

Phosphorylated Insulin Like Growth Factor-I Receptor Expression and Its Clinico-Pathological Significance in Histologic Subtypes of Human Thyroid Cancer

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Overexpression of insulin-like growth factor-I receptor (IGF-IR) is seen in a multitude of human thyroid cancers and correlates with poor prognosis. However, recent studies suggest that low phospho-IGF-IR (pIGF-IR) expression rather than its overexpression may be an indicator of poorly differentiated disease. No previous study has evaluated the expression of pIGF-IR to determine if activation or loss of expression of this receptor is associated with thyroid tumor progression. Accordingly, a quantitative immunohistochemical (IHC) method was used to evaluate the clinico-pathological significance of pIGF-IR expression in archival samples of human thyroid carcinomas. Quantitative analysis of pIGF-IR levels revealed a significant difference in the median index of pIGF-IR between different histological subtypes of thyroid cancer ($P < 0.001$). Specifically,

the median pIGF-IR index of differentiated thyroid cancers was significantly higher than the median index of other poorly differentiated thyroid cancer ($P < 0.001$). This was further confirmed in individual tumor sections of thyroid carcinoma where anaplastic and differentiated components co-existed. No significant difference was noted in the pIGF-IR index of tumors grouped by size or stage but a trend towards lower mean pIGF-IR index was noted in older patients. Our data indicates that pIGF-IR is upregulated in a majority of follicular thyroid carcinomas, suggesting it may be a potential target for therapy for patients with this disease. In addition, since low pIGF-IR expression was found to correlate with aggressive human thyroid carcinoma, it also suggests that IGF-IR may not be needed for progression of anaplastic thyroid carcinoma possibly because other cell signaling pathways are activated, obviating the need for IGF-IR signaling. However, more mechanistic studies would be necessary to substantiate the possibility that pIGF-IR may be important for differentiation of thyroid tissues and is lost with disease progression. *Exp Biol Med* 234:372–386, 2009

Key words: phospho-IGF-IR; phospho-insulin receptor; insulin like growth factor-I; thyroid carcinoma; tyrosine kinase receptor; prognostic factor; tissue array; Image Pro

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Background

According to recent American Cancer Society estimates, the incidence of thyroid cancer is rapidly rising in the United States (1). From approximately 8,000 new patients in 1975 to the current number of approximately 33,500 per year translates to an increase of 4% every year in the past 20 years (2). Most of this increase is thought to be the result of

intensification of surveillance through the use of thyroid ultrasound which detects small thyroid nodules that might not otherwise be detectable. Almost 95% of all thyroid tumors are of follicular epithelial cell origin and of differentiated phenotype. In general, differentiated thyroid carcinomas pursue protracted clinical course with a 10-year survival rate of 92%. A small subset may recur and metastasize to distant sites resulting in death of the patient within years of diagnosis. The remaining 5% are medullary thyroid carcinomas derived from neuroendocrine 'C' cells. They can spread via the bloodstream and/or the lymphatics. The 5-year survival rate for medullary thyroid carcinomas is approximately 50%. Anaplastic thyroid carcinomas, on the other hand, are almost invariably fatal. They rapidly invade critical structures in the neck, and frequently metastasize to distant sites (3). Anaplastic thyroid carcinomas may occur either *de novo* or they may arise from follicular or papillary carcinomas. Very little is known about the mechanisms or factors that lead to aggressive transformation or development of anaplastic thyroid carcinoma.

Recent efforts have focused on the identification of genes that may play a central role in thyroid cancer progression using comparative genomic hybridization (CGH) (4). This approach has helped to identify numerous potential markers that have since been applied to *in vitro* and *in vivo* thyroid model systems (5). However, thyroid cancer is an indolent and heterogeneous disease with a multitude of genetic and epigenetic changes in and between the tumor types. Thus genome-wide approaches such as CGH are less effective in precisely identifying specific genes that facilitate the progression of this complex and multi-factorial disease. A pathway-specific approach however, may help identify molecular genetic targets that are associated with malignancy, onset and progression and can be used to determine their effects on the network of effectors and modifiers involved in the process.

Recent studies suggest that numerous growth factors and their receptors may be abnormally overexpressed or constitutively activated in thyroid tumors and may influence their biological behavior (6–8). The insulin like growth factor-I (IGF-I) and the insulin like growth factor-I receptor (IGF-IR) axis is a promising candidate. Reports on IGF-I production from normal thyroid cells and thyroid adenomas can be traced back to 1987 (9, 10). However, overexpression of IGF-IR has been implicated more recently in the development of thyroid cancer (11, 12). The IGF-IR is a heterodimeric transmembrane tyrosine kinase that plays a crucial role in organ development during embryogenesis and regulation of mitogenesis. Signaling through this receptor leads to suppression of apoptosis and stimulation of proliferation, constituting an important cell survival pathway (13–15). Phosphorylation of IGF-IR occurs following binding of its two ligands, the IGF-I and II, followed by the recruitment of several effector molecules, which, in turn modulate cellular transformation and proliferation (16). There are several papers showing that

activation of IGF-IR is essential for the mitogenic effects of thyroid stimulating hormone (TSH) and for its effects to stimulate thyroid function (17–19). Unlike other growth factor receptors, IGF-IR and insulin receptor (IR) are not inhibitory to thyroid function and they cooperate with TSH to stimulate growth.

Inappropriate IGF-IR signaling has been implicated in the development and progression of several human malignancies (20–22), including those of the thyroid (23–25), and often correlates with poor prognosis (26, 27). There are several mechanisms by which IGF-IR signaling is deregulated in human tumors. It can be constitutively activated through autocrine or paracrine signaling (26, 28). Alternatively, ligand-independent mechanisms can result in the activation of the receptor (29). By far, the most common occurrence is overexpression of IGF-IR. A recent study in thyroid carcinoma (12) corroborated the above findings and demonstrated that overexpression of IGF-IR was upregulated in poorly differentiated thyroid cancers. However, the same study also indicated that several thyroid cancer cell lines, despite their high IGF-IR content, failed to autophosphorylate the IGF-IR when treated with IGF-I. Additionally, anti-IGF-IR immunotherapy studies also indicated that failure of these cell lines to respond to anti IGF-IR treatment was linked to their pIGF-IR levels and, in turn, their inability to activate the downstream signaling cascade. These data support a paradoxical hypothesis that loss of pIGF-IR expression rather than its overexpression may be an indicator of poorly differentiated disease. Since thyroid cancer is a heterogeneous disease, both the hypotheses may be relevant to specific subsets or stages of the disease.

Although, several studies have evaluated the role of IGF-IR expression in different human tumors, none to our knowledge has reported on pIGF-IR expression in thyroid cancer. Our objective, therefore, was to evaluate pIGF-IR expression in a large cohort of formalin fixed paraffin embedded human thyroid cancer specimens and determine, if, it is an indicator of their clinical behavior.

Materials and Methods

Patient Samples. Samples used in this study were from thyroid cancer patients who had undergone initial surgery at The University of Texas M. D. Anderson Cancer Center between 1992 and 2002. A total of 85 such patients were identified whose tissue samples and follow-up information were available. Follow-up was updated through September 2006 by reviewing medical records. Six cases of normal thyroid epithelium, 15 anaplastic cancers (ATC), 30 follicular thyroid tumors (FTC), 13 Hurthle cell carcinoma (HCC), 18 medullary (MTC) and 12 papillary carcinomas (PTC) were included in the tissue microarray block. The use of all archival paraffin-embedded tissue blocks and chart reviews were approved by the institutional review board of M. D. Anderson Cancer Center. Demographic and survival data for each specimen were entered into a comprehensive

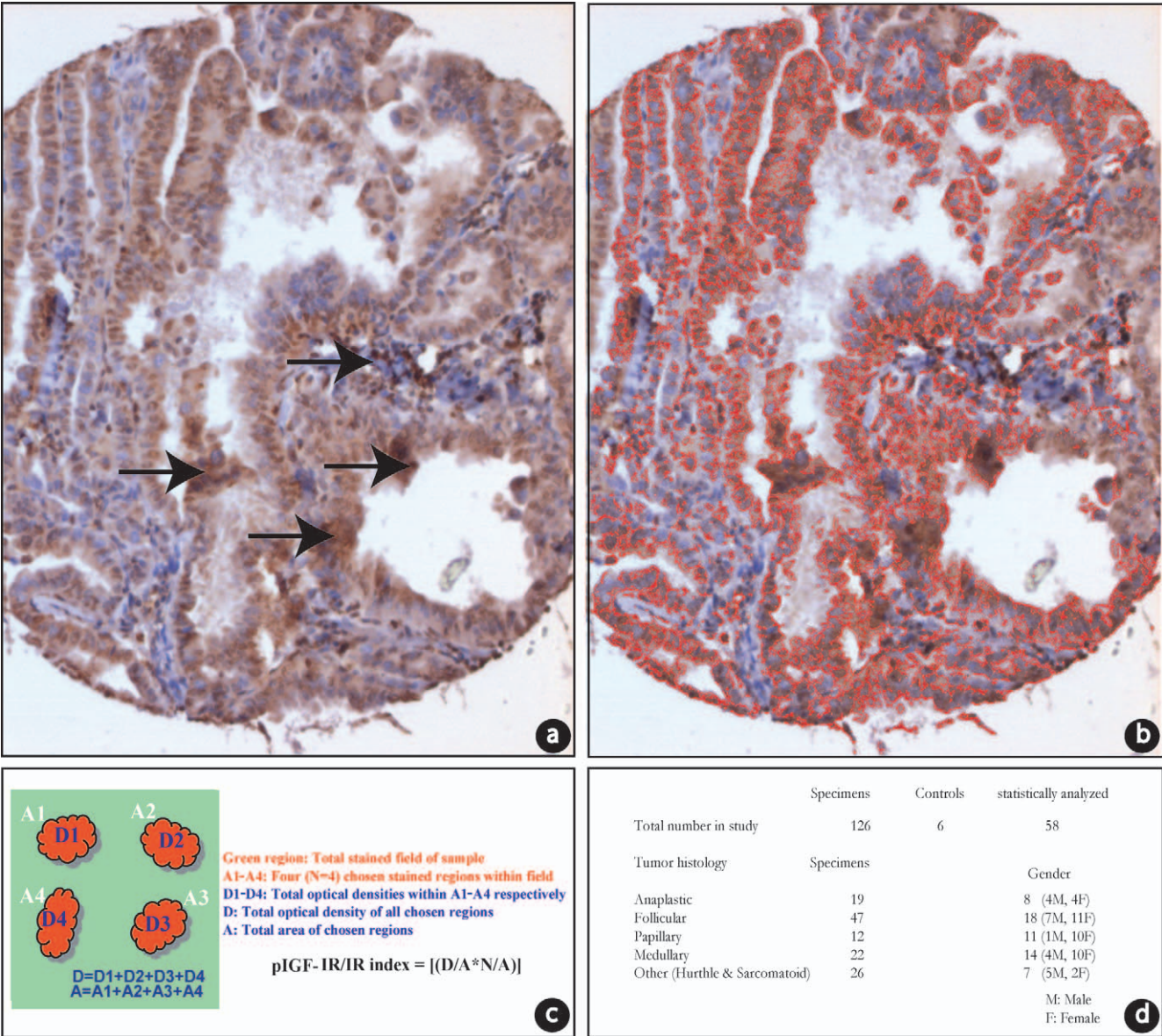


Figure 1. Representative images of thyroid carcinoma specimen from the tumor tissue array analyzed by immunohistochemistry to quantitate the pIGF-IR index. a) Immunoperoxidase visualization (brown color) of pIGF-IR in a follicular thyroid carcinoma tissue. Arrows highlight the non-specifically stained regions in the field. b) The same section after selecting the different parameters outlined in the Methods section and applying the various filters. By choosing appropriate parameters, regions included in the quantitative analysis and those excluded from it are clearly highlighted through pixilation. Red pixels denote the included regions, while the regions that do not have red pixels denote the excluded regions. Arrows indicate staining artifacts also excluded from the analysis. c) Schematic illustrating the methodology used to calculate the pIGF-IR index of each specimen in the thyroid tissue array. d) Summary of thyroid carcinoma samples used for statistical analysis of pIGF-IR index of thyroid tumor microarray samples. A color version of the figure is available in the online journal.

database created with Microsoft Excel (version 2000). Histopathologic diagnoses were provided by an in-house pathologist. For statistical analysis, FTC, PTC and HCC were grouped as “well differentiated” while ATC and MTC were grouped as “all other thyroid cancers”. Similarly, tumors with a T1 or T2 diagnosis were grouped together and T3 and T4 were grouped together.

Construction of Tissue Microarrays. To construct the two tissue microarrays used in this study, formalin-fixed, paraffin-embedded archival tissue blocks

and their matching hematoxylin and eosin-stained slides were retrieved, reviewed and screened for representative tumor regions by a pathologist. The tissue microarrays were constructed with a tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA) as described previously (30). The composition of tumors in the tissue microarrays is listed in Figure 1d. Each tumor or normal thyroid control was sampled from representative areas of the donor blocks using a 1.0-mm punch and arrayed onto tissue microarray blocks. Because many of our tumors exhibited a high degree of

heterogeneity (e.g., the same block encompassing a PTC or FTC with an anaplastic component), more than one punch biopsy was obtained from the same block and analyzed as an independent sample for histochemical analysis. This was to ensure uniform statistical criteria across all samples.

Antibodies. The following primary and secondary antibodies were used at the specified dilutions: anti-IGF-IR β antibody (31) (C-20) (1:100), anti-IR β antibody (C-19) (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phosphorylated IGF-IR β (Tyr 1131)/IR (Tyr 1146) antibody (1:200), (Cell Signaling Technology, Beverly, MA); anti-phosphotyrosine clone 4G10 (Upstate), anti-phospho-insulin receptor pY972 (Biomol), peroxidase-conjugated goat anti-rabbit IgG (1:500) (Jackson ImmunoResearch Laboratories, West Grove, PA); biotinylated goat anti-rabbit IgG (Biocare Medical, Walnut Creek, CA); and streptavidin horseradish peroxidase (Dako A/S).

Controls. FTC tumor grown in nude mice that overexpresses human wild-type IGF-IR (wt-IGF-IR) or constitutively activated IGF-IR (Ca-IGF-IR; construction of pBabe puro-Ca-IGF-IR has been described previously (32) and was a kind gift from Dr. Lee) were included as positive controls with each analysis. A transplanted FTC tumor with no detectable pIGF-IR (tumors derived from vector control cells) was also included as negative control.

FTC Cell Lines and Culture Conditions. Thyroid cell lines used in this study were treated and maintained as monolayer cultures in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine, penicillin, sodium pyruvate, and nonessential amino acids (Life Technologies, Inc.) except otherwise indicated. Adherent monolayer cultures were maintained on plastic and incubated at 37°C in 5% carbon dioxide and 95% air.

Immunofluorescence Staining for pIGF-IR on Cultured Cells. 1×10^5 FTC133 cells or poorly differentiated Wro cells were plated in 6-well dishes containing coverslips and cultured overnight in regular 10% serum growth media. Next morning, the regular media was replaced with serum-free media and cells were cultured for the next 24 hrs. Fifteen minutes before harvesting, cells on one of the coverslips of each cell line were treated with 10 ng/mL IGF-I for 15 min, washed with PBS and fixed in 4% paraformaldehyde solution for 15–20 min. Excess aldehyde was quenched by incubating in 1 mg/mL sodium borohydride solution pH 8 for 15 min. Fixed cells were blocked overnight (Tris buffered saline pH 7.4 with 0.1% Tween 20 and 1% BSA) and treated with primary antibody (1:250, pIGF-IR Try1131/Try1146) diluted in blocking buffer for 2 hrs at room temperature (RT). Alexa 488 anti-rabbit secondary Ab (Molecular Probes-Invitrogen) was used for 2 hrs at RT in the dark. After washing, the coverslips were mounted with Slowfade containing DAPI (Invitrogen) and sealed in place with clear nail polish. Images were acquired with a CoolSnap HQ CCD camera (Roper Scientific, Tucson, AZ) coupled to a motorized Nikon 2000U inverted microscope (Nikon,

Inc.) controlled by Metamorph software (Molecular Devices Corp., Downingtown, PA). Images were then deconvolved using AutoDeblur (Media Cybernetics, Silver Spring, MD).

Immunohistochemical Staining. Standard immunoperoxidase based staining techniques were used to analyze the expression of IGF-IR (31) and pIGF-IR in 36 samples of thyroid cancer and 6 normal thyroid tissues. Details on the optimization of the staining with the pIGF-IR antibody are provided in supplemental methods section and in Supplemental Figure 1. (Supplementary material is available online.) The level of staining intensity was assigned a grade 0 = negative, 1 = weak, 2 = moderate, and 3 = strong. Non-specific staining (additional negative control) was obtained by omitting the primary antibody and replacing it with normal rabbit serum from Dako. A positive reaction was visualized by incubating the slides with stable DAB for 3–5 min and counter-stained with Gill's hematoxylin. The sections were mounted on Universal Mount (Research Genetics, Huntsville, AL). For immunostaining the arrays, slides were backed overnight at 60°C before dewaxing. This was followed by immunostaining for pIGF-IR using procedures described above. Relative levels of pIGF-IR were quantified to obtain their pIGF-IR index as described below.

Western Blot to Detect pIGF-IR Expression in Follicular Thyroid Carcinoma Cells. There is considerable evidence of interaction and cross-talk between IGF-IR and other RTK signaling pathways (33–35) and this antibody is reported to cross react with some of those. To ensure that the antibody was not picking up cross reactive bands, lysates from thyroid cancer cells and immortalized thyrocytes cultured in low IGF-I media (10 ng/ml) were analyzed by Western blot as described (12). Additionally, the same cell lysates were analyzed for insulin receptor beta and phospho insulin receptor beta (pIR β) expression. Similarly the ability of this antibody to detect IGF-I-induced tyrosine phosphorylation of IGF-IR was also tested using Wro clones overexpressing IGF-IR. For this analysis cells were plated in 10% serum (un-starved), washed the next day and supplemented with serum-free medium for 24 hrs (serum-starved). Cells were then activated with recombinant human IGF-I (10 ng/mL) for 15 min, washed with PBS, and scraped with lysis buffer and analyzed per previously described procedures (12). Immunoprecipitation was performed as per procedures described in Pold *et al.* (2004) (36).

Digital Analysis of Immunostained Sections. Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD) was used to obtain a quantitative index of pIGF-IR expression as elaborated later in the Methods section. The "Irregular Area of Interest" feature was used to select the area to be measured. The tumor area is chosen arbitrarily for different samples to encompass stained regions (see Supplemental Fig. 2 in the online version for delineation of irregular area of interest). To define the number and area of the pixels to be identified, the command

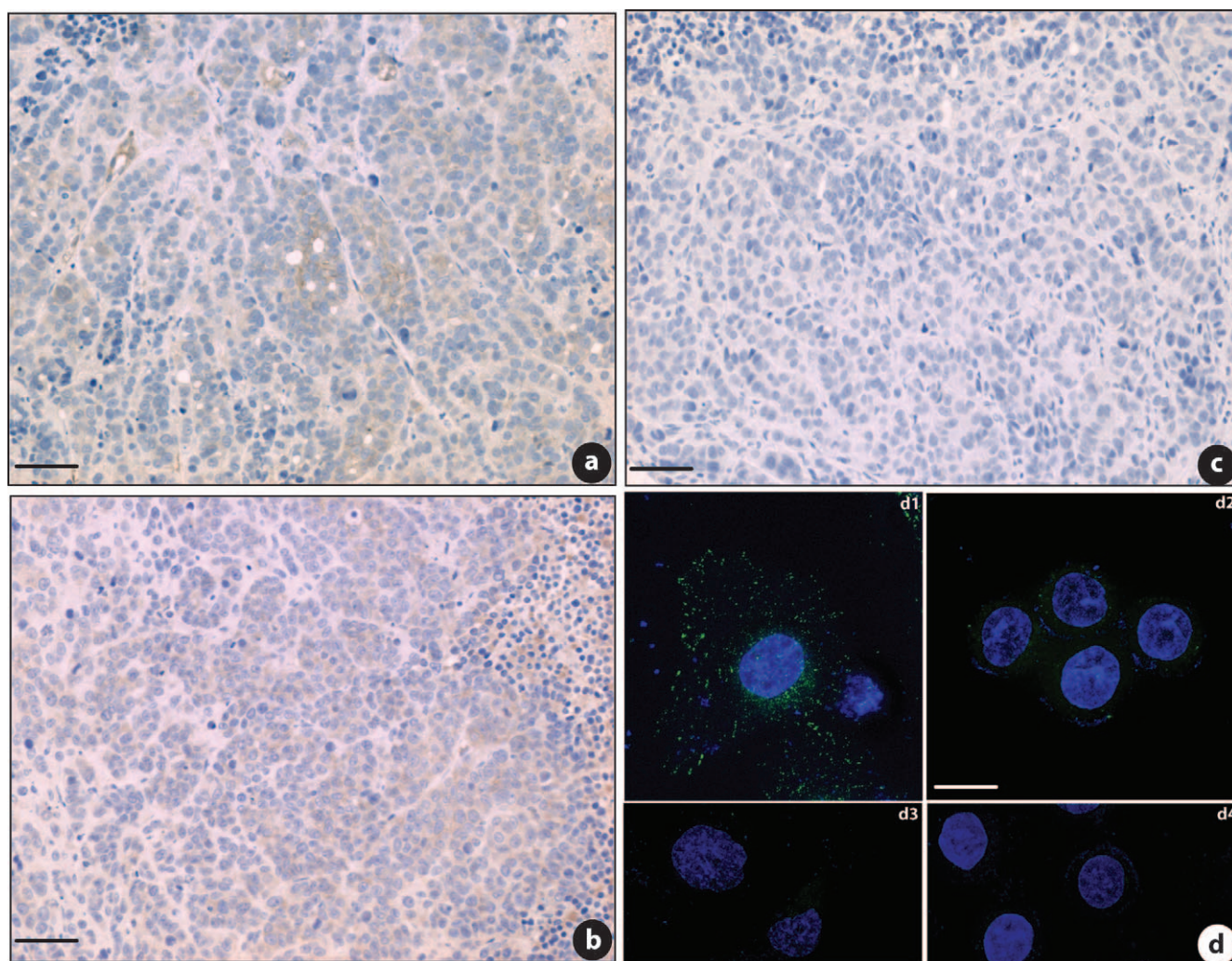


Figure 2. Sensitivity and specificity of phosphorylated IGF-IR Tyr-1131/IR-Tyr-1146 antibody in immunohistochemical, immunofluorescence, Western blot and immunoprecipitation assays. a) Phosphorylated IGF-IR expression by IHC in nude mice derived human thyroid tumors overexpressing the Wt-IGF-IR receptor (b) Ca-IGF-IR or (c) vector transduced WRO cells. Bar represents 100 μ m; d) deconvolved images of pIGF-IR expression in follicular thyroid carcinoma cell lines. Immunolocalization of endogenous pIGF-IR (green) and DAPI stained nuclei (blue) in FTC133 (d1 & d2) and Wro (d3 & d4) cells. Note the membranous localization of pIGF-IR in FTC133 cells (panel d1) when treated with 10 nM IGF-I for 15 min and compare it with cells in panel (d3) that were cultured in serum-free medium (SFM). To further establish the specificity of pIGF-IR staining, another follicular thyroid carcinoma cell line, Wro, was stained with anti pIGF-IR antibody when they were treated with (d3) or without 10 nM IGF-I (d4). Note the minimal staining for membranous pIGF-IR even when Wro cells are treated with 10 nM IGF-I (d3). Scale bar represents 25 μ m; note cells were serum-starved for 24 hrs in serum-free media before being activated with IGF-I. e) Phosphorylated IGF-IR and total IGF-IR expression assessed by Western analysis in Wro clones used to generate the nude mice tumors in serum-free media or serum-free media supplemented with IGF-I (10 ng/ml) for 30 and 60 min. Please note that the vector control or the wt-IGF-IR cell lysates do not show phosphorylated IGF-IR expression under serum-starved conditions. Addition of IGF-I induces barely detectable expression of pIGF-IR in the vector control cells but readily detectable levels of pIGF-IR in wt-IGF-IR cells. Lysates from Ca-IGF-IR cells exhibit constitutive activation of pIGF-IR, albeit at a much lower level. Data are representative of many separate experiments. f) Western analysis of phosphorylated IGF-IR, IGF-IR β , pIR and IR β protein expression assessed after culturing thyroid cancer cell lines in serum-free media for 24hrs and subsequent stimulation with low dose IGF-I (10 ng/mL) for 15 min. g) 200 μ g total protein from IGF-I (10 ng/mL) or insulin (5 ng/mL) treated Dro cells was immunoprecipitated (IP) with pIGF-IR/IR, pIR^{Y972} or IR β antibody and immunoblotted (IB) with pTry 4G10, IGF-IR β or IR β . Purified rabbit IgG were used for mock immunoprecipitation. A color version of the figure is available in the online journal.

“Select Measurements” in the “Measure” menu in the “Count/Size” dialogue box was used. To manually set the thresholds of the color range representing brown immunostaining, the command “Select Colors” in the “Count/Size” dialogue box was used. The histogram-based mode of color thresholding was used. Digitized images were converted from the RGB color mode to the HSI (Hue, Saturation, and Intensity) color mode and the three HSI parameters were

manually gated while the accuracy was checked with a pseudo-color. In this way, the thresholding could be set to exclusively encompass the specific staining (see representative images before and after pseudo-coloring in Fig. 1 (panels a & b)). The command “Count” from the “Count/Size” dialogue box was used to calculate the percentage of stained area. Under the “View” menu in the “Count/Size” dialogue box, data were presented in the “Statistics-Sum”

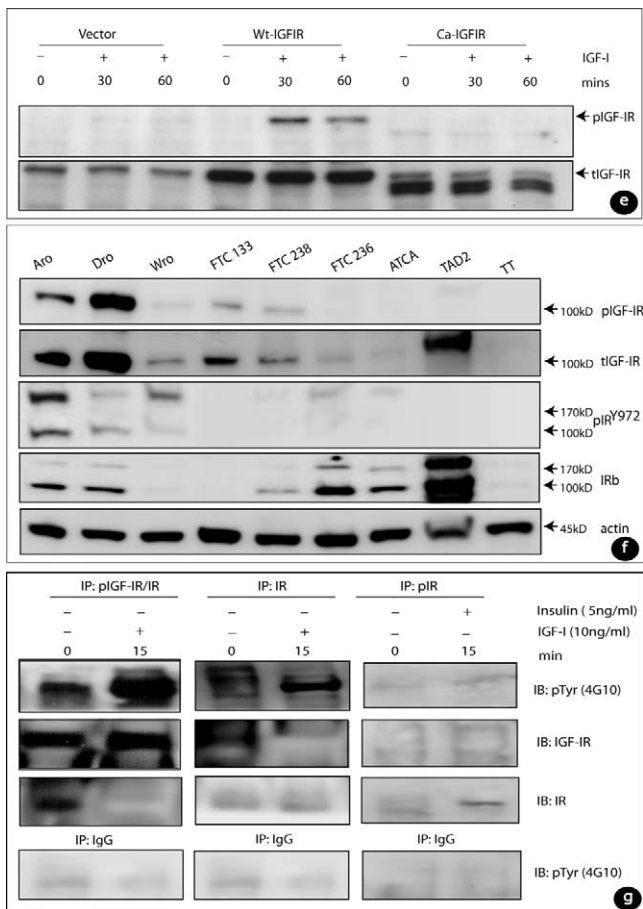


Figure 2. Continued.

window. The functions “count”, “Area” and “density sum” output from the software were used to compute the pIGF-IR index of each specimen. Background correction was achieved in three consecutive steps. 1) Correction for background illumination: The background image was stored as a temporary image and subtracted from each captured area of interest (AOI). Each individual AOI (minus the background image) was stitched together into one large composite TIFF image for further quantitation. 2) Color based delineation of feature of interest from background: In the saved TIFF image, each selected tissue feature of interest was assigned a unique color (segmentation) using the Hue, Saturation, and Intensity (HSI) color model as described above. The remaining regions were automatically treated as background and were filtered out during actual measurements. 3) Size and intensity threshold filters: Thresholds for size (size filter) and chosen intensity levels were set to eliminate any remaining background staining. These features were saved as a master file and recalled every time a new sample was analyzed.

Quantitation of pIGF-IR Index Using Image Pro 6.0. Immunostaining of the pIGF-IR was quantified using a Leica DM LA microscope coupled to a Cool SNAP Pro Color camera. Images of stained tissue sections were

acquired at $\times 100$ magnification. The digitized images were saved as TIFF files and analyzed using Image-Pro 6.0 software (Media Cybernetics, Silver Spring, MD, USA) as described above. The intensity of staining for pIGF-IR was quantified using the product of two parameters characteristic of each sample. The first parameter was defined as the average optical density per field of a tissue section. It was calculated as the ratio (D/A), where D is the sum of the optical density values in the threshold area within the tissue section and A is the total area of thresholded region of interest. The values of both D and A were output by the software. The second parameter was defined as the number of stained cells per unit area of the chosen field and was calculated as (N/A), where N is the count function obtained from the software (thresholding parameters pertaining to the nuclei output a number that represents cell number) and A is the stained area. This parameter accounted for the variability in the number of cells stained amongst different histological subtypes. The product [(D/A)*(N/A)] referred to as pIGF-IR index (see schematic in Fig. 1c) was statistically analyzed to obtain the distribution of pIGF-IR staining amongst histotypes of thyroid cancer. To ensure that there is no change in the light intensity of the microscope light source while capturing the images, each specimen from an array was photographed on the same day under identical conditions.

Data Analysis. pIGF-IR index of different thyroid cancer (differentiated vs. poorly differentiated) or its association with tumor size, stage or lymph node status was compared with Kruskal-Wallis and Wilcoxon rank-sum test, as appropriate. A two-tailed $P < 0.05$ was considered significant. All statistical analyses were performed using Stata 9 (StataCorp, College Station, TX, US).

Results

Expression of IGF-I Axis Components in Different Thyroid Carcinomas. Since IGF-signaling pathway is involved in the development and progression of many solid tumors, analysis of IGF-I signaling components (IGF-IR/pIGF-IR) was undertaken in thyroid cancer specimens to select a subgroup for evaluating the pIGF-IR antibody for immunohistochemical studies. Seven normal thyroid specimens were also evaluated to obtain the baseline expression level of these markers. Initial studies were restricted to standardizing the immunohistochemical assays to detect the expression of IGF-IR and pIGF-IR in archival paraffin embedded thyroid cancer samples.

Immunohistochemical detection of pIGF-IR expression in human tumors has not been reported previously. Thus pilot studies were undertaken to assess 1) the specificity of the pIGF-IR antibody, 2) the type and nature of tissue (frozen vs. paraffin) that would provide optimum evaluation of the marker, and 3) if, standard immunohistochemical methods can be used to detect its expression in archival samples of follicular thyroid carcinoma. Since pIGF-IR Ab

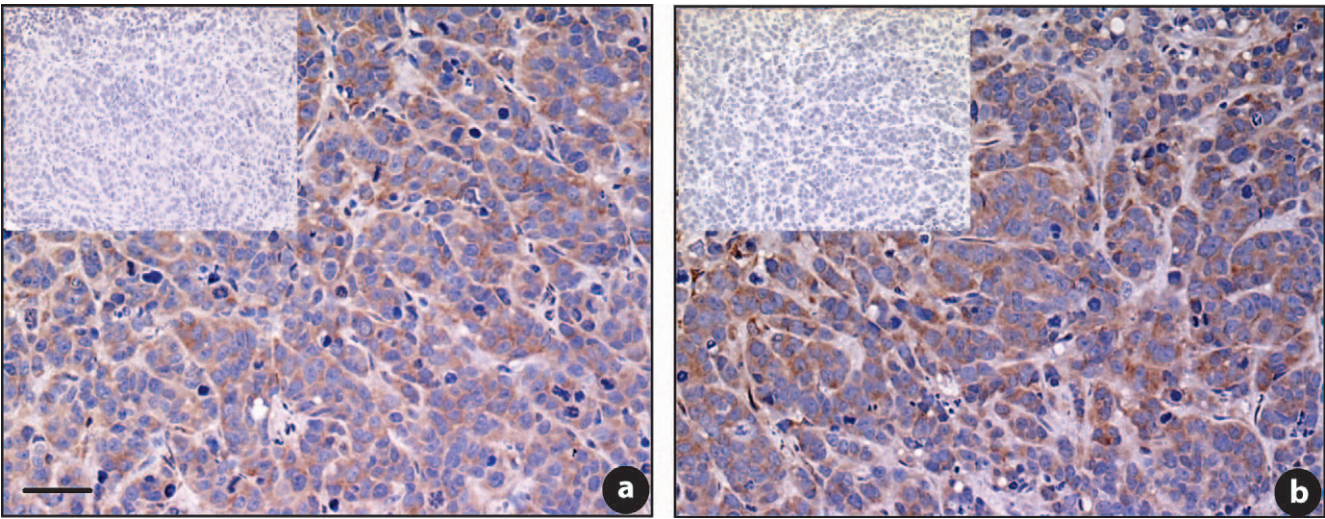


Figure 3. Comparison of immunohistochemical detection of pIGF-IR expression on paraffin embedded vs. frozen thyroid carcinoma specimen. This tumor was processed for both paraffin embedding and frozen section. Sections from the paraffin embedded tissue (a) or frozen section (b) were immunostained with anti-pIGF-IR/IR Ab. pIGF-IR staining was fairly uniform under both conditions indicating the suitability of the antibody for archival assessment. Adjacent sections were treated with normal rabbit serum for use as negative control as described in Materials and Methods and are shown here as the insets. Please note that the negative control sections are photographed at lower magnification to consolidate the very faint brown background staining seen under the microscope. Scale bar represents 50 μ m. A color version of the figure is available in the online journal.

from cell signaling has been used for the detection of pIGF-IR expression (12) by Western analysis, this antibody was further evaluated for its specificity in IHC studies. Human thyroid tumors grown in nude mice were used as positive and negative controls to standardize the IHC assays. As seen in Figure 2c, the vector control cell derived tumor had no detectable expression of pIGF-IR or other cross reactive receptor tyrosine kinases. But nude mouse tumors derived from Wro cells transduced with wt-IGF-IR (Fig. 2a) or ca-IGF-IR construct (Fig. 2b) had cytoplasmic and/or membranous expression of pIGF-IR. These findings could be corroborated by Western analysis as well (Fig. 2e), where vector control cells show barely detectable expression of pIGF-IR, but cells transduced with retroviral constructs expressing wt-IGF-IR or ca-IGF-IR readily demonstrate a band co-migrating with the 97 kD marker or the truncated form of the receptor as the case may be (Fig. 2e). The

specificity of the antibody was further tested by immunofluorescence staining of formalin fixed FTC133 and Wro cells with or without IGF-I treatment (Fig. 2d). Although the antibody localized the phosphorylated form of the receptor mostly in the membrane region (Fig. 2-d1), faint cytoplasmic and perinuclear staining was also observed in occasional fields (Fig. 2-d2) of FTC 133 cells. Wro cells on the other hand were negative in the immunofluorescence assay (Fig. 2-d3 & d4) and corroborated the results of our Western analysis.

However, apart from specificity issues, since the relative abundance of IGF-IR and IR could also influence the interpretation of this study, a panel of thyroid cancer cell lines were grown in low IGF-I containing media and analyzed for relative expression of pIGF-IR, IGF-IR β , pIR, and IR β . With the exception of TAD2 and at times FTC cell lines 236 and 238, all other thyroid cancer cell lines either

Table 1. Immunohistochemical Survey of Expression of IGF-IR and pIGF-IR in Histological Subtypes of Thyroid Cancer

	No. of samples	Negative	Weak	Moderate	Strong	% positive cases
IGF-IR						
Normal	6	-	6	-	-	100
Papillary	4	-	-	-	4	100
Follicular	2	-	-	-	2	100
Anaplastic	37	1	6	7	23	97
pIGF-IR						
Normal	6	6	-	-	-	0
Papillary	4	-	4	-	-	100
Follicular	2	-	-	2	-	100
Anaplastic	17	16	1	-	-	6

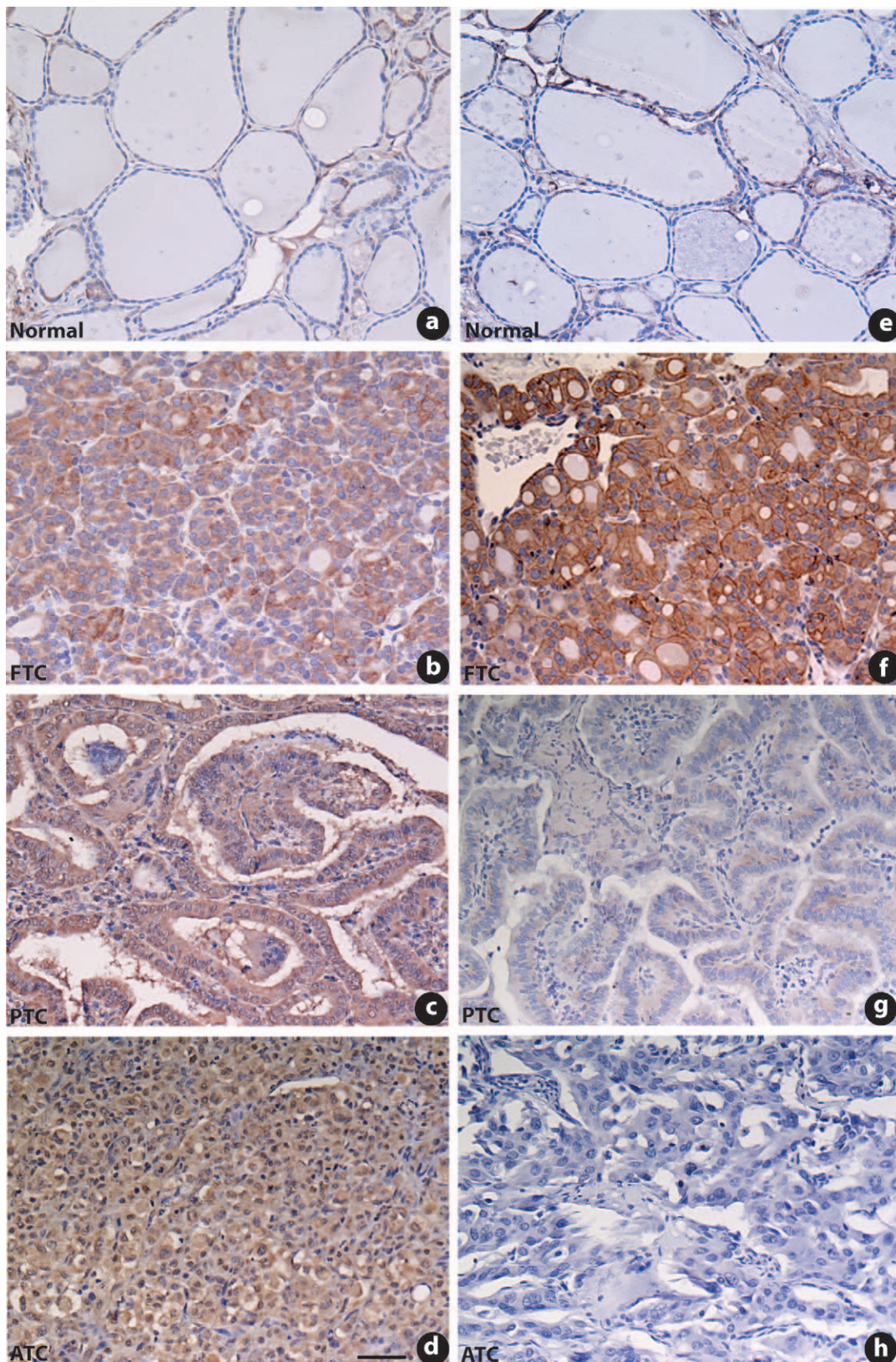


Figure 4. Representative paraffin-embedded sections of normal and histological subtypes of thyroid carcinoma treated with the anti-IGF-IR antibody (a–d) and anti-pIGF-IR/IR Ab (e–h). Note that the normal tissue has very low levels of IGF-IR or pIGF-IR as compared to the adjacent panels showing intense staining in tumor tissues. Additionally, only follicular thyroid carcinoma samples and few papillary carcinomas were more often positive for anti-pIGF-IR antibody staining even though all the tumor tissue types had detectable IGF-IR expression. Scale bar represents 50 μ m. A color version of the figure is available in the online journal.

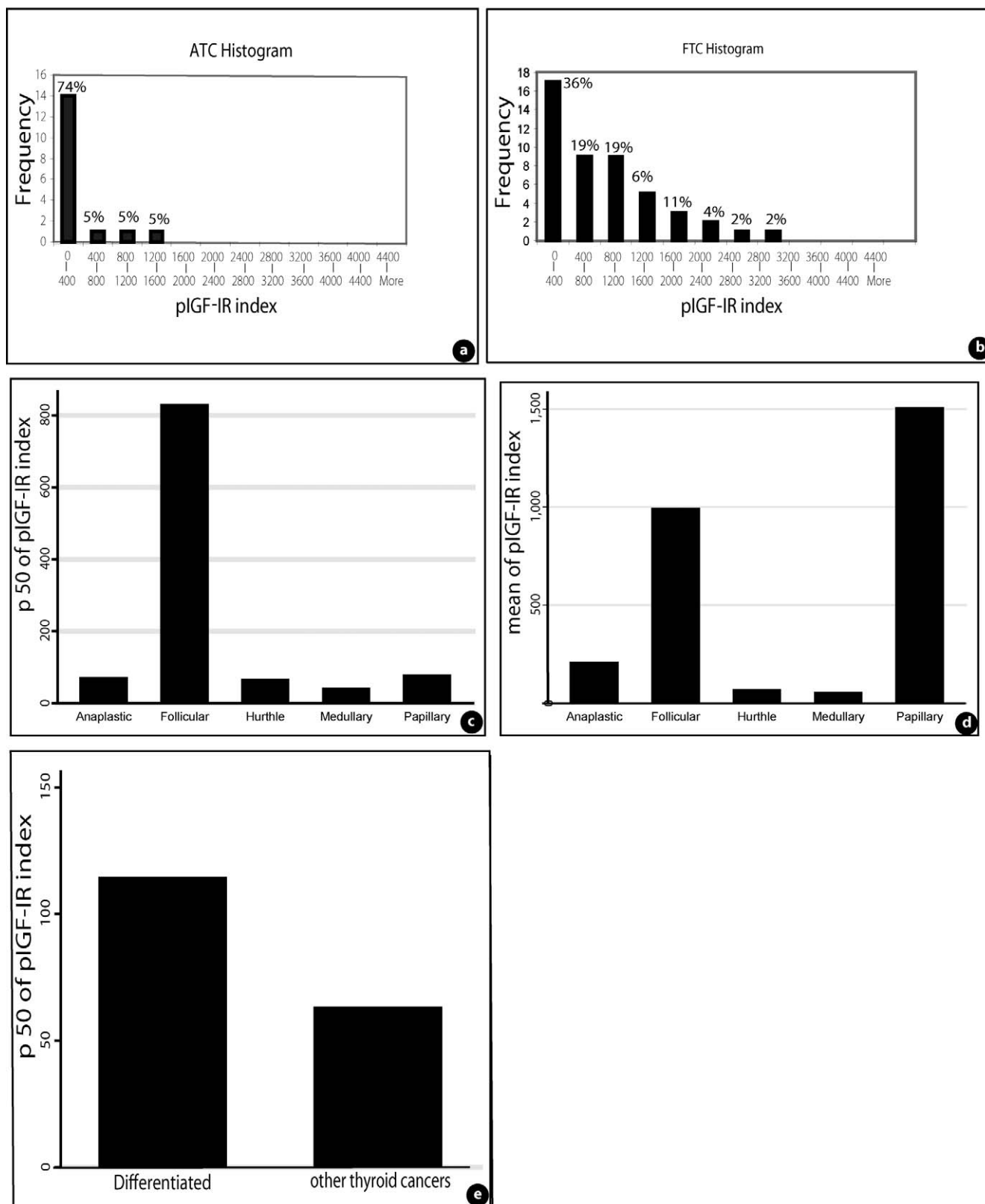


Figure 5. pIGF-IR index of thyroid cancer specimens: frequency distribution of pIGF-IR index in a total of 17 anaplastic (a) and 47 follicular (b) thyroid carcinoma cases. Please note that for this analysis each punch biopsy was treated as an independent sample even if it came from the same patient. The median pIGF-IR index varied substantially amongst histological subtypes of thyroid carcinomas (c) ($P < 0.001$, chi-square = 25.734 with 4 d.f., chi-square with ties = 25.735 with 4 d.f.). Post-hoc multiple comparison analyses with Kruskal-Wallis test demonstrated significant difference in median pIGF-IR index of follicular vs. Hurthle ($P < 0.001$), follicular vs. medullary ($P < 0.001$), follicular vs. papillary ($P \leq$

show a single band with this antibody on Western or do not have detectable pIGF-IR expression. Similarly total IGF-IR level varied greatly amongst the cell lines but was detectable in all the cell lines tested. Contrarily, pIR expression was much lower in the FTC cell lines but moderately expressed in the anaplastic cell lines Aro and Dro. Most cell lines also demonstrated a band above 170 kD with both IR antibodies and may represent the precursor form of IR. Occasionally, this higher band is also detected with the pIGF-IR antibody in some cell lines. Comparatively, total IR expression was detectable in all the cell lines except for FTC133 cells (Fig. 2f). These data suggest that low levels of pIR may not contribute to the phosphorylation signal obtained with the pIGF-IR antibody in follicular thyroid carcinoma samples. To test this paradigm further, Dro cells that have high levels of endogenous pIGF-IR and moderate IR were stimulated with either 10 ng/mL IGF-I or 5 ng/mL insulin. Lysates from IGF-I treated samples were immunoprecipitated with either pIGF-IR Ab or IR β antibody and immunoblotted with phosphotyrosine, IGF-IR or IR antibodies (Fig. 2g, left and center panels). Similarly, the insulin treated cells were lysed and immunoprecipitated with pIR antibody and immunoblotted with phosphotyrosine, IGF-IR or IR antibodies (Fig. 2g, right panel). As seen in the Figure 2g, pIGF-IR immunoprecipitation from IGF-I treated samples pulled down much higher levels of phosphorylated proteins (left panel) as compared to the IR antibody (central panel). Additionally, when the phosphotyrosine blots were stripped and immunoblotted with IGF-IR and IR antibodies, only the IGF-IR blot had an appreciable signal. No signal was detected with IR antibody, suggesting pIGF-IR antibody did not pull down IR receptor (Fig. 2g, left panel). This was confirmed in the reverse assay, where the IGF-I treated lysates were immunoprecipitated with IR antibody and immunoblotted with phosphotyrosine, IGF-IR or IR antibodies. Once again IR antibody pulled down IR during immunoprecipitation but no IGF-IR. Furthermore, in our hands, low levels of insulin stimulation of Dro cells failed to phosphorylate the IGF-IR as the immunoprecipitates from these lysates only pulled down IR and no IGF-IR despite the fact that it was overexpressed. These data suggest that the phosphorylation signal obtained with the pIGF-IR antibody can be assumed to reflect pIGF-IR expression in thyroid carcinoma cells and IR receptor only marginally influences the phosphorylation signal after IGF-I stimulation. Finally, no visual difference was apparent in the staining intensity of frozen vs. paraffin sections, suggesting this antibody was ideal for archival assessment (Fig. 3).

In accordance with published reports, staining intensity of IGF-IR was remarkably high in ATC, FTC and PTC specimens as compared to controls (Fig. 4, compare panels

b–d with a and also see Table 1). But contrary to current belief, none of the ATC tumors retained pIGF-IR expression while some FTC and PTC specimens exhibited moderate to high expression of pIGF-IR, respectively (Fig. 4, panels f & g, also see summary in Table 1). These results suggested that low expression of growth-signaling components of the IGF system, particularly low pIGF-IR expression may be associated with malignant phenotype or more aggressive tumor behavior of thyroid cancer. To test this hypothesis, pIGF-IR expression was next analyzed in archival thyroid tumor microarrays containing specimens with 10 to 12 year follow up. Use of tumor tissue microarrays ensured rapid screening of multiple tissue samples under uniform staining and scoring conditions.

Low pIGF-IR Expression Correlates with More Aggressive Thyroid Cancers. Thyroid tumor tissue arrays, with 120 specimens on one and 84 specimens on the other, were used for this study. Only 126 samples were analyzed for their pIGF-IR expression. The remaining samples could not be used because of either tissue disappearance or partial/complete lack of neoplastic cells available for analysis. A total of 58 specimens could be used for final statistical analysis. The array also had six pairs of normal and tumor tissue from the same patient. In some paired normal and tumor samples, pIGF-IR content of the tumor was higher in differentiated tumor components as compared to the corresponding adjacent normal tissue specimen.

Histogram analysis of morphological histotypes of thyroid cancer indicated that 74% of ATC had a pIGF-IR index below 400 as opposed to only 34% of the FTC (Fig. 5a & b). As these results obtained with tissue microarrays were very similar to the results obtained using the test group of tumors, further statistical comparisons to assess the clinico-pathological significance of pIGF-IR were carried out using the pIGF-IR index of the array specimen. As predicted from the pilot study described earlier, a significant difference was noted in the median index of pIGF-IR between the different histological subtypes of thyroid cancer ($P < 0.001$) (Fig. 5c). Additionally, when all thyroid cancers were stratified as differentiated (FTC, PTC and HCC) or other thyroid cancers (ATC and MTC), the median pIGF-IR index of differentiated thyroid cancer was significantly higher than the median index of other thyroid cancer (114 vs. 63, $P < 0.001$, Fig. 5e). These results confirmed our hypothesis that low pIGF-IR expression correlates with aggressiveness of thyroid cancers.

pIGF-IR Expression in Anaplastic Foci Associated with DTC. Although ATC follow a lethal course and FTC follow an indolent course, paradoxical as it may seem, there are cases where there is evidence of evolution of an

← 0.001) and anaplastic vs. follicular thyroid cancer ($P = 0.0025$). The mean pIGF-IR index (d) of the tumors also varied substantially amongst the tumors and was substantially higher for follicular and papillary thyroid cancer. e) pIGF-IR index of differentiated thyroid carcinoma was significantly higher than that of all other thyroid cancers ($P < 0.001$) (Mann-Whitney U test).

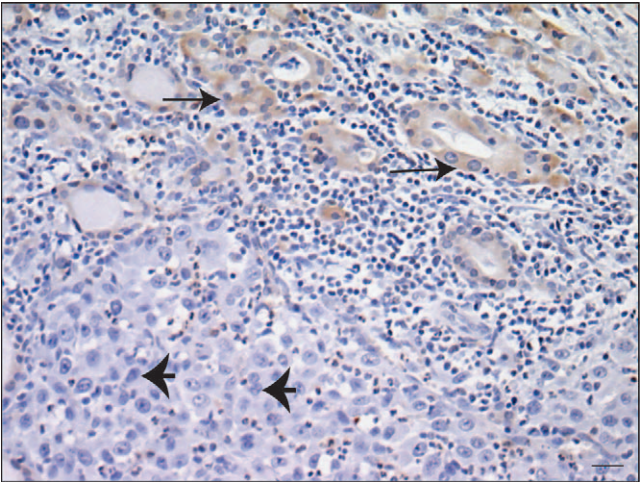


Figure 6. pIGF-IR expression in a thyroid carcinoma sample that harbors both an anaplastic and a differentiated thyroid tumor foci. Photomicrograph depicting staining specific to pIGF-IR. Note the intense brown staining in the differentiated component (pointed here by long arrows) while the adjacent anaplastic region (pointed here by short arrows) is completely devoid of any staining characteristic of pIGF-IR expression. Scale bar represents 50 μ m. A color version of the figure is available in the online journal.

anaplastic carcinoma from the DTC component (37). If this is true, our hypothesis can be further tested using thyroid carcinoma specimens where both the components co-exist. Accordingly, five such cases were identified and analyzed to see if pIGF-IR was differentially expressed in these two compartments. As expected, there was negligible staining for pIGF-IR in the anaplastic region but the differentiated regions stained well for cytoplasmic pIGF-IR expression (Fig. 6). This trend was noted in 3/5 samples analyzed. These data once again confirmed our observation that loss of pIGF-IR correlates with poorly differentiated disease and that this may be a late event in the progression of thyroid carcinoma.

pIGF-IR Expression and Clinico-Pathological Associations. To make the predictive power of our analysis more significant, we next analyzed additional parameters such as age, tumor size, tumor grade and lymph node metastasis, along with pIGF-IR index of the specimen. Age is an important prognostic indicator of thyroid cancer. Thus when patients with PTC or FTC were stratified according to their age, a trend towards a lower mean pIGF-IR index was noted in patients above 45 years of age. As seen in Table 2A, the mean pIGF-IR index of differentiated thyroid cancer patients above 45 years of age was significantly lower than the mean pIGF-IR index of patients below 45 years of age. However, statistical significance couldn't be reached due to the small sample size of our study. No significant difference was noted in the pIGF-IR index of tumors grouped by size or stage. Although lymph node (LN) metastasis is not a good prognostic indicator in thyroid cancer, it does indicate recurrence and local control. Thus, when tumors were stratified based on their LN status,

Table 2A. pIGF-IR Index Variability by Age in Differentiated Thyroid Cancers

		Median pIGF-IR	Mean pIGF-IR	P value
Follicular	<45 yrs (7)	948	1,273	0.84
	>45 yrs (10)	708	794	
Papillary	<45 yrs (7)	91	2,341	0.25
	>45 yrs (4)	64	59	
Differentiated	<45 (14)	99	1,807	0.71
	>45 (19)	125	451	

our analysis indicated that among those patients with differentiated thyroid cancer, those without lymph node metastases have a significantly ($P = 0.03$) higher pIGF-IR index. But no significant difference was noted among those patients with poorly differentiated thyroid cancer with or without lymph node metastasis ($P = 0.12$) (Table 2B).

Discussion

Auto-phosphorylation of IGF-IR receptor is the first step in a cascade of reactions that results in the stimulation of cell proliferation, apoptosis, or regulation of mitogenesis in response to IGF-I treatment. To our knowledge, no previous study has looked at pIGF-IR expression in human thyroid cancers or analyzed its role in the progression of the disease. In this retrospective histopathologic and phenotypic study, we have evaluated the clinico-pathological significance of pIGF-IR expression in a large cohort of different thyroid carcinomas. Our results indicate a strong association between the loss of pIGF-IR expression and poorly differentiated thyroid carcinoma, suggesting that pIGF-IR levels may be a useful predictive marker of thyroid tumor progression.

Since all commercially available pIGF-IR antibodies cross react with phosphorylated insulin receptor (pIR), our initial efforts were focused on testing the cross reactivity of phosphorylated IGF-IR β (Tyr 1131)/IR (Tyr 1146) antibody (Ab) from cell signaling with other known tyrosine kinase receptors. In our hands this Ab detected a single band of 97 kD specific to pIGF-IR in majority of thyroid carcinoma cell lines studied when grown in the presence of low IGF-I containing media (Fig. 2f) or 10% serum. But it is likely that the gel resolution was not high enough to separate the 95 kD band of pIR from the 97 kD band of pIGF-IR. Moreover the two proteins are so highly homologous that immunohistochemical methods cannot distinguish the two receptors. Then there was the issue: Is pIR expressed in thyroid tissues and cell lines in high enough levels to interfere with our analysis? How does the pattern of pIR compare to pIGF-IR in thyroid cancer cell lines? Further investigations into these issues have demonstrated that pIR expression in FTC cell lines was lower compared to pIGF-IR (Fig. 2f) and that the pIGF-IR signal obtained with this antibody was essentially resulting from IGF-IR and not the IR receptor (Fig. 2g). These results suggested that the phosphorylation signal

Table 2B. p-Insulin Like Growth Factor Receptor I Tyrosine Kinase Expression vs. Patient Outcome

Patient group	n	Median pIGF-IR index	P value
Differentiated thyroid cancer with no lymph node metastasis	21	471.3	0.0329*
Differentiated thyroid cancer with lymph node metastasis	12	78.51	
Poorly differentiated thyroid cancer with no lymph node metastasis	7	37.5	0.1208
Poorly differentiated thyroid cancer with lymph node metastasis	15	69.07	

* Statistically significant ($P < 0.05$).

obtained in our study with the pIGF-IR/IR antibody can be assumed to reflect pIGF-IR expression in thyroid carcinoma cells and that IR receptor only marginally influences the phosphorylation signal after IGF-I stimulation. This assumption is certainly substantiated by the immunoprecipitation studies (Fig. 2g), where a large amount of total protein was being pulled down with the IR antibody and yet it failed to demonstrate a phosphorylation signal that was in any way near that obtained with the pIGF-IR antibody. When this is compared to IHC assays, it is important to understand that the pIGF-IR antibody encounters much lower concentration of the protein per cell basis in IHC assays. Moreover, the protein is distributed on the surface of individual cells and only membrane specific pIGF-IR is amenable to histochemical detection, and hence, it is expected that pIR signal would be considerably less pronounced than that seen in immunoprecipitation experiments. Nonetheless, even if the IHC data obtained with this antibody were to be interpreted to encompass staining specific to both pIGF-IR and pIR, it would not affect the outcome of this study as it has been established that IGF-I can signal through IGF-IR, IR and IGF-IR/IR hybrid receptors (38, 39).

It has also been reported that this Ab cross reacts with other receptor tyrosine kinases such as PDGF, FGF and EGF receptors, ErbB2 and c-Met in cells treated with 100 nM IGF-I. However, it is important to recognize that tumor cells grow in conditions better recapitulated by thyroid cancer cell lines grown in 10% serum or low IGF-I environment rather than those grown in the presence of high IGF-I levels. Accordingly, when majority of thyroid cancer cell lines detected a single band of 97 kD with this Ab when cultured in low IGF-I containing media, we continued to use this Ab for archival assessment once its specificity was established under different experimental conditions.

Deconvolution imaging of IGF-I treated cells on coverslips with this antibody localized the phosphorylated receptor on the membrane supporting its physiological role

of providing docking sites for other downstream regulators and tethering them to the membrane as has also been reported in *Pcmt1*^{-/-} mice particularly within the subgranular zone of the dentate gyrus (40). Occasionally, some fields also exhibited cytoplasmic or perinuclear staining of pIGF-IR. Since the perinuclear staining was also seen in cells cultured in serum-free media, it is possible that part of this staining may be the result of non-specific binding of the Alexa 488 conjugated antibody. Although, perinuclear staining of many receptors, such as the PDGF receptor, is often observed and is consistent with early endosomal/recycling membrane associated compartments in cells (41). Membranous localization of pIGF-IR was also evident in many FTC and some PTC samples analyzed in this study.

Accurate quantitative immunohistochemical (IHC) data are important to reach correct treatment decisions in today's clinical paradigm of individualized medicine. Thus, for pIGF-IR expression to be a reliable indicator of disease progression, there is a clear need to perform IHC staining that can be quantitatively interpreted. Accordingly, efforts were made to carry out independent experimental analysis to unequivocally establish the correlation between pIGF-IR antigen expression and IHC as demonstrated by Lehr *et al.* in their estrogen receptor (ER) quantitation studies (42). However, lack of a reliable quantitative method for phospho-IGF-IR expression and the confounding problem of possible IR cross reactivity, made it difficult for us to adopt their technique for these studies. Moreover, ER-staining was exclusively nuclear with almost all the nuclei being of relatively uniform size. Therefore, it was perhaps easier to automate the comparison of staining intensities within and across different samples using standard software like Photoshop. In contrast, pIGF-IR staining in the present study varied considerably both within and amongst samples. The stained regions involved cytoplasmic, membranous and/or occasionally perinuclear/nuclear components. Hence, just measuring the nuclei or selecting the stain intensity alone was not sufficient. Instead, additional parameters such as the total area (A), count (N) and the stain density (D) had to be measured and normalized to obtain per unit area values (D/A & N/A) that allowed uniform comparisons within and across samples. However, this in itself was not sufficient either. Due to significant variations in cell types or cell numbers across the array samples, even the normalized D/A or the N/A parameters could vary between samples, thereby making them unreliable measures by themselves. This was overcome by defining the pIGF-IR index as the product of two individual parameters output by the Image Pro software [(D/A)*(N/A)]. We also envisaged that combining the advantages of tissue microarrays that allow rapid screening of multiple tissue samples under uniform staining and scoring conditions with those of digital analysis and quantitation of TIFF images using the powerful tools of the Image Pro 6.0 would yield the most unbiased values of the product of D/A and N/A. But despite our best efforts, it is very likely that the pIGF-IR index may not have a linear

relationship with staining intensity. Although, the close agreement between the visual semi-quantitative findings for pIGF-IR (Fig. 3 and Table 1) and the results of quantitative image analysis detailed in Figure 5 appear to establish the validity of our approach.

Using this quantitative analysis, the mean pIGF-IR index of FTC and PTC was found to be 1,000 and 1,500, respectively, as opposed to <400 for most anaplastic and medullary thyroid carcinomas, thereby suggesting that inactivation/loss of expression of the receptor correlates with poorly differentiated thyroid carcinoma. Furthermore, detailed histochemical analysis revealed that in normal and pathological human thyroid specimens, pIGF-IR was expressed to various degrees. While it was virtually absent in anaplastic thyroid tumors and was very low in normal thyroid specimens, we observed major differences in the expression and sub-cellular localization of pIGF-IR between different thyroid carcinomas. A majority of the FTC and some PTC showed high cytoplasmic and membranous pIGF-IR expression. Interestingly, some FTC showed nuclear localization of pIGF-IR. As these sections were not analyzed confocally, the nuclear localization claims could not be substantiated. However, there is one report in rat testis where Colon *et al.* have demonstrated phosphorylated IGF-IR staining in the nucleus of Leydig cells, Sertoli cells and spermatogonia (43). Since all of these cells are highly proliferative, it is possible that in certain cell types, pIGF-IR is translocated to the nucleus in response to hormonal or other external stimuli to modulate cell proliferation. However, the authors did not comment on the nuclear localization of the protein in their report. A similar logic may be applicable for some follicular thyroid cancers, where phosphorylated IGF-IR may move to the nucleus to modulate the proliferative or apoptotic potential of these cells.

In agreement with previous published reports (6, 23), despite differential expression of pIGF-IR between different thyroid cancers, the total IGF-IR levels were unaltered between different thyroid tumors and were always higher than the normal adjacent tissue. It is intriguing to note that although the receptor is expressed at high levels in all the histological subtypes of thyroid cancer, it is functional only in some follicular or papillary thyroid carcinoma. Some of the possibilities of why the ligand receptor communication is interrupted may be: 1) the receptor is mutated in late stage cancers; 2) there is activation of phosphatases or additional mutations that render the receptor dephosphorylated or that 3) the receptor phosphorylation is blocked by unknown mechanisms. However, pIGF-IR upregulation in FTC is an interesting observation on its own as it may well be a potential target for therapy for patients with this disease. Although with the limited number of FTC cases available for statistical analysis in this study, it would be premature to draw this conclusion.

Anaplastic thyroid cancer unlike other thyroid carcinomas (FTC, PTC and HCC) quickly metastasize to distant

sites (3). Little is known about the molecular determinants of aggressive growth in these tumors. At present, there are no prognostic factors that are useful in an individual patient that help in distinguishing a subset of patients with aggressive disease. If such prognostic factors can be identified, more targeted antitumor treatment can be carried out in this subset to improve survival. Accordingly, we sought to determine if anaplastic tumors evolve from their differentiated counterparts and if pIGF-IR levels are indicative of this transition. To address this, pIGF-IR expression was analyzed in specific thyroid tumors where the anaplastic and differentiated thyroid carcinomas co-existed. Interestingly, pIGF-IR expression was intact in the differentiated component but was lost in anaplastic areas. This was true for the three of the five cases analyzed; once again reaffirming our observation that low pIGF-IR level may be a reliable indicator of aggressive disease. This could be further substantiated by comparing the pIGF-IR index of differentiated and all other thyroid tumors. Due to the small numbers of individual tumors available for statistical comparisons, the differentiated tumors (follicular, papillary and Hurthle) were grouped together and all the other thyroid cancers (anaplastic and medullary) were grouped into a separate group for comparison. Based on this stratification, our analysis indicated that the median pIGF-IR index of differentiated thyroid cancers was significantly higher than the median index of most other poorly differentiated thyroid carcinoma. Similarly, median pIGF-IR index of differentiated thyroid tumors with no lymph node metastasis was higher than those with lymph node metastasis, indicating that IGF-IR acts directly to activate downstream signaling rather than through transactivation of other tyrosine kinase membrane receptors. A role for pIGF-IR in patient response has been suggested in previous studies (44). Ulfarsson *et al.* (2005) reported that growth of craniopharyngioma cell lines with low IGF-IR expression was only slightly affected by IGF-IR inhibition (45). Similarly, herceptin resistance has been shown to occur through the activation of IGF-IR (46). Taken together with our results, it may be that analysis of pIGF-IR expression and downstream signaling may be critical for an accurate assessment of potential anti-IGF-IR or similar targeted therapy response of thyroid cancer patients. On the other hand, because of the low expression of pIGF-IR in normal thyrocytes, loss of expression of pIGF-IR in poorly differentiated thyroid cancer poses a challenge to use it as a diagnostic marker, especially when simple histology can provide the same information. The promise of the present study, however, lies in incorporating pIGF-IR screening to guide the choice of optimal adjuvant treatment after surgery for those thyroid carcinoma patients who may benefit from anti-IGF-IR or similar targeted therapy.

In summary, our analysis indicates that pIGF-IR is upregulated in a majority of follicular thyroid carcinomas, suggesting it may be a potential target for therapy for patients with this disease. In addition, since low pIGF-IR

expression was found to correlate with aggressive human thyroid carcinoma, our data also suggests that IGF-IR may not be needed for progression of anaplastic thyroid carcinoma, possibly because other cell signaling pathways are activated obviating the need for IGF-IR signaling. Finally, none of our observations overrules the possibility that pIGF-IR may be important for differentiation of thyroid tissues and is lost with disease progression. Additional mechanistic studies would be necessary to substantiate this hypothesis.

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