Anatomic Pathology / BRAF Mutation Detection Using Three Methods

Detection of BRAF V600E Mutation With Thyroid Tissue Using Pyrosequencing

Comparison With PNA-Clamping and Real-Time PCR

Seong-Ho Kang, MD, PhD,¹ Ju Yeon Pyo, MD,² Seok-Woo Yang, MD,² and Soon Won Hong, MD, PhD²

Key Words: BRAF, Peptide nucleic acid; Pyrosequencing; Quantification; Sensitivity

Abstract

We used pyrosequencing, peptide nucleic acid (PNA)–clamping polymerase chain reaction (PCR), and real-time PCR to detect the BRAF V600E mutation and to investigate the prognostic effect of the BRAF V600E mutation in paraffin block specimens from 100 patients diagnosed with papillary thyroid carcinoma. Positive rates of PNA-clamping PCR, real-time PCR, and pyrosequencing were 66%, 70%, and 68%, respectively. Pyrosequencing and PNA-clamping PCR detected mutant type in a 99:1 (wild-type:mutant) DNA concentration, and PNA-clamping PCR detected mutant type in a 99.5:0.5 DNA concentration. Clamping PCR showed higher κ value than real-time PCR (0.729 vs 0.626). The BRAF V600E mutation was associated with an advanced stage of cancer (P = .045) and was found to be associated with poor prognostic factors. This study suggests that pyrosequencing can be as sensitive as real-time PCR and that PNA-clamping PCR is a sensitive and reliable method to detect the BRAF V600E mutation.

Thyroid carcinoma is the most common endocrine tumor, and papillary thyroid carcinoma (PTC) is the most common type of thyroid carcinoma.¹ Diagnosis of PTC is made with fine-needle aspiration biopsy (FNAB) of the thyroid nodule and, in Korea, by evaluating for the BRAF V600E mutation. The BRAF mutation was first detected in malignant melanoma, colon cancer, and other tumors.² The V600E mutation is the most common type of BRAF mutation in PTC.³,⁴ The incidence of the BRAF V600E mutation in Korean patients with PTC is higher than that of patients in other countries.⁵,⁷ Whether the BRAF V600E mutation is a poor prognosticator of PTC is controversial. Some researchers have reported that BRAF V600E is correlated with a poor outcome of PTC,⁸,⁹ but others have not reported such a correlation.¹⁰ Various methods, such as direct sequencing, allele-specific polymerase chain reaction (PCR), real-time PCR, and pyrosequencing, are used to detect the BRAF V600E mutation. Direct sequencing is considered the reference method but requires PCR and dye purification and is therefore labor intensive. Direct sequencing is also less sensitive than other methods because mutant DNA can only be detected when it comprises more than 15% to 20% of a sample.¹¹,¹² Allele-specific PCR is reported to be a simple, rapid, and reliable method to detect the BRAF mutation,¹³,¹⁴ but it requires gel loading, which may cause contamination, and reading of the gel may be difficult when the bands of PCR are faint. Real-time PCR does not require gel loading, and interpretation of results is easier than with allele-specific PCR. Peptide nucleic acid (PNA)–mediated clamping PCR (PNA-clamping PCR) is based on the principle that PNA inhibits wild-type by hybridizing normal sequences, and therefore mutant DNA is preferentially amplified.¹⁵ This was recently introduced.
as a method to detect the BRAF V600E mutation. Pyrosequencing can quantify the amount of mutant type present and does not need purification of PCR product and sequencing dye. The present study aims to compare PNA-clamping PCR and real-time PCR with pyrosequencing and to investigate the prognostic effect of the BRAF V600E mutation.

Materials and Methods

One hundred patients diagnosed with PTC were enrolled in the study, and informed consent was obtained. The study was approved by the Gangnam Severance Hospital (Seoul, Korea) institutional review board. DNA was extracted from paraffin block using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Elution volume was 40 μL. To evaluate the limit of detecting mutants among wild-types, wild-type DNA (HeLa cell line) was mixed with mutant DNA (Colo205 cancer cell line). The relative concentrations (wild-type: mutant) of DNA were 0:100, 50:50, 80:20, 90:10, 95:5, 99:1, 99.5:0.05, and 100:0.

PNA-Mediated Clamping PCR

We used the PNA-Clamp BRAF mutation detection kit (Panagene, Daejeon, Korea) to detect the BRAF V600E mutation. All reactions totaled 20 μL in volume and contained template DNA, primers, a PNA probe, and SYBR green PCR master mix. Real-time PCR reaction of PNA-clamping PCR was performed using a CFX96 real-time PCR system (Bio-Rad, Pleasanton, CA). PCR cycling conditions included a 5-minute hold at 94°C followed by 40 cycles of 30 seconds at 94°C, 20 seconds at 70°C, 30 seconds at 63°C, and 30 seconds at 72°C.

In this assay, the PNA probe and primers were separate oligonucleotides, and the PNA probe was located between forward and reverse primers in the template. Positive signals were detected by intercalation of SYBR green fluorescent dye. The PNA probe sequences, which are complementary to wild-type (V600), enhance preferential amplification of mutant sequences by competitively inhibiting amplification of wild-type sequences (Figure 1). PCR efficiency was determined by measuring the cycle threshold (Ct) value. Ct values for the control and mutation assays were obtained by observing the SYBR green amplification plots. The delta Ct (ΔCt) value was calculated by subtracting the Ct value of a tested sample from the standard Ct value of a clamping control sample ([Standard Ct] – [Sample Ct] = ΔCt). The cutoff for a positive sample was ΔCt = 2.

Real-Time PCR Using Anyplex BRAF V600E Real-Time Detection System

The BRAF V600E mutation was also detected using the Anyplex BRAF V600E real-time detection system (Seegene,
Seoul, Korea). For 1 reaction, the mixture contained 2 μL of ×10 BRAF Oligo Mix, 3 μL of 8-Mop (8-methoxypsoralen) solution to prevent carryover contamination, and 10 μL of Anyplex PCR master mix containing DNA polymerase, buffer, and deoxynucleoside triphosphates. The reaction tube was either inverted 5 times or quickly vortexed to mix materials. Fifteen microliters of the reaction mixture was dispensed into 0.2-mL PCR tubes. Five microliters of each sample’s nucleic acid was added to the reaction mixture tube to bring the total reaction volume to 20 μL. Real-time PCR was performed on a CFX96 real-time PCR system or StepOne real-time PCR systems (Applied Biosystems, Carlsbad, CA) under the following conditions: hold for 15 minutes at 95°C, followed by 45 cycles of 30 seconds at 95°C and 30 seconds at 60°C.

The Ct in real-time PCR assay is defined as the number of cycles at which the fluorescent signal exceeds the threshold. A sample and internal control with a Ct value below 40 were considered positive. Each run contained a positive control and negative control.

**Pyrosequencing**

Quantitative estimation of BRAF V600E mutants using pyrosequencing was performed as described previously. Briefly, PCR amplification was performed with a forward primer (5'-GAAGACCTCACAGTAAAAATAG-3') and a reverse primer (5'-biotin-ATAGCCTCAATTCTTACCATCC-3') on a PTC-200 thermal cycler (MJ Research, Waltham, MA). The pyrosequencing reaction was done with a sequencing primer (5'-biotin-ATAGCCTCAATTCTTACCATCC-3') on a Pyromark Q24 instrument (Qiagen). Pyrogram outputs were analyzed with the PyroMark Q24 software (Qiagen) to determine the percentage of mutant vs wild-type alleles according to relative peak height.

**Statistics**

The Student t test was used to analyze differences in mean age and mean tumor size between the BRAF V600E–positive and –negative groups. Differences in multifocality, extrathyroidal invasion, and lymph node metastasis were analyzed with a χ² test. Differences in stage were analyzed with the Fisher exact test. Univariate and multivariate logistic regression analyses were performed to determine the association between BRAF V600E mutation and clinicopathologic features. SPSS version 12.0 (SPSS, Chicago, IL) was used for the statistical analysis.

**Results**

**Patient Characteristics**

The mean age ± standard deviation (SD) at diagnosis was 42.7 ± 10.8 years (range, 16-80 y). The mean tumor size ± SD was 0.98 ± 0.77 cm (range, 0.2-5.0 cm). Of 100 patients, 56 (56%) had lymph node metastasis, 56 (56%) had extrathyroidal extension, and 25 (25%) had multicentric tumors. Of 100 patients, the pathology results of 8 (8%) revealed follicular variant papillary thyroid carcinoma, 4 (4%) revealed diffuse sclerosing variant papillary thyroid carcinoma, and the remainder were conventional type.

**Detection Limit of the Three Methods**

Several concentrations of wild-type: mutant DNA were tested to evaluate the detection limit of the 2 real-time PCR methods. Aforementioned concentrations of wild-type: mutant DNA were each tested 3 times. PNA-clamping PCR was positive 3 times at a concentration of 99.5:0.5 (wild-type: mutant) DNA. Anyplex real-time PCR was positive 2 of 3 times at the DNA concentration of 99:1. Anyplex real-time PCR was negative 3 times at the DNA concentration of 99:5:0.5.

To obtain a cutoff value for pyrosequencing, samples containing wild-type: mutant DNA concentrations of 100:0, 99.5:0.5, and 99:1 were each tested 3 times. Pyrosequencing peaks (percentage of mutant A peak height compared with the nearest normal peak height) of 100:0 DNA were 2.03%, 2.12%, and 2.31%, those of 99.5:0.5 DNA were 2.27%, 2.45%, and 2.69%, and those of 99:1 DNA were 3.22%, 3.99%, and 4.69%. Cutoff value was defined as 3.2%. DNA concentration of 90:10 was also tested 3 times using pyrosequencing. Pyrosequencing peaks of the 90:10 DNA were 9.47%, 9.37%, and 9.01%, suggesting that a 10% concentration of mutant DNA corresponds to a 10% pyrosequencing peak.

**Figure 2** Detection limits of peptide nucleic acid (PNA)–clamping polymerase chain reaction (PCR). DNA concentrations of 0:100, 50:50, 80:20, 90:10, 95:5, 99:1, 99.5:0.05, and 100:0 (wild-type: mutant) were tested. The threshold cycle (Ct) value of the control sample was 35. The cutoff for a positive sample was ∆Ct = 2. PNA-clamping PCR was positive at a DNA concentration of 99.5:0.5 (wild-type: mutant). RFU, relative fluorescence units.
Detection of the BRAF Mutation Among 100 Patients With PTC

PNA-clamping PCR detected the BRAF V600E mutation in 66 (66%) of 100 patients. Anyplex real-time PCR detected the BRAF V600E mutation in 70 (70%) of 100 patients. Pyrosequencing detected the BRAF V600E mutation in 68 (68%) of 100 patients. The BRAF V600E mutation was detected in 77 patients (77%) using 1 or more of the 3 methods. Of 8 patients with the follicular variant, 3 (37.5%) were BRAF V600E mutation positive. Of 4 patients with the diffuse sclerosing variant, 3 (75%) were BRAF V600E mutation positive.

Pyrosequencing was the reference method of BRAF mutation detection in the present study.

The κ value of PNA-clamping PCR compared with pyrosequencing was 0.729, and that of Anyplex real-time PCR compared with pyrosequencing was 0.626. The accuracy of PNA-clamping PCR compared with pyrosequencing was 88%, and that of Anyplex real-time PCR compared with pyrosequencing was 84%. Discrepant results between PNA-clamping PCR and pyrosequencing ranged from 2.46% to 5.41% of the pyrosequencing peak, and discrepant results between Anyplex real-time PCR and pyrosequencing ranged from 1.76% to 6.41% of the pyrosequencing peak.

Table I
Clinical Features According to BRAF V600E Mutation Status

<table>
<thead>
<tr>
<th>Clinical Feature</th>
<th>BRAF V600E–Positive (n = 77)</th>
<th>BRAF V600E–Negative (n = 23)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age ± SD, y</td>
<td>43.7 ± 10.3</td>
<td>39.4 ± 11.8</td>
<td>.529</td>
</tr>
<tr>
<td>Mean tumor size ± SD, cm</td>
<td>0.97 ± 0.77</td>
<td>1.01 ± 0.90</td>
<td>.144</td>
</tr>
<tr>
<td>Multifocality, %</td>
<td>18 (23.4)</td>
<td>7 (30.4)</td>
<td>.471</td>
</tr>
<tr>
<td>Extrathyroidal extension, No. (%)</td>
<td>47 (61.0)</td>
<td>9 (39.1)</td>
<td>.063</td>
</tr>
<tr>
<td>Lymph node metastasis, No. (%)</td>
<td>47 (61.0)</td>
<td>9 (39.1)</td>
<td>.063</td>
</tr>
<tr>
<td>Stage, No. (%)</td>
<td></td>
<td></td>
<td>.045</td>
</tr>
<tr>
<td>I</td>
<td>46 (60.0)</td>
<td>20 (87.0)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>26 (34.0)</td>
<td>3 (13.0)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>5 (6.0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>III + IV</td>
<td>31</td>
<td>3</td>
<td>.023</td>
</tr>
</tbody>
</table>

Table II
Univariate and Multivariate Analysis of BRAF V600E Mutation and Clinicopathologic Features of Papillary Thyroid Carcinoma

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate Odds Ratio (95% CI)</th>
<th>Multivariate Odds Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multifocality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Yes</td>
<td>0.697 (0.248-1.959)</td>
<td>0.753 (0.242-2.342)</td>
</tr>
<tr>
<td>Extrathyroidal extension</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Yes</td>
<td>2.437 (0.938-6.330)</td>
<td>1.512 (0.433-5.274)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Yes</td>
<td>2.437 (0.938-6.330)</td>
<td>2.437 (0.938-6.330)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>III and IV</td>
<td>4.493 (1.229-16.422)</td>
<td>5.021 (1.123-22.440)</td>
</tr>
</tbody>
</table>

CI, confidence interval.
1.23-22.440) Table 2I and that BRAF V600E mutation is independently associated with poor prognostic factors.

Discussion

In the current study, the detection rate of the BRAF V600E mutation was 66% with PNA-clamping PCR, 70% with Anyplex real-time PCR, and 68% with pyrosequencing. The rate of BRAF V600E mutation detection with any of the 3 methods was 77%. Previous studies used thyroid tissue to evaluate the prevalence of the BRAF V600E mutation in Korean patients with PTC. They reported a detection range of 58% to 81%. These studies used direct sequencing to detect the BRAF V600E mutation, which has been used as the reference method. However, pyrosequencing was reported to be more sensitive than direct sequencing, and direct sequencing cannot detect the presence of mutant alleles when the mutant: wild-type ratio is less than 1:5.12

Pyrosequencing is a rapid and sensitive method compared with direct sequencing for quantifying the BRAF V600E mutation. This method has been used to detect the BRAF V600E mutation in FNAB specimens of thyroid nodules in prior studies. The current study found the mean pyrosequencing peak to be 8.8% in patients with the BRAF V600E mutation, which is rather low. About 30% of patients with the BRAF mutation had a pyrosequencing peak below 6%, which is lower than the detection limit of direct sequencing. These pyrosequencing results allow for an evaluation of the sensitivity and accuracy of PNA-clamping PCR and real-time PCR. PNA-clamping PCR demonstrated a better κ value and accuracy than Anyplex real-time PCR. Discrepant results between PNA-clamping PCR and pyrosequencing ranged from 1.76% to 6.41%, whereas discrepan-ant results between Anyplex real-time PCR and pyrosequencing ranged from 2.46% to 5.41% of the pyrosequencing peak, and discrepant results between PNA-clamping PCR and pyrosequencing ranged from 1.76% to 6.41% of the pyrosequencing peak. PNA-clamping PCR demonstrated a narrower range of pyrosequencing peak in the discrepant results compared with Anyplex real-time PCR (2.46% to 5.41% vs 1.76% to 6.41%). These results imply that PNA-clamping PCR may offer a sensitive and reliable alternative method to pyrosequencing, particularly for the detection of a small amount of mutant. The use of PNA-clamping PCR to detect the BRAF V600E mutation has not been reported in prior studies.

We used pyrosequencing and PNA-clamping PCR successfully to detect DNA concentration of 99:1 (wild-type:mutant). These results suggest that pyrosequencing and PNA-clamping PCR are more sensitive than allele-specific PCR, which detected wild-type:mutant DNA at a ratio of 98:2. Allele-specific PCR does not require special equipment such as a real-time PCR machine, sequencer, or pyrosequencer; therefore, it is easier to perform than other methods. However, the reading of PCR bands can be difficult if the bands are faint, and contamination during gel loading is possible.

Previous studies that focused on Korean patients suggested that the BRAF V600E mutation is useful for predicting clinical recurrence and that the mutation is associated with aggressiveness.1,9,20 We investigated the prognostic effect of the BRAF V600E mutation in PTC. We found that 61% of patients in the BRAF V600E–positive group experienced extrathyroidal extension and lymph node metastasis, whereas only 39% of patients in the BRAF V600E–negative group experienced these complications. However, this difference did not reach statistical significance (P = .063). The Fisher exact test demonstrated that the BRAF V600E mutation was significantly associated with advanced stage (P = .045).

Of the 8 patients with follicular variant papillary thyroid carcinoma studied herein, 37.5% were positive for the BRAF V600E mutation. A previous study reported that the incidence of the BRAF V600E mutation in follicular variant papillary thyroid carcinoma was 31%, which is lower than in conventional PTC.23 The results of the current study support this previous finding.

In conclusion, the current study demonstrates that pyrosequencing and real-time PCR are equally sensitive and that PNA-clamping PCR is a sensitive and reliable method to detect the BRAF V600E mutation. The BRAF V600E mutation was found to be associated with poor prognostic factors.

From the 1Department of Laboratory Medicine, Chosun University Medical School, Gwangju, Korea; and the 2Department of Pathology, Gangnam Severance Hospital, Yonsei University, Seoul, Korea.

This study was supported by research funds from Chosun University (2012), Gwangju, Korea.

Address reprint requests to Dr Hong: Dept of Pathology, Gangnam Severance Hospital, Yonsei University, 211 Eonju-ro, Gangnam-gu, 133-720, Korea; soonwonh@yuhs.ac.

References


