A High Percentage of \( \text{BRAF}^{\text{V600E}} \) Alleles in Papillary Thyroid Carcinoma Predicts a Poorer Outcome

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Context: \( \text{BRAF}^{\text{V600E}} \) is considered a negative prognostic marker in papillary thyroid carcinoma (PTC), but unexplained conflicting results are present in the literature. In light of the new finding that most PTC consist of a mixture of tumor cells with wild-type and mutant \( \text{BRAF} \), we examined the associations between the percentage of \( \text{BRAF}^{\text{V600E}} \) alleles and both the clinicopathological parameters at time of diagnosis and the disease outcome in a large series of PTCs.

Study Design: Tumors from 168 patients with PTC were genotyped for \( \text{BRAF}^{\text{V600E}} \) using BigDye Terminator sequencing and pyrosequencing, and the clinical parameters were analyzed. The associations between clinicopathological characteristics, including disease recurrence at follow-up (median 5.1 yr) and the percentage of mutant \( \text{BRAF} \) alleles were assessed.

Results: The observed prevalence of \( \text{BRAF}^{\text{V600E}} \) was higher when using pyrosequencing than when using BigDye Terminator sequencing (53.6 vs. 36.9%). In the PTC positive for \( \text{BRAF}^{\text{V600E}} \), the percentage of mutant alleles ranged from 5.1 to 44.7% of the total \( \text{BRAF} \) alleles, with a median of 20.6%. The presence or the percentage of \( \text{BRAF}^{\text{V600E}} \) alleles did not correlate significantly with sex, multicentricity, lymph node metastasis, or tumor stage. The percentage of \( \text{BRAF}^{\text{V600E}} \) alleles directly correlated with age at diagnosis and tumor volume \((R^2 = 0.223, P = 0.039, \text{and } R^2 = 0.166, P < 0.001, \text{respectively})\). The percentage of \( \text{BRAF}^{\text{V600E}} \) alleles \((P = 0.014)\), tumor volume \((P = 0.012)\), and lymph node metastasis \((P = 0.008)\) predicted the disease outcome. The odds ratio of recurrence for PTC with \( \text{BRAF}^{\text{V600E}} \) alleles of 30% or greater, compared with that for PTC with \( \text{BRAF}^{\text{V600E}} \) alleles of less than 30%, was 5.31 \((P = 0.002)\).

Conclusions: A high percentage of \( \text{BRAF}^{\text{V600E}} \) alleles defines a PTC molecular subtype and predicts a poorer disease outcome. The analysis of \( \text{BRAF} \) mutations by pyrosequencing is useful to refine the risk stratification of patients with PTC. (J Clin Endocrinol Metab 97: 2333–2340, 2012)

The somatic point mutation T1799A in the \( \text{BRAF} \) gene, which results in a valine-to-glutamate substitution at residue 600 \( (\text{BRAF}^{\text{V600E}}) \), is the most frequent genetic alteration occurring in papillary thyroid carcinoma (PTC). The associations of \( \text{BRAF}^{\text{V600E}} \) with the clinicopathological features of PTC patients and the impact of this mutation on clinical outcome have been extensively investigated, with conflicting results. In an early study that considered 219 patients from different geographic areas, Xing et al. (1) observed a correlation between \( \text{BRAF}^{\text{V600E}} \)
and extrathyroidal extension, lymph node metastasis, and advanced stage (III/IV). Since then, a number of studies have been performed from different groups, but many failed to demonstrate a correlation between the BRAF mutation status and more advanced disease (2–5). A large meta-analysis performed by Lee et al. (6) including 1168 patients from 12 selected studies revealed a correlation between the BRAF mutation and both extrathyroidal extension and advanced disease stage. More importantly, regarding the impact on the management of PTC, some studies that included large numbers of cases found an association between the BRAF mutation and a poorer outcome (1, 7–9). Although the studies in favor of a correlation between the BRAF mutation and more aggressive PTC prevail, the results of many studies are discordant, and there is no definitive explanation for these inconsistent results.

Very recently we demonstrated that completely clonal PTC harboring BRAFV600E is a rare occurrence; more frequently this cancer consists of a mixture of tumor cells with wild-type and mutant BRAF (10). In light of this heterogeneity, the association between the BRAF mutation and the clinicopathological features of PTC must be reevaluated, taking into account the clonal/subclonal status of the BRAF mutation. In the present study, we examined the association between the percentage of BRAFV600E alleles and both the clinicopathological parameters and disease outcome in a large series of PTC, demonstrating that a high percentage of mutant BRAF alleles strongly predicts a reduced disease-free survival.

Patients and Methods

Patients, tissues, and clinicopathological data collection

A total of 168 patients who underwent total thyroidectomy for PTC at the Ospedale S. Paolo and Fondazione IRCCS Ca’ Granda (Milan, Italy) from 1994 to 2010 were randomly and consecutively selected. Most patients also underwent pretracheal and paratracheal lymph node dissection. Most patients gave consent to make their thyroid tissue available for experimental studies at the time of surgical treatment. A minority of patients gave informed consent at enrollment for the genetic analysis of the archival paraffin embedded tissues. All patients gave consent for inclusion of the clinical data in scientific studies. The institutional review board of the centers involved approved the present study after obtaining the above-mentioned consents.

The clinical records of the patients were retrospectively reviewed, and the patients were followed up a period of 3–195 months. All patients, with the exception of those with pT1N0 and a tumor size less than 1 cm, were submitted to radioiodine ablation. All patients were followed up according to the European and American guidelines for the management of differentiated thyroid cancer (11, 12). In particular, thyroid residue ablation was assessed by a recombinant human thyroid stimulating hormone (rhTSH) test (Thyrogen, Genzyme Corp., Cambridge, MA) 10–12 months after radioiodine therapy. Patients were maintained on TSH-suppressive levothyroxine treatment, unless disease remission was documented, and were shifted to lower doses, with the aim of reaching TSH levels between 0.5 and 1.0 mU/liter. Patients in remission were monitored yearly by neck ultrasound and thyroglobulin (Tg) and anti-Tg antibody (Ab-Tg) evaluation while taking levothyroxine treatment. The levels of Ab-Tg were measured using the liaison kit (Byk-Sangtec Diagnostica, Dietzenbach, Germany), and the Tg levels were measured using a highly sensitive (minimal detectable concentration = 0.18 μg/liter) immunometric assay (Delfia hTg; Wallac, Turku, Finland). Patients with persistent or relapsing disease were treated by surgery and/or radioiodine treatment.

Briefly, the criteria used to identify persistent/recurrent disease, which have already been reported extensively (13), included the presence of at least one of the following: 1) a basal Tg level greater than 2 μg/liter on consecutive determinations; 2) a Tg response to rhTSH greater than 2 ng/ml in a single test or greater than 1 ng/ml for two rhTSH tests performed within 10–12 months; 3) the presence of neck or body masses with a cytology and/or Tg washout levels positive for metastasis of thyroid cancer; 4) the presence of radioiodine uptake; or 5) the persistence of Ab-Tg antibodies for more than 4 yr with an increasing trend or sudden rise in the levels of autoantibodies. Of note, in patients who did not undergo radioiodine ablation, the Tg response to rhTSH indicating persistence/recurrence had to be higher than 2 ng/ml or had to show an increasing trend over two rhTSH tests performed within 10–12 months. Moreover, the cutoff values for both basal (during thyroid hormone therapy) and rhTSH-stimulated Tg levels have been established based on the experience derived from the use of the same Tg assay on a long-term basis. The clinicopathological data of the patients are reported in Supplemental Table 1, published on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org.

Thyroid tumor tissues and DNA extraction

Primary PTC tissues (147 conventional and 21 follicular variant PTC) had been routinely fixed overnight in buffered formalin and embedded in paraffin or alternatively snap frozen at the time of surgery and stored at −80 C. Tumors were classified according to the World Health Organization classification, and the American Joint Committee on Cancer stage was determined for each patient (14). All tissue samples were carefully microdissected under a dissecting microscope to exclude surrounding normal tissue. Patients with concomitant Hashimoto’s thyroiditis or with the presence of evident lymphoid infiltrate in the tissues were excluded. For genomic DNA extraction from paraffin-embedded tissues, 5-μm sections were immersed in xylene for 30 min to remove the paraffin and washed in absolute ethanol and then in 70% ethanol. Frozen samples were thawed, and all samples were subjected to digestion with 0.5% sodium dodecyl sulfate and 0.5 mg/ml proteinase K at 37 C overnight, extracted with phenol, and precipitated with ethanol in the presence of sodium acetate. The final pellet was resuspended in 50 μl of diethylpyrocarbonate-treated water. The DNA concentration was quantitated using the A260 absorbance measured with a BioPhotometer (Eppendorf, Hamburg, Germany).
Detection of the BRAF mutation

For BigDye Terminator sequencing (PE Applied Biosystems, Foster City, CA), the DNA was amplified by PCR with specific primers (Supplemental Table 2) according to the following protocol: a 10-min denaturation at 98 C, 35 three-step cycles (60 C for 1 min, 72 C for 2 min, and 94 C for 1 min), and 10 min at 72 C in a TouchDown thermal cycler (Hybaid, Middlesex, UK). The PCR products were directly sequenced after the removal of the unincorporated deoxynucleotide triphosphates and primers using a sequencing by synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Aliquots of 3–10 ng per 100 bp of purified DNA and 3.2 pmol of either the forward or reverse primer were used in standard cycle sequencing reactions with an ABI PRISM BigDye Terminator kit and run on an ABI PRISM 310 genetic analyzer (both PE Applied Biosystems). The cycle-sequence conditions consisted of 25 cycles of 96 C for 30 sec, 50 C for 15 sec, and 60 C for 4 min.

For pyrosequencing, PCR reactions were performed with 50–100 ng of genomic DNA, forward primer and reverse 5′-biotinylated primers (Supplemental Table 2) at concentrations of 10 μM and 2.5 U of recombinant Taq polymerase (VWR, Milan Italy). All PCR reactions were performed separately in a TC-4000 thermal cycler (Bibby Scientific, Milan, Italy) using an initial denaturation of 5 min at 94 C followed by denaturation for 20 sec at 94 C, annealing for 20 sec at 61 C and extension for 30 sec at 72 C. The PCR products were electrophoresed in a 2% agarose gel containing ethidium bromide to confirm the successful amplification of the PCR products. The preparation of the single-stranded DNA template for pyrosequencing was performed using the PSQ vacuum prep tool (Diatech, Ancona, Italy) according to the manufacturer’s instructions. Twenty microliters of biotinylated PCR product were immobilized on streptavidin-coated Sepharose high-performance beads (Diatech) and processed to obtain single-stranded DNA using the PSQ 96 sample preparation kit (Diatech) according to the manufacturer’s instructions; the samples were incubated under shaking at room temperature for 10 min in binding buffer. Subsequently, samples were hybridized to 13.5 μM sequencing primers (Supplemental Table 2) in annealing buffer at 80 C for 2 min in a PSQ96 plate followed by cooling to room temperature. The sequencing-by-synthesis reaction of the complementary strand was automatically performed on a PSQ 96MA instrument (Biotage, Uppsala, Sweden) at room temperature using PyroGold reagents (Diatech). As the nucleotides were dispensed, a light signal was generated proportional to the amount of each incorporated nucleotide. These light signals were detected by a charge-coupled device camera and converted into peaks in a sequencing pyrogram that was automatically generated in real time for each sample. The sequencing primer was designed to include the analysis of codons 599 to 602 to provide internal positive control peaks for nucleotide incorporation and to screen for mutations in alternate codons. The coefficient of variation for the percentage of the mutant allele, calculated from quadruplicate pyrosequencing analysis, was 0.57% (range 0–1.4%). The cutoff was set at 5%, corresponding to the mean percentage of normal tissues plus 2 sd. As shown previously, using this cutoff, benign nodules and 15 follicular thyroid carcinomas were invariably negative (10).

Statistics

Statistical procedures included ANOVA, χ² analysis, simple and rank correlation analysis, and logistic regression analysis. The P value was considered statistically significant when P < 0.05 and borderline significant when P > 0.05 and P < 0.10. The data are presented as the prevalence for categorical data and the mean or median with the range for continuous data, as appropriate. Odds ratios were calculated by exponentiation of logistic regression analysis and are reported with the 95% confidence intervals (CI). The statistical significance between Kaplan-Meier curves was analyzed by log-rank test. Disease outcome was defined as a recurrence of one or more of the above-listed criteria in patients who were disease free at the initiation of follow-up.

Results

BRAF mutation analysis

The BRAF mutation statuses of 168 PTC were analyzed by BigDye Terminator sequencing and pyrosequencing for the T1799A transversion in exon 15, and results of the two techniques were compared (Fig. 1 and Table 1). DNA sequencing was successful by both methods in all of the samples. The BRAF mutation identified by BigDye Terminator sequencing was present in 62 PTC (36.9%). Ninety PTC (53.6%) were found to harbor the mutation by pyrosequencing analysis. The BRAFV600E allele accounted for 5.1–44.7% of the total BRAF alleles, with a mean and a median of 21.9 and 20.6%, respectively. Only three of the 62 samples positive for BRAFV600E by BigDye Terminator sequencing were found to be BRAFwild-type by pyrosequencing (95.2% concordance), whereas 31 of the 90 samples positive for BRAFV600E by pyrosequencing were found to be BRAFwild-type by BigDye Terminator sequencing (65.5% concordance) (Supplemental Table 3).

BRAF mutation and clinical parameters at diagnosis

The BRAF mutation status, determined by either method, was not significantly associated with sex, age at diagnosis, multifocality, tumor volume, or extrathyroidal extension (Table 1). The BRAF mutation status determined by BigDye Terminator sequencing was significantly associated with lymph node metastasis (P = 0.012). The
positive association between the presence of the mutant BRAF alleles and the American Joint Committee on Cancer stage was significant when using BigDye Terminator sequencing and borderline significant when using pyrosequencing.

Percentage of BRAFV600E alleles and clinical parameters at diagnosis

Four arbitrary groups were defined on the basis of the percentage of BRAFV600E alleles by pyrosequencing: less than 5% (without mutation), 5–14%, 15–29%, and 30% or greater (Table 2). The cutoffs were chosen such that there was one group without mutation (BRAFV600E <5% as per the definition described in Patients and Methods) and three groups consisting of adequate numbers of patients with progressively higher percentages of the BRAFV600E allele. The sex distribution, age, tumor volume, multifocality, and extrathyroidal extension were not significantly different among groups. There was a borderline significantly positive trend between the percentage of the mutant BRAF alleles and lymph node metastasis.

If only the tumors with the BRAFV600E mutation were considered, the percentage of BRAFV600E alleles was directly correlated with the patient age (R² = 0.223 in simple regression analysis; \( P = 0.039 \)) (Fig. 2A) and with the tumor volume at diagnosis (R² = 0.166 in simple regression analysis, \( P = 0.001 \); R² = 0.073 in Spearman rank correlation \( P = 0.017 \)) (Fig. 2B).

BRAF mutation and recurrence at follow-up

PTC recurrence occurred in 22.8% of patients after a median follow-up of 5.1 yr (range 3–195 months) (Supplemental Table 4). Recurrence was 2.1 times more frequent among the BRAFV600E-positive patients than among the BRAFV600E-negative patients identified by BigDye Terminator sequencing (33.3% and 15.6%, \( P = \) 0.012).

TABLE 1. BRAF mutation status and clinicopathological characteristics of PTC

<table>
<thead>
<tr>
<th></th>
<th>BigDye Terminator sequencing</th>
<th>Pyrosequencing</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>BRAFV600E</td>
<td>BRAFwild type</td>
</tr>
<tr>
<td>Number of patients (%)</td>
<td>62 (36.9%)</td>
<td>106 (63.5%)</td>
</tr>
<tr>
<td>Percentage men</td>
<td>21.3%</td>
<td>20.8%</td>
</tr>
<tr>
<td>Age at diagnosis (yr), median range)</td>
<td>46.4 (14–81)</td>
<td>45.8 (21–82)</td>
</tr>
<tr>
<td>Tumor volume (ml), mean range)</td>
<td>13.3 (0.01–178)</td>
<td>15.1 (0.01–86.5)</td>
</tr>
<tr>
<td>Multifocality (%)</td>
<td>47.4%</td>
<td>36.3%</td>
</tr>
<tr>
<td>Extraplyroidal extension (%)</td>
<td>54.5%</td>
<td>42.2%</td>
</tr>
<tr>
<td>Lymph node metastasis (%)</td>
<td>72.2%</td>
<td>49.4%</td>
</tr>
<tr>
<td>AJCC stages (%)</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>50.0%</td>
<td>52.9%</td>
</tr>
</tbody>
</table>

P values are for comparison by ANOVA (continuous variables) or \( \chi^2 \) analysis (categorical variables) of BRAFV600E with BRAFwild type by BigDye Terminator sequencing and by pyrosequencing, respectively. Samples with BRAFV600E less than 5% by pyrosequencing were negative as per definition in Patients and Methods. n.s., Not significant (\( P > 0.05 \)); AJCC, American Joint Committee on Cancer.

TABLE 2. Clinicopathologic profile by level of BRAFV600E (four arbitrary groups) by pyrosequencing analysis

<table>
<thead>
<tr>
<th>BRAFV600E groups</th>
<th>1 (&lt;5%)</th>
<th>2 (5–14%)</th>
<th>3 (15–29%)</th>
<th>4 (≥30%)</th>
<th>Linearity</th>
<th>Contrast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>78</td>
<td>27</td>
<td>40</td>
<td>23</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Percentage men</td>
<td>24.4%</td>
<td>18.5%</td>
<td>20.0%</td>
<td>13.0%</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Age at diagnosis (yr), median</td>
<td>45.2</td>
<td>42.5</td>
<td>47.4</td>
<td>51.0</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Tumor volume (ml), mean</td>
<td>18.6</td>
<td>12.0</td>
<td>6.0</td>
<td>21.3</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Multifocality (%)</td>
<td>32.9%</td>
<td>51.9%</td>
<td>45.0%</td>
<td>45.0%</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Extraplyroidal extension (%)</td>
<td>43.2%</td>
<td>40.0%</td>
<td>52.5%</td>
<td>52.6%</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Lymph node metastasis (%)</td>
<td>54.5%</td>
<td>50.0%</td>
<td>60.0%</td>
<td>82.4%</td>
<td>0.080</td>
<td>n.s.</td>
</tr>
<tr>
<td>AJCC stages (%)</td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>69.3%</td>
<td>76.9%</td>
<td>57.5%</td>
<td>50.0%</td>
<td>n.s.</td>
<td>0.045</td>
</tr>
<tr>
<td></td>
<td>4.0%</td>
<td>0.0%</td>
<td>2.5%</td>
<td>10.0%</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>13.3%</td>
<td>23.1%</td>
<td>27.5%</td>
<td>40.0%</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>13.3%</td>
<td>0.0%</td>
<td>12.5%</td>
<td>0.0%</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

n.s., Not significant; AJCC, American Joint Committee on Cancer.
0.040, respectively). The trend was not significant when the BRAF mutation status was determined by pyrosequencing (26.8 and 17.3%, \( P > 0.5 \)).

When the four groups listed in Table 2 were considered, PTC persistence or recurrence occurred in 17.3% of group 1, 20.0% of group 2, 15.6% of group 3, and 52.6% of group 4 (\( P = 0.024 \) by analysis of linearity and \( P = 0.009 \) by analysis of contrast). Figure 3 shows the Kaplan-Meier analysis of the association between BRAF\(_{V600E}\) and disease recurrence over time. Among stage I PTC, 12.8% of PTC had percentages of the BRAF\(_{V600E}\) allele of 30% or greater, 40% of which recurred. Importantly, the percentage of patients experiencing recurrence of stage I PTC with percentages of the BRAF\(_{V600E}\) alleles less than 30% was only 11.8%. The sample number was small, and this difference was not significant (\( P = 0.06 \)). Table 3 reports the univariate odds ratio of BRAF for recurrence of PTC during follow-up in comparison with other variables at the time of diagnosis. Larger tumor volume and the presence of lymph node metastasis predicted a poorer disease outcome. In tumors with percentage of BRAF\(_{V600E}\) alleles of 30% or greater, the odds ratio of recurrent PTC was 5.31-fold higher than in tumors with percentages of BRAF\(_{V600E}\) alleles less than 30% (\( P = 0.002 \)).

**Discussion**

Although the BRAF mutation and its correlations with clinical features at diagnosis and with outcome are the subject of controversy, most studies have proposed that PTC harboring the BRAF\(_{V600E}\) mutation exhibit greater incidences of extrathyroidal extension, lymph node metastasis, and advanced stages and, more importantly, seem to have a poorer clinical outcome. These conclusions resulting from clinical studies are supported by experimental studies in animal models and in cell culture. The transforming activity of BRAF mutations in the thyroid has been demonstrated in transgenic mice, in which the mutated gene induces the development of tumors with features of aggressive papillary cancer (15–17). The expression of BRAF\(_{V600E}\) in thyroid cells in culture demonstrated a stronger oncogenic potential and increased matrix cell invasion with respect to RET/PTC, the other most frequent genetic alteration in thyroid cancer, thus supporting the clinical observation that PTC harboring the BRAF mutation is a more aggressive cancer (18, 19). Moreover, BRAF\(_{V600E}\) inhibits characteristic thyroid functions such as iodide trapping, rendering the PTC less sensitive to \( ^{131} \)I treatment with a consequently increased cancer recurrence rate (1, 20, 21). Nonetheless, many studies failed to confirm the association between the BRAF mutation and high-risk clinicopathological factors or poorer outcome (2, 4, 22–26). Although many factors have been considered, including the small number of samples, the incompleteness of the patient records, the variability of the histological subtype, and the methods used to detect BRAF mutation, there is no definitive explanation for these inconsistent results.

**FIG. 2.** Correlation between patient age or tumor volume and the percentage of mutant BRAF alleles. PTC harboring 5–47.5% BRAF\(_{V600E}\) are plotted vs. patient age (A) and tumor volume (B). The significances were \( P = 0.039 \) and \( P < 0.001 \), respectively.

**FIG. 3.** Kaplan-Meier curves for disease-free survival for papillary thyroid cancer patients. Follow-up of patients with PTC with or without the BRAF mutation (left panel) or with a percentage of the BRAF\(_{V600E}\) allele less than 30% or 30% or greater (right panel). The BRAF mutation status was determined by pyrosequencing in tumor tissues. A log-rank test indicated that the curves were significantly different in the right panel (BRAF\(_{V600E}\) < 30% vs. \( \geq 30\%\), log rank = 6.71, \( P = 0.009 \)) but not in the left panel (BRAF\(_{V600E}\) vs. BRAF\(_{\text{wild-type}}\), log-rank = 0.87, \( P = 0.350 \)).
Multifocality, extrathyroidal extension, and lymph node metastasis were more frequent in the tumors with percentages of the \( \text{BRAF}^{V600E} \) allele of 30% or greater than in those harboring the \( \text{BRAF}^{\text{wild-type}} \) allele, although the differences were not significant. Although the overall number of patients in the study was quite large (\( n = 168 \)), the reduced number of samples in each group (only 24 for the group with \( \text{BRAF}^{V600E} \geq 30\% \)) might account for the absence of significance in the statistical analysis. A larger study could finally address this issue.

An interesting finding of our analysis is the direct correlation between the percentage of \( \text{BRAF}^{V600E} \) alleles and the tumor volume. If PTC cells harboring \( \text{BRAF}^{V600E} \) grow faster or survive longer than their counterparts harboring \( \text{BRAF}^{\text{wild-type}} \), the \( \text{BRAF}^{V600E} \) to \( \text{BRAF}^{\text{wild-type}} \) ratio would increase over time, and older tumors would be larger and would contain a higher percentage of cells with the \( \text{BRAF} \) mutation. This explanation fits with the observation that a higher percentage of \( \text{BRAF}^{V600E} \) alleles is present in the tumors of older subjects. However, it is also possible that the \( \text{BRAF}^{V600E}/\text{BRAF}^{\text{wild-type}} \) ratio remains constant over time and that the paracrine secretion of the \( \text{BRAF}^{V600E} \)-harboring cells stimulates the entire tumor to grow larger. According to this hypothesis, many factors, including chemokines, are secreted by PTC cells with the \( \text{BRAF} \) mutation, and these factors exert both autocrine and paracrine effects (27).

The most important finding of this study is the demonstration that determining the percentage of mutant \( \text{BRAF} \) alleles in surgical PTC specimens predicts the disease outcome. The finding that this association is restricted to tumors with a high percentage of mutant \( \text{BRAF} \) alleles reinforces the concept that PTC are heterogeneous tumors and that the clonal, subclonal, or oligoclonal occurrence of this mutation defines tumors with different biological and clinical features. Previous studies analyzed the possible correlation between the \( \text{BRAF} \) mutation status and the recurrence or persistence of the disease. In these studies, the tumor recurrence was variably associated with both clinicopathological features and disease outcome.

The postsurgical knowledge of the quantitative \( \text{BRAF} \) mutation status in tissue samples may be used to determine the most appropriate postoperative medical treatments and follow-up procedures in PTC patients. These procedures include \( {^{131}}\text{I} \) remnant ablation, long-term TSH sup-

### TABLE 3. Association with recurrence

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (yr)</td>
<td>1.01</td>
<td>0.983–1.039</td>
<td>n.s.</td>
</tr>
<tr>
<td>Male gender</td>
<td>0.96</td>
<td>0.345–2.677</td>
<td>n.s.</td>
</tr>
<tr>
<td>Tumor volume(^a)</td>
<td>1.02</td>
<td>1.006–1.045</td>
<td>0.012</td>
</tr>
<tr>
<td>Log tumor volume(^b)</td>
<td>2.16</td>
<td>1.252–3.740</td>
<td>0.006</td>
</tr>
<tr>
<td>Multifocality</td>
<td>2.86</td>
<td>1.132–7.210</td>
<td>0.026</td>
</tr>
<tr>
<td>Extrathyroidal extension</td>
<td>2.01</td>
<td>0.810–4.987</td>
<td>n.s.</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>7.72</td>
<td>1.699–35.117</td>
<td>0.008</td>
</tr>
<tr>
<td>AJCC stages</td>
<td>1.65</td>
<td>1.124–2.424</td>
<td>0.011</td>
</tr>
<tr>
<td>( \text{BRAF}^{V600E} ) continuous</td>
<td>1.04</td>
<td>1.008–1.071</td>
<td>0.014</td>
</tr>
<tr>
<td>( \text{BRAF}^{V600E} ) vs. ( \text{BRAF}^{\text{wt}} )</td>
<td>1.75</td>
<td>0.717–4.251</td>
<td>0.220</td>
</tr>
<tr>
<td>( \text{BRAF}^{V600E} &lt;30% ) vs. ( \geq30% )</td>
<td>5.31</td>
<td>1.888–14.929</td>
<td>0.002</td>
</tr>
</tbody>
</table>

This was a simple logistic regression analysis. \( \text{BRAF} \) status was evaluated by pyrosequencing. CI, Confidence interval; AJCC, American Joint Committee on Cancer.

\(^a\) Odds ratio per milliliter of tumor volume.

\(^b\) Odds ratio per 10 ml tumor volume.

The recent finding of intratumoral heterogeneity with respect to \( \text{BRAF} \) mutation in PTC offers a new explanation for this controversy (10). In the present study, the percentage of mutant \( \text{BRAF} \) alleles, the clinicopathological features, and the disease outcomes were analyzed in a large cohort of PTC patients, demonstrating that the correlation with the disease outcome is not a feature of any PTC harboring the \( \text{BRAF} \) mutation but that this correlation is instead restricted to PTC with a high percentage of \( \text{BRAF}^{V600E} \) alleles. The direct sequencing of \( \text{BRAF} \) using the BigDye Terminator method was revealed to be less sensitive than pyrosequencing, with \( \text{BRAF}^{V600E} \) detected in 36.9 or 53.6% of PTC, respectively. BigDye Terminator sequencing is an analytical method, and the interpretation of the results can be difficult when the genetic material is not homogeneous. In general, the sensitivity of this method does not allow the definitive identification of a single-point mutation when the mutation is present in less than 20% of the DNA in a mixture (5). Because of this limit, a conservative interpretation of the results of direct sequencing preferentially recognizes as positive tumors with a higher percentage of \( \text{BRAF}^{V600E} \) alleles. In our analysis, the median percentage of mutant \( \text{BRAF} \) alleles in the samples considered positive by direct sequencing was higher than the median percentage for samples considered positive by pyrosequencing (24.1 and 20.6%, respectively), indicating that samples with a lower percentage of mutant alleles were considered wild type by direct sequencing. A less conservative interpretation of direct sequencing analysis that would also consider as positive the samples with a low percentage of the mutation might explain why even some large studies failed to find a correlation between \( \text{BRAF}^{V600E} \) and clinicopathological features.
pression by T₄, basal and/or stimulated thyroglobulin monitoring, neck ultrasonography, and radiiodine whole-body scanning (12, 30). In general, patients with PTC have a very good prognosis, and for this reason, the need for radiiodine ablation, the degree of TSH suppression, and the follow-up visit intervals are still debated, especially in low-risk patients. Nevertheless, the current clinicopathological criteria used to classify PTC as low or high risk appear in some cases to be inadequate. In the future, molecular subtyping will be useful for the clinic to refine the risk stratification and to tailor the postoperative management of patients with PTC. BRAFV600E detection by pyrosequencing or by other quantitative methods can be used for the molecular subtyping of PTC, provided that a careful microscopic observation of the sample excludes the presence of a large nontumoral cell component. Preoperative BRAFV600E analysis was demonstrated to be useful as an adjunctive diagnostic tool along with conventional cytological evaluation. Recently the pyrosequencing method of detection has been applied to BRAFV600E in thyroid cytology samples (31–33). Quantitative preoperative knowledge of the percentage of BRAFV600E alleles determined by pyrosequencing may also help to determine the PTC risk category, thus indicating the best follow-up strategy and, in particular, the appropriate extents of thyroidectomy and central compartment dissection, the need for ¹³¹I remnant ablation, and the appropriate degree of TSH suppression (28, 34–36).

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