Highly Prevalent Genetic Alterations in Receptor Tyrosine Kinases and Phosphatidylinositol 3-Kinase/Akt and Mitogen-Activated Protein Kinase Pathways in Anaplastic and Follicular Thyroid Cancers

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Context: Genetic alterations in receptor tyrosine kinases (RTKs) and phosphatidylinositol 3-kinase (PI3K)/Akt and MAPK pathways have not been fully defined in anaplastic and follicular thyroid cancers [anaplastic thyroid cancer (ATC), follicular thyroid cancer (FTC)].

Objective: The objective of the study was to explore a wide-range genetic basis for the involvement of these pathways in ATC.

Design: We examined mutations and copy number gains of a large panel of genes in these pathways and corresponding phosphorylation of ERK (p-ERK) and Akt.

Results: We found frequent copy gains of RTK genes, including *EGFR*, *PDGFR*α and -β, *VEGFR1* and *2*, *KIT*, and *MET* and in *PIK3Ca*, *PIK3Cb*, and *PDK1* genes in the PI3K/Akt pathway. Mutations of *Ras*, *PIK3Ca*, *PTEN*, and *BRAF* genes and *RET/PTC* rearrangements were common, whereas mutations in *PDK1*, *Akt1*, *Akt2*, and RTK genes were uncommon in ATC. Overall, 46 of 48 ATC (95.8%) harbored at least one genetic alteration, and coexistence of two or more was seen in 37 of 48 ATC (77.1%). These genetic alterations were somewhat less common in FTC. Genetic alterations that could activate both the PI3K/Akt and MAPK pathways were found in 39 of 48 ATC (81.3%). RTK gene copy gains were preferentially associated with p-Akt, suggesting their dominant role in activating the PI3K/Akt pathway. The phosphorylation of Akt was far more common than p-ERK in FTC, and both were relatively common and often coexisted in ATC.

Conclusions: Genetic alterations in the RTKs and PI3K/Akt and MAPK pathways are extremely prevalent in ATC and FTC, providing a strong genetic basis for an extensive role of these signaling pathways and the development of therapies targeting these pathways for ATC and FTC, particularly the former. **(*J Clin Endocrinol Metab* 93: 3106–3116, 2008)**

Follicular cell-derived thyroid cancer is the most common endocrine malignancy and can be classified into well-differentiated thyroid cancer and anaplastic thyroid cancer (ATC) (1). Well-differentiated thyroid cancer includes papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC) and may sometimes progress to ATC. ATC is the most common cause of death from thyroid cancer, which usually occurs soon after the diagnosis (2). Several recent studies have investigated the role of genetic alterations in the phosphatidylinositol 3-kinase (PI3K)/Akt and MAPK pathways in thyroid cancers, including ATC

0021-972X/08/$15.00/0

Printed in U.S.A.

doi: 10.1210/jc.2008-0273 Received February 5, 2008. Accepted May 7, 2008.

First Published Online May 20, 2008

Abbreviations: ATC, Anaplastic thyroid cancer; EGFR, epidermal growth factor receptor; FTC, follicular thyroid cancer; KIT, another name for stem cell factor receptor; MET, another name for hepatocyte growth factor receptor; p-Akt, phosphorylation of Akt; PDGFR, platelet-derived growth factor receptor; PDK1, phosphoinositide-dependent kinase 1; p-ERK, phosphorylation of ERK; PI3K, phosphatidylinositol 3-kinase; PIP_3, phosphatidylinositol-3, 4, 5-trisphosphate; PTC, papillary thyroid cancer; PTEN, phosphatase and tensin homolog deleted from chromosome 10; RTK, receptor tyrosine kinase; VEGFR, vascular endothelial growth factor receptor.

(3–7). Because of the relatively small size of the series or the small number of genes investigated in these studies, the full extent of the genetic alterations and their relationship with these pathways in ATC remain unknown.

The PI3K/Akt pathway plays a fundamental role in the regulation of cell growth, division, survival, and, when disarranged, tumorigenesis (8, 9). Signaling of this pathway involves PI3K-catalyzed generation of phosphatidylinositol-3, 4, 5-trisphosphate (PIP_3). PIP3 recruits phosphoinositide-dependent kinase 1 (PDK1), which activates the Ser/Thr protein kinase Akt through phosphorylation at the cell membrane. Activated Akt in turn phosphorylates and regulates downstream effector proteins and promotes cell proliferation and survival. phosphatase and tensin homolog deleted from chromosome 10 (PTEN) negatively regulates this pathway by acting as a lipid phosphatase that degrades PIP_3 and terminates the signaling (10). PI3K is composed of a heterodimer of a p85 regulatory subunit and one of several p110 catalytic subunits, such as PIK3Ca and PIK3Cb. Ras is also an important regulator of the PI3K/Akt pathway through its interactions with the Ras-binding domains of PI3K subunits. The Ras → Raf → MAPK kinase (MEK) → ERK/MAPK pathway similarly plays a fundamental role in the regulation of cell proliferation and survival and its aberrant activation is associated with tumorigenesis (11, 12). Genetic alterations in the *Ras*, *BRAF*, *PIK3Ca*, and *PTEN* genes are common in the PI3K/Akt and MAPK pathways in human cancers (11, 12), including thyroid cancer (3–7, 13). Signalings of the PI3K/Akt and MAPK pathways can both start at various receptor tyrosine kinases (RTKs). Consequently, RTKs play an important role in a variety of cellular processes and tumorigenesis (14). Many RTKs are involved in tumor progression and metastasis through mutations or genetic amplifications. These include epidermal growth factor receptor (EGFR) (15–17), platelet-derived growth factor receptor (PDGFR) (17–20), vascular endothelial growth factor receptor (VEGFR) (17, 20, 21), stem cell factor receptor (SCFR or KIT) (17, 18, 22), and hepatocyte growth factor receptor (HGFR or MET) (23, 24). Many of the genetically activated RTKs are potential therapeutic targets for human cancers (14).

Based on the increasing accumulation of genetic alterations in the PI3K/Akt and MAPK pathways from low-grade thyroid tumor to ATC, we have recently proposed a model in which dual involvement of both pathways may play a key role in the development of ATC (5). This was based, however, on the analysis of limited number of genes in these signaling pathways. Consequently, the extent of the involvement of the PI3K/Akt and MAPK pathways remains uncertain in ATC. To further explore the genetic bases for potential development of novel therapy for ATC targeting RTKs and their coupled PI3K/Akt and MAPK pathways, in the present study we extensively investigated a large panel of genes in these signaling pathways for genetic alterations in ATC and, for comparison, also in FTC.

Materials and Methods

Human thyroid samples and DNA isolation

Paraffin-embedded thyroid tumor samples, including 51 ATC and 64 FTC, were originally obtained and prepared for genomic DNA isolation with institutional review board approvals as described previously (5). Briefly, after xylene treatment for 8–10 h at room temperature to remove paraffin, tissues were digested with 1% sodium dodecyl sulfate-proteinase K at 48 C for 36–48 h and 3–5 μl of 20% sodium dodecyl sulfate-proteinase K was added to facilitate the digestion. DNA was subsequently isolated following standard phenol-chloroform extraction and ethanol precipitation protocols.

Detection of mutations in RTKs

Genomic DNA sequencing was performed for mutation analysis on the genes for specific RTKs or their subtypes that were found to harbor mutations in other cancers. The PCR primers used are summarized in Table 1. For *EGFR* gene, we selected exons 18–21 (tyrosine kinase domain) for mutation analysis because most functional mutations are clustered within this domain (25). Genomic DNA was amplified by PCR as follows: after 5 min denaturing at 95 C, the reaction mixture was run for 35 cycles at 94 C, 62 C, and 72 C, each for 30 sec, followed by an elongation at 72 C for 10 min (for all the four exons). For *PDGFR*α gene, most mutations have been identified in exon 12 (juxtamembrane domain) and exon 18 (tyrosine kinase domain) in human cancers (19). We therefore selected these two exons for mutation analysis. The two exons were amplified by PCR as follows: after a 5-min denature at 95 C, the reaction mixture was run for 35 cycles at 94 C, 60 C, and 72 C, each for 30 sec, followed by an elongation at 72 C for 10 min. For *VEGFR2* gene, mutations have been reported to be mostly in exon 26 (kinase domain) (21), which we amplified for mutation analysis in the present study using the same PCR condition as for *PDGFR*α gene. For *KIT* gene, we selected exons 9 and 11 for analysis as they most commonly harbored mutations in other cancers (22). The PCR conditions were the same for the two exons and are as follows: after a 5-min denature at 95 C, the reaction mixture was run for 35 cycles at 94 C, 64 C, and 72 C, each for 30 sec, followed by an elongation at 72 C for 10 min. After PCR amplification of the specific exons, Big-dye reaction was pursued for DNA sequencing as described previously (5).

Detection of mutations in the PI3K/Akt and MAPK pathways

For *Ras*, *PIK3Ca*, *PTEN*, and *BRAF* genes, genomic DNA was amplified by using the same primers and PCR condition as described previously (5). For *PDK1* gene, exons 10 and 14 (kinase domain) were previously found to have mutations (26) and were analyzed here. A nested PCR procedure was used to amplify exon 10. The first round of PCR involved an initial 5-min denature at 95 C. The reaction mixture was then run for 35 cycles at 94 C, 62 C, and 72 C each for 30 sec, followed by an elongation at 72 C for 10 min. The product was diluted 1:100 and amplified for the second round with the second pairs of primers by using the same PCR condition. For exon 14, PCR was performed as follows: after a 5-min denature at 95 C, the reaction mixture was run for 35 cycles at 94 C, 60 C, and 72 C, each for 30 sec, followed by an elongation at 72 C for 10 min. For *AKT1* gene, mutations were previously reported in the pleckstrin homology domain in several human cancers. The primers and PCR conditions for amplifying this domain were as previously described (27), and we designed our own sequencing primer. For *AKT2* gene, most mutations were previously identified in exons 10 and 11 within its kinase domain (26). We designed primers for these two exons and used the following conditions to amplify them: exon 10 was amplified by using the same conditions as for the *PDK1* exon 14; for exon 11, we used nested PCR for amplification and the conditions were same as for *PDK1* exon 10 except for 60 C as the annealing temperature. The primers used in PCR amplification and Big-dye sequencing for these genes are summarized in Table 1.

Analysis of gene copy number gains with quantitative real-time PCR

We used quantitative real-time PCR technique to analyze copy number of RTK genes and several genes in the PI3K/Akt and MAPK path-

TABLE 1. PCR primers and probes used in gene mutation and copy number analysis

PCR primers used for gene mutation analysis

Gene name	Exon	Product size, bp	Forward primer (5′-3′)	Reverse primer (5′-3′)	Sequencing primer (5′-3′)
EGFR	18	250	ATGGTGAGGGCTGAGGTGAC	TTGCAAGGACTCTGGGCTC	CTCCCCACCAGACCATGAG
EGFR	19	251	AGCATGTGGCACCATCTCAC	AGCAGGGTCTAGAGCAGAGCAG	AGCAGGGTCTAGAGCAGAGCAG
EGFR	20	367	TGCGTCTTCACCTGGAAGG	TCCCCATGGCAAACTCTTG	TCCCCATGGCAAACTCTTG
EGFR	21	388	TCGACGTGGAGAGGCTCAG	GCGAGCTCACCCAGAATG	GCGAGCTCACCCAGAATG
PDGFRα	12	330	TGCTGCTTCAGTGAAGCTCTG	GCAAATTTCCATTGCCTAGTTC	GCAAATTTCCATTGCCTAGTTC
PDGFRα	18	326	TGGATCAGCCAGTCTTGCAG	TGACCCCTTGAAATCTATTGATG	TGACCCCTTGAAATCTATTGATG
VEGFR2	26	368	GCCTCTGGAGTATGGGACTCTG	TGGCTGAGAGGATGGCATG	GCCTCTGGAGTATGGGACTCTG
KIT	9	182	AAAGTATGCCACATCCCAAGTG	CATGGTCAATGTTGGAATGAACT	CATGGTCAATGTTGGAATGAACT
KIT	11	127	CGTAGCTGGCATGATGTGC	AAACAAAGGAAGCCACTGGAG	CGTAGCTGGCATGATGTGC
PIK3Ca	9	487	GATTGGTTCTTTCCTGTCTCTG	CCACAAATATCAATTTACAACCATTG	TTGCTTTTTCTGTAAATCATCTGTG
PIK3Ca	20	525	TGGGGTAAAGGGAATCAAAAG	CCTATGCAATCGGTCTTTGC	TGACATTTGAGCAAAGACCTG
PTEN	5	314	CTTATTCTGAGGTTATCTTTTTACC	CTCAGATCCAGGAAGAGGA	CTTATTCTGAGGTTATCTTTTTACC
PTEN	6	202	TTGGCTTCTCTTTTTTTTCTG	ACATGGAAGGATGAGAATTTC	TTGGCTTCTCTTTTTTTTCTG
PTEN	7	428	ACAGAATCCATATTTCGTGTA	TAATGTCTCACCAATGCCA	ACAGAATCCATATTTCGTGTA
PTEN	8	159	ACACATCACATACATACAAGTC	GTGCAGATAATGACAAGGAATA	ACACATCACATACATACAAGTC
H-ras	3	109	AGGTGGTCATTGATGGGGAG	AGGAAGCCCTCCCCGGTGCG	AGGAAGCCCTCCCCGGTGCG
K-ras	2	163	GGCCTGCTGAAAATGACTGAA	GGTCCTGCACCAGTAATATGC	GGCCTGCTGAAAATGACTGAA
N-ras	1	252	AAAGTACTGTAGATGTGGCTCGCC	CAGAATATGGGTAAAGATGATCCG	CAGAATATGGGTAAAGATGATCCG
N-ras	3	174	TCTTACAGAAAACAAGTGGT	GTAGAGGTTAATATCCGCAA	TCTTACAGAAAACAAGTGGT
PDK1	10.1	278	TCACCCTGGCAGTGACTTG	GGGCATGAAGCCCAGTG	
PDK1	10.2	269	TGGCAGTGACTTGTCTTGATTG	CATGAAGCCCAGTGCACG	CATGAAGCCCAGTGCACG
PDK1	14	420	CTTGTGTGAATAACCGTCACACC	GTGGTTGGGTGTTTTTTTTCTTC	AATGCTGCAAGGTTTCCG
AKT2	10	229	CCAGCTGTTCCCAGGTACC	TAGTCATTGTCCTCCAGCACC	GCTGGAAACACACAGGTCTGG
AKT2	11.1	574	ATCAAGATCACTGACTTTGGCCTC	ACACACTGCGACCCTACAAG	
AKT2	11.2	395	GTGTGAGGCCAAGGGTAGG	AGGGACAGTGGCAGCAGCTG	CCAGACCTGTGTGTTTCCAG

Primers and probes used in quantitative real-time PCR for gene copy number analysis

Gene name	Exon	Product size, bp	Forward primer (5′-3′)	Reverse primer (5′-3′)	Probe (5′-3′)
EGFR	3	103	CTG CAG ATC ATC AGA GGA AAT ATG	CAGTCCGGTTTTATTTGCATCATAG	CGAAAATTCCTATGCCTTAG
PDGFRα	4	100	TGTAGCCTTTGTACCTCTAGGAATG	AGGAGTCTCGGGATCAGTTG	ATGATGATTCTGCCATTATACCTTGTCGC
PDGFRß	4	102	ATGATGCCGAGGAACTATTCAT	ACCAGCTGTGGGTCTGTTACTC	CACGGAAATAACTGAGATCA
VEGFR1	9	90	GTT ACC TGC GAC TGA GAA ATC TG	TAA TTC CCT GCA TCC TCT TCA GTT	CTATTTGACTCGTGGCTACT
VEGFR2	30	100	GGCTACCAGTCCGGATATCAC	CGGTTTGCACTCCAATCTCT	CCGATGACACAGACACCACCGTGTA
MET	2	100	ACC TGC CAG CGA CAT GTC TT	GAC ACT GGC TGG GCT CTT CTA TC	CCACAATCATACTGCTGACA
KIT	2	100	TGA GAT CCT GGA TGA AAC GAA TG	GTG TTT GTT GGT GCA CGT GTA TT	AAGCAGAATGAATGGATCAC
PIK3Ca	21	81	AAATGAAAGCTCACTCTGGATTCC	TGTGCAATTCCTATGCAATCG	CACTGCACTGTTAATAACTCTCAGCAGGCAAA
PIK3Cb	5	100	TGGAATGCTGCAAGATCAAG	GGTGGTAATGGAAGAGGAAGATT	CCATAGAGGCTGCCATAAATCGAAATTCA
PDK1	11	100	AGGCAGCAACATAGAGCAGTAC	CTTCTCCAACAACAACCTCTTCT	TTCACGATCTGGACTCGAACTCCTTTG
AKT1	5	100	CAACCGCCATCCAGACTGT	TCTTCAGCCCCTGAGTTGTC	ACTTCCGGTCGGGCTCACCC
AKT2	11	100	GGCTGGGTGTGGTCATGTAC	CGGATCTCTTCCATGAGGAT	ACCACGAGCGCCTCTTCGAGCT
β-actin	4	109	TCACCCACACTGTGCCCATCTACGA	TCGGTGAGGATCTTCATGAGGTA	ATGCCCTCCCCCATGCCATCC

ways, as listed in Table 1, using the ABI 7900HT PCR system (Applied Biosystems, Foster City, CA) following the manufacturer's instruction. Real-time PCR is a widely used robust and efficient method in genomic copy number gain analysis. This method was well established and used in gene copy number analysis for thyroid cancers in our and others' previous studies (4, 5, 7, 28). Specific primers and probes (Table 1) were designed using Primer Express 3.0 (Applied Biosystems) to amplify and detect both the specific genes and β-actin. The probes were labeled with 5′ fluorescent reporter dye (6FAM) and 3′ quencher dye (TAMRA). The reaction of quantitative real-time PCR for each gene was repeated twice, and *β-actin* was run in parallel to standardize the input DNA. Standard curve was established by using normal leukocyte DNA with a quantity range of 0.02–10 ng/μl.

Analysis of *RET/PTC* rearrangement using RT-PCR

Formalin-fixed, paraffin-embedded tissue sections of anaplastic thyroid carcinoma were used for RNA extraction using the RNeasy FFPE Kit (QIAGEN, Valencia, CA) following the manufacturer's protocol. Three micrograms total RNA was reverse transcribed by using random hexamer primers and superscript II RT (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The primers used for the analysis of *RET/PTC1* and *RET/PTC3* were as described previously (29). cDNA made from RNA extracted from TPC1 cell lines harboring RET/PTC1 and cDNA from a tumor sample carrying *RET/PTC3* rearrangement (a kind gift from Dr. Yuri E Nikiforv, University of Pittsburgh, Pittsburgh, PA) were used as positive controls. All samples were assessed for the adequacy and integrity of RNA by amplification of the *β-actin* gene. The PCR products were electrophoresed on a 1.5% agarose for visualization by ethidium bromide.

Analysis of phospho-ERK and phospho-Akt by immunohistochemistry

Tumor tissue sections were dewaxed, soaked in alcohol, and incubated in 3% hydrogen peroxide for 15 min to inactivate endogenous peroxidase activity after microwave treatment in an antigen-unmasking solution (Vector Lab, Burlingame, CA). Tissue sections were then incubated overnight at 4 C with anti-phospho-ERK (Thr 202/Tyr 204) or anti-phospho-Akt (Ser473 IHC specific) antibodies from Cell Signaling Technology (Beverly, MA). Immunostaining was performed with Vectastain Universal Quick kit (Vector Lab) following the manufacturer's protocol. Peroxidase activity was revealed using 3,3 diaminobenzidine. Negative control was performed by omission of primary antibodies. For positive control we used samples from previously examined thyroid cancer tissues positive for phospho-ERK or phospho-Akt.

Results

Uncommon mutation but common copy number gain of RTK genes in thyroid cancers

RTK gene mutations have been reported in many human cancers. We selected the *EGFR*, *PDGFRα*, *VEGFR2*, and *KIT* genes to analyze for thyroid cancers, which had been commonly found to be mutated in other cancers. As shown in Table 2, we did not find any mutation in the hot-spot exons of these genes in 51 ATC and 64 FTC samples, suggesting that RTK gene mutations are not common in ATC and FTC. In contrast, we found genomic copy number gains in many RTK genes with relatively high prevalences in these thyroid cancers. The prevalences of these copy gains were generally higher in ATC than FTC (Table 2).

Common genetic alterations in the PI3K/Akt pathway in thyroid cancers

We and others had previously reported mutations in *Ras*, *PIK3Ca* and *PTEN* genes in the PI3K/Akt pathway (3–5, 7, 30).

TABLE 2. Prevalences of genetic alterations in ATC and FTC (n/N [%])

Genetic alterations	Gene	ATC (%)	FTC (%)
Copy number gain	EGFR[15,16,17]	19/41 (46.3)	19/59 (32.2)
	PDGFRα[17,18,20]	11/46 (23.9)	4/52 (7.7)
	PDGFRß	14/37 (37.8)	8/59 (13.6)
	VEGFR1	20/44 (45.5)	26/59 (44.1)
	VEGFR2[17,20]	8/46 (17.4)	2/52 (3.8)
	KIT[17,18,20]	10/46 (21.7)	6/61 (9.8)
	MET[23,24]	5/42 (11.9)	5/58 (8.6)
	PIK3Ca[5,7,30]	18/47 (38.3)	15/63 (23.8)
	PIK3Cb[32]	16/42 (38.1)	25/55 (45.5)
	PDK1	8/40 (20)	14/58 (24.1)
	AKT1	9/48 (18.8)	5/61 (8.2)
	AKT2	0/44 (0)	13/58 (22.4)
Mutation	EGFR[16]	0/47 (0)	0/64 (0)
	PDGFRα[19]	0/47 (0)	0/64 (0)
	VEGFR2[21]	0/47 (0)	0/64 (0)
	KIT[22]	0/47 (0)	0/64 (0)
	PIK3Ca[3,7]	6/50 (12)	4/64 (6.3)
	RAS[5,7,12,13,30]	4/51 (7.8)	13/64 (20.3)
	PTEN[5,7,30]	8/48 (16.7)	3/64 (4.69)
	PDK1[26]	0/47 (0)	0/64 (0)
	AKT1[27]	0/47 (0)	0/64 (0)
	AKT2[26]	0/47 (0)	0/64 (0)
	BRAF[4,6,7]	14/50 (28)	
Rearrangement	RET/PTC[29]	3/20 (15)	

Numbers in superscript are references that represent some previous studies in which the indicated genetic alterations had been investigated in other cancers or, in some cases, also thyroid cancers. n, The number of cases positive for the genetic event; N, the number of cases examined for the genetic event.

Here we extended the mutation analysis to include several other important genes in the PI3K/Akt pathway and also examined their genomic copy number. We found no mutation in the hotspot exons of *PDK1*, *AKT1*, and *AKT2* genes in all the ATC and FTC specimens (Table 2). Mutations were relatively common in the *PIK3Ca* and *PTEN* genes in ATC and in the *Ras* gene in FTC. Copy number gains were common in the *PIK3Ca*, *PIK3Cb*, and *PDK1* genes but not in *Akt1* and *Akt2* genes in ATC and FTC (Table 2).

BRAF mutation and *RET/PTC* rearrangement in the MAPK pathway in ATC

BRAF mutation and *RET/PTC* rearrangements are the most common activating genetic alterations in the MAPK pathway in thyroid cancer. Because they virtually do not occur in FTC, we analyzed them only in ATC samples. *BRAF* mutation was present in 14 of 50 ATC (28%). We had 20 ATC samples available for RNA extraction that were successfully analyzed for *RET/PTC* rearrangements. One of the 20 samples (5%) harbored *RET/PTC1* and two of 20 samples (10%) harbored *RET/PTC3* rearrangement, with an overall prevalence of three of 20 (15%). Two of these three ATC samples also contained PTC tissues, and it is therefore possible that *RET/PTC* in these two samples could actually be from PTC. One ATC sample harbored both *BRAF* mutation and *RET/PTC* rearrangement.

High prevalence of overall genetic alterations in thyroid cancers

Taking together, in all the RTK genes and the genes in the PI3K/Akt and MAPK pathways examined in this study, we found at least one mutation in 26 of 51 ATC (51%) and 17 of 64 FTC (26.6%) and at least one copy number gain in 41 of 51 ATC (80.4%) and 51 of 64 FTC (79.7%). Taking together, all the genetic alterations, including mutation and copy gain, 46 of 51 ATC (90.2%) and 55 of 64 FTC (85.9%) harbored at least one genetic alteration. Due to insufficient tumor or DNA specimens, some samples only had a partial analysis for some of the genetic alterations. When excluding these samples but including only the samples that had complete data, 46 of 48 ATC (95.8%) and 55 of 59 FTC (93.2%) harbored at least one genetic alteration. Coexistence of genetic alterations was common; 37 of 51 ATC (72.5%) and 41 of 64 FTC (64.1%) harbored two or more genetic events. When the cases without complete data on all the genes were excluded, coexistence of genetic alterations (two or more) was found in 37 of 48 ATC (77.1%) and 41 of 59 FTC (69.5%). This coexistence involved mostly copy gains (Tables 3-5). Gene mutations were largely mutually exclusive among themselves in both ATC and FTC; 20 of 26 ATC (76.9%) and 14 of 17 FTC (82.4%) that harbored mutations harbored only one mutation in individual tumors (Tables 3 and 4).

High prevalence of genetic alterations that could activate both PI3K/Akt and MAPK pathways in ATC

When considering the cases harboring genetic alterations in RTKs or Ras that can dually activate the PI3K/Akt and MAPK pathways and the cases concurrently harboring other genetic alterations that can activate both pathways (*e.g.* coexistence of *PIK3Ca* copy gain and *BRAF* mutation), 39 of 51 (76.5%) ATC harbored genetic alterations that could potentially activate both pathways (Table 3). If the three cases of ATC without complete genetic analysis were excluded, 39 of 48 (81.3%) ATC harbored genetic alterations that could potentially activate both pathways.

Relationship of genetic alterations with phosphorylation of Akt and ERK1/2 in ATC and FTC

To explore the relevance of genetic alterations in the PI3K/Akt and MAPK pathways in activating the two pathways, we performed immunohistochemical analysis of phosphorylation of Akt (p-Akt) and phosphorylation of ERK (p-ERK) in selected tumor tissues (Fig. 1). We examined 27 ATC samples available for this analysis. Representative cases of immunohistochemical staining are shown in Fig. 1 and detailed data are summarized in Table 5. When looking at the phosphorylation of proteins and its relationship with genetic alterations, 16 of 27 ATC (59.3%) showed p-Akt immunoreactivity, and of the 16 p-Akt samples, 13 (81.3%) harbored genetic alterations in the PI3K/Akt pathway. Eight of 27 ATC (29.6%) showed p-ERK1/2 immunoreactivity and all the eight samples (100%) harbored genetic alterations in the MAPK pathway. Six of 27 ATC (22.2%) showed immunoreactivity for both p-Akt and p-ERK1/2, and all six samples (100%) harbored genetic alterations in both PI3K/Akt and MAPK pathways. Thus, most of the ATC tumors that were positive for p-Akt or p-ERK1/2 immunoreactivity correspondingly harbored genetic alterations in the PI3K/Akt or MAPK pathway, consistent with the expected function of these genetic alterations.

When looking at the specific genetic alterations and their relationship with the activation of PI3K/Akt and MAPK pathways as shown in Table 5, five of seven ATC (71.4%) that harbored *BRAF* mutation showed immunoreactivity for p-ERK1/2. In PI3K/Akt pathway, 10 of 15 ATC (66.6%) with *PIK3Ca* copy gain, four of seven ATC (57.1%) with *PIK3Cb* copy gain and three of five ATC (60%) with *PDK1* copy gain showed p-Akt immunoreactivity. Both of the two cases of ATC with *PTEN* mutation stained positively for p-Akt. Thus, the genetic alterations in the PI3K/Akt pathway were mostly associated with p-Akt, reflecting the activation of this pathway. Among the RTK genes, eight of 11 (72.7%) and four of 11 ATC with *EGFR* copy gains (36.4%), three of four (75%) and one of four ATC with *PDGFRα* copy gains (25%), seven of 10 (70%) and four of 10 ATC with *VEGFR1* copy gains (40%), and three of four (75%) and two of four ATC with *VEGFR2* copy gains (50%) showed p-Akt and p-ERK1/2 immunoreactivity, respectively. These data showed clearly a preferential activation of the PI3K/Akt pathway over the MAPK pathway by the RTK gene copy number gains. A few genetic alterations, such as copy gains of *PDGFRβ*, *KIT*, *MET*, *AKT1*, and *AKT2* genes were less commonly associated with p-Akt or p-ERK1/2 (Table 5). In fact, nine of 27 ATC (33.3%) did not show either p-Akt or p-ERK1/2 immunoreactivity, although all nine samples harbored at least one genetic alteration (Table 5). Two cases showed p-Akt immunoreactivity, but no genetic alterations were identified in them, consistent with possibly a yet unidentified genetic alteration in the PI3K signaling pathway in these tumors.

We similarly performed immunohistostaining analysis for p-

TABLE 3. Genetic alterations in individual ATC samples

	EGFR	PDGFRα	PDGFRß	VEGFR1	VEGFR2	KIT	MET	PIK3Ca	PIK3Cb	PDK1	AKT1	AKT2	Ras	PIK3Ca	PTEN	BRAF	RET/PTC
						Copy number gain								Mutation			Rearrangement
A1	5.0/+	3.1		27.7/+	3.9/+	13.6/+		8.3/+	5.5/+	1.1	3.6/+					+	
A2	2.3	4.9/+	3.0	2.5	3.2	1.9	1.7	3.9/+	3.1	2.1	2.4	2.1					
A3	2.5	4.5/+	2.6	2.6	2.7	1.9	1.3	3.1	3.2	2.9	2.9	2.1					
A4	4.8/+	2.3	3.5/+	8.9/+	2.3	5.5/+	2.6	5.2/+	4.1/+	0.8	3.3	2.8					
A5	2.6	2.1	2.1	2.8	1.4	1.6	1.1	2.9	2.7	2.4	2.7	1.8	+	+			
A6		2.0	2.7	2.9	1.9	2.1	1.9	4.5/+	2.4	3.0	2.7	2.2		+		+	+
A7	3.7/+	2.9	4.3/+	2.9	2.6	2.0	1.7	3.9/+	3.1	3.6/+	3.0	2.4					
A8	2.2	3.4	2.4	2.7	2.8	1.8	1.8	2.5									+
A9		3.2	4.4/+	2.5	1.9	1.8	1.8		3.3	2.6	2.7	2.2				+	
A10	3.6/+	1.7		3.8/+	3.6/+	2.9	2.9	4.7/+	4.5/+			2.3					
A11	3.0	1.4	2.7	2.4	1.3	1.4	2.0	2.7	2.0	3.1	2.7	1.9					
A12	3.3	3.1	4.6/+	3.6/+	2.5	2.1	2.1	4.5/+	2.9	3.7/+	3.3	2.3					
A13									4.1/+	2.8	2.1						
A14	4.0/+		3.4	5.2/+	2.3	2.4	1.3	4.8/+	2.9		3.2	2.1				+	
A15	4.5/+	1.8		5.2/+	3.9/+	1.8	1.7	6.5/+	5.8/+	1.9	3.3	2.5					
A16		4.2/+			3.2				5.3/+	6.1/+	3.1	2.7			+		
A17		1.7			2.9	5.3/+		4.2/+		2.7	2.1					+	
A18	4.4/+	1.8	2.8	2.9	2.6	1.8	2.2	2.3	3.0	2.6	3.4	2.0			+		
A19	2.5	3.2		2.8	2.1	1.9	1.6	4.4/+	3.0	1.9	2.6	2.6					
A20	8.1/+	6.7/+	5.3/+	7.7/+	1.9	4.3/+	8.7/+		5.0/+	5.7/+	3.8/+	2.3			+		
A21	2.0	2.9	2.1	2.4	2.3	2.4	2.2	2.7	2.9	4.5/+	2.7	2.0					+
A22	4.4/+		3.0	4.4/+		1.7	1.3	3.6/+	4.3/+		2.6	2.4	+				
A23	2.2	2.2		2.4	2.0	1.3	1.3	1.8	2.5	1.3	2.8	1.6					
A24	4.9/+	5.1/+	4.1/+	5.4/+	3.9/+	2.7	2.7	7.3/+	3.2	2.6	3.3	3.0		+			
A25	2.5	2.4	1.9	2.6	2.2	1.9	2.2	2.8	3.0	4.3/+	2.7	2.0				+	
A26	4.5/+	2.3	3.2	6.0/+		2.6	2.2	4.5/+	3.4	1.8	2.2	2.0					
A27	3.8/+	2.9	2.8	4.8/+	3.4	2.2	2.0	4.9/+	2.8	1.2	2.5	2.2					
A28	2.2	1.8	2.5	3.1	1.8	1.8	1.8	2.3	2.9	2.7	2.7	2.0				+	
A29		1.8			2.9			3.0	2.3		3.9/+	2.3		+			
A30	6.6/+		8.4/+	16.3/+				12.9/+			6.6/+	2.1		+	+		
A31	5.3/+	1.9	5.9/+	6.6/+	3.0	5.7/+	1.6	1.7	3.8/+		6.3/+	3.4					
A32	5.9/+	2.6	2.8	5.3/+	3.1	2.5	3.3	2.0			3.7/+	1.9			+	+	
A33	2.7	2.4	2.5	3.3	2.2	2.4	1.8	2.3	3.0	1.7	2.9	2.1					
A34	2.8	2.6	2.2		1.9	2.3	2.9	1.8	2.6	2.2	2.4	2.8					
A35	2.1	3.3	1.7	2.2	1.6	1.7	1.7	2.3	2.5	1.5	2.1	1.7				+	
A36		1.1		5.7/+	4.0/+	2.7	3.5/+	1.8		1.3	2.8			+			
A37		3.8/+	4.0/+	6.1/+	2.3	3.6/+	4.5/+	3.2	4.7/+	1.7	1.9	2.2				+	
A38	3.8/+	2.1	4.3/+	4.7/+	2.4	3.3		1.8	2.8	1.1	2.5						
A39	3.1	2.1	2.5	2.8	2.2	2.0	1.3	2.5	2.9	1.6	2.5	2.2	+				
A40		3.3	2.9		3.3	6.9/+		2.7	3.6/+	2.9	2.4	1.8					
A41	5.3/+	6.5/+		9.5/+	5.4/+	5.0/+	8.6/+	10.4/+	3.8/+	4.5/+	5.0/+	2.6			+	+	
A42	2.9	4.1/+	4.5/+	2.8	2.2	1.8	1.3	2.8	4.0/+		2.9	2.0				+	
A43	2.4	4.0/+		3.4	3.0	2.4	1.1	3.3		1.4	3.7/+	2.6	+		+		
A44	3.1	2.2	4.7/+	2.7	2.0	1.6	1.5	2.5	3.8/+	1.7	2.9	2.0				+	
A45	2.9	2.2	3.9/+	3.3	2.0	2.0	1.4	2.0			3.4	2.2					
A46	4.9/+	4.1/+		8.4/+	3.5/+	4.2/+	7.5/+	9.3/+	2.6	4.6/+	3.4	2.5			+		
A47	1.8	3.2	3.0	2.0	2.4	6.8/+	2.1	1.7	3.6/+	1.8	2.1	2.1					
A48	4.0/+	1.2	4.3/+	3.5/+	2.9	3.0	1.2	2.0		1.0	3.6/+	1.9					
A49								3.1									
A50	2.6	1.7	2.0	2.7	1.9	1.8	1.7	1.5	3.9/+	1.4	1.9	2.5					
A51	1.3	6.5/+	2.8	1.5	3.9/+	3.3	1.5	3.3	2.6	3.4	2.3	2.1				+	

The numerical values represent copy numbers of the indicated genes. Gene copy number gain, as designated with a +, is defined as a copy number of 4 (rounded up from 3.5 to 3.9) or higher.

TABLE 4. Genetic alterations in individual FTC samples

	EGFR	PDGFRα	PDGFRß	VEGFR1	VEGFR2	KIT	MET	PIK3Ca	PIK3Cb	PDK1	AKT1	AKT2	Ras	PIK3Ca	PTEN
						Copy number gain								Mutation	
F1	3.0		2.7	2.4	1.5	1.6	1.8	2.6	2.8	3.6/+	3.5/+	2.6			
F2	4.2/+		2.9	3.7/+		2.1	2.0	3.4	4.3/+	4.4/+	2.8	3.1			
F3	3.2	2.3	2.3	3.2	1.9	2.1	1.8	2.2	3.7/+	3.2	3.1	2.9	+		
F4	3.7/+		3.2	5.2/+		3.4	2.3	3.3	4.8/+	3.7/+	3.0	3.4	+		
F5	5.0/+	4.4/+	3.9/+	5.4/+	3.1	2.1	2.6	2.9	5.2/+	2.7	2.8	4.8/+			
F6	3.9/+	1.6	2.8	4.7/+		2.3	2.1	2.6	4.2/+	4.8/+	3.0	3.0			
F7	3.1		2.6	3.7/+		2.2	1.8	1.8	3.6/+	3.2	2.5	3.1			+
F8	4.0/+	1.8	2.4	3.6/+	2.7	2.4	2.1	3.2	3.2	2.8	2.1	3.2			
F9	4.1/+		2.5	3.2		2.3	2.5	3.3	4.0/+	3.1	2.8	2.7			
F10	2.2	2.5	2.4	2.4	2.9	2.0	1.9	1.5	2.7	4.6/+	2.6	2.4			
F11	1.7	2.4	1.7	2.8	2.3	2.8	1.8	1.7	2.5	3.5/+	2.5	2.3	+	+	
F12	1.4	2.0	1.5	2.0	2.1	1.7	1.4	1.9	2.6	2.8	2.2	2.4			
F13	2.6	1.3		4.2/+	1.5	1.8	1.6	2.3	4.9/+	2.5	2.8	3.2	+		
F14	3.2	2.4	2.1	3.1	1.6	2.1	2.8	2.9		2.1	1.8	2.0			
F15	3.7/+	3.1	1.8	3.8/+		2.5	2.2	2.7	3.3	1.5	1.6	2.5			
F16								2.2							
F17	2.1		2.1	2.5	2.1	1.8	1.6	1.6	2.2	2.8	2.3	3.0			
F18	2.3	3.2	1.9	3.3	14.8/+	2.2	1.5		2.6	2.8	2.3	2.5			
F19	1.6	2.5	2.4	2.1	1.8	2.2	2.3	1.6	2.2	2.4	2.0	2.7			
F20	2.3		2.4	2.8	2.3	2.2	1.7	2.3	2.3	2.8	2.6	2.7			
F21	1.6	2.0	2.2	2.0	2.0	2.4	2.1	1.5	1.9		2.2	2.5	+		
F22	2.4	2.5	2.2	2.3	2.6	2.6	2.5	1.4	2.2	4.5/+	2.5	3.0	+		
F23	2.7	1.8	2.1	2.5	3.5/+	2.9	2.3	1.9	2.5	3.3	2.2	2.8			
F24	2.0	2.7	3.1	2.2	2.2	1.8	1.7	1.2	1.9	2.4	1.2	2.2			
F25	3.6/+	1.9	2.2	2.0	2.8	2.2	1.8	1.2	1.9		1.9	2.2			
F26	2.2	2.3	1.9	1.9	2.1	1.9	1.6	1.0	1.9	2.5	1.7	2.1	+		
F27	2.2	2.4	2.5	2.3	2.5	2.9	2.3	1.7	2.8	4.3/+	2.7	2.4			
F28	9.4/+	5.8/+		8.1/+		3.9/+	1.8	2.0	4.4/+		5.8/+	6.1/+			
F29	3.6/+	2.9	2.2	2.8	2.5	3.0	1.6	3.4	3.4	3.5/+	2.5	1.9	+		
F30	2.5	3.0	2.3	2.5	3.0	2.2	1.6	3.3	2.8	3.9/+	2.4	2.3			
F31	2.1	2.3	1.7	2.3	2.5	1.8	1.7	2.0	1.9	2.3	1.9	2.6			
F32	3.6/+	2.3	3.8/+	4.2/+	2.8	2.4	1.5	2.4	4.7/+	2.2	3.6/+	4.2/+		+	+
F33	3.4	2.6	2.8	3.8/+	2.1	2.2	1.7	3.1	4.3/+		2.9	4.4/+			
F34	3.2	2.4	4.1/+	5.0/+	2.4	2.6	2.1	5.7/+	3.6/+	3.2	3.8/+	4.3/+			
F35	2.7	2.0	3.3	3.7/+	2.0	2.6	1.7	3.2	2.3	2.9	2.7	3.3	+		+
F36	2.7	2.0	3.0	3.9/+	2.5	1.4	2.3	3.4	3.0	2.6	2.6	3.6/+			
F37	3.6/+	2.1	3.2	3.8/+	2.7	2.3	1.9	3.8/+	4.7/+	3.6/+	3.5/+	3.7/+			
F38	3.1	2.8	3.4	3.3	2.7	2.3	1.8	3.2	2.0	2.8	2.7	2.8		+	
F39	4.5/+	2.0	4.8/+	4.1/+	1.8	1.9	3.1	4.1/+	4.1/+	2.6	2.7	4.7/+			
F40	3.2	2.7	3.6/+	4.1/+	3.0	2.7	1.6	4.5/+	3.9/+	2.6	3.1	3.4	+		
F41	5.9/+		3.6/+	6.1/+	2.1	2.0	2.7	5.3/+	7.5/+	3.2	3.1	6.6/+			
F42								4.5/+	19.9/+	0.8					
F43	2.8	2.2	2.2	2.7	2.1	2.1	2.2	4.5/+	3.4	2.4	2.5	3.1	+		
F44	2.9	2.5	2.6	4.0/+	1.7	2.1	1.9	4.5/+		3.4	3.0	4.8/+			
F45	2.9	2.9	2.3	3.6/+	2.2	4.8/+	9.8/+	2.4	4.0/+	1.8	2.0	2.9			
F46		1.5	2.7		1.7	3.1		2.6		2.0	2.8	3.9/+		+	
F47		2.6	1.3		1.9			3.9/+	5.9/+	1.8	1.7	2.6			
F48	2.7	2.1	2.0	3.3	2.5	2.5	2.4	3.9/+	6.2/+	2.5	2.4	2.9			
F49	5.1/+	2.8	2.4	2.8	3.0	2.7	2.7	4.3/+	4.1/+	2.0	2.3	3.4			
F50	2.7	2.2	2.0	2.6	1.8	2.3	2.3	3.2	2.2	2.2	2.1	2.1	+		
F51	3.8/+	2.5	2.7	3.0	2.2	3.7/+		3.4	4.8/+	2.5	2.7	3.8/+	+		
F52	3.0	3.9/+	1.7	2.5	3.3	2.1	2.2	2.7	3.1	2.1	1.8	3.0			
F53		3.1			2.4	4.0/+	4.0/+	2.5	3.4	4.1/+	3.1				
F54	3.0	2.8	2.0	2.1		3.0	2.4	2.2	2.5	2.4	2.0	2.6			
F55	4.4/+	1.4	2.4	3.1		15.2/+	21.4/+	3.3		2.5	2.6				
F56	3.2	2.5	2.9	5.3/+	2.2	2.8	2.4	4.5/+	3.9/+	4.5/+	3.4	4.7/+			
F57	4.9/+	2.3	2.5	4.6/+	2.0	2.1	2.9	1.7		2.2	2.4				
F58	3.2	1.9	3.3	2.8	1.8	2.9	2.9	4.0/+		1.9	1.9	3.2			
F59	3.0	2.5	1.9	3.4	2.0	7.4/+	4.8/+	4.9/+	4.6/+	3.0	2.2	2.2			
F60	4.7/+		2.8	6.7/+		3.3	4.0/+	2.7							
F61	2.9	2.2	4.2/+	4.8/+	2.4	3.4	2.9	2.1	4.8/+	3.2	3.0	2.7			
F62	3.3	2.2	2.5	3.6/+	1.6	1.8	2.4	2.3	1.9	2.2	3.2	2.8			
F63	1.8		4.5/+	2.7	1.6	3.1		2.5		2.0	2.5	2.6			
F64	3.3	3.7/+	3.4	4.3/+	1.5	2.3	2.7	4.5/+	2.1	4.0/+	2.9	3.0			

The numerical values represent copy numbers of the indicated genes. Gene copy number gain, as designated with a +, is defined as a copy number of 4 (rounded up from 3.5–3.9) or higher.

TABLE 5. Immunohistochemical staining of p-Akt and p-ERK and corresponding genetic alterations in individual ATC and FTC samples

	Immunohistochemistry		Genetic alterations		
	p-Akt	p-ERK	Copy number gain	Mutation	Rearrangement
A1	+	+	EGFR, VEGFR1, VEGFR2, KIT, PIK3Ca, PIK3Cb, AKT1	BRAF	
A2	+	−	PDGFRα, PIK3Ca		
A3	+	−	PDGFRα		
A4	−	−	EGFR, PDGFRß, VEGFR1, KIT, PIK3Ca, PIK3Cb		
A5	−	−		PIK3Ca, RAS	
A6	+	+	PIK3Ca	PIK3Ca, BRAF	RET/PTC3
A7	+	−	EGFR, PDGFRß, PIK3Ca, PDK1		
A8	+	−			RET/PTC1
A9	−	−	PDGFRß	BRAF	
A10	+	−	EGFR, VEGFR1, VEGFR2, PIK3Ca, PIK3Cb		
A11	+	−			
A12	+	−	PDGFRß, VEGFR1,PIK3Ca, PDK1		
A13	−	−	PIK3Cb		
A14	+	+	EGFR, VEGFR1, PIK3Ca	BRAF	
A15	+	+	EGFR, VEGFR1, VEGFR2, PIK3Ca, PIK3Cb		
A16	+	+	PDGFRα, PIK3Cb, PDK1	PTEN	
A17	−	−	KIT, PIK3Ca	BRAF	
A18	+	−	EGFR	PTEN	
A19	−	−	PIK3Ca		
A21	−	−	PDK1		RET/PTC3
A22	−	−	EGFR, VEGFR1, PIK3Ca, PIK3Cb	RAS	
A23	+	−			
A24	−	−	EGFR, PDGFRα, PDGFRß, VEGFR1, VEGFR2, PIK3Ca	PIK3Ca	
A25	−	+	PDK1	BRAF	
A26	+	−	EGFR, VEGFR1, PIK3Ca		
A27	+	+	EGFR, VEGFR1, PIK3Ca		
A28	−	+		BRAF	
F33	+	−	VEGFR1, PIK3Cb, AKT2		
F35	−	+	VEGFR1	RAS, PTEN	
F36	+	+	VEGFR1, AKT2		
F37	−	−	EGFR, VEGFR1, PIK3Ca, PIK3Cb, PDK1, AKT1, AKT2		
F38	−	+		PIK3Ca	
F39	+	−	EGFR, PDGFRß, VEGFR1, PIK3Ca, PIK3Cb, AKT2		
F41	+	−	EGFR, PDGFRß, VEGFR1, PIK3Ca, PIK3Cb, AKT2		
F42	+	−	PIK3Ca, PIK3Cb		
F43	+	−	PIK3Ca	RAS	
F44	+	−	VEGFR1, PIK3Ca, AKT2		
F45	+	−	VEGFR1, KIT, MET, PIK3Cb		
F47	+	−	PIK3Ca, PIK3Cb		
F48	+	−	PIK3Ca, PIK3Cb		
F50	−	+		RAS	
F51	+	+	EGFR, KIT, PIK3Cb, AKT2	RAS	
F52	−	−	PDGFRα		
F53	+	+	KIT, MET, PDK1		
F54	−	−			
F55	+	−	EGFR, KIT, MET		
F57	−	−	EGFR, VEGFR1		
F60	−	−	EGFR, VEGFR1, MET		
F61	+	−	PDGFRß, VEGFR1, PIK3Cb		
F62	−	−	VEGFR1		
F63	+	−	PDGFRß		
F64	+	−	PDGFRα, VEGFR1, PIK3Ca, PDK1		

+, Positive for immunohistochemical staining of p-Akt or p-ERK; −, negative for immunohistochemical staining of p-Akt or p-ERK.

Akt and p-ERK in 25 selected FTC samples (Fig. 1). As shown in Table 5, 16 of 25 FTC (64%) showed p-Akt immunoreactivity, and six of 25 FTC (24%) showed p-ERK1/2 immunoreactivity. All 16 FTC that showed p-Akt immunoreactivity harbored genetic alterations in the PI3K/Akt pathway. When looking at the specific genetic alterations and their relationship with the activation of PI3K/Akt pathway as shown in Table 5, eight of nine FTC with *PIK3Ca* copy gain (88.9%), nine of 10 FTC with *PIK3Cb* copy gain (90%), and six of seven FTC with *Akt2* copy gain (85.7%) showed p-Akt immunoreactivity. Among the RTK genes, four of seven FTC with *EGFR* copy gains (57.1%), four of four FTC with *PDGFR*β copy gains (100%), eight of 13 with

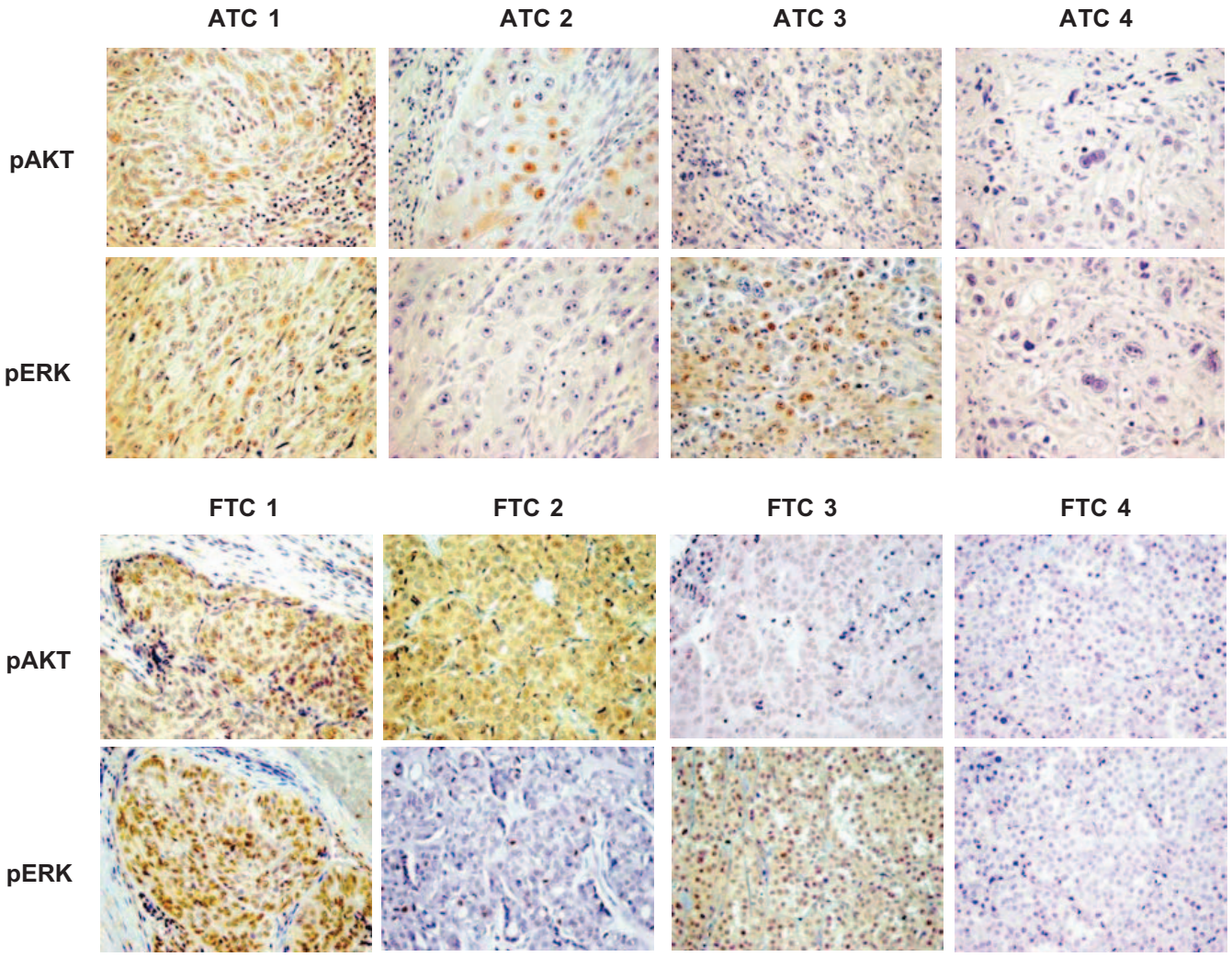

FIG. 1. Immunohistochemical analysis of p-Akt and p-ERK in ATC and FTC tissues. Shown are representative samples of ATC and FTC. ATC1 and FTC1 show significant staining with both anti p-Akt and p-ERK antibodies. ATC2 and FTC2 show p-Akt but not p-ERK immunostaining. ATC3 and FTC3 show p-ERK, but not p-Akt immunostaining. ATC4 and FTC4 show no significant staining for either p-Akt or p-ERK.

VEGFR1 copy gains (61.5%), four of four with *KIT* copy gains (100%), and three of four FTC with *MET* copy gains (75%) showed p-Akt immunoreactivity. Many cases had some normal thyroid tissues adjacent to the tumor tissues on this immunohistostaining analysis, and these areas of normal thyroid tissues did not show p-Akt or p-ERK.

Discussion

ATC is one of the most pernicious and rapidly progressive human malignancies and accounts for most of the fatalities caused by thyroid cancer. At present, no effective treatment for ATC is available and virtually all patients die rapidly once the diagnosis is established. In recent years, genetic alteration-targeted treatment has emerged to be a highly promising therapeutic approach to human cancers as represented by RTKs, which, with their mutations and copy number gains, are promising therapeutic targets in many cancers (14). One such specific example is the EGFR, whose activating mutations and genetic copy gains have become the basis for predictable effectiveness of treatments targeted at EGFR in non-small-cell lung cancer (31).

Because activating genetic alterations are commonly seen in the PI3K/Akt and MAPK pathways coupled to RTKs, these two major signaling pathways have also emerged to be important therapeutic targets in the development of novel therapy for human cancers (8, 11, 12). A limited number of genes in these signaling pathways have been recently explored in ATC and shown to frequently harbor genetic alterations, such as *PIK3Ca* copy gain and *BARF* mutation (3, 5). It remains unclear, however, how common the genetic alterations can be in other critical genes in these signaling pathways and hence how extensive these pathways are involved in ATC tumorigenesis. To provide a full-scale genetic basis for the understanding of the role of RTKs and their coupled PI3K/Akt and MAPK pathways in ATC, we extended previous studies to explore genetic alterations in a large and comprehensive panel of genes in these pathways in ATC.

We for the first time demonstrated a high prevalence of copy number gains in several major RTK genes in ATC and FTC, including the *EGFR*, *PDGFRα*, *PDGFRβ*, *and VEGFR1* genes

(Table 2). These findings, as in several other aggressive human cancers (31), underscore that copy number gain of RTK genes is an important genetic event and may represent a potentially effective therapeutic target in ATC. It is interesting to note that copy number gains of these RTK genes were more commonly associated with p-Akt than p-ERK in both ATC and FTC, suggesting that genetically activated RTKs preferentially use the PI3K/Akt pathway to promote ATC and FTC tumorigenesis and invasiveness. We also, for the first time, demonstrated a high prevalence of *PIK3Cb* copy gain in both ATC and FTC (Table 2), suggesting that this genetic alteration, like the *PIK3Ca* copy gain commonly seen in FTC and ATC (Table 2) (5), may play an important role in thyroid tumorigenesis through the PI3K/Akt pathway. The *PIK3Cb* copy gain has recently been found in other cancers such as ovarian cancer (32). Unlike *PIK3Ca*, *Ras*, and *PTEN* genes, mutations were uncommon in other genes examined in the PI3K/Akt pathway. Our real-time PCR approach could not definitively indicate genomic amplification *vs.* polysomy as the cause of gene copy number gains. However, regardless of the mechanism for their occurrence, copy gains of these tumor growth-promoting genes most likely play a role in the aberrant activation of PI3K/Akt and MAPK pathways in thyroid tumors as evidenced by their association with p-Akt or p-ERK in most cases.

The classical MAPK pathway-activating *BRAF* mutation was relatively common in ATC (Table 2). A few cases of ATC also harbored *RET/PTC*, but some of them contained PTC tissues, which could be the source of *RET/PTC*. Overall, most of these genetic alterations were correlated with increased p-Akt or p-ERK, consistent with their expected functionality. However, genetic alterations in some specimens were not associated with p-Akt or p-ERK. One explanation for this difference is that not all the genetic alterations found in this study may be functional in ATC, although these genetic alterations have been found to be functionally significant in other cancers. This could be due to a possibly differential role of these genetic alterations in different tissues. Alternatively, the lack of p-Akt or p-ERK might reflect the loss of phosphoproteins in tissue specimens due to inappropriate handling during the initial pathological processing and preserving process.

It is striking that up to 95.8% of ATC harbored at least one of the genetic alterations in RTKs and the PI3K/Akt and MAPK pathways and 81.3% of ATC had genetic alterations that could activate both PI3K/Akt and MAPK pathways. The coexistence of two or more of these genetic alterations was seen in most ATCs, particularly between mutation and copy number gains and among copy number gains (Table 3), underlying a possible genetic mechanism for amplifying and enhancing the aberrant signaling of these pathways. Although the prevalence of the genetic alterations found in the present study is already high, the results are likely still an underestimate as the number of the genes analyzed is till relatively limited and some genetic alterations, such as mutations, were only partially analyzed on these genes (*i.e.* only on certain exons). The overall genetic alterations and their coexistence in RTKs and the PI3K/Akt and MAPK pathways were more commonly seen in ATC than FTC (Tables 2–4). These genetic findings strongly suggest that RTKs and their coupled PI3K/Akt and MAPK pathways may play an extensive and fundamental role in ATC tumorigenesis and aggressiveness. Unlike the mutual exclusivity between mutations and the *PIK3Ca* copy gain in FTC (Table 4) (5, 30), gene mutations commonly coexisted with copy number gains of RTK genes in this cancer (Table 4). Our genetic analysis with a large panel of additional genes in RTKs and PI3K/Akt pathways in the present study revealed a significantly higher overall prevalence of genetic alterations in FTC than we previously reported on a limited number of genes (5, 30). These genetic alterations were mostly associated with p-Akt (Table 5). These data further support the notion that the PI3K/Akt pathway also plays an important role in FTC tumorigenesis.

In summary, by exploring genetic alterations in a large and comprehensive panel of genes in RTKs and their coupled PI3K/Akt and MAPK pathways, we extensively extended previous genetic observation in these pathways in thyroid cancers, including the first demonstration of copy number gains of RTKs genes and *PIK3Cb* in ATC and FTC, particularly the former. In ATC, we demonstrated a strikingly high prevalence of genetic alterations (involving virtually every tumor) and the common presence of genetic alterations that could activate both the PI3K/Akt and MAPK pathways. The data thus provided strong genetic evidence suggesting that genetically altered RTKs and their coupled PI3K/Akt and MAPK pathways may play an extensive role in the tumorigenesis and aggressiveness of ATC. Specific genotype-based targeting at these signaling pathways may be an effective therapeutic strategy for ATC.

Acknowledgments

We thank Dr. Yuri E Nikiforv for kindly providing us with the cDNA for RET/PTC3 analysis.

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This work was partly supported by American Cancer Society Grant RSG-05-199-01-CCE (to M.X.).

Disclosure Statement: The authors have nothing to disclose.

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