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Clinical Implication of Hot Spot *BRAF* Mutation, V599E, in Papillary Thyroid Cancers

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Activating mutations in the *BRAF* kinase gene have recently been reported in human cancers. The aim of the present study was to determine the frequency of *BRAF* mutations in thyroid cancer and their correlation with clinicopathological parameters. We analyzed exons 11 and 15 of *BRAF* gene in six human thyroid cancer cell lines and 207 paraffin-embedded thyroid tumor tissues. A missense mutation was found at T1796A (V599E) in exon 15 in four of the six cell lines and 51 of 207 thyroid tumors (24.6%; 0 of 20 follicular adenoma, 0 of 11 follicular carcinoma, 49 of 170 papillary carcinomas, and 2 of 6 undifferentiated carcinomas). Activation of MAPK kinase-MAPK pathway was observed in cell lines harboring *BRAF* mutation. *BRAF* mutation-associated enhanced cell growth was suppressed by MAPK kinase inhibitor, U0126. Examination of 126 patients with papillary thyroid cancer showed that *BRAF* mutation correlated significantly with distant metastasis ($P = 0.033$) and clinical stage ($P = 0.049$). Our results indicate that activating mutation of *BRAF* gene could be a potentially useful marker of prognosis of patients with advanced thyroid cancers. (*J Clin Endocrinol Metab* 88: 4393–4397, 2003)

THE BIOLOGICAL BEHAVIOR of thyroid cancer varies widely from indolent microcarcinoma, growing slowly with little or no invasion, to invasive cancer that metastasizes and can potentially cause death (1, 2). Previous studies using multifactorial analysis of clinical risk factors in thyroid cancer showed that metastasis, age, completeness of resection, invasion, and tumor size are useful prognostic factors of differentiated thyroid cancer (3). Despite vigorous molecular analysis performed over the past 10 yr, limited prognostic biomarkers are currently available for human thyroid cancers. *Ret/PTCs*, *Ret* protooncogene rearrangements, are specifically found in papillary cancers but do not correlate with the grade of malignancy (4). In contrast, our previous studies and those of other investigators have shown that mutations of *p53* gene are exclusively found in undifferentiated thyroid cancers (5). Analysis of *p53* gene is, therefore, a useful tool to detect undifferentiated thyroid cancers. Considered collectively, there is a desire to identify more reliable prognostic markers such as oncogenes, activated signaling pathways, and other basic mechanisms that are specifically relevant to thyroid cancers.

The Ras/Raf/MAPK kinase (MEK)/MAPK pathway is a classic signal pathway known to mediate cellular proliferation in various cell types. Activating mutations of *ras* gene are identified in approximately 30% of human thyroid tumors, suggesting that the kinase pathway is involved in thyroid tumorigenesis (6, 7). Recently, activating mutations in the *BRAF* kinase gene were described in a broad range of other human malignancies (8). The frequency of *BRAF* mutations varies widely in human cancers from more than 80% in melanomas and nevi (9, 10), to as little as 0–18% in other tumors, such as 1–3% in lung cancers and 5% in colorectal cancers (11–13). Herein, we investigated the frequency of *BRAF* mutations and the relationship between the mutation and clinical stage of human thyroid cancers. We detected *BRAF* mutation, V599E, in four of six human thyroid cancer cell lines and in 51 of 207 thyroid tumor tissues. The correlation analysis using various clinicopathological parameters revealed that *BRAF* mutation was significantly associated with advanced thyroid cancers.

Abbreviations: FBS, Fetal bovine serum; MEK, MAPK kinase.

Materials and Methods

Cell culture and materials

Four human thyroid cancer cell lines, ARO, FRO, NPA, and WRO, were kindly provided by Dr. G. Juillard (University of California–Los Angeles, Los Angeles, CA). Another papillary thyroid cancer cell line, TPC-1, and anaplastic carcinoma cell line, KTC-1, were kindly provided by Dr. Sato (Cancer Institute, Kanazawa University, Japan) and Dr. Kurebayashi (Kawasaki Medical School, Kawasaki, Japan) (14), respectively. All cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and grown at 37 C in 5% CO_2-95% air environment.

Thyroid tumor tissues were selected from 207 paraffin blocks (20 follicular adenomas, 170 papillary carcinomas, 11 follicular carcinomas, and six undifferentiated carcinomas) filed at the Department of Pathology, Nagasaki University School of Medicine (Nagasaki, Japan) and Ishigaki Thyroid Clinic (Hamamatsu, Japan). All thyroid tumors were independently reclassified by two experienced pathologists based on the histopathological typing of the World Health Organization as papillary carcinoma, follicular carcinoma, undifferentiated carcinoma, or follicular adenoma (15).

Correlations between *BRAF* mutation and various clinicopathological parameters were clinically and retrospectively analyzed in 126 patients

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who consented to the study. Clinical staging of thyroid cancer cases was classified according to the Tumor Node Metastasis (TNM) classification of the International Union Against Cancer (UICC). The study protocol was approved by the Human Ethics Review Committees of Nagasaki University School of Medicine.

Immunoblot analysis

All cells were seeded at a density of 1×10^6 cells in 10-cm dishes. The cells were incubated in RPMI 1640 with 10% FBS for 24 h, and then the medium was changed to RPMI 1640 with 2% FBS. After 24 h, the cells were harvested with RIPA buffer. In the next step, 40 μg of whole cell lysates were separated by electrophoresis in 10% SDS-PAGE, and then blotted onto nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). To quantitate the levels of MEK, phospho-MEK, MAPK, and phosphor-MAPK, the blots were incubated for 60 min with the respective antibody against human MEK, phospho-MEK, MAPK and phosphor-MAPK (Cell Signaling Technology, Beverly, MA). The antigen-antibody complexes were visualized with horseradish peroxidase-conjugated antirabbit IgG antibody and the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Cell growth assays

The kinetics of cell growth were examined using a cytometer as follows. Cells were seeded at a density of 0.1 or 0.5×10^5 cells per well in 12-well culture plates. They were counted at d 2, 3, 4, and 5. The experiments were performed at least three times. Cells were cultured with or without 5 μM U0126 (Cell Signaling Technology), or 0.1% dimethyl sulfoxide and counted at 24 h after treatment.

DNA isolation and sequencing

Genomic DNA was extracted from cell lines using the Wizard Genomic Purification Kit (Promega, Madison, WI) and amplified for analysis of mutations in exons 11 and 15 of *BRAF* gene (8) and the regions containing codons 12, 13, 59, and 61 of *H, K,* and *N-ras* genes by PCR using specific primers (11). DNA from 207 paraffin-embedded thyroid tumor specimens was prepared from five 10-μm-thick sections after microdissection, resulting in selection of more than 90% tumor cells. Genomic DNA was isolated using DXPAT (Takara Co., Kyoto, Japan), and *BRAF* exons 11 and 15 were amplified by PCR. The following intron-based PCR primers were designed to amplify the exons 11 and 15: BRAF exon 11, forward-TCCCTCTCAGGCATAAGGTAA, reverse-CGAACAGTGAATATTTCCTTTGAT; BRAF exon 15, forward-TCATAATGCTTGCTCTGATAGGA, reverse-GGCCAAAAATTTAAT-CAGTGGA. PCRs were performed using standard PCR conditions (95 C × 5 min; 94 C × 30 sec, 58 C × 30 sec, 72 C × 30 sec, for 40 cycles; then 70 C × 10 min). The amplified products were purified by MinElute PCR Purification Kit (Qiagen, Chatsworth, CA) and sequenced on an ABI PRISM 3100 automated capillary DNA Sequencer using the BigDye terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA).

Statistical analysis

Data (shown in Table 3) were analyzed using the Mann-Whitney *U* test or χ^2 for independence test. A *P* value <0.05 denoted the presence of a significant difference.

Results

BRAF mutation and MAPK activation in thyroid cancer cell lines

To detect mutations in the *BRAF* gene in human thyroid cancer cells, we first performed sequence analysis of *BRAF* exons 11 and 15 using genomic DNA extracted from six human thyroid cancer cell lines. We found the missense mutation T1796A (V599E) in four of six thyroid cancer cell lines. Among the four cell lines harboring the mutation, homologous mutation was detected in two cell lines, FRO and NPA, and heterologous mutation in the other two cell lines, ARO and KTC-1 (Fig. 1A).

To confirm the enhanced activity of the downstream pathway of BRAF, phosphorylation of MEK and MAPK was examined in six cell lines by immunoblot analysis (Fig. 1B). Although the phosphorylation of MEK in cell lines with homologous *BRAF* mutation was stronger than in cells with heterologous mutation, increased phosphorylation of MEK was noted in the four cell lines harboring *BRAF* mutation. Thus, the V559E mutation itself was associated with an activated form of BRAF protein. Similarly, MAPK was strongly phosphorylated in all cell lines exhibiting *BRAF* mutation. Although increased phosphorylation of MAPK was also identified in one cell line free of *BRAF* mutation, TPC-1, which has *RET/PTC-1* rearrangement, Carlomagno *et al.* (16) recently demonstrated that RET/PTC fusion protein can activate MAPK. Neither *RET/PTC-1* nor *RET/PTC-3* rearrangement was observed in other cell lines by RT-PCR method (data not shown).

Next, we examined whether activated RAS was involved in the activation of MEK-MAPK pathway. Sequence analysis confirmed no activating mutations of *H, K,* and *N-ras* genes in all cell lines used in this study. Table 1 summarizes the results of *BRAF*, *ras*, and *RET* genes mutation analyses in the six human thyroid cancer cell lines. Furthermore, to investigate whether the *BRAF* mutation affects cell proliferation, cell growth assays were performed. Cell lines with *BRAF* mutation showed more rapid cell growth than the WRO cell line, which does not harbor *BRAF* mutation or *RET/PTC* rearrangement (Fig. 1C). Twenty-four-hour treatment of cells with 5 μM U0126, a MEK1/2 inhibitor, showed significant suppression of cell growth in *BRAF* mutation cell lines, ARO and FRO, but not in non-*BRAF* mutation cell lines, TPC-1 and WRO (Fig. 1D). These results suggest that *BRAF* mutation promotes cell growth directly through the MEK-MAPK pathway in these thyroid cancer cell lines.

BRAF mutation in paraffin-embedded thyroid tumor tissues

We studied *BRAF* exons 11 and 15 in 207 paraffin-embedded thyroid tumors, including 20 follicular adenomas, 11 follicular carcinomas, 170 papillary carcinomas, and six undifferentiated carcinomas, of 165 female and 42 male patients aged from 12–85 yr (mean, 52 yr) at the time of operation. Of 207 thyroid tumors studied, there were 51 cases (24.6%) with *BRAF* mutation. Although we examined both *BRAF* exons 11 and 15, the mutations were limited to the T1796A (V599E) in exon 15. No mutations of *BRAF* were detected in the normal thyroid tissues surrounding malignant tissue in the six examined *BRAF* mutation-positive thyroid cancers, suggesting that the mutations were somatically acquired. Further analysis according to tumor type showed that of the 51 thyroid tumors with *BRAF* mutation (Table 2), none was follicular adenoma or follicular carcinoma, 49 were papillary carcinomas, and two were undifferentiated carcinomas.

Correlation analysis between BRAF mutation and clinical parameters

We examined the correlation between *BRAF* mutation and various clinicopathological parameters in 126 patients with

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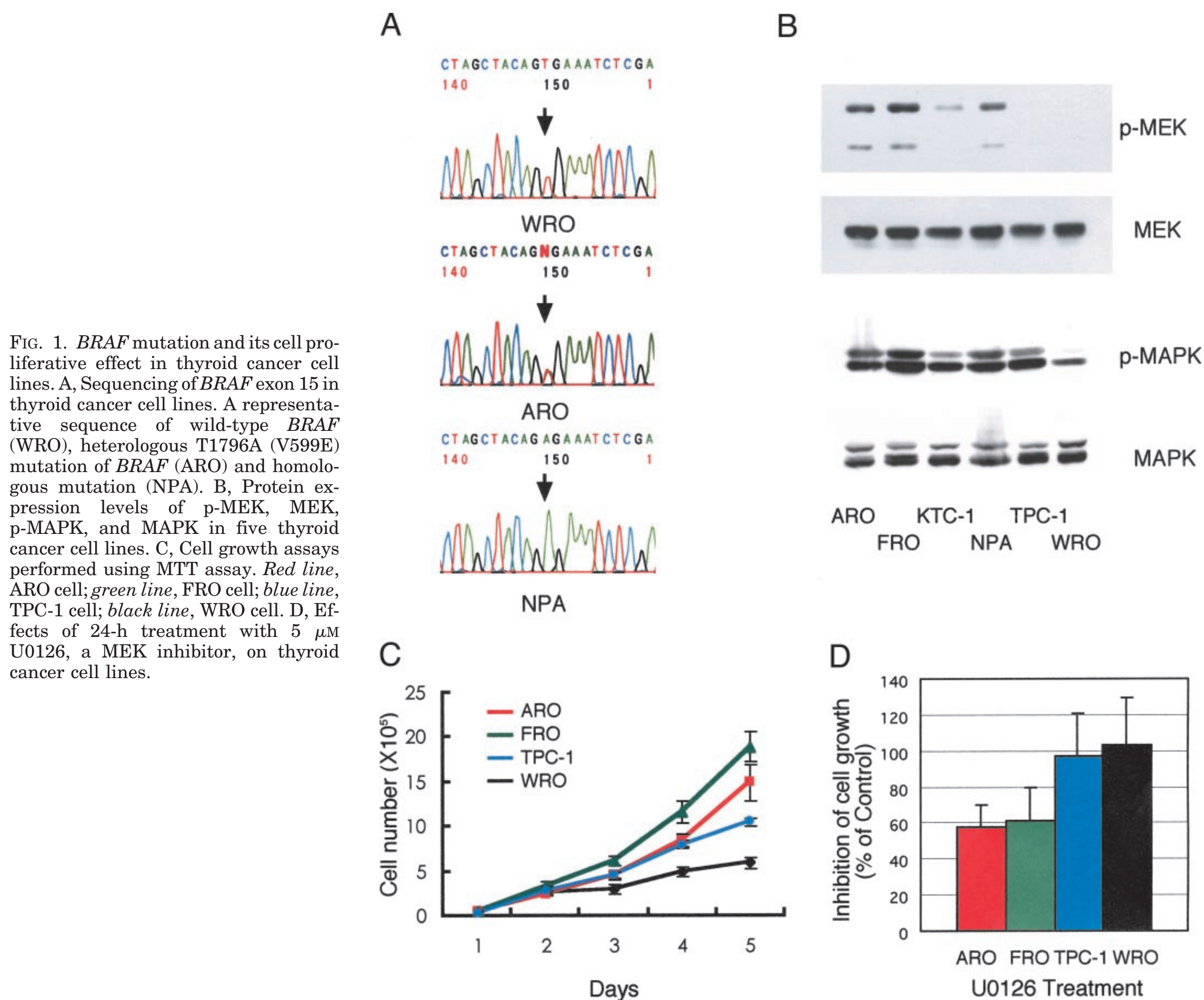

FIG. 1. *BRAF* mutation and its cell proliferative effect in thyroid cancer cell lines. A, Sequencing of *BRAF* exon 15 in thyroid cancer cell lines. A representative sequence of wild-type *BRAF* (WRO), heterologous T1796A (V599E) mutation of *BRAF* (ARO) and homologous mutation (NPA). B, Protein expression levels of p-MEK, MEK, p-MAPK, and MAPK in five thyroid cancer cell lines. C, Cell growth assays performed using MTT assay. *Red line*, ARO cell; *green line*, FRO cell; *blue line*, TPC-1 cell; *black line*, WRO cell. D, Effects of 24-h treatment with 5 μM U0126, a MEK inhibitor, on thyroid cancer cell lines.

TABLE 1. Results of mutation analysis of *BRAF*, *ras*, and *RET* genes in six human thyroid cancer cell lines

Genes	Human thyroid cancer cell lines					
	ARO	FRO	KTC-1	NPA	TPC-1	WRO
BRAF exon 11	–	–	–	–	–	–
BRAF exon 15	V599E hetero	V599E homo	V599E hetero	V599E homo	–	–
H-ras	–	–	–	–	–	–
K-ras	–	–	–	–	–	–
N-ras	–	–	–	–	–	–
RET/PTC1	–	–	–	–	+	–
RET/PTC3	–	–	–	–	–	–

Codons 12, 13, 59, and 61 of *ras* genes were examined.
–, No mutation.

papillary thyroid cancer (Table 3). There was no significant correlation between *BRAF* mutation and sex, age, nodal metastasis, or extrathyroidal invasion at a median postoperative follow-up period of 6 yr. However, there was a significant

TABLE 2. *BRAF* mutations according to tumor type in 207 thyroid tumors

Tumor type	No. of cases	Mutation of *BRAF*	
		Positive (%)	Negative (%)
Follicular adenoma	20	0 (0)	20 (100)
Follicular carcinoma	11	0 (0)	11 (100)
Papillary carcinoma	170	49 (28.8)	121 (71.2)
Undifferentiated carcinoma	6	2 (33.3)	4 (66.7)

correlation between *BRAF* mutation and clinical stage (P = 0.049; Mann-Whitney U test) and distant metastasis to lung or bone (P = 0.033; χ^2 for independence test).

Discussion

Here, mutations of *BRAF* exons 11 and 15 were examined in six human thyroid cancer cell lines and 207 thyroid tumor tissues. These two exons were specifically selected because all previously reported *BRAF* mutations were identified within these two exons (9). Unlike with other types of cancer,

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TABLE 3. Correlation between *BRAF* mutation and various clinicopathological parameters in 126 papillary thyroid cancers

	BRAF mutation				*P*
	Positive		Negative		
	n	(%)	n	(%)	
Age					
<45 yr	5	13.2	18	19.3	0.331
≥45 yr	33	86.8	70	80.7	
Gender					
Female	27	71.1	69	78.4	0.374
Male	11	28.9	19	21.6	
Tumor size					
<10 mm	6	15.8	10	11.4	
10–40 mm	26	68.4	68	77.2	>0.99
>40 mm	6	15.8	10	11.4	
Extrathyroidal invasion					
No	24	63.2	64	72.7	0.286
Yes	14	36.8	24	27.3	
Nodal metastasis					
No	17	44.7	34	38.6	0.523
Yes	21	55.3	54	61.4	
Stage					
I	5	13.2	22	25.0	0.049
II	7	18.4	20	22.7	
III	20	52.6	41	46.6	
IV	6	15.8	5	5.7	
Distant metastasis					
No	31	84.2	83	94.3	0.033
Yes	7	15.8	5	5.7	

only T1796A (V599E) missense mutation was observed in four of six thyroid cancer cell lines and 51 of 207 thyroid tumors. Of 51 thyroid tumors with *BRAF* mutation, there were 49 papillary thyroid carcinomas and two undifferentiated carcinomas. Because undifferentiated carcinoma arises from differentiated carcinoma, including papillary carcinoma, *BRAF* mutation seemed to be papillary phenotype-specific in thyroid tumors. In contrast, *BRAF* mutation was observed in different types of melanocytic nevi and melanomas at high rates, which indicates that the mutation is a critical initiation step in melanocytic neoplasia (10). Thus, our results and those of previous studies suggest that *BRAF* mutation plays a different role in the development of tumors in a tissue-type specific manner.

In this study, we found the significant correlation between with *BRAF* mutation and clinical stage. Thyroid cancers with *BRAF* mutation were characterized as advanced cancers with metastasis. Consistent with our finding, Webb *et al.* (17) demonstrated experimentally that the Raf/MEK/MAPK pathway mediates metastasis as well as tumor growth. Our results suggest that *BRAF* mutation could be a useful marker of poor prognosis of patients with thyroid cancer.

Because activating *ras* mutations exist in about 30% of thyroid tumors (18), we examined the *ras* gene mutations in *BRAF* mutation-positive tumors. No *H, N*, and *K-ras* mutations were detected in these tumors (data not shown). Mutations of *ras* genes have been described in both follicular adenomas and follicular carcinomas, suggesting that *ras* activation is an early step in thyroid tumorigenesis (19). In contrast, *BRAF* mutation is mainly associated with the papillary phenotype of differentiated thyroid cancers and cancers of clinically advanced stage. These results suggest that activation of RAS and that of BRAF play different roles in thyroid tumorigenesis, although both molecules activate MAPK. Sirakawa *et al.* (20) have demonstrated that activation of RAS induces apoptosis of thyroid cells. Activated RAS may affect not only MAPK but also other pathway(s) predisposed to apoptosis. Thus, it seems that the comprehensive effects of constitutive activation of MAPK pathway and other intracellular signals, which are simultaneously activated by the mutation of component genes forming the Ras/Raf/MEK/MAPK pathway, determine the histopathological phenotype and/or aggressiveness of human thyroid tumors.

Because MAPK is thought to be essential for cellular growth in various cancers, this pathway is a target for pharmacological intervention in proliferative diseases (21). In particular, inhibition of MEK represents a suitable target for therapy because of its substrate specificity. In this study, U0126, which inhibits phosphorylation of MEK1/2, suppressed cell growth in *BRAF* mutation-positive cell lines. Small molecule inhibitors of MEK1/2 have already been developed, and one of them induces potent growth inhibition of colorectal tumors *in vivo* (22). Such inhibitors may be used orally as noncytotoxic agents for clinical management of patients with thyroid advanced cancers in the near future.

In conclusion, our study provided clinical evidence that *BRAF* mutation, V599E, correlates with advanced pathological stage in papillary thyroid cancers. The search for *BRAF* mutation seems to be useful and valuable for evaluation of prognosis of patients with papillary thyroid cancer.

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