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High Prevalence of BRAF Gene Mutation in Papillary Thyroid Carcinomas and Thyroid Tumor Cell Lines[1]

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ABSTRACT

The RAS-RAF-MEK-ERK-MAP kinase pathway mediates the cellular response to extracellular signals that regulate cell proliferation, differentiation, and apoptosis. Mutation of the *RAS* proto-oncogene occurs in various thyroid neoplasms such as papillary thyroid carcinomas (PTCs), follicular thyroid adenomas and carcinomas. A second genetic alteration frequently involved in PTC is *RET/PTC* rearrangements. Recent studies have shown that *BRAF*, which is a downstream signaling molecule of RET and RAS, is frequently mutated in melanomas. This study tests whether *BRAF* is also mutated in thyroid tumors and cell lines. We analyzed *BRAF* gene mutation at codon 599 in thyroid tumors using mutant-allele-specific PCR and in 10 thyroid tumor cell lines by DNA sequencing of the PCR-amplified exon 15. We found that *BRAF* was mutated in 8 of 10 thyroid tumor cell lines, including 2 of 2 papillary carcinoma cell lines, 4 of 5 anaplastic carcinoma cell lines, 1 of 2 follicular carcinoma cell lines, and 1 follicular adenoma cell line. *BRAF* mutation at codon 599 was detected in 21 of 56 PTC (38%) but not in 18 follicular adenomas and 6 goiters. *BRAF* mutation occurred in PTC at a significantly higher frequency in male patients than in female patients. To test whether *BRAF* mutation may cooperate with *RET/PTC* rearrangements in the oncogenesis of PTC, we tested whether *BRAF*-mutated PTCs were also positive for *RET/PTC* rearrangements. Immunohistochemical staining was conducted to evaluate *RET/PTC* rearrangements by using two different anti-RET antibodies. Surprisingly, we found that a large number of *BRAF*-mutated PTCs (8 of 21) also expressed RET, indicating that the *RET* proto-oncogene is rearranged in these *BRAF*-mutated PTCs. These observations suggest that mutated *BRAF* gene may cooperate with *RET/PTC* to induce the oncogenesis of PTC.

INTRODUCTION

Thyroid cancer is the most common endocrine neoplasm in the United States. Tumors derived from thyroid epithelial cells display diverse neoplastic phenotypes, including benign follicular adenomas, well-differentiated papillary and follicular carcinomas, and aggressive anaplastic carcinomas (1). Both genetic and epigenetic alterations are involved in the initiation and progression of thyroid carcinomas. Mutation of the thyroid hormone receptor gene (2) or *GSP* (3–5) and all three *RAS* proto-oncogenes (*KRAS*, *HRAS*, and *NRAS*) are found in both benign and malignant thyroid neoplasms (6–18). Mutation and activation of these genes play an important role in the early steps of thyroid tumor progression (19). In contrast, rearrangement and point mutation of the protein tyrosine kinase receptors, *RET* and *TRK*, are not found in benign follicular adenomas but are restricted to PTCs[3] (19–24). *TP53* tumor suppressor gene mutations are not present in benign adenomas nor in papillary carcinomas but are present in poorly differentiated and anaplastic carcinomas (25–28). Epigenetic alterations of tumor suppressor genes *P15INK4B* and *P16INK4A* through aberrant DNA methylation that silences gene expression is common in primary thyroid tumors and is an early event in thyroid tumorigenesis (29). *p27*, a potential tumor suppressor gene that belongs to the Kip/Cip cyclin-dependent kinase inhibitor family, is not mutated in thyroid tumors, but its expression is retained in the cytoplasm, which eliminates its effect in blocking cell cycle progression (30).

Davies *et al.* (31) recently reported that somatic missense *BRAF* mutation occurs in 66% of malignant melanomas and at low frequencies in many human cancers, including ~11% of colon cancers, 14% of borderline ovarian cancers, and 0.5% of sarcomas. Mutations are restricted to the kinase domain of the *BRAF* gene at multiple sites located in exons 11 and 15. However, >80% of these mutations are attributable to a single-base thymidine substitution at the nucleotide position 1796 with adenosine, which leads to the conversion of valine at codon 599 to glutamic acid. These investigators did not find *BRAF* mutation in 33 breast, 23 prostate, 14 lung, and 19 head and neck squamous cell carcinomas, and 15 gliomas. It appears that *BRAF* mutations tend to occur in tumors that harbor *RAS* mutations at a higher frequency. Because *RAS* oncogenes are mutated in ~30% of spontaneous thyroid tumors (6, 32), it is possible that *BRAF* may be mutated in thyroid neoplasms at high frequency too. Here, we report that *BRAF* mutation at V599E occurred in 21 of 56 papillary thyroid carcinomas (38%). However, *BRAF* mutation at this site was not detected in any of 18 FTAs examined. These observations suggest that *BRAF* mutation may be involved in the oncogenesis of papillary thyroid carcinomas but not involved in the formation of benign FTAs.

MATERIALS AND METHODS

Tumor Specimens and Patient Information. Paraffin-embedded tumor blocks from thyroidectomy specimens of patients with thyroid neoplasms were retrieved for analysis of *BRAF* gene mutation upon approval by the Institutional Review Board of Rush Presbyterian St. Luke's Medical Center. A total of 80 specimens, all with adequate clinical and pathological information, was studied. These included 56 papillary carcinomas, 18 follicular adenomas, and 6 benign nodular goiters. The presence of metastases was determined by reviewing the patients' medical records, pathology reports, and subsequent clinical courses. Patients were staged using the tumor-node-metastasis system and classified according to the presence of extrathyroidal extension, cervical nodes, and distant metastases. Nontumor tissue blocks (lymph nodes, parathyroid, and thyroid) from 20 patients with *BRAF*-mutated PTC were sectioned and used for genomic DNA extraction followed by PCR analysis of *BRAF* mutation.

Cell Lines. Ten thyroid tumor cell lines were used in this study (Table 1). One follicular adenoma (KAK-1), 1 papillary carcinoma (KAT-10), and 3 anaplastic (KAT-4, KAT-18, and SW1736) carcinoma cell lines were originally established in Dr. Ain's laboratory and have been described previously (33). The other 5 thyroid tumor cell lines, including 1 papillary (NPA87), 2 follicular (WRO82 and MRO87), and 2 anaplastic carcinomas (ARO81 and DRO90), were kindly provided by Dr. Guy J. F. Juillard at University of California at Los Angeles. All thyroid tumor cell lines were grown in complete RPMI 1640 containing 10% fetal bovine serum.

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[3] The abbreviations used are: PTC, papillary thyroid carcinoma; FTA, follicular thyroid adenoma; IHC, immunohistochemistry; MASA, mutant-allele-specific amplification; mAb, monoclonal antibody.

Table 1 *BRAF gene mutation in a panel of thyroid tumor cell lines*

Cell line	Tumor type	Passages	Metastasis	*BRAF* mutation
KAK-1	FTA	50	No	Yes
KAT-4	ATC[a]	16	Yes	Yes
KAT-18	ATC	35	Yes	No
SW1736	ATC	>9	Yes	Yes
ARO81	ATC	65	Yes	Yes[b]
DRO90	ATC	35	Yes	Yes[b]
MRO87	FTC	46	Unknown	Yes
WRO82	FTC	67	Yes	No
KAT-10	PTC	36	Yes	Yes
NPA87	PTC	44	Unknown	Yes

[a] ATC, anaplastic thyroid carcinoma; FTC, follicular thyroid carcinoma; NPA87, a poorly differentiated PTC cell line.
[b] Homozygous/hemizygous *BRAF* mutation.

Genomic DNA Extraction and PCR Amplification of Exon 15 of the BRAF Gene and Exons 1 and 2 of the *NRAS* Gene. Genomic DNA from paraffin-embedded tissues were extracted using a standard protocol. Briefly, one set of slides was stained with H&E and examined microscopically by a pathologist with expertise in thyroid disease. Tumor areas were marked and matched with a dewaxed but unstained set of slides. Tumor areas were dissected from unstained slides and transferred into an Eppendorf tube followed by proteinase K digestion at 56°C overnight and DNA extraction with phenol. Genomic DNA was precipitated with ethanol in the presence of glycogen. Genomic DNA extraction from thyroid tumor cell lines and blood of one patient was conducted by using Qiagen genomic DNA extraction kit following the manufacturer's instructions. DNA concentration was quantitated by A^{260} absorbance in a spectrophotometer. Exon 15 of the *BRAF* gene was amplified with two primers in the introns flanking it. The sequence of the forward primer is 5′-TCATAATGCTTGCTCTGATAGGA-3′, the sequence of the reverse primer is 5′-GGCCAAAATTTAATCAGTGGA-3′. Genomic DNA (100 ng/sample) was used as the template to amplify exon 15. The PCR reaction was set with an initial denaturation of 2 min at 94°C and subsequent denaturation for 30 s at 94°C, annealing for 45 s at 55°C, and extension for 45 s at 72°C. Thirty-five cycles were used to apply the PCR product with the expected size of 215 bp. Exon 1 of the *NRAS* gene was similarly amplified with a forward primer (5′-GACTGAGTACAAACTGGTGG-3′) and a reverse primer (5′-GGGCCTCACCTCTATGGTG-3′); exon 2 of the *NRAS* gene was amplified with a forward primer (5′-GGTGAAACCTGTTTGTTGGA-3′) and a reverse primer (5′-ATACACAGAGGAAGCCTTCG-3′). *BRAF* and *NRAS* mutations were detected by direct sequencing of the PCR products at the CRC-DNA sequencing facility of the University of Chicago.

MASA. MASA-PCR was conducted in principle as previously described (34, 35). A forward primer flanking the sequence of exon 15 (5′-TAGGTGATTTTGGTCTAGCTACAGT-3′) was used as a positive control to amplify wild-type as well as mutant *BRAF*. A second primer with substitution of two bases at the 5′-end (GGTGATTTTGGTCTAGCTACAAA-3′) was designed to amplify mutant *BRAF* gene only. The sequence of the reverse primer is 5′-GGCCAAAATTTAATCAGTGGA-3′. The PCR reaction was set with an initial denaturation of 2 min at 94°C and subsequent denaturation for 30 s at 94°C, annealing for 45 s at 52°C, and extension for 45 s at 72°C. Thirty-five cycles were used to amply the PCR product with the expected size of 129 bp. All negative samples were reexamined for *BRAF* mutation by PCR under the same condition but with 40 cycles. To confirm *BRAF* mutation detected by MASA, 9 *BRAF*-mutated DNA samples were randomly selected among 21 MASA-positive samples and used as the templates to amplify exon 15 of the *BRAF* gene. PCR products were extracted and sequenced.

RET/PTC Rearrangement Analyzed by IHC Staining. Wild-type RET is not expressed in normal follicular thyroid epithelial cells nor in papillary thyroid carcinomas, whereas the rearranged *RET/PTC* driven by a promoter of its fusion partner is readily expressed and can be detected by IHC staining with the antibodies against the COOH terminus of RET. Several recent studies have shown that *RET/PTC* rearrangements detected by IHC staining are consistent with that identified by Southern blot or by reverse transcription-PCR analysis followed by Southern hybridization (36–38). To test whether *RET/PTC* rearrangements take place in *BRAF*-mutated PTC, we conducted IHC to analyze RET/PTC expression in 56 PTC specimens to determine whether *BRAF* mutation was overlapping with *RET/PTC* rearrangements in some PTC specimens. Tissue sections were dewaxed with xylene and rehydrated. Slides were then heat inactivated in 10 mM sodium citrate (pH 6.0) in a microwave for 3 min. Cooled slides were rinsed with PBS and then incubated with 1% H_2O_2 in methanol for 30 min at room temperature. Sections were then blocked with 5% normal goat serum in PBS for 30 min at room temperature followed by 1 h incubation with an anti-RET rabbit serum (Santa Cruz Biotechnology, Santa Cruz, CA) (1:300 dilution) in PBS. Slides were washed and then incubated with goat antirabbit antibody-biotin conjugate (PharMingen, San Diego, CA) diluted at 1:300 in PBS/5% normal goat serum. Strepavidin-horseradish peroxidase conjugate (Zymed, San Francisco, CA) diluted at 1:200 in PBS with 5% normal goat serum was added and incubated for 45 min at room temperature. Color development was done with 3,3′-diaminobenzidine substrate (Sigma, St. Louis, MO). Slides were counterstained with Mayer's hemotoxylin for 2 min, dehydrated, and mounted. RET/PTC expression was graded by two investigators in a blinded fashion. The expression of RET/PTC in these specimens was additionally confirmed by IHC using a monoclonal antibody against the extreme COOH terminus of RET (1:40; Novocastra Laboratories Ltd., Burlingame, CA) with a Catalyzed Signal Amplification system (Dako Corp., Carpinteria, CA) following the manufacture's protocol.

Statistical Analysis. Correlation between HPR1 gene expression and the histological diagnosis or clinicopathological parameters of the thyroid neoplasms was determined by a χ^2 test.

RESULTS

BRAF Gene Mutation in Thyroid Tumor Cell Lines. We first analyzed *BRAF* gene mutation in 10 thyroid tumor cell lines by sequencing the PCR-amplified exon 15 of the *BRAF* gene. As shown in Fig. 1 and Table 1, 2 anaplastic thyroid carcinoma cell lines, ARO81 and DRO90, harbored a homozygous *BRAF* mutation at the nucleotide position 1796 where an adenosine was replaced by a thymidine, leading to the substitution of valine at 599 with glutamic acid. In the other 6 thyroid tumor cell lines (Table 1; 1 follicular adenoma, 2 anaplastic, 2 papillary, and 1 follicular carcinoma cell lines), only one allele of the *BRAF* gene was mutated at this site (Fig. 1 and Table 1). No mutation at T1796 was detected in an anaplastic cancer cell line (KAT18) and a follicular carcinoma cell line (WRO82; Fig. 1 and Table 1). No other point mutation within exon 15 was detected. Overall, our results show that 8 of 10 thyroid tumor cell lines originally established in two laboratories (Dr. Guy J. F. Juillard, University of California at Los Angeles; Dr. Kenneth B. Ain, University of Kentucky Medical Center) contained *BRAF* gene mutation at the T1796 nucleotide position. This suggests that *BRAF* may be mutated at high frequency in thyroid neoplasms.

Somatic BRAF Mutation in Thyroid Neoplasms. We then sought to analyze *BRAF* mutation in benign follicular adenomas and papillary thyroid carcinomas using PCR-based MASA to detect the hot-spot *BRAF* mutation at the T1796 site. A PCR primer with two mismatches at the 3′-end was used to amplify mutant *BRAF*, whereas a control primer derived from wild-type *BRAF* gene that amplifies both wild-type and T1796 mutant *BRAF* was used as a control. We first tested the feasibility of this method to detect *BRAF* mutation at T1796 site in well-characterized thyroid tumor cell lines. As shown in Fig. 1*B*, the *BRAF* exon 15 was amplified by using the mismatched primer in 4 *BRAF*-mutant thyroid tumor cell lines (DRO90 and ARO81 with homozygous mutation; NPA87 and MRO87 with heterozygous mutation) but not in 2 wild-type cell lines (WRO82 and KAT18). A PCR reaction that amplifies *BRAF* exon 15 was included as a positive control. *BRAF* exon 15 was amplified in all samples. These results suggest that screening of *BRAF* mutation using this MASA-PCR is specific, and this approach can be used to rapidly screen *BRAF* mutation at codon 599 in a large number of tumor samples.

We next analyzed *BRAF* mutation in microdissected thyroid tumor tissues by using MASA. As shown in Fig. 2*A*, *BRAF* mutation at

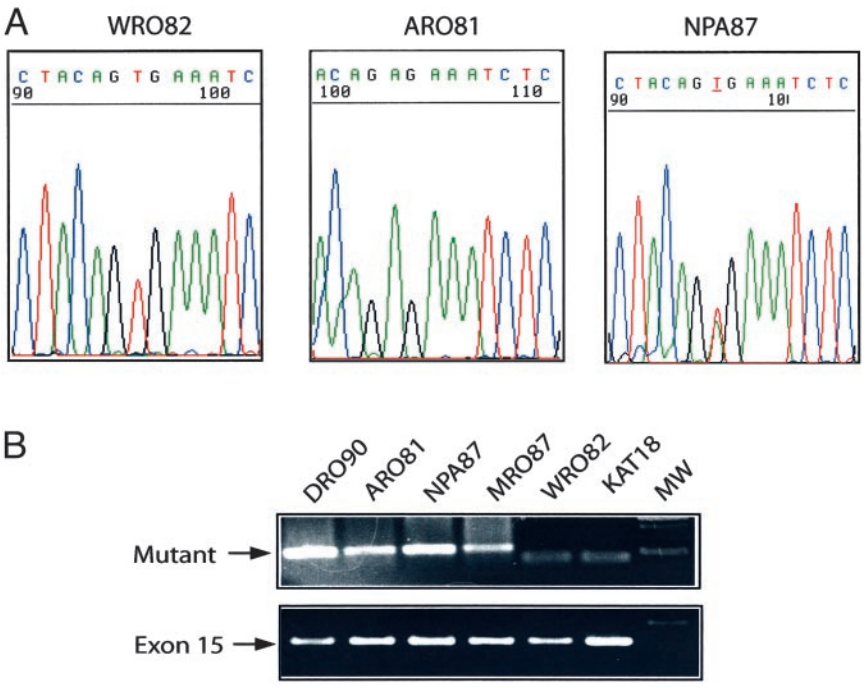

Fig. 1. *BRAF* gene mutation analysis in thyroid tumor cell lines. *A*, DNA sequencing. Genomic DNA from 10 thyroid tumor cell lines was extracted and used as a template to amplify a DNA fragment flanking exon 15. The amplified PCR product was sequenced with a forward primer to amplify exon 15 of the *BRAF* gene. Three sequences representing the wild-type *BRAF* gene (WRO81), homozygous/hemizygous *BRAF* mutation (ARO81), and heterozygous *BRAF* mutation (NPA87) are shown. *B*, MASA analysis of *BRAF* gene mutation in thyroid tumor cell lines. Genomic DNA samples of 6 representative thyroid tumor cell lines were used as templates in PCR reactions to specifically amplify mutant *BRAF* gene by using a mutant-specific primer. Exon 15 was amplified as a positive control by using two intron primers flanking it. The PCR conditions were 94°C, 2 min (94°C 30", 52°C 45"; 72°C 45") × 35 cycles; 72°C, 8 min. PCR products were analyzed in a 3% agarose gel and stained with ethidium bromide.

T1796 was detected in 21 of 56 papillary carcinomas but not detected in 18 benign follicular adenomas and 6 nodular goiters. All negative DNA samples were reanalyzed for *BRAF* mutation by PCR reaction with 40 cycles, no *BRAF* mutation was found. Nine of 21 positive samples were randomly selected for DNA sequencing to confirm *BRAF* mutation. All 9 samples were positive with *BRAF* mutation. Shown in Fig. 2*B* are three representative DNA sequences of *BRAF*-mutated samples, two of which had almost equal peak areas of A and T nucleotides, indicating a high homogeneity of tumor cells in these two microdissected sections. A third sample had a smaller A peak than T peak, reflecting heterogeneity of tumor cells and nontumor cells in the microdissected section. Nevertheless, these sequencing data additionally suggest that *BRAF* mutation detected by MASA is reliable and specific.

To test whether *BRAF* mutation in these PTC is somatically acquired, MASA-PCR was carried out to analyze *BRAF* mutation using the genomic DNA from normal tissues such as lymph nodes, parathyroid, or thyroid, which were separately embedded in paraffin tissue blocks. There was no *BRAF* mutation detected in all 20 normal tissues derived from patients with *BRAF*-mutated PTC (data not shown). One patient with *BRAF*-mutated PTC also developed melanoma at the time of thyroidectomy and thus was highly suspicious of a germ-line *BRAF* mutation. However, direct sequencing of PCR-amplified exon 15 of the *BRAF* gene derived from normal tissue confirmed that there was no germ-line *BRAF* mutation. In another patient with *BRAF*-mutated PTC, genomic DNA from peripheral blood was used in MASA-PCR to analyze for *BRAF* mutation. Again, no germ-line *BRAF* mutation was present in this patient. Thus, we concluded that *BRAF* mutation in all 21 PTC cases was somatic.

Higher Frequency of *BRAF* Mutation in Male Patients than in Female Patients with PTC. We analyzed whether *BRAF* mutation correlated with patient age, gender, and tumor stage. Interestingly, we found that *BRAF* mutation occurred in 9 of 14 male PTC patients and in 12 of 42 female patients (Table 2). Statistical analysis revealed that *BRAF* mutation occurred at a significantly higher frequency in male patients than in female patients ($P < 0.05$). In addition, we found that *BRAF* mutation occurred in 15 of 33 PTC patients older than 40 years old and in only 6 of 23 PTC patients younger than 40 years old. *BRAF* mutation in patients with PTC tended to occur at a higher frequency in older patients than in younger patients, but this was not a significant difference ($P > 0.05$). We did not find that *BRAF* mutation was associated with tumor volume (Table 2). *BRAF* was mutated at a higher rate in PTC with invasive and metastatic potential (Table 2) than in the noninvasive tumors, but again, this was not statistically significant ($P > 0.05$).

Overlapping of *BRAF* Mutation with other Genetic Alterations in PTC. *RET/PTC* rearrangements frequently occur in papillary thyroid carcinomas. To test whether *BRAF* mutation concurs with *RET/PTC* rearrangements, we conducted IHC to determine the status of *RET/PTC* rearrangement. IHC analysis using an anti-RET rabbit serum revealed that RET/PTC expression was abundantly present in the cytoplasm of the tumor cells in a *BRAF*-mutated specimen (Fig. 3*A*, *inset*) and a PTC with wild-type *BRAF* but not in the neighboring normal thyroid follicular cells (Fig. 3*A*). Similar results were obtained when an anti-RET mAb was used (Fig. 3, *C* and *D*). Normal mouse IgG and normal rabbit serum included as negative controls did not show any positive signal (data not shown). All samples analyzed for RET/PTC expression using anti-RET rabbit serum were additionally tested for RET/PTC expression by IHC with an anti-RET mAb. We found that RET/PTC expression was consistent in 85% of the specimens. The samples graded as having RET/PTC expression with either method were considered as RET/PTC positive.

We next analyzed whether *RET/PTC* rearrangements were overlapping with *BRAF* mutation in PTC. As shown in Table 3, we found that 21 of 56 PTCs (38%) were RET/PTC positive. Among them, 13 of 35 PTCs (37%) with wild-type *BRAF* were RET/PTC positive. Unexpectedly, we found that 8 of 21 *BRAF*-mutated samples (38%) were also RET/PTC positive, suggesting that *BRAF* mutation and *RET/PTC* rearrangements are overlapping in a large number of PTC.

All three *RAS* oncogenes (*NRAS*, *KRAS*, and *HRAS*) were equally mutated in thyroid neoplasms. In this study, we selectively analyzed *NRAS* mutation in *BRAF*-mutated PTC to determine whether *BRAF* and *NRAS* mutations were overlapping. Exons 1 and 2 of the *NRAS* gene were amplified individually, and the PCR products were directly

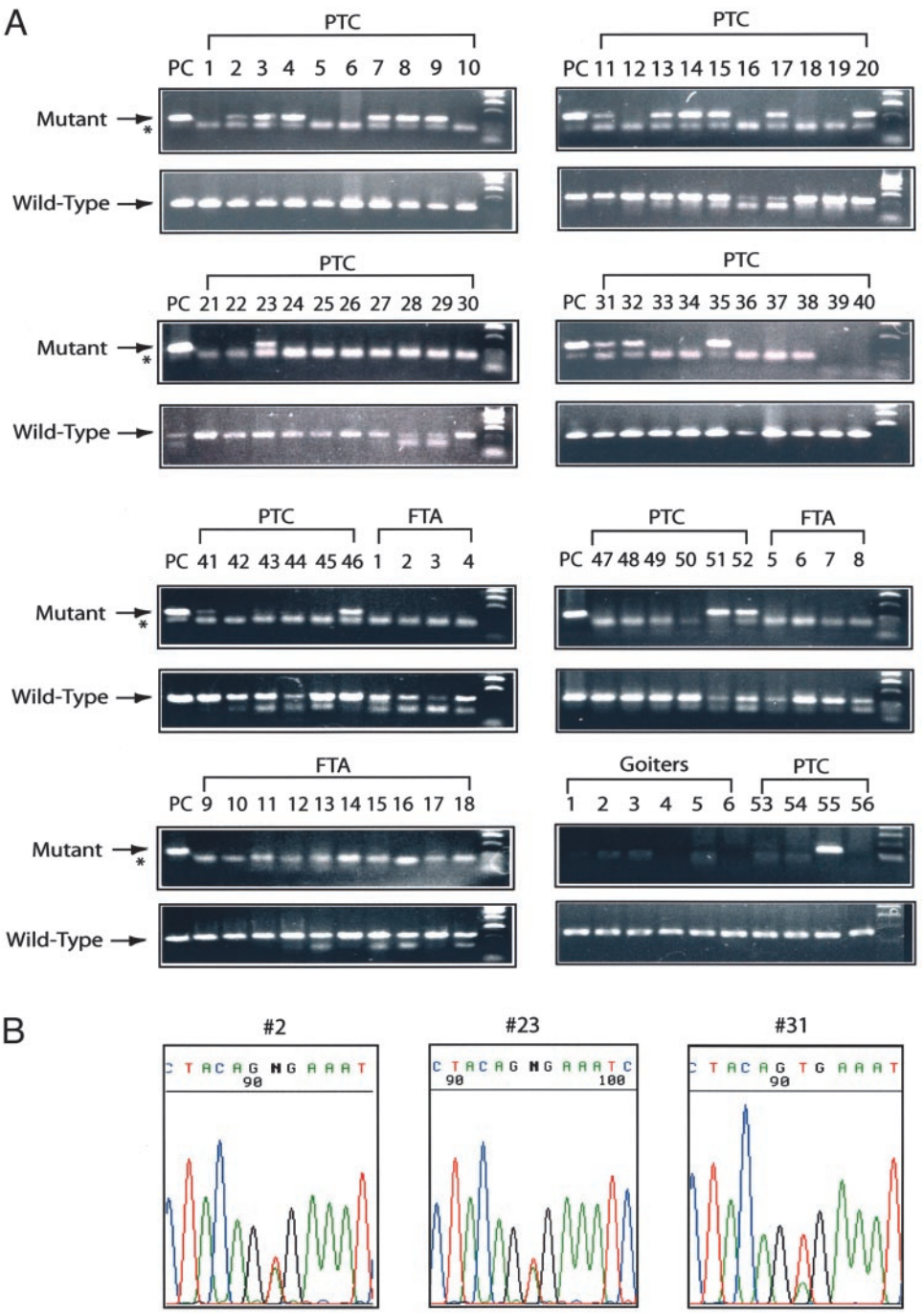

Fig. 2. *BRAF* mutation in thyroid tumors. *A*, MASA analysis of *BRAF* mutation. Genomic DNA was extracted from microdissected tumor sections and used as templates in PCR reactions with wild-type or mutant-specific primer as described in the "Materials and Methods." The PCR conditions were 94°C, 2 min, (94°C 30", 52°C 45"; 72°C 45") × 35 cycles; 72°C, 8 min. PCR products were analyzed in a 3% agarose gel and stained with ethidium bromide. Genomic DNA extracted from the DRO81 cell line was used as a positive control (PC). *, nonspecific amplification of a smaller DNA fragment. *B*, *BRAF* mutation confirmed by DNA sequencing. Exon 15 of the *BRAF* gene was PCR-amplified using genomic DNA as a template from 8 random PCR-positive samples shown in (*A*) and sequenced. Three representative DNA sequences are shown.

sequenced to monitor *NRAS* mutation at codons 12 and 13 in exon 1 and at codon 61 in exon 2. As shown in Table 3, we did not find any *NRAS* mutations in all 21 *BRAF*-mutated specimens, indicating that there is no overlapping in *NRAS* and *BRAF* mutations in these PTC. Because *KRAS* and *HRAS* mutations were not analyzed in this study, we could not exclude the possibility that *KRAS* and *HRAS* mutation might be overlapping with *BRAF* mutation. However, a recent study by Kimura *et al.* (39) demonstrated that 28 *BRAF*-mutated PTCs did not contain any form of mutated *RAS* genes.

DISCUSSION

Recent studies have demonstrated that *BRAF* mutation at codon 599 comprises ~80–90% of all mutations detected in several types of tumors, in particular in melanomas and colon cancers (31, 40). Several other sites in the kinase domain of BRAF in exons 11 and 15 can also be mutated but with much lower frequency (31, 40). Our present study using MASA demonstrated that *BRAF* mutation at the hot-spot codon 599 occurred in 21 of 56 papillary carcinomas (38%). While our manuscript was being revised, Kimura *et al.* (39) reported that *BRAF* mutation occurs in 28 of 78 PTCs (35.8%) and that all mutations are located at codon 599. Thus, these observations collectively suggest that the rate of *BRAF* mutation in PTC is the second highest to that in melanomas (>60%; Refs. 31, 41, 42) and is much higher than other cancers such as colorectal adenocarcinomas (5–10%; Refs. 31, 40, 43) and lung cancers (1.8%; Ref. 44).

Although our present study revealed that *BRAF* was mutated in 38% of PTC at codon 599, we did not find *BRAF* mutation at this site

Table 2 *BRAF gene mutation in thyroid neoplasms*

	No.	Positive (%)	*P*
Tumor type			
Follicular adenoma	18	0 (0%)	
Papillary carcinoma	56	21 (38%)	0.006
Tumor stage (PTC)			
T_1	10	4 (40%)	>0.05
T_2	35	13 (37%)	
T_3-T_4	11	4 (36%)	
Age (PTC)			
<40 years	23	6 (26%)	
>40 years	33	15 (46%)	0.233
Gender (PTC)			
Male	14	9 (64%)	
Female	42	12 (27%)	0.038
Node metastasis (PTC)			
Yes	13	7 (54%)	
No	43	14 (32%)	0.288

in any of 18 follicular adenomas studied. Statistical analysis revealed that *BRAF* was differentially mutated in these two different types of thyroid neoplasms (Table 1; $P = 0.006$). Consistent with this observation, Kimura *et al.* (39) reported that no *BRAF* mutation was detected in 14 FTAs, as well as in 10 follicular thyroid carcinoma. These observations are in sharp contrast to previous studies showing that the upstream activators of BRAF, the *RAS* genes (*HRAS*, *KRAS*, and *NRAS*), are mutated in both benign FTAs and papillary thyroid carcinomas (10, 45, 46). It is not clear why *BRAF* is only mutated in papillary thyroid carcinomas but not in FTAs, whereas *RAS* is mutated in both benign and malignant thyroid neoplasms. Nevertheless, a higher *BRAF* mutation rate in carcinomas than in adenomas was also observed in another type of tumor: Rajagopalan *et al.* (40) reported that *BRAF* is mutated in 30 of 276 clinical colon cancers (11%) but is mutated in only 2 of 54 colon adenomas (3.8%); Similarly, Yuen *et al.* (43) reported that *BRAF* is mutated in 11 of 215 colorectal adenocarcinomas (5.1%) and in 3 of 108 (2.8%) sporadic adenomas.

The *RET* proto-oncogene is rearranged in ~40% of spontaneous PTC when analyzed by IHC staining and/or reverse transcription-PCR followed by Southern hybridization (36–38) and with a much higher rate in radiation-associated PTC (56–84%) as well as in pediatric PTCs (70%; Ref. 47). A few prior studies demonstrated that combined *RET/PTC* rearrangements or *TSHR* and *RAS* mutation can occur in papillary thyroid carcinomas (18, 46, 48–50). For example, Bounacer *et al.* (48) reported that combined *RAS* mutation and *RET/PTC* rearrangements are detected in 2 of 28 radiation-associated thyroid tumors. In another study, Sugg *et al.* (50) analyzed genetic alterations in 20 PTCs and found one of them harboring both *NRAS* mutation and a *RET/PTC3* rearrangement. In this study, we tested whether *BRAF* mutation would occur together with *RET/PTC* rearrangements in PTC. To our surprise, IHC analysis revealed that a large number (8 of 21) of *BRAF*-mutated PTC-expressed RET, indicating that the *RET* proto-oncogene is rearranged in these tumors. This observation is totally unexpected not only because it contradicts recent studies showing that *BRAF* mutation at codon 599 does not overlap with *RET/PTC* rearrangements nor with *RAS* mutation (39) in PTC, as well as in other malignancies such as melanomas and colorectal adenocarcinomas (31, 40, 43) but also because two combined genetic alterations in the same signaling pathway appear to be redundant and therefore to be unnecessary. Nevertheless, based on our observation that 38% of the 21 *BRAF*-mutated PTCs also harbored *RET/PTC* rearrangements, we speculate that *RET/PTC* may cooperate with mutated *BRAF* to induce clinically overt PTC (24). In fact, both mutant *BRAF* gene and *RET/PTC* have a relatively low oncogenic potential. For example, the oncogenic potential of mutant *BRAF* gene is ~50-fold lower than V12 HRAS when tested in NIH3T3 cells (31), whereas *RET/PTC1* is unable to fully transform the rat thyroid PC Cl 3 cells (*e.g.*, unable to grow in soft agar and to develop tumorigenicity in athymic mice) but can cooperate with *RAS* oncogenes to fully transform PC Cl3 cells (24). It is possible that *BRAF* may cooperate with *RET/PTC* to fully transform thyroid epithelial cells, leading to the progression of occult

Table 3 *Overlapping of BRAF mutation with other genetic alterations in PTC*

BRAF mutation	No. of tumors	RET/PTC positive	NRAS mutation[a]
Yes	21	8 (38%)	0 (0%)
No	35	13 (37%)	ND[b]
Total	56	21 (38%)	ND[b]

[a] Exons 1 and 2 of the *NRAS* gene were PCR amplified individually and sequenced to detect NRAS mutation at codons 12, 13, and 61.
[b] ND, not done.

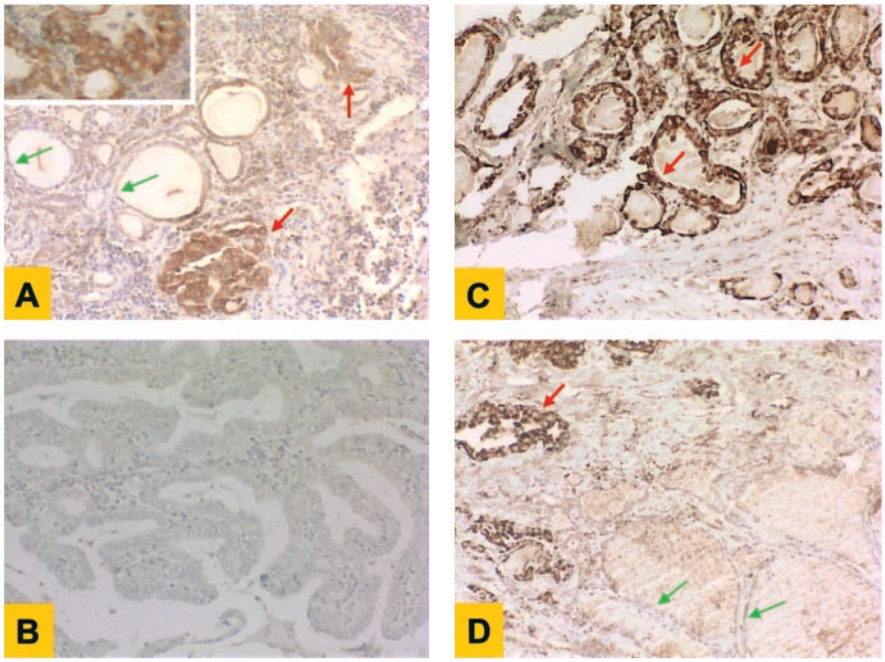

Fig. 3. IHC analysis of RET/PTC expression in PTC. *RET/PTC* rearrangements were analyzed for RET/PTC expression using IHC staining with anti-RET rabbit serum or anti-RET mAb as described in the "Materials and Methods" section. *A*, strong RET/PTC expression detected by IHC with an anti-RET rabbit antiserum in the cytoplasm of tumor cells (*red arrows*) but not in normal thyroid follicles (*green arrows*); *inset*, RET/PTC expression in a *BRAF*-mutated PTC (400× amplification); *B*, a RET/PTC-negative PTC showing no signal present in papillary carcinoma cells. *C* and *D*, strong RET/PTC expression in the cytoplasm and membrane of two PTC specimens detected by IHC with an anti-RET mAb. *D*, no signal present in the cytoplasm and membrane of normal follicular cells in a RET/PTC-positive PTC specimen. *Red arrows*, strong RET/PTC signal; *green arrows*, no signal present in normal thyroid follicles.

microcarcinomas, which have a very high rate of *RET/PTC* rearrangements (>70%), into clinically overt papillary thyroid carcinomas. Bearing in mind that concomitant *BRAF* mutation and *RET/PTC* rearrangements were not confirmed in a similar study recently published by Kimura *et al.* (39), this double-hit model should be taken with caution.

Detailed mutation analyses in a variety of tumors by Davies *et al.* (31) demonstrated that *BRAF* is generally mutated at a higher frequency in tumor cell lines than that in their corresponding tumor samples. For example, these investigators reported that *BRAF* is mutated in 7 of 40 colon cancer cell lines (18%), 5 of 59 sarcoma cell lines (9%), 4 of 38 glioma cell lines, and 4 of 131 lung cancer cell lines. In contrast, *BRAF* mutation is only detected in 4 of 33 colon cancers (12%), 1 of 182 sarcomas (0.5%), and none of the 15 gliomas and 14 lung cancers (31). Our present study demonstrated that *BRAF* was mutated overall in 8 of 10 thyroid tumor cell lines (80%), including one follicular adenoma cell line, whereas *BRAF* was only mutated in 38% of papillary thyroid carcinomas. The higher *BRAF* mutation rate in tumor cell lines than in their corresponding tumor specimens may be attributable to: (*a*) the cell lines from the original tumors with *BRAF* mutations may be selectively established because of an advantage in cell growth (31) and an antiapoptotic effect (51); (*b*) established cell lines are homogenous, whereas microdissected tumor sections contain many normal cells such as stromal cells, endothelial cells, and infiltrating immune cells. Therefore, a low percentage of tumor cells in a specimen may lower the sensitivity of the methods used to detect *BRAF* mutation, resulting in a false negative result; and (*c*) *BRAF* mutation in cell lines may be gained during long-term *in vitro* cell culture.

The molecular mechanisms by which *BRAF* is mutated at high frequency in melanoma and papillary thyroid carcinomas are not known. Davies *et al.* (31) proposed that *BRAF* mutation in melanoma is somehow related to features of melanocyte biology. This supposition is based on the observations that besides the RAS signaling pathway, BRAF in melanocytes can be directly activated by cyclic AMP, a secondary messenger regulated by binding of α-melanocyte stimulating-hormone, and other proopiomelanocortin-derived peptides to their melanocortin receptor I (52, 53). This alternative *BRAF* activation pathway plays a critical role in controlling melanocyte proliferation and differentiation, particularly in response to UVB radiation (52, 53). Interestingly, in thyrocytes, thyroid-stimulating hormone strongly activates cyclic AMP and promotes thyrocyte cell proliferation (54–57). Therefore, it is likely that mutation of *BRAF* in melanocytes and thyrocytes may be related to cyclic AMP, although how exactly cyclic AMP results in *BRAF* gene mutation remains elusive. In colon cancer, Rajagopalan *et al.* (40) reported that *BRAF* mutation is associated with the deficiency of mismatch repair. Although it is not clear whether mismatch repair activity is also compromised or deficient in papillary thyroid carcinomas, β-catenin, another gene frequently mutated in colon cancer because of the deficiency of mismatch-repair, is also mutated in papillary thyroid carcinomas (58). Therefore, it is possible that *BRAF* mutation in papillary thyroid carcinomas may, in part, result from the defective DNA mismatch repair.

Another interesting observation in this study is that among 8 mutant thyroid tumor cell lines, 6 were heterozygous (Table 2). The other two cell lines (ARO81 and DRO90) were either homozygous because of gene conversion or hemizygous because of the loss of a second allele of the *BRAF* gene. Similar to this observation, Rajagopalan *et al.* (40) found that 2 of 28 colon adenocarcinomas with *BRAF* mutation at residue 599 were not heterozygous. The underlying mechanism for this nonheterozygous *BRAF* gene mutation and its role in trigging cell transformation and tumorigenesis remain to be defined.

In summary, our present study demonstrates that *BRAF* was mutated at a high frequency in papillary thyroid carcinomas but not in follicular adenomas and that a large number of *BRAF*-mutated PTC also harbored *RET/PTC* rearrangements. These observations provide a molecular basis for the oncogenesis of PTC and suggest that PTC and FTA may be developed through the mutation of different oncogenes within the same signaling pathway. It will be interesting to find out whether quantitative and/or qualitative differences in the RAS/BRAF-signaling pathway determine the type of thyroid neoplasm.

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REFERENCES

1. Gimm, O. Thyroid cancer. Cancer Lett., *163:* 143–156, 2001.
2. Saavedra, H. I., Knauf, J. A., Shirokawa, J. M., Wang, J., Ouyang, B., Elisei, R., Stambrook, P. J., and Fagin, J. A. The RAS oncogene induces genomic instability in thyroid PCCL3 cells via the MAPK pathway. Oncogene, *19:* 3948–3954, 2000.
3. Lyons, J., Landis, C. A., Harsh, G., Vallar, L., Grunewald, K., Feichtinger, H., Duh, Q. Y., Clark, O. H., Kawasaki, E., Bourne, H. R., *et al.* Two G protein oncogenes in human endocrine tumors. Science (Wash. DC), *249:* 655–659, 1990.
4. 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.
5. Suarez, H. G., du Villard, J. A., Caillou, B., Schlumberger, M., Parmentier, C., and Monier, R. gsp mutations in human thyroid tumours. Oncogene, *6:* 677–679, 1991.
6. Esapa, C. T., Johnson, S. J., Kendall-Taylor, P., Lennard, T. W., and Harris, P. E. Prevalence of Ras mutations in thyroid neoplasia. Clin Endocrinol. (Oxf), *50:* 529–535, 1999.
7. Krohn, K., Reske, A., Ackermann, F., Muller, A., and Paschke, R. Ras mutations are rare in solitary cold and toxic thyroid nodules. Clin. Endocrinol. (Oxf), *55:* 241–248, 2001.
8. Lemoine, N. R., Mayall, E. S., Williams, E. D., Thurston, V., and Wynford-Thomas, D. Agent-specific ras oncogene activation in rat thyroid tumours. Oncogene, *3:* 541–544, 1988.
9. Lemoine, N. R., Mayall, E. S., Wyllie, F. S., Farr, C. J., Hughes, D., Padua, R. A., Thurston, V., Williams, E. D., and Wynford-Thomas, D. Activated ras oncogenes in human thyroid cancers. Cancer Res., *48:* 4459–4463, 1988.
10. 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.
11. Namba, H., Gutman, R. A., Matsuo, K., Alvarez, A., and Fagin, J. A. H-ras protooncogene mutations in human thyroid neoplasms. J. Clin. Endocrinol. Metab., *71:* 223–229, 1990.
12. Park, K. Y., Koh, J. M., Kim, Y. I., Park, H. J., Gong, G., Hong, S. J., and Ahn, I. M. Prevalences of Gs α, ras, p53 mutations and ret/PTC rearrangement in differentiated thyroid tumours in a Korean population. Clin. Endocrinol. (Oxf), *49:* 317–323, 1998.
13. Wright, P. A., Lemoine, N. R., Mayall, E. S., Wyllie, F. S., Hughes, D., Williams, E. D., and Wynford-Thomas, D. Papillary and follicular thyroid carcinomas show a different pattern of ras oncogene mutation. Br. J. Cancer, *60:* 576–577, 1989.
14. Wright, P. A., Williams, E. D., Lemoine, N. R., and Wynford-Thomas, D. Radiation-associated and 'spontaneous' human thyroid carcinomas show a different pattern of ras oncogene mutation. Oncogene, *6:* 471–473, 1991.
15. Shi, Y. F., Zou, M. J., 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.
16. Suarez, H. G., Du Villard, J. A., Caillou, B., Schlumberger, M., Tubiana, M., Parmentier, C., and Monier, R. Detection of activated ras oncogenes in human thyroid carcinomas. Oncogene, *2:* 403–406, 1988.
17. Suarez, H. G., du 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.
18. Said, S., Schlumberger, M., and Suarez, H. G. Oncogenes and anti-oncogenes in human epithelial thyroid tumors. J. Endocrinol. Investig., *17:* 371–379, 1994.
19. Klugbauer, S., and Rabes, H. M. The transcription coactivator HTIF1 and a related protein are fused to the RET receptor tyrosine kinase in childhood papillary thyroid carcinomas. Oncogene, *18:* 4388–4393, 1999.
20. Beimfohr, C., Klugbauer, S., Demidchik, E. P., Lengfelder, E., and Rabes, H. M. NTRK1 re-arrangement in papillary thyroid carcinomas of children after the Chernobyl reactor accident. Int. J. Cancer, *80:* 842–847, 1999.
21. Tallini, G., and Asa, S. L. RET oncogene activation in papillary thyroid carcinoma. Adv. Anat. Pathol., *8:* 345–354, 2001.
22. Grieco, M., Santoro, M., Berlingieri, M. T., Melillo, R. M., Donghi, R., Bongarzone, I., Pierotti, M. A., Della Porta, G., Fusco, A., and Vecchio, G. PTC is a novel rearranged form of the ret proto-oncogene and is frequently detected in vivo in human thyroid papillary carcinomas. Cell, *60:* 557–563, 1990.

23. Santoro, M., Carlomagno, F., Hay, I. D., Herrmann, M. A., Grieco, M., Melillo, R., Pierotti, M. A., Bongarzone, I., Della Porta, G., Berger, N., *et al*. Ret oncogene activation in human thyroid neoplasms is restricted to the papillary cancer subtype. J. Clin. Investig., *89:* 1517–1522, 1992.
24. Santoro, M., Melillo, R. M., Grieco, M., Berlingieri, M. T., Vecchio, G., and Fusco, A. The TRK and RET tyrosine kinase oncogenes cooperate with ras in the neoplastic transformation of a rat thyroid epithelial cell line. Cell Growth Differ., *4:* 77–84, 1993.
25. Dobashi, Y., Sakamoto, A., Sugimura, H., Mernyei, M., Mori, M., Oyama, T., and Machinami, R. Overexpression of p53 as a possible prognostic factor in human thyroid carcinoma. Am. J. Surg. Pathol., *17:* 375–381, 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. Investig., *91:* 179–184, 1993.
27. Ito, T., Seyama, T., Mizuno, T., Tsuyama, N., Hayashi, Y., Dohi, K., Nakamura, N., and Akiyama, M. Genetic alterations in thyroid tumor progression: association with p53 gene mutations. Jpn. J. Cancer Res., *84:* 526–531, 1993.
28. Thiagalingam, A., De Bustros, A., Borges, M., Jasti, R., Compton, D., Diamond, L., Mabry, M., Ball, D. W., Baylin, S. B., and Nelkin, B. D. RREB-1, a novel zinc finger protein, is involved in the differentiation response to Ras in human medullary thyroid carcinomas. Mol. Cell. Biol., *16:* 5335–5345, 1996.
29. Elisei, R., Shiohara, M., Koeffler, H. P., and Fagin, J. A. Genetic and epigenetic alterations of the cyclin-dependent kinase inhibitors p15INK4b and p16INK4a in human thyroid carcinoma cell lines and primary thyroid carcinomas. Cancer (Phila.), *83:* 2185–2193, 1998.
30. Baldassarre, G., Belletti, B., Bruni, P., Boccia, A., Trapasso, F., Pentimalli, F., Barone, M. V., Chiappetta, G., Vento, M. T., Spiezia, S., Fusco, A., and Viglietto, G. Overexpressed cyclin D3 contributes to retaining the growth inhibitor p27 in the cytoplasm of thyroid tumor cells. J. Clin. Investig., *104:* 865–874, 1999.
31. Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley, W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B. A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G. J., Bigner, D. D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J. W., Leung, S. Y., Yuen, S. T., Weber, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C. J., Wooster, R., Stratton, M. R., and Futreal, P. A. Mutations of the *BRAF* gene in human cancer. Nature (Lond.), *417:* 949–954, 2002.
32. Fagin, J. A. Molecular genetics of human thyroid neoplasms. Annu. Rev. Med., *45:* 45–52, 1994.
33. Ain, K., Taylor, K., Rofiq, S., and Venkataraman, G. Somatostatin receptor subtype expression in human thyroid and thyroid carcinoma cell lines. J. Clin. Endocrinol. Metab., *82:* 1857–1862, 1997.
34. Hasegawa, Y., Takeda, S., Ichii, S., Koizumi, K., Maruyama, M., Fujii, A., Ohta, H., Nakajima, T., Okuda, M., Baba, S., *et al*. Detection of K-ras mutations in DNAs isolated from feces of patients with colorectal tumors by mutant-allele-specific amplification (MASA). Oncogene, *10:* 1441–1445, 1995.
35. Linard, B., Bezieau, S., Benlalam, H., Labarriere, N., Guilloux, Y., Diez, E., and Jotereau, F. A ras-mutated peptide targeted by CTL infiltrating a human melanoma lesion. J. Immunol., *168:* 4802–4808, 2002.
36. Bongarzone, I., Vigneri, P., Mariani, L., Collini, P., Pilotti, S., and Pierotti, M. A. RET/NTRK1 rearrangements in thyroid gland tumors of the papillary carcinoma family: correlation with clinicopathological features. Clin. Cancer Res., *4:* 223–228, 1998.
37. Sugg, S. L., Ezzat, S., Rosen, I. B., Freeman, J. L., and Asa, S. L. Distinct multiple RET/PTC gene rearrangements in multifocal papillary thyroid neoplasia. J. Clin. Endocrinol. Metab., *83:* 4116–4122, 1998.
38. Tallini, G., Santoro, M., Helie, M., Carlomagno, F., Salvatore, G., Chiapetta, G., Carcangiu, M. L., and Fusco, A. RET/PTC oncogene activation defines a subset of papillary thyroid carcinomas lacking evidence of progression to poorly differentiated or undifferentiated tumor phenotypes. Clin. Cancer Res., *4:* 287–294, 1998.
39. Kimura, E. T., Nikiforova, M. N., Zhu, Z., Knauf, J. A., Nikiforov, Y. E., and Fagin, J. A. High prevalence of *BRAF* mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-*BRAF* signaling pathway in papillary thyroid carcinoma. Cancer Res., *63:* 1454–1457, 2003.
40. Rajagopalan, H., Bardelli, A., Lengauer, C., Kinzler, K. W., Vogelstein, B., and Velculescu, V. E. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature (Lond.), *418:* 934, 2002.
41. Pollock, P. M., Harper, U. L., Hansen, K. S., Yudt, L. M., Stark, M., Robbins, C. M., Moses, T. Y., Hostetter, G., Wagner, U., Kakareka, J., Salem, G., Pohida, T., Heenan, P., Duray, P., Kallioniemi, O., Hayward, N. K., Trent, J. M., and Meltzer, P. S. High frequency of *BRAF* mutations in nevi. Nat. Genet., *33:* 19–20, 2003.
42. Pollock, P. M., and Meltzer, P. S. A genome-based strategy uncovers frequent *BRAF* mutations in melanoma. Cancer Cell, *2:* 5–7, 2002.
43. Yuen, S. T., Davies, H., Chan, T. L., Ho, J. W., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Tsui, W. W., Chan, A. S., Futreal, P. A., Stratton, M. R., Wooster, R., and Leung, S. Y. Similarity of the phenotypic patterns associated with *BRAF* and KRAS mutations in colorectal neoplasia. Cancer Res., *62:* 6451–6455, 2002.
44. Naoki, K., Chen, T. H., Richards, W. G., Sugarbaker, D. J., and Meyerson, M. Missense mutations of the *BRAF* gene in human lung adenocarcinoma. Cancer Res., *62:* 7001–7003, 2002.
45. Namba, H., Rubin, S. A., and Fagin, J. A. Point mutations of ras oncogenes are an early event in thyroid tumorigenesis. Mol. Endocrinol., *4:* 1474–1479, 1990.
46. Suarez, H. G. Genetic alterations in human epithelial thyroid tumours. Clin. Endocrinol. (Oxf.), *48:* 531–546, 1998.
47. Nikiforov, Y. E., Rowland, J. M., Bove, K. E., Monforte-Munoz, H., and Fagin, J. A. Distinct pattern of ret oncogene rearrangements in morphological variants of radiation-induced and sporadic thyroid papillary carcinomas in children. Cancer Res., *57:* 1690–1694, 1997.
48. Bounacer, A., Wicker, R., Caillou, B., Cailleux, A. F., Sarasin, A., Schlumberger, M., and Suarez, H. G. High prevalence of activating ret proto-oncogene rearrangements, in thyroid tumors from patients who had received external radiation. Oncogene, *15:* 1263–1273, 1997.
49. Russo, D., Arturi, F., Schlumberger, M., Caillou, B., Monier, R., Filetti, S., and Suarez, H. G. Activating mutations of the TSH receptor in differentiated thyroid carcinomas. Oncogene, *11:* 1907–1911, 1995.
50. Sugg, S. L., Ezzat, S., Zheng, L., Freeman, J. L., Rosen, I. B., and Asa, S. L. Oncogene profile of papillary thyroid carcinoma. Surgery (St. Louis), *125:* 46–52, 1999.
51. Erhardt, P., Schremser, E. J., and Cooper, G. M. B-Raf inhibits programmed cell death downstream of cytochrome *c* release from mitochondria by activating the MEK/Erk pathway. Mol. Cell. Biol., *19:* 5308–5315, 1999.
52. Busca, R., Abbe, P., Mantoux, F., Aberdam, E., Peyssonnaux, C., Eychene, A., Ortonne, J. P., and Ballotti, R. Ras mediates the cAMP-dependent activation of extracellular signal-regulated kinases (ERKs) in melanocytes. EMBO J., *19:* 2900–2910, 2000.
53. Halaban, R. The regulation of normal melanocyte proliferation. Pigm. Cell Res., *13:* 4–14, 2000.
54. Kimura, T., Van Keymeulen, A., Golstein, J., Fusco, A., Dumont, J. E., and Roger, P. P. Regulation of thyroid cell proliferation by TSH and other factors: a critical evaluation of *in vitro* models. Endocr. Rev., *22:* 631–656, 2001.
55. Medina, D. L., and Santisteban, P. Thyrotropin-dependent proliferation of in vitro rat thyroid cell systems. Eur. J. Endocrinol., *143:* 161–178, 2000.
56. Pomerance, M., Abdullah, H. B., Kamerji, S., Correze, C., and Blondeau, J. P. Thyroid-stimulating hormone and cyclic AMP activate p38 mitogen-activated protein kinase cascade. Involvement of protein kinase A, rac1, and reactive oxygen species. J. Biol. Chem., *275:* 40539–46, 2000.
57. Tsygankova, O. M., Saavedra, A., Rebhun, J. F., Quilliam, L. A., and Meinkoth, J. L. Coordinated regulation of Rap1 and thyroid differentiation by cyclic AMP and protein kinase A. Mol. Cell. Biol., *21:* 1921–1929, 2001.
58. Garcia-Rostan, G., Camp, R. L., Herrero, A., Carcangiu, M. L., Rimm, D. L., and Tallini, G. β-Catenin dysregulation in thyroid neoplasms: down-regulation, aberrant nuclear expression, and CTNNB1 exon 3 mutations are markers for aggressive tumor phenotypes and poor prognosis. Am. J. Pathol., *158:* 987–996, 2001.