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BRIEF REPORT

BRAF Mutations in Papillary Thyroid Carcinomas Inhibit Genes Involved in Iodine Metabolism

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Context: BRAF mutations are common in papillary thyroid carcinomas (PTCs). By affecting the expression of genes critically related to the development and differentiation of thyroid cancer, they may influence the prognosis of these tumors.

Objective: Our objective was to characterize the expression of thyroid-specific genes associated with BRAF mutation in PTCs.

Design/Setting and Patients: We examined the expression of key markers of thyrocyte differentiation in 56 PTCs with BRAF mutations (BRAF-mut) and 37 with wild-type BRAF (BRAF-wt). Eight samples of normal thyroid tissue were analyzed as controls. Quantitative PCR was used to measure mRNA levels for the sodium/iodide symporter (NIS), apical iodide transporter (AIT-B), thyroglobulin (Tg), thyroperoxidase (TPO), TSH receptor (TSH-R), the transcription factor PAX8, and glucose transporter type 1 (Glut1). NIS protein expression and localization was also analyzed by immunohistochemistry.

Results: mRNA levels for all thyroid-specific genes were reduced in all PTCs *vs.* normal thyroid tissues. NIS, AIT-B, Tg, and TPO expression was significantly lower in BRAF-mut tumors than in the BRAF-wt group. Glut-1 transcript levels were increased in all PTCs, and additional increases were noted in BRAF-mut tumors. In both tumor subsets, the NIS protein that was expressed was abnormally retained in the cytoplasm.

Conclusion: BRAF V600E mutation in PTCs is associated with reduced expression of key genes involved in iodine metabolism. This effect may alter the effectiveness of diagnostic and/or therapeutic use of radioiodine in BRAF-mut PTCs. (***J Clin Endocrinol Metab* 92: 2840–2843, 2007**)

GAIN-OF-FUNCTION MUTATIONS in the BRAF oncogene are detected in approximately 45% of all sporadic papillary thyroid carcinomas (PTCs) (1–3). Studies of small series of BRAF-mutant (BRAF-mut) PTCs have revealed changes in the expression of the sodium/iodide symporter (NIS) (4, 5), the apical iodide transporter (AIT) (5), and/or thyroperoxidase (TPO) (6), but the global gene expression profile of these tumors has never been investigated. To determine whether BRAF mutation might have negative effects on functional PTC differentiation, we analyzed the expression of mRNA for thyroid-specific genes: NIS; AIT; thyroglobulin (Tg); TPO; TSH receptor (TSH-R); the nuclear factor PAX8, which regulates the latter genes' expression; and glucose transporter type-1 (Glut-1), which is highly expressed in thyroid tumors with enhanced glucose metabolism (7).

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Abbreviations: AIT, Apical iodide transporter; BRAF-mut, BRAF-mutant; BRAF-wt, wild-type BRAF; NIS, sodium/iodide symporter; PTC, papillary thyroid carcinoma; RT, room temperature; TPO, thyroperoxidase.

Patients and Methods

We examined surgical specimens of 93 nonconsecutive sporadic PTCs that were negative for ret/PTC 1 and ret/PTC 3 rearrangements. Normal thyroid tissues from eight patients undergoing surgery for benign thyroid disease were examined as controls. The tissues were snap-frozen and stored at −80 C until use. All 93 tumors were histologically diagnosed as PTCs (classic form or variants, as shown in Table 1). Patients' charts were reviewed to define the clinical features of each case. When possible, tumors were staged according to the criteria of the American Joint Committee on Cancer (8). The study was approved by the local medical ethics committee. Before surgery, each study participant provided written, informed consent to the collection of fresh thyroid tissue for genetic studies.

Exclusion of ret/PTC 1 and ret/PTC 3 rearrangements was based on preliminary RT-PCR analysis with breakpoint-spanning primers, as previously described (1). BRAF mutations were identified by single-stranded conformation polymorphism screening of products obtained by RT-PCR amplification of exon 15, and results were confirmed by means of sequence analysis (1). Total RNA was extracted with Trizol (Invitrogen Corp., Carlsbad, CA). First-strand cDNA synthesis was performed using 2 μg of each RNA sample primed with random hexamers with 200 U Invitrogen Superscript II reverse transcriptase. Quantitative PCR Assays-on-Demand Gene Expression Products (Applied Biosystems, Foster City, CA) were used to evaluate expression of the NIS, the AIT-B, Tg, TPO, TSH-R, PAX8, and Glut-1. Endogenous controls, *i.e.* glyceraldehyde-3-phosphate dehydrogenase, β2-microglobulin, and β-actin, were purchased from Applied Biosystems as predeveloped TaqMan assay reagents (VIC dye-labeled). Details of the reaction have been described elsewhere (9).

TABLE 1. Clinicopathological features of BRAF-mut and BRAF-wt PTC subsets[a]

	BRAF-mut (n = 56)	BRAF-wt (n = 37)	*P* value
Patient age (yr)	53.41 ± 17.6	43.95 ± 14.2	<0.006
Females	38/56 (67.9%)	28/37 (75.7%)	0.41
Tumor size (cm)	2.0 ± 0.9	2.0 ± 1.1	0.85
Extrathyroidal invasion[b]	14/56 (25.0%)	12/36 (33.3%)	0.38
Nodal metastasis[c]	16/55 (29.1%)	12/37 (32.4%)	0.73
Distant metastasis[d]	3/37 (7.5%)	6/31 (19.4%)	0.13
Stage[e]			
I	25/43 (58.1%)	22/32 (68.8%)	0.36[f]
II	3/43 (7.0%)	3/32 (9.4%)	
III	4/43 (9.3%)	1/32 (3.1%)	
IV	11/43 (25.6%)	6/32 (18.7%)	
Histology			
Classic PTC	48/56 (85.7%)	18/37 (48.6%)	0.0001[g]
Follicular PTC	7/56 (12.5%)	11/37 (29.7%)	
Other types of PTC	1/56 (1.8%)	5/37 (13.5%)	
Dedifferentiated PTC	0/56 (0%)	3/37 (8.2%)	

[a] Twenty-seven of the BRAF-mut PTCs and 12 of those that were BRAF-wt were included in the series reported in Ref. 1.
[b] Information unavailable for one BRAF-wt tumor.
[c] Information unavailable for one BRAF-mut tumor.
[d] Information unavailable for 16 BRAF-mut tumors and six BRAF-wt tumors.
[e] Stage could not be determined for 13 BRAF-mut tumors and five BRAF-wt tumors.
[f] *P* was calculated comparing the percentage of stage I plus II and stage III plus IV tumors in BRAF-wt *vs.* BRAF-mut groups.
[g] Comparing classic variant of PTC with respect to all other variants.

Immunohistochemistry was performed to evaluate the subcellular localization of the NIS protein in PTC samples [12 BRAF-mut tumors and 12 wild-type BRAF (BRAF-wt) tumors]. Normal thyroid tissues were used as positive controls. Dewaxed tissue sections were treated for 10 min at room temperature (RT) with 0.1 mol/liter Tris buffer 1× (pH 7.2) and 0.03% H_2O_2 to block endogenous peroxidase activity. Sections were then processed in a microwave oven with 1 mmol/liter EDTA buffer (pH 8) (three 5-min cycles), cooled at RT, and incubated for 30 min at RT in blocking solution (3% BSA plus 0.05% Triton X-100 diluted in Tris 1×). Excess blocking solution was eliminated, and the sections were incubated for 60 min at RT with the primary polyclonal NIS antibody diluted 1:1000 in CheMate antibody diluent (DakoCytomation, Glostrup, Denmark). After three 5-min washes in Tris-HCl 1× buffer (pH 7.6), sections were incubated for 30 min with peroxidase-conjugated secondary antibody (peroxidase antirabbit En Vision System, DakoCytomation). After two 5-min washes with Tris-HCl 1× buffer and one with Tris 1×, activity was visualized using diaminobenzidine chromogen (DakoCytomation). The sections, lightly counterstained with Mayer's hematoxylin, were dehydrated, mounted, and examined under a microscope. Negative controls were prepared without the primary antibody.

Statistical analysis

Correlations between BRAF-mut and clinicopathological parameters were analyzed in all 93 patients unless otherwise indicated. Results are presented as means ± SD or as percentages, as appropriate. We used the *t* test to compare continuous variables and the χ^2 test to determine *P* values in 2 × 2 contingency tables. Quantitative PCR results are expressed as means ± SE, and differences were analyzed with the Mann-Whitney nonparametric test. *P* values of <0.05 were considered significant.

Results

Thirty-seven of the 93 PTCs had wild-type BRAF (BRAF-wt group). The 56 tumors with BRAF mutations (BRAF-mut group) included 55 with a heterozygous T→A transversion at nucleotide 1799 that causes a valine-to-glutamic-acid substitution at position 600 ($BRAF^{V600E}$) and one with an in-frame insertion at position 1796 of a codon for valine ($BRAF^{V599Ins}$). This mutation has recently been shown to cause a gain of function that is in all respects similar to $BRAF^{V600E}$ (10). BRAF mutations were significantly associated with the classic PTC phenotype ($P = 0.0001$ *vs.* all other variants) and with older age ($P < 0.006$) (Table 1).

Table 2 shows the gene expression levels in normal thyroid tissue and in the BRAF-wt and BRAF-mut tumors. Expression of the thyroid-specific genes was significantly reduced in all 93 tumors (*vs.* normal tissues), and for certain markers of differentiation, this underexpression was markedly accentuated in the BRAF-mut subgroup. For example, transcript levels of AIT, NIS, and TPO in BRAF-mut tumors were reduced by 86, 82, and 90%, respectively ($P < 0.0001$ *vs.* values for BRAF-wt tumors), and there was a smaller but still significant reduction in Tg expression (46%, $P = 0.0014$). The two subgroups displayed no significant differences in terms of TSH-R or PAX8 expression. Glut-1 mRNA levels were significantly increased in all PTCs *vs.* normal tissues ($P < 0.0001$), and additional increases were noted in the BRAF-mut tumors ($P < 0.0001$ *vs.* BRAF-wt tumors).

Immunohistochemistry revealed intense anti-NIS staining in normal tissues that was located almost exclusively at the plasma membrane. Staining was appreciably less intense in tumor tissues, particularly those from the BRAF-mut group. In both BRAF-mut and BRAF-wt tumors, most, if not all, of the NIS was confined to the cytoplasm (data not shown).

Discussion

The prognosis for PTCs is generally good unless the tumor presents a poorly differentiated phenotype. Dedifferentiation is associated with molecular alterations of proteins that allow the thyrocyte to concentrate the iodine, which render the tumor more or less refractive to radioiodine therapy (11, 12). In most cases, the defects involve the transcription of NIS and its translocation to plasma membrane (13, 14), but there are also reports of reduced mRNA levels for AIT, which plays an unidentified role in the transfer of iodide to the colloid, and for TPO, which is responsible for iodide organification (12). The gain-of-function BRAF V600E mutation might play

TABLE 2. Gene expression levels for molecular markers of differentiation in normal and tumoral thyroid tissues

Gene	Normal tissue	PTC (n = 93)	BRAF-wt (n = 37)	BRAF-mut (n = 56)	*P* value
NIS	1 ± 0.253	0.078 ± 0.022	0.153 ± 0.052	0.027 ± 0.008	<0.0001[a] <0.0001[b]
AIT-B	1 ± 0.087	0.133 ± 0.029	0.276 ± 0.066	0.038 ± 0.010	<0.0001[a] <0.0001[b]
TPO	1 ± 0.123	0.191 ± 0.055	0.418 ± 0.128	0.042 ± 0.011	<0.0001[a] <0.0001[b]
Tg	1 ± 0.084	0.392 ± 0.037	0.544 ± 0.068	0.292 ± 0.036	<0.0001[a] 0.0014[b]
TSH-R	1 ± 0.086	0.679 ± 0.048	0.743 ± 0.087	0.638 ± 0.056	0.0003[a] 0.3667[b]
PAX8	1 ± 0.11	0.384 ± 0.044	0.384 ± 0.054	0.388 ± 0.034	<0.0001[a] 0.9562[b]
Glut-1	1 ± 0.07	1.636 ± 0.102	1.204 ± 0.129	1.921 ± 0.134	<0.0001[a] <0.0001[b]

For each gene, the mRNA level (mean ± SE) found in normal tissue is the reference value (the latter corresponding to 1.0).
[a] PTC *vs.* normal.
[b] BRAF-mut tumors *vs.* BRAF-wt tumors.

an important role in tumor dedifferentiation. Our study is the first attempt to obtain a complete profile of the changes in thyroid-specific gene expression produced in PTCs by BRAF mutation.

In all 93 of the PTCs we examined, levels of mRNA for PAX8 and for all of the thyroid-specific genes studied were significantly reduced (*vs.* normal tissues). Furthermore, the limited amounts of NIS protein that were expressed in these tumors (roughly 10 times lower than those of normal thyroid tissues) were confined almost exclusively to the cytoplasm rather than being targeted to the plasma membrane. Although defective NIS translocation was similar in the two subgroups of tumors, levels of NIS transcript in BRAF-mut PTCs were about five times lower than those observed in BRAF-wt tumors. Similar differences emerged for AIT and TPO expression. These *in vivo* findings indicate that abnormal activation of the ras/raf pathway in human thyroid tumors compromises differentiation via effects exerted mainly at the mRNA level (transcription or mRNA stabilization), which is fully consistent with hypotheses advanced by other investigators based on *in vitro* data and results observed in animal models (15, 16). These dedifferentiating effects are not dependent on the underexpression of PAX8, which was similar in both BRAF-mut and BRAF-wt PTCs. Transcription of thyroid-specific genes (especially the NIS) is regulated by numerous other factors, including some acting on the promoter methylation (14, 17). Additional study is needed to identify the one(s) that mediate(s) BRAF-induced dedifferentiation.

The picture of BRAF-mut PTCs that emerges from our study is that of a less differentiated tumor with severely compromised expression of a variety of iodide-metabolizing genes. This feature was unrelated to tumor stage and histology (data not shown). Indeed, a similar picture was also observed in the 66 patients with classic PTC phenotype. Overall, these findings suggest that histological features of differentiation not always correlate with functional differentiation, and in this regard, BRAF mutation may play a role. These results are consistent with the two recent reports associating BRAF mutation with loss of radioiodine avidity and treatment failure in recurrent PTC (4, 18). Our BRAF-mut PTCs also exhibited increased Glut-1 expression, an alteration that has been associated with *in vitro* loss of differentiation in thyroid cancer cells (19). It probably reflects a BRAF-induced abnormal glucose metabolism, which is associated with increased cell growth and more aggressive behavior. Although other groups have reported evidence of the increased aggressiveness of BRAF-mut PTCs (18, 20), our study and others (3) found no association between this mutation and extrathyroid invasion or metastases at the time of diagnosis. It is interesting to note that increased Glut-1 levels in thyroid tumors has been associated with the absence of detectable radioiodine uptake (7). Furthermore, Riesco-Eizaguirre *et al.* (4) found that recurrent thyroid tumors with BRAF mutations were almost always undetectable on ^{131}I scans, although some could be visualized with 18-fluorodeoxyglucose positron emission tomography. Due to the limited number of patients with metastatic disease in this series, we were unable to evaluate the possible impact of BRAF mutation on the diagnostic or therapeutic use of radioiodine. This issue should be explored in larger studies that include longer follow-ups and assessment of mortality.

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The authors have nothing to declare.

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