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G Protein Mutations in Pituitary Tumors: A Study on Turkish Patients

Beki Kan[1], Christopher Esapa[2,,†], Tammam Sipahi[1], Cevdet Nacar[1], Fahir Özer[3], Nur Buyru Sayhan[4], Mehmet Yaşar Kaynar[4], Ali Çetin Sarıoğlu[3,4], and Philip Edward Harris[2]*

[1]*Department of Biophysics, Marmara University School of Medicine, Istanbul, Turkey;* [2]*Guy's, King's and St Thomas' College School of Medicine Department of Endocrinology, London, UK;* [3]*Amiral Bristol Hospital Department of Neurosurgery, Istanbul, Turkey;* [4]*Istanbul University Cerrahpaşa School of Medicine, Istanbul, Turkey*

Abstract. **Activating mutations of the G proteins, $G_{s\alpha}$ (*gsp*) and $G_{i2\alpha}$ (*gip*) have been reported in subsets of pituitary tumors. The objective of the study was to assess the frequency of *gsp* and *gip* mutations in pituitary tumors from Turkish patients and to investigate the possibility of mutations of protein kinase A catalytic subunit (PKAC) that activates the downstream effectors of adenylyl cyclase. PCR-amplified genomic DNA was analyzed for the presence of mutations in codons 201 and 227 of $G_{s\alpha}$, codon 179 and 205 of $G_{i2\alpha}$ and codon 196 of PKAC, by single strand conformation polymorphism analysis, allele-specific oligonucleotide hybridization and DNA sequencing.**

Twenty-two patients from Turkey, 15 females and 7 males were investigated; 7 somatotroph adenomas, 7 clinically non-functioning tumors, 7 prolactinomas and 1 corticotroph adenoma. G protein mutations were identified in 6 of 22 (27.3%) pituitary tumors. Four tumors (3/7 somatotroph adenomas, 43%, 1/7 clinically non-functioning tumor) demonstrated *gsp* mutations at codon 201 arginine to cysteine and one recurrent somatotroph adenoma demonstrated a mutation of the $G_{i2\alpha}$ gene at codon 193 lysine to arginine. One tumor exhibited a C to T variation in the intervening sequence between codons 179 and 205 of the $G_{i2\alpha}$ gene. No mutations at codon 227 of $G_{s\alpha}$, codons 179 and 205 of $G_{i2\alpha}$ and codon 196 of the PKAC gene were identified.

Key Words. **pituitary tumors, G protein mutations, adenylyl cyclase, protein kinase A**

Introduction

Heterotrimeric G-proteins play an essential role in cellular transduction by coupling to cell membrane—bound receptors. G-proteins are composed of three subunits: α, β and γ. The α-subunit, which is believed to confer receptor and effector specificity, binds guanosine 5′-triphosphate (GTP) when the G-protein is activated by a ligand-bound receptor. The intrinsic GTPase activity of the α-subunit returns the G protein complex to the resting or inactive state. Inhibition of the GTPase activity of the G-protein α-subunit stabilizes the α-subunit in its active GTP-bound conformation and induces ligand-independent activation of the signaling pathway [1,2]. Point mutations, which constitutively activate adenylyl cyclase by impairing the GTPase activity of the α-subunit of G_s were first described in growth hormone secreting pituitary adenomas [3,4] and autonomously functioning thyroid nodules [5,6]. These mutations (*gsp*) replace arginine-201 with cysteine or histidine and serine, or glutamine-227 with arginine or leucine, converting the $G_{s\alpha}$-subunit into a putative oncoprotein. Homologous mutations of the α-subunit of the inhibitory G-protein, $G_{i2\alpha}$ at codons 179 and 205 (*gip*) have been described in a small number of invasive pituitary macroadenomas [5,7]. Protein Kinase A (PKA) is the downstream effector of adenylyl cyclase. Random mutagenesis of the catalytic subunit (PKAC) has demonstrated that replacement of histidine 87 with glutamine and tryptophan 196 with arginine blocks the interaction of C with the regulatory subunit, R [8]. These mutations, which protect C from inhibition by R, can be expected to mimic the upstream activation of adenylyl cyclase. In this study, we examined pituitary tumors from 22 Turkish patients for *gsp* and *gip* mutations as well as for mutations in the PKAC gene using allele-specific oligonucleotide hybridization, single strand conformation polymorphism (SSCP) analysis and DNA sequencing.

Materials and Methods

Patients

Approval for the study was obtained from the Ethical Committee of Marmara University School of Medicine.

*These authors have contributed equally to this work.

†Current address: Oxford University Department of Pharmacology, Oxford, UK.

Address correspondence to: Beki Kan, Ph.D., Department of Biophysics, Marmara University School of Medicine, Tibbiye Caddesi No. 49, Haydarpasa, 34716, Istanbul Turkey. Tel./Fax: 90 216 3480585; E-mail: bekikan@marmara.edu.tr

Table 1. *Clinical data of patients studied*

Patient no., age, sex	Diagnosis	Tumor size	Immunohistochemistry	SSCP	ASOH	Sequencing
1, 26, M	Prolactinoma	Macro	PRL	–	–	
2, 21, F	Prolactinoma	Macro	PRL	–	–	
3, 26, F	Cushings	Micro	ACTH, (GH, PRL)	–	–	
4, 24, M	Prolactinoma	Macro	NP	–	–	
5, 34, F	Acromegaly*	Macro	NP	+(179/205)	–	W193R
6, 34, F	Acromegaly	Micro	GH, (PRL)	–	–	
7, 65, F	Non-functioning	Macro		–	–	
8, 42, F	Prolactinoma	Macro	PRL	–	–	
9, 43, F	Acromegaly*	Macro	GH, (PRL)	+(201)	R201C	R201C
10, 53, F	Acromegaly	Macro	GH	+(201)	R201C	R201C
11, 45, M	Non-functioning	Macro		–	–	
12, 48, F	Acromegaly	Macro	GH	+(179/205)	–	Intron, C/T
13, 51, M	Acromegaly	Macro	GH, (PRL)	–	–	
14, 41, M	Non-functioning	Macro		+(179/205)	–	Wild type
15, 44, M	Non-functioning	Macro		–	–	
16, 56, M	Non-functioning	Macro		–	–	
17, 34, F	Prolactinoma	Micro	NP	–	–	
18, 38, F	Prolactinoma	Macro	PRL	–	–	
19, 23, F	Non-functioning	Macro		–	–	
20, 25, F	Non-functioning	Macro		+(201)	R201C	R201C
21, 26, F	Prolactinoma	Macro	PRL	–	–	
22, 30, F	Acromegaly	Macro	GH	+(201)	R201C	R201C

Clinical and histopathological data from 22 patients with pituitary tumors analyzed in this study. Immunohistochemistry (IHC) was performed using antibodies to GH, PRL, ACTH, FSH, LH and TSH. Minor immunoreactivity has been indicated in parenthesis. NP indicates IHC was not performed. *, repeat surgery.

Clinical data from 22 patients are shown in Table 1. There were 7 males and 15 females with an age range of 21–65 years. The tumors comprised of 7 GH-secreting adenomas (acromegaly), 7 non-functioning adenomas (NFT), 7 prolactinomas and 1 ACTH-secreting adenoma (Cushing's disease). The patients did not receive radiotherapy following surgery. Two patients had repeat surgery.

DNA extraction

Genomic DNA was extracted from frozen tissue using a standard protocol for rapid DNA preparation [9]. Briefly, frozen tissue was homogenized in a buffer containing 0.1 M NaCl, 0.2 M sucrose, 10 mM EDTA and 0.3 M Tris-HCl, pH 8.0. SDS (0.05%) was added and the homogenate was incubated at 65°C for 30 min. After the addition of 1 M sodium acetate, the sample was incubated on ice for 60 min, centrifuged at 5000 × *g* for 10 min at 4°C. DNA was extracted from the supernatant with phenol-chloroform, followed by ethanol precipitation.

Polymerase chain reaction (PCR)

DNA was PCR-amplified for $G_{s\alpha}$ (exon 8, encompassing codon 201 and exon 9, encompassing codon 227), $G_{i2\alpha}$ (exons 5 and 6, encompassing codons 179 and 205 plus intervening intron) and the PKAC (codon 196) genes. Amplification reactions were carried out in a 100 μl-volume containing 1 μg genomic DNA, 2.5 U Taq Polymerase (Bioline, UK or Promega, USA), 20 pmol of each primer, 0.2 mM of each dNTP, NH_4 buffer provided by the manufacturer and $MgCl_2$ at a concentration of 1.5–2.5 mM. The cycle parameters were as follows: 30 sec at 94°C, 30 sec at 60°C and 90 sec at 72°C for 30 cycles for exons 8 and 9; 60 sec at 94°C, 120 sec at 58°C and 180 sec at 72°C for 30 cycles for exons 5 and 6 plus intervening intron; 30 sec at 94°C, 45 sec at 61°C and 45 sec at 72°C for 30 cycles for exons 5 and 6; and 30 sec at 94°C, 60 sec at 54°C and 30 sec at 72°C for 35 cycles for PKAC. Negative controls were included in every reaction. For PCR of codons 201 and 227, the primers used were sense 5′ CTACTCCAGACCTTTGCTTTAG 3′ and antisense 5′ ACAGCTGGTTATTCCAGAGGGA 3′, sense 5′ GTTTCTTGACATTCACCCCAGT 3′ and antisense 5′ AGCGACCCTGATCCCTAACAAC 3′, respectively. For amplification of $G_{i2\alpha}$, the primers, which yielded a single product spanning codons 179 and 205 were sense 5′ ATTGCACAGAGTGACTACATCCCC 3′ and antisense 5′ GGCGCTCAAGGCTACGCAGAA 3′. The primers used to amplify codons 179 and 205 separately were: 179-sense 5′ CCCAGCTACCTGAACGACCTGG 3′ and antisense 5′ CTCGCTCACTTGAAGTGTAGGTC 3′; 205-sense 5′ CATCTGCAGGATGTTTGATGTGG 3′ and antisense 5′ CATCCTCTCACCATCTCCTCG 3′. The primers for PKAC were sense 5′ GTGACAGAGTTCGGTTTCGC 3′ and antisense 5′ TTTGCTCAGGATAATCTCAG 3′.

SSCP analysis

PCR products were heat-denatured in a buffer containing 95% formamide, 10 mM NaOH, 0.05% bromophenol blue and 0.05% xylene cyanol at 95°C for 5 min. The denatured samples were chilled on ice and loaded onto either a 6% non-denaturing polyacrylamide gel (14 × 13 cm)

or onto a 0.5 × mutation detection enhancement (MDE) gel (20 × 41 cm) with or without 5% glycerol. Polyacrylamide gels were run at 200 V for 2–6 hours. Electrophoresis of samples on MDE gels was either at 8 W for 15–17 hours (with glycerol) or at 600 V for 17 hours (without glycerol). Gels were fixed in 10% ethanol/5% acetic acid, stained with 0.1% silver nitrate and developed in 1.5% NaOH/0.15% formaldehyde and in 0.75% sodium carbonate.

Allele-specific oligonucleotide hybridization

PCR-amplified DNA denatured with 0.4 M NaOH and 25 mM EDTA at 99°C for 5 min and chilled on ice was blotted onto HYBOND-N^+ membranes (Amersham, UK) and prehybridized in a fluid containing 5 × SSPE, 0.5% SDS, 5 × Denhardt's solution and 0.1 mg/ml sheared salmon sperm DNA at 55°C for 3 hours. Membranes were hybridized with [^{32}P]-labeled synthetic oligonucleotide probes (20-base), degenerate or specific for single-base mutations at codons 201 and 227 of $G_{s\alpha}$, codons 179 and 205 of $G_{i2\alpha}$ for three hours and codon 196 of PKAC overnight at 55°C. The membranes were washed with 3 M TMAC, 0.2% SDS, 50 mM Tris-HCl, pH 8.0 at 55°C for 10 min and with the same solution at 60°C (PKA, codons 201, 179 and 205) and at 67°C (codon 227) for 15 min for high stringency. The membranes were autoradiographed for 8–16 hours at −70°C. To provide positive controls for each mutation, amplified sequences were synthesized by PCR using primers containing single base mutations in Arg 201 and Gln 227 of $G_{s\alpha}$, Arg 179 and Gln 205 of $G_{i2\alpha}$ and Trp 196 of PKAC.

Sequencing

PCR products positive on slot blots or SSCP were subcloned into pGEM-T vector using the T4 cloning kit (Promega, UK) and transformed into JM109 cells. Recombinant plasmid DNA (miniprep) was extracted from single clones and sequenced using the T7 Sequenase v2.0 kit (Amersham, UK) according to the manufacturer's instructions. Direct sequencing of PCR products was also done using a dye terminator sequencing kit on the ABI automated sequencer.

Results

PCR

The DNA of pituitary tumors was amplified by PCR for the $G_{s\alpha}$, $G_{i2\alpha}$ and PKAC genes. The length of the products for exon 8 (codon 201) and exon 9 (codon 227) of $G_{s\alpha}$ were 241 bp and 187 bp, respectively. PCR for $G_{i2\alpha}$ yielded a single product spanning codons 179 and 205 and the intervening sequence (604 bp). When exons 5 and 6 of $G_{i2\alpha}$ were amplified separately, the lengths of the products were 165 bp and 175 bp, respectively. The product for the PKAC gene was 96 bp. All amplified products gave a single distinct band on 2% agarose gels (data not shown).

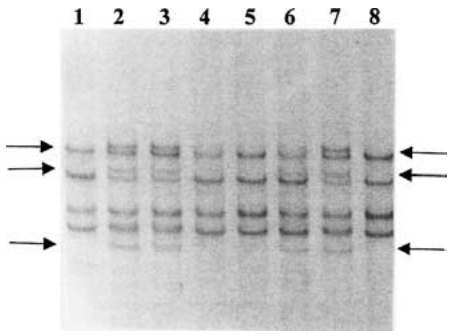

Fig. 1. *Identification of the mutation in exon 8 of the $G_{s\alpha}$ gene by SSCP analysis. Amplified DNA was denaturated at 95°C for 5 min and resolved in* 0.5 × *MDE gel without glycerol. The figure is a silver-stained gel. Lane1, normal control; lane 2, tumor 9; lane 3, tumor 10; lane 4, tumor 12; lane 5, tumor 17; lane 6, tumor 20; lane 7, tumor 22; lane 8, normal control. The arrowheads point to bands, which exhibit differences in migration (Lanes 2, 3, 6, 7).*

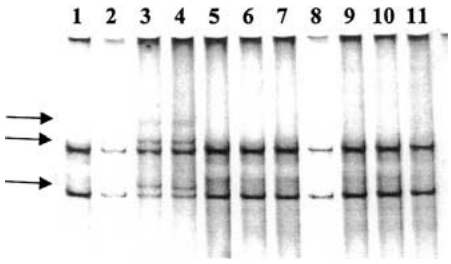

Fig. 2. *SSCP analysis of PCR-amplified genomic DNA for mutations of the $G_{i2\alpha}$ gene. DNA amplified to yield a single product spanning exons 5 and 6 and the intervening sequence was heat-denatured and electrophoresed through a 0.5 × MDE gel without glycerol. Lane 1, tumor 2; lane 2, tumor 5; lane 3, tumor 12; lane 4, tumor 14; lane 5, tumor 16; lane 6, tumor 17; lane 7, tumor 18. The arrowheads point to bands, which exhibit differences in migration (Lanes 2, 3, 4).*

SSCP analysis

In total, seven tumor samples exhibited abnormal SSCP banding patterns. Comparison of the mobility of tumor and normal DNA showed differences in four samples from exon 8 of $G_{s\alpha}$ (Fig. 1). Three samples displayed additional bands when amplified to yield a single product spanning exons 5 and 6 of $G_{i2\alpha}$ (Fig. 2). No band shifts were identified for codon 196 of the PKAC gene.

Allele-specific oligonucleotide hybridization

All four tumor samples with an SSCP shift for exon 8 of $G_{s\alpha}$, hybridized to oligonucleotide probes confirming a point mutation at codon 201 of $G_{s\alpha}$, encoding a change from arginine to cysteine (CGT to TGT) (Fig. 3). No mutations were detected at codons 227, 179 and 205 of G proteins and codon 196 of PKAC (data not shown). None of the samples with an additional SSCP band for the

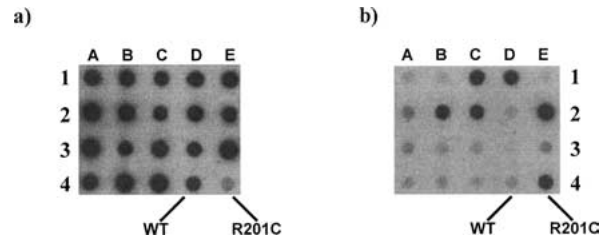

Fig. 3. *The figure is an autoradiogram of DNA samples hybridized to the wild type probe (a) and the probe for the Arg to Cys mutation (b). The controls for wild type (WT) and codon 201 mutation (R201C) are shown. Figure 3b also shows four samples (C1, tumor 9; D1, tumor 10; C2, tumor 20; E2, tumor 22) and one sample (B2) from a different study which hybridized to the mutant probe.*

exon 5/6 PCR product were positive for codons 179 and 205. All samples hybridized with the wild-type probe, indicating that the mutations were heterozygous.

Sequencing

DNA sequencing of the PCR products for samples that hybridized to a mutant probe for codon 201 of the $G_{s\alpha}$ gene confirmed the mutation as CGT (arginine, wild type) to TGT (cysteine) (Fig. 4). Three of the tumors demonstrating *gsp* mutations were GH-secreting and the other was a NFT. Direct sequencing of PCR products and automated sequencing of plasmid DNA prepared from PCR products for exon 5/6 of $G_{i2\alpha}$ (samples 5, 12 and 14) did not show any mutations at codons 179 and 205. Sample 5 which displayed an SSCP shift for exon 5/6 of $G_{i2\alpha}$ demonstrated a novel mutation at codon 193, encoding a change from lysine to arginine (AAG to AGG) (data not shown). A nucleotide change from C to T was determined in the tenth nucleotide of the intervening sequence between exons 5 and 6 of $G_{i2\alpha}$ in Sample 12 (data not shown).

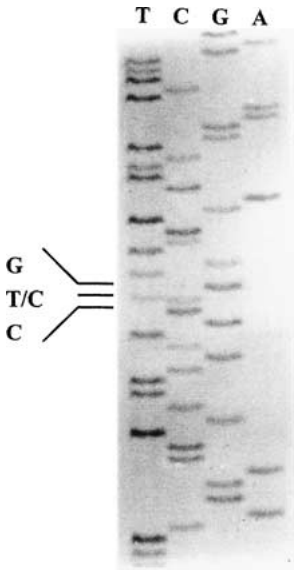

Fig. 4. *Direct DNA sequencing of sample 9 confirming the mutation of $G_{s\alpha}$ at codon 201, encoding a change from arginine to cysteine (CGT to TGT). Sequencing was performed using a T7 Sequeanase Quick-Denature Plasmid Sequencing kit (Amersham U.K.). Samples from sequencing reactions were electrophoresed on 6% denaturing polyacrylamide gels.*

Discussion

This study demonstrates the presence of G protein mutations in 6 of 22 (27.3%) pituitary tumors from Turkish patients. The types of *gsp* mutations determined in the current study were similar to those found previously, with mutation of arginine 201 to cysteine being the most common. The *gsp* oncogene has been identified in approximately 30 to 45% of GH-secreting adenomas in Western countries [4,5,10–13]. Two studies have demonstrated lower prevalence rates (4–9%) of the *gsp* oncogene in Japanese acromegalic patients [14,15], raising the possibility of a geographical difference in the occurrence of the mutation. On the other hand, results of a study in Korean acromegalic patients point to a similar frequency to those of Caucasian patients [16]. Three of the seven (43%) GH-secreting tumors examined in the present study displayed a point mutation at Arg 201, suggesting that the frequency of $G_{s\alpha}$ mutations in Turkish acromegalic patients is similar to those in Caucasian and Korean patients.

There are some discrepancies among studies published to date on the incidence of G protein mutations in clinically NFTs. Tordjman et al. [17] have demonstrated *gsp* mutations in 10% of NFTs but have not identified any tumors harboring *gip* mutations. Williamson et al. [7] have demonstrated the presence of G protein mutations in 3 of 22 (13%) NFTs with *gsp*. Two of these tumors with particularly aggressive growth characteristics, bore dual *gsp* and *gip* mutations. On the other hand, two previous studies have not identified G protein mutations in NFTs [5,18]. Of the 7 NFTs analyzed in this study, we identified 1 tumor bearing the *gsp* mutation, R201C.

A number of studies have investigated whether *gsp* positive and *gsp* negative somatotroph adenomas vary in the expression of the $G_{s\alpha}$ gene. Landis and co-workers [4] have shown that genomic DNA obtained from GH-secreting pituitary adenomas with *gsp* mutations contained equal amounts of wild type and mutant $G_{s\alpha}$ genes. On the other hand, Vallar [19] indicated that GH-secreting pituitary adenomas carrying *gsp* mutations expressed reduced levels of mutant $G_{s\alpha}$ protein. Ballaré et al. [20] have also shown that the content of $G_{s\alpha}$ protein was barely detectable in tumors bearing the *gsp* mutation although the level of $G_{s\alpha}$ mRNA determined by semi quantitative RT-PCR was not different in *gsp* positive and *gsp* negative tumors. Recently, Barlier et al. [21] demonstrated by Northern blot analysis decreased

levels of $G_{s\alpha}$ mRNA expression in GH-secreting adenomas carrying *gsp* mutations. The reduced level of $G_{s\alpha}$ protein in GH-secreting adenomas with *gsp* mutations represents one mechanism that counterbalances the constitutive activation of the cAMP signaling pathway. Another feedback mechanism involving a compensatory increase in phosphodiesterase activity in *gsp* positive GH-secreting tumors has recently been described by Lania et al. [22] who demonstrated stimulation of intracellular cAMP accumulation in the presence of PDE inhibitors. This feedback loop may explain in part the lack of obvious phenotypic differences between tumors with and without *gsp* mutations [23]. Evidence from previous studies indicates that patients bearing the $G_{s\alpha}$ mutations do not differ markedly from those that express wild type tumors in terms of distinct clinical features, sex, age distribution and tumor size [11,13,24,25]. Although there are no clear-cut differences distinguishing *gsp* positive and *gsp* negative tumors, results from *in vitro* and *in vivo* studies indicate that the presence of the *gsp* oncogene could have an impact on targeting of therapy by somatostatin analogues [21,24,26]. cAMP-regulated factor CREB is thought to be involved in the mechanism by which cAMP stimulates proliferation. CREB stimulates the expression of cAMP-responsive genes [23] upon being phosphorylated by protein kinase A at serine 133 [27]. Based on the assumption that mutations, which protect the catalytic subunit of the PKA from inhibition by the regulatory subunits, may mimic the upstream activation of adenylyl cyclase, we examined the tumors for the W196R mutation, known to prevent reassociation of the catalytic and regulatory subunits of the PKA gene. None of the tumor samples examined were found to harbor the W196R mutation. This suggests that such a mutation is unlikely to be of etiological significance in the pathogenesis of pituitary tumors. This finding provides further support to a recent study by Esapa and Harris [28] which reported the absence of activating mutations of the PKAC gene at codons 87 and 196 in thyroid and pituitary tumors. Mutations at codons 179 and 205 of the $G_{i2\alpha}$ gene were not detected in any of the tissues studied. Two GH-secreting and one NFT, however, exhibited abnormal SSCP banding patterns when amplified to yield a single product spanning exons 5 and 6. One of the samples (sample 5, recurrent GH-secreting tumour) harbored a mutation at codon 193, AAG to AGG, which encoded a change in amino acid from lysine to arginine. This neutral amino acid change is unlikely to be of functional significance. A change in a single nucleotide was determined in the intervening sequence between exons 5/6 in another sample (Sample 12, GH-secreting tumor). None of the seven prolactinomas and the corticotroph adenomas included in the study exhibited a G protein mutation.

In summary, we have demonstrated *gsp* mutations in GH-secreting and NFTs from Turkish patients. These data are consistent with previous reports. We have detected a novel mutation coding for a neutral change in amino acid at codon 193, and an intronic mutation, neither of which are likely to be of functional significance.

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