Clinicopathological Analysis of Papillary Thyroid Cancer with *PIK3CA* Alterations in a Middle Eastern Population

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Context: Genetic aberration in phosphatidylinositol 3-kinase (PI3K)/AKT pathway has been detected in numerous and diverse human cancers. *PIK3CA*, which encodes for the catalytic subunit of p110α of PI3K, is amplified in some cases of papillary thyroid cancer (PTC). Mutations in the PIK3CA have also been identified in thyroid cancers and, although relatively common in anaplastic thyroid carcinoma, are uncommon in PTC.

Objective: The objective of the study was to investigate genetic alterations like *PIK3CA* gene mutation, *PIK3CA* amplification, *RAS*, and *RAF* mutations and to further explore the relationship of these genetic alterations with various clinicopathological characteristics in Middle Eastern PTC.

Design: We used the fluorescence *in situ* hybridization technique for analysis of PIK3CA amplification from 536 PTC cases, and selected amplified samples were further validated by real-time quantitative PCR. Mutation analysis was done by direct DNA sequencing of *PIK3CA*, *N2-RAS*, and *BRAF* genes.

Results: PIK3CA amplification was seen in 265 of 499 PTC cases analyzed (53.1%); PIK3CA gene mutations in four of 207 PTC (1.9%); *N2-RAS* mutations in 16 of 265 PTC (6%); and BRAF mutations in 153 of 296 PTC (51.7%). N-RAS mutations were-associated with an early stage ($P = 0.0465$) and lower incidence of extrathyroidal extension ($P = 0.027$), whereas BRAF mutations were-associated with metastasis ($P = 0.0274$) and poor disease-free survival ($P = 0.0121$) in PTCs.

Conclusion: A higher incidence of *PIK3CA* alterations and the possible synergistic effect of *PIK3CA* alterations and *BRAF* mutations suggest their major role in Middle Eastern PTC tumorigenesis and argue for therapeutic targeting of PI3K/AKT and MAPK pathways. **(*J Clin Endocrinol Metab* 93: 611–618, 2008)**

Papillary thyroid carcinoma (PTC) is the most common malignant thyroid tumor, representing 80–90% of all thyroid malignancies. PTC is usually well differentiated; however, the clinical behavior of PTC varies widely. For example, incidental microcarcinomas grow very slowly and are noninvasive or minimally invasive. On the other hand, invasive PTC with metastasis can be lethal. PTC often recurs many years after surgical removal. The prognosis for PTC is often favorable; however, approximately 20% of PTC tumors recur, and some reach advanced stages (1). Several clinicopathological variables including stage, cancer invasion, and distant metastasis are used for prognostication and treatment selection for PTC (2, 3). A better understanding of the factors and mechanisms determining the aggressive behavior of some papillary carcinomas is critical in developing new treatment.

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Abbreviations: BAC, Bacterial artificial chromosome; FISH, fluorescence *in situ* hybridization; MEK, mitogen-activated kinase; PI3K, phosphatidylinositol 3-kinase; PTC, papillary thyroid carcinoma; RAS, rat sarcoma viral oncogene homolog; TMA, tissue microarray.

Increased mitogenic signaling through receptors has proven to play a major role in thyroid cancer. One of the major downstream mediators of signaling initiated by these receptors is the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. Genetic aberration in the PI3K/AKT pathway has been detected in numerous and diverse human cancers (4). *PIK3CA*, which encodes for the catalytic subunit of p110α of class IA PI3K, is amplified in some cases of papillary thyroid cancer (5–7). Mutations in the *PIK3CA* have also been identified in thyroid cancers and, although relatively common in anaplastic thyroid carcinoma, are uncommon in PTC (5–7).

Rat sarcoma viral oncogene homolog (RAS) proteins are located on the inner surface of the plasma membrane and are attached to the membrane by a farnesyl residue. RAS proteins transmit extracellular signals that promote the growth, proliferation, differentiation, and survival of cells. The signaling cascade starts from the plasma membrane in which the growth factor (*e.g.* epidermal growth factor) binds to its enzyme-linked receptor, causing receptor dimerization. The major downstream target of RAS-GTP is MAPKs, but it is also known to activate other targets like PI3K (8). Activation of MAPK occurs through specific phosphorylation of both a threonine and tyrosine separated by a single amino acid. The first component of the MAPK cascade is called raf proto-oncogene serine/threonine protein kinase (RAF), which is activated on the plasma membrane by RAS-GTP. RAF phosphorylates mitogen-activated kinase (MEK) 1/2, which activates the ERK 1/2 kinase or p44/42 MAPK by phosphorylation. ERK1/2 phosphorylates a variety of downstream targets, which results in changes in several key growth factors: the catalytic activities of enzymes and protooncogenes that transduce signals promote growth and differentiation through this cascade (9).

The RAS-RAF-MEK-ERK pathway is hyperactivated in 30% of human cancer (10). In PTC, activating mutation in RAS has been identified in 0–10% of PTC cases (7, 11). RAS mutation can promote thyroid tumorigenesis through the RAS-RAF-MEK-ERK pathway or through its interaction with PI3K/AKT pathway (8, 9). Similarly, activating mutation in *BRAF* (one of the three *RAF* genes in humans) is seen in 29–69% of PTC cases, making *BRAF* mutations the most common defined genetic abnormality in PTC.

We have shown that aberrant PI3K/AKT signaling may play a role in Middle Eastern PTC tumorigenesis and progression (12). However, other thyroid tumor-related genetic alterations like PIK3CA gene mutation, amplification, *RAS*, and *RAF* mutations are yet to be studied in the Middle Eastern population. The present study was conducted to investigate these genetic alterations and their relationship to various clinicopathological characteristics in Middle Eastern PTC.

Patients and Methods

Patient selection and tissue microarray construction

A total of 536 patients with papillary carcinoma of the thyroid, diagnosed between 1988 and 2004, were selected from the files of the King Faisal Specialist Hospital and Research Centre. All samples were analyzed in a tissue microarray (TMA) format. TMA construction was performed as described earlier (13). Briefly, tissue cylinders with a diameter of 0.6 mm were punched from representative tumor regions of each donor tissue block and brought into recipient paraffin block using a modified semiautomatic robotic precision instrument (Beecher Instruments, Woodland, WI). Two cores of papillary carcinoma of the thyroid were arrayed from each case. Patients were reclassified into three histology subtypes of papillary carcinoma: classical papillary carcinoma, follicular variant of papillary carcinoma, and tall cell variant, according to well-established histopathological criteria. All PTC cases showing presence of distant metastasis to organs like lung, liver, bone, *etc.* were classified as M1 (American Joint Committee on Cancer definition). Presence of cervical lymph node metastasis was not considered as M1. Disease-free survival was defined as the time from thyroidectomy to the first event of either metastasis (M1) or death. Extrathyroidal extension was defined as extension of the tumor outside the capsule as documented in histopathology reports. The Institutional Review Board of the King Faisal Specialist Hospital and Research Centre approved the study. In addition four PTC cell lines were chosen for this study: ONCO DG-1, B-Cap, 8505-C, and CGTH-W-1.

DNA isolation

Genomic DNA was extracted from tissue microarray punches obtained from paraffin-embedded tumor block using a modified version of Gentra kit protocol (Minneapolis, MN).

Fluorescence *in situ* hybridization (FISH) methodology

FISH on tissue microarray was performed as previously described (14). Briefly, the search for FISH probe was done by browsing Ensemble Genome Browser (http://ensemble.org/) for bacterial artificial chromosome (BAC) corresponding to the *PIK3CA* gene. BAC RP11–245 C23 was purchased from Children's Hospital Oakland Research Institute (Oakland, CA), cultured, and DNA isolated. BAC DNA probe was labeled with digoxigenin using the digoxigenin-nick translating kit from Roche (Mannheim, Germany). FISH was performed with a digoxigenin-labeled BAC DNA probe, containing the *PIK3CA* gene and a Spectrum Orange-labeled chromosome 3 centromeric probe as a reference (purchased from Vysis, Abbott Park, IL). TMA sections were treated according to the paraffin pretreatment reagent kit protocol (Vysis) before hybridization. For the thyroid cancer TMA study, hybridization and posthybridization washes were according to the Vysis LSI procedure. Probe visualization using fluorescent isothiocyanate-conjugated sheep antidigoxigenin (Roche Diagnostics, Indianapolis, IN) was as described (15). Slides were then counterstained with 125 ng/ml 1,4′,6-diamino-2-phenylindole in an antifade solution and screened with a BX51 fluorescent microscope (Olympus, Tokyo, Japan). Tissue samples were classified with a PIK3CA to centromere 3 ratio of 1.0 as normal and between 1.0 and 2.0 as having PIK3CA gains. A PIK3CA to centromere 3 ratio of more than 2.0 was considered as amplified.

Analysis of *PIK3CA*, *RAS*, and *BRAF* genes for mutations

Because the vast majority of *PIK3CA* gene mutations in human cancers were reported in exons 9 and 20, we focused our mutation analysis on these exons (16). Sequencing of *PIK3CA* exons 9 and 20 was done by PCR amplification and direct sequencing of both strands for all PTC cases as previously described (17). In brief, step-down PCR was performed as follows: after a 10-min denaturing at 95 C, the PCR was run with each temperature for 1 min at five step-down steps, for two cycles each. The denaturing temperature was 95 C, and extension temperature was 72 C for each step, with the annealing temperature of 66, 64, 62, 60, and 58 C from the first to the last step. The PCR was finally run at 95, 58, and 72 C each for 1 min for 35 cycles, followed by an elongation at 72 C for 5 min. PCR was performed in a total volume of 25 μl using 50 ng of genomic DNA, 2.5 μl 10× Taq buffer, 1.5 μl $MgCl_2$ (25 mM), 0.05 μl deoxynucleotide triphosphate (10 mM), 0.2 μl Taq polymerase (1 U/μl) (all reagents were from QIAGEN Inc., Valencia, CA), 1 μl of each primer (2.5 μM), and water. Primer pairs flanking *PIK3CA* exons 9 and

20 were selected to avoid the frequent cross-amplification of chromosome 22q (known *PIK3CA* pseudogene) observed with those previously reported. The same step-down cycling condition was used for BRAF T1799A transversion mutation in exon 15 of the BRAF gene (18, 19).

Because a majority of *RAS* mutations were found in exon 2 of the *NRAS* gene (*N2-RAS*) in papillary thyroid, we focused our mutation analysis on *N2-RAS* (5, 7). The PCR mixture contained the same components as in PCR for the *PIK3CA* gene. The PCR condition was as follows: after a 10-min denaturation at 95 C, 30 sec of annealing at 53 C, and 1 min of extension at 72 C, with an extension of 72 C for 7 min at the last step (7). The efficiency and quality of the amplification PCR were confirmed by running the PCR products on a 2% agarose gel. The PCR products were subsequently subjected to direct sequencing PCR with BigDye terminator version 3.0 cycle sequencing reagents (Applied Biosystems, Foster City, CA). The samples were finally analyzed on an ABI PRISM 3100xl genetic analyzer (Applied Biosystems).

Immunohistochemistry

Immunohistochemical studies on formalin-fixed, paraffin-embedded tissue sections were performed as described in earlier studies (12, 20). TMA sections were processed and stained manually. For antigen retrieval, target retrieval solution (pH 9.0) (catalog no. S2368; Dako, High Wycombe, UK) was used, and the slides were microwaved at 750 W for 5 min and then at 250 W for 30 min. IHC for p-AKT was performed by staining 3- to 4-μm-thick tissue microarray sections with the p-AKT (Ser 473) antibody using phospho-AKT (Ser 473) IHC detection kit (Cell Signaling Technology, Beverly, MA; product 8100). Endogenous peroxidase activity was quenched using 3% H_2O_2. Endogenous biotin was blocked and all slides were counterstained, with hematoxylin, dehydrated, cleared, and coverslipped with premount. Only fresh-cut slides were stained simultaneously to minimize the influence of slide ageing and maximize repeatability and reproducibility of the experiment. Two types of negative controls were used. One was the negative control in the kit in which the primary antibody was omitted. A preabsorption experiment using p-AKT Ser 473 blocking peptide (Cell Signaling Technology; product 1140) was used as the second negative control.

Quantitative real-time PCR

Thyroid tumors with increased copy number by FISH of the PIK3CA gene were selected for validation by quantitative real-time PCR. DNA content was normalized to that of long interspersed elements (LINE1), a repetitive element for which copy number per haploid genome are similar both in normal DNA sample and neoplastic cells. Primers were designed by Primer express 3.0 software (Applied Biosystems) hybridized to sequences of genomic DNA for *PIK3CA* and *LINE 1*. Primers to genomic sequences were: PIK3CA forward, 5′-TATGGTTGTCTGTCAATCGGTGA-3′, reverse, 5′-GCCTTTGCAGTGAATTTGCAT); and LINE1 forward, 5′-CCGCTCAACTACATGGAAACTG-3′, reverse, 5′-GCGTCCCAGAGATTCTGGTATG-3′. Conditions for all PCRs were optimized in gradient cycler (MJ Research, Waltham, MA) with regard to *Taq* DNA polymerase, forward and reverse primers, $MgCl_2$ concentrations, deoxynucleotide triphosphate concentrations, and various annealing temperatures (55–65 C). Specificity of the PCR product was confirmed by agarose gel electrophoresis. Optimized results were transferred on the following LightCycler PCR protocol (Roche).

All reactions were performed in glass capillaries (Roche) with a final reaction volume of 10 μl of 1× LightCycler-FastStart DNA master SYBR Green I reaction mixture (Roche) containing FastStart *Taq*, reaction buffer, and deoxynucleoside triphosphate, 1 mM $MgCl_2$, and final concentrations of 0.5 μM for each primer. $MgCl_2$ concentrations were optimized for each target gene (varied from 2 to 4 mM). Thermocycling and detection were performed on the LightCycler (Roche). An initial preheating step of 10 min at 95 C was used to activate the DNA polymerase and then a touchdown procedure, consisting of 10 sec at 95 C, annealing for 5 sec at temperatures decreasing from 63 to 59 C, and ending with an extension step at 72 C for 10 sec. A total of 45 cycles were performed, followed by melting curve program (60–95 C with a heating rate of 0.1 C/sec and a continuous fluorescence measurement), and finally a cooling step to 40 C.

Pfaffle method for relative quantification was used to calculate fold of changes for normal and thyroid cancer samples (21). The relative copy number ratio of a target gene is calculated based on efficiency and crossing point deviation of samples (normal) *vs.* (thyroid tumor) and expressed in comparison with a reference gene (LINE1). For a normal cell, the copy number of a gene per haploid genome should be 1.

Results

PIK3CA mutations and amplification in PTC and human thyroid cell lines

FISH analysis revealed the presence of PIK3CA amplification in 265 of 499 PTC cases analyzed (53.1%). Selected amplified samples were further validated by real-time PCR (Fig. 1). Two cell lines (CGTH-W-1 and ONCO-DG-1) showed PIK3CA amplification by FISH as well as real-time PCR. Mutational analysis revealed missense mutation in 4 of 207 PTC patients (1.9%). The specific nucleotide change and the corresponding amino acid substitution are shown in Table 1. This is higher than what has been reported before in different ethnic groups (5, 6).

Correlation of PIK3CA alterations (mutation plus amplifications) with *RAS* and *BRAF* mutations

A total of 265 of the PTCs included in this study had been analyzed for N2-RAS mutations, whereas 296 of the PTCs had

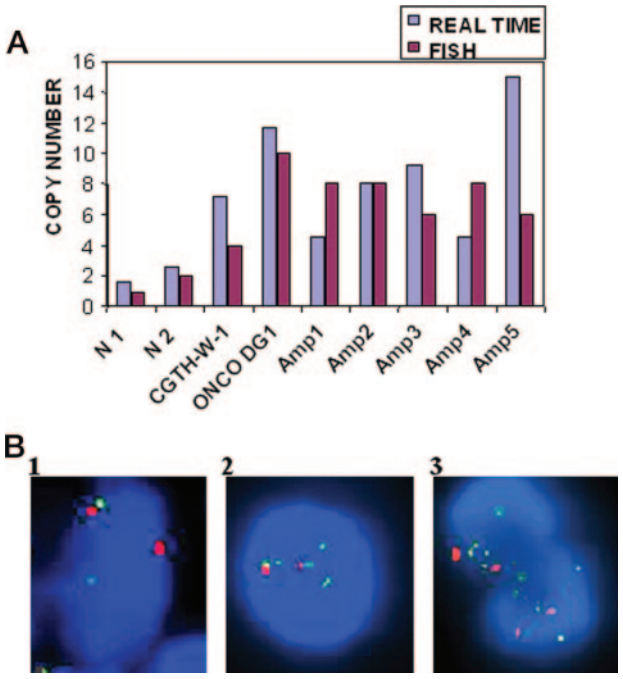

FIG. 1. Determination of PIK3CA gene copy number by quantitative PCR in tumors with PIK3CA gene amplification. Sample N1 and N2, are normals. Cell lines with amplification CGTH-W-1 and ONCO DG1 and samples Amp 1–5 are PIK3CA-amplified thyroid tumor samples selected according to FISH (A). FISH images show cell nuclei (*blue*) from selected cases, hybridized with probes directed against PIK3CA gene (*green*, RP11–245 C23) and centromere 3 (*red*) (B) (1). Normal cell (*blue*) shows centromeric signals (2, *red*) and PIK3CA signals (2, *green*), whereas 2 and 3 representative cells show amplification two centromeric signals (*red*) and PIK3CA amplified signals (*green*).

TABLE 1. Summary of individual cases of PTC with genetic alterations in PIK3CA, BRAF, and N2-RAS genes

Case no.	N2-RAS mutation			PIK3CA	PIK3CA mutation			T1799A
	Exon/ codon	Nucleotide substitution	Amino acid	Amplification	Exon/ codon	Nucleotide substitution	Amino acid	BRAF mutation
1	2/61	C**A**A>C**G**A	GLn>Arg	−				−
2				+	20/1049	**G**GT>**C**GT	Gln>Arg	+
3	2/61	C**A**A>C**G**A	GLn>Arg	−				−
4	2/61	C**A**A>C**G**A	GLn>Arg	+				−
5	2/61	C**A**A>C**G**A	GLn>Arg	+				−
6				−	9/539	C**C**T>C**G**T	Pro>Arg	+
7	2/61	C**A**A>C**G**A	GLn>Arg	−				−
8	2/61	C**A**A>C**G**A	GLn>Arg	+				−
9	2/61	C**A**A>C**G**A	GLn>Arg	−				−
10				+	9/542	**G**AA>**A**AA	Gln>Lys	+
11				+	20/1047	C**A**T>C**G**T	His>Arg	+
12	2/61	C**A**A>C**G**A	GLn>Arg	+				−
13	2/61	C**A**A>C**G**A	GLn>Arg	+				−
14	2/61	C**A**A>**A**AA	GLn>Lys	+				−
15	2/61	C**A**A>C**G**A	GLn>Arg	−				−
16	2/61	C**A**A>C**G**A	GLn>Arg	+				−
17	2/61	C**A**A>C**G**A	GLn>Arg	−				−
18	2/61	C**A**A>C**G**A	GLn>Arg	−				−
19	2/61	C**A**A>C**G**A	GLn>Arg	+				−
20	2/61	C**A**A>C**G**A	GLn>Arg	−				−

Bold letters indicate nucleotide substitution in the mutated codon.

been analyzed for *BRAF* mutations. We found *N2-RAS* mutations in only 16 of 265 PTC (6%), whereas *BRAF* mutation was found in 153 of 296 PTC (51.7%). PIK3CA mutational analysis was performed in 207 PTC cases. Because PI3KCA mutations were seen in only a small number (four cases), and PIK3CA amplification was commonly overlapped with PIK3CA gene mutations in PTC (three of the four cases that harbor the mutations showed PIK3CA amplification), we combined these two groups. Henceforth, we will refer to PTCs that showed PIK3CA alterations (either mutation or amplification) as one group. We analyzed the relationship of PIK3CA alterations with each of the other gene mutations as shown in Table 1. No statistically significant association was found between *RAS* ($P = 0.9954$) or *BRAF* ($P = 0.3708$) oncogenic activation and *PIK3CA* alterations in PTC. Three percent of the PTCs with PIK3CA amplification (eight of 265) were mutated at RAS and 53.33% of the PTCs harboring RAS mutations also had PIK3CA amplification (eight of 15). The total number of samples with data available for PIK3CA alterations was 155. Of these 155 PTCs, only 89 samples contained *PIK3CA* alteration (mutation and amplification). However, *BRAF* mutations were analyzed in 296 PTCs and 153 cases carried *BRAF* mutations. Of these 153 PTCs, PIK3CA amplification data were not available in seven cases, and PIK3CA amplification was seen in 84 of the remaining 146 BRAF mutated PTCs (Table 2). Interestingly, all the four cases with *PIK3CA* mutation also showed a *BRAF* mutation. As expected, no PTC was simultaneously mutated at *RAS* and *BRAF* because both these are recognized alternative events in thyroid tumorigenesis (22). Thus, more than half of PTCs with *PIK3CA* alterations were mutated at *RAS* or at *BRAF*.

AKT activation in PTC and its correlation with PIK3CA alterations, N-RAS, and BRAF mutations

The level of expression of activated AKT as a downstream effector of PIK3CA gene product was analyzed in our own TMA of PTC. Phospho-AKT staining was considered indicative of AKT activation and the case was recorded to be positive for phospho-AKT when it was 2+ or 3+ (Fig. 2). Phospho-AKT was detected in 55.1% of PTC cases. The correlation between the level of phospho-AKT expression and mutational status of *N2-RAS*, *BRAF* genes, and *PIK3CA* alteration was explored. No statistical effect was observed between PTCs that harbored *PIK3CA* alteration or *BRAF* mutations and AKT activation. However, 11 of 13 PTCs with *N2-RAS* mutations showed AKT activation and that association was statistically significant ($P = 0.0082$).

Correlation of PIK3CA alterations, RAS, BRAF mutations, and clinicopathological features in PTC

There were no significant associations of PIK3CA alterations with the histology types and any clinicopathological features. *N2-RAS* mutations in PTCs were associated with an early American Joint Committee on Cancer stage ($P = 0.0465$) and a lower incidence of extrathyroidal extension ($P = 0.0313$). Metastasis (M1) was seen 13.7% (21 of 153 PTC) with BRAF mutation, compared with only 6.3% of PTC (9 of 143 without BRAF mutations). Thus, the presence of *BRAF* mutations in PTCs was significantly associated with metastasis ($P = 0.0274$). Also noted was the significant association of BRAF mutations with a poor disease-free survival ($P = 0.0121$, Table 2 and Fig. 3). To investigate the hypothesis of synergistic effect of BRAF mutations and PIK3CA amplifications, we stratified our PTC cases into two groups depending on the status of *PIK3CA* amplifications and

TABLE 2. Clinical characteristics and BRAF mutation of patients with PTC

		BRAF mutation, absent		BRAF mutation, present		
	Total	n	%	n	%	*P* value
No. of patients	296	143	48.3	153	51.7	
Age (yr)						
≤45	180	90	50.0	90	50.0	0.4686
>45	116	53	45.7	63	54.3	
Sex						
Female	223	101	45.3	122	54.7	0.0690
Male	73	42	57.5	31	42.5	
Histology type						
Papillary, classical and FVPC	283	138	48.8	145	51.2	0.4650
Tal-cell variant	13	5	38.5	8	61.5	
pT						
pT1	88	41	46.6	47	53.4	0.3658
pT2	51	30	58.8	21	41.2	
pT3	138	62	44.9	76	55.1	
pT4	11	6	54.6	5	45.4	
pN						
pN0	96	45	46.9	51	53.1	0.9642
pN1	176	83	47.2	93	52.8	
pM						
pM0	257	131	51.0	126	49.0	0.0274
pM1	30	9	30.0	21	70.0	
Stage						
I	170	89	52.4	81	47.6	0.3233
II	21	8	38.1	13	61.9	
III	15	8	53.3	7	47.0	
IV	70	29	41.4	41	58.6	
Extrathyroidal extension						
Present	147	70	47.6	77	52.4	0.8130
Absent	149	73	49.0	76	51.0	
Surgical margins						
Positive	42	20	47.6	22	52.4	0.7005
Negative	76	39	51.3	37	48.7	
p-AKT intensity						
Low (0–1)	128	55	43.0	73	57.0	0.2289
High (2–3)	135	68	50.4	67	49.6	
PIK3CA mutation						
Present	4	0	0.0	4	100.0	0.0247
Absent	152	72	47.4	80	52.6	
N-RAS mutation						
Present	16	16	100.0	0	0.0	<0.0001
Absent	246	110	44.7	136	55.3	
PIK3CA, FISH						
Amplified	152	68	44.7	84	55.3	0.2285
Nonamplified	129	67	51.9	62	48.1	

FVPC, follicular variant of papillary carcinoma; pT, pathological assessment of primary tumor; pN, pathological assessment of regional lymph nodes; pM, pathological assessment of distant metastasis.

BRAF mutations: one group with *PIK3CA* gene amplification and *BRAF* mutation and the second group consisted of PTC cases with no abnormality or abnormality in either *PIK3CA* or *BRAF*. The PTC group with both *PIK3CA* amplification and *BRAF* mutation showed a significant association with larger tumor size ($P = 0.0479$), metastasis ($P = 0.0264$), and a significantly poor disease-free survival ($P = 0.0070$, Fig. 3).

Discussion

Similarities in the prevalence of *PIK3CA* mutations in Asian and Western thyroid cancer (5, 7) have raised an interest to study this activating mutation in other ethnic groups. As expected, our data suggest similar frequencies of *PIK3CA* mutation in the Middle Eastern PTCs, which might indicate that *PIK3CA* gene mutation is not a common mechanism in the activation of PI3K/AKT in papillary thyroid tumors. Interestingly, the frequency of *PIK3CA* amplification (53.1%) was higher than that usually observed in Western and Asian populations (5, 7). The frequency of amplification remained high (14.99%), even after raising the amplification cutoff by a number 10 or greater. Ethnic difference is one possible explanation that we propose for this higher incidence of *PIK3CA* amplification in the Middle Eastern PTCs, compared with earlier reports (7). *PIK3CA* amplification has also been reported to be present in some benign adenomas as well

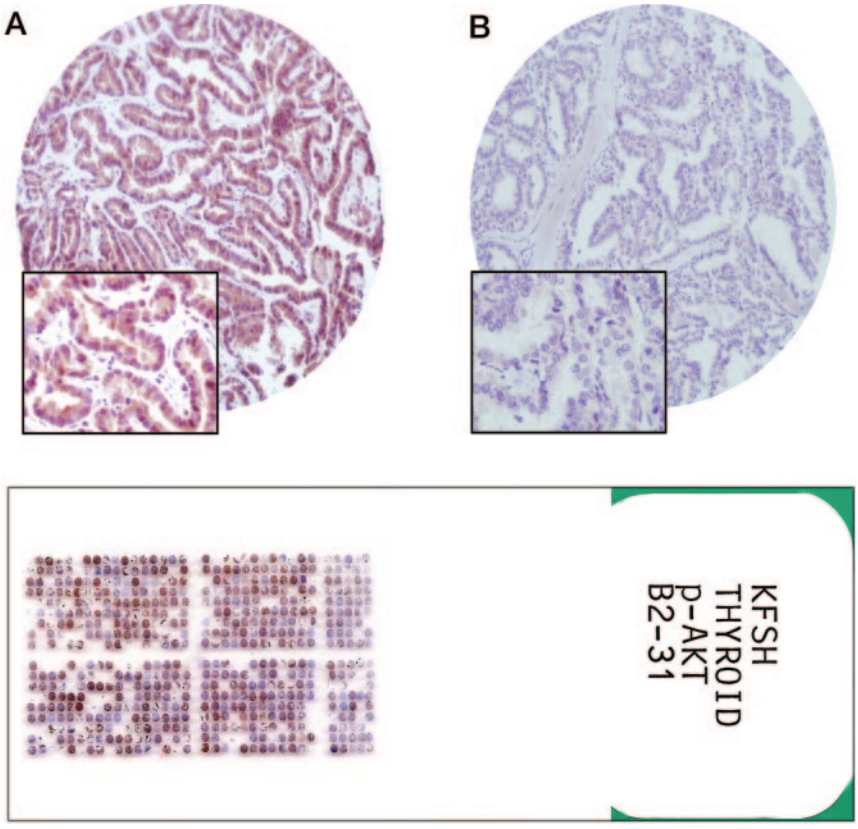

FIG. 2. Immunohistochemical analysis of p-AKT expression in PTC. A, Overexpression of p-AKT in a PTC TMA specimen. B, Negative staining for p-AKT (no expression) in another PTC TMA specimen (× 20 magnifications with the *inset* showing a ×100 magnified view of the same). An overview of the TMA hematoxylin and eosin-stained slide is shown at the *bottom*.

as thyroid cell lines (6, 7). These findings are of potential practical interest and might highlight the important role of this genetic alteration in tumorigenesis in the Middle Eastern PTCs. The fact that PIK3CA amplification has also been reported in the benign adenomas might suggest that this alteration might be involved as tumor initiating event in thyroid tumorigenesis (6, 7).

As a readout of PI3K functional activation, we tested AKT phosphorylation (activation) in PTC. Our findings show that AKT is activated in a large proportion of PTCs (55%), regardless of the presence of PIK3CA alterations, *i.e.* mutations and/or PIK3CA amplification status. Currently multiple pathways have been implicated as having role in AKT activation. Previously we found strong correlation between hepatocyte growth factor receptor (*cMET*) expression and AKT activation in a large subset of PTC (12). Therefore, *cMET* overexpression seems to be able to activate AKT signaling by a mechanism independent of the PI3K pathway. Moreover, the oncogenic *Ras* mutation may also represent another alternative mechanism for PI3K/AKT activation in PTC through a cross talk with the Ras/Raf/MAPK signaling pathway. Our study indeed confirms this hypothesis because a strong association between *Ras* mutation and AKT activation is seen ($P = 0.0082$). The fact that the *Ras* mutation is seen in PTC subset with lower incidence of extrathyroidal extension ($P = 0.0313$) and its existence in the early stage ($P = 0.0465$) might suggest that activation of the PI3K/AKT pathway by *Ras* mutations particularly occur as an early genetic event in PTC.

In examining the relationship between *Ras* mutations and *PIK3CA* amplification, we found *PIK3CA* amplification in eight of 15 samples that harbored *Ras* mutation. Although mutual exclusivity between *Ras* mutation and *PIK3CA* amplification was reported by other authors (5, 7), our data failed to show that correlation. A possible explanation for this lack of exclusivity is that *Ras* may be able to activate the AKT signaling pathway in PI3K-independent pathway and also in a PI3K-dependent pathway. The coexistence of *Ras* mutation and *PIK3CA* amplification might indicate that *PIK3CA* amplification is a less potent activator of the PI3K pathway in PTC.

We also studied another potent MAPK signaling pathway, *BRAF* mutation, and its correlation with *PIK3CA* alterations. *BRAF* mutation is seen in more than half of PTC samples studied (51.7%). This is in accordance with previous reports in which activating *BRAF* mutation was found in 35–70% of PTC (23–25), which indicates that *BRAF* mutation through the MAPK pathway plays an important role in tumorigenesis of PTC. Our study shows significant association of BRAF mutation with metastasis ($P = 0.0274$) and a decreased disease-free survival ($P = 0.012$). Several other studies have confirmed the association between *BRAF* mutation and poor clinicopathological behavior (19, 26).

Furthermore, mutual exclusivity between *BRAF* mutation and *Ras* mutation was seen in our PTCs. This finding is also in agreement with previous reports (22, 27, 28). These and other studies (29, 30) support the idea that each of these two genetic alterations (either *BRAF* mutation or *Ras* mutation) in PTC might be sufficient to cause thyroid tumorigenesis. The mutual exclusivity among these genetic alterations in PTC is not surprising, though because the signaling pathway of these activating genetic alterations share the common MAPK pathway at different steps. A single oncogenic alteration along this pathway is sufficient to drive thyroid cell transformation. Thus, we can conclude that activating mutations can occur at several levels in MAPK pathway in PTC. The presence of the *Ras* mutation in the early stages of PTC suggests that the acquisition of mutations in this pathway occurs early and may be even a founder event of PTC tumorigenesis.

The PI3K/AKT pathway is known to have a close cross talk with the RAS/RAF/MAPK signaling cascade (31). Many of our BRAF-mutated PTCs showed coexisting *PIK3CA* amplifications, which could reflect the putative cooperation of PI3K signaling and BRAF signaling in tumorigenesis and thus might have important implication in the behavior of this subset of PTC. To investigate the hypothesis of synergistic effect of

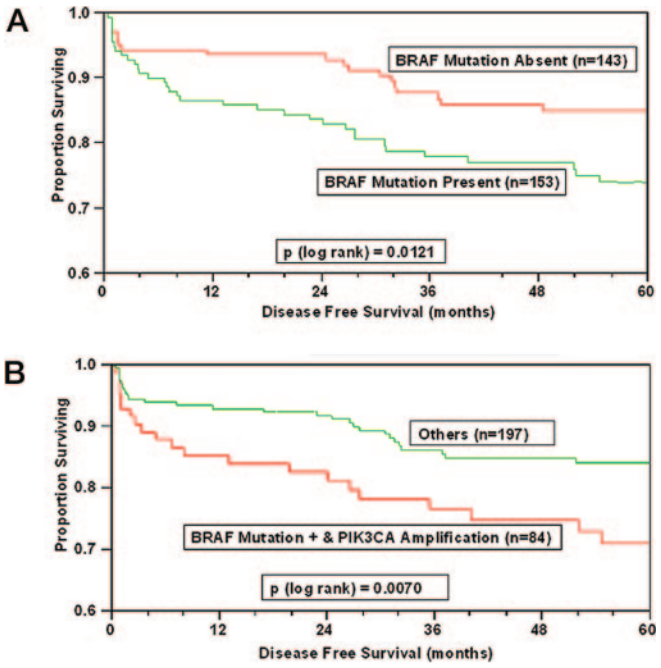

FIG. 3. PIK3CA alteration and BRAF mutational status and disease-free survival of patients with PTC. A, Kaplan-Meier disease-free survival estimates of PTC with and without BRAF mutations. B, Kaplan-Meier disease-free survival estimates of PTC grouped into PIK3CA amplification and BRAF mutation group and other abnormalities group.

these two mutations, we stratified our PTC cases into two groups, depending on the status of *PIK3CA* amplifications and *BRAF* mutations: one group with the *PIK3CA* gene amplification and *BRAF* mutation and the second group consisted of PTC cases with no abnormality or abnormality in either *PIK3CA* or *BRAF*. The PTC group with both *PIK3CA* amplification and *BRAF* mutation showed a significant association with larger tumor size ($P = 0.0479$), metastasis ($P = 0.026$), and a significantly poor disease-free survival ($P = 0.007$, Fig. 3). From these observations we can speculate on the existence of a synergism between BRAF mutations and PIK3CA amplifications in PTC tumorigenesis.

A higher incidence of *PIK3CA* alterations in the Middle Eastern PTC probably attributed to ethnic variation and a possible synergistic effect of *PIK3CA* alterations and *BRAF* mutations in the tumorigenesis of PTC are some of the significant findings of our study. This study raises the possibility that inhibition of PI3K/AKT and MAPK might have significant therapeutic activity and could be developed into an effective anticancer combination regimen.

Acknowledgments

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