Factors influencing the detection of the \textit{BRAF} T1799A mutation in papillary thyroid carcinoma

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\textbf{Abstract.} The \textit{BRAF} T1799A mutation is a heterozygous point mutation and its reported prevalence in papillary thyroid carcinoma (PTC) has varied from 29 to 83%, with an overall mean of 44%. In Korea, the reported mutation rate reached 83% in PTC and 52% in micropapillary carcinoma. We hypothesized that the differences in prevalence may be influenced by the methods of mutation analysis, the sizes of tumor and ethnic differences. Three types of DNA samples from the same PTC mass (0.4-1.6 cm, mean 0.83 cm sized) per each patient (n=17) were isolated. The first type was obtained from frozen PTC tissues using laser-captured microdissection (Frozen-laser, n=17), the second was obtained from frozen tissue by manual tumor margin dissection using a blade (Frozen-blade, n=17) and the third was obtained from formalin-fixed, paraffin-embedded tissue by manual margin dissection (Paraffin-blade, n=15, 2 failed). The mutation rates of the three-matched DNA samples were compared by the SNP mode and AQ mode of pyrosequencing, and direct DNA sequencing. Both the AQ mode of pyrosequencing and the direct DNA sequencing detected the \textit{BRAF} T1799A mutation in nearly all Korean PTCs, and even in micro-PTCs. For the accurate detection of the \textit{BRAF} T1799A mutation from small PTCs, fresh or frozen tissues and more cautious microdissection are required, and the AQ mode of pyrosequencing assay is preferred.

\textbf{Introduction}

Papillary thyroid carcinoma (PTC) is the most common malignant tumor of the thyroid gland. Recently, the detection of impalpable small PTCs has markedly increased due to the rapid development of high-resolution ultrasound technology and ultrasound-guided fine needle aspiration cytology (1). PTC is a slowly progressing tumor with low mortality, but it is often multifocal, it can be present in the bilateral lobes and it spreads to regional lymph nodes early in its course (2-4). These characteristics caused some anxiety for both patients and doctors. Based on the increased amount of evidence, American Thyroid Association (ATA) recently revised the management guidelines for patients with differentiated thyroid cancer (5). Moreover, recent dramatic advances in the understanding of the molecular pathogenesis of PTC lead us to expect the development of effective molecular pathogenesis-based therapy (6,7). For the personalized management of PTC, more exact molecular diagnosis may be needed in future.

Mutations of the gene encoding B-type RAF kinase (BRAF) have been found in various human cancers, including malignant melanoma, some colorectal and ovarian cancers and most PTCs (8). BRAF activates the mitogen-activated protein kinase (MAP kinase) pathway in the middle of a cascade of RET-RAS-RAF-MEK-ERK-MAP kinase signal transduction (9). The most prevalent \textit{BRAF} mutation in human cancers, including PTC, is a heterozygous T1799A transition in exon 15, leading to a substitution of glutamic acid for valine at position 600 (V600E) (8,10,11). \textit{In vitro}, this \textit{BRAF} mutation leads to a 10-fold increase in the activation of ERK over that seen with wild-type BRAF (9). The \textit{BRAF} T1799A mutation is highly prevalent in PTC, and especially in Koreans. Thus, the \textit{BRAF} T1799A mutation may play a critical role in PTC tumorigenesis and it may be a powerful target for treatment as well as a useful diagnostic and prognostic marker (12).

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Fine-needle aspiration (FNA) cytology is the most sensitive and specific method for identifying PTCs, and the detection of *BRAF* T1799A mutation on FNA specimens adds to the diagnostic accuracy (13-16). Because the mutation involves a single nucleotide change that is easy to identify in genomic DNA and it is consistently found in primary tumors, molecular evidence of the *BRAF* T1799A mutation in a FNA specimen is highly specific for PTC (13-19). However, analyses of cytological specimens and paraffin-embedded tissue samples have sometimes yielded discordant results (11). The discrepancy is probably caused by insufficient levels of tumor DNA during preparation of PTC samples from the specimens (12), suggesting that the sensitivity of finding the *BRAF* T1799A mutation may be limited.

In this study, we showed that the *BRAF* T1799A mutation was detected in all the PTCs isolated by laser-captured microdissection of frozen tissues, but it was omitted in some of the specimens isolated by conventional scalpel dissection of frozen tissues and in many more of the formalin-fixed, paraffin-embedded tissues. These results indicate that the previous studies may have underestimated the true prevalence of *BRAF* T1799A mutation because of the methods of specimen preparation.

**Materials and methods**

**Tissue samples.** PTC samples were obtained from 17 patients who underwent total thyroidectomies at Daejeon St. Mary's Hospital of the Catholic University of Korea in 2007. During the operation, the main PTC mass was resected, and a portion was used to prepare formalin-fixed paraffin blocks for pathological examination. The remaining tissue was frozen at -80°C. The included patients were 14 females and 3 males and they ranged in age from 31 to 68 years (mean age, 48), and they had PTCs with sizes ranging from 0.4 to 1.6 cm (mean, 0.83 cm). All the tumors except one, which was the follicular variant, had the typical histology of papillary carcinoma. Blood was collected from 14 of 17 patients and from 12 healthy persons. All the samples were obtained along with written informed consent, and the study was approved by the hospital Internal Review Board.

**PTC isolation from frozen or formalin-fixed paraffin tissue.** After clinical use, the frozen or formalin-fixed paraffin tissues from the same PTC mass were used to isolate genomic tumor DNA by three different methods. The first and second preparations were obtained from frozen PTC tissue, while the third was from formalin-fixed, paraffin-embedded tissue. They consisted of 8 µm-thick sections of matched formalin-fixed paraffin-embedded tissue, and they also had been manually dissected along the tumor margin with scalpels after H&E staining.

**Genomic DNA preparation.** Genomic DNA from PTCs isolated by the Frozen-laser or Frozen-blade methods were purified using the QIAamp DNA Micro Kit (Qiagen GmbH, Hilden, Germany). The specimens from the Paraffin-blade method were dewaxed by washing in xylene and then by rinsing in ethanol. The dried tissues were digested with proteinase K and subjected to DNA extraction using phenol-chloroform-isooamyl alcohol and ethanol precipitation. Blood DNA was also purified using the QIAamp DNA Blood Midi Kit (Qiagen GmbH). The concentrations of all the purified DNA preparations were measured using the Nanodrop ND-1000 Spectrophotometer (Nanodrop technologies, Wilmington, NC, USA).

**Pyrosequencing assay for the *BRAF* T1799A mutation.** A total of 2 ng of PTC DNA was amplified by polymerase chain reaction (PCR) in a 25-µl reaction mixture containing a forward primer (5'-TGAGGACCTCACTGAAATATGGTG-3') and a biotinylated reverse primer (5'-biotin-CCCAAATAAGGTAGTTGACTACA-3') designed to yield a 102-bp product containing the nucleotide position 1799 of the *BRAF* gene. All the amplification reactions were performed as follows: 94°C for 2 min, 40 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 40 sec and followed by 72°C for 5 min. The PCR products were run on a 2% agarose gel to ensure an adequate yield and purification. Pyrosequencing assays were performed according to the manufacturer's instructions using the sequencing primer (5'-TGATTTTTGGTCTAGTCA-3') on the PSQ HS 96A System (Biotage AB, Sweden). The sequence at the nucleotide position 1799 of the BRAF gene was analyzed by the proportions of the mutant A-allele peak in the AQ mode and also in the SNP mode. Each analysis was performed in triplicate.

**Direct DNA sequencing analysis of *BRAF* T1799A.** Each 2-ng sample of PTC DNA was also amplified to examine the nucleotide sequence at position 1799 of the *BRAF* gene, and the following PCR primer set was used: forward primer, 5'-TTGACTCTAAGAGGAAAGATGAA-3'; reverse primer, 5'-TAGTGTGAGACCTTCAATGACTTT-3'. The assay was designed to yield a 357-bp product. The sequencing reactions for each PCR product were bi-directionally performed with the forward and reverse primers used with the Big Dye terminator V1.1 cycle sequencing kit (Applied Biosystems, CA, USA) as follows: 96°C for 1 min, then 35 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. The product was analyzed by an ABI PRISM® 3700 genetic analyzer (Applied Biosystems).

**Statistical analysis.** The proportion of the mutant A-allele in the *BRAF* 1799 position, as assessed by the AQ mode of pyrosequencing, was used to express the presence of the
**Results**

**Discrimination of the BRAF T1799A mutation on pyrosequencing assay.** When the BRAF T1799A mutation is present in PTC in a heterozygous condition, the proportion of the A-mutant allele (i.e., the mutation) would be theoretically close to 50% on the AQ mode of the pyrosequencing assay. On the other hand, background noise in a pyrosequencing assay can be detected as a false positive mutation, even in normal cells. Therefore, to optimize the sensitivity for the BRAF T1799A mutation, a threshold proportion must be set above the level of the background noise. When the proportion of A-allele exceeds this threshold, the test for the mutation is considered positive. In this study, the frequency of the A-allele in the blood DNA was 4.3-8.7% (mean ± SD, 6.7±1.39%) in the 12 healthy volunteers and 5.9-10.1% (mean ± SD, 7.3±1.38%) in 14 patients (the left most panel of Fig. 2), and this did not significantly differ between the two groups. This result shows that the A-allele frequency may appear as high as 10%, due to background noise, even in the absence of the BRAF T1799A mutation. Therefore, 10% was set as the threshold frequency for defining a positive test for the mutation.

**Detection of BRAF T1799A in the PTCs isolated by different methods.** Fig. 2 shows the representative pyrosequencing results for the BRAF T1799A mutation in the PTCs isolated from two patients. In the normal thyroid cells isolated by the Frozen-laser method, the A-allele frequencies were detected...
in the AQ mode at 8 and 7.2% in each patient, respectively. In fact, the mean A-allele frequency in the normal thyroid cells from the 17 PTC patients was 7.54±1.2%, which was not much different from those in the blood DNA from the patients and healthy controls. This result shows that the normal cells around the PTC did not carry the BRAF T1799A mutation, based on the 10% threshold. On the other hand, the A-allele frequencies in the PTC cells isolated by the Frozen-laser method were 38.9 and 40.5% in each of two patients, respectively (Fig. 2) and this ranged from 31.1 to 44.6% (mean ± SD, 39.84±2.91%) in the 17 PTCs. These results indicate that all the PTCs carried a positive BRAF T1799A mutation at a frequency well above the 10% threshold (Fig. 3A). Fig. 2 also shows the pyrograms of the PTCs prepared by the Frozen-blade and Paraffin-blade methods in the same patient. The A-allele was detected with these two methods at 35.5% and 34.3%, respectively, in case no. 16 (i.e., positive for the mutation), but at 8.1 and 5.8%, respectively, in case no. 11 (i.e., negative for the mutation). These results suggest that in case no. 11 the PTC was mixed with non-PTC cells during isolation by the Frozen-blade or Paraffin-blade methods. The case no. 11 had a lymphocyte infiltration due to chronic lymphocytic thyroiditis and moderate fibrosis within the tumor.

In each patient of the 15 patients without missing data, the proportions of the A-allele among the three types of DNA preparation were significantly different (p=0.00001). The result by the Frozen-laser was significantly higher than the results by the Frozen-blade (p=0.0001) and Paraffin-blade methods (p=0.0001). Also, the result by the Frozen-blade was significantly higher than the one by the Paraffin-blade (p=0.023) (Fig. 3). The proportions of the A-allele of the PTC isolated by the Frozen-blade method ranged from 8.1 to 38.17% (mean ± SD, 28.33±7.99%) in the 17 PTCs; only one case (5.9%) failed to meet the 10% cut-off in the AQ mode (case no. 11 in Figs. 2 and 3A), and three cases (17.6%) were called as ‘T/T’ or ‘mutation-free’ in the SNP mode by the machine (Table I). In the 15 PTCs prepared by the Paraffin-blade method, the A-allele frequencies ranged from 5.7 to 36.3% (mean ± SD, 21.2±12.5%) (Fig. 3A); as many as five PTCs (33.3%) were below the 10% threshold in the AQ mode, and nine (60%) failed to be called as T/A in the SNP mode (Table I).

Detection of BRAF T1799A in the PTCs by direct sequencing.
We evaluated the efficacy of pyrosequencing vs. direct DNA sequencing for detecting the mutation in the isolated PTCs. In case no. 11, a distinct A-allele peak was detected only in the PTC from Frozen-laser and thus it was read as ‘N’ according to base labeling in the DNA sequencing technique, but an A-allele peak was not detectable in the Frozen-blade or Paraffin-blade preparations (Fig. 2), showing that these results from DNA sequencing were the same as those results from pyrosequencing. Yet in case no. 16, a distinct A-allele peak was detected by pyrosequencing and direct sequencing in all the PTC preparations, but according to base labeling on direct sequencing, the nucleotides were read as ‘T’ in the

Table I. The detection rate of the BRAF T1799A mutation based on the sample preparation, the analysis tool and the discrimination criteria.

<table>
<thead>
<tr>
<th>Method</th>
<th>Criteria for BRAF T1799A (%)</th>
<th>Frozen-laser (n=17) (%)</th>
<th>Frozen-blade (n=17) (%)</th>
<th>Paraffin-blade (n=15) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrosequencing</td>
<td>AQ mode (A &gt;10%)</td>
<td>17 (100)</td>
<td>16 (94)</td>
<td>10 (67)</td>
</tr>
<tr>
<td></td>
<td>SNP mode (T/A heterozygote)</td>
<td>17 (100)</td>
<td>14 (82)</td>
<td>6 (40)</td>
</tr>
<tr>
<td>Direct DNA sequencing</td>
<td>Detectable ‘A’ peak</td>
<td>17 (100)</td>
<td>16 (94)</td>
<td>10 (67)</td>
</tr>
</tbody>
</table>
Frozen-blade and Paraffin-blade PTC preparations (bottom panel in Fig. 2). This result indicates that the height of the A-allele peak by direct DNA sequencing sometimes corresponded to the proportion of the A-alleles in the pyrogram, but when the A-allele peak was below about 40% it was often missed according to base labeling in the sequencing analysis. On the basis of the visible A-allele peak regardless of base labeling, direct sequencing showed the same sensitivity as that of the AQ mode of pyrosequencing for the detection of BRAF T1788A mutation.

Discussion

Since the BRAF T1799A mutation is the most prevalent molecular event in sporadic PTC pathogenesis in humans, it may be a powerful target for diagnosis and treatment. Various methods have been evaluated for detecting the BRAF T1799A mutation in specimens from thyroid nodules, including direct DNA sequencing (13-18), RFLP (20) and real-time, allele-specific PCR analysis (18,19). More recently, pyrosequencing has been demonstrated to be a rapid and sensitive method for detecting the BRAF T1799A mutation (21). In this study, using the AQ mode of pyrosequencing analysis, we found the background noise for the A-allele to be at a maximum of 10% at the nucleotide position 1799 of the BRAF gene in the normal DNA taken from blood and the result corresponds well to a previous study that demonstrated that the background signal from pyrosequencing analysis ranged from 2 to 11% with an average of 6% (22). Thus, we established that the frequency of the allele must exceed the threshold of 10% to be considered a positive test for the mutation.

Based on this criterion, we showed that the BRAF T1799A mutation was clearly detected in all the PTCs prepared by the Frozen-laser method. Of the seventeen small PTCs tested in this study, six (35.3%) had multifocal tumors, seven (41.2%) had metastasized to regional lymph nodes and eight (47.1%) had invaded the thyroid capsule and/or surrounding tissue. Five patients had accompanying chronic lymphocytic thyroiditis and a lymphocyte infiltration in the PTC mass. Fourteen PTC masses contained a mild to moderate amount of fibrotic tissue. In spite of these pathological differences, our results indicate that the BRAF T1799A mutation may be a common characteristic in all PTCs. If a PTC carries the BRAF T1799A mutation in a heterozygous state, but the adjacent normal cells or other cells do not have the mutation, then the Frozen-laser method ensures PTC homogeneity of approximately 60 to 90%. On the other hand, the BRAF T1799A mutation was not detected in parts of the PTCs prepared by the Frozen-laser and paraffin-preparation methods from the same samples. In the same manner, the PTC homogeneity may be inferred to be 16-76% in the samples from the Frozen-laser method or 11-72% from the Paraffin-blade method. Our results indicated that the PTCs prepared by the Frozen-blade and paraffin-blade methods were variously mixed with stromal or normal cells and they showed a low yield of tumor DNA in some cases, which resulted in negative tests for the BRAF T1799A mutation in 1 of 17 Frozen-blade samples and in 5 of 15 Paraffin-blade samples. The extraction of high quality DNA from archival, formalin-fixed, paraffin-embedded tissue is problematic (26). Although our series of paraffin-blocks were not stored for a long time, the quality of tumor DNA extracted from them would not be as high as that from the frozen tissues. The differences may be amplified by the small size of the PTCs. A lower prevalence of the mutation (52-63%) was reported in micropapillary carcinoma than the 78-83% prevalence of the mutation in the overall PTCs in Koreans (20,24,25). The prevalence of mutation in the overall PTCs in Koreans was similar with the percents in the paraffin-blade preparation of our small PTCs. One recent report from Italy using manual microdissection of paraffin-blocks showed that the identification of BRAF T1799A was decreased according to the size of tumor: BRAF T1799A was identified in 17.6% of 0.5-4 mm sized incidental microcarcinomas, in 38.3% of the identified microcarcinomas (<1 cm) and in 45% of the clinically evident PTCs (26).

To examine the prevalence of the BRAF T1799A mutation in PTC, the previous studies generally used the method of manual microdissection with a scalpel to isolate PTC from the formalin-fixed, paraffin-embedded tissue (what we called the Paraffin-blade method). The prevalence of the BRAF T1799A mutation in PTC has been reported to be from 29 to 83% with an average of 44% worldwide (11). The current study demonstrates that the Paraffin-blade method may not be suitable to confirm the BRAF T1799A mutation in small PTC due to the low quality of tumor DNA.

In addition, it is unlikely that the SNP mode in pyrosequencing analysis is useful for measuring the mutation because the T/A heterozygote in PTC can often be read as the T/T homozygote when the tumor is mixed with large numbers of adjacent normal cells, as was noted in our results with the Frozen-blade and paraffin-blade methods. Our data also showed that as the BRAF T1799A mutation was examined by direct DNA sequencing, the A- and T-allele peaks were clear or recorded as ‘N’ on the electropherograms of all the PTCs from the Frozen-laser method, while the A-allele peak was often ambiguous on the electropherograms of some PTCs from the Frozen-blade and Paraffin-blade methods. Therefore, our results demonstrate that when direct DNA sequencing is used, the Frozen-laser method is much more effective than the other two methods for detecting the BRAF T1799A mutation in PTC.

In summary, the variable results of the prevalence of BRAF T1799A in PTC could be influenced by the poor quality of the tumor DNA obtained from formalin-paraffin tissues, the dilution of tumor DNA due to manual margin dissection, the amplification of the afore-mentioned differences in small-sized tumor and the ambiguity when interpreting the A-allele peak from the results of direct sequencing or pyrosequencing, in addition to ethnic differences in the prevalence of BRAF T1799A mutation.

In conclusion, we showed that the BRAF T1799A mutation was clearly detected in all the PTCs isolated by the Frozen-laser method. Thus, the Frozen-laser method is considered superior to the others, such as the Frozen-blade and Paraffin-blade methods; these other methods may in fact account for the discrepancies in the prevalence reported in the literature. We conclude that the BRAF T1799A mutation in PTC may have been underestimated in previous studies. Further analysis is needed to confirm the true prevalence of
the \textit{BRAF} T1799A mutation in a large sample of PTCs with using the Frozen-laser method.

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\section*{References}