Adrenal Hyperplasia and Adenomas Are Associated with Inhibition of Phosphodiesterase 11A in Carriers of PDE11A Sequence Variants That Are Frequent in the Population


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Abstract

Several types of adrenocortical tumors that lead to Cushing syndrome may be caused by aberrant cyclic AMP (cAMP) signaling. We recently identified patients with micronodular adrenocortical hyperplasia who were carriers of inactivating mutations in the 2q-located phosphodiesterase 11a (PDE11A) gene. We now studied the frequency of two missense substitutions, R804H and R867G, in conserved regions of the enzyme in several sets of normal controls, including 745 individuals enrolled in a longitudinal cohort study, the New York Cancer Project. In the latter, we also screened for the presence of the previously identified PDE11A nonsense mutations. R804H and R867G were frequent among patients with adrenocortical tumors; although statistical significance was not reached, these variants affected significantly enzymatic function in vitro with variable increases in cAMP and/or cyclic guanosine 3',5'-monophosphate levels in HeLa and HEK293 cells. Adrenocortical tissues carrying the R804H mutation showed 2q allelic losses and higher cyclic nucleotide levels and cAMP-responsive element binding protein phosphorylation. We conclude that missense mutations of the PDE11A gene that affect enzymatic activity in vitro are present in the general population; protein-truncating PDE11A mutations may also contribute to a predisposition to other tumors, in addition to their association with adrenocortical hyperplasia. We speculate that PDE11A genetic defects may be associated with adrenal pathology in a wider than previously suspected clinical spectrum that includes asymptomatic individuals. (Cancer Res 2006; 66(24): 11571-5)

Introduction

Aberrant cyclic AMP (cAMP) signaling has been linked to genetic forms of cortisol excess that lead to Cushing syndrome (1, 2). Macronodular bilateral adrenocortical hyperplasia may be due to GNAS mutations associated with McCune-Albright syndrome (2). Micronodular hyperplasia and primary pigmented nodular adrenocortical disease may be caused by germ line inactivating mutations of the PRKAR1A (3–6). However, over the last years, it has become apparent that several forms of adrenal hyperplasia are not caused by mutations in PRKAR1A or GNAS. Recently, after running a whole-genome association study, we reported inactivating mutations of the phosphodiesterase 11A (PDE11A) in a subgroup of patients with primary pigmented nodular adrenocortical disease and other forms of bilateral hyperplasia (7). PDE11A is a dual-specificity phosphodiesterase; it is expressed in several endocrine tissues, including the adrenal cortex (8–12). The PDE11A gene was mapped to the 2q31–35 chromosomal region and the adrenal tumors from patients with PDE11A-inactivating mutations showed 2q allelic losses, although a cause-and-effect relationship could not be shown because the study was a loss-of-heterozygosity and association one (7). In addition to the PDE11A nonsense mutations, we identified two missense substitutions and previously unknown silent polymorphisms (7).

In the present investigation, we examined the frequency of these variants in the population and in our cohort of patients with adrenal tumors. We also examined the frequency of all PDE11A-inactivating sequence variants in a large cohort of normal subjects between the ages of 30 and 60 years who were enrolled in the New York Cancer Project (NYCP), a long-term prospective study that monitors the effect of cancer risk behaviors and screening practices in the New York Metropolitan area (13). In tissue from carriers of the variants, we examined cAMP and cyclic guanosine 3',5'-monophosphate (cGMP) levels and cAMP-responsive element binding protein (CREB) phosphorylation. Our data suggest that PDE11A genetic defects act as low-penetrance alleles and raise the potential for the involvement of PDE11A in neoplasms of other tissues.

Materials and Methods

Clinical studies and sample collection. The institutional review boards of National Institute of Child Health and Human Resources, NIH, Mayo Clinic, and Hospital Cochin, Paris, approved the investigation of patients under National Institute of Child Health and Human Resources protocols 95-CH-0059 and 00-CH-0160 after informed consent. In addition to the
controls previously described (7), 745 randomly selected individuals from the NYCP study who have been described elsewhere (13) were also genotyped. All the patients with adrenal tumors in this study were negative for PKRARA mutations, screened as described elsewhere (14).

Complete details of all the methods are available online (Supplementary Methods).

Allelic losses, loss of heterozygosity, and protein studies. The RP11-428114 BAC containing a large part of the PDE11A gene was used for fluorescent in situ hybridization (FISH), as described (7). Loss of heterozygosity (LOH) analysis of rs1435572 and rs1997207 was obtained from the Affymetrix 10K Genechip data, as we published elsewhere (7). Comparison of the intensities of the peripheral and tumor DNA signals was determined.

Table 1. PDE11A sequence changes among patients with adrenocortical tumors and subjects from the population

<table>
<thead>
<tr>
<th>Sequence change</th>
<th>No. unrelated patients with adrenal tumors</th>
<th>No. unrelated Coriell normal controls</th>
<th>No. unrelated NYCP normal controls</th>
<th>Patients vs Coriell controls*</th>
<th>Patients vs NYCP controls</th>
<th>Coriell controls vs NYCP controls</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>c.171Tdel/fs41X</td>
<td>17</td>
<td></td>
<td>17</td>
<td></td>
<td>745</td>
<td></td>
</tr>
<tr>
<td>c.919C&gt;T</td>
<td>17</td>
<td></td>
<td>17</td>
<td></td>
<td>745</td>
<td></td>
</tr>
<tr>
<td>p.R307X</td>
<td>17</td>
<td></td>
<td>17</td>
<td></td>
<td>745</td>
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<tr>
<td>c.1657TCT</td>
<td>17</td>
<td></td>
<td>17</td>
<td></td>
<td>745</td>
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<tr>
<td>p.1657TCT/2delCCIns/fs15X</td>
<td>17</td>
<td></td>
<td>17</td>
<td></td>
<td>745</td>
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<tr>
<td>Combined stop codon variations</td>
<td>3</td>
<td></td>
<td>14</td>
<td></td>
<td>273</td>
<td></td>
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<tr>
<td>c.2411G&gt;A</td>
<td>17</td>
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<td></td>
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<td>p.R804H</td>
<td>17</td>
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<td>17</td>
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<tr>
<td>p.R867G</td>
<td>17</td>
<td></td>
<td>17</td>
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</tbody>
</table>

Allelic losses, loss of heterozygosity, and protein studies. The RP11-428114 BAC containing a large part of the PDE11A gene was used for fluorescent in situ hybridization (FISH), as described (7). Loss of heterozygosity (LOH) analysis of rs1435572 and rs1997207 was obtained from the Affymetrix 10K Genechip data, as we published elsewhere (7). Comparison of the intensities of the peripheral and tumor DNA signals was determined.

Generation of sense, mutant, and antisense PDE11A cDNA constructs. The 2.8-kb PDE11A cDNA was amplified using specific primers containing XhoI and NotI sites (forward, 5'-GCTAGCTCTCAAGGAAAG-CAGCTCTGTCGTGACaat-3'; reverse, 5'-GCTAGCCGCACCTGCGCACTGTGATGCCGCTTAGT-3'), digested, and cloned in pcDNA expression vector (Promega, Madison, WI). The R804H and R867G mutations were introduced using overlapping PCR. The antisense constructs were generated using the sense sequence but cloned in inverted orientation. To evaluate the effects of the missense substitutions on cAMP and cGMP levels, pCl (empty vector), pCl-PDE11A sense and antisense, and pCl-PDE11A-R804H/B867G were transfected in subconfluent HEK293 and HeLa cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Results

Frequency of the PDE11A sequence variants. In the expanded set of DNA samples that included 745 NYCP subjects, we searched for the five PDE11A sequence variants that were described by Horvath et al. (7): three truncating mutations (c.171Tdel/fs41X, c.919C>T/p.R307X, and c.1657TCT/2delCCIns/fs15X) and two missense substitutions [c.2411G>A (R804H) and c.2599C>G (R867G)] located in highly conserved regions of the gene (Supplementary Table S1). All five PDE11A variants were identified among >2,000 alleles studied (Table 1). The truncating mutations were not present in at least 273 healthy controls of mostly European descent; however, they were found among 745 normal subjects enrolled in the NYCP cohort: 12 carried one mutant allele each, with a combined frequency of 1.6%. These protein-truncating mutations were significantly more frequent among patients with adrenal tumors compared with the NYCP subjects (P < 0.0001; odds ratio, 13.1; 95% confidence interval, 3.3–51.6). One of these subjects reported a history of ovarian cancer. Six subjects reported a history of cancer in family members. These subjects are not available for further studies.

The two PDE11A missense substitutions (R804H and R867G) were found more frequently among patients with adrenocortical tumors (12% each) compared with both the normal controls in the NYCP cohort (4% and 3.6%, respectively) and the other normal control set (2.4% and 3%, respectively), but the observed differences did not reach statistical significance.

PDE11A missense substitutions: phenotype, LOH, and functional studies. The R804H mutation was found in family CAR545 (Fig. 1). The proband (CAR545.01) had an infant with micronodular adrenal hyperplasia and cyclical Cushing syndrome that was reported by Gunther et al. (16). She had inherited her PDE11A sequence change from her father, CAR545.01; she was studied by imaging and biochemical investigations and was found to have mild adrenal hyperplasia bilaterally but no other clinical or biochemical abnormalities (Fig. 1A). Other members of this family who were found to be carriers of the R804H mutation were clinically and biochemically normal (data not shown). The R804H mutation was also identified in one patient (CAR600.02) with adrenal hyperplasia, classic Cushing syndrome, and pathologic...
findings similar to those of primary pigmented nodular adrenocortical disease. It was also identified in an unrelated patient with macronodular adrenal hyperplasia, classic Cushing syndrome, and family history of this disease (CAR73.01).

The identification of PDE11A mutations and their familial inheritance in CAR545 led us to investigate LOH by SNP or polymorphic marker studies. Two informative PDE11A SNPs on the mutation-bearing haplotype were retained by the tumor in adrenocortical samples from CAR545.03, whereas the alleles on the wild-type were specifically lost in the tumor tissue (Fig. 1C). Analysis of the microsatellite marker D2S1776 and other markers in the proximity of the PDE11A gene showed retention of the mutant allele in the tumor tissue (data not shown).

The tumor tissue from patient CAR545.03 who carried the R804H substitution and had allelic losses of the PDE11A-containing BAC and LOH of PDE11A SNPs also had higher cAMP and cGMP levels (Fig. 2B). In vitro studies suggested that the R804H mutation inhibits PDE11A activity (Fig. 2C and D); introduction of the full-length PDE11A cDNA in HEK293 and HeLa cells decreased baseline cAMP and cGMP levels, an effect that was abolished by introduction of the full-length PDE11A antisense construct. When a construct bearing the c.2411G>A (R804H) substitution was introduced, cAMP levels increased, whereas cGMP levels decreased in HEK293 but not HeLa cells, suggesting that this substitution inhibits the activity of the enzyme for cAMP (possibly dominant-negative manner) but has a differential effect on cGMP levels in vitro, which may be tissue specific or dependent on other factors, such as the PDE milieu of a given cell. The experiments with a construct bearing the R867G variant showed similar changes of the cGMP but not cAMP levels (Supplementary Fig. S1); there was no tissue available from the patients that carried this mutation to study cyclic nucleotide levels in vivo.

We next examined the effect of the R804H mutation on the phosphorylation status of CREB in tumor tissues from the patient with the mutation compared with normal adrenal samples both by Western blotting and immunohistochemistry (Fig. 3). The ratio of phosphorylated CREB (P-CREB) to CREB in the mutant-PDE11A–carrying samples was increased compared with normal adrenal tissues. Nuclear staining for P-CREB was also greater than that for CREB in the tumor with the mutation and was even further increased within the nodular tissue.

**Discussion**

The data presented here are supportive of PDE11A association with low penetrance predisposition to the development of adrenal tumors. PDE11A mutations identified in CAR545 led to the investigation of LOH by SNP or polymorphic marker studies. Two informative PDE11A SNPs on the mutation-bearing haplotype were retained by the tumor in adrenocortical samples from CAR545.03, whereas the alleles on the wild-type were specifically lost in the tumor tissue (Fig. 1C). Analysis of the microsatellite marker D2S1776 and other markers in the proximity of the PDE11A gene showed retention of the mutant allele in the tumor tissue (data not shown).

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**Discussion**

The data presented here are supportive of PDE11A association with low penetrance predisposition to the development of adrenal
hyperplasia and/or adenomas, and, possibly, other tumors. For the two PDE11A missense substitutions (R804H and R867G), one cannot be certain: they may be low-penetrance alleles that occur relatively frequently in the general population or they may be clinically inconsequential despite their unequivocal in vitro effects. It is noteworthy that their frequency approximates that of adrenal nodules identified incidentally or in autopsy (17). It is tempting to speculate that PDE11A is indeed the gene that predisposes to this adrenal pathology in the general population.

The present study shows that the previously identified PDE11A protein-truncating mutations can also be found among normal subjects. Although the frequency of these mutations in normal individuals was significantly less than that in patients with adrenal hyperplasia, the question that arises is whether these genetic changes occur frequently de novo or whether in fact they cause adrenal hyperplasia or are simply predisposing factors to a variety of tumors including adrenocortical neoplasms. It will be of interest to follow subjects in the NYCP cohort to see if these mutations are associated with cancer incidents in the future. Clearly, further studies need to address not only the prospective clinical screening of individuals carrying PDE11A mutations but also their genetic origin (de novo or inherited).

The number of factors likely to affect tumorigenicity by these mutations may be the reason for their apparent low penetrance; as in the adrenal cortex, these factors are likely to be developmental, hormonal, and gender related. For example, adrenocortical tumors and Cushing syndrome are generally more frequent in females, and almost all of the probands studied here or in our previous investigations (7) were females. On the other hand, in all cases where inheritance of the PDE11A mutation could be proved, the asymptomatic carrier was the father (see Fig. 1A). In addition, the presence of allelic losses of the corresponding normal allele in adrenal tissues seems to be a determining factor in the development of an adrenal tumor as it is suggested by the PDE11A-associated tumor genetic studies.

In conclusion, PDE11A-inactivating mutations are present in the general population and may be risk factors for a variety of tumors.

**Acknowledgments**

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**Figure 2.** A. HEK293 cell lysates were assayed for PDE11A protein content at baseline (Mock) and after transfection with pCI plasmids bearing the PDE11A antisense open reading frame (ORF; PDE11A4/AS), the wild-type PDEA11A ORF (PDE11A), and the mutant c.2411G>A form (PDE11A4/R804H). The antisense construct completely abolished endogenous expression of the PDE11A gene, whereas similar PDE11A protein levels were expressed from the pCI-PDE11A4 and pCI-PDE11A4-R804H plasmids. B, cAMP and cGMP activity in CAR545.03’s adrenal tumor. Tissue lysates from three normal adrenal glands (Normal) and those from CAR545.03 were assayed separately for cAMP and cGMP content; all experiments were repeated at least twice and each sample was run in triplicate. C, cAMP levels in HEK293 cells after transfection with the wild-type PDE11A decreased, as expected; they increased following transfection with the antisense and R804H constructs. cGMP levels in HEK293 cells also decreased after transfection with the wild-type PDE11A, as expected; transfection of the antisense ORF-bearing construct led to an increase of cGMP levels, and that of the R804H construct to a decrease, suggesting that the mutation affects PDE11A catalysis of cAMP and cGMP differently. D, cAMP and cGMP levels in HeLa cells after identical experiments; cGMP levels were the same as mock after transfection with the R804H construct, indicating that the same mutation has different effects on cGMP levels in HeLa cells. NS, nonsignificant. *, P < 0.05; **, P < 0.05, all comparisons for this transfection were significant, except for that with the wild-type construct (P > 0.1).
Figure 3. CREB and P-CREB levels in the patients’ adrenal tumors. A, tissue lysates from six normal adrenal glands (Normal) and those from CAR545.03 (Mutant) were tested by Western blotting with commercially available antibodies for CREB and P-CREB. The ratios were calculated after scanning the individual protein bands and correcting for β-actin absorbance; the y-axis measures random absorbance units. Each sample was tested at least twice, each time in duplicate. B, representative immunostaining for P-CREB from one normal adrenal and the CAR545.03 tissue sample; these data are included in (A). C and D, samples from CAR545.03 and three normal adrenal glands were stained using commercially available CREB and P-CREB antibodies and following the manufacturer’s instructions; only the results on CAR545.03’s adrenal are shown. Staining for CREB (C) was less intense than that for P-CREB (D), especially within the nodule (>10) where intense, nuclear-specific staining is evident.* P < 0.001.

References


Adrenal Hyperplasia and Adenomas Are Associated with Inhibition of Phosphodiesterase 11A in Carriers of \textit{PDE11A} Sequence Variants That Are Frequent in the Population

Anelia Horvath, Christoforos Giatzakis, Audrey Robinson-White, et al.