Clinical Significance of BRAF (V600E) Mutation and Ki-67 Labeling Index in Papillary Thyroid Carcinomas

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Abstract. Background: Activating mutations of the BRAF gene have recently been reported in thyroid carcinomas. In particular, V600E mutation is highly prevalent in papillary thyroid carcinoma (PTC). Patients and Methods: In this study, the BRAF (V600E) mutation in 54 PTCs was investigated and the relationship between the BRAF mutation and clinicopathological features such as age, gender, tumor size, extrathyroid extension, lymph node metastasis, and distant metastasis was analyzed. Additionally, Ki-67 labeling index (LI) was determined to evaluate tumor cell proliferative activity. Results: The BRAF mutation was detected in 26 (65%) of 40 primary and 12 (85.7%) of 14 recurrent PTCs. The BRAF mutation was significantly related to older age (57.4 vs. 43.1 years, p=0.012), extrathyroid extension (76.9% vs. 35.7%, p=0.026), and lymph node metastasis (88.5% vs. 57.1%, p=0.044). Moreover, the mean Ki-67 LI was significantly higher in BRAF-positive patients than in BRAF-negative patients (1.01% vs. 0.135%, p=0.014). The BRAF mutation was common in PTCs classified as advanced TNM stage. Eighteen of 20 (90%) patients in TNM stages III and IV were positive for this gene mutation. Similarly, the BRAF mutation was investigated in 14 recurrent PTCs and was detected in 85.7% (12 of 14). The BRAF mutation was also common in patients with regional lymph node recurrence. Conclusion: These results suggest that PTCs with BRAF (V600E) mutation are more aggressive than those with wild-type BRAF. This mutation may be important for predicting a worse prognosis in patients with PTC.

Papillary thyroid carcinoma (PTC) is the most common malignant tumor of the thyroid (1). Several genetic events involved in the initiation of PTC have been investigated (2, 3). Recently, an activating point mutation of the BRAF gene has been identified as a common genetic event in PTC (29%-83%) (4-14). BRAF mutations are unique to PTC, because BRAF mutation has not been observed in other thyroid neoplasms such as follicular, Hürthle cell, and medullary carcinoma, or in benign thyroid tumors (4, 5).

The BRAF gene codes a cytoplasmic serine/threonine kinase that plays an initial role in the enzymatic cascade of the MAP kinase pathway (15), which is a classic signal pathway known to mediate cellular proliferation in various cell types.

Almost all cases of BRAF mutation in thyroid carcinomas involve the same missense thymine (T) to adenine (A) transversion at nucleotide 1799 in exon 15, resulting in the substitution of a valine by glutamate at residue 600 (V600E) (4, 8). This mutation is considered to mimic the phosphorylation in the activation segment by insertion of an acidic residue close to a site of regulated phosphorylation at serine 599 (16).

In this study, the BRAF (V600E) mutation in 40 primary and 14 recurrent PTCs and the relationship between the BRAF mutation and clinicopathological features were investigated. Moreover, the Ki-67 labeling index (LI), which expresses the proliferative activity (17-19), was compared between the BRAF mutation-positive and -negative patients.

Patients and Methods

Tumor samples. Snap-frozen tumor samples from 54 PTCs (40 primary and 14 recurrent tumors) were used in this study. Recurrent tumors consisted of regional node recurrences, recurrences in the residual thyroid gland, and other sites. The tissues were obtained surgically at the Department of Thyroid Surgery, Kanagawa Cancer Center. All patients signed an informed consent form before the surgery. Histologic diagnosis was confirmed by an experienced pathologist.

Tissue processing. Formalin-fixed paraffin-embedded tissues from each patient were cut into sections 4 μm thick and mounted on adhesive-coated slides. Sections were deparaffinized, rehydrated, and subjected to antigen retrieval by heating in 10 mM citrate buffer (pH 6.0) for 15 minutes in a microwave oven. After blocking endogenous peroxidase with 3% hydrogen peroxide, the sections were incubated with anti-Ki-67 monoclonal antibody (DAKO, Carpinteria, CA) at a dilution of 1:100 for 60 minutes at room temperature. After washing, sections were incubated with a biotinylated antimouse antibody (DAKO) for 40 minutes, followed by an avidin-biotin complex (DAKO) for 40 minutes. The immunoreaction was visualized with a 3,3-diaminobenzidine substrate kit (DAKO), and the sections were counterstained with hematoxylin. The Ki-67 LI was determined by counting 1000 tumor cells in the most proliferative areas of the tissue sections. The LI was expressed as the percentage of Ki-67-positive tumor cells.

Mutation analysis. Genomic DNA was extracted from snap-frozen tissue specimens using a DNeasy tissue kit (Qiagen, Valencia, CA) and quantified by UV spectrophotometry. The BRAF V600E mutation was analyzed by direct sequencing of the BRAF V600 codon in the DNA extracted from formalin-fixed, paraffin-embedded thyroid specimens after reverse transcription-PCR amplification of the BRAF gene. Samples with a sufficient amount of DNA were subjected to DNA amplification and sequencing. Primers for BRAF gene amplification were forward CGCATAGAGTGATGTCAGCC and reverse CTAGAGCAAGTTGCTGCTG. The PCR product was purified and sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI3730 Genetic Analyzer (Applied Biosystems). DNA sequencing was performed in both directions to confirm the authenticity of the sequences.

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Detection of BRAF (V600E) mutation. Mutation in the BRAF (V600E) gene was detected by direct sequencing. Genomic DNA was extracted from the tissues using a QIAamp DNA mini kit (QIAGEN, Hilden, Germany) and BRAF exon 15 was amplified by polymerase chain reaction (PCR). The following PCR primers were designed to amplify the target exon: forward: GGAAGACATTCCACCTCACC, reverse: GTAACCTAGCGACATCTCAGGGGC. Amplifications were carried out for 35 cycles (95°C for 30 s, 60°C for 30 s, 72°C for 60 s). PCR products were purified using a Wizard SV Gel and PCR Clean-up System kit (Promega, WI, USA), and sequenced using a DTCS Kit (Beckman Coulter, CA, USA); the forward primer was used as the sequencing primer. Sequencing products were purified using ethanol precipitation and analyzed using a CEQ 2000XL (Beckman Coulter).

Analysis of Ki-67 labeling index (LI). Ki-67 LI as a measure of proliferative activity was investigated by immunohistochemical staining using the monoclonal antibody for Ki-67 antigen (MIB-1; Immunotech, Marseille, France) (20). Immunohistochemistry was performed on paraffin-embedded sections (4-μm thick) after microwave pretreatment using the common avidin-biotin-peroxidase complex method (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA, USA). MIB-1 was used at 1:100 dilution. The reaction products were developed by incubating the sections with 3,3′-diaminobenzidine tetrahydrochloride (DAB), and then the slides were counterstained with hematoxylin. The Ki-67 LI was calculated as the percentage of Ki-67-positive cells (those having brown nuclei) by counting more than 2000 tumor cells in clearly stained fields. Ki-67 LI was analyzed only in primary PTCs.

Evaluation of clinicopathological features. Clinicopathological features were extracted from the chart review, including pathological reports. Tumor-node-metastasis (TNM) staging was determined based on the 6th edition of the American Joint Committee on Cancer (AJCC)/International Union Against Cancer (UICC) TNM classification system (21). Age, gender, tumor size, extrathyroid extension (tumor stage), lymph node metastasis and distant metastasis were evaluated as clinicopathological features.

Statistical analysis. Clinicopathological features and Ki-67 LI were compared between BRAF-negative (wild-type) and BRAF-positive (V600E mutation) patient groups. Results are expressed as means ± standard deviation (SD). Statistical analysis was performed with the use of Student’s t-test or the Mann-Whitney test, as appropriate. Frequencies were compared with the Chi-square test and Fisher’s exact probability test. Differences were considered significant when p-values were less than 0.05. Statistical analyses were performed with the Statistical Package for Social Sciences for Windows (SPSS, Inc., Chicago, IL, USA).

Results

As shown in Figure 1, BRAF mutation was investigated by direct sequencing analysis. The V600E BRAF mutation was detected in 26 (65%) of 40 primary PTCs. The results are shown in Table I, which summarizes the relationship between the BRAF mutation and clinicopathological features in 40 primary PTCs. The BRAF-positive patients were significantly older than the BRAF-negative patients (57.4 vs. 43.1 years, p=0.012). There were no significant differences concerning gender or tumor size. Extrathyroid extension was found at a significantly higher rate in BRAF-positive patients compared to BRAF-negative patients (76.9% vs. 35.7%, p=0.026). The frequency of BRAF-positive patients was related to advanced tumor stage. Lymph node metastasis was more common in the BRAF-positive patients than in BRAF-negative patients, although there was no significant difference between the BRAF-positive and -negative groups. Advanced TNM stages were found in a significantly higher fraction of BRAF-positive patients (69.2% vs. 14.3%, p=0.002). The mean Ki-67 LI was significantly higher in BRAF-positive patients than in BRAF-negative patients (1.01% vs. 0.135%, p=0.014). Immunohistochemical staining for Ki-67 LI analysis is shown in Figure 2.

Similarly, BRAF mutation was investigated in 14 recurrent PTCs and was detected in 85.7% of them (12 of 14), as shown in Table II. The BRAF mutation was common in patients with regional lymph node recurrence.

Discussion

We analyzed the BRAF (V600E) mutation in primary and recurrent PTCs. Our results revealed the high prevalence of the BRAF mutation in both primary and recurrent PTCs, consistent with the findings of previous investigations (4-14).
The BRAF mutation found in thyroid carcinoma is almost exclusively the T1799A transversion mutation in exon 15. This study focused on the T1799A BRAF mutation. This particular BRAF mutation has only been found in papillary and anaplastic thyroid carcinoma (ATC). The coexistence of papillary and anaplastic components in the same tumor has been found and both harbored the BRAF mutation (4, 22, 23). This finding suggests that ATC with the BRAF mutation might be transformed from PTC with the BRAF mutation. On the other hand, BRAF mutation has never been found in follicular or medullary thyroid carcinomas, or in benign thyroid tumors (4-6, 8, 9, 12). Moreover, Knauf et al. reported that the formation of PTC could be induced in transgenic mice in which expression of the BRAF (V600E) mutant was targeted to thyroid cells (24). Thus, the BRAF mutation in PTCs is considered to be a specific genetic event and may play a fundamental role in the initiation or progression of tumorigenesis of PTC.

Previous investigations found the BRAF mutation to be associated with adverse clinicopathological features. In the current study, the BRAF mutation was also associated with older age, extrathyroid extension, lymph node metastasis, and advanced TNM staging. The BRAF mutation was detected in 18 of 20 (90%) patients in stages III and IV. Nikiforova et al. found that the BRAF mutation was significantly related to older age, extrathyroid extension and advanced TNM stages (4). Namba et al. concluded that the BRAF mutation was significantly related to distant metastasis and advanced TNM stages (8). Kim et al. found the BRAF mutation to be significantly associated with lymph node metastasis (11). On the other hand, the BRAF mutation was not significantly related to tumor size in previous reports. Our results were in accordance with these findings. These findings suggest that the BRAF (V600E) mutation may have relevance to biological aggressiveness, including tumor invasion and metastasis to distant sites.

Moreover, we analyzed the relationship between the BRAF mutation and Ki-67 LI. The Ki-67 antigen is expressed in the nucleus of proliferating cells (25). Assessment of the tumor cell proliferative activity by Ki-67 staining has been performed in various types of human neoplasms. It is known that the Ki-67 LI in PTCs is lower than that in breast, lung, stomach and colon adenocarcinomas (17). As expected, the mean Ki-67 LI was low, but was significantly higher in the BRAF-positive patients than in the BRAF-negative patients. These findings suggest that the BRAF mutation may activate the MAP kinase pathway, resulting in activated tumor cell proliferation. Activation of the MAP kinase pathway by the BRAF (V600E) mutation may play an important role in thyroid carcinogenesis and tumor progression. Therapeutic agents targeting this pathway are considered to be a promising novel treatment modality for thyroid carcinomas, and a specific inhibitor targeting the Raf kinase has been reported (26, 27). It is hoped that this inhibitor will be therapeutically useful for patients with the BRAF mutation, particularly for those who have incurable disease.

We also found high prevalence of the BRAF (V600E) mutation in recurrent PTCs. Of these, 35.7% (12 of 14) had the BRAF mutation. This finding indicates that the BRAF mutation may have an effect on tumor aggressiveness in PTCs. In particular, BRAF mutation was common in patients with regional lymph node recurrence. Vasko et al. reported the high prevalence of BRAF mutation in lymph node metastasis from BRAF mutation-positive primary tumors (28). They proposed that the local milieu of lymph nodes may specially favor the selection and survival of thyroid cancer cells that harbor BRAF mutation. This may be a reason why the BRAF mutation was common in regional lymph node recurrence in our series. The frequency of lymph node metastasis was significantly higher in BRAF mutation-positive patients than in BRAF mutation-negative patients. These findings suggest that the BRAF mutation may have a significant relationship to nodal status at initial treatment or during the follow-up period for patients with PTC.

In summary, we found that the BRAF (V600E) mutation was common in both primary and recurrent PTCs and was significantly related to adverse clinicopathological features such as extrathyroid extension, lymph node metastasis, and advanced TNM staging. These results suggest that PTCs with the BRAF (V600E) mutation are more aggressive than those with wild-type BRAF. This mutation may be important for predicting a worse prognosis in patients with PTC.

References

Figure 1. Sequence chromatograms of PCR products from primary PTC samples. The mutation (T → A) appeared as a double peak at position 1799 (arrow) in BRAF exon 15 (mutant-type), in contrast to the single peak of the wild-type sample.

Figure 2. A representative example of immunohistochemical staining of PTC with monoclonal antibody MIB-1 directed against the Ki-67 antigen present in proliferating cells. The cells with brown nuclei are regarded as MIB-1 positive. This case was BRAF-positive PTC and the Ki-67 LI was 5.11% (the highest rate in this study). (Magnification x200)
Table II. **BRAF (V600E) mutation in primary and recurrent PTCs.**

<table>
<thead>
<tr>
<th>Category</th>
<th>Value (% of Total)</th>
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<tbody>
<tr>
<td>Primary PTC (n=40)</td>
<td>26 (65.0%)</td>
</tr>
<tr>
<td>Recurrent PTC (n=14)</td>
<td>12 (85.7%)</td>
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<tr>
<td>Regional lymph node (n=8)</td>
<td>8 (100%)</td>
</tr>
<tr>
<td>Residual thyroid gland (n=2)</td>
<td>1 (50.0%)</td>
</tr>
<tr>
<td>Other (n=4)</td>
<td>3 (75.0%)</td>
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