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Original Article

Diagnostic value of BRAF^{V600E} mutation analysis in fine needle aspiration for evaluation of thyroid nodules

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Objectives: Ultrasound-guided fine-needle aspiration (FNA) is routinely used in the evaluation of thyroid nodules. However, it has several pitfalls, as has been noted in nondiagnostic and indeterminate cases. This study aims to investigate the value of BRAF^{V600E} mutation co-testing in FNA cytology.
Method: A total of 310 patients underwent BRAF^{V600E} mutation co-testing in FNA cytology on thyroid nodules between June 2013 and June 2014. Of the 310 patients, 69 patients who had undergone a surgery for thyroid nodules were included in this study. The presence of the BRAF^{V600E} mutation was determined by allele-specific polymerase chain reaction amplification of exon 15 of the BRAF gene.
Results: Of 69 cases, 33 (47.8%) were BRAF^{V600E} mutation positive. The BRAF^{V600E} mutation was not significantly associated with high-risk features such as tumor size, lymph node metastasis, and pathological stage. The respective diagnostic performance of FNA (P = 0.02), BRAF^{V600E} mutation (P = 0.03), and ultrasonographic (P = 0.00) findings was statistically significant.
The sensitivity, specificity and positive predictive value of FNA was 64.9%, 83.3%, and 94.8%. The sensitivity, specificity and positive predictive value of BRAF^{V600E} mutation was 56.1%, 91.7%, and 96.9% and the US features was 91.2%, 91.7%, and 98.1% respectively. However, sensitivity of FNA with BRAF^{V600E} mutation (77.2%) was lower than FNA with US (92.9%) and combination all together (92.9%).
Conclusion: In this study, we found that US features were the most useful in preoperative differential diagnosis of thyroid nodules. BRAF^{V600E} mutation co-testing in FNA cytology was also useful for diagnosis of thyroid tumors.

Key Words: BRAF^{V600E} mutation, Fine-needle aspiration, Thyroid nodules, Ultrasound features

Thyroid nodules represent a common disease, as they are palpated at an average rate of 3%-7% of normal adults,[1,2] and they are diagnosed at a rate of about 20%-76% during thyroid ultrasonography.[3,4] Most thyroid nodules are benign and fewer than 5% of these nodules are malignant.[3-5] It has been recently reported that the incidence rate of thyroid cancer is dramatically increasing worldwide, and the rise in South Korea is so high that thyroid cancer has ranked at the top in terms of the cancer incidence rate in women as of 2011. In the diagnosis of thyroid nodules, ultrasound (US)-guided

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fine-needle aspiration (FNA) is a simple, accurate, and cheap method, but it is non-diagnostic in 30%-45% of cases, while atypical follicular lesions of undetermined significance (AUS), suspected follicular carcinoma, or Hurthle cell carcinoma may not be diagnosed by FNA.[6]

Thyroid ultrasonography, which plays an important role in the diagnosis and treatment of thyroid diseases, is the standard and most recommended test method for thyroid nodules.[7] Clinical studies have shown various parameters for the determination of malignancy on the basis of ultrasonographic findings. The specific ultrasonographic finding of benign thyroid nodules is the spongiform appearance of multiple microcystic components separated by thin septa inside thyroid nodules; the specificity of the finding is 99.7%-100%.[8] The findings that suggest malignant nodules include thyroid nodules that are taller (rather than wide) in appearance, spiculate boundaries, a marked hypoechoic shadow, microcalcifications, and extracapsular invasion. Among these findings, the specificity of thyroid nodules that are taller, rather than wide, in appearance is 93%; that of spiculate boundaries is 92%, that of marked hypoechoic shadow is 92%-94%, and that of microcalcification is 86%-95%. Moreover, about 70%-90% of thyroid nodules may be differentiated if the parameters of ultrasonography are used at a positive predictive value of 42%-94%.[8,9]

With the recent development of molecular biological methods, various molecular biological markers are used for the diagnosis, prognosis, prediction, and postoperative follow-up of thyroid nodules. The Revised American Thyroid Association Management Guidelines for Patients with Thyroid Nodules and Differentiated Thyroid Cancer published in 2009 and the Revised Korean Thyroid Association Management Guidelines for Patients with Thyroid Nodules and Thyroid Cancer published in 2011 suggested that molecular markers including V-raf murine sarcoma viraloncogene homolog B1 (BRAF), rat sarcoma viral oncogene homolog (RAS), rearranged during transfection/papillary thyroid cancer (RET/PTC), paired box gene 8/peroxisome proliferator-activated receptor (PAX8/PPAR-γ), galectin-3, and cytokeratin serve as expert recommendations to increase the accuracy of thyroid nodule diagnosis.[7,10]

Raf kinase is an important factor in the signaling pathway, Ras → Raf → MAPK kinase → ERK/MAPK, involved in the growth, differentiation, and proliferation of cells. Among the various isotypes of Raf kinase, the B type RAF (BRAF) is the most powerful activator of the MAPK signaling pathway, and the gene of the B type RAF (BRAF) is located on chromosome 7.[11] $BRAF^{V600E}$, which is the most common mutation of BRAF, accounts for 90% of BRAF mutations; it is caused by the transversion of the 1799 nucleotide on exon 15 of the gene from thymine to adenine.[12] Among thyroid carcinomas, the $BRAF^{V600E}$ mutation is particularly common in thyroid papillary carcinoma, and it is most frequently expressed in conventional papillary carcinoma among the various subtypes of papillary carcinomas. It has been reported that with respect to thyroid

cancer, Koreans shows a higher incidence rate of papillary carcinomas and a higher positive response rate of BRAF^{V600E} in comparison with other countries.[13] Since it has been known that the BRAF^{V600E} mutation is specific to papillary carcinoma, and given that it is highly correlated with the prognosis of thyroid cancer, a BRAF test is often performed in FNA for the diagnosis of thyroid nodules or as an additional test if diagnosis by FNA is unclear. However, an assessment of the effect of the BRAF test is required because there are reports that the BRAF mutation test yields false-positive results, although this is very rare.[14]

The present study was conducted to investigate the clinical significance of the BRAF^{V600E} mutation, as well as the effect of ultrasonography, the FNA test, and the BRAF^{V600E} mutation test on the differentiation of malignancy and benignancy of nodules in the diagnosis of thyroid nodules by performing each of the tests independently or in combination.

MATERIALS AND METHODS

Subjects

This study was conducted using 69 surgery cases, of a total of 310 cases, where the BARF^{V600E} test was performed during US-guided FNA test in the Endocrine System Center at Kosin University Gospel Hospital between June 2013 and June 2014. The subjects included 12 cases of benign carcinoma (five cases of nodular proliferating carcinoma, five cases of follicular adenoma, and two cases of Hashimoto's thyroiditis) and 57 cases of malignant nodules (53 cases of conventional papillary carcinoma, one case of follicular mutant papillary carcinoma, and three cases of follicular carcinoma). The 69 subjects consisted of 12 males (17.4%) and 57 females (72.6%). The average age of the male subjects was 53.0 ± 14.3 years (range: 31-75 years) and that of the female subjects was 45.9 ± 10.8 years (range: 24-67 years). The present study was approved by the Institutional Review Board of Kosin University Gospel Hospital.

Methods

1) Ultrasonography and Evaluation

A high-resolution 5-12 MHz ultrasonography instrument (Philips Healthcare IU 22; Bothell, WA, USA) was used to observe the number, size, internal echo type, marginal zone type, and calcification type of the nodules. Based on the ultrasonographic criteria provided by the Thyroid Research Society of the Korean Society of Neuroradiology, the nodules were classified as probable benign nodules, indeterminate nodules or suspicious malignant nodules.[8] The US reading was performed by a doctor from the Department of Endocrine Internal Systems who is skilled in ultrasonography.

2) US-guided FNA

The US-guided FNA was performed by extending the anterior cervical part of a patient while in the supine position by supporting the patient's shoulders with a pillow. Under the guidance of a high-resolution ultrasonography instrument (Philips Healthcare IU 22; Bothell, WA, USA), a

25-gauge needle attached to a 10 mL syringe was fixed at the center of the lesion, and then aspiration was performed by applying negative pressure. The aspirate was fixed in 99% alcohol by smearing it on a glass slide. The fixed smear sample was observed with an optical microscope after performing Papanicolau dyeing. The FNA diagnosis followed the Bethesda criteria.[15]

3) Detection of the $BRAF^{V600E}$ Mutation

The $BRAF^{V600E}$ mutation was detected by allele-specific polymerase chain reaction (PCR).

(1) DNA Extraction

The genomic DNA was extracted from the aspirate in the syringe after the FNA by using the QIAamp DNA mini kit (Qiagen, Chatsworth, CA, USA), according to the instructions provided by the manufacturer.

(2) Amplification

The PCR amplification of BRAF exon 5 was performed using the F:5'-TTCATGAAGACCTCACAG TAAAAA-3'R: 5'-CCACAAAATGGATCCAGACA-3' primer. The allele-specific PCR analysis was performed using the GeneAmp 9700 PCR machine (Applied Biosystems, Foster City, CA) based on the Seeplex BRAF ACE detection system (Seegene, Seoul, Korea) designed for the detection of V600E. The PCR mixtures included 5 x BRAF primer (4 μL), extracted DNA (10 ng/μL) 3 μL, 8-methoxypsoralen solution 3 μL, and 23 multiplex master mix (Seegene) 10 μL; and the total volume was 20 μL. The PCR was performed by initially culturing the mixtures at 94°C for 15 minutes, and repeating this process a total of 35 times. The cycle consisted of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 60 seconds using the Thermo cycler (Applied Biosystems, Foster City, CA); the cycle ended after the final extension at 72°C for 10 minutes.

(3) Sequencing

The amplified PCR products were analyzed using the Screen Tape system (Lab901 Ltd., Edinburgh, Scotland, UK) based on the Seegene Viewer software. The level of internal control/V600E amplicon band intensity was recorded each time the mutation rate exceeded 10%.

Statistical Analysis

The data used for the analysis included the FNA findings, the presence of a $BRAF^{V600E}$ mutation, and the ultrasonographic findings. When two of the indicators were used in combination (the FNA findings, the presence of the $BRAF^{V600E}$ mutation, and the ultrasonographic findings), a positive response from either of the two was considered a positive response. The data analysis was performed using SPSS version 18.0, and the significance level was set to $P < 0.05$. The correlation between the presence of the $BRAF^{V600E}$ mutation and the clinicopathological parameters in papillary thyroid carcinomas was analyzed by performing the χ^2 test and Fisher's exact test. The sensitivity, specificity, positive predictive value, and negative predictive value of FNA, the ultrasonographic findings, and the presence of the $BRAF^{V600E}$ mutation were respectively calculated. The sensitivity, specificity, positive predictive value, and negative

predictive value were also calculated for the combination of FNA and the presence of the BRAF^{V600E} mutation, as well as for the combination of FNA and the ultrasonographic findings. Statistical significance was analyzed by performing the χ^2 test.

RESULTS

Comparison of the Presence of BRAF^{V600E} and FNA with Histopathologic Examination Results

Of the 69 cases that were included in this study, 36 (52.2%) were BRAF^{V600E} mutation negative and 33 (47.8%) were positive. Of the 36 BRAF^{V600E} mutation negative cases, papillary carcinoma was found in 22 cases (61.6%), while follicular carcinoma was found in 2 cases. Of the 33 BRAF^{V600E} mutation positive cases, malignant carcinoma was found in 31 papillary carcinoma cases (93.9%), in one follicular carcinoma case, and in one case of follicular adenoma - a benign carcinoma that was identified as a papillary carcinoma by surgery. Of the seven AUS cases, a papillary carcinoma was found in six cases, among which four were BRAF^{V600E} mutation positive. Finally, of the 39 cases diagnosed with a suspicious papillary carcinoma or a papillary carcinoma, papillary carcinoma was confirmed by histopathologic examination in 33 cases (84.6%), among which 25 cases were BRAF^{V600E} mutation positive (Table 1).

Correlation between the BRAF^{V600E} Mutation and Various Clinicopathological Parameters in Papillary Thyroid Carcinomas

There was no significant difference in the expression of the BRAF^{V600E} mutation in papillary thyroid carcinoma with respect to either the subject's age group or sex. No significant difference was found in the size of the carcinoma. Nodule calcification was observed in 19 cases (61.3%) of the BRAF^{V600E} mutation positive group, while it was observed only in eight cases (34.8%) of the BRAF^{V600E} mutation negative group, indicating that there was a significant difference between the two groups (P = 0.04). None the findings were associated with any of the factors that impact thyroid cancer prognosis (including extrathyroidal invasion, lymph node metastasis, and TNM stage); these factors were also not correlated with the presence of the BRAF^{V600E} mutation (Table 2).

Comparison of Diagnostic Ability among FNA, BRAF^{V600E} Mutation, and Ultrasonographic Findings

The sensitivity, specificity, and positive predictive value of FNA were 64.9%, 83.3%, and 94.8%, respectively, and those of the BRAF^{V600E} mutation test were 56.1%, 91.7%, and 96.9%, respectively. The BRAF^{V600E} mutation test showed a lower sensitivity and a higher specificity when compared with FNA. The sensitivity, specificity, and positive predictive value of ultrasonographic findings were 91.2%, 91.7%, and 98.1%, respectively, all of which were higher than those of an independent FNA and an independent BRAF^{V600E} mutation test. All three test methods, FNA (P = 0.02), ultrasonography

Table 1. Fine-needle aspiration, BARF^{V600E}, and final pathologic diagnosis with operation in thyroid nodules

FNA result	BRAF^{V600E} mutation	Numbers	Pathology (n = 69)
Nondiagnostic (n = 3)	Negative	3	PTC (n = 3)
	Positive	0	None
Benign (n = 17)	Negative	14	NH (n = 3), FA (n = 3), FC (n = 2), FVPTC (n = 1), PTC (n = 5)
	Positive	3	FA (n = 1), PTC (n = 2)
AUS (n = 7)	Negative	3	HT (n = 1), PTC (n = 2)
	Positive	4	PTC (n = 4)
Suspicious for FN (n = 3)	Negative	2	NH (n = 1), FA (n = 1)
	Positive	1	FC (n = 1)
Suspicious for PTC (n = 7)	Negative	4	NH (n = 1), HT (n = 1), PTC (n = 2)
	Positive	3	PTC (n = 3)
PTC (n = 32)	Negative	10	PTC (n = 10)
	Positive	22	PTC (n = 22)

FAN, fine needle aspiration; AUS, atypia undetermined significance; FN, follicular neoplasm; PTC, papillary thyroid carcinoma; NH, nodular hyperplasia; FA, follicular adenoma; HT, Hashimoto's thyroiditis; FC, follicular carcinoma; FVPTC, follicular variant papillary thyroid carcinoma

(P = 0.00), and the BRAF^{V600E} mutation test (P = 0.03), were significant in terms of their ability to differentiate between a malignant carcinoma and a benign carcinoma found by histopathologic examination (Table 3).

Comparison of Diagnostic Ability between a Combination of FNA and the BRAF^{V600E} Mutation and a Combination of FNA and Ultrasonographic Findings

The sensitivity, specificity, and positive predictive value of the combination of FNA and the BRAF^{V600E} mutation test were 77.2%, 75.0%, and 93.6%, respectively, indicating that the sensitivity was higher and the specificity was lower in comparison with independently performed FNA and the BRAF^{V600E} mutation test. The sensitivity, specificity, and positive predictive value of the combination of FNA and ultrasonographic findings were 92.9%, 83.3%, and 96.3%, respectively, indicating that the specificity was lower than that of independent ultrasonography (Table 3).

DISCUSSION

Since it was first attempted by Lipton et al.[16] in 1944 for a case of thyroid disease, FNA has

Table 2. Correlation between $BRAF^{V600E}$ mutation and various clinicopathological parameters in papillary thyroid carcinomas

	Mutation of $BRAF^{V600E}$, n (%)		X^2	*P*-value
	Wild (n = 23)	Mutant (n = 31)		
Age				
〈 45 years	9 (39.1)	12 (38.7)	0.001	0.59
≥ 45 years	14 (60.9)	19 (61.3)		
Gender				
Male	5 (21.7)	5 (16.1)	0.275	0.42
Female	18 (78.3)	26 (83.9)		
Tumor size				
≤ 10 ㎜	21 (91.3)	24 (77.4)	1.833	0.16
〉 10 ㎜	2 (8.7)	7 (22.6)		
Calcification				
No	15 (65.2)	12 (38.7)	3.711	0.04
Yes	8 (34.8)	19 (61.3)		
ETE				
No	14 (60.9)	15 (48.4)	0.827	0.26
Yes	9 (39.1)	16 (51.6)		
Lymphocytic thyroiditis				
No	17 (73.9)	24 (77.4)	0.089	0.50
Yes	6 (26.1)	7 (22.6)		
Nodal metastasis				
Negative (N0)	19 (82.6)	19 (61.3)	2.878	0.08
Positive (N1a + N1b)	4 (17.4)	12 (38.7)		
pTMN staging				
I + II	20 (87.0)	25 (80.6)	0.379	0.40
III + IV	3 (13.0)	6 (19.4)		

ETE; extrathyroidal extension. According to the TNM staging system: N1a indicates lymph node metastasis to level VI (pretracheal, tracheal and prelaryngeal nodes); N1b indicates metastasis to unilateral, bilateral, contralateral cervical or superior mediastinal nodes. Calculated by the x^2-test and Fisher's exact test

been frequently performed in clinical settings since this procedure is simple and adverse effects are rare. A meta-analysis conducted by Gharib et al.[17] with 18,000 specimens showed that the number of patients undergoing thyroidectomy had decreased by 25%, and the incidence rate of detecting postoperative thyroid cancer had increased by 15%-30% since the introduction of FNA in the diagnosis of thyroid nodules. Afterward, US-guided FNA was introduced, and it was found capable not only of diagnosing small nodules, but also of detecting deep nodules; this method became a standard diagnostic method for the diagnosis of thyroid nodules. However, since the diagnosis by US-guided FNA is dependent on a small number of smeared cells, the rate of false-negative responses is high in samples without intranuclear inclusion or grooves, which are characteristic of

Table 3. Sensitivity, specificity, positive predictive value and negative predictive value according to diagnostic modalities

	Sensitivity %	Specificity %	PPV %	NPV %	*P*-value
Cytology	64.9 (37/57)	83.3 (10/12)	94.8 (37/39)	66.7 (20/30)	0.02
$BRAF^{V600E}$	56.1 (32/57)	91.7 (11/12)	96.9 (32/33)	30.1 (11/36)	0.03
US assessment	91.2 (52/57)	91.7 (11/12)	98.1 (52/53)	68.8 (11/16)	0.00
Cytology with $BRAF^{V600E}$	77.2 (44/57)	75.0 (9/12)	93.6 (44/47)	40.9 (9/22)	0.00
Cytology with US	92.9 (53/57)	83.3 (10/12)	96.3 (53/55)	71.4 (10/14)	0.00
All combination	92.9 (53/57)	75.0 (9/12)	94.6 (53/56)	69.2 (9/13)	0.00

PPV; positive predictable value, NPV; negative predictable value

papillary carcinoma and are mostly found in only a specific part of tumor cells. In addition, it is generally accepted that a follicular carcinoma may not be diagnosed only by cytological findings because a follicular carcinoma is differentiated only when capsular or vascular invasion is confirmed by a histological examination.[18] In the present study, 53.7% of subjects were diagnosed with suspicious malignant carcinoma or malignant carcinoma by a preoperative FNA test, indicating that the sensitivity of FNA was lower than that of previous studies. Three nondiagnostic cases of the preoperative FNA were diagnosed with a papillary carcinoma, and eight cases (47.0%) out of 17 cases that were diagnosed as benign in FNA were diagnosed as papillary carcinoma, indicating that FNA is not sufficient for differentiating malignant nodules.

To overcome the shortcomings of FNA, various molecular biological markers have been discovered and used in the diagnosis of thyroid nodules. Among these markers, the $BRAF^{V600E}$ mutation is the most frequently observed marker in thyroid cancer, and it is currently used for the diagnosis of thyroid cancer. It has been reported that the $BRAF^{V600E}$ mutation is found in about 29%-83% of papillary carcinomas, and in more than 80% of papillary thyroid cancer cases in South Korea.[19] Different clinical characteristics of the $BRAF^{V600E}$ mutation have been reported in previous studies. For instance, Namba et al.[20] and Nikiforva et al.[21] reported that the $BRAF^{V600E}$ mutation was correlated with stage 3 and 4 thyroid cancer by TNM staging, and Kim et al.[14] stated that this mutation is correlated with remote metastasis. However, Puxeddu et al.[22] reported that the $BRAF^{V600E}$ mutation is not correlated with the stage of a carcinoma, local infiltration, or lymph node metastasis. The results of the present study showed that the presence of the $BRAF^{V600E}$ mutation was not correlated with the clinical characteristics of thyroid carcinoma, with the exception of the presence of calcification.

It is well known that the positive predictive value of the $BRAF^{V600E}$ mutation test in combination with FNA is known to be 99% or higher.[23] In the present

study, 31 cases (93.9%) of the 33 cases showing a positive response during the preoperative BRAFV600E mutation test were diagnosed as papillary carcinomas, whereas 22 cases (61.1%) of papillary carcinoma were found out of the 36 cases that showed a negative response during the preoperative BRAF^{V600E} mutation test, indicating that the sensitivity of the BRAF^{V600E} mutation test was as low as 56.1%, but its positive predictive value was as high as 96.9%. In addition, the BRAF^{V600E} mutation was found by the FNA test in two cases of benign nodules and four cases of AUS, all of which were diagnosed as papillary carcinoma by surgery, indicating that performing the BRAF^{V600E} mutation test in those cases where diagnosis by FNA is not distinctive may help to diagnose malignant nodules. On the other hand, as the BRAF^{V600E} mutation test is frequently performed, the percentage of false-positive responses has been reported to be about 2%. In the present study, a false-positive response was observed in one case of follicular adenoma by the BRAF^{V600E} mutation test.

In this study, the sensitivity of the FNA test was 64.9% when it was performed independently, but its sensitivity increased to 77.2% when it was performed in combination with the BRAF^{V600E} mutation test. In the study conducted by Moon et al.,[24] a positive response on the BRAF^{V600E} mutation test was found in 141 (82.9%) of 170 cases of papillary carcinoma, and the sensitivity of the independent FNA test was 81.8%, which increased to 94.1% by adding the BRAF^{V600E} mutation test. In the study conducted by Seo et al.,[25] the sensitivity of the independent FNA test was 66%, which was increased to 84.7% by adding the BRAF^{V600E} mutation test, indicating that the addition of the BRAF^{V600E} mutation test to the FNA test may help to increase the diagnosis rate.

Although different results have been obtained in previous studies that used ultrasonographic findings to identify malignant nodules - which are thyroid nodules that are taller (rather than wide) in appearance, and which feature spiculate boundaries, a marked hypoechoic shadow, microcalcification, and extracapsular invasion - Koike et al.[26] reported that the sensitivity, specificity, and positive predictive value of US were 76.0%, 92.2%, and 80.6%, respectively, while Kim et al.[9] reported that they were 93.8%, 66.0%, and 56.1%, respectively. In the present study, the sensitivity, specificity, and positive predictive value of ultrasonography were 91.2%, 91.7%, and 98.1%, respectively, indicating that the sensitivity and specificity were higher than those of previous studies. The sensitivity and specificity of these ultrasonographic findings were higher than those of the FNA test and the BRAF^{V600E} mutation test, which showed that ultrasonographic findings are important in the diagnosis of thyroid nodules. The sensitivity of the combination of FNA, ultrasonographic findings, and the BRAF^{V600E} mutation test was 98.2% in the study by Moon et al.[24] and 96.2% in the investigation by Seo et al.[25] In this study, the sensitivity of the combination of FNA, ultrasonographic findings, and the BRAF^{V600E} mutation test was 92.9%. This result demonstrates the im-

portance of ultrasonographic findings.

In conclusion, the present study showed that ultrasonographic findings were most helpful in providing a differential diagnosis of preoperative thyroid cancer. Furthermore, combining the $BRAF^{V600E}$ mutation test with US-guided FNA may be clinically helpful, particularly since a positive response on the $BRAF^{V600E}$ mutation test in combination with US-guided FNA is indicative of malignancy.

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