Jpn. J. Cancer Res. 86, 737–742, August 1995

Point Mutations of *ras* and *Gsα* Subunit Genes in Thyroid Tumors

Hidetaka Horie,[1,3] Yutaka Yokogoshi,[1] Masaru Tsuyuguchi[2] and Shiro Saito[1]

[1]*First Department of Internal Medicine, School of Medicine, The University of Tokushima, 3-18-15 Kuramoto-cho, Tokushima 770 and* [2]*Department of Surgery, Tokushima Municipal Hospital, 2-34 Kitajosanjima-cho, Tokushima 770*

We studied 43 thyroid tumors including 5 adenomatous goiters, 7 follicular adenomas, 22 papillary carcinomas, and 9 medullary carcinomas with regard to the presence of point mutations in the genes of Gs alpha subunit (*Gsα*), Gi2 alpha subunit (*Gi2α*), H-*ras*, K-*ras*, and N-*ras* by a polymerase chain reaction-direct sequencing method. An adenomatous goiter and a follicular adenoma showed double mutations at codon 227 and 231, and 4 papillary carcinomas showed mutation at codon 231 of the *Gsα* gene. An adenomatous goiter, a follicular adenoma, and a papillary carcinoma showed a missense mutation in codon 13 of the K-*ras* gene. There were no such missense mutations of these G-protein or *ras* genes in medullary carcinomas. These data indicate that the genetic events involved in the oncogenesis of parafollicular C-cells are different from those of thyroid follicular cells, in which missense mutations of *Gsα* and *ras* genes seem to play important roles in tumorigenesis.

Key words: *Gsα* — *Gi2α* — *ras* — Thyroid tumor

Thyroid tumor is a common neoplasm found in 4–7% of human adults[1] or in 50% of autopsied cases.[2] Thyroid tumors are classified mainly into two groups: epithelial tumors derived from thyroid follicular cells and medullary tumors derived from parafollicular C-cells. These tumors account for more than 90% and 3–10% of thyroid carcinomas, respectively.[3] Since thyroid tumors are thought to show stepwise progression of hyperplasia to solitary nodule, differentiated carcinoma and anaplastic carcinoma, they are an attractive model in which to identify genetic changes involved in tumorigenesis.

Many factors have been reported to contribute to thyroid tumorigenesis including irradiation,[4] iodine deficiency,[5] inherited defects of the enzymes involved in thyroid hormone synthesis,[6] anti-thyroid drugs,[6] and genetic changes,[7,8] but no critical events that may disrupt the intracellular signaling systems in thyroid cells have been reported. Since the proliferation and function of thyroid follicular cells and parafollicular cells depend on different signaling mechanisms,[9] there may be differences in the genetic events involved in their neoplastic transformation.

In this study, we investigated whether thyroid tumors which developed from thyroid follicular cells or parafollicular C-cells showed common genetic changes or any predisposition for *ras* and G-protein gene mutations. Ras and G-proteins serve as molecular switches coupling to many cell-surface receptors for transduction of extracellular signals to intracellular signaling systems.[10] Mutated forms of Ras and G-proteins have been reported in some endocrine tumors and in several pathological states involving impaired intracellular signaling.[11,12]

Therefore, we studied 43 thyroid tumors to analyze the genetic changes in *Gsα*, *Gi2α*, H-*ras*, K-*ras* and N-*ras* genes, using direct sequencing of DNA after amplification from tumor specimens by use of the polymerase chain reaction (PCR).

MATERIALS AND METHODS

Patients and thyroid tissues Human thyroid tumors were obtained by surgery at the University of Tokushima Hospital and the Tokushima Municipal Hospital with adjacent normal tissues or peripheral blood leukocytes, and stored at −80°C. The thyroid tumors included 5 adenomatous goiters, 7 follicular adenomas, 22 papillary carcinomas and 9 medullary carcinomas.

DNA extraction and PCR High-molecular-weight DNA was extracted from tumor tissues and peripheral leukocytes by proteinase K digestion and phenol/chloroform extraction. Oligonucleotide primers were synthesized by the phosphoramidite method using a 392 DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). A set of PCR primers flanking exons 7 and 8 of the *Gsα* gene (total 13 exons), exons 5 and 6 of the *Gi2α* gene (total 8 exons), and exons 1 and 2 of H-*ras*, N-*ras*, and K-*ras* genes were used to generate a single specific amplification product from each sample. The nucleic acid sequence of each primer and conditions for PCR amplification are shown in Table I. The PCR reaction was performed in a 100 μl reaction mixture containing 10–20 ng of template DNA, a pair of primers, 60 μmol/liter of each deoxynucleotide triphosphate, 20 mmol/liter Tris-HCl buffer (pH 8.8), 1.5 mmol/liter $MgCl_2$, 25 mmol/liter KCl and 5 units of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). The template DNA was initially denatured at 95°C

[3] To whom requests for reprints should be addressed.

Table I. PCR Primers and Conditions

Gene	Exon	Primer set	pmol /tube	Annealing	Extension	Cycle	Size of PCR product (bp)
Gsα	8, 9	5′-GTGATCAAGCAGGCTGACTATGTG-3′ 5′-GCTGCTGGCCACCACGAAGATGAT-3′	36	55°C, 1 min	72°C, 1 min	40	541
Gi2α	5, 6	5′-CCCCCCATCCCCAGCTACCT-3′ 5′-TCTCACCATCTCCTCGTCCTC-3′	36	55°C, 1 min	72°C, 1 min	40	579
H-*ras*	1	5′-CAGGCCCCTGAGGAGCGATG-3′ 5′-TTCGTCCACAAAATGGTTCT-3′	90	60°C, 1 min	72°C, 2 min	40	110
	2	5′-TCCTGCAGGATTCCTACCGG-3′ 5′-GGTTCACCTGTACTGGTGGA-3′	90	60°C, 1 min	72°C, 2 min	50	87
N-*ras*	1	5′-GACTGAGTACAAACTGGTGG-3′ 5′-GGGCCTCACCTCTATGGTG-3′	100	60°C, 1 min	72°C, 2 min	50	118
	2	5′-GGTGAAACCTGTTTGTTGGA-3′ 5′-ATACACAGAGGAAGCCTTCG-3′	100	60°C, 1 min	72°C, 2 min	50	103
K-*ras*	1	5′-GGCCTGCTGAAAATGACTGA-3′ 5′-GTCCTGCACCAGTAATATGC-3′	100	55°C, 1 min	72°C, 2 min	50	162
	2	5′-TTCCTACAGGAAGCAAGTAG-3′ 5′-CACAAAGAAAGCCCTCCCCA-3′	100	55°C, 1 min	72°C, 2 min	50	128

for 10 min, followed by successive cycles of annealing for 1 min, extension at 72°C for 2 min and denaturation at 95°C for 1 min in each PCR procedure.

Direct sequencing The nucleotide sequences of PCR-amplified fragments were analyzed as described previously.[13] Briefly, the PCR products were subjected to electrophoresis in 1.6% agarose gels, and the amplified products were excised and purified with a Gene Clean kit (Bio101, La Jolla, CA). The purified double-stranded PCR products (0.5 ng/ 26 μl) were sequenced with a dsDNA cycle sequencing system using 7-deaza-dGTP (Gibco BRL, Canada) according to the manufacturer's protocol.

RESULTS

Clinical history and data of patients All patients had not been irradiated, not received any drug treatment and were in a euthyroid state at surgery. Two papillary carcinoma patients were complicated with Basedow's disease and three papillary carcinoma patients with Hasimoto's disease. An adenomatous goiter patient was complicated with Basedow's disease. Nine medullary carcinomas consisted of a MEN 2A, five MEN 2B and three sporadic types.

Mutations of *Gsα* and *Gi2α* genes Missense mutation of the *Gsα* gene was identified at codon 227, where CAG (glutamine) was mutated to CTG by an A-to-T transversion, in an adenomatous goiter which was not complicated with Basedow's disease and a follicular adenoma from patients in a clinically euthyroid state (Fig. 1A). The adenomatous goiter and the follicular adenoma which showed the mutation at codon 227 also showed a mutation at codon 231, CGC (arginine) to CTC (leucine), i.e., they exhibited double mutations of codons 227 and 231. Four papillary carcinomas which were not complicated with Basedow's or Hasimoto's disease showed mutation at codon 231, CGC (arginine) to CTC (leucine). One of these four papillary carcinomas showed an additional mutation at codon 239, AAC (asparagine)

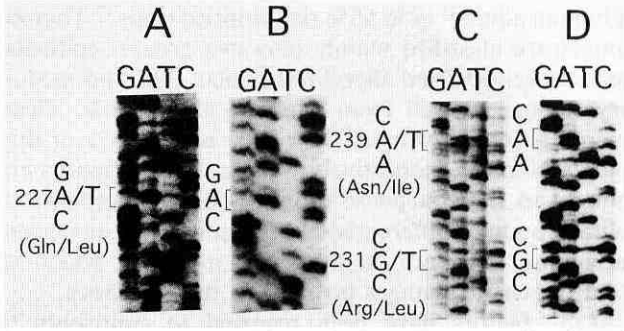

Fig. 1. Missense mutation of the *Gsα* gene in thyroid follicular neoplasms. PCR-amplified DNA fragments from the tumor tissue were purified and sequenced by the dideoxy termination method using Taq polymerase. A: Result of direct sequencing of a DNA fragment amplified from a follicular adenoma showing substitution of CTG for CAG at codon 227. B: Result of direct sequencing of a DNA fragment amplified from normal thyroid tissue of the follicular adenoma patient. C: Result of direct sequencing of a DNA fragment from a papillary carcinoma showing double mutations substituting CTC for CGC at codon 231 and ATC for AAC at codon 239. D: Result of direct sequencing of DNA fragment from normal thyroid tissue of the papillary carcinoma patient.

to ATC (isoleucine) (Fig. 1C). There has been no previous report of mutations at codons 231 and 239 of the *Gsα* gene. No mutation was identified at codon 201. The *Gi2α* gene, including codons 179 and 205, showed no mutation. The 9 medullary carcinomas showed no *Gsα* or *Gi2α* mutations.

Mutations of *ras* genes Missense mutations of K-*ras* gene were identified at codon 13; GGC (glycine) was converted to GCC (alanine) by a G-to-C transversion in an adenomatous goiter, a follicular adenoma and a papillary carcinoma (Fig. 2A). A neutral mutation substituting GGC for GGA at codon 10 was identified in 2 follicular adenomas and 4 papillary carcinomas (Fig. 2B). Since DNAs from non-tumor thyroid tissues and peripheral leukocytes showed similar mutations, this mutation is thought to be a polymorphism. There was no mutation of H-*ras* or N-*ras* genes, including codons 12, 13 and 61, in our series. Nine medullary carcinomas showed no missense mutations of the *ras* genes except a neutral mutation of K-*ras* substituting GAT for GAC at codon 30 (Fig. 2C).

Table II summarizes the incidence of missense mutations of G protein gene and *ras* genes in thyroid tumors.

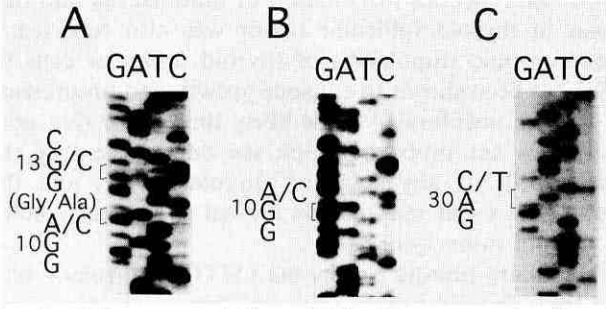

Fig. 2. Missense mutation of the K-*ras* gene in thyroid follicular neoplasms. PCR-amplified DNA fragments from tumor tissues were purified and sequenced by the dideoxy termination method using Taq polymerase. A: Result of direct sequencing of a DNA fragment amplified from a follicular adenoma showing missense mutation at codon 13 substituting GCC for GGC and a neutral mutation at codon 10 substituting GGC for GGA. B: Result of direct sequencing of a DNA fragment from a follicular adenoma showing the neutral mutation at codon 10 only. C: Result of direct sequencing of a DNA fragment from a medullary carcinoma showing a neutral mutation at codon 30 substituting GAT for GAC.

DISCUSSION

Growth and differentiation of thyroid cells depend on many factors which interact with cell surface receptors to activate intracellular signal transduction pathways. The *ras* gene product (c-Ras) mediates signal transduction from growth factor binding to receptor tyrosine kinases to the mitogen-activated protein (MAP) kinases,[14] and heterotrimeric G-proteins transduce the signal of TSH from seven-transmembrane domain receptors to adenylate cyclase. The $\beta\gamma$-subunit of heterotrimeric G proteins activates MAP kinase in a Ras-dependent manner[15] and Ras and heterotrimeric G-proteins interact cooperatively in the transmission of the proliferative signal from the cytoplasmic membrane to the nucleus. Thus, we analyzed the DNA of various thyroid tumors for mutations of representative G-protein genes, H-*ras*, K-*ras*, N-*ras*, *Gsα* and *Gi2α*, simultaneously. Mutations of Arg^{201} and Gln^{227} of Gs-protein are known to reduce intrinsic GTPase activity and to cause constitutive activation of adenylate cyclase, leading to tumorigenesis of some endocrine tumors.[12] It is also known that Arg^{179} and Gln^{205} of Gi2-protein are equivalent to Arg^{201} and Gln^{227} of Gs-protein. Since exon 7 of *Gsα* gene contains codon 201, exon 8 of *Gsα* gene codon 227, exon 5 of *Gi2α* gene codon 179 and exon 6 of *Gi2α* gene codon 205, we analyzed exons 7 and 8 of the *Gsα* gene and exons 5 and 6 of the *Gi2α* gene.

There have been many studies on genetic changes in thyroid neoplasia, including chromosomal abnormalities[16] and mutations of *ras*,[17–21] *Gsα*,[22–25] *p53*,[26] *ptc/ret*[27] and *trk*[28] genes. For example, rearrangement of the *ret* proto-oncogene in 19% of papillary thyroid carcinomas,[27] mutations at the cysteine-rich extracellular domain of the *ret* proto-oncogene in 97% of multiple

Table II. Missense Mutations of G-Protein Genes in Thyroid Tumors

Gene	Codon	Mutation	Adenomatous goiter	Follicular adenoma	Papillary carcinoma	Medullary carcinoma
Gsα	227	CAG→CTG	1/5[a]	1/7[a]	0/22	0/9
	231	CGC→CTC	1/5[a]	1/7[a]	4/22[b]	0/9
	239	AAC→ATC	0/5	0/7	1/22[b]	0/9
K-*ras*	13	GGC→GCC	1/5	1/7	1/22	0/9

a) An adenomatous goiter and a follicular adenoma showed double mutations at codons 227 and 231 of the *Gsα* gene.
b) A papillary carcinoma showed double mutations at codons 231 and 239 of the *Gsα* gene.

endocrine neoplasia (MEN) 2A and 86% of familial medullary thyroid carcinoma[29] and mutations at the tyrosine kinase domain of the *ret* proto-oncogene in 100% of MEN 2B and 33% of sporadic medullary thyroid carcinoma[30] have been reported. However, consistent data have not been reported on the incidences of these genetic changes. Most previous reports have utilized indirect methods such as PCR-dot blot hybridization using allele-specific oligoprobes or PCR-single strand conformation polymorphism (PCR-SSCP) methods for the detection of mutated genes. However, these indirect methods of DNA sequence analysis may afford false-positive or false-negative results. For example, we found a *Gsα* gene mutation of the pituitary adenoma which could not be detected by PCR-SSCP.[31] In this study, therefore, we analyzed the nucleotide sequences of all samples from follicular and parafollicular neoplasms of the thyroid directly using a PCR-direct sequencing method. This is the first report of oncogene analysis of thyroid tumors using this direct sequencing method without prior screening.

We found missense mutations of *ras* genes and the *Gsα* gene in 3/34 (9%) and 6/34 (18%), respectively, of the follicular tumors (Table II). On the other hand, medullary thyroid carcinoma showed no missense mutation of *ras* or G-protein genes, and only a neutral mutation in codon 30 of the K-*ras* gene was detected. Although we did not have the opportunity to examine any anaplastic carcinomas or functioning adenomas of the thyroid, in which very high incidences of *ras* and *Gsα* gene mutations have been reported, respectively, our data on the incidence of mutated *ras* gene in follicular neoplasms do not differ greatly from previous reports.[19–21] With regard to the *Gsα* gene, we found the incidence of mutation to be several-fold higher as compared with previous reports[23–25]; we detected a missense mutation in codon 231 of CGC (coding for the polar amino acid arginine) to CTC (non-polar leucine) in 4/22 (18%) papillary carcinomas. This region does not contain the GTP binding site or the intrinsic GTP activity site, but is well conserved in the G-protein family, indicating functional importance of this region. As for mutation in codon 239, the polar amino acid Asn is replaced by non-polar Ile. Therefore, these mutations may reduce intrinsic GTPase activity and contribute to tumorigenesis of the thyroid follicular cells. We also found double mutations of codons 227 and 231 in an adenomatous goiter and a follicular adenoma. The Gln^{227} of Gs-protein is equivalent to Gln^{61} of $p21^{ras}$, a known site of oncogenic mutation in *ras*. Point mutations in codon 227 of the *Gsα* gene reduce intrinsic GTP activity and probably contribute to the tumorigenesis. Although the functional consequences of these mutations should be determined by expression analysis, our results showed the superiority of the PCR-direct sequencing methods to dot-blot or primer-introduced restriction analysis, which cannot detect novel mutations.

Several authors have tried to correlate the presence of a mutated gene with clinical parameters, namely mutated *ras* and metastatic capability,[32] or mutated *Gsα* and hypersecretion of thyroid hormone.[12, 22] Others have suggested that *ras* or *Gsα* gene mutations play no major role in the development or progression of thyroid neoplasms because of their low frequency.[20, 25] Both of these viewpoints depend on data from rather small numbers of cases using indirect screening methods. As the prevalence of oncogene mutation may vary with various factors, such as genetic predisposition to mutation, environmental exposure to radiation, dietary supply of iodine, serum levels of TSH and exposure to other mutagenic environmental or infectious agents, care is needed in interpreting the results. Moreover, thyroid adenoma and carcinoma were reported to be monoclonal in origin,[33] but a heterogenous distribution of mutated *ras* and *Gsα* genes in thyroid follicular tumor was also reported.[34] Since chronic stimulation of thyroid follicular cells by TSH has been shown to enhance growth and progression of thyroid neoplasms,[35] it is likely that *ras* or *Gsα* gene mutations act supportively on the development of the tumor cells at any stage of thyroid tumor, and the mutational event may not be limited to a specific stage of thyroid tumorigenesis.

Medullary thyroid carcinoma (MTC) is a tumor arising from thyroid parafollicular C-cells originating from neural crest cells, and has distinctive biochemical and genetic features.[3, 36] It occurs sporadically, familially, or as a component of MEN type 2. Despite extensive genetic analysis, MTC showed no genetic changes in *ras, myc* or *p53* genes,[37–39] such as are found commonly in human neoplasms. Our results also showed the absence of activating mutations of *ras* and *Gsα* genes in MTC. These results suggest two possibilities; parafollicular cells may be protected from mutational events to a greater extent than follicular cells, or activation of *ras* and *Gsα* genes may be beneficial to the tumorigenic process in follicular cells but not in parafollicular cells.

In this respect, the effects of transfection of TT cells, a cell line derived from a sporadic MTC patient, with the v-*ras* gene are very interesting. Expression of the v-*ras* gene in TT cells resulted in their dramatic differentiation, characterized by slowing of cell growth, increased transcription of the calcitonin gene, and the appearance of abundant mature cytoplasmic neurosecretory granules.[40] These events are associated with stimulation of the protein kinase C (PKC) pathway and increased expression of the nuclear oncogene c-*jun*.[36] As activation of the PKC pathway and expression of c-*jun* were also observed in fibroblasts transformed by v-*ras*, this complete discrep-

ancy of cell response to v-*ras* between TT cells and fibroblasts was thought to be due to differences at a more distal point of the signal transduction pathway.[36] These findings suggest that the Ras-dependent signaling pathway transmits the signal for differentiation but not for proliferation in thyroid parafollicular cells. Therefore, the absence of G-protein mutation in MTC may be due to failure of tumor development in the parafollicular cells harboring activated G-protein genes. This suggests the presence of cell type-specific regulatory mechanisms of proliferation and differentiation between follicular cells and parafollicular cells. The underlying molecular mechanisms of these differences should be studied further.

In conclusion, PCR-direct sequencing analysis showed missense mutations of *Gsα* and *ras* genes in 6 of 34 (18%) and in 3 of 34 (9%) thyroid follicular neoplasms, respectively. On the other hand, 9 MTC did not show any missense mutation of G-protein genes. Our results suggest that the presence of activated *Gsα* and *ras* genes may be beneficial to the development of thyroid follicular tumor, but not MTC.

(Received February 27, 1995/Accepted May 25, 1995)

REFERENCES

1) Rojeski, M. T. and Gharib, H. Nodular thyroid disease. Evaluation and management. *N. Engl. J. Med.*, **313**, 428–436 (1985).
2) Mortensen, J. D., Woolner, L. B. and Bennett, W. A. Gross and microscopic findings in clinically normal thyroid glands. *J. Clin. Endocrinol. Metab.*, **15**, 1270–1280 (1955).
3) Uribe, M., Grimes, M., Fenoglio-Preiser, C. M. and Feind, C. Medullary carcinoma of the thyroid gland. Clinical, pathological, and immunohistochemical features with review of the literature. *Am. J. Surg. Pathol.*, **9**, 577–594 (1985).
4) Parker, L. N., Belsky, J. L., Yamamoto, T., Kawamoto, S. and Keehn, R. J. Hiroshima shi, Japan. Thyroid carcinoma after exposure to atomic radiation. A continuing survey of a fixed population, Hiroshima and Nagasaki, 1958–1971. *Ann. Intern. Med.*, **80**, 600–604 (1974).
5) Belfiore, A., La Rosa, G. L., Padova, G., Sava, L., Ippolito, O. and Vigneri, R. The frequency of cold thyroid nodules and thyroid malignancies in patients from an iodine-deficient area. *Cancer*, **60**, 3096–3102 (1987).
6) Thomas, G. A. and Williams, E. D. Evidence for and possible mechanisms of non-genotoxic carcinogenesis in the rodent thyroid. *Mutat. Res.*, **248**, 357–370 (1991).
7) Frauman, A. G. and Moses, A. C. Oncogenes and growth factors in thyroid carcinogenesis. *Endocrinol. Metab. Clin. North Am.*, **19**, 479–493 (1990).
8) Fagin, J. A. Genetic basis of endocrine disease 3 — molecular defects in thyroid gland neoplasia. *J. Clin. Endocrinol. Metab.*, **75**, 1398–1400 (1992).
9) Smith, P. and Wynford-Thomas, D. Control of thyroid follicular cell proliferation — cellular aspects. *In* "Thyroid Tumors: Molecular Basis of Pathogenesis," ed. D. Wynford-Thomas and E. D. Williams, pp. 66–90 (1984). Churchill Livingstone, New York.
10) Johnson, G. L. and Dhanasekaran, N. The G-protein family and their interaction with receptors. *Endocr. Rev.*, **10**, 317–331 (1989).
11) Maenhaut, C., Roger, P. P., Reuse, S. and Dumont, J. E. Activation of the cyclic AMP cascade as an oncogenic mechanism: the thyroid example. *Biochimie*, **73**, 29–36 (1991).
12) Lyons, J., Landis, C. A., Harsh, G., Vallar, L., Grünewald, K., Feichtinger, H., Duh, Q.-Y., Clark, O. H., Kawasaki, E., Bourne, H. R. and McCormick, F. Two G protein oncogenes in human endocrine tumors. *Science*, **249**, 655–659 (1990).
13) Hosoi, E., Yokogoshi, Y., Hosoi, E., Horie, H., Sano, T., Yamada, S. and Saito, S. Analysis of the Gs α gene in growth hormone-secreting pituitary adenomas by the polymerase chain reaction-direct sequencing method using paraffin-embedded tissues. *Acta Endocrinol.*, **129**, 301–306 (1993).
14) Blenis, J. Signal transduction via the MAP kinases: proceed at your own RSK. *Proc. Natl. Acad. Sci. USA*, **90**, 5889–5892 (1993).
15) Crespo, P., Xu, N., Simonds, W. F. and Gutkind, J. S. Ras-dependent activation of MAP kinase pathway mediated by G-protein $\beta\gamma$ subunits. *Nature*, **369**, 418–420 (1994).
16) Herrman, M. A., Hay, I. D., Bartelt, D. H., Jr., Ritland, S. R., Dahl, R. J., Grand, C. S. and Jenkins, R. B. Cytogenetic and molecular genetic studies of follicular and papillary thyroid cancers. *J. Clin. Invest.*, **88**, 1596–1604 (1991).
17) Lemoine, N. R., Mayall, E. S., Wyllie, F. S., Williams, E. D., Goyns, M., Stringer, B. and Wynford-Thomas, D. High frequency of *ras* oncogene activation in all stages of human thyroid tumorigenesis. *Oncogene*, **4**, 159–164 (1989).
18) Suarez, H. G., Villard, J. A., Severino, M., Caillou, B., Schlumberger, M., Tubiana, M., Parmentier, C. and Monier, R. Presence of mutations in all three *ras* genes in human thyroid tumors. *Oncogene*, **5**, 565–570 (1990).
19) Shi, Y., Zou, M., Schmidt, H., Juhasz, F., Stensky, V., Robb, D. and Farid, N. R. High rates of *ras* codon 61 mutation in thyroid tumors in an iodide-deficient area. *Cancer Res.*, **51**, 2690–2693 (1991).
20) Karga, H., Lee, J.-K., Vickery, A. L., Jr., Thor, A., Gaz, R. D. and Jameson, J. L. *ras* oncogene mutations in benign and malignant thyroid neoplasms. *J. Clin. Endocrinol. Metab.*, **73**, 832–836 (1991).

21) Yoshimoto, K., Iwahana, H., Fukuda, A., Sano, T., Katsuragi, K., Kinoshita, M., Saito, S. and Itakura, M. *ras* mutations in endocrine tumors: mutation detection by polymerase chain reaction-single strand conformation polymorphism. *Jpn. J. Cancer Res.*, **83**, 1057–1062 (1992).
22) O'Sullivan, C., Barton, C. M., Staddon, S. L., Brown, C. L. and Lemoine, N. R. Activating point mutations of the *gsp* oncogene in human thyroid adenomas. *Mol. Carcinog.*, **4**, 345–349 (1991).
23) Suarez, H. G., Villard, J. A., Caillou, B., Schlumberger, M., Parmentier, C. and Monier, R. *gsp* mutations in human thryoid tumors. *Oncogene*, **6**, 677–679 (1991).
24) Matsuo, K., Friedman, E., Gejman, P. V. and Fagin, J. A. The thyrotropin receptor (TSH-R) is not an oncogene for thyroid tumors: structural studies of the TSH-R and the α-subunit of Gs in human thyroid neoplasms. *J. Clin. Endocrinol. Metab.*, **76**, 1446–1451 (1993).
25) Yoshimoto, K., Iwahana, H., Fukuda, A., Sano, T. and Itakura, M. Rare mutations of the Gs alpha subunit gene in human endocrine tumors. *Cancer*, **72**, 1386–1393 (1993).
26) Fagin, J. A., Matsuo, K., Karmakar, A., Chen, D. L., Tang, S.-H. and Koeffler, H. P. High prevalence of mutations of the p53 gene in poorly differentiated human thyroid carcinomas. *J. Clin. Invest.*, **91**, 179–184 (1993).
27) Santoro, M., Carlomagno, F., Hay, I. D., Herrmann, M. A., Grieco, M., Melillo, R., Pierotti, M. A., Bongarzone, I., Porta, G. D., Berger, N., Peix, J. L., Paulin, C., Fabien, N., Vecchio, G., Jenkins, R. B. and Fusco, A. Ret oncogene activation in human thyroid neoplasms is restricted to the papillary cancer subtype. *J. Clin. Invest.*, **89**, 1517–1522 (1992).
28) Bongarzone, I., Pierotti, M. A., Monzini, N., Mondellini, P., Manenti, G., Donghi, R., Pilotti, S., Grieco, M., Santoro, M., Fusco, A., Vecchio, G. and Porta, G. D. High frequency activation of tyrosine kinase oncogenes in human papillary thyroid carcinoma. *Oncogene*, **4**, 1457–1462 (1989).
29) Mulligan, L. M., Eng, C., Healey, C. S., Clayton, D., Kwok, J. B. J., Gardner, E., Ponder, M. A., Frilling, A., Jackson, C. E., Lehnert, H., Neumann, H. P. H., Thibodeau, S. N. and Ponder, B. A. J. Specific mutations of the RET proto-oncogene are related to disease phenotype in MEN 2A and FMTC. *Nature Genet.*, **6**, 70–74 (1994).
30) Hofstra, R. M. W., Landsvater, R. M., Ceccherini, I., Stulp, R. P., Stelwagen, T., Luo, Y., Pasini, B., Höppener, J. W. M., van Amstel, H. K. P., Romeo, G., Lips, C. J. M. and Buys, C. H. C. M. Mutation in the RET proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. *Nature*, **367**, 375–376 (1994).
31) Hosoi, E., Yokogoshi, Y., Hosoi, E., Yokoi, K., Sano, T. and Saito, S. A pituitary specific point mutation of codon 201 of the Gsα gene in a pituitary adenoma of a patient with multiple endocrine neoplasia (MEN) type I. *Endocrinol. Jpn.*, **39**, 319–324 (1992).
32) Stringer, B. M., Rowson, J. M., Parkar, M. H., Seid, J. M., Hearn, P. R., Wynford-Thomas, D., Ingemansson, S., Woodhouse, N. and Goyns, M. H. Detection of the H-*ras* oncogene in human thyroid anaplastic carcinomas. *Experientia*, **45**, 372–376 (1989).
33) Namba, H., Matsuo, K. and Fagin, J. A. Clonal composition of benign and malignant human thyroid tumors. *J. Clin. Invest.*, **86**, 120–125 (1990).
34) Goretzki, P. E., Lyons, J., Stacy-Phipps, S., Rosenau, W., Demeure, M., Clark, O. H., McCormick, F., Röher, H.-D. and Bourne, H. R. Mutational activation of ras and gsp oncogenes in differentiated thyroid cancer and their biological implications. *World J. Surg.*, **16**, 576–582 (1992).
35) Edmonds, C. J. and Tellez, M. Hyperthyroidism and thyroid cancer. *Clin. Endocrinol.*, **28**, 253–259 (1988).
36) Nelkin, B. D., de Bustros, A. C., Mabry, M. and Baylin, S. B. The molecular biology of medullary thyroid carcinoma. A model for cancer development and progression. *J. Am . Med. Assoc.*, **261**, 3130–3135 (1989).
37) Yang, K.-P., Castillo, S. G., Nguyen, C. V., Hickey, R. C. and Samaan, N. A. c-*myc*, N-*myc*, N-*ras* and c-*erb*-B: lack of amplification or rearrangement in human medullary thyroid carcinoma and a derivative cell line. *Anticancer Res.*, **10**, 189–192 (1990).
38) Moley, J. F., Brother, M. B., Wells, S. A., Spengler, B. A., Biedler, J. L. and Brodeur, G. M. Low frequency of *ras* gene mutations in neuroblastomas, pheochromocytomas, and medullary thyroid cancers. *Cancer Res.*, **51**, 1596–1599 (1991).
39) Yana, I., Nakamura, T., Shin, E., Karakawa, K., Kurahashi, H., Kurita, Y., Kobayashi, T., Mori, T., Nishisho, I. and Takai, S. Inactivation of the p53 gene is not required for tumorigenesis of medullary thyroid carcinoma or pheochromocytoma. *Jpn. J. Cancer Res.*, **83**, 1113–1116 (1992).
40) Nakagawa, T., Mabry, M., de Bustros, A., Ihle, J. N., Nelkin, B. D. and Baylin, S. B. Introduction of v-Ha-*ras* oncogene induces differentiation of cultured human medullary thyroid carcinoma cells. *Proc. Natl. Acad. Sci. USA*, **84**, 5923–5927 (1987).