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Evaluation of the Real-Q *BRAF* V600E Detection Assay in Fine-Needle Aspiration Samples of Thyroid Nodules

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Recently, several molecular assays for detecting the *BRAF* V600E mutation in fine-needle aspiration (FNA) specimens have been developed. Herein, we tested 294 consecutive FNA samples from patients with thyroid nodules with the Real-Q *BRAF* V600E detection assay (Real-Q). These results were compared with an allele-specific PCR-based kit using dual-priming oligonucleotides (AS-PCR). Any discordant results between the two tests were also analyzed by mutant enrichment with 3′-modified oligonucleotide sequencing. A total of 128 cases were confirmed histologically; of these, 121 were diagnosed as papillary thyroid carcinoma (PTC). The *BRAF* mutation was detected by Real-Q and AS-PCR testing in 80.2% (95% CI, 71.9%—86.9%) and 76.9% (95% CI, 68.3%—84.0%), respectively, of FNA specimens with PTC. Combining the *BRAF* V600E molecular assays (Real-Q and AS-PCR) with cytological diagnoses of malignant and suspicious for malignant cells, the detection rates (sensitivity) of Real-Q and AS-PCR for diagnosis of PTC increased to 94.2% (95% CI, 88.4%—97.6%) and 92.6% (95% CI, 86.4%—96.5%), respectively. In conclusion, the detection of *BRAF* V600E mutations in PTC by Real-Q is compatible to that of AS-PCR. *(J Mol Diagn 2015, 17: 431—437; http://dx.doi.org/10.1016/j.jmoldx.2015.03.006)*

The B-type Raf kinase (BRAF) is a cytoplasmic serine/threonine kinase that is regulated by binding to RAS. The *BRAF* V600E mutation is one of the most common genetic alterations in papillary thyroid carcinoma (PTC).[1] This mutation causes constitutive activation of BRAF and, thus, induces the RAS/RAF/MAPK/ERK pathway.[1] A recent genomic study on PTC by The Cancer Genome Atlas Research Network reported that the fraction of PTC with *BRAF* V600E is approximately 60%.[2] The *BRAF* V600E mutation has also been associated with the development and progression of PTC.[3] Previous studies reported that *BRAF* V600E is associated with aggressive histopathological features of PTC, tumor recurrence, and tumor-related mortality.[4]

Fine-needle aspiration (FNA) is the most common diagnostic tool for the evaluation of thyroid nodules. FNA cytology provides a conclusive diagnosis; however, approximately 25% of FNA specimens yield indeterminate cytological findings that cannot distinguish malignant neoplasms from benign diseases.[4] Molecular testing, including *BRAF* V600E, can be helpful for evaluating indeterminate cytological nodules.[4] However, molecular detection from FNA specimens requires a sensitive and specific technique because these specimens often contain fewer mutation-carrying cells than tumor tissue specimens, and samples can also include many nontumor cells. Recently, various molecular assays have been developed with enhanced sensitivity and specificity for detecting the *BRAF* V600E mutation in FNA specimens.[5—10]

Herein, we prospectively analyzed a series of 294 FNA specimens for the *BRAF* V600E mutation. We correlated detection of the *BRAF* V600E mutation and clinicalpathological features between real-time PCR with TaqMan minor groove-binding probes and allele-specific PCR (AS-PCR) using dual-priming oligonucleotides.

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Materials and Methods

Patients and Specimens

A total of 294 consecutive FNA cytology samples were prospectively collected from patients with ultrasonography features suspicious for malignancy from July 2011 to March 2012 at the Samsung Medical Center (Seoul, Republic of Korea). The study protocol was approved by the Institutional Review Board.

FNA was performed by interventional radiologists who specialize in ultrasound-guided thyroid FNA. The FNA specimens were obtained from thyroid nodules in two or four passes using a 22- or 23-gauge needle attached to a 10-mL syringe. The FNA samples were smeared onto glass slides and fixed in 95% alcohol for Papanicolaou and hematoxylin and eosin staining. After acquisition of an adequate amount of sample for cytological examination, the remaining material was collected for *BRAF* V600E mutation analysis.

Cytological Diagnosis, Histological Diagnosis, and Clinical Monitoring

Cytological diagnosis was performed by a specialized pathologist (Y.L.O.), blinded to the molecular findings. More than two specialists were consulted when there were questionable specimens. The cytological diagnosis was classified according to the Bethesda System for Reporting Thyroid Cytopathology recommendations,[11] as follows: benign, atypia of undermined significance/follicular lesion of undetermined significance (AUS/FLUS), follicular or oncocytic (Hürthle cell) neoplasm/suspicious for follicular or oncocytic (Hürthle cell) neoplasm, suspicious for malignant cells (SMCs), malignant, and unsatisfactory.

The results of cytological diagnosis and *BRAF* V600E mutation analysis in the FNA specimens were reviewed from all patients who were enrolled. For patients who underwent subsequent thyroidectomy, histopathological findings, including tumor size, lymph node metastasis, extrathyroidal extension, multicentricity, and other pathological findings (chronic lymphocytic thyroiditis and nodular hyperplasia), were reviewed. For patients who did not undergo thyroidectomy, we reviewed clinical observations with serial ultrasound observations, repeated FNA cytology, or repeated *BRAF* V600E mutation analysis in the FNA.

BRAF V600E Mutation Analysis

On specimen arrival at the molecular laboratory, the FNA specimens were immediately centrifuged at 16,000 × *g* for 1 minute; then, the supernatant and red blood cells were removed. Genomic DNA from the remaining FNA specimens was extracted using the QIAamp DNA minikit (Qiagen, Chatsworth, CA), according to the manufacturer's instructions. *BRAF* V600E mutation analysis was performed by real-time PCR with minor groove-binding probes and AS-PCR using dual-priming oligonucleotides. Discordant results between the two tests or between cytological and histological diagnoses were additionally analyzed by mutant enrichment with 3′-modified oligonucleotide (MEMO) sequencing, which is a test developed in our laboratory. We previously reported that the detection limit of MEMO sequencing is lower than that of Sanger sequencing (0.1% versus 20%, respectively).[10]

For real-time PCR, the Real-Q BRAF V600E detection kit (Real-Q; Biosewoom, Seoul, Republic of Korea) was used with TaqMan minor groove-binding probes. For *BRAF* V600E amplification, this assay uses VIC fluorescence in a region of exon 15. A region of exon 8 with 6-carboxyfluorescein fluorescence is used for amplification as the internal control. Real-time PCR was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. The PCR conditions were as follows: 50°C for 2 minutes (one cycle), 95°C for 10 minutes (one cycle), and 95°C for 15 seconds (one cycle), followed by 62°C for 45 seconds (40 cycles).

AS-PCR using dual-priming oligonucleotides was performed using the Seeplex BRAF ACE detection system (Seegene, Seoul, Republic of Korea). This commercial kit uses two separate priming regions joined by a polydeoxyinosiline linker; the longer segment binds to the template DNA, whereas the shorter segment selectively binds to its target site and blocks non-specific annealing.[12] AS-PCR was performed on a GeneAmp 9700 PCR machine (Applied Biosystems) with 15 minutes of incubation at 94°C and 35 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 60 seconds, with a final extension at 72°C for 10 minutes.

MEMO sequencing uses a blocking primer, which matches the wild-type DNA, and therefore the extension of the normal allele does not occur. Information on the primers and PCR conditions for MEMO-PCR was described in our previous study.[10]

Analysis of LOD

To evaluate the limit of detection (LOD) of Real-Q, mutant DNA (100 ng/μL) obtained from a *BRAF* V600E-positive cell line (SNU-790) was serially diluted into *BRAF* V600E-negative DNA. Eight replicates at each of the eight different concentrations (0.001% to 1.50%) were tested. The LOD was calculated using probit analysis of SPSS Statistics version 21.0 (IBM Corp., Armonk, NY).

Statistical Analysis

Statistical analysis was performed using MedCalc software version 13.1 (MedCalc Software bvba, Ostend, Belgium). Sensitivity, specificity, positive predictive value, and negative predictive value, with 95% CIs (Clopper-Pearson exact method), were calculated using MedCalc software. We defined the following: true positives had PTC on the basis of