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Detection of Plasma BRAF^{V600E} Mutation Is Associated with Lung Metastasis in Papillary Thyroid Carcinomas

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Purpose: The BRAF^{V600E} mutation represents a novel indicator of the progression and aggressiveness of papillary thyroid carcinoma (PTC). The purpose of this study was to determine the clinical significance of free circulating mutant BRAF^{V600E} in predicting the advanced disease of PTC. **Materials and Methods:** Seventy seven matched tumor and plasma samples obtained from patients with both benign and PTC were analyzed for BRAF^{V600E} mutation using a peptide nucleic acid (PNA) clamp real-time polymerase chain reaction (PCR). **Results:** The BRAF^{V600E} mutation was absent in tumor DNA samples obtained from patients with benign follicular adenomas or adenomatous goiter. In contrast, 49 of 72 (68.1%) PTC tumors were positive for the BRAF^{V600E} mutation. Among them, 3 (6.1%) patients with PTC were positive for BRAF^{V600E} mutation in plasma and tumor. However, all 3 patients (100%) had lateral lymph node and lung metastasis. **Conclusion:** These findings suggest that the BRAF^{V600E} mutation can be detected using a PNA clamp real-time PCR in the blood of PTC patients with lung metastasis. Future studies are warranted to determine clinical significance of serum BRAF^{V600E} mutation in large prospective studies.

Key Words: BRAF, papillary thyroid carcinoma, plasma, DNA, biomarker

INTRODUCTION

Papillary thyroid carcinoma (PTC) is the most common malignant thyroid tumor and comprises about 90% of thyroid malignancies. The overall 10-year survival rate for middle-aged adults with thyroid carcinomas is about 80% to 95% worldwide.[1] However, cervical lymph node metastases are common even when the primary tumor is small and have been reported in up to 90% of patients with PTC.[2,3] Loco-regional recurrences occur in 5% to 20% of patients with PTC. The most common site of recurrence is cervical lymph nodes, which comprise the majority of all recurrences. In many patients, however, lymph node metastases in the central compartment do not appear abnormal preoperatively with imaging such as computed tomography and ultrasonography or by inspection at the time of sur-

gery.[4] In addition, the overall incidence of distant metastases is approximately 10% in patients with PTC.[5,6] Therefore, it is important to identify patients who are at risk of developing distant metastasis or recurrence that necessitate more aggressive surgical treatment or more intensive radioactive iodine therapy.

A thymidine-to-adenosine transversion at exon 15 nucleotide 1799 (T1799A) of the BRAF gene, resulting in the replacement of valine with glutamic acid at position 600 ($BRAF^{V600E}$) occurs exclusively in PTC and PTC-derived anaplastic thyroid cancers. The $BRAF^{V600E}$ mutation has been correlated even with PTC recurrence in patients with conventionally low-risk clinicopathological factors. Thus, $BRAF^{V600E}$ mutation represents a novel indicator of the progression and aggressiveness of PTC.[7] Recently, $BRAF^{V600E}$ was detected by allele-specific real-time polymerase chain reaction (PCR) in the blood of PTC patients with residual or metastatic disease and also detectable using a gap-ligase chain reaction technique in the plasma samples from patients with PTC.[8,9] Therefore, detection of circulating $BRAF^{V600E}$ might allow diagnosis of some PTC patients with a blood test, help to identify patients with unrecognized postoperative minimal residual disease, and prove useful in selection of patients who are at risk of developing distant metastasis or recurrence that necessitate more aggressive treatment.

In Korea, the prevalence of the $BRAF^{V600E}$ mutation in PTC is much higher (73–90%) than that in Western countries.[10-12] The peptide nucleic acid (PNA)-mediated PCR clamping method is highly sensitive and is efficiently applicable to the detection of BRAF mutations in a clinical setting.[12,13] Therefore, we have hypothesized that $BRAF^{V600E}$ mutation is more common in peripheral blood in Korean patients with advanced and aggressive PTC and detectable using a PNA clamp real-time PCR. We also evaluated the association of the presence of $BRAF^{V600E}$ mutation with clinicopathologic risk factors for papillary thyroid cancers.

MATERIALS AND METHODS

Ethics statements

The protocol for the collection of tumor and plasma samples was approved by the Institutional Review Board of Pusan National University Hospital, Busan, Korea (Number: 2011161). Peripheral bloods were obtained preoperatively after written informed consent was obtained. The biospecimens for this study were provided by the Pusan National University Hospital, a member of the National Biobank of Korea, which is supported by the Ministry of Health, Welfare and Family Affairs. All of the data were securely protected, made available only to investigators and analyzed anonymously.

Patient population

Seventy-seven matched tumor and plasma DNA samples from patients with thyroid neoplasms (72 PTCs, 1 follicular adenoma, and 4 adenomatous goiters) were obtained from the tumor bank at the Division of Head and Neck Cancer Research, Department of Otolaryngology Head and Neck Surgery, Pusan National University Hospital. Surgical therapy had been total thyroidectomy and central node or lateral node dissection in patients with PTC. Clinicopathologic features were analyzed retrospectively.

DNA extraction

For tissue DNA isolation, a total of fresh thyroid samples including tumor tissues and benign thyroid mass were included in the study, 5 benign and 72 malignant. Briefly, a portion of each lesion was removed on resection, immediately snap frozen in liquid nitrogen and stored at -80°C. DNA was extracted from frozen fresh tissue with QIAamp DNA Mini kits (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

For plasma DNA isolation, two 5 mL-aliquots of peripheral blood were collected in EDTA tubes, transported within one hour to the laboratory and centrifuged twice at 4°C for 10 min (1600 rcf and 14000 rcf; or at 415 g or 1660 g). Plasma was aliquoted into 1.5 mL tubes after centrifugation, and stored at -80°C until genetic analysis. DNA was extracted from 500 μL of plasma, using the MinElute Virus Vacuum Kit (Qiagen) and RNAse digestion to prevent RNA interference during assay reaction. Plasma DNA was extracted with the QIAamp DNA Blood Midi Kit (Qiagen) according to the manufacturer's instruction. The extracted DNA yields were similar in the respective tumor and plasma specimens.

PNA-mediated clamping polymerase chain reaction for detection of BRAF

The assay for the detection of BRAF was carried out with the PNA Clamp™ BRAF Mutation Detection kit (Panagene, Daejeon, Korea) according to the manufacturer's instructions. Briefly, PCR amplification was performed in a total volume of 20 μL that contained 50 ng of DNA, 13 μL of real-time SYBR Green PCR master mix and each of the

primers and PNA probes for codon 600. The PCR control lacked a PNA probe and contained the wild type template. The PCR cycling conditions were at 94°C for 5 minutes followed by 40 cycles of four temperature steps (94°C for 30 seconds, 70°C for 20 seconds, 63°C for 30 seconds, and 72°C for 30 seconds), and a final extension of 72°C for 5 minutes. The PNA probe was designed to hybridize completely to the wild type B-raf allele. PNA probe hybridization securely inhibits the amplification of the wild-type B-raf allele, while the PNA/mutant-type allele hybrid is unstable due to base pair mismatch, and therefore, it does not inhibit *Taq* polymerase from extension. The threshold cycle (Ct) was automatically calculated from the PCR amplification plots in which fluorescence was plotted against the number of cycles. Delta-Ct values (ΔCt) were calculated as the Ct value of the PCR with the PNA control minus the Ct value of the PCR of the samples. The higher ΔCt means that the mutant was efficiently amplified. The cutoff value of 2.0 was used for determining the presence of mutant DNA.

DNA sequencing

PCR were performed in a final volume of 50 uL containing 25 μL of 2x PCR premix, 3 μL of extracted DNA, 2 μL of BRAF_230F, 5'-AAACTCTTCATAATGCTTGCTCTG-3', BRAF_230R, 5'-GGCCAAAAATTTAATCAGTGGA-3'. PCR cycling commenced with a 5-minute incubation at 94°C, followed by 40 cycles of 94°C for 30 seconds, 63°C for 30 seconds, 72°C for 60 seconds, and then a final incubation at 72°C for 5 minutes. The amplified products were purified with a MinElute PCR Purification Kit (Qiagen) and were sequenced in duplicate in the forward and reverse directions with a BigDye terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3730XL sequencer (Applied Biosystems).

Statistical analysis

The statistical analysis was performed using the SPSS (ver 15.0 for Windows, SPSS Inc., Chicago, IL, USA) software package. Numeric data were expressed as mean±SD. Categorical data were presented as frequency and percentage. Student t-test for testing differences of nominal variables between BRAF positive group and BRAF negative group was performed. Pearson's chi-square test was used to compare innominal variables of clinicopathologic characteristics between two groups. Statistical significance was defined as a $p<0.05$.

RESULTS

In this study, 72 matched tumor and plasma DNA samples were obtained. Table 1 summarized characteristics of the study population and tumor status. There were 12 men and 62 women with an average age of 54.7±14.5 years (range, 34–77 years) in PTC group. There were 5 women with an average age of 50.3±14.0 years (range, 30–60 years) in benign thyroid tumor group. Among 72 PTCs which were evaluated for the BRAF mutation status, 49 PTC tumor samples (68.0%) were determined to harbor the BRAF mutation. However, three of these cases who were positive for BRAF mutation in primary tumors had also detectable BRAF mutation in plasma (Fig. 1). Mutational results of PNA clamp real time PCR was confirmed by direct sequencing (Fig. 2). In our study population, therefore, only 6.1% of patients (3/49) diagnosed with PTC contained the BRAF mutation, and had also circulating DNA positive for the BRAF mutation. Clinical data for four patients with lung metastasis and status of BRAF mutation are presented in Table 2, and clinicopathologic data for patients according to BRAF mutation status in tumor tissue DNA are presented in Table 3. The patients with

Table 1. Characteristics of Study Population and Tumor Status

Pathology	No. of cases	Age (yrs)	Female (%)	No. of tumor with BRAF mutation (%)	No. of paired tumor and plasma with BRAF mutation (%)
PTC (total)	72	54.7±14.5	60 (83.3)	49 (68.1)	3 (6.1)
Stage I	22	44.0±15.8	18 (81.8)	12 (54.5)	0
Stage II	1	75	1 (100)	1 (100)	0
Stage III	18	59.1±8.4	16 (88.9)	12 (66.7)	0
Stage IVA	27	58.9±11.3	22 (81.5)	20 (74.1)	0
Stage IVC	4	66.0±14.1	3 (75.0)	4 (100)	3 (75.0)
Benign	5	50.3±14.0	5 (100)	0	0

No., number; PTC, papillary thyroid carcinoma; Stage, TNM stage.

positive BRAF mutation had bigger tumor size and more advanced TNM stage was compared with the patients with negative BRAF mutation. However, there was no significant difference in extrathyroidal extension, lymph node (LN) metastasis, and multifocality between the two groups.

DISCUSSION

In this study, the $BRAF^{V600E}$ mutation was detected using the peptide nucleic acid-mediated PCR clamping method in the tissue DNA obtained from 68.1% (49/72) and plasma DNA obtained from 6.1% (3/49) of patients diagnosed with PTC. In contrast, patients with benign thyroid neoplasms had no detectable BRAF mutation in the primary tissue or plasma DNA samples. Plasma $BRAF^{V600E}$ mutation was detected in three patients with lung metastasis.

PTC is associated with an excellent prognosis compared with other cancers. In addition, PTC typically has a prolonged disease course and is asymptomatic for long periods. However, regional lymph node metastases are frequently detected at diagnosis ranging from 20 to 90%. Cervical lymph node metastases have a poor prognostic factor on survival in patients with follicular thyroid carcinoma (FTC) and in patients with PTC over 45 years.[14] Also, about 10% of patients with papillary carcinoma and up to 25% of those with FTC develop distant metastasis or tumor invades the neck aggressively, half of which are present at the time of diagnosis.[15] Therefore, it is crucial to identify patients who are at risk of developing distant metastasis or loco-regional recurrence that necessitate more aggressive therapy. In view of this, more convenient and reliable biomarkers are needed to identify high risk patients.

Association of $BRAF^{V600E}$ mutation with aggressive clinicopathologic characteristics and high tumor recurrence has been demonstrated, although the results are controversial.[16-21] $BRAF^{V600E}$ mutation represents a novel indicator of the progression and aggressiveness of PTC.[7,22] Therefore, detection of circulating $BRAF^{V600E}$ mutation in the blood could determine if patients are disease-free or if they still harbor residual disease. Also, $BRAF^{V600E}$ mutation status in the blood may be useful biomarkers for selection of patients with residual and recurrent disease for radioactive iodine remnant ablation, radioactive iodine therapy, or other adjuvant treatments, especially in a situation in which a patient had a positive anti-thyroglobulin (Tg) antibody.

Recently, $BRAF^{V600E}$ mutation was detected in the blood of PTC patients with residual or metastatic disease by allele-specific real-time PCR[8] and gap-ligase chain reaction technique.[9] Cradic, et al.[8] reported that circulating $BRAF^{V600E}$ mutation was detected in 20 of 173 PTC patients (11.6%), and that $BRAF^{V600E}$ mutation positivity in blood correlated

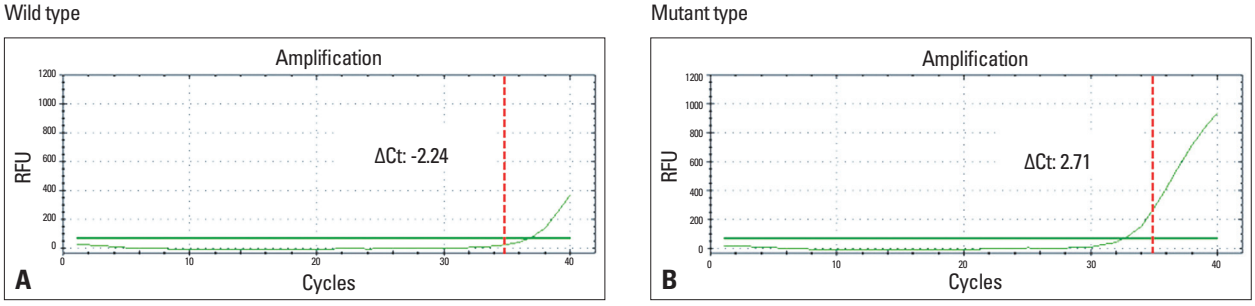

Fig. 1. PNA Clamp™ standard curves in tumor DNA samples and plasma DNA sample. The detection signal was obtained by intercalation of SYBR green fluorescent dye of real-time PCR. A PNA/DNA hybrid with a single base-pair mismatch did not suppress annealing of the PCR primer (A) and amplification of mutant alleles (B). PNA, peptide nucleic acid; PCR, polemerase chain reaction; RFU, relative fluorescence units.

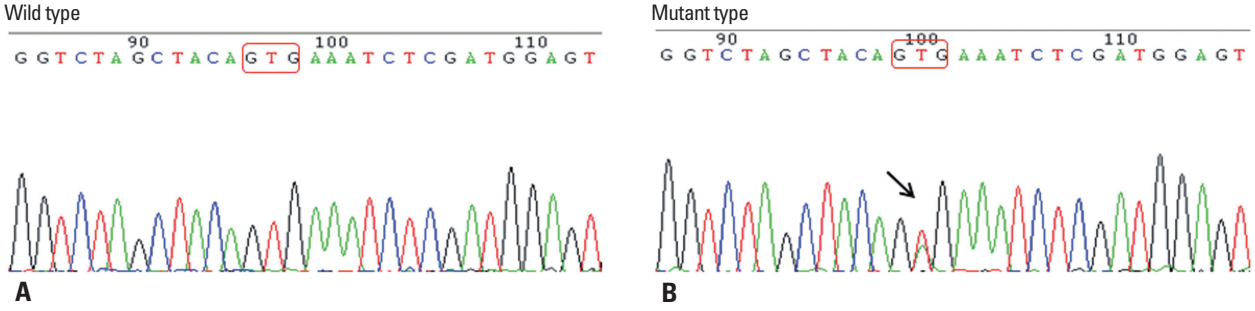

Fig. 2. Using the direct sequencing of DNA, representative sequence chromatographs from BRAF axon 15 showing wild type (A) and mutation (B) in papillary thyroid carcinomas. Arrows indicate mutations.

Table 2. Clinical Characteristics of Patients with Distant Metastasis and BRAF Mutation Status in Plasma DNA

Age	Sex	BRAF mutation status in plasma	Tumor size and description	LN metastasis	Distant metastasis	Detection method of distant metastasis	Tg post RAI Tx (ng/mL)	Anti-Tg antibody (U/mL)	RAI lung uptake after RAI Tx	Stimulated Tg 1 year after RAI Tx (ng/mL)
77	Female	V600E	3.2 cm, multifocal with ETE	Central and lateral LN	Lung, micronodular	FU chest CT	476.5	38.4	Positive	320.5
53	Female	V600E	3.0 cm, multifocal with ETE	Central and lateral LN	Lung, macronodular	Preoperative PET-CT	68.1	12.5	Positive	182.2
67	Female	V600E	2.1 cm, with ETE	Central and lateral LN	Lung, macronodular	Preoperative chest CT	216.3	19.2	Positive	120.9
55	Male	Wild type	2.5 cm, multifocal with ETE	Central and lateral LN	Lung, micronodular	Diagnostic ^{131}I WBS	34.6	16.9	Positive	148.2

CT, computed tomography; ETE, extrathyroidal extension; FU, follow-up; LN, lymph node; PET, positron emission tomography; RAI, radioactive iodine; Tg, thyroglobulin; Tx, treatment; WBS, whole body scan.
Reference range of anti-Tg antibody: 0–60 U/mL

with the presence of active disease at the time of the blood draw. Chuang, et al.[9] also demonstrated that 3 of 5 patients (60%) with PTC were positive for BRAF^{V600E} mutation in serum and tumor. The results in the present study were consistent with previous studies showing that this plasma BRAF^{V600E} mutation was found only in PTC.[8,9]

It is well known that high iodine intake is a risk factor for BRAF^{V600E} mutation.[23] In Korea, dietary iodine intake seems to be higher than in other countries.[24] The prevalence of the BRAF^{V600E} mutation in PTC is much higher (73–90%) than that in Western countries.[10-12] The prevalence of BRAF^{V600E} mutation in tumor tissue was 49 of 72 PTC patients (68.1%), which was consistent with previous Korean data.[10-12] PNA clamp technology is the PNA-based PCR clamping that selectively amplifies only the mutated target DNA sequence in the presence of wild type DNAs. Also, PNA-based clamp method has no nonspecific amplications of DNAs. Thus, it is very useful to detect low-level mutant DNAs.[12,13] Recently, PNA-mediated PCR clamping method was found to be highly sensitive and efficiently applicable to the detection of BRAF in patients with PTC.[12,25] Jeong, et al.[12] reported that the PNA clamp real-time PCR method for the BRAF^{V600E} mutation detection was sensitive in comparison to sequencing. They found that the delta Ct value of the 0.5% mutant was larger than the cutoff value of 2.0 and the kit sensitively detected 0.5% mutation. For the sequencing, an automatic reading detected 20% of the mutant in the background of the wild type. In addition, Kang, et al.[25] recently reported that pyrosequencing using Food and Drug Administration (FDA) approved method and PNA clamping PCR detected mutant type in a 99:1 (wild-type:mutant) DNA concentration, and PNA-clamping PCR detected mutant type in a 99.5:0.5 DNA concentration for the detection of BRAF^{V600E} mutation with thyroid tissue. PNA clamping PCR showed higher κ value than allele specific real-time PCR. This study suggested that PNA clamping PCR was a sensitive and reliable method to detect the BRAF^{V600E} mutation.

Thus, we have expected higher positivity of plasma BRAF^{V600E} mutation in patient with advanced papillary thyroid carcinoma. However, the current study showed only 3 of 49 patients (6.1%) with plasma positive for the BRAF^{V600E} mutation. In the present study, although the number of patients is small plasma BRAF^{V600E} mutation positivity was associated with lung metastasis in patients with PTC. In addition, among 31 patients with lateral LNM (N1b), plasma BRAF mutation was detected in 3 patients with lung metastasis. Thus, plasma BRAF mutation might be useful for predic-

Table 3. Comparison between Disease Status and BRAF Mutation in Tumor Tissue

Variables	BRAF mutation positive (n=49)	BRAF mutation negative (n=23)	*p* value
Age (yrs)*	57.2±11.9	51.3±15.6	0.103
Size (cm)*	1.85±0.82	1.35±1.1	0.043
Extrathyroidal extension (%)†	30 (61.2)	13 (56.5)	0.269
LN metastasis (%)†	36 (73.4)	14 (60.9)	0.192
Lateral LN metastasis (%)†	24 (39.7)	6 (26.1)	0.110
Advanced Stage (%)†	32 (65.3)	9 (39.1)	0.022
Multifocality (%)†	24 (48.9)	8 (34.8)	0.279

n, number; SD, standard deviation; LN, lymph node.
Advanced stage: stage III+stage IV. Data are expressed as mean±SD for continuous variables and frequency (%) for categorical variables.
*Student t-test.
†Chi-square test.

tion of lung metastasis in patients with lateral LNM.

$BRAF^{V600E}$ positivity in blood seems to be correlated with active disease at the time of the blood draw. Among 4 patients with stage IVC, the initial pathologic stage of a patient with negative plasma $BRAF^{V600E}$ mutation was pT3N-1bMx, stage IVA. However, this patient (a 55-year-old man) underwent diagnostic ^{131}I whole body scan (WBS) and stimulated Tg 1 year after iodine ablation therapy. ^{131}I WBS showed minimal diffuse uptake in both lung fields, and chest CT showed micronodular lung metastasis in both lung fields. Thus, we regarded this patient as final TNM stage IVC. Thyroid tumor tissue and plasma samples of this patient were collected at initial surgery. However, circulating DNA was not extracted on the day of the draw. Although the reason for this has not clear, the lower prevalence of serum $BRAF^{V600E}$ mutation in this study might be explained by different analytical method in the detection of $BRAF^{V600E}$ mutation, the time interval between extraction of circulating DNA and blood draw, minimal disease activity at the time of the blood sampling, and small sample size. Thus, these results in our study should be further elucidated in future large prospective studies.

$BRAF^{V600E}$ mutation in PTC has been shown to correlate with aggressive clinicopathologic characteristics and poor outcomes.[16-20] In the current study, the patients with positive BRAF mutation had bigger tumor size and more advanced TNM stage than the patients with negative BRAF mutation. However, previous reports indicated no significant difference in extrathyroidal extension, LN metastasis, and multifocality between the two groups.[26-28] We think that the lack of association between the $BRAF^{V600E}$ mutation and some poor clinicopathologic factors might have resulted from the small number of cases and/or large number of micro-PTCs (28 of 72, 38.9%), and different prevalence of $BRAF^{V600E}$ mutation. Therefore, further studies with a larger patient population may be necessary to determine clinical utilities such as diagnosis and prognosis in patients with PTC.

In conclusion, this study demonstrated that circulating DNA harboring the $BRAF^{V600E}$ mutation was detected in patients with PTC by PNA-mediated PCR clamping method. Although the number of patient is small, the current study showed that detection of plasma $BRAF^{V600E}$ mutation was associated with lung metastasis. These results suggest the possibility of circulating BRAF mutation as a biomarker in the detection of advanced thyroid cancer. Further investigation for the clinical significance of detecting the $BRAF^{V600E}$ mutation in the blood of patients with PTC is warranted.

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