Concomitant *RAS*, *RET/PTC*, or *BRAF* Mutations in Advanced Stage of Papillary Thyroid Carcinoma

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Background: *RET/PTC* rearrangement, *RAS*, and *BRAF* mutations are considered to be mutually exclusive in papillary thyroid carcinoma (PTC). However, although concomitant mutations of *RET/PTC*, *RAS*, or *BRAF* have been reported recently, their significance for tumor progression and survival remains unclear. We sought to examine the prognostic value of concomitant mutations in PTC.

Methods: We investigated 88 PTC for concomitant mutations. Mutation in *BRAF* exon 15, *KRAS*, *NRAS*, and *HRAS* were studied by polymerase chain reaction (PCR)-sequencing of tumor DNA; *RET/PTC* rearrangement was determined by reverse transcription (RT)-PCR-sequencing of tumor cDNA.

Results: *BRAF*^{V600E} was detected in 39 of 82 classic PTC (CPTC) and in all three tall-cell variants (49%, 42/85). *KRAS* mutation (p.Q61R and p.S65N) was detected in two CPTC (2%, 2/88) and *NRAS*^{Q61R} in one CPTC and two follicular variant PTC (FVPTC; 3%, 3/88). *KRAS*^{S65N} was identified for the first time in thyroid cancer and could activate mitogen-associated protein kinase (MAPK). *RET/PTC-1* was detected in nine CPTC, one tall-cell variant, and two FVPTC. Concomitant *BRAF*^{V600E} and *KRAS*, or *BRAF*^{V600E} and *RET/PTC-1* mutations were found in two CPTC, and six CPTC and one tall-cell variant, respectively. In total, 11 concomitant mutations were found in 88 PTC samples (13%), and most of them were in the advanced stage of disease (8/11, 73%; $p < 0.01$).

Conclusions: Our data show that concomitant mutations are a frequent event in advanced PTC and are associated with poor prognosis. The concomitant mutations may represent intratumor heterogeneity and could exert a gene dosage effect to promote disease progression. *KRAS*^{S65N} can constitutively activate the MAPK pathway.

Introduction

THYROID CARCINOMA IS THE MOST COMMON type of endocrine malignancy and can be classified into papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), and anaplastic thyroid carcinoma (ATC). PTC is the most common type of differentiated thyroid carcinoma, accounting for more than 80% of thyroid malignancies (1). Genetic alterations in the mitogen-associated protein kinase (MAPK) pathway play an important role in the initiation and progression of PTC. Notable among them are *RAS* point mutations, *RET/PTC* oncogene rearrangements, and *BRAF* point mutations. *BRAF* mutations are the most common genetic alteration in PTC from 28% to 83%, with an overall rate of 44% (2–4).

It is generally believed that genetic alterations in *RET/PTC*, *RAS*, and *BRAF* are mutually exclusive in PTC, and mutations at more than one of these genes are unlikely to provide an additional biological advantage (2,5,6). However, recent studies have shown concomitant mutations in PTC (7–10). Di

Cristofaro *et al.* reported concomitant *RAS* and *RET/PTC1* mutations in 1/24 (4%) follicular variant PTC (FVPTC), and *BRAF* and *RET/PTC3* mutations in 1/26 (4%) classic PTC (CPTC) (8). Costa *et al.* showed concomitant *BRAF* and *KRAS* mutations in 4/35 PTC (11%) (10), whereas Henderson *et al.* reported concomitant *BRAF* and *RET/PTC* mutations in 5/54 (9.3%) recurrent PTC (7). Zhu *et al.* reported concomitant mutations of *RET/PTC* and *RAS* or *BRAF* in 8/15 (53%) subclonal or nonclonal PTC but none in clonal PTC (9). It is not clear whether concomitant mutations are associated with aggressive disease behavior and poor prognosis. In the present study, we investigated concomitant mutations in *RET/PTC*, *RAS*, and *BRAF* in 88 PTC from Saudi Arabia and its association with disease progression and prognosis.

Materials and Methods

Thyroid tumor specimens and cell lines

All tumor tissues were obtained at surgery with informed consent, and were immediately frozen in liquid nitrogen and

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stored at -70°C until processed. The clinical staging of thyroid cancer was based on the Tumor, Node, Metastasis (TNM) classification (11). Eighty-eight PTC diagnosed between 1987 and 2006 were selected randomly and included in the study: 82 CPTC, three tall-cell variants, and three FVPTC. The mean follow-up interval was 12 years. The following cell lines were studied to determine whether they have dual mutations in *RET/PTC*, *RAS*, or *BRAF*: K1, BCPAP, 8305C, 8505C, HTC-C3, BHT101, CAL62, TPC-1, and FRO. TPC-1 and FRO were kindly provided by Dr. James Fagin (Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY). Other cell lines were purchased from DSMZ (Braunschweig, Germany). ATC cell lines such as 8305C, 8505C, BHT101, FRO, and CAL62 were screened for concomitant mutations, since all of them carry either *BRAF* or *RAS* mutations, and it is not clear whether they have dual mutations in the MAPK pathway. The study was reviewed and approved by the Institutional Review Board.

Detection of *BRAF* and *RAS* mutations

Tumor tissue was obtained by standard sectioning, and DNA was extracted by standard proteinase-K treatment followed by phenol/chloroform extraction. *BRAF* exon 15 was amplified by PCR and directly sequenced as described previously (12). Mutations of *KRAS*, *NRAS*, and *HRAS* were analyzed by polymerase chain reaction (PCR) sequence analysis of exon 2 (for codon 12/13), exon 3 (for codon 61), and exon 4 in *KRAS* and *NRAS*; and exon 1 (codon 12/13), exon 2 (codon 61) and exon 3 in *HRAS*. Exon 4 was screened for non-hotspot mutations. PCR primers are listed in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/thy). The PCR conditions were 95°C for 5 min followed by 35 cycles of amplification (95°C for 40 s, 50 – 62°C for 40 s, and 72°C for 40 s) with final extension at 72°C for 5 min. The resulting PCR products were analyzed by gel electrophoresis. Each successfully amplified fragment was directly sequenced using the BigDye Terminator V3.1 Cycle Sequencing kit and ABI prism 3100 sequencer (Applied Biosystems, Foster City, CA). DNA from paraffin blocks of patients 27 and 78 was extracted as described previously (13).

Detection of *RET/PTC* rearrangements

Total RNA was extracted from tumor specimens and cell lines by the guanidine thiocyanate-phenol-chloroform method. The integrity of RNA was verified by denaturing gel electrophoresis. Two μg of total RNA were reverse-transcribed into cDNA using the Promega reverse transcription (RT) system (Promega, Madison, WI). Nested RT-PCR was used to amplify transcripts of *RET/PTC* rearrangements, using two sets of primers listed in Supplementary Table S1 and PCR conditions described above. The resulting PCR products were analyzed by gel electrophoresis and directly sequenced. GAPDH cDNA was used as an internal control for RNA quality. To rule out PCR contamination, positive samples were confirmed by repeating PCR using different batches of same specimen.

Screening for *PAX8/PPAR γ* rearrangement in FVPTC

A fusion gene (*PAX8/PPAR γ*) between the DNA binding domain of the thyroid-restricted transcription factor paired

box gene 8 (*PAX8*) and the peroxisome proliferator-activated receptor γ gene (*PPAR γ*) has been detected in about 10% of FVPTC. *PAX8/PPAR γ* rearrangement was screened by RT-PCR in FVPTC as described previously (14).

Cloning and expression of mutant *KRAS*^{S65N} and *NRAS*^{S65C}

The full-length *KRAS* and *NRAS* cDNA clones were obtained from GenScript (Piscataway, NJ) and cloned into pcDNA3.1 under the control of the CMV promoter (Invitrogen, Carlsbad, CA). *KRAS*^{S65N} and *NRAS*^{S65C} mutants were obtained by site-directed mutagenesis and were verified by DNA sequencing. Wild-type *KRAS* and *NRAS* were used as controls. Equal amounts of the constructs were transfected into HEK293, BCPAP, and CAL62 cell lines using Lipofectamine (Invitrogen). Cells were collected 48 hours after transfection for MAPK and cAMP response element binding protein (CREB) activity.

Western blot analysis

Forty μg of protein from transfected cells was loaded onto a 12% SDS-polyacrylamide gel. Proteins were transferred to a polyvinylidene (PVDF) membrane and subject to Western blot analysis using anti-phospho-MEK1/2, anti-phospho-ERK 1/2 antibody, or anti-phospho-CREB (1:1000; Cell Signaling Technology, Inc., Danvers, MA).

Cell proliferation assay

Cell proliferation was measured by a non-radioactive MTS assay kit according to the manufacturer's procedure (Promega). Briefly, cells were plated in triplicate into 96-well plates (5×10^3 cells/well) containing 1% or 10% serum respectively for 48 h. At the final 4 h of incubation, 20 μL of CellTiter 96[®] AQueous One Solution reagent was added into each well for measurement of cell viability.

Statistical analysis

Fisher's exact test (two-tailed) was used. A *p*-value of ≤ 0.05 was considered significant.

Results

BRAF mutation

BRAF^{V600E} mutation was detected in 39 of 82 classic PTC (48%) and in all three tall-cell variants (Fig. 1 and Table 1). No mutation was detected in FVPTC. The *BRAF*^{V600E} mutation occurred more frequently in the advanced stages (stage III or IV) compared to the early stages (stage I or II) of CPTC: 17/26 (65%) versus 22/56 (39%) (Table 1; *p* < 0.05, Fisher's exact test). The *BRAF*^{V600E} mutation was also detected in 8305C, 8505C, HTC-C3, BHT101, BCPAP, K1, and FRO cells.

RAS mutation

KRAS mutation was detected in two CPTC: c.182A > G (p.Q61R) with stage III cancer, and c.194 G > A (p.S65N) with stage I cancer (Fig. 1). *NRAS*^{Q61R} was detected in one CPTC case and two FVPTC cases. No mutation was found in *HRAS*. The overall rate of *RAS* mutation was 6% (5/88) in the cohort studied (Table 2). *KRAS*^{G12R} was found in the CAL62

FIG. 1. Detection of *BRAF*, *RAS*, and *RET/PTC-1* mutations in papillary thyroid carcinoma (PTC). (A) *RET/PTC-1* rearrangement was detected by reverse transcription polymerase chain reaction (RT-PCR) from patients. The PCR product was run on a 2% agarose gel. GAPDH cDNA was used as an internal control for RNA quality. (B) DNA sequence analysis of PCR products from three patients with concomitant mutations. The *RAS* and *BRAF* mutations are indicated by an arrow. The breakpoint of *RET/PTC-1* rearrangement is indicated by a vertical line.

cell line. *KRAS*^{S65N} has not been reported before in thyroid cancer. The significance of the mutation was further investigated first in matched normal tissue from paraffin blocks. No mutation was found in normal tissue, suggesting that it is not a germ-line polymorphism (data not shown). Multiple protein sequence alignments around codon 65 showed that serine⁶⁵ is highly conserved across different species (data not shown). Next, we used PolyPhen-2 to predict the possible effect of S65N substitution (<http://genetics.bwh.harvard.edu/pph/>). PolyPhen-2 predicted a possible damaging effect with a score of 0.919 of 1.0 (sensitivity: 0.81; specificity: 0.94). To confirm that *KRAS*^{S65N} or *NRAS*^{S65C} (reported previously in neuroendocrine lung cancer and melanoma but not characterized by functional studies) can activate the MAPK signaling pathway, we cloned and expressed *KRAS*^{S65N} and *NRAS*^{S65C} in HEK293 cells. As shown in Figure 2A, both *KRAS*^{S65N} and *NRAS*^{S65C} caused increased p-MEK and p-ERK expression in HEK293 cells. *KRAS*^{S65N} can also increase MAPK activity and cell proliferation in both low- and high-serum conditions (Fig. 2B and C). We further investigated the effect of *KRAS*^{S65N} on MAPK and CREB activity in the PTC cell line BCPAP and the ATC cell line CAL62. CREB is activated by phosphorylation at Ser133 by various signaling pathways, including the MAPK/ERK pathway. As shown in Figure 2B, the expression of both phospho-ERK and phospho-CREB was increased following *KRAS*^{S65N} ex-

pression. The basal level of phospho-ERK was also higher in BCPAP and CAL62 as compared to HEK293, probably due to the fact that BCPAP and CAL62 contain *BRAF*^{V600E} and *KRAS*^{G12R} respectively.

RET/PTC rearrangements

A *RET/PTC-1* rearrangement was detected in nine CPTC (9/82, 11%), one tall-cell variant, two FVPTC, and the TPC-1 cell line (Fig. 1 and Table 2). The overall rate of *RET/PTC-1* was 14% (12/88). *RET/PTC-2* and -3 were not detected in this series of samples. *RET/PTC-1* occurred more frequently in the late stage (7/29, 24%) than in the early stage of PTC (5/59, 9%; $p = 0.0548$).

PAX8/PPAR γ rearrangement

No rearrangement was detected in the three FVPTC cases (data not shown).

Concomitant *BRAF*^{V600E}, *RAS*, or *RET/PTC-1* mutations

Two CPTC samples had both *BRAF*^{V600E} and *KRAS*^{Q61R} or *KRAS*^{S65N}. Six CPTC and one tall-cell variant contained both *BRAF*^{V600E} and *RET/PTC-1*. Two FVPTC samples had both *NRAS*^{Q61R} and *RET/PTC-1* (Table 1). None of the samples were found to have triple mutations. In total, concomitant

TABLE 1. *BRAF*, *RAS*, OR *RET/PTC* MUTATION IN PAPILLARY THYROID CARCINOMA

Case #	Age/sex	Type/stage	KRAS	NRAS	BRAF	RET/PTC
1	18/M	PTC/I	ND	ND	V600E	ND
2	21/F	PTC/I	ND	ND	ND	ND
3	23/F	PTC/I	ND	c.182 A > G, p.Q61R	ND	ND
4	24/M	PTC/I	ND	ND	ND	ND
5	28/F	PTC/I	ND	ND	ND	ND
6	36/F	PTC/I	ND	ND	ND	PTC-1
7	38/F	PTC/I	ND	ND	ND	ND
8	39/F	PTC/I	ND	ND	ND	ND
9	40/M	PTC/I	ND	ND	ND	PTC-1
10	40/F	PTC/I	ND	ND	ND	ND
11	40/M	PTC/I	ND	ND	ND	ND
12	43/F	PTC/I	ND	ND	ND	ND
13	12/F	MET PTC/I	ND	ND	ND	ND
14	18/F	MET PTC/I	ND	ND	ND	ND
15	20/F	MET PTC/I	ND	ND	ND	ND
16	21/F	MET PTC/I	ND	ND	ND	ND
17	21/F	MET PTC/I	ND	ND	ND	ND
18	22/F	MET PTC/I	ND	ND	ND	ND
19	24/F	MET PTC/I	ND	ND	ND	ND
20	24/F	MET PTC/I	ND	ND	ND	ND
21	25/F	MET PTC/I	ND	ND	V600E	ND
22	25/F	MET PTC/I	ND	ND	V600E	ND
23	26/F	MET PTC/I	ND	ND	ND	ND
24	26/F	MET PTC/I	ND	ND	V600E	PTC-1
25	26/M	MET PTC/I	ND	ND	ND	ND
26	27/F	MET PTC/I	ND	ND	ND	ND
27	28/F	Met PTC/I	c.194 G > A, p.S65N	ND	V600E	ND
28	29/F	MET PTC/I	ND	ND	V600E	ND
29	30/F	MET PTC/I	ND	ND	V600E	ND
30	32/F	MET PTC/I	ND	ND	V600E	ND
31	33/F	MET PTC/I	ND	ND	ND	ND
32	33/F	MET PTC/I	ND	ND	V600E	ND
33	34/F	MET PTC/I	ND	ND	ND	ND
34	34/F	MET PTC/I	ND	ND	V600E	ND
35	35/F	MET PTC/I	ND	ND	V600E	ND
36	36/F	MET PTC/I	ND	ND	ND	ND
37	37/F	MET PTC/I	ND	ND	V600E	ND
38	37/F	MET PTC/I	ND	ND	ND	ND
39	39/M	MET PTC/I	ND	ND	ND	ND
40	39/M	MET PTC/I	ND	ND	V600E	ND
41	40/F	MET PTC/I tall-cell variant	ND	ND	V600E	ND
42	41/F	MET PTC/I	ND	ND	V600E	ND
43	41/F	MET PTC/I	ND	ND	V600E	ND
44	41/F	MET PTC/I	ND	ND	V600E	ND
45	42/F	MET PTC/I	ND	ND	ND	ND
46	42/F	MET PTC/I	ND	ND	V600E	ND
47	43/M	FVPTC/I	ND	ND	ND	ND
48	44/F	MET PTC/I	ND	ND	V600E	ND
49	27/F	MET PTC/II	ND	ND	ND	ND
50	27/F	MET PTC/II	ND	ND	ND	PTC-1
51	30/F	MET PTC/II	ND	ND	V600E	ND
52	45/F	FVPTC/II	ND	c.182 A > G, p.Q61R	ND	PTC-1
53	50/M	PTC/II	ND	ND	ND	ND
54	50/F	PTC/II	ND	ND	ND	ND
55	53/M	PTC/II	ND	ND	ND	ND
56	57/F	PTC/II	ND	ND	V600E	ND
57	60/F	PTC/II	ND	ND	V600E	ND
58	62/F	PTC/II	ND	ND	V600E	ND

(continued)

TABLE 1. (CONTINUED)

Case #	Age/sex	Type/stage	KRAS	NRAS	BRAF	RET/PTC
59	76/F	PTC/II	ND	ND	ND	ND
60	45/F	PTC/III	ND	ND	ND	ND
61	46/M	PTC/III	ND	ND	ND	ND
62	46/F	PTC/III	ND	ND	V600E	ND
63	50/M	PTC/III	ND	ND	ND	ND
64	52/M	PTC/III	ND	ND	V600E	ND
65	55/F	PTC/III	ND	ND	V600E	PTC-1
66	58/F	PTC/III	ND	ND	ND	ND
67	59/F	PTC/III	ND	ND	V600E	PTC-1
68	60/F	PTC/III	ND	ND	ND	ND
69	60/M	PTC/III	ND	ND	V600E	ND
70	65/M	PTC/III	ND	ND	V600E	ND
71	66/M	PTC/III	ND	ND	V600E	ND
72	66/M	PTC/III	ND	ND	ND	ND
73	71/M	PTC/III	ND	ND	V600E	PTC-1
		tall-cell variant				
74	72/F	PTC/III	ND	ND	V600E	ND
75	72/F	PTC/III	ND	ND	V600E	PTC-1
76	73/F	PTC/III	ND	ND	V600E	ND
77	78/M	PTC/III	ND	ND	V600E	ND
		tall-cell variant				
78	82/M	PTC/III	c.182A > G, p.Q61R	ND	V600E	ND
79	48/M	PTC/IVA	ND	ND	ND	ND
80	49/M	PTC/IVB	ND	ND	V600E	PTC-1
81	52/M	PTC/IVC	ND	ND	V600E	PTC-1
82	58/F	PTC/IVA	ND	ND	V600E	ND
83	60/M	PTC/IVC	ND	ND	ND	ND
84	62/M	PTC/IVB	ND	ND	ND	ND
85	63/M	PTC/IVC	ND	ND	V600E	ND
86	68/F	FVPTC/IVC	ND	c.182A > G, p.Q61R	ND	PTC-1
87	70/F	PTC/IVB	ND	ND	V600E	ND
88	77/M	PTC/IVB	ND	ND	V600E	ND
Cell lines						
8305C		ATC	ND	ND	V600E	ND
8505C		ATC	ND	ND	V600E	ND
HTC-C3		ATC	ND	ND	V600E	ND
BHT101		ATC	ND	ND	V600E	ND
FRO		ATC	ND	ND	V600E	ND
CAL62		ATC	<i>KRAS</i> ^{G12R}	ND	ND	ND
BCPAP		PTC	ND	ND	V600E	ND
K1		PTC	ND	ND	V600E	ND
TPC-1		PTC	ND	ND	ND	PTC-1

MET PTC refers to stage I or II tumors with local lymph node metastasis. Dual mutations are highlighted in bold.
PTC, papillary thyroid carcinoma; ND, not detected.

TABLE 2. FREQUENCY OF *BRAF*, *RAS*, OR *RET/PTC* MUTATIONS IN DIFFERENT STAGES OF PTC

Stage	Histology subtype	Cases	<i>BRAF</i> only	<i>KRAS</i> only	<i>NRAS</i> only	<i>RET/PTC-1</i> only	<i>BRAF</i> and <i>RAS</i>	<i>BRAF</i> and <i>RET/PTC-1</i>	<i>RAS</i> and <i>RET/PTC-1</i>
I	CPTC	46	16 (35)	0	1 (2)	2 (4)	1 (2)	1 (2)	0
	Tall cell	1	1 (100)	0	0	0	0	0	0
	FVPTC	1	0	0	0	0	0	0	0
II	CPTC	10	4 (40)	0	0	1 (10)	0	0	0
	FVPTC	1	0	0	0	0	0	0	1 (100)
III	CPTC	17	7 (41)	0	0	0	1 (6)	3 (18)	0
	Tall cell	2	1 (50)	0	0	0	0	1 (50)	0
IV	CPTC	9	4 (44)	0	0	0	0	2 (22)	0
	FVPTC	1	0	0	0	0	0	0	1 (100)
Total (%)		88	33 (38)	0	1 (1)	3 (3)	2 (2)	7 (8)	2 (2)

Numbers in parentheses indicate percentage of mutation in the histology subtype or group. *HRAS* mutation was not detected in the series.

FIG. 2. Characterization of *KRAS*^{S65N} and *NRAS*^{S65C} on mitogen-associated protein kinase (MAPK) activity. **(A)** The mutant and wild-type constructs (*KRAS*^{S65N}, *NRAS*^{S65C}, and *KRAS*^{WT}) were transfected into HEK293 cells. Cells were collected 48 h after transfection for Western blot analysis. Increased pMEK1/2 and pERK 1/2 expression was detected by an anti-pMEK1/2 and anti-pERK 1/2 antibody respectively in cells transfected with mutant *RAS* constructs. The membrane was reprobed with total ERK1/2 and GAPDH (37Kd) antibody to monitor protein loading. Quantification of the Western blot results is shown in Supplementary Figure S1. **(B)** The *KRAS*^{WT}, *KRAS*^{S65N}, and *NRAS*^{S65C} were transfected into HEK293 cells. Cells were cultured in serum-free medium for an additional 2 days 24 h after transfection and collected for Western blot analysis. Increased pERK 1/2 and pMEK 1/2 expression was detected in both *KRAS*^{S65N} and *NRAS*^{S65C} transfected cells following 48 h serum starvation. **(C)** Cell proliferation of HEK293 cells transfected with *KRAS*^{S65N} or *KRAS*^{WT}. The cells were plated into 96-well plates (5×10^3 cells/well) containing 1% or 10% serum respectively for 48 h. Data are expressed as mean $\pm$ standard error of the mean (SEM) of triplicates and are representative of three different experiments (* $p < 0.01$). **(D)** Increased MAPK and cAMP response element binding protein (CREB) activity in thyroid cancer cell lines following *KRAS*^{S65N} transfection. Both phospho-CREB and-ERK1/2 expressions were increased in CAL62 and BCPAP cell lines. *HRAS*^{G12V} was transfected into CAL62 and BCPAP cell lines as a positive control. The basal level of pERK1/2 was also higher in CAL62 and BCPAP as compared to HEK293.

TABLE 3. *BRAF* AND *RAS* MUTATION IN DIFFERENT TUMOR SECTIONS

Case #	Age/sex	Type/stage	Tumor sections	BRAF	RAS	Frequency of mutant RAS allele in 50 clones (%)
27	28/F	PTC/I	PT-A	V600E	NT	0/50
			PT-B	V600E	NT	3/50 (6)
			PT-C	V600E	NT	4/50 (8)
			LNM-A	V600E	<i>KRAS</i> ^{S65N}	17/50 (34)
			LNM-B	V600E	<i>KRAS</i> ^{S65N}	14/50 (28)
78	82/M	PTC/III	PT-A	V600E	NT	6/50 (12)
			PT-B	V600E	<i>KRAS</i> ^{Q61R}	12/50 (24)
			PT-C	V600E	<i>KRAS</i> ^{Q61R}	Not done
			PT-D	V600E	<i>KRAS</i> ^{Q61R}	Not done

PT, primary tumor; LNM, lymph node metastasis; NT, not detected by direct Sanger sequencing.

mutations were found in 11/88 PTC samples (13%). Among them, three were found in early stages of PTC (3/59, 5%), and eight (8/29, 28%) in an advanced stage. The association of concomitant mutations with advanced stage was statistically significant ($p < 0.01$). Eight cases with concomitant mutations were older than 45 years. Concomitant mutations were not found in nine cell lines studied. Co-existence of *RET/PTC* with *RAS* or *BRAF* has been reported in multiclonal PTC (9). We next examined different tumor sections of paraffin blocks for concomitant *BRAF* and *RAS* mutations found in our two cases. As shown in Table 3, the *BRAF* mutation was present in all tumor sections, whereas the *RAS* mutation was detected only in some sections. This may indicate intratumor heterogeneity. Histologic heterogeneity was not observed. Due to the limited sensitivity of Sanger sequencing to detect a low frequency of mutant alleles, we cloned the PCR fragments from tissue sections with negative *RAS* mutation into a TA vector, and screened 50 clones from each fragment for *RAS* mutation by direct Sanger sequencing. As shown in Table 3, a *RAS* mutation was detected in two sections that were negative by direct Sanger sequencing. We further evaluated the sensitivity of Sanger sequencing to detect *KRAS* mutations by mixing different amounts of wild-type and mutant *KRAS* DNA. As shown in Figure 3, about 20% mutant alleles are required in the sample to be reliably detected by Sanger sequencing (the detection limit is about 20%). In the lymph node metastases, the *RAS* mutation was significantly enriched and can be easily detected (Table 3).

To determine whether concomitant mutations were associated with a poor clinical outcome, we analyzed disease-free survival of the patients who had concomitant mutations versus those without mutation or those who had only one mutation. As shown in Figure 4, the Kaplan–Meier curve shows a significant difference in the recurrence-free probability over a 10-year period among patients with no mutation, a single mutation, and double mutations (log-rank test $\chi^2 = 18.2$, $p < 0.0001$), and between patients with single and double mutations (log-rank test $\chi^2 = 4.2$, $p < 0.041$). These data indicate that patients with concomitant mutations had much poorer clinical outcomes than those with a single or no mutation.

Discussion

In the present study, we found 11 cases of PTC (13%) with concomitant *BRAF*, *RAS*, or *RET/PTC-1* mutations. Concomitant mutations occurred more frequently in advanced stages of disease. Long-term follow-up showed that patients with concomitant mutations had a poor response to treatment and shorter disease-free survival.

We also report the first case of *KRAS*^{S65N} in PTC (1%). Mutation in *KRAS* codon 65 has been reported in one case of cervical cancer (1/107, 1%) (15). A codon 65 mutation in *NRAS* has been reported in 2/12 (17%) neuroendocrine lung cancers and 1/35 (3%) melanomas (16). The melanoma with *NRAS*^{S65C} also had *BRAF*^{V600E} (16). The significance of codon 65 mutations in *RAS* has not been investigated previously, although the residue is highly conserved across

FIG. 3. Sensitivity of Sanger sequencing to detect *KRAS* mutations. Different amounts of wild-type and mutant *KRAS* DNA were mixed together in a total concentration of 100 ng DNA for PCR. PCR products were directly sequenced. At least 20% mutant alleles need to be present in the sample to be reliably detected by Sanger sequencing.

FIG. 4. Kaplan–Meier analysis for recurrence-free probability. The Kaplan–Meier curves shows a significant difference in the recurrence-free probability over a 10-year period after the diagnosis among patients with no mutation, a single mutation, and double mutations (log-rank test $\chi^2=18.2$, $p<0.0001$), and also between patients with single mutation and double mutations (log-rank test $\chi^2=4.2$, $p<0.041$).

different species. We demonstrate in the present study that codon 65 mutations in both *KRAS* and *NRAS* can constitutively activate the MAPK pathway. To our knowledge, this is the first report showing the functional significance of a *RAS* codon 65 mutation. The prevalence of a codon 65 mutation appears to be low in human cancers. However, many studies have only screened *RAS* mutations at codon 12/13 and 61, and may miss this mutation. Two of three FVPTC (66.7%) in our study have *NRAS* mutations. A *NRAS* mutation is more frequent in FTC (17) and FVPTC (8), supporting the notion that FVPTC shares many molecular features of FTC.

The overall rate of *RAS* mutation is 6% (5/88) in the cohort studied. The frequency of *RAS* mutation in PTC is variable depending on the cohort studied, ranging from 0% to 16% (2,8,18), with an overall rate of 11% (17). Higher frequencies have been reported in poorly differentiated thyroid carcinomas (23%) (19) and FTC (20%) (17). In the study by Wang *et al.* (18), a *RAS* mutation was found in 3 of 31 FTC (10%) and none of the 141 PTC in a Chinese population. Similar results were also reported by Di Cristofaro *et al.* in a French population (8). In contrast, Kimura *et al.* reported a *RAS* mutation in 11 of 67 PTC (16%). As discussed by Vasco *et al.* (17), multiple factors may contribute to such a wide range of variations, including the methodology used to detect mutations (the overall rate of mutations was significantly lower when estimated with direct sequencing than without: 12.3% vs. 17%), environmental factors (iodine deficiency, radiation exposure, and food-borne carcinogens), geographic variation, and tumor heterogeneity. Based on the pooled literature analysis by Vasco *et al.* (17), the most common mutation in PTC is *NRAS*^{Q61R} (5%), whereas the *KRAS* and *HRAS* mutation rate is about 3% and 2.6% respectively.

Concomitant *RAS* and *BRAF* mutations have been reported previously in PTC, poorly differentiated and anaplastic thyroid carcinomas (10), colorectal cancers (20), and cell lines (21). By using pyrosequencing to quantitate *BRAF* and *RAS* mutations, Guerra *et al.* demonstrated simultaneous subclonal *BRAF* and *RAS* mutations in two PTC (3%) (22). The

frequency of concomitant *KRAS* and *BRAF* mutations increased along progression of colorectal cancers, suggesting that activation of both genes may have a synergistic effect for disease progression (20). In our two cases with concomitant *RAS* and *BRAF* mutations (one of them was a stage I PTC at diagnosis), they responded poorly to treatment. The disease relapsed within five years after surgery and progressed aggressively. Given that the *BRAF* mutation is present in all tumor sections and the *RAS* in some sections, it is likely that the *RAS* mutation occurred later than the *BRAF* and may play a role in disease progression. In the four cases of concomitant *RAS* and *BRAF* mutations reported by Costa *et al.* (10), two cases show a negative *RAS* and a positive *BRAF* mutation in some primary tumor sections, but concomitant *RAS* and *BRAF* mutations are present in all sections of lymph node metastasis, supporting the role of *RAS* in disease progression. *RAS* can promote thyroid tumorigenesis through both the MAPK and PIK3CA/AKT pathways. Concomitant *RAS* and *BRAF* mutations may allow simultaneous activation of both signaling pathways in cancer cells to gain growth advantage. Indeed, activation of *AKT* has frequently been found in PTC and is associated with cancer progression (23,24). *PIK3CA* and *AKT* mutations also frequently coexist with *RAS* and *BRAF* mutations in patients with advanced cancers (10,25,26). Although occurring less frequently, *AKT1*, *AKT3*, and *PIK3CA* mutations have been reported in PTC (27,28).

BRAF inhibitors have been undergoing clinical trials for thyroid cancer with mixed results (29,30). Recent studies have demonstrated that *BRAF* inhibitors may paradoxically increase MAPK activation in the presence of mutated *RAS* or wild-type *BRAF* (31–33). Therefore, patients with concomitant *RAS* and *BRAF* mutations may not be suitable for *BRAF* inhibitor treatment. Reactivation of MAPK or activation of PIK3CA/AKT (34) plays an important role in *BRAF* inhibitor resistance (35). MAPK reactivation can occur via *NRAS* mutations, *COT* overexpression, *BRAF*^{V600E} alternative splicing or amplification, and *MEK1* mutation, whereas PIK3CA/AKT can be activated through overexpression of

receptor tyrosine kinases (35). *RET/PTC* can stimulate both PIK3CA/AKT and MAPK pathways (6,36,37). For patients with concomitant *RET/PTC* and *BRAF* mutations, it remains to be determined whether *RET/PTC* plays any role in *BRAF* inhibitor resistance, since cancer cells may escape inhibition via PIK3CA/AKT survival pathway. Targeting both MAPK and PIK3CA/AKT pathways may offer better long-term therapeutic effects. Selection of patients based on their mutation profile for clinical trials or treatment strategies may be warranted.

RET/PTC-1 was detected in 12 cases of PTC (14%). The presence of a *RET/PTC* rearrangement is not an indication of malignancy, as it occurs in benign thyroid nodules and is associated with a high growth rate of these nodules (38). Papillary carcinomas are often multifocal, and individual tumor foci in patients with multifocal PTC often arise from independent clonal origins (39). Based on the methodology used in the current study, we cannot distinguish whether dual mutations are present in the same cell or in different cells of the same tumor. We found concomitant mutations of *RET/PTC-1* and *BRAF* or *RAS* in nine cases of PTC but none in the studied cell lines, suggesting intratumor heterogeneity or multiclonal origin in tumor samples and monoclonal origin of cell lines. The mutual exclusivity of gene mutations in the MAPK pathway may still hold if tumors are of monoclonal origin. A recent study of a single kidney cancer has shown remarkable diversity in the genetic changes that have taken place in different parts of the tumor (40). In advanced thyroid carcinoma, multiple genetic changes may occur in different parts of the tumor, resulting in concomitant mutations. Among nine cases with concomitant *RET/PTC-1* and *BRAF* or *RAS* mutations, seven cases (78%) are in the late stage of cancer, indicating that *RET/PTC-1* may occur late in subclonal PTC and is involved in tumor progression, as suggested previously by Unger *et al.* (41). Recently, Guerra *et al.* also reported concomitant subclonal *BRAF* and *RET/PTC* mutations in 19.4% (14/72) case of PTC (42). *RAS* mutations may have been underestimated in thyroid cancer. Using sensitive allele-specific competitive blocker-PCR (ACB-PCR), which can detect less than 1% mutant alleles, Myers *et al.* detected *KRAS* mutations in 30% of PTC (43). Although Sanger sequencing is less sensitive than pyrosequencing (detection limit is about 5%) or ACB-PCR to detect *RAS* mutations, mutations detected by Sanger sequencing indicate that at least 20% mutant alleles are present in the tumor sample, which may play a more significant role in promoting tumor progression. The oncogenic effects of *RET/PTC-1* are mediated via *RAS*, which allows growth promoting signals through both the MAPK and PIK3CA/AKT pathways, and concomitant mutations of *RET/PTC-1* and *BRAF* or *RET/PTC-1* and *RAS* in subclonal/multiclonal PTC may provide a synergistic effect for disease progression.

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Author Disclosure Statement

All authors have nothing to declare.

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