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BRAF Mutation Predicts a Poorer Clinical Prognosis for Papillary Thyroid Cancer

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Context: Use of *BRAF* mutation in papillary thyroid cancer (PTC) has the potential to improve risk stratification of this cancer.

Objective: The objective of the study was to investigate the prognostic value of *BRAF* mutation in patients with PTC.

Design, Setting, and Subjects: In a multicenter study of 219 PTC patients, data on their clinicopathological characteristics and clinical courses between 1990 and 2004 were retrospectively collected, and their tumor *BRAF* mutation status was determined. Associations of *BRAF* mutation with initial tumor characteristics and subsequent recurrence were analyzed.

Main Outcome Measure: Relationships between the *BRAF* mutation status and clinicopathological outcomes, including recurrence, were measured.

Results: We found a significant association between *BRAF* mutation and extrathyroidal invasion ($P < 0.001$), lymph node metastasis ($P < 0.001$), and advanced tumor stage III/IV ($P = 0.007$) at initial surgery. This association remained significant on multivariate analysis, adjusting for conventional clinicopathological predictors of recurrence excluding the histological PTC subtype, but was lost when the tumor subtype was included in the model. *BRAF* mutation was also significantly associated with tumor recurrence, 25 *vs.* 9% with and without mutation, respectively ($P = 0.004$), during a median of 15 (interquartile range, 3–29) months of follow-up. This association remained significant on multivariate analysis adjusting for conventional clinicopathological predictors of recurrence, even including the PTC subtype (odds ratio, 4.0; 95% confidence interval, 1.1–14.1; $P = 0.03$). *BRAF* mutation was even an independent predictor of recurrence in patients with stage I/II disease, 22 *vs.* 5% with and without *BRAF* mutation, respectively ($P = 0.002$). *BRAF* mutation was also more frequently associated with absence of tumor I-131 avidity and treatment failure of recurrent disease.

Conclusions: In patients with PTC, *BRAF* mutation is associated with poorer clinicopathological outcomes and independently predicts recurrence. Therefore, *BRAF* mutation may be a useful molecular marker to assist in risk stratification for patients with PTC. **(*J Clin Endocrinol Metab* 90: 6373–6379, 2005)**

RAF KINASE IS a component of the RAS→RAF→MAPK kinase→ERK/MAPK signaling pathway, which plays a central role in the regulation of cell growth, division, and proliferation (1, 2). Among several isoforms of RAF kinase, the B-type (BRAF) is the strongest activator of the downstream MAPK signaling. When this pathway is constitutively activated, it causes tumorigenesis. Mutations of the *BRAF* gene have been found in a variety of human cancers, most notably in melanomas (3). The most common *BRAF* mutation is the T1799A transversion mutation (formerly named *BRAF* T1796A mutation) in exon 15 of the gene, which causes a V600E (formerly named V599E) amino acid substitution in the protein and consequent constitutive activation of the kinase (3). Recent studies have shown that this *BRAF* mutation occurs in papillary thyroid cancer (PTC) with a prevalence ranging from 29 to 83% (4–19). The T1799A *BRAF* mutation occurs exclusively in PTC and PTC-derived anaplastic thyroid cancers but not in normal thyroid tissue or benign thyroid neoplasms or follicular or medullary thyroid cancers.

PTC is the most common thyroid cancer, accounting for 80% or more of thyroid malignancies (20, 21). Although PTC is usually indolent and curable with surgical thyroidectomy, often followed by radioiodine treatment, many patients suffer disease recurrence, which in some cases proves to be incurable and fatal (22–27). Therefore, risk stratification is

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Abbreviations: BRAF, B-type RAF kinase; CI, confidence interval; PTC, papillary thyroid cancer.

important to identify patients at higher risk of recurrence so more aggressive therapy and monitoring can be implemented. This prognostication has traditionally been based on the presence or absence of certain clinical and histopathological risk factors, such as patient age and gender, tumor size and histological subtype, and extrathyroidal and metastatic spread of disease (22–27). However, these criteria often leave uncertainty regarding the risk of tumor progression and recurrence. Moreover, histopathological criteria are typically not defined preoperatively, and consequently, they cannot contribute to decision making about the optimal extent of initial surgery.

Because *BRAF* mutation appears to play an important role in PTC tumorigenesis, it has been postulated that this mutation might have prognostic value. Namba *et al.* (9) reported an association of *BRAF* mutation with distant metastasis of PTC. Nikiforova *et al.* (10) found an association of *BRAF* mutation with higher prevalences of extrathyroidal invasion and advanced pathological stage of PTC. Kim *et al.* (18) found an association of *BRAF* mutation with lymph node metastasis. Other studies (*e.g.* Refs. 16 and 19), however, found no association of *BRAF* mutation with high-risk pathological characteristics. There has been no clear explanation for this controversy. Different sample sizes may contribute to the inconsistent results in these studies. The variable involvement of various confounding factors in these studies appears to be a more important cause for the inconsistent findings on the role of *BRAF* mutation in PTC. However, there has been no study using appropriate methods, such as multivariate analysis, to exclude the effects of confounding factors. Moreover, the probability of PTC recurrence and the aggressiveness of the recurrent tumor in relation to *BRAF* mutation status have not been previously evaluated. We here conducted a multicenter study in a large series of PTC patients using multivariate analysis to define the associations of *BRAF* mutation with clinicopathological characteristics and tumor recurrence of PTC and to determine the prognostic value of this novel genetic marker for PTC.

Patients and Methods

Patients and clinicopathological data collection

The study involved The Johns Hopkins University School of Medicine; The Yale University School of Medicine; The Hospital for Endocrine Surgery in Kiev, Ukraine; and The University of Bologna Hospital in Bologna, Italy, which contributed 141, 17, 29, and 32 patients, respectively, to comprise the 219 cases included in this study. Histologically, these included classical (n = 125), follicular variant (n = 77), tall cell (n = 16), and columnar variant (n = 1) PTC subtypes. With approval from the institutional review boards of the four medical institutions and appropriate consenting where required, we retrospectively reviewed the clinical records of these patients, who underwent thyroidectomy for PTC and were followed up over a period of 14 yr (between 1990 and 2004) at these medical institutions and whose thyroid tumor tissues were available for *BRAF* mutation analysis. The patients were randomly and consecutively selected. Information was abstracted from their records using a standard protocol to define the histopathological features of the tumor, and the demographic characteristics and clinical course of the patients (*i.e.* tumor recurrence and history of radioiodine treatment). The histological diagnosis was made by experienced pathologists (W.H.W., G.T., and V.V.) with hematoxylin and eosin staining. Anaplastic/undifferentiated thyroid cancers were excluded. Thyroid cancer recurrence was confirmed by positive radioiodine body scan, serum thyroglobulin detectability after the cure of initial disease, and/or investigation of a tumor mass that was confirmed cytologically or pathologically to be thyroid cancer. Children under the age of 18 yr at diagnosis of thyroid cancer were excluded.

To assure that all patients included were adults with sporadic PTC and avoid confounding effects of radiation-induced thyroid cancers in the Ukrainian patients, patients who were younger than 20 yr at the time of the Chernobyl nuclear accident were excluded. The *BRAF* mutation status of a portion of the tumors has already been reported in our previous studies (5, 11–14). *BRAF* mutation data from a previous study in which fine-needle aspiration specimens from patients whose biopsied thyroid nodule was definitively correlated with a subsequent histological diagnosis were also used (13). All of these PTC patients underwent total or near total thyroidectomy. The clinical follow-up interval for those patients who had cancer recurrence was defined as the time from initial thyroid surgery to detection of their tumor recurrence. For those patients who did not have cancer recurrence, the follow-up interval was defined as the time from the initial thyroid surgery to the most recent clinical evaluation at their respective medical institutions.

Thyroid tumor tissues and DNA isolation

Fresh frozen or paraffin-embedded PTC samples from patients were microdissected and DNA isolated as previously described (28). After 8 h treatment at room temperature with xylene for tissues dissected from paraffin-embedded specimens, samples were subjected to digestion with 1% sodium dodecyl sulfate and 0.5 mg/ml proteinase K at 48 C for 48 h. To facilitate the digestion, a midinterval addition of a spiking aliquot of concentrated sodium dodecyl sulfate-proteinase K was added to the sample tubes. DNA was then isolated from the digested tissues by standard phenol-chloroform extraction and ethanol precipitation procedures.

Detection of BRAF mutation

Because the T1799A transversion mutation is virtually the only *BRAF* mutation that has been described in PTC with a high prevalence in previous studies, we sought this particular mutation in various subtypes of PTC in the present study. The *BRAF* T1799A mutation was analyzed using genomic DNA by direct sequencing and a colorimetric method using the Mutector kit (TrimGen, Baltimore, MD) as described previously (11, 13). For direct DNA sequencing, exon 15 of the *BRAF* gene was amplified by PCR, followed by Big Dye terminator cycle sequencing reaction and sequence reading on an ABI PRISM 3730 genetic analyzer (Applied Biosystems, Foster City, CA). The colorimetric method for *BRAF* mutation was based on the technique of shifted termination assay, which was demonstrated to have a 100% sensitivity and specificity for the detection of *BRAF* mutation (13).

Statistical analysis

Categorical data were summarized using frequencies and percents. Distributions of the continuous variables were assessed, and all but age at diagnosis were found to not be normally distributed. Therefore, these data were summarized with medians and interquartile ranges. Group comparisons of categorical variables were performed using the χ^2 test or, for small cell sizes, Fisher's exact test. Nonparametric statistics were used to compare the continuous variables. Comparisons of two groups were evaluated with the Wilcoxon rank sum test, and comparisons of three groups were done using the Kruskal-Wallis test. Multivariate logistic regression analyses were performed to assess the independent associations of *BRAF* mutation with extrathyroidal invasion, cervical lymph node metastasis, tumor stages as defined previously (26), and recurrence of the tumor, with adjustment for various established clinicopathological prognostic factors. The analysis of the effect of *BRAF* mutation on tumor recurrence was adjusted also for I-131 treatment. Product-limit survival analysis (29) and log-rank test were used to evaluate the effect of *BRAF* mutation on cancer recurrence. Proportional hazards regression analysis on tumor recurrence (30), with adjustment for the same variables as the dichotomous outcome, was performed to examine the risk for cancer recurrence associated with *BRAF* mutation. We compared baseline demographics and clinical characteristics by site. Gender, age at diagnosis, presence of *BRAF* mutation, tumor multifocality, stage and size, and follow-up time did not differ by site. Type of

tumor, lymph node metastasis, extrathyroidal invasion, tumor recurrence, and I-131 dose did differ by site. When site was included in the multivariate analyses, there was minimal change in the odds ratio for *BRAF* mutation, and we report the results without adjusting for site. Confidence intervals (CIs) were computed by standard methods. All reported *P* values are two sided. Analysis was performed using SAS software (versions 9.0 and 9.12; SAS Institute, Cary, NC).

Results

Association of BRAF mutation with high-risk histopathological features and recurrence of PTC

Consistent with previous reports (4–19), this series of PTC showed a high prevalence of *BRAF* mutation, 49% (95% CI, 42–56%), which was not different among the four medical institutions (Table 1). The overall prevalence of *BRAF* mutation in the present study was lower than that reported in one of our previous studies (5). This was because a significant number of follicular variant PTC were included in the present study, whereas the previous study comprised mainly classical and tall cell PTC, with the former known to harbor *BRAF* mutation with a low prevalence and the latter two with a high prevalence of *BRAF* mutation.

Overall analysis of these patients revealed significant associations of the *BRAF* mutation with extrathyroidal invasion, neck lymph node metastasis, and more advanced initial tumor stage (Table 2). These are three pathological features conventionally associated with a higher risk of thyroid cancer recurrence. There was no significant association of *BRAF* mutation with patient age or gender or tumor multifocality.

Tumor recurrence also occurred significantly more often in the *BRAF* mutation-positive group than the mutation-negative group of PTC, 25 *vs.* 9% ($P = 0.004$), respectively. Most tumor recurrences were in the cervical, thyroid bed, and paratracheal regions except in five patients who also had mediastinal, pulmonary, or bone metastases. There was no significant difference in clinical follow-up durations between the *BRAF* mutation-positive and -negative groups, with a median of 16.5 months (interquartile range, 5–30) and 14 months (interquartile range, 2–28) for the two groups, respectively ($P = 0.40$). There was also no significant difference in the proportion of *BRAF* mutation-positive and -negative patients who received postoperative radioiodine-131 treatment (Table 2). It is notable that the tumor sizes in *BRAF* mutation-positive patients were significantly smaller than that of the mutation-negative group (Table 2), suggesting that the *BRAF* mutation-associated tumor aggressiveness might occur at a relatively early tumor stage. Analysis of the Hopkins group alone revealed a *P* value that fell just short of significance for some parameters examined (data not shown), in contrast to the overall analysis of the larger number of all the cases from the four medical institutions.

TABLE 1. Case contribution and prevalence of BRAF mutation by study site

	No. of cases	No. of BRAF mutation-positive cases	Prevalence of BRAF mutation (%)
Site[a]			
Johns Hopkins	141	64	45
Yale	17	8	47
Ukraine	29	16	55
Italy	32	19	59
Total	219	107	49

[a] Fisher's exact test, $P = 0.47$.

TABLE 2. Correlation between clinicopathological characteristics and BRAF mutation status in patients with PTC

	BRAF+	BRAF−	*P* value
n (total)	107	112	
Age at diagnosis (yr)	43 (35–56)	45 (38–55)	0.41
Gender, male	29 (27)	29 (26)	0.84
Tumor size (cm)	2.0 (1.4–3.0)[a]	2.4 (1.5–3.5)[b]	0.009
Multifocality	45 (42)	42 (38)	0.49
Extrathyroidal invasion	44 (41)	18 (16)	<0.001
Lymph node metastasis	58 (54)	24 (21)	<0.001
Tumor stage[c]			0.002
I	44 (42)	39 (35)	
II	30 (29)	57 (51)	
III	29 (28)	15 (13)	
IV	2 (2)	1 (1)	
Tumor stage III/IV	31 (30)[c]	16 (14)	0.007
Tumor recurrence	23 (25)[d]	9 (9)[e]	0.004
No. of I-131 treatments			0.69
0	19 (22)	26 (28)	
1	68 (77)	67 (71)	
2	1 (1)	1 (1)	
Total I-131 dose (mCi)	100 (32–100)[d]	100 (0–101)[e]	0.96
Total follow-up (months)	16.5 (5–30)[d]	14.0 (2–28)[e]	0.40

Median (interquartile range) or n (%).

[a] Seven cases had no information on tumor size.

[b] Two cases had no information on tumor size.

[c] Two cases had insufficient data to define tumor stage in the *BRAF* mutation-positive group. If, on a conservative assumption, both cases had a tumor stage less than III/IV and were included in the analysis, the prevalence of tumor stages III/IV in *BRAF* mutation-positive group would be 29% (instead of 30%) and the *P* value would be 0.008 (instead of 0.007).

[d] Fifteen cases had no information on tumor recurrence, treatment, and follow-up.

[e] Sixteen cases had no information on tumor recurrence, treatment, and follow-up.

BRAF mutation was associated with PTC subtypes with high-risk pathological features and more frequent recurrence. The prevalences of BRAF mutation, extrathyroidal tumor invasion, cervical lymph node metastasis, advanced tumor stages, and tumor recurrence differed among the PTC subtypes in a three-way comparison. All of these features were more common in tall cell PTC, followed by classical PTC, and less frequent in follicular variant PTC (Table 3). This three-way comparison did not show significant differences in the patient age and gender, tumor multifocality, follow-up duration, and radioiodine treatments among the three PTC subgroups.

Pair-wise analysis of tumor types on *BRAF* mutation, extrathyroidal invasion, lymph node metastasis, advanced tumor stage, and tumor recurrence further confirmed a significant relationship between *BRAF* mutation and these high-risk pathological and clinical characteristics (Table 4). Specifically, the frequency of each of these high-risk pathological features and cancer recurrence was higher in *BRAF* mutation-prevalent tall cell and classical PTC than it was in *BRAF* mutation-infrequent follicular variant PTC. The prevalences of *BRAF* mutation, extrathyroidal tumor invasion, lymph node metastasis, advanced tumor stage, and tumor recurrence tended to be more frequent in tall cell PTC than classical PTC (Table 3), but the difference on pair-wise analysis was not statistically significant

TABLE 3. Three-way comparison of the clinicopathological characteristics and *BRAF* mutation status in various subtypes of PTC

Characteristic	Classic	Follicular	Tall cell	*P* value
n (total)	125	77	16	
BRAF+ mutation	81 (65)	11 (14)	14 (88)	<0.001
Age at diagnosis (yr)	44 (38–56)	44 (35–52)	64 (39–73)	0.06
Gender, male	35 (28)	17 (22)	5 (31)	0.58
Tumor size (cm)	2.0 (1.5–2.7)[a]	2.8 (1.5–3.5)	2.3 (1.9–3.5)	0.004
Multifocality	55 (44)	25 (32)	7 (44)	0.25
Extrathyroidal invasion	47 (38)	5 (6)	10 (63)	<0.001
Lymph node metastasis	65 (52)	7 (9)	9 (56)	<0.001
Tumor stage[b]				0.003
I	48 (39)	31 (40)	4 (25)	
II	43 (35)	39 (51)	4 (25)	
III	30 (24)	6 (8)	8 (50)	
IV	2 (2)	1 (1)	0 (0)	
Tumor stage III/IV	32 (26)[b]	7 (9)	8 (50)	<0.001
Tumor recurrence	21 (21)[c]	5 (7)[d]	5 (31)	0.02
No. of I-131 treatments				0.83
0	25 (26)	16 (24)	3 (20)	
1	71 (72)	52 (76)	12 (80)	
2	2 (2)	0 (0)	0 (0)	
Total I-131 dose	100[c] (0–103)	100[d] (25–100)	100 (50–100)	0.42
Total follow-up (months)	16.0 (5–30)[c]	13.0 (2–27)[d]	20.5 (8–34)	0.55

Median (interquartile range) or n (%).
[a] Nine cases had no information on tumor size.
[b] Two cases had insufficient data to define tumor stage in the classic PTC group.
[c] Twenty-three cases had no information on tumor recurrence, treatment, and follow-up.
[d] Eight cases had no information on tumor recurrence, treatment, and follow-up.

(Table 4), perhaps due to the already relatively high prevalences of these events in both groups. Similarly, no significant association of *BRAF* mutation with these high-risk pathological parameters was seen within the classical PTC subgroup alone (data not shown).

Association of BRAF mutation with poorer tumor prognosis was independent of classical clinicopathological risk factors in PTC. Because patient age at diagnosis, gender, tumor size, multifocality, and histological type of PTC have been previously shown to be associated with clinical outcomes for patients with thyroid cancer (22–27), we performed a multivariate analysis adjusting for these known factors to identify independent correlations between *BRAF* mutation status and each of the three high-risk pathological features (*i.e.* tumor extrathyroidal invasion, neck lymph node metastasis, and advanced tumor stages) and tumor recurrence. For tumor recurrence, an additional adjustment for radioiodine treatment was also made because such treatment may alter the clinical outcome. This multivariate analysis showed significantly increased odds of the three high-risk pathological features and tumor recurrence for those positive for the *BRAF* mutation with adjustments for patient age, gender, tumor size, and multifocality (Table 5). Even with additional adjustment for extrathyroidal tumor invasion and tumor stage III/IV, the association of *BRAF* mutation with lymph node metastasis was still highly significant, suggesting that

TABLE 4. Pair-wise comparison of the *BRAF* mutation and clinicopathological characteristics among the three subtypes of PTC (*P* values)[a]

	Tall cell *vs.* follicular variant	Classical *vs.* tall cell	Classical *vs.* follicular
BRAF mutation	<0.001	0.09	<0.001
Extrathyroidal invasion	<0.001	0.06	<0.001
Lymph node metastasis	<0.001	0.80	<0.001
Tumor stages III/IV	<0.001	0.07	0.003
Tumor recurrence	0.02	0.34	0.02

[a] *P* values of pair-wise comparisons are from Fisher's exact test on those high-risk clinicopathological features and tumor recurrence that were significantly different on three-way comparison (Table 3).

TABLE 5. Multivariate analyses of the association of *BRAF* mutation with the high-risk clinicopathological outcomes of patients with PTC

Clinicopathological outcomes	*BRAF* mutation		
	Odds ratio	95% Confidence interval	*P* value
Extrathyroidal invasion[a]	4.0	2.0–7.9	<0.001
Lymph node metastasis[a]	5.0	2.6–9.5	<0.001
Tumor stage III/IV[a]	5.8	2.2–15	<0.001
Tumor recurrence[b]	4.8	1.7–14	0.003
Extrathyroidal invasion[c]	1.9	0.89–4.2	0.10
Lymph node metastasis[d]	3.1	1.5–6.5	0.002
Tumor stage III/IV[e]	2.9	0.87–9.5	0.09
Tumor recurrence[f]	4.4	1.3–14	0.02
Extrathyroidal invasion[g]	1.2	0.5–2.8	0.68
Lymph node metastasis[g]	1.9	0.8–4.3	0.13
Tumor stage III/IV[g]	3.0	0.8–11.0	0.10
Tumor recurrence[h]	4.0	1.1–14.1	0.03

[a] Adjusted for age at diagnosis, gender, multifocality, and tumor size.
[b] In addition to *footnote a*, also adjusted for I-131 treatment.
[c] In addition to *footnote a*, also adjusted for lymph node metastasis and tumor stage III/IV.
[d] In addition to *footnote a*, also adjusted for extrathyroidal invasion and tumor stage III/IV.
[e] In addition to *footnote a*, also adjusted for extrathyroidal invasion and lymph node metastasis.
[f] In addition to *footnote b*, also adjusted for extrathyroidal invasion, lymph node metastasis, and tumor stage III/IV.
[g] In addition to *footnotes c, d, and e,* also adjusted for PTC subtypes (classic, follicular-variant, and tall-cell subtypes).
[h] In addition to *footnote f*, also adjusted for PTC subtypes.

BRAF mutation is an independent factor that predicts, and perhaps contributes to, lymph node metastasis. When tumor stages III/IV and lymph node metastasis were additionally adjusted in the multivariate analysis for the association of *BRAF* mutation with extrathyroidal tumor invasion and when extrathyroidal tumor invasion and lymph node metastasis were additionally adjusted in the analysis for the association of *BRAF* mutation with tumor stage III/IV, these relationships fell short of statistical significance. This is not surprising because the statuses of extrathyroidal invasion and lymph node metastasis had already been considered in determining tumor stages. When the histological subtypes (classic, follicular variant and tall cell) of PTC were added to the multivariate analysis, the significance of *BRAF* mutation association with these high-risk pathological characteristics was lost (Table 5). This is most likely because the *BRAF* mutation most often occurs in the PTC subtypes (classic and tall cell variants) that are most often associated with high-risk pathological features of the tumor (Tables 3 and 4), supporting the idea that *BRAF* mutation contributes to the aggressiveness of these PTC subtypes known to be more clinically aggressive.

Remarkably, the significant association of *BRAF* mutation with tumor recurrence was sustained even with adjustment for all of the pathological factors known to be associated with poor thyroid cancer outcome, including extrathyroidal tumor invasion, lymph node metastasis, advanced tumor stage, and subtype of PTC ($P = 0.03$; Table 5). Because tall cell PTC is well known to be associated with a poorer prognosis, we further performed logistic regression with tall cell PTC excluded from the analysis and a significant association of *BRAF* mutation with tumor recurrence was still seen in the remainder of the tumors ($P = 0.02$). These data thus demonstrate that *BRAF* mutation is a strong and independent predictor for a poorer prognosis of PTC.

Kaplan-Meier survival analysis revealed a significantly lower tumor recurrence-free probability in PTC patients with *BRAF* mutation than in patients without the mutation (Fig. 1). Cox proportional hazards regression analysis, adjusting for age at diagnosis, gender, tumor size and multifocality, and total dose of I-131 treatment revealed a risk ratio of 3.2 (95% CI, 1.3–7.7; $P = 0.01$) for *BRAF* mutation-associated tumor recurrence. When extrathyroidal invasion, lymph node metastasis, and advanced tumor stage were additionally adjusted, the risk ratio was 2.4 (95% CI, 0.97–5.9; $P = 0.06$), just marginally short of statistical significance. These results further demonstrate the prognostic value of *BRAF* mutation in predicting a poorer course of PTC.

Recurrent thyroid cancer was more common, even in patients with conventionally low-grade initial disease and was more aggressive when BRAF mutation was present. To define whether *BRAF* mutation could predict the clinical course for patients with lower initial disease stages, we examined the frequency of thyroid cancer recurrence among the subgroup of patients who had initial clinicopathological stages I and II. Thyroid cancer recurred more often in *BRAF* mutation-positive than *BRAF* mutation-negative patients with stage I/II disease, 14/64 (22%) *vs.* 4/83 (5%), respectively ($P = 0.002$). For these patients with low initial tumor stages, a model of the recurrence probability adjusting for age, gender, multifocality, tumor size, cumulative I-131 dose, lymph node metastasis, extrathyroidal invasion, and PTC subtypes still revealed a significant association between *BRAF* mutation and thyroid cancer recurrence (odds ratio, 11.6; 95% CI, 2.2–62.6; $P = 0.004$).

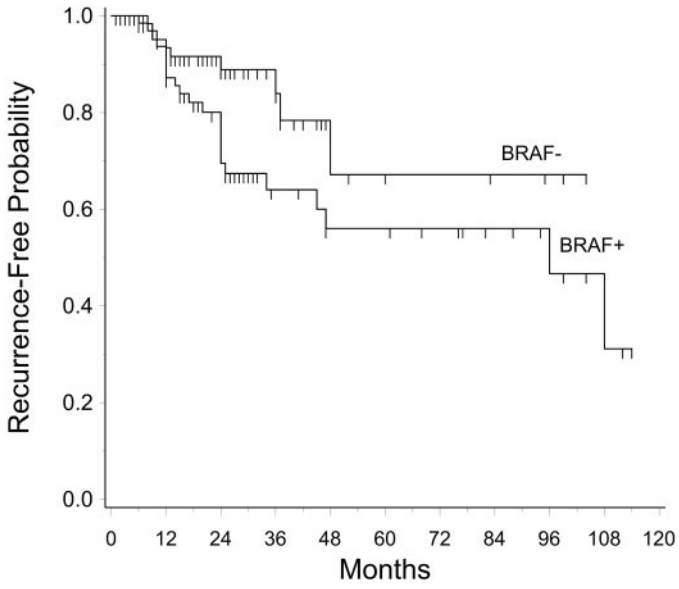

FIG. 1. Kaplan-Meier estimate of cancer recurrence-free probability in *BRAF* mutation-positive and -negative papillary thyroid cancers. *Short vertical lines* indicate censored observations (months of follow-up for those that have not had a recurrence). Log-rank test; $\chi^2 = 4.0$, $P = 0.04$.

We performed a further analysis to determine the extent of recurrent disease and modes of required treatment in the subgroup of patients from Johns Hopkins, for which more clinical information was available. Twenty recurrent PTC patients had been thoroughly evaluated for recurrent thyroid cancer with sufficient information for further analysis. Recurrent disease was more extensive and needed more aggressive treatments (surgical and external radiation therapies) in the *BRAF* mutation-positive patients than in the mutation-negative patients. In addition to radioiodine treatment, nine of 13 (69%) recurrent patients with *BRAF* mutation needed at least one additional surgery and/or external radiation therapy whereas only one of seven (14%) recurrent patients without the mutation needed additional surgery and no radiotherapy ($P = 0.057$). Moreover, seven of 13 (54%) patients in the *BRAF* mutation-positive group *vs.* none of seven (0%) in the *BRAF* mutation-negative group lacked I-131 avidity in their foci of recurrent tumor ($P = 0.04$). The patients who lost I-131 avidity in the recurrent tumor continued to have active disease, even after repeated surgeries or external radiotherapy. In the *BRAF* mutation-negative group of patients with recurrent disease, six patients with recurrent tumors were cured by repeating I-131 treatment alone, and the remaining one patient in this group was cured by an additional surgery and one further I-131 treatment.

Discussion

Follicular epithelial cell-derived thyroid cancers are the most common and increasingly incident endocrine malig-

nancies. PTC accounts for the vast majority of thyroid cancers (20, 21). Although PTC is generally an indolent cancer with a favorable long-term survival rate with the current standard treatments (22–27), many patients suffer recurrence, and some become incurable and die of this disease. To reduce the morbidity and mortality of PTC and optimize management of afflicted patients, their initial extent of disease is routinely defined and the risk of recurrence predicted. Older patient age at diagnosis and male gender portend a poorer prognosis. Pathologically, larger tumor size, extrathyroidal invasion, distant metastasis, cervical lymph node metastases, and tumor multifocality have all been shown to predict higher rates of subsequent recurrence. Despite the prognostic value of these clinical and pathological prognostic factors, however, there remains uncertainty regarding an individual patient's likelihood of tumor recurrence. Moreover, complete risk evaluation based on current pathological criteria is typically not possible until the thyroid surgery has been performed, when the full characteristics of the tumor become known. Consequently, it is often difficult to define recommendations for optimal initial surgical treatment (*e.g.* bilateral *vs.* unilateral thyroidectomy and neck lymph node dissection *vs.* no dissection), even though appropriate extent of initial surgery has been associated with a distinct outcome advantage (23, 31). Postoperatively, the need for radioiodine treatment and intensity of TSH-suppressive thyroxine therapy must also be determined based on the clinicopathological risk evaluation. Finally, clinicians must define the degree of vigilance to be applied in postoperative monitoring. Consequently, the availability of a novel informative prognostic marker, such as the *BRAF* mutation, which can now be readily detected even preoperatively in fine-needle-aspirated cytological materials (11, 13, 32, 33), has the potential to improve risk stratification and recurrence prediction in patients with PTC, better informing decisions about initial and long-term management.

The T1799A transversion *BRAF* mutation is the most common known genetic alteration in thyroid cancer and occurs exclusively in PTC and PTC-derived anaplastic thyroid cancers (4–19). It has been hypothesized that presence of this oncogenic mutation would be associated with the behavior of PTC and possibly its clinical outcome. Among PTC patients in the present study, we demonstrated strong associations of the *BRAF* T1799A mutation with several high-risk pathological features and tumor recurrence. The association of the *BRAF* mutation with high-risk pathological features persisted after adjusting for all the previously known clinical and pathological risk factors but the PTC subtype; the association was lost when PTC subtype was additionally adjusted. This is consistent with the distribution pattern of the *BRAF* mutation and the high-risk pathological features among the different subtypes of PTC; *BRAF* mutation occurred more frequently in those PTC subtypes (tall cell and classical variants) that are more often associated with extrathyroidal invasion, lymph node metastasis, and advanced tumor stage as seen in the present study and many previous studies (33).

These data support the idea that *BRAF* mutation may play an important role in the formation, progression, and aggressiveness of the classically known high-risk subtypes of PTC. Importantly, logistic regression adjusting for all the clinical and pathological confounding factors, including PTC subtypes, still showed an independent association of *BRAF* mutation with tumor recurrence and a lower probability of recurrence-free survival, demonstrating the incremental information provided by *BRAF* mutation status in predicting the clinical course of patients with PTC. A significant association of *BRAF* mutation with thyroid cancer recurrence was observed, even in patients with only stage I/II initial disease, and this significance remained after adjustment for all the confounding factors including PTC subtypes. In addition, the recurrent disease in patients with *BRAF* mutation tended to be more extensive and was more likely to have lost iodine avidity, limiting the applicability of this treatment modality. In fact, *BRAF* mutation-positive patients required more aggressive surgical and external radiation treatments for their recurrent disease. This is consistent with the two recent interesting reports of the strong associations of *BRAF* mutation with the silencing of the genes in PTC for the apical iodide transporter SLC5A8 (34) and the key iodide-metabolizing enzyme thyroid peroxidase (35). Thus, *BRAF* mutation is a genetic indicator that independently predicts a poorer prognosis for PTC. These clinical data closely resemble the recent results in transgenic mouse studies in which *BRAF* mutation was shown to initiate the formation and promote the aggressiveness of PTC (36).

Controversy has existed among previous reports regarding the association of *BRAF* mutation with high-risk features of PTC. Studies with relatively large sample sizes have generally shown an association (9, 10), whereas those with smaller sample sizes have not (16, 19). One recent report on *BRAF* mutation analysis in a large number of various benign and malignant thyroid tumors also showed no association of *BRAF* mutation with high-risk tumor features (37). However, in this report, the relationship of *BRAF* mutation with tumor features was analyzed only within subgroups of PTC with relatively small sample sizes. Multivariate analyses in our present study suggest that, in addition to differences in sample sizes, confounding risk factors involved, particularly different compositions of PTC subtypes, may well explain these inconsistent findings regarding the relationship of *BRAF* mutation with clinicopathological features in previous studies, which uniformly lacked adjustment for these confounding factors.

In summary, our study confirmed the association of *BRAF* mutation with high-risk subtypes of PTC and the implication of its role in the formation and progression of these subtypes of PTC. More importantly, we demonstrate that *BRAF* mutation is independently associated with PTC recurrence, even in patients with low initial disease stages, and with greater aggressiveness of the recurrent tumor. Therefore, *BRAF* mutation is a novel prognostic marker that complements traditionally used prognostic factors for PTC. Consequently, *BRAF* mutation adds a new dimension to risk assessment of patients with this most common form of thyroid cancer. Its value as a prognostic indicator is enhanced by the fact that it can be determined preoperatively on cytological materials aspirated from thyroid nodules, unlike pathological characteristics, including histological PTC subtypes, currently used in postoperative risk stratification. Characterization of *BRAF* mutation may help optimize both initial treatment and long-term monitoring of disease recurrence for patients with PTC.

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