BRAF\textsuperscript{V600E} Mutation Testing in Fine Needle Aspirates of Thyroid Nodules: Potential Value of Real-Time PCR

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Abstract. The BRAF\textsuperscript{V600E} mutation is a valuable adjunctive diagnostic tool to ultrasound (US)-guided fine-needle aspiration (US-FNA). The objective of this study was to investigate the potential value of real-time PCR to detect BRAF\textsuperscript{V600E} mutation. This study included 447 thyroid nodules in 420 patients who underwent US-FNA and BRAF\textsuperscript{V600E} mutation analysis using dual priming oligonucleotide-based multiplex polymerase chain reaction (DPO-PCR) and real-time PCR. We calculated and compared the diagnostic performances of DPO-PCR and real-time PCR to detect BRAF\textsuperscript{V600E} mutation in the thyroid nodules. Receiver operating characteristic (ROC) analysis was used to quantify the cut-off value of the Ct values of BRAF\textsuperscript{V600E} mutation on real-time PCR. Optimal thresholds were determined (Youden index). We also compared the diagnostic performances between DPO-PCR and real-time PCR after applying the cut-off value on real-time PCR. Sensitivity, accuracy, and NPV were significantly higher in real-time PCR than DPO-PCR. When the optimal cut-off value of 32.4 at Ct values of BRAF\textsuperscript{V600E} mutation was adjusted on real-time PCR, sensitivity was 66.2% and specificity was 100%. Sensitivity, accuracy, and NPV of real-time PCR were also significantly higher than DPO-PCR. In contrast, specificity and PPV were not significantly different between DPO-PCR and real-time PCR. Real time PCR can be a promising diagnostic method in detecting BRAF\textsuperscript{V600E} mutation using optimal cut-off value.

Key words: real-time PCR, DPO-PCR, BRAF, papillary thyroid cancer

Introduction

Papillary thyroid carcinoma (PTC) is the most common malignancy involving the endocrine system, and its incidence has sharply increased due to the widespread usage of high resolution ultrasound (US) for medical surveillance [1]. US-guided fine needle aspiration (US-FNA) is a highly sensitive and accurate method in the diagnosis of thyroid nodules. However, cytologic evaluation is not always accurate in documenting the nature of thyroid nodules because of non-diagnostic, indeterminate, false-negative, and false-positive results [2,3]. Indeed, cytology results are influenced by many factors, including the performer’s skill, technique of preparation, and experiences of interpreting cytologists [4].

As an adjunctive diagnostic tool of FNA, BRAF\textsuperscript{V600E} mutation can help recognize PTC in some equivocal cases, especially in thyroid nodules with “non-diagnostic” or “suspicious for malignancy” cytologic results [5,6]. Several methodologies have been introduced in detecting a BRAF\textsuperscript{V600E} mutation in thyroid nodules. Among the various methods, pyrosequencing and dual priming oligonucleotide-based multiplex polymerase chain reaction (DPO-PCR) have been widely accepted in detecting BRAF\textsuperscript{V600E} mutation in thyroid nodules. Most studies have demonstrated a specificity of 100% in detecting BRAF\textsuperscript{V600E} mutation in thyroid cancer, except for
recent studies showing false-positive results when using highly sensitive methodologies, such as pyrosequencing and DPO-PCR [7,9-11].

Real-time PCR was introduced to quantify mRNA expression easily [12] and has been used in various genetic fields [13,14]. Recently, real-time PCR has been used to detect genetic mutations including the BRAFV600E mutation in colorectal cancer and melanoma [15,16]. Another study used quantitative real-time PCR analyzing BRAFV600E mutation in thyroid nodules, but this study had limitations because a small sample size was included and nodules with undetermined cytology had been included [17]. Therefore, we investigated the potential value of real-time PCR in detecting BRAFV600E mutation in a relatively large sample and compared it with DPO-PCR.

Materials and Methods

The Institutional Review Board approved of this study and required neither patient approval nor informed consent for our review of patient images and records. However, informed consent was obtained from all patients for US-FNA and BRAFV600E mutation analysis prior to each procedure.

Study Population. This study was performed at Severance Hospital (a referral center) from June 2009 to October 2010. During the study period, US-FNA and BRAFV600E mutation analysis using DPO-PCR were performed on aspiration specimens from 1,114 thyroid nodules. The BRAFV600E mutation analysis was performed by clinicians’ request or radiologists’ recommendations based on factors such as the presence of suspicious US features. Of the nodules, 558 nodules were excluded because they did not undergo further evaluation such as surgery, follow-up FNA, and follow-up US. Additional 71 nodules were excluded because they had increased in size during follow-up US. Thirty-eight nodules were also excluded because they had inconclusive cytologic results after repeated FNA. Among these, we selected 447 consecutive FNA samples to be studied with real-time PCR, of these: (a) 345 underwent thyroid surgery regardless of cytologic results, (b) 28 were PTC on US-FNA without surgery, (c) 48 had benign cytology at least twice without surgery, and (d) 26 had benign cytology without interval change or decreased in size at follow-up US within a year. Finally, this study included 447 thyroid nodules in 420 patients (346 women and 74 men). 394 patients had one thyroid nodule, 25 had two nodules, and 1 had three nodules. Mean age of the patients was 48 years of age (range, 17 – 78 years). The mean size of nodules was 9.2 mm (range, 3 - 52 mm).

US Analysis. US images were obtained using iU22 5–12 MHz linear probe (Philips Medical Systems, Bothell, WA) or a 6–13 MHz linear probe (Hitachi Medical, Tokyo, Japan) in the evaluation of thyroid glands and neck areas. Compound imaging was obtained in all images from the iU22 machine. Real-time US imaging was performed by 7 board-certified radiologists with 1 - 15 years of experiences in thyroid imaging.

US features of all thyroid nodules that underwent US-FNA were prospectively recorded according to the internal component, echogenicity, margin, calcification, and shape at the time of US examination. Microcalcifications, irregular or microlobulated margins marked hypoechochogenicity, and taller than wide shape are the US characteristics that are considered malignant in our institution. Suspicious malignant nodules were defined as those showing one or more of the suspicious US findings presented above [18].

US- FNA and Cytologic Analysis. After US examinations, US-FNA was performed by the same radiologists who evaluated the thyroid gland with US. At our institution, US-FNAs were performed on either the thyroid nodule with suspicious US features or the largest thyroid nodule if no suspicious US features were detected. US-FNA was performed with a 23-gauge needle attached to a 2-mL disposable plastic syringe with free hand technique. Each lesion was aspirated at least twice. Materials obtained from aspiration were expelled onto glass slides and smeared. All smears were placed immediately in 95% alcohol for Papanicolaou staining. Cytopathologists were not on site during the biopsy. Additional special staining was done on a case-by-case basis according to the cytopathologist’s needs. One of five cytopathologists specializing in thyroid cytology interpreted the
smears obtained from US-FNA. In our institution, cytology reports were divided into the following 5 categories from June 2009 to November 2009: 1) benign, 2) indeterminate cytology, 3) suspicious for papillary thyroid carcinoma (PTC), 4) malignant, and 5) inadequate [19]. After December 2009, the Bethesda classifications have been used in the classification of cytology results: category 1) nondiagnostic, category 2) benign, category 3) atypia, category 4) follicular neoplasm or suspicious for follicular neoplasm, category 5) suspicious for malignancy, and category 6) malignant [20].

The material remaining in the syringe after cytological preparation was collected for BRAF V600E mutation analysis. DNA was extracted immediately upon arrival at the laboratory and stored at -20°C until testing for real-time PCR analysis.

Detection of BRAF V600E Mutation.

Dual priming oligonucleotide-based multiplex polymerase chain reaction (DPO-PCR). BRAF V600E mutation analysis using the DPO technology was performed using the Seeplex BRAF ACE detection system (Seegene, Seoul, Korea) as described previously [9].

Real-time PCR. Real-time PCR was performed using Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) under the following conditions: at 50°C for 2 min for one cycle, denaturation at 95°C for 10 min for one cycle and then denaturation at 95°C for 15 sec, and one step of annealing and elongation at 62°C for 45 sec for 40 cycles. Real-Q BRAF V600E Detection Kit (BioSewoom, Korea) was used to carry out PCR reaction. The Real-Q BRAF Detection Kit constitutes a ready-to-use kit for the detection of the BRAF V600E (1799T>A) somatic mutation in the BRAF oncogene in a background of wild type genomic DNA in a multiplex real time PCR assay based on TaqMan MGB probe system. The kit supplies two assays. The BRAF mutation assay is labeled with VIC; it contains allele specific forward primer for the discrimination of the V600E mutation. The internal control assay, labeled with FAM, is used to check for nucleic acid isolation and possible PCR inhibition. It amplifies a region of exon 8 of the BRAF gene. The primers and probe have been designed to avoid any known BRAF polymorphisms.

In assay with clinical sample, the positive reaction of BRAF V600E mutation was determined as instructions for use of Real-Q BRAF V600E Detection Kit. If FAM signal (control assay) and VIC signal (mutation assay) were observed simultaneously, \( \Delta C_T \) values by subtracting control Ct value from mutation Ct value were calculated. Samples which showed \( \Delta C_T \) over 13 cycles were determined as a negative reaction of BRAF V600E mutation, according to instructions for use of Real-Q BRAF V600E Detection Kit. Using 120 normal genomic DNA samples not containing the BRAF
V600E mutation, we performed tests of BRAF diagnostic specificity on Real-Q BRAFV600E Detection Kit. Five DNA samples among 120 DNA samples were amplified between $C_t$ 32.58 and $C_t$ 34.19 in BRAF V600E mutation assay. However, these samples were verified as BRAF V600E negative because it was identified that they calculated $\Delta C_t$ (mutation $C_t$-control $C_t$) for more than 13 cycles. These 5 samples were amplified between $C_t$ 17.95 and $C_t$ 18.76 in control assay and showed $\Delta C_t$ between 14.38 and 16.02. As a result, all of 120 samples were identified BRAF V600E negative.

The 242 bp of partial BRAF gene containing V600E region was amplified from human melanoma cell line, SK-MEL-28 (ATCC, Manassas, VA, USA) with BRAFV600E mutation and it was inserted into the pZEM-T Easy Vector (Promega, Madison, WI, USA) to produce the BRAFV600E plasmid DNA. The analytical sensitivity was evaluated using the plasmid clone BRAFV600E mutation and the 95% positive cut-off value (limit of detection; LOD) was calculated at 21.5 copy/ul by the Probit analysis.

To determine the cut-off $C_t$ value of BRAF V600E test, 100 repeats of low-positive concentrations of the BRAF V600E plasmid DNA of 64.5copy/ul representing a BRAF V600E concentration equivalent to 3x the 95% positive cut-off concentration was used. The positive rate was 100% and the average $C_t$ value of the 100 repeats was 39.9 cycle. Based on this test, the cut-off point for BRAF V600E positive was set to $C_t$ 40.

**Data and Statistical Analysis.** The Gold standard for data analysis was based on pathologic results (n=345) (Figure 1). Among the remaining 102 nodules which were not confirmed on pathology, follow-up cytology and US were used to determine gold standard (Figure 2). Categorical data was summarized using frequencies and percentages. We used the $\chi^2$ test to compare malignancy to categorical variables. An independent two-sample $t$ test was used to evaluate the association of malignancy to continuous variables.

We calculated the diagnostic performances of DPO-PCR and real-time PCR to detect the BRAFV600E mutation at the thyroid nodules. In this study, we considered cytopathologic results as the gold standard. We compared BRAF status to the gold standard. Detection of BRAFV600E mutation in the aspirates that were cytopathologically confirmed as malignant, were considered as TP when calculating the diagnostic performances of BRAFV600E mutation testing. Detection of BRAFV600E mutation in the aspirates that were cytopathologically confirmed as benign were categorized as FP. Failure to detect BRAFV600E mutation group on the benign lesions were considered as TN, and failure to detect BRAFV600E mutation group in the malignant lesions was categorized as
FN, the following statistical values were calculated as: Sensitivity = TP/(TP + FN) x 100; specificity = TN/(TN + FP) x 100; positive predictive value (PPV) = TP/(TP + FP) x 100; negative predictive value (NPV) = TN/(TN + FN) x 100; accuracy = (TP + TN)/(TP + TN + FP + FN) x 100. Receiver operating characteristic (ROC) analysis was used to quantify cut-off value of the Ct values of BRAFV600E mutation on real-time PCR. Optimal thresholds were determined by the Youden index. To compare the diagnostic performances between DPO-PCR and real-time PCR, we used logistic regression with GEE (generalized estimating equation) method. We also compared the diagnostic performances between DPO-PCR and real-time PCR after applying the cut-off value on real-time PCR.

Analysis was performed using SAS software (version 9.1.3; SAS Institute, Cary, NC). Statistical significance was assumed when the P value was less than 0.05. All reported P values are 2-sided.

Results

Of the 447 nodules, 361 nodules (80.8%) were malignant and 86 (19.2%) were benign. Of the 345 nodules which had been operated on, 333 were diagnosed as malignant. They included conventional PTC (n = 306), follicular variant of PTC (n = 23), Warthin’s tumor like variant of PTC (n=1), minimally invasive follicular carcinoma (n = 2), and medullary carcinoma (n = 1). The 12 benign operated nodules were adenomatous hyperplasia (n=9), follicular adenoma (n=2), and Hurthle cell adenoma (n=1). The mean size of the benign nodules was 12 ± 8.8 mm, which was significantly larger than that of malignant nodules, which were 8.5 ± 5.7 mm (P < .001). Patients with malignant nodules were significantly younger (47.3 ± 11.5 years) than those with benign nodules (50.7 ± 10.9 years, P = .013). There was no statistically significant relationship between gender and malignancy.

Sensitivity, accuracy, and NPV were significantly higher when using real-time PCR (77.8%, 81%, and 50.3%, respectively) than DPO-PCR (53.2%, 62%, and 33.5%, respectively) (Table 1). When the optimal cut-off value of 32.4 at Ct values of BRAFV600E mutation was adjusted on real-time PCR, sensitivity was 66.2% and specificity was 100%. Sensitivity, accuracy, NPV, and the area under the ROC curve (A\textsubscript{z}) of real-time PCR were also significantly higher than DPO-PCR (Table 1, Figure 3).

In this study, 245 out of the 361 cancers were preoperatively diagnosed as malignancy on cytology.
Among the remaining 116 cancers, the BRAFV600E mutation using DPO-PCR was not detected in 82 nodules. Out of those 82 nodules, of which were detected the BRAFV600E mutation using real time PCR at 17 nodules when Ct cut-off value was 32.4.

In contrast, specificity and PPV were not significantly different between DPO-PCR and real-time PCR.

Discussion

The BRAFV600E mutation is the most common genetic event observed in PTC of adults and is seen in approximately 80% of Korean patients [7,21]. Because the BRAFV600E mutation occurs exclusively in PTC and PTC-derived anaplastic thyroid cancers and shows high prevalence in Korea, the BRAFV600E mutation has been used as a complementary diagnostic tool to US-FNA in the diagnosis of malignancy in a thyroid nodule [6,9-11,19,22-26]. Recently, highly sensitive diagnostic methods for detecting the BRAFV600E mutation, such as pyrosequencing and DPO-PCR, have been introduced and lead us believe that they are a promising diagnostic tool to overcome the limitations of US-FNA [7-11].

Because many reports demonstrated 100% specificity of BRAFV600E mutation testing in detecting malignancy, recent detection methods such as pyrosequencing and DPO-PCR have focused on improving diagnostic sensitivity [9,11,24,27,28]. However, recent studies showed an exceptionally high diagnostic accuracy (more than 0.9 of A2 values) using highly sensitive pyrosequencing and DPO-PCR (Figure 3), resulting in false positive results of BRAFV600E mutation [7,8]. The false-positive results of BRAFV600E mutation may be explained by the trade-off of improved sensitivity by sacrificing specificity [7,8,10]. A2 value nearly reached 0.9 even when using direct DNA sequencing, showing a high diagnostic accuracy with a case showing false positive result (however the sample size was small, necessitating further studies) [23]. To avoid false-positive results of BRAFV600E mutation test, an optimal cut-off value should be applied to reach 100% specificity, albeit sacrificing sensitivity of the test [10]. In this study, we tried to detect BRAFV600E mutation using real-time PCR, and compare it with DPO-PCR. When the optimal cut-off value of 32.4 at Ct values of BRAFV600E mutation was adjusted on real-time PCR, sensitivity, accuracy, and NPV of real-time PCR were also significantly higher than DPO-PCR (Table 1). Comparing with Yeo et al's study using pyrosequencing, A2 value of in our study using real-time PCR was higher than that reported in Yeo’s study (Figure 3). In this study, 116 out of the 361 cancers were preoperatively not definitely diagnosed as malignant on cytology. BRAFV600E mutation using

![Figure 3. Receiver operating characteristic (ROC) curve of BRAFV600E mutation testing in fine-needle aspirates from several studies and this study.](image)

1. A2 value was 0.926 by pyrosequencing [7]
2. A2 value was 0.909 by DPO-PCR [8]
3. A2 value was 0.895 by direct DNA sequencing [23]
4. A2 value was 0.688 by direct DNA sequencing [32]
5. A2 value was 0.759 by pyrosequencing [10]*
6. A2 value was 0.76 by DPO-PCR in this study.
7. A2 value was 0.831 by real-time PCR in this study.

*Diagnostic performances were calculated using optimal cut-off value.
†The cut-off value of threshold cycle was 32.4.

DPO-PCR, dual priming oligonucleotide-based multiplex PCR.
DPO-PCR were not detected in 82 nodules of which were detected the BRAF\(^{V600E}\) mutation using real time PCR at 17 nodules. Therefore, the BRAF\(^{V600E}\) mutation test using real time PCR can help these patients to avoid delayed diagnosis.

In this study, we found false-positive results in highly sensitive methods (both DPO-PCR and real-time PCR). False-positive results can be due to faint low density of mutation band associated with an extensive contamination with wild-type DNA in DPO-PCR [8,29], and non-specific DNA amplification in real-time PCR [12]. On real-time PCR, \(C_t\) value is very important in an accurate and reproducible quantification [30], and \(C_t\) values decrease linearly with increasing input target quantity, which can be used as a quantitative measurement of the input target. In this study, we evaluated the optimal cut-off value using \(C_t\) values in real-time PCR to obtain 100% specificity of real-time PCR. In a real-time PCR setting, we usually regard it negative when the \(C_t\) values were larger than or equal to 40. When we regarded the optimal cut-off value of 32.4 in \(C_t\) values of BRAF\(^{V600E}\) mutation, specificity was 100% and sensitivity was 66.5%. The sensitivity was higher than those of another study using optimal cut-off value of pyrosequencing [10], suggesting it a promising method in detecting BRAF\(^{V600E}\) mutation.

There are several potential limitations in this study. First, the nodules which had not undergone follow-up or surgery were included, based on the cytology results. This may have affected our results in ways of false-negative or false-positive cytologic results [2,31]. Second, the number of benign nodules included in this study was relatively small for determining their specificity.

In conclusion, real time PCR can be a promising diagnostic method in detecting BRAF\(^{V600E}\) mutation using optimal cut-off value.

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References


