Mutation analysis of protein kinase A catalytic subunit in thyroid adenomas and pituitary tumours

Christopher T Esapa and Philip E Harris

Department of Diabetes, Endocrinology and Internal Medicine, Guy’s, King’s and St Thomas’ School of Medicine, Denmark Hill Campus, Bessemer Road, London SE5 9PJ, UK

(Correspondence should be addressed to P E Harris; E-mail: philip.harris@kcl.ac.uk)

Abstract

Objective: The adenylyl cyclase system plays an important role in the control of both thyroid follicular and anterior pituitary cell function. Activating mutations affecting important pathway components such as the TSH receptor and Gsα occur in the majority of autonomously functioning thyroid nodules. Only a small proportion of other types of thyroid tumours, however, have been reported to harbour these mutations. Activating mutations of Gsα have been reported to occur in up to 40% of pituitary somatotroph adenomas. As the majority of cold thyroid nodules and pituitary tumours are unaffected by these mutations, we have investigated the possibility of activating mutations occurring in protein kinase A (PKA), which is another key component of the adenylyl cyclase pathway.

Design: Genomic DNA and cDNA were analysed for the presence of PKA Cα mutations by allele-specific oligonucleotide hybridisation and single strand conformation polymorphism analysis.

Patients: A total of 171 tissue samples were investigated. These comprised 66 benign and 24 malignant thyroid neoplasms, 21 somatotroph adenomas, 35 non-functioning pituitary adenomas, 2 corticotroph adenomas, 1 malignant prolactinoma, and 22 normal pituitary tissue samples.

Results: No mutations of PKA Cα were identified using either allele-specific oligonucleotide hybridisation or single strand conformation polymorphism analysis.

Conclusions: It appears that PKA Cα mutations at the codons investigated do not represent an oncogenetic mechanism in the development of thyroid and pituitary neoplasms.

Introduction

The adenylyl cyclase (AC) system plays an important role in the control of thyroid follicular cell growth and function and also in anterior pituitary cell function. The effects of thyrotrophin (TSH) on the thyroid, and growth hormone-releasing hormone (GHRH) on somatotrophs are mediated via the cAMP-dependent protein kinase A (PKA) (Fig. 1).

The significance of this pathway has been highlighted by the identification of activating mutations in key pathway components. In the thyroid, activating mutations of the TSH receptor and its coupled G protein Gsα (gsp) have been reported in up to 80% and 25% respectively of autonomously functioning thyroid nodules (1–4), but only in a small proportion of other types of thyroid tumours (5–9). In the pituitary, gsp mutations have been reported to occur in 30–40% of somatotroph adenomas (10, 11), 10–13% of non-functioning pituitary adenomas (12, 13) and 6% of corticotroph adenomas (14). Elevated levels of Ser133 phosphorylated cAMP response element binding protein (CREB) have been reported in 15/15 somatotroph adenomas (15). This activation of CREB was associated with gsp mutations and over-expression of wild type Gsα in only 4/15 and 2/15 tumours respectively. This suggests that activation of the AC pathway occurs distally to Gs in a majority of these tumours. As the majority of thyroid and pituitary neoplasms do not have gsp or receptor mutations, we have investigated the possibility of activating mutations occurring in PKA, which is another key component of the AC pathway.

Protein kinase A consists of two regulatory (R) and two catalytic (C) subunits. When inactive, the holoenzyme exists as a tetramer. Binding of two cAMP molecules to each R subunit causes the release of active C subunits. Although the structural components required to maintain the inactive conformation are not fully understood, point mutations of the Cα isoform have been generated by random chemical mutagenesis at codons 87 (His to Glu) and 196 (Trp to Arg), which prevent binding and sequestration of Cα by the regulatory subunits, without compromising catalytic activity (16). In vivo, these mutations may be
expected to mimic gsp mutations, in terms of their effects on distal intracellular signalling mechanisms. We have, therefore, investigated a series of thyroid and pituitary tumours for the presence of PKA Cα mutations at codons 87 and 196.

Materials and methods
Ethical approval for the study was obtained from the Hospital Ethics Committee. A total of 66 benign (25 nodular goitres, 38 follicular adenomas and 3 Hürthle cell adenomas) and 24 malignant thyroid neoplasms (6 follicular carcinomas, 12 papillary carcinomas, 1 follicular variant of papillary carcinoma, 3 minimally invasive follicular carcinomas, 1 anaplastic carcinoma and 1 papillary carcinoma metastasis), 21 somatotroph adenomas, 35 non-functioning pituitary tumours, 2 corticotroph adenomas, 1 malignant prolactinoma and 22 normal pituitary tissue samples were analysed.

DNA and RNA extraction
Genomic DNA was extracted from fresh/frozen tissue using the Puregene DNA isolation kit (Flowgen, Lichfield, Staffs, UK) according to the manufacturer’s instructions. Formalin-fixed and paraffin-embedded tissue was extracted as previously described (5). Total RNA was extracted from fresh/frozen tissue using TRI reagent (Sigma, Gillingham, Dorset, UK) or RNazol B (Biogenesis, Poole, Dorset, UK).

PCR
Genomic DNA was amplified by PCR across codon 196 using primers based on the cDNA sequence of PKA Cα (codons 182 to 213): forward, 5’-GTGACA-GACTCCGTTTCGC-3’; reverse, 5’-TTTGCTCAGGA-TAATCTCAG-3’. The reaction was carried out in 100 μl volume containing 1 μg genomic DNA, 2.5 U Taq polymerase (Bioline, London, UK), 0.2 mmol/l each dNTP, 10 mmol/l Tris–HCl (pH 8.8), 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.1% Triton X-100, and 50 pmol of each primer. PCR conditions were as follows: denaturation at 94 °C for 2 min, 35 cycles (94 °C for 30 s, 54 °C for 1 min, 72 °C for 30 s) and a final elongation step of 5 min at 72 °C. RNA (1–5 μg) extracted from 9 pituitary tumours was reverse transcribed using oligo dT random primers and 200 U Superscript II (Life Technologies, Paisley, Renfrewshire, UK). The resulting cDNA was used as template for PCR using the following primers: forward, 5’-CAG-AAGGGCAGCGAGCA-3’; reverse, 5’-TCAGAAAGCTCC- TTGCCA-3’. PCR conditions were 35 cycles of 95 °C for 1 min, 52 °C for 1 min 30 s and 72 °C for 2 min. Fifty picomols of each primer were used with 1 mmol/l MgCl₂. Codon 87 could not be amplified from genomic
DNA, presumably due to the presence of an intronic sequence between the primers.

**Allele-specific oligonucleotide hybridisation**

The resulting PCR products, together with mutant and wild-type controls were slot-blotted onto Hybond N+ membrane and hybridised with 32P-labelled wild-type and mutant oligonucleotide probes for codons 87 and 196. The membranes were washed at high stringency and autoradiographed at −70°C for 18–24 h.

**Single strand conformation polymorphism (SSCP) analysis**

The PCR product spanning codon 196 (codons 182–213) was also used for SSCP analysis. Ten microlitres of the PCR reaction mixture were added to 9 μl buffer (95% formamide, 10 mmol/l NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol) and heated at 95°C for 5 min. The denatured DNA was chilled on ice and loaded onto 0.5 × mutation detection enhancement gel (Flowgen) containing 5% glycerol. Electrophoresis was performed at 6 watts for 15–18 h, and the gel was stained with 0.1% silver nitrate.

**Results and discussion**

**Allele-specific oligonucleotide hybridisation**

All thyroid tumours were wild-type at codon 196 (Fig. 2). Pituitary tumours were wild-type at both codons 196 and 87.

**SSCP**

No SSCP band shifts were identified using the PCR product spanning codons 182 to 213.

Protein kinase A has a central role in the AC signalling pathway. In view of this, mutations in PKA may be expected to result in alterations in cellular function and proliferation. We hypothesised that activating mutations in PKA Ca may be present in thyroid and pituitary tumours. We have, however, been unable to demonstrate potential activating mutations of PKA Ca at codon 196 (16), using allele-specific oligonucleotide hybridisation and SSCP. In addition, 9 pituitary tumours analysed by RT-PCR were wild-type at codon 87. These data do not, however, exclude the possibility of mutations at other sites of the C-subunits or indeed the R-subunits of PKA. As far as we are aware, activating mutations of PKA have not been reported previously in endocrine tumours.

Although activating mutations of components of the AC system have been described in thyroid disease (2, 3) and in pituitary tumours (10–14), their role(s) as sole mediators of abnormal cellular function and proliferation is open to question. It is likely that counter-regulatory mechanisms will occur within the cell which will tend to negate the effects of AC activation (17, 18). Activation of the phosphodiesterase system has in fact been demonstrated in FRTL5 cells (18) and in somatotroph adenomas (19). Similarly, if activating mutations of PKA do occur in vivo, counter-regulation may also be expected to occur.

In summary, we have been unable to demonstrate potential activating mutations of PKA Ca subunit in thyroid and pituitary neoplasia, although mutations could occur at other sites of PKA. Current data suggest that the AC pathway may be activated distal to AC in some tumours. The presence of counter-regulatory mechanisms within the cell, however, suggest that other oncogenic mutations are likely to occur in addition to simple activating mutations such as gsp.

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