

## ***ras* Mutations in Endocrine Tumors: Mutation Detection by Polymerase Chain Reaction-Single Strand Conformation Polymorphism**

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To elucidate the molecular basis for endocrine tumorigenesis, *ras* mutations in human endocrine tumors were analyzed using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis. Mutations of the H-, K-, N-*ras* genes were examined in genomic DNAs from 169 successfully amplified primary endocrine tumors out of 189 samples. Four out of 24 thyroid follicular adenomas analyzed contained mutated N-*ras* codon 61, and one contained the mutated H-*ras* codon 61. One of the 19 pheochromocytomas revealed mutation of the H-*ras* codon 13. No mutations of the *ras* gene were detected in pituitary adenomas, parathyroid tumors, thyroid cancers, endocrine pancreatic tumors, and adrenocortical tumors. Based on these findings we conclude that activation of the *ras* gene may play a role in the tumorigenesis of a limited number of thyroid follicular adenomas and pheochromocytomas, and that mutation of the *ras* gene is not frequent in other human endocrine tumors.

Key words: *ras* Gene — Missense mutation — Thyroid follicular adenoma — Pheochromocytoma

Both activation of protooncogenes and inactivation of tumor suppressor genes are involved in the tumorigenesis of various human tumors, but aberrations of oncogenes and suppressor genes including *ras*, *ret*, *gsp*, *gip2*, *p53* genes have been reported in only a few human endocrine tumors.<sup>1-14)</sup> Thus the molecular and genetic basis for tumorigenesis of the majority of relatively well-differentiated endocrine tumors remains to be elucidated.

Mutations of the *ras* genes are observed at the highest frequency in human malignancies. The genes of this family, including H-*ras*, K-*ras*, and N-*ras* genes, code for closely related 21-kDa proteins which presumably play a role in signal transduction. The proteins, designated as p21<sup>ras</sup>, acquire a transforming potential when a single amino acid is substituted at a critical codon, 12, 13, or 61.<sup>15)</sup>

Mutational activation of the *ras* genes has been found in a wide range of human malignancies. The frequency of *ras* mutations varies widely between different tumor types.<sup>15)</sup> The reported frequency of *ras* mutations varied between 5 and 15% in all human tumors. In pancreatic cancers, mutated K-*ras* genes were found in over 90% of the cases. In colon cancers, *ras* genes are activated in about 50% of the cases. On the other hand, *ras* mutations in breast cancers are rare. In several human cancers with *ras* mutations, a bias exists for a particular member of the

*ras* family. For example, H-*ras* mutations predominate in human urinary bladder carcinoma, K-*ras* mutations frequently occur in lung or colon carcinoma, and N-*ras* mutations are particularly associated with hematologic malignancies.

Although activation of *ras* genes by point mutations has been reported to be frequent in human cancers, few studies, except those on thyroid tumors<sup>1-8)</sup> and only one pituitary tumor,<sup>9)</sup> have investigated *ras* mutations in human endocrine tumors. In this study, we screened *ras* mutations in 169 human endocrine tumors to elucidate the molecular basis for endocrine tumorigenesis. The examined samples included tumors of the pituitary, thyroid, parathyroid, endocrine pancreas, adrenal cortex and medulla. Exons 1 and 2 of the H-, K-, and N-*ras* genes, i.e. the regions encompassing codons 12, 13 and 61, were amplified by means of polymerase chain reaction (PCR), and screened by single strand conformation polymorphism (SSCP)<sup>16)</sup> for *ras* mutations.

### MATERIALS AND METHODS

**Human tissue samples** One hundred and eighty-nine specimens from patients with endocrine tumors were studied. Eighty were frozen samples and 109 were formalin-fixed and embedded in paraffin. Among the paraffin-embedded specimens, DNAs from 89 specimens were successfully amplified for all *ras* genes analyzed, and DNAs from the remaining 20 specimens, which did

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not show enough amplification of at least one locus of the *ras* genes, were excluded from this study. The 169 human endocrine tumors consisted of 53 pituitary adenomas (43 growth hormone (GH)-producing adenomas, 1 prolactinoma, 9 non-functioning adenomas), 60 thyroid tumors (24 follicular adenomas, 26 papillary carcinomas, 1 poorly differentiated carcinoma, 8 medullary carcinomas, 1 anaplastic carcinoma), 12 parathyroid tumors (7 hyperplasia, 4 adenomas, 1 carcinoma), 11 endocrine pancreatic tumors (9 adenomas, 2 carcinomas), 14 adrenocortical tumors (12 adenomas, 2 hyperplasia), and 19 pheochromocytomas. Primary tumors together with adjacent noncancerous tissue, or peripheral blood leukocytes were obtained at the time of surgery.

**DNA extraction** High-molecular-weight DNA was prepared from the tissues by proteinase K digestion and phenol/chloroform extraction as previously described.<sup>17)</sup> Genomic DNA was isolated from the tissue embedded in paraffin as previously described.<sup>12)</sup> Briefly, the tumor regions were precisely located by microscopic observation of hematoxylin and eosin-stained samples. Sections 10  $\mu\text{m}$  in width were scraped from a glass microscope slide with a razor blade, placed in an Eppendorf centrifuge tube, deparaffinized by washing twice in xylene and twice in 95% ethanol, and finally dried at reduced pressure. The sample was treated with proteinase K (200 mg/liter) in 100  $\mu\text{l}$  of digestion buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5% Tween 20), at 37°C overnight. After inactivation of proteinase K by incubation at 95°C for 10 min, the samples were used directly for PCR analysis.

**PCR-SSCP analysis** Oligonucleotide primers were synthesized by the phosphoramidite method using a 392 DNA synthesizer (Applied Biosystems Inc., Foster City, CA). The nucleotide sequences of the primers used in this work were the same as described previously.<sup>18)</sup> PCR was performed using a thermal cycler (Astek, Fukuoka) with 50 ng of genomic DNA in a total volume of 5  $\mu\text{l}$  containing 0.5  $\mu\text{l}$  of [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol; 10 mCi/ml).<sup>19)</sup> Thirty cycles consisting of 1 min at 95°C for denaturation, 1 min at 55°C for annealing, and 2 min at 72°C for extension were performed. The PCR mixture (5  $\mu\text{l}$ ) was heated at 80°C with 245  $\mu\text{l}$  of a formamide dye mixture (95% formamide: 20 mM EDTA: 0.05% xylene cyanol; 0.05% bromophenol blue), then 1  $\mu\text{l}$  of the mixture was applied to a 6% polyacrylamide gel containing 45 mM Tris-borate (pH 8.3) and 4 mM EDTA.<sup>16)</sup> Glycerol (5%) was also added when specified. Electrophoresis was performed at 40 W for 2 to 4 h with cooling by a fan at room temperature or at 4°C. The gel was dried on filter paper and exposed to an X-ray film for 10–24 h at –80°C with an intensifying screen. PCR-SSCP was repeated twice to ensure that the results were reproducible in each case showing a mobility shift.

**Direct sequencing** Abnormal bands detected by SSCP analysis were excised from the dried gel, placed in 100  $\mu\text{l}$  of distilled water, and incubated at 37°C. An aliquot (1–3  $\mu\text{l}$ ) of the supernatant was then used as a DNA template in the PCR reaction.<sup>20)</sup> For direct sequencing, the primers were the same as those used in the original amplification, except that the 5' end of one primer contained M13, and the other contained the M13 reverse sequence. The double-stranded PCR product resulting from this amplification was purified by two centrifugal washes using a SUPREC™-2 microconcentrator (Takara Shuzo, Kyoto). DNA sequences of the PCR products were determined by fluorescence-based dideoxy sequencing using *Taq* polymerase in a thermal cycler, and fluorescently labeled M13 universal or reverse sequencing primers, followed by gel electrophoresis, data collection and analysis on an Applied Biosystems model 373A automated sequencer (Applied Biosystems, Inc.).

## RESULTS

**Screening of point mutations of the *ras* genes by PCR-SSCP** PCR-SSCP analysis of exon 2 of the N-*ras* gene from genomic DNA of 4 thyroid follicular adenomas disclosed extra bands with altered migrations relative to those amplified from normal leukocytes. Fig. 1A shows two different PCR-SSCP patterns. One pattern was detected in two thyroid follicular adenomas, and was

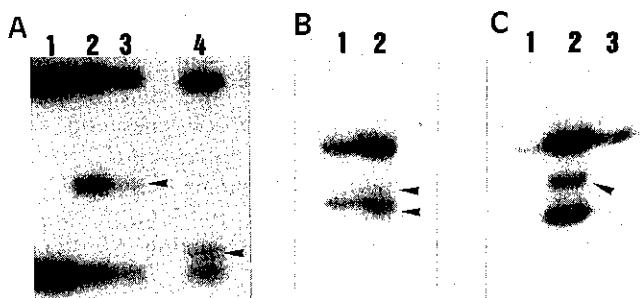


Fig. 1. PCR-SSCP analysis of exon 2 of the N-*ras* gene, exon 2 of the H-*ras* gene, and exon 1 of the H-*ras* gene in genomic DNA. DNA was PCR-amplified with respective primers for the *ras* genes. Panels A, B, and C show exon 2 of the N-*ras* gene, exon 2 of the H-*ras* gene, and exon 1 of the H-*ras* gene, respectively. A. Lane 1; normal leukocytes, lane 2; human fibrosarcoma cell line, HT1080 cells, lane 3; thyroid follicular adenoma, and lane 4; thyroid follicular adenoma. B. Lane 1; normal leukocytes, lane 2; thyroid follicular adenoma. C. Lane 1; patient's leukocytes, lane 2; pheochromocytoma, and lane 3; normal leukocytes. Electrophoresis was performed in a 6% polyacrylamide gel without glycerol at room temperature. Arrowheads denote the bands with altered migration relative to controls.

similar to that of a human fibrosarcoma cell line, HT1080 having the N-*ras* codon 61 mutation (CAA to AAA).<sup>21)</sup> Another PCR-SSCP pattern was found in exon 2 of the H-*ras* gene from one thyroid follicular adenoma, which does not have a corresponding example (Fig. 1B). PCR-SSCP of exon 1 of the H-*ras* gene from one pheochromocytoma also showed an extra band compared to those amplified from the patient's leukocytes (Fig. 1C). In all of these 6 tumors (5 thyroid follicular adenomas and 1 pheochromocytoma), bands with migration similar to those of the normal controls were observed, which sug-

gests the presence of a normal *ras* allele. In other endocrine tumors, no extra bands with altered migration were detected by PCR-SSCP of the H-, K-, N-*ras* genes.

**Sequencing of the *ras* genes** All the *ras* genes which revealed extra bands in PCR-SSCP were sequenced. Fig. 2A shows the sequence of exon 2 of the N-*ras* gene of four thyroid follicular adenomas which showed extra bands in SSCP analysis. Mutations of codon 61 of the N-*ras* gene were identified at 2 different sites. In two thyroid follicular adenomas, codon 61 of CAA for glutamine was mutated to AAA for lysine by a C to A transversion of the first letter (Fig. 2A, 1), as in HT1080. In the other two thyroid follicular adenomas, codon 61 of CAA for glutamine was mutated to CGA for arginine by an A to G transition of the second letter (Fig. 2A, 2). In one thyroid follicular adenoma, codon 61 of CAG for

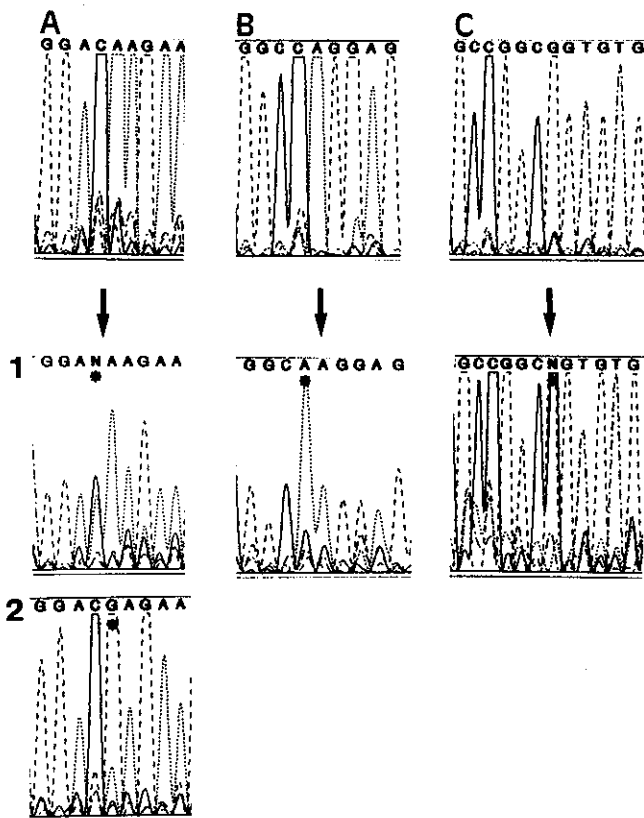


Fig. 2. Nucleotide sequence analysis of the *ras* genes from genomic DNA. DNA fragments that showed a mobility shift on SSCP, and DNA from normal leukocytes were PCR-amplified and sequenced. A. Genomic sequence and sequences of variant SSCP allele at exon 2 of the N-*ras* gene from two thyroid follicular adenomas. The top panel shows the normal sequence of codons 60-62. The middle panel (1) shows the direct genomic sequences from a thyroid follicular adenoma. The designation "N" at the first letter of codon 61 shows the co-presence of C and A. The bottom panel (2) shows the sequences of variant SSCP allele in another thyroid follicular adenoma with C to G transversion at codon 61. Mutated bases are indicated by asterisks. B. Sequence of the variant allele at exon 2 of the H-*ras* gene from thyroid follicular adenoma. The top panel shows the normal sequence of codons 60-62. The bottom panel shows the C to A transversion at codon 61. Mutated bases are indicated by asterisks. C. The genomic sequence of exon 1 of the H-*ras* gene from pheochromocytoma. The top panel shows the normal sequence of codons 11-14 in a patient's leukocytes. The bottom panel shows the G to C transversion at codon 13. The designation of "N" in direct sequencing with signals for G and C of similar magnitudes demonstrate the heterogeneity of mutation. Mutated bases are indicated by asterisks.

Table I. *ras* Gene Mutations in Human Endocrine Tumors

Patient	Age	Sex	Pathology	<i>ras</i> gene mutation		
				site	sequence	amino acid
1	42	F	thyroid follicular adenoma	N-61	CAA→AAA	Gln→Lys
2	21	F	thyroid follicular adenoma	N-61	CAA→AAA	Gln→Lys
3	51	F	thyroid follicular adenoma	N-61	CAA→CGA	Gln→Arg
4	57	F	thyroid follicular adenoma	N-61	CAA→CGA	Gln→Arg
5	39	M	thyroid follicular adenoma	H-61	CAG→AAG	Gln→Lys
6	51	F	pheochromocytoma	H-13	GGT→CGT	Gly→Arg

glutamine in the *H-ras* gene was mutated to AAG for lysine by a C to A transversion of the first letter (Fig. 2B, 1). In one pheochromocytoma, codon 13 of GGT for glycine in the *H-ras* gene was mutated to CGT for arginine by a G to C transversion of the first letter (Fig. 2C). Thus four different amino acid changes were obtained in 5 thyroid follicular adenomas, and 1 pheochromocytoma, as summarized in Table I.

## DISCUSSION

Mutation of the *ras* gene alone or in combination with another oncogene leads to various phenotypic consequences according to the cell type.<sup>15,22</sup> A mutated *ras* gene transforms NIH3T3 or other primary rodent fibroblasts. Retroviral infection of a rat pheochromocytoma cell line, PC12, or a human medullary thyroid carcinoma cell line, TT, with the viral homologues of *H-ras* or *K-ras* induces differentiation and cessation of cell division.<sup>23,24</sup> Microinjected antibodies to *ras* proteins inhibited differentiation in PC12.<sup>25</sup> Thus *ras* genes may play a role in both proliferation and differentiation processes in different cell types.

Mutational activation of the *ras* protooncogene in human thyroid tumors occurs at various frequencies.<sup>1-8</sup> The frequency of mutations ranged from 0-62% in papillary carcinoma, 0-53% in follicular carcinoma, and 0-46% in follicular adenoma. Even in the subtypes of follicular adenoma, the reported frequency of mutations ranges from 0-100% in a macrofollicular type and 12.5-50% in a microfollicular type. In our study, mutations in the *N-ras* and *H-ras* genes were found in 5 of 24 follicular adenomas (21%), but no *ras* mutation was detected in 26 papillary, 8 medullary, 1 poorly differentiated carcinoma, and 1 anaplastic carcinoma of the thyroid.

The explanations for the different frequencies of *ras* mutations include the following. First, detection of *ras* mutations by the frequently used method of oligonucleotide hybridization needs carefully controlled conditions to discriminate positive from negative. The absence of positive controls for *ras* mutations may also reduce the credibility. Second, the differences in the prevalence of *ras* mutations may arise from various factors such as genetic predisposition to *ras* mutations, environmental exposure to radiation, dietary supply of iodine, serum levels of TSH, and exposure to other mutagenic environmental or infectious agents. For example, the *ras* mutation rate in follicular adenomas and carcinomas was significantly higher in individuals living in an iodine-deficient area than that in an iodine-sufficient area.<sup>8</sup> The rate of *K-ras* mutation is significantly higher in radiation-associated follicular carcinomas than that in spontaneous follicular carcinomas.<sup>9</sup> Although contamination with noncancerous cells in our study may have caused under-

estimation of the frequency of *ras* mutations, focussing on the tumor tissue for DNA extraction in paraffin-embedded samples should have minimized the underestimation. In addition, a mutated sequence could be identified by PCR-SSCP when it was present in more than 10% of the total DNA.<sup>18</sup> Thus, underestimation of the frequency of *ras* mutations in our study is unlikely.

Moley *et al.* reported that *ras* mutations were not detected in 10 human pheochromocytomas.<sup>26</sup> In our study, we found *H-ras* codon 13 mutation in one of 19 pheochromocytomas. This pheochromocytoma with *ras* mutation was from a 51-year-old woman who had labile hypertension and increased urinary norepinephrine and epinephrine excretions of 1000 and 670  $\mu\text{g}/\text{day}$ , respectively. A right adrenal mass detected by CT scan was resected. The tumor, weighing 40 g, showed a typical histology of benign pheochromocytoma. The clinical and pathological features of this tumor were not different from those of the other 18 pheochromocytomas. Thus the significance of activated *ras* gene for the differentiation process of pheochromocytoma is currently unknown.

Since the initial analyses of human cancers, most of the point mutations have been localized at codons 12 and 61 in the *ras* gene family. Although mutations at codon 13 of *N-ras* have been detected at a relatively high frequency in myelogenous malignancies, mutations at codon 13 of *K-* and *H-ras* have been rare. One pheochromocytoma in our study revealed a G to C transition in the first letter at codon 13 of *H-ras* as shown in Fig. 2C. Mutations of *H-ras* codon 13 were reported in a cell line of oral squamous cell carcinoma and one of urinary bladder carcinoma.<sup>27-29</sup> Thus the detection of *H-ras* codon 13 mutation in one pheochromocytoma in our study is interesting for its rarity, but its significance remains to be elucidated.

In several tumors, *ras* mutations were found in both carcinomatous and adenomatous stages.<sup>15</sup> In our study, patients 2 and 4 in Table I had two thyroid follicular adenomas, though *ras* mutation was detected in only one of them. Therefore, the *ras* gene may participate in the initiation of tumor development in some tumors even in its adenomatous stage, but not necessarily in all tumors of the same histology found in the same patients.

Interaction of the mutant *ras* protein with the mutant p53 has been reported in transforming primary embryonic fibroblasts,<sup>30</sup> human lung carcinoma cell lines, and primary colorectal tumors.<sup>31,32</sup> We did not detect any associated mutations of the p53 genes in 6 endocrine tumors having *ras* mutations (data not shown), which is consistent with the findings reported for esophageal tumors.<sup>33</sup> Thus, the interaction of *ras* oncogenes and the p53 gene is an unlikely cause of the tumorigenesis of these endocrine tumors.

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