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META-ANALYSIS

The association of the mitochondrial DNA OriB variant (16184–16193 polycytosine tract) with type 2 diabetes in Europid populations

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Abstract
Aims/hypothesis The association between the mitochondrial DNA 16181–16193 polycytosine variant (known as the OriB variant as it maps to the OriB origin of replication) and type 2 diabetes has not been reliably characterised, with studies reporting conflicting results. We report a systematic review of published literature in Europid populations, new data from the Norfolk Diabetes Case–Control Study and a meta-analysis to help quantify this association.
Methods We performed a systematic review identifying all the studies of the OriB variant and type 2 diabetes in Europid populations published before January 2013. We typed the OriB variant by pyrosequencing and sequencing in the Norfolk Diabetes Case–Control Study, which comprised 5,574 type 2 diabetes cases and 6,950 population-based controls.
Results Overall, the meta-analysis included eight published studies plus the current new results, with a total of 11,794 type 2 diabetes cases and 14,465 controls. In the Norfolk Diabetes Case–Control Study, the OR for type 2 diabetes for the OriB variant was 1.09 (95% CI 0.96, 1.24). In a combined analysis, the relative risk for type 2 diabetes for the OriB variant in Europid populations was 1.10 (95% CI 1.01, 1.20; $p=0.03$)
Conclusions/interpretation Results from this systematic review and meta-analysis suggest that the mitochondrial DNA OriB variant is modestly associated with an increased risk of type 2 diabetes in Europid populations, with an effect size comparable with that of recently identified variants from genome-wide association studies.

Keywords Europid populations · Meta-analysis · Mitochondrial DNA · OriB variant · Pyrosequencing · Systematic review · Type 2 diabetes

Abbreviations

EPIC	European Prospective Investigation of Cancer
GWAS	Genome-wide association studies
mtDNA	Mitochondrial DNA
NCR	Non-coding region
polyC	Polycytosine

Introduction

Recent genome-wide association studies (GWAS) have identified many common variants in the nuclear genome that are associated with type 2 diabetes [1–3]. However,

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the identified variants explain only a small proportion of the heritability of type 2 diabetes. The unexplained heritability could be due partly to interactions with environmental and lifestyle factors, rare variants, epigenetic factors or possibly mitochondrial DNA (mtDNA) variants. Mutations in mtDNA such as the 3243A\G mutation in the mtDNA-encoded $tRNA^{Leu(UUR)}$ gene (also known as *MTTL1*) are associated with maternally inherited diabetes. Even though this mutation has a prevalence of 1 in 400, it causes fewer than 2% of type 2 diabetes cases [4, 5]. As with many other monogenic forms of diabetes, it is possible that extreme functional mutations in mitochondrial DNA cause syndromic forms of diabetes while less severe common variants are more weakly associated with typical type 2 diabetes.

A common mtDNA variant that results in a T to C substitution at the nucleotide position 16189 (also called the 16189 variant) lies in the hypervariable D-loop region of mtDNA for replication and transcription [6]. The mtDNA 16189 T>C transition frequently produces an uninterrupted polycytosine (polyC) tract between nucleotide positions 16184 and 16193 that is susceptible to replication errors in the important regulatory D-loop region. Because this variant maps precisely to the OriB origin of replication, we renamed it the 'OriB variant'. In common with other homopolymeric C tracts in mtDNA [7], when the unbroken tract exceeds 11 bp it generates heteroplasmic length variation. As well as this alteration, the mtDNA becomes less susceptible to DNase I, though this may be because of changes in DNA secondary structure or protein binding [8].

The frequency of the OriB variant differs in ethnic groups, with 10% in Europids, 30% in Asians, 50% in Pima Indians and >95% in Polynesians; this distribution has intriguing similarities with the prevalence of diabetes [9]. The association of this variant with type 2 diabetes has been studied previously, with apparently conflicting results [8–27]. A previous review of four studies with a total of 1,455 type 2 diabetes cases and 3,132 controls reported that the OriB variant was not associated with type 2 diabetes in European populations [18]. However, most of those data were from relatively small studies and although the meta-analysis was able to exclude a very strong association in European populations, it could not exclude the possibility that the variant was modestly associated. A modest association can only be detected by large, well-powered studies.

Our reassessment of the association of the OriB variant with type 2 diabetes used the following approach to maximise power and minimise bias: (1) we investigated the association between the OriB variant and type 2 diabetes in a large European population (the Norfolk Diabetes Case–Control Study), which included 5,574 type 2 diabetes cases and 6,950 healthy controls; and (2) we conducted a meta-analysis of studies of the OriB variant with type 2 diabetes in Europid populations (e.g. European continental ancestry), involving a total of 11,794 type 2 diabetes cases and 14,465 controls, over five time as many participants as in the previous review [18].

Methods

Norfolk Diabetes Case–Control Study The Norfolk Diabetes Case–Control Study is a study of men and women with type 2 diabetes in Norfolk. All type 2 diabetes patients identified through general practice diabetes registers in Norfolk and local hospital diabetes clinic and retinal screening programme patient registers were invited to participate. A total of 5,574 cases reporting British, Irish and/or other white ethnic origin were included in the current analyses. Other forms of diabetes were excluded by restricting cases to those without insulin use during the first year of diagnosis, and those without cystic fibrosis, chronic pancreatitis or long-term steroid use. A total of 6,950 controls free of known diabetes at baseline or during follow-up were randomly selected from European Prospective Investigation of Cancer (EPIC)-Norfolk participants. Diabetes was excluded in controls based on self-report (self-reported history of diabetes, doctor-diagnosed diabetes and/or use of glucose-lowering drugs), linkage to primary care registers, secondary care registers, hospital admissions and mortality data. The Norfolk study was approved by the Norwich Local Research Ethics Committee and written informed consent was obtained from all participants.

Detection of the OriB variant We characterised the OriB variant using initial Sanger cycle sequencing in 92 cases and 92 controls in the Norfolk study using Seq2 and Seq5 PCR primers as previously described [28], but extended to include 21 M13/M13 Rev sequences. The primers were supplied by IDT (Leuven, Belgium), PCR reagents by Qiagen (Crawley, UK) and sequencing primers, reagents and equipment by Applied Biosystems (Warrington, UK). The population-specific variation was then used to develop a pyrosequencing assay based on the method previously described [29]. Primers were supplied by IDT, Streptavidin Sepharose High Performance beads were from GE Healthcare Life Sciences (Little Chalfont, UK) and all other reagents and equipment were from Qiagen.

The same individuals selected for the initial Sanger cycle sequencing were sequenced with the pyrosequencing assay. The sequencing calls for the OriB region obtained by Sanger cycle sequencing and pyrosequencing were 100% concordant in these samples.

Following the successful validation of the pyrosequencing assay, we used this method to screen the OriB region in Norfolk Diabetes Case–Control Study participants, blinded to case and control status, using 10 ng purified genomic

DNA; 248 of these individuals were run in duplicate. For samples with a homopolymeric C-tract sequence <10 we had a concordance of 97.8% and for the homopolymeric C-tract sequence ≥10 a concordance of <95% was achieved. The difficulty in determining the precise number of Cs in the homopolymeric C tract from PCR templates because of the loss of linearity in long homopolymeric sequences and heteroplasmic tract inflation has been previously discussed [29]. In order to resolve the low level of sequence concordance we repeated the sequence verification using the Sanger cycle sequencing method for all 1,252 individuals with a homopolymeric C-tract sequence ≥10. Again, we were unable to get precise resolution of the homopolymeric C tract. However, we were able to categorise these into four groups by discriminating the polyT sequence prior to the homopolymeric C tract as previously described [29]. When grouped together into all individuals with homopolymeric C tract ≥10, concordance of 100% was achieved. Samples for a further 392 individuals failed with the initial pyrosequencing reaction and 27 samples were uncategorised using pyrosequencing. All these samples were re-sequenced by Sanger cycle sequencing. The final call rate for the 12,795 was 99% and the concordance of the sequence in the duplicate samples was 98.3%. Major haplogroups for the 16189 variant were also estimated based on European mtDNA haplogroups and haplogroups previously constructed for the UK population [15].

Literature search and data extraction We sought to identify all studies published until 9 January 2013 describing the association of the OriB variant with type 2 diabetes in Europid populations. We also undertook a separate analysis in Asians. Articles were identified through electronic searches of MEDLINE and the Chinese National Knowledge Infrastructure Database by scanning the reference lists of articles identified for all relevant studies and review articles (including meta-analyses), and by correspondence with the lead authors of included studies. PRISMA guidelines were followed. Electronic literature searches combined search terms related to the OriB variant (mitochondrial DNA or mtDNA or mt DNA or mitochondrial polymorphism or polycytosine tract or polyC tract) and diabetes (diabetes or glucose or metabolic syndrome or hyperglycaemia) without language restriction (Fig. 1). The following data were extracted from each study according to a fixed protocol: study design; geographical location; ethnic group of participants; number of cases and controls; genotyping methods; and mean age of type 2 diabetes cases (electronic supplementary material [ESM] Table 1).

Statistical analysis The association between the OriB variant and type 2 diabetes in the Norfolk Diabetes Case–Control Study was tested using logistic regression models adjusted for age, sex and body mass index. Summary ORs

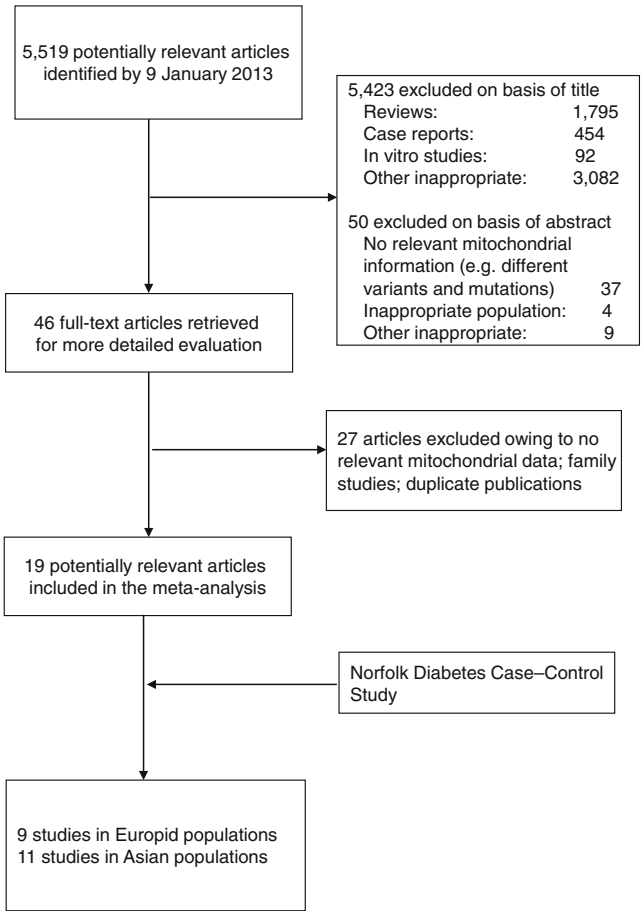

Fig. 1 Flow diagram of studies identified in the literature search for the association between the OriB variant and the risk of type 2 diabetes

for type 2 diabetes and the OriB variant were calculated using a random-effects model that included between-study heterogeneity. Consistency of findings across studies was assessed using the I^2 statistic [30]. Heterogeneity was assessed by using the Q statistic [31]. Publication bias was assessed using funnel plots and the Egger test [32]. All analyses were performed using Stata Statistical Software, Release 11 (StataCorp LP, College Station, TX, USA). All statistical tests were two-sided, with a significance level of $p<0.05$, except where indicated.

Results

Norfolk Diabetes Case–Control Study The characteristics of participants in the Norfolk Diabetes Case–Control Study are described in Table 1. The mean age was 60.7 (SD 11.4) years in cases and 59.3 (SD 9.3) years in controls. Women comprised 41% of cases and 51.8% of the control population. BMI was higher in the diabetes patients than in controls and a greater proportion of the cases than controls reported having a family history of diabetes. The frequency

Table 1 Baseline characteristics of participants of the Norfolk Diabetes Case–Control Study

Characteristic	Type 2 diabetes cases	Controls
N	5,574	6,950
Age (years)[a]	60.7 (11.4)	59.3 (9.3)
Women (%)	41.0	51.8
BMI (kg/m^2)[a]	30.1 (5.7)	26.2 (3.8)
Family history of diabetes (%)	23.3	13.4

[a] Mean (SD)

of the OriB variant in the Norfolk Diabetes Case–Control Study is summarised in ESM Table 2. The frequency of the OriB variant was higher in the type 2 diabetes cases (579 of 5,574, 10.4%) than in controls (675 of 6,950, 9.7%). Individuals with this variant are mainly within four haplogroups: H, T, U and X. Overall, 12.1% of individuals were categorised as 'other variants', which comprised 21 different variants. From these 21 different variants, we have identified six new variants (variants 16–21) that have not been previously reported. Individuals within other variants are mainly within three haplogroups: H, T and U. Within the other variants, the most common one (variant 15) is within the T haplogroup (ESM Table 2). The OriB variant was not significantly associated with type 2 diabetes (OR 1.09; 95% CI 0.96, 1.24; p=0.20) (ESM Table 3). There was no interaction between the OriB variant and BMI for type 2 diabetes risk (OR 1.00; 95% CI 0.99, 1.02; p=0.28).

Systematic review and meta-analysis We identified 5,519 articles from literature searches (Fig. 1). After exclusions [8, 12, 14, 17, 19–23, 25, 26, 33–36], eight studies in Europid populations were included. For other ethnic groups, we identified 11 studies in Asian populations; genotype frequencies were unavailable in one Asian population study [33], which was excluded for meta-analysis. A further three studies were excluded because the data duplicated or overlapped with reports already included in the review [34–36]. Our meta-analysis included eight genetic association studies in Europid populations plus the new data from the Norfolk Diabetes Case–Control Study, giving a total of 11,794 type 2 diabetes cases and 14,465 controls. Eleven studies in Asian populations included a total of 4,691 type 2 diabetes cases and 3,530 controls. Three studies used sequencing/pyrosequencing methods to identify the OriB variant and the remaining studies used PCR/RFLP and sequencing (ESM Table 1).

There was little evidence of heterogeneity between the nine Europid population studies (I^2=0%; 95% CI 0%, 65%; p=0.68). Overall, the summary OR for type 2 diabetes risk for the OriB variant in Europid populations was 1.10 (95% CI 1.01, 1.20; p=0.03; Fig. 2). When we excluded one study that typed 16189 variant without probing tract homogeneity [24], the summary OR for type 2 diabetes risk for the OriB variant in Europid populations was 1.13 (95% CI 1.02, 1.25; p=0.02). A funnel plot (not shown) did not indicate the presence of publication bias in these studies (Begg's test, p=0.40). There was evidence of a moderate degree of heterogeneity between the 11 Asian studies (I^2=41%; 95% CI 0%, 71%; p=0.08). The summary OR for type 2 diabetes risk for the OriB variant in the Asian population was 1.49 (95% CI 1.27, 1.74; $p=6.4\times10^{-7}$) (ESM Fig. 1).

Discussion

Previous genetic studies and earlier meta-analyses of the OriB variant have been dominated by small studies that are susceptible to various forms of bias. A previous meta-analysis concluded that the OriB variant was not associated with type 2 diabetes in the European population, but it did not include sufficient numbers of cases or controls to exclude a modest association of this variant with diabetes [18]. Methods used by other studies were not specific for the homopolymeric tract and hence underestimated the magnitude of the association [24]. Given the absence of large individual studies in Europid populations, we have conducted an updated systematic review and meta-analysis of new and published data, involving a total of 11,794 type 2 diabetes cases and 14,465 controls, which provides the most comprehensive assessment to date of the association of the OriB variant with type 2 diabetes in Europid populations. Our new data include the single largest study so far, the Norfolk Diabetes Case–Control Study, with a total of 5,574 type 2 diabetes cases and 6,950 controls. Our meta-analysis suggests that there is a statistically significant association of modest effect size between the OriB variant and the risk of type 2 diabetes in Europid populations. Our findings also suggest that the magnitude of the association may vary by ethnic background. The magnitude of the measure of association is comparable with that seen for variants identified by GWAS and associated with type 2 diabetes. The level of significance in this study is not as low as that seen in GWAS, but this is a function of the much smaller sample size, which, in itself, is a reflection of the much greater technical difficulty of typing the OriB variant. The accepted GWAS statistical thresholds for limiting false-positive associations do not apply to this study as only the OriB variant has been typed and this is not a genome-wide analysis. On the basis of the magnitude of the association and its overall statistical significance in Europid populations, the association of the OriB variant with type 2 diabetes deserves further investigation in additional case–control studies, including in other ethnic groups where the higher frequency of the OriB variant should increase the power to detect a true association.

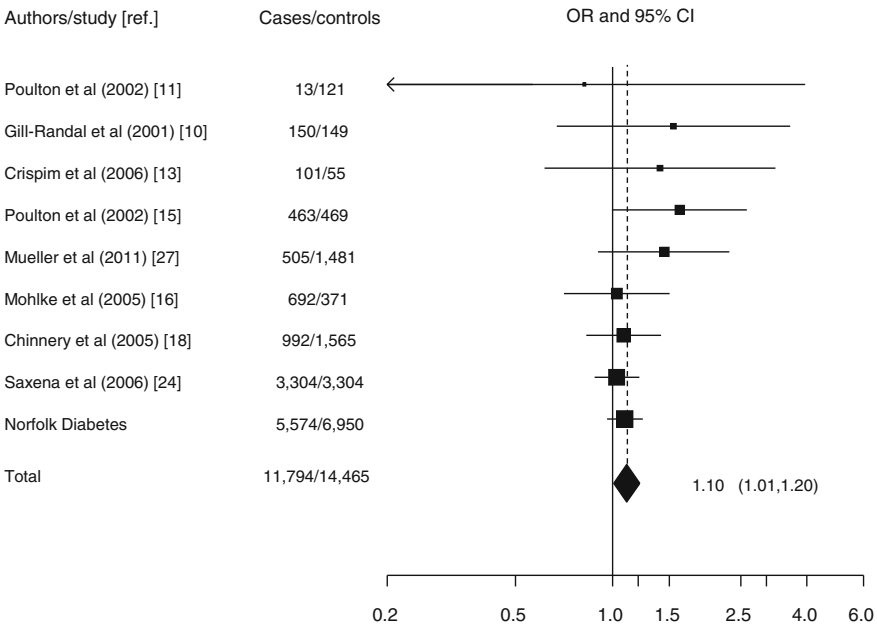

Fig. 2 The association of the mtDNA OriB variant with type 2 diabetes in Europid populations

Replication and transcription of mtDNA is complex, being mediated by nuclear-encoded proteins. The mechanisms of human mtDNA replication are far from resolved, and several origins of replication have been proposed. One of them, OriB, maps near one end of the major non-coding region (NCR) of mammalian mtDNA [37]. Many molecules of mtDNA contain a triple strand or D-loop region, and OriB is located close to the 3′ end of a D-loop. The D-loop may act as a platform for recruiting essential components of the replisome to mtDNA, such as the β subunit of the mitochondrial γ polymerase [38]. Therefore, the D-loop might regulate recruitment of replication initiation factors to OriB. Nevertheless, current understanding of the physiological roles of the various mtDNA origins is limited and nothing is known about the role of OriB in mtDNA replication in the beta cell [37, 39]. OriB appears to be more important in mtDNA replication during recovery from transient mtDNA depletion than in mtDNA replication required for maintaining appropriate mtDNA during cell doubling in culture. The mtDNA content of blood cells is slightly lower in healthy individuals with the OriB variant than in those with wild type, suggesting that sequence variants might influence this origin's activity [40]. Furthermore, the sequence appears to influence the access of proteins to this region. For instance, the variant both restricts the DNase I footprint in the region [9] and reduces the binding of the mitochondrial single-strand DNA binding protein (mtSSB) [37]. These results suggest that the OriB variant may alter protein binding in the NCR and thereby affect mtDNA replication. Thus, our findings in the present meta-analysis should stimulate further investigation of the functional abnormalities underlying our observation, in particular the role of OriB and the homopolymeric C tract in the aetiology of type 2 diabetes.

Given that the prevalence of the OriB variant is about 10% in Europid populations, the small increase (10%) in diabetes risk associated with it would explain only a small proportion of people with type 2 diabetes. However, given that the prevalence of the variant is higher in Asian populations and that the magnitude of association with diabetes is also greater, it is possible that the population-attributable fraction related to this variant could differ markedly by ethnic group. Hence, it might explain an important component of variance in the worldwide distribution of diabetes [9]. It has also been proposed that the association of the OriB variant with diabetes might be stronger in certain subgroups, such as those with obesity [17]. Our analysis of the Norfolk Diabetes Case–Control Study does not provide support for the existence of such interactions with BMI or other characteristics. As our meta-analysis was based on published results and not individual participant data, we were unable to explore interactions by participant-level characteristics in the meta-analysis.

The strengths and limitations of the current study merit consideration. Our meta-analysis involved five times more data than the earlier review. These data provided greater power than previously available to quantify the magnitude of association. Although we have also identified a large effect of the OriB variant and type 2 diabetes in Asian populations, the sample sizes in the majority of studies in Asian populations were very small. Meta-analysis of these smaller studies is more likely to overestimate such associations and lead to biased results. There was evidence of heterogeneity identified between Europids and Asians (I^2=45%; 95% CI 7%, 71%; p=0.02). Therefore, in this report, we focused on the association of the OriB variant and type 2 diabetes in Europid populations, which could

give us more reliable results. In contrast to earlier studies that used PCR/RFLP and sequencing methods, we made use of a combination of pyrosequencing and Sanger sequencing methods to accurately genotype over 12,000 individuals for the OriB variant in the Norfolk Diabetes Case–Control Study, which should be less liable to misclassification bias in the OriB variant. Although the Norfolk study is the single largest study so far, it is still insufficient alone to enable reliable assessment of the moderate association between the OriB variant and the risk of type 2 diabetes in Europid populations. However, in a meta-analysis of this and other published studies, we were able to demonstrate a modest association. It is possible that studies that did not accurately identify the variations around the homopolymeric tract could have underestimated its effect [24]. Because we did not have access to individual data in the published studies, we could not control for population stratification, adjust for possible confounding factors, explore heterogeneity by individual-level characteristics or perform haplogroup analyses.

In summary, this meta-analysis of new and previously published data suggests that the mitochondrial DNA OriB variant is modestly associated with an increased risk of type 2 diabetes in Europid populations. Further studies to investigate the functional abnormalities underlying this association are required, as are additional epidemiological investigations of ethnic group differences.

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Duality of interest The authors confirm that there is no duality of interest associated with this manuscript.

Contribution statement ZY researched the data and wrote the manuscript. CG and MS acquired the data and carried out the pyrosequencing and Sanger sequencing. K-TK contributed to the study design and acquired the data. MP contributed to the interpretation of data and provided the recombinant polyC-tract sequencing control. JP contributed to the interpretation of data. CL supervised the study and contributed to the study design and interpretation of data. NJW obtained funding, was responsible for the conception of the study, supervised the study and contributed to the study design and interpretation of data. CG, MS, K-TK, MP, JP, CL and NJW reviewed and/or edited the manuscript. All authors approved the final version.

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