal inheritance of non-insulin-dependent diabetes mellitus (NIDDM). We therefore investigated the relationship between this variant, insulin resistance and other risk factors in a cohort which had been well characterised with respect to diabetes. Blood DNA was screened from 251 men born in Hertfordshire 1920--1930 in whom an earlier cohort study had shown that glucose tolerance was inversely related to birthweight. The 16189 variant (T- > C transition) in the first hypervariable region of mitochondrial DNA was detected using the polymerase chain reaction and restriction digestion. DNA analysis showed that 28 of the 251 men (11 %) had the 16 189 variant. The prevalence of the 16189 variant increased progressively with fasting insulin concentration ($p < 0.01$). The association was independent of age and body mass index and was present after exclusion of the patients with NIDDM or impaired glucose tolerance. We found that insulin resistance in adult life was associated with the 16 189 variant. This study provides the first evidence that a frequent mitochondrial variant may contribute to the phenotype in patients with a common multifactorial disorder. [Diabetologia (1998) 41: 54--58]

Keywords Insulin resistance, mitochondrial DNA, mitochondrial diabetes.

Mutations in mitochondrial DNA (mtDNA) are of potential importance in the pathophysiology of non-insulin-dependent diabetes mellitus (NIDDM) [1]. As mtDNA is exclusively maternally inherited, maternal transmission of mtDNA mutations could explain the excess mother to offspring transmission of NIDDM which has been observed in several populations. The commonest single mutation of mtDNA which may lead to diabetes is located at bp 3243G:C [1] relative to the reference sequence [2], and is associated with mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) [3]. However, the 3243 mutation which has been found in up to 2 % of Caucasian familial NIDDM patients [4] is too rare to account for the excess of maternal over paternal transmissions.

There are several potential mechanisms whereby mitochondrial dysfunction could cause diabetes. Since mtDNA encodes respiratory chain enzymes, mtDNA mutations may impair the production of ATP which plays a central role in insulin release [5, 6], and impede beta-cell development [7]. Secondly, defects in oxidative phosphorylation in insulin-sensitive tissues could impair insulin action. Thirdly, because insulin has a central role in fetal growth and because inherited defects in insulin action have been shown to impair fetal growth, maternally inherited mtDNA defects could provide a novel genetic mechanism explaining the recently described association between reduced fetal growth and NIDDM in adult life [8].

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Abbreviations: NIDDM, Non-insulin-dependent diabetes mellitus; MELAS, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes; IGT, impared glucose tolerance.

Wild type (normal) sequence	ccccctcccc	No length variation
Example 1 with 16189 variant	{ ccccccccc { cccccccc { ccccccccccc { cccccccccccc	Length variation with modal length 10
Example 2 with 16189 variant	{ cccccccccccc { ccccccccc { cccccccccc { ccccccccccc { cccccccccccc { ccccccccccccc	Length variation with modal length 12
Example 3 with two point mutations t → c at 16189 c → t at 16192	ccccccctcc	No length variation

Fig. 1. Examples of DNA sequences around bp 16 189 including two examples of the 16 189 variant. This figure shows examples of variations from the wild type (normal) mtDNA sequence from 16 184 to 16 193 in the region around bp 16 189 in three individuals to illustrate heteroplasmic length variation identified in earlier studies [11]. A t- > c transition at bp 16 189 often generates heteroplasmic (that is more than one distinct population of mtDNAs in the same individual) length variation (for instance, C tracts of lengths 9,10,11 and 12, mode 10 as in example 1; or lengths 9, 10, 11, 12, 13 and 14, mode 12 as in example 2) which we name the 16 189 variant. Note that this contrasts with heteroplasmic mtDNA mutations in that the wild type (normal sequence) is not present. The distribution of length variants is very similar in all tissues. The length variation can be stabilised in individuals with a c- > t mutation elsewhere in the tract as in example 3

We recently sequenced 400 bp of the large non-coding region of mtDNA, in order to investigate the role of commoner mtDNA variants (polymorphisms) in a group of patients with the 3243G:C mutation associated with MELAS, many of whom had mitochondrial diabetes [9]. We showed that a variant at bp 16 189, which gives rise to both an uninterrupted tract of 8--12 cytosines and to heteroplasmic length variation (mtDNAs of different lengths in the same individual, see Fig. 1), had a much higher incidence in these patients (5/9 or 55 %) than in control subjects (25/273 or 9 % [10]) or in other patients with mitochondrial disease [11]. We suggested that the 16 189 variant may indicate a propensity for the occurrence or persistence (fixation) of the 3243G:C mutation, and perhaps of other $tRNA^{leu}$ mutations and mutations associated with diabetes [11]. Unlike the 3243G:C mutation, the 16 189 variant is common. It is found in approximately 9 % of haplotypes in the United Kingdom and in up to 96 % in some Pacific populations [10], where NIDDM may be very common. We therefore decided to assess the prevalence of this mutation in a cohort of men aged 64 years born in Hertfordshire, UK, who had undergone oral glucose tolerance tests and whose birth and infant weights had been recorded [8].

Subjects and methods

In the county of Hertfordshire, from 1911 onwards the midwives who attended the birth of each child recorded the birthweight. Health visitors visited the babies periodically throughout infancy and recorded their weight at one year. We have previously reported the associations between low birth weight and raised blood pressure and glucose tolerance in a group of 370 men born during 1920–1930 [8].

Blood for DNA analysis was available on 251/370 (68 %) of these 64-year-old men from this cohort [8], of whom 59/251 (23 %) had impaired glucose tolerance (IGT) or NIDDM defined by WHO criteria [8] which is comparable with other studies [12]. DNA was extracted using a Nucleon Kit (Scotlab, Scotlab -- Coatbridge, Lanarkshire, Scotland) and amplified using PCR as previously [11]. For the purposes of this study we have used the term "the 16 189 variant"" to identify individuals with an uninterrupted C tract around bp 16 189. This mutation appears to destabilise the mtDNA in this region as it usually generates length variation (Fig. 1) [11, 13]. Sequence analysis [9] was used in 61 cases (including 22 of 59 (32 %) of the diabetic patients and 12 of 28 (43 %) of the patients with the 16 189 variant). In the remaining 190 cases, PCR and restriction enzyme digestion were used to detect the 16 189 variant, because this technique is less labour intensive. PCR and restriction digestion with *Mnl* 1 were carried out as previously described [11]. The presence of the 3243G:C mutation was investigated in the 28 men with the 16 189 variant using standard methods [11, 14].

Clinical variables were previously investigated in this cohort [8]. The subjects' blood pressures were measured at home with an automated recorder (Dinamap, Dinamap, model 18465X; Criticon, Tampa, Florida, USA). Their heights were also measured, with a portable stadiometer, and their weights with a portable SECA scale. The ratio of waist to hip circumference was recorded as a marker of central obesity. Smoking habits and alcohol consumption were recorded. Following the home visit the men attended a clinic at 09.00 hours in the morning, after an overnight fast, and a standard 75 g oral glucose tolerance test was performed. Plasma glucose concentrations were measured by the hexokinase method and plasma insulin, proinsulin and 32--33 split proinsulin concentrations were determined by specific two-site immunometric assays.

Statistical analysis. We analysed the data using tabulation of means and two-sample *t*-tests. Concentrations of insulin and glucose were transformed to normality in the analysis by using logarithms. Homeostasis model assessment (HOMA) which is

Table 1. Age, obesity, glucose tolerance and blood pressure in 251 men with and without the 16 189 variant

	Age (years)	BMI (kg/m^2)	Waist to hip ratio	Fasting insuln (pmol/l)	Proinsulin (pmol/l)	32--33 split pro-insulin (pmol/l)	HOMA insulin resistance	HOMA beta-cell function	Fasting glucose (mmol)	2 h plasma glucose (mmol)	Systolic blood pressure (mm Hg)
Normal mtDNA (n = 223)	64.1 ± 3.1	26.9 ± 3.6	0.93 ± 0.05	40.5 ± 1.9[a]	2.9 ± 2.0[c]	3.1 ± 2.1[c]	1.92 ± 1.9[b, c]	58.0 ± 1.9[c]	6.18	6.6 ± 1.4[c]	163 ± 22
16 189 variant (n = 28)	64.0 ± 3.1	27.0 ± 3.3	0.94 ± 0.07	57.4 ± 1.8[a, c]	3.2 ± 2.2[c]	3.5 ± 2.3[c]	2.67 ± 2.1[b, c]	75.7 ± 1.6[c]	6.03	7.2 ± 1.4[c]	165 ± 27

Data are mean ± SD
[a] $p < 0.01$; [b] $p < 0.02$; [c] Geometric standard deviation

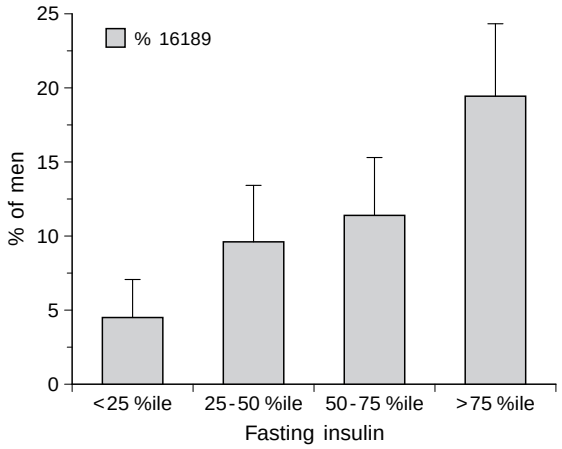

Fig. 2. Prevalence of the 16 189 variant by fasting insulin quartiles. Bar chart showing that the proportion of individuals with the 16 189 variant increases by fasting insulin quartile (comprising 3, 6, 7 and 12 individuals out of 63 from lower to upper quartile). Error bars represent one standard error. There is a significant difference between the quartiles (p = 0.008)

based on fasting glucose and insulin concentrations [15] was used to assess insulin secretion. It was also used to assess insulin sensitivity as glucose clamping was not feasible in a cohort study. The sum of the fasting insulin, proinsulin and 32--33 split proinsulin concentrations was used to estimate the total immunoreactive insulin concentrations required in this model [16]. Multiple logistic and linear regression analysis were used to assess the relationship between the prevalence of the 16 189 variant and glucose tolerance and to allow for the effect of confounding variables. *P* values were calculated by using the variables as continuous where appropriate. As the prior hypothesis of this study was to investigate any association between the 16 189 variant and intermediate phenotypes and risk factors for NIDDM, no correction for multiple testing was required.

Results

Blood was available for DNA analysis on 251 of 370 (68 %) of the men in this cohort who were representative of the whole group on all parameters investigated. The age range was 59--70 (mean 64 years) and 28 of 251 (11.2 %) had the 16 189 variant. The variant was present in 19 of 192 or 9.9 % of the normoglycaemic men compared with 9 of 59 or 15 % of the men with NIDDM or IGT ($\chi^2 = 1.31$, $p = 0.25$). The prevalence of NIDDM or IGT was 9 of 28 (32 %) in men with the 16 189 variant compared with 50 of 223 (22 %) of the remainder (comprising 3/28 compared with 13/223 with established NIDDM and 6/38 and 35/223 with IGT, respectively). The prevalence of the 16 189 variant was 3 of 18 (17 %) in established NIDDM, 6 of 41 (15 %) in men with IGT and 19 of 192 (9.9 %) in men with normal glucose tolerance. As the tendency for individuals with the 16 189 variant towards NIDDM or IGT did not reach significance (odds ratio 1.64 [0.64--4.12]) due to the small size of the cohort, intermediate phenotypes were investigated for an association. Compared with the normal subjects, men with the 16 189 variant had higher fasting insulin levels (40.5 and 57.4 pmol/l respectively, $p < 0.01$, odds ratio for having a fasting insulin above the mean = 2.42 [1.05--5.57]). Men with the 16 189 variant were more insulin resistant as defined by HOMA analysis (using logarithmic transformation, $p < 0.02$) although they were of similar age and degree of obesity (Table 1). The proportion of men with the 16 189 variant increased with higher fasting insulin quartiles (Fig. 2) and the association between the variant and fasting insulin remained significant after exclusion of the 59 men with NIDDM and IGT (mean fasting insulin in men with and without the variant was 53.2 and 38.2 pmol/l, respectively, $p = 0.03$).

The levels of proinsulin, split proinsulin and the 2 h plasma glucose and insulin levels were higher but not statistically different in men with the 16 189 variant. The variant was unrelated to insulin secretion. Using logistic regression, the association between the variant and either fasting insulin or HOMA was independent of age, body mass index, waist to hip ratio and the presence of glucose intolerance or diabetes.

The 16 189 mutation was unrelated to either birthweight or infant weight. We used multiple regression to explore the simultaneous relationships of fasting insulin with the 16 189 variant, obesity, age and birthweight as follows. The association between the mito-

chondrial mutation and fasting insulin remained statistically significant ($p = 0.009$), as did body mass index (BMI, $p = 0.001$), waist to hip ratio ($p = 0.004$) but not age or birthweight. However, in a further regression analysis exploring the relationship between 2 h plasma glucose, birthweight age, obesity and the variant, the 2 h plasma glucose in the men was related to birthweight ($p = 0.01$), age ($p = 0.02$), BMI ($p = 0.04$), waist to hip ratio ($p = 0.04$) but not the 16 189 variant. The 3243G:C mutation was not detected in any men (sensitivity < 1 % of the blood mtDNA).

Discussion

In this cohort of men [8] we have found that the 16 189 variant (an mtDNA polymorphism) is associated with two related measures of insulin resistance, raised fasting insulin and HOMA score ($p < 0.01$). However, we found no relation between the variant and birthweight. The 16 189 variant was associated with fasting insulin but not glucose intolerance, while reduced fetal growth was associated with IGT but not with fasting insulin. The effects of the mitochondrial polymorphism appear to be independent of birth weight, current obesity, its known association with the 3243 G:C mutation or other confounding variables. It is unlikely that inclusion of a distinctive genetic stock with a high prevalence of the 16 189 variant within the cohort underlies the association as almost all were Anglo-Saxon Caucasian. This suggests that mtDNA plays a substantial role in the genetic component of insulin resistance, but not in the relationship between fetal growth and NIDDM. This is consistent with current views on the multifactoral aetiology of NIDDM.

While the association between the 16 189 variant and insulin resistance was significant, the association between 16 189 and IGT or NIDDM was not, presumably because of the small size of the study. However, when we compared the prevalences of diabetes [10, 17--19] and of the 16 189 variant we observed a significant positive association in high and low income populations (Pearson correlation $p < 0.01$ and < 0.05 respectively, unpublished). The 16 189 variant is hence a candidate genetic determinant of the thrifty genotype [20]. This suggests that patients with the 16 189 variant may progress to IGT or NIDDM in the presence of additional factors, such as obesity or impaired insulin release.

Potential mechanisms for the association between the 16 189 variant and insulin resistance could be causal or indirect. Firstly the 16 189 variant may be causal because it is mutagenic, either by its ability to generate local length variation or by destabilising distant sites. In bacteria, length variation in repetitive sequences in gene control regions can generate profound changes in the expression of gene products, within a single generation, and hence promote evolutionary flexibility. By analogy, if the region around bp 16 189 lay within a hitherto unrecognised control region for mtDNA, mutability could enable metabolic thrift. Alternatively, the 16 189 variant could be a causal risk factor for a diabetogenic mtDNA mutation, perhaps by influencing the mutation rate elsewhere in the genome. It is clear that point mutations in nuclear DNA can alter local DNA stability and hence mutation rate. By analogy with work on minisatellite repeats, single base pair changes may alter the mutation rate at a distant site profoundly, perhaps by influencing the binding of proteins involved in recombination/repair of DNA. The 16 189 variant would thus be a premutation for a pathogenic mtDNA mutation.

On the other hand, the relationship between insulin resistance and the 16 189 variant may be indirect by association with diabetogenic mitochondrial or nuclear factors. The 16 189 variant may lie within a single major mtDNA haplotype which carries a diabetogenic mtDNA mutation elsewhere in the genome (much as a nuclear polymorphism might be in linkage disequilibrium with a diabetogenic gene). This is unlikely because we found several different haplotypes in a preliminary study of the 16 189 patients in this series. Finally, the association between the 16 189 variant and insulin resistance may be indirect, both being secondary to a nuclear-encoded factor, for instance, variants in nuclear genes involved DNA repair.

Previous studies have demonstrated both beta cell dysfunction and decreased insulin sensitivity in patients with diabetes associated with the 3243 mutation [21]. Sequential follow-up of such patients documented decreased sensitivity to insulin prior to the development of insulin deficiency (and hyperglycaemia-related reversible insulin resistance was clearly not the prime mechanism) [21]. If insulin-sensitive glucose uptake into skeletal muscle were ATP-dependent, one could envisage that reduced sensitivity to insulin could be a consequence of minor mitochondrial dysfunction, and deficiency only occur with a severe defect. Possible mechanisms include a reduction in hexokinase II activity. Recently, the ATP-sensitive K channel has been implicated in mediating the action of insulin: variants of the Kir 6.2 component of the channel (which is expressed in skeletal muscle as well as beta cells) appear to influence insulin sensitivity rather than insulin release in normal control subjects [22]. ATP availability in skeletal muscle could similarly influence insulin sensitivity. Furthermore, peripheral effects of insulin may be involved as increased expression of mitochondrial genes has been demonstrated in skeletal muscle of diabetic patients compared with control subjects.

In conclusion, our study suggests that the 16 189 mitochondrial variant contributes to insulin resistance in adult life, which is important in the

development of NIDDM. This study is the first evidence that mitochondrial variants may contribute towards the phenotype in a substantial proportion of patients with a common multifactorial disorder.

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