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BRIEF REPORT

Influence of the BRAF V600E Mutation on Expression of Vascular Endothelial Growth Factor in Papillary Thyroid Cancer

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Context: The BRAF mutation may influence the expression patterns of molecular markers that are related to the development and progression of thyroid cancer.

Objective: The objective of the study was to investigate the effects of the BRAF V600E mutation on expression of galectin-3, cyclooxygenase-2, cyclin D1, p53, and vascular endothelial growth factor (VEGF) in papillary thyroid cancer (PTC).

Design, Setting, and Subjects: One hundred sixty-three PTC and 28 nodular hyperplasia patients were selected retrospectively. The presence of the BRAF V600E mutation and the level of expression of the molecular markers were determined.

Results: Of 161 PTC patients, 102 patients (63.4%) were BRAF V600E(+), and these cases had significantly larger tumor sizes ($P = 0.01$), compared with V600E(−) cases (n = 59, 36.6%). Although PTC tissues had higher expression levels of the selected molecular markers than nodular hyperplasia tissues, expression levels of several molecular markers in BRAF V600E(+) PTC were not significantly different from those of BRAF V600E(−) PTC. But VEGF was significantly up-regulated in BRAF V600E(+) PTC, compared with BRAF V600E(−) PTC. VEGF expression levels were strongly positively correlated to tumor size ($P < 0.001$), extrathyroidal invasion ($P = 0.02$), and tumor stage ($P = 0.04$). Multivariate analysis clearly showed that VEGF expression was up-regulated in BRAF V600E(+) PTC (odds ratio 2.5, confidence interval 1.1–5.6; $P = 0.03$).

Conclusions: BRAF V600E(+) PTC tended to have larger tumor volumes and higher expression of VEGF. The level of VEGF expression was closely correlated with tumor size, extrathyroidal invasion, and stage. The relatively high levels of VEGF expression may be related to poorer clinical outcomes and recurrences in BRAF V600E(+) PTC. **(*J Clin Endocrinol Metab* 91: 3667–3670, 2006)**

PAPILLARY THYROID CARCINOMA (PTC) is the most frequent of the histological subtypes of thyroid cancers. The majority of PTCs are caused by constitutive activation of oncogenic signaling pathways such as RET/PTC, NTRK1, and the serine/threonine kinase BRAF V600E (1, 2). Among these genetic abnormalities, BRAF V600E is the most common genetic alteration, occurring in half of the sporadic PTCs, particularly in the relatively aggressive subtypes such as the tall-cell PTC (3).

Many clinical studies have been conducted to characterize the behavior of BRAF V600E(+) PTC. However, a number of studies have produced controversial findings on the usefulness of BRAF V600E as a prognostic indicator (4). A recent multicenter trial showed that BRAF V600E is associated with factors that predict a high risk of recurrence and poor clinical outcomes (5). Because mutation of BRAF plays an important role in tumorigenesis and determination of clinical phenotypes, it is possible that BRAF V600E(+) PTC possesses characteristic expression patterns of markers that could be correlated with disease prognosis.

Several molecular markers, such as galectin-3 (6), cyclooxygenase (COX)-2 (7), cyclin D1 (8), p53 (9), and vascular endothelial growth factor (VEGF) (10), have been examined both for establishing a diagnosis and predicting prognostic outcomes in thyroid cancers. The expression of these molecular markers may be perturbed by activation of the ras/raf signaling pathway. We therefore measured the effects of the BRAF V600E mutation on expression levels of these molecular markers in PTC.

Patients and Methods

Selection of patients and analysis of clinicopathological data

Thyroid tissue specimens were obtained from 191 patients [28 nodular hyperplasia (NH) and 163 PTC, including 142 conventional, 12 follicular variant, four tall cell variant, and five columnar cell variant PTC cases] who underwent surgery from 2004 to 2005 at the Center for

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Abbreviations: CI, Confidence interval; COX, cyclooxygenase; IHC, immunohistochemical; NH, nodular hyperplasia; PTC, papillary thyroid cancer; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor.

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Endocrine Surgery, Chungnam National University Hospital (Daejeon, Korea). All protocols were approved by the institutional review board. Patient information and clinicopathological parameters were analyzed retrospectively. Tumor node metastasis staging and histotypes were assessed according to the World Health Organization classifications.

DNA isolation and sequencing

Genomic DNA from paraffin-embedded thyroid tissue specimens was prepared. Exon 15 of the BRAF gene was amplified by PCR with the following primers: forward, 5′-ATGCTTGCTCTGATAGGAAA-3′ and reverse, 5′-ATTTTTGTGAATACTGGGGAA-3′. RET/PTC1 and RET/PTC3 rearrangements were verified using previously described methods (11). The purified PCR products were sequenced on an ABI PRISM 3100 automated capillary DNA sequencer using the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA).

Tissue microarray construction and immunohistochemical staining

Paraffin-embedded thyroid tissue samples were arrayed on duplicate blocks to minimize loss of tissue. Immunohistochemical (IHC) staining for galectin-3, COX-2, cyclin D1, p53, and VEGF was performed in NH and PTC samples. Tissue sections were incubated with primary antibodies against galectin-3 (NovoCastra Laboratories Ltd., Tyne, UK; 1:100), COX-2 (Assay Designs, Ann Arbor, MI; 1:50), cyclin D1 (Neomarker, Fremont, CA; 1:50), p53 (DakoCytomation, Glostrup, Denmark; A/S, 1:50), and VEGF (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200). Negative controls were incubated with PBS instead of a primary antibody, and positive control tissues were also stained. Staining was scored as follows: 0, no staining; 1, weak or focal staining; 2, moderate staining in most cells; and 3, strong staining in most cells.

Statistical analysis

Group comparisons of categorical variables were performed using the χ^2 test or linear-by-linear association. Bonferroni's adjustment was used as a more conservative measure of significance for multiple comparisons. Comparisons of means were evaluated with the independent-samples *t* test. Multivariate logistic regression analysis was performed to assess association of the BRAF V600E mutation with molecular markers and clinicopathological outcomes of patients with PTC and assess association of VEGF expression with clinicopathological outcomes. Confidence intervals (CIs) were computed by standard methods. All reported *P* values are two tailed. Analyses were performed using SPSS (version 12.0 for Windows; SPSS Inc., Chicago, IL).

Results

BRAF mutation status in relation to clinicopathological characteristics

The BRAF V600E mutation was not found in any of the 28 NH cases but was detected in 102 of 161 (63.4%) of the PTC cases. DNA from the 59 PTC that were BRAF V600E(−) was examined for the RET/PTC1 and RET/PTC3 rearrangements, but none were found. Consistent with previous studies, the follicular variant PTC had a low prevalence of BRAF V600E(+) cases (two of 12, 16.7%).

Next, we analyzed the clinicopathological characteristics of the PTC cases (Table 1). BRAF V600E(+) PTC had a larger tumor size than BRAF V600E(−) PTC (mean size 2.7 ± 1.2 *vs.* 2.3 ± 0.9 cm, respectively; $P = 0.01$) and showed a tendency to undergo extrathyroidal invasion ($P = 0.08$). However, there was no significant

TABLE 1. BRAF V600E mutation in relation to clinicopathological characteristics and molecular markers

	BRAF V600E mutation		*P* value
	Positive	Negative	
Age (yr)	45.1 ± 13.7	44.2 ± 13.6	0.67[a]
Female:male	84 (82.4):18 (17.6)	50 (84.7):9 (15.3)	0.7[b]
Tumor size (cm)	2.7 ± 1.2	2.3 ± 0.9	0.01[a]
Extrathyroidal invasion			
No:yes	34 (33.3):68 (66.7)	28 (47.5):31 (52.5)	0.08[b]
Nodal metastasis			
No:yes	53 (52):49 (48)	32 (54.2):27 (45.8)	0.78[b]
Stage			
I	68 (66.7)	38 (64.4)	0.95[c]
II	11 (10.8)	10 (16.9)	
III	22 (21.6)	10 (16.9)	
IV	1 (1)	1 (1.7)	
Distant metastasis			
No:yes	101 (99):1 (1)	58 (98.3):1 (1.7)	0.69[b]
Galectin-3 staining intensity[d]			
0/1/2/3	11/19/20/25	5/14/13/11	0.7[b]
COX-2 staining intensity[d]			
0/1/2/3	27/30/18/0	23/13/6/1	0.13[b]
Cyclin D1 staining intensity[e]			
0/1/2/3	47/17/11/2	24/13/5/1	0.8[b]
p53 staining intensity[e]			
0/1/2/3	59/10/8/0	37/5/1/0	0.12[b]
VEGF staining intensity[f]			
0/1/2/3	2/17/51/32	2/22/27/7	0.001[b]

Data in *parentheses* are percents.
[a] Independent samples *t* test.
[b] Pair-wise comparisons from the Pearson χ^2 test.
[c] Comparisons of three or four groups using linear by linear association.
[d] BRAF V600E mutation status could not be determined in one of 119 cases.
[e] BRAF V600E mutation status could not be determined in one of 121 cases.
[f] BRAF V600E mutation status could not be determined in two of 162 cases.

correlation between the presence of the BRAF V600E mutation and any component of the staging system.

Expression of molecular markers in NH and PTC

Galectin-3 staining was present in 119 cases of PTC. The intensity of IHC staining was moderate or strong in 69 (57.9%) cases, all of which displayed a pancytoplasmic staining pattern. In contrast, only four cases of NH showed galectin-3 staining, which was focal or moderate in intensity ($P < 0.001$). IHC staining for COX-2 revealed that 68 of 119 (57.1%) PTC cases were positive, whereas two cases of NH were positive ($P < 0.01$). Although the percentage of immunopositive cells was higher in PTC, the distribution of COX-2 reactivity was not restricted to tumor cells. COX-2 expression was observed in normal thyroid tissue adjacent to PTC. For cyclin D1, three of 121 (2.5%) PTC cases showed strong staining intensity, 16 (13.2%) cases stained with moderate intensity, and 30 (24.8%) cases showed a weak staining intensity. Cyclin D1 immunoreactivity was evident in the nuclei of cancer cells in all positive cases, and there was a statistically significant difference between NH and PTC staining intensity ($P = 0.04$). IHC staining of 121 cases of PTC revealed p53-positive nuclear staining in 24 cases (19.8%); NH samples did not show any detectable immunoreactivity ($P = 0.017$).

Differential cytoplasmic VEGF expression was detected by IHC in 162 PTC, and the positive reactions in adjacent normal thyroid tissues were usually faint. VEGF was also detected in endothelial cells of the stromal vessels. A moderate or strong staining intensity of VEGF was detected in 119 of 162 cases of PTC (73.5%), and VEGF expression was significantly up-regulated in PTC, compared with NH ($P < 0.001$).

Differential expression of molecular markers in BRAF V600E(+) PTC

Despite a tendency for various promising molecular markers to be more strongly stained in PTC relative to NH, there were no significant differences in expression level of any of these markers in BRAF V600E(+) PTC *vs.* BRAF V600E(−) PTC (Table 1). A slightly higher amount of moderate staining intensity for p53 was detected in BRAF V600E(+) PTC, but the two groups were not significantly different. Interestingly, a higher percentage of BRAF V600E(+) PTC cases (83 cases, 80.5%) exhibited a moderate to strong VEGF staining intensity, compared with BRAF V600E(−) PTC cases (34 cases, 59.6%).

Multivariate logistic regression analyses were performed to assess the association of the BRAF V600E mutation with molecular markers and clinicopathological parameters. After adjustments for age, sex, and tumor size, the BRAF V600E mutation showed a significant association with moderate to strong VEGF staining (Table 2). Even when additional adjustments for extrathyroidal invasion, nodal metastasis, tumor stage III/IV, and distant metastasis were included, the association of the BRAF V600E mutation with VEGF expression was still highly significant [odds ratio 2.5, CI 1.1–5.6, $P = 0.03$].

VEGF expression and clinicopathological characteristics

Next, we compared the levels of VEGF expression to important clinicopathological parameters. VEGF immunoreactivity was positively correlated to tumor size ($P < 0.001$, supplemental Table S1, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org), extrathyroidal invasion ($P = 0.02$, supplementary Table S1), and tumor stage ($P = 0.04$, supplementary Table S1). The association of extrathyroidal invasion with higher VEGF expression was also highly significant after adjustments for age at diagnosis, sex, tumor size, nodal metastasis, tumor stage III/IV, distant metastasis, and histotypes (odds ratio 2.7, CI 1.2–5.9, $P = 0.02$, supplemental Table S2).

TABLE 2. Multivariate analysis of the association of the BRAF mutation with clinicopathological outcomes and molecular markers

	BRAF V600E mutation		
	Odds ratio	95% CI	*P* value
Extrathyroidal invasion[a]	1.6	0.8–3.1	0.18
Nodal metastasis[a]	1.0	0.5–2.0	0.97
Stage III/IV[a]	0.8	0.3–2.4	0.75
Distant metastasis[a]	0.3	0.01–6.4	0.35
Galectin-3 staining intensity 2–3[a]	1.1	0.5–2.5	0.8
COX-2 staining intensity 2–3[a]	1.7	0.6–4.6	0.32
Cyclin D1 staining intensity 2–3[a]	1.2	0.4–3.6	0.7
p53 staining intensity 2–3[a]	5.6	0.6–49.1	0.12
VEGF staining intensity 2–3[a]	2.6	1.2–5.8	0.02
VEGF staining intensity 2–3[b]	2.5	1.1–5.6	0.03
VEGF staining intensity 2–3[c]	2.5	1.1–5.6	0.03

[a] Adjusted for age, sex, and tumor size.
[b] In addition to adjustment 1, adjusted for extrathyroidal invasion, nodal metastasis, tumor stage, and distant metastasis.
[c] In addition to adjustment 2, adjusted for histotypes.

Discussion

In this study, we confirmed that the BRAF V600E mutation is the leading genetic event correlated with sporadic PTC (4). DNA from the 59 PTC that were BRAF V600E(−) were examined for RET/PTC1 and RET/PTC3 genomic rearrangements, but none were found. The prevalence of RET/PTC rearrangements in PTC (especially RET/PTC1 and RET/PTC3) has been reported to be around 30%. However, the prevalence data from Japan, Taiwan, and Korea are quite low, compared with Italy and other Western countries (12, 13). All of the control subjects had no detectable RET/PTC1 or RET/PTC3 rearrangements using a genomic PCR method (11), but it is possible that other forms of RET/PTC mutations exist and may not have been detected by this method.

The expression of several molecular markers was examined in this study. Galectin-3, COX-2, and cyclin D1 have been implicated in invasion, malignant progression, and metastasis, respectively (6–8). Expression of p53 is more frequent in poorly differentiated carcinomas and undifferentiated carcinomas than in well-differentiated carcinomas (9). In this study, the percentage of p53-positive PTC seems to be higher than in other reports, but the intensity of p53 immunoreactivity was lower than that of the other three molecular markers. Although the expression of these markers was more frequently up-regulated in PTC than NH, none of the markers showed differential expression between BRAF V600E(+) and (−) PTC.

VEGF is a major regulator of angiogenesis and has become a target in cancer therapy (14). VEGF expression was signifi-

cantly higher in BRAF V600E(+) PTC, and higher VEGF expression levels were correlated with extrathyroidal invasion. This finding suggests that VEGF plays an important role in tumor aggressiveness and that BRAF-mediated activation of specific signaling pathways may be responsible for the enhanced expression of VEGF in BRAF V600E(+) PTC. We could not evaluate the differential expressions of molecular markers according to PTC histotypes because most of the BRAF V600E(+) subjects in this study carried conventional PTC.

VEGF expression can be up-regulated by hypoxia-inducible factor-1 (15), which binds the hypoxia response element sites of the VEGF promoter and mediates up-regulation of VEGF expression in hypoxia. Recently several studies discovered that nonhypoxic signals may also regulate hypoxia-inducible factor-1 expression and suggest that constitutive activation of BRAF and its downstream Raf/MEK/ERK pathway could increase the expression of VEGF in BRAF V600E(+) PTC (16). Additionally, the VEGF promoter contains binding elements for signal transducer and activator of transcription 3 (STAT3), which is involved in VEGF up-regulation induced by various oncogenic tyrosine kinases, including v-Src and RET/PTC (17, 18). The activation of the ERK/MAPK pathway in response to both insulin and osmotic shock also resulted in the serine phosphorylation of STAT3 (19). These observations also suggest that constitutive activation of BRAF may induce serine phosphorylation of STAT3 and activation of the VEGF promoter. In previous studies, quantitation of VEGF expression demonstrated higher VEGF expression in metastatic cancers (10). Additionally, higher VEGF expression was positively correlated with tumor size (20). Thus, VEGF, which is up-regulated in BRAF V600E(+) PTC, may provoke an increase in tumor growth and tumor vasculature.

Although the BRAF V600E mutation is the most frequent genetic abnormality found in PTC, its long-term prognostic significance is not fully understood because there have been a number of contradictory findings. The contradictory observations may be due to heterogeneity in PTC at the molecular level or overlapping phenotypes from different genetic alterations. Here we found a higher level of VEGF expression in BRAF V600E(+) PTC, and the VEGF expression was correlated with extrathyroidal invasion. However, this cross-sectional study could not evaluate the long-term outcome of patients with BRAF V600E(+) PTC. In the future, the prognostic importance of BRAF V600E and high VEGF expression should be evaluated based on long-term outcomes.

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