Mutation Analysis of Gsα, Adrenocorticotropin Receptor and p53 Genes in Japanese Patients with Adrenocortical Neoplasms: Including a Case of Gsα Mutation

HIROMASA KOBAYASHI*,#, TAKESHI USUI**, JUNICHI FUKATA***, TAKAAKI YOSHIMASA*††, YUTAKA OKI### AND KAZUWA NAKAO*

* Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan
** Clinical Research Institute, Kyoto National Hospital, Kyoto 612-8555, Japan
*** First Department of Medicine, Kochi Medical School, Nangoku 783-8505, Japan
# Department of Medicine, Kobe City General Hospital, Kobe 650-0046, Japan
## Department of Medicine, Shizuoka City Hospital, Shizuoka 420-3192, Japan
### Second Division Department of Medicine, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan

Abstract. While the mechanisms of tumorigenesis for adrenocortical neoplasms remain unknown, several genes, such as Gsα, ACTH receptor (MC2-R), p53, and p16 tumor suppressor genes, are considered to be candidates for adrenocortical neoplasms. Mutation analysis studies have documented these genes in adrenocortical neoplasms, but these studies focused on the mutation of only one of these genes. In the present study we examined the mutations of three of these genes (Gsα, MC2-R, and p53) in adrenocortical neoplasms in Japanese patients. We amplified these genes using polymerase chain reaction and directly sequenced them in 30 functioning adrenocortical neoplasms. As for Gsα, we identified a heterogeneous substitution of glutamine to histidine at codon 227 and a gain of an Nru I restriction endonuclease site. The mutation was restricted to adenomatous tissue, and did not occur in the adjacent normal adrenal tissue or leukocytes of the patient. We did not find any mutations in MC2-R and p53. In conclusion, although the contribution of these three genes to adrenocortical tumorigenesis remains to be determined, it is suggested that the mutation of Gsα might play a role in functional adrenocortical neoplasms.

Key words: gsp, MC2-R, p53, Mutation, Adrenocortical neoplasms

UNTIL recently, steroid-secreting human adrenocortical neoplasms were considered to be rare. With recent advances in imaging techniques, such as computed tomography, ultrasound sonography, and magnetic resonance imaging, the prevalence of clinically silent, incidentally detected adrenal neoplasms is actually much higher than previously assumed. Some of these neoplasms produce a small amount of cortisol without further development of Cushing's syndrome. However, the molecular mechanisms of tumorigenesis and steroidogenesis of adrenal neoplasms have not been extensively studied.

G proteins, which are involved in signal transduction after ligand-receptor binding, are candidate factors for the endocrine hyperfunctioning state. The gsp oncogene results from mutations at codons 201 and 227, two critical sites involved in the intrinsic guanosine triphosphatase activity of the Gsα protein. These mutations lead to constitutive activation of adenylyl cyclase [1]. Mutations in Gsα, the stimulatory regulatory protein of adenylyl cyclase, have been found in up to 40% of GH-secreting adenomas [1, 2].
As cAMP proliferates in GH cells, a gsp mutation is considered to be an oncogene. These mutations take place less often in other pituitary adenomas [3, 4] and in some thyroid tumors [5-7]. The gsp mutations have not been found in adrenocortical neoplasms [5, 8], except for one sporadic aldosterone-secreting tumor [9], nodular adrenal hyperplasia [10], and in the very peculiar condition called McCune-Albright syndrome [11].

Several G-protein-coupled receptors are mitogenic and capable of transforming cells in vitro in the presence of the appropriate agonist [12]. When such receptors are constitutively activated by mutations, they transform cells in an agonist-independent fashion. Consistent with these findings, several up-regulatory somatic mutations have been reported in the carboxyl-terminal portion of the third cytoplasmic loop of the TSH receptors isolated from hyperfunctioning thyroid adenomas [13]. Activating mutations of the LH receptor cause familial male precocious puberty, an autosomal dominant disorder in which affected males show signs of virilization often by age four or even earlier [14]. A heterozygous activating mutation of the PTH/PTHrP receptor gene was identified in a subject with a rare form of dwarfism termed Jansen-type metaphyseal chondrodysplasia [15].

The p53 tumor suppressor gene is mutated in about half of almost all types of cancers [16, 17]. The mutations are mainly missense mutations, clustered in four of the five highly conserved domains of the protein; they are believed to alter its conformation. These changes exert a dominant negative effect by decreasing the functionality of tetramers. They also increase the intranuclear stability of the p53 protein [17, 18]. Two recent studies analyzing p53 in adrenocortical tumors [19, 20] found that mutations are frequent, particularly in benign tumors [19]. More recently, the involvement of the p16 tumor suppressor gene in adrenocortical malignant tumors has been reported [21].

To date, however, there have been no reports concerning mutations of several candidate genes in the same adrenocortical neoplasms. We studied three of these candidate genes (Gsa, MC2-R, and p53) in Japanese patients with hyperfunctioning adrenal disease and found a gsp mutation in one tumor in a patient who had preclinical Cushing's syndrome.

Materials and Methods

Patients

We studied 30 Japanese patients with adrenocortical neoplasms (Table 1). Twenty-eight tumor samples were obtained at surgery or autopsy, and two samples of primary pigmented nodular adrenocortical disease (PPNAD) were obtained as paraffin-embedded specimens. Except for the paraffin-embedded specimens, all tissue samples were immediately placed in liquid nitrogen and stored at −70°C until DNA extraction. All functional adrenal tumors were diagnosed by clinical and hormonal data and confirmed by tumor histology at individual institutions.

DNA Preparation and Analysis

DNA was prepared from frozen neoplasms or paraffin-embedded tissues. It was used as a template for amplification by polymerase chain reaction (PCR) of exons 8 and 9 of the Gsa gene, the exon that

<table>
<thead>
<tr>
<th>Type of neoplasm</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol-producing adenoma (Cushing's syndrome)</td>
<td>8</td>
</tr>
<tr>
<td>Cortisol-producing adenoma (preclinical Cushing's syndrome)</td>
<td>13</td>
</tr>
<tr>
<td>Primary pigmented nodular adrenocortical disease</td>
<td>2</td>
</tr>
<tr>
<td>Macronodular adrenocortical hyperplasia</td>
<td>1</td>
</tr>
<tr>
<td>Aldosterone-producing adenoma</td>
<td>5</td>
</tr>
<tr>
<td>Cortisol-producing adrenal carcinoma</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>30</td>
</tr>
</tbody>
</table>
encodes the entire coding region of MC2-R, and exons 4, 5, 6 and 7 of the p53 gene. The following primers were used for Gsa: sense, 5'-GGCAATTATTACTGT TTCGG-3' and antisense, 5'-GTCAGACA GGCACCTGTGATCC-3'. The following pairs of flanking primers were used to amplify the MC2-R gene; sense, 5'-AAGT CCAAGTAACATCCC-3'; and antisense, 5'-TAAGCAACACACAGTG C-3', and sense, 5'-CAGCCTGCTGATC TTGC-3'; and antisense, 5'-TGGATTCTAAAACCAGGG-3'. Amplification of the p53 gene was performed as previously described [20]. Amplification was allowed for 30 cycles at 94°C for 30 sec, 55°C for 1 min and 72°C for 3 min with AmpliTaq (PE Biosystems, Foster City, CA) or Pfu (STRATAGENE, La Jolla, CA) polymerase. The PCR products were purified by using Wizard PCR prep (Promega, Madison, WI) and used for PCR direct sequencing. Sequencing was performed by using the dye-terminator cycle sequencing method and the 373 DNA sequencer (PE Applied Biosystems).

Case Report of Gsa Mutation

A 41-year-old woman was admitted for further examination of adrenal tumor. Abdominal computed tomography revealed right adrenal tumor (2.0 cm × 2.0 cm). Physical examination revealed hypertension, but no abdominal obesity, abdominal striae, or muscle weakness. The patient's plasma corticotropin concentration was 5.6 pg/ml (normal, 10 to 55 pg/ml) at 8 a.m. The plasma cortisol concentration was 10.6 μg/dl at 8 a.m. and 3.3 μg/dl on the morning after the administration of 8 mg of dexamethasone at midnight. The plasma aldosterone concentration, measured while the patient was supine, was 137 pg/ml (normal, 57 to 150 pg/ml), plasma renin activity was 1.6 ng/ml/h (normal, 0.50 to 1.58 ng/ml/h), and the plasma potassium concentration was 3.8 mEq/l. An abnormal accumulation

<table>
<thead>
<tr>
<th>Type of neoplasm</th>
<th>Gsaα</th>
<th>p53</th>
<th>MC2-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol-producing adenoma (Cushing’s syndrome)</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>Cortisol-producing adenoma (preclinical Cushing’s syndrome)</td>
<td>1/13</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>Primary pigmented nodular adrenocortical disease</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Macronodular adrenocortical hyperplasia</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>Aldosterone-producing adenoma</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Cortisol-producing adrenal carcinoma</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>Total</td>
<td>1/30</td>
<td>0/18</td>
<td>0/18</td>
</tr>
</tbody>
</table>
was detected in the tumor by dexamethasone suppression scintigraphy.

Results

In 18 patients, the Gsa, MC2-R, and p53 genes were successfully amplified with PCR and shown to have the expected size on agarose gel electrophoresis (data not shown). Direct sequencing analysis of the Gsa gene revealed a single heterogeneous missense mutation on amino acid 227 in one patient, whose clinical data showed preclinical Cushing’s syndrome (Table 2). In this case, a point mutation at codon 227 of Gsa converts glutamine to histidine (CAG to CAT) (Fig. 1A). The mutation was confirmed by restriction endonuclease Nru I digestion. In the mutated DNA, the PCR product was digested to a 256 bp fragment and a 110 bp fragment (Fig. 2, lane 3). The mutation was not seen in adjacent normal adrenal tissue (Fig. 1, lanes 1 and 2, Fig. 2B) or leukocytes (data not shown). Because the clinical data of the patient having the gsp mutation were compatible with preclinical Cushing’s syndrome, we analyzed an additional 12 tumors from patients with the disease, but found no mutations of gsp in these tumors. On the other hand, direct sequencing of the MC2-R and the p53 gene showed results identical to the reported sequences in all neoplasms studied (Table 2). These results are summarized in Table 2.

Discussion

Mutations of the Gsa gene (gsp) have been reported to be involved in some endocrine tumors, such as pituitary adenoma [5, 6]. Two specific amino acids, codon 201 and codon 227, were mainly affected by these mutations. Hyperfunctioning states are believed to be caused by the constitutive activation of cAMP synthesis, which occurs when GTPase activity is disturbed by these mutations. Activating gsp mutations have been described in some functional endocrine tumors, including GH-producing pituitary adenomas [1, 2] and thyroid adenomas [5-7]. The gsp mutations are also well characterized in McCune-Albright syndrome, which may be the result of a single, early, post-zygotic somatic mutation [10]. Boston et al. reported the gsp mutation in an infant with Cushing’s syndrome and nodular adrenal hyperplasia [10]. In this study, we found a single nucleotide substitution in adrenocortical adenoma tissue, which alters codon 227 (Q227H), in one allele in one patient whose phenotype was preclinical Cushing’s syndrome. The mutation of codon 227 of Gsa has not been reported in adrenocortical neoplasms, but an identical nucleotide substitution and amino acid substitution at this position have been reported in one thyroid tumor [6]. The prevalence of gsp mutations in adrenocortical neoplasms is far less than in pituitary adenomas or thyroid tumors, but gsp mutations might contribute to some functioning adrenocortical neoplasms. Gsp in adrenocortical cells could lead to cellular proliferation and nodule formation [11]. Gsp would also account for autonomous cortisol secretion by adrenocortical cells, because ACTH-stimulated cortisol synthesis and secretion are mediated by activation of Gsa.

Latronico et al. [22] and Light et al. [23] also reported that there were no mutations in the coding region of the MC2-R gene in adrenocortical neoplasms. Our findings confirm this. Although it is
conceivable that they might occur in occasional cases, abnormalities in the coding region of the MC2-R gene are not a frequent mechanism of human hyperfunctioning adrenocortical disease.

Concerning p53 gene, Lin et al. [19] found p53 mutations in 11 of 16 benign adrenocortical tumors, 78% of them in exon 4, Reincke et al. [20] found p53 mutations in three of 16 adrenocortical carcinomas and none in five benign adrenocortical adenomas, although they did not investigate exon 4. We did not find any mutations in exons 4 through 7 of the p53 gene. Reincke et al. also could not detect p53 mutations, including on exon 4 in samples obtained from Caucasians [24]. From this data, we could suppose that the frequency of p53 mutations in adrenocortical tumors might be different between races.

In summary, we analyzed mutations in the Gsα, MC2-R and p53 genes in Japanese patients with adrenocortical neoplasms. We found no mutation in the coding region of the MC2-R gene or in exons 4 through 7 of the p53 gene. We did find one somatic heterogeneous gsp mutation in one adrenocortical adenoma from a patient with preclinical Cushing's syndrome. However, as gsp may play a role in some functioning adrenocortical neoplasms, these results suggest that abnormalities in these three genes are not frequent mechanisms of adrenocortical neoplasms in the Japanese population.

Acknowledgements

We thank Dr. Yoshitaka Taguchi, Dr. Makoto Sakamoto, and Dr. Toshihiro Yakura for providing the tissue samples. We also thank Ms. Keiko Matsuda for her excellent technical assistance. This work was supported in part by research grants from the Naito Foundation and the Uehara Memorial Foundation.

References


