Haplotype Analysis of the Promoter Region of Phosphodiesterase Type 8B (*PDE8B*) in Correlation with Inactivating *PDE8B* Mutation and the Serum Thyroid-Stimulating Hormone Levels

Anelia Horvath,¹ Fabio Faucz,^{1,2} Gabriela P. Finkelstein,¹ Maria Eleni Nikita,¹ Anya Rothenbuhler,¹ Madson Almeida,¹ Veronica Mericq,³ and Constantine A. Stratakis¹

Background: Human phosphodiesterase (PDE) type 8B (*PDE8B*) is located at 5q14.1 and is known as the PDE with the highest affinity to cAMP. We recently described a family with bilateral micronodular adrenocortical disease that was apparently caused by an inactivating *PDE8B* mutation (H305P). As a result of a genome-wide study, a strong association between six polymorphic variants in the *PDE8B* promoter and serum levels of the thyroid-stimulating hormone (TSH) has been recently reported. Despite an extended analysis of the regions surrounding 5q14.1, no other potential genetic variants that could be responsible for the associated TSH levels were found.

Methods: In this study, we genotyped by polymerase chain reaction the described six polymorphic variants in the *PDE8B* promoter in the family with micronodular adrenocortical disease and inactivating *PDE8B* mutation and analyzed their correlation with individual TSH values in the family members.

Results: We observed complete segregation between the reported association and individual TSH values in the family we studied. Haplotype analysis showed that the haplotype associated with the high TSH levels is different from the one that segregated with H305P, suggesting that the mutation most probably has arisen on an allele independent of the high TSH-associated allele.

Conclusions: The proposed mechanism by which *PDE8B* may influence TSH levels is through control of cAMP signaling. Our analysis revealed separate segregation of an inactivating *PDE8B* allele from the high-TSH-allele and showed low TSH levels in persons who carry an inactivating *PDE8B* allele. These data suggest that, indeed, *PDE8B* may be involved in regulation of TSH levels.

Introduction

HUMAN PHOSPHODIESTERASE (PDE) type 8B (*PDE8B*) is located at 5q14.1, regulates cAMP signaling, and has the highest affinity for cAMP compared with all other known PDEs (1). We recently described a family with bilateral micronodular adrenocortical disease (MAD); the latter was caused by an inactivating *PDE8B* mutation (H305P) (2). MAD is a distinct form of corticotrophin (ACTH)-independent adrenal hyperplasia characterized by multiple nodules of up to 1 cm in diameter that are surrounded by hyperplastic cortical tissue

(3–5). This form of adrenocortical hyperplasia is one of the rare genetic causes of Cushing syndrome that affects primarily children and young adults and is linked to molecular defects of the cAMP signaling pathway (6,7).

As a result of a genome-wide study, a strong association between six polymorphic variants in the *PDE8B* promoter (rs461497, rs6453293, rs13158164, rs4704397, rs6885099, and rs2046045) and serum levels of the thyroid-stimulating hormone (TSH) has been recently reported (8). Despite an extended analysis of regions surrounding 5q14.1, no other potential sequence variants were found that could be linked to the

¹Program on Developmental Endocrinology and Genetics, Section of Endocrinology and Genetics, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland.

²Laboratory of Molecular Genetics, Center for Healthy and Biological Science, Pontificia Universidade Catolica do Parana, Curitiba, Brazil.

³Faculty of Medicine, Institute of Maternal and Child Research, University of Chile, Santiago, Chile.

regulation of the TSH levels (4,8). TSH regulates the thyroid function through the cAMP pathway, and its serum level is a sensitive predictor for thyroid activity, even within the normal range (9). Although TSH levels show certain heritability (at least in some ethnic groups), the genetic mechanism by which specific gene variants influence TSH levels remains unknown (9,10).

In this study, we genotyped the described six polymorphic variants in the *PDE8B* promoter (8) in a family with MAD that we have described before (2); its members carry an inactivating *PDE8B* mutation that leads to Cushing syndrome. We then analyzed segregation of these *PDE8B* alleles with individual TSH values in family members.

Materials and Methods

Subjects and their samples

The institutional review boards of National Institute of Child Health and Human Development (NICHD), National Institutes of Health (NIH), approved the genetic investigation of patients with adrenocortical tumors under NICHD protocols 95-CH-0059 and 00-CH-0160 after the patients had given informed consent. The clinical data for the herein studied family are presented in Table 1. The proband (CAR 559.03) was a girl found to have Cushing syndrome due to isolated MAD at age 2 years (2,7). In addition to characteristic Cushing syndrome signs (hypercholesterolemia and other metabolic abnormalities, obesity, and growth retardation) she presented also with elevated TSH that was unusual for her degree of hypercortisolemia. She continued to run relatively high TSH levels with negative antibodies, after her cure with surgery. She inherited the *PDE8B* inactivating mutation (H305P) from her father (CAR 559.01), who had mild hyperplasia of the adrenal glands on computed tomography, and moderate hypercholesterolemia and hypertension (2,7). In contrast to the proband, his TSH levels were in the low normal range. Similarly, the sister of the proband (CAR 559.04), who was also carrier of the H305P mutation in *PDE8B*, had normal-to-low TSH serum values; she was clinically normal and did not undergo extensive testing. The mother (CAR 559.02) was negative for the *PDE8B* H305H mutation and presented with intermediate TSH levels; she was positive for microsomal antibodies, but had no history of thyroid disease and no goiter.

DNA extraction and sequence analysis

Blood samples were collected; DNA was extracted from whole blood using standard procedures (7). All the primers used for the polymerase chain reaction amplification of the *PDE8B* promoter regions containing the single-nucleotide polymorphism (SNPs) of interest are listed in Table 2. After the amplification, the polymerase chain reaction products were agarose gel purified (Minelute; Qiagen, Valencia, CA) and bidirectionally sequenced on a 3130x1 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were analyzed using Vector NTI 10 software (Invitrogen, Carlsbad, CA).

In silico analysis

The promoter region of *PDE8B* was analyzed for potential functional or expressed variants using online available tools: for detection of potential initiation exons we applied *First exon finder* (11); for the detection of possible splice sequences we applied *Splice port* (12) and *Genescan* (13).

Results

We first genotyped all four family members for the six *PDE8B* promoter variants; the results are presented in Figure 1. A schematic representation of the six polymorphic sites in relation to the *PDE8B* locus is shown on Figure 2. We determined the haplotype linked to the H305P mutation: rs461497 [g], rs6453293 [a], rs13158164 [g], rs4704397 [a], rs6885099 [a], rs2046045 [g], or [g-a-g-a-a-g], which was different from the one associated with increased TSH [g-a-c-a-g-g] (8), and thus, rejected our initial hypothesis of linkage between the high-TSH-associated haplotype and the inactivating mutation H305P in *PDE8B*. Next, we analyzed the segregation between the reported high-TSH-associated alleles and the individual TSH values in the members of the family we studied. Our results were completely consistent with the reported association: the proband (CAR 559.03), who carried both the H305P-linked and high-TSH-associated alleles (see Fig. 1), had elevated TSH levels (5.45 mIU/L, normal range 0.4–4). In contrast, her father (CAR 559.01) and sister (CAR 559.04), both positive for the H305P mutation, did not carry the high-TSH-linked allele and presented with TSH levels in the lower interval of the normal range (1.13 and 0.59 mIU/L, respectively). The mother (CAR 559.02), who shared the high-TSH-linked

TABLE 1. CLINICAL DATA OF THE FAMILY POSITIVE FOR INACTIVATING MUTATION IN PHOSPHODIESTERASE TYPE 8B (H305P)

Patient code	Family relation	Thyroid hormones				PDE8B genotype
		TSH (normal range, 0.4–4 mIU/L)	FT4 (µg/dL)	T3 (ng/mL)	Microsomal antibodies (normal range, <35.0 IU/mL)	
CAR 559.03	Proband	5.45 mIU/L	1.5	129	NA	wt/H305P
CAR 559.01	Father	1.13 mIU/L	1.3	NA	NA	wt/H305P
CAR 559.02	Mother	2.3 mIU/L	6.9 ^a	NA	510 IU/mL	wt/wt
CAR 559.04	Sister	0.59 mIU/L	1.3	1.4	NA	wt/H305P

Value in bold: outside the normal range.

^aFor CAR 559.02, the total T4 has been measured.

FT4, free thyroxine; *PDE8B*, phosphodiesterase (PDE) type 8B; T3, triiodothyronine; TSH, thyroid-stimulating hormone; N/A, non-available.

TABLE 2. PRIMERS USED TO GENOTYPE THE SIX POLYMORPHIC VARIANTS IN THE PHOSPHODIESTERASE TYPE 8B PROMOTER REGION

Poly	Amplicon	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')	PCR product size (bp)
rs4361497	PDE8B poly1	gaagccagctgtggcattt	tggccttagataggcgctcat	485
rs6453293	PDE8B poly2	tgaggggcagtggtctataaa	taatggaaaatggccaaagg	600
rs13158164 and rs4704327	PDE8B poly3&4	ccttgacgtccctttgctat	gctttgagctccaggcttag	844
rs6885099	PDE8B poly5	gggaatgtcacctttgttgg	gaacgagaaaacaggccaaa	592
rs2046045	PDE8B poly6	ggcttgtctctccagaatgc	cagctactcgaaggctgag	472

PCR, polymerase chain reaction.

allele with the proband, had intermediate TSH levels (2.3 mIU/L).

The correlation between the TSH levels and the high-TSH-linked allele was also supported by the cumulative number of high-TSH-associated alleles—9 (out of maximum possible 12) in the proband versus 5 and 7 in the father and the sister, respectively. When only the three SNPs showing the genome-wide significant association with TSH levels were taken into

account (rs4704397, rs6885099, and rs2046045) (8), their cumulative number in the proband was 5 (out of a maximum of 6) compared to 3 in both the father and the sister, and 4 in the mother (see Fig. 1).

To search for closely positioned coding or functional *PDE8B* regions, we performed an *in silico* analysis on 75,000 bp from the 3' region of the gene that contained the promoter, the previously recognized exon 1 and intron 1, the novel

FIG. 1. *PDE8B* polymorphic variants that were associated with TSH levels in a family with inactivating *PDE8B* mutation (H305P). The asterisks indicate association with TSH levels. The haplotypes associated with the H305P mutation and with the high TSH levels are shown in boxes. *PDE8B*, phosphodiesterase (PDE) type 8B; TSH, thyroid-stimulating hormone.

FIG. 2. Schematic representation of the region between nucleotides 764,490,000 and 767,500,000 of chromosome 5. The positions of the six polymorphic sites in the *PDE8B* promoter are indicated. *PDE8B* exons are represented by white rectangles.

alternative exon 1 (7), and all six high-TSH-associated SNPs (11–13). Our analysis predicted with the highest confidence the two already detected *in vivo* initiation exons: the longest exon of the gene (isoforms 1–4), and the novel exon encoding 12 alternative AA (isoform 6) that was recently described (7,14,15); no other sequences from the region were scored reasonably high to initiate an analysis of their expression *in vivo*.

Discussion

The inactivating *PDE8B* mutation H305P was found on a novel isoform of the enzyme *PDE8B* (*PDE8B6*) that expresses a previously unknown initiating exon from the 3' region of the promoter of the gene (7). In the present study, we determined the phase of the alleles for an inactivating *PDE8B* sequence alteration and six *PDE8B* promoter polymorphic variants that are reported to be associated with high TSH serum levels (8). The observed individual TSH values in the family we studied support the association of high TSH with the reported alleles. The haplotype associated with the high TSH levels was different from the one that segregated with H305P, suggesting that the mutation has arisen on an allele independent of the high TSH-associated allele.

In contrast to the proband, the mother, who was also harboring the high-TSH-associated allele, presented with intermediate TSH levels. Unfortunately, the mother was not available for further clinical investigation. Nevertheless, the observed genotype–phenotype correlations in the described family represent an informative example of the combined action of the known genetic factors that influence thyroid function.

The proposed mechanism by which *PDE8B* may influence TSH levels is through control of cAMP signaling. In this study, an inactivating *PDE8B* allele was present in association with both the high-TSH-allele (in the proband and her mother) and the low-TSH-level-associated alleles (in the father and the sister). Thus, *PDE8B* inactivation by this mutation that causes increased cAMP signaling in the adrenal cortex does not seem to lead to increased TSH in all subjects. Although tissue-specific effects need to be considered, it appears that the identified genetic variants that are associated with TSH levels in the population have a different effect that, at least in the case of this mutation, exceeds the increase of cAMP signaling mediated by *PDE8B* partial inactivation.

TSH production by the pituitary gland is regulated by the hypothalamic factors TSH-releasing hormone (TRH) and so-

matostatin (16,17). In addition, TRH and TSH secretion are regulated through a negative feedback mechanism by thyroid hormones (17). Since *PDE8B* is expressed primarily in the thyroid and significantly less in the pituitary and the hypothalamus, it is likely that it affects TSH responsiveness at the level of the thyroid cells more than at the level of pituitary production (16,17): if the identified genetic variants affect *PDE8B* activity in the thyroid, a decrease in cAMP levels there would lead to lower T3 and T4 production, which in turn would increase TSH levels.

Other molecular factors that may influence TSH levels in the population include the loci encoding the TSH and the TSH receptor, the latter harboring also the genes for thyroid transcription factor 1 (*NKX2-1*) and iodothyronine deiodinase type 2 (*DIO2*) (18,19). These genes are on different chromosomes and were not studied in the present investigation.

In conclusion, we report separate segregation of high TSH levels from a *PDE8B* inactivating allele in a family with bilateral adrenocortical hyperplasia. Our results support the suggested association (8) between the reported *PDE8B* haplotype and elevated TSH levels.

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Disclosure Statement

The authors declare that no competing financial interests exist.

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Address correspondence to:

Constantine A. Stratakis, M.D., D(Med.)Sc.

Program on Developmental Endocrinology and Genetics

Section of Endocrinology and Genetics

National Institute of Child Health and Human Development

National Institutes of Health

Building 10, CRC

Room 1-3330, 10 Center Drive, MSC1103

Bethesda, MD 20892

E-mail: stratak@c@mail.nih.gov