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GH-secreting pituitary adenomas infrequently contain inactivating mutations of PRKAR1A and LOH of 17q23–24

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Summary

OBJECTIVE The molecular events leading to the development of GH-secreting pituitary tumours remain largely unknown. Gsα (*GNAS1*) mutations are found in 27–43% of sporadic GH-secreting adenomas in the Caucasian population, but the frequency of *GNAS1* mutations in Japanese and Korean acromegalic patients was reported to be lower, 4–9% and 16%, respectively. Other genes responsible for the tumourigenesis of GH-secreting pituitary adenomas have not been detected yet. *PRKAR1A*, which codes for the RIα regulatory subunit of cyclic AMP-dependent protein kinase A (PKA) on 17q23–24, was recently reported to contain inactivating mutations in some Carney complex families, which involved GH-secreting adenomas in about 10%. We re-evaluated the frequency of *GNAS1* mutations and investigated *PRKAR1A* on the hypothesis that it might play a role in the tumourigenesis of GH-secreting adenomas.

DESIGN We analysed exons 8 and 9 of *GNAS1* and all exons and the exon–intron boundaries of PRKAR1A with the PCR and by direct sequencing using genomic DNA extracted from 32 GH-secreting pituitary adenomas (30 GH-secreting adenomas, two GH and PRL-secreting adenomas) and 28 corresponding peripheral blood samples, and performed loss of heterozygosity (LOH) analysis of 17q23–24 with four microsatellite markers and intragenic markers of *PRKAR1A*.

RESULTS Seventeen of 32 (53·1%) tumours showed somatic-activating mutations of *GNAS1*: 16 (53·3%) of 30 GH-secreting adenomas and one of two GH and PRL-secreting adenomas. Neither inactivating somatic mutations of *PRKAR1A* nor LOH of 17q23–24 were detected in any of the tumours examined.

CONCLUSION We reconfirm the important role of activating mutations of *GNAS1* in GH-secreting adenomas, and conclude that *PRKAR1A* does not play a significant role in the tumourigenesis.

Pituitary adenomas are monoclonal benign tumours that account for about 10% of all intracranial neoplasms. Little is known about the pathogenetic mechanisms driving pituitary adenomas. The most frequent alterations are oncogenic mutations of Gsα protein (Landis *et al*., 1989; Lyons *et al*., 1990), which are detected in a subset of GH-secreting adenomas, and reduced expression of the tumour suppressor gene $p16^{INK4a}$ due to methylation (Woloschak *et al*., 1996). In contrast, somatic mutations of the *MEN1* gene are rare in pituitary adenomas (Zhuang *et al*., 1997; Tanaka *et al*., 1998). Although other candidate genes for tumourigenesis of pituitary adenomas have been studied, genetic changes are infrequently found in pituitary adenomas.

G proteins are transducers that link extracellular receptor-bound ligands to intracellular secondary messenger systems. Adenylyl cyclase activity is under stimulatory control by Gs and inhibitory control by Gi. Activating mutations of α-subunits of Gs and Gi2 that convert these subunits into putative oncoproteins, which are termed *gsp* and *gip*, respectively, have been described (Landis *et al*., 1989). Point mutations at codon 201 (Arg → Cys or His) in exon 8 or 227 (Gln → Arg or Leu) in exon 9 in the Gsα gene (*GNAS1*) result in the constitutive activation of adenylyl cyclase, and are frequently found in GH-secreting adenomas. In previous reports, heterozygous missense mutations of *GNAS1* were present in 27–43% of sporadic GH-secreting adenomas in the Caucasian population (Landis *et al*., 1990; Lyons *et al*., 1990; Barlier *et al*., 1998; Salvatori *et al*., 2001), but the frequency of *GNAS1* mutations in Japanese and Korean acromegalic patients was reported to be lower, 4–9% (Hosoi *et al*., 1993; Yoshimoto *et al*., 1993) and 16% (Kim *et al*., 2001), respectively. Based on these results, it has been suggested that activation of the cyclic

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AMP (cAMP) signalling pathway could act in concert with other factors to promote tumour development.

Carney complex is an autosomal dominant syndrome characterized by multiple neoplasias, including myxomas at various sites, endocrine tumours, and lentiginosis. About 10% of patients with Carney complex have a GH-secreting pituitary adenoma that results in acromegaly (Stratakis, 2001). Genetic defects of Carney complex are mapped to chromosomal region 2p16 (Stratakis *et al*., 1996) and 17q22–24 (Casey *et al*., 1998). The *PRKAR1A* gene encoding the R1α regulatory subunit of cAMP-dependent protein kinase A (PKA) was mapped to 17q22–24, and several mutations of the gene were detected in some kindreds of individuals with Carney complex (Casey *et al*., 2000; Kirschner *et al*., 2000a). Based on these results, we hypothesized that *PRKAR1A* could function as a tumour suppressor gene in some sporadic GH-secreting adenomas.

In the present study, we re-evaluated the frequency of *GNAS1* mutations and analysed the role of *PRKAR1A* by sequencing *PRKAR1A* and loss of heterozygosity (LOH) of 17q23–24 in the tumourigenesis of sporadic GH-secreting adenomas.

Materials and methods

Patients and DNA extraction

Tumour tissue samples were collected from 32 patients operated on at Toranomon Hospital, Tokyo. Twenty-eight blood samples were also obtained. Fully informed consent was obtained in accordance with institutional guidelines. The study was also conducted in accordance with the provisions of the Declaration of Helsinki. There were 18 males and 14 females, with a mean age of 45·0 (range 25–73) years. Tumours included 30 GH-secreting and two GH and PRL-secreting adenomas. The other clinical information is described in Table 1. All samples were frozen at −70 °C, and stored for later use. Genomic DNA was extracted from frozen tissue and peripheral blood with standard Proteinase K and phenol–chloroform extraction in cases 26–32, and DNeasy Tissue Kit (Qiagen K.K., Tokyo, Japan) for tumours and Mr GenTLE™ (TaKaRa, Kusatsu, Japan) for peripheral blood in cases 1–25, according to the manufacturer's instructions.

Primer design of GNAS1 and PRKAR1A

Nucleotide sequences of exons 8 and 9 of *GNAS1* were obtained from a bacterial artificial chromosome (BAC) clone RP4–543J19 (GenBank accession number, AL109840). We designed primers for each exon and its flanking intronic regions: Exon 8: 5′-GGCAATTATTACTGTTTCGGTTGGC-3′/5′-GACTGGGGT-GAATGTCAAGAAACC-3′; Exon 9: 5′-TTCTTGACATTCAC-CCCAGTCC-3′/5′-CTAACAACACAGAAGCAAAGCG-3′.

The human *PRKAR1A* was composed of 11 exons, not 10 exons as reported by Solberg *et al*. (1997). Nucleotide sequences of exons 3–11 of *PRKAR1A* were obtained from a BAC clone hRPK.62_F_10 (AC005799). In addition, nucleotide sequences of exon 2 and exons 1a (noncoding exon) and 1b (noncoding exon) were obtained from a BAC clone RP11–120M18 (AC079210) and 5′ flanking region of PRKAR1A (Y07641), respectively. We synthesized oligonucleotide primers for exons 3–11 and their flanking intronic regions according to the report by Casey *et al*. (2000), but designed new oligonucleotide primers for exons 1a, 1b and 2: Exon 1a: 5′-AGGAGTCGCCCACCTGTCAT-CTGA3′/5′CTTATCCACAGCAGTTTCCTCACG-3′; Exon 1b: 5′-ACGTC AGTAGCCGAACGCTGATTG-3′/5′-ACGCCAT-CTTGGATCGGTCCAGCT-3′; Exon 2: 5′-TCCCTGTGAAT-CAGTTGTCTAAT-3′/5′-ATGTAACAACTGTCACAATCACC-3′.

PCR and sequence analysis

Genomic DNA (100 ng) was subjected to 35 cycles of PCR in a reaction mixture containing 2 μl of 10 × PCR buffer with 1·6 μl of dNTP, 1·0 unit of AmpliTaq Gold (Applied Biosystems, Tokyo, Japan), 10 nmol each of a sense primer and antisense primer, and 11·3 μl of H_2O. Each PCR cycle contained a denaturation step at 95 °C for 30 s, annealing step at 56 °C for 30 s, and extension step at 72 °C for 60 s for *GNAS1*; and a denaturation step at 94 °C for 20 s, annealing step at 58 °C for 30 s, and extension step at 72 °C for 45 s for *PRKAR1A*, in a GeneAmp PCR system 9700 (Applied Biosystems). The PCR products were electorophoresed in an 8% polyacrylamide gel to confirm amplification. After treatment of PCR products with exonuclease I and shrimp alkaline phosphatase (Amersham Bioscience, Tokyo, Japan), samples were subjected to direct sequencing in sense and antisense directions using an ABI PRISM BigDye™ terminators v3·0 cycle sequencing kit (Applied Biosystems) and analysed on an ABI PRISM 3700 analyser (Applied Biosystems).

LOH analysis

For LOH analysis, genomic DNAs from 28 paired DNA samples of patient's peripheral blood and tumour tissues were analysed with polymorphic 17q23–24 markers: D17S942, D17S807, D17S795, and D17S789 (Dib *et al*., 1996). One of each oligonucleotide primer pair was labelled with 6-FAM fluorescent dye (Applied Biosystems). PCR, gel electrophoresis, and data analysis were performed as described (Shintani *et al*., 1995). To minimize the effects of possible contamination by DNA from normal tissue, the number of PCR cycles was either 25 or 30, but never more than this. LOH was considered present when the signal intensity of one allele was reduced by more than 50% in comparison with the corresponding allele in normal DNA. In addition, intragenic polymorphic markers of *PRKAR1A* were analysed at direct sequencing.

Table 1 Clinical data of 32 patients

Case number	Sex	Age	Basal GH (μg/l)	Basal PRL (μg/l)	Provocation by GHRH (%)	Immunohistochemistry	Hardy
1	F	26	16		380	GH	III-C
2	M	34	22		285	GH	III-0
3	F	26	23		141	GH	II-0
4	F	28	78		294	GH	III-C
5	F	65	4·7		156	GH	I-0
6	F	36	46	26·1	1403	GH	III-B
7	M	42	11		219	GH	III-0
8	M	46	47		131	GH	III-A
9	M	38	80		140	GH	III-A
10	M	73	9		–	GH	I-0
11	M	25	78·5		261	GH	II-A
12	M	43	22		338	GH	III-A
13	F	59	67		286	GH	II-A
14	F	30	38		161	GH	IV-B
15	M	63	17		771	GH	III-A
16	M	28	60	38·3	242	GH	III-A
17	M	42	112		146	GH	III-0
18	F	45	5·7		620	GH	II-0
19	F	64	22		123	GH	II-0
20	M	48	43		186	GH	III-0
21	M	56	35·9		78	GH	II-A
22	F	67	104	42·2	111	GH	III-0
23	M	28	80	24·8	207	GH	III-0
24	M	63	7·8		212	GH	I-0
25	M	51	7·1		1397	GH	I-0
26	F	44	88·5		–	GH	III-0
27	M	47	370		–	GH	III-0
28	M	38	28	28	178	GH	III-0
29	M	59	19·6		497	GH	II-0
30	F	35	21		–	GH	II-0
31	F	46	9·6	52	–	GH/PRL	II-0
32	F	45	19	56	165	GH/PRL	III-0

Provocation by GHRH: Maximum stimulation ratio of serum GH by GHRH; –: not examined; Hardy: Hardy's classification.

Data analysis

The Mann–Whitney rank sum test was used to test for differences of basal serum GH levels and GH levels provocated by GH-releasing hormone (GHRH) between patients with mutant and wild-type *GNAS1*.

Results

Somatic mutations of GNAS1

Based on the hypothesis that inactivating mutations of *PRKAR1A* might occur independent of *GNAS1* mutations, tumours were first screened for activating mutations of *GNAS1*. Because activating *GNAS1* mutations are located in exon 8 or 9, we screened exons 8 and 9 of *GNAS1*. Seventeen of 32 (53·1%) cases showed somatic mutations of *GNAS1*: 16 (53·3%) of 30 GH-secreting adenomas, and one of two GH and PRL-secreting adenomas (Table 2). Mutations at codon 201 replaced Arg (CGT) with either Cys (TGT) in 15 cases, or His (CAT) in one case. Another mutation replaced Gln (CAG) with Arg (CGG) at codon 227. Both mutations were mutually exclusive. We also investigated *GNAS1* mutations in peripheral blood DNA (15 cases out of 17), but did not detect any.

Average basal serum GH levels in patients with and without *GNAS1* mutations were 64·9 μg/l and 32·6 μg/l, respectively. There was not significant difference between the two groups ($P = 0{\cdot}1084$). On GHRH provocation test, we calculated the stimulation ratio as maximum GH levels/basal GH levels. The ratios in groups with and without mutations were 173% and 544% on average, respectively. The stimulation ratios were significantly lower in patients with mutations than without ($P < 0{\cdot}05$).

Table 2 The results of sequencing of *GNAS1* and *PRKAR1A* and LOH analysis of 17q23–24

Case number	*GNAS1*		*PRKAR1A* polymorphisms		Loss of heterozygosity				
	201 Arg (CGT)	227 Gln (CAG)	Exon	Exon 4 IVS-5	D17S807	D17S942	D17S795	D17S789	IGM
1	wt	wt		4T/5T	RH	RH	RH	RH	RH
2	Cys (TGT)	wt		4T/4T	NI	RH	RH	RH	NI
3	Cys (TGT)	wt		4T/4T	–	–	–	–	NI
4	wt	wt		4T/5T	–	–	–	–	RH
5	Cys (TGT)	wt	exon 6 546 G/A†	5T/5T	RH	NI	RH	RH	RH
6	wt	wt		4T/5T	RH	NI	NI	RH	RH
7	wt	wt		4T/5T	NI	RH	RH	NI	RH
8	Cys (TGT)	wt	exon 1a 103 A/C*	4T/5T	RH	RH	RH	RH	RH
9	Cys (TGT)	wt		4T/4T	NI	RH	RH	RH	NI
10	wt	wt		4T/4T	RH	RH	RH	NI	NI
11	wt	wt		4T/5T	RH	NI	RH	NI	RH
12	wt	wt	exon 1a 103 A/C*	4T/4T	NI	RH	RH	NI	RH
13	Cys (TGT)	wt		4T/5T	RH	NI	RH	RH	RH
14	wt	wt		4T/4T	–	–	–	–	NI
15	wt	wt		4T/5T	NI	NI	RH	RH	RH
16	wt	Arg (CGG)		4T/5T	–	–	–	–	RH
17	His (CAT)	wt		4T/5T	RH	RH	RH	RH	RH
18	wt	wt	exon 1a 103 A/C*	4T/4T	RH	NI	RH	NI	RH
19	Cys (TGT)	wt	exon 6 546 G/A†	5T/5T	RH	RH	RH	RH	RH
20	wt	wt	exon 1a 103 A/C*	4T/4T	NI	NI	RH	NI	RH
21	Cys (TGT)	wt		4T/4T	NI	RH	RH	RH	NI
22	Cys (TGT)	wt		4T/5T	RH	RH	RH	NI	RH
23	Cys (TGT)	wt		4T/5T	RH	NI	RH	RH	RH
24	Cys (TGT)	wt		4T/4T	NI	RH	RH	RH	NI
25	wt	wt		4T/5T	NI	RH	NI	NI	RH
26	wt	wt		4T/4T	RH	RH	RH	RH	NI
27	Cys (TGT)	wt		4T/4T	RH	RH	RH	RH	NI
28	Cys (TGT)	wt		4T/5T	RH	RH	NI	NI	RH
29	wt	wt		4T/5T	RH	RH	RH	NI	RH
30	Cys (TGT)	wt		4T/5T	RH	NI	RH	NI	RH
31	wt	wt		4T/5T	RH	RH	RH	RH	RH
32	Cys (TGT)	wt		4T/4T	RH	RH	RH	NI	NI

wt: wild-type; *For exon 1a of *PRKAR1A*, nucleotide numbering starts from the beginning of exon 1a. †For exon 6, nucleotide numbering starts from the first codon of ATG in cDNA. 4T and 5T show the number of Ts' repeat on exon 4 IVS-5. IGM: intragenic markers; RH: retention of heterozygosity; NI: not informative; –: not performed.

Sequence analysis of PRKAR1A

Although we analysed the nucleotide sequence of *PRKAR1A* of genomic DNA from tumour tissue and peripheral blood, neither somatic nor germline mutations were detected except for two types of single nucleotide polymorphisms (SNPs) in six cases and T insertion at intronic sequences (exon 4 IVS −5) in 19 cases (Table 2). One of SNPs was an A to C heterozygous change in exon 1a (5′untranslated region) in four tumours and another was a G to A heterozygous change in exon 6 (cDNA position 546) in two tumours, which did not cause any change of amino acid. On database, four Ts are consecutively located from −8 to −5 at 5′intronic sequences to the start of exon 4; however, consecutive five Ts caused by a T insertion were detected in 19 cases. These 4T/5T polymorphisms were heterozygous in 17 cases, and homozygous in 15 cases. These nucleotide changes were observed in the respective leukocyte DNA. Because every nucleotide change was also detected in peripheral blood samples, the nucleotide changes suggest polymorphism. The frequency of these polymorphisms was analysed in 50 unrelated anonymous DNA samples from healthy individuals. In the A/C polymorphism on exon 1a, the frequency of A and C was 98% and 2%, respectively. In the G/A polymorphism on exon 6, the frequency of G and A was 98% and 2%, respectively. In the 4T/5T

polymorphism on exon 4 IVS-5, the frequency of 4T and 5T was 71% and 29%, respectively. These polymorphisms were not reported by Kirschner *et al*. (2000b).

LOH analysis

Although we analysed LOH of 17q23–24 with 4 microsatellite markers, no LOH was revealed in 28 cases (Table 2). Because peripheral blood samples were not always available, information on LOH in four cases was not obtained. In addition, we analysed LOH with intragenic markers: exon 1a 103 A/C, exon 6 546 G/A, and T insertion at exon 4 IVS-5. Direct sequencing showed no LOH in 22 cases who showed heterozygosity in at least one of three markers (Table 2).

Discussion

The proliferation of pituitary somatotroph cells and secretion of GH are under dual hypothalamic regulation, stimulated by GHRH and inhibited by somatostatin. These hormones bind to specific cell surface receptors that interact with the heterotrimeric G proteins Gs (GHRH) or Gi (somatostatin) to stimulate or inhibit the production of cAMP by adenylyl cyclase, respectively. cAMP stimulates PKA, which in turn phosphorylates many proteins that can regulate gene expression, including cAMP-responsive element binding protein (CREB).

Gsα, the gene for which is located on chromosome 20, is a subunit of the Gs signalling protein. The *GNAS1* mutation of codon 201 (Arg → Cys or His) or 227 (Gln → Arg or Leu) produces independent activation of the adenylyl cyclase system (Landis *et al*., 1989; Lyons *et al*., 1990; Spada *et al*., 1993; Shimon & Melmed, 1997). The *GNAS1* mutations have been demonstrated in 27–43% of GH-secreting pituitary tumours in Caucasian patients (Barlier *et al*., 1998; Landis *et al*., 1990; Lyons *et al*., 1990; Salvatori *et al*., 2001), 16% in Korean patients (Kim *et al*., 2001), and 4–9% in Japanese patients (Hosoi *et al*., 1993; Yoshimoto *et al*., 1993) with acromegaly. This discrepancy in the frequency of *GNAS1* mutations has been thought to reflect a geographical or ethnic influence. The present study demonstrated that *GNAS1* mutation was detected in 53·1% of sporadic GH-secreting adenomas and the frequency of mutations in Japanese patients was 53·3% (16 of 30 cases), because patients 9 and 18 were a Vietnamese and a Taiwanese, respectively. The frequency in this study was higher than that in other reports on Japanese patients (Hosoi *et al*., 1993; Yoshimoto *et al*., 1993). Although we cannot provide a clear explanation on the difference in frequency, the cases examined in this study did not overlap with those in the previous study (Yoshimoto *et al*., 1993). Further large-scale research seems to be necessary to re-evaluate the frequency.

Landis *et al*. (1990) found *GNAS1*-activating mutations in 10 of 25 (40%) GH-secreting pituitary adenomas. They demonstrated that patients in the mutation-positive group came to surgery with smaller tumours and had lower serum GH levels. On the other hand, Barlier *et al*. (1998) reported that adenomas with *GNAS1* mutations appeared to secrete significantly more GH than negative adenomas. Kim *et al*. (2001) reported that mean tumour size was significantly smaller in patients with *GNAS1* mutation than without, but basal serum GH levels were not different in the two groups. In our cases, the average basal serum GH level in patients with *GNAS1* mutations was about twice that in patients without mutations. In cases with Gsα mutations, basal GH levels was thought to be higher than those without mutations because the cAMP signalling pathway was continuously activated and additional GHRH stimulation might be less effective. On GHRH provocation test, the ratio in patients with mutations was significantly lower than that in patients without mutations. Recently, Yasufuku-Takano *et al*. (1999) reported that a heterozygous *GNAS1* mutation renders ion channels of GH-secreting pituitary adenomas unresponsive to GHRH. Regarding tumour size in Hardy's classification, there was no significant difference between the two groups.

The pathogenesis of the remaining GH-secreting adenomas is unclear. Interestingly, phosphorylation of CREB is promoted not only in GH-secreting adenomas with *GNAS1* mutations, but also in adenomas with wild-type *GNAS1* (Bertherat *et al*., 1995) therefore other factors activating the cAMP signalling pathway seem to contribute to the tumourigenesis of GH-secreting adenomas.

GH-secreting pituitary adenomas are encountered in about 10% of cases of Carney complex (Stratakis, 2001), an autosomal dominant multiple neoplasia syndrome which has been linked to loci on 2p16 (Stratakis *et al*., 1996) and 17q22–24 (Casey *et al*., 1998). Kirschner *et al*. (2000a) and Casey *et al*. (2000) reported that *PRKAR1A*, which codes for the RIα regulatory subunit of PKA, is located on 17q23–24 and is found to be mutated in some Carney complex families. Each of the *PRKAR1A* mutations is predicted to lead to the production of truncated protein products by missense mutations, frameshifts, or splice site mutations (Casey *et al*., 2000; Kirschner *et al*., 2000a). In a patient with Carney complex, LOH was found on 17q22–24 in a GH-secreting pituitary tumour (Kirschner *et al*., 2000a). LOH in tumours suggests that *PRKAR1A* is a tumour suppressor gene.

Hormonally mediated increases in intracellular cAMP levels lead to the dissociation of a catalytically inactive complex of PKA (R2C2) containing a cAMP-binding regulatory subunit (R) and free (catalytic active) subunit (C); the active catalytic subunit then phosphorylates multiple target proteins including CREB. Four distinct R isoforms (RIα, RIβ, RIIα, RIIβ) showed tissue-specific expression patterns (McKnight, 1991). Loss of the RIα subunit of PKA is expected to activate PKA and subsequent downstream signalling such as GH hypersecretion and tumourigenesis of somatotroph cells.

In the present study, we analysed the nucleotide sequence of *PRKAR1A* by direct sequencing of all exons and their flanking intronic sequences, and LOH on 17q23–24 with microsatellite markers and intragenic markers to investigate whether *PRKAR1A* mutations play a role in the tumourigenesis of sporadic GH-secreting adenomas. Neither inactivating mutations of *PRKAR1A* nor LOH on 17q23–24 were detected in GH-secreting adenomas. From these results, inactivation of *PRKAR1A* by mutations or LOH occurs infrequently if at all in sporadic GH-secreting pituitary adenomas.

Because GHRH acts by binding to the GHRH receptor, which activates adenylyl cyclase and promotes the growth and formation of somatotroph cells, Salvatori *et al*. (2001) and Lee *et al*. (2001) hypothesized that activating mutations of the GHRH receptor gene might occur in a subset of GH-secreting pituitary adenomas, and analysed 26 and 54 tumours, respectively. However, they found several SNPs and somatic mutations that did not increase intracellular cAMP. They concluded that activating mutations of the GHRH receptor gene in GH-secreting adenomas are rare or absent. Only Gsα among elements in the cAMP signalling pathway plays an important role in the tumourigenesis of GH-secreting adenomas, but other genes responsible remain to be elucidated in tumours lacking activating *GNAS1* mutations.

In the present study, *GNAS1* mutations were detected in 53·1% of GH-secreting pituitary adenomas; however, *PRKAR1A* mutations were not detected in any of the 32 cases. We reconfirm that frequent somatic mutations of *GNAS1* occur in GH-secreting adenomas, and conclude that *PRKAR1A* does not play a significant role in the tumourigenesis of GH-secreting adenomas.

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