Targeted next-generation sequencing of cancer-related genes in thyroid carcinoma: A single institution's experience

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Abstract. Thyroid carcinoma (TC) has characteristic genetic alterations, including point mutations in proto-oncogenes and chromosomal rearrangements that vary by histologic subtype. Recent developments in next-generation sequencing (NGS) technology enable simultaneous analysis of cancer-associated genes of interest, thus improving diagnostic accuracy and allowing precise personalized treatment for human cancer. A total of 50 patients who underwent thyroidectomy between 2014 and 2016 at Hokuto Hospital were enrolled. Total DNA was extracted from formalin-fixed, paraffin-embedded tissue sections and quantified. Targeted regions of 24 cancer-associated genes were amplified by PCR, barcoded and sequenced using an Illumina MiSeq platform. Subjects included 30 patients with papillary carcinoma (PC), two with PC tall cell variant (TVPC), two with PC follicular variant (FVPC), eight with follicular carcinoma, seven with poorly differentiated carcinoma (PDC), and one with anaplastic carcinoma (AC). The *BRAF* V600E mutation was present in 25 of 30 (83%) patients with PC, 2 of 2 (100%) patients with TVPC, 6 of 7 (86%) patients of PDC, and one patient with AC. *PIK3CA* mutations were present in 3 of 30 (delPV104P, A1046T and C420R; 10%) patients with PC and 1 of 7 (H1047R; 14%) patients with PDC. The *TP53* mutation was present in 1 of 30 (R306*; 3.3%) patients with PC and 1 of 7 (Q152*; 14%) patients with PDC. The *NRAS* mutation was present in 1 of 2 (Q61K, 50%) patients with FVPC. Statistical analysis showed that patients without the *BRAF* V600E mutation had advanced pathologic T and N stages compared with those with the mutation (P=0.047 and P=0.019, respectively). The *BRAF* V600E mutation was not correlated with overall and disease-free survival in patients with PC. A patient with PC with a mutation in *EGFR* (K852Q) and the *PIK3CA* mutation had an aggressive course with multiple bone and lung metastases. Detection of mutations in cancer-associated genes using NGS could enhance the understanding of the clinical behavior of TC.

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Abbreviations: TC, thyroid carcinoma; PC, papillary carcinoma; TVPC, papillary carcinoma tall cell variant; FVPC, papillary carcinoma follicular variant; FC, follicular carcinoma; PDC, poorly differentiated carcinoma; AC, anaplastic carcinoma; BRAF, v-raf murine sarcoma viral oncogene homolog B1; PI3K/Akt, phosphatidylinositol-3 kinase/v-Akt murine thymoma viral oncogene; PIK3CA, phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit alpha isoform; EGFR, epidermal growth factor receptor; NGS, next-generation sequencing

Key words: thyroid carcinoma, papillary carcinoma, BRAF, PIK3CA, next-generation sequencing

Introduction

Thyroid carcinoma (TC) is the most common malignant tumor in endocrine organs, and its incidence has increased in recent decades (1). A major histologic subtype of TC is papillary carcinoma (PC), which has a good prognosis after surgical treatment. However, we rarely encounter PC patients with an aggressive clinical course such as bone or lung metastasis at the first clinic visit. Poorly differentiated thyroid carcinoma (PDC) represents an aggressive variant of TC with an incidence of 0.8 to 15%, depending on the defining criteria and geographic location (2). Anaplastic carcinoma (AC) accounts for <1% and has a median survival of 3 to 5 months (3). The initiation and progression of TC are associated with the accumulated genetic and epigenetic changes. The observed genetic changes frequently lead to activation of the MAPK or PI3K/Akt signaling pathways. Approximately 70% of TC cases demonstrated one of four

genetic abnormalities: Point mutations in the *BRAF* or *RAS* genes or one of two chromosomal rearrangements (*RET/PTC* or *PAX8/PPARγ*) (4). PDC and AC are thought to arise from pre-existing PC or follicular carcinoma (FC) through additional genetic alterations, including *CTNNB1* and *TP53* mutations (5).

BRAF, a serine-threonine kinase and downstream signaling molecule of Ras and RET, is a potent activator of the MAPK signaling pathway (1,6). *BRAF* mutations have previously been reported in a broad range of human cancers, with the highest prevalence observed in melanoma and TC (6). A T1799A transversion mutation, which occurs in the kinase domain of *BRAF*, located on chromosome 7, results in a single amino acid substitution of valine to glutamic acid (V600E). The *BRAF* V600E mutation potently increases the kinase activity of BRAF by evoking a 480-fold increase in phosphorylation of ERK1/2 compared with wild-type BRAF, resulting in the expression of a number of genes that are involved in cell proliferation, differentiation, survival, tumorigenesis and promotion of epithelial-mesenchymal transition (7).

PIK3CA, the α-type isoform of the catalytic subunit of phosphatidylinositol-3-kinase (PI3K), has been shown to harbor oncogenic mutations in human cancers (8). However, little is known about the role of *PIK3CA* gene mutations in patients with TC (9,10). EGFR is a tyrosine kinase of the ErbB family that regulates signaling pathways for cellular proliferation and survival. Although many types of somatic mutations in the *EGFR* gene have been reported in non-small cell lung carcinoma (NSCLC), few reports have described such mutations in patients with TC (11).

Next-generation sequencing (NGS) technology enables the simultaneous analysis of hundreds of genes of interest using targeted sequence panels. NGS has been used in molecular tumor classification, and the prediction of recurrence and metastasis in some human cancers (12). NGS data are also useful in patient's management, facilitating risk stratification of patients based on the risk of malignancy. In the present report, we describe a patient with rare mutations and the results of mutational analysis using NGS. We attempted to correlate these mutations with clinicopathologic features of patients with TC.

Patients and methods

Patients. The study group consisted of 50 Japanese patients (45 females and 5 males) with a median age of 65 years (range, 26 to 86 years) who underwent curative surgery between 2012 and 2016 at Hokuto Hospital. Patients were classified according to the 8th edition of the AJCC/TNM staging system (13). Histological diagnosis was reviewed by the two experienced pathologists. PDC was diagnosed according to the Turin criteria (14). Written informed consent for publication of clinical details was obtained from all patients. Sampling, storage, and analysis of the tumor samples included in the present study were approved by the internal review board on ethical issues of Hokuto Hospital, Obihiro, Japan (Hokuto Hospital Institutional Ethics Committee no. 83).

Genetic analysis. Surgical specimens were obtained from 50 patients with TC who underwent thyroidectomy. Genetic analysis was performed according to the manufacturer's instructions (15,16). Briefly, total DNA was extracted from 5-μm-thick formalin-fixed paraffin-embedded (FFPE) tissue sections of TC specimens and areas of no pathology using a Maxwell 16 FFPE Plus LEV DNA purification kit (Promega, Madison, WI). The quality of genomic DNA was assessed using a Qubit dsDNA BR assay kit (Life Technologies, Carlsbad, CA) and a GeneRead DNA QuantiMIZE assay kit (Qiagen, Valencia, CA). The GeneRead DNAseq Targeted Panels V2 Human Clinically Relevant Tumor Panel (NGHS-101X; Qiagen) was used for amplicon sequencing of targeted regions of 24 cancer-related genes (*AKT1, ALK, AR, BRAF, CTNNB1, DDR2, EGFR, ERBB2, FGFR3, GNA11, GNAQ, IDH1, IDH2, KIT, KRAS, MAP2K1, MET, NRAS, PDGFRA, PIK3CA, PTEN, RET, STK11, TP53*). Library quality was assessed using an Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA) and GeneRead Library Quant kit (Qiagen). The libraries were sequenced using an Illumina MiSeq (Illumina, San Diego, CA). Raw read data obtained from the amplicon sequencing were processed using online analytical resources from the GeneRead DNAseq Variant Calling Service for analysis of mutations.

Statistical analysis. The significance of differences between two groups was evaluated using Fisher's exact test and summarized with the appropriate P-value. A P-value <0.05 was considered indicative of statistical significance. Odds ratios and 95% confidence intervals were also calculated. Overall survival time was measured from the date of diagnosis to the date of death or date of last follow-up visit. Disease-free survival time was measured from the date of surgical removal of tumor to the date of first relapse or the date of last follow-up. The probability of overall and disease-free survival was calculated using the Kaplan-Meier method and compared using the log-rank test.

Results

Clinicopathologic features. Clinicopathologic features and mutational pattern in 50 patients with TC are listed in Table I. TC subtypes included 30 (60%) patients with PC, 2 (4%) with papillary carcinoma tall cell variant (TVPC), 2 (4%) with papillary carcinoma follicular variant (FVPC), 8 (16%) with FC, 7 (14) with PDC, and 1 (2%) with AC. Tumor size ranged from 0.6 to 7.5 cm with a median size of 2.3 cm. A total of 22 patients (44%) were stage I, 17 (34%) were stage II, 8 (16%) were stage III, and 3 (6%) were stage IVB. Hemi-thyroidectomy with routine central compartment and lateral neck lymph node dissection were performed in 29 (58%) and 4 (8%) patients, respectively. Total thyroidectomy with routine central compartment and lateral neck lymph node dissection were performed in 8 (16%) and 9 (18%) patients, respectively. Disease pathologic T classification of was T1a in 6 (12%) patients, T1b in 4 (8%), T2 in 7 (14%), T3a in 3 (6%), T3b in 16 (32%), T4a in 13 (26%) and T4b in 1 (2%). The pathologic N classification was N0 in 24 (48%) patients, N1a in 12 (24%), and N1b in 14 (28%). Pathologic extrathyroidal extension and multifocal tumors were observed in 30 (60%) and 23 (46%) patients, respectively. Follow-up period ranged from 8 to 78 months, with a median duration of 39 months for

Table I. Clinicopathological features and mutational pattern in 50 patients with thyroid carcinoma.

					Pathologic findings				Mutation			
No.	Age/sex	Histology	Tumor size, cm	Stage	pT	pN	Extension	Multifocality	*BRAF*	*PIK3CA*	*TP53*	Other
1	61/F	PC	2.0	I	1b	0		+	V600E			
2	54/F	PC	1.7	I	3b	0	+		V600E			
3	65/F	PC	1.6	I	3b	0	+		V600E			
4	66/F	PC	1.9	I	3b	0	+		V600E			
5	69/F	PC	2.4	I	3b	0	+		V600E			
6	77/F	PC	3.4	I	2	0			V600E			
7	36/F	PC	1.9	I	4a	1a	+	+	V600E			
8	75/F	PC	2.5	I	2	0		+	V600E			
9	52/M	PC	1.7	I	3b	1a	+	+	V600E			
10	54/F	PC	0.8	I	1a	0		+	V600E			
11	56/F	PC	2.5	II	3b	1a	+		V600E			
12	56/M	PC	4.0	II	2	1a			V600E			
13	72/F	PC	0.7	II	3b	0	+	+	V600E			
14	67/F	PC	2.3	II	3b	1a	+		V600E			
15	63/F	PC	3.1	II	3b	1b	+	+	V600E			
16	71/F	PC	5.0	II	3b	1b	+	+	V600E			
17	62/F	PC	2.2	II	3b	1a	+		V600E			
18	55/F	PC	1.6	III	4a	1a	+		V600E			
19	73/F	PC	2.3	III	4a	1a	+		V600E			
20	73/F	PC	2.3	III	4a	0	+	+	V600E			
21	80/F	PC	2.1	III	4a	1b	+		V600E			
22	56/F	PC	1.4	III	4a	1b	+	+	V600E			
23	86/F	PC	1.1	III	4a	0	+		V600E			
24	77/F	PC	2.7	III	4a	0	+		V600E	delPV104P		
25	77/F	PC	3.2	II	3b	1b	+	+	V600E	A1046T	R306*	*FGFR3* (G382R)
26	86/F	PC	5.0	IVB	4b	1b	+	+		C420R		*EGFR* (K852Q)
27	49/F	PC	5.9	I	4a	1b	+					
28	83/F	PC	1.0	III	4a	1b	+					
29	78/F	PC	1.1	II	3b	0	+	+				
30	51/M	PC	1.5	I	4a	1b	+	+				
31	26/F	TVPC	0.8	I	3b	1b	+	+	V600E			
32	83/F	TVPC	4.5	II	3b	1b	+	+	V600E			
33	85/F	FVPC	4.4	II	3a	0						
34	56/F	FVPC	1.7	I	1b	0		+				*NRAS* (Q61K)
35	49/F	FC	3.2	I	2	0						
36	76/F	FC	3.6	I	2	0						
37	71/F	FC	1.0	I	1a	0						
38	56/F	FC	3.4	I	2	0						
39	55/F	FC	7.5	I	1a	0						
40	54/F	FC	3.6	I	2	0						
41	74/F	FC	6.5	II	3a	0						
42	71/M	FC	4.6	II	3a	0						
43	76/F	PDC	1.6	II	1b	1a		+	V600E			
44	81/F	PDC	4.2	IVB	4a	1b	+	+	V600E			
45	74/F	PDC	1.4	II	1b	1a			V600E			
46	74/F	PDC	2.0	II	3b	0	+		V600E			
47	51/F	PDC	2.5	I	4a	1a	+	+	V600E			

Table I. Continued.

No.	Age/sex	Histology	Tumor size, cm	Stage	Pathologic findings				Mutation			
					pT	pN	Extension	Multifocality	*BRAF*	*PIK3CA*	*TP53*	Other
48	28/M	PDC	1.0	I	1a	1b		+		V600E	H1047R	
49	67/F	PDC	0.7	II	1a	1a		+				
50	86/F	AC	0.6	IVB	1a	1b		+		V600E		Q192*

F, female; M, male; PC, papillary carcinoma; TVPC, papillary carcinoma tall cell variant; FVPC, papillary carcinoma follicular variant; FC, follicular carcinoma; PDC, poorly differentiated carcinoma; AC, anaplastic carcinoma; pT, pathological T stage; pN, pathological N stage.

all patients. Forty-five (90%) of the patients are alive without disease. Three (6%) patients (PC: 1, PDC: 1, and AC: 1) died of disease due to distant metastasis. One patient with PC was alive with neck lymph node recurrence, and 1 patient with PC was alive with lung metastasis at the time of this report.

Mutational analysis. The *BRAF* V600E mutation was present in 25 (83%) of 30 patients with PC, in 2 (100%) of 2 with TVPC, in 6 (86%) of 7 with PDC, and in 1 AC patient (100%). *PIK3CA* mutations were present in 3 (delPV104P, A1046T, and C420R; 10%) of 30 patients with PC and 1 (H1047R; 14%) of 7 with PDC. *TP53* mutations were present in 1 (R306*; 3.3%) of 30 patients with PC and 1 (Q152*; 14%) of 7 with PDC. An *NRAS* mutation (Q61K) was present in 1 of 2 patients with FVPC. An *FGFR3* mutation (G382R) was present in 1 of 30 patients with PC, and an *EGFR* mutation (K852Q) was present in 1 of 30 patients with PC.

Correlation of BRAF V600E mutation with clinicopathologic factors in PC. Statistical analyses of the 30 patients with PC showed no significant correlation between the *BRAF* V600E mutation and clinicopathologic factors such as age, sex, tumor size, stage, extrathyroidal extension, and multifocal tumor (Table II). However, patients without the *BRAF* V600E mutation had more advanced pathologic T and N stages compared to patients with the mutation (P=0.047 and P=0.019, respectively). Kaplan-Meier analysis showed that *BRAF* V600E mutation was not significantly correlated with overall (P=0.299, Fig. 1A) or disease-free survival (P=0.401, Fig. 1B) in patients with PC.

Case presentation of patient no. 26. An 86-year-old female complained of dyspnea and suffered from pathologic fracture of the left femur. Enhanced computed tomography (CT) scan revealed a thyroid tumor with invasion of the trachea and esophagus (Fig. 2A). CT and fluorodeoxyglucose (FDG)-positron emission tomography (PET)/CT scans showed multiple bone metastases, including to the cranial bone (Fig. 2B), humerus, and femur (Fig. 2C), as well as multiple lung metastases (Fig. 2D). Histologic analysis of specimens from the thyroid tumor indicated PC. Genetic analysis of the thyroid tumor using NGS showed that the patient harbored *EGFR* (K852Q) and *PIK3CA* (C420R) mutations but no *BRAF* mutation. After total thyroidectomy with tracheal resection, the patient died, 34 months after the first clinic visit.

Discussion

BRAF mutations in TC have been vigorously investigated since the early 2000s (17,18). The frequency of the *BRAF* V600E mutation reportedly ranges from 32 to 80% in patients with PC (4,19-21). Several large-scale multicenter studies reported that the average frequency of the *BRAF* V600E mutation in PC is approximately 48% (22,23). In the present study, the frequency of the *BRAF* V600E mutation in PC was 83%, which was higher than that previously reported. The frequency in the present study may be biased due to the small number of patients analyzed. However, the higher frequency could also be attributed to tumors in patients from specific geographic locations and to methodologic differences. Recent studies from eastern Asia demonstrated a higher frequency of approximately 80% for the *BRAF* V600E mutation in PC, which is consistent with our results (24-27). Residents in eastern Asia commonly consume seaweeds as a part of their regular diet. The region where our hospital is located, and in which all the patients involved in this study resided is well known for seaweed production and consumption. Iodine intake has been linked with a higher frequency of *BRAF* mutations in Korean patients with PC (28). Guan *et al* (29) reported that high iodine intake is associated with a higher prevalence of the *BRAF* V600E mutation in Chinese patients with PC. Elisei *et al* (30) suggested that iodine supplementation might be associated with the increasing trend of *BRAF* mutation in PC.

The frequency of *BRAF* mutations reported in the literature has increased significantly over the years (31). This may be related to innovations in methodologies used to detect mutations. The use of NGS could be associated with the higher frequency of the *BRAF* V600E mutation in patients with PC noted in the present study. To date, the frequency of the *BRAF* V600E mutation in patients with PC has been analyzed using Sanger sequencing (SGS) with FFPE (21,26), SGS with frozen tissue (32,33), pyrosequencing (34) and real-time PCR (27). In the present study, we analyzed FFPE tissue sections obtained from 50 patients with TC using an Illumina Miseq sequencer. Since 2013, only 2 reports concerning PC and 2 reports concerning PDC and AC were published describing results of mutational analyses using NGS with FFPE tissue sections (2,35-37). Tumor samples are histologically heterogeneous (15), and tumor-specific DNA contains varying proportions of contaminating DNA from normal and inflammatory cells. NGS methods enables the analysis of somatic

Table II. Correlation of *BRAF* V600E mutation with clinicopathologic factors in 30 patients with papillary carcinoma.

Variables	No. of patients	*BRAF* V600E mutation		P-value	OR	95% CI
		+ (n=25)	- (n=5)			
Age, years						
<55	6	4	2	0.254	1.00	
≥55	24	21	3		0.29	0.04-2.30
Sex						
Male	3	2	1	0.434	1.00	
Female	27	23	4		0.35	0.02-4.80
Tumor size, cm						
<2	14	11	3	0.642	1.00	
≥2	16	14	2		0.52	0.07-3.70
Stage						
I, II	21	18	3	0.622	1.00	
III, IVB	9	7	2		1.71	0.23-12.60
pT						
1a-3b	19	18	1	0.047	1.00	
4a, 4b	11	7	4		10.29	1.00-109.00
pN						
0, 1a	21	20	1	0.019	1.00	
1b	9	5	4		16.00	1.45-177.00
Extrathyroidal extension						
-	5	5	0	0.556	1.00	
+	25	20	5		1.25	1.02-1.52
Multifocality						
-	16	14	2	0.642	1.00	
+	14	11	3		1.91	0.27-13.50

P-values were calculated with Fisher's exact test. OR, odds ratio; CI, confidence interval; pT, pathological T stage; pN, pathological N stage.

mutation using a small amount of tumor-specific DNA (38). NGS can detect a broad range of mutations, including single nucleotide substitutions, small insertions and deletions, and large genomic duplications. Moreover, targeted NGS is more cost efficient and faster than SGS (39). In general, the detection sensitivity of NGS reported in previous studies is >94% (40), which is greater than that of SGS.

Results from numerous studies and meta-analyses have associated the *BRAF* V600E mutation with high-risk clinicopathologic features, such as larger tumor size, extrathyroidal extension, higher stage at presentation, and lymph node and distant metastases in patients with PC (4,20,22,30,31,41-43). However, these associations remain controversial. A number of other reports have suggested that there is no significant association between the *BRAF* V600E mutation and high-risk clinicopathologic features in patients with PC (25,34,44-46). In the present study, contrary results were obtained, in that PC patients without the *BRAF* V600E mutation had more advanced pathologic T and N stages compared to patients with the mutation. There have been few reports that support our results. The much lower number of patients without the *BRAF* V600E mutation (n=5) in our study compared with the number of patients with the mutation (n=25) could have affected our results; that is, the *BRAF* V600E mutation-negative group could have been biased. However, 1 of the 5 PC patients without the *BRAF* V600E mutation was previously described in the case presentation as having *PIK3CA* and *EGFR* mutations. Three other patients without the *BRAF* V600E mutation had copy number alterations (CNAs) in either the *PIK3CA*, *PTEN*, *DDR2*, *STK11*, or *ERBB2* genes whereas only 2 of 25 patients with *BRAF* V600E mutation had the alterations. We hypothesize that the accumulation of other genetic alterations except for the *BRAF* V600E mutation might have contributed to the advanced pathologic stages in these patients.

A recent retrospective analysis of 1849 PC patients found a mortality rate of 5.3% in *BRAF* V600E mutation-positive patients vs. 1.1% in mutation-negative patients (43). In contrast, Pelttari *et al* (47) suggested that there was no association between the *BRAF* V600E mutation and recurrence following primary treatment with total thyroidectomy and radioiodine remnant ablation in patients with PC. A study of non-high-risk PC patients in Japan found no prognostic impact of the *BRAF* V600E

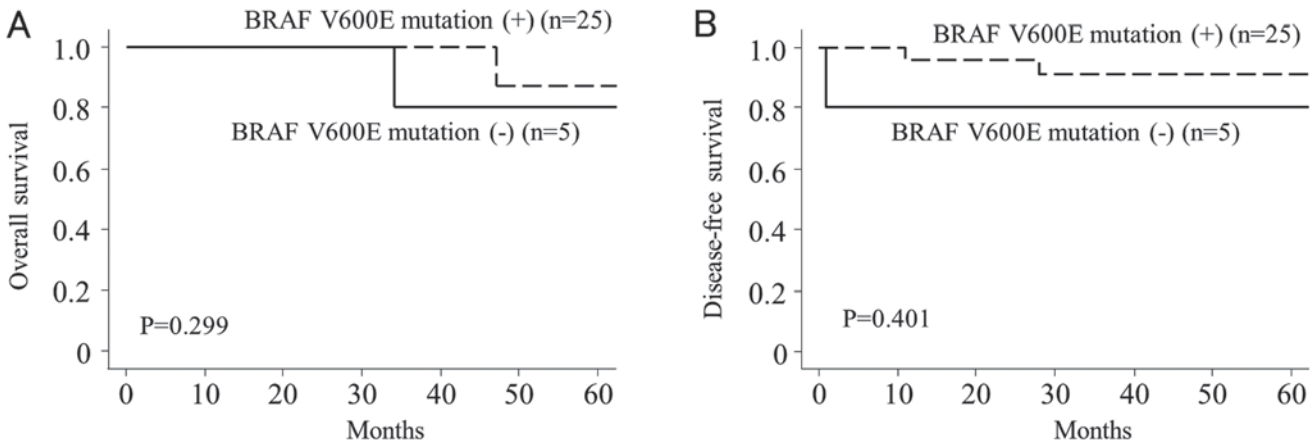

Figure 1. Kaplan-Meier survival curves for papillary carcinoma patients with *BRAF* V600E mutation. There were no statistically significant differences in overall (A) or disease-free (B) survival between patients with and without the *BRAF* V600E mutation.

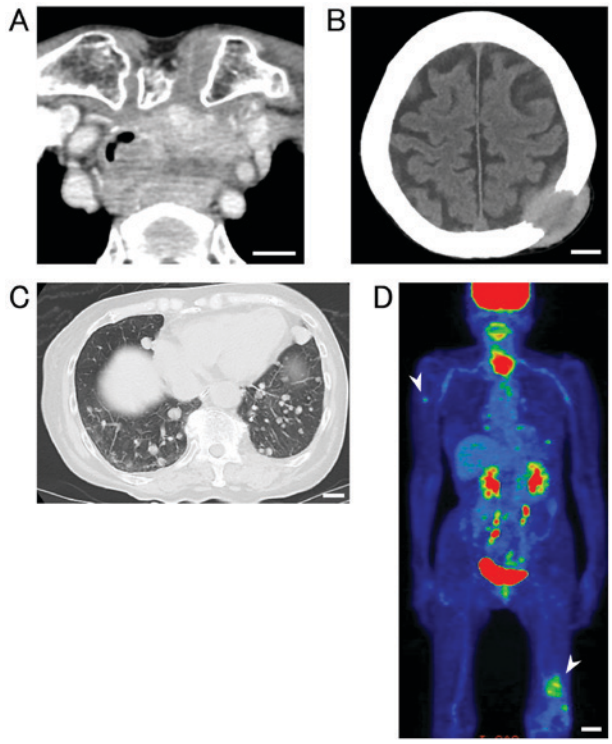

Figure 2. Imaging findings for patient no. 26 with *EGFR* (K852Q) and *PIK3CA* (C420R) mutations but without the *BRAF* V600E mutation. (A) Enhanced neck CT scan revealed invasion of the thyroid tumor into the trachea and esophagus. CT scan indicated metastasis of tumors to the cranial bone (B) and the lung (C). Scale bar, 2 cm. (D) FDG-PET/CT scan indicated metastasis of tumors to the right humerus and left femur (arrow heads). Scale bar, 5 cm.

mutation on lymph node recurrence-free, distant recurrence-free, or cause-specific survival (46). In the present study, there was no correlation between the *BRAF* V600E mutation and overall and disease-free survival in patients with PC. We obtained inconsistent results, in that PC patients without the *BRAF* V600E mutation had more advanced pathologic T and N stages but did not show poor survival. That appropriate surgery was performed depending on the extention of T and N stages in these subjects could explain this inconsistency. Otherwise, other markers except for *BRAF* V600E mutation may be associated with poorer prognosis. Shimamura *et al* (48) suggested that the *BRAF* V600E mutation alone is not sufficient for development of PC. This, however, does not mean that *BRAF* V600E is not the driver mutation, but rather that additional genetic and/or epigenetic changes may be required for full transformation in PC. Several other studies have agreed with this hypothesis, reporting associations between development of PC and increased expression of several tumor promoting molecules, including vimentin (49), matrix metalloproteinase (50), nuclear factor-κB (51), prohibitin (52), vascular endothelial growth factor (53), and hepatocyte growth factor receptor (54). A recent report indicated that the telomerase reverse transcriptase (*TERT*) promoter is a poor prognostic factor in patients with PC (55).

In the present study, 2 patients with TVPC harbored the *BRAF* V600E mutation, whereas 2 patients with FVPC and 8 patients with FC did not harbor the *BRAF* V600E mutation. TVPC, a subtype of PC, is characterized by a predominance of tall and oncocytic tumor cells. Patients with TVPC exhibit a higher recurrence rate and decreased disease-specific survival (56). The *BRAF* V600E mutation is reportedly common in approximately 80% of TVPC cases (4). By contrast, in FVPC, another subtype of PC, the *BRAF* V600E mutation is less common, reportedly found in only approximately 10% of patients (4,57). FVPC is instead characterized by a high prevalence of mutations other than *BRAF* V600E, such as mutations in *RAS* and other factors, which has been associated with follicular-pattern thyroid tumors, including FC and follicular adenoma (4). The *BRAF* V600E mutation also occurs in PDC and AC arising from PC (4,18). In the present study, 7 PDCs and 1 AC were pathologically diagnosed as derived from PC.

Mutations in *PIK3CA* that enhance PI3K/Akt signaling are associated with tumor progression and dedifferentiation in some human cancers and occur at an early stage in tumorigenesis in TC (9). Using an NGS approach, Nikiforova *et al* (35) showed that *BRAF* mutations are the most frequent (59%), followed by mutation in *PIK3CA* (11%), *TP53* (7%), and *NRAS* (4%). Lee *et al* (26) also demonstrated *BRAF* mutations in 79.2% of PC patients and *PIK3CA* mutations in 10.4%. These data are consistent with our results demonstrating that the second most frequent genetic mutations occurred in *PIK3CA*

in 10% of patients with PC. Over 90% of the mutations in the *PIK3CA* gene in human cancers occur in 4 regions: The p85 binding (exons 1 and 2), C2 (exon 7), helical (exon 9), and catalytic (exon 20) domains (58). Four mutations in *PIK3CA* we identified were located within these regions, as previously reported. *PIK3CA* mutations are related to tumor development, progression and more aggressive behavior in TC (9). Therefore, detecting *PIK3CA* mutations in patients with PC is also critical (59).

In the case presentation, we presented a patient with *EGFR* and *PIK3CA* mutations who exhibited an aggressive clinical course. *EGFR* mutations are commonly found in NSCLC, but they are less common in PC. The most common genetic alterations in the *EGFR* gene are in-frame deletions in exon 19 and point mutations in exon 21 in the intracellular tyrosine kinase domain (60,61). The role of *EGFR* mutation in TC remains unclear. Masago *et al* (11) reported 8PC patients with in-frame deletion and/or L858R mutations in *EGFR*. One of the 8 patients showed distant metastasis as the initial manifestation. A study of Korean patients found *EGFR* mutations and increased copy number in 14 of 23 analyzed samples, suggesting that *EGFR* genetic alterations are correlated with the biological dedifferentiation process in TC (62). Of the 30 patients with PC in the present study, the patient who showed multiple bone and lung metastases at the first clinical visit and died with the disease had an *EGFR* mutation. We hypothesize that *EGFR* mutations in patients with PC are related to aggressive tumor behaviors such as multiple lung and bone metastases.

There are some limitations to the present study. First, we analyzed only 50 patients with TC, which was an insufficient number of patients to correlate mutational status with clinical significance. Most studies conducted to date were carried out at a single institution using specific subtypes of TC with small sample sizes. To overcome this limitation, multicenter studies examining TC by geographic location will be required. Second, we used a commercially available panel that targets only 24 cancer-related genes in the NGS analysis. The panel was not specific for TC and not able to elucidate the underlying mechanism of tumorigenesis in TC. Nikiforova *et al* has already conducted an analysis of gene fusions, CNA, and abnormal gene expression as well as mutational analysis of more than 100 genes with the latest panel ThyroSeq v3 for thyroid tumor (63). In this way, a thyroid cancer-specific gene panel that targets a larger number of cancer genes should be employed in conjunction with NGS. Furthermore, analysis of rearrangements in *RET*/*PTC* and *PAX8*/*PPAR*γ in TC should be carried out. Comprehensive molecular testing of both gene mutations and rearrangements using new sequencing technologies will contribute to the development of new screening systems for predicting clinical outcome and assist in the development of new molecular target treatments.

In conclusion, NGS analysis of 24 cancer-related genes using FFPE tissue sections from 50 patients with TC revealed the *BRAF* V600E mutation in 83% of patients with PC and 86% of patients with PDC. Statistical analyses showed that patients without this *BRAF* mutation had more advanced pathologic T and N stages. A PC patient with *EGFR* and *PIK3CA* mutations but without the *BRAF* V600E mutation showed an aggressive course including multiple bone and lung metastases. Analysis of cancer-related genes using NGS approaches can enhance our understanding of the biological behavior of TC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

NB, TG, MK, HI and KS performed surgery and acquired data. TA, TS and TY performed the mutational analyses. HNa and YuK confirmed the mutational analysis data. HNi and YaK performed the pathologic diagnoses. HK and YH conceived the study design. NB drafted the manuscript and analyzed the clinical data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Written informed consent for publication of clinical details was obtained from all patients. Sampling, storage, and analysis of the tumor samples included in the present study were approved by the internal review board on ethical issues of Hokuto Hospital, Obihiro, Japan (Hokuto Hospital Institutional Ethics Committee no. 83).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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