Association between $BRAF^{V600E}$ Mutation and Mortality in Patients with Papillary Thyroid Cancer

Mingzhao Xing, M.D., Ph.D., 1 Ali S. Alzahrani, M.D., 1 Kathryn A. Carson, Sc.M., 2 David Viola, M.D., 3 Rossella Elisei, M.D., 3 Bela Bendlova, Ph.D., 4 Linwah Yip, M.D., 5 Caterina Mian, M.D., 6 Federica Vianello, M.D., 7 R. Michael Tuttle, M.D., 8 Eyal Robenshtok, M.D., 8 James A. Fagin, M.D., 8 Efisio Puxeddu, M.D., Ph.D., 9 Laura Fugazzola, M.D., 10 Agnieszka Czarniecka, M.D., 11 Barbara Jarzab, M.D., Ph.D., 12 Christine J. O’Neill MBBS(Hons) MS, 13 Mark S. Sywak, M.D., 13 Alfred K Lam, M.D., Ph.D., 14 Garcielaso Riesco-Eizaguirre, M.D., Ph.D., 15,16 Pilar Santisteban, Ph.D., 16 Hirotaka Nakayama, M.D., 17 Ralph P. Tufano, M.D., 18 Sara I Pai, M.D., Ph.D., 18 Martha A Zeiger, M.D., 19 William H Westra, M.D., 20 Douglas P. Clark, M.D., 20 Roderick Clifton-Bligh, Ph.D., 21 David Sidransky, M.D., 18 Paul W. Ladenson, M.D., 22 and Vlasta Sykorova, Ph.D. 4

From:
1 Laboratory for Cellular and Molecular Thyroid Research, Division of Endocrinology and Metabolism, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA (M.X., A.S.A.);

2 Department of Epidemiology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD 21205, USA (K.A.C.);

3 Department of Endocrinology and Metabolism, University of Pisa, Pisa 56100, Italy (D.V., R.E.);

4 Department of Molecular Endocrinology, Institute of Endocrinology, Prague, the Czech Republic (B.B., V.S.);

5 Department of Surgery, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213 (L.Y.);

6 Operative Unit of Endocrinology, Department of Internal Medicine-DIMED; University of Padua, Italy (C.M.);

7 Veneto Institute of Oncology (IOV), Instituto di Ricovero e Cura a Carattere Scientifico (IRCCS); Padua, Italy (F.V.);

8 Department of Medicine, Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10065, USA (R.M.T., E.R., J.A.F.);

9 Department of Internal Medicine, University of Perugia, Perugia 06126, Italy (E.P.);

10 Department of Clinical Sciences & Community Health, University of Milan and Endocrine Unit, Fondazione IRCCS Cà Granda, Milan 20122, Italy (L.F.);
Key words: thyroid cancer, papillary thyroid cancer, \textit{BRAF} mutation, mortality, prognostic molecular marker, \textit{BRAF}^{V600E}

Address Correspondence to:
Michael Mingzhao Xing, M.D., Ph.D.
Division of Endocrinology and Metabolism
The Johns Hopkins University School of Medicine
1830 East Monument Street, Suite 333
Baltimore, MD 21287, U.S.A
Email: mxing1@jhmi.edu
ABSTRACT

Context $BRAF^{V600E}$ is a prominent oncogene in papillary thyroid cancer (PTC), but its role in PTC-related patient mortality has not been established.

Objective To investigate the relationship between $BRAF^{V600E}$ mutation and PTC-related mortality

Design, Setting, and Participants Retrospective multicenter study of 1,849 patients (1,411 women and 438 men) with a median age of 46 years (interquartile range: 34-58 years) and an overall median follow-up time of 33 months (interquartile range: 13-67 months) after the initial treatment.

Main Outcome Measures Patient deaths specifically caused by PTC

Results Mortality was 5.3% (45/845; 95% CI, 3.9-7.1%) versus 1.1% (11/1004; 95% CI, 0.5-2.0%) (P<0.001) in $BRAF^{V600E}$-positive versus –negative patients and 12.87 (95% CI, 9.61-17.24) vs. 2.52 (95% CI, 1.40-4.55) deaths per 1000 person years [relative risk(RR) 5.10, 95% CI, 2.64-9.87]. This association remained significant after adjustment for patient age and gender and stratification by center [hazard ratio (HR)=2.66, 95% CI,1.30-5.43]. Similar results were obtained from subgroup analyses of conventional and follicular variant PTCs. Significantly higher $BRAF^{V600E}$-associated patient mortality was also observed in many clinicopathological categories. For example, in patients with distant tumor metastasis the mortality was 18.2% (8/44; 95% CI,8.2-32.7%) in $BRAF^{V600E}$-negative versus 51.5% (34/66; 95% CI,38.9-64.0%) in mutation-positive groups (RR 2.72; 95% CI, 1.26-5.87). There was a significant additive interaction between $BRAF^{V600E}$ and lymph node metastasis (LNM), distant metastasis, disease
stage IV, and older patient age, as exemplified by the mortality of 11.1% (39/351; 95% CI, 8.0-14.9%) in patients with both \textit{BRAF}^{V600E} and LNM versus 2.6% (8/307; 95% CI, 1.1-5.1%) in patients with LNM but no \textit{BRAF}^{V600E} and 1.0% (4/410; 95% CI, 0.3-2.5%) in \textit{BRAF}^{V600E}-positive patients without LNM (Synergy Index=4.46; 95% CI, 1.76-11.32).

**Conclusions** In this preliminary report, the presence of \textit{BRAF}^{V600E} mutation was significantly associated with increased cancer-related mortality among patients with PTC.
INTRODUCTION

Papillary thyroid cancer (PTC) is the most common endocrine malignancy and accounts for 85-90% of all thyroid cancers.\textsuperscript{1,2} There are several variants of PTC, the majority of which are conventional PTC (CPTC) and follicular variant PTC (FVPTC), with the former typically showing papillary structures and the latter follicular structures in addition to the characteristic nuclear features of PTC. The overall 5-year patient survival rate for PTC is 95-97\%\textsuperscript{2}. A major clinical challenge is how to reliably distinguish patients who need aggressive treatments to reduce mortality from those who do not. This represents a widely controversial issue in today’s thyroid cancer medicine, particularly because of the relatively low overall mortality of this cancer. The issue has become even more challenging given the high, and still rapidly rising, annual incidence of PTC.\textsuperscript{1,2} Several clinicopathological risk factors have been used in the stratification of PTC, including older age of the patient at diagnosis, larger tumor size, cervical lymph node metastasis (LNM), extrathyroidal invasion, distant metastasis, and high levels on disease staging.\textsuperscript{3-5} Although these factors are known to be associated with a higher risk for progression of PTC, they often lack accuracy in helping tailor the extent of treatment of PTC to balance treatment-associated benefit and risk.

The T1799A nucleotide transversion in the \textit{BRAF} gene (GenBank of NCBI accession number: NM_004333) is a prominent oncogenic mutation in PTC\textsuperscript{6-11} and occurs, on average, in 45\% of cases.\textsuperscript{12} This mutation causes a valine-to-glutamic acid change in codon 600 of the \textit{BRAF} protein, resulting in \textit{BRAF}^{V600E} that possesses elevated serine/threonine protein kinase activities and constitutively activates the MAP kinase signaling pathway in human cancer.\textsuperscript{13} Many studies have shown an association of the \textit{BRAF}^{V600E} mutation with aggressive clinicopathological characteristics of PTC, including LNM, extrathyroidal invasion, loss of
radioiodine avidity and hence failure of radioiodine treatment and disease recurrence. Consequently, $BRAF^{V600E}$ mutation has drawn considerable attention and interest as a potential prognostic factor for PTC. However, the clinical significance of this mutation in PTC-related mortality has not been established. If an association is demonstrated, $BRAF^{V600E}$ would be useful in the death risk stratification of PTC. We undertook the present multicenter study to examine and define the prognostic value of $BRAF^{V600E}$ mutation in PTC-related mortality.

**METHODS**

**Study organization**

This study was conducted in 13 medical centers in 7 countries including the Johns Hopkins Medical Institution, University of Pittsburgh Medical Center, and Memorial Sloan Kettering Cancer Center in the United States; medical centers at the University of Pisa, University of Perugia, University of Milan, and University of Padua in Italy; Kanagawa Cancer Center, Yokohama in Japan; Maria Sklodowska-Curie Memorial Cancer Centre and Institute of Oncology in Poland; medical centers at Griffith Medical School and University of Sydney in Australia; Hospital La Paz Health Research Institute (IdiPAZ) in Spain; and Institute of Endocrinology, Prague in the Czech Republic.

**Study patients**

All patients had been treated and followed for PTC at the participating institutions and their collaborating medical centers. Patients at each center were consecutively selected, from different time periods at the 13 centers, which overall spanned 1978-2011. All patients were
treated with surgical total thyroidectomy for PTC, and therapeutic neck dissection was performed in patients with standard indications. Standard pathological diagnoses of PTC were based on World Health Organization (WHO) criteria and documented in our peer-reviewed publications.\textsuperscript{16-29} Postoperative treatments included, as guided by standard criteria, conventional thyroid-stimulating hormone (TSH) suppression at appropriate levels and radioiodine-131 ablation (supplemental Table S1), except for Kanagawa Cancer Center where no radioiodine-131 treatment was used for thyroid cancer patients. Follow-up or survival time was defined as the time from the initial surgical treatment to patient death or, for those who survived, to the most recent clinic visit.

**Study design**

This was a retrospective study approved by the institutional review board (IRB) of each center, with informed patient consent obtained where required; patient consent was waived in some cases following IRB-approved procedures in the collection of pathological data. The study only involved the use of thyroid tumor tissues and clinicopathological information of patients. The $BRAF^{V600E}$ mutation status of primary PTC tumors was determined after the surgical and medical (e.g., radioiodine) treatments in all cases and did not affect the decision on selection of treatments. Genomic DNA isolated from primary PTC tumors was used to analyze the sequence of exon 15 of the $BRAF$ gene for $BRAF^{V600E}$ as described in our published studies.\textsuperscript{16-29} Clinicopathological information was obtained from the medical records using a uniform protocol designed for this study. PTC-specific death was defined as death that occurred as a result of incurable advanced PTC disease that invaded and compromised vital organs. Data from
the 13 centers were pooled for the analysis of the relationship of $BRAF^{V600E}$ with PTC-specific mortality in various clinicopathological categories.

**Statistical analyses**

PTC-specific mortality was calculated by dividing the number of deaths due to PTC by the total number of patients. Fisher’s exact test was used to compare mortality by $BRAF^{V600E}$ mutation status. Person-year rates were calculated by dividing the number of PTC-specific deaths by the total follow-up time and Poisson regression was used to calculate the relative risk (RR) and 95% confidence intervals (CIs). Kaplan-Meier survival curves and log-rank tests, censoring patients at time of last follow-up or 15 years, and Cox proportional hazards regression analyses, censoring patients at time of last follow-up, were used to compare PTC-specific survival by $BRAF^{V600E}$ mutation status. Proportional hazards regressions were adjusted for age at diagnosis, gender, and medical center. A second model also adjusted for LNM, extrathyroidal invasion and distant metastasis. The covariates were tested for the proportional hazards assumption using the “Assess” statement in SAS version 9.3 (SAS Institute, Inc., Cary, NC). As medical center violated the proportional hazards assumption, stratified models were used. Additive interactions of $BRAF^{V600E}$ mutation status with other factors were tested using the synergy index (SI) and 95% CI described by Hosmer and Lemeshow. Exact binomial CIs for percent mortality were calculated using Stata/IC 12.1 (StataCorp, College Station, TX). All other analyses were performed using SAS version 9.3. All reported P values are two-sided and significance was set at $P<0.05$.

**RESULTS**
Relationship between $BRAF^ {V600E}$ and PTC-related mortality

The number, gender, and age of patients from each center and country are summarized in Table 1. A total of 1,849 patients (1,411 women and 438 men) with a median age of 46 years (interquartile range: 34-58 years) were included, with an overall median follow-up time of 33 months (interquartile range: 13-67 months) after the initial treatment. The overall prevalence of $BRAF^{V600E}$ was 45.7% (845/1,849; 95% CI, 43.4-48.0%) (Table 1), which is within the range of published $BRAF^{V600E}$ mutation rates.\textsuperscript{12,14,15} There were 56 PTC-related deaths among the 1,849 patients, representing an overall mortality of 3.0% (95% CI, 2.3-3.9%), which is consistent with the general mortality rate of PTC.\textsuperscript{2} Among these deaths, 45 cases (80.4%) were positive for $BRAF^{V600E}$. Percent mortality and deaths per 1000 person years for different types of PTC are reported in Table 2. The overall mortality of all PTCs was 5.3% (45/845; 95% CI, 3.9-7.1%) in the $BRAF^{V600E}$–positive patients vs. 1.1% (11/1004; 95% CI, 0.5-2.0%) in the mutation-negative patients ($P<0.001$). Significantly higher mortality was also observed for the $BRAF^{V600E}$–positive patients compared to the $BRAF^{V600E}$–negative patients when the analysis was restricted to CPTC or FVPTC. The total person years of follow-up for all PTC cases were 7856.75 years. Deaths per 1000 person years were 12.87 (95% CI, 9.61-17.24) for $BRAF^{V600E}$–positive vs. 2.52 (95% CI, 1.40-4.55) for $BRAF^{V600E}$–negative patients (RR 5.10; 95% CI, 2.64-9.87). Similar results were found when restricting to CPTC and FVPTC (Table 1).

Proportional hazards regression analysis adjusting for patient age at diagnosis and gender and stratifying by medical center demonstrated a significant increased risk of death associated with $BRAF^{V600E}$ mutation [hazard ratio (HR)=2.66; 95% CI, 1.30-5.43]. Significant results were also seen when the sample was restricted to CPTC patients (HR=3.53; 95% CI, 1.25-9.98). No significant result was observed for the FVPTC group which had low numbers of cases and
patient deaths (HR=1.67; 95%, CI 0.06-47.49). When aggressive tumor features of LNM, extrathyroidal invasion and distant metastasis were also included in the model, the association of \(BRAF^{V600E}\) with mortality was attenuated (HR=1.21; 95% CI 0.53-2.76 for all PTC and HR=1.51; 95% CI, 0.50-4.57 for CPTC). Kaplan-Meier survival curves of patients for all PTC and CPTC are displayed in Fig 1A and B, respectively. \(BRAF^{V600E}\) mutation-positive patients had significantly poorer survival in each panel.

PTC-related mortality, total person years, and rates by \(BRAF^{V600E}\) mutation by clinicopathological categories are presented in Table 3. Significant RR associated with \(BRAF^{V600E}\) for PTC mortality was seen within most of the categories. The highest mortality occurred in patients with distant metastasis and advanced American Joint Committee on Cancer stage IV, and \(BRAF^{V600E}\) was also associated with a significantly higher mortality within these two conventionally high-risk categories. The association of \(BRAF^{V600E}\) with mortality of patients with diseases of stages I, II, and III was not statistically significant. When tumors were stratified by size, the absolute magnitude of mortality rose from smaller to larger tumors, particularly in the \(BRAF^{V600E}\)-positive groups. \(BRAF^{V600E}\) had a significant association with mortality of micro-PTC (≤1.0 cm), but the absolute mortality rate was low.

We found that therapeutic doses of radioiodine used in the treatments of patients were comparable between the \(BRAF^{V600E}\)-positive and -negative groups except in some centers where the \(BRAF^{V600E}\) group received higher doses (supplemental Table 1S).

**Interaction of \(BRAF^{V600E}\) with conventional clinicopathological risk factors**

We observed a significant interaction of \(BRAF^{V600E}\) with several conventional clinicopathological risk factors in affecting PTC-related mortality, as reflected by a significant SI
(supplemental Table S2). These included LNM, distant metastasis, and stage IV disease. The SI was not statistically significant for extrathyroidal invasion. As shown in Table 3, mortality for co-existing LNM and $BRAF^{V600E}$ was 11.1% (39/351; 95% CI, 8.0-14.9%), whereas mortality was 2.6% (8/307; 95% CI, 1.1-5.1%) in LNM-positive but $BRAF^{V600E}$-negative patients and 1.0% (4/410; 95% CI, 0.3-2.5%) in LNM-negative but $BRAF^{V600E}$-positive patients. Co-existence of distant metastatic disease and $BRAF^{V600E}$ had a mortality of 51.5% (34/66; 95% CI, 38.9-64.0%), whereas mortality was 18.2% (8/44; 95% CI, 8.2-32.7%) in $BRAF^{V600E}$-negative patients with distant metastasis and 1.4% (11/772; 95% CI, 0.7-2.5%) in $BRAF^{V600E}$-positive patients without distant metastasis. Similarly, co-existence of stage IV disease and $BRAF^{V600E}$ had a mortality of 31.4% (38/121; 95% CI, 23.3-40.5%), whereas mortality was 13.0% (10/77; 95% CI, 6.4-22.6%) in $BRAF^{V600E}$-negative patients with stage IV disease and 0.9% (6/699; 95% CI, 0.3-1.9%) in $BRAF^{V600E}$ mutation-positive patients without stage IV disease. The common pattern of these relationships is that the mortality rate associated with co-existence of $BRAF^{V600E}$ and a conventional risk factor was higher than the addition of the two mortalities associated with either alone (Table 3), further supporting the synergistic interactions of $BRAF^{V600E}$ with these risk factors demonstrated by the SI test (supplemental Table S2). This pattern of interaction of $BRAF^{V600E}$ with clinicopathological factors in affecting PTC-related mortality was also demonstrated in the Kaplan-Meier survival curves (Fig 2A, 2B, and 2C).

**$BRAF^{V600E}$ and patient age in PTC-related mortality**

As shown in Table 3, in both $BRAF^{V600E}$-positive and –negative patients, there was an increasing mortality with age and this was particularly evident in the $BRAF^{V600E}$-positive patients. Specifically, the mortalities in $BRAF^{V600E}$-positive patients aged <45 and ≥45 years
were 1.4% (5/346; 95% CI, 0.5-3.3%) and 8.0% (40/499; 95% CI, 5.8-10.8%) \( (P<0.001) \), respectively, versus 0.4% (2/530; 95% CI, 0.0-1.4%) and 1.9% (9/474; 95% CI, 0.9-3.6%) \( (P=0.03) \) in \textit{BRAF}^{V600E}-negative patients in these age groups with a significant synergistic interaction (SI 3.15; 95% CI, 1.37-7.27). Using an age cutpoint of ≥60 years produced similarly higher mortality in the older age group, particularly in the \textit{BRAF}^{V600E}-positive patients (Table 3), displaying a significant synergistic interaction of \textit{BRAF}^{V600E} with the older patient age on the mortality (SI 3.40; 95% CI, 1.52-7.62). This positive interaction of \textit{BRAF}^{V600E} with patient age in affecting PTC mortality was also shown in Kaplan-Meier survival curves (Figs 2D and 2E).

When analyzing patients with conventional PTC, \textit{BRAF}^{V600E} was similarly associated with higher patient mortalities within various clinicopathological risk categories (supplemental Table S3).

\textit{Mortality in \textit{BRAF}^{V600E} mutation-negative conventionally low-risk patients}

As shown in Table 3, the overall mortality was low in conventionally low-risk patients, i.e., those with tumor size ≤1.0 cm, stage I-III diseases, or patient age < 45 years. The mortality rate was lowest in the \textit{BRAF}^{V600E}-negative patients of these groups, ranging from 0-0.4%. A uniform 0% mortality was observed in \textit{BRAF}^{V600E}-negative patients of these groups when the analysis was restricted to only CPTC (supplemental Table S3). A moderate increase in the mortality was seen in the presence of \textit{BRAF}^{V600E} in some of these groups, but they were not statistically significant (Table 3 and supplemental Table S3).

\textbf{COMMENT}
In this large multicenter study, we demonstrate a significant association of $BRAF^{\text{V600E}}$ mutation with PTC-related mortality both in the analysis of all PTC patients and in the analysis of CPTC or FVPTC variants. The RR of $BRAF^{\text{V600E}}$ for mortality was high across several clinicopathological risk categories and the vast majority of the death cases harbored this mutation. These results suggest the importance of $BRAF^{\text{V600E}}$ in PTC-related mortality. We also observed a significant additive interaction between $BRAF^{\text{V600E}}$ and several conventional clinicopathological factors in affecting the magnitude of PTC-related mortality, including older patient age, LNM, distant metastasis, and advanced disease stage IV. Most of these factors alone had only a modest mortality risk, which was significantly increased by co-existing $BRAF^{\text{V600E}}$. Thus, the widely known mortality risk associated with the conventional high-risk clinicopathological factors of PTC is closely related to the co-existing $BRAF^{\text{V600E}}$ mutation. It should be noted that the significance of RR of $BRAF^{\text{V600E}}$ for mortality needs to be interpreted in clinical relevance. For example, as shown in Table 3, $BRAF^{\text{V600E}}$ was associated with an increase in mortality from 0.3% (3/944) to 1.4% (11/772) ($P=0.01$) in patients without distant metastasis and from 18.2% (8/44) to 51.5% (34/66) ($P<0.001$) in patients with distant metastasis. Although the RR was statistically significant in both situations, there would be only one additional patient death in the former versus 33 additional deaths in the latter associated with $BRAF^{\text{V600E}}$ in 100 patients. In some of the conventionally low-risk categories, such as tumors $\leq 1.0$ cm, $BRAF^{\text{V600E}}$ also had an association with mortality, consistent with previous findings that this mutation could be associated with aggressive tumor features even in conventionally low-risk patients.\textsuperscript{14,18,31} It is also clinically important to note, on the other hand, that absence of $BRAF^{\text{V600E}}$ mutation was associated with a mortality of nearly 0% in conventionally low-risk patients, providing a
negative predictive power that may further assure an excellent survival of these patients when $BRAF^{V600E}$ is absent.

The explanation for a role of $BRAF^{V600E}$ in PTC-related mortality may lie in the molecular mechanisms by which $BRAF^{V600E}$ promotes aggressive molecular pathogenesis of PTC. For example, $BRAF^{V600E}$ causes de-differentiation of PTC, resulting in the loss of expression of thyroid genes involved in thyroid iodide concentration and hence failure of radioiodine treatment.\textsuperscript{14,15} $BRAF^{V600E}$ strongly up-regulates many classical angiogenic and tumor-promoting molecules (e.g., vascular endothelial growth factor, matrix metalloproteinases, c-MET, and nuclear transcription factor κB) and is associated with hypermethylation and hence inactivation of tumor suppressor genes (e.g., tissue inhibitor of matrix metalloproteinase-3, death-associated protein kinase, and SLC5A8)\textsuperscript{14,31} as well as extracellular pro-tumor micro-environmental changes.\textsuperscript{32} $BRAF^{V600E}$ also causes genome-wide alterations in methylation and hence aberrant expression of prominent genes in thyroid cancer\textsuperscript{33} as well as in melanoma.\textsuperscript{34} It is conceivable that through these unique molecular mechanisms $BRAF^{V600E}$ promotes aggressive tumor behaviors such as LNM, tumor invasion and distant metastasis, renders the tumor resistant to radioiodine treatment, and expedites tumor progression, hence aggravating the mortality risk of PTC. PTC-related deaths are ultimately caused by these aggressive tumor behaviors; without them, patients would not die from PTC. Thus, $BRAF^{V600E}$ cannot be independent of such tumor behaviors in affecting patient mortality and, consequently, multivariate analyses adjusting for these aggressive tumor behaviors to look for an “independent” association of $BRAF^{V600E}$ with PTC-related mortality would be biologically invalid. In fact, we performed such an analysis and did show that the impact of $BRAF^{V600E}$ was misleadingly attenuated. In contrast, stratification by center
and adjustment for patient age and gender did not significantly affect the association of \( \text{BRAF}^{V600E} \) with mortality.

The large number of cases and multicenter nature represent a prominent strength of this study. The treatments, including total thyroidectomy, therapeutic neck dissection, and appropriate postoperative TSH suppression were pursued following accepted standards at the participating centers and the pathological diagnoses of tumors were formally documented in our peer-reviewed publications.\textsuperscript{16-29} Although patient follow-up durations after the initial treatment varied between centers, this was not different by \( \text{BRAF}^{V600E} \) status within centers and on overall analysis. Moreover, we employed Cox proportional hazards regression and Kaplan-Meier survival analysis as well as person-year mortality rates to account for different durations. It is worth noting that all the classically known relationship patterns of clinicopathological risk factors with PTC-related mortality were accurately reproduced in the present study; for example, all the conventional high-risk clinicopathological factors were associated with a greater PTC-specific mortality in the present study, further attesting the validity of the study. It is noteworthy that at some centers \( \text{BRAF}^{V600E} \) patients received higher doses of radioiodine for treatments when retrospectively analyzed after the \( \text{BRAF}^{V600E} \) testing. This likely reflects the fact that \( \text{BRAF}^{V600E} \) patients tended to present with more aggressive clinicopathological behaviors of PTC, prompting more aggressive radioiodine treatment. This may have caused an underestimate of the association of \( \text{BRAF}^{V600E} \) with PTC mortality in the present study since radioiodine treatment can decrease mortality of thyroid cancer, particularly in conventionally high-risk patients,\textsuperscript{3} which, as the present study showed, is where \( \text{BRAF}^{V600E} \) has the most significant association with mortality.
There are a few limitations of the present study. First, the low number of PTC-specific deaths, as is generally seen for PTC, reduced the power to find associations and resulted in wide confidence intervals for some of the estimates. Additionally, stratified analyses were performed with no adjustment for multiple comparisons. Therefore, some of these stratified analyses should be considered exploratory and hypothesis-generating. Secondly, many of the patients had a relatively short clinical follow-up. This may have caused an underestimate of the association of \(BRAF^{V600E}\) with PTC-specific mortality, as suggested by the observation that this association became clearer after a longer time of follow-up (Figs 1 and 2). Another limitation is that we only captured PTC-specific deaths in our data, censoring patients that died of other causes at the time of last follow-up. Therefore, we could not look at overall mortality.

In summary, this large multicenter study demonstrates that the presence of \(BRAF^{V600E}\) mutation is significantly associated with increased cancer-related mortality among patients with PTC, particularly when co-existing with conventional clinicopathological risk factors. As such, \(BRAF^{V600E}\) mutation as a genetic prognostic molecular marker may add to current risk stratification of PTC in appropriate clinical settings.

**Author Contributions:** M. Xing had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. M. Xing conceived, designed, and coordinated the overall research; all the authors participated in the part of the research at their corresponding centers and contributed research materials or data from the individual centers for overall analysis; M. Xing, A.S. Alzahrani, and K.A. Carson organized and analyzed the overall data; K.A. Carson provided statistical assistance; M. Xing wrote the report;
all authors provided feedbacks/suggestions on the report, revised the report, and approved its final version.

**Conflict of Interest Disclosures:** All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Drs M. Xing and D. Sidransky reported receiving royalties as co-holders of a licensed US patent related to \( \text{BRAF}^{V600E} \) mutation in thyroid cancer. No other authors reported disclosures.

**Funding/Support:** This project was supported by U.S.A. National Institutes of Health (NIH) RO-1 R01CA134225 to M. Xing. The statistical effort of K.A. Carson for this project was supported by Grant UL1 RR 025005 from the National Center for Advancing Translational Sciences of NIH and NIH Roadmap for Medical Research. In addition, the studies at individual centers were supported as follows:

The Ministry of Science and Higher Education Research grants N N403 194340 and N N401 612440 to A. Czarniecka and B. Jarza, respectively (Poland); grant NIDCR/NCI SPORE P50DE019032 to D. Sindransky (U.S.A.); grants BFU-2010-16025, RD06/0020/0060 FIS, and S2011/BMD-2328 TIRONET to P. Santisteban and G. Riesco-Eizaguirre (Spain); NIH-RO1-CA50706 and the Byrne Foundation funding to J. A. Fagin (U.S.A.); grants from Fondazione Cassa di Risparmio di Perugia and Associazione Italiana per la Ricerca sul Cancro (IG 9338) (Italy) and the Beadle Family Foundation (San Antonio, Texas, U.S.A.) to E. Puxeddu; grant IGA MH CR NT 13901-4 to V. Sýkorová and B. Bendlová (the Czech Republic); and grants from the New South Wales Cancer Institute to C. J. O’Neill and from Cancer Council of New South Wales to R. Clifton-Bligh (Australia).
**Role of the Sponsor:** The funding organizations had no role in the design and conduct of the study; the collection, management, analysis, and interpretation of the data; or the preparation, review, or approval of the manuscript.

**Disclaimer:** The content of this article is solely the responsibility of the authors and does not necessarily reflect the official views of the US NIH or the funding entities of the individual centers participating in this study.
References


Figure Legends

**Figure 1** Kaplan-Meier Survival Curves of PTC-specific Survival by $BRAF^{V600E}$ Mutation status

Comparison of patient survival, represented by the indicated log-rank and p values in each panel, was performed between $BRAF^{V600E}$-negative (blue line) and –positive (red line) groups for all patients (A) and patients with conventional PTC (B). Follow-up time is truncated at 12 years. The number of patients in each stratum at risk is displayed.

**Figure 2** Kaplan-Meier Survival Curves of the Interaction of $BRAF^{V600E}$ Mutation with Clinicopathological Risk Factors in Affecting Disease-specific Survival of PTC Patients

Color lines in panels A, B, and C represent: blue—patients negative for both $BRAF^{V600E}$ mutation and the indicated clinicopathological risk factor (lymph node metastasis in A, tumor extrathyroidal invasion in B, and tumor distant metastasis in C), orange—patients positive for the indicated clinicopathological factor but negative for $BRAF^{V600E}$ mutation, green—patients negative for the indicated clinicopathological factor but positive for $BRAF^{V600E}$ mutation, and red—patients positive for both the indicated clinicopathological factor and $BRAF^{V600E}$ mutation.

Color lines in panel D represent: blue—patients <45 years of age and negative for $BRAF^{V600E}$, orange—patients ≥45 years of age and negative for $BRAF^{V600E}$, green—patients <45 years of age and positive for $BRAF^{V600E}$, and red—patients ≥45 years of age and positive for $BRAF^{V600E}$.

Color lines in panel E represent: blue—patients <60 years of age and negative for $BRAF^{V600E}$, orange—patients ≥60 years of age and negative for $BRAF^{V600E}$, green—patients <60 years of age and positive for $BRAF^{V600E}$, and red—patients ≥60 years of age and positive for $BRAF^{V600E}$.

In all the panels, follow-up time is truncated at 12 years and the number of patients in each
stratum at risk is displayed. In each panel, p values are from the log-rank test adjusted for multiple comparisons comparing each stratum to patients positive for both the $BRAF^{V600E}$ mutation and the indicated clinicopathological factor (panels A, B, and C) or patient age $\geq 45$ years (panel D) or $\geq 60$ years (panel E).
<table>
<thead>
<tr>
<th>Medical Center or Country</th>
<th>Number of Patients</th>
<th>Age at Diagnosis in years, median (IQR)</th>
<th>Male Gender, n (%)</th>
<th>BRFV600E Mutation n (%)</th>
<th>PTC-related Deaths, n (%)</th>
<th>Follow-up months for all patients, median (IQR)</th>
<th>Follow-up months for survivors, median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Johns Hopkins Hospital</td>
<td>387</td>
<td>45 (35-57)</td>
<td>101 (26.1)</td>
<td>151 (39.0)</td>
<td>8 (2.1)</td>
<td>12 (1-30)</td>
<td>12 (1-28)</td>
</tr>
<tr>
<td>University of Pittsburgh</td>
<td>169</td>
<td>52 (38-63)</td>
<td>42 (24.8)</td>
<td>101 (59.8)</td>
<td>1 (0.6)</td>
<td>19 (11-26)</td>
<td>19 (11-26)</td>
</tr>
<tr>
<td>Memorial Sloan-Kettering Cancer Center</td>
<td>135</td>
<td>50 (35-63)</td>
<td>44 (32.6)</td>
<td>64 (47.4)</td>
<td>11 (8.2)</td>
<td>96 (1-144)</td>
<td>90 (1-144)</td>
</tr>
<tr>
<td>University of Pisa</td>
<td>189</td>
<td>38 (28-51)</td>
<td>47 (24.9)</td>
<td>65 (34.4)</td>
<td>9 (4.8)</td>
<td>72 (24-180)</td>
<td>84 (24-180)</td>
</tr>
<tr>
<td>University of Perugia</td>
<td>117</td>
<td>49 (37-59)</td>
<td>32 (27.4)</td>
<td>76 (65.0)</td>
<td>5 (4.3)</td>
<td>22 (6-39)</td>
<td>22 (6-40)</td>
</tr>
<tr>
<td>University of Milan</td>
<td>110</td>
<td>42 (34-55)</td>
<td>24 (21.8)</td>
<td>38 (34.6)</td>
<td>1 (0.9)</td>
<td>48 (24-64)</td>
<td>48 (24-64)</td>
</tr>
<tr>
<td>University of Padua</td>
<td>135</td>
<td>48 (39-57)</td>
<td>32 (23.7)</td>
<td>87 (64.4)</td>
<td>1 (0.7)</td>
<td>26 (22-30)</td>
<td>26 (22-30)</td>
</tr>
<tr>
<td>Kanagawa Cancer Center</td>
<td>49</td>
<td>55 (41-65)</td>
<td>16 (32.6)</td>
<td>33 (67.4)</td>
<td>9 (18.4)</td>
<td>68 (31-78)</td>
<td>65 (33-76)</td>
</tr>
<tr>
<td>MSC Memorial Cancer Centre and Institute of Oncology</td>
<td>99</td>
<td>49 (33-59)</td>
<td>10 (10.1)</td>
<td>42 (42.4)</td>
<td>1 (1.0)</td>
<td>48 (42-53)</td>
<td>48 (43-53)</td>
</tr>
<tr>
<td>Griffith Medical School</td>
<td>76</td>
<td>40 (34-56)</td>
<td>20 (26.3)</td>
<td>34 (44.7)</td>
<td>0 (0)</td>
<td>42 (4-82)</td>
<td>42 (4-82)</td>
</tr>
<tr>
<td>University of Sydney</td>
<td>95</td>
<td>44 (34-59)</td>
<td>20 (21.0)</td>
<td>55 (57.9)</td>
<td>5 (5.3)</td>
<td>103 (63-135)</td>
<td>104 (64-137)</td>
</tr>
<tr>
<td>Hospital La Paz Health Research Institute (IdiPAZ)</td>
<td>66</td>
<td>42 (32-54)</td>
<td>11 (16.7)</td>
<td>28 (42.4)</td>
<td>2 (3.0)</td>
<td>41 (30-57)</td>
<td>42 (30-57)</td>
</tr>
<tr>
<td>Institute of Endocrinology,Prague</td>
<td>222</td>
<td>47 (31-60)</td>
<td>39 (17.6)</td>
<td>71 (32.0)</td>
<td>3 (1.4)</td>
<td>50 (30-85)</td>
<td>50 (30-85)</td>
</tr>
<tr>
<td>USA</td>
<td>691</td>
<td>47 (36-59)</td>
<td>187 (27.1)</td>
<td>316 (45.7)</td>
<td>20 (2.9)</td>
<td>17 (2-36)</td>
<td>16 (2-32)</td>
</tr>
<tr>
<td>Italy</td>
<td>551</td>
<td>44 (34-56)</td>
<td>135 (24.5)</td>
<td>266 (48.3)</td>
<td>16 (2.9)</td>
<td>33 (20-70)</td>
<td>34 (20-72)</td>
</tr>
<tr>
<td>Japan</td>
<td>49</td>
<td>55 (41-65)</td>
<td>16 (32.6)</td>
<td>33 (67.4)</td>
<td>9 (18.4)</td>
<td>68 (31-78)</td>
<td>65 (33-76)</td>
</tr>
<tr>
<td>Poland</td>
<td>99</td>
<td>49 (33-59)</td>
<td>10 (10.1)</td>
<td>42 (42.4)</td>
<td>1 (1.0)</td>
<td>48 (42-53)</td>
<td>48 (43-53)</td>
</tr>
<tr>
<td>Australia</td>
<td>171</td>
<td>43 (34-57)</td>
<td>40 (23.4)</td>
<td>89 (52.0)</td>
<td>5 (2.9)</td>
<td>75 (32-118)</td>
<td>76 (33-118)</td>
</tr>
<tr>
<td>Spain</td>
<td>66</td>
<td>42 (32-54)</td>
<td>11 (16.7)</td>
<td>28 (42.4)</td>
<td>2 (3.0)</td>
<td>41 (30-57)</td>
<td>42 (30-57)</td>
</tr>
<tr>
<td>Czech Republic</td>
<td>222</td>
<td>47 (31-60)</td>
<td>39 (17.6)</td>
<td>71 (32.0)</td>
<td>3 (1.4)</td>
<td>50 (30-85)</td>
<td>50 (30-85)</td>
</tr>
<tr>
<td>Overall</td>
<td>1,849</td>
<td>46 (34-58)</td>
<td>438 (23.7)</td>
<td>845 (45.7)</td>
<td>56 (3.0)</td>
<td>33 (13-67)</td>
<td>33 (13-65)</td>
</tr>
</tbody>
</table>

**Notes:** IQR, interquartile range
Table 2. Papillary Thyroid Cancer (PTC)-related Mortality, Person Years and Relative Risk (RR) for $BRAF^{V600E}$ Mutation-positive vs. -negative Patients

<table>
<thead>
<tr>
<th>Type of PTC</th>
<th>Mortality Percent</th>
<th>Total Person Years</th>
<th>Deaths per 1000 Person Years (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overall</td>
<td>BRAF+</td>
<td>BRAF-</td>
</tr>
<tr>
<td>All PTC</td>
<td>56/1849 (3.0%)</td>
<td>45/845 (5.3%)</td>
<td>11/1004 (1.1%)</td>
</tr>
<tr>
<td>Conventional PTC</td>
<td>39/1233 (3.2%)</td>
<td>33/659 (5.0%)</td>
<td>6/574 (1.0%)</td>
</tr>
<tr>
<td>Follicular Variant PTC</td>
<td>6/411 (1.5%)</td>
<td>4/82 (4.9%)</td>
<td>2/329 (0.6%)</td>
</tr>
</tbody>
</table>
Table 3. Papillary Thyroid Cancer (PTC)-related Mortality and Relative Risks (RR) in $BRAF^{V600E}$ Mutation-positive vs. –negative Patients in Various Clinicopathological Categories of All PTCs

<table>
<thead>
<tr>
<th>Category</th>
<th>Mortality Percent</th>
<th>Total Person Years</th>
<th>Deaths per 1000 Person Years (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BRAF+, n/N (%)</td>
<td>BRAF-, n/N (%)</td>
<td>P value</td>
</tr>
<tr>
<td>All patients</td>
<td>12.87 (9.61-17.24)</td>
<td>2.52 (1.40-4.55)</td>
<td>5.10 (2.64-9.87)</td>
</tr>
<tr>
<td>Age &lt; 45 yrs</td>
<td>3.19 (1.33-7.66)</td>
<td>0.81 (0.20-3.24)</td>
<td>3.94 (0.76-20.29)</td>
</tr>
<tr>
<td>Age ≥ 45 yrs</td>
<td>20.75 (15.22-28.29)</td>
<td>4.76 (2.48-9.14)</td>
<td>4.36 (2.12-8.99)</td>
</tr>
<tr>
<td>Age &lt; 60 yrs</td>
<td>5.27 (3.12-8.89)</td>
<td>1.34 (0.56-3.21)</td>
<td>3.94 (1.42-10.94)</td>
</tr>
<tr>
<td>Age ≥ 60 yrs</td>
<td>37.03 (26.04-52.65)</td>
<td>9.68 (4.35-21.56)</td>
<td>3.82 (1.60-9.17)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>2.43 (0.91-6.47)</td>
<td>1.10 (0.36-3.42)</td>
<td>2.20 (0.49-9.83)</td>
</tr>
<tr>
<td>Extrathyroidal invasion</td>
<td>5.24 (2.98-9.23)</td>
<td>0.58 (0.15-2.32)</td>
<td>9.04 (2.02-40.38)</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>7.02 (5.24-10.03)</td>
<td>11.04 (5.75-21.22)</td>
<td>2.45 (1.17-5.13)</td>
</tr>
<tr>
<td>Multifocality</td>
<td>2.08 (0.93-4.62)</td>
<td>0.25 (0.04-1.80)</td>
<td>8.20 (0.99-68.14)</td>
</tr>
<tr>
<td>Stage IV disease</td>
<td>69.97 (50.91-96.16)</td>
<td>32.38 (17.42-60.18)</td>
<td>2.16 (1.08-4.34)</td>
</tr>
<tr>
<td>Stage I</td>
<td>0.52 (0.07-3.67)</td>
<td>0.33 (0.05-2.37)</td>
<td>1.55 (0.10-24.76)</td>
</tr>
<tr>
<td>Stage II</td>
<td>3.48 (0.49-24.71)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td>5.99 (2.25-15.96)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Tumor ≤1.0 cm</td>
<td>4.65 (2.46-17.46)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Tumor 1.0- 2.0 cm</td>
<td>3.69 (1.66-8.21)</td>
<td>0.49 (0.49-7.2)</td>
<td>2.42 (0.61-9.68)</td>
</tr>
<tr>
<td>Tumor 2.0-3.0 cm</td>
<td>13.32 (7.73-22.93)</td>
<td>2.49 (0.80-7.73)</td>
<td>5.34 (1.52-18.73)</td>
</tr>
<tr>
<td>Tumor 3.0-4.0 cm</td>
<td>17.54 (9.13-33.7)</td>
<td>6.09 (2.28-16.22)</td>
<td>2.88 (0.89-9.36)</td>
</tr>
<tr>
<td>Tumor ≥ 4.0 cm</td>
<td>29.20 (16.96-50.29)</td>
<td>8.67 (3.61-20.83)</td>
<td>3.37 (1.20-9.45)</td>
</tr>
</tbody>
</table>

Note: Percentages and mortality rates are calculated based on the number of deaths and person-years at risk.