Sequence analysis of the catalytic subunit of PKA in somatotroph adenomas

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Abstract

Objective: The pathogenetic mechanisms of sporadic somatotroph adenomas are not well understood, but derangements of the cAMP pathway have been implicated. Recent studies have identified L206R mutations in the alpha catalytic subunit of protein kinase A (*PRKACA*) in cortisol-producing adrenocortical adenomas and amplification of the beta catalytic subunit of protein kinase A *PRKACB* in acromegaly associated with Carney complex. Given that both adrenocortical adenomas and somatotroph adenomas are known to be reliant on the cAMP signalling pathway, we sought to determine the relevance of the L206R mutation in both *PRKACA* and *PRKACB* for the pathogenesis of sporadic somatotroph adenomas.
Design: Somatotroph adenoma specimens, both frozen and formalin-fixed, from patients who underwent surgery for their acromegaly between 1995 and 2012, were used in the study.
Methods: The DNA sequence at codon 206 of *PRKACA* and *PRKACB* was determined by PCR amplification and sequencing. The results were compared with patient characteristics, the mutational status of the *GNAS* complex locus and the tumour granulation pattern.
Results: No mutations at codon 206 of *PRKACA* or *PRKACB* were found in a total of 92 specimens, comprising both WT and mutant *GNAS* cases, and densely, sparsely and mixed granulation patterns.
Conclusions: It is unlikely that mutation at this locus is involved in the pathogenesis of sporadic somatotroph adenoma; however, gene amplification or mutations at other loci or in other components of the cAMP signalling pathway, while unlikely, cannot be ruled out.

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Introduction

Genetic studies of the pathogenesis of sporadic somatotroph adenomas have identified a role for the cAMP signalling pathway, and conversely syndromes that feature a germline mutation in the components of this pathway can be associated with the formation of somatotroph adenomas. Thus, sporadic somatotroph adenomas are often associated with mutations at the *GNAS* complex locus, encoding the *GSP* oncogene. These mutations implicate the cAMP pathway in the pathogenesis of sporadic somatotroph adenomas in 15–58% of cases (1, 2, 3), although the tumourigenic mechanism is unclear. The McCune–Albright syndrome results from post-zygotic germline mutations in *GNAS* and is often characterised by endocrine neoplasia: it is associated with somatotroph adenomas and acromegaly in 10–20% of cases (4). Carney complex is an autosomal dominant syndrome characterised by multiple, often endocrine tumours, including somatotroph adenomas, in ~10% of cases (5). In over 60% of cases of Carney complex, a mutation is found in the regulatory subunit 1A of protein

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kinase A (*PRKAR1A*), an intrinsic part of cAMP signalling, although such mutations are not found in sporadic pituitary adenomas (6, 7, 8). Recently, whole-exome sequencing studies have identified a mutation in the active-site cleft of catalytic subunit A of PKA (*PRKACA*) in cortisol-producing adrenocortical adenomas, most commonly R206 (9, 10, 11). This mutation was observed in 37–66% of cases and resulted in constitutive activation of PKA that was neither suppressed by the PKA regulatory subunit nor increased by addition of cAMP (either directly or indirectly by forskolin treatment) (10, 11). A recent report of a patient with Carney complex with acromegaly, spotty pigmentation and myxomas, but without Cushing's syndrome, has shown the presence of a triplication of chromosome 1p31.1 (containing *PRKACB*), leading to increased expression of *PRKACB* (12). The authors propose that amplification of *PRKACB* may be responsible for non-adrenal manifestations of Carney complex. Given the involvement of the cAMP pathway in sporadic somatotroph adenoma pathogenesis, we sought to determine whether mutations in the catalytic subunit of PKA might also occur in these lesions and be involved in the pathogenesis of these tumours. We therefore amplified and sequenced the region of *PRKACA* and *PRKACB* containing codon 206 in a large series of sporadic somatotroph adenomas. The findings were compared with patient characteristics, the mutational status of *GNAS* in a sub-group and the granulation pattern of the specimens.

Subjects and methods

Patients ($n=92$) with a diagnosis of acromegaly were included. None of the patients had a clinical history of FIPA, MEN1 or SDH-associated acromegaly, but germline mutations in *MEN1* and *SDH* were not sought routinely in all individuals. The tumour samples, either formalin fixed and paraffin embedded (FFPE; $n=43$) or frozen ($n=49$), were retrieved from the surgical neuropathology archive of the Oxford Brain Bank and the Tissue Bank of the Department of Endocrinology at Barts and the London School of Medicine. The specimens were excluded if the samples were too small or the DNA was of insufficient quality to enable successful amplification of the regions containing codon 206 of *PRKACA* and *PRKACB*. The specimens were preferentially included if previous analysis had shown them to be *GNAS* WT. The findings of Sato *et al*. (11) demonstrate that mutations in *PRKACA* and *GNAS* are mutually exclusive in adrenocortical adenomas and so we speculated that any *PRKACA* mutations identified might only be present in *GNAS*-mutation negative specimens. All studies were conducted on linked-anonymised samples under multi-site and local Research Ethics Committee (REC) approval.

DNA was extracted from either 5×10 μm sections of FFPE tissue (QiaAmp FFPE DNA Kit, Qiagen) or from homogenised fresh-frozen specimens (QiaAmp DNA Mini Kit or DNeasy Blood and Tissue Kit (Qiagen)) according to manufacturer's instructions. PCR was carried out to generate amplicons including codon 206 of *PRKACA* and *PRKACB*. The primers were designed using Primer-Blast (www.ncbi.nlm.nih.gov/tools/primer-blast). For FFPE specimens primers were as follows: *PRKACA* sense (5′-GGTGACAGACTTCGGTTTCGC-3′) and antisense (5′-CCTTGTTGTAGCCCTGGAGCA-3′); *PRKACB* sense (5′-TGGTTTTATTTCTTTGCAGTGAGC-3′) and antisense (5′-CCTGGATATAGCCTTGATGGTCA-3′). The primers for gDNA from frozen specimens were as follows: *PRKACA* sense (5′-CAACTGCCTGTTCTTGTGCC-3′) and antisense (5′-AGTCCACGGCCTTGTTGTAG-3′); *PRKACB* sense (5′-AAACTTTCAACGTAGGTGCAAT-3′) and antisense (5′-CAAAAGTCCATAGGGATGCATGT-3′). The primers for cDNA from frozen specimens were as follows: *PRKACA* sense (5′-CTGCACTCGCTGGATCTCAT-3′) and antisense (5′-CAGAGCTGAAGTGGGAAGGG-3′) and *PRKACB* sense (5′-GCAGCTCAGATAGTGCTAACATTC-3′) and antisense (5′-GGTCTGCAAAGAATGGGGGATA-3′).

For FFPE specimens, DNA template (100 ng) was added to 10× PCR buffer solution (10% v/v; Qiagen), $MgCl_2$ (final concentration 4 mmol/l, for *PRKACB* only), dNTPs (200 μmol/l) (Promega), 0.5 U of Taq polymerase (HotStarTaq Plus, Qiagen) and 400 nmol/l each of forward and reverse primers. The cycling conditions were as follows: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s; annealing temperature for 30 s (61 °C for *PRKACA* and 57 °C for *PRKACB*) and 72 °C for 40 s, followed by a single 1 min extension. Total reaction volume was 20 μl. For frozen specimens, gDNA template ((100 ng) was added to 10× Taq reaction buffer (10% v/v; (New England Biolabs (NEB), Hitchin, UK)), dNTPs (200 μmol/l) (New England Biolabs), 0.12 U of Taq polymerase (New England Biolabs) and 200 nmol/l each of forward and reverse primers. The cycling conditions were as follows: 95 °C for 2 min, followed by 35 cycles at 95 °C for 30 s, annealing temperature for 30 s (67.6 °C for *PRKACA* and 62.5 °C for *PRKACB*) and 68 °C for 30 s, followed by a single 5 min extension. Total reaction volume was 25 μl.

RNA was extracted from frozen specimens using the RNeasy Kit (Qiagen), according to manufacturer's instructions. RT was performed using 500 ng RNA, random hexamer primers (0.5 μg/l, Promega) and M-MLV reverse

Table 1 Cases included in the study. Cases 1–21 have previously been reported (13).

Case	Sex	Age	Material	Preservation	Size	Granulation	*GNAS*	*PRKACA*	*PRKACB*
1	F	39	gDNA	FFPE	Macro	S	WT	WT	WT
2	F	64	gDNA	FFPE	Micro	D	WT	WT	WT
3	F	54	gDNA	FFPE	Macro	D	WT	WT	WT
4	F	41	gDNA	FFPE	Micro	D	WT	WT	WT
5	F	57	gDNA	FFPE	Macro	D	WT	WT	WT
6	M	49	gDNA	FFPE	Macro	D	WT	WT	WT
7	F	56	gDNA	FFPE	Micro	M	WT	WT	WT
8	M	35	gDNA	FFPE	Macro	ND	R201C	WT	WT
9	F	66	gDNA	FFPE	Macro	S	ND	WT	WT
10	F	23	gDNA	FFPE	Macro	S	ND	WT	WT
11	F	47	gDNA	FFPE	Macro	S	WT	WT	WT
12	M	43	gDNA	FFPE	Micro	D	WT	WT	WT
13	F	44	gDNA	FFPE	Macro	S	WT	WT	WT
14	F	62	gDNA	FFPE	Macro	M	WT	WT	WT
15	M	52	gDNA	FFPE	Macro	S	R102C	WT	WT
16	F	54	gDNA	FFPE	Macro	M	WT	WT	WT
17	F	26	gDNA	FFPE	Macro	S	ND	WT	WT
18	F	33	gDNA	FFPE	Macro	ND	WT	WT	WT
19	F	53	gDNA	FFPE	Macro	ND	WT	WT	WT
20	F	59	gDNA	FFPE	Micro	D	WT	WT	WT
21	M	56	gDNA	Frozen	Macro	D	R201C	WT	WT
22	F	49	gDNA	FFPE	ND	S	ND	WT	WT
23	M	31	gDNA	FFPE	ND	S	ND	WT	WT
24	F	45	gDNA	FFPE	ND	ND	ND	WT	WT
25	F	49	gDNA	FFPE	ND	ND	ND	WT	WT
26	M	34	gDNA	FFPE	Micro	S	ND	WT	WT
27	M	55	gDNA	FFPE	Micro	S	ND	WT	WT
28	M	43	gDNA	FFPE	Micro	ND	ND	WT	WT
29	F	46	gDNA	FFPE	Macro	ND	ND	WT	WT
30	F	61	gDNA	FFPE	Macro	S	ND	WT	WT
31	F	19	gDNA	FFPE	Macro	ND	ND	WT	WT
32	F	41	gDNA	FFPE	Macro	D	ND	WT	WT
33	M	52	gDNA	FFPE	ND	ND	ND	WT	WT
34	F	29	gDNA	FFPE	Micro	ND	ND	WT	WT
35	M	40	gDNA	FFPE	ND	ND	ND	WT	WT
36	M	69	gDNA	FFPE	ND	ND	ND	WT	WT
37	M	18	gDNA	FFPE	Macro	ND	ND	WT	WT
38	F	41	gDNA	FFPE	Micro	D	ND	WT	WT
39	F	25	gDNA	FFPE	Macro	S	ND	WT	WT
40	F	29	gDNA	FFPE	Macro	S	ND	WT	WT
41	F	67	gDNA	FFPE	Macro	D	ND	WT	WT
42	M	53	gDNA	FFPE	Macro	ND	WT	WT	WT
43	F	30	gDNA	FFPE	Macro	S	WT	WT	WT
44	F	36	gDNA	FFPE	Macro	S	WT	WT	WT
45	F	57	gDNA	Frozen	Macro	ND	ND	WT	WT
46	M	48	gDNA	Frozen	Micro	ND	ND	WT	WT
47	F	42	gDNA	Frozen	Macro	ND	ND	WT	WT
48	M	50	gDNA	Frozen	Micro	ND	WT	WT	WT
49	M	80	gDNA	Frozen	Macro	ND	ND	WT	WT
50	F	66	gDNA	Frozen	Macro	ND	ND	WT	WT
51	F	37	gDNA	Frozen	Macro	ND	ND	WT	WT
52	M	32	gDNA	Frozen	Macro	ND	ND	WT	WT
53	M	71	gDNA	Frozen	Macro	ND	ND	WT	WT
54	F	53	gDNA	Frozen	ND	ND	ND	WT	WT
55	M	42	gDNA	Frozen	Macro	ND	ND	WT	WT
56	F	33	gDNA	Frozen	Macro	ND	ND	WT	WT
57	M	44	gDNA	Frozen	Macro	ND	ND	WT	WT
58	F	38	gDNA	Frozen	Macro	ND	ND	WT	WT
59	M	48	gDNA	Frozen	ND	ND	ND	WT	WT
60	F	28	gDNA	Frozen	Macro	ND	ND	WT	WT
61	M	40	gDNA	Frozen	ND	ND	ND	WT	WT

Table 1 *Continued*

Case	Sex	Age	Material	Preservation	Size	Granulation	*GNAS*	*PRKACA*	*PRKACB*
62	F	33	gDNA	Frozen	Micro	ND	ND	WT	WT
63	F	43	gDNA	Frozen	Macro	ND	ND	WT	WT
64	F	26	gDNA	Frozen	Micro	ND	ND	WT	WT
65	M	63	gDNA	Frozen	Macro	ND	ND	WT	WT
66	F	57	gDNA	Frozen	Macro	ND	ND	WT	WT
67	M	48	gDNA	Frozen	Micro	ND	ND	WT	WT
68	F	46	gDNA	Frozen	ND	ND	ND	WT	WT
69	F	32	gDNA	Frozen	Macro	ND	ND	WT	WT
70	F	25	gDNA	Frozen	Macro	ND	ND	WT	WT
71	M	25	gDNA	Frozen	Macro	ND	ND	WT	WT
72	M	10	gDNA	Frozen	Macro	ND	ND	WT	WT
73	F	14	gDNA	Frozen	Macro	ND	ND	WT	WT
74	M	62	gDNA	Frozen	Macro	ND	ND	WT	WT
75	F	48	gDNA	Frozen	Micro	ND	ND	WT	WT
76	F	63	gDNA	Frozen	Macro	ND	ND	WT	WT
77	F	57	gDNA	Frozen	ND	ND	ND	WT	WT
78	M	34	gDNA	Frozen	Macro	ND	ND	WT	WT
79	M	26	gDNA	Frozen	Macro	ND	ND	WT	WT
80	F	28	gDNA	Frozen	ND	ND	ND	WT	WT
81	F	40	gDNA	Frozen	Macro	ND	ND	WT	WT
82	F	78	gDNA	Frozen	Micro	ND	ND	WT	WT
83	M	39	gDNA	Frozen	Macro	ND	ND	WT	WT
84	M	53	gDNA	Frozen	Macro	ND	ND	WT	WT
85	M	53	cDNA	Frozen	Macro	ND	WT	WT	WT
86	M	ND	cDNA	Frozen	Macro	ND	WT	WT	WT
87	M	69	cDNA	Frozen	Macro	ND	WT	WT	WT
88	M	62	cDNA	Frozen	Micro	ND	R201C	WT	WT
89	M	40	cDNA	Frozen	Macro	ND	ND	WT	WT
90	M	52	cDNA	Frozen	Macro	ND	ND	WT	WT
91	F	41	cDNA	Frozen	Macro	ND	ND	WT	WT
92	F	64	cDNA	Frozen	Macro	ND	ND	WT	WT

ND, not determined; DG, densely granulated; MG, mixed granulation pattern; SG, sparsely granulated; FFPE, formalin fixed, paraffin embedded; macro, macroadenoma; micro, microadenoma.

transcriptase (100 U) (Life Technologies). For amplification of *PRKACA*, cDNA template (100 ng) was added to 5× Q5 reaction buffer (20% v/v; (New England Biolabs)), 0.25 μl DNA polymerase (Q5 High-Fidelity DNA polymerase, New England Biolabs) 5× Q5 High GC Enhancer (20% v/v, New England Biolabs) dNTPs (200 μmol/l) and 500 nmol/l each of forward and reverse primers. The cycling conditions were as follows: 98 °C, 30 s, followed by 35 cycles of 98 °C for 10 s, 60 °C for 20 s, 72 °C for 20 s followed by a single extension for 2 min. Total reaction volume was 25 μl. For amplification of *PRKACB*, reaction and cycling conditions were identical to those for gDNA except that 400 μmol/l dNTPs were added and the annealing temperature was 62 °C.

The products were examined by agarose gel separation and purified (MinElute PCR Purification Kit (Qiagen)). Bidirectional sequencing of amplicons was carried out using BigDye Terminator chemistry (v3.1) and an ABI-3730 capillary sequencer (Applied Biosystems). The sequences were compared with the WT sequence (RefSeq NM_002730.3 (*PRKACA*) and NM_182948.2 (*PRKACB*)).

The tumours were categorised as sparsely granulated (SG), densely granulated (DG) or of mixed granulation pattern (MG). A mixed phenotype was assigned when more than 30% of tumour cells deviated from the dominant Cam5.2 pattern. The immunohistochemical methods for determination of granulation pattern and the sequencing of *GNAS* have been previously described (13).

Results

In this study, a total of 92 sporadic somatotroph adenoma specimens (54 from females) were used. Age at diagnosis varied between 10 and 80 years, and 77% of samples were obtained from patients with macroadenomas (where tumour size was known; $n=77$). There were mutations at the *GNAS* complex locus, G-protein α subunit codon 201 (R201C), in four of 26 cases for which sequences

were available (20 from FFPE specimens and six from frozen specimens) (Table 1). In the remaining FFPE specimens ($n=23$), DNA quality was not sufficient to allow amplification of codons 201 and 227 of G-protein α subunit, so such data for these cases were not available.

We did not find a mutation at codon 206 of *PRKACA* or *PRKACB* in either the gDNA or cDNA of any of the sporadic somatotroph adenomas. Although information regarding the mutational status of *GNAS* and the granulation pattern was not available for all cases, we did not find a mutation in either *GNAS* mutant or WT cases (mutational status was established in 26 of 92 specimens) or in tumours with any type of granulation pattern.

Discussion

The lack of a mutation in either *PRKACA* or *PRKACB* in our series suggests that constitutive activation of *PRKACA* or *PRKACB* by mutation of codon 206 is unlikely to be involved in the pathogenesis of sporadic somatotroph adenomas. However, as we did not sequence all exons of *PRKACA* or *PRKACB*, we cannot rule out mutations at other loci, although this seems unlikely.

Both adrenal adenomas and pituitary somatotroph adenomas are known to contain mutations in components of the cAMP signalling pathway in a proportion of cases, and mutations in *PRKAR1A* can underlie the development of both of these lesions. However, while mutation of *PRKACA* appears to be important for the development of up to two-thirds of cortisol-producing adrenal adenomas (9, 10), it does not appear to be the case for somatotroph adenomas, even though mutation of the cAMP signalling pathway has been shown to be involved in the pathogenesis of this tumour type (1, 2, 13, 14, 15, 16, 17, 18, 19). We did not determine whether there was amplification of either the *PRKACA* or *PRKACB* gene in this series. Amplification of chromosome 19p (containing *PRKACA*) and 1p31.1 has been shown to underlie impaired inhibition of the catalytic subunit by the PKA regulatory subunit and increased basal PKA activity (9, 12). In the absence of a *PRKACA* or *PRKACB* mutation, amplification could lead to increased cAMP signalling that might result in somatotroph tumourigenesis.

In adrenocortical adenomas, mutations in *PRKACA* have been found to be mutually exclusive with mutations in *GNAS* (11). In this series, we found no mutation in codon 206 of *PRKACA* or *PRKACB* in 22 specimens which were *GNAS* WT and four specimens that had *GNAS* R201C mutations. Where *GNAS* status was known, we preferentially included WT specimens in this series. However, there is a lack of information concerning the mutational status of *GNAS* in the remaining cases ($n=66$). Given the reported prevalence of *GNAS* mutations in somatotroph adenomas (1, 2, 3), we could expect 15–58% of them (between ten and 38 specimens) to harbour a *GNAS* mutation; this suggests that although the majority would be negative for GNAS mutation, GNAS mutation in a subset may preclude the finding of a *PRKACA* or *PRKACB* mutation.

Previously, we and others have suggested that DG and SG somatotroph adenomas may have differing clinical characteristics and responses to treatment (1, 18, 20, 21, 22). The genetic basis for this difference has yet to be determined, and so a series containing representative samples from all granulation subtypes was chosen for this study.

The findings of several groups (9, 10, 11, 12) support a central role for the cAMP signalling pathway in the pathogenesis of adrenocortical adenomas and somatotroph adenomas. Although we did not demonstrate the presence of a recurring mutation in either *PRKACA* or *PRKACB*, mutations in other components of this pathway cannot be ruled out and warrant further investigation. At present, any mutational event pathogenetic in the majority of sporadic somatotroph tumours remains elusive.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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