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Identification of novel genetic variants in phosphodiesterase 8B (*PDE8B*), a cAMP specific phosphodiesterase highly expressed in the adrenal cortex, in a cohort of patients with adrenal tumors

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Summary

Background—Genetic aberrations in various components of cAMP signalling pathway predispose to endocrine tumors. Growing evidence has shown that mutations in the phosphodiesterases (PDEs) are involved in the predisposition to adrenocortical neoplastic conditions.

Objective—Screen for genetic variations in *PDE8B* among patients with different types of adrenocortical tumors.

Design and Subjects—Case-control study followed by functional analyses. 216 unrelated patients with different types of adrenocortical tumors and 192 healthy control individuals.

Methods—Bi-directional Sanger sequencing, *in vitro* cell line transfection, *in silico* modelling.

Results—Nine different *PDE8B* sequence changes, 6 novel and 3 previously reported, were identified in our patients and controls. Two of the variations, seen only in the patient group, showed significant potential to impair protein function, both *in vitro* and *in silico*.

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Conclusion—*PDE8B* is another gene in which variations may contribute to predisposition of adrenocortical tumors.

Key terms

PDE8B variants; adrenal tumors; cAMP signalling pathway

Introduction

Genetic aberrations in various components of the adenosine 3',5'-cyclic monophosphate (cAMP) signalling pathway have been found to predispose to different types of adrenocortical tumors. By regulating cAMP degradation, phosphodiesterases (PDEs) play a critical role in maintaining its intracellular levels and thus control multiple instants of the signal transduction.

Among the numerous phosphodiesterases, PDE8B is known to hydrolyze cAMP with the highest affinity, and *PDE8B* mutations have been recently implicated in adrenal tumorigenesis.[1–3] Several lines of studies further support the essential role of PDE8B for normal adrenal functioning and suggest it as another key molecule in predisposition to adrenocortical neoplasms. First, *PDE8B* is highly expressed in the adrenal gland where it functions as a major regulator of steroidogenesis.[2,3] *Pde8b* knockout mouse models have demonstrated significantly higher corticosterone levels; shRNA silencing of the Pde8b leads to increased corticosterone synthesis in an adrenal cell line.[3] Next, another cAMP-hydrolyzing phosphodiesterase, PDE11A, has been recently found mutant in adrenocortical and other endocrine tumors, including testicular germ cell tumors (TGCT) and prostate cancer (PCa).[4–7] The *PDE8B* locus had the second highest linkage score (after the *PDE11A* locus) in a genome-wide association (WGA) study of patients with early onset bilateral adrenocortical hyperplasia and Cushing syndrome.[4] Finally, a severe case of adrenocortical hyperplasia and cortisol hypersecretion leading to Cushing syndrome due to inactivating *PDE8B* mutation has been reported.[1] Herein, we report two novel damaging *PDE8B* genetic variations in patients with adrenocortical tumors. Our data support the notion that PDE8B, a key cAMP-specific PDE, may play a role in predisposition to adrenocortical tumors.

Subjects and methods

Subjects' selection

The institutional review boards of the participating research centres - NIH, Bethesda, MD, USA and Hôpital Cochin, Paris, France - have approved the genetic and clinical analyses of all the patients and controls included in the study. All study participants signed informed consent for genetic testing and for the analysis of the collected data. 216 patients with adrenocortical tumors were included in our study: 84 patients with bilateral primary pigmented adrenal disease(PPNAD), 54 patients with ACTH-independent macronodular adrenocortical hyperplasia (AIMAH), 50 patients with secreting adrenocortical adenomas (40 corticol producing and 10 aldosteron producing), 10 patients with non secreting tumors and 18 with adrenocortical carcinoma (ACC). Prior to the study, all patients or tumor tissue were tested and found negative for mutations in *PRKAR1A, PDE11A and GNAS* - the three genes from the cAMP signalling pathway that have been associated with adrenocortical tumors.

A control group of 192 healthy individuals with no personal or family history of endocrine tumor were recruited by Cochin Hospital, Paris, France, as part of a clinical protocol that studies the genetic predisposition to endocrine tumors. These individuals were specifically

examined by a senior endocrinologist to exclude any clinical signs suggestive of a genetic syndrome or endocrine tumor.

Sequencing analysis

The *PDE8B* coding regions and the exon-intron junctions of the above described patients and controls were analyzed by classical bi-directional Sanger sequencing on germline DNA. In the *PDE8B* mutation carriers, tumor DNA, when available, was sequenced to look for possible Loss of heterozygosity in the locus of identified genetic change, as previously described.[2] All the identified variations were confirmed on a second independent DNA aliquot.

RNA extraction

The total RNA extracted from tumor was treated with DNase and further purified with the RNeasy Mini kit and RNase-free DNase Set (Qiagen) according to the manufacturer's instructions.

Functional studies

For transfection experiments, the *PDE8B* open reading frame was cloned into pCR3.1, and the missense mutations (R121H, H391A, P660L, V697I and D755N) were introduced by overlapping PCR, as described previously.[5] The human embryonic kidney (HEK) 293 cells were transiently transfected using Lipofectamine 2000 (Invitrogen), following the manufacturers protocol. Cells were transfected with 6 μg of plasmid DNA expressing either the wild-type (WT) or the mutated form of PDE8B, harvested 48 hours after the transfection, and subjected to cAMP level and PDE activity assays as previously described.[6] All functional studies were done in triplicates, and an average was calculated for each value; each experiment was repeated at least twice.

Reverse Transcribed Polymerase Chain Reaction (RT-PCR)

Purified RNA was reverse transcribed with SuperScript™ II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions and amplified using DyNAzyme™ II DNA Polymerase (Finnzymes) by PCR. Nucleotide sequences of the specific primers used were: 12F: 5'-GGAAAGAGTCCATTGACGTG-3'; 13F: 5'GGATCCACTCCATGACCATC-3'; 14R: 5'-CAGGCCTCCAACAAGATCAC-3'; 16R: 5'-GGCATTGCAAGGTGACTGTG-3'. These primer pairs yielded PCR products of 370bp (12F-16R), of 284 bp (12F-14R), of 283 bp (13F-16R), and of 197 bp (13F-14R), respectively.

All amplified samples were examined by agarose gel electrophoresis to confirm successful amplification of each exon. Direct sequencing of the purified fragments was then done using the Genetic Sequencer ABI3100 Applied Biosystems apparatus.

In silico analyses

Three independent *in silico* software tools were utilized to predict the pathogenic potential of the identified missense variants in *PDE8B*: Polyphen2 (http://coot.embl.de/polyphen), SIFT (http://sift.jcvi.org/) and SeattleSeqAnnotation (http://gvs.gs.washington.edu/SeattleSeqAnnotation/).[8,9]. The potential effect of the splice variant was modeled using the SplicePort online prediction tool (http://spliceport.cs.umd.edu/).[10]

Results

We identified six *PDE8B* coding sequence alterations in eight unrelated individuals from our cohort of 216 patients with adrenal tumors; one individual presented with two different mutations. All mutations were found in a heterozygote state on germline DNA (Table 1). Five of the variations were missense and resulted in aminoacid substitutions: c.362G>A/p.R121H, c.1171C>A/p.H391A, c.1979C>T/p.P660L, c.2089G>A/p.V697I, and c.2263G>T/p.D755N, and one was a splice variant located five nucleotides upstream of exon 14 (c.1365-5g/a). Two of the five missense variants (R121H and D755N) were previously described in the public databases, and three were novel. Three additional *PDE8B* sequence variations were identified in the control group – one reported polymorphic variant (c.1267A>G/p.I423V), and 2 previously unknown missense substitutions (c.971G>A/p.R324Q and c.1032G>A/p.V344I), each of the three present in one control individual in a heterozygote state. Of the six *PDE8B* variants identified in the patients with adrenal tumors, only one, R121H, was seen among the controls; it was present in 4 out of the 192 unrelated control individuals studied. The total allelic frequency of variant *PDE8B* alleles was comparable in the patients with adrenocortical tumors and the controls (1.85% vs 1.56%, respectively).

Three independent *in silico* models (Polyphen2, SIFT and SeattleSeqAnnotation) predicted mild or no effect on the PDE8B protein function for the two known (R121H and D755N) and one of the novel missense variants (V697I) in the patient group, as well as for all three missense substitutions found only in the control group ‘R324Q, V344I and I423V). The H391A and P660L mutations that were seen only in the patient group were predicted respectively as possibly damaging and probably damaging. The splice site variant modelling (SplicePort) predicted a 20% decrease in the probability of the mutant junction sequence to serve as a donor site (Table 1).

Seven of the mutation-positive individuals carried a single *PDE8B* change, and one patient – with ACC - presented with 2 mutations: R121H and c.1365 -5 g/a. Apart from this patient, the R121H variant was identified in one more patient with an PPNAD. This patient did not have a *PRKAR1A* mutation. The c.1365-5g/a splice variation was seen in two more patients – one with AIMAH, and one with a cortisol-producing adrenocortical adenoma. The reported missense variation, D755N, and the novel H391A were each seen in one female with AIMAH, the P660L variant was identified in a male patient with a non-secreting adrenal adenoma and the V697I variant in a patient with a cortisol-producing adenoma.

To look for potential loss of heterozygosity, we sequenced 4 tumor DNAs that were available - 2 cortisol-producing adenomas (one with the c.1365 -5 g/a splice site variant and one with the V697I substitution), one AIMAH samples (with the c.1365 -5 g/a splice site variant) and one ACC (with both R121H and V697 mutations). Loss of the wild type allele was detected in the two cortisol-producing adenomas; the AIMAH and ACC retained both the normal and the mutant PDE8B alleles.

To assess the potential effect on the PDE8B protein function, transfection experiments with expression vectors harbouring the R121H, H391A, P660L, V697I and D755N substitutions were conducted on HEK293 cell line (Fig. 1, A and B). A statistically significant decrease in PDE activity and an increase in cAMP levels relative to the WT were measured in the H391A and P660L transfected cells, indicating a reduced ability of the mutant PDE8B protein to degrade cAMP. The most significant reduction of PDE activity and increase in cAMP levels was observed after transfection with the P660L-harbouring construct. This observation is concordant with the *in silico* prediction of a high potential for the P660L substitution to impair the protein function.

In order to determine if alternative splice isoforms were caused by the intronic variant (c. 1365-5 g/a) we performed RT-PCR in 2 tumor samples harbouring the splice site variant and 3 WT tumor samples, as controls. No difference in gel migration of PCR product between WT and mutated tumors were detected. By sequencing the PCR products, no alternative cDNA species were found in mutated tumors (data not shown) and only the wild-type sequence was present.

Discussion

In this study of 216 patients with adrenal tumors, we identified four novel variations affecting the coding sequence of *PDE8B*: three missense substitutions H391A, p.P660L and V697I and the c.1365-5 g/a splice variant; none of them were present among the 192 controls negative for endocrine disorders. This is the first systematic screening for *PDE8B* genetic defects in a heterogeneous cohort of patients with adrenocortical tumors, including benign and malignant, secreting and silent, unilateral and bilateral tumors, and nodular and hypertrophic adrenal lesions. In consistence with our prior findings, damaging germline *PDE8B* mutations were present in patients with adrenocortical tumors, including AIMAH, PPNAD, secreting and non-secreting adrenal adenomas, and adrenocortical carcinomas. Two novel and one reported *PDE8B* missense variants, all three benign, were seen in the control group. The frequency of *PDE8B* variant alleles was comparable among the controls and the patients. Of note, although all the individuals from the control group were carefully examined and proven negative for any endocrine abnormality or a family history of such, no hormonal and imaging data were available for this group.

We have previously reported PDE genetic variations in individuals with mild or no obvious adrenal phenotype, likely due to the PDE redundancy.[5,11] From the evolutionary point of view, PDE redundancy, through compensatory action, can potentially protect the cell from possible fatal consequences of single deleterious events affecting such an essential process as the cAMP degradation.

While a significant difference in the distribution of *PDE8B* variants between the patient and control groups was not seen, a difference in the pathogenic potential of the identified variants was apparent: only the H391A and P660L variants were seen exclusively in the patients' group and these mutations showed a potential to impair the protein function, both *in silico* and *in vitro*. This observation is in line with the sometimes modest, but constant higher number of PDE variations, in patients with endocrine tumors compared to controls.[5–7,11] Genetic changes in another phosphodiesterase, *PDE11A*, may act as a modifying genetic factor toward the development of testicular and adrenal tumors in Carney complex patients with *PRKAR1A* -inactivating mutations.[12]

Interestingly, the only patient who presented with two different *PDE8B* variations - the missense R121H and the splice c.1365-5 g/a – had adrenocortical carcinoma. R121H and c. 1365-5 g/a are the two most frequent *PDE8B* variations in our cohort of patients and controls, and, apart from this patient, they have never been seen together, thus likely being carried by different alleles. Adrenal tumor clonal analyses have suggested a multi-step adrenocortical tumorigenesis progressing from normal to adenomatous and eventually maliganant phenotype.[13,14] Whether or not the malignant phenotype in this patient is related to the possible lack of a wild type *PDE8B* allele in this patient is hard to say given the absence of available tissue for study.

The *PDE8B* splice variant (c.1365-5 g/a) did not lead to the generation of detectable alternative mRNA, as shown by RT-PCR and sequencing of the resulting amplified cDNA products. Such alternative mRNA was expected based on the length of exon 14 (165bp),

which was predicted to be spliced out leading to an in-frame deletion end expression of shorter PDE8B isoform. Lack of presence of such an alternate PDE8B mRNA species might be due to the relatively low rate of predicted mutant *PDE8B* molecules or, nonsense-mediated decay (NMD) of the mutant mRNA.

Strong support for the involvement of impaired PDE8B functioning in adrenococrtical tumorigenesis comes from a recent study of a knockout mouse model and shRNA silencing.[3] The authors demonstrated that both genetic ablation and chemical/pharmacological inhibition of PDE8B potentiate adrenocortical steroidogenesis and that PDE8B has its greatest effect under low ACTH conditions. This phenomenon is likely related to the high affinity of PDE8B to cAMP and implies its involvement in fine cAMP regulation; the authors demonstrated that PDE8B is a major regulator of one or more pools of cAMP that promote steroidogenesis, via both acute and chronic mechanisms. The link between cAMP dysregulation and adrenal tumorigenesis is well established.[5,15,16]

In summary, we identified two novel damaging *PDE8B* variants (H391A and P660L) in two patients with respectively AIMAH and a non-secreting adrenal tumor. These two genetic defects were absent in the control group. The presented data support the possibility that *PDE8B*, together with other genetic factors or on its own in some cases, involved in the predisposition to adrenocortical tumor formation.

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References

1. Horvath A, Mericq V, Stratakis CA. Mutation in PDE8B, a cyclic AMP-specific phosphodiesterase in adrenal hyperplasia. New England Journal of Medicine. 2008; 358:750–752. [PubMed: 18272904]
2. Horvath A, Giatzakis C, Tsang K, et al. A cAMP-specific phosphodiesterase (PDE8B) that is mutated in adrenal hyperplasia is expressed widely in human and mouse tissues: a novel PDE8B isoform in human adrenal cortex. European Journal of Human Genetics. 2008; 16:1245–1253. [PubMed: 18431404]
3. Tsai LC, Shimizu-Albergine M, Beavo JA. The high affinity cAMP-specific phosphodiesterase 8B (PDE8B) controls steroidogenesis in the mouse adrenal gland. Molecular Pharmacology. 2010; 79:639–648. [PubMed: 21187369]
4. Horvath A, Boikos S, Giatzakis C, et al. A genome-wide scan identifies mutations in the gene encoding phosphodiesterase 11A4 (PDE11A) in individuals with adrenocortical hyperplasia. Nature Genetics. 2006; 38:794–800. [PubMed: 16767104]
5. Horvath A, Giatzakis C, Robinson-White A, et al. Adrenal hyperplasia and adenomas are associated with inhibition of phosphodiesterase 11A in carriers of PDE11A sequence variants that are frequent in the population. Cancer Research. 2006; 66:11571–11575. [PubMed: 17178847]
6. Horvath A, Korde L, Greene MH, et al. Functional phosphodiesterase 11A mutations may modify the risk of familial and bilateral testicular germ cell tumors. Cancer Research. 2009; 69:5301–5306. [PubMed: 19549888]
7. Faucz FR, Horvath A, Rothenbuhler A, et al. Phosphodiesterase 11A (PDE11A) genetic variants may increase susceptibility to prostatic cancer. Journal of Clinical Endocrinology and Metabolism. 2011; 96:135–140.
8. Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. Nature Methods. 2010; 7:248–249. [PubMed: 20354512]

9. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nature Protocols. 2009; 4:1073–1081.

10. Dogan RI, Getoor L, Wilbur WJ, et al. SplicePort--an interactive splice-site analysis tool. Nucleic Acids Research. 2007; 35:285–291.

11. Libe R, Fratticci A, Coste J, et al. Phosphodiesterase 11A (PDE11A) and genetic predisposition to adrenocortical tumors. Clinical Cancer Research. 2008; 14:4016–4024. [PubMed: 18559625]

12. Libe R, Horvath A, Vezzosi D, et al. Frequent phosphodiesterase 11A gene (PDE11A) defects in patients with Carney complex (CNC) caused by PRKAR1A mutations: PDE11A may contribute to adrenal and testicular tumors in CNC as a modifier of the phenotype. Journal of Clinical Endocrinology and Metabolism. 2011; 96:208–214.

13. Beuschlein F, Reincke M, Karl M, et al. Clonal composition of human adrenocortical neoplasms. Cancer Research. 1994; 54:4927–4932. [PubMed: 7915195]

14. Gicquel C, Leblond-Francillard M, Bertagna X, et al. Clonal analysis of human adrenocortical carcinomas and secreting adenomas. Clinical Endocrinology (Oxford). 1994; 40:465–477. [PubMed: 7910530]

15. Kirschner LS, Carney JA, Pack SD, et al. Mutations of the gene encoding the protein kinase A type I-alpha regulatory subunit in patients with the Carney complex. Nature Genetics. 2000; 26:89–92. [PubMed: 10973256]

16. Groussin L, Kirschner LS, Vincent-Dejean C, et al. Molecular analysis of the cyclic AMP-dependent protein kinase A (PKA) regulatory subunit 1A (PRKAR1A) gene in patients with Carney complex and primary pigmented nodular adrenocortical disease (PPNAD) reveals novel mutations and clues for pathophysiology: augmented PKA signaling is associated with adrenal tumorigenesis in PPNAD. American Journal of Human Genetics. 2002; 71:1433–1442. [PubMed: 12424709]

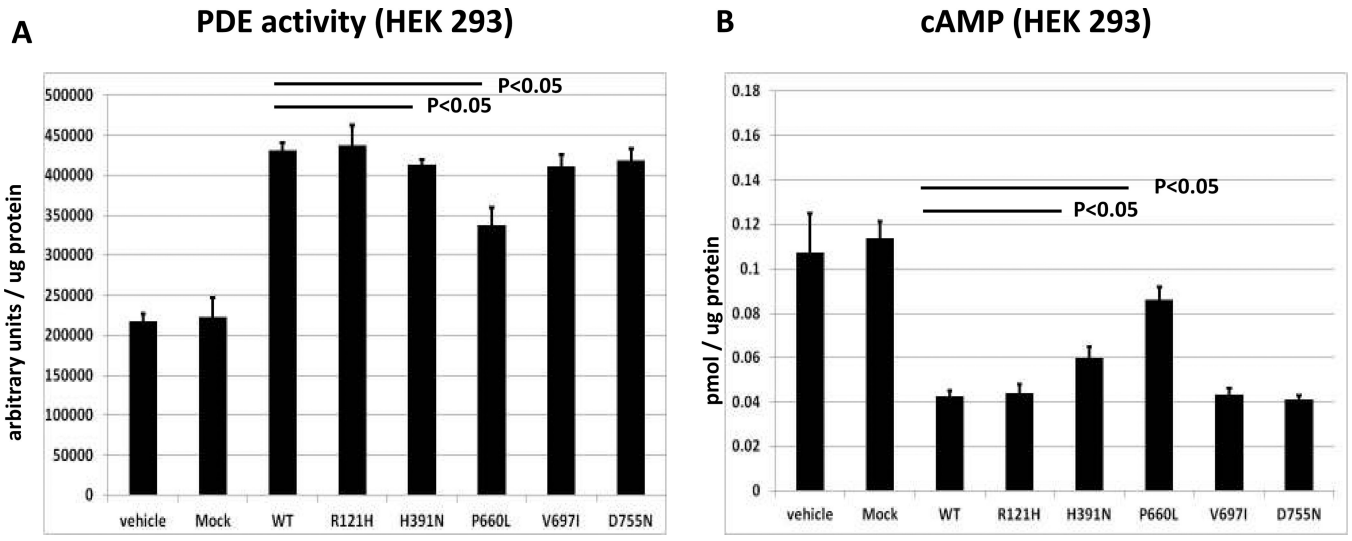

Figure 1.

PDE (A) and cAMP (B) activity after transfection of HEK293 cells with WT and mutant *PDE8B* expression vectors.

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Table 1

PDE8B mutations seen among the studied patients with adrenocortical tumors and in control individuals

***PDE8B*mutation**	**Type**	**Novel (Y/N)**	**In silico prediction**	**Tumor type**	**# Patients (out of 216)**	**#Controls (out of 192)**
c.362 G>A / p.R121H	missense	N	benign	ACC, PPNAD	2	4
c.971G>A / p.R324Q	missense	Y	benign	na	0	1
c.1032G>A / p.V344I	missense	Y	benign	na	0	1
c.1171C>A / p.H391A	missense	Y	damaging	MMAH	1	0
c.1267A>G / p.I423V	missense	N	benign	na	0	1
c.1365-5 g/a	splice	Y	20% decrease donor features	ACC, MMAH, AA (cortisol producing)	3	0
c.1979C>T / p.P660L	missense	Y	damaging	AA (non-secreting)	1	0
c.2089G>A / p.V697I	missense	Y	benign	AA (non-secreting)	1	0
c.2263G>T / p.D755N	missense	N	benign	MMAH	1	0
Total					9	7

ACC: adrenocortical carcinoma; PPNAD: primary pigmented nodular adrenal disease; MMAH: macronodular adrenocortical hyperplasia; AA: adrenocortical adenoma