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

Xiulong Xu,2 Roderick M. Quiros, PaoloGattuso, Kenneth B. Ain, and Richard A. Prinz

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 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 by using two different anti-RET antibodies. Surprisingly, we found that a large number of BRAF-mutated PTC (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.

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 (29). 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.
BRAF Gene and Exons 1 and 2 of the poorly differentiated PTC cell line.

DNA concentration was quantitated followed by proteinase K digestion at 56°C overnight and DNA extraction with a dewaxed but unstained set of slides. Tumor areas were matched with that identified 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 manufacturer’s protocol.

Statistical Analysis. Correlation between HRPI gene expression and the histological diagnosis or clinicopathological parameters of the thyroid neoplasms was determined by a χ2 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, AR081 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 the BRAF gene was similarly amplified with a forward primer (5'-GCTGAGTACAAACTGGTG-3') and a reverse primer (5'-GGTGAAACCTGTTTGTTGGA-3') as a positive control. 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 with a forward primer flanking the sequence of exon 15 (5'-TACCAGTTTGTTGCTACTACGT-3') and a reverse primer (5'-GGTGAAACCTGTTTGTTGGA-3'). CRAF and NRAS mutations were detected as a positive control to amplify wild-type as well as mutant BRAF. A second primer with substitution of two mismatches at the 3' -end (GGTGATTTTGGTCTACTACAC-3') was used to amplify mutant BRAF only. The sequence of the reverse primer is 5'-GGGAGCTTACCCTTAGG-3'; exon 2 of the NRAS gene was amplified with a forward primer (5'-GGTGAAACCTGTTTGTTGGA-3') and a reverse primer (5'-ATACACAGAGAGAGCCTTTG-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.

Somatic BRAF Mutation in Thyroid Neoplasms. We 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. 1B, the BRAF exon 15 was amplified by using the mismatched primer in 4 BRAF-mutant thyroid tumor cell lines (DRO90 and AR081 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. 2A, BRAF mutation at
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. 2B 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 non tumor 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. 3A, inset) and a PTC with wild-type BRAF but not in the neighboring normal thyroid follicular cells (Fig. 3A). Similar results were obtained when an anti-RET mAb was used (Fig. 3C 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
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...
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 carcinomas. 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 PC13 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 PC13 cells (24). It is possible that BRAF may cooperate with RET/PTC to fully transform thyroid epithelial cells, leading to the progression of occult PTC.

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**Table 2. BRAF gene mutation in thyroid neoplasms**

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>No.</th>
<th>Positive (%)</th>
<th>( P )</th>
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<tr>
<td>Follicular adenoma</td>
<td>18</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Papillary carcinoma</td>
<td>56</td>
<td>21 (38%)</td>
<td>0.006</td>
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</tbody>
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**Table 3. Overlapping of BRAF mutation with other genetic alterations in PTC**

<table>
<thead>
<tr>
<th></th>
<th>BRAF mutation</th>
<th>No. of tumors</th>
<th>RET/PTC positive</th>
<th>NRAS mutation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>21</td>
<td>8 (38%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>35</td>
<td>13 (37%)</td>
<td>ND*</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
<td>21 (38%)</td>
<td>ND*</td>
<td></td>
</tr>
</tbody>
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*Exons 1 and 2 of the NRAS gene were PCR amplified individually and sequenced to detect NRAS mutation at codons 12, 13, and 61.

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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 sarcoma cell lines (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 antiaiportotic 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 thyocytes, thyroid-stimulating hormone strongly activates cyclic AMP and promotes thyocyte cell proliferation (54–57). Therefore, it is likely that mutation of BRAF in melanocytes and thyocytes 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 triggering 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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