Mitochondrial transcription factors TFA, TFB1 and TFB2: A search for DNA variants/haplotypes and the risk of cardiac hypertrophy

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Abstract. Mitochondrial transcription factors mtTFA, mtTFB1 and mtTFB2 are required for the replication of mitochondrial DNA (mtDNA), regulating the number of mtDNA copies. Mice with a mtTFA deletion showed a reduced number of mtDNA copies, a reduction in respiratory chain activity, and a characteristic dilated cardiomyopathy. DNA variants in these genes could be involved in the risk for cardiac hypertrophy (HCM).

We determined the variation in the TFAM, TFB1M, and TFB2M genes (using SCA, DHPLC, and direct sequencing) in a total of 200 HCM-patients from Spain and Germany, and in 250 healthy controls. We found several common polymorphisms that defined haplotype blocks in these genes, with frequencies that did not differ between patients and controls. We also found four novel variants in patients which were absent in the controls: −91 C > A (5'-UTR) and Ala105 > Thr in TFAM, and Thr211 > Ala and Arg256 > Lys in TFB1M. The three missense changes were in highly conserved amino acids, and could be involved in HCM-risk.

In conclusion, common variants in the mitochondrial transcription factors were not associated with the risk for HCM. However, rare DNA variants (putative mutations) could be involved in the pathogenesis of HCM in a reduced number of cases.

Keywords: Cardiac hypertrophy, mitochondria DNA, transcription factors, mutations

1. Introduction

Mitochondria are the cellular organelles that generate energy as adenosine triphosphate (ATP). Mitochondria contain their own DNA (mtDNA) and their own machinery for RNA and protein synthesis. The mitochondrial genome consists of 37 intronless genes, and encodes 13 subunits of the electron-transfer chain, 2 ribosomal RNAs (rRNA), and 22 transfer RNAs (tRNA). However, most of the mitochondrial proteins are encoded by genes in the nucleus and imported from the cytoplasm. Expression of the mitochondrial genome is
initiated in the D-loop regulatory region of the mtDNA, and the basic transcription machinery consists of a mitochondrial RNA (mtRNA) polymerase molecule, a mt transcription factor A (mtTFA), and two related transcription factors B1 and B2 (mtTFB1 and mtTFB2) [13, 15,37]. All these mitochondrial transcription factors are encoded by nuclear genes, TFAM, TFB1M and TFB2M. The mtTFBs interact with the C-terminal tail of mtTFA, facilitating the interaction between the heavy and light-strand promoters and the initiation of the transcription [6,11]. In addition to its activity as a transcription factor, mtTFB1 belongs to the family of RNA adenine methyltransferases, a group of proteins that methylate small subunit rRNAs at a conserved stem loop [6].

Mutations in mtDNA are frequently linked to multisystemic diseases and follow a maternal transmission (mitochondria are inherited from the mother), while mutations in the nuclear genes encoding mitochondrial proteins cause typical mendelian traits [2,10]. These mtDNA mutations have an effect on cellular energy metabolism, and more severely affect those organs that are highly energy dependent, such as muscles and the nervous system. Cardiac muscle contraction is highly dependent on the energy supplied by mitochondria. The accumulation of mtDNA deletions is responsible for the age-related decrease in cardiac function, and some mtDNA mutations have been linked to hypertrophic cardiomyopathy (HCM), either isolated or as part of a multisystemic syndrome [19,21,33,40,41,44]. In addition to rare highly penetrant mutations, mtDNA is highly polymorphic and some single nucleotide polymorphisms (SNPs) could contribute to the expression of mitochondrial-related diseases [7,20].

Left ventricular hypertrophy (LVH) is caused by several factors that impose overwork to the heart, such as hypertension, valvular disease, cardiomyopathy, and myocardial infarction [26,29,36]. Hypertrophic cardiomyopathy (HCM) is the Mendelian autosomal dominant form of LVH, and is primarily a disorder of the cardiac sarcomere linked to mutations in genes that encode components of the sarcomere, although other non-sarcomere genes could be involved in this disease [3, 4,30,38]. Thus, polymorphisms in the genes encoding components of the renin-angiotensin and the endothelin systems, among others, could be involved in the risk of developing cardiac hypertrophy [5,8]. Further, some authors have reported the association between mtDNA variation and HCM [2,7,33].

Every cell contains hundreds of mitochondria, and each mitochondriom about five mtDNA molecules. The number of copies of mtDNA is directly dependent on the levels of mtTFA [23,24]. Mice heterozygous for a TFAM deletion showed a significant reduction in mtDNA copies [28]. The overexpression of mtTFA increased the number of copies, but did not affect respiratory chain activity or mitochondrial mass [11]. Mice with a deletion of TFAM limited to the heart reproduced the pathophysiological features of mitochondrial cardiomyopathy, and showed a significant reduction of mtDNA copies in cardiomyocytes with a partial reduction in respirator chain activity [27,42,43]. The overexpression of mtTFB2 is accompanied by an increased amount of mtDNA, while cells that overexpressed mtTFB1 showed significantly increased mitochondrial mass [9,12,31,43].

TFAM, TFB1M and TFB2M are thus candidate to be involved in the risk for developing cardiac hypertrophy. Therefore, we searched for DNA variation in these transcription factors in a cohort of well characterized HCM patients, and analysed the association of putative mutations with the disease through a case-control study.

2. Methods

2.1. Patients and controls

This study was part of a research to analyse the contribution of DNA variation to HCM. A total of 150 patients with HCM were recruited at the Cardiology Department of Hospital Central Asturias. The inclusion criteria were a left ventricular wall thickness > 13 mm of unexplained cause: the hypertrophy was not secondary to other cardiac diseases capable of producing LVH, such as hypertension, valvular disease, and myocardial infarction. These patients were negative for mutations in the most frequently mutated sarcomeric genes (MYH7, TNNT2, MYBPC3, TNNI2, and TPM1). The main clinical and anthropometric characteristics of the 150 patients are summarised in Table 1. We also searched for TFAM, TFB1M, and TFB2M variants in a cohort of 50 HCM patients from Germany with the same clinical and genetic inclusion criteria as the Spanish cohort.

A total of 250 healthy individuals, aged 20 to 75 years, were used as population controls for the genetic association studies. These controls were recruited through the blood bank and the Cardiology Dept. of Hospital Central Asturias and did not have a history of HCM. In 110 of these controls we performed elec-
trocardiographic examination to exclude the existence of HCM or other cardiovascular diseases; in 140 controls, electro or echocardiographic studies were not performed to exclude the presence of asymptomatic LVH. All the Spanish patients and controls were Caucasians from the same region (Asturias, Northern Spain, total population 1 million). All the individuals participating in the study gave their informed consent, and the study was approved by the Ethical Committee of Hospital Central Asturias.

2.2. Genetic analysis of TFAM, TFB1M and TFB2M

Genomic DNA was obtained from 10 ml of blood, and the coding regions of TFAM (7 exons), TFB1M (7 exons), and TFB2M (8 exons) were PCR-amplified in fragments 180 to 327 bp long. Each PCR consisted of 100 ng of DNA, 2 mM MgCl₂, 2 mM of each dNTP, 10 pmol of each primer pair, and 0.5 U of Taq DNA-polymerase, in a final volume of 20 µl, and for a total of 33 PCR cycles (30 s − 95°C, 60 s-annealing temperature, 60 s − 72°C). Primer-sequences and PCR conditions are available upon request to the corresponding author. Nucleotide positions relative to the gene sequences were numbered according to the Ensembl database (www.ensembl.org). To search for sequence variants, the PCR fragments were amplified from all the patients and controls and analysed through single strand conformation analysis (SSCA) and denaturing high performance liquid chromatography (DHPLC).

For the SSCA, five µl of each PCR were mixed with 30 µl of formamide, denatured for 5 min at 95°C, and 5 µl were loaded on a 6% SSCA-polyacrylamide gel (5.8% acrylamide/0.2% bisacrylamide; 50 cm long). After 18 h of electrophoresis at 25 W, gels were silver-stained to visualise the electrophoretic patterns. For the DHPLC analysis, the PCR-products were injected in Helix DVB polymeric reversed-phase columns in a Varian Helix system, and eluted with a linear binary gradient created with buffers Varian Helix A (triethylammonium amine – TEAA) and B (TEAA + 25% acetonitrile) (www.varianinc.org). The DHPLC elution temperatures and buffers gradient for the PCR fragments were calculated with the DHPLC Melt Program (http://insertion.stanford.edu/melt.html), and are available upon request to the corresponding author.

To identify the nucleotide changes responsible for the observed genotypes, PCR fragments of cases representative for the different electrophoretic patterns were sequenced using BigDye chemistry in an automated ABI310 capillary system (Applied Biosystems, Foster City, CA, USA).

2.3. Genotyping, haplotypes and linkage disequilibrium

Because each allele gave a characteristic SSCA-pattern, we could assign the genotype for each variant in all the patients and controls. Allele and genotype frequencies for the TFAM, TFB1M and TFB2M polymorphisms were determined in the Spanish patients and controls by SSCA-genotyping.

The frequencies for all the possible haplotypes defined by each pair of loci were calculated with the Cubic exact solutions for the estimation of pairwise haplotype frequencies (www.oegree.org/software/cubex). This program estimates the haplotype frequencies, the normalised linkage disequilibrium (LD) parameter (D’), and the LD correlation coefficient between two loci (r²) [17].

2.4. Statistical analysis

Allele and genotype frequencies in patients and controls were compared through a chi-square test. Odds ratios (OR) with 95% confidence intervals (CI) were obtained to calculate the relative risk of HCM associated with the genotypes. The Chi² was also used to analyse the deviation from the Hardy–Weinberg equilibrium of the genotype frequencies. All statistical analyses were performed with the SPSS statistical package (v.11.0).

3. Results

We searched for TFAM, TFB1M and TFB2M variants in a total of 200 HCM patients and 250 healthy controls. The coding exons and flanking intronic sequences were analysed through SSCA (Fig. 1), DHPLC (Fig. 2), and direct sequencing.
Table 2

| TFAM variants  | Change | Allele frequencies |
|----------------|--------|--------------------|
|                |        | Patients | Controls |
| New, exon 1    | 5'UTR, −91 C > A | < 1% * | 0 |
| rs1937, exon 1 | 35 C/G, S12T | C: 0.93 | 0.91 |
| rs17710934, exon 2 | 183 T/C, S61S | T: 0.95 | 0.93 |
| rs34084383, intron 3 | IVS3, −13 ins/del T | T: 0.20 | 0.22 |
| new, exon 4    | 445 G > A, A105T | < 1% * | 0 |
| rs41283688, intron 6 | IVS6, +12 G/A | A: 0.10 | 0.11 |

*These variants were found in one patient and none of the 250 controls.

Fig. 1. (A) SSCA of polymorphism rs5782396 in TFB2M, showing the three genotypes for the IVS +26 ins/del T (intron 6). (B) SSCA of the exon 5 fragment, showing the different electrophoretic patterns for the 633 G/A polymorphism (rs324356).

3.1. TFAM

In the TFAM gene we identified four previously reported polymorphisms (Table 2). Among the two exon polymorphisms, rs17710934 was a silent change, and rs1937 was the only missense variant (S12T). Allele and genotype frequencies did not differ between patients and controls for the TFAM variants. We also determined the haplotype frequencies and linkage disequilibrium between these loci in the 150 patients and 250 controls from Spain. None of the TFAM polymorphisms were in significant LD, and the estimated haplotype frequencies did not differ between patients and controls (Fig. 3).

In addition to the 4 common polymorphisms, we also found two rare TFAM variants in one patient and none of the healthy controls. The two changes were conserved among species. This patient was a 39 years old male with a left ventricular septum of 17 mm and was heterozygous for two nucleotide changes: −91 C > A in the 5’-non translated (5’-UTR) sequence of exon 1, and a missense change in exon 4, A105T. This patient did not have symptoms of muscular or neurological disease. His mother was 82 years old and had a left ventricular septum (LVS) of 19 mm. She was heterozygous for A105T, and in this way the patient should have the two rare variants in different chromosomes. No other family members were affected (the father died at the age of 60 without symptoms of HCM).

3.2. TFB1M

In the TFB1M gene we found five previously reported polymorphisms (Table 3). We also found two novel missense variants, a G > C change in codon 120 (exon 3; A120 > P), and a G > A change in codon 189 (exon 5; G189 > R). These two changes were found in two patients and one control and therefore regarded as rare polymorphisms. Allele and genotype frequencies for the five common polymorphisms did not differ between patients and controls. The three exon 1 variants were in complete LD ($D^* = 1.0$, $r^2 = 1.0$), and in almost complete LD with rs324356. The estimated haplotype frequencies for each pair of loci did not differ between patients and controls (Fig. 3).
Table 3
TFB1M polymorphisms, and allele frequencies in our population. Nucleotides in exons were numbered considering +1 as the first base in codon 1 of the transcript (ENST00000367166). Nucleotides in introns were numbered according to the genomic sequence (ENSG000000029639).

| TFB1M variants | Change | Allele frequencies |
|----------------|--------|--------------------|
|                |        | Patients | Controls |
| rs1334687, exon 1 | 5'UTR, −75 G/T | G: 0.56 0.58 | T: 0.44 0.42 |
| rs1334688, exon 1 | 5'UTR, −62 C/T | C: 0.56 0.58 | T: 0.44 0.42 |
| rs162981, exon 1  | 5'UTR, −23 G/T | G: 0.58 0.58 | T: 0.42 0.42 |
| New, exon 3      | 358 G/C, A120 > P | C < 0.01 C < 0.01 |
| New, exon 5      | 621 G > A, G189 > R | A < 0.01 A < 0.01 |
| rs324356, exon 5 | 633 G/A, T211T | A: 0.42 0.42 |
|                 |        | G: 0.58 0.58 |
| New, exon 5      | 631 A > G, T211 > A | G < 1%* 0 |
| New, exon 6      | 767 G > A, R256 > Q | A < 1%* 0 |
| rs3734322, intron 6 | IVS6, −30 G/T | G: 0.59 0.58 |

* these variants were found in one patient and none of the 250 controls.

Fig. 2. DHPLC profile of the TFB1M exon 5 nucleotide variant (A631 > G), corresponding to the putative mutation Thr211 > Ala. The PCR-amplified fragment of the heterozygous carrier eluted as two peaks, compared to the single peak in the wild-type homozygous.

Two sporadic cases had missense changes that were not found among the 250 controls. A 35 years old male with a LVS of 16 mm was heterozygous for a missense change in exon 5 (631A > G; T211 > A). A 25 years old female with a LVS of 15 mm was heterozygous for R256 > Q in exon 6 (767 G > A). These
two TFB1M changes affected amino acids conserved among species.

3.3. **TFB2M**

In the TFB2M gene we found a total of 11 variants (Table 4). Three were novel polymorphisms: A64T (exon 1, 190 G/A), IVS4 + 40 del/ins GT (intron4), and T392T (exon 8, 1176 C/T). All the TFB2M variants were found in patients and controls, and A64 > T was a rare polymorphism found in two patients and one control. Allele, genotype, and haplotype frequencies for the TFB2M variants did not differ between patients and controls. LD analysis between pairs of loci showed three haplotype blocks: one defined by six polymorphisms between rs34850511 (intron 4) and rs3124131 (intron 6) and the other by rs3129568 and rs3124119 (intron 7) (Fig. 3).

4. **Discussion**

MtTFA facilitates the transcription of mtDNA, and is also important for the package of mtDNA in nucleoid particles [16,18,24,25,34]. Recent studies with transgenic mice demonstrated that mtTFA is important to maintain the number of mtDNA-copies, and its deletion was associated with a deficient respiratory chain activity and symptoms of dilated cardiomypathy [42]. Moreover, this pathological phenotype was rescued by the expression of human mtTFA in the transgenic mice [22]. These findings suggested that mtTFA dysfunction could be involved in the development of cardiac hypertrophy in humans, and TFAM gene variants could be linked to the risk for HCM. Because mtTFA interacts with the mitochondrial transcription factors B1 and B2, and the cellular overexpression of these
TFBs increases the amount of mtDNA and mitochondrial mass, mutations in TFB1M and TFB2M could also influence the risk for HCM [9, 12, 31].

We searched for TFAM, TFB1M and TFB2M gene variants in a cohort of HCM patients and controls. We found several common polymorphisms, with frequencies that did not differ between patients and controls, and thus would not contribute to the risk for cardiac hypertrophy. We also found four nucleotide changes in three patients, but none of the healthy controls. Nucleotide changes in candidate genes could be considered as putative mutations if they introduced amino acid changes that alter protein function, affect gene expression (promoter variation), or RNA-processing (intronic variants). In opposition to polymorphisms, mutations are rare among healthy controls, affect amino acids conserved among species, and segregate with the disease in affected families. Most of the patients in our study were sporadic cases, or from families with a limited number of patients (not big enough to perform a statistically significant linkage). In this way, the main reason to consider the two TFAM (−91 C > A and A105 > T) and TFB1M (T211 > A and R256 > Q) as putative mutations was their absence among the ethnically matched controls and the fact that they were highly conserved among species. The −91 C > A in TFAM was in a region that contains binding sites for transcription factors such as the nuclear respiratory factors. Amino acid 105 was in a high mobility group (HMG) domain of mtTFA. These HGM domains show high affinity binding for DNA sequences, and the Ala to Thr change could affect the binding of mtTFA to mtDNA [11, 35].

Human mtTFBs are closely related to RNA adenine methyltransferases, a group of proteins that methylate adenine residues at a conserved stem loop of rRNA molecules. This mtTFB dual-function as transcription activators and rRNA modifiers represents a potential link between transcription, ribosomal biogenesis, and translation [39]. There are no obvious reason why the two putative mutations in TFB1M could have a functional effect, but amino acid 211 is in a beta-sheet of the protein close to the seventh RNA methyltransferase motif. These changes could have effects on mtTFA and mtTFB1 expression/function, but functional studies are absolutely necessary to confirm this point. In contrast with these rare variants found in the patients, the TFAM S12T alleles were frequent in patients and controls, and this amino acid was not conserved between species (S/T in human, K in mice). In addition, both variants would have similar mitochondrial signal peptide characteristics, hydropathy-scores, and average and local net charge [14].

In addition to TFB1M T211 > A and R256 > Q, found in one patient and none of the controls, we also
found three rare variants in two patients and one control: A120 > P and G189 > R in TFB1M, and A64 > T in TFB2M. Amino acids 120 and 189 of mtTFB1 are highly conserved in mammals. However, their presence in one healthy control suggests that they could be rare variants not linked to HCM. The screening of more patients and controls should be necessary to define the association between these rare missense changes and HCM.

Finally, in addition to describe the variation at the TFAM, TFB1M and TFB2M in a Caucasian population, we also reported the linkage disequilibrium between the different polymorphisms at each gene. Haplotype frequencies and LD values did not differ between patients and controls, suggesting a non significant contribution to cardiac hypertrophy. However, the population genetics reported in our study could be useful for future studies with diseases in which these mitochondrial transcription factors have been implicated, such as Alzheimer’s [1].

Our work has several limitations. First, the main argument to consider the rare variants found in the patients as putative mutations, was their absence in the healthy controls. In addition, they affected amino acids conserved among species, suggesting a limitation for missense changes at these protein residues. However, functional studies are necessary to conclude that these variants affect the mitochondrial function. Second, the patients who had these rare variants were sporadic cases or from families with a reduced number of affected. This made impossible to establish a familial segregation of the disease with these putative mutations. Third, the patients in our study had an essential form of left ventricular hypertrophy, and we can not exclude that the TFAM, TFB1M and TFB2M variants are involved in the risk for hypertrophy secondary to hypertension, cardiac valvular disease, exercise, or any other recognised cause of LVH. Fourth, SSCA and DH-PLC are indirect techniques for detecting nucleotide changes in PCR-amplified fragments, and the existence of nucleotide changes that are not detected (approximately 5% of false negatives) is the main limitation for these methods. It is thus possible that some mutations/polymorphisms in the mitochondrial transcription factors were not identified in our study.

In conclusion, our work suggests that common DNA polymorphisms at the TFAM, TFB1M and TFB2M have no effect on risk for cardiac hypertrophy. We found rare variants in the TFAM and TFB1M that could contribute to the risk of developing HCM. However, the study of larger series of patients is necessary to clarify the role of mitochondrial transcription factors mutations in HCM. In addition, functional studies should be necessary to determine the biological effect of these rare putative mutations.

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