Genetic Alterations in the Ras/Raf/Mitogen-Activated Protein Kinase and Phosphatidylinositol 3-Kinase/Akt Signaling Pathways in the Follicular Variant of Papillary Thyroid Carcinom...

ARTICLE *in* CANCER · JUNE 2010

Impact Factor: 4.89 · DOI: 10.1002/cncr.25061 · Source: PubMed

6 AUTHORS, INCLUDING:

Francesco Trimarchi
Università degli Studi di Messina
317 PUBLICATIONS **3,973** CITATIONS

SEE PROFILE

Original Article

Genetic Alterations in the Ras/Raf/Mitogen-Activated Protein Kinase and Phosphatidylinositol 3-Kinase/Akt Signaling Pathways in the Follicular Variant of Papillary Thyroid Carcinoma

Libero Santarpia, MD, PhD[1]; Jeffrey N. Myers, MD, PhD[2]; Steven I. Sherman, MD[1]; Francesco Trimarchi, MD[3]; Gary L. Clayman, MD, PhD[2]; and Adel K. El-Naggar, MD, PhD[4]

BACKGROUND: The follicular variant of papillary thyroid carcinoma (FVPTC) is the second most common histotype among papillary thyroid cancers (PTCs). Although the prognosis of FVPTC is similar to the conventional phenotype, differential diagnostic difficulties may not be uncommon with other follicular thyroid neoplasms, and little is known about their genetic alterations. Defining these alterations may lead to the identification of diagnostic and biologic markers. **METHODS:** In this study, the authors evaluated genetic alterations and downstream-activated signals of the Ras/Raf–mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/v-akt murine thymoma viral oncogene (Akt) (PI3K/Akt) signaling pathways in 30 FVPTC tissue specimens. Tumors and matched normal thyroid samples were tested for *RAS*, for the v-raf murine sarcoma viral oncogene (*BRAF*) substitution of valine (V) for glutamate (E) at codon 600 (the V600E mutation), for phosphatase and tensin homolog (*PTEN*), for catalytic PI3k p110 subunit alpha (*PIK3CA*), for *AKT*, and for the presence of rearranged during transfection (ret) proto-oncogene/PTC (*RET-PTC*) and paired box-8 (PAX8)/peroxisome proliferator-activated receptor γ (PPARγ) fusion protein (*PAX8-PPAR*γ) rearrangements by direct sequencing and reverse transcriptase-polymerases chain reaction analyses, respectively. Western blot analysis was used to assess the effects of these gene abnormalities on the activation of the 2 pathways. **RESULTS:** Genetic alterations were identified in 70% of FVPTCs. Activation of the MAPK and PI3K pathways was observed in 74% and 22% of tumors, respectively. The alterations that were identified in the genes of the 2 pathways were mutually exclusive. Chromosomal *RET-PTC* and *PAX8-PPAR*γ rearrangements were observed in 20% and 17% of tumors, respectively. It was noteworthy that some FVPTCs with RET-PTC had the coactivation of both pathways. **CONCLUSIONS:** *RET-PTC* and *PAX8-PPAR*γ rearrangements and mutations of the neuroblastoma RAS viral oncogene homolog *N-RAS* at codon 61 were the most common genetic alterations in FVPTCs. Activation of the MAPK pathway was a frequent event in FVPTCs, and the PI3K signaling pathway could be coactivated in *RET-PTC* tumors. These findings may have important therapeutic implication in patients with FVPTC. ***Cancer*** **2010;116:2974–83.** © *2010 American Cancer Society*.

KEYWORDS: follicular variant papillary thyroid cancer, genetic alterations, mitogen-activated protein kinase pathway, phosphatidylinositol 3-kinase pathway, phosphorylation activation, *RET-PTC* rearrangements, *PAX8-PPAR*γ translocations.

The follicular variant papillary thyroid carcinoma (FVPTC) is the second most common morphologic subtype in patients with papillary thyroid carcinoma (PTC). Studies of this histologic subtype have demonstrated that certain clinicopathologic and molecular features are shared with papillary and follicular neoplasms, suggesting that FVPTC represents a

Corresponding author: Adel K. El-Naggar, MD, PhD, Department of Pathology, Unit 85, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030-4009; Fax: (713) 792-5532; anaggar@mdanderson.org

[1]Department of Endocrine Neoplasia and Hormonal Disorders, The University of Texas M. D. Anderson Cancer Center, Houston, Texas; [2]Department of Head and Neck Surgery, The University of Texas M. D. Anderson Cancer Center, Houston, Texas; [3]Department of Endocrinology, University of Messina, Policlinico University, Messina, Italy; [4]Department of Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

Libero Santarpia's current address: Translational Research Unit, Department of Oncology, Hospital of Prato and Istituto Toscana Tumori, Florence, Italy.

We thank the Foundation Carrozza-Pollicino for research support and the American Thyroid Association for a Thy-Ca Grant (to Dr. Santarpia), Dr. Marina Nikiforova for kindly providing the complementary DNA for the RET-PTC3 analysis, Lei Feng for the statistical analysis support, and Danielle Litofsky for her assistance in data collection.

DOI: 10.1002/cncr.25061, **Received:** July 1, 2009; **Revised:** September 4, 2009; **Accepted:** September 22, 2009, **Published online** March 30, 2010 in Wiley InterScience (www.interscience.wiley.com)

hybrid of these entities. However, the genetic alterations underlying the pathogenesis of FVPTC remain not well clarified.[1,2]

Alterations in several signal-transduction pathways are associated with thyroid tumorigenesis, including the Ras/Raf–mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/v-akt murine thymoma viral oncogene (Akt) (PI3K/Akt) signaling pathways.[3-5] The most commonly altered members of these 2 signaling pathways are the *RAS*, v-raf murine sarcoma viral oncogene (*BRAF*), catalytic PI3k p110 subunit alpha (*PIK3CA*), phosphatase and tensin homolog (*PTEN*), paired box-8 (PAX8)/peroxisome proliferator-activated receptor γ (PPARγ) (*PAX8-PPARγ*), and rearranged during transfection (ret) proto-oncogene/papillary thyroid carcinoma (*RET-PTC*) genes. The *RAS* gene family was among the first to be associated with thyroid carcinoma and is responsible for abnormal cell growth and differentiation. Both in vivo and in vitro studies have implicated the *RAS* genes in thyroid tumorigenesis,[6,7] and mutations of the Harvey rat sarcoma, Kirsten rat sarcoma, and neuroblastoma viral RAS oncogenes (*H-RAS*, *K-RAS*, and *N-RAS*), respectively) have been reported in different thyroid tumors histotypes.[8-10]

Similarly, the *BRAF* gene, a member of the RAF family of serine/threonine kinases, plays an important role in thyroid tumorigenesis. It is noteworthy that the *BRAF* substitution of valine (V) for glutamate (E) at codon 600 (the V600E mutation) has been associated with aggressive features and a poor outcome in patients with PTC.[11] This mutation is reported mainly in the conventional and tall cell variants of PTC. We demonstrated previously that *BRAF* mutations are infrequent in FVPTC, suggesting a different molecular signature within the PTC histologic variants.[12] FVPTC often has different mutation types and a variable frequency of *BRAF* mutations.[2,13-15]

Studies on *RET-PTC* rearrangements support an early role of these abnormalities in the early tumorigenesis of PTC. These genetic rearrangements result from the juxtaposition of the tyrosine kinase domain coding region of RET with the 5′-promoter regions of a variety of unrelated genes. It is noteworthy that these rearrangements have been detected frequently in tumors from children with radiation exposure.[16,17] Although different rearrangements have been reported in thyroid carcinomas, *RET-PTC1* (coiled-coil domain-containing protein 6 [CCDC6]; H4/D10S170-tyrosine kinase RET) and *RET-PTC3* (nuclear receptor coactivator 4 [NcoA4]; RET fused gene/[RFG/ELE1]-tyrosine kinase RET) comprise almost 95% of all rearrangements. Another type of chromosomal translocation between the thyroid transcription factor *PAX8* and the *PPARγ* gene (*PAX8-PPARγ* t[2;3][q13;25]) reportedly occurs with variable frequency in FVPTC and follicular thyroid carcinoma and less frequently in follicular adenoma.[1,18-20] Studies of individual fusion transcripts in follicular thyroid tumorigenesis and/or progression have been conducted, but the biologic significance and the underlying factors for the development of these rearrangements in thyroid cancer remain uncertain.

It also has been demonstrated that the activation and dysregulation of the PI3K-AKT-mammilian target of rapamycin pathway plays a significant role in the development and progression of thyroid cancers.[21] *PTEN*, a tumor suppressor gene and a member of the PI3K pathway, plays an important role in the regulation of this pathway. Mutations of this gene have been associated with Cowden syndrome and may be involved in thyroid tumorigenesis.[22] However, the frequency of *PTEN* mutations and their role in FVPTC are not known. Several investigators have demonstrated that the activation of the phosphatidyl inositol phosphate p110 subunit, which encodes the *PIK3CA* gene, plays an important role in thyroid tumorigenesis.[23] Moreover, the activation of PI3K downstream AKT signaling has been linked to thyroid tumor progression,[24] and mutations of the *PIK3CA* gene have been observed in differentiated, poorly differentiated, and undifferentiated thyroid cancers.[3-5,24-27] Similar studies on the role of PIK3CA and AKT in FVPTC are needed.

The objective of the current study was to investigate the genetic alterations within the MAPK and PI3K signaling pathway genes in FVPTC tumors. We include an analysis of different types of chromosomal rearrangements (*RET-PTC* and *PAX8-PPARγ*) and genetic alterations in genes that encode downstream signaling molecules within the MAPK and PI3K signaling pathways.

MATERIALS AND METHODS

Specimen Selection

We analyzed 56 fresh-frozen thyroid tissues. Thirty FVPTC tissue specimens and 26 matched, adjacent, normal thyroid tissues derived from the same unaffected thyroid lobe were collected between 1990 and 2006 and stored in the Head and Neck Tissue Bank in the Department of Pathology, The University of Texas M. D. Anderson Cancer Center and formed the material for this study.

Each diagnosis was made based on World Health Organization tumor classification criteria.[28] The selection of specimens was described in our previously published report.[12] The study was approved by the Institutional Review Board at The University of Texas, M. D. Anderson Cancer Center.

Assessment of Genetic Alterations

The presence of *PAX8-PPARγ* rearrangements (exons 7-9 of *PAX8* with exon 1 of *PPARγ*), *RET-PTC* rearrangements 1 and 3, *RAS* (*K-RAS*, *H-RAS*, and *N-RAS*) hotspot mutations (codons 12, 13, and 61), the *BRAF* V600E mutation, *PTEN* (exons 5-8), *AKT* (*AKT-1*, *AKT-2*, and *AKT-3*) hot spot mutations, and *PIK3CA* (exons 9 and 20) gene mutations and amplification were analyzed by using molecular techniques. The activation status of the MAPK and PI3K pathways was determined by evaluating the levels of phosphorylation in the 2 main signals, extracellular signal-regulated kinase [ERK] and AKT, using Western blot analysis. Twenty-six additional histologically matched, normal thyroid tissues that were collected also were analyzed as biologic controls.

Acid Nucleic Extraction

Total RNA was extracted from fresh-frozen tumor specimens and normal thyroid tissues. Approximately 200 mg of tumor tissue was homogenized in 1 mL of TRIzol reagent (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions. The absorbance of the extracted RNA was quantified using the Beckman Coulter DU 60 spectrophotometer (Beckman Coulter, Fullerton, Calif). RNA integrity and quality were evaluated on 2% agarose gels by observing the 18 and 28 S bands. Genomic DNA was extracted from the same set of tumor tissues with the Qiagen kit (Qiagen, Germantown, Md) according to the manufacturer's instructions.

Reverse Transcriptase-Polymerase Chain Reaction Assay to Detect PAX8-PPARγ Transcripts

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis (Promega Corp., Madison, Wis) was performed using 0.5 μg of extracted RNA treated with Dnase I (Invitrogen) in a final mixture of 50 μL consisting of nuclease-free water, 2 μL of 25-mM $MgSO_4$, 1 μL avian myeloblastosis virus (AMV) reverse transcriptase (5 U/μL), 10 μL AMV/*Thermus flavus* (Tfl) 5X reaction buffer, 1 μL 10-mM deoxyribonucleotide (dNTP) mix, 1 μL Tfl DNA polymerase (5 U/μL), and 50-pmol of both primers. Initial primers that were used for RT-PCR have been published previously.[29] The RT-PCR consisted of an initial cycle at 45°C for 45 minutes and 94°C for 2 minutes; followed by 39 cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 68°C for 60 seconds; and a final cycle of 68°C for 8 minutes. The band sizes of the final PCR products, once rearranged, were from 68 base pairs (bp) to 362 bp (exon 7 of PAX8 and exon 1 of PPARγ), from 67 bp to 172 bp (exon 8 of PAX8 and exon 1 of PPARγ), and 72 bp (exon 9 of PAX8 and exon 1 of PPARγ) and were detected by gel electrophoresis on 2.5% MetaPhor agarose gels (Cambrex Biosciences, Rockland, Me). The phosphoglycerate kinase 1 gene (*PGK-1*) was used for housekeeping. For positive controls, we used RNA from thyroid tumors known as positive for the *PAX8-PPARγ* transcript.

RT-PCR Assay to Detect RET-PTC Rearrangements

RET-PTC rearrangements 1 and 3 were detected with minor modifications in the PCR reactions as described previously.[30] Briefly, RT-PCR was performed with 1 μg of extracted RNA treated with Dnase I (Invitrogen) in a final mixture of 50 μL nuclease-free water containing 2 μL of 25-mM $MgSO_4$, 1 μL AMV RT (5 U/μL), 10 μL AMV/Tfl 5X reaction buffer, 1.5 μL 10-mM dNTP mix, 1.5 μL Tfl DNA polymerase (5 U/μL), and 50-pmol of both primers. The RT-PCR cycle was an initial cycle at 45°C for 45 minutes and 94°C for 2 minutes; followed by 39 cycles at 94°C for 30 seconds, 56°C for 45 seconds, and 68°C for 60 seconds; and a final cycle at 68°C for 9 minutes. Complementary DNA (cDNA) from the TPC-1 cell line was included as a positive control for RET/PTC-1 analysis. For ret/PTC-3, cDNA from a tumor with the *RET/PTC3* transcript was used (a kind gift from Dr. Marina Nikiforova, University of Pittsburgh, Pittsburgh, Pa). *PGK-1* was used in all reactions as a housekeeping gene.

Analysis of the H-RAS, K-RAS, N-RAS, and BRAF Genes

Point mutations in codons 12/13 and codon 61 (hot spots) of the *H-RAS*, *K-RAS*, and *N-RAS* genes were detected as described previously.[9] The *BRAF* gene was amplified with the primers 5′-TCATGAAGACCTCAC AGTAAAAAT-3′ (forward) and 5′-TGGATCCAAGAC AACTGTTCAA-3′ (reverse). PCR was performed in a 25-μL mixture containing 20 pmol of each primer, 200 μM of each dNTP, 0.5 U of Taq DNA polymerase, and 30 ng to 50 ng of genomic DNA. PCR conditions consisted of initial denaturation at 95°C for 5 minutes

followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 60 seconds, and extension at 72°C for 30 seconds. The final extension step was performed at 72°C for 8 minutes. The samples were analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, Calif).

PTEN, PIK3CA, *and* AKT *Mutation Analysis and Quantitative Real-Time PCR to Detect* PIK3CA *Amplification*

Abnormalities in the *PTEN* and *PIK3CA* genes were assessed as described previously.[4] *AKT* mutations were assessed by direct sequencing of exon 3 with specific primers that were designed to amplify hot-spot regions of *AKT*. To detect *AKT1* mutation at nucleotide 49-exon 3 resulting in a replacement of a lysine (K) with glutamic acid (E) at amino acid 17 (E17K), we used the following PCR and sequencing primers: forward, 5′-ACATCT GTCCTGGCACAC-3′; reverse, 5′-GCCAGTGCTTG TTGCTTG-3′. Analogous hot-spot mutations in *AKT2* and *AKT3* also were analyzed as reported previously.[31] We detected *PIK3CA* copy number gains by using quantitative real-time PCR. Ribonuclese P (Applied Biosystems) was used as the reference control. Data were analyzed by using the Delta-Delta cycle threshold (ΔΔCt) method.

Analysis of the MAPK and PI3K Activation Signaling Pathways by Western Blot Analysis

We used Western blot analyses to assess and evaluate the protein phosphorylation status of AKT in the PI3K pathway and of ERK in the MAPK pathway as key indicators of pathway activation. Preparation of total cell lysates and Western blots were carried out as described previously.[12] Briefly, Western blot analysis was performed using 40 μg to 50 μg of protein electrophoresed on 10% to 12% sodium dodecyl sulfate-polyacrylamide electrophoresis gels using the antibodies antiphosphorylated ERK (p-ERK1/2), anti-total AKT, and antiphosphorylated AKT (p-AKT; serine [Ser] 473) (Cell Signaling Technology, Beverly, Mass). The assay was run twice for each tumor. After normalization, the band intensities from Western blot analyses for p-ERK and p-AKT in tumors were compared with basal levels of matched normal thyroid tissues and were analyzed using the LiCOR Odyssey Imaging System software package (Biosciences, Lincoln, Neb) and NIH image software (National Institutes of Health, Bethesda, Md).

RESULTS

Twenty-one of the 30 FVPTC tumor specimens had at least 1 genetic alteration, and 9 had no genetic abnormalities in any of the genes of interest. It is noteworthy that the 9 "negative" tumors were indistinguishable morphologically from the other FVPTCs that were tested; accordingly, we believe that a genetic screening of other exons of the aforementioned genes or a broader genetic approach (eg, microarray expression analysis) may help to define the genetic background of this subgroup of tumors. Only 1 tumor had the concomitant presence of 2 gene alterations: *RAS* mutation and *PAX8-PPARγ* rearrangement. All histologically normal thyroid tissues were negative for alterations in any of the genes that were evaluated. Table 1 presents the genetic alterations in this group of FVPTC specimens.

Genetic Alterations

Mutations

N-RAS mutations were identified in 5 of 30 FVPTC specimens (17%), but no mutations were identified in the *H-RAS* or *K-RAS* genes. Figure 1A shows the *N-RAS* wild type. All mutations in the *N-RAS* gene were localized at codon 61 with the substitution of adenine to a guanine (CAA→CGA) in 3 tumors and the substitution of cytosine to adenine (CAA→AAA) in 1 tumor (Fig. 1B,C). An uncommon mutation that led to the replacement of an adenine with a thymine, thus substituting glutamine with leucine, also was identified (Fig. 1D).

BRAF was mutated in 4 of the 30 FVPTC specimens (13%). Three of these mutations were identified at codon 600 (*BRAF* V600E). A novel *BRAF* point mutation in FVPTC that was reported previously by our group was included in this study.[12] This mutation led to the replacement of an alanine by a valine in the BRAF kinase activation C3 domain (*BRAF* A598V).

Table 2 presents the variable frequency of the *BRAF* V600E mutation and of other different *BRAF* mutations at exon 15 in diverse series of FVPTC from the English literature. No mutations in the *PTEN*, *PIK3CA*, or *AKT* genes were detected. Two tumors had an amplification of the *PIK3CA* gene.

Rearrangements

RET-PTC rearrangements.

Figure 2A is a schematic representation of the RET proto-oncogene structure with the structure of the kinase domain. Six of 30 FVPTC specimens (20%) had rearrangements for *RET-PTC*, including 3 tumors that had

Table 1. The Frequency of Different Genetic Alterations and Phosphorylation of Downstream Signaling of the Mitogen-Activated Protein Kinase and Phosphoinositide 3-Kinase Signal-Transduction Pathways in Follicular Variant Papillary Thyroid Carcinomas

FVPTC Tumors	*K-RAS*, *H-RAS*, *N-RAS* Mutations	*BRAF* Mutations	*PTEN* Mutations	*PIK3CA* Alterations	*AKT* Mutations	*RET-PTC* Rearrangements	*PAX8-PPARγ* Rearrangements	*MAPK*	*PI3K*
1	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	N/A	N/A
3	–	V600E	–	–	–	–	–	+	–
4	–	–	–	–	–	RET-PTC1	–	+	–
5	–	–	–	–	–	–	–	N/A	N/A
6	Q61R	–	–	–	–	–	–	+	–
7	–	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	PAX8-PPARγ	+	–
9	–	V600E	–	–	–	–	–	+	–
10	Q61K	–	–	–	–	–	–	+	–
11	Q61R	–	–	–	–	–	–	+	–
12	–	–	–	–	–	–	–	N/A	N/A
13	–	–	–	–	–	RET-PTC3	–	+	+
14	Q61L	–	–	–	–	–	–	+	–
15	–	–	–	–	–	–	–	+	–
16	–	–	–	–	–	–	PAX8-PPARγ	+	–
17	–	–	–	–	–	RET-PTC3	–	+	+
18	–	–	–	–	–	–	–	–	–
19	–	–	–	–	–	–	PAX8-PPARγ	–	+
20	–	–	–	Amplified	–	–	–	–	+
21	–	V600E	–	–	–	–	–	+	–
22	–	–	–	–	–	RET-PTC1	–	+	+
23	–	A598E	–	–	–	–	–	+	–
24	–	–	–	–	–	–	PAX8-PPARγ	–	–
25	–	–	–	–	–	RET-PTC3	–	+	–
26	–	–	–	–	–	–	–	+	–
27	Q61R	–	–	–	–	–	PAX8-PPARγ	+	–
28	–	–	–	Amplified	–	–	–	–	+
29	–	–	–	–	–	–	–	+	–
30	–	–	–	–	–	RET-PTC1	–	+	–
No./total (%)	5/30 (17)	4/30 (13)	0 (0)	2/30 (7)	0 (0)	6/30 (20)	5/30 (17)	(74)	(22)

FVPTC, indicates follicular variant of papillary thyroid carcinoma; *H-RAS*, *K-RAS*, *N-RAS*, Harvey rat sarcoma, Kirsten rat sarcoma, and neuroblastoma viral RAS oncogenes, respectively; *BRAF*, v-raf murine sarcoma viral oncogene; *PTEN*, phosphatase and tensin homolog; *PIK3CA*, phosphoinositide 3-kinase, catalytic; *AKT*, v-akt murine thymoma viral oncogene; *RET-PTC*, rearranged during transfection (ret) proto-oncogene/papillary thyroid carcinoma; *PAX8-PPARγ*, paired box-8(PAX8)/peroxisome proliferator-activated receptor (PPAR)-γ fusion protein; *MAPK*, mitogen-activated protein kinase; *PI3K*, phosphoinositide 3-kinase; –, negative; N/A, not available or not enough protein; V600E, substitution of valine (V) for glutamate (E) at codon 600; +, positive; Q61R, substitution of glutamine (Q) for arginine (R) at codon 61; Q61K, substitution of glutamine for lysine (K) at codon 61; Q61L, substitution of glutamine (Q) for lysine (L) at codon 61; A598E, substitution of alanine (A) for glutamate (E) at codon 598.

fusion of the tyrosine kinase RET proto-oncogene with the gene *CCDC6* (H4/D10S170; *RET-PTC1*) (Fig. 2B) and 3 tumors that had rearrangement of *RET-PTC3* consisting of the fusion between *NcoA4* (RFG/ELE1) and the tyrosine kinase domain of RET (Fig. 2C). None of the tumors had different concurrent rearrangements of these genes.

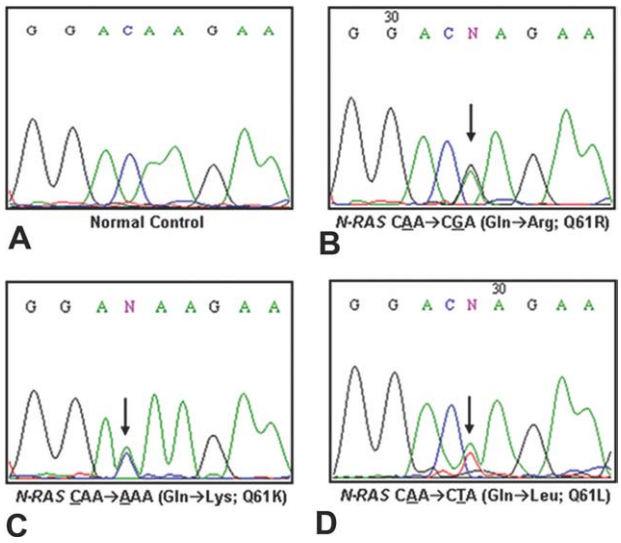

Figure 1. Neuroblastoma RAS viral oncogene homolog (*N-RAS*) mutations at codon 61 are illustrated in different follicular variants of papillary thyroid carcinoma. The predominant mutation was observed with the translocation of cytosine, adenine, adenine to cytosine, guanine, adenine (CAA→CGA), replacing the amino acid glutamine with arginine (protein; Q61R). A single *N-RAS* mutation CAA→AAA replaced a cytosine with an adenine (protein; substitution of glutamine [Q] for lysine [K] at codon 61 [Q61K]). In 1 tumor, an uncommon mutation CAA→CTA replaced an adenine with thymine (protein; substitution of glutamine [Q] with leucine [L] at codon 61 [Q61L]).

PAX8-PPARγ Rearrangements.

In 5 of the 30 FVPTC specimens (17%), *PAX8-PPARγ* rearrangements were present (Fig. 3A). One tumor (20%) had a single transcript, and 4 tumors (80%) had the concurrent presence of multiple *PAX8-PPARγ* transcripts, and the most common translocation was between exon 7 of PAX-8 and exon 1 of PPARγ (Fig. 3B). One positive rearranged tumor was positive for *N-RAS* (Table 1).

Activation of the MAPK and PI3K Downstream Target Proteins

Western blot analysis on proteins extracted from all specimens and normal tissues revealed elevated phosphorylation of ERK (74%) in contrast to AKT (22%) in FVPTCs (Table 1). We also observed that the genetic abnormalities were associated with the activation of downstream signals, suggesting a specific gene-pathway regulatory function. The 2 tumors with amplification of *PIK3CA* had AKT phosphorylation, confirming activation of the PI3K pathway. Three tumors with *PAX8-PPARγ* rearrangements had ERK phosphorylation, and 1 tumor had AKT activation. Two tumors with *RET-PTC3* rearrangements and 1 tumor with *RET-PTC1* rearrangement had AKT and ERK phosphorylation, suggesting coactivation of both pathways (Fig. 4). Two tumors had ERK phosphorylation independent of the mutation status of any of the genes that were studied.

DISCUSSION

The current study, which is the first to our knowledge that simultaneously analyzes several genetic abnormalities in

Table 2. Studies That Reported Different Mutations From the Classic *BRAF* Substitution of Valine for Glutamate at Codon 600 in Follicular Variant Papillary Thyroid Carcinomas

Report	FVPTC Tumors Analyzed	*BRAF* V600E	Different *BRAF* Mutations (No.)	Study
1	32	0	K601E (3)	Trovisco 2004[13]
2	40	0	K601E (3), G474R (1)	Castro 2006[2]
3	114	21	K601E (1), T599I-VKSR(600-603)del (1)	Lupi 2007[15]
4	3	0	V600-S605 (VKSRWS)del (1)	Barzon 2008[14]
5	47	9	K601del mutation (1)	Oler & Cerutti 2008[35]
6	30	3	A598E (1)	Santarpia 2009[12]
Total (%)	266	33/266 (12)	12/266 (5)	

FVPTC indicates follicular variant papillary thyroid carcinoma; *BRAF*, v-raf murine sarcoma viral oncogene; V600E, substitution of valine (V) for glutamate (E) at codon 600; K601E, substitution of lysine (K) for glutamate at codon 601; G474R, substitution of glycine (G) for arginine (R) at codon 474; T599I-VKSR(600-603)del, substitution of threonine (T) for isoleucine (I) and deletion of valine 600 (V), lysine 601 (K), serine 602 (S), and arginine 603 (R); V600-S605 (VKSRWS)del, replacement of amino acids from valine (V) 600 to serine (S) 605 (ie, V 600, K 601, S 602, R 603, tryptophan [W] 604, S 605); K601del, deletion of lysine (K) at codon 601; A598E, substitution of alanine (A) for glutamic acid (E) at codon 598.

the MAPK and PI3K signaling cascades in FVPTC, demonstrates frequent genetic alterations in several genes of these pathways. These data support the finding that FVPTC shares certain abnormalities with both papillary and follicular carcinomas and, thus, may be considered an intermediate phenotypic form between PTC and follicular neoplasms. The results also reveal that the MAPK pathway can be activated independent of the genetic alterations in few tumors, suggesting the involvement of other gene signal-activating pathway. Our phosphoprotein analysis of ERK and AKT also supports the overriding activation of the MAPK pathway (74%) compared with the PI3K pathway (22%). The results indicate that, of all the RAS gene isoforms, *N-RAS* mutation at codon 61 is the only genetic event in FVPTC.

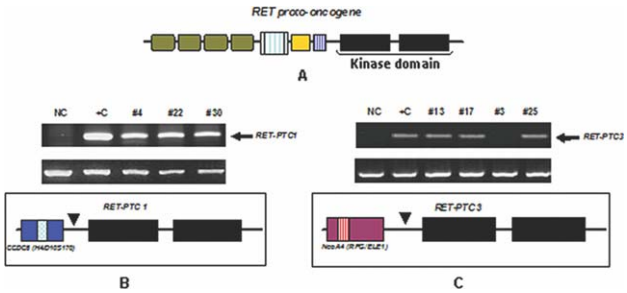

Figure 2. The presence of rearranged during transfection (ret) proto-oncogene/papillary thyroid carcinoma (*RET-PTC*) rearrangements in the follicular variant of papillary thyroid carcinoma (FVPTC) is illustrated. (A) This schematic represents the RET proto-oncogene with all different domains, including the tyrosine kinase domain. (B) The presence of *RET-PTC1* rearrangement is observed in the positive control (+C), the TPC-1 thyroid cancer cell line, and in 3 FVPTCs (tumors 4, 22, and 30). The graph below shows the *RET-PTC1* "rearranged chimera." (C) The presence of *RET-PTC3* rearrangements is observed in a positive control (+C) and in 3 FVPTC samples (tumors 13, 17, and 25). Tumor 3 was negative for *RET-PTC3.* The graph below shows the *RET-PTC3* "rearranged chimera." NC indicates negative control.

It is noteworthy that approximately 20% of human tumors have activating *RAS* point mutations: *K-RAS* accounts for about 85%, and *N-RAS* and *H-RAS* account for only 15% and for <1% of all *RAS* mutations, respectively.[32] In those studies, mutation of the *RAS* genes appeared to be tumor-specific; frequent *K-RAS* mutations were reported in the colon, pancreas, and lung; and an increased incidence of *N-RAS* mutations was reported in myeloid leukemias and cutaneous melanomas.[32] Accordingly, specific *RAS* mutations also have been reported in different thyroid carcinomas histotypes, with *K-RAS* and *N-RAS* mutations associated mainly with PTC and follicular thyroid carcinoma or FVPTC, respectively.[1,4,33] Moreover, although it was reported previously that *RAS* mutations were associated primarily with aggressive cancer behavior in poorly differentiated and undifferentiated thyroid cancers,[9,34] we confirmed that these genetic alterations also appear to be frequent findings in FVPTC. The restricted alteration to *N-RAS* in FVPTC is interesting and may underlie a role in the follicular manifestation of this phenotype.

In the current study, we noted both the low frequency of genetic alterations in the PI3K pathway and the absence of mutations in any of the 3 PI3K pathway genes (*PTEN*, *PIK3CA,* and *AKT*). Only 2 tumors had a copy number gain of the *PIK3CA* gene (≥4 copies), and only 1 had an aggressive phenotype. Typically, an increase in copy number of *PIK3CA* or a molecular mutation of this gene leads to a gain of function reflected by higher or constitutive activation of PI3K activity and to oncogenic transformation. However, the oncogenic role of *PIK3CA*

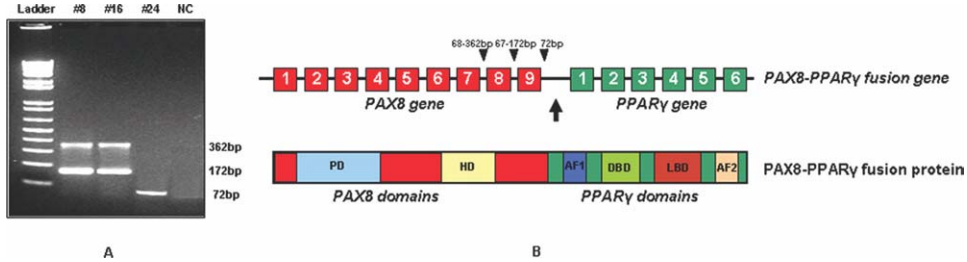

Figure 3. Different types of paired box-8 (PAX8)/peroxisome proliferator-activated receptor γ (PPARγ) (*PAX8-PPARγ*) fusions are shown in samples of the follicular variant of papillary thyroid carcinoma (FVPTC). (A) Two tumors (no. 8 and 16) had the concurrent presence of different *PAX8-PPARγ* translocations, and another FVPTC tumor (no. 24) had a single translocation. Normal thyroid was used as negative control (NC) for any *PAX8-PPARγ* transcripts. (B) This graph illustrates all of the possible types of translocations between the thyroid transcription factor PAX8 domains and PPARγ. PD indicates paired domain-DNA binding domain; HD, homeodomain; AF, activation function domains; DBD, DNA-binding domain; LBD, ligand-binding domain/heterodimerization domain.

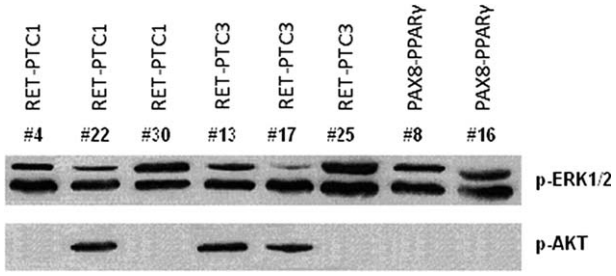

Figure 4. Western blot analysis of tumors that had the rearranged during transfection (ret) proto-oncogene/papillary thyroid carcinoma (*RET-PTC*) and paired box-8 (PAX8)/peroxisome proliferator-activated receptor γ (PPARγ) fusion protein (*PAX8-PPARγ*) rearrangements revealed activation of the mitogen-activated protein kinase (MAPK) (extracellular signal-regulated kinase [ERK] phosphorylation [p-ERK]) and the phosphoinositide 3-kinase (PIK3)-protein kinase B (AKT) (AKT phosphorylation [p-AKT]) pathways in different follicular variant of papillary thyroid carcinoma (FVPTC) samples. Three RET-PTC tumors had phosphorylation of both ERK1/2 and AKT.

amplification in differentiated thyroid tumors, including FVPTC, remains to be determined, and larger series with long-term follow-up are needed. We also noted a relatively low incidence of *BRAF* V600E mutation in FVPTC (10%), confirming the infrequency of the mutation in this PTC subtype. *BRAF* V600E has been demonstrated mainly in PTC and in other phenotypic variants with prominent papillary growth patterns, whereas different *BRAF* mutations have been reported in FVPTC.[2,12-15,35] We contend that restricting the genetic analysis of papillary thyroid subtypes to the *BRAF* V600E mutation (in tumor specimens and/or fine-needle aspiration biopsies) may lead to overlooking other activating *BRAF* mutations, at least in FVPTC.

An important finding in our study was the high incidence of *PAX8-PPARγ* and *RET-PTC* rearrangements in FVPTC. However, linking these events to the development of FVPTC requires further investigations. It is possible, however, that these genetic abnormalities, which are formed by the fusion of different broken chromosomal segments, may reflect a genomic instability of this variant. It is noteworthy that we identified concurrent *PAX8-PPARγ* isoform rearrangements in several tumors that had ERK or AKT phosphorylation, in support of the finding that this subset of rearranged PTCs most likely either directly or indirectly activates or regulates ERK and/or AKT during tumorigenesis. Within the MAPK pathway, the ERK cascade is a major signal that regulates cell proliferation, survival, and several other cellular functions. This contention is evidenced further by the strong nuclear PPARγ expression observed in thyroid carcinomas, indicating that the activation of PPARγ often is modulated by MAPK signals or by nucleocytoplasmic compartmentalization through the ERK activators MEK1/2.[19,20] A reciprocal regulation effect of *ERK/PAX8-PPARγ* could occur in thyroid cancer cells whether the mechanisms by which PAX8-PPARγ might activate AKT remain to be elucidated. Together, all of these concepts support the presence of a tight crosstalk between all these signals. This rearranged protein and its function deserve further investigations.

The current results also demonstrate that a subset of tumors has either *RET-PTC1* or *RET-PTC3* rearrangements. These rearrangements, along with activation of the RAS-BRAF-ERK cascade,[36] may trigger the activation of additional signals. Accordingly, we noted that *RET-PTC* rearrangements tended to be associated with coactivation of the PI3K-AKT pathway. Two RET-PTC3 tumors and 1 RET-PTC1 tumors had consistent elevation of AKT protein phosphorylation. Several studies have indicated that the oncogenic potential of *RET/PTC* rearrangements is not limited to their intrinsic tyrosine kinase activity but also includes enhanced phosphorylation and the activation of different substrates. It has been demonstrated that signaling pathways are activated by RET-PTC and that activation of the PI3K/AKT signaling pathway is 1 of the most important events in RET-mediated cell transformation.[37-39] RET activation may lead to a double activation of the MAPK and PI3K pathways. One of the mechanisms responsible for this coactivation is a 1 single tyrosine residue, RET Y1062, which is a RET docking site for Shc and for the PI3K-p85 regulatory subunit, which activate the MAPK and PI3K pathways, respectively. Therefore, this site appears to be essential for the activation of both pathways, and both are important for promoting cell survival.[40,41]

Activation of the PI3K pathway in thyroid cancer cells that have RET-PTC or RET activation often is potentiated by the interaction of different protein adaptors, eg, RAI (ribonuclease/angiogenin inhibitor), GAB1 (growth factor receptor-bound 2 [GRB2]-associate binding protein 1), or XB130 (a 130-kDa tissue-specific adaptor protein), some of which belong to the PI3K pathway, along with RET.[42-44] These types of interactions make RET able to activate and potentiate the PI3K pathway through the p85/p110 subunits. Consequently, the activated PI3K pathway may regulate Ret-dependent and Ret-independent survival signals. A thorough understanding of the signaling pathways mediated by RET activation may have an impact on the management of subsets of PTCs and medullary thyroid carcinomas.

FVPTCs usually respond properly to standard therapy (surgery and radioiodine); however, few patients progress to more aggressive forms or present ab initio with a more aggressive phenotype. Unfortunately, clinical data and long-term follow-up on patients were not available for all selected tumors, and we were unable to perform any correlation studies between genetic findings and clinical outcomes. Although we observed that genetic abnormalities in the MAPK and PI3K pathway genes appeared to be mutually exclusive in FVPTCs, few tumors with RET abnormalities had the coactivation of downstream signals in both pathways. Genetic alterations of RET (eg, *RET-PTC* rearrangements, *RET* mutations) or in general RET activation make this proto-oncogene capable of activating the 2 pathways, inducing the cells to acquire additional alterations that may disable antiapoptotic signal effectors or inhibitory effect on cell growth. Although the activation of both pathways seems to be a rare event in differentiated thyroid carcinomas, this occurrence certainly is more common in differentiated thyroid carcinomas that progress to undifferentiated forms, in anaplastic thyroid carcinomas, and in several thyroid cancer cell lines.

Altogether, these data suggest that a subset of patients with FVPTC certainly may benefit from treatments with MAPK signal inhibitors, whereas selected patients who have tumors with RET-PTC rearrangement or RET activation may benefit from the concomitant use of PI3K-AKT inhibitors. Moreover, because of the interaction and cross-regulation between PPARγ and ERK/MEK and AKT and because of the presence of PPARγ activation in several thyroid carcinomas, further studies are warranted.

Additional and larger studies will be needed to establish the relation between *RET-PTC* rearrangements, RET activation, and mechanisms of interaction through specific PI3K-AKT pathway gene subunits.

CONFLICT OF INTEREST DISCLOSURES

This work was supported in part by the Kenneth D. Müller Professorship, National Cancer Institute Grant U01 DE019765, and National Cancer Institute Specialized Program of Research Excellence (SPORE) grant in head and neck cancer. Research support was received from the Foundation Carrozza-Pollicino and American Thyroid Association (to Dr. Santarpia).

REFERENCES

1. Nikiforova MN, Lynch RA, Biddinger PW, et al. RAS point mutations and PAX8-PPAR gamma rearrangement in thyroid tumors: evidence for distinct molecular pathways in thyroid follicular carcinoma. *J Clin Endocrinol Metab*. 2003; 88:2318-2326.
2. Castro P, Rebocho AP, Soares RJ, et al. PAX8-PPARgamma rearrangement is frequently detected in the follicular variant of papillary thyroid carcinoma. *J Clin Endocrinol Metab*. 2006;91:213-220.
3. Hou P, Liu D, Shan Y, et al. Genetic alterations and their relationship in the phosphatidylinositol 3-kinase/Akt pathway in thyroid cancer. *Clin Cancer Res*. 2007;13:1161-1170.
4. Santarpia L, El-Naggar AK, Cote GJ, Myers JN, Sherman SI. Phosphatidylinositol 3-kinase/akt and ras/raf-mitogen-activated protein kinase pathway mutations in anaplastic thyroid cancer. *J Clin Endocrinol Metab*. 2008;93:278-284.
5. Liu Z, Hou P, Ji M, et al. Highly prevalent genetic alterations in receptor tyrosine kinases and PI3K/Akt and MAP kinase pathways in anaplastic and follicular thyroid cancers. *J Clin Endocrinol Metab*. 2008;93:3106-3116.
6. Bond JA, Wyllie FS, Rowson J, Radulescu A, Wynford-Thomas D. In vitro reconstruction of tumour initiation in a human epithelium. *Oncogene*. 1994;9:281-290.
7. Knauf JA, Ouyang B, Knudsen ES, Fukasawa K, Babcock G, Fagin JA. Oncogenic RAS induces accelerated transition through G2/M and promotes defects in the G2 DNA damage and mitotic spindle checkpoints. *J Biol Chem*. 2006; 281:3800-3809.
8. Lemoine NR, Mayall ES, Wyllie FS, et al. Activated ras oncogenes in human thyroid cancers. *Cancer Res*. 1988;48: 4459-4463.
9. Vasko V, Ferrand M, Di Cristofaro J, Carayon P, Henry JF, de Micco C. Specific pattern of RAS oncogene mutations in follicular thyroid tumors. *J Clin Endocrinol Metab*. 2003;88:2745-2752.
10. Garcia-Rostan G, Zhao H, Camp RL, et al. Ras mutations are associated with aggressive tumor phenotypes and poor prognosis in thyroid cancer. *J Clin Oncol*. 2003;21:3226-3235.
11. Xing M. BRAF mutation in papillary thyroid cancer: pathogenic role, molecular bases, and clinical implications. *Endocr Rev*. 2007;28:742-762.
12. Santarpia L, Sherman SI, Marabotti A, Clayman GL, El-Naggar AK. Detection and molecular characterization of a novel BRAF activated domain mutation in follicular variant of papillary thyroid carcinoma. *Hum Pathol*. 2009;40:827-833.
13. Trovisco V, Vieira de Castro I, Soares P, et al. *BRAF* mutations are associated with some histological types of papillary thyroid carcinoma. *J Pathol*. 2004;202:247-251.
14. Barzon L, Masi G, Merante Boschin I, et al. Characterization of a novel complex BRAF mutation in a follicular variant papillary thyroid carcinoma. *Eur J Endocrinol*. 2008; 159:77-80.
15. Lupi C, Giannini R, Ugolini C, et al. Association of BRAF V600E mutation with poor clinicopathological outcomes in 500 consecutive cases of papillary thyroid carcinoma. *J Clin Endocrinol Metab*. 2007;92:4085-4090.
16. Santoro M, Melillo RM, Fusco A. RET/PTC activation in papillary thyroid carcinoma: European Journal of Endocrinology Prize Lecture. *Eur J Endocrinol*. 2006;155:645-653.
17. Nikiforov YE. Thyroid carcinoma: molecular pathways and therapeutic targets. *Mod Pathol*. 2008;21(suppl 2):S37-S43.
18. Cheung L, Messina M, Gill A, et al. Detection of the PAX8-PPAR gamma fusion oncogene in both follicular thyroid carcinomas and adenomas. *J Clin Endocrinol Metab*. 2003;88:354-357.

19. Sahin M, Allard BL, Yates M, et al. PPARγ staining as a surrogate for PAX8/PPARγ fusion oncogene expression in follicular neoplasms: clinicopathological correlation and histopathological diagnostic value. *J Clin Endocrinol Metab*. 2005;90:463-468.
20. Placzkowski KA, Reddi HV, Grebe SK, Eberhardt NL, McIver B. The role of the PAX8/PPARgamma fusion oncogene in thyroid cancer. *PPAR Res*. 2008;2008:672829-672829.
21. Paes JE, Ringel MD. Dysregulation of the phosphatidylinositol 3-kinase pathway in thyroid neoplasia. *Endocrinol Metab Clin North Am*. 2008;37:375-387, viii-ix.
22. Gustafson S, Zbuk KM, Scacheri C, Eng C. Cowden syndrome. *Semin Oncol*. 2007;34:428-434.
23. Shinohara M, Chung YJ, Saji M, Ringel MD. AKT in thyroid tumorigenesis and progression. *Endocrinology*. 2007; 148:942-947.
24. Wang Y, Hou P, Yu H, et al. High prevalence and mutual exclusivity of genetic alterations in the PI3K/Akt pathway in thyroid tumors. *J Clin Endocrinol Metab*. 2007;92:2387-2390.
25. Abubaker J, Jehan Z, Bavi P, et al. Clinicopathological analysis of papillary thyroid cancer with PIK3CA alterations in a Middle Eastern population. *J Clin Endocrinol Metab*. 2008;93:611-618.
26. Garcia-Rostan G, Costa AM, Pereira-Castro I, et al. Mutation of the PIK3CA gene in anaplastic thyroid cancer. *Cancer Res*. 2005;65:10199-10207.
27. Ricarte-Filho JC, Ryder M, Chitale DA, et al. Mutational profile of advanced primary and metastatic radioactive iodine-refractory thyroid cancers reveals distinct pathogenetic roles for BRAF, PIK3CA, and AKT1. *Cancer Res*. 2009;69: 4885-4893.
28. Li Volsi, Albores-Saavedra J, Asa SL, et al. Papillary carcinoma. In: DeLellis LA, Lloyd RV, Heitz PU, Eng C, eds. Pathology and Genetics of Tumors of the Endocrine Organs. World Health Organization Classification of Tumors. Lyon, France: IARC Press; 2004:57-72.
29. Nikiforova MN, Biddinger PW, Caudill CM, Kroll TG, Nikiforov YE. PAX8-PPARgamma rearrangement in thyroid tumors: RT-PCR and immunohistochemical analyses. *Am J Surg Pathol*. 2002;26:1016-1023.
30. Nakazawa T, Kondo T, Kobayashi Y, et al. RET gene rearrangements (RET/PTC1 and RET/PTC3) in papillary thyroid carcinomas from an iodine-rich country (Japan). *Cancer*. 2005;104:943-951.
31. Kim MS, Jeong EG, Yoo NJ, Lee SH. Mutational analysis of oncogenic AKT E17K mutation in common solid cancers and acute leukaemias. *Br J Cancer*. 2008;98:1533-1535.
32. Downward J. Targeting RAS signalling pathways in cancer therapy. *Nat Rev Cancer*. 2003;3:11-22.
33. Di Cristofaro J, Marcy M, Vasko V, et al. Molecular genetic study comparing follicular variant versus classic papillary thyroid carcinomas: association of N-ras mutation in codon 61 with follicular variant. *Hum Pathol*. 2006;37:824-830.
34. Volante M, Rapa I, Gandhi M, et al. RAS mutations are the predominant molecular alteration in poorly differentiated thyroid carcinomas and bear prognostic impact. *J Clin Endocrinol Metab*. 2009;94:4735-4741.
35. Oler G, Cerutti JM. High prevalence of BRAF mutation in a Brazilian cohort of patients with sporadic papillary thyroid carcinomas: correlation with more aggressive phenotype and decreased expression of iodide-metabolizing genes. *Cancer*. 2009;115:972-980.
36. Melillo RM, Castellone MD, Guarino V, et al. The RET/PTC-RAS-BRAF linear signaling cascade mediates the motile and mitogenic phenotype of thyroid cancer cells. *J Clin Invest*. 2005;115:1068-1081.
37. Hennige AM, Lammers R, Arlt D, et al. Ret oncogene signal transduction via a IRS-2/PI 3-kinase/PKB and a SHC/Grb-2 dependent pathway: possible implication for transforming activity in NIH3T3 cells. *Mol Cell Endocrinol*. 2000;167:69-76.
38. Segouffin-Cariou C, Billaud M. Transforming ability of MEN2A-RET requires activation of the phosphatidylinositol 3-kinase/AKT signaling pathway. *J Biol Chem*. 2000;275: 3568-3576.
39. Miyagi E, Braga-Basaria M, Hardy E, et al. Chronic expression of RET/PTC 3 enhances basal and insulin-stimulated PI3 kinase/AKT signaling and increases IRS-2 expression in FRTL-5 thyroid cells. *Mol Carcinog*. 2004;41:98-107.
40. van Weering DH, de Rooij J, Marte B, Downward J, Bos JL, Burgering BM. Protein kinase B activation and lamellipodium formation are independent phosphoinositide 3-kinase-mediated events differentially regulated by endogenous Ras. *Mol Cell Biol*. 1998;18:1802-1811.
41. De Vita G, Melillo RM, Carlomagno F, et al. Tyrosine 1062 of RET-MEN2A mediates activation of Akt (protein kinase B) and mitogen-activated protein kinase pathways leading to PC12 cell survival. *Cancer Res*. 2000;60:3727-3731.
42. Pelicci G, Troglio F, Bodini A, et al. The neuron-specific Rai (ShcC) adaptor protein inhibits apoptosis by coupling Ret to the phosphatidylinositol 3-kinase/Akt signaling pathway. *Mol Cell Biol*. 2002;22:7351-7363.
43. De Falco V, Guarino V, Malorni L, et al. RAI(ShcC/N-Shc)-dependent recruitment of GAB 1 to RET oncoproteins potentiates PI3-K signalling in thyroid tumors. *Oncogene*. 2005;24:6303-6313.
44. Lodyga M, De Falco V, Bai XH, et al. XB130, a tissue-specific adaptor protein that couples the RET/PTC oncogenic kinase to PI 3-kinase pathway. *Oncogene*. 2009;28:937-949.