

# Overexpression and Overactivation of Akt in Thyroid Carcinoma<sup>1</sup>

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## ABSTRACT

Enhanced activation of Akt occurs in Cowden's disease, an inherited syndrome of follicular thyroid, breast, colon, and skin tumors, via inactivation of its regulatory protein, PTEN. Whereas PTEN inactivation is uncommon in sporadic thyroid cancer, activation of growth factor pathways that signal through Akt is frequently identified. We hypothesized that Akt overactivation could be a common finding in sporadic thyroid cancer and might be important in thyroid cancer biology. We examined thyroid cancer cell lines and benign and malignant thyroid tissue for total Akt activation and isoform-specific Akt expression. In thyroid cancer cells, Akt 1, 2, and 3 proteins were expressed, total Akt was activated by insulin phosphatidylinositol 3'-kinase, and inhibition of phosphatidylinositol 3'-kinase reduced cell viability. In human thyroid tissue, increased levels of phosphorylated total Akt were identified in follicular but not papillary cancers compared with normal tissue. Levels of Akt 1 and 2 proteins and Akt 2 RNA were elevated only in the follicular cancers. In paired samples, Akt 1, 2, 3, and phospho-Akt levels were higher in five of six cancers, including three of three follicular cancers. These data suggest that Akt activation may play a role in the pathogenesis or progression of sporadic thyroid cancer.

## INTRODUCTION

Thyroid cancer accounts for ~1% of all of the malignancies in the United States (1). Although most patients have an excellent prognosis, ~1200 patients develop progressive metastatic disease and die from thyroid cancer yearly. Typically, these progressive cancers either lose or have reduced responsiveness to radioactive iodine and demonstrate TSH<sup>3</sup>-independent growth, rendering them resistant to standard therapeutic approaches (2). Because no effective alternative therapies for thyroid cancer have been identified, there is a critical need to develop novel approaches to treat thyroid cancer.

Activation of tyrosine kinase receptors and/or their downstream signaling molecules are frequently identified in thyroid cancer. Specifically, mutations and gene rearrangements involving Ret (3–7); overexpression of c Met; (8); overexpression of fibroblast growth factor (9), insulin (10), and insulin-like growth factor-I receptors (11, 12); and mutations in p21<sup>ras</sup> (13) occur commonly in thyroid cancer. Moreover, both thyroid-specific and generalized overexpression of *Ret/PTC* oncogenes cause thyroid cancer *in vivo* (14, 15). The downstream signaling pathways activated by these oncogenes include those mediated by mitogen-activated protein kinase and PI3k (16–22). However, the specific pathways responsible for the transforming,

growth promoting, and antiapoptotic properties of these oncogenes in thyroid cells have not been fully characterized.

Recently, the genetic basis for Cowden's syndrome, an autosomal dominant disorder characterized by breast cancer, colon cancer, benign and malignant follicular thyroid tumors, and skin tumors, was identified as inactivation of the tumor suppressor, PTEN (23, 24). PTEN is a dual function lipid phosphatase that negatively regulates PI3k signaling (25–28). Thus, tumors with inactivation of PTEN have enhanced PI3k signaling, resulting in constitutive activation of the serine threonine kinase, Akt (29–32). Activated Akt phosphorylates downstream effectors that inhibit apoptosis, induce cell growth, enhance glucose uptake and utilization, and increase protein synthesis.

Although Cowden's syndrome suggests a unique association between PTEN and thyroid cancer, PTEN inactivation is reported to be uncommon in sporadic thyroid cancer (23, 33). However, overactivation of Akt by activated tyrosine kinase receptors and p21<sup>ras</sup> may be central to the transforming capabilities of many thyroid oncogenes. Therefore, Akt may be a common central regulator of thyroid oncogene function.

In benign thyroid cells, Akt appears to have both growth activating and apoptotic inhibitory effects (34–36). Akt activation appears to be the principle mediator of growth factor activation of thyroid cell growth and inhibition of apoptosis. Overexpression of a constitutively activated form of Akt 1 (myristoylated Akt) in thyroid cells results in serum-independent growth, sensitizes them to the effects of TSH, and leads to resistance to cell death, although it appears to be insufficient to transform the thyroid cells (35, 36). These data imply that additional genetic "hits" or activation of additional signaling pathways may be required to develop thyroid cancer. However, a key consequence of Akt activation may also be that it represents an important pathway for the progressive growth of thyroid cancer cells in the absence of TSH, thus accounting for tumor progression during TSH-suppressive therapy. Finally, an alternative explanation for the lack of transformation *in vivo* is that other forms of Akt (*i.e.*, Akt 2 or 3) are activated and may have unique transforming effects on thyroid cells.

Three isoforms of Akt have been cloned, and although there are no certain differences in the function and activation of the isoforms (37–40), unique tissue expression patterns have been described. In particular, overexpression of Akt 2 has been described in ovarian and pancreatic cancers (37, 41–44), whereas Akt 3 is overexpressed in poorly differentiated breast and prostate cancers (39, 45). Akt 1 appears to be more widely expressed and is clearly involved in growth factor signaling in a wide variety of other tissues but has not been shown to be overexpressed in malignancies.

In the present study, our goals were to determine whether Akt signaling is enhanced in human thyroid cancer and to identify the expression patterns of Akt isoforms in benign and malignant thyroid tissue. In human thyroid cancer cell lines, we demonstrated that Akt is activated by insulin and is important for cell survival. In human thyroid tissue, we showed that Akt 2 mRNA and Akt 1 and 2 protein levels are increased in differentiated thyroid cancer (particularly follicular thyroid cancer) compared with normal thyroid tissue and that levels of phosphorylated Akt (activated Akt) are increased in thyroid cancer. These data suggest that overexpression and overactivation of

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<sup>3</sup> The abbreviations used are: TSH, thyrotropin; PI3k, phosphatidylinositol 3'-kinase; Akt, protein kinase B; WM, wortmannin; MTT, 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide; C<sub>T</sub>, Threshold Cycle; PBS-T, 0.190Tween 20 in PBS.

Akt proteins are common in sporadic thyroid cancer and that Akt 2, in particular, may be important in the pathogenesis and/or progression of thyroid cancer.

## SUBJECTS AND METHODS

**Study Subjects and Samples.** Thyroid tissue samples were obtained at the time of surgery for known thyroid cancer based on preceding fine needle aspiration of a solitary or dominant thyroid nodule from 24 patients with thyroid cancer (11 with follicular and 13 with papillary cancer). Normal thyroid samples were obtained from 19 additional patients from whom malignant tissue was not obtained. Adjacent normal tissue was obtained from 10 of the patients with tumor tissue samples (paired samples). Tissue samples were snap frozen in liquid nitrogen. Frozen sections were prepared from each sample and reviewed with a single pathologist to confirm the histological diagnosis from the sample before analysis. In all of the cases,  $\geq 90\%$  of cells within the section were thyrocytes.

Total RNA was prepared from all of the samples. Protein was prepared from eight follicular, nine papillary, and 11 normal tissue samples, including 6 paired samples. This protocol was approved by the Institutional Review Board at The Washington Hospital Center and by the Department of Clinical Investigation at Walter Reed Army Medical Center.

**Cell Culture.** Three human thyroid cancer cell lines, ARO, WRO, and NPA (provided by Dr. R Julliard, UCLA, Los Angeles, CA) were propagated in DMEM in 10% FCS. Once the cells achieved 70% confluence, the medium was removed, the cells were washed with PBS, and the medium was changed to include no serum for 24 h. After 24 h, cells medium was aspirated, and the cells were placed in new DMEM containing 10  $\mu\text{g/ml}$  insulin,  $1 \times 10^{-10}$  M TSH, or 5% FCS, with or without 100 nM WM (Sigma Chemical Co., St. Louis, MO), an inhibitor of PI3k. Protein and RNA were isolated and analyzed as noted below after 1 and 24 h of incubation. Human malignant melanoma cell line Mel 28 (American Type Culture Collection, Manassas, VA) was grown in DMEM in 10% FCS. On reaching confluence, total RNA and protein were isolated as noted below. The RNA and protein from MEL 28 cells were used as positive controls for isoform-specific analysis of Akt mRNA and protein levels, because they are known to express all three Akt isoforms (data not shown).

**Cell Viability Assay.** Cells were plated in 24-well culture plates in an initial concentration of  $5 \times 10^2$ /well in culture medium to allow the cells to adhere and grow in the cell culture environment. Experiments were performed when cells reached 50% confluence. They were then exposed to increasing concentrations of WM from 4 through 72 h. Cell viability was measured by MTT assay as described previously (46). Briefly, 50  $\mu\text{l}$  of 5 mg/ml MTT was added to the culture medium and cultured for 2 h. After MTT incubation, the supernatant was discarded, and 200  $\mu\text{l}$  of stop solution (90% isopropyl and 10% HCl) was added to each well. A 100- $\mu\text{l}$  sample of each well was transferred to 96-well plate for  $A_{450\text{ nm}}$  (Revelation 4.02; DYNEX Technology, Chantilly, VA).

**RNA Extraction.** Frozen tissues ( $\sim 200$  mg) were homogenized with a Polytron PT-10 (Brinkmann Instruments, Westbury, CT) in TRIzol LS reagent (Life Technologies, Inc., Gaithersburg, MD) for RNA isolation. Total RNA was isolated as per the manufacturer's recommendations. The extraction yields were quantified spectrophotometrically, and quality was assessed by determining the absorbance ratio at 260 and 280  $\text{\AA}$ .

**Determination of Akt Isoform mRNA Levels Using Real-Time PCR.** Intron-spanning Akt isoform-specific oligonucleotide primers and internal Taqman probes were designed using Primer Express (Applied Biosystems, Burlingame, CA). Sequences are shown below and were confirmed to be sequence-specific by BLAST search. All of the Akt probes were fluorescently labeled with 6-carboxyfluorescein.

Akt 1 Primers: S (5'CAAGCCCAAGCACCGC3');  
AS (5'GGATCACCTTGCCGAAAGTG3')  
Probe: (5'AGTTTGGAGTACCTGAAGCTGCTGGGCAA3')  
Akt 2 Primers: S (5'GCAAGGCACGGGCTAAAG3');  
AS (5'CCCAGCACCAGGATGACTT3')  
Probe: (5'ATGAATGACTTCGACTATCTCAAACCTCTTGGC3')  
Akt 3 Primers: S (5'GAAGAGGAGAGAATGAATTGTAGTCCA3');  
AS: (5'AGTAGTTTCAAATAGTCAAATCATTTCATTG3');

Probe: (5'TTCACAAATTGATAATATAGGAGAGGAAGAGATGGATGC3')

Total RNA (700 ng) for each sample was reverse transcribed in a 35- $\mu\text{l}$  reaction using 0.75 units/ $\mu\text{l}$  Moloney murine leukemia virus reverse transcriptase and reverse transcriptase buffer containing 5.5 mM  $\text{MgCl}_2$ , 500  $\mu\text{M}$  each deoxynucleotide triphosphates, 2.5  $\mu\text{M}$  random hexamers, 0.4 units/ $\mu\text{l}$  RNase inhibitor, and RNase-free  $\text{H}_2\text{O}$ .

Quantitative PCR was performed in 96 sample plates separately for each Akt isoform. cDNA equivalent of 100 ng of total RNA (5  $\mu\text{l}$  of room-temperature reaction mixture)/25  $\mu\text{l}$  tube containing TaqMan PCR Universal Master Mix (Applied Biosystems), 100 nM of Akt probe, and 200 nM of each Akt primer were used. As a control for RNA integrity and for assay normalization, 18S rRNA was amplified using TaqMan rRNA control reagents kit (Applied Biosystems). Initial template, primer, and probe dilution studies were performed to determine the optimal template amount and component concentrations to preserve assay sensitivity but also to allow for  $< 5\%$  difference in the slopes of the linear portion of the PCR amplification curve between the Akt isoforms and 18S. On the basis of these experiments, cDNA equivalent of 0.25 ng total RNA was used as a template per 25- $\mu\text{l}$  well containing 40 nM of 18S probe and 20 nM of 18S primers in TaqMan PCR Universal Master Mix (Applied Biosystems).

PCR was performed in the following manner: after an initial 10 min denaturation at 95°C, samples were subjected to 40 cycles of a two-step amplification protocol that included 15 s of denaturation at 95°C and a 1-min annealing-elongation step using the standard protocol of the manufacturer. Akt 1, 2, 3, and 18S were amplified from all tumor samples in duplicate in three separate reactions. Interassay variability was  $< 5\%$ . Negative controls are included for the entire reverse transcription-PCR and for the PCR alone in each reaction.

Normalized results for Akt 1, 2, and 3 are calculated using the mean threshold cycle ( $C_T$  Akt) of all reactions for each sample and the mean threshold cycle of 18S ( $C_T$  18S) amplification for each sample by calculating  $2^{-(C_T \text{ Akt} - C_T \text{ 18S})}$  as recommended by the manufacturer (Applied Biosystems). Several samples were run on electrophoreses through agarose gels, and all showed a single unique band at the expected size for each amplicon.

**Protein Extraction.** For cell culture, cells were propagated in 10-cm<sup>2</sup> dishes after rinsing twice with 1 ml of ice-cold PBS, 0.5 ml of ice-cold cell lysis buffer [20 mM TRIS-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium  $\text{PP}_i$ , 1 mM  $\beta$ -glycerophosphate, 1 mM  $\text{Na}_3\text{VO}_4$  (Sigma Chemical Co.), 1% Triton X-100 (Sigma Chemical Co.), 20  $\mu\text{M}$  phenylmethylsulfonyl fluoride (Roche Molecular Biochemicals, Indianapolis, IN), and 0.3  $\mu\text{M}$  Okadaic acid (Sigma Chemical Co.)] was added, and the dish was incubated on ice for 10 min. The cells were scraped and centrifuged at 12,000  $\times$  g for 10 min at 4°C. The supernatant was saved and stored at  $-80^\circ\text{C}$ .

For fresh human tissue,  $\sim 500$  mg of frozen thyroid tissue was homogenized in 500  $\mu\text{l}$  of lysis buffer (see above) and centrifuged at 12,000  $\times$  g for 15 min at 4°C. The supernatant was saved and stored at  $-80^\circ\text{C}$ . Protein was quantified using bicinchoninic acid kit (Pierce, Rockford, IL).

**SDS-PAGE and Autoradiography.** Anti-Akt 1, 2, and 3 (Upstate Biotechnologies, Lake Placid, NY), anti-phospho Akt (ser473; Cell Signaling Technology, Beverly, MA), and anti- $\alpha$  tubulin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies were used as primary antibodies. Twenty  $\mu\text{g}$  of total protein lysate were suspended in reduced SDS sample buffer after boiling for 5 min. Protein lysates were subjected to SDS-PAGE (7%), and the separated proteins were transferred to polyvinylidene difluoride or nitrocellulose membranes (0.2- $\mu\text{m}$  pore size; Invitrogen, Carlsbad, CA) by electrophoretic blotting (Invitrogen). Nonspecific binding was prevented by blocking the membrane with PBS-T (0.1% Tween 20 in PBS) containing 10% nonfat dry milk overnight at 4°C. Immunoblotting was performed in the following manner. In brief, membranes were washed 4 times (10 min/wash) with PBS-T, incubated with the primary antibody in the same buffer for 2 h at room temperature and washed 4 times (15 min/wash). Membranes were then incubated with the secondary antibody conjugated with peroxidase in PBS-T containing 5% nonfat dry milk for 1 h at room temperature. After washing with PBS-T 4 times (15 min/wash), immunodetection was performed by using SuperSignal West Pico staining kit (Pierce). On each gel, proteins isolated from MEL 28 melanoma cells were run as positive controls because they express all three isoforms of Akt.

Relative quantitation of proteins was determined in the following manner:

autoradiographs were prepared at exposure times ranging from 15 s to 5 min. Films were scanned, and the density of bands of the appropriate size for the targets were measured using Imagegauge software (Fuji Photo Film Co., LTD, Tokyo, Japan). Background measures were obtained from each lane and subtracted from the measured band. A linear range of density *versus* time was obtained for each blot. Interblot differences were calculated to be <10% during this linear phase by comparing the densities of the control blot MEL-28 cells included on each blot. Immunoblots of the same protein samples were performed for  $\alpha$  tubulin for normalization. Quantitation was performed using blots that fell within the linear portion of the curve and was calculated as the density of Akt isoform or phospho Akt divided by the density of  $\alpha$  tubulin.

**Statistical Analysis.** To examine WM effects on ARO cell viability, ANOVA analysis was initially performed using Stat View Program (Abacus Concept, Inc., Berkeley, CA). When the ANOVA exhibited significance, each group was compared using a post-hoc Fisher PDSL test. To compare protein and gene expression in thyroid tissues, Wilcoxon's rank-sum test was performed. In both cases,  $P < 0.05$  was considered statistically significant.

**RESULTS**

**Akt Is Activated by PI3K in Human Thyroid Cancer Cells.** We tested two human thyroid cancer cell lines, NPA (papillary cancer) and WRO (follicular cancer), that express the TSH receptor (47, 48) for total Akt expression and activation using nonisoform-specific anti-Akt and phospho-Akt antibodies, respectively. Cells were serum-starved for 24 h before stimulation with TSH ( $10^{-10}$  M) or insulin (10  $\mu$ g/ml) in the presence or absence of 100 nM of WM, an inhibitor of PI3k. Similar to nonmalignant rat thyroid cells (34–36), insulin stimulated total Akt phosphorylation (Fig. 1A) in a PI3k-sensitive manner. TSH did not induce Akt phosphorylation. Phosphorylation (activation) of total Akt was not detected in the absence of serum, and levels of PTEN expression were similar in the cells (data not shown), making constitutive activation of Akt unlikely in these cells. Serum

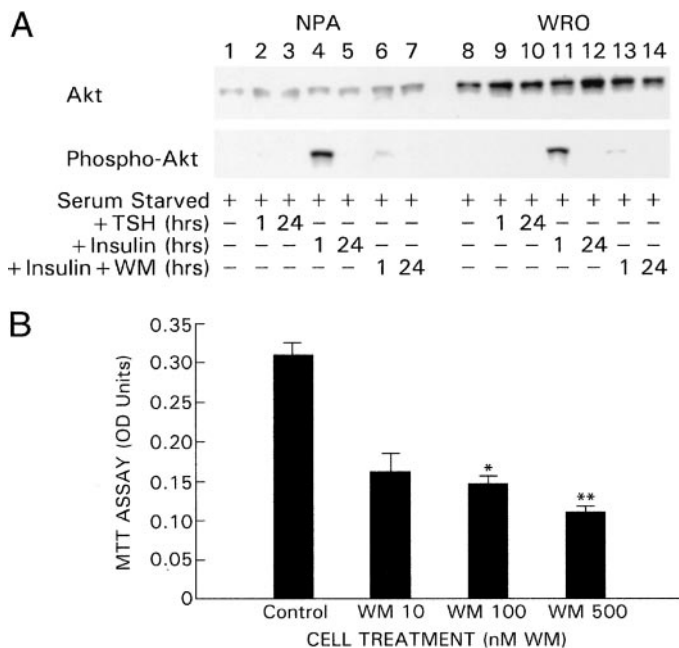


Fig. 1. Akt activation in WRO and NPA thyroid cancer cells. A, cells were cultured in the absence of serum and then stimulated with 10  $\mu$ g/ml insulin,  $1 \times 10^{-10}$  M TSH, or both, with or without WM. After stimulation, lysates were prepared and immunoblotted using nonisoform-specific anti-Akt and anti-phospho Akt (Ser473) antibodies. No basal or TSH-stimulated Akt activity was detected. Insulin activated Akt after 1 h of incubation. This effect is lost after 24 h and is inhibited by WM. B, inhibition of PI3k reduced cell viability of anaplastic thyroid cancer cells. ARO cancer cells were grown continuously in medium supplemented with 5% FCS. Cells were then incubated with WM for 48 h at the noted concentrations. Cells were washed, and cell viability was assessed by MTT assay. WM reduced ARO cell viability in a concentration-dependent manner.

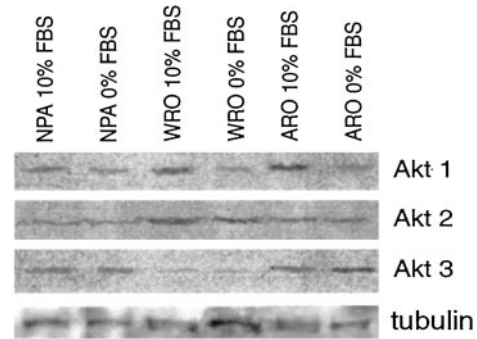


Fig. 2. Expression pattern of Akt isoforms in human thyroid cancer cell lines. Whole cell lysates were isolated from ARO, NPA, and WRO thyroid cancer cells grown in the absence or presence of serum for 24 h. Protein (20  $\mu$ g) was electrophoresed by SDS-PAGE and transferred to PVDF membranes and analyzed using polyclonal anti-Akt 1, 2, and 3 antibodies. Only levels of Akt 1 protein were increased by serum, Akt 2 levels were highest, and Akt 3 levels were lowest in the follicular cancer cell line (WRO).

also activated Akt in the NPA, WRO, and ARO (anaplastic thyroid cancer cell line) cells in a PI3k-dependent manner (data not shown).

**Inhibition of PI3k Signaling Reduces Thyroid Cancer Cell Viability.** To test the functional role of PI3k signaling in thyroid cancer cells, we measured the effect of PI3k inhibition on thyroid cancer cells grown in serum. Thyroid cancer cells were incubated with increasing concentrations of the PI3k inhibitor WM for 48 h, washed, and cell viability was assessed by MTT assay (46). WM reduced the viability of ARO cells grown in serum (Fig. 1B). Similar results were obtained for the WRO and NPA cell lines (data not shown). There was no effect of WM in the absence of serum (data not shown) consistent with the serum dependence of PI3k-dependent Akt activation in these cells (Fig. 1A).

**Isoforms of Akt Are Differentially Expressed in Human Thyroid Cancer Cells.** Isoform-specific overexpression of Akt 1, 2, and 3 has been described in nonthyroid malignancies (see "Introduction") but had not been assessed in thyroid cancer. We initially measured the expression of the Akt isoforms in WRO, NPA, and ARO human thyroid cancer cells lines using isoform-specific antibodies. Akt 1 and 2 proteins are expressed in all three cell lines on Western blot of whole cell lysates, but only Akt 1 expression is influenced by serum at 24 h. Levels of Akt 3 were much higher in the NPA and ARO cells than in the WRO cells (Fig. 2).

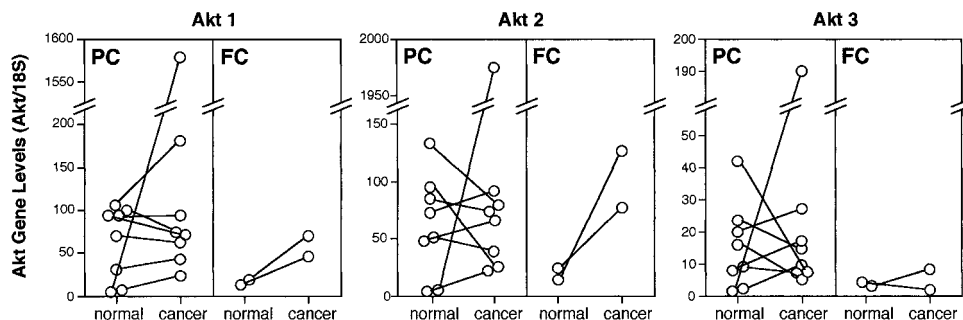
**Akt Is Overexpressed and Overactivated in Thyroid Cancer.** After demonstrating Akt expression and activation in thyroid cancer cell lines, we determined levels of Akt isoform mRNA from human thyroid tissue samples using real-time quantitative PCR. Histologically confirmed papillary ( $n = 13$ ) and follicular ( $n = 11$ ) thyroid cancers, as well as normal thyroid tissue ( $n = 19$ ), including all cancer cases used for protein analysis (see below), were analyzed. For 10 cases (8 papillary and 2 follicular cancers), RNA isolated from paired normal and malignant tissue was available.

RNA from NPA thyroid cancer cells was used to create standard curves for quantitative analysis of Akt 1, 2, and 3 and 18S expression (interassay variability <5%). cDNA was prepared from each reverse transcriptase reaction to allow for duplicate experiments and for quantitative amplification of 18S. Results are, therefore, quantified as units of mRNA/18S RNA. Standard curves and samples were run in duplicate in the same reactions on at least three separate occasions, and values are the mean quantities from those experiments.

Overall, Akt mRNA levels were variable in cancer and normal samples (data not shown). Statistically, only Akt 2 was increased in cancers compared with normal ( $P < 0.05$ ). In paired samples (Fig. 3), Akt 2 was increased in both follicular cancers but only in four of eight papillary cancers. Akt 1 and 3 were slightly elevated in follicular



Fig. 3. Akt RNA levels in paired samples. Akt RNA levels were determined by real-time reverse transcription-PCR, normalized for interassay variance and also by 18S quantitation. Paired data from eight papillary cancers (PC) and two follicular cancers (FC) are shown. Akt 1, 2, and 3 are all increased in follicular cancers compared with normal, particularly Akt 2. Papillary cancers show more variable results. The one case with very high levels corresponds to the aggressive tumor with very high levels of Akt proteins.



cancers (Fig. 3). For samples from which protein and mRNA were analyzed ( $n = 27$ ), a statistical correlation was identified for only Akt 2 ( $P = 0.02$ ).

We next assessed levels of Akt isoform protein expression in benign and malignant thyroid cancer tissue in eight follicular cancer, nine papillary cancer, and 11 normal tissue samples. For Western blot analysis, paired normal and cancer tissue from the same patient were available from three papillary and three follicular cancers. Positive and negative control experiments with NIH 3T3 cell membrane proteins treated acutely with platelet-derived growth factor in the presence or absence of WM and experiments performed in the presence or absence of phosphatase inhibitors during protein preparation confirmed the specificity of the phospho-Akt antibody (data not shown).

To semiquantify and normalize levels of Akt protein and phospho-Akt levels attributable to possible higher amounts of noncellular colloid protein being present in the normal tissue samples,  $\alpha$  tubulin was used as an internal cellular protein control (49). Ratios of normalized Akt isoform (or phospho-Akt): $\alpha$  tubulin were obtained within the linear range for each sample.

Relative amounts of Akt 1, 2, and phosphorylated Akt were higher in follicular cancer than normal tissue [ $P < 0.01$ , 0.005, and 0.05, (Figs. 4 and 5)]. For papillary cancers, Akt isoforms were not statistically elevated ( $P = 0.08$  for Akt 1 and 2), but there was significant variability in the results, with particularly high levels of all three isoforms in one locally aggressive follicular variant of papillary carcinoma. Because of differences in levels of Akt among normal tissue samples, paired samples were analyzed in six cases. In these samples, normalized levels of phospho-Akt were significantly higher ( $P < 0.05$ ) in malignant compared with normal tissue, despite the small number of cases. Levels were higher in all three follicular cancers and in two of three papillary cancers compared with normal tissue (Figs. 4 and 5). Paired follicular cancers had a mean increase of 80%, whereas papillary cancers had a 30% increase in phospho-Akt.

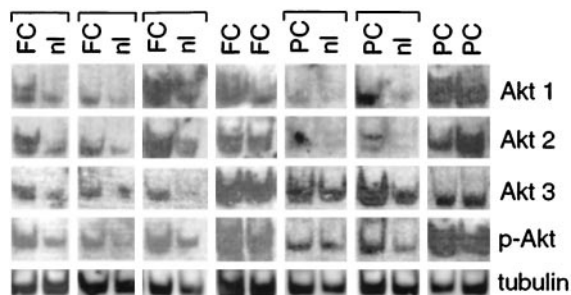


Fig. 4. Representative Western blots of Akt 1, 2, 3, and phospho-Akt in thyroid samples (PC, papillary cancer; FC, follicular cancer; nl, normal thyroid tissue). Whole cell lysates were isolated in the presence of phosphatase inhibitors okadaic acid and  $\text{Na}_3\text{VO}_4$ . Protein (20  $\mu\text{g}$ ) was electrophoresed by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed using polyclonal anti-Akt 1, 2, and 3 antibodies, and anti-phospho Ser 473 Akt antibodies.

Akt isoform expression was higher for all three isoforms in all of the follicular cancers and in two of three papillary cancers, including one patient whose tumor was associated with a very aggressive clinical course.

DISCUSSION

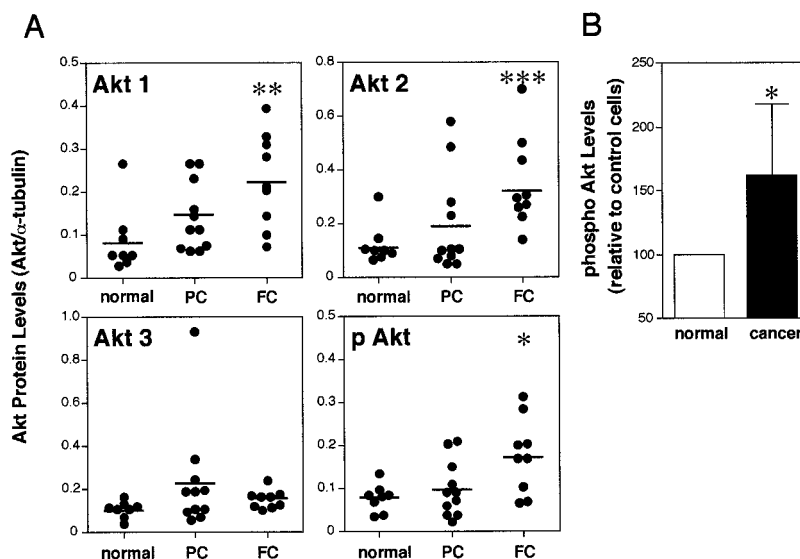
Akt is a critical mediator of growth factor-activated PI3k signaling that appears central to the regulation of benign thyroid cell growth and survival (16, 34–36). An important pathogenic role for Akt in thyroid tumorigenesis is suggested by the high frequency of follicular adenomas and carcinomas in Cowden’s syndrome in which Akt is activated by loss of PTEN activity (24, 25, 50). However, PTEN inactivation appears to occur rarely in sporadic thyroid cancers (23, 33). Akt activation is also known to play a role in cell signaling for a wide variety of growth factors, many of which are activated in sporadic thyroid cancers by a variety of mechanisms (8, 16, 18, 22, 36, 51–54). Thus, we hypothesized that activation of Akt, whether mediated by PTEN inactivation or growth factor signaling activation, might represent an important common pathway in the pathogenesis or progression of sporadic thyroid carcinoma. The goal of the present study was to assess the level of activation of Akt in sporadic thyroid cancers and to determine the expression patterns of Akt isoforms in these tumors.

We first demonstrated that insulin was able to activate Akt in the three poorly differentiated thyroid cancer cell lines. These lines derive from papillary (NPA), follicular (WRO), and anaplastic (ARO) thyroid cancers that contain mutations in p53 (48). In these thyroid cancer cell lines, the time course of the Akt phosphorylation was similar to serum-induced activation of Akt in benign thyroid cell models (35, 36).

To begin to assess the functional relevance of this pathway in thyroid cancer, we measured the effect of pharmacological inhibition of PI3k in these cells. Inhibition of PI3k signaling resulted in reduced cancer cell viability (Fig. 1B) at similar concentrations of WM to those reported to inhibit the growth of nonmalignant thyroid cells (16, 34–36). These data suggest that, in contrast to TSH signaling, PI3k/Akt signaling remains important in even poorly differentiated thyroid cancer cells, suggesting it may be a potential target for antitumor therapy.

The expression of Akt proteins was also assessed in these cells. Levels of the three isoforms varied among the samples. Interestingly, whereas levels of Akt 1 protein appeared to be influenced by serum after 24 h of exposure, levels of Akt 2 and 3 were the same as baseline. This suggests unique regulation of expression of the Akt isoforms in these cell lines, although more formal time course experiments are needed to better characterize these potential differences. Because Akt 2 has been the predominant isoform shown to be overexpressed in a variety of other cancers (37, 41, 42, 44, 55) and may have unique transforming capabilities (42, 43), we focused on isoform-specific expression in the actual thyroid cancer tissue samples.

Fig. 5. Relative expression of Akt isoforms and phospho-Akt. Western blots were performed to detect all Akt isoforms, phospho-Akt, and  $\alpha$  tubulin. Semiquantitative, normalized values (Akt/ $\alpha$  tubulin) were calculated as described in the text and were used to compare levels of Akt expression and activation between normal and malignant tissue samples. A, Akt 1 and 2, and phospho-Akt levels were elevated overall for follicular cancers compared with normal tissue. A similar trend was noted for papillary cancers ( $P = 0.08, 0.08,$  and  $0.06,$  respectively). B, because of variation in amounts of Akt protein detected between individual samples, we evaluated paired normal and malignant thyroid samples from the same patient. Levels of Akt 1, 2, and 3, and phospho-Akt were increased in three of three follicular cancers, whereas levels of phospho-Akt and Akt 2 were increased in two of three papillary cancers (data not shown). Overall, in the paired samples, the thyroid cancers had higher levels of phospho-Akt than normal thyroid tissue ( $P < 0.05,$  panel B).



To determine whether there is isoform specificity of Akt expression in thyroid cancer, we quantitatively assessed levels of Akt 1, 2, and 3 mRNA and protein in benign and malignant thyroid tissue. Levels of Akt 1 and 2 protein and Akt 2 RNA were higher in follicular cancer compared with normal tissue. Similar differences were identified in only a subset of papillary cancers. Levels of expression were variable among samples, even in the normal tissues; therefore, paired samples were separately analyzed. Higher levels of Akt mRNA and protein were identified in all of the follicular and most papillary cancers compared with paired normal tissue. The difference was seen for all isoforms but was most striking for Akt 2.

Normalization of the Western blot analysis was performed using  $\alpha$  tubulin to avoid introducing a potential bias attributable to possible increased cellularity of the tumor *versus* normal tissue, in which a larger amount of noncellular thyroglobulin protein from colloid might be present. Interblot variation was minimal, as assessed by comparison of expression of a control cell line run on each Western blot.

Levels of total Akt activation were measured using a nonisoform-specific antibody directed against phosphorylated (Ser 473) Akt. Increased levels of phosphorylated Akt were higher in cancer compared with normal tissue, but this was most impressive for follicular carcinomas (Figs. 4 and 5), the subtype of thyroid cancer most frequently found in patients with Cowden's syndrome. The mechanism for this activation has not yet been determined but could include overexpression or mutations of Akt, alterations in kinases or phosphatases that regulate its action, PTEN inactivation, mutations in Ras, and mutations and/or overexpression of a variety of tyrosine kinase receptors.

These data suggest an association between Akt overactivation and thyroid cancer, particularly for follicular cancers. Although the number of samples is small, and larger studies are planned, the association with follicular carcinoma is consistent with the phenotype of Cowden's syndrome in which follicular rather than papillary carcinomas are frequently identified (24, 27, 56). The results are still somewhat surprising because of the relative rarity of mutations of tyrosine kinase receptors in follicular cancer compared with papillary cancer (4, 7, 8, 12, 14, 57). In many series, however, activating mutations of Ras, another upstream regulator of PI3k and Akt, are commonly demonstrated in follicular thyroid cancers (13, 54). These mutations or other unknown cell signaling alterations could explain the increased activation of Akt in the follicular cancers analyzed in the present study. Additional studies correlating the phosphorylation of downstream

effectors of Akt with the present results are ongoing; however, it is important to recognize that Akt effectors may also be regulated by other pathways.

The relative overexpression of Akt 2 mRNA and protein in follicular carcinomas is also of interest. These data are similar to those reported previously for ovarian, breast (37, 41, 58), and pancreatic carcinomas (42, 44, 59), in which Akt 2 appears to be the predominant isoform in the malignant tissue, related in some cases to gene amplification (37, 42, 44, 59). However, the increase of Akt proteins in the present series of thyroid cancer samples appears to be more impressive and consistent than the increased levels of Akt RNA, suggesting a more important role for post-transcriptional and post-translational alterations. Our data also suggest that the Akt 2 isoform is frequently activated by the presence of a second, slower migrating band, of which the presence is dependent on phosphatase inhibition (data not shown); however, Akt 2 kinase activity assays will be required to confirm this result. The published data suggesting that the myristoylated Akt 1 is not sufficient for malignant transformation of thyroid cells (35, 36) may need to be reexamined with an activated form of Akt 2 if this isoform is principally activated in thyroid carcinomas.

We were unable to evaluate benign follicular adenomas for Akt protein expression or activation in the present study because of difficulty obtaining fresh tissue in these cases. The distinction between follicular adenomas and carcinomas requires careful histological analysis; thus, the procurement of frozen tissue from follicular neoplasms might affect the diagnosis and was not performed in this study (1). It may be of interest to determine whether Akt is activated in benign follicular adenomas to determine whether Akt overactivation is an early event in thyrocyte malignant transformation. In addition, immunohistochemistry was attempted to define the pattern of expression of Akt isoforms in the samples, but results were not interpretable, presumably related to the suitability of the primary antibodies for these types of experiments.

In summary, we have demonstrated that Akt, a central regulator of nonmalignant thyroid cell growth and apoptosis, can be activated by serum and insulin in poorly differentiated human thyroid cancer cells in a PI3k-dependent manner and that PI3k activation is critical for the survival of these cells. We have also shown that Akt 1, 2, and 3 mRNAs and proteins are present in human thyroid cancer cells and that the expression patterns differ in the cell lines. In human thyroid cancer tissue, we identified increased levels of Akt 2 mRNA and protein and overactivation of total Akt compared with normal tissue.

Overactivation of Akt was most impressive in follicular thyroid cancers similar to the phenotype of Cowden's syndrome. Therefore, it appears that increased levels of Akt 2 mRNA and protein and overactivation of total Akt are associated with thyroid cancer, particularly follicular carcinoma. Additional studies evaluating the effects of Akt 2 activation on thyrocytes and Akt inhibition on thyroid cancer cells are warranted to determine whether disruption of Akt signaling might represent a target for cancer therapy.

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