Association of the T1799A BRAF mutation with tumor extrathyroidal invasion, higher peripheral platelet counts, and overexpression of platelet-derived growth factor-B in papillary thyroid cancer

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Abstract

The relationship among BRAF mutation, platelet counts, and platelet-derived growth factor (PDGF) with respect to clinicopathological outcomes of papillary thyroid cancer (PTC) may play a role in PTC pathogenesis but remains undefined. We examined the T1799A BRAF mutation by direct genomic DNA sequencing in 108 primary PTC samples from a Chinese cohort and analyzed its relationship with clinicopathological, hematological, and other laboratory results as well as the levels of expression of PDGF in tumors. We found that the BRAF mutation was significantly associated with extrathyroidal invasion and advanced tumor stages III and IV. Specifically, extrathyroidal invasion was seen in 30/54 (56%) PTC with BRAF mutation versus 18/54 (33%) PTC without the mutation ($P<0.02$). Tumor stages III and IV were seen in 16/54 (30%) PTC with BRAF mutation versus 7/54 (13%) PTC without the mutation ($P<0.04$). The BRAF mutation was also significantly associated with a higher platelet count, with $249.28 \pm 53.76 \times 10^9/l$ in the group of patients with BRAF mutation versus $207.79 \pm 58.98 \times 10^9/l$ in the group without the mutation ($P=0.001$). An association of higher platelet counts with extrathyroidal invasion was also seen, with $242.66 \pm 51.75 \times 10^9/l$ in patients with extrathyroidal invasion versus $218.49 \pm 59.10 \times 10^9/l$ in patients without extrathyroidal invasion ($P=0.03$). The BRAF T1799A-positive PTC tissues harbored a significantly higher level of PDGF-B than BRAF T1799A-negative PTC tissues. The data suggest that the BRAF T1799A mutation is associated with aggressive pathological outcomes of PTC in which high platelet counts and increased PDGF production may play a role.

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Introduction

Thyroid cancer is the most common endocrine malignancy with a rapidly rising incidence worldwide in recent decades (Hundahl et al. 1998, Liu et al. 2001, Davies et al. 2002, Davies & Welch 2006, Mazzaferri 2006, Sandeep et al. 2006). This increase is virtually completely attributed to the diagnosis of papillary thyroid cancer (PTC), which accounts for ~80% of all thyroid cancers. As in other human cancers, genetic alterations are the driving force for PTC tumorigenesis. Recent several years have seen a major progress in understanding the genetic basis of PTC. In particular, numerous studies have consistently shown a high prevalence of the T1799A point mutation in exon 15 of
the BRAF gene, which is the most common oncogenic genetic alteration in PTC and plays an important role in the tumorigenesis of this cancer (Xing 2005).

Since the initial report of BRAF mutations in human cancer (Davies et al. 2002), more than 40 point mutations in BRAF gene have been identified, with the T1799A mutation accounting for about 90% of them (Garnett & Marais 2004). This mutation causes a valine-to-glutamic acid amino acid change in codon 600 of the BRAF protein kinase, confers BRAFV600E oncogenic function by constitutively activating the kinase activity, and leads to tumorigenesis through aberrant activation of the Ras→Raf→MEK→> MAP kinase/ERK pathway (Davies et al. 2002, Garnett & Marais 2004). In thyroid tumors, the T1799A BRAF mutation occurred virtually only in PTC with an average prevalence of 44% (Xing 2005). Its tumor-initiating function for PTC tumorigenesis was well demonstrated in transgenic mouse model (Knauf et al. 2005). Most clinicopathological studies have showed its close association with aggressive clinicopathological characteristics and tumor recurrence (Namba et al. 2003, Nikiforova et al. 2003, Xing et al. 2005, Kim et al. 2006, Xing 2007).

In the present report, we investigated the relationship of the T1799A BRAF mutation with clinicopathological characteristics of PTC and peripheral platelet counts which are often associated with aggressiveness of other human cancers (Hernandez et al. 2000, Ikeda et al. 2002, Verheul & Pinedo 2003, Shimada et al. 2004, Brown et al. 2005, Bensalah et al. 2006) to examine the role of this mutation in PTC in Chinese where this mutation has not been well studied.

Materials and methods

Tumor samples and clinicopathological and laboratory data collection

With institutional approval, a series of 108 paraffin-embedded primary PTC samples were collected from the Affiliated Hospital of Qingdao University School of Medicine in Qingdao, China, including 94 conventional, 11 tall cell, and 3 follicular-variant PTC subtypes. The histopathological diagnoses and subtype classification of PTC tumors were examined and agreed upon by two experienced pathologists (Xianlu Sun & Yujun Li). Anaplastic and undifferentiated thyroid cancers were excluded from this study. Children under the age of 18 years at diagnosis of thyroid cancer were also excluded. The clinicopathological and laboratory data of the corresponding patients, as shown in the tables, were collected by a retrospective review of the records of the patients who underwent total or near-total thyroidectomy from 1996 to 2005. Preoperative hematological panel, including peripheral blood platelet count, was routinely performed within 3 days prior to thyroid surgery using an automated particle counter (CELL-DYN-1700). Thyroid-stimulating hormone, free tri-iodothyronine (FT3), free thyroxine (FT4), thyroid peroxidase antibody (TPO-Ab), and thyroglobulin antibody (TG-Ab) were determined by RIA, with the range of intra- and inter-assay coefficient of variability being 2.5–5.5% and 5.0–12.0% respectively.

Genomic DNA isolation

Paraffin-embedded PTC samples were microdissected and DNA isolated as previously described (Xing et al. 2005). Briefly, after an 8-h treatment at room temperature with xylene to remove paraffin, samples were subjected to protein digestion with a digestion solution containing 1% SDS and 0.5 mg/ml proteinase K at 48 °C for 48 h. A few mid-interval spiking aliquots of concentrated SDS-proteinase K were added to facilitate the digestion. DNA was subsequently isolated by standard phenol–chloroform extraction and ethanol precipitation.

Detection of BRAF mutation

The T1799A transversion BRAF mutation in exon 15 was analyzed by direct DNA sequencing. Using genomic DNA as the template, exon 15 of the BRAF gene was PCR amplified using the following primers: ACCTAAACTCTTTCAAATGCTTCTG (forward) and CTGATTTTTGTGAATACTGGGAACT (reverse). This PCR was run with a step-down protocol: 95 °C for 5 min for one cycle; 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min for two cycles; 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min for two cycles; and 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min for 35 cycles; followed by a final extension at 72 °C for 5 min. The reaction mixture, in a 30 μl volume, contained, in final concentration, 16.6 mm ammonium sulfate, 67 mm Tris (pH 8.8), 10 mm 2-mercaptoethanol, 1.5 mm each deoxynucleotide triphosphate, 6.7 mm MgCl2, 5% dimethylsulfoxide, 1.67 mm each primers (forward and reverse), 60 ng genomic DNA, and 0.5 U platinum DNA Taq polymerase (Life Technologies Inc., Gaithersburg, MD, USA). The specificity and integrity of the PCR were confirmed by a single band of PCR product with expected molecular weight on a 1.5% agarose gel. The PCR products were subsequently subjected to sequencing PCR with Big Dye terminator V 3.0 cycle sequencing reagents (Applied Biosystems, Foster City, CA, USA) with the following cycles: 95 °C for 30 s for one cycle, and 95 °C for 15 s, 50 °C for 15 s, and 60 °C for 4 min for 35 cycles. The samples were analyzed on an ABI PRISM...
3700 DNA Analyzer (Applied Biosystems) for mutation identification.

**Tissue immunohistochemical (IHC) staining**

IHC staining for platelet-derived growth factor-A (PDGF-A) and PDGF-B was performed in PTC samples. For the analysis of PDGF expression by IHC staining, 30 BRAF T1799A-positive and 30 BRAF T1799A-negative samples were randomly selected. Tissue sections were incubated with 1:500 primary antibodies against PDGF-A or PDGF-B (Boster Bioengineering Ltd, Wuhan, China) at 37 °C for 3 h. Development of color was achieved with DBA after incubation with the secondary antibody at 37 °C for 20 min. Negative controls were processed similarly except with the removal of the primary antibodies. Staining intensity was expressed as IHC scores as follows: 0, no staining; 1, weak or focal staining; 2, moderate staining in most cells; and 3, strong staining in most cells.

**Statistical analysis**

Categorical data were summarized using frequencies and percents. Parametric continuous data were expressed as mean±s.d. TPO-Ab, TG-Ab, and tumor sizes were summarized with medians and interquartile as they lacked normal distribution. Comparisons of two groups of categorical variables were performed using χ²-test. Comparisons of two groups of continuous parametric data were performed using t-test. Comparisons of non-parametric data of two groups with continuous variables were performed by Mann–Whitney U test. Multivariate Cox regression analysis was used to examine the independent significance of certain parameters. Linear regression analysis was used to determine the correlation between two factors. A P value <0.05 was considered to be statistically significant. All statistical analyses were performed using the SPSS statistical package (11.5, Chicago, IL, USA).

**Results**

**Association of BRAF mutation with aggressive clinicopathological characteristics of PTC**

The T1799A BRAF mutation is mostly associated with more aggressive tall cell and conventional PTC and is rarely seen in follicular variant PTC in various ethnic populations (Xing 2005). This distribution pattern has not been well examined in the Chinese population. We therefore first used the current Chinese cohort to explore further this issue. As shown in Table 1, we found the T1799A BRAF mutation in 45/94 (48%) conventional PTC, 8/11 (73%) tall cell PTC, 1/3 (33%) follicular variant PTC, and 54/108 (50%) total PTC in this Chinese cohort, being most common in the aggressive tall cell PTC. To further explore the role of the BRAF mutation in PTC tumorigenesis in the Chinese population, we analyzed its relationship with various clinicopathological characteristics of PTC, including the patient age at diagnosis, gender, tumor size, multifocality, extrathyroidal invasion, lymph node metastasis, and tumor stages. As shown in Table 2, the T1799A BRAF mutation was significantly associated with extrathyroidal invasion and advanced tumor stages III and IV. Specifically, extrathyroidal invasion was seen in 30/54 (56%) PTC with BRAF mutation versus 18/54 (30%) PTC without the mutation (P=0.02). Tumor stages III and IV were seen in 16/54 (30%) PTC with BRAF mutation versus 7/54 (13%) PTC without the mutation (P=0.04). Except for older patient age, no significant association of BRAF mutation with other clinicopathological characteristics of PTC was seen (Table 2).

**Association of BRAF mutation in PTC with higher peripheral blood platelet counts**

Prompted by previous findings that elevated peripheral blood platelet count was associated with some human cancers and their aggressiveness (Hernandez et al. 2000, Ikeda et al. 2002, Verheul & Pinedo 2003, Shimada et al. 2004, Brown et al. 2005, Bensalah et al. 2006), we analyzed the relationship of the BRAF mutation with platelet count and several other hematological and biochemical parameters. This represented an attempt to see if a higher platelet count played any role in BRAF mutation-associated tumor aggressiveness of PTC. As shown in Table 3, BRAF mutation was significantly associated with a higher peripheral blood platelet count. Specifically, the platelet count was 249.28 ± 53.76×10⁹/l in PTC patients harboring the T1799A mutation versus 207.79 ± 58.98×10⁹/l in patients without the mutation (P=0.001). The BRAF mutation was not associated with other hematological parameters, including blood leukocyte, neutrophil, erythrocyte, and hemoglobin. Similarly, no association of BRAF mutations was seen with several other clotting parameters.
thyroid function parameters and thyroid autoimmune antibodies (Table 3). These results suggest that the association of the T1799A BRAF mutation with higher platelet counts was a specific phenomenon.

**Association of extrathyroidal invasion of PTC with higher platelet counts**

Since BRAF mutations were associated with both poor pathological behaviors of PTC and higher peripheral blood platelet counts as shown above, we next examined whether there was a correlation between elevated platelet counts and poor clinicopathological characteristics of PTC. Indeed, as shown in Table 4, extrathyroidal invasion of PTC was closely associated with higher peripheral blood platelet counts. Specifically, the platelet count was 242.66 \( \pm \) 51.85 \( \times \) 10^9/l in PTC patients with extrathyroidal invasion versus 218.49 \( \pm \) 59.10 \( \times \) 10^9/l in PTC patients without extrathyroidal invasion (\( P = 0.03 \)).

Other hematological or thyroid function parameters were not associated with extrathyroidal invasion of PTC (Table 4), suggesting a specific role of high platelet counts in the development of this aggressive pathological behavior of PTC. Except for BRAF mutation and extrathyroidal invasion, none of other factors was significantly associated with increased platelet count, including patient age and gender, tumor size, multifocality, lymph node metastasis, tumor stage, and blood leukocyte. We performed multivariate analysis on the association with extrathyroidal invasion for these factors. After adjustment for all these factors, including platelet count, the odd ratios for the association of BRAF mutation with extrathyroidal invasion was 4.410 (95% CI: 1.571–12.377; \( P = 0.005 \)). When adjusted for these factors, including BRAF mutation, the odd ratios for the association of platelet count with extrathyroidal invasion was 1.121 (95% CI: 1.010–1.210; \( P = 0.012 \)). Although this latter association was statistically significant, the low odd ratios suggest that increased platelet count plays a role largely secondary to BRAF mutation in the development of extrathyroidal invasion of PTC. None of the remaining factors was significantly associated with extrathyroidal invasion.

**Association of BRAF mutation with PDGF-B expression**

Having demonstrated the association of BRAF mutation with higher platelet counts, we next explored whether the expression of platelet-released PDGF, a well-known tumor-promoting growth factor, is altered in relation to BRAF mutation. Using IHC staining, we examined...
expression of both PDGF-A and PDGF-B. We found no difference in the expression of PDGF-A in PTC between BRAF mutation-positive and BRAF mutation-negative groups (Table 5). In contrast, a significant association of BRAF mutation with the expression of PDGF-B in PTC was observed (Table 5 and Fig. 1). Specifically, a moderate to strong staining of IHC scores 2–3 for PDGF-B was seen in 27/30 (90.0%) PTC with BRAF mutation versus 16/30 (53.3%) PTC without the mutation ($P = 0.03$). This difference was apparently not due to other biological factors, such as patient age or gender, which could potentially confound the comparison of the two groups (Table 5). Linear regression analysis showed that increased platelet count was also marginally associated with increased PDGF-B expression ($r = 0.383$, $P = 0.048$). However, this association disappeared when cases without BRAF mutation were excluded ($r = 0.064$, $P = 0.737$). This could be expected given the result that BRAF mutation was associated with, and perhaps a major cause of, increased platelet count or PDGF-B (Tables 3 and 5).

### Discussion

Association of the T1799A BRAF mutation with poor clinicopathological outcomes in PTC has been reported in various ethnic populations (Namba et al. 2003, Nikiforova et al. 2003, Xing et al. 2005, Adeniran et al. 2006, Frasca et al. 2006, Kim et al. 2006, Lee et al. 2006, Riesco-Eizaguirre et al. 2006, Xing 2007), consistent with an important oncogenic role of this mutation in the tumorigenesis and progression of PTC. The present study investigated this role of BRAF mutation in a Chinese cohort by examining its relationship with clinicopathological characteristics of PTC. To this end, we also investigated the relationship of BRAF mutation with peripheral blood platelet counts as thrombocytosis is associated with various human cancers and their poor prognosis (Hernandez et al. 2000, Ikeda et al. 2002, Verheul & Pinedo 2003, Shimada et al. 2004, Brown et al. 2005, Bensalah et al. 2006). We found a close association of the T1799A BRAF mutation with extrathyroidal invasion and advanced tumor stages (III and IV) in this Chinese cohort of PTC. These results are similar to the findings on BRAF mutation in PTC in other ethnic populations (Nikiforova et al. 2003, Xing et al. 2005, Adeniran et al. 2006, Frasca et al. 2006, Lee et al. 2006, Riesco-Eizaguirre et al. 2006, Xing 2007), suggesting that BRAF mutation plays a similar role in promoting the progression and aggressiveness of PTC in the Chinese population.

Interestingly, we for the first time observed also a significant association of BRAF mutation with higher peripheral blood platelet counts in PTC patients. The BRAF mutation and higher platelet counts were both individually associated with extrathyroidal invasion of PTC. When BRAF mutation and platelet count were adjusted for each other, in addition to the adjustment for

### Table 4 Relationship of extrathyroidal invasion of papillary thyroid cancer (PTC) with platelet counts and other laboratory parameters (mean ± s.d.)

<table>
<thead>
<tr>
<th>Extrathyroidal invasion (+)</th>
<th>Extrathyroidal invasion (−)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>($N=48$)</td>
<td>($N=60$)</td>
<td></td>
</tr>
<tr>
<td>Platelet ($\times 10^9/l$)</td>
<td>242.66 ± 51.85</td>
<td>218.49 ± 59.10</td>
</tr>
<tr>
<td>Blood leukocyte ($\times 10^9/l$)</td>
<td>6.53 ± 2.18</td>
<td>6.65 ± 1.81</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>56.11 ± 9.87</td>
<td>56.66 ± 14.15</td>
</tr>
<tr>
<td>Erythrocyte ($\times 10^{12}/l$)</td>
<td>4.28 ± 0.48</td>
<td>4.21 ± 0.57</td>
</tr>
<tr>
<td>Hemoglobin (g/l)</td>
<td>129.26 ± 16.07</td>
<td>131.25 ± 12.17</td>
</tr>
<tr>
<td>TSH (mu/l)</td>
<td>4.13 ± 1.88</td>
<td>4.03 ± 2.66</td>
</tr>
<tr>
<td>TPO-Ab (u/l; median (interquartile ranges))</td>
<td>15.60 (9.94–21.81)</td>
<td>18.27 (11.61–24.94)</td>
</tr>
<tr>
<td>TG-Ab (u/l; median (interquartile ranges))</td>
<td>12.72 (9.73–15.72)</td>
<td>13.26 (10.02–16.50)</td>
</tr>
<tr>
<td>FT$_3$ (pmol/l)</td>
<td>5.29 ± 1.75</td>
<td>5.41 ± 1.20</td>
</tr>
<tr>
<td>FT$_4$ (pmol/l)</td>
<td>13.98 ± 2.98</td>
<td>14.05 ± 3.25</td>
</tr>
</tbody>
</table>

### Table 5 Correlation between BRAF T1799A mutation and platelet-derived growth factor (PDGF) expression in papillary thyroid cancer (PTC)

<table>
<thead>
<tr>
<th></th>
<th>$BRAF$ T1799A mutation ($N=30$)</th>
<th>Without $BRAF$ mutation ($N=30$)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years; mean ± s.d.)</td>
<td>48.13 ± 15.012</td>
<td>44.33 ± 15.248</td>
<td>0.335</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>7/23</td>
<td>4/26</td>
<td>0.506</td>
</tr>
<tr>
<td>PDGF-A (IHC scores 2–3; n (%))</td>
<td>27 (90.0%)</td>
<td>26 (86.7%)</td>
<td>1.00</td>
</tr>
<tr>
<td>PDGF-B (IHC scores 2–3; n (%))</td>
<td>27 (90.0%)</td>
<td>16 (53.3%)</td>
<td>0.03</td>
</tr>
</tbody>
</table>
other multiple factors on the multivariate analysis, it was mainly BRAF mutation that was still left to be significantly associated with extrathyroidal invasion. These data suggest that increased platelet count may be a mediating factor involved in BRAF mutation-promoted extrathyroidal invasion and aggressiveness of PTC. Among various clinicopathological characteristics of PTC, extrathyroidal invasion is a major risk factor that predicts tumor progression and aggressiveness (Mazzaferrri & Jhiang 1994), and BRAF mutation is most commonly associated with, among various clinicopathological characteristics, extrathyroidal invasion (Xing 2007). Transgenic mouse study clearly demonstrated the driving force of the BRAF mutation in promoting extrathyroidal invasion of PTC (Knauf et al. 2005). Therefore, it is convincing that BRAF mutation plays an important role in driving extrathyroidal invasion. One important molecular mechanism involved in this process is BRAF mutation-promoted methylation and hence silencing of the tissue inhibitor of metalloproteinase-3 (TIMP-3; Hu et al. 2006) and over-expression of metalloproteinases (Melillo et al. 2005, Mesa et al. 2006, Palona et al. 2006). TIMP-3 is not only an inhibitor of metalloproteinases, which promote interstitial matrix destruction and hence cancer invasion, but also an antagonist of the potent angiogenic molecule vascular endothelial growth factor (VEGF; Qi et al. 2003). BRAF mutation was shown to be associated with over-expression of VEGF in PTC (Jo et al. 2006). We now also demonstrated the association of BRAF mutation with over-expression of PDGF-B in PTC in the present study. One mechanism for the increased PDGF-B expression in PTC could be through elevation in platelets counts but alternative mechanisms such as autocrine production by PTC tumor itself may also exist. It is therefore possible that both VEGF and PDGF play a role in BRAF mutation-promoted pathogenesis of PTC, such as extrathyroidal invasion, which involves and requires vigorous vascular formation. Elevated platelet count in cancer is associated with increased circulating VEGF and PDGF which can all be released from platelets (Salven et al. 1999, Verheul & Pinedo 2003). The rise in platelet count in cancer is explained by a model in which intra-tumor activation of platelets leads to release of thrombopoietin, which specifically and potently stimulates megakaryocytes in the bone marrow to produce platelets (Verheul & Pinedo 2003). Based on these previous and our present data, we propose that BRAF mutation-promoted PTC invasion involves the production of pro-tumor molecules VEGF and PDGF-B from elevated peripheral platelet counts. Therefore, BRAF mutation and platelets seem to complete a vicious cycle in which extrathyroidal invasion of PTC promoted by BRAF mutation promotes platelet production which in turn enhances the pathologic progression of the tumor through accelerated production of angiogenic and pro-tumor molecules by platelets. The invasive regions of PTC contain gene expression changes that are consistent with epithelial-to-mesenchymal transition (EMT; Vasko et al. 2007) and a PDGF autocrine loop is required for EMT in hepatocellular carcinomas initiated by activation of the MAP kinase pathway (Fischer et al. 2007). It would therefore be interesting to investigate whether the BRAF mutation, platelet, PDGF model proposed here may play a role in the development of EMT in PTC.

In summary, we investigated the relationship of the T1799A BRAF mutation with clinicopathological outcomes and peripheral blood platelet counts in PTC in a Chinese cohort. We observed an association of BRAF mutation with extrathyroidal invasion, advanced tumor
stages, higher peripheral platelet counts, and increased expression of PDGF-B in PTC. These results, together with previous data, support a model in which BRAF mutation and cancer-associated thrombocytosis coordinate promotes pathologic progression and aggressiveness of PTC. Therefore, adjunctive interference in the platelet system may be a helpful therapeutic strategy in the treatment of aggressive BRAF mutation-harboring PTC.

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