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Evaluation of the Anyplex *BRAF* V600E Real-Time Detection Assay Using Dual-Priming Oligonucleotide Technology in Fine-Needle Aspirates of Thyroid Nodules

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Background: Several molecular assays have been developed to detect the *BRAF* V600E mutation in fine needle aspirates (FNAs) for the diagnosis of papillary thyroid cancer. Using a multiplex PCR technique, we evaluated the Anyplex *BRAF* V600E Real-time Detection (Anyplex) assay and compared its efficacy with that of the Seeplex *BRAF* V600E ACE Detection (Seeplex) method.

Methods: We tested 258 consecutive FNA specimens using the Seeplex and Anyplex assays. Any conflicting results between the two assays were confirmed by using mutant enrichment with 3′-modified oligonucleotide (MEMO) sequencing. The limits of detection (LODs) and reproducibility for each assay were evaluated with serially diluted DNA from a *BRAF* V600E-positive cell line.

Results: The *BRAF* V600E mutation was detected in 36.4% (94/258) FNA specimens by either the Seeplex or Anyplex assay. Results for the two assays showed 93.4% (241/258) agreement, with a kappa value of 0.861 (95% confidence interval, 0.798-0.923). Of the eight specimens that were *BRAF* V600E-positive by the Anyplex assay but not by the Seeplex assay, five were found to be *BRAF* V600E-positive by MEMO sequencing. The mutation detection rate of the Seeplex and Anyplex assays was 79.0% and 84.0%, respectively, in the FNA specimens diagnosed as malignant (n=81). The LOD as determined by probit analysis was 0.046% (95% confidence interval, 0.019-0.532%).

Conclusions: The Anyplex assay performed better than the Seeplex assay with respect to the detection of the *BRAF* V600E mutation.

Key Words: *BRAF* V600E, Evaluation, Fine-needle aspiration, Real-time PCR, Seeplex, Anyplex

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INTRODUCTION

Thyroid cancer is one of the most common malignancies worldwide [1]. Among the numerous genetic alterations that have been identified to play a fundamental role in the tumorigenesis of various thyroid cancers, the c.1799T>A mutation in the *BRAF* gene is of particular interest. This mutation results in the expression of the *BRAF* V600E mutant protein, and causes the constitutive activation of a serine/threonine kinase [2, 3]. This serine/threonine kinase is found in up to 80% of papillary thyroid carcinomas (PTCs) and 50% of anaplastic thyroid carcinomas, but not in follicular neoplasms or benign nodules [4-6]. The advent of molecular diagnostic methods has allowed for easier detection of the *BRAF* V600E mutation in thyroid fine-

needle aspirates (FNAs), in clinical pathology and clinical oncology practices. These molecular methods complement routine cytopathological examinations and are of significant value in identifying diagnostic and prognostic molecular markers and therapeutic targets for thyroid cancer [2, 7-11].

Numerous *BRAF* assays employing various technologies are presently in use. These technologies encompass conventional Sanger sequencing, pyrosequencing, LightCycler PCR (Roche Diagnostics, Mannheim, Germany) with melting curve analysis, the colorimetric Mutector assay (TrimGen, Sparks, MD, USA), restriction fragment length polymorphisms, the amplification refractory mutation system, and dual-priming oligonucleotide (DPO) PCR [12]. The *BRAF* V600E mutation might only be present in malignant cells of thyroid FNA cytology specimens; however, these specimens contain a mixture of cell types and are of variable quality [13]. Therefore, a sensitive assay is required to detect the *BRAF* V600E mutation in FNA specimens [12]. Although conventional Sanger sequencing is the standard method used to detect the *BRAF* V600E mutation, it is not sensitive enough (approximately 20%) to detect the mutation if it is present at a low frequency in specimens [14]. Other molecular methods with mutation enrichment that have a higher sensitivity than conventional Sanger sequencing, such as DPO PCR and mutant enrichment with 3′-modified oligonucleotide (MEMO) sequencing, have also been developed [12, 14, 15].

In the present study, we evaluated a new real-time PCR assay using DPO technology, the Anyple *BRAF* V600E Real-time Detection system (Seegene, Seoul, Korea), and compared the results with those obtained from the Seeplex *BRAF* V600E ACE Detection system (Seegene), which is based on conventional DPO PCR technology.

METHODS

1. Patients

We collected 258 consecutive FNA specimens from Korean patients (131 women and 127 men; patients' age showed normal distribution with 51.0±11.2 yr, mean±SD) who were found to have malignant or indeterminate thyroid nodules by ultrasonography. After informed consent was granted by each patient, fine-needle aspiration was performed under ultrasonographic guidance by experienced radiologists.

2. Methods

The aspirates were placed onto glass slides and fixed in 95% alcohol for cytological examination. Remaining materials were collected for *BRAF* V600E mutation analysis. On the basis of cytology, each specimen was classified into one of six categories [16]: benign, follicular lesion of undetermined significance/atypia of undetermined significance (AUS/FLUS), follicular neoplasm (FN)/suspicious for FN (SFN), suspicious for malignancy (SMC), malignant, and nondiagnostic because of unsatisfactory sample quality. For patients who underwent a thyroidectomy, histopathological examination was performed by specialized pathologists.

Genomic DNA was extracted from the aspirated thyroid cells by using a QIAamp DNA Micro Kit (QIAGEN, Chatsworth, CA, USA) according to the manufacturer's instructions. All FNA specimens were tested for the *BRAF* V600E mutation by using the Seeplex and Anyplex methods. Any conflicting results, including invalid results (n=17), between the two assays were confirmed with MEMO sequencing. [12]. The Seeplex assays were carried out on a GeneAmp 9700 PCR thermal cycler (Applied Biosystems, Foster City, CA, USA) as previously described [17]. The Anyplex assays were performed according to the manufacturer's instructions. In brief, the reaction mixture contained 2 µL of 10× BRAF Oligo Mix including amplification and detection reagents, 3 µL of 8-methoxypsoralen solution to prevent carryover contamination, 10 µL of 2× Anyplex PCR master mix including DNA polymerase, and buffer with deoxynucleoside triphosphates. We added 5 µL of template DNA to each reaction mixture to achieve a final reaction volume of 20 µL. The real-time PCR was performed by using a CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA). The thermal cycling conditions involved an initial denaturation step at 95°C for 15 min, followed by 15 amplification cycles (95°C for 15 sec and 60°C for 30 sec), and then a further 35 amplification cycles (95°C for 30 sec and 60°C for 32 sec). For real-time PCRs, the cycle threshold (Ct) was defined as the cycle at which a significant increase in fluorescence was detected. Specimens and the internal control, with a Ct value <33 were considered positive. Each assay contained a positive and a negative control. MEMO sequencing was carried out as previously described [12].

Correlations between cytological and histological findings were determined, and *BRAF* V600E mutation detection results for the Seeplex and Anyplex assays were compared. To evaluate the limit of detection (LOD) for each assay, mutant DNA (100 ng/µL) obtained from a *BRAF* V600E-positive cell line (SNU-790) was serially diluted with a V600E-negative cell line (DMPK-M). Eight replicates at eight different concentrations (0.001-3.000%) were tested and, the LOD was determined by probit analysis.

3. Statistical analysis

Statistical analyses were performed by using SPSS 20.0 (SPSS Inc., Chicago, IL, USA) and MedCalc software (MedCalc, Ostend, Belgium). We calculated kappa inter-rater agreement values with 95% confidence intervals (CIs) to compare the detection rates of *BRAF* V600E mutations between the Seeplex and Anyplex assays. Kappa values were interpreted as follows: 0-0.20 as slight; 0.21-0.40 as fair; 0.41-0.60 as moderate; 0.61-0.80 as substantial; and 0.81-1 as almost perfect agreement [18].

Table 1. Comparison of *BRAF* V600E mutation-detection results for the Seeplex and Anyplex assays

		Anyplex			
		Negative	Positive	Inconclusive	Total
Seeplex	Negative	156	8	1	165
	Positive	1	85	0	86
	Inconclusive	7	0	0	7
	Total	164	93	1	258

Kappa value=0.861 (95% confidence interval: 0.798-0.923).

RESULTS

The *BRAF* V600E mutation was detected in 36.4% (94/258) of FNA specimens by either the Seeplex or Anyplex assay. These included 86 (33.3%) cases as determined by Seeplex and 93 (36.0%) cases by Anyplex (Table 1). The results of the Seeplex and Anyplex assays showed 93.4% (241/258) agreement, with a kappa value of 0.861 (95% CI: 0.798-0.923). We observed more invalid results for the Seeplex (2.7%, n=7) than for the Anyplex (0.4%, n=1) assays.

According to our cytology results, 31.4% (81/258) of cases were diagnosed as malignant. These included 80 PTCs and one medullary thyroid cancer (MTC). Of the 81 specimens diagnosed as malignant, the *BRAF* V600E mutation was detected in 64 (79.0%) and 68 (84.0%) specimens by Seeplex and Anyplex, respectively (Table 2). Among the 22 specimens classified as SMC, the detection rates of the *BRAF* V600E mutation by Seeplex and Anyplex were 86.4% (19/22) and 90.9% (20/22), respectively.

Thyroidectomies and post-surgical histopathological examinations were performed for 35.3% (91/258) of samples, with four classified as benign, six as AUS/FLUS, 18 as SMC, and 63 as

Table 2. Comparison of *BRAF* V600E mutation detection using the Seeplex and Anyplex assays, with cytological diagnoses (n=258) and post-thyroidectomy histological diagnoses (n=91)*

	Seeplex			Anyplex			Total (%)
	Positive (%)	Negative (%)	Inconclusive (%)	Positive (%)	Negative (%)	Inconclusive (%)	
FNA cytology							
Benign	1 (0.8)	114 (95.8)	4 (3.4)	2 (1.7)	116 (97.5)	1 (0.8)	119 (46.1)
AUS/FLUS	2 (12.5)	12 (75.0)	2 (12.5)	3 (18.8)	13 (81.3)	0 (0.0)	16 (6.2)
FN/SFN	0 (0.0)	4 (100)	0 (0.0)	0 (0.0)	4 (100)	0 (0.0)	4 (1.6)
SMC	19 (86.4)	3 (13.6)	0 (0.0)	20 (90.9)	2 (9.1)	0 (0.0)	22 (8.5)
Malignant	64 (79.0)	17 (21.0)	0 (0.0)	68 (84.0)	13 (16.0)	0 (0.0)	81 (31.4)
Unsatisfactory	0 (0.0)	15 (93.8)	1 (6.3)	0 (0.0)	16 (100)	0 (0.0)	16 (6.2)
Total	86 (33.3)	165 (64.0)	7 (2.7)	93 (36.0)	164 (63.6)	1 (0.4)	258 (100)
Histology							
Benign	0 (0.0)	4 (100)	0 (0.0)	0 (0.0)	4 (100)	0 (0.0)	4 (4.4)
PTC	67 (78.8)	18 (21.2)	0 (0.0)	72 (84.7)	13 (15.3)	0 (0.0)	85 (93.4)
Other cancers†	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)	2 (100)	0 (0.0)	2 (2.2)
Total	68 (74.7)	23 (25.3)	0 (0.0)	72 (79.1)	19 (20.9)	0 (0.0)	91 (100)

*Two specimens contained the *BRAF* V600E mutation as detected by mutant enrichment with 3′-modified oligonucleotide (MEMO) sequencing; however the mutation was not detected by dual-priming oligonucleotide (DPO) PCR or DPO real-time PCR, and these were diagnosed as benign on FNA cytology. Histopathological evaluations with surgical thyroidectomy for these cases were not performed; †Other cancers included one minimally-invasive follicular carcinoma and one medullary thyroid carcinoma.

Abbreviations: FNA, fine needle aspirates; AUS/FLUS, atypia of undetermined significance/follicular lesion of undetermined significance; FN/SFN, follicular or oncocytic (Hürthle cell) neoplasm/suspicious for follicular or oncocytic (Hürthle cell) neoplasm; SMC, suspicious for malignant cells; PTC, papillary thyroid cancer; NT, not tested.

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Table 3. Conflicting results between Seeplex and Anyplex assays (n=9) were resolved using MEMO sequencing, FNA cytology, and post-thyroidectomy histological analyses

Case No.	Seeplex	Anyplex (Ct)*	MEMO sequencing	FNA cytology	Histology
70	Positive	Negative (-)	Negative	Benign	Follicular carcinoma†
24	Negative	Positive (28.26)	Negative	Benign	NT
138	Negative	Positive (32.60)	Negative	Benign	NT‡
33	Negative	Positive (30.50)	Negative	AUS	NT
185	Negative	Positive (25.13)	Positive	Suspicious for malignancy	Papillary microcarcinoma
47	Negative	Positive (25.28)	Positive	Papillary thyroid carcinoma	Papillary microcarcinoma
175	Negative	Positive (26.68)	Positive	Papillary thyroid carcinoma	Papillary microcarcinoma
133	Negative	Positive (26.37)	Positive	Papillary thyroid carcinoma	Papillary microcarcinoma
155	Negative	Positive (26.64)	Positive	Papillary thyroid carcinoma	Papillary microcarcinoma

*Ct values <33 were considered positive; †Minimally-invasive follicular carcinoma; ‡Post-thyroidectomy histological findings were unavailable; however, follow-up FNA cytology results after 18 months indicated this case was benign.
Abbreviations: Ct, cycle threshold; MEMO, mutant enrichment with 3′-modified oligonucleotide; FNA, fine needle aspirates; NT, not tested; AUS, atypia of undetermined significance.

malignant by FNA cytology. These samples included 85 PTCs, four benign thyroid nodules (three benign nodular hyperplasias and one follicular adenoma), and two other thyroid carcinomas (one follicular thyroid carcinoma and one MTC). Of the 85 specimens diagnosed as PTCs, the *BRAF* V600E mutation was detected in 67 (78.8%) cases by Seeplex and 72 (84.7%) cases by Anyplex assays.

As shown in Table 3, conflicting results were observed in nine cases. For one case, the *BRAF* V600E mutation was detected by Seeplex only, while for the other eight cases, the mutation was detected by the Anyplex assay only. Of the eight *BRAF* V600E-positive samples by Anyplex, five were also positive according to MEMO sequencing, cytology results, and post-surgical pathologic findings consistent with PTC. Unfortunately, thyroidectomy was not performed for three cases that were positive by Anyplex but negative by Seeplex and MEMO sequencing. Therefore, confirmatory information for the histopathological findings regarding PTC was not available.

All specimens with invalid results by Seeplex and Anyplex were diagnosed as benign or FLUS on FNA cytological examination, except for one specimen that was *BRAF* V600E-positive by MEMO sequencing, and yielded unsatisfactory results by FNA cytological examination. Histopathological examinations following thyroidectomy were not performed. No specimens were identified as having the *BRAF* V600E mutation by MEMO sequencing and Seeplex, but not by Anyplex, confirming the higher sensitivity of the Anyplex assay over the Seeplex assay.

All results were positive at a concentration of 0.05%. The LOD as determined by probit analysis was 0.046% (95% CI: 0.019-0.532) (Table 4).

Table 4. The limits of detection for the Anyplex assay

Concentration of mutant DNA (%)*	Positives/total runs	Mutation detection rate (%)
3.000	8/8	100
2.000	8/8	100
1.500	8/8	100
1.000†	8/8	100
0.500	8/8	100
0.100	8/8	100
0.050	8/8	100
0.010	4/8	50
0.005	4/8	50
0.001	1/8	12.5

*The SNU-790 *BRAF* V600E-positive cell line was serially diluted with the DMPK-M *BRAF* V600E-negative cell line; †The limit of detection claimed by the manufacturer was 1.000% of *BRAF* mutations. All results were positive when the concentration was 0.050%. The limit of detection as determined by probit analysis was 0.046% (95% confidence interval:0.019-0.532). Reproducibility was verified at 1.000% and lower concentrations.

DISCUSSION

We evaluated the performance of the Anyplex assay in detecting the *BRAF* V600E mutation using clinical FNA specimens collected from Korean patients with malignant or indeterminate thyroid nodules. In specimens diagnosed as PTC, detection rates for the *BRAF* V600E mutation were higher with the Anyplex assay than with the Seeplex assay. More specimens diag-

nosed with PTC contained the *BRAF* V600E mutation as determined by the Anyplex assay and MEMO sequencing, including conflicting results. Therefore, we concluded that the Anyplex assay was more sensitive than the Seeplex assay.

In the current study, histopathological findings were not used to discriminate between true positives and true negatives for the *BRAF* V600E mutation, in evaluating the mutation detection sensitivity and specificity between diagnostic methods. Conventional Sanger sequencing is the standard method by which the *BRAF* V600E mutation is usually detected. However, this method is not sensitive enough to detect the mutation when it occurs at low frequencies in specimens. Therefore, highly sensitive MEMO sequencing has been used to detect the *BRAF* V600E mutation [12]. We used this as the reference method to analyze samples that provided conflicting results in the Seeplex and Anyplex assays. Our Anyplex results strongly corresponded with the Seeplex results, with a kappa value of 0.861 indicating almost perfect agreement. The clinical sensitivity (detection rate) of the Anyplex assay for *BRAF* V600E detection was higher than that of the Seeplex assay, for the 81 FNA specimens diagnosed as malignant and the 22 specimens diagnosed as SMC. In addition, among the 85 specimens diagnosed as PTCs on the basis of post-thyroidectomy histopathological findings, detection rates were 78.8% (67/85) by Seeplex and 84.7% (72/85) by Anyplex. The analytical sensitivity test revealed that Anyplex had a higher sensitivity than Seeplex, but a lower sensitivity than MEMO sequencing. These results were comparable with previous studies, which found that DPO real-time PCR and MEMO sequencing were more sensitive than DPO PCR [12, 19].

In this study, one case that was *BRAF* V600E-positive by Seeplex, but negative by the Anyplex assay and MEMO sequencing was finally diagnosed as minimally invasive follicular carcinoma on histopathological examination. Considering that only about 1% of follicular carcinomas reportedly contain the *BRAF* V600E mutation [20], and that analytical sensitivities of the Anyplex assay and MEMO sequencing were higher than that of Seeplex, the *BRAF* mutation status of this sample was questionable and could be a false positive. In three samples, the *BRAF* V600E mutation was detected only by Anyplex, not by Seeplex or MEMO sequencing; however, none of these patients underwent thyroidectomy. There were no false positive results from the Anyplex assay when post-thyroidectomy histopathological results were available to be evaluated as references.

The LOD of the Anyplex assay in this study was 0.046%. The LOD claimed by the manufacturer and determined by our comparison test was 1.0%, which was better than that of the Seeplex assay (10.0%), but it was not good as that for MEMO sequencing (0.1%). The reproducibility of the Anyplex assay was verified and found to be close to the LOD claimed by the manufacturer. The analytical sensitivity of the three diagnostic methods was estimated by using the SNU-790 *BRAF* V600E-positive cell line, which could be considered as a limitation of this study. Another limitation might be that over 66% of the conflicting results could not be solved because surgical histopathological results could not be obtained.

In conclusion, the real-time PCR-based Anyplex method was more sensitive than the Seeplex assay in detecting the *BRAF* V600E mutation in FNA specimens from thyroid nodules. Anyplex assay could be more useful in diagnosis of PTCs harboring the *BRAF* V600E mutation.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69-90.
2. Xing M. Molecular pathogenesis and mechanisms of thyroid cancer. Nat Rev Cancer 2013;13:184-99.
3. Millington GW. Mutations of the *BRAF* gene in human cancer, by Davies et al. (Nature 2002; 417: 949-54). Clin Exp Dermatol 2013;38:222-3.
4. Li C, Lee KC, Schneider EB, Zeiger MA. *BRAF* V600E mutation and its association with clinicopathological features of papillary thyroid cancer: a meta-analysis. J Clin Endocrinol Metab 2012;97:4559-70.
5. Lee JH, Lee ES, Kim YS. Clinicopathologic significance of *BRAF* V600E mutation in papillary carcinomas of the thyroid: a meta-analysis. Cancer 2007;110:38-46.
6. Rivera M, Ricarte-Filho J, Knauf J, Shaha A, Tuttle M, Fagin JA, et al. Molecular genotyping of papillary thyroid carcinoma follicular variant according to its histological subtypes (encapsulated vs infiltrative) reveals distinct *BRAF* and *RAS* mutation patterns. Mod Pathol 2010;23:1191-200.
7. Xing M, Westra WH, Tufano RP, Cohen Y, Rosenbaum E, Rhoden KJ, et al. BRAF mutation predicts a poorer clinical prognosis for papillary thyroid cancer. J Clin Endocrinol Metab 2005;90:6373-9.
8. Hertzman Johansson C and Egyhazi Brage S. BRAF inhibitors in cancer therapy. Pharmacol Ther 2014;142:176-82.

http://dx.doi.org/10.3343/alm.2015.35.6.624

9. Nikiforov YE, Steward DL, Robinson-Smith TM, Haugen BR, Klopper JP, Zhu Z, et al. Molecular testing for mutations in improving the fine-needle aspiration diagnosis of thyroid nodules. J Clin Endocrinol Metab 2009; 94:2092-8.
10. Kim SW, Lee JI, Kim JW, Ki CS, Oh YL, Choi YL, et al. *BRAF* V600E mutation analysis in fine-needle aspiration cytology specimens for evaluation of thyroid nodule: a large series in a BRAF V600E-prevalent population. J Clin Endocrinol Metab 2010;95:3693-700.
11. Poller DN. New technology in thyroid fine-needle aspiration. J Clin Pathol 2014;67:457.
12. Lee ST, Kim SW, Ki CS, Jang JH, Shin JH, Oh YL, et al. Clinical implication of highly sensitive detection of the *BRAF* V600E mutation in fine-needle aspirations of thyroid nodules: a comparative analysis of three molecular assays in 4585 consecutive cases in a BRAF V600E mutation-prevalent area. J Clin Endocrinol Metab 2012;97:2299-306.
13. Dyhdalo K, Macnamara S, Brainard J, Underwood D, Tubbs R, Yang B. Assessment of cellularity, genomic DNA yields, and technical platforms for *BRAF* mutational testing in thyroid fine-needle aspirate samples. Cancer Cytopathol 2014;122:114-22.
14. Milbury CA, Li J, Makrigiorgos GM. PCR-based methods for the enrichment of minority alleles and mutations. Clin Chem 2009;55:632-40.
15. Lee ST, Kim JY, Kown MJ, Kim SW, Chung JH, Ahn MJ, et al. Mutant enrichment with 3′-modified oligonucleotides a practical PCR method for detecting trace mutant DNAs. J Mol Diagn 2011;13:657-68.
16. Baloch ZW, LiVolsi VA, Asa SL, Rosai J, Merino MJ, Randolph G, et al. Diagnostic terminology and morphologic criteria for cytologic diagnosis of thyroid lesions: a synopsis of the National Cancer Institute Thyroid Fine-Needle Aspiration State of the Science Conference. Diagn Cytopathol 2008;36:425-37.
17. Kwak JY, Kim EK, Kim JK, Han JH, Hong SW, Park TS, et al. Dual priming oligonucleotide-based multiplex PCR analysis for detection of BRAFV600E mutation in FNAB samples of thyroid nodules in BRAF V600E mutation-prevalent area. Head Neck 2010;32:490-8.
18. Landis JR and Koch GG. The measurement of observer agreement for categorical data. Biometrics 1977;33:159-74.
19. Kwak JY, Han KH, Yoon JH, Kim EK, Moon HJ, Kim YL, et al. BRAF V600E mutation testing in fine needle aspirates of thyroid nodules: potential value of real-time PCR. Ann Clin Lab Sci 2012;42:258-65.
20. Forbes SA, Bhamra G, Bamford S, Dawson E, Kok C, Clements J, et al. The Catalogue of Somatic Mutations in Cancer (COSMIC). Curr Protoc Hum Genet 2008;Chapter 10:Unit 10 1.