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Priority Report

Functional Phosphodiesterase 11A Mutations May Modify the Risk of Familial and Bilateral Testicular Germ Cell Tumors

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Abstract

Inactivating germline mutations in phosphodiesterase 11A (*PDE11A*) have been implicated in adrenal tumor susceptibility. *PDE11A* is highly expressed in endocrine steroidogenic tissues, especially the testis, and mice with inactivated *Pde11a* exhibit male infertility, a known testicular germ cell tumor (TGCT) risk factor. We sequenced the *PDE11A* gene-coding region in 95 patients with TGCT from 64 unrelated kindreds. We identified 8 nonsynonymous substitutions in 20 patients from 15 families: four (R52T, F258Y, G291R, and V820M) were newly recognized, three (R804H, R867G, and M878V) were functional variants previously implicated in adrenal tumor predisposition, and one (Y727C) was a known polymorphism. We compared the frequency of these variants in our patients to unrelated controls that had been screened and found negative for any endocrine diseases: only the two previously reported variants, R804H and R867G, known to be frequent in general population, were detected in these controls. The frequency of all *PDE11A*-gene variants (combined) was significantly higher among patients with TGCT (P = 0.0002), present in 19% of the families of our cohort. Most variants were detected in the general population, but functional studies showed that all these mutations reduced PDE activity, and that PDE11A protein expression was decreased (or absent) in TGCT samples from carriers. This is the first demonstration of the involvement of a PDE gene in TGCT, although the cyclic AMP signaling pathway has been investigated extensively in reproductive organ function and their diseases. In conclusion, we report that *PDE11A*-inactivating sequence variants may modify the risk of familial and bilateral TGCT. [Cancer Res 2009;69(13):5301–6]

Introduction

The molecular causes of testicular germ cell tumor (TGCT), the most common malignancy in Caucasian men ages 15 to 45 years, remain elusive (1, 2). A genetic basis for TGCT is supported by familial clustering, younger-than-usual age at diagnosis, and an increased risk of bilateral disease (3, 4). Thus, a positive family history is an accepted and major risk factor, with an estimated relative risk of 8 to 10 for brothers of patients with TGCT, significantly greater than the 2- to 3-fold increase in familial risk observed in most adult solid tumors (5–7). Two consortium-based linkage analyses have identified genomic regions of modest statistical interest, but the data provide no definitive evidence of a single, rare, high-penetrance susceptibility gene (8–11). A consensus is emerging that familial susceptibility to TGCT may be best explained by the combined interaction of multiple, more common, low-penetrance genes.

We recently described adrenocortical tumors in patients who carry germline inactivating mutations of isoform 4 of *PDE11A* (*PDE11A4*), an essential regulator of cyclic AMP (cAMP) signaling in adrenal and other steroidogenic tissues (12, 13). Several lines of evidence suggested that *PDE11A* might be a candidate gene for TGCT: (*a*) we have observed high expression of the *PDE11A4* isoform in human testes; testicular tissue was also the only tissue to express all four known isoforms of *PDE11A* (12); (*b*) nongerm cell testicular tumors have been linked to genetic aberrations of the cAMP signaling pathway, including somatic activating mutations of the G-stimulatory subunit of the G-protein (*GNAS1*) in Leydig cell hyperplasia and tumors and in McCune-Albright syndrome, and germline inactivating *PRKAR1A* mutations that are responsible for large cell calcifying Sertoli cell tumors in the context of Carney complex (14, 15); and (*c*) male-limited infertility has been reported in the *Pde11a*$^{-/-}$ mouse, and is a known risk factor for TGCTs in humans (16–18).

We analyzed the *PDE11A* coding sequence in patients with familial and bilateral TGCT and various groups of control individuals. We then assessed the functional effects of the identified variants *in vitro*, evaluated their segregation with the TGCT phenotype in families, and studied PDE11A protein expression in TGCTs. Our data suggest that germline *PDE11A* mutations may modify the risk of familial and bilateral TGCTs.

Materials and Methods

The *PDE11A* gene was analyzed in 95 patients with TGCT from 64 families enrolled in National Cancer Institute Clinical Genetics Branch

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

A. Horvath and L. Korde have contributed equally to this work and are thus sharing the first authorship.

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Table 1. Allele frequency (minor allele) of sequence variations in *PDE11A* in patients with testicular cancer and control individuals

Sequence Change		TGCT patients (# alleles 128)	Endo-negative controls (# alleles 384)	TGCT patients vs Endo-negative controls	
				χ^2	*P*
Splice	c.1072-3c/t	24 (0.19)	33 (0.09)	10.01	0.0016
	c.1577-3c/t	33 (0.26)	133 (0.35)	3.43	0.06
	c.1644+26insGTTTATA	33 (0.26)	133 (0.35)	3.43	0.06
Synonymous / Poly-s	c.147A>C / p.L49L	0 (0.0)	1 (0.002)	0.00	>0.1
	c.690C>T / p.C230C	6 (0.05)	5 (0.01)	3.75	0.05 †
	c.792C>A / p.T264T	23 (0.18)	NA	NA	NA
	c.799C>T / p.L267L	1 (0.01)	0 (0.0)	1.73	>0.1 †
	c.1263A>G / p.E421E	21 (0.16)	39 (0.10)	3.62	0.06
	c.1626A>G / p.A542A	33 (0.26)	133 (0.35)	3.43	0.06
	c.2758ins / p.920Sins	70 (0.55)	243 (0.63)	2.98	>0.1 †
Missense	c.155G>C / p.R52T	1 (0.01)	0 (0.0)	0.33	>0.1 †
	c.773T>A / p.F258Y	1 (0.01)	0 (0.0)	0.33	>0.1 †
	c.871G>C / p.G291R	1 (0.01)	0 (0.0)	0.33	>0.1 †
	c.1045G>A / p.A349T	0 (0.00)	1 (0.002)	0.33	>0.1 †
	c.1825A>G / p.D609N	0 (0.00)	0 (0.0)	—	—
	c.2180A>G / p.Y727C ‡	3 (0.02)	0 (0.0)	5.48	0.02 †
	c.2411G>A / p.R804H	3 (0.02)	5 (0.01)	0.17	>0.1 †
	c.2458 G>A / p.V820M	1 (0.01)	0 (0.0)	0.33	>0.1 †
	c.2599C>G / p.R867G	3 (0.02)	5 (0.01)	0.17	>0.1 †
	c.2632A>G / p.M878V	2 (0.02)	0 (0.0)	2.68	>0.1 †
All missense		15 (0.09)	11 (0.03)	13.83	0.0002 †
Stop	c.171Tdel FS41X	0 (0.0)	0 (0.0)	0.00	>0.1 †
	c.919C>T / p.R307X	0 (0.0)	0 (0.0)	0.00	>0.1 †
	c.1655_1657del/inFS15X	0 (0.0)	0 (0.0)	0.00	>0.1 †
All nonsense		0 (0.0)	0 (0.0)	0 (0.0)	>0.1 †
All nonsense and misssense		15 (0.09)	11 (0.03)	13.83	0.0002 †

Abbreviation: NA, not available.

* Between 508 and 754 NYCP individuals were genotyped for the presence of the different variants; the results are presented as allele frequency in the individual cells.

† χ^2 is calculated after Yates corrections for continuity (Yates correction was applied for all calculations having number<10 in any cell of the contingency table).

‡ Y727C is a frequent polymorphic variant listed in the databases.

Familial Testicular Germ Cell Study (Protocol 02-C-0178). Written informed consent was obtained from all participants, and the study was approved by the institutional review boards of the participating centers (NCI, NIH, Bethesda, MD, USA and Hôpital Cochin, Paris, France). The study population includes 19 males with bilateral testicular cancer and no affected relatives (included because of the known excess of bilateral TGCT in familial testicular cancer), 6 males with both bilateral cancer and an affected family member, and 70 males from multiple-case TGCT families. Of the multiple-case families, a majority (80%) had 2 affected family members; the remaining families had 3 to 5 affecteds. The identified variants were compared with those found in three different control groups: 192 individuals specifically examined at Hôpital Cochin, Paris, France, who were selected because they had no clinical evidence for any endocrine tumors (designated the "endo-negative" group) plus a negative family history of endocrine disorders; 95 unselected individuals negative for the most common adult diseases from the Coriell Institute database (the "Coriell" group); and, finally, 745 unselected healthy individuals enrolled in the New York Cancer Project (NYCP), who were already genotyped for the 5 *PDE11A* variants that we published previously (c.171Tdel/fs41X, R307X, c.1655_1657TCTdelCCins/fs15X, R804H, and R867G; ref. 12). For the first two groups, the complete *PDE11A* coding sequence was analyzed as previously described (12). The individuals from the third group were genotyped for all identified *PDE11A* coding variants, applying Sequenom mass spectrometry, as published (13).

For transfection experiments, the *PDE11A* open reading frame was cloned into pCR3.1, and the missense mutations (R52T, F258Y, G291R, A349T, D609N, Y727C, V820M, and M878V) were introduced by overlapping PCR, as previously described (13). Primers used for vector generation are described in Supplementary Table S1. The HEK293 and murine Leydig tumor cell (MLTC-1) were transiently transfected using Lipofectamine 2000 (Invitrogen), following the manufacturer's protocol. Cells were transfected with 6 μg of plasmid DNA expressing either the wild-type (WT) or the mutated form of PDE11A, harvested 48 h after the transfection, and subjected to cAMP level and PDE activity assays. cAMP levels were determined using the cAMP [3H] Biotrak Assay System (GE Healthcare Life Science), following the manufacturer's instructions. PDE activity was measured using BIOMOL GREENTM Reagent supplied by QuantiZymeTM Assay System, BIOMOL International LP, according to recommended protocol. All functional studies were done in triplicates, and an average was calculated for each value; each experiment was repeated at least twice.

To assess the PDE11A protein expression in the affected tumor tissues, we applied immunohistochemistry using rabbit polyclonal antibody specific for PDE11A (Abcam), as described previously (13, 19). Paraffin-embedded

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Table 1. Allele frequency (minor allele) of sequence variations in *PDE11A* in patients with testicular cancer and control individuals (Cont'd)

Coriell controls (# alleles 190)	TGCT patients vs Coriell controls		NYCP controls*	TGCT patients vs NYCP controls	
	χ^2	*P*	χ^2	χ^2	*P*
40 (0.21)	0.25	>0.1	NA	NA	NA
49 (0.26)	0.00	>0.1	NA	NA	NA
49 (0.26)	0.00	>0.1	NA	NA	NA
0 (0.0)	0.00	>0.1	NA	NA	NA
7 (0.04)	0.02	>0.1 †	NA	NA	NA
30 (0.16)	0.26	>0.1	NA	NA	NA
1 (0.01)	0.19	>0.1 †	NA	NA	NA
28 (0.15)	0.16	>0.1	NA	NA	NA
49 (0.26)	0.00	>0.1	NA	NA	NA
111 (0.59)	0.43	>0.1	0.66	3.81	>0.1
0 (0.0)	0.04	>0.1 †	0.003	0.05	>0.1 †
0 (0.0)	0.04	>0.1 †	0.0	2.63	>0.1 †
0 (0.0)	0.04	>0.1 †	0.0	2.63	>0.1 †
1 (0.01)	0.19	>0.1 †	0.003	0.21	>0.1 †
1 (0.01)	0.04	>0.1 †	0.003	0.22	>0.1 †
6 (0.03)	0.01	>0.1 †	0.05	1.00	>0.1 †
2 (0.01)	0.20	>0.1 †	0.02	0.13	>0.1 †
0 (0.0)	0.04	>0.1 †	0.003	0.22	>0.1 †
5 (0.03)	0.03	>0.1 †	0.02	0.03	>0.1 †
1 (0.01)	0.12	>0.1 †	0.02	0.08	>0.1 †
16 (0.08)	0.95	>0.1	0.08	1.86	>0.1
2 (0.01)	0.19	>0.1 †	0.0007	2.63	>0.1 †
0 (0.0)	0.00	>0.1	0.01	0.05	>0.1
1 (0.01)	0.04	>0.1 †	0.001	0.89	>0.1 †
3 (0.02)	0.7	>0.1 †	0.008	0.19	>0.1
19 (0.1)	0.24	>0.1	0.05	1.67	>0.1

tissue slides were stained in all cases for both H&E and PDE11A expression, and were compared with negative controls and normal tissue.

Results

We found 17 different sequence changes in the TGCT cases: three splice variants, one in-frame *ins/del* polymorphism, five synonymous, and eight nonsynonymous substitutions (Table 1). Missense changes F258Y and G291R were encountered only among TGCT patients; they were not detected in the 1,032 individuals from any of the three control groups. The two previously identified terminating mutations (c.171Tdel/FS41X and c.1655_1657del/insFS15X) were found in the newly studied Coriell control panel, and all three known *PDE11A* nonsense mutations were present in the NYCP controls, as previously published (12, 13).

We first compared the combined frequency of *PDE11A* sequence variants in the TGCT patients with the three control groups. A higher prevalence of missense and nonsense mutations was observed in the TGCT patients compared with both the endo-negative and Coriell controls, but the difference reached statistical significance only for the first group ($P = 0.0002$). When compared with the NYCP controls, all identified variants were seen with similar prevalence and were only slightly more frequent among the TGCT patients (see Table 1).

Analyzed individually, only two mutations—the novel substitutions F258Y and G291R—showed significant differences between the TGCT patients and each of the three control groups. Notably, both F258Y and G291R reside in the longest coding exon of *PDE11A* (exon 3) that is expressed only in the *PDE11A4* isoform that is most common in steroidogenic tissues. A third novel substitution (R52T) was detected in the same exon; the combined frequency of these three *PDE11A4*-specific mutations was substantially greater among the TGCT cases compared with each of the control groups, as well as all controls combined ($P = 0.0005$; odds ratio, 12.96; 95% confidence interval, 2.87–58.54).

Splice variants and synonymous substitutions showed similar frequency in all studied groups, with two exceptions: the known polymorphism C230C and the splice variant c.1072-3c/t were found more often in patients with TGCT than in the endo-negative controls. The reason for this is unclear; because this was not seen when compared with other control groups, it may reflect the slightly different ethnic background of the studied populations; it may also reflect the small number of TGCT cases we have in this study.

Transfection experiments in HEK293 and MLTC-1 cells with expression vectors harboring the substitutions R52T, F258Y, G291R, A349T, D609N, Y727C (the latter tested only in MLTC-1 cells), V820M, and M878V were conducted along with the R804H and R867G for which we previously showed impaired PDE11A function (13). Higher (relative to the WT) cAMP levels were measured in both tumor cell lines for all *PDE11A* missense mutations, indicating a reduced ability of the variant PDE11A proteins to degrade cAMP

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(Fig. 1*A* and *C*). Posttransfection changes in PDE activity were negatively correlated with cAMP levels: PDE activity was lower versus WT *PDE11A*-transfected HEK 293 and MLTC-1 cells for all mutation-bearing constructs (Fig. 1*B*). The observed differences in both cAMP levels (increased) and PDE activity (decreased) ranged between 10% and 200% of normal, and were greater in MLTC-1 cells than HEK293 cells.

To assess the cosegregation with the TGCT phenotype, we analyzed the distribution of *PDE11A* missense changes in 11 families carrying a *PDE11A* variant (Fig. 2). In general, the presence of TGCT and the family mutation were concordant: 17 of 21 (81%) of TGCT patients were heterozygous for a missense mutation. In two of the three families with affected father and sons, both were carriers of that family's *PDE11A* mutation (Families 2 and 7); in four of the five families involving affected siblings, each brother carried the *PDE11A* mutation (families 3–6). In one family (family 1), R804H was independently transmitted from two unrelated family branches and was present in three of the four available for analysis male individuals (individuals #05, #11, and #14): two of these patients had TCGT, and the one who did not have the disease (#14) was 18 years old at the time of his investigation.

In total, 22 unaffected individuals from these 11 families were positive for a *PDE11A* mutation; 11 were females, and 5 were males under age 35 years. Although these latter individuals did not have TGCT, they often presented with a genitourinary abnormality—primarily, cryptorchidism or testicular microcalcifications, both of which are associated with increased risk of TGCT.

Immunohistochemistry in tumor tissue from four patients with TGCT who carried a *PDE11A* mutation showed very low or no PDE11A protein expression in tumor cells compared with surrounding normal testicular tissue (Fig. 3*A–C*, representative stained sections from an M878V-related tumor). In contrast, TGCT tumor tissue from a patient with the normal *PDE11A* gene sequence (Fig. 3*D–F*) showed high PDE11A expression.

Discussion

In this study, we observed a significantly higher frequency of *PDE11A* coding changes in patients with TGCT compared with healthy controls. There was strong but not perfect concordance between the presence of a tumor and the presence of a mutation. Transfection experiments showed that each of the missense mutations resulted in lower levels of PDE and higher levels of cAMP. The mutations had a greater effect in a murine testicular Leydig cell tumor (MLTC-1) cell line known to be cAMP-responsive. Immunohistochemistry documented low or absent PDE expression in testicular cancer cells from men harboring these genetic abnormalities. Finally, there was a suggestion that cancer-free family members who carried these mutations manifested nonmalignant GU abnormalities that have been associated with increased risk of TGCT, as well as diverse nontesticular cancers. The biological plausibility of this association is further supported by the recent discovery that male *Pde11a*$^{-/-}$ mice are sterile; male infertility is a known risk factor for human TGCT (16–18).

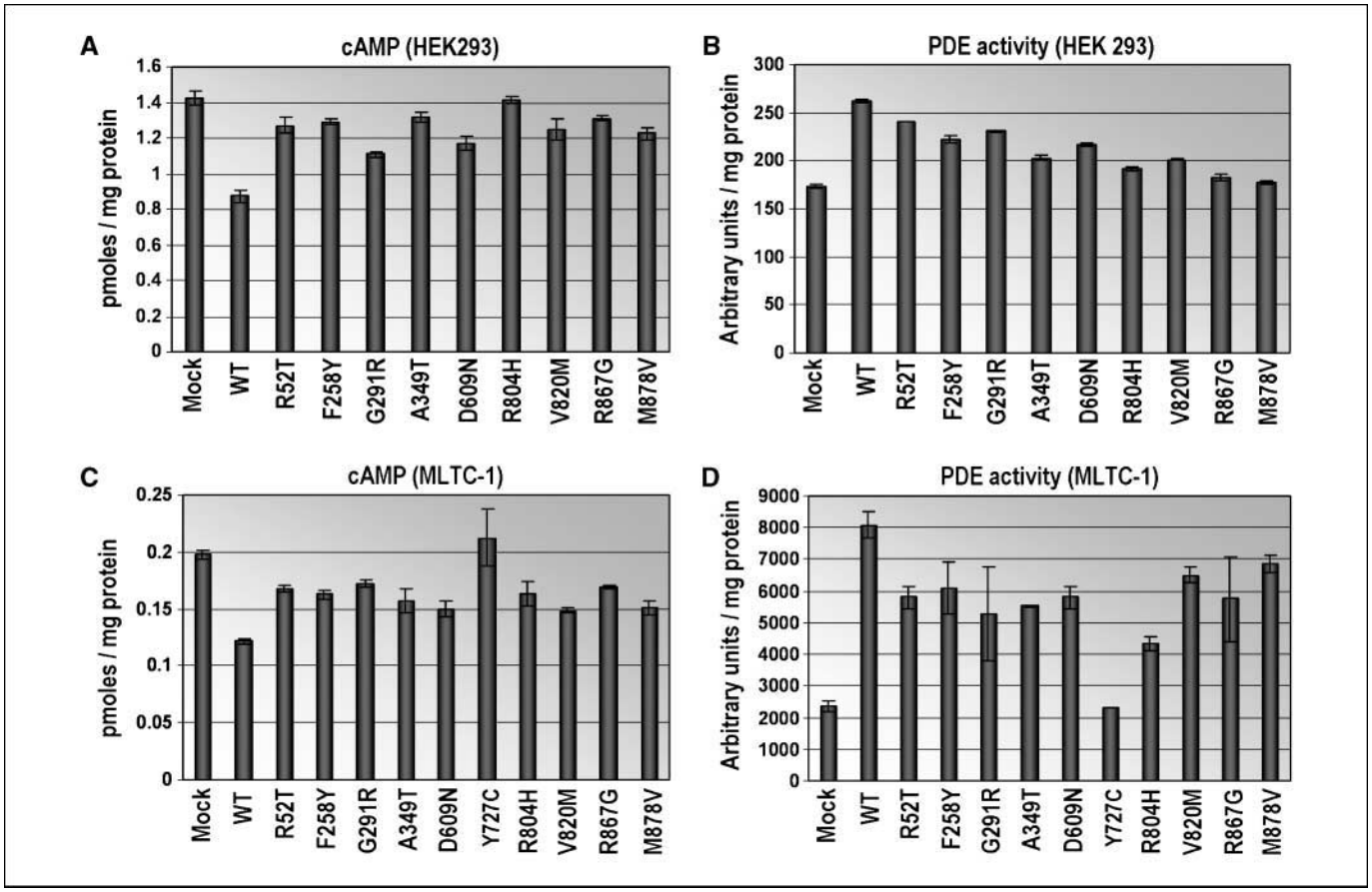

Figure 1. cAMP (*A*) and PDE (*B*) activity after transfection of HEK293 cells with WT and mutant *PDE11A4* expression vectors; cAMP (*C*) and PDE (*D*) activity after transfection of MLTC-1 cells with WT and mutant *PDE11A* expression vectors.

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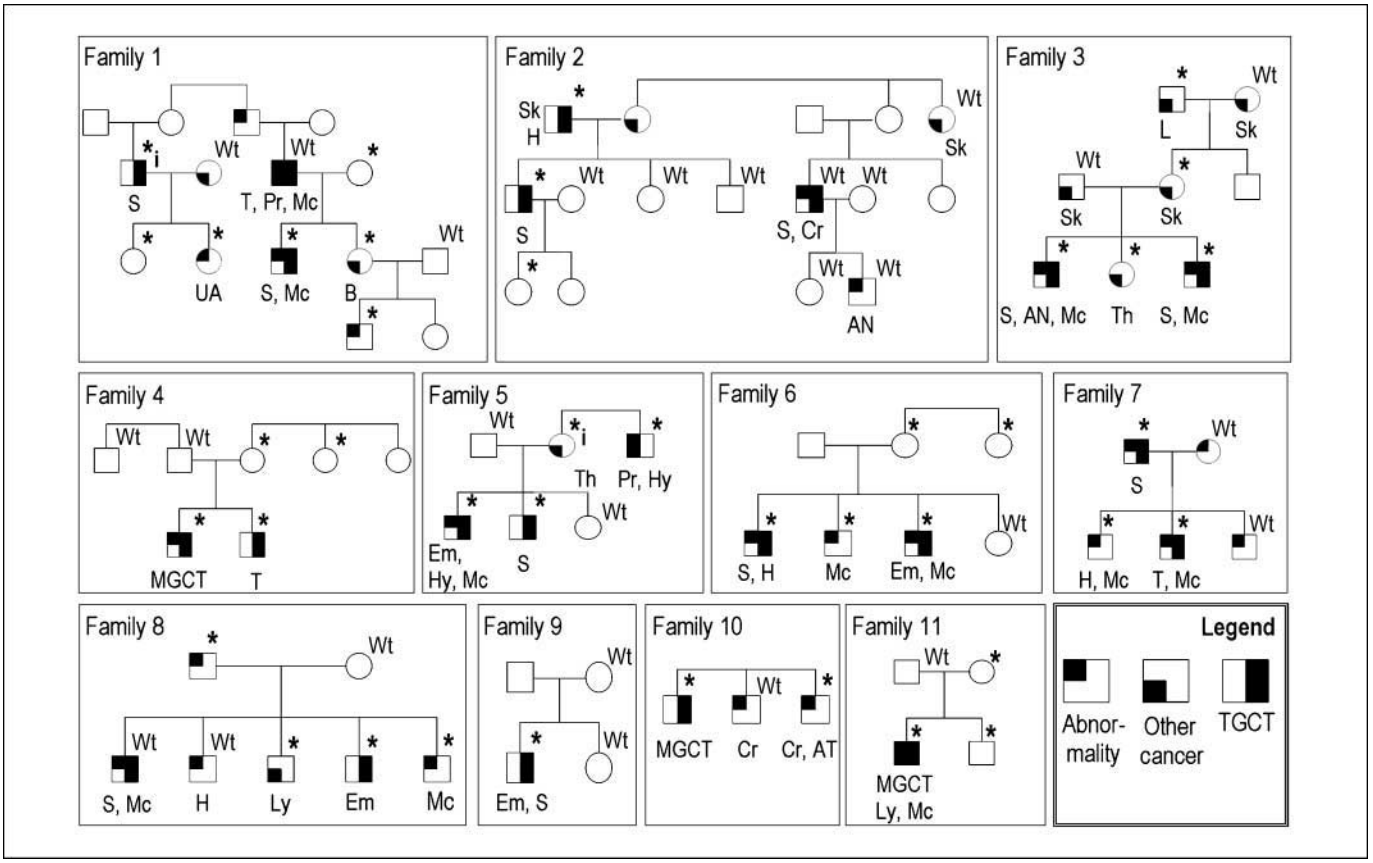

Figure 2. Segregation of the *PDE11A* mutations with the TGCT phenotype. *Families 1* to *8,* multiple-case kindred; *Families 9* to *11,* sporadic bilateral testicular cancers. The genotype is indicated above each symbol: *Wt,* WT alleles; *, mutation carrier; **i,* mutation carrier (inferred). Family mutations: families 1, 8, and 11, R804H; family 2, M878V; family 3, F258Y; families 4, 6, and 9, R867G; family 5: R52T; family 7, V820M; family 10, G291R. The phenotype is indicated below each symbol: *S,* seminoma; *T,* teratocarcinoma; *Em,* embryonal; *MGCT,* mixed germ cell tumor; *Pr,* prostate cancer; *B,* breast cancer; *Th,* thyroid cancer; *Sk,* skin cancer; *L,* lung cancer; *Ly,* lymphoma; *H,* inguinal hernia; *Mc,* microlithiasis; *UA,* uterine abnormality; *AN,* accessory nipple; *AT,* absence testes (bilateral orchiectomy for undescended testes); *Cr,* cryptorchidism.

Collectively, these data suggest that *PDE11A*-inactivating defects may confer susceptibility to familial and bilateral TGCT.

A polygenic model for susceptibility to TGCT has been proposed (1, 2, 7–10). Our *in vitro* studies suggest that impaired cAMP degradation and, consequently, increased cAMP signaling, analogous to that observed in benign testicular tumors of the Carney complex and McCune-Albright syndromes (14, 15), may promote TGCT formation. We confirmed the wide spectrum of *PDE11A* variants, including both missense and nonsense mutations, previously reported in unselected population controls (12, 13), a finding consistent with the limited segregation of the mutant allele with TGCT in affected families. Because this and previous studies clearly showed that the mutant variants alter PDE function, their presence may produce compensatory changes in other members of the PDE family and/or additional factors that regulate cAMP levels and PKA activity, thereby offering a possible explanation for their reduced penetrance. The comparison between *PDE11A* variants associated with testicular and adrenocortical tumors showed a similar frequency distribution (Supplementary Table S2; ref. 20). It is tempting to speculate that other cAMP-sensitive tissues would also be possible targets of a PDE defect.

It is noteworthy that different classes of *PDE11A* gene variants have variable distributions between the multiple "control" groups in this study: nonsense mutations were absent among the 192 individuals in whom endocrine neoplasms were aggressively sought and excluded, whereas they were detected in our other two control groups from the general population (see Supplementary Table S1). In addition, *PDE11A* missense substitutions were significantly less frequent in the endocrine-negative cohort (20). This may be a consequence of the presence in the general population of individuals with a very mild (and, thus, unrecognizable without targeted testing) endocrine phenotype. This pattern may bias the disease/variant association toward the null when compared with the unselected population controls.

In conclusion, we present for the first time data suggesting that *PDE11A* may be one of the hypothesized multiple genetic factors that, in concert, contribute to inherited TGCT susceptibility. Additional investigations are needed to confirm this observation, and to elucidate the mechanism by which increased cAMP levels affect the risk of TGCT.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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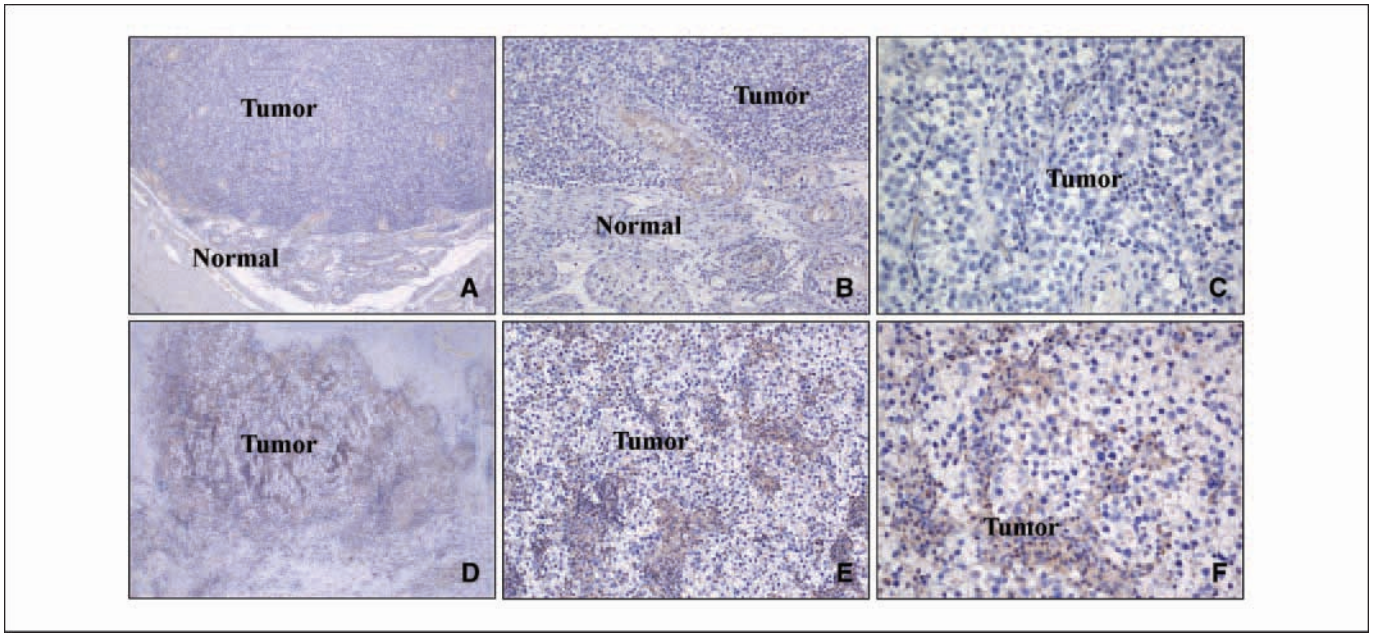

Figure 3. *A,* expression of PDE11A in testicular tumor tissue and surrounding normal cells of patient positive for *PDE11A* missense mutation (M878V), ×100; *B,* seminiferous tubule showing positive cytoplasmic staining, ×150; *C,* negative lymphocytes (×200); *D,* expression of PDE11A in testicular tumor cells of patient with wt *PDE11A* genotype (×100); *E,* densely cellular region from inside the tumor showing negative staining for PDE11A, 150×; *F,* tumor regions positive for PDE11A, ×200.

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