Phosphatidylinositol 3-Kinase/Akt and Ras/Raf-Mitogen-Activated Protein Kinase Pathway Mutations in Anaplastic Thyroid Cancer

Libero Santarpia, Adel K. El-Naggar, Gilbert J. Cote, Jeffrey N. Myers, and Steven I. Sherman

Departments of Endocrine Neoplasia and Hormonal Disorders (L.S., G.J.C., S.I.S.), of Pathology (A.K.E.-N.), and of Head and Neck Surgery and Cancer Biology (J.N.M.), The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030-4009

Context: Anaplastic thyroid carcinoma (ATC) can occur in the setting of differentiated thyroid carcinoma (DTC), which suggests a continuum in malignant progression from DTC to ATC. The Ras/Raf-MAPK and the phosphatidylinositol 3-kinase/Akt signaling pathways play critical roles in DTC tumorigenesis, but their roles in the pathogenesis of ATC are poorly defined.

Objective: Our objective was to explore the potential contributions of these two pathways in ATC pathogenesis.

Design, Setting, and Subjects: The mutational status of *BRAF*, *PIK3CA, PTEN,* and *RAS* genes was analyzed in genomic DNA from microdissected tumor specimens of 36 cases of ATC, and in 16 samples of paired-matched lymph node metastases. *PIK3CA* copy number gain was assessed by real-time quantitative PCR. We performed immunohistochemistry for phospho-ERK and phospho-AKT in 26 cases of ATC.

Results: DTC was present in half of the cases. *BRAF* V600E mutation was identified in nine of 36 (25%) ATCs; seven cases had identical mutations in both the ATC and DTC components. *PIK3CA* kinase domain mutations were found in five (14%) ATCs, one of which had mutations in both differentiated and anaplastic areas. *RAS* and *PTEN* mutations were each found in two (6%) ATCs. *PIK3CA* gain copy number was found notably increased in 14 (39%) ATCs.

Conclusions: *BRAF* mutations appear to play a role in the tumorigenesis of a subset of ATCs, and the majority of lymph node metastases. *PIK3CA* alterations occur preferentially in the later stages of ATC and were the most relevant events during thyroid cancer progression. The activation of both pathways suggests an important role in ATC dedifferentiation. **(*J Clin Endocrinol Metab* 93: 278–284, 2008)**

Thyroid carcinoma is the most common malignancy of the endocrine system (1). The common differentiated follicular-cell derived tumors include papillary (PTC) and follicular (FTC) carcinoma. Poorly differentiated thyroid carcinomas (PDTCs) occur less commonly, and undifferentiated thyroid carcinoma is represented by anaplastic thyroid carcinoma (ATC), which is a high-grade carcinoma that is nearly always fatal. Previous reports suggest that some cases of ATC may derive from differentiated thyroid carcinoma (DTC) (2–4). Evidence of this progression includes the coexistence of ATC and PDTC within the same tumor specimens and the fact that some cases of treated DTC have recurred as ATC (5). Moreover, a subset of ATC may present within a component of a larger DTC or may contain microscopic foci of differentiated carcinoma, typically of the papillary phenotype (6). Although these findings suggest that dedifferentiation occurs through a continuum, little is known about the mechanisms underlying this process. Alternatively, due to the rarity, rapid growth, and presence of undifferentiated fetal cells in ATC, the "tumor stem cells" model should also be considered (7). These two mechanisms perhaps are not mutually exclusive.

A role for abnormalities in Ras/Raf-MAPK signaling has been established in DTC tumorigenesis, but a similar role in ATC

0021-972X/08/$15.00/0

Printed in U.S.A.

doi: 10.1210/jc.2007-1076 Received May 14, 2007. Accepted October 26, 2007.

First Published Online November 7, 2007

Abbreviations: ATC, Anaplastic thyroid carcinoma; DTC, differentiated thyroid carcinoma; FTC, follicular thyroid carcinoma; LN, lymph node metastases; p-AKT, phosphorylated AKT; PDTC, poorly differentiated thyroid carcinoma; p-ERK, phosphorylated ERK; PI3K, phosphatidylinositol 3-kinase; PTC, papillary thyroid carcinoma; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

pathogenesis is less clear. RAS family oncogenes are important regulators of cell growth (8) and have a role in thyroid tumor differentiation (9). Downstream of RAS are multiple signal transduction pathways responsible for the execution of RAS-mediated cellular effects. The most widely validated ones that contribute to human tumorigenesis include the Raf-MAPK and phosphatidylinositol 3-kinase (PI3K)/Akt pathways (10).

The *BRAF* gene belongs to the RAF family of serine/threonine kinase, playing a central role in the regulation of cell growth, division, and proliferation (11). The gene has been variably mutated in PTC and ATC (12). Recent reports of the restriction of *BRAF* mutations to a subset of ATC derived from PTC did not agree on the real rate of *BRAF* mutation in this type of tumor, and previous studies have shown a variable mutation frequency in *BRAF* of 10, 62.5, and 20% (3, 4, 7).

Alterations in components of the PI3K/Akt pathway have been demonstrated in a variety of human cancers (13). The *PIK3CA* gene encoding the catalytic subunit p110α of class IA PI3K has acted as an oncogene because of its elevated kinase activity and frequent genomic amplification in cancer (13, 14). Common *PIK3CA* gene amplifications and mutations have been demonstrated in thyroid cancer (15–18), providing evidence that aberrant activation of the PI3K/Akt pathway plays an important role in thyroid tumorigenesis, but the role of these genetic alterations in the progression from DTC to ATC is still unclear.

PTEN is a tumor suppressor gene that antagonizes signaling through the PI3K pathway. The function of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) protein is to dephosphorylate the phosphatidylinositol-3,4,5-triphosphate, which reduces the downstream activity of protein kinase B/Akt kinase, thereby inducing cell-cycle arrest, apoptosis, or both (19). *PTEN* has been mutated or deleted in different types of thyroid tumors (20), but the real incidence of these alterations and their role in ATC have not been well established.

The pathogenesis of ATC and its possible progression from preexisting DTC are subjects of debate. No published studies have simultaneously addressed the mutational activation of *RAS, BRAF, PIK3CA*, and *PTEN*, in the PI3K and RAS pathways in the malignant transformation of ATC. The purpose of this study was to evaluate how these events are involved in ATC and in dedifferentiation from DTC to ATC. Understanding the function and the interaction of these signaling pathways is crucial to comprehending the regulation of subsequent signaling events: cell metabolism, cell cycle progression, cell survival, and cell growth in thyroid cancer progression.

In this study we analyzed a series of 36 ATC cases, 50% of which showed coexisting DTC, for alterations in the PI3K and RAS pathways. We found evidence of different and increasing molecular alterations during thyroid cancer progression.

Materials and Methods

Human thyroid tissues, laser-capture microdissection, and DNA isolation

ATC specimens paraffin embedded from 36 patients were obtained from the Department of Pathology at The University of Texas M. D. Anderson Cancer Center. The study protocol was approved by the institutional review board. The tumor tissue specimens were evaluated for histological features. Among the 36 ATCs, 18 coexisting DTCs (15 PTCs, two FTCs, and one Hürthle cell carcinoma) and four concomitant PDTCs were present. Moreover, 10 cases of PTC and 10 cases of FTC without an undifferentiated component were examined as controls.

Areas within the primary tumor that had different microscopic appearances with the same histological grade (primarily spindle cell/sarcomatoid, giant cell epithelioid, or mixed) or that were high grade and poorly differentiated were separately analyzed. Sixteen samples of matched lymph node metastases (LN) (11 from primary tumors containing both ATC and PTC and five from primary tumor containing ATC) and 10 noncontiguous foci of PTC within the ATC samples with coexisting PTC were also analyzed in this study.

The paraffin-embedded tumor sections were microdissected by laser capture in a way to ensure that the cancer cells represented at least 80% of the cells in each section. DNA was isolated from both undifferentiated and differentiated components of the tissue sections as well as from paired lymph nodes. A total of 84 tumor specimens for the ATC cases was analyzed: 36 undifferentiated components, 18 differentiated components, 16 lymph nodes, 10 additional foci of PTC, and four foci of PDTC. Samples of normal thyroid tissue from 10 of the cases were available and analyzed as well. All slides were reviewed according to established histological criteria (5).

Genomic DNA was isolated from 5-μm sections containing both thyroid tumor and normal thyroid tissue when available. The sections were dewaxed with two xylene washes (30 min at 55 C each), one 100% ethanol wash, two 70% ethanol washes, and two distilled water washes (6 min at 55 C for each step), and then incubated with proteinase K (20 mg/ml) and digestion buffer [100 mM NaCl/10 mM Tris-HCl (pH 8.0) 25 mM EDTA (pH 8.0), and 0.5% sodium dodecyl sulfate]. We analyzed the DNA sequences of the genes *BRAF* (exon 15), *PIK3CA* (exons 9 and 20), *PTEN* (exon 5–8), and H-, N-, and K-*RAS* (exons 1 and 2) in all 84 tumor specimens and the 10 paired normal thyroid tissue samples.

Mutational analysis for *BRAF, PIK3CA, RAS,* and *PTEN* genes

Genes were amplified by PCR, and direct sequenced with forward and reverse amplification primers. The *BRAF* gene was amplified with the primers 5′-TCATGAAGACCTCACAGTAAAAAT-3′ (forward) and 5′-TGGATCCAAGACAACTGTTCAA-3′ (reverse). PCR was performed in a 25-μl mixture containing 20 pmol each primer, 200 μM each deoxynucleotide triphosphate, 0.5 U *Taq* DNA polymerase, and 30- to 50-ng genomic DNA. PCR conditions consisted of the initial denaturation (95 C for 5 min), followed by 36 cycles of denaturation at 94 C for 30 sec, annealing at 54 C for 60 sec, and extension at 72 C for 30 sec. For *PIK3CA,* we restricted mutation analyses to the helical and kinase domain "hot spots," in which 80–90% of mutations are reported to cluster. The *PIK3CA* gene was amplified for exon 9 as previously reported (16) and for exon 20 with the primers 5′-TGACATTTGAGCAAAGACCTG-3′ (forward) and 5′-ATCAAACCCTGTTTGCGTTT-3′ (reverse). PCR was performed in a total volume of 25 μl containing 20 pmol each primer pair, 50- to 60-ng genomic DNA, 200 μM deoxynucleotide triphosphates, 50 mM PCR buffer containing 2.0 mM $MgCl_2$, and 0.5 U *Taq* DNA polymerase. The PCR consisted of the initial denaturation step of 8 min at 95 C, followed by 36 cycles of 1 min at 94 C, 30 sec at 55 C, and 30 sec at 72 C. The *PTEN* gene was amplified for exons 5–8, where most identified mutations are reported to cluster (17, 18). For the three *RAS* genes, we used direct sequencing after PCR amplification of exons 1 and 2, as previously described (21). All PCR products were run onto 2% agarose gel to establish the quality of the PCR reaction and purified using a DNA clean-up kit (Promega, Madison, WI).

TABLE 1. Total number of mutations of *BRAF*, *PIK3CA, PTEN*, and *RAS* in 36 ATC cases

Gene	Total no. of mutations (%)	ATC (n = 18)	ATC + DTC (n = 18)[a]
BRAF	9 (25)	2[b]	7
PIK3CA	5 (14)	4[c]	1
PTEN	2 (6)	2	0
RAS (H-, N-, K-)	2 (6)	2[c]	0

[a] Mutation in both components.

[b] One case showed the presence of PTC in the lymph node.

[c] One tumor with concomitant mutation in the PDTC.

Copy number analysis of PIK3CA by real-time quantitative PCR

Genomic DNA was assessed for copy number gain of *PIK3CA* in all 36 ATCs, in the concurrent DTC when present, and in three ATC cell lines (ARO, KAT4, and C643).

Real-time PCR was run on the StepOne system (Applied Biosystems, Foster City, CA), and the reactions were all performed in triplicate using: forward primer, 5′-AGTGAATTGAATTCTGTCCCTTCCAA-3′; primer reverse, 5′-CGATTTCTGGATAGTGGGCAATAAGG-3′; and probe, 5′-FAM-GAATTTACCAGTGGAT-NFQ-3′. Ribonuclease P (Applied Biosystems) was used as a reference control. Data were analyzed by the ΔΔCt method.

Immunohistochemical staining for phospho-ERK p44/42 and phospho-AKT

To examine the activation status of MAPK/Erk1/2 and Akt, 4-μm paraffin-embedded sections from 26 cases of ATC, including DTC when present, were immunostained with antibodies to phospho-p44/42 MAPK (Thr202/Tyr204) (dilution 1:200) and phospho-Akt (Ser473) (dilution 1:100) (Cell Signaling Technology, Beverly, MA). After deparaffinization and rehydration, sections were incubated at 4 C overnight with the antibodies. Staining was performed using the Vectastain Universal Quick kit (Vector Laboratories, Burlingame, CA). The peroxidase reaction was developed with 3,3′-diaminobenzidine, and the slides were counterstained with hematoxylin.

Immunoreactivity was expressed as the percentage of positive-stained cells in different categories: −, staining; +, low/weak; 2+, moderate/distinct; and 2++, high/intense. The immunohistostaining score for the intensity of the staining was assigned in a blind fashion without knowing the mutational status of the genes.

Results

To assess the frequency of *BRAF, PIK3CA, PTEN,* and *RAS* hot spot mutations in ATC, we sequenced genomic DNA derived from ATC primary and metastatic tumor specimens, including DTC and PDTC foci within or adjacent to the ATC tumor specimens. Collectively, these mutations were identified in 15 of the 36 (42%) primary ATC tumors (Tables 1 and 2).

The transversion T1799A (V600E) *BRAF* mutation was found in nine (25%) tumors. Seven (78%) of these were found to have PTC and ATC areas with identical *BRAF* mutations (Fig. 1). The remaining two *BRAF*-mutated ATC cases did not contain any papillary architecture, although a focus of PTC was noted in a lymph node in one case (case 18). The *BRAF* mutations were not uniformly identified within different PTC foci with similar morphological features. Among 10 noncontiguous foci of PTC present within the ATC tumors, four had the *BRAF* mutation, whereas the remaining six had wild-type *BRAF*, suggesting different clonal cell origins within the same tumor.

PIK3CA mutations were found in five of the 36 (14%) tumors, affecting codons 1030 (AAA>GAA, Lys>Glu), 1031 (ACT>ATT, Thr>Ile), 1047 (CAT>CGT, His>Arg), and 1048 (CAT>TAT, Hys>Ile). The same codon 1031 mutation was found in two samples. In our ATC cases, *PIK3CA* mutations

TABLE 2. Mutation locations of *BRAF*, *PIK3CA*, *PTEN*, and *RAS* genes in ATC cases with matched components of ATC, DTC, and lymph nodes and *PIK3CA* gene copy gain

Case no.	BRAF status			PIK3CA status			PTEN status			RAS status (H-, K-, and N-Ras)			PIK3CA copy gain	
	ATC	DTC	LN	ATC	DTC	LN	ATC	DTC	LN	ATC	DTC	LN	ATC	DTC
3	**MUT**	**MUT**	**MUT**	WT	WT	WT	WT	WT	WT	WT	WT	WT	+	−
7	**MUT**	**MUT**	n/a	**MUT**	WT	n/a	WT	WT	n/a	WT	WT	n/a	+	−
8[a,b]	WT	np	WT	**MUT**	np	WT	WT	np	WT	WT	np	WT	+	−
9	**MUT**	**MUT**	np	WT	np	np	WT	np	np	WT	np	np	+	−
14[a,c]	WT	np	WT	**MUT**	np	**MUT**	WT	np	WT	**MUT**	np	WT	−	−
15	**MUT**	**MUT**	**MUT**	**MUT**	**MUT**	**MUT**	WT	WT	WT	WT	WT	WT	+	−
16	**MUT**	np	np	WT	np	np	WT	np	np	WT	np	np	+	−
18	**MUT**	np	**MUT**	WT	np	WT	WT	np	WT	WT	np	WT	+	−
19	WT	WT	np	WT	WT	np	WT	WT	np	**MUT**	WT	np	−	−
21	**MUT**	**MUT**	n/a	WT	WT	n/a	WT	WT	n/a	WT	WT	n/a	−	−
24	**MUT**	**MUT**	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	+	−
26	**MUT**	**MUT**	**MUT**	WT	WT	WT	WT	WT	WT	WT	WT	WT	+	+
31	WT	np	np	**MUT**	np	np	WT	np	np	WT	np	np	+	−
32	WT	np	np	WT	np	np	**MUT**	np	np	WT	np	np	−	−
35	WT	WT	np	WT	WT	np	**MUT**	WT	np	WT	WT	np	−	−

Bold regions indicate coexistence of two or more alterations. MUT, Mutated; n/a, not available due to the absence of DNA or poor DNA quality; np, not present; WT, wild type.

[a] Coexistence of a PDTC.

[b] *PIK3CA* mutated also in the PDTC.

[c] *RAS* mutated also in the PDTC.

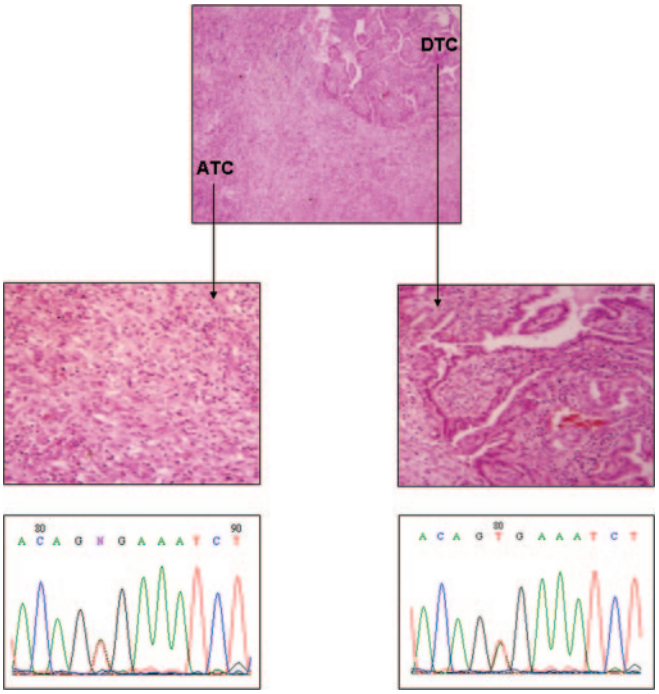

FIG. 1. Hematoxylin and eosin-stained sections and the results of sequencing analysis in case 21. *Lower panels*, High magnification of the upper-section areas that were microdissected. The *BRAF* V600E mutation was identified in both the ATC and in the adjacent area within the DTC-PTC. Magnification, *upper panel*, ×20; extracted micrographs, ×40.

were restricted exclusively to the kinase domain. When there was coexisting DTC, the mutations were identified in DNA isolated from only the ATC components except in one case, in which the mutation was found in both the ATC and DTC components, as well as in a paired lymph node (case 15). In another case the same mutation was identified in both the ATC and PDTC (case 8). *PIK3CA* mutations were identified in regions of ATC tumors with divergent microscopic phenotypical architecture, such as spindle cell/sarcomatoid and giant cell epithelioid (Fig. 2). The analysis of phenotypically different areas with the same histological grade within the same tumor showed that the *PIK3CA* mutations were heterogeneously distributed within the specimen, illustrating the intratumor heterogeneity of the ATC.

Two exon 5 (6%) *PTEN* mutations were found within the anaplastic areas of the tumor. One mutation was the AGA>GGA mutation at codon 161 (Arg>Gly), and the other was the transversion mutation TAT>TGT at codon 155 (Tyr>Cys).

Only two (6%) cases of K-*RAS* mutations were found. One mutation was the transversion mutation GGT>GAT at codon 12 (Gly>Asp) within an ATC, including the coexistent PDTC. The mutation GGC>GAC at codon 13 (Gly>Asp) was found in another tumor only within the undifferentiated component.

Finally, two cases (cases 7 and 15) harbored both *BRAF* and *PIK3CA* mutations (Fig. 3), and one case harbored both *PIK3CA* and *RAS* mutations (case 14) (Table 2).

None of the mutations identified appeared in the analyzed normal thyroid tissues. In contrast, in the 16 samples of matched LN, four harbored *BRAF* mutations in ATC with accompanying PTC samples, and two had *PIK3CA* mutations (one case was

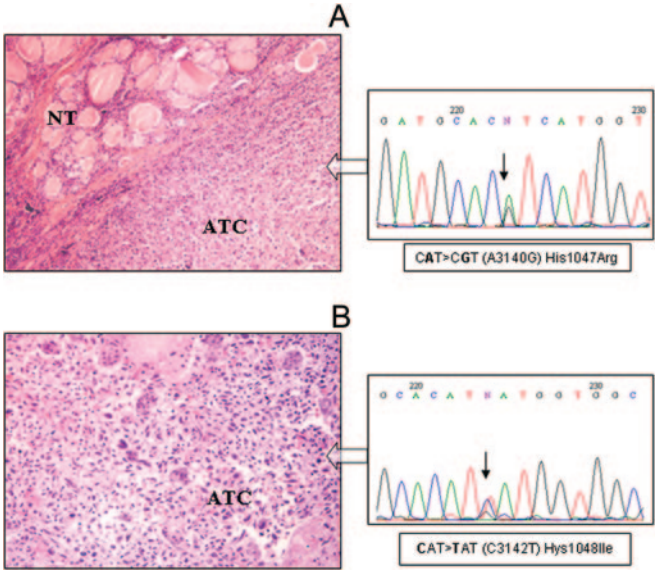

FIG. 2. Status of *PIK3CA* mutations in different ATC samples. A, Case 14, normal thyroid tissue (NT) *vs.* ATC. Magnification, ×20. B, Case 31, ATC-giant cell mutated at *PIK3CA* gene. Magnification, ×40.

ATC, and another was ATC + PTC); none had *RAS* or *PTEN* mutations (Table 2). Mutations of both *BRAF* and *PIK3CA* were found in one lymph node (case 15). Among the 20 cases of DTC with no coexisting undifferentiated components, the *BRAF* V600E mutation was found in four cases of PTC (40% of PTC cases); no mutations were found in FTC. No mutations in the *PIK3CA* or *PTEN* genes were found in both PTC and FTC.

PIK3CA gains (with a gene copy number of three or more) were found in 22 (61%) ATC samples. *PIK3CA* gene copies were notably increased (four copies or more) in 14 (39%) ATCs. Four of five (80%) *PIK3CA* mutated cases also had an increase in gene copy number (Table 2). Among the DTC within the ATC, only

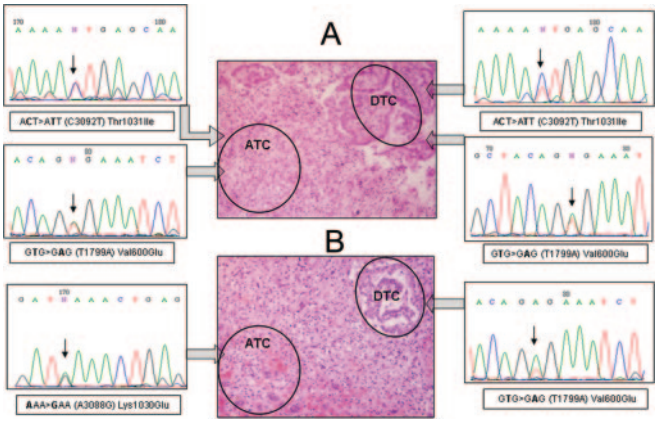

FIG. 3. Hematoxylin and eosin-stained sections and sequence analysis chromatograms showing the mutation status of the two most frequently mutated genes, *BRAF* (T1799/A, V600E) and *PIK3CA*, in ATC with accompanying DTC. *Circled areas* indicate the microdissected section of the tumor by laser-capture microdissection. A, Case 15, ATC *vs.* PTC (*right*), mutations of both the *BRAF* and *PIK3CA* genes in both components. B, Case 7, ATC *vs.* PTC (*right*), mutation of *BRAF* in both components, and mutation of *PIK3CA* only in the ATC. Areas indicated by *clear arrows* were microdissected, and the sequence of *BRAF* and *PIK3CA* gene are shown. *Solid black arrows* in the chromatograms indicate the mutation. Magnification, ×20. WT, Wild type.

two of 18 (11%) showed moderate gene copy gain. Two ATC cell lines, ARO and KAT4, showed a high level of *PIK3CA* amplification, whether C643 did not show any significant gene copy gain.

Activation of AKT (phosphorylation) was detected in 22 of 26 (85%) ATCs. High levels of phosphorylated AKT (p-AKT) were found in five (23%) ATCs, moderate staining in 12 (54%) ATCs, and low levels in five (23%) ATCs. In all positive cases, the activation of AKT was demonstrated in the cytoplasm. In 17 (77%) ATC (and in one PTC) samples, nuclear p-AKT was noted in conjunction with cytoplasmic staining (Fig. 4). Only cytoplasmatic localization of p-AKT was observed in DTC.

Activation of ERK was found in 17 of 26 (65%) ATC cases. Slight phosphorylated ERK (p-ERK) immunostaining was noted in 12 (71%) ATCs without a noteworthy difference within the concomitant DTC. Moderated focally ERK activation was noted in five (29%) ATCs. Both DTC and ATC components had p-ERK localized at cytoplasmic level. Of note, p-ERK was present within some endothelial cells (Fig. 4).

Discussion

Our study showed that both the Ras/Raf-MAPK and PI3K/Akt signaling pathways are involved in the pathogenesis and progression of ATC. We showed a low frequency of genetic alterations in these two aforementioned pathways in DTC. In contrast, we found an increase in the frequency of genetic abnormalities in both pathways during thyroid cancer progression. We identified a large proportion of ATCs with mutational activation of the RAS and PI3K signaling pathways.

In our study, when DTC coexisted with ATC, the only mutations that overlapped were *BRAF* and *PIK3CA*. Increasing of any genetic mutations became more common as the dedifferentiation progressed. We found the *BRAF* V600E mutation mainly in the anaplastic area and in the accompanying differentiated areas (PTC) within the same tumor specimen. The transversion T1799A, leading to a Glu substitution for Val at codon 600 (V600E), accounts for approximately 90% of *BRAF* mutations in thyroid carcinomas, and it has been found to be mutated in approximately 44% of PTC cases and 24% of ATC cases (11). The *BRAF* V600E mutation appears to play a pivotal role in thyroid cancer initiation because it has been mutated in microcarcinomas (11), and *in vitro* and *in vivo* models have demonstrated that overexpression of activated BRAF induces malignant transformation and aggressive tumor behavior (22–24). We have confirmed that some cases of ATC can occur from anaplastic transformation of DTC. Our data support findings from prior studies (2–4).

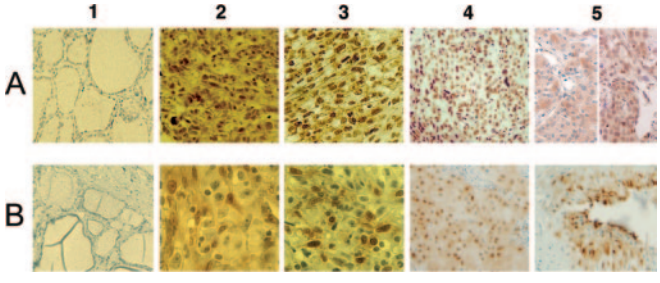

FIG. 4. p-AKT and p-ERK in several ATCs. A1, Expression of p-AKT in normal thyroid tissue. A2, Nuclear and cytoplasmatic expression of p-AKT in a case of ATC mutated at *PIK3CA*. A3, Nuclear and cytoplasmatic expression of p-AKT in a case of ATC wild type at *PIK3CA*. A4, Activation of p-AKT in another case of ATC. A5, FTC (*left panel*) and PTC (*right panel*) present within two different ATCs; in both cases the expression of p-AKT was limited to the cytoplasm. B1, Expression of p-ERK in normal thyroid tissue. B2, Cytoplasmatic staining of p-ERK in a case of ATC mutated at *BRAF*. B3, Cytoplasmatic staining of p-ERK in a case of ATC wild type at *BRAF*. B4, Focal activation of p-ERK in a case of PDTC. B5, Endothelial cells showing immunoreactivity for p-ERK. Magnification, ×20; B2 and B3, ×40.

However, we found that two of the nine (22%) cases of ATC with *BRAF* mutations had no apparent coexisting differentiated component, which was probably due to the loss of papillary or any follicular-epithelial architecture or to the DTC component being completely obscured by the extensive anaplastic tumor. Of note, one of these tumors was mutated for *BRAF* in the lymph node (case 18). When they were present, other distinct noncontiguous foci of PTC within the same primary tumor were analyzed, and we found that *BRAF* mutations were restricted to only some of the foci, supporting the idea that PTC multifocality is due to different cell clone origins instead of spreading from the same tumor. Our finding that 19% of ATC cases had B*RAF* mutations in both the differentiated and undifferentiated areas of thyroid tumor specimens suggests a role for oncogenic *BRAF* in disease progression as well. However, the fact that *BRAF* mutations were found in both components of ATC and in control PTC suggests that these mutations are most likely an early event during dedifferentiation, and that these events are probably insufficient by themselves to initiate anaplastic dedifferentiation but may predispose the tumor cells to gain additional genetic alterations that activate other pathways and lead to dedifferentiation. Although, *BRAF* V600E mutation was the leading event in LN (four of 16, 25%) (Table 2).

We found that the *PIK3CA* gene was mutated in 14% of the ATC cases and was restricted mainly to the undifferentiated thyroid component. We confirmed the preferential role of *PIK3CA* mutation in the later stages of thyroid cancer (17), suggesting an important role of this event in ATC rather than DTC.

The importance of the *PIK3CA* mutation in thyroid cancer might be its ability to activate other genetic markers, such as p53, which was recently activated in a subset of ATC cases derived from PTC (4), and PIK3 signaling could be an important endogenous inducer of p53 in thyroid cancer transformation.

Copy gains of *PIK3CA* gene seem to be the most relevant event in ATC. Our results show that a change in the gene copy number of *PIK3CA* occurs almost exclusively in the undifferentiated component compared with paired DTC within the same tumor. This suggests that the activation of the PI3K-Akt pathway through *PIK3CA* copy gain contributes to thyroid tumor progression in ATC. Moreover, our data provide evidence that gain of gene copy number and somatic mutations may exist in parallel in ATC, most likely reinforcing the activation of the PI3K-Akt pathway.

Increased PI3K expression results in the enhanced activity of AKT, which is a marker downstream of PI3K that, once activated, regulates apoptosis, proliferation, and migration in thy-

roid cells (25) contributing to thyroid cancer progression (26, 27).

A *PTEN* mutation was present in the 6% of our ATC series. The role of *PTEN* in tumor suppression has been linked to the down-regulation of the PI3K/Akt pathway by PTEN′s lipid phosphatase activity (19). The low prevalence of *PTEN* mutations in our study may be due to the fact that our series included only two cases of ATC with coexisting FTC. No overlapping mutations between *PTEN* and *PIK3CA* were found, suggesting that only one of these mutations is sufficient to promote tumorigenesis and that each event may play a distinct role in thyroid tumorigenesis. PTEN is often lost or down-regulated in ATC, leading to AKT activation, in the same way gain of function mutations in *PIK3CA* may induce the activation of PI3K with effects on cell proliferation.

We noted a low frequency of *RAS* mutations in our cases (6%) (one case of ATC with coexisting PDTC, and in one case of ATC only), both in K-*RAS*. Constitutive activation of H-, K-, and N-*RAS* mutations has been reported as markers for aggressive thyroid cancer behavior (28).

In a survey study of *RAS* mutations involving 39 reports and 269 mutations, N-*RAS* (exon 1) and K-*RAS* (exon 2) had a mutation frequency less than 1%, and H-*RAS* mutation (codons 12 and 13) was 2–3% of all thyroid tumors types (18). From this report also emerge the importance of RAS in FTC and the concept that mutation frequency in *RAS* isoforms is tumor phenotype related, supporting the idea that most likely, the activity of specific RAS oncogenes in thyroid is histotype dependent, and there is a possibility that K-*RAS* could be more important in PTC-ATC phenotypes.

Our finding of both *RAS* and *PIK3CA* mutations in the same tumor specimen implies that *R*AS mutations can occur coincident with *PIK3CA* mutations and reinforces the concept that cross talk between the two signaling pathways exists.

An important finding was that the activation of ERK and AKT was noted at higher frequency in ATC, and that sometimes activation of these pathways occurred also in the absence of PI3K or RAS mutations. Furthermore, the presence of mutation or amplification was not correlated with higher staining intensity. These results imply that activation of these pathways play an important role in ATC progression and also may be a target through other mechanisms, *e.g.* epidermal growth factor or fibroblast growth factor.

The increasing of any genetic alterations in the Ras/Raf-MAPK and PI3K/Akt pathways was noted almost exclusively in the undifferentiated tumor components. Thus, even though only one pathway is needed to initiate thyroid cancer pathogenesis, both pathways seem to be involved during thyroid cancer dedifferentiation. Although we reported a high prevalence (42%) of genetic mutations in ATC, this is likely an underestimate because not all the coding regions of the genes were examined, and other important genes such as *p53* were not analyzed.

On the basis of all these considerations and the results of the experimental assays, we conclude that the Ras and PIK3 signal transduction pathways play important roles in thyroid tumor initiation and progression. Cross talk between the PI3K and RAS signaling pathways may occur at several stages, and both pathways are implicated in the anaplastic transformation of thyroid cancer. Elucidating how these pathways interact and clarifying the genetic background of the thyroid tumor components will lead to an understanding of thyroid carcinogenesis and, perhaps, the development of therapeutic strategies based on targeting both pathways.

Acknowledgments

We thank Dr. Rakesh Kumar for his assistance with the immunohistochemistry assay.

Address all correspondence and requests for reprints to: Steven I. Sherman, Department of Endocrine Neoplasia and Hormonal Disorders, Unit 435, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030-4009. E-mail: sisherma@mdanderson.org.

Disclosure Statement: The authors have nothing to disclose.

References

1. **Figge J** 1999 Epidemiology of thyroid cancer. In: Wartofsky L, ed. Thyroid cancer: a comprehensive guide to clinical management. Totowa, NJ: Humana Press; 77–83
2. **Pasieka JL** 2003 Anaplastic thyroid cancer. Curr Opin Oncol 15:78–83
3. **Nikiforova MN, Kimura ET, Gandhi M, Biddinger PW, Knauf JA, Basolo F, Zhu Z, Giannini R, Salvatore G, Fusco A, Santoro M, Fagin JA, Nikiforov YE** 2003 BRAF mutations in thyroid tumors are restricted to papillary carcinomas and anaplastic or poorly differentiated carcinomas arising from papillary carcinomas. J Clin Endocrinol Metab 88:5399–5404
4. **Quiros RM, Ding HG, Gattuso P, Prinz RA, Xu X** 2005 Evidence that one subset of anaplastic thyroid carcinomas are derived from papillary carcinomas due to BRAF and p53 mutations. Cancer 103:2261–2268
5. **Rosai J, Carcangiu ML, DeLellis RA** 1992 Tumors of the thyroid gland. In: Atlas of tumor pathology. Washington, DC: Armed Forces Institute of Pathology
6. **Wiseman SM, Loree TR, Rigual NR, Hicks Jr WL, Douglas WG, Anderson GR, Stoler DL** 2003 Anaplastic transformation of thyroid cancer: review of clinical, pathologic, and molecular evidence provides new insights into disease biology and future therapy. Head Neck 25:662–670
7. **Takano T, Ito Y, Hirokawa M, Yoshida H, Miyauchi A** 2007 BRAF (V600E) mutation in anaplastic thyroid carcinomas and their accompanying differentiated carcinomas. Br J Cancer 96:1549–1553
8. **Crespo P, Leon J** 2000 Ras proteins in the control of the cell cycle and cell differentiation. Cell Mol Life Sci 57:1613–1636
9. **Rochefort P, Caillou B, Michiels FM, Ledent C, Talbot M, Schlumberger M, Lavelle F, Monier R, Feunteun J** 1996 Thyroid pathologies in transgenic mice expressing a human activated Ras gene driven by a thyroglobulin promoter. Oncogene 12:111–118
10. **Luo J, Manning BD, Cantley LC** 2003 Targeting the PI3K-Akt pathway in human cancer: rationale and promise. Cancer Cell 4:257–262
11. **Mercer KE, Pritchard CA** 2003 Raf proteins and cancer: B-Raf is identified as a mutational target. Biochim Biophys Acta 1653:25–40
12. **Xing M** 2005 BRAF mutation in thyroid cancer. Endocr Relat Cancer 12: 245–262
13. **Vivanco I, Sawyers CL** 2002 The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat Rev Cancer 2:489–501
14. **Samuels Y, Diaz Jr LA, Schmidt-Kittler O, Cummins JM, Delong L, Cheong I, Rago C, Huso DL, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE** 2005 Mutant PIK3CA promotes cell growth and invasion of human cancer cells. Cancer Cell 7:561–573
15. **Wu G, Mambo E, Guo Z, Hu S, Huang X, Gollin SM, Trink B, Ladenson PW, Sidransky D, Xing M** 2005 Uncommon mutation, but common amplifications, of the PIK3CA gene in thyroid tumors. J Clin Endocrinol Metab 90:4688–4693
16. **Garcia-Rostan G, Costa AM, Pereira-Castro I, Salvatore G, Hernandez R, Hermsem MJ, Herrero A, Fusco A, Cameselle-Teijeiro J, Santoro M** 2005

Mutation of the PIK3CA gene in anaplastic thyroid cancer. Cancer Res 65: 10199–10207
17. **Hou P, Liu D, Shan Y, Hu S, Studeman K, Condouris S, Wang Y, Trink A, El-Naggar AK, Tallini G, Vasko V, Xing M** 2007 Genetic alterations and their relationship in the phosphatidylinositol 3-kinase/Akt pathway in thyroid cancer. Clin Cancer Res 13:1161–1170
18. **Wang Y, Hou P, Yu H, Wang W, Ji M, Zhao S, Yan S, Sun X, Liu D, Shi B, Zhu G, Condouris S, Xing M** 2007 High prevalence and mutual exclusivity of genetic alterations in the PI3K/Akt pathway in thyroid tumors. J Clin Endocrinol Metab 92:2387–2390
19. **Cantley LC, Neel BG** 1999 New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci USA 96:4240–4245
20. **Dahia PL, Marsh DJ, Zheng Z, Zedenius J, Komminoth P, Frisk T, Wallin G, Parsons R, Longy M, Larsson C, Eng C** 1997 Somatic deletions and mutations in the Cowden disease gene, PTEN, in sporadic thyroid tumors. Cancer Res 57:4710–4713
21. **Vasko V, Ferrand M, Di Cristofaro J, Carayon P, Henry JF, de Micco C** 2003 Specific pattern of RAS oncogene mutations in follicular thyroid tumors. J Clin Endocrinol Metab 88:2745–2752
22. **Melillo RM, Castellone MD, Guarino V, De Falco V, Cirafici AM, Salvatore G, Caiazzo F, Basolo F, Giannini R, Kruhoffer M, Orntoft T, Fusco A, Santoro M** 2005 The RET/PTC-RAS-BRAF linear signaling cascade mediates the motile and mitogenic phenotype of thyroid cancer cells. J Clin Invest 115:1068–1081
23. **Mitsutake N, Knauf JA, Mitsutake S, Mesa Jr C, Zhang L, Fagin JA** 2005 Conditional BRAFV600E expression induces DNA synthesis, apoptosis, dedifferentiation, and chromosomal instability in thyroid PCCL3 cells. Cancer Res 65:2465–2473
24. **Knauf JA, Ma X, Smith EP, Zhang L, Mitsutake N, Liao XH, Refetoff S, Nikiforov YE, Fagin JA** 2005 Targeted expression of BRAFV600E in thyroid cells of transgenic mice results in papillary thyroid cancers that undergo dedifferentiation. Cancer Res 65:4238–4245
25. **Shinohara M, Chung YJ, Saji M, Ringel MD** 2007 AKT in thyroid tumorigenesis and progression. Endocrinology 148:942–947
26. **Ringel MD, Hayre N, Saito J, Saunier B, Schuppert F, Burch H, Bernet V, Burman KD, Kohn LD, Saji M** 2001 Overexpression and overactivation of Akt in thyroid carcinoma. Cancer Res 61:6105–6111
27. **Frisk T, Foukakis T, Dwight T, Lundberg J, Hoog A, Wallin G, Eng C, Zedenius J, Larsson C** 2002 Silencing of the PTEN tumor-suppressor gene in anaplastic thyroid cancer. Genes Chromosomes Cancer 35:74–80
28. **Garcia-Rostan G, Zhao H, Camp RL, Pollan M, Herrero A, Pardo J, Wu R, Carcangiu ML, Costa J, Tallini G** 2003 ras mutations are associated with aggressive tumor phenotypes and poor prognosis in thyroid cancer. J Clin Oncol 21:3226–3235