APPENDIX

Amplification and resequencing of TRB3 coding region:

Genomic DNA was extracted using a DNA isolation kit (Roche Diagnostic).

To amplify the coding region of TRB3 (exons 2-4) set of primers (Table 1) were designed according to the genomic sequence available at NCBI GenBank accession number NT-011387.

All PCR reactions were performed in 25 μl reaction volumes containing 50 ng of genomic DNA, 25 pmol of each oligonucleotide primer, 0.2 mmol/l, 2U of *Taq Gold* (Applied Biosystem) in 1.5 mmol/l MgCl2. PCR conditions were as follows: denaturation at 94°C for 12 min, 35 cycles of: denaturation (94°C, 30 sec), annealing (30 sec at annealing temperatures specified for each amplimer in table 1), and extension (72°C, 30sec), and a final extension at 72°C for 7 minutes. Sequencing reactions were performed with the same primer sets used for amplification and big dyes v.3.1 sequencing reagents (Applied Biosystem) and then run on Automated DNA sequencer Aby Prism 3100 (Applied Biosystem).

Genotyping of Q84R variant:

The TRB3 Q84R variant, due to an A>G base change at 251 nucleotide position in exon 2, creates a restriction sites for MspI enzyme.

PCR reactions were performed in 25 μl reactions volume, as previously described. PCR products were then digested with MspI restriction enzyme (New England Biolabs) and electrophoresed on 2.5% agarose gel yielding one 593bp band for the uncutting, wild type AA genotype, two, 593bp and 297bp, bands for heterozygous AG genotype and one, 297bp, cutted band for the homozygous GG genotype.

Construction of the expression plasmid

Amplification of TRB3 full lenght was performed using following primer set: FW 5' CACACAAAGCTTGATTAGCTCCGGTTTGCATC 3' and RV 5' CACACACTCGAGCTTCTGGAAGGCACTGAAGG 3' with HindIII and XhoI recognition sites in their 5' end, respectively. After enzymatic digestion the fragment was cloned in the pcDNA3.1 Myc-His vector (Invitrogen) for expression in mammalian cells. Mutagenesis of TRB3 R84 gene variant was performed by Quick Change Site Direct Mutagenesis Kit (Stratagene).

Table 1. TRB3 coding region PCR primers and conditions

Exon	Primers	Annealing Temperature	Amplimer size (bp)
EX2	**FW 5'** GGCCACCAAGCAGTCTCAC **3'**	66°C	593bp
	RV 5' CGCCCATGATCCCTAAGTTC **3'**		
EX3	**FW 5'** CAGTGAAGCGCTTGGTGCG **3'**	62°C	521bp
	RV 5' ACTTCTTGCCTAAGTTACCTTG **3'**		
EX4A	**FW-A 5'** CATGGGGGTTCTGGGTAGGA **3'**	63°C	314bp
	RV-A 5' CTGCAGGCAAGGCGTAGG **3'**		
EX4B	**FW-B 5'** GTCTGGTTCGCTGCCTCCT **3'**	61°C	300bp
	RV-B 5' AAGGTTTGGCTCAGTTCAGAGG **3'**		