

# Prevalence of Gs alpha mutations in Korean patients with pituitary adenomas

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## Abstract

The reported frequencies of Gs $\alpha$  mutations (*gsp* mutations) in growth hormone (GH)-secreting pituitary adenomas are variable (ranging from 4.4 to 43%), and the presence of these mutations in the other pituitary adenomas is still a matter of controversy. Previous clinical and biochemical analyses of patients with GH-secreting pituitary adenomas and *gsp* mutations produced conflicting results and did not demonstrate obvious characteristics. Therefore, we investigated the prevalence of *gsp* mutations in Korean patients with pituitary adenomas and elucidated the characteristics of these patients. Forty-four GH-secreting adenomas, 7 prolactin (PRL)-secreting adenomas and 32 clinically non-functioning adenomas were examined for the presence of point mutations in codon 201 and 227 of the Gs $\alpha$  gene using a nested PCR and direct sequencing of DNA extracted from fresh tissue or paraffin-embedded pituitary adenoma samples. Seven of the 44 GH-secreting pituitary adenomas had point mutations

at codon 201 or 227; of these, five mutations were in codon 201 and two were in codon 227. In patients with *gsp* mutations, mean tumor size was significantly smaller than in patients without *gsp* mutations ( $15.9 \pm 8.7$  mm *vs.*  $24.9 \pm 14.9$  mm,  $P < 0.05$ ). Age, sex, basal GH levels, GH response to oral glucose loading, GH response to octreotide and surgical outcome were not different in the two groups. One of the 32 clinically non-functioning pituitary adenomas had a point mutation at codon 201; none of the seven prolactinomas had these mutations. These results show that *gsp* mutations are not rare in Korean acromegalic patients and mean tumor size is significantly smaller in acromegalic patients with *gsp* mutations. Our results also confirm the low frequency of *gsp* mutations in clinically non-functioning pituitary adenomas and the absence of *gsp* mutations in prolactinoma.

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## Introduction

The mutations of the gene for the  $\alpha$ -subunit of G protein (Gs $\alpha$ ) have been identified in human growth hormone (GH)-secreting pituitary adenomas (Vallar *et al.* 1987, Landis *et al.* 1989, Clementi *et al.* 1990, Lyons *et al.* 1990) and clinically non-functioning pituitary adenomas (Tordjiman *et al.* 1993, Williamson *et al.* 1994). The Gs $\alpha$  mutations (*gsp* mutations) inhibit the guanine triphosphatase activity of Gs $\alpha$ . Inhibition of guanine triphosphatase leads to persistent activation of adenylyl cyclase and continually elevated intracellular cAMP levels in pituitary tissue, leading to cellular proliferation, differentiation and hypersecretion. Thus, *gsp*

mutations in pituitary tissues result in hyperfunctioning and non-functioning gland adenomas (Landis *et al.* 1989, Dhanasekaran *et al.* 1995). These mutations are detected in either codon 201 in exon 8 (arginine replaced by cysteine, serine or histidine) or codon 227 in exon 9 (glutamine replaced by arginine or leucine) of the Gs $\alpha$  gene.

The reported frequencies of *gsp* mutations in patients with GH-secreting pituitary adenomas ranged from 4.4 to 43% of examined cases (Lyons *et al.* 1990, Hosoi *et al.* 1993). It is reported that the prevalence of *gsp* mutations in GH-secreting pituitary adenomas varies by geographic location and the genetic background of the population (Hosoi *et al.* 1993). In Caucasians, the prevalence of *gsp* mutations was 27–43% of GH-secreting pituitary

adenomas (Lyons *et al.* 1990, Barlier *et al.* 1998), but in Japanese, the prevalence of *gsp* mutations was considerably lower (4.4–9.3%) (Hosoi *et al.* 1993, Yoshimoto *et al.* 1993). By contrast, Yang *et al.* (1996) reported that mutation of the *gsp* gene of GH-secreting adenomas in Korean acromegalic patients is as common as that found in Caucasian patients.

Although several investigators have demonstrated the characteristics of *gsp* mutations in individuals with GH-secreting pituitary adenomas, available published information does not adequately describe the characteristics of such mutations. Some studies have suggested that the *gsp*-positive tumors are smaller (Landis *et al.* 1990, Spada *et al.* 1990, Boggild *et al.* 1994) and have lower levels of GH (Landis *et al.* 1990), but these have not been confirmed by other studies (Harris *et al.* 1992, Adams *et al.* 1993, Yang *et al.* 1996). Several results have shown that the GH nadir was significantly lower in *gsp*-positive adenomas during acute octreotide testing (Faglia *et al.* 1996, Yang *et al.* 1996). Recently Barlier *et al.* (1998) reported that *gsp*-positive tumors should have a better prognosis and that *gsp* mutation could provide a marker for tumor response to the somatostatin analog.

In addition to GH-secreting pituitary adenomas, *gsp* mutations have also been reported in clinically non-functioning pituitary adenomas (Tordjman *et al.* 1993, Williamson *et al.* 1994). These *gsp* mutations were detected in about 10% of the clinically non-functioning pituitary adenomas examined, and were located at codons 201 and 227.

In this study, we investigated the prevalence of *gsp* mutations between codons 184 and 251 in Korean patients with pituitary adenomas and analyzed the characteristics of patients with pituitary adenomas and *gsp* mutations.

## Materials and Methods

### Subjects

Eighty-three patients with pituitary adenomas who underwent trans-sphenoidal adenomectomy at Seoul National University Hospital from April 1994 to July 1998 were involved in this study. Of these, there were 44 patients with acromegaly, 7 patients with prolactinoma and 32 patients with clinically non-functioning pituitary adenoma. None had previously undergone radiation therapy. Informed consent was obtained from all subjects. Ethical approval was given for the study protocol by the appropriate University authority.

Diagnosis of acromegaly was on the basis of clinical features, a failure to suppress GH to  $<5 \mu\text{g/l}$  during an oral glucose tolerance test (OGTT) and immunohistochemical staining of the tumors for GH. The duration of disease was estimated by carefully questioning patients and relatives. Prolactinoma was confirmed by elevated

serum prolactin (PRL) levels of  $>200 \mu\text{g/l}$  and immunological staining techniques. Diagnosis of clinically non-functioning pituitary adenoma was on the basis of the lack of clinical and laboratory evidence of hormonal hypersecretion.

### Hormone assays and light microscopic findings

In all patients, anterior pituitary functions were evaluated before surgery and re-evaluated post-operatively. Basal pituitary function, and if indicated, dynamic testing of the pituitary-adrenal axis were assessed in all patients. Serum GH, cortisol, thyrotropin, PRL, luteinizing hormone and follicle-stimulating hormone were measured using the appropriate specific radioimmunoassay. The size and extent of each pituitary tumor were evaluated by magnetic resonance imaging and operative findings. Tumor size was measured as maximal diameter. Tumor size and extension were also graded according to Hardy's classification (Hardy & Vezina 1976). Tissue samples were confirmed as adenoma by histological examination. Hormone secretion was confirmed by immunohistochemistry.

The basal serum GH levels were taken in the early morning after an overnight fast. Serum GH levels were also expressed as serum GH corrected for tumor size (GH/tumor size). Dynamic GH endocrine studies were performed in 25 acromegalic patients on separate days. Blood samples for GH were obtained at 30-min intervals immediately before and over a 2-h period following an oral 75 g glucose load. After a single 100  $\mu\text{g}$  subcutaneous injection of the somatostatin analog, octreotide (Sandostatin; Sandoz, Switzerland), blood samples were obtained at 0, 3, and 4 h to measure serum GH. Nadir GH levels were obtained at any time during an OGTT and after octreotide injection, and the percentage suppression of GH was calculated. Acromegalic patients were considered to be good responders when the percentage suppression of GH by octreotide was  $>80\%$  of the basal level. Post-operative normalization of basal GH level ( $<2 \mu\text{g/l}$ ) was defined as a surgical cure.

### DNA extraction

From 83 tumors, 48 fresh tissue samples obtained directly from the operating room were immediately frozen in liquid nitrogen and stored at  $-70^\circ\text{C}$  until DNA extraction. The remaining 35 tumors were examined from paraffin-embedded tissue obtained from Seoul National University Hospital Department of Pathology. Genomic DNA was extracted from frozen tissue or paraffin embedded tissue by proteinase K digestion using a QIAamp Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. In the case of paraffin-embedded tissue, sections ( $5 \mu\text{m}$ ) were sliced and de-paraffinized with xylene, and DNA extracted according to the manufacturer's protocol.

### Nested PCR

PCR amplification of the exon 8–10 region of the *Gsa* gene was performed from human genomic DNA using the following primers: first PCR, 5'-GCG CTG TGA ACA CCC CAC GTG TCT-3' (sense, 1F) and 5'-CGC AGG GGG TGG GCG GTC ACT CCA-3' (antisense, 1R); and second PCR, 5'-GTG ATC AAG CAG GCT GAC TAT GTG-3' (sense, 2F) and 5'-GCT GCT GGC CAC CAC GAA GAT GAT-3' (antisense, 2R).

The first PCR was carried out with 5 µl of genomic DNA, using *Taq* polymerase and 0.5 µmol/l of the 1F and 1R primers in a volume of 20 µl and a deoxynucleotide concentration of 0.5 mmol/l. The second PCR was performed with primers 2F and 2R and a 1:1000 dilution of the first PCR product as a template. Nested PCR was carried out in a thermal cycler 2400 (Perkin-Elmer/Cetus, Norwalk, CT, USA) as follows: 30 cycles at 95 °C for 20 s, 50 °C for 20 s and 72 °C for 30 s for the first PCR; 35 cycles at 95 °C for 20 s, 57 °C for 30 s and 72 °C for 40 s for the second PCR. Each of the PCRs was preceded by 3 min of denaturation at 95 °C and followed by 5 min of chain elongation at 72 °C.

After PCR amplification, the PCR products were analyzed by gel electrophoresis on a 1.6% ethidium bromide-stained agarose gel and the DNA visualized under short wavelength UV light.

The double-stranded PCR products resulting from this amplification were purified using a Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA).

### Direct sequencing

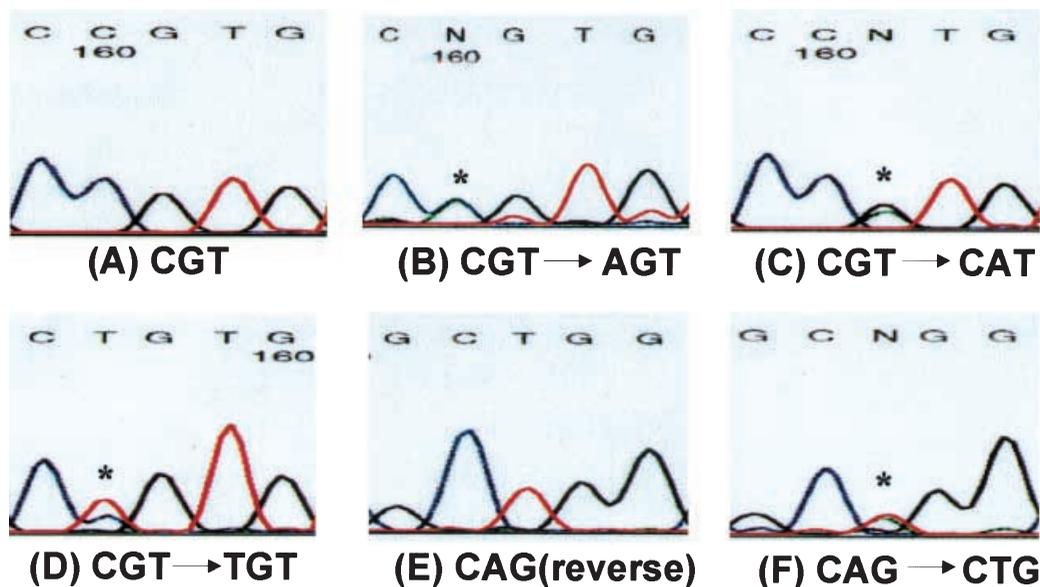
The purified double-stranded PCR products were analyzed to determine the sequence of genomic DNA. The primers were the same as those used in the second PCR. DNA sequences of the PCR products were determined by fluorescence-based dideoxy sequencing using *Taq* polymerase in a thermal cycler, followed by gel electrophoresis, data collection and analysis on an Applied Biosystems model 373A automated sequencer (Applied Biosystems, Foster City, CA, USA).

### Statistical analysis

Results are expressed as means ± s.d. Data were analyzed by unpaired Student's *t*-test and Fisher's exact test.  $P < 0.05$  was considered statistically significant.

### Results

The *Gsa* gene, containing codons 201 and 227, was amplified from each sample by nested PCR. Of the 83 pituitary adenomas, all 83 PCR products gave a single distinct band on gel electrophoresis. The purified double-stranded PCR products were then sequenced to determine the point mutations of the *Gsa* gene. *Gsp* mutations were detected in 7 of 44 GH-secreting pituitary adenomas and 1 of 32 clinically non-functioning pituitary adenomas. No mutations were detected in seven prolactinomas.



**Figure 1** Nucleotide sequence analysis of the *Gsa* codon 201 and 227 from genomic DNA. (A) Normal codon 201; (B) R201S; (C) R201P; (D) R201C; (E) Normal codon 227; (F) Q227L; \*mutated position.

**Table 1** Pre-operative clinical features of acromegalic patients with and without *gsp* mutations

	<i>gsp</i> -positive (n=7)	<i>gsp</i> -negative (n=37)
Age (y)	41.0 ± 13.0	37.5 ± 10.8
Sex (M/F)	2/5	15/22
BMI (kg/m <sup>2</sup> )	25.2 ± 4.3	25.6 ± 3.5
Duration of disease (y)	5.4 ± 4.0	10.6 ± 16.7
Basal GH (µg/l)	72.0 ± 81.2	109.8 ± 132.4
Suppression of GH by octreotide (%)	63.3 ± 40.5	80.0 ± 23.4
Suppression of GH by glucose (%)	21.8 ± 23.7	9.8 ± 43.0
Basal PRL (µg/l)	53.9 ± 97.4	49.8 ± 80.8
Tumor size (mm)	15.9 ± 8.7*	25.9 ± 14.9
GH/size (µg/l per mm)	4.0 ± 2.6	4.1 ± 4.0
Tumor grade (1/2/3/4) <sup>a</sup>	2/1/2/2	3/3/16/15

\*,  $P < 0.05$  compared to the patients without *gsp* mutations.

BMI, body mass index; PRL, prolactin.

<sup>a</sup>Adenomas were classified by grade on the basis of the classification of Hardy and Vezina: grade 1, microadenoma (<10 mm); grade 2, tumors ≥10 mm with an enlarged sella; grade 3, local destruction of the sella floor; grade 4, diffuse destruction of the sella floor.

Of the seven patients with GH-secreting pituitary adenoma with *gsp* mutations, five mutations were in codon 201 (arginine was replaced by cysteine in three tumors, by serine in one and by histidine in one), and two were in codon 227 (glutamine was replaced by leucine). A single clinically non-functioning pituitary adenoma was identified as having a *gsp* mutation in codon 201 (arginine was replaced by cysteine). Figure 1 illustrates examples of the sequencing results of the PCR products.

Table 1 shows the pre-operative clinical characteristics of patients with GH-secreting pituitary adenomas with and without *gsp* mutations. In patients with *gsp* mutations, mean tumor size was significantly smaller than in patients without mutations (15.9 ± 8.7 vs. 25.9 ± 14.9 mm,  $P < 0.05$ ). However, basal GH levels, tumor grade and GH/size were not significantly different between the patients with and without *gsp* mutations. There were no significant differences in the mean percentage suppressions of GH by octreotide and glucose between the two groups (63.3 ± 40.5 vs. 80.0 ± 23.4% ( $P > 0.05$ ), 21.8 ± 23.7 vs. 9.8 ± 43.0 ( $P > 0.05$ ), respectively). Three of five patients with *gsp* mutations and 16 of 20 patients without *gsp* mutations were good responders. There was no significant difference in age, sex, body mass index, duration of disease and basal PRL levels between the two groups (Table 1). No statistically significant differences were observed in the post-operative serum levels of GH and insulin-like growth factor-I and the surgical cure rate (Table 2).

## Discussion

In this study, we investigated the prevalence of *gsp* mutations in Korean patients with pituitary adenomas and

**Table 2** Outcome of surgery in patients with and without *gsp* mutations

	<i>gsp</i> -positive (n=7)	<i>gsp</i> -negative (n=37)
Post-operative		
Basal GH (µg/l)	25.8 ± 55.8	24.7 ± 51.5
IGF-I (µg/l)	402.7 ± 260.4	569.1 ± 415.4
Surgical cure rate (%) <sup>a</sup>	28.6	22.2

No statistical difference was observed.

<sup>a</sup>Postoperative normalization of basal GH levels (<2 µg/l) was defined as a surgical cure.

the clinical characteristics of patients with *gsp* mutations. *Gsp* mutations were detected in 7 of 44 GH-secreting pituitary adenomas (15.9%) and 1 of 32 clinically non-functioning pituitary adenomas (3.1%). There were no *gsp* mutations in seven prolactinomas.

Several studies have demonstrated the prevalence of *gsp* mutations in GH-secreting pituitary adenomas. *Gsp* mutations were present in 27–43% of somatotroph adenomas in the Caucasian population (Landis *et al.* 1989, Lyons *et al.* 1990, Barlier *et al.* 1998), but the reported frequency of *gsp* mutations in Japanese acromegalic patients was 4–9% (Hosoi *et al.* 1993, Yoshimoto *et al.* 1993). Thus, race differences have been suggested to be responsible for the different occurrence frequencies of *gsp* mutations. However, Yang *et al.* (1996) reported that *gsp* mutations of GH-secreting adenomas in Korean acromegalic patients were as common as in Caucasian patients. In this study, we found *gsp* mutations in 15.9% of the GH-secreting pituitary adenomas we examined; *gsp* mutations are not rare in Korean GH-secreting pituitary adenoma. Although we cannot provide a clear explanation, it is thought that the different prevalence of *gsp* mutations in GH-secreting pituitary adenomas is most likely due to the low number of patients examined in the different studies and in the different countries. To further examine the cause of this difference, it will be necessary to investigate the prevalence of *gsp* mutations in a large number of patients with GH-secreting pituitary adenoma. *Gsp* mutations in clinically non-functioning pituitary adenomas have been reported in approximately 10% of tumors examined (Tordjman *et al.* 1993, Williamson *et al.* 1994). In this study, we detected *gsp* mutations in only 1 of 32 clinically non-functioning pituitary adenomas, which indicates that *gsp* mutations are rare in Korean clinically non-functioning pituitary adenomas.

In this study, of seven GH-secreting pituitary adenomas with *gsp* mutations, five mutations were in codon 201 (three arginine to cysteine, R201C; one arginine to histidine, R201H; one arginine to serine, R201S) and two were in codon 227 (both glutamine to leucine, Q227L), which are similar to those found previously (Landis *et al.* 1989, Lyons *et al.* 1990, Yang *et al.* 1996). *Gsp* mutations

R201C and R201H are frequently observed in human GH-secreting pituitary adenomas (Landis *et al.* 1989, Lyons *et al.* 1990, Yang *et al.* 1996). On the other hand, there have been few reports on the *gsp* mutations R201S and Q227L in human GH-secreting pituitary adenomas (Clementi *et al.* 1990, Yang *et al.* 1996, Yasufuku-Takano *et al.* 1999). Specifically, R201S is a very rare type of mutation. Only two cases have been reported in previous studies, one case from a Korean study (Yang *et al.* 1996) and the other case from a recent Japanese study (Yasufuku-Takano *et al.* 1999). Q227L mutation is also a rare type of mutation. Four cases of Q227L mutation have been reported, three of which were in an Italian study (Clementi *et al.* 1990) and the other in a Japanese study (Yasufuku-Takano *et al.* 1999). Of 32 clinically non-functioning pituitary adenomas tested, one contained a point mutation in codon 201 (arginine to cysteine, R201C). Williamson *et al.* (1994) reported that *gsp* mutations R201C and Q227R were identified in 2 of 22 tumors. In another study of clinically non-functioning pituitary adenomas, two tumors were found to have *gsp* mutations R201C and Q227L (Tordjman *et al.* 1993).

A number of authors have investigated the relationship between the clinical and biochemical characteristics of patients with GH-secreting pituitary adenomas and *gsp* mutations, including basal serum GH levels, tumor size and serum GH response to oral glucose or somatostatin analog. Some studies reported that mean tumor size was significantly smaller in tumors with *gsp* mutations than those in wild-types (Landis *et al.* 1990, Spada *et al.* 1990). By contrast, other studies have demonstrated that tumor size was not significantly different (Harris *et al.* 1992, Adams *et al.* 1993). In this study, GH-secreting pituitary adenomas with *gsp* mutations were smaller than those without *gsp* mutations. No differences in age, sex, BMI, and duration of disease were observed between the two groups. These results are in agreement with those of others (Spada *et al.* 1990, Harris *et al.* 1992, Adams *et al.* 1993, Yang *et al.* 1996).

Previous studies have indicated that *gsp* mutations are associated with better octreotide sensitivity. Adams *et al.* (1995) reported that the absence of *gsp* oncogenes was often associated with resistance to octreotide *in vitro* cell culture studies, and data on the relationship between the presence of *gsp* oncogenes and the sensitivity to somatostatin have been reported (Yang *et al.* 1996, Barlier *et al.* 1998). Yang *et al.* (1996) found that octreotide-induced GH suppression was significantly greater in patients with the *gsp* oncogene mutation than in those without this mutation. In a long-term study *in vivo*, it is reported that the percentage inhibition of GH hypersecretion was higher in *gsp*-positive adenomas (Barlier *et al.* 1998). In contrast to previous reports, which showed that the somatostatin analog, octreotide, powerfully inhibited GH secretion in patients with GH-secreting pituitary

adenomas and *gsp* mutations, there were no significant differences in the mean percentage suppression of GH by octreotide and the number of good responders to octreotide between the patients with and without *gsp* mutations. The limitations of our study are that we could not subject all patients with *gsp* mutations to octreotide testing and that we observed only an acute response to octreotide. The effect of the former was probably minimal in our results, but further study in a large number of patients is required. In regard to the latter, additional studies will be required to determine whether improved long term octreotide responsiveness occurs in patients with *gsp* mutations *in vivo*.

In this study, the surgical cure rate was not different in patients with GH-secreting pituitary adenomas with and without *gsp* mutations. Spada *et al.* (1990) also reported that the cure rate was similar in acromegalic patients bearing tumors with and without *gsp* mutations. Recently, it was reported that *gsp*-positive tumors should have a better prognosis because GH hypersecretion was controlled in all patients with *gsp* mutations, even in those with tumoral tissue remaining after surgery (Barlier *et al.* 1998). In our study, patients bearing active tumor remnants were not yet treated by the long-term administration of somatostatin agonists after surgery; thus the effect of these agents needs further investigation.

Only one of 32 patients with clinically non-functioning pituitary adenomas had a *gsp* mutation. Arginine in codon 201 was changed to cysteine (R201C) by mutating codon CGT to TGT, no other mutation was identified at the other codons. The patient was a 32-year-old woman with a pituitary adenoma, which was 45 mm in diameter, and of Hardy stage 4. She was initially treated by transphenoidal adenomectomy. After surgery, she was given radiotherapy with a total dose of 5040 cGy. Now, she is receiving estrogen- and progesterone-based hormone replacement therapy. We did not examine in detail the characteristics of patients with clinically non-functioning pituitary adenoma and *gsp* mutation because of the limited number of subjects.

In summary, we have shown that *gsp* mutations are not rare in Korean acromegalic patients and mean tumor size is significantly smaller in acromegalic patients with *gsp* mutations. No significant differences were found between patients with GH-secreting pituitary adenomas with and without *gsp* mutations, in terms of their *in vivo* acute GH-responses to octreotide-induced GH suppression. We also report the low frequency of *gsp* mutations in clinically non-functioning pituitary adenomas and the absence of *gsp* mutations in prolactinoma.

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