Clinicopathological Features of Rare BRAF Mutations in Korean Thyroid Cancer Patients

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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 reverse (5’-TCATAATGCTTGCTCTGATAGGA-3’).
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_1798insAGAGCTTACACA and c.1794_1795insGT; 4) 2 cases with in-frame deletions, c.1797T > 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; 1797T > A], c.[1796C > G; 1799T > 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,
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).

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.

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).

**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.
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 mutation (2). 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.

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 prognosis is a matter of further research.

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       | FV, Classic       | 0.8-9.4% of FVPTCs | (3, 4, 7, 9) |
| c.1799_1801del    | p.Val600_Lys601delinsGlu | PTC       | Solid             | 1             | (23) |
| c.1796_1809delinsTC | p.Thr599_Arg603delinsIle | PTC       | Classic           | 4             | (14, 16, 24, 25) |
| c.1794_1799delinsTT | p.Ala598_Thr599insVal | PTC       | Classic           | 1             | (20) |
| c.1792_1799delinsCT | p.Val600delAspPheGlyLeuAlaThr | PTC       | NA                | 1             | (15) |
| c.1793>T          | p.Ala598Val       | PTC       | FV                | 1             | (26) |
| c.1792C>T         | p.[Thr599Val;Val600delinsAsp615Thr] | 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_1800delinsTACA | p.Val600delinsTyrMet | PTC       | NA                | 2             | (17) |
| c.1791_1797del    | p.Thr599dup       | ATC*      | PTC, aggressive   | 11            | (30) |
| 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.
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.

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