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Clinicopathological Features of Rare *BRAF* Mutations in Korean Thyroid Cancer Patients

Uiju Cho,[1] Woo Jin Oh,[1] Ja Seong Bae,[2] Sohee Lee,[2] Young Sub Lee,[1] Gyeong Sin Park,[1] Youn Soo Lee,[1] and Chan Kwon Jung[1]

Departments of [1]Hospital Pathology and [2]Surgery, College of Medicine, The Catholic University of Korea, Seoul, Korea

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Address for Correspondence:
Chan Kwon Jung, MD
Department of Hospital Pathology, The Catholic University of Korea, Seoul St. Mary's Hospital, 222 Banpo-daero, Seocho-gu, Seoul 137-701, Korea
Tel: +82.2-2258-1622, Fax: +82.2-2258-1627
E-mail: ckjung@catholic.ac.kr

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The most common *BRAF* mutation in thyroid cancer is c.1799T > A (p.Val600Glu), and other *BRAF* mutations are rarely reported. We investigated the clinicopathological features of thyroid cancer with rare *BRAF* mutations. A total of 2,763 patients with thyroid cancer underwent molecular testing by direct DNA sequencing for mutations in *BRAF* exon 15. Among them, 2,110 (76.4%) had *BRAF* mutations. The c.1799T > A mutation was found in 2,093 (76.9%) of 2,722 papillary carcinomas and in one of 7 medullary carcinomas. Sixteen cases (0.76%) harbored rare mutation types. Five cases had single-nucleotide substitutions, 5 cases had small in-frame deletion or insertion, and one harbored a two-nucleotide substitution. Of these mutations, 2 were novel (c.1797_1798insGAGACTACA, c.[1799T > A; 1801_1812del]). The c.1801A > C mutation was identified in 4 follicular variant papillary carcinomas and one follicular carcinoma. None of the patients with the c.1801A > C mutation showed extrathyroidal extension or lymph node metastasis. The prevalence of rare *BRAF* mutations was 0.76% of all *BRAF*-positive thyroid cancers, and the rare mutations were associated with less aggressive pathologic features. Although *BRAF* mutations are detected exclusively in papillary carcinoma, they are also found in medullary carcinoma and follicular carcinoma.

Keywords: Thyroid Neoplasms; *BRAF*; Mutation; Pathology; Biomarkers

INTRODUCTION

Mutations in the *BRAF* gene are the most common genetic alteration in papillary thyroid carcinomas (PTC) and are present in 30%-87% of these carcinomas in different populations (1). The spectrum of mutations in this gene includes point mutations, small "in-frame" deletions or insertions, and chromosomal rearrangements. Among those, the most common mutation is a point mutation that involves a thymine to adenine substitution at position c.1799 of the *BRAF* gene, which results in a valine-to-glutamate substitution at amino acid codon 600 (p.Val600Glu; V600E). *BRAF* V600E represents almost 99% of all *BRAF* mutations in thyroid cancer (2). We previously reported that *BRAF* mutations were found in 835 (80.2%) of 1,041 Korean patients with PTC (2). Most PTCs with the *BRAF* V600E mutation showed papillary growth pattern, either classic or tall cell variants (3, 4). The second most common mutation was a single nucleotide substitution of adenine to guanine at position 1801 (c.1801A > C), which leads to lysine to glutamate substitution at residue 601 (p.Lys601Glu; K601E). *BRAF* K601E has been reported in about 1% of PTCs, especially the follicular variant of PTC (FVPTC), and in 2 cases of follicular adenoma (3, 5). Other *BRAF* point mutants are very rare in thyroid cancers.

We investigated the type and prevalence of rare *BRAF* mutations and their clinicopathologic characteristics in a large number of thyroid cancer cases. Furthermore, we report novel complex mutations of the *BRAF* gene that were identified in PTC.

MATERIALS AND METHODS

Patients

A total of 2,763 consecutive patients with thyroid cancers who had surgery at Seoul St. Mary's Hospital between October 2008 and June 2013 were retrospectively reviewed including the 1,041 PTC patients used in our previous study (2). Thyroid cancer slides were reviewed by an endocrine pathologist and classified according to the World Health Organization classification.

Genomic DNA was extracted from two 10-μm sections of formalin-fixed, paraffin-embedded archival tissue blocks. The representative tumor areas were marked and manually microdissected under a stereomicroscope. The largest tumors were chosen for the study from cases with multifocal lesions. After deparaffinization, genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

A 224-bp fragment of exon 15 of the *BRAF* gene was amplified using polymerase chain reaction (PCR) with the following primers: forward (5´-TCATAATGCTTGCTCTGATAGGA-3´) and

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reverse (5´-GGCCAAAAATTTAATCAGTGGA-3´). Thermo cycling was performed as follows: 35 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. Amplicons were evaluated by 2% agarose gel electrophoresis and purified by QIAquick PCR purification kit (Qiagen).

Mutational analysis of *BRAF* genes

We performed direct Sanger sequencing using the previously reported primers (2). For the tumors with rare mutations, the following additional methods were used to confirm the authenticity: 1) re-amplification of the exon and bidirectional direct sequencing on a different day, 2) repeat DNA sequencing with the re-isolated genomic DNA from the same tissue block and different tissue block (if available), 3) bidirectional sequencing using 2 new primer sets specific to the 209 bp and 191-bp fragments of *BRAF* exon 15. In order to identify the nucleotide composition of novel mutations, PCR amplicons were cloned using the TOPO®TA Cloning Kit (Invitrogen, Carlsbad, CA, USA), as we described previously (2).

Nomenclature of the novel mutations

The descriptions of the mutations are assigned according to "Guidelines for mutation nomenclature" from Human Genome Variation Society (6). For *BRAF* gene analysis, NBCI reference sequences-NG_007873.1 and NM_004333.4 were used (www.ncbi.nlm.nih.gov/nuccore).

Ethics statement

The study protocol was approved by the institutional review board of Seoul St. Mary's Hospital, The Catholic University of Korea (KC14RISI0016). Informed consent was exempted by the board.

RESULTS

The cohort consisted of 2,722 (98.5%) PTCs, 33 (1.2%) follicular carcinomas, 7 medullary carcinomas, and one undifferentiated carcinoma. Of a total of 2,763 patients with thyroid cancers, 2,110 (76.4%) had *BRAF* mutations, which were found in 2,108 (77.4%) of 2,722 PTCs, one of 33 follicular carcinoma, and one of 7 medullary carcinomas. Nearly all *BRAF* mutations were the c.1799T > A (V600E) mutation except for 16 cases (0.76%). Other types of rare mutations were as follows: 1) 5 cases with single nucleotide substitution, c.1801A > C (K601E); 2) 3 cases with silent mutation, c.[1797A > G; 1799T > A]; 3) 2 cases with in-frame insertions, c.1797_1798insGAGACTACA and c.1794_1795insGTT; 4) 2 cases with in-frame deletions, c.[1799T > A; 1801_1812del] and c.1799_1801del; and 5) 4 cases with rare mutation types that we previously reported (c.[1770_1795dup26; 1795_1796insA], c.[1742-10T > C; 1799T > A], c.[1796C > G; 1799 T > A], and c.1799_1800TG > AA (2).

The clinicopathologic features of the 16 patients with rare types of mutation are summarized in Table 1. The c.1801A > C (K601E) mutation was found in one case of minimally invasive follicular carcinoma and four cases of encapsulated follicular variant of PTC. All PTCs with *BRAF* K601E mutations were encapsulated follicular variant and did not show extrathyroidal extension or lymph node metastasis. The patients were all younger than 45 yr.

We previously reported 3 novel complex mutations (cases 13,

Table 1. Clinicopathologic features of thyroid carcinomas with rare *BRAF* mutations

Case No.	Nucleotide change*	Amino acid change*	Age (yr)	Sex	Tumor size (cm)	Diagnosis	Histologic variant	Extrathyroid extension	Multifocality	LN meta
1	c.1801A > C	p.Lys601Glu	38	Female	1.2	PTC	EFV	Absent	Absent	NA
2	c.1801A > C	p.Lys601Glu	43	Female	1.3	PTC	EFV	Absent	Absent	NA
3	c.1801A > C	p.Lys601Glu	43	Male	4.5	PTC	EFV	Absent	Absent	Absent
4	c.1801A > C	p.Lys601Glu	42	Female	4.5	PTC	EFV	Absent	Present	Absent
5	c.1801A > C	p.Lys601Glu	70	Male	8	FTC	Minimally invasive	Absent	Absent	NA
6	c.[1797A > G; 1799T > A]	p.[(= ; Val600Glu)]	46	Female	0.7	PTC	Classic	Present	Present	Present
7	c.[1797A > G; 1799T > A]	p.[(= ; Val600Glu)]	60	Male	0.7	PTC	Classic	Present	Absent	Present
8	c.[1797A > G; 1799T > A]	p.[(= ; Val600Glu)]	55	Female	1	PTC	Classic	Present	Absent	Absent
9	c.1797_1798insGAGACTACA†	p.Thr599_Val600insGluThrThr†	47	Female	2	PTC	EFV	Present	Present	Present
10	c.1794_1795insGTT	p.Ala598_Thr599insVal	54	Female	1.2	PTC	EFV	Present	Absent	Present
11	c.[1799T > A; 1801_1812del]†	p.[(Val600Glu; Lys601_Trp604del)]†	50	Female	0.9	PTC	Classic	Present	Present	Absent
12	c.1799_1801del	p.Val600_Lys601delinsGlu	54	Male	3.3	PTC	EFV	Absent	Absent	Absent
13	c.[1770_1795dup26; 1795_1796insA]	p.[Lys591_Ala598dup; Ala598_Thr599insLys]	46	Female	0.8	PTC	Classic	Absent	Absent	Absent
14	c.[1742-10T > C; 1799T > A]	p.Val600Glu	38	Female	1.5	PTC	Classic	Absent	Absent	Absent
15	c.[1796C > G; 1799 T > A]	p.[(Thr599Arg; Val600Glu)]	44	Female	0.8	PTC	Classic	Absent	Present	Absent
16	c.1799_1800TG > AA	p.Val600Glu	56	Female	0.3	PTC	Classic	Absent	Absent	Present

*Nomenclatures are assigned according to the system of the Human Genome Variation Society (www.hgvs.org/mutnomen); †These are novel complex mutations that have not been reported in thyroid cancer. PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; EFV, encapsulated follicular variant; LN meta, lymph node metastasis.

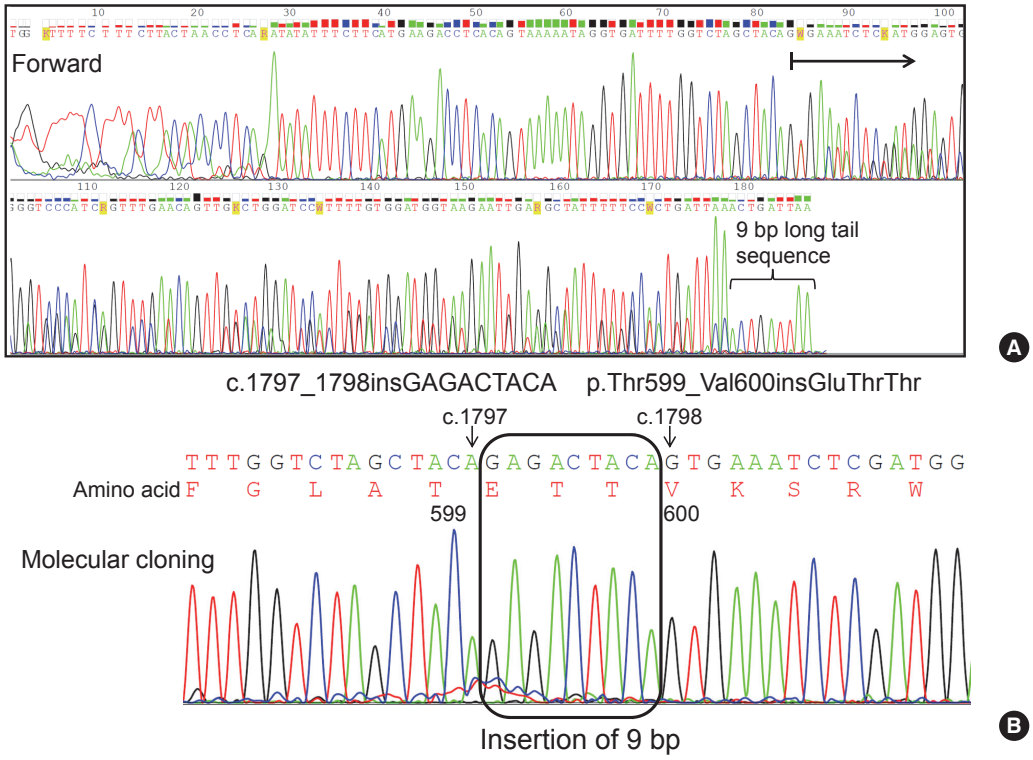

Fig. 1. Electropherograms of case 9 harboring a mutation of c.1797_1798insGAGACTACA. (**A**) Direct sequencing of *BRAF* exon 15 PCR product shows 9-bp tail sequence in its electropherograms. (**B**) Subcloning demonstrates newly inserted nucleotides (GAGACTACA) in between nucleotides positions c.1797 and c.1798.

14 and 15) and additionally found 2 novel *BRAF* mutations, c.1797_1798insGAGACTACA and c.[1799T > A; 1801_1812del] (case 9 and case 11, respectively). Sequence analysis of case 9 showed 9-nucleotide GAGACTACA insertion between positions c.1797 and c.1798 (c.1797_1798insGAGACTACA). This mutation leads to the insertion of 3 amino acids, glutamate-threonine-threonine, between codons 599 and 600 (p.Thr599_Val600insGluThrThr) (Fig. 1). The mutation in case 11 consisted of the usual T to A substitution at position c.1799 (c.1799T > A), followed by deletion of 12 nucleotides from c.1801 to c.1812. These mutations lead to a substitution from valine to glutamate at codon 600 and in-frame deletion of 4 amino acids from codons 601 to 604 (p.[(Val600Glu; Lys601_Trp604del)]) (Fig. 2). Cloning and subsequent sequence analysis of the PCR amplicon demonstrated that both mutations were located within the same allele.

Interestingly, a 61-yr-old female with medullary carcinomas at bilateral lobes showed *BRAF* c.1799T > A mutation (Fig. 3). The tumors were confirmed as pure medullary carcinomas based on the immunohistochemical findings of diffuse positivity for calcitonin, carcinoembryonic antigen and chromogranin (Fig. 3).

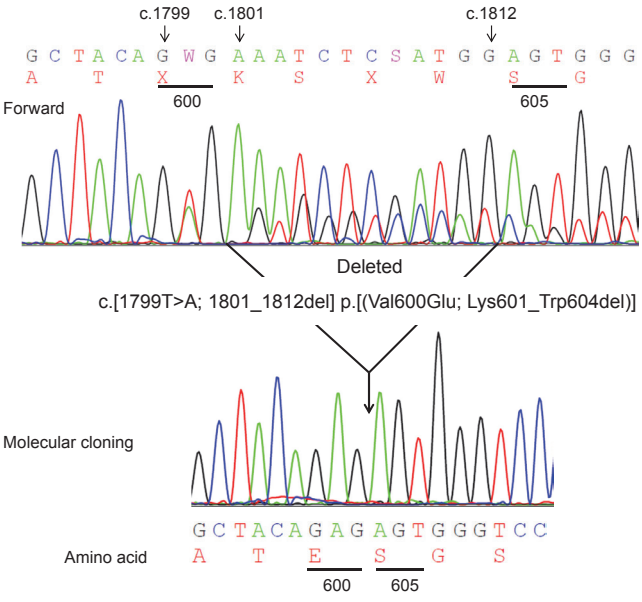

Fig. 2. Electropherograms of case 11, harboring a mutation of c.[1799T>A; 1801_1812del]. Direct sequencing shows a complex mutation of c.1799T>A and c.1801_1812del overlapped with the wild-type allele peaks (above). Deletion of 12 nucleotides (*AAATCTCGATGG) and, a substitution at nucleotide position c.1799 are located on the same allele. Subcloning reveals substitution and deletion mutations of the mutant clone, confirming that both mutations are located on the same allele (below).

DISCUSSION

Our study summarizes data on 16 patients with rare mutations in *BRAF*, detected by direct sequencing of *BRAF* exon 15, in 2,763 thyroid cancer samples. Thus, 0.76% of *BRAF*-mutated tumors exhibited a rare mutation type.

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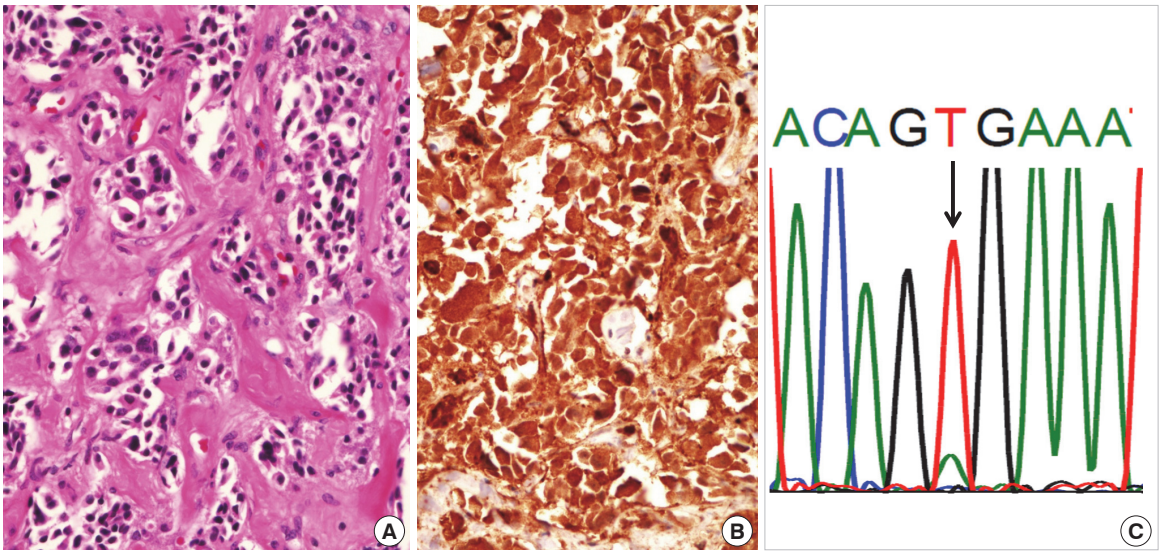

Fig. 3. Histological and immunohistochemical aspects of the medullary thyroid carcinoma case (**A**, **B**) and electropherogram of its *BRAF* mutation (**C**). (**A**) The tumor shows a characteristic appearance with the presence of round to polygonal tumor cells with fibrosis and amyloid deposition (Hematoxylin and eosin stain, × 200). (**B**) Calcitonin immunohistochemical stain reveals diffuse and strong positivity in tumor cells (×200). (**C**) Forward electropherogram show overlapping peak at the nucleotide position c.1799. Mutant peak demonstrates a T to A transversion.

Regarding the prevalence of mutation other than *BRAF* V600E, *BRAF* K601E was found the most common mutation, which is in agreement with a past report (1). The K601E mutation results from a substitution of A to G at the base position 1801 and has been reported to be associated with a FVPTC. In the FVPTC, the *BRAF* V600E mutation rate ranged from 9.6% to 26%, and the rate of *BRAF* K601E mutation has been reported to be as high as 9% (3, 4, 7). In our study, there were 4 cases of FVPTC harboring a *BRAF* K601E mutation. Another case, which was also mentioned in our previous report, was minimally invasive follicular carcinoma harboring the *BRAF* K601E mutation (2). Overall, the incidence of FVPTC with *BRAF* K601E mutation in Korea seems to be lower than incidences reported in Western countries (8). In our study, all 5 thyroid cancers with *BRAF* K601E showed less aggressive pathologic features (e.g., extrathyroidal extension and lymph node metastasis) compared with those harboring *BRAF* V600E. It is well known that there are 2 types of FVPTC: infiltrative and encapsulated forms. These forms show different clinical behaviors, and the presence of tumor capsule in the encapsulated follicular variant (EFV) is associated with excellent prognosis (4). All *BRAF* K601E-mutated tumors in our series belonged to EFV. Castro et al. (9) analyzed somatic mutation of 40 FVPTCs and found 3 cases harboring *BRAF* K601E. The tumors with *BRAF* K601E had no extrathyroidal extension, multifocality nor vascular invasion.

Penelli et al. (10) reported a case of follicular carcinoma with *BRAF* K601E and *PIK3CA* E545I mutations in a 78-yr-old male. The tumor showed capsular and multiple vascular invasions and a poorly differentiated component. Schulten et al. (11) reported the *BRAF* K601E in one case each from minimally invasive follicular carcinoma, classic PTC, and a follicular variant of PTC. We found a *BRAF* K601E in 70-yr-old male patient with an 8 cm-sized minimally invasive follicular carcinoma (Table 1, case 5). The tumor was composed of microfollicles and focally invaded the fibrous tumor capsule. No angioinvasion or extrathyroidal extension was found. These results suggest a link between *BRAF* K601E and follicular histology subtypes encompassing PTC and follicular carcinoma. *BRAF* mutation should be included as part of the molecular pathogenesis of follicular carcinoma although the mutation itself rarely occurs in the tumor.

Three classic PTCs were associated with nucleotide substitution at the base position 1797 and 1799, resulting in the common V600E mutation. The tumors were all less than 1 cm in size, but showed extrathyroidal extension. Among these cases, lymph node metastasis was found in 2 cases (Table 1).

In our series, one of 7 medullary carcinomas had the *BRAF* V600E genetic alteration. Hereditary medullary carcinoma, arising in multiple endocrine neoplasia type 2, harbored activating germ-line mutations of the RET proto-oncogene. Somatic RET mutations were detected in 46%-60% of sporadic medullary carcinomas, and somatic RAS mutations were also found in the tumors. However, *BRAF* mutation of medullary carcinoma has been reported only in a study by Goutas et al. (12). A Greek medullary carcinoma cohort of 44 patients analyzed *BRAF* status with an enriched PCR-restriction fragment length polymorphism method (PCR-RFLP) and found the *BRAF* V600E mutation in 30 samples (68.2%). One possible reason for such high frequency may be that the erroneous high mutation rate was made by the technical defect of PCR-RFLP method. PCR-RFLP utilizes restriction endonuclease for digestion of wild type and mutant

type PCR productions. Therefore, when enzymatic activity is insufficient on the PCR products, false positive result may occur. In the sequencing analysis studies of large number of MTC samples (total 95 cases), no mutation of *BRAF* was found (13). However, the reason for such disparity remains to be clarified. In the present study, authenticity of the mutation was confirmed by repeat bidirectional DNA sequencing with the re-isolated genomic DNA using 2 different primer sets.

Table 2 summarizes rare exon 15 *BRAF* mutations previously reported in the literature with description of nucleotide changes. All rare *BRAF* mutations in thyroid cancers, except for the K601E, have been detected in PTC. Next to *BRAF* K601E mutation, 3-nucleotide (thymidine-guanine-adenine) deletions from the base positions 1799 to 1801 were found in 7 cases of PTC. This mutation results in deletion of 2 amino acids (p.Val600_Lys601) and insertion of glutamate. Interestingly, in one case studied by Oler et al. (14), the mutation was present exclusively in lymph node metastases. The authors suggested that it could be an additional cumulative genetic event in tumor progression or a result of metastasis from a different primary focus.

BRAF is a serine-threonine kinase and is a part of MAPK signaling pathway. Once *BRAF* is translocated to the cell membrane and activated by RAS, it phosphorylates and activates MAPK pathway. This signaling pathway regulates various processes including cell proliferation, differentiation and survival (1). Oncogenic mutations of *BRAF* act by constitutive activation of MAPK pathway and they mostly affect residues located within the kinase domain of the protein; glycine-rich phosphate-binding loop (P loop, residues 462-471) and activation loop (A loop, residues 593-622). Activation of wild-type *BRAF* kinase needs phosphorylation of residues Thr599 and/or Ser602 which takes position in the activation segment. Under basal condition, hydrophobic interactions between A-loop and P-loop stabilize the protein. Oncogenic *BRAF* mutations of residue Val600 destabilize this inactive conformation, thereby triggering constitutive activation of the enzyme. Cancer-associated *BRAF* mutations can be divided into 3 categories according to their function. High kinase activity group (exemplified by V600E and K601E in A loop), low kinase activity group (exemplified by Gly464Glu in P loop and Phe595Leu in A loop), and rare impaired activity group (exemplified by Asp594Val in A loop). In the case of *BRAF* K601E, although it is in the high activity group, its kinase activity is 40% of that of *BRAF* V600E (15). Hou et al. (16) functionally characterized mutation caused by deletion and insertion (c.1799delinsATTTTGGTCTAGCTACAG). Like classical *BRAF* V600E, the new mutation resulted in constitutive activation of the kinase activity and also caused transformation of transfected cells (16). Several researchers also analyzed functional activity of the rare *BRAF* mutations, suggesting their important role in PTC tumorigenesis (17-20). These results support that the nucleotide sequence around position c.1799 is vulnerable to genetic alterations and disturbance of the electric charge in amino acid in this region converts *BRAF* into the oncogenic kinases (1).

There has been an association between *BRAF* V600E mutation and aggressive histologic characteristics of PTC, including extrathyroidal extension, lymph node metastasis, more advanced stage at the time of diagnosis and poor prognosis (4). Moreover, this mutation has been associated with a higher recurrence in low-risk stage I-II PTC patients (21). Although such results has not been found in some studies (7) and the conclusion is still a matter of debate, the clinical significance of the *BRAF* mutation in regard to tumor aggressiveness and as a poor prognos-

Table 2. Clinicopathologic features of rare *BRAF* mutations of thyroid tumors previously reported in the literature

Nucleotide change	Amino acid change	Diagnosis	Histologic variant	Reported cases	Reference
c.1801A > C	p.Lys601Glu	PTC FTC	FV, Classic NA	0.8-9.4% of FVPTCs 2	(3, 4, 7, 9) (10, 11)
c.1799_1801del	p.Val600_Lys601delinsGlu	PTC PTC PTC PTC	Solid Classic Trabecular EFV	1 4 1 1	(23) (14, 16, 24, 25) (4) (24)
c.1796_1809delinsTC	p.Thr599_Arg603delinsIle	PTC	EFV	2	(19)
c.1794_1795insGTT	p.Ala598_Thr599insVal	PTC	Classic	1	(20)
c.1799delinsATTTTGGTCTAGCTACAG	p.Val600delinsAspPheGlyLeuAlaThr	PTC	NA	1	(16)
c.1793C > T	p.Ala598Val	PTC	FV	1	(26)
c.[1796C > T;1798_1799insCTT]	p.[Thr599Ile;Val600delinsAspLeu	PTC	Solid	1	(27)
c.1799_1814delinsATGT	p.Val600_Ser605delinsAspVal	PTC	FV	1	(28)
NA	p.Gly474Arg	PTC	FV	1	(9)
c.1834C > T	p.Gln612Ter	PTC	NA	1	(29)
c.1798delinsTACA	p.Val600delinsTyrMet	PTC	Classic	4	(18)
c.1799_1800TG > AA	p.Val600Glu	PTC	NA	2	(17)
c.1795_1797dup	p.Thr599dup	ATC* PTC	- aggressive	11	(30) (11)
c.1794_1796delTAC	p.Thr599del	FA	NA	1	(11)

*The tumor was mixed ATC/PTC type. FV, follicular variant; PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; EFV, encapsulated follicular variant; LN meta, lymph node metastasis; ATC, anaplastic thyroid carcinoma; NA, not available; FA, follicular adenoma.

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tic factor is generally accepted. However, only a small number of cases have been reported regarding the clinicopathologic features of the rare *BRAF* mutations and further studies are needed to determine the significance of these mutations.

Less is known about the exact cause of PTC carcinogenesis, but one of the well-established risk factors is an ionizing radiation exposure. Among thyroid cancer mutation mechanisms, chromosomal rearrangements are strongly associated with ionizing radiation exposure (1). There have been only a few studies on the molecular epidemiology of thyroid cancer. According to a recent study, the increase in the incidence of thyroid cancer over the past 4 decades is strongly related to the increase in *BRAF* mutation in classic PTC and the increase in RAS mutation in FVPTC. In contrast, the frequency of thyroid cancer-specific chromosomal rearrangements is decreasing (22). Along with this general epidemiologic trend, the exceptionally high frequency of *BRAF* mutation in PTC of the Korean population implies that the recent increase in PTC incidence in Korea may be associated with the causes of *BRAF* mutation. There is some evidence supporting the role of environmental factors, including iodine diet and chemical influence, in *BRAF* mutagenesis (1), but further studies are still needed.

Direct sequencing is accepted as the gold standard method for the detection of genetic alterations and it can detect mutations at any gene position. However, other assay methods (e.g., pyrosequencing, colorimetric assay or shifted termination assay, real-time PCR, etc.) are mostly designed to detect point mutations in *BRAF* codon 600. The present study demonstrates that point mutations in codon 600 comprise more than 99% of all *BRAF* mutations. Based on this result, assay methods that screen for the "hot spot" *BRAF* c.1799T > A mutation seem to be reasonably sufficient for *BRAF* mutation screening in Korean thyroid cancer patients.

In conclusion, several *BRAF* mutation types other than *BRAF* V600E mutation exist but their prevalence is very low at around 0.76% among all *BRAF* mutation positive Korean thyroid cancers. *BRAF* K601E is the most common type of rare mutations. Although limited by the small number of *BRAF* K601E-mutated tumors, *BRAF* K601E may be associated with less aggressive pathologic features, when taken together with previous reports. We add 2 novel mutations to the list of *BRAF* mutations found in PTC. Further studies are needed to characterize the roles of rare *BRAF* mutations in thyroid cancers.

DISCLOSURE

The authors declare that no competing financial interests exist.

ORCID

Uiju Cho *http://orcid.org/0000-0002-6229-8418*
Woo Jin Oh *http://orcid.org/0000-0001-6130-9210*
Ja Seong Bae *http://orcid.org/0000-0003-3904-6519*
Sohee Lee *http://orcid.org/0000-0003-1559-8853*
Young Sub Lee *http://orcid.org/0000-0003-0841-1122*
Gyeong Sin Park *https://orcid.org/0000-0002-9727-5566*
Youn Soo Lee *https://orcid.org/0000-0002-1653-6315*
Chan Kwon Jung *http://orcid.org/0000-0001-6843-3708*

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