Three Common Intronic Variants in the Maternal and Fetal Thiamine Pyrophosphokinase Gene (TPK1) are Associated with Birth Weight

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Three Common Intronic Variants in the Maternal and Fetal Thiamine Pyrophosphokinase Gene (TPK1) are Associated with Birth Weight

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Summary

Extreme variations in birth weight increase immediate postnatal mortality and morbidity, and are also associated with the predisposition to metabolic diseases in late adulthood. Birth weight in humans is influenced by yet unknown genetic factors. Since the 7q34-q35 region showed linkage with birth weight in a recent human genome scan ($p = 8.10^{-5}$), this study investigated the *TPK1* (thiamine pyrophosphokinase) gene locus, located in 7q34-36. Having found no coding variants in the *TPK1* gene, we genotyped 43 non coding SNPs spanning a region of 420kb, and used the QTDT method to test their association with birth weight in 964 individuals from 220 families of European ancestry. Family-based tests detected association of 8 SNPs with birth weight ($p<0.008$), but after correction for multiple tests only rs228581 C/T ($p = 0.03$), rs228582 A/G ($p = 0.04$) and rs228584 C/T ($p = 0.03$) were still associated with birth weight, as well as their T-A-T haplotype ($p = 0.03$). In addition, we found an association between maternal rs228584 genotype and offspring birth weight ($p = 0.027$).

These observations suggest that genomic variations in the fetal and maternal *TPK1* gene could contribute to the variability of birth weight in normal humans.

Keywords: TPK1, birth weight, association

Introduction

Birth weight is a complex multifactorial trait. It is an important risk factor not only for infant mortality and morbidity (Guyer *et al*. 1999), but also for adult metabolic disorders (Barker, 1990) such as obesity, type 2 diabetes (T2D) or cardiovascular diseases, independent of gestational age and of potential confounding factors such as social class or smoking, an observation that has been replicated in several populations from different countries. Birth weight risk associations are not confined to differences between the smallest and large infants, but relate to a continuum of variable risk throughout the whole range of birth weights. Faced with low birth weight or macrosomia, paediatricians often declare them "idiopathic" since environmental and genetic factors remain elusive in the vast majority of cases. The current study is a preliminary step into the genetics of human birth weight variability.

Family and twin studies provide evidence that genetic factors influence birth weight (Johnston *et al*. 2002; Magnus, 1984; Magnus *et al*. 1984; Morton, 1955). A recent genome scan for birth weight in 220 Caucasian families (Fradin *et al*. 2006) indicated the highest degree of linkage in 7q34-q35 with a LOD score of 3.1 ($p = 0.00008$). According to the stringent linkage criteria proposed by Lander & Kruglyak (1995), this result strongly suggests that the 7q34-35 region contains allelic variations influencing normal birth weight variability.

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Among the more than forty genes located within 7q34-q35, we elected to study *TPK1* (thiamine pyrophosphokinase). TPK1 is a cellular enzyme, abundantly expressed in maternal, placental and fetal tissues (Bellyei *et al*. 2005), which catalyzes the conversion of thiamine, a form of vitamin B1, to thiamine pyrophosphate (TPP). TPP is an active cofactor for enzymes involved in glycolysis and energy production, including transketolase, pyruvate dehydrogenase, and alpha-ketoglutarate dehydrogenase. Vitamin B1 is an essential vitamin for vertebrates. Our reasons for testing *TPK1* as a candidate gene for birth weight control was: i) *TPK1* harbours a number of common variants in Caucasians; ii) *TPK1* is implicated in thiamine metabolism, and maternal thiamine deficiency causes intra-uterine growth retardation in rats (Butterworth, 1993b; Roecklein *et al*. 1985), and children born to mothers suffering from hyperemesis gravidarum, a disease often accompanied by thiamine deficiency, are known to have a decreased birth weight (Dornhorst & Girling, 1995; Langer *et al*. 1989).

The *TPK1* gene maps to chromosome 7q34-q36, contains at least 9 exons, and spans approximatively 420kb (NM_022445) (Zhao *et al*. 2001). According to the db SNP public database, *TPK1* has numerous intronic polymorphisms but no coding variants.

The current study analyzed sequence variations of the human *TPK1* gene and performed family-based and haplotype-based association tests to determine whether genetic polymorphisms within this gene could contribute to the normal variability of birth weight in humans.

Materials and Methods

Cohort

The study design and recruitment procedures have been published before (Fradin *et al*. 2006). Briefly, inclusion criteria were: 1) a birth weight at term exceeding 2000g (10 patients excluded) and below 5000g (4 patients excluded); 2) gestational age strictly between 39 to 41 weeks, determined by reference to the last menstrual period; 3) healthy gestation (mothers were considered healthy if they had no known diabetes, hypertension, medical condition or addiction associated with impaired fetal growth); 4) access to at least one sib; 5) parental data and DNA available. The study population included 220 families, 154 pedigrees of 2 sibs, 53 pedigrees of 3 sibs, 9 pedigrees of 4 sibs, 3 pedigrees of 5 sibs and 1 pedigree of 6 sibs, leading to a total of 412 sib-pairs. All participants were French Caucasian of European origin. The studied sample included 964 individuals who showed the same distribution of birth weight as the French general population (440 parents, 240 male children and 284 female children). Our cohort can therefore be considered a representative healthy sample of the population living in a developed European country. Term and weight measurements were obtained from the Carnet de Santé completed by maternity paediatricians at birth. French children have to be precisely measured at birth to be included in the obligatory follow-up system of "Carnet de Santé de l'Enfance" (Child Health Bulletin). The study was approved by the Cochin University Institutional Review Board. All of the participants signed an informed-consent document.

Sequencing

We sequenced the complete *TPK1* coding sequence (NM_022445) in ten individuals randomly selected from our cohort. Primers used to amplify each *TPK1* exon are listed in Table 1. Amplification was carried out in 96 well microtiter plates (Abgen) : each 50μl reaction contained DNA (200ng), $MgCl_2$ (1.5 mM), 1X PCR Buffer (Invitrogen), dNTPs (0.2 mM each), primers (1 μM each) and *Taq* polymerase (1.25 units, Invitrogen). PCR products were purified using a QIAGEN kit before sequencing. PCR products were sequenced using a BigDye Terminator v3.1 sequencing standard kit. All sequence determinations were performed in duplicate.

Genotyping

Forty-three SNPs *TPK1* were selected from public databases (HapMap project) based on the following criteria : 1) reported to dbSNP by various sources; 2) validation status in Caucasians; 3) high degree of heterozygosity (Table 2, Figure 1). These 43 SNPs were successfully genotyped by Illumina technology in all individuals (parents and offspring, n = 964). The Illumina Golden Gate Assay employs highly multiplexed, allele-specific extension and ligation

Table 1 Primers used to amplify the TPK1 coding region

Exon	Primer forward	Primer reverse
1	5′ACGAAGCCTTGGAGACTTGA3′	5′CTATGAGGTCCGAGGGGTTG3′
2	5′CTCCCGTGCTGTTGGTTC3′	5′AAAATGAGACCGGCGAGAG3′
3	5′GGACCCGTTTATGTATATGC3′	5′CCACTTTTTAGTCAGGTTTCAGG3′
4	5′TAGCGTGACTCTGTGCGTCT3′	5′AACATGGTTAAGCAGCTCAGG3′
5	5′TGCACTTGGCCACAGTTTTA3′	5′TTTGTGAAAAAGTGTAAGTTTCCAT3′
6	5′TGTCCCATGTGAAATAAAAA3′	5′CATCCTTGGAATAACCACCAA3′
7	5′GGTTCTGTTGGTGCTTGGTT3′	5′TTGTGAAGGAAAGGGCTCAC5′
8	5′TTAAGTCGGGTGAAGGTTCC3′	5′TGACCCAGCAATTCCTCCTA3′
9	5′CCCATTTTAAAGCCATACATCA3′	5′CCCCTTCCCCCAATATTTA3′
	5′GGTTGGAACCTAATGGGAATC3′	5′GAGTAGGGCTCCACGAATCA3′
	5′TGATTCGTGGAGCCCTACTC3′	5′TCAGCCCTATGACTAAGTG3′
	5′GCAGTCATAGGGCTGATAATACA3′	5′TCTCACACTGTCATAGCACCAA3′

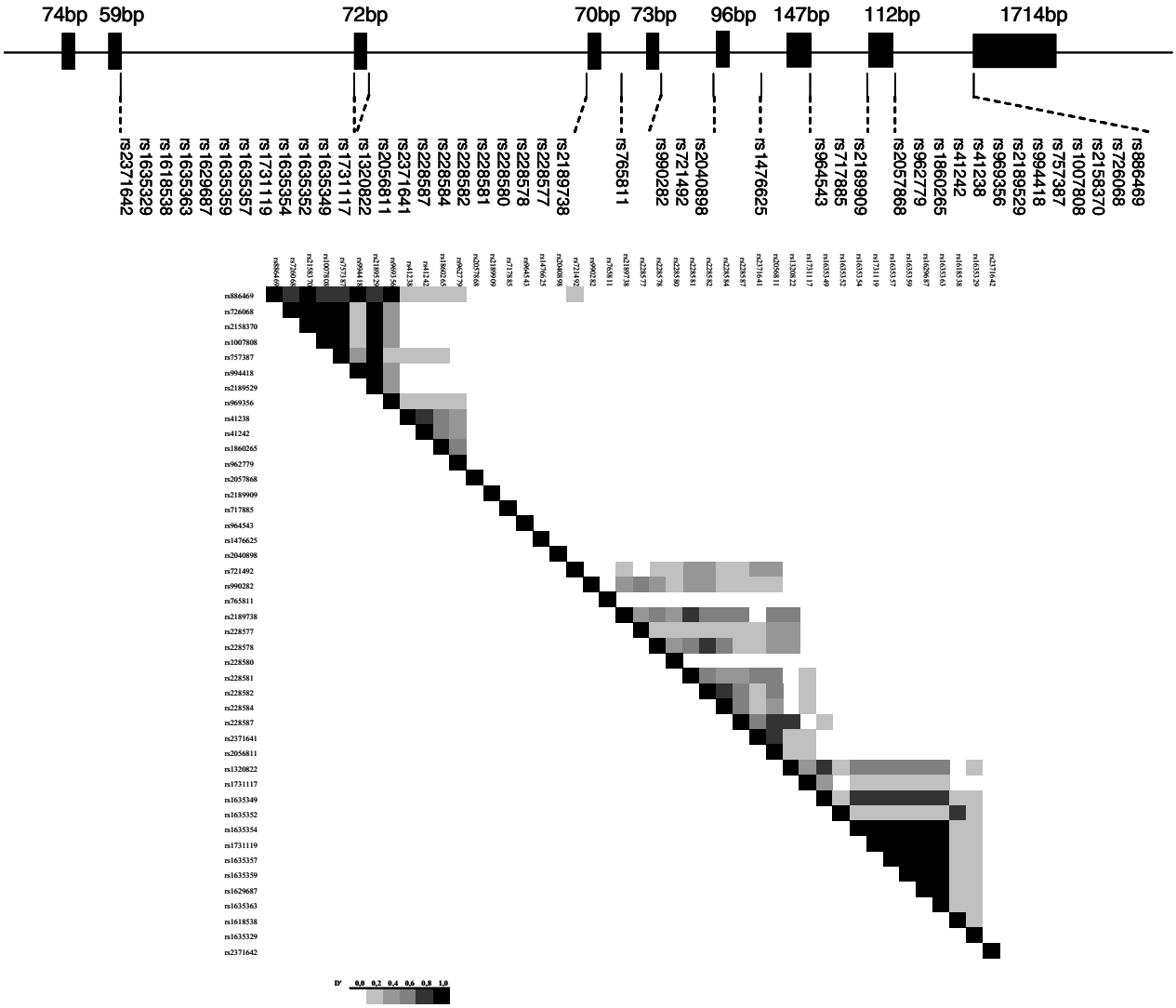

Figure 1 Localisation and linkage disequilibrium of the 43 SNPs that were genotyped in the studied cohort.

Table 2 Details of the 43 SNPs that were genotyped in the studied cohort

SNPs	Position	Frequencies
rs886469	143589702	C = 0.325
rs726068	143591093	A = 0.325
rs2158370	143592701	A = 0.203
rs1007808	143596709	A = 0.194
rs757387	143597931	G = 0.208
rs994418	143601739	T = 0.333
rs2189529	143603643	A = 0.208
rs969356	143610971	G = 0.333
rs41238	143624003	A = 0.292
rs41242	143634663	G = 0.292
rs1860265	143652570	T = 0.3
rs962779	143657052	C = 0.281
rs2057868	143662292	A = 0.208
rs2189909	143695944	G = 0.067
rs717885	143710050	A = 0.092
rs964543	143722498	G = 0.05
rs1476625	143738373	T = 0.017
rs2040898	143759559	C = 0.15
rs721492	143771803	T = 0.25
rs990282	143774546	T = 0.373
rs765811	143806013	A = 0.308
rs2189738	143835631	G = 0.05
rs228577	143838310	T = 0.449
rs228578	143846081	T = 0.275
rs228580	143848813	T = 0.467
rs228581	143849129	T = 0.342
rs228582	143857525	G = 0.467
rs228584	143861007	T = 0.475
rs228587	143867027	T = 0.417
rs2371641	143870995	C = 0.407
rs2056811	143892673	T = 0.43
rs1320822	143900057	C = 0.424
rs1731117	143903560	A = 0.492
rs1635349	143909046	C = 0.267
rs1635352	143913243	A = 0.492
rs1635354	143916310	T = 0.225
rs1731119	143916417	C = 0.467
rs1635357	143918197	T = 0.467
rs1635359	143918736	A = 0.467
rs1629687	143924149	A = 0.466
rs1635363	143926945	C = 0.467
rs1618538	143929388	C = 0.457
rs1635329	143939212	T = 0.225
rs2371642	143951197	-

methodology and universal-primer PCR amplification reactions. SNP interrogation occurs before amplification using genomic DNA as template, and therefore specificity is not hybridization-based. The Bead Array Technology involves quantitatively pooled libraries of 3-micron beads, each containing multiple copies of covalently attached oligonucleotide probes, assembled into etched fiber optic bundles. Array bundles are fabricated into matrices whose configuration matches the wells of a standard 96-well microtiter plate. The average genotyping error rate estimated through blind duplicated sampling was less than 0.01%.

Haplotypes Reconstruction

Merlin software (Abecasis *et al*. 2000a) was used to analyze linkage disequilibrium (LD) and reconstruct rs228581-rs228582-rs228584 haplotypes based on data obtained from the studied families. Information about gene flow in a pedigree can be used to reconstruct the more likely haplotypes for families and individuals. The physical distances of the genetic markers required for haplotype reconstruction were derived from the Genethon map.

Statistical Analyses

The Mann-Whitney test and the Kruskal-Wallis analysis of variance by rank were applied to compare, respectively, two and several independent samples when a classical *t*-test or an ANOVA test could not be used. Data were evaluated using Statview 5.0 (SAS Institute). Results were corrected for multiple tests by Bonferroni correction. Values are reported as means ± sd.

Quantitative Transmission Disequilibrium Test (QTDT)

Birth weight was analyzed as a continuous trait adjusted for sex. To test for both linkage and association with birth weight variation, we used the QTDT method (Abecasis *et al*. 2000b) implemented in Merlin (Abecasis *et al*. 2002). The orthogonal model of Abecasis *et al*. was used in our analyses, where the total association is partitioned into orthogonal within-and between-family components ($ß_b$ and $ß_w$, respectively). The between-family component is specific for each nuclear family and could be confounded by population stratification. However, the within-family component is significant only in the presence of linkage disequilibrium caused by close linkage. Thus, a QTDT test of the significance of $ß_w$ based on allelic transmission is robust to

population stratification. Sex was used as a covariate in each analysis of birth weight. The null model was "Means = Mu+ sex+$ß_b$" and the full model was "Means = Mu+sex+ $ß_b$+ $ß_w$". We used Bonferroni's correction for multiple tests.

Transcription Element Search System (TESS)

TESS, Transcription Element Search Software (http://agave.humgen.upenn.edu/utess/tess), is a web-based software tool for locating possible transcription factor binding sites in DNA sequence and for browsing the TRANSFAC® (a database of eukaryotic transcription factors, their genomic binding sites and DNA-binding profiles). It provides functionality beyond that of the TRANSFAC flat files and website. TESS allows the user to search sequence for possible binding sites using either cis-element strings or weight matrix models (Schug & Overton, 1997).

Alternative Splicing Database (ASD)

The ASD Project aims to understand the mechanism of alternative splicing on a genome-wide scale by creating a database of alternative splice events and the resultant isoform splice patterns of human genes (Thanaraj *et al*. 2004). Web-based software from the ASD site can be used for intron analysis (donor or acceptor sites, branching points, polypyrimidine tracts etc., http://www.ebi.ac.uk/asd-srv/wb.cgi).

Results

We identified no common coding variants in the *TPK1* gene after re-sequencing all known exons in our cohort (Figure 1). We therefore studied a panel of 43 SNPs located within 420kb of the *TPK1* gene locus (Table 2). All 43 SNPs were located in intronic regions of the *TPK1* gene and all were frequent (m.a.f > 0.10) (Table 2). None of them was located in classical splice donor or acceptor sites. We found that 3/43 SNPs were associated with birth weight after correction for multiple tests: rs228581 (C/T; p = 0.03), rs228582 (A/G; p = 0.04) and rs228584 (C/T; p = 0.03). Allele T of rs228581 was over-transmitted with low birth weight, as well as allele A of rs228582 and allele T of rs228584 (Table 3). We tested a model of dominance for these 3 SNPs and replicated the association between rs228581, rs228582 and rs228584 and birth weight (p = 0.001, p = 0.004 and p = 0.001 respectively). The three associated SNPs are located in intron 3 of the *TPK1* gene. D′, the linkage disequilibrium parameter, was less than 0.5, supporting the quasi independence of effects due either to rs228581, rs228582 or rs228584.

Table 3 QTDT analysis of 43 SNPs located in the TPK1 gene region in 220 Caucasian families

Marqueurs	p-value	p-value corrected*
rs964543	0.008	0.34
rs765811	0.004	0.17
rs228577	0.003	0.13
rs228581	**0.0007**	**0.03**
rs228582	**0.001**	**0.04**
rs228584	**0.0007**	**0.03**
rs228587	0.002	0.09
rs2371641	0.002	0.09

*For multiple tests.

The reconstruction of haplotypes formed by these three SNPs allowed us to identify only 4 haplotypes (C-A-C, C-A-T, C-G-C and T-A-T, respectively, ordered rs228581, rs228582 and rs228584). Using QTDT we confirmed that the T-A-T haplotype was associated with low birth weight (3362 ± 558g *versus* 3477 ± 532g for all other pooled haplotypes, p = 0.03).

Since imprinting is an important mechanism of fetal growth regulation, we tested if the parental origin of *TPK1* alleles had an effect on the observed association. We found no difference between paternal and maternal alleles of the studied SNPs and haplotypes.

As several maternal genes are known to regulate birth weight, we tested whether variation in the maternal *TPK1* genotype was associated with offspring birth weight. We found an association between the maternal rs228584 genotype and offspring birth weight (ANOVA p-value = 0,027), but no association with other SNPs. To differentiate maternal and offspring genotypes in their effect on birth weight, we tested for an association between the untransmitted maternal rs228584 allele and offspring birth weight. We found a significant and relatively large difference in birth weight when the untransmitted maternal allele was C (3283 ± 560g) and when the untransmitted maternal allele was T (3522 ± 500g, p = 0.005). Therefore, the association between

rs228584 and birth weight could be partly explained by the maternal genotype at this locus.

Discussion

Following our genome scan analysis for birth weight (Fradin *et al*. 2006) we tested *TPK1* as a candidate gene in the 7q34-q36 region. We used a nuclear family-based approach using the QTDT method. The QTDT directly tests for linkage disequilibrium between a trait and a marker locus, and prevents false-positive results in genetic studies due to population stratification. The more commonly used population-based association approaches are open to the risk of spurious associations due to recent admixture or to population samples that are stratified with respect to genetically heterogeneous groups (Deng, 2001). To avoid these problems the QTDT test uses parental genotypes to construct well-matched controls. On the other hand, while the linkage approach designed to identify genomic regions harbouring QTLs for continuous traits is often of limited statistical power to detect genes of small effects (Risch & Merikangas, 1996; Sham *et al*. 2000), the QTDT is considered much more powerful for testing linkage of specific candidate genes to complex traits (Allison, 1997).

The current sample reflects the continuous distribution of birth weight in normal infants born to healthy Caucasian mothers, with a mean value of 3341g and a standard deviation of 537g, which is comparable to most studies in developed countries (Wilcox, 1993). Because mortality augments both sides of the birth weight distribution, evolution has stabilized birth weight symmetrically within a relatively narrow range, around a mean value of 3200–3400g at term (Wilcox, 1993). While mean birth weight depends partly on environmental factors, it is expected to depend on a number of genes (Magnus 1984; Magnus *et al*. 1984). Each of these can be expected to contribute only a small fraction of the trait variance (Farrall, 2004). This contribution can be postulated to be 0.5 to 10 percent of the trait variance if the trait is oligogenic (Farrall, 2004), an order of magnitude consistent with the statistical strength of the association observed in the current study.

Using splice sequence analysis we were not able to assign the three intronic SNPs to positions that are known to be critical for the splicing process, for regulation of expression (Mizumoto *et al*. 1997), or for nucleosome formation. The only positive finding was that allele T of rs228581 could bind the N-Oct-3 factor, which belongs to a large family of transcription factors that bind the octameric ATGCAAAT motif. The functional mechanisms through which the three SNPs could affect gene splicing or gene expression thus remains to be established. It is also unknown whether the three SNPs are associated with gain-or loss-of-function of TPK1 enzyme activity, nor are there any physiological data allowing us to document genotypic differences in the thiamine status of the studied mothers or infants.

Because of this association finding, it will be important to examine thiamine metabolism in a prospective genotype-phenotype study. There is an increased requirement for thiamine during pregnancy, which often results in a relative deficiency in the mother (Heinze & Weber, 1990). During normal pregnancies the mean thiamine value in maternal blood cells falls from 230 nmol/l in the 28th week to 170 nmol/l in the 39th week (Heinze & Weber, 1990). In cases of severe fetal hypotrophy the corresponding thiamine levels have been found to be 140 nmol/l in the 30th week and 130 nmol/l in the 39th week of gestation (Heinze & Weber, 1990).

Three key enzymes of glucose metabolism, pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and transkelotase, are thiamine dependent (Butterworth, 1993a; Hsu & Chow, 1960; McCandless *et al*. 1968). Thiamine deficiency, and consequently TPP deficiency, results in an overall reduction of tissue glucose utilization and impairment of the production of insulin (Rathanaswami *et al*. 1991; Rathanaswami & Sundaresan, 1988; 1989; 1991). The maternal allele T of rs228584 was associated with higher birth weight of offspring in our study. If the *TPK1* maternal genotype affects TPK1 enzyme activity, which the current study did not show, it could influence the pool of TPP. Whether it could modify blood glucose and thus influence fetal growth remains entirely speculative.

On the other side, low birth weight infants are at increased risk of metabolic disorders in later life. It is intriguing in this respect that the current 7q34-q36 region has been linked to adult metabolic traits in several studies. Suggestive linkage was found between this 7q region and BMI in 160 pairs (Borecki *et al*. 1994).

Significant linkage with BMI to D7S1824 (a microsatellite located on 7q36) was reported in 157 white subjects (Platte *et al*. 2003), while a LOD score of 2.3 for abdominal fat was found in the same region in 215 subjects (Rice *et al*. 2002). The region 7q35-q36 also harbours a QTL linked to the variation of plasma triglyceride levels (LOD score = 3.7) (Sonnenberg *et al*. 2004). A LOD score of 1.8 for triglyceride levels was also found in this region of chromosome 7 in another study (Shearman *et al*. 2000).

In summary, several common polymorphisms of the *TPK1* gene appear to be associated with birth weight. It cannot be formally excluded that the currently associated SNPs are only markers of neighbouring unidentified functional SNPs that are responsible for the observed association. Also, our study is underpowered to identify associations with polymorphisms that are rare or exert only modest effects. Finally, the fact that we were not able to reconstruct all haplotypes in all families reduced the size of the haplotype groups and thus the level of significance of our observations. Confirmation of the current association and new physiological insights are therefore necessary before genetic relationships between thiamine metabolism and human fetal growth can be established. Since thiamine deficiency remains an important public health issue in some populations, the study of thiamine status during the third trimester of pregnancy may help to investigate the postulated effects of the *TPK1* genotype in these populations.

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