의학과 석사학위논문

유두상 갑상선암에서 *BRAF*^{V600E} 돌연변이의 동정을 위한 면역 조직 화학 염색과 직접 염기 서열 분석 방법의 비교

Comparison of immunohistochemistry and direct sequencing methods for identification of the *BRAF*^{V600E} mutation in papillary thyroid carcinoma

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Background: *BRAF*^{V600E} mutation is the most common somatic variant in papillary thyroid carcinoma (PTC) and is associated with aggressive prognostic factors. The conventional detection methods for *BRAF* mutations is polymerase chain reaction followed by Sanger sequencing. Recently, an immunohistochemistry (IHC) method-using a *BRAF*^{V600E} specific antibody (VE1) has been developed and widely adopted in the clinics. However, there is lack of evidence regarding the comparability of the IHC and Sanger sequencing methods.

Methods: We retrospectively reviewed the clinical data of patients who underwent

thyroidectomy in Seoul National University Hospital from January 2013 to October 2016. Among a total of 3584 patients, samples from 886 cases had been analyzed by both IHC and direct sequencing. We excluded cases in which the above tests were performed with tissues obtained lymph node metastasis or histology of other PTC subtypes. Finally, patients with classic PTC, where both tests had been performed in primary tumor tissue, were included in the study (n=697).

For IHC staining, we used a commercially available *BRAF* ^{V600E} mutation-specific antibody (clone VE1, Spring Bioscience, Pleasanton, CA, USA). IHC was performed using paraffin-embedded tissue microarrays (TMAs). Briefly, TMA sections were moved to adhesive slides and placed in a drying oven at 60℃ for 30 min. Using the Benchmark XT system, specimens were deparaffinized with EZ prep solution (Ventana Medical Systems, Inc, Tucson, AZ, USA). During the pretreatment step, cell-conditioning solutions containing EDTA (Ventana) were applied for 64 min. Subsequently, antibody was applied to the slides, and incubated for 32 min. And a positive control was attached to the same slide. Finally, stained tissue sections were examined by a microscopy.

PCR amplification and direct Sanger sequencing were performed to detect the *BRAF*^{V600E} mutation in 697 PTC samples. In cases with multifocal PTC, the largest tumor was selected for sequencing analysis. The target region was marked on hematoxylin and eosin-stained slides. We extracted DNA with Maxwell instrument. Briefly, *BRAF* exon 15, potentially containing the T1799A transversion (encoding *BRAF*^{V600E}) was amplified by PCR from genomic DNA. After purification of the PCR products, direct bidirectional sequencing was performed using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). We performed IHC and direct sequencing methods simultaneously on the sample.

Results: *BRAF* mutation was detected in 90.0% (627/697) of samples using IHC and 83.4% (581/697) by direct sequencing. The diagnostic parameters of IHC compared with Sanger

sequencing were as follows: 100% sensitivity (581/581), 60.3% specificity (70/116), 92.7% positive predictive value (581/627), and 100% negative predictive value (70/70). No false negative results were recorded using IHC. The overall concordance rate between the two methods was 93.4% (651/697). Discordant results were found in 46 samples (6.6%), 29 of which were from cases with small tumors (< 6mm), eight were from cases with low tumor cellularity, and nine were specimens yielding low quality DNA.

To investigate clinical and pathological differences among patients with discordant results, we split patients into three groups: Group 1 contained patients with positive results in both IHC and direct sequencing (n = 581); group 2 included patients with positive results by IHC but negative results using direct sequencing (n = 46); and group 3 consisted of patients with negative results from both IHC and direct sequencing (n = 70). We compared the sex ratio, age, gross ETE, tumor size, lymph node metastasis, tumor multifocality, thyroiditis, tumor lymphatic invasion, and tumor angio-invasion among the three subgroups. When these three groups were compared, group 3 patients had larger tumors than those in group 1, and group 1 patients had larger tumors than those in group 2 ($p < 0.001$). An increased frequency of lymph node metastasis was observed in group 1 compared with group 2 ($p = 0.007$), however, more group 2 than group 1 patients had thyroiditis ($p = 0.001$). Comparisons of tumor lymphatic invasion, and tumor angio-invasion that group 1 and 3 patients exhibited a higher incidence of these phenomena than those in group 2 ($p = 0.005$ and $p = 0.006$ respectively).

Univariate and multivariate logistic regression analysis showed that tumor size and thyroiditis were independent predictors for discordance between IHC and direct sequencing. Of these factors, tumor size is the most powerful predictor, decreasing the odds ratio (OR) of discordance between IHC and sequencing to 0.249 (95% confidence interval [CI], 0.113-0.548) in multivariate analysis. Thyroiditis is also predictor, increasing the OR of discordance between these methods to 2.825 (95% CI, 1.516-5.266) in multivariate analysis.

Conclusions: IHC using the VE1 antibody is a reliable and highly sensitive method to detect

the *BRAF*^{V600E} mutation in classic PTC. When we analyzed the results according to the discordancy of the IHC and sequencing, the smaller tumor size and the presence of thyroiditis did not match the results of the two tests. Because the results of this study suggest that the sequencing results were likely to be false negative if the two test results were inconsistent, IHC is an acceptable test method in patients with small sized thyroid cancer or thyroiditis.

Keywords: *BRAF*^{V600E}, papillary thyroid cancer, immunohistochemistry, sensitivity, specificity

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Introduction

Papillary thyroid cancer (PTC) is the most common endocrine malignancy and comprises the vast majority (90%) of thyroid malignancies [1]. The most common somatic driver mutations of PTC are in the *BRAF* gene, which encodes an activator of the mitogen-activated protein kinase signaling pathway[2]. The most frequent *BRAF* mutation in PTC is a substitution of valine for glutamic acid at amino acid 600, *BRAF* ^{V600E} [3]. *BRAF* mutation appears to be important in PTC carcinogenesis and, may have prognostic value [4]. *BRAF* mutation is associated with aggressive clinical features, such as distant metastasis, extrathyroidal extension (ETE), advanced pathologic T stage, and lymph node metastasis [5-7].

Several techniques can be used for detection of *BRAF* mutations including high resolution melting analysis, pyrosequencing, the cobas test, next generation sequencing, and Sanger sequencing. These methods are based on specific polymerase chain reaction (PCR) amplification of the *BRAF* gene[8-9]. Among these techniques, Sanger sequencing has been widely used[8], however such approaches are expensive, time-consuming, and involve multiple steps[10]. Recently, immunohistochemical (IHC) visualization of the *BRAF*^{V600E} mutated protein using a mutation-specific antibody, VE1, raised against a synthetic peptide representing the *BRAF*^{V600E} mutated amino acid sequence (amino acids 596-606, GLATEKSRWSG) has been developed[10]. IHC staining is used widely in routine diagnostic pathology laboratories and compared with molecular techniques, it is less time-consuming and potentially cheaper[10-11].

In routine clinical practice, several techniques for detecting the *BRAF*^{V600E} mutation in tumors have been introduced. Most methods are based on specific PCR, including sequencing, allele-PCR, peptide nucleic acid (PNA)-mediated clamping PCR[9, 12]. These methods are expensive, multi-step, and time consuming[10]. According to recent studies, IHC was shown to have high sensitivity and specificity[10, 11]. In addition, the IHC method is less expensive and less time consuming than PCR based methods[10, 11]. Although IHC is a useful and established

diagnostic clinical tool, whether testing with VE1 antibody can replace PCR-based *BRAF* mutation tests remains unclear, and the diagnostic accuracy of IHC for detection of the *BRAF* mutation has not yet been clearly established[10, 12]. In the present study, we compared IHC and direct sequencing methods to evaluate the clinical utility of IHC for detection of the $BRAF^{V600E}$ mutation in patients with classic PTC.

Materials and Methods

Case selection

In Seoul National University Hospital, we have performed direct sequencing and IHC in all PTC tissues routinely since July 29th, 2015. Before that period, IHC first has been performed and direct sequencing underwent optionally. These indications were BRAF mutation negative results in IHC, small size tumor cases, and it was better to do it in the opinion of a pathologist.

We retrospectively reviewed the clinical data of patients who underwent thyroidectomy in Seoul National University Hospital from January 2013 to October 2016. Among a total of 3584 patients, samples from 886 cases had been analyzed by both IHC and direct sequencing. We excluded cases in which the above tests were performed with tissues obtained lymph node metastasis or histology of other PTC subtypes. Finally, patients with classic PTC, where both tests had been performed in primary tumor tissue, were included in the study (n=697). Median follow-up period after surgery was 8 months. Cancer stage was calculated according to the American Joint Committee on Cancer (AJCC) staging system (8th edition). This study was approved by the Seoul National University Hospital Institutional Review Board (1609-001-787).

$BRAF^{V600E}$ Immunochemistry

For IHC staining, we used a commercially available *BRAF* ^{V600E} mutation-specific antibody (clone VE1, Spring Bioscience, Pleasanton, CA, USA). IHC was performed using paraffin-embedded tissue microarrays (TMAs). Briefly, TMA sections were moved to adhesive slides and placed in a drying oven at 60℃ for 30 min. Using the Benchmark XT system, specimens were deparaffinized with EZ prep solution (Ventana Medical Systems, Inc, Tucson, AZ, USA). During the pretreatment step, cell-conditioning solutions containing EDTA (Ventana) were applied for 64 min. Subsequently, antibody was applied to the slides, and incubated for 32

min. And a positive control was attached to the same slide. Finally, stained tissue sections were examined by a microscopy. IHC results were classified into one of three categories by a specialist pathologist as follows: negative-no staining, weak positive-faint staining intensity, strong positive-diffuse staining intensity.

Direct sequencing

PCR amplification and direct Sanger sequencing were performed to detect the $BRAF^{V600E}$ mutation in 697 PTC samples. In cases with multifocal PTC, the largest tumor was selected for sequencing analysis. The target region was marked on hematoxylin and eosin-stained slides. We extracted DNA with Maxwell instrument. PCR amplification conditions: 94 ℃ for 10 min, followed by 34 cycles of 94 ℃ for 1 min, 60 ℃ for 1 min, 72 ℃ for 1 min, with a final extension of 10 min at 72 ℃. The PCR products were purified using a QIAquick gel extraction kit (Qiagen) and sequenced with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). The cycling conditions were as follows: 96 ℃ for 1 min, followed by 24 cycles of 96 ℃ for 10 s, 50 ℃ for 5 s, 60 ℃ for 4 min, with a final extension at 60 ℃ for 1 min[13]. Briefly, *BRAF* exon 15, potentially containing the T1799A transversion (encoding $BRAF^{V600E}$) was amplified by PCR from genomic DNA. After purification of the PCR products, direct bidirectional sequencing was performed using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). We performed IHC and direct sequencing methods simultaneously on the sample.

Statistical analyses

SPSS software version 20.0 (IBM Corporation., Armonk, NY, USA) was used for statistical analysis. Chi-square and Fisher's exact tests were used to evaluate differences between categorical variables. *T*- and ANOVA tests were used to compare mean values, and *p*-values < 0.05 were considered statistically significant.

Results

Clinical and pathological characteristics

The clinical and pathological characteristics of PTC patients included in this study are presented in Table 1. Mean age (±standard deviation) was 47.8±12.9 years. Of included patients, 551 (79.1%) were female and 146 (20.9%) were male. Surgical interventions included thyroid lobectomy, total thyroidectomy, and total thyroidectomy with modified radical neck dissection (MRND), which were performed in 211 (30.3%), 411 (59.0%), and 75 (10.8%), respectively. The median follow-up periods were 8 months (range 1-46 months), and the mean largest tumor size was 1.2 cm (range 0.1-7.7 cm). Other clinical and pathological variables are presented in Table 1.

Comparison between IHC and direct sequencing for detection of the BRAF ^{V600E} mutation

The results of *BRAF* mutation analysis, and concordance between IHC staining and direct sequencing are presented in Table 2. The *BRAF* ^{V600E} mutation was detected in 90.0% (627/697) of patients by IHC and, 83.4% (581/697) by direct sequencing. Among *BRAF* ^{V600E} IHC positive results, 614 strong positive and 13 were weak positives. The concordance between IHC and direct sequencing was 93.6% (575/614) for IHC samples exhibiting strong positive staining, 46.2% (6/13) in samples with weak-positive by IHC, 100% (70/70) for samples mutation-negative by IHC. Overall concordance between IHC and direct sequencing was 93.4% (651/697). Forty-six PTC samples were IHC positive (weak or strong), but negative for the *BRAF*^{V600E} mutation by direct sequencing.

The sensitivity and specificity of the IHC method relative to Sanger sequencing were 100% (581/581) and 60.3% (70/116), and the positive predictive and negative predictive values for IHC were 92.7% (581/627) and 100% (70/70), respectively. No false negatives were

detected using the IHC tests (Table 2).

Factors associated with discordance between IHC and direct sequencing

Discordant results for *BRAF* status between IHC and direct sequencing were identified in samples from 46 patients (6.6%). All 46 samples exhibited IHC staining (weak positive or strong positive), but were negative for the *BRAF* ^{V600E} mutation by direct sequencing analysis. To investigate clinical and pathological differences among patients with discordant results, we split patients into three groups: Group 1 contained patients with positive results in both IHC and direct sequencing (n = 581); group 2 included patients with positive results by IHC but negative results using direct sequencing (n = 46); and group 3 consisted of patients with negative results from both IHC and direct sequencing (n = 70) (Table 3). We compared the sex ratio, age, gross ETE, tumor size, lymph node metastasis, tumor multifocality, thyroiditis, tumor lymphatic invasion, and tumor angio-invasion among the three subgroups.

When these three groups were compared, group 3 patients had larger tumors than those in group 1, and group 1 patients had larger tumors than those in group 2 ($p < 0.001$). An increased frequency of lymph node metastasis was observed in group 1 compared with group 2 ($p = 0.007$), however, more group 2 than group 1 patients had thyroiditis ($p = 0.001$). Comparisons of tumor lymphatic invasion, and tumor angio-invasion that group 1 and 3 patients exhibited a higher incidence of these phenomena than those in group 2 ($p = 0.005$ and $p = 0.006$ respectively). No correlations were observed among these three groups with regards to patient sex, age, gross ETE, or multifocality (Table 3).

Predictive factors for discordance between IHC and direct sequencing

Univariate and multivariate logistic regression analysis showed that tumor size and thyroiditis were independent predictors for discordance between IHC and direct sequencing (Table 4). Of these factors, tumor size is the most powerful predictor, decreasing the odds ratio (OR) of discordance between IHC and sequencing to 0.249 (95% confidence interval [CI],

0.113-0.548) in multivariate analysis. Thyroiditis is also predictor, increasing the OR of discordance between these methods to 2.825 (95% CI, 1.516-5.266) in multivariate analysis. Other factors including male sex, age, gross ETE, lymph node metastasis, tumor multifocality, tumor lymphatic invasion, and tumor angioinvasion were not associated with the development of discordance between IHC and direct sequencing.

Discussion

The *BRAF*^{V600E} mutation is a well-known driver mutation in PTC and acts as a diagnostic marker for PTC among the various types of thyroid cancers. In addition, *BRAF*^{V600E} mutation is associated with factors indicating poor prognosis factors in PTC [10, 14]. Sanger sequencing is the most commonly used method for detection of gene mutations [8, 10]. However, this type of test generally requires high quality DNA, is time-consuming and expensive, and requires special reagents or equipment [10]. By contrast, IHC is routinely used in diagnostic pathology laboratories, due to advantages of speed, simple processing, and low cost; these benefits have led to increasing use of IHC to detect mutant proteins in recent years [10]. The diagnostic accuracy of IHC for detection of the *BRAF* mutation is not yet fully established [10, 12] and was, therefore, compared with that of Sanger sequencing in this study. Since 2015, we have routinely performed IHC and Sanger sequencing in PTC patients, allowing a retrospective review of relevant data.

The *BRAF*^{V600E} mutation was detected by IHC in 90.0% (627/697) of classic PTC cases. *BRAF*^{V600E} mutations have previously been reported in 29-69% of PTC patients [2, 15, 16]; therefore, our *BRAF*^{V600E} mutation-positive rate was higher than those of prior studies. There are three possible explanations for this finding. The first, relates to a form of selection bias. This study was a retrospective review of data from 2013 January to 2016 October. Among the 3584 thyroid patients who received thyroid surgery over this time period, we enrolled only 697, for whom *BRAF* tests had been performed, and cases without *BRAF* tests were not included in this study. For this reason, the *BRAF* mutation-positive rate would be expected to be higher than that of the whole thyroid cancer population. Second, the proportion of patients with samples positive for the *BRAF*^{V600E} mutation has actually been increasing over recent years. For example, Aarti *et al*. [17] reported that the rate of *BRAF*^{V600E} mutation in PTC patients had increased over a 15 year period at their institution. *BRAF*^{V600E} mutation detected in 88% of PTC patients in 2001-2005[17]. The high rate of *BRAF*^{V600E} mutations observed in our study may be explained this trend. The

third reason is an ethnic characteristics. According to a recent study reported in Korea, Jung *et al.*[18] reported *BRAF*^{V600E} mutations were found in 839 (80.6%) patients with overall PTC and in 85.3% patients with classic PTC. And Lee *et al.*[19] showed *BRAF*^{V600E} mutations were found in 80.8% patients with overall PTC patients. These results showed that the prevalence of *BRAF*^{V600E} mutation is higher in Korean patients with papillary thyroid cancer compared to other races. The results of our study are also consistent with prior studies.

In this study, concordance between IHC and direct sequencing was 93.4% (651/697). The results are similar to previous studies [10, 12]. Compared with direct sequencing, the specificity of IHC was 60.3% (70/116), which was slightly lower than reported in some prior studies [12, 20-22]. Zagzag *et al.*[10] reported that the specificity of IHC was 100% and Zhu *et al.*[12] presented that the specificity of IHC was 82.2%. However, these studies had small sample sizes. In this study, no *BRAF*^{V600E} mutation was detected by direct sequencing, 46 cases that were mutation-positive by IHC (weak or strong staining), giving a positive predictive value of 92.7% (581/627); however, the sensitivity of *BRAF*^{V600E} detection by IHC was 100% (581/581). No false negatives were detected using IHC. Overall, our results demonstrate that IHC is a reliable, highly sensitive, alternative method for detection of the *BRAF*^{V600E} mutation.

For many years, direct sequencing has been considered the reference method for identification of acquired mutations in tumors [23]; however, because of its relatively low sensitivity, detection of mutations from tumor DNA requires a high percentage of tumor cells within the samples, a requirements that cannot always be met in routine diagnostic testing of human samples [24]. In this study, the *BRAF*^{V600E} mutation was detected in 46 patients by IHC but not using direct sequencing. Of these 46 patients, 29 had small tumors (< 0.6 cm), hence it is possible that direct sequencing produced false negative results as a consequence of the small tumor size. A further eight patients had large tumors (> 1 cm); however, their tissue block samples had very low tumor to normal cell ratios, which may also have resulted in false negative results by direct sequencing, due to low tumor DNA content below the detection

threshold required to amplify the *BRAF* gene using PCR-based techniques. A further 9 of the 46 tumors were calcified or fibrotic, according to pathology results, which may also indicate low numbers of cancer cells. Hence, the ability of IHC to detect the $BRAF^{V600E}$ mutation in these particular types of tissue blocks may explain the observed discordance. Moreover, all of the discordant results for *BRAF* status between IHC and direct sequencing in this study could be explained by the higher sensitivity of the IHC test compared with was direct sequencing analysis.

IHC can be used to evaluate tissues with very low tumor content [22]. In this study, samples in group 2 (those generating discordant data) (Table 3) were from patients with less lymph node metastasis, lymphatic invasion, and angio-invasion than those in other groups. Moreover, group 2 patients had smaller tumors than those assigned to group 1 and 3. These results demonstrated that $BRAF^{V600E}$ mutations could be detected by IHC in patients who had early cancer lesions, but not using direct sequencing. Moreover group 2 patients more frequently had thyroiditis tissue than those in group 1, demonstrating that inflammatory tissue dose not interfere with IHC test results. In multivariate logistic regression analysis, when the size of the tumor was small and thyroiditis was present, there was a difference result between the two tests. Overall, these data indicate that IHC is an acceptable test method in patients with small sized thyroid cancer or thyroiditis.

There are several prognostic molecular markers in thyroid cancer, including mutations in *RAS, PIK3CA, PTEN, P53, ALK, TERT*, and *BRAF* genes [25]. There are studies showing that PTC showed a poor prognosis in the co-existence of $BRAF^{V600E}$ mutation and *TERT* C228T mutations or TP53 mutation cooperatively [26, 27]. However, many studies have shown the role of $BRAF^{V600E}$ mutation alone in tumor aggressiveness [28-30] and even patient mortality in PTC [31]. Therefore, the $BRAF^{V600E}$ mutation seems to be one of the predictors of the prognosis of patients with PTC. In this study, IHC showed excellent results in detecting $BRAF^{V600E}$ mutation, so that it can play a major role in predicting the prognosis of the patient and establishing the treatment

plan.

This study has several limitations, including retrospective data collection and the lack of inclusion of control group. A study including prospective data collection will be important to support our findings indicating that the IHC test is superior to PCR-based approaches. Additionally, the number of patient samples with weak-positive IHC staining (n = 13) was small in this study; therefore, we could not determine the usefulness of IHC for quantification of the expression of the $BRAF^{V600E}$. Finally, 7 cases were in which IHC results were weak positive in the two groups with discordant results. In this case, it is possible that the IHC came out as a false positive. This may lead to false-positive results.

In conclusion, our study demonstrates that the IHC test is a reliable, highly sensitive method for detection of the $BRAF^{V600E}$ mutation in classic PTC, and is an acceptable test method in patients with small sized thyroid cancer or thyroiditis.

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Table 1. Clinicopathological characteristics

Characteristics	**Patients (N = 697)**
Age	47.8
≥ 55 (n, %)	211 (30.3)
< 55 (n, %)	486 (69.7)
Sex	
Male	146 (20.9)
Female	551 (79.1)
Surgical extent	
Lobectomy	211 (30.3)
Total thyroidectomy with/without CLND	411 (59.0)
Total thyroidectomy and MRND	75 (10.8)
Largest tumor size (cm)	1.2
Tumor multifocality	238 (34.1)
Thyroiditis	185 (26.5)
Tumor lymphatic invasion	256 (36.7)
Tumor angioinvasion	10 (1.4)
Tumor margin involvement	32 (4.6)
pT stage	
T1	577 (82.8)
T2	52 (7.5)
T3	54 (7.7)
T4	14 (2.0)
pN stage	
N0	391 (56.1)
N1	306 (43.9)
AJCC cancer stage (8^{th} edition)	
I	599 (85.9)
II	89 (12.8)
III	9 (1.3)
IV	0 (0.0)
RAI therapy	310 (44.5)

Data are expressed as *n* (%) or mean values.

CLND, central lymph node dissection; MRND, modified radical neck dissection; AJCC, American Joint Committee on Cancer; RAI, radioactive iodine.

Table 2. Comparison of $BRAF^{V600E}$ detection in PTC by IHC and direct sequencing

Sequencing	IHC			N (697)
	-	Weak +	Strong +	
BRAF mutation	0	6	575	581
No mutation	70	7	39	116
Concordance rate	100% (70/70)	46.2% (6/13)	93.6% (575/614)	93.4% (651/697)

Data are expressed as total numbers or percentages.

Table 3. Comparison of clinicopathological status of samples divided into three groups based on concordance of $BRAF^{V600E}$ mutation detection by IHC and Sanger sequencing

	Group 1 (a), (n = 581)	Group 2 (b), (n = 46)	Group 3 (c), (n = 70)	p-value	Scheffe
Male	130 (22.4)	5 (10.9)	11 (15.7)	0.096	
Age	47.9 ± 13.6	49.3 ± 11.3	45.6 ± 16.2	0.270	
Gross ETE	51 (8.8)	1 (2.2)	7 (10.0)	0.269	
Tumor size (cm)	1.1 ± 0.8	0.8 ± 0.7	1.5 ± 1.4	< 0.001	c > a > b
LN metastasis	265 (45.6)	10 (21.7)	30 (42.9)	0.007	a > b
Tumor multifocality	374 (64.4)	32 (69.6)	53 (75.7)	0.144	
Thyroiditis	139 (23.9)	21 (45.7)	25 (35.7)	0.001	b > a
Tumor lymphatic invasion	215 (37.0)	8 (17.4)	33 (47.1)	0.005	a,c > b
Tumor angioinvasion	6 (1.0)	0 (0.0)	4 (5.7)	0.006	a,c > b

Data are expressed as *n* (%) or mean values. Group 1, patients with positive results in both IHC and direct sequencing; group 2, patients with positive results by IHC but negative results using direct sequencing; group 3, patients with negative results from both IHC and direct sequencing.

Scheffe, Scheffe's method; ETE, extrathyroidal extension, LN, lymph node.

Table 4. Predictive factors for discordance between IHC and direct sequencing

Covariates	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*
Male sex	0.546 (0.207-1.440)	0.221		
Age	0.331 (0.143-0.766)	0.010	1.009 (0.983-1.036)	0.514
Gross ETE	0.510 (0.065-4.016)	0.523		
Tumor size	0.261 (0.121-0.562)	0.001	0.249 (0.113-0.548)	0.001
LN metastasis	0.679 (0.308-1.496)	0.337		
Tumor multifocality	0.888 (0.442-1.785)	0.738		
Thyroiditis	2.586 (1.376-4.860)	0.003	2.825 (1.516-5.266)	0.001
Tumor lymphatic invasion	0.760 (0.313-1.846)	0.544		
Tumor angioinvasion	0.000 (0.000)	0.999		

IHC, immunohistochemistry; 95% CI, 95% confidence interval; ETE, extrathyroidal extension; LN, lymph node.

유두상 갑상선암에서 *BRAF*^{V600E} 돌연변이의 동정을 위한 면역 조직 화학 염색과 직접 염기 서열 분석 방법의 비교

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국문초록

배경: *BRAF*^{V600E} 돌연변이는 유두상 갑상선암에서 가장 흔한 체세포 돌연변이이며 공격적인 예후 인자와 관련이 있다. *BRAF*^{V600E} 돌연변이에 대한 통상적인 검출 방법은 생어 시퀀싱에 의한 중합 효소 연쇄 반응이다. 최근 *BRAF*^{V600E} 돌연변이 특이 항체를 이용한 면역 조직 화학법이 개발되어 임상적으로 널리 쓰이고 있다. 하지만 면역 조직 화학법과 생어 시퀀싱 방법의 비교에 대한 연구 결과가 거의 없다.

방법: 2013년 1월부터 2016년 10월까지 서울대 병원에서 갑상선 수술을 받은 환자들의 임상 정보를 후향적으로 분석하였다. 상기 기간 동안 총 3584명의 환자가 갑상선 수술을 받았으며, 이 중에 886명의 환자 검체에 면역 조직 화학법과 시퀀싱 방

법을 두 방법을 모두 이용하여 *BRAF*^{V600E} 돌연변이 유무를 분석하였다. 전이된 임파선 검체를 이용하였거나 다른 아형의 유두암이었던 검체를 제외하고 총 697개의 검체를 본 연구의 최종대상으로 등록하였다.

면역 조직 화학법은 *BRAF*^{V600E} 돌연변이 특수 항체를 구입하여 사용하였고, 파라핀 검체를 대상으로 하였다. 먼저 파라핀 검체를 슬라이드로 옮긴 뒤 오븐에 60℃로 30분간 두었다. Benchmark XT system과 EZ prep 용액을 이용하여 검체의 파라핀을 벗겨내었다. 전처치를 64분간한 뒤, *BRAF*^{V600E} 돌연변이 특수 항체를 슬라이드에 위치시키고 32분간 두었다. 같은 슬라이드에 양성 대조군을 배치하였다. 이렇게 만들어진 조직 단면을 현미경으로 관찰하였다.

697개의 검체 모두 시퀀싱을 시행하였다. 다발성 병변일 경우 가장 큰 종양 조직을 이용하였다. Maxwell 장비를 이용하여 DN를 추출하여 *BRAF*^{V600E} 돌연변이가 있는 15번 엑손을 증폭시켰다. DNA 증폭이 끝나면, 3730 DNA analyzer를 이용하여 양방향으로 시퀀싱을 시행하였다.

결과: *BRAF*^{V600E} 돌연변이는 면역 조직 화학법을 사용했을 때 90.0% (627/697)가 검출되었고, 직접 시퀀싱을 이용하였을 때는 83.4% (581/697)에서 검출되었다. 면역 조직 화학법의 민감도는 100% (581/581), 특이도는 60.3% (70/116), 양성 예측도는 92.7% (581/627), 음성 예측도는 100% (70/70)이었다. 면역 조직 화학법을 사용하여 위음성

결과가 기록되지 않았다. 두 방법간의 결과의 전체 일치율은 93.4% (651/697)였다. 불일치 결과는 46개 샘플 (6.6%)에서 발견되었으며, 그 중 29개는 종양이 작은 (6mm 미만) 경우였고, 8개는 종양 세포성이 낮은 경우였고, 9개는 낮은 품질의 DNA 를 산출하는 표본이었다.

두 검사 결과에 따라 환자를 세그룹으로 분류하였다. 그룹 1은 면역 조직 화학법 및 시퀀싱 둘 다에서 $BRAF^{V600E}$ 돌연변이가 관찰되었던 환자군 (n = 581), 그룹 2는 면역 조직 화학법에서는 $BRAF^{V600E}$ 돌연변이가 관찰되었으나 시퀀싱에서는 그렇지 않았던 환자군 (n = 46), 그룹 3은 면역 조직 화학법 및 시퀀싱 둘 다에서 $BRAF^{V600E}$ 돌연변이가 관찰되지 않았던 환자군 (n = 70), 총 3그룹으로 분류하였다. 세 그룹의 성비, 연령, 종야의 육안적 갑상선외 조직 침범 여부, 종양의 크기, 림프절 전이 여부, 다발성 종양 여부, 갑상선염 유무, 종양의 림프절 및 혈관 침범 여부 등에 대하여 분석하였다. 그 결과, 그룹 3의 종양 크기가 그룹 1보다 컸고, 그룹 1의 종양 크기가 그룹 2보다 컸다 ($p < 0.001$). 그룹 1이 그룹 2에 비하여 림프절 전이가 많이 관찰 되었고 ($p = 0.007$), 그룹 2에서 그룹 1보다 갑상선염이 더 빈번히 나타났다 ($p = 0.001$). 종양의 림프절, 혈관 침범 여부는 그룹 1과 그룹 3에서 그룹 2에 비해 더 높은 발생률을 보였다 ($p = 0.005$, $p = 0.006$).

두 검사 결과의 불일치 정도를 종속 변수로 하여 단변량, 다변량 로지스틱 회귀분석을 시행하였더니, 종양의 크기와 갑상선염이 독립 변수로 나타났다. 이들 변수중,

종양의 크기가 가장 연관성이 큰 인자로, 0.249의 상대 위험도가 다변량 분석에서 나타났다 (95% 신뢰 구간, 0.113-0.548). 갑상선염은 다변량 분석에서 2.825의 상대 위험도를 보였다 (95% 신뢰 구간, 1.516-5.266).

결론: VE1 항체를 사용하는 면역 조직 화학법은 고전적인 유두상암에서 *BRAF*^{V600E} 돌연변이를 검출하는 신뢰할 수 있고 매우 민감도가 높은 방법이다. 면역 조직 화학법과 시퀀싱 결과의 불일치를 분석하였을 때 종영의 크기가 작고, 갑상선염이 있는 경우 두 검사 결과가 일치하지 않는 경향을 보였다. 본 연구 결과에서 두 검사 결과가 일치하지 않는 경우에 시퀀싱에서 위음성이 나왔을 가능성이 높으므로, 면역 조직 화학법은 초기 갑상선암 또는 갑상선염이 있는 환자에서 고려해볼 수 있겠다.

주요어: *BRAF*^{V600E} 돌연변이, 유두상 갑상선암, 면역 조직 화학법, 민감도, 특이도

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