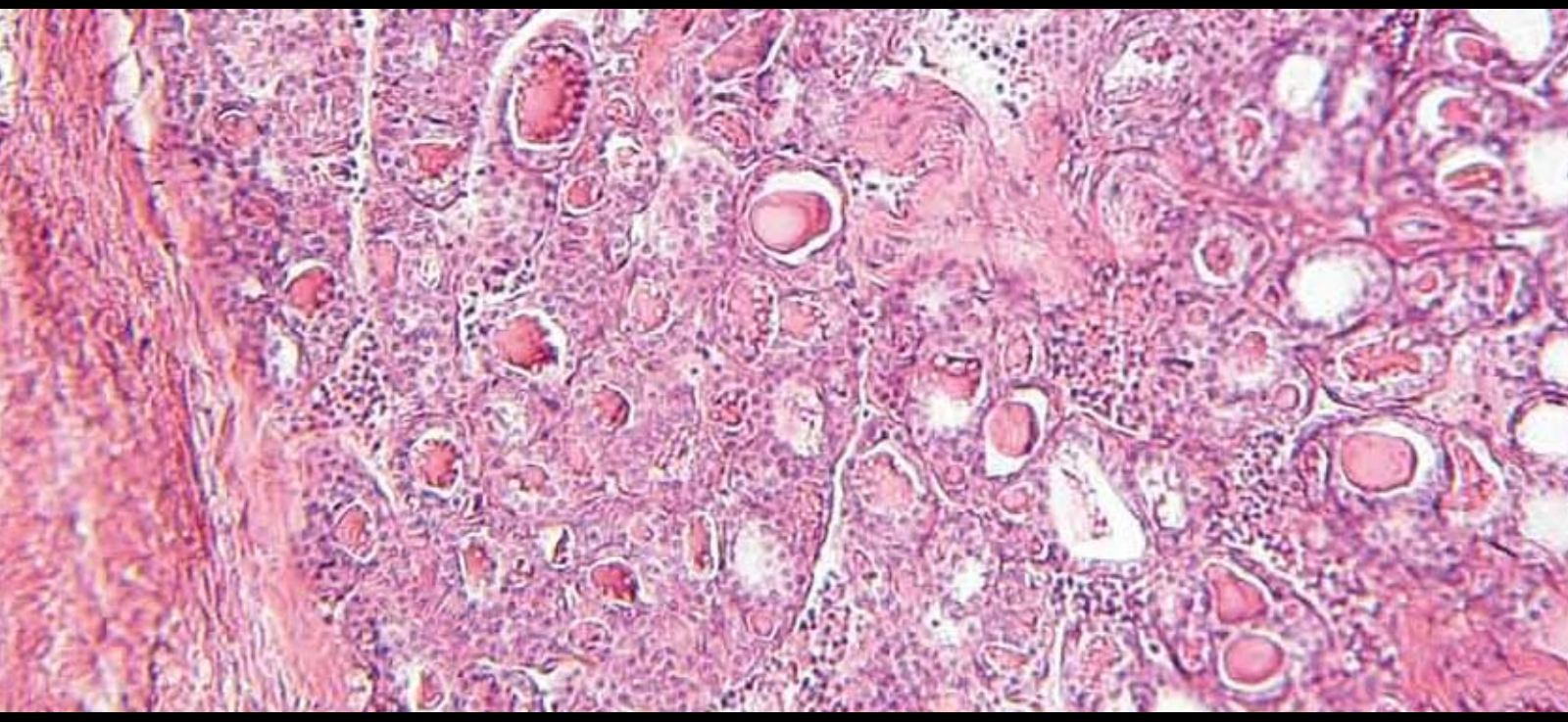
Journal of Thyroid Research

Biomarkers in Thyroid Tumor Research: New Diagnostic Tools and Potential Targets of Molecular-Based Therapy

Guest Editors: Oliver Gimm, Maria Domenica Castellone, Cuong Hoang-Vu, and Electron Kebebew



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Editorial

Biomarkers in Thyroid Tumor Research: New Diagnostic Tools and Potential Targets of Molecular-Based Therapy

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Thyroid cancer's incidence has increased dramatically over the last years and it now accounts for 2.6% of all new cancers when epithelial skin cancers are excluded. This increase has been partially attributed to improved screening methods, mainly ultrasonography identifying subclinical tumors. However, the evidence that also the number of more advanced tumors and thus thyroid-cancer-associated mortality is increasing suggests that the underlying cause is not completely understood [1]. The molecular basis of thyroid carcinogenesis has been widely investigated, leading to the discovery of oncogenes such as *BRAF*, *RAS*, and *RET* as major players in tumor development and progression. Recently, however, it has been shown that the rate of *BRAF* mutations that have been associated with a more aggressive tumor type in papillary thyroid carcinomas has increased over time [2]. This oncogenic addiction to specific genetic changes has highlighted thyroid cancer as an ideal model for targeted therapy using biological inhibitors and small molecule inhibitors of *RET* and *BRAF*, which have already reached the clinic [3]. Our ability to detect persistent and recurrent malignant thyroid tumors has also improved as a result of more sensitive biochemical tumor marker assays, although it is unclear whether these higher rates indicate clinically significant disease.

The articles published in this special issue shed new light on the molecular mechanisms involved in thyroid tumors and propose novel diagnostic and therapeutic targets against this disease. The results could have important clinical ramifications in the management of patients with thyroid tumors.

Thyroid follicular neoplasms represent a diagnostic challenge, as thyroid fine-needle aspiration biopsy cannot distinguish benign from malignant tumors in up to 30% of cases. An important goal would be to identify diagnostic and prognostic markers that could help avoid unnecessary surgeries, which can result in complications such as recurrent laryngeal nerve palsy and, in the case of bilateral thyroid surgery, hypoparathyroidism. The main preoperative diagnostic challenge concerns follicular thyroid lesions. Traditionally, the distinction between follicular thyroid adenomas and carcinomas is made histologically when there is evidence of capsular and/or vascular invasion. Preoperative markers are, therefore, sought after. Prabakaran and colleagues analyzed the expression levels of various genes in archival thyroid tissue. They found that *RAP2A*, a member of the ras family that is closely related to *Ras*, was significantly associated with higher expression in microdissected carcinoma cells that have invaded through the thyroid capsule and entered blood vessels than in thyroid tumor cells growing under the capsule. They concluded that *RAP2A* may be a biomarker associated with invasion of thyroid follicular cells. If their finding can be confirmed in larger studies, the evaluation of this marker in fine-needle aspiration aspirates may be warranted and informative.

In another study, T. Kobawala and colleagues report on the clinical utility of interleukin-8 (IL-8) and interferon-alpha in the diagnosis of thyroid diseases. IL-8 is a well-characterized chemotactic cytokine that is produced by macrophages and other cell types such as epithelial cells. Interferon-alpha is an antiviral and anti-proliferative agent that can stimulate both macrophages and natural killer cells to elicit an anti-viral response. It has also been shown to be active against tumors. The authors have analyzed 88 patients with various types of thyroid diseases. They found overall increased levels of serum IL-8 in patients with thyroid carcinoma as compared to various benign disorders. While there is a large overlap between the serum IL-8 levels in patients with malignant and benign thyroid diseases, these data support the idea that chronic inflammatory processes may play an important role in the development and progression of cancer and provide new therapeutic targets in malignant thyroid tumors.

Among the novel diagnostic markers proposed in this issue, M. Hedayati and colleagues report on leptin levels in 83 patients with papillary thyroid and 90 healthy control persons. Leptin is a neuroendocrine hormone that has a variety of different effects including effects on the immune system and the thyroid gland. The authors found significantly higher serum leptin level in patients with thyroid cancer as compared to the control group including a difference by sex.

In another article, l. Giovanella and colleagues have investigated thyroglobulin as a marker of recurrent or persistent disease by analyzing the levels of thyroglobulin in lymph nodes in order to analyze the presence of metastatic thyroid tissue. Thyroglobulin is a protein synthesized by the thyroid gland and stored in the follicular lumen. Since, under normal conditions, thyroglobulin can only be found inside the thyroid gland, it has been used for decades as a sensitive tumor marker to detect persistent and recurrent disease in patients with differentiated thyroid cancer of follicular cell origin (the most common types of thyroid cancer). Thyroglobulin can, however, also be used preoperatively to guide the extent of surgery in patients with persistent or recurrent disease. They found that thyroglobulin levels higher than 1.1 ng/mL in aspirates from cervical lymph nodes were highly sensitive for the presence of metastases. Both sensitivity and specificity were higher than that of cytology from fine-needle aspirates. This approach can thus help guide the need and extent of lymph node dissection in patients who have suspicious lymph nodes on ultrasound but with inconclusive cytologic findings either in the preoperative setting or during follow up.

Finally, N. Burrows and colleagues report on the role of hypoxia-inducible factor-1 (HIF-1) in thyroid carcinoma aggressiveness. HIF-1 regulates the expression of several genes that have been shown to be involved in tumor cell survival, progression, metastasis, and even resistance to both chemotherapy and radiotherapy. In this study, the authors show that both hypoxia and oncogenic signaling pathways can induce HIF-1 in thyroid carcinoma. Based on their analysis, they also suggest that targeting HIF-1 might improve the poor therapeutic response of advanced thyroid carcinoma to radiotherapy.

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Maria Domenica Castellone
Cuong Hoang-Vu
Electron Kebebew

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Research Article

Rap2A Is Upregulated in Invasive Cells Dissected from Follicular Thyroid Cancer

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The development of molecular biomarkers (BMs) of follicular thyroid carcinoma is aimed at advancing diagnosis of follicular neoplasm, as histological examination of those tumors does not lend itself to definitive diagnosis of carcinoma. We assessed the relative levels of expression of 6 genes: *CCND2, PCSK2, PLAB, RAP2A, TSHR, and IGF-1R* in archived thyroid tissue. The quantitative real-time PCR analysis revealed a significant change in 3 genes: *PSCK2* (a 22.4-fold decrease, $P = 2.81E - 2$), *PLAB* (an 8.3-fold increase, $P = 9.81E - 12$), and *RAP2A* (a 6.3-fold increase, $P = 9.13E - 10$) in carcinoma compared with adenoma. Expression of *PCSK2* was equally low, *PLAB* was equally high, whereas *RAP2A* expression was significantly higher (25.9-fold, $P = 0.039$) in microdissected carcinoma cells that have invaded through the thyroid capsule and entered blood vessels than in thyroid tumor cells growing under the capsule. Thus, *RAP2A* appeared as a unique and worthy of further evaluation candidate BM associated with invasion of thyroid follicular cells.

1. Introduction

Differentiated thyroid carcinomas originating from the follicular epithelium have a papillary (range, 65–88%) and a follicular (range, 9–23%) histotype [1]. Although follicular thyroid carcinomas (FTCs) are the second most common differentiated thyroid cancers, they are more aggressive than papillary thyroid carcinomas (PTCs) and invade into the capsule (minimally invasive) and veins (angioinvasive) within the thyroid gland. Importantly, mortality is related to the degree of invasion [2]. Furthermore, FTC has a greater rate of recurrence and is frequently associated with distant metastasis to the lung, bone, brain, and liver [3, 4]. Total thyroidectomy represents the dominant method of surgical treatment for follicular neoplasms diagnosed preoperatively by fine needle aspirates (FNAs). Distinguishing follicular adenoma from minimally invasive or encapsulated angioinvasive carcinoma in FNA can be extremely challenging [3, 5]. Gene and microRNA (miRNA) expression profiling are being investigated to identify potential BMs differentiating benign from malignant follicular tumors [6, 7]. Such BMs might be clinically useful to help predicting follicular thyroid malignancy and reduce the frequency of surgical procedures by identifying those patients with benign lesions who do not require surgical excision. So far, however, global genetic screens have not improved preoperative diagnosis of FTC. Hence, novel approaches are necessary to identify potential preoperative molecular BMs to facilitate the diagnosis of FTC. One of the approaches could be discovering specific molecular BMs associated with invasion of thyroid follicular cells.

2. Materials and Methods

2.1. Thyroid Tissue. Cases of follicular-patterned thyroid cancer are quite rare; even lesser is the number of remaining samples available for research. For this study, a unique cohort of patients diagnosed with follicular-patterned thyroid cancer was identified on review of medical records from the Hospital of University of Pennsylvania between 1992 and 2007.

After reexamination of 16 available formalin-fixed, paraffin-embedded (FFPE) tissues (for histological presence of vascular and/or capsular invasion) and initial determination of integrity of total RNA in the tissue scrapes, we found that two samples had degraded RNA, one sample had too little RNA to be amplified by *in vitro* transcription (IVT), in two samples the areas of invasion had already been cut through, and 10 specimens fully met study's criteria. Subsequently, the study was performed in specimens from 8 patients diagnosed with FTC, 1 patient diagnosed with FTC-Hürthle cell carcinoma (HCC), 1 patient diagnosed with HCC, and 10 patients diagnosed with follicular thyroid adenoma (FTA). Groups of patients with FTA (mean age, 52.4 ± 16.2 SD years) and follicular thyroid malignancy (mean age, 50.8 ± 13.1 SD, years) were age matched (Table 1). Ten normal FFPE thyroid samples were from patients who underwent surgery after diagnosis of larynx squamous cell carcinoma (mean age, 62.4 ± 7.0 SD, years). Histopathological analysis of all tissues was performed by a surgical pathology fellow (JG) and confirmed by a thyroid pathologist (Dr. Virginia LiVolsi). The study protocol was approved by the University of Pennsylvania Institutional Review Board committee.

2.2. Thyroid Tissue Analysis: RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR (Q-RT-PCR). RNA was extracted from the normal, adenoma, and cancer tissue scrapes using the Absolutely RNA FFPE kit (Stratagene, La Jolla, CA). In addition, RNA was extracted from a snap frozen thyroid carcinoma using the High Pure RNA Tissue kit (Roche Diagnostics, Indianapolis, IN) to use as a positive control and generate a standard curve for all subsequent PCR reactions. Integrity of RNA from a snap frozen tissue was determined by 260 to 280 nm ratio using a DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA). Integrity of the scraped tissue RNA was assessed by Q-RT-PCR using 3′*ACTB* and 5′*ACTB* primers (Table 2) and the Paradise Sample Quality Assessment Kit (Molecular Devices, Sunnyvale, CA). 10–100 ng of the scraped tissue RNA or 500 ng of a positive control RNA were reverse-transcribed into single-stranded cDNA using the first-strand cDNA synthesis kit (Roche Diagnostics, Indianapolis, IN). cDNA synthesis was carried out in a 20 μL reaction mix containing 5 mM $MgCl_2$, 1 mM dNTPs, 0.04 units of random primers $p(dN)_6$, 50 units of RNase inhibitor, and 20 units of Avian Myeloblastosis Virus (AMV) reverse transcriptase. Q-RT-PCR was performed using 3 μL of the first-strand cDNA with 1 μM of the housekeeping gene, *ACTB*, or target gene-specific primers (Table 2) using the LightCycler 2.0 (Roche Molecular Biochemicals, Mannheim, Germany) instrument and the LightCycler Fast Start DNA Master^{PLUS} SYBR Green 1 kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. PCR parameters were a 10 min preincubation time at 95°C followed by 45 cycles of denaturation (10 sec at 95°C), annealing (10 sec at 55°C), and extension (25 sec at 72°C). A standard curve for each of the target and housekeeping gene was generated for every PCR run to determine levels of gene expression. All reactions were performed in duplicates with at least three repeats. Relative expression of each target gene in all samples was determined as a ratio of mRNA of target gene to mRNA of the housekeeping gene as described in [8].

2.3. Laser-Capture Microdissection (LCM). LCM was performed as in the frozen thyroid tissue samples [9] with modifications. Briefly, FFPE blocks of FTC were cut into 7 μm thick sections, mounted on RNase-free membrane slides (MMI, Manchester, NH), deparaffinated with d-limonene, rehydrated with sequential washes of 100%, 95%, and 75% ethanol, and then washed in nuclease-free water. Next, slides were stained with Paradise staining solution (Arcturus Engineering Inc., Mountain View, CA), dehydrated in Xylene for a minimum of 5 min, and air dried. Cells from areas of angioinvasion, capsular invasion, and tumor under the capsule were dissected onto Capsure HS LCM Caps (MMI, Manchester, NH) using a Laser Capture Micro-dissection microscope Nikon ECLIPSE TE 2000-S and MMI Cell Tools software (MMI, Manchester, NH).

2.4. Dissected Thyroid Cancer Cell Analysis: RNA Extraction and Amplification, cDNA Synthesis, and Q-RT-PCR. RNA was extracted from laser-captured microdissected cancer cells using the Absolutely RNA FFPE kit (Stratagene, La Jolla, CA). Assessment of the integrity of cellular RNA was performed by Q-RT-PCR using 3′*ACTB* and 5′*ACTB* primers. Amplification of RNA from laser-captured microdissected cells was performed using the Ambion MessageAmp II aRNA kit (Ambion, Austin, TX). We used the IVT method which is based on the linear amplification protocol developed and validated previously [10, 11]. The advantage of such a technique is that the product of the reaction is unable to act as template and the yield of any individual species within a mixed population is for the most part determined by the template concentration that is not changed. Amplification was linear when at least 1ng of LCM RNA was used as the input for IVT. Two rounds of linear amplification of the mRNA fraction of at least 1 ng total cellular RNA were performed. First-strand cDNA synthesis yielded cDNA incorporating a T-7 promoter sequence. This cDNA was converted to a double-stranded transcription template by a second-strand synthesis reaction utilizing exogenous primers that yielded double-stranded cDNA. Double-stranded cDNA was then used as a template for IVT with T7 RNA polymerase to generate amplified antisense RNA (aRNA). Integrity of aRNA samples was determined as described above. aRNA samples with a 3′*ACTB* to 5′*ACTB* ratio of ≤20 or a 260 to 280 nm ratio between 1.8 and 2.2 were used for further experiments. 10–100 ng of aRNA was converted to cDNA using 1 μM target gene-specific primers and the first-strand cDNA synthesis kit (Roche Diagnostics, Indianapolis, IN). Q-RT-PCR was then performed for the housekeeping gene, *ACTB*, and target genes as described above. After all the reactions were performed in duplicates with at least three repeats, relative expression of target genes was determined.

2.5. Statistical Analysis. Data were reported as mean ± standard error of the mean (SEM). Comparisons between

Table 1: Clinical data of patients from whom follicular thyroid tumor tissue samples were collected.

Gender	Age, years	FNA diagnosis	Nodule size, cm	Final diagnosis	Invasion vascular/capsular
F	48	Follicular neoplasm	5.6 × 4.0 × 2.4	FTC	+/+
M	64	NA	NA	FTC	+/+
F	51	Follicular neoplasm	2.0 × 1.5 × 1.0	FTC	+/+
F	48	Follicular neoplasm	3.6 × 3.0 × 2.2	FTC	+/+
F	56	NA	NA	FTC	+/+
M	25	NA	NA	FTC	+/+
F	40	Follicular neoplasm	3.0 × 2.5 × 1.5	FTC	+/+
F	59	Follicular neoplasm	3.8 ×1.8 × 1.7	HCC, angioinvasive	+/+
M	45	Follicular neoplasm	3.8 × 3.1 × 2.5	FTC	+/+
M	72	Benign goiter	7.0 × 5.3 × 4.3	FTC and HCC	−/+
M	45	Follicular neoplasm	3.2 × 3.0 × 3.0	FTA	−/−
M	76	Follicular neoplasm	3.1 × 5.3 × 2.6	FTA	−/−
M	41	Follicular neoplasm	3.1 × 2.1 × 1.7	FTA	−/−
F	76	Follicular neoplasm	3.1 × 2.0 × 1.5	FTA	−/−
M	64	Follicular neoplasm	4.5 × 4.4 × 3.3	FTA	−/−
M	60	NA	3.8 × 3.0 × 5.0	FTA	−/−
F	28	Follicular neoplasm	2.5 × 2.2 × 2.0	FTA	−/−
F	52	Follicular neoplasm	5.0 × 4.0 × 3.5	FTA	−/−
F	44	Follicular neoplasm	4.5 × 3.5 × 2.9	FTA	−/−
F	38	Follicular neoplasm	3.1 × 1.9 × 1.5	FTA	−/−

N/A: records were not available.

Table 2: A list of tested genes and encoded by them proteins, including the gene and protein accession numbers and corresponding intron-spanning primers used for Q-RT-PCR.

Gene	GenBank	Primer sequence	Protein	Swiss-Prot
CCND-2	AY888219	S_CAC TTG TGA TGC CCT GAC TG AS_ACG GTA CTG CTG CAG GCT AT	G1/S-specific cyclin-D2	P30279
PCSK2	BC040546	S_AGC ATA CAA CTC CAA GGT TGC AS_GCT GTA GAT GTC AAT CAG CTG TG	Proprotein convertase subtilisin/kexin type 2	Q8IWA8
PLAB	BC008962	S_CAA CCA GAG CTG GGA AGA TT AS_AGA GAT ACG CAG GTG CAG GT	Placental bone morphogenetic protein	Q99988
RAP2A	NM 021033	S_AGA TCA TCC GCG TGA AGC AS_CCC CAC TCT TCA GCA AGG	Ras-related protein-2a	P10114
TSHR	BC024205	S_GGA TAT GCT TTC AAT GGG ACA AS_GCA TCT TTG TCA ATA ACT GTC AGG	Thyroid-stimulating hormone receptor	P16473
IGF1R	NM000875	S_GTG AAA GTG ACG TCC TGC ATT TC AS_CCT TGT AGT AAA CGG TGA AGC TGA	Insulin-like growth factor I receptor	P08069
3′ACTB	NP001092	S_TCC CCC AAC TTG AGA TGT ATG AAG AS_AAC TGG TCT CAA GTC AGT GTA CAG G	Actin, cytoplasmic 1	P60709
5′ACTB	NP001092	S_ATC CCC CAA AGT TCA CAA TG AS_GTG GCT TTT AGG ATG GCA AG	Actin, cytoplasmic 1	P60709

S: sense, forward primer 5′ to 3′; AS: antisense, backword primer 3′ to 5′.

normal, benign, and cancer groups were made by using one-way analysis of variance (ANOVA). A value of $P < .05$ was considered as statistically significant.

3. Results and Discussion

In this exploratory study, we investigated the expression of the potential thyroid cancer-discriminating genes: *CCND2, PCSK2, PLAB, RAP2A, TSHR*, and *IGF-1R* (Table 2) by comparing their expression at the mRNA levels in the normal thyroid tissue, benign follicular lesions, and follicular carcinomas. The target genes have been chosen based on importance of abnormal expression and activities of the thyroid-stimulating hormone receptor (TSHR) and insulin- like growth factor type I receptor (IGF-IR) in thyroid tumorigenesis [12, 13] and the results of gene micro-array analysis showing differential expression of *CCND2, PCSK2, PLAB, RAP2A* in FTC [14, 15]. We found no statistically significant difference in *CCND2, TSHR*, and *IGF-1R* mRNA expression between the groups of normal thyroid, benign and malignant thyroid cancer (Figure 1(a)). There was no difference between the levels of mRNA expression of *PCSK2, PLAB*, and *RAP2A* between normal thyroid and FTA (Figure 1(b)). Interestingly, however, in the Q-RT-PCR analysis of FTA and cancer, *PSCK2* was markedly downregulated (22.4-fold), whereas *PLAB* and *RAP2A* were notably upregulated (8.3- and 6.3-fold, resp.) in cancer. Furthermore, a comparative mRNA expression analysis revealed a statistically significant difference in *PCSK2* ($P = 2.81E-2$), *PLAB* ($P = 9.81E-12$) and *RAP2A* ($P = 9.13E-10$) expression between groups of benign and malignant thyroid tumors (Figure 1(b)). Thus, in tested age-matched cohort of 20 patients diagnosed with follicular-patterned thyroid neoplasm, the levels of *CCND2, TSHR*, and *IGF-1R* were not significantly different; *PLAB* and *RAP2A* were significantly increased, whereas *PCSK2* was significantly decreased in cancer compared with adenoma. Overexpression of *PLAB* and *PCSK2* as well as down-regulation of *CCND2* has been found in frozen sections of FTC [15]. Weber et al. proposed that a combination of those three genes allowed the accurate molecular classification of FTC versus FTA with a high specificity and sensitivity. However, Shibru et al. were unable to confirm the diagnostic accuracy of the 3-gene assay either in frozen tissue or in FNAs [16]. The difference is likely attributed to the difference in types of analyzed tissue, as Shibru et al. compared a benign group represented by hyperplastic nodule, FTA, Hürthle cell adenomas (HCAs) with a collective group of thyroid malignancies, including FTC, PTC, follicular variant of PTC, HCC. Although FTC and HCC may carry similar molecular alterations [17], PTC has distinct genetic features (somatic alterations such as *RET/PTC* translocation and *BRAF* mutations) that distinguish them from FTC [6]. Our data are in close agreement with the findings reported by Weber et al. except that the observed down-regulation of *CCND2* in cancer has not reached statistical significance.

Intratumoral heterogeneity is well-recognized phenomenon [5, 18, 19], so it is plausible that within areas of invasion tumor cells are genetically different from the rest of tumor.

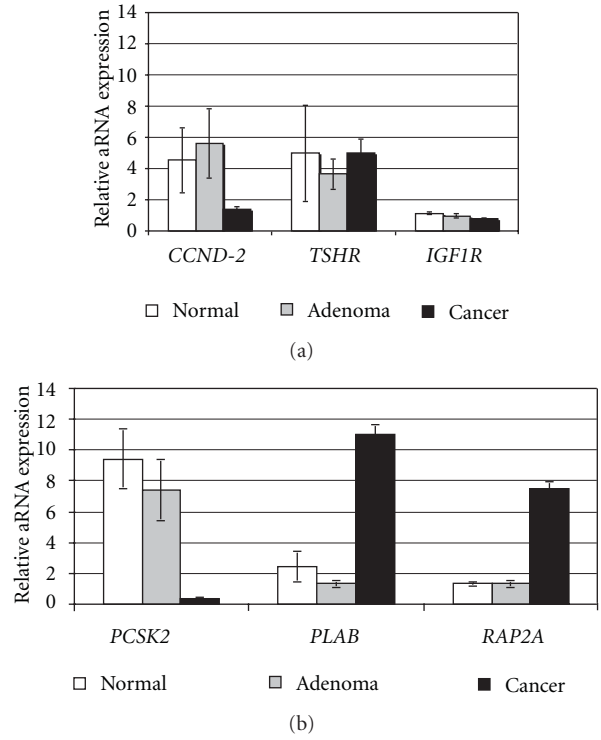

Figure 1: Evaluation of *CCND2, TSHR, IGF-1R* (a) and *PCSK2, PLAB, RAP2A* (b) mRNA expression by Q-RT-PCR in tissue samples from the human thyroid (normal, benign, cancer). All PCR reactions were performed in duplicates with at least three repeats. Mean of normalized expression level of mRNA in each analyzed group ($n = 10$) are shown. Bar, SEM. $^{*}P = 2.81E-2$, $^{**}P = 9.81E-12$, and $^{***}P = 9.13E-10$.

Here, we tested the hypothesis that in thyroid malignancy differential expression of molecular BMs may be detected in thyroid follicular cancer cells invaded through the tumor capsule and entered into vasculature. The three genes (*PCSK2, PLAB*, and *RAP2A*) were selected for in-depth analysis because of their significantly different expression in cancers compared with adenomas (Figure 1(b)). To ensure the presence of invasion in freshly cut 7 μm thick sections of cancer tissue, a thyroid pathologist reviewed slides stained with hematoxylin and eosin and marked the areas of invasion using diagnostic criteria adopted in our institution [3]. Nine angioinvasive samples of thyroid cancers had both capsular and vascular invasion; one minimally invasive specimen had only capsular invasion. Eight out of ten specimens had more than one invasive focus. To selectively isolate population of thyroid carcinoma cells that have invaded the capsule to enter blood vessels and to compare them to the cells remained in the main tumor mass, we applied an LCM method as illustrated in Figure 2(a). After dissecting multiple areas, the captured cells from each of the cancer specimens were pooled in to two matched groups: (i) remained under the tumor capsule ("noninvasive" group) and (ii) invaded the capsule and/or entered blood vessels ("invasive" group). The total RNA was assessed in the captured tumor cells, and samples with an adequate amount of input RNA (>1.0 ng)

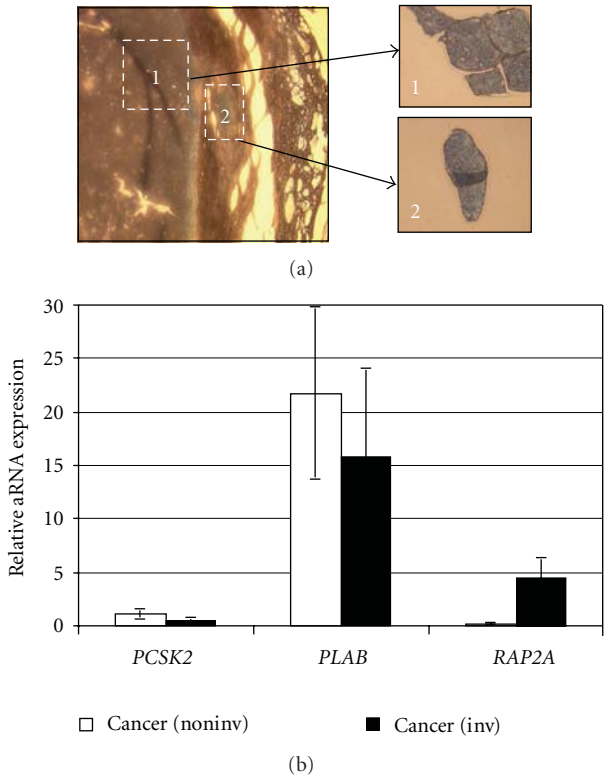

FIGURE 2: An example of laser-capture micro-dissection in follicular thyroid carcinoma. (a) FTC tissue before (left) and after collecting the groups of cells from the main tumor mass (inset 1) and angioinvasive area (inset 2). Original magnification, 200x. (b) Histogram, relative expression of *PCSK2, PLAB, RAP2A* in the cells collected from matched noninvasive and invasive areas of the same specimens ($n = 4$). Mean of normalized expression levels of aRNA in each group is shown. Bar, SEM. $^*P = 0.039$.

were subjected to two rounds of linear amplification by *in vitro* transcription to further increase the amount of RNA. Twice-amplified aRNA of high quality only was used for a cDNA preparation and Q-RT-PCR with specific primers for target genes. As expected from the analysis of tissue scrapes, *PSCK2* expression was low in the cells from the main tumor mass; it was insignificantly different ($P = 0.322$) in invasive cells dissected from the same specimens (Figure 2(b)). Likewise, *PLAB* expression was equally high in both types of dissected cells ($P = 0.698$). The results of the Q-RT-PCR analysis for *RAP2A* aRNA were intriguing, as the relative level of *RAP2A* expression was 25.9-fold higher ($P = 0.039$) in the cells dissected from areas of invasion. *RAP2A* encodes Ras-related protein 2a (Rap-2a), a member of the Ras family of small GTPases (Rap1a/b and Rap2a/b/c) that has been reported to induce cytoskeleton rearrangements promoting cell rounding and cell migration [20, 21]. Although activating mutations of Rap have not been reported, up regulation of Rap activating guanine nucleotide exchange factors [22, 23] and down regulation of Rap GTPase-activating proteins promoting Rap inactivation [24, 25] have been found in human tumors including thyroid carcinomas [26]. High levels of expression of Rap2, but not Rap1, have been detected in human thyroid cancer cell lines. Importantly, Rap2 protein expression was several fold higher in anaplastic than in well-differentiated papillary thyroid cancer cells [27]. Furthermore, increased Rap activity has been shown to promote carcinoma cells invasion *in vitro* and *in vivo* [28, 29]. We found up regulation of human gene encoding Rap-2a in follicular thyroid cancer tissue, particularly in the regions enriched with invasive cancer cells. It could be speculated that thyroid tumor cells "require" the genetic changes in *RAP2A*, in addition to *PSCK2* and *PLAB*, to allow them to invade and/or "maintain and flourish" in the nonnative areas of the tumor capsule and blood vessels.

4. Conclusions

We demonstrated the feasibility of combining LCM and Q-RT-PCR for analysis of gene expression in microscopic clusters dissected from FFPE thyroid tissue. Our study is a first and important step in the assessment of novel molecular BMs associated with invasion of follicular thyroid carcinoma cells, despite the relatively small sample size. Validation of diagnostic applicability of *RAP2A* requires a follow-up work in larger tissue sample sets.

Conflict of Interests

The authors declare that they have no competing interests.

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Research Article

Leptin: A Correlated Peptide to Papillary Thyroid Carcinoma?

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Introduction. Leptin as an adipose-tissue-related peptide hormone contributes to the control of food intake, energy expenditure, and other activities such as cell proliferation. Therefore, association of leptin level with thyroid cancer has been suggested recently. Considering that thyroid cancer is the most common endocrine cancer, the aim of this study was evaluation of leptin levels in thyroid cancer. *Materials and Methods*. 83 patients with papillary thyroid cancer (35 males and 48 females) with 90 healthy persons as control group (40 male and 50 females) were selected. serum thyroxine, thyrotropin, and leptin levels were determined in both groups. As a body fat tissue affects leptin level, so height and weight were measured and body mass index was calculated too. *Results*. There was no statistically significant difference in age, serum Thyroxine, and Thyrotropin levels. BMI in women was more than in men in both groups. Serum leptin levels in thyroid cancer group were significantly higher than control group ($P < 0.05$). *Conclusion*. The results of this study showed an acceptable association between the hormone Leptin levels with papillary thyroid cancer, so it may be considerad as a correlated peptide which may help in the diagnosis or confirmation of thyroid cancer beside in other specific tumor markers.

1. Introduction

leptin with 16 kDa molecular weight mainly produces by white adipose tissue cells [1]. So its level is proportional to the adipose tissue mass [2]. leptin as a neuroendocrine hormone has effects on the glucose metabolism, sexual maturation, reproduction, pituitary-adrenal axis, immune system, thyroid, and growth hormones level [3–6]. The association between this neuroendocrine hormone with obesity and some cancers has been proposed. Probably this hormone is an important risk factor in carcinogenesis, because obesity itself can promote tumorgenesis and is a risk factor for cancer over time [7, 8]. On the other hand, leptin plays an important role in the oxidation reactions such as fatty acid oxidation [9] and angiogenesis [10]. There are many reports concerning the effect of leptin on stimulation of cell mitosis and its involvement in carcinogenic stages of breast, clone, prostate, lung, kidney, and ovary cells [11–16]. Studies have shown that leptin by increase of cell proliferation and inhibition of apoptosis is involved in creating certain types of tumors [17–19].

leptin acts through its receptor on the cell surface and its receptor expression also increases following the activity of PI3K/AKT pathway and increases the activity of antiapoptosis molecules such as Bcl-XL and XIAP [20]. In some cancer cells, expression of leptin receptor levels and stimulation by leptin will lead to increase of cell proliferation [21]. leptin stimulates expression of some molecules such as CyclinD1, CDK2 and c-Myc that result in cell cycle progression and cell proliferation [21, 22].

The Important molecular pathways, such as JAK/STAT3, PI3K/AKT, ERK/MAPK, in many cancer cells can be activated by leptin/leptin receptors [21–24]. Furthermore, leptin with the induction of VEGF and VEGF-R2 molecules expression plays an important role in the tumorigenesis [25]. These molecules are involved in many malignancies such as colon, stomach, endometrial, ovarian, and breast cancer [26–30]. Additionally, increased serum levels of

leptin and its receptor have been associated with distant metastases, disease recurrence, and lower survival in patients with breast cancer [31]. Increased expression of leptin and its receptor in papillary thyroid cancer has been proved. This hormone probably through its receptor and activation of the PI3K/AKT pathway plays an important role in papillary thyroid cancer pathogenesis. It also seems that the oncogenic effects of leptin on papillary thyroid carcinoma cells are related to the stimulating cell proliferation and apoptosis inhibition. Involvement of thyroid hormones on basal metabolism and regulating appetite and weight control in many scientific reports is given explicitly [10, 32–34]. The most common endocrine malignancy is thyroid cancer, and papillary form of thyroid cancer is the most common type of thyroid cancer (80–90%) [32]. The aim of this study was determining the serum leptin levels in patients with papillary thyroid cancer and its comparison with healthy subjects.

2. Materials and Methods

2.1. Subjects

Patients. The case population consisted of 83 individuals, including 35 males and 48 females, 14 to 62 years (mean age 38.6 years) with papillary thyroid cancer (PTC). They were referred to Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Science. Also, 90 persons were selected as control group (40 male and 50 females) from referred to the laboratory with normal thyroid function tests (TSH: 0.3 = 3.5 mIU/L, T4: 4.5 = 12.5 μg/dL, T = Up: 25 = 35% and T3: 75 = 210 ng/mL) with age, sex, BMI matched with case group. Both groups were also matched for age and sex. The participants were included in the survey after obtaining an informed consent. Also, the clinical examination was performed by endocrinologist. The diagnosis of PTC was confirmed by histopathologic documents. This study has been approved by Institutional Review Board and Ethics Committee of Obesity Research Center, Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences.

2.2. Methods. Blood sampling was performed in both studied groups. For preparation of serum, 3 mL of whole blood was collected from antecubital vein in sitting position and was incubated 10 min in RT for coagulation. Then sera separated by 10 min centrifugation at 3000 rpm and the obtained sera were aliquoted in three 0.5 mL microtubes. The isolated serum samples from each individual were stored in 1 mL Eppendorf microtubes at −80°C (Japan's Sanyo C Company).

Anthropometric characteristics, including height and weight of patients and control group, were measured by height measuring scaled balance (Seca, German company); height with 0.5 cm and weight with 250 g sensitivity were reported. These data were used to calculate the body mass index (kg/m^2). Demographic profiles, including age, and sex were also recorded. Those individuals, who were using drugs affecting thyroid function and obesity drugs, were excluded.

In groups thyroxine, thyrotropin, and leptin hormones were measured by ELISA method. The used kits were prepared from the Canadian company (DBC Company, Ontario, Canada). The ELISA reader was from Tecan Austrian Company and Sunrise Model. Human thyrotropin and leptin hormones were determined based on sandwich ELISA method, whereas a thyroxin hormone was measured according to the competitive EIA method. The sensitivity of thyroxine, thyrotropin, and leptin kits was 0.6 μg/dL, 0.1 mU/L, and 0.4 ng/mL, respectively. Additionally, the coefficients of variation for these assays were 6.2%, 7.1%, and 6.5%, respectively.

2.2.1. Statistical Analysis. According to the normal distribution of data obtained by testing Kolmogorov-Smirnov (KS) ($P = 0.68$ for case group and $P = 0.52$ for control group), the frequency, mean and standard deviation were used to describe characteristics. All the data were in normal distribution (except leptin after normalization). The independent t-test was used to compare mean (except leptin with geometric mean and CI 95%) of variables between two groups. Comparison of qualitative data was done with Chi-square test. Further, data was analyzed using statistical software (SPSS 15), and significant level was considered at 0.05.

3. Results

Demographic profiles and anthropometric characteristics of participants are provided in Table 1. The results of thyroid hormones test, including thyroxine and thyrotropin in both control and patients groups, are given in Table 2. Since the leptin hormone secreted from adipose tissue is different in male and female, therefore the different levels of measured leptin hormone in two groups are shown in Table 3 (gender based). Height, weight, and body mass index between males and females of both groups were significant ($P < 0.05$). In addition, a significant difference ($P < 0.05$) was observed between the leptin hormone levels in males and females in both healthy and cancer groups. The amount of leptin hormone in cancer patients was higher than that in normal individuals, significantly ($P < 0.05$).

4. Discussion

Our data showed that the serum leptin levels of Iranian patients with papillary thyroid carcinoma were significantly higher than those in control group subjects. This increased level was observed in both males and females with papillary thyroid carcinoma. As this increased level was observed in both gender and different ages, so it could be related to thyroid carcinoma and it is independent of sex and age. Even though in this study the leptin level was higher in females than males in both groups, this is probably related to more adipose tissue mass in women. Both leptin and thyroid hormones cause thermogenesis and reduce body weight therefore maybe it is considered as a first association between the two hormones. The hunger reduces the leptin

Table 1: Anthropometric characteristics of participants.

Group	Sex	Height (cm)	Weight (kg)	BMI (kg/m^2)	Mean age (year)
Control	Female (50)	159.5 ± 12.1	61.9 ± 0.3	24.5 ± 2.3	38.1 ± 12.5
	Male (40)	169.2 ± 10.5	66.3 ± 0.2	23.2 ± 2.0	37.9 ± 15.8
Case	Female (48)	160.2 ± 11.6	63.7 ± 0.3	24.9 ± 2.7	39.1 ± 13.7
	Male (35)	170.4 ± 11.3	68.4 ± 0.2	23.7 ± 2.4	37.5 ± 17.0

Table 2: Serum levels of thyroxin and thyrotropin hormones in participants.

Group	Thyroxin (μg/dL)	Thyrotropin (mU/L)
Control	9.1 ± 2.9	2.4 ± 1.2
Case	8.9 ± 3.0	2.6 ± 1.0

Table 3: Serum leptin levels in participants.

Group	Females serum leptin level (ng/mL)	Males serum leptin level (ng/mL)
Control	4.3 ± 6.9	2.2 ± 5.6
Case	19.6 ± 23.3 ($P < 0.05$)	10.4 ± 17.3 ($P < 0.05$)

and thyroid hormones levels [35]. High levels of thyroid hormones decrease leptin expression in adipose tissue. But the most studies have not shown significant changes in leptin levels in hypothyroidism and hyperthyroidism disorders [36, 37]. However, increased leptin level in postpartum thyroiditis has been reported [38]. Akinci et al. reported that leptin levels increased in papillary thyroid carcinoma in Turkish population [39]. But in their study only 34 cases were investigated, the status of thyroid function in patients and healthy group was not evaluated, and age-matching was not considered [39]. In our study, 83 persons were matched for age, sex, and BMI. Assessing thyroid function in patients and healthy individuals was performed, and no significant difference was observed in both groups.

In both above studies, BMI in women was higher than in men, which was quite predictable. In both studies leptin levels in women were higher than those in men that is because of increased fat mass in women. In one study Cheng et al. showed that expression of leptin and/or leptin receptor in papillary thyroid cancer was associated with neoplasm aggressiveness, including tumor size and lymph node metastasis [40]. Interestingly, in another study, Uddin et al. demonstrated that leptin plays an important role in papillary thyroid cancer pathogenesis through PI3K/AKT pathway via its receptor (Ob-R) and is a potential prognostic marker associated with an aggressive phenotype and poor survival [32].

One of the limitations of our study was inability to followup the patients after surgery. Therefore, reduction or normalization of high leptin levels in thyroid cancer patients was not assessed. However, a significant increase of serum leptin levels in Iranian patients with papillary thyroid carcinoma maybe used as a reliable marker to diagnose or confirm papillary thyroid cancer. In addition if the leptin levels in cancer patients decrease after thyroidectomy, it will be used for the followup treatment, possibly. So a before-after study is recommended for future investigations instead of case control study. Thus, leptin level measurement can be used to followup the treatment of patients.

Strongly high leptin level in papillary thyroid cancer patients in comparison with health subject potentially suggests leptin as a peptide marker of papillary thyroid cancer. It means that adipose tissue secreted hormones, proteins, and peptides potentially may have application in diagnosis, confirmation, and/or treatment followup.

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Review Article

Hypoxia-Inducible Factor in Thyroid Carcinoma

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Intratumoural hypoxia (low oxygen tension) is associated with aggressive disease and poor prognosis. Hypoxia-inducible factor-1 is a transcription factor activated by hypoxia that regulates the expression of genes that promote tumour cell survival, progression, metastasis, and resistance to chemo/radiotherapy. In addition to hypoxia, HIF-1 can be activated by growth factor-signalling pathways such as the mitogen-activated protein kinases- (MAPK-) and phosphatidylinositol-3-OH kinases- (PI3K-) signalling cascades. Mutations in these pathways are common in thyroid carcinoma and lead to enhanced HIF-1 expression and activity. Here, we summarise current data that highlights the potential role of both hypoxia and MAPK/PI3K-induced HIF-1 signalling in thyroid carcinoma progression, metastatic characteristics, and the potential role of HIF-1 in thyroid carcinoma response to radiotherapy. Direct or indirect targeting of HIF-1 using an MAPK or PI3K inhibitor in combination with radiotherapy may be a new potential therapeutic target to improve the therapeutic response of thyroid carcinoma to radiotherapy and reduce metastatic burden.

1. Introduction

The hypoxia-inducible factors (HIFs) are transcription factors that function under low oxygen tensions (hypoxia) and are, therefore, active in a number of diseases associated with low oxygen (O_2) environments. These include ischemic disorders, atherosclerosis, and importantly cancer. HIF drives the survival and development of cancer cells by activating and repressing a multitude of genes that promote tumour cell survival, proliferation, invasion, and disease progression. As a result, hypoxia and HIF are associated with poor prognosis in many tumour types [1–3]. Hypoxia occurs in the majority of solid tumours, thus functional HIF is present in most tumour types indicating the importance of this signalling pathway in cancer. There is little known, however, about the role of HIF in thyroid carcinoma. Here, we summarise current literature that supports the potential significance of the HIF signalling pathway in progression and aggressiveness of thyroid carcinoma. Current data proposes that the HIF pathway may be a novel therapeutic target in reducing local tumour growth, metastatic burden, and resistance to chemo/radiotherapy.

2. Oxygen-Dependent Regulation of HIF-1

There are three known isoforms of HIF: HIF-1, 2, and 3. HIF-1 is expressed in all cells and is the most extensively researched, whereas the expression of the other isoforms is restricted to certain tissues. HIF-1 is a heterodimeric protein consisting of a constitutively expressed HIF-1β (also known as the ary lhydrocarbon receptor nuclear translocator; ARNT) subunit and an oxygen-labile HIF-1α subunit. Under conditions of low oxygen, HIF-1α is stabilised, heterodimerises with HIF-1β through the Per-ARNT-Sim (PAS) A and PAS B domains, and translocates to the nucleus. The complex then binds to the hypoxia-responsive element (HRE; consensus sequence G/ACGTG), in the promoter region of target genes via the basic helix-loop-helix (bHLH) DNA-binding domain and activates transcription. This process involves binding of the coactivators CREB-binding protein (CBP) and p300 [4, 5].

Under normoxia, HIF-1α is hydroxylated on proline residues 402 and/or 564 in the oxygen-dependent degradation domain (ODD). This process is carried out by specific oxygen-dependent enzymes known as proline hydroxylase domain proteins (PHDs). There are 3 PHDs: 1, 2, and 3. PHD2 is specifically involved in the hydroxylation of HIF-1α. The PHDs use O_2 and 2-oxoglutarate (2-OG) as substrates. Upon hydroxylation, von Hippel-Lindau (VHL), a tumour-suppressor protein, binds HIF-1α and recruits the E3 ubiquitin ligase, leading to ubiquitination and proteosomal degradation of HIF-1α (Figure 1). Activation of asparaginyl hydroxylases such as factor inhibiting HIF-1 (FIH-1) represents an additional oxygen-dependent mechanism of inhibition of HIF-1α activity. FIH-1 hydroxylates asparagine-803 in the C-terminal transcriptional activation domain (C-TAD) of HIF-1α. This modification inhibits the interaction of C-TAD with the transcriptional co-activators CBP/p300, and thus inhibits the transcriptional activity of HIF-1 (Figure 1). Under hypoxia, the level of HIF-1α hydroxylation is reduced via inhibition of the PHD2 enzyme, resulting in stabilisation and accumulation of HIF-1α protein [4, 5]. An additional oxygen-sensitive mechanism of HIF-1 regulation is the generation of reactive oxygen species (ROS) from mitochondria. ROS inactivate PHD2 resulting in direct stabilisation of HIF-1α [6].

HIF-2α is likewise regulated by oxygen-dependent hydroxylation and dimerises with HIF-1β to form the functional HIF-2 complex [4, 7]. Both isoforms are similar in structure and function but have differences, particularly in the N-TADs [8]. This suggests that both isoforms may differ in the activation of target genes and the recruitment of coactivators. There are also fewer HIF-2α-regulated genes compared to HIF-1α. For example, in MCF-7 breast carcinoma cells, 80% of hypoxia-regulated genes were dependent on HIF-1α. A small group were dependent on HIF-2α, and the regulation of these genes was due to the interaction of HIF-2α with the transcription factor Elk-1 [9]. This interaction with Elk-1 is unique to HIF-2α.

Although HIF-1α and -2α show some overlap of target genes, the proteins do have distinct downstream targets. HIF-1 predominantly regulates the expression of genes encoding glycolytic proteins such as lactate dehydrogenase-A (LDH-A) and carbonic anhydrase-9 (CA-9), whereas in certain tissues expressing both HIF-1 and -2α, expression of genes such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO) is mainly regulated by HIF-2α [7, 10, 11]. In support of this, high expression of HIF-2α but not -1α has been found in well-vascularised areas of neuroblastoma and is associated with aggressiveness [12]. Although HIF-1α is accepted as the most important of the HIFs, there is increasing evidence suggesting that HIF-2α may be of equal significance. The expression of HIF-2α is both tissue and cell-type specific, and the regulation of target genes differs depending on tissue type, tumour type, and coexpression with HIF-1α. A better understanding of how these factors lead to cell-specific differences in HIF-dependent gene regulation may help in the development of more effective therapeutics for diseases highly dependent on hypoxia. The HIF-3α isoform is also hypoxia regulated in a HIF-1-dependent manner and is an inhibitor of HIF-1 function [13].

3. Oxygen Independent Mechanisms of HIF Activation

3.1. Mutations in VHL. VHL targets all the HIF-αs for rapid proteosomal degradation and, as a result, plays a central role in molecular oxygen sensing [14]. Studies into the phenotype of VHL knockout (KO) mice provide direct evidence for the physiological relevance of the HIF pathway and VHL-regulated expression of HIF-1α in both normal foetal development and cancer progression. Genetic knock-out of VHL in the murine germ line results in embryonic lethality in mid-gestation due to abnormal vasculature formation thought to be HIF dependent [15]. In certain hereditary cancers such as clear cell renal carcinomas, Chuvash polycythemia, pheochromocytoma of the central nervous system or hemangioblastoma of the retina, normoxic degradation of HIF are impaired due to mutations in VHL. These tumours are usually well vascularised, a characteristic largely attributed to deregulated HIF-1α signalling [16, 17].

3.2. Metabolic Signalling Pathways. Metabolic signalling pathways such as the tricarboxylic acid (TCA) cycle are also necessary for normal regulation of HIF-1α. In addition to O_2, PHDs require the substrates 2-OG and ascorbic acid as a cofactor, to catalyze hydroxylation of HIF-1α. This reaction produces succinate and CO_2 as byproducts. 2-OG is a metabolite of the TCA cycle. As 2-OG is required for the functionality of PHDs, activity of the TCA cycle regulates PHD activity and thus HIF-1α stability. Furthermore, inactivating mutations in enzymes of the TCA cycle leads to direct stabilisation of HIF-1α protein [18, 19]. Mutations in fumarate hydratase (FH) and succinate dehydrogenase (SDH) lead to accumulation of fumarate and succinate. Both fumarate and succinate inhibit PHDs by competing with 2-OG for binding to the active site [20, 21]. Inactivating mutations in SDH have been found in a range of cancers associated with enhanced HIF-1 and VEGF signalling. These include renal cell carcinomas, gastrointestinal stromal (GIST) tumours, and Carney's syndrome [22, 23].

3.3. Growth Factor Signalling Pathways. For thyroid cancer the most important mutational changes occur in the MAPK/Ras—extracellular signal-regulated kinase (ERK) and the PI3K/AKT signalling cascades [24]. These pathways are dominant regulators of many cellular processes including growth, metabolism, cell survival, and angiogenesis and are important in the coordinated interaction of cells with the microenvironment [25, 26]. Both the MAPK and PI3K signalling pathways increase HIF-1 signalling in many cancer types including thyroid [27–30].

3.4. HIF-1 and the MAPK Pathway. A number of MAPK (p38 and p38γ) and ERK1/2 (p42 and p44) isoforms have been found to regulate HIF-1α activity in a cell-specific manner. MAPK signalling enhances the transactivation of

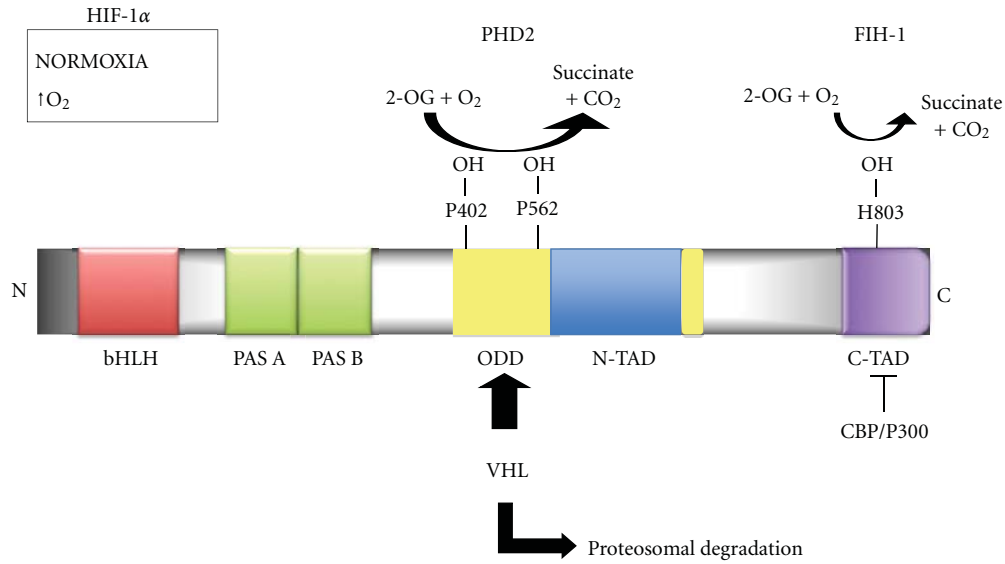

Figure 1: Structure of HIF-1α and the oxygen-dependent regulation of HIF-1α protein stabilisation and activation: The N-terminal regions contain the basic helix-loop-helix (bHLH) domain involved in DNA-binding and the Per-Arnt-Sim (PAS) A and B domains required for heterodimerisation with HIF-1β. The oxygen-dependent degradation domain (ODD) is where PHD2 hydroxylates proline residues P402 and P562, which enables binding of VHL and proteosomal degradation. The terminal transcriptional activation domains (TADs) are responsible for transactivation of target genes. The N-TAD is located towards the N-terminus, with the C-TAD located at the extreme C-terminus. Factor inhibiting HIF-1 (FIH-1) hydroxylates asparagine H803 in the C-TAD, preventing the binding of coactivators CBP/p300, thus inhibiting activation of HIF-1α. Both hydroxylation processes use 2-oxoglutarate (2-OG) and O_2 as substrates and produce succinate and carbon dioxide (CO_2) as byproducts.

HIF-1α by phosphorylation of HIF-1α co-activators and by direct phosphorylation of HIF-1α itself. MAPK stimulation leads to the phosphorylation of p300. This phosphorylation facilitates binding of p300/CBP to the C-TAD and promotes the transactivation activity of both p300/CBP and HIF-1α. Studies suggest that MAPK does not phosphorylate FIH, and therefore, this signalling cascade is not involved in the oxygen-dependent regulation of HIF-1α [31]. Thus, even though MAPK signalling does not depend on sensing oxygen tension, activation of this signalling cascade augments the HIF response.

Studies have shown that MAPK can directly phosphorylate the TADs of HIF-1α. Sang et al. found that MAPK signalling leads to indirect phosphorylation of C-TAD constructs by MAPK and direct phosphorylation of N-TAD constructs, when expressed in *Escherichia coli*. However, this direct phosphorylation of the TADs was not necessary for binding of p300 and activation of HIF-1 [31]. Other studies suggest that direct phosphorylation of HIF-1α by MAPKs has functional consequences on HIF-1 activity. In HeLa and CCL39 cells, p42/44 MAPK (but not p38 MAPK and JNK) increased the transcriptional activity of HIF-1 by phosphorylation of HIF-1α and not by increasing the level of HIF-1α protein. This was blocked by the MEK inhibitor PD098059 [32]. Conversely, in Hep3B and HEK293 cells, activation of the small GTPase Rac1 by hypoxia leads to Rac1-dependent p38 MAPK signalling resulting in both enhanced phosphorylation of the N-TAD and transcriptional activity of HIF-1α [33, 34]. Another study, however, has shown that Rac1 reduces HIF-1 activity by activating NAPDH oxidases resulting in enhanced generation of ROS leading to reduced HIF-1 in both normoxia and hypoxia [35]. The effect of Rac1 on MAPK signalling was not shown in this study. Furthermore, the latter group used 8% O_2 for hypoxia, whereas the first study used 5 and 1% O_2 for hypoxia. These functional differences observed by Rac1 may, therefore, be dependent upon O_2 concentration. Furthermore, different cell lines were used in these studies suggesting that these differences in Rac1 signalling may be dependent on the activating mutation status of the MAPK pathway in different cell lines. Studies in HeLa cells have also shown that direct phosphorylation of HIF-1α by p42/44 MAPKs not only leads to enhanced HIF-1 transcriptional activity, but also promotes nuclear accumulation of HIF-1α [36]. Thus, depending on the cell type, the MAPK signalling cascade can enhance HIF-1 activity via activation of selective kinases which either directly phosphorylate HIF-1α or co-activators of HIF-1α (Figure 2).

In addition to MAPK signalling leading to enhanced activity of HIF-1, studies have also shown that the MAPK pathway may increase HIF-1α protein synthesis in certain cancer types. Fukuda et al. showed that MAPK signalling can increase HIF-1α protein synthesis in HCT116 colon carcinoma cells. Inhibitors of MAPK blocked HIF-1α protein synthesis, and the overexpression of a constitutively active MAPK kinase (MEK2) induced HIF-1α protein [37] (Figure 2).

Genetic mutations in the v-raf murine sarcoma viral oncogene homolog B1 (BRAF) gene, which encodes a serine-threonine kinase, result in hyperactive MAPK signalling.

$BRAF^{V600E}$ is the most common genetic mutation within papillary thyroid carcinomas (PTCs). On average, approximately 50% of all PTCs harbour the $BRAF^{V600E}$ mutation [38]. This BRAF mutant is constitutively active and phosphorylates MEK1/2 leading to hyperactive MAPK signalling (Figure 2). It is thought that BRAF and, thus, MAPK signalling are important in the early stages of thyroid tumour development and predispose cells to become dedifferentiated tumours [39–41]. This has recently been substantiated in a mouse model of a thyroid-specific knock-in of oncogenic BRAF (LSL-BRAFV600E/TPO-Cre), which leads to a high frequency of invasive PTCs. These mice had high levels of thyroid stimulating hormone that acted cooperatively with oncogenic BRAF to drive tumour initiation [42].

The GTPase V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) is one of three members of the RAS family of GTPases. KRAS interacts with and activates a number of effector proteins including BRAF and PI3K. Genetic mutations in RAS leading to constitutive activity results in hyperactivation of the PI3K/MAPK pathways, promoting tumour progression in many cancer types including thyroid [43]. Recently, it has been shown that mutations in KRAS or BRAF differentially regulate HIF-1α and HIF-2α in colon carcinoma cells. Mutant KRAS enhanced expression of HIF-1α only, whereas mutant BRAF enhanced expression of both HIF-1α and -2α. KRAS-induced HIF-1α was dependent on PI3K activation, whereas only HIF-2α was inhibited by the MEK inhibitor PD089059 in BRAF mutant cells [44]. These data highlight the complexity of HIF induction by oncogenic signalling cascades and may contribute to the phenotypic differences observed not only in colon cancer but other cancers harbouring these mutations such as thyroid.

The interplay and activation of MAPK by additional signalling pathways promotes HIF-1 activity. In colon cancer cells, loss of the tumour suppressor SMAD4 (mothers against decapentaplegic (MAD) and the *Caenorhabditis elegans* protein SMA-4) led to enhanced HIF-1α activity through the activation of MEK-ERK and p38 MAPK by Transforming growth factor-β (TGF-β) [45].

Stimulation of the MAPK cascade enhances HIF-1 signalling mainly by increasing the transcriptional activity of HIF-1α. This will lead to increased expression of HIF-1 downstream targets under normoxic conditions and in the presence of hypoxia will lead to a cooperative enhancement of expression of HIF-1 target genes.

3.5. HIF-1 and the PI3K Pathway. Aberrant activation of the PI3K pathway occurs by (a) hyperactive stimulation of receptor tyrosine kinases (RTKs) including the insulin-like growth factor-1 receptor (IGF-IR), the human epidermal growth factor receptor 2 ($HER^{2neu}R$), and the epidermal growth factor receptor (EGFR) and (b) by mutational events that occur in negative regulators of PI3K signalling such as those in Phosphatase and tensin homolog (PTEN) and p53. Activation of the PI3K pathway is pivotal in the development and progression of thyroid cancer aggressiveness and appears to be predominantly involved in the metastatic behaviour of these tumours [41]. Hyperactive PI3K signalling leads to stabilisation of HIF-1α in normoxia. This depends on the activation of AKT and subsequently mammalian target of rapamycin (mTOR), which increases the translation and activity of HIF-1α [37, 41, 46, 47].

mTOR is a serine/threonine kinase consisting of multiple protein-binding motifs that enable interaction and phosphorylation of many proteins and is considered a central signalling molecule for convergence and crosstalk between multiple pathways. mTOR phosphorylates and activates the translational machinery proteins: eukaryotic translation initiation factor 4E- (eIF-4E-) binding protein (4E-BP1) and p70 S6 kinase. Upon phosphorylation, 4E-BP1 no longer interacts with and represses eIF-4E, and $p70^{S6}$ phosphorylates and activates the 40 S ribosomal protein. Activation of both eIF-4E and the 40 S ribosomal protein initiates translation of HIF-1α mRNA (Figure 3) [48, 49].

Stimulation of PI3K signalling by activation of different RTKs in a variety of cancer types induces protein synthesis of HIF-1α. This has been shown in colon and breast cancer cells via activation of IGF-1R and $HER2^{nue}R$ by IGF-1 and heregulin, respectively, [37, 50]. Stimulation of PI3K/AKT/mTOR induces the activation of the translational machinery proteins and protein synthesis of HIF-1α. $HER2^{neu}$ is an indicator for poor prognosis in breast cancer and tumours overexpressing HER2 have increased angiogenesis due to increased expression of the HIF-1 target gene VEGF. Similarly, overexpression of HIF-1α and VEGF has been found in prostate cancer cells that have hyperactive PI3K/AKT/mTOR activity as a result of enhanced EGFR signalling [51].

Depending on the type of RTK that is activated and on the type of cancer, increased HIF-1α protein may be PI3K dependent even in the presence of activated MAPK signalling or may be dependent on both signalling pathways. Oestrogen-dependent tumours usually display hyperactive, PI3K, HIF-1α, and VEGF signalling. Studies on the effect of oestrogen induced HIF-1α and VEGF in rat uterine tissue have shown that although oestrogen receptor signalling stimulates both the PI3K and MAPK signalling cascades, induction of HIF-1α and VEGF was only dependent upon PI3K signalling [52]. However, in HCT116 colon cancer cells, IGF-1 induced HIF-1α protein synthesis involved both the PI3K and MAPK signaling pathways [37]. Enhanced activity of HIF-1 by growth factor receptor signalling pathways is both receptor and cell specific and highlights the complexity of HIF-1 regulation by these signalling cascades.

Loss of negative regulators of the PI3K pathway including PTEN, p53 and the tumour-suppressor proteins tuberous sclerosis proteins TSC1 (hamartin), TSC2 (tuberin), and promyelocytic leukemia (PML) leads to hyperactive PI3K-signalling. PTEN is a phosphatase that dephosphorylates the PI3K products, and thus inhibits PI3K downstream signalling. Loss of PTEN or loss of function of PTEN has been found in a range of cancers including prostate, glioma, and thyroid [53–55]. Loss of PTEN has been associated with enhanced angiogenesis and cancer progression possibly through PI3K-induced HIF-1α and downstream targets including VEGF. Loss of function or deletion of the tumour suppressor p53 is common in many cancer types including

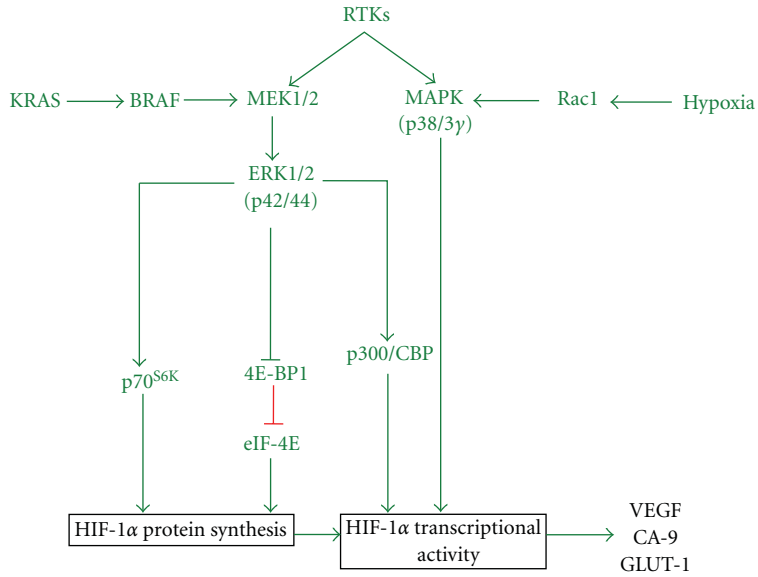

Figure 2: Regulation of HIF-1α by the MAPK pathway. Stimulation of receptor tyrosine kinases (RTKs) by growth factors, or activating mutations in the RAS family of GTPases, such as KRAS, results in activation of a range of MAPK-signalling proteins. Activation of ERK1/2 by MEK1/2 increases HIF-1 signalling (a) by phosphorylating and promoting the interaction of the coactivator p300 with HIF-1α and (b) by phosphorylating and activating the translational regulatory protein $p70^{S6K}$ and by phosphorylating and disrupting the repressive interaction of 4E-BP1 with eIF-4E. Activation of these translational machinery proteins leads to increased protein synthesis of HIF-1α. Activation of MAPKs (p38/p38γ) leads to the direct phosphorylation and activation of HIF-1α. Additionally, under hypoxia, activation of the small GTPase Rac1 leads to a Rac1 dependent increase in HIF-1α activity via Rac-1 induced activation of p38 MAPK. Genetic mutations in members of the MAPK signalling pathway such as those in BRAF ($BRAF^{V600E}$) lead to hyperactive MEK/ERK signalling and enhanced HIF-1α activity.

thyroid. p53 negatively regulates the PI3K pathway by inhibiting transcription of the PIK3CA gene that encodes the catalytic subunit of PI3K; p110α and by activating transcription of PTEN and TSC2 [56, 57]. Furthermore, p53 can directly interact with HIF-1α and recruit MDM2, which targets HIF-1α for ubiquitination and degradation [28, 58]. mTOR activity is negatively regulated by the heterodimer TSC1 (hamartin) and TSC2 (tuberin). This complex is disrupted and functionally inactivated by AKT leading to enhanced activity of mTOR and upregulation of HIF-1α. This has recently been confirmed in irradiated tumours where HIF-1α is activated through this pathway [59]. HIF-1α is not only stimulated through PI3K/AKT activation of mTOR via loss of the TSC1/2 complex, but also via mutational changes leading to loss of TCS2 function. Subsequently, HIF-1α downstream targets including VEGF and Glucose transporter-1(GLUT-1) are increased [60]. Hypoxia inhibits mTOR activity in both a HIF-dependent and independent manner [61]. Under hypoxia, PML negatively regulates mTOR by directly interacting with and preventing activation of mTOR by the small GTPase Rheb1. Loss of PML is observed in a number of sporadic tumours and correlates with increased VEGF and HIF-1α expression via attenuation of hypoxic mTOR inhibition [62]. Characteristically, patients with mTOR-associated hamartoma and tumours harbouring mutations in TSC2 and PML are highly vascularised, a common phenotype of tumours arising from mutations in VHL, which have hyperactive HIF-1 and VEGF signalling [60, 62]. Collectively, these tumour types highlight the importance of HIF-1 in tumour angiogenesis and progression.

Studies in human embryonic kidney cells suggest that mTOR may directly phosphorylate HIF-1α, promoting binding of p300/CBP and enhancing HIF-1α activity. mTOR interacts with the scaffold protein raptor (regulatory-associated protein of mTOR). Raptor directly interacts with HIF-1α under conditions favouring stabilisation of HIF-1α, that is, hypoxia, resulting in enhanced activity, which was not due to mTOR induced stabilisation of HIF-1α protein [63].

The pathophysiology behind increased HIF-1α in many solid tumours is not only restricted to hypoxia, growth factor signalling cascades, or mutations in components of the oxygen-dependent regulatory mechanism of HIF-1, but also may be mediated by other signalling pathways. A hyperactive Wnt/β-catenin signalling cascade may similarly result in increased activity of HIF-1 due to the interaction of β-catenin with HIF-1α [64, 65]. Collectively, these data highlight that many pathways are involved in the fine tuning of HIF-1α regulation by oncogenic signalling cascades such as the ERK-MAPK and PI3K/AKT pathways.

4. The Role of HIF in Cancer

The majority of solid tumours encounter hypoxic stress as a result of (a) limited oxygen diffusion due to the rapid proliferation of tumour cells which outgrow the existing vascular network and (b) by perfusion deficits mediated by

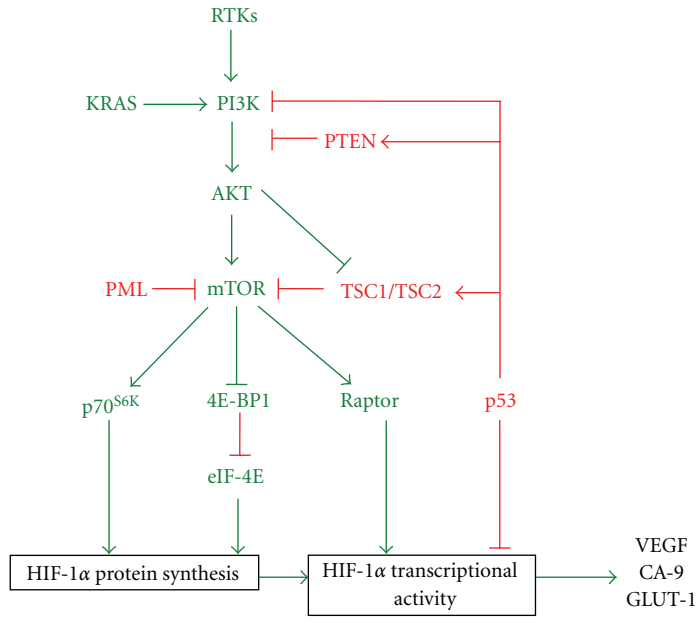

Figure 3: Regulation of HIF-1α by the PI3K pathway. Stimulation of receptor tyrosine kinases (RTKs) by growth factors or activating mutations in the RAS family of GTPases, such as KRAS, results in activation of PI3K. Activated PI3K enables the phosphorylation and activation of AKT. AKT in turn activates mammalian mTOR by disrupting the inhibitory interaction of the TSC1/TSC2 complex with mTOR. mTOR enhances HIF-1 signalling in a multimechanistic way (a) By activating the translational machinery proteins $p70^{S6K}$ and EIF-4E leading to enhanced protein synthesis of HIF-1α and (b) By promoting the interaction of HIF-1α with the coactivator p300, possibly by direct phosphorylation of HIF-1α via interaction with the scaffold protein raptor. Additionally, deletion or loss of function of negative regulators of the PI3K pathway leads to enhanced signalling and activation of HIF-1. These include PTEN, p53, PML, and TSC1/2. The phosphatase PTEN dephosphorylates the products of PI3K, inhibiting activation of AKT and subsequent downstream targets. p53 inhibits PI3K signalling at multiple levels; p53 inhibits transcription of the catalytic subunit of PI3K (p110α), enhances transcription of PTEN and TSC2, interacts with HIF-1α, and recruits MDM2, targeting HIF-1α for proteosomal degradation. PML inhibits mTOR under hypoxia and TSC1/2 interacts with and prevents activation of mTOR in the absence of PI3K activation.

abnormal blood vessel structure and function within the tumour [3, 66, 67]. Exposure to low oxygen tensions results in enhanced expression and activity of HIF-1α leading to increased tumour cell survival and growth under stressful environments. Over one hundred HIF target genes have been identified. HIF promotes tumour cell survival and progression by regulating multiple genes including those involved in angiogenesis (VEGF), anaerobic metabolism (GLUT-1), control of intracellular pH (CA-9), regulation of cell cycle, DNA damage response, proliferation and apoptosis (p21 and p27) and extracellular matrix remodelling and cell migration (lysyl oxidase (LOX), and matrix-metalloprotease-1 and -9 (MMP-1 and -9)) [68–71].

Clinically, hypoxia and HIF-1 has been associated with poor prognosis in a range of cancers including uterine, breast, and nonsmall cell lung cancer as well as poor treatment response in cancers such as nasopharyngeal cancer [1–3, 72]. Additionally, coexpression of HIF-1α with other pro-oncogenic proteins has been shown to be a significant prognostic predictor of survival. In patients with nonsmall cell lung cancer, coexpression of HIF-1α with Snail or TWIST1 has been shown to reduce overall survival and recurrence-free survival [73]. Expression of HIF-2α and coexpression with EGFR and insulin-like growth factor-binding protein-2 (IGFBP-2) has also been linked to reduced survival in patients with higher grade astrocytomas [74].

The HIFs act as the most important sensors of oxygen homeostasis. Constant HIF activation is a common feature in many cancers and is becoming increasingly recognized as a target for therapeutic intervention.

5. HIF and Chemo/Radiotherapy

5.1. HIF and Chemoresistance. HIF-1α has been found to upregulate expression of the ATP-binding cassette (ABC) transporter family of proteins and multidrug resistance related proteins (MRPs) in certain tumours and cancer cell lines. The ABC-transporter proteins can efflux chemotherapeutic agents out of the cell, namely, taxanes or anthracyclines resulting in reduced cytotoxicity and cell death [75–77].

The HIF-1 downstream target VEGF has also been implicated in chemoresistance. Blockade of VEGF signalling can promote normalisation of the tumour vasculature resulting in enhanced delivery of chemotherapeutics into the tumour. This has been observed in anaplastic thyroid carcinoma xenografts, where bevacizumab (inhibitory monoclonal antibody against VEGF) lowered tumour interstitial pressure and reduced vascular permeability [78]. Due to enhanced VEGF

signalling, the tumour vasculature is erratic, dysfunctional, and leaky, resulting in poorly perfused regions and high tumour interstitial pressure. This can hinder diffusion of chemotherapeutics into the tumours [79]. Qayum et al. recently showed that blockade of the EGFR, RAS, and PI3K pathways leads to long-term morphological changes that promote increased perfusion and allow prolonged and enhanced drug delivery to the tumour. These changes may also improve tumour response to radiotherapy [80]. In addition to VEGF being a HIF-1 downstream target, VEGF itself can induce HIF-1α and promote HIF-1 activity leading to enhanced chemo-resistance.

Other HIF-1 target genes implicated in chemo-resistance include CA-9. CA-9 plays a role in acidification of the tumour microenvironment by catalysing the hydration of CO_2 to bicarbonate and protons. Due to this, anticancer drugs that are weakly basic are unable to be taken up effectively into tumour cells as a result of raised intracellular pH and low extracellular pH, leading to reduced cytotoxicity [81–83]. Additionally, our group has shown that inhibition of HIF-1α either genetically or pharmacologically can enhance chemo-sensitivity when used in combination with drugs that lack efficacy in hypoxic cells such as etoposide [84].

Collectively, these data highlight the importance of HIF-1 in chemo-resistance and suggest that combination treatments with HIF inhibitors may be useful in improving therapeutic response to existing drugs and especially those associated with hypoxic chemo-resistance.

5.2. HIF and Radioresistance. Tumour hypoxia is associated with radioresistance due to the lack of oxygen leading to a reduction in the level of radiation-induced free radicals. These free radicals induce single- and double-stranded DNA breaks, leading to cell death by necrosis, apoptosis, or mitotic catastrophe [85, 86]. Within a tumour, the level of hypoxia has been shown to inversely correlate with radiosensitivity [66]. HIF-1 signalling promotes cell survival following exposure to ionising radiation. Clinically, high HIF-1 activity after radiation is associated with poor prognosis [2, 87]. HIF-1 activity has been found to increase with increasing doses of radiation in tumour xenograft models. No effect of radiation on HIF-1 activity was observed in tumour cells in vitro, suggesting that increased activity is dependent on radiation-induced physical changes within the intact tumour [88]. Conversely, other studies suggest that radiation itself can increase HIF-1α expression in addition to hypoxia in vitro. HIF-1 reporter activity was found to increase in hypoxic cells following exposure to radiation in range of carcinoma cell lines [89]. As a result of radiation-induced HIF-1 signalling, expression of HIF-1 downstream targets such as VEGF, plasminogen activator inhibitor-1, and CA-9 are increased and promote radio-resistance [90].

Within a tumour, mechanisms thought to contribute to radiation induced increases in HIF-1 include tumour reoxygenation. Radiation increases the level of O_2 within a tumour. This is due to cell death of well-oxygenated cells that frees up O_2 molecules. Additionally, as there are fewer tumour cells due to cell death, the vasculature is able to expand and grow, leading to enhanced oxygenation. This reoxygenation causes the generation of free radical such as reactive nitrogen species that lead to inhibition of the PHDs, thus reducing proteosomal degradation of HIF-1α [91].

Hyperactivity of growth factor signalling pathways such as EGFR, RAS, and PI3K/AKT cascades have been linked to radio-resistance and tumour cell survival following radiotherapy [92, 93]. As HIF-1 is a downstream target of PI3K, PI3K-induced HIF-1 activity may contribute to radio-resistance. Therefore, it is feasible to assume that tumour cells associated with hyperactive PI3K signaling, and thus, HIF-1 activity may respond better to radiotherapy when used in combination with a PI3K inhibitor. Currently, inhibition at the level of the EGFR in combination with radiotherapy has been used clinically and with success in certain tumour types [92, 94]. However, as there are many different points of activation within the PI3K signalling pathway, inhibition of PI3K itself may be useful in a whole range of tumours that have hyperactive PI3K signalling which is not dependent on EGFR activation but are dependent on other RTKs. Enhanced radiosensitivity has been observed in colon carcinoma cells treated with the selective PI3K inhibitor PI-103 [95] and in glioma xenografts treated with selective small molecule inhibitors of p110α [96]. Thus, these data provide promising evidence that in addition to inhibiting the downstream effects of PI3K that promote cell survival after radiation, indirect targeting of HIF-1 by PI3K inhibitors may significantly contribute to enhanced radiosensitivity.

6. HIF and Thyroid Hormones

Thyroid hormones play a key role in growth, metabolism and development. The effects of these hormones are brought about, namely, by the thyroid hormone ligand 3,3,5-triiodothyronine (T3) binding to the nuclear thyroid hormone receptors (TR) β1, β2, or α1. The ligand-nuclear receptor complex recruits additional proteins and acts as transcription factors that regulate gene expression by binding to the thyroid hormone response elements (TREs) in the promoter region of target genes. These genomic actions of thyroid hormones usually involve the binding of T3 to monomers, homodimers, or as heterodimers of the TRs with another member of the nuclear hormone receptor family such as the retinoid X receptor. This results in shedding of corepressors and recruitment of co-activators leading to alterations in transcription of thyroid hormone responsive genes [97, 98]. This contrasts to the rapid, nongenomic actions of thyroid hormones on the activity of ion pumps, cytosolic signalling, mitochondria, and the intracellular protein trafficking from cytosol to nucleus [98].

Thyroid hormones can activate both the PI3K and MAPK-signalling cascades [98–101]. Furthermore, thyroid hormones have been found to directly regulate expression of HIF-1α via activation of these signal transduction pathways. Microarray analysis of T3 regulated genes in human skin fibroblasts revealed an upregulation of HIF-1α and HIF-1 target genes GLUT1, platelet-type phosphofructokinase (PFK) and monocarboxylate transporter-4 (MCT-4) via

T3/TRβ signalling. The specificity of this upregulation by T3 was confirmed in fibroblasts from patients with an inactivating mutation in TRβ. These fibroblasts did not show any such changes in gene expression of HIF-1α and target genes [99]. The direct upregulation of HIF-1α by T3 resulted in a T3 indirect increase of GLUT-1, PFK, and MCT-4 expression via enhanced HIF-1α activity. This direct increase in HIF-1α activity was due to T3/TRβ activation of PI3K (Figure 4). Other studies have also shown that a TRβ mutant (TR$\beta^{PV/PV}$) interacts with the PI3K-regulatory subunit p85α leading to enhanced PI3K signalling, promoting thyroid carcinogenesis in a mouse model of spontaneous follicular thyroid cancer (thyroid hormone receptor$^{PV/PV}$ mice) [100]. This may lead to enhanced HIF-1α signalling in vivo.

T3 can also increase HIF-1α expression through interaction with the TRα members. In endothelial cells, T3 promotes the interaction of TRα1 with the PI3K subunit p85α, leading to increased phosphorylation and activation of AKT and endothelial nitric oxide synthase (eNOS). This is thought to contribute to the vasodilatory and cardiovascular protective effects of thyroid hormones [101]. It is likely that this T3-induced PI3K activity in endothelial cells would enhance HIF-1α activity, which may contribute to the vascular effects of thyroid hormone, as eNOS is a HIF-1 regulated gene [102]. In glioma cells, stimulation of PI3K/AKT by T3 induces shuttling of TRα from the cytoplasm to the nucleus, and promotes transcription of HIF-1α mRNA (Figure 4). This T3-dependent process was inhibited by treatment with the PI3K inhibitor LY294002 but not by the ERK inhibitor PD098095 [98]. T3-induced expression of HIF1-α and the downstream target VEGF has been verified in a gastric cancer cell line and *in vivo* mouse model. Accumulation of HIF-1α was due to T3 dependent activation of the PI3K signalling cascade. Interestingly, in addition to PI3K-signalling, HIF-1α overexpression by T3 may be regulated by fumarate accumulation; this accumulation was enhanced by T3-mediated inactivation of fumarate hydratase and reduced in the presence of 2-oxoglutarate [103] (Figure 4). These data link thyroid hormones with metabolic proteins and the HIF-1 signalling pathway in carcinogenesis and provide a means for understanding the adaptive mechanisms of tumour cells to metabolic stress.

Interestingly, T4 but not T3 has been found to impact on HIF-1α expression via interaction with the TRβ1 in glioblastoma (U-87) cells. This process was dependent on MAPK (ERK1/2) signalling as PD098095 inhibited T4-induced HIF-1α expression [98]. In CV-1 cells transfected with TRβ1, T4-activated MAPK signalling led to the interaction of MAPK with TRβ1 and enhanced phosphorylation and TRβ1 signalling [104]. Other studies have shown that both T3 and T4 can promote HIF-1α activity by activating the PI3K pathway. In hepatoma cells, T3 and to a lesser extent T4 increased expression of the HIF-1 target genes EPO and adrenomedullin (ADM), by stimulating the PI3K pathway, thus leading to increased translation of HIF-1α mRNA. No effects on HIF-1α protein stability/proteosomal degradation were observed [105].

In addition to the rapid nongenomic induction of HIF-1α by thyroid hormones via activation of growth factor signalling cascades, thyroid hormones have been shown to induce HIF-1α via the classic genomic actions of TRs. T3 can indirectly increase HIF-1α mRNA in a range of carcinoma cell lines that express hepatic leukemia factor (HLF). This regulation appears to be independent of a major protein kinase pathway, as the inhibition of members of both the MAPK and PI3K kinase pathways, including mTOR, had no effect on T3-mediated HIF-1α expression. T3 induction of HIF-1α was dependent on HLF-mediated gene expression of HIF-1α. This stimulation of HLF was in turn mediated by T3-activation of the TRβ-retinoid X receptor α heterodimer. Increased HIF-1α was, therefore, solely at the mRNA level and independent of translation [106] (Figure 4).

A close link between thyroid hormones and HIF is further substantiated by data on the activity of deiodinase type 3 (DIO3). Hypoxia induces DIO3, the physiological inactivator of T3, which catalyses the conversion of T3 to the metabolically inactive T2 (Figure 4). As this effect was also mimicked by hypoxia mimetics such as cobalt chloride, a direct action of HIF-1 was suspected. HIF-1α directly interacts with the DIO3 promoter, and thus stimulates DIO3 activity. This, in turn, induces a reduction of T3-dependent activation of metabolic pathways and represents a protective mechanism to reduce metabolic rate in tissues subjected to hypoxia [107].

The effect of T3 and T4 on HIF-1α is both receptor and cell specific. Furthermore, interaction of these hormones with additional signalling cascades provides insight into the importance of thyroid hormones in driving carcinogenesis by upregulating prosurvival pathways and those involved in the adaptation to the stressful conditions of the tumour microenvironment. An understanding of how these hormones and their receptors differentially regulate HIF-1α in both normal tissue and cancer may help identify the most appropriate drug treatment to improve therapeutic response.

7. HIF and Thyroid Cancer

7.1. Clinical Significance of HIF-1 and Thyroid Carcinomas. Recently we expanded on the potential pathophysiological importance of HIF-1α in thyroid carcinomas by showing that HIF-1α protein was variably expressed in primary thyroid carcinomas associated with advancing tumour grade. Tumour samples were representative of papillary (PTC), follicular (FTC), and anaplastic (ATC) thyroid carcinomas [26]. HIF-1α expression was absent from normal thyroid tissue. Interestingly and consistent with previous findings in a range of cancers, HIF-1α expression was highest in the most aggressive dedifferentiated anaplastic thyroid carcinomas (ATCs). However, analysis of vessel number and distribution via Von Willebrand factor (vWF) immunostaining were unable to link HIF-1α expression and vessel distribution. This may have been expected due to the short half-life of HIF-1α and transient regions of hypoxia that occur as a result of the erratic and dysfunctional vasculature, HIF-1α expression may not always be detected. Similarly to HIF-1α, highest expression of GLUT-1 was observed in ATCs and cell lines derived from ATCs, with lower expression observed

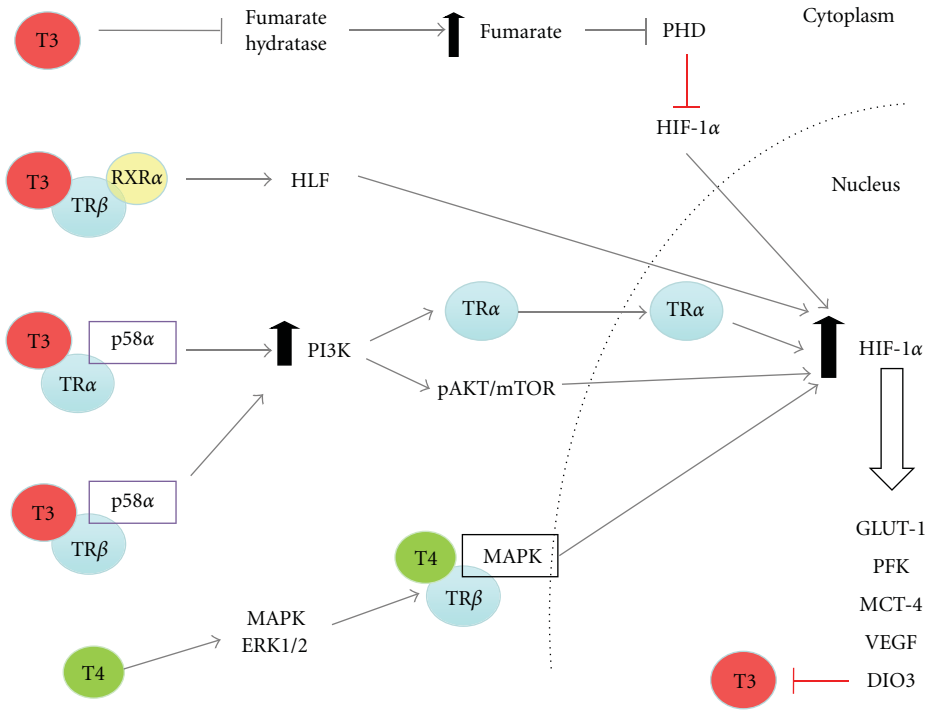

Figure 4: Thyroid hormone-dependent activation of HIF-1α: T3 and T4 induce HIF-1α activity by both genomic and nongenomic mechanisms. Genomically, T3 indirectly increases HIF-1α mRNA by increasing expression of the transcription factor hepatic leukemia factor (HLF), which initiates transcription of HIF-1α. Non-genomically, T3 stimulates PI3K signalling by promoting the interaction of both TRα and β with the PI3K regulatory subunit p85 leading to enhanced PI3K/AKT/mTOR activity and translation of HIF-1α mRNA. T3-induced PI3K promotes nuclear shuttling of TRα leading to increased HIF-1α expression. T3-induced PI3K signalling by either TRα or β is cell specific. T3 inhibits the enzyme fumarate dehydrogenase resulting in the accumulation of fumarate. Fumarate inhibits PHD2 leading to reduced hydroxylation of HIF-1α and increased protein stabilisation. T4 increases HIF-1α by stimulating MAPK signalling, leading to enhanced T4/TRβ activity and expression of HIF-1α. Activated HIF-1α by T3/T4 results in the upregulation of target genes, known to promote tumour cell survival and progression. These include GLUT-1, PFK, MCT-4, and VEGF. Additionally, HIF-1 upregulates DIO3, which inhibits T3 by catalysing the conversion of T3 to the metabolically inactive T2.

in cell lines derived from differentiated tumours. These increases, however, were only slight and were not significant. In contrast to GLUT-1, the HIF-1 downstream target CA-9 closely mimicked the pattern of HIF-1α expression with significantly higher levels observed in the ATCs. This suggests that CA-9 as in other cancer types may serve as a potential new biomarker of aggressive disease in thyroid carcinomas. This observation has been supported by previous work by Jubb et al. who reported high expression levels of HIF-1α in all thyroid carcinoma types particularly in FTCs [29, 108]. Additionally, data from a preliminary study in our group looking at HIF-1α and CA-9 in PTC, FTC, and ATC xenografts showed a similar correlation to that seen in clinical samples; both HIF-1α and CA-9 expression was highest in the tumours derived from ATC (8505c) cells. Furthermore, tumours with high expression of HIF-1α and CA-9 had a greater number of spontaneous metastatic colonies to the lungs (Figure 5). Collectively, these data from primary tumour tissue and xenografts suggest that HIF-1α expression is involved in the adaption of thyroid carcinomas to hypoxia and supports a pathophysiological role for thyroid tumour progression, aggressiveness and metastasis.

7.2. Hypoxia and HIF-1 in Thyroid Carcinomas. A number of functional studies in thyroid carcinoma cell lines and in immortalized cells derived from normal thyroid tissue substantiate the importance of hypoxia-induced HIF-1 in promoting the expression of proteins that drive thyroid tumour progression. HIF-1α was predominantly localized in the cytoplasm under normoxia but stabilised and translocated to the nucleus in hypoxia [29]. Enhanced nuclear localisation of HIF-1α has also been observed in the BcPAP cell line harbouring the $BRAF^{V600E}$ mutation versus a cell line with WT BRAF under normoxia [30]. The functional response of both normal and carcinoma thyroid cell lines to hypoxia was further confirmed as graded hypoxia-induced a marked increase in expression of HIF-1α and downstream targets CA-9, VEGF and GLUT1. Hypoxia induced HIF-1α was further supported by HIF-1α reporter activation in cells exposed to lowering oxygen tensions. Furthermore, the degree of hypoxia induced HIF-1α activity related to the level of thyroid carcinoma aggressiveness; lowest activity was observed in PTC cell lines, with highest seen in ATC cells. We have also confirmed that hypoxia induces expression of HIF-2α in follicular and anaplastic thyroid carcinoma cell lines (unpublished, data not shown). This is of interest,

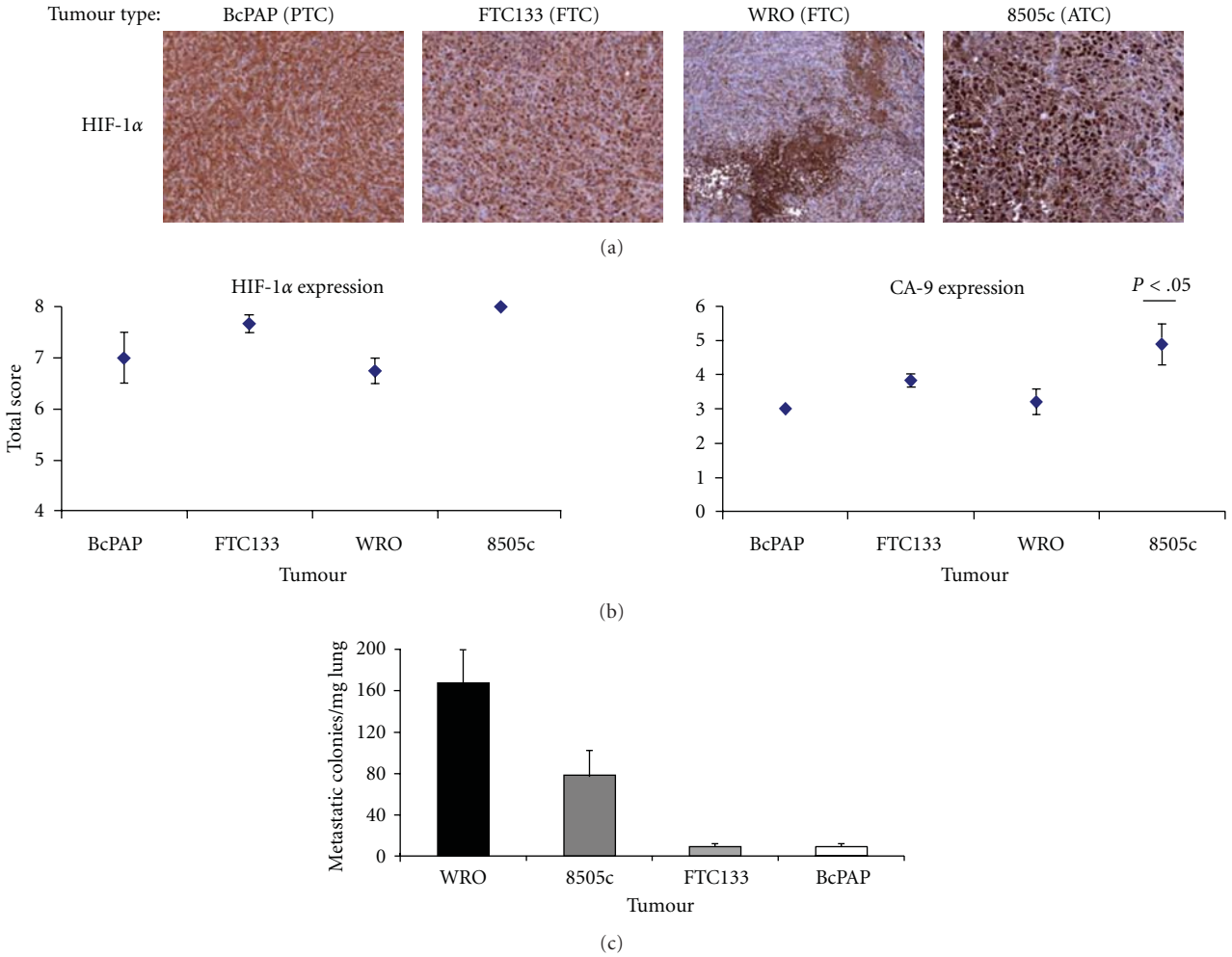

Figure 5: HIF-1α and CA-9 protein expression and metastatic potential are highest in tumours derived from follicular (WRO) and anaplastic (8505c) cell lines. Briefly, BcPAP, FTC133, WRO and 8505c cells were implanted sub-cutaneously in female CBA nu/nu nude mice. Once tumours reached 450 mm^3, tumour tissue and fresh lung tissue were excised. Tumour sections were immuno-stained for HIF-1α. Freshly excised lung tissue was enzymatically digested, plated out in serial dilution and left until visible colonies formed. Colonies were stained, counted and number of metastatic colonies per mg lung tissue calculated. (For full methods, see supplementary materials and methods which is located at doi: 10.406/2011/762905). (a) Immunohistochemical staining for HIF-1α (brown staining). One example of HIF-1α staining for each tumour type: BcPAP, FTC133, WRO and 8505c are displayed. (b) Semiquantitative analysis of HIF-1α and CA-9 immunostaining quantified according to Allred et al. [109]. The total score is representative of intensity and proportion of staining. HIF-1α was highly expressed in all tumour types with highest expression observed in the 8505c tumours. CA-9 was also expressed in all tumour types with significantly higher expression observed in 8505c tumours ($P < .05$, one way Anova, with Tukey post test). (c) The number of spontaneous metastatic colonies cultured from enzymatically digested lung tissue that was excised from mice bearing WRO, 8505c, FTC133, and BcPAP tumours. The highest number of colonies counted were from the lungs of mice bearing FTC (WRO) and ATC (8505c) tumours. Data are representative of the mean ± S.D. of 5 independent mice for each tumour type.

as the HIFs are known to regulate genes differentially in many different cancer types. Therefore, expression in thyroid carcinoma may account for some of the phenotypic differences seen between the different classifications of thyroid carcinoma.

7.3. Growth-Factor Signalling Pathways and HIF-1 in Thyroid Carcinomas. We found that in addition to hypoxia, growth factor-signalling pathways also induced HIF-1α expression and activity in thyroid carcinoma cell lines. Induction of HIF-1α by pathways such as the MAPK and PI3K signalling cascades have been described in a range of cancer types (see oxygen-independent regulation of HIF) [110]. The majority of thyroid carcinomas have mutations in growth factor signalling pathways. Mutations in the BRAF gene ($BRAF^{V600E}$) leading to hyperactive MAPK/ERK signalling have been found in approximately 50% of all PTCs [38]. Recently, this mutation has been reported to increase the transcription and expression of HIF-1α protein [30].

KRAS has been found to differentially regulate HIFs in certain cancer types [44]. Activating mutations in the RAS family of GTPases, such as those found in KRAS,

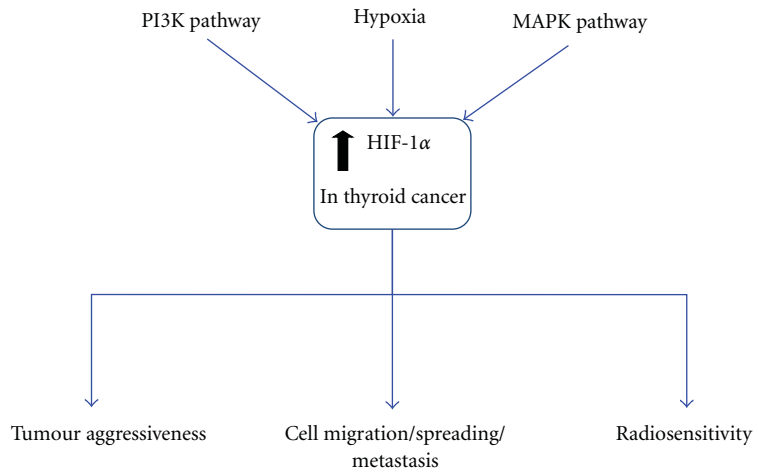

Figure 6: Mechanisms of induction of HIF-1α and downstream effects of HIF-1 signalling in thyroid carcinomas. HIF-1 activity is induced not only by hypoxia but by the oncogenic MAPK and PI3K signalling pathways. Clinically, expression of HIF-1α and the downstream target CA-9 have been linked with thyroid carcinoma aggressiveness. It is well known that HIF-1 plays an important role in tumour development, metastasis, and aggressiveness and is ultimately correlated with poor prognosis. Enhanced HIF-1 signalling by hypoxia and the PI3K/MAPK pathways may play an important role in the metastatic characteristics of thyroid carcinoma. HIF-1-induced MET upregulation has been implicated in promoting invasiveness of PTC. HIF-1 signalling may also play a prominent role in the therapeutic response to radiotherapy in thyroid carcinoma.

have been found in approximately 20% of PTCs and more specifically the follicular variant of PTC suggesting the mutation promotes aggressive phenotype [43]. As KRAS activates both BRAF and PI3K, it would be interesting to determine the role of KRAS in the context of HIF activity and aggressive phenotype in thyroid tumours and thyroid carcinoma cell lines harbouring this mutation.

Studies have shown that highest levels of HIF-1α expression and activity were detected in thyroid tumours and tumour cell lines harbouring classic mutations in BRAF and PI3K [29, 30]. Thus, the current data supports the importance of oncogenic activation of HIF-1 in thyroid cancer and aggressive disease [46]. Direct intervention with pharmacological inhibitors of these pathways (LY294002 for PI3K, PD098059 for ERK/MAPK, and sorafenib for inhibition of RAF-1 kinase/BRAF) or by genetic modulation; (re-expression of PTEN in PTEN null cell lines and silencing of mutant BRAF), further support the close dependency of HIF expression on oncogenic signalling. Interestingly, inhibition of HIF-1α reporter activity was much more substantial as a consequence of LY294002 treatment under varying O_2 tensions compared to PD98059 even in cell lines with no known mutation in the PI3K pathway. These findings are particularly interesting with regard to the frequent mutations in the PI3K/AKT pathway found in up to 15% of PTC and approximately 50% of FTC [111, 112], where PI3K-pathway mutations have been directly linked to tumour aggressiveness [41]. HIF-1α silencing reduced expression of HIF-1 target genes like CA-9. Thus, blocking HIF-1α, either directly or through inhibition of the PI3K pathway in thyroid carcinomas, may decrease aggressiveness and thus open new therapeutic options [46].

7.4. HIF and VEGF in Thyroid Carcinomas. VEGF serum levels are elevated in thyroid carcinomas, and this is associated with poor tumour prognosis [113–116]. As one of the most important downstream targets of HIF-1 signalling, VEGF has not yet been linked clinically to HIF activity in thyroid carcinomas. A number of studies using antagonists of VEGF signalling for the treatment of advanced thyroid carcinomas have been reported with some positive results ([117–120]. However, no data are currently available on the effect on VEGF levels by direct targeting of HIF-1. We have reported that VEGF levels are significantly increased by anoxia in thyroid carcinoma cells lines derived from PTC, FTC and ATC. Additionally, highest basal levels of VEGF were observed in the PTEN—null FTC133 follicular thyroid carcinoma cell lines [29]. This suggests that the two different signalling pathways may act synergistically to increase VEGF. The anoxia-induced increases in VEGF expression did not, however, match the pattern of HIF-1α reporter activity in varying thyroid carcinoma cell lines. This suggests that the level of dependency on either the HIF-1 or growth factor-signalling pathways to induce VEGF is specific to the type of thyroid carcinoma cell line. Current unpublished data from our group supports a prominent role and an important link between the PI3K and HIF-1 pathways in the regulation of VEGF. Furthermore, we have found that inhibition of these pathways has profound effects on VEGF expression in thyroid carcinoma cell lines. These data provide a new angle for therapeutic approaches of thyroid carcinoma as drugs which target HIF-1 signalling may be more efficacious in downregulating VEGF dependent signalling in these tumours.

7.5. HIF-1, Migration, and Metastasis in Thyroid Carcinomas. Hypoxia and HIF-1 have been associated with increased migration and metastasis in a range of cancer types [121–123]. We have found that hypoxia significantly increased migration in cell lines derived from PTCs and FTCs. Additionally, we have exciting data that supports a prominent role of PI3K and HIF-1 signalling in metastatic characteristics of FTC and ATC (unpublished).

Studies have shown a link between hypoxia/HIF-1α and MNNG HOS transforming gene (MET) upregulation in clinical PTCs. MET is a receptor tyrosine kinase that is stimulated by hepatocyte growth factor (HGF). Stimulation results in the activation of signal transduction pathways such as PI3K, which promote migration, invasion, and the release of cytokines and proangiogenic factors such as VEGF [124, 125]. Additionally, MET activation promotes migration and invasion by stimulating the translocation of β-catenin from the cytoplasm to the nucleus [126]. MET is highly expressed in PTC, and this high expression of MET is thought to be due to HIF-1 induced upregulation in hypoxic regions of PTCs. Furthermore, higher expression of MET and HIF-1α mRNA was found at the periphery of tumours (in cells located at the invading front) compared to the centre in 44% of PTCs [127]. These were characterised to have a more aggressive phenotype. Collectively, these data suggest that HIF-1 may play a role in promoting migration and aggressiveness in PTC.

Taken together, these data highlight the importance of hypoxia, HIF-1α and PI3K signalling as a means of promoting metastatic characteristics and driving aggressive disease in thyroid carcinomas (Figure 6).

8. HIF-1 and Sensitivity to Radiotherapy in Thyroid Carcinomas

There is increasing evidence for the role of HIF in tumour cell sensitivity to chemo- and importantly radiotherapy [77, 84, 128]. As discussed above, HIF-1 stabilisation and action depend on growth factor-signalling pathways. Thus, many inhibitors of growth factor signalling affect HIF-1 expression and downstream targets. Anticancer drugs that target these pathways possess antiangiogenic properties and include the BCR-ABL kinase inhibitor imatinib (Gleevec), EGFR inhibitors such as gefitinib, erlotinib, and cetuximab, the $HER2^{neu}$ inhibitor trastuzumab, and the mTOR inhibitors rapamycin, temsirolimus, and everolimus. Collectively, these drugs all target the translation of HIF-1α mRNA into protein [129]. Modulation of HIF-1α expression can increase tumour sensitivity, in particular to radiotherapy in certain cancer types (see HIF and radio-resistance). Thus, HIF-1 signalling may be a particularly important feature for the sensitivity of dedifferentiated thyroid carcinomas to external beam radiation. This has yet to be investigated.

It has been shown that tumour cell apoptosis is increased when targeted radioiodide therapy is combined with angiogenic inhibitors, which represents an indirect approach to circumvent HIF-1—dependent radio-resistance [130]. Data discussed here support the view that targeting of HIF-1 may serve as a more direct approach to overcome HIF dependent adaptation of thyroid carcinoma cells to radiotherapy. Preliminary data from our group in FTC and ATC cell lines supports this notion of HIF-1 signalling playing a role in radio-resistance (unpublished). As described earlier, PI3K has been found to play an important role in HIF-1α induced expression and activity in thyroid carcinoma cell lines under both normoxia and anoxia. Therefore, targeting HIF-1 either directly or by use of a PI3K-inhibitor may improve the therapeutic response of thyroid carcinoma to radiotherapy.

Currently, there is no data available on whether inhibition of HIF-1 may reflect on the sensitivity of more differentiated tumours to standard radiotherapy. It is conceivable that in patients receiving external beam radiation treatment and particularly in patients with a poor response but positive uptake of radioiodine, any augmentation of the sensitivity towards irradiation-induced apoptosis would be favourable (Figure 6). Studies in our group on animal models of metastatic follicular and anaplastic thyroid carcinomas will hopefully allow us to answers some of these questions in the future.

9. Conclusions

HIF-1 is potently induced by both hypoxia and oncogenic signalling pathways in thyroid carcinoma, and its expression and activity have been correlated with aggressiveness. Current literature suggests that PI3K and MAPK pathways promote aggressive and metastatic disease in part via the upregulation of HIF-1 activity. With the known effects of hypoxia, PI3K/MAPK pathways, and HIF-1 on desensitisation to radiotherapy, HIF-1 may be a new and important therapeutic target in reducing local tumour growth, metastatic burden, and radio-resistance in thyroid carcinoma.

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Research Article

Lymph Node Thyroglobulin Measurement in Diagnosis of Neck Metastases of Differentiated Thyroid Carcinoma

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Aim. Enlarged cervical lymph nodes (LNs) in patients with thyroid cancer are usually assessed by fine-needle aspiration cytology (FNAC). Thyroglobulin (Tg) is frequently elevated in malignant FNAC needle wash specimens (FNAC-Tg). The objectives of the study were to (1) determine an appropriate diagnostic cut-off for FNAC-Tg levels (2) compare FNAC and FNAC-Tg results in a group of 108 patients affected by differentiated thyroid carcinoma (DTC). *Methods*. A total of 126 consecutive FNACs were performed on enlarged LNs and the final diagnosis was confirmed by surgical pathology examination or clinical follow-up. The best FNAC-Tg cut-off level was selected by receiver operating curve analysis, and diagnostic performances of FNAC and FNAC-Tg were compared. *Results*. The rate of FNAC samples adequate for cytological examination was 77% in contrast FNAC-Tg available in 100% of aspirates ($P < .01$). The sensitivity, specificity, and accuracy of FNAC were 71%, 80%, 74%, 100%, 80%, and 94%, respectively. The most appropriate cut-off value for the diagnosis of thyroid cancer metastatic LN was 1.1 ng/mL (sensitivity 100%, specificity 100%). *Conclusions*. The diagnostic performance of needle washout FNAC-Tg measurement with a cut-off of 1.1 ng/mL compared favorably with cytology in detecting DTC node metastases.

1. Introduction

The prognosis of patients who receive appropriate treatment for thyroid carcinoma is usually favourable, especially for differentiated thyroid carcinoma (DTC). However, although most patients have a long-term survival rate, 5% to 20% of patients will develop recurrence during the followup, primarily in the cervical lymph nodes (LNs) [1, 2]. These LNs metastases may be detected clinically, but now are most often discovered on ultrasonography (US) [3]. It is of great importance to differentiate accurately LN metastases from benign reactive lymph nodes in order to avoid unnecessary treatment, but also to treat metastatic patients without delay. As consequence, diagnostic procedures must offer good sensitivity, but also high negative predictive value. US criteria distinguishing benign from metastatic or suspicious LNs have been described but they lack accuracy [4]. US-guided fine needle cytology (FNAC) proved to be a reliable method in examining the neck in patients who were previously treated for thyroid cancer [5, 6]. However, sensitivity of FNAC is far from excellent, varying from 75% to 85%, and altered by high rate of nondiagnostic or false-negative samples. To improve the diagnostic yield of FNAC, several authors have proposed measurement of Tg in aspirates (FNAC-Tg), particularly in the cases involving small, partially cystic, lymph nodes [7–12]. On the basis of prior studies, an increased Tg level in the needle washout has been shown to directly reflect the status of metastatic lymph nodes in patients affected by differentiated thyroid carcinoma. However, controversies still persist concerning some issues. First, there are few studies to validate the benefit of FNAC-Tg over FNAC alone, and, second, cut-off values ranging from 0.9 to 39 ng/mL have been suggested for FNAC-Tg, depending on the method used for the measurement [7, 13–15]. As consequence, the exact place for FNAC-Tg in the management of DTC patients is still debated.

This study was then undertaken to (1) determine a diagnostic cut-off value for washout Tg in patients treated by total thyroidectomy for detecting recurrences, and (2) to compare the performance of the Tg cut-off value to US-guided FNAC for detecting DTC recurrences.

2. Materials and Methods

Our institutional review board approved our research study, and all subjects gave written informed consent.

2.1. Patients. Between January 2006 and February 2009 a total of 126 consecutive US-guided FNACs were performed on enlarged LNs. The samples were obtained from 108 patients (19 males, 89 females; age 42.7 ± 18.2 years; 91 patients with 1, 94 with 2, and 5 with 3 lesions, resp.). All patients had histologically confirmed primary DTC (papillary, $n = 99$, including two tall-cell variants; follicular, $n = 9$, including 2 Hürtle cells carcinomas). The primitive carcinoma was classified pT1 in 34 cases, pT2 in 43 cases, pT3 in 24 cases, and pT4 in 7 cases. Forty-five patients (42%) had LN metastases at diagnosis. All patients underwent (near) total thyroidectomy and subsequent radioiodine ablation (administered activity from 1.85 to 3.70 GBq). The US criteria for possible malignant infiltration of lymph nodes were rounded contour, irregular internal echogenicity, punctate calcifications, fluids components, and abnormal colour Doppler pattern. Patients with positive cytology and/or FNAC-Tg measurement ($n = 86$, 96 lesions) underwent surgery and, if necessary, further radioiodine treatments. The diagnosis was confirmed in all cases by surgical pathology examination. Patients with negative FNAC-Tg measurement and cytology ($n = 22$, 30 lesions) underwent further follow up by serial clinical examinations, serum Tg measurements, neck US, and, whenever necessary, additional imaging procedures (i.e., radioiodine scan, ^{18}FDG-PET/CT). No DTC recurrence was detected among these patients (follow up: mean 36 months, range 15–42 months).

2.2. US-Guided FNAC Procedure. All US-guided FNAC procedures were performed on supine patients with the neck hyperextended under continuous real-time US guidance with a high-resolution transducer (ACUSON ×150, Siemens, Erlangen, Germany). Each lesion was aspirated at least twice by a 21 G needle. The needle was inserted obliquely within the transducer plane of view, and moved back and forth through the nodule to compensate for patient movement and needle deflection. Gradual aspiration was applied by a 20 mL syringe connected to Cameco's device. Contents of needles were expelled onto glass slides and smeared with a second slide to spread fluid across the surface. Slides were fixed in 95% ethanol, papanicolaou stained to identify cellular details, and read by our cytopathologist. Following collection of cytology samples the needles were washed by 1 mL of normal saline in a plain serum tube (Vacutainer Systems, Plymouth, UK) and the washout directly sent to the laboratory [16]. Cytological examinations were performed by experienced cytopathologists and expressed as (1) positive: presence of epithelial cells with atypical cytological characteristics, or with cytological features of papillary carcinoma; (2) negative: reactive lymphadenitis and absence of malignant cells; (3) inadequate or nondiagnostic: absence of cells or presence of blood cells.

2.3. Tg Measurement. Thyroglobulin was measured in fine-needle washouts using an immunoradiometric assay (IRMA) based on coated tubes with monoclonal antibodies directed against distinct epitopes of the molecule of Tg (DYNO test Tg-plus, BRAHMS Diagnostic GmbH, Berlin, Germany). With this measurement, analytic sensitivity, defined as the detectable minimum concentration different from zero (mean value + 2 standard deviation), and functional sensitivity, defined as the lowest value that was measured with the precision of a maximum 20% interassay variance, were 0.08 ng/mL and 0.2 ng/mL, respectively. We did not measure Tg antibodies (FNAC-TgAb) because the clinical performance of FNAC-Tg is unaffected by serum TgAb [17].

2.4. Data Analysis. Diagnostic performance (i.e., sensitivity, specificity, positive predictive value, negative predictive value, and accuracy) of FNAC and FNAC-Tg was evaluated by comparing the results of the two procedures to the status of the patients defined as follows: malignant lymph node from thyroid cancer was proved by histological examination of surgically resected LNs; benign lymph node was proved by negative histological examination of surgically respected LNs, or if disappearance or absence of evolution on imaging modalities was demonstrated at 12 months or more follow up.

The Chi-square (χ^2) test, performed with SAS version 9.1 for Windows (SAS Institute, Cary, NC, US) was employed to compare the diagnostic rate of FNAC and FNAC-Tg. The FNAC-Tg receiver operating characteristic curve was developed using MedCalc 6.1 software (MedCalc Software, Mariakerke, Belgium). The cut-off values which maximise the sum of sensitivity plus specificity were determined as the points in the upper left hand corner. A P value $< .05$ was considered to indicate statistical significance.

3. Results

Patients characteristics and cytological, pathological, and biochemical data are displayed in Table 1. Of the 126 lymph node lesions assessed for postoperative recurrences by US-guided FNAC and FNAC-Tg, 86 (68%) lesions were finally diagnosed as malignant and the remaining 40 (32%) lesions were diagnosed as benign LNs, respectively. The final diagnosis of the 86 malignant and 8 benign LN was established by surgical pathology; the remaining 32 benign lesions were diagnosed based on imaging follow up after at least 1 year. The time from thyroid ablation to US-FNAC was 19.5 ± 14.31 and 19.4 ± 13.76 months in patients with benign and malignant lesions, respectively (P not significant). Serum Tg levels were higher in patients with malignant lesions (median 4.20 ng/mL, range <0.2–27.10 ng/mL) than those with benign lesions (median .80 ng/mL, range <0.2–2.70 ng/mL; $P < .0001$). Serum TgAb were positive in 11

Table 1: Patients characteristics and cytological, pathological, and biochemical data.

Patients	Histology	pTNM	Duration (months)	Tg (ng/mL)	TgAb (IU/mL)	Lesions	Sites	FNA		Final diagnosis
								Cytology	Tg (ng/mL)	
1	PTC	pT1N1Mx	6	1.0	<60	1	R II	ND	<0.2	B
2	PTC	pT1NxMx	15	4.6	<60	1	L III	Negative	435.2	M
3	PTC	pT1NxMx	18	1.7	<60	1	R IV	CTM	85.3	M
4	PTC	pT1NxMx	30	0.4	<60	1	R III	ND	<0.2	B
5	PTC	pT1N0Mx	6	3.1	<60	1	L II	ND	97.2	M
6	PTC	pT2N0Mx	19	9.1	<60	2	R IV, VI	CTM	118.5	M
7	PTC	pT2NxMx	47	<0.2	334	1	L IV	CTM	879.4	M
8	PTC	pT2N1Mx	6	0.9	78	1	VI	Negative	1.1	B
9	PTC	pT1N1Mx	9	9.8	<60	1	L II	ND	1348.6	M
10	PTC	pT2N0Mx	14	1.5	<60	1	L III	CTM	358.5	M
11	PTC	pT1NxMx	8	12.6	<60	3	R II	CTM	2387.4	M
12	PTC	pT2NxMx	16	9.7	<60	1	R III	CTM	875.9	M
13	PTC	pT1NxMx	9	6.3	<60	1	R II	CTM	958.6	M
14	PTC	pT1N0Mx	11	1.0	<60	1	L III	Negative	48.7	M
15	PTC	pT2N1Mx	39	9.5	<60	1	L IV	CTM	>3000	M
16	PTC	pT1NxMx	12	0.8	<60	1	L III	CTM	107.5	M
17	PTC	pT2NxMx	19	2.7	<60	3	R III-IV	ND, CTM	541.6	RIII B, IV M
18	PTC	pT4N1Mx	6	1.6	<60	1	L III	CTM	31.8	M
19	PTC	pT1N1Mx	15	0.5	289	1	R IV	ND	<0.2	B
20	PTC	pT2N1Mx	3	2.3	<60	1	L III	ND	540.7	M
21	PTC	pT1NxMx	34	4.1	<60	1	L III	CTM	650.6	M
22	PTC	pT2N1Mx	8	<0.2	98	1	L II	ND	5.8	M
23	PTC	pT1NxMx	9	0.8	<60	2	R II, R III	CTM	87.5	M
24	PTC	pT4N0Mx	10	1.4	755	1	R II	CTM	196.4	M
25	PTC	pT2NxMx	21	11	<60	1	R III	ND	585.3	M
26	PTC	pT1NxMx	9	<0.2	<60	1	R III	Negative	<0.2	B
27	PTC	pT4N1M	12	1.1	160	1	L III	Negative	0.3	B
28	PTC	pT1N0Mx	23	5.3	<60	1	R III	CTM	784.2	M
29	PTC	pT2N1Mx	5	<0.2	>1000	1	R III	ND	<0.2	B
30	PTC	pT2N0Mx	22	4.9	<60	1	R III	CTM	641.5	M
31	PTC	pT1NxMx	36	0.3	367	1	R IV	Negative	<0.2	B
32	PTC	pT1NxMx	15	7.1	<60	1	R III	ND	665.4	M
33	PTC	pT2N0Mx	41	<0.2	<60	1	L II	Negative	<0.2	B
34	PTC	pT1NxMx	24	2.6	<60	1	R III	CTM	570.6	M
35	PTC	pT4N0Mx	31	1.4	>1000	3	L IV, VI	CTM	96.7	M
36	PTC	pT4N1M	12	27.1	<60	1	R II	CTM	2987.3	M
37	PTC	pT1N1Mx	54	6.8	<60	1	L III	CTM	875.6	M
38	PTC	pT2NxMx	12	<0.2	149	1	R IV	Negative	<0.2	B
39	PTC	pT2N0Mx	9	0.9	<60	1	R II	Negative	<0.2	B
40	PTC	pT1N0Mx	34	2.4	89	1	L III	ND	347.4	M
41	PTC	pT2NxMx	14	5.1	<60	1	L IV	ND	1655.7	M
42	PTC	pT4N1M	8	7.7	<60	1	L III	Negative	2076.5	M
43	PTC	pT1NxMx	61	1.1	<60	1	VI	Negative	<0.2	B
44	PTC	pT2N0Mx	22	1.0	<60	1	L II	CTM	90.6	M

Table 1: Continued.

Patients	Histology	pTNM	Duration (months)	Tg (ng/mL)	TgAb (IU/mL)	Lesions	Sites	FNA		Final diagnosis
								Cytology	Tg (ng/mL)	
45	PTC	pT2N1Mx	12	6.4	<60	2	R III-IV	CTM, ND	458.6	RIII M, IV B
46	PTC	pT1NxMx	45	0.6	<60	1	R IV	Negative	<0.2	B
47	PTC	pT4N0Mx	36	4.3	<60	1	R III	CTM	766.4	M
48	PTC	pT2NxMx	11	1.9	<60	1	L II	ND	306.5	M
49	PTC	pT3NxMx	18	7.0	<60	1	R III	CTM	955.6	M
50	PTC	pT2NxMx	10	1.2	<60	1	L III	Negative	<0.2	B
51	PTC	pT1N1Mx	12	3.9	<60	1	R III	CTM	564.7	M
52	PTC	pT3N0Mx	25	6.4	190	1	R III	ND	1078.6	M
53	PTC	pT2N1Mx	16	9.5	<60	1	L IV	CTM	759.4	M
54	PTC	pT1NxMx	9	0.6	<60	1	R III	Negative	<0.2	B
55	PTC	pT3N1Mx	60	<0.2	107	2	L II-III	CTM	137.5	M
56	PTC	pT1N0Mx	11	5.3	<60	1	R II	CTM	654.8	M
57	PTC	pT2N1Mx	18	1.1	860	1	L III	ND	<0.2	B
58	PTC	pT1NxMx	9	0.9	<60	1	R IV	Negative	<0.2	B
59	PTC	pT2NxMx	28	1.1	<60	1	R III	ND	99.6	M
60	PTC-TCV	pT3NxMx	13	4.6	<60	1	L II	CTM	436.8	M
61	PTC	pT1NxMx	2	2.7	<60	1	R II	CTM	194.5	M
62	PTC	pT3NxMx	7	21.9	<60	3	R III, L II	Neg., CTM	>3000	RIII B, LII M
63	PTC	pT1NxMx	18	1.1	<60	1	R III	CTM	27.4	M
64	PTC	pT3NxMx	10	3.5	<60	1	VI	CTM	147.4	M
65	PTC	pT2N0Mx	29	<0.2	>1000	1	R IV	Negative	<0.2	B
66	PTC	pT3N1Mx	6	5.6	<60	1	L III	CTM	<0.2	M
67	PTC	pT1N1Mx	11	2.1	<60	1	R III	ND	<0.2	M
68	PTC	pT3N0Mx	18	0.7	<60	1	R III	ND	<0.2	B
69	PTC	pT2N0Mx	23	1.7	<60	1	L IV	CTM	75.6	M
70	PTC	pT1NxMx	9	3.5	<60	1	L II	ND	72.6	M
71	PTC	pT3N0Mx	18	7.1	<60	2	R IV, VI	CTM	386.2	RIV B, VI M
72	PTC	pT1NxMx	7	1.8		1	L II	Negative	<0.2	B
73	PTC	pT2NxMx	11	3.6	<60	1	R III	ND	245.7	M
74	PTC	pT2NxMx	29	2.2	<60	2	L III-IV	CTM	67.4	IV M, III B
75	PTC	pT2N1Mx	14	5.7	<60	1	L II	CTM	198.7	M
76	PTC	pT1N0Mx	8	1.5	<60	1	R IV	CTM	26.1	M
77	PTC	pT2NxMx	10	2.1	<60	1	VI	CTM	59.7	M
78	PTC	pT1NxMx	18	0.7	<60	1	R IV	Negative	<0.2	B
79	PTC	pT3N1Mx	34	12.3	<60	1	R III	ND	678.4	M
80	PTC	pT1N1Mx	16	1.8	<60	1	L II	Negative	<0.2	B
81	PTC	pT2N0Mx	52	0.9	<60	1	R III	Negative	<0.2	B
82	PTC	pT2N1Mx	25	9.4	<60	1	L III	ND	563.6	M
83	PTC	pT1NxMx	9	4.1	<60	1	VI	CTM	64.7	M
84	PTC	pT2N1Mx	18	3.1	<60	1	R IV	CTM	116.4	M
85	PTC-TCV	pT2NxMx	20	15.5	<60	3	R IV-L III	CTM	432.9	M
86	PTC	pT3N0Mx	8	<0.2	56	1	VI	ND	<0.2	B
87	PTC	pT1N0Mx	31	2.9	<60	1	R IV	CTM	39.3	M
88	PTC	pT2NxMx	11	1.7	<60	1	R III	Negative	<0.2	B
89	PTC	pT2NxMx	54	6.5	<60	1	L IV	ND	467.7	M
90	PTC	pT1N1Mx	28	0.3	651	1	R II	Negative	<0.2	B
91	PTC	pT2NxMx	12	2.1	<60	1	L III	CTM	88.6	M

Table 1: Continued.

Patients	Histology	pTNM	Duration (months)	Tg (ng/mL)	TgAb (IU/mL)	Lesions	Sites	FNA		Final diagnosis
								Cytology	Tg (ng/mL)	
92	PTC	pT3N0Mx	17	9.4	<60	2	R IV, VI	CTM	156.2	IV M, VI B
93	PTC	pT1NxMx	22	1.0	<60	1	L IV	Negative	<0.2	B
94	PTC	pT2N1Mx	9	4.3	<60	1	VI	Negative	53.8	M
95	PTC	pT2N0Mx	26	<0.2	<60	1	R IV	Negative	<0.2	B
96	PTC	pT1N0Mx	12	0.9	<60	1	R III	ND	2.7	M
97	PTC	pT2NxMx	17	8.1	<60	1	L II	ND	356.4	M
98	PTC	pT2NxMx	9	0.9	<60	1	R III	CTM	19.6	M
99	PTC	pT3N1Mx	12	1.1	<60	1	L III	ND	0.6	B
100	FTC	pT1N1Mx	45	4.9	<60	1	R III	CTM	117.5	M
101	FTC	pT3N0Mx	36	1.4	<60	1	R III	Negative	<0.2	B
102	FTC	pT2N1Mx	11	<0.2	>1000	2	R III-IV	Negative	<0.2	B
103	FTC (HC)	pT1NxMx	72	10.4	<60	1	R IV	ND	278.9	M
104	FTC	pT2N1Mx	11	0.7	<60	1	R III	Negative	<0.2	B
105	FTC	pT1N0Mx	18	2.1	<60	1	R IV	CTM	74.4	M
106	FTC (HC)	pT1N0Mx	6	1.9	<60	1	L III	Negative	0.8	B
107	FTC	pT2N1Mx	25	7.8	<60	1	R II	ND	116.4	M
108	FTC	pT1NxMx	19	4.6	<60	2	L II-III	CTM	89.5	M

FNA, fine-needle aspiration; PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; TCV, tall-cell variant; HC Hürtle cell; R, right, L, left; duration, time from thyroid ablation to FNA; II-III-IV, upper, middle, lower neck lateral compartment, IV, central neck compartment.

Table 2: Diagnostic performance of FNAC cytology as compared to final diagnosis.

FNAC	Final diagnosis	
	Malignant LNs ($n = 86$)	Benign LNs ($n = 40$)
Positive	61	0
Negative	4	32
Inadequate	21	8

Table 3: Diagnostic performance of FNAC-Tg as compared to final diagnosis.

	Malignant LNs ($n = 86$)	Benign LNs ($n = 40$)
FNAC-Tg >1.1 ng/mL	86	0
FNAC-Tg ≤1.1 ng/mL	0	40

Table 4: Figures of merits of FNAC cytology and FNAC Tg.

FNAC	Sensitivity	Specificity	PPV	NPV	Accuracy
Cytology	71%	80%	88%	56%	74%
Tg	100%	100%	100%	100%	100%

of 40 patients with benign lesions and 7 of 67 patients with malignant lesions ($P < .001$). The rate of FNAC samples adequate for cytological examination was 77% (97 samples) in contrast FNAC-Tg available in 100% of aspirates ($P < .01$). As shown in Table 2 cytological examination correctly identified 61 malignant LNs, was negative in 4, and inadequate in 21. For benign LNs, FNAC was negative in 32 and inadequate in 8. It showed no false-positive results. Thus, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of FNAC were 71%, 80%, 88%, 56%, and 74%, respectively. The ROC curve analysis demonstrated that the most appropriate cut-off value for the diagnosis of thyroid cancer metastatic lesions was 1.1 ng/mL (sensitivity 100%, specificity 100%, PPV 100%, NPV 100%, accuracy 100%; Figure 1, Tables 3, and 4). Basing on this cut-off level, the FNAC-Tg results correctly concluded all 25 malignant (100%) and 8 benign (100%) cases with false-negative ($n = 4$) and nondiagnostic ($n = 29$) FNAC results, respectively (Table 5). The FNAC-Tg levels were significantly higher in malignant (median 513.8 ng/mL, range 1.7-> 3000 ng/mL) than benign (median <0.2 ng/mL, range <0.2–1.1 ng/mL) lesions, respectively ($P < .0000001$). Particularly, specimen Tg levels were undetectable (i.e., <0.2 ng/mL) in 36 cases and were 0.3, 0.6, 0.8, and 1.1 ng/mL in remaining 4 cases with benign lesions.

Table 5: Rate of positive FNAC-Tg values (i.e., >1.1 ng/mL) in patients with inadequate or misdiagnosed FNAC-cytology.

Final status	FNAC inadequate ($n = 29$)	FNAC false-negative ($n = 4$)
Malignant LNs ($n = 25$)	21/21	4/4
Benign LNs ($n = 8$)	0/8	—

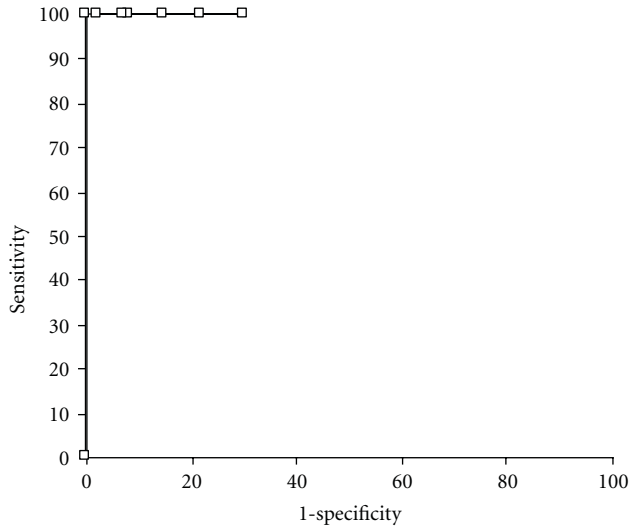

Figure 1: FNAC-Tg: ROC curve analysis.

4. Discussion

An accurate discrimination between metastatic and reactive LNs is essential in the management of thyroid cancer. Cytological examination of FNAC samples disclosed by US has been the most accurate method to diagnose a cervical LN. However, as showed also in our study, its sensitivity is negatively impacted by the rate of nondiagnostic samples although FNAC procedures are performed by US-experienced physicians and dedicated cytopathologists [5, 6, 15]. In the present study 23% of samples were nondiagnostic; this perfectly conforms with previously reported data [18]. Cystic metastasis and partial LN involvement comprise most of the inadequate/nondiagnostic FNAC-cytology cases and could be misinterpreted as a benign cervical cystic mass or branchial cleft cysts and could therefore delay the correct diagnosis and a further radical neck lymphadenectomy. The immunocytochemical Tg staining on FNAC samples from of neck nodes was previously evaluated in patients with DTC. Because an adequate FNAC sample is required, however, the practical impact of this technique is limited in clinical practice [19]. Recently, the FNAC-Tg measurement has been proposed to be a useful diagnostic technique in the management of patients with thyroid cancer. Because Tg is produced only by follicular thyrocyte-derived cells, measurement of Tg in FNAC specimens of nonthyroidal tissues enables the detection of persistence, recurrence, or metastasis of differentiated thyroid carcinoma. In our study FNAC-Tg analysis was more sensitive for detecting metastasis when compared with FNAC alone, and allows the accurate diagnosis in samples with inconclusive cytology. Our results perfectly conforms those recently reported by Bournaud and colleagues [18]. By contrast Tg could be determined in all aspirates and a sensitivity of 100% was achieved in our series, that is at the higher end of previously reported data (81%–100%) [7, 9, 14, 17, 18]. Although the performance of FNAC-Tg is well established, the Tg threshold value remains controversial. The Tg assays employed and methods for determining the cut-off value differed from one study to another, resulting in a large range, from 0.9 ng/mL to values as high as 39 ng/mL, proposed in the literature.

In our study the best Tg threshold was determined at 1.1 ng/mL by ROC curve analysis. Using a threshold of 1.1 ng/mL we observe neither false-positive results in non-malignant LNs nor false-negative results in malignant LNs at final diagnosis. Additionally, FNAC-Tg results correctly classified as malignant 4 lesions that tested negatively in cytological examination. All in all, our results are in accordance with those of Snozek and colleagues that used a Tg assay with a functional sensitivity at 0.1 ng/mL and proposed a cut-off level of 1.00 ng/mL: basing on their results these authors suggested that FNAC-Tg should be substituted for FNAC in many cases [7]. Of importance, our samples were obtained in a population of well-differentiated thyroid carcinomas (i.e., only two Hürtle cell and two tall-cell variants among 108 DTC cases). Several authors reported, however, that FNAC-Tg levels could be undetectable in some types of thyroid cancers (i.e., poorly differentiated thyroid carcinomas) [13, 17]. This correspond to the fact that amount and intensity of Tg expression parallel with differentiation of the tumor and could produce false-negative results. As a consequence, caution is needed, and a combination of FNAC and FNAC-Tg should remain the standard, especially in patients harboring less differentiated thyroid carcinomas.

5. Conclusions

The diagnostic performance of needle washout FNAC-Tg measurement with a cut-off of 1.1 ng/mL compared favourably with cytology and allowed accurate diagnosis in all cases in whom cytology was nondiagnostic.

Conflict of Interests

The author report that there are no conflicts of interests.

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Research Article

Clinical Utility of Serum Interleukin-8 and Interferon-Alpha in Thyroid Diseases

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Serum interleukin-8 (IL-8) and interferon-alpha (IFN-α) levels have been estimated from a total of 88 individuals of which 19 were disease-free healthy individuals, and 69 were patients with thyroid diseases: goitre ($N = 21$), autoimmune diseases ($N = 16$), and carcinomas ($N = 32$). Both IL-8 and IFN-α were significantly higher in all the patients as compared to healthy individuals. Serum IL-8 levels showed significant positive correlation with disease stage in thyroid cancer patients. Higher serum IL-8 levels were associated with advanced disease stage while no significant correlation was observed between serum IFN-α levels and any of the clinicopathological parameters. IL-8 and IFN-α significantly correlated with each other in anaplastic carcinoma patients. Finally concluding, monitoring the serum IL-8 and IFN-α levels can help differentiate patients with thyroid diseases from healthy individuals, and IL-8 seems to have a role in the pathogenesis of thyroid diseases and may represent a target for innovative diagnostic and therapeutic strategies.

1. Introduction

Although thyroid problems are among the most common medical conditions, because their symptoms often appear gradually, they are commonly misdiagnosed. The three most common thyroid problems are the underactive thyroid, the overactive thyroid, and thyroid nodules [1]. Based on these problems, the disorders of the thyroid gland include: goitre, autoimmune thyroid diseases, as well as thyroid carcinoma.

Thyroid cancer is the fastest growing and most common cancer of the total endocrine malignancies, accounting for 94.5% of the total new endocrine cancers and 65.9% of the deaths due to endocrine cancers. It is the endocrine tumor that bears the highest incidence with 33 550 new cases per year in the US [2]. The vast majority of patients with thyroid diseases are curable using present treatment modalities. However, accumulating evidences indicate that follicular cell-derived thyroid cancer constitutes a biological continuum progressing from the highly curable well-differentiated thyroid cancer to the universally fatal anaplastic thyroid cancer [3, 4]. An association between thyroid cancer and a history of several benign diseases has been observed in most studies [5, 6]. However, the molecular mechanisms underlying thyroid cancer progression remain ill-defined. So it is important to decipher these mechanisms, since poorly differentiated and anaplastic carcinomas account for the majority of thyroid cancer-related deaths.

Moreover, accumulated epidemiologic studies support that chronic inflammatory diseases are frequently associated with increased risk of cancers [7–9]. It is estimated that

underlying infections and inflammatory responses are linked to 15–20% of all deaths from cancer worldwide [7]. There are many triggers of chronic inflammation that increase the risk of developing cancer. Such triggers include microbial infections, autoimmune diseases, and inflammatory conditions of unknown origin. The hallmarks of cancer-related inflammation include the presence of inflammatory cells and inflammatory mediators (e.g., chemokines, cytokines, and prostaglandins) in tumor tissues, tissue remodeling and angiogenesis similar to that seen in chronic inflammatory responses, and tissue repair. Indeed, inflammatory cells and mediators are present in the microenvironment of most, if not all, tumors, irrespective of the trigger for development [10]. Cytokine shedding by tumor cells into the local microenvironment is an important modulator of tumorigenesis [11]. Thus, the relationship between the immune system and cancer is complex, and cytokines, chemokines, and growth factors in the tumor environment play a key role in this interaction [12].

Cytokines are a category of signaling proteins and glycoproteins that, like hormones and neurotransmitters, are used extensively in cellular communication, which mediate and regulate immunity, inflammation, and hematopoiesis. Thus, cytokines are a group of polypeptides produced mainly by inflammatory cells, but also by nonimmune cells, and have a key role in triggering and coordinating inflammatory and immune reactions [13].

However, the relationships between cytokines and cancer are multiple and bidirectional. On one hand, cytokines may directly influence carcinogenesis and metastasis by modifying the tumor phenotype while, on the other hand during tumor progression, modifications of the cytokine expression in the tumor environment may be induced by the tumor cells [14]. Thus, a better understanding of the basis of molecular talk between tumor cells and the immune system would be helpful in developing immunotherapeutic approaches.

Interleukin-8 (IL-8) is one of the best-characterized members of C-X-C subfamily of the chemotactic cytokines [15, 16]. It is a proinflammatory cytokine produced by macrophages and other cell types such as epithelial cells. As it serves as a chemical signal that attracts neutrophils at the site of inflammation, it is also called "neutrophil chemotactic factor". Interleukin (IL)-8 is produced by a wide variety of normal cells as well as tumor cell and its principal role is in the initiation and amplification of acute inflammatory reactions. IL-8 has also been implicated in chronic inflammatory processes and diseases with a chronic inflammatory component such as cancer. IL-8 has been shown to contribute to human cancer progression through its potential functions as a mitogenic, angiogenic, and mitogenic factor [17, 18]. Recent studies have demonstrated that IL-8 regulates tumor cell growth and metastasis in melanoma [19], carcinoma of breast [20], stomach [21], pancreas [22], and liver [22, 23]. Elevated serum level of IL-8 was found to be a prognostic marker in soft tissue sarcoma [24], B-cell chronic lymphocytic leukemia [25], primary gastrointestinal non-Hodgkin's lymphoma [26], and malignant melanoma [27].

So, understanding the mechanisms of IL-8 expression can be helpful in designing potential therapeutic strategies of targeting IL-8 to control tumor growth and metastasis. On the other hand, according to current data, endocrine system is closely related to immune system, and interferons play an important role in this relationship. Interferons can inhibit cell proliferation or control apoptosis [28].

Interferon-alpha (IFN-α) is a small protein released by macrophages, lymphocytes, and tissue cells infected with a virus, it has pleiotropic properties and exerts a wide range of immunomodulatory activities [29]. It is predominantly characterized as an antiviral and antiproliferative agent which stimulates both macrophages and natural killer cells to elicit an antiviral response, and it is also active against tumors. Besides, a very important property of IFN-α is its ability to arrest the cell in the G1-G0 phase, which is what determines static effect of IFN-α on growth of many tumors [28]. Moreover, IFN-α is a potent activator of monocytic functions. But, since human monocytes are major producers of neutrophil chemotactic cytokine: IL-8, we have been tempted to study levels of both these cytokines (IL-8 and IFN-α) in sera of patients with thyroid disorders by ELISA. Moreover, it is much more convenient to quantitate serum levels of these cytokines than to evaluate their expression in tissue specimens as the former approach is noninvasive and reproducible. Furthermore, it does not require tumor tissue specimens.

So, the aim of this study was to explore the occurrence of interleukin-8 and interferon-alpha in sera of patients with various thyroid diseases (goiter, autoimmune disorders, and thyroid carcinoma) and to correlate the results with clinicopathological parameters in thyroid cancer patients.

2. Materials and Methods

Our study included a group of 88 individuals, out of which 69 were patients with thyroid disorders (goiter: $N = 21$, autoimmune thyroid diseases: $N = 16$, and thyroid carcinoma: $N = 32$) and 19 were age-matched disease-free healthy individuals (Table 1). The mean age of healthy individuals included in the study was 30.57 years (range: 18–56 years). Goiter occurred in patients at an earlier age (mean age: 34.23 years, range: 18–58 years) while the occurrence of autoimmune thyroid disease (mean age: 42.81 years, range: 26–61 years; $P = .04$) and thyroid carcinoma (mean age: 43.96 years, range: 18–78 years; $P = .034$) was found in more elderly people. But as in the American Joint Committee on Cancer (AJCC) TNM staging system, the patients are staged on the basis of their age (<45/≥45 years), we have also grouped our patients into a younger (<45 years) and an older group (≥45 years) (Table 1).

Fasting blood samples were collected in the morning from disease-free healthy individuals and patients with thyroid diseases in vacuettes with gel for serum separation. All patients with thyroid disorders were never previously treated with chemotherapy, surgery, or radiotherapy at the time of blood collection. Moreover, none of these patients were ever before diagnosed with any other autoimmune

Table 1: Characterization of patients with thyroid diseases and healthy individuals.

Subjects	N (%)	Gender		Age	
		Male	Female	<45 years	≥45 years
Healthy individuals	19	6	13	15	4
Total Patients	69	16	53	40	29
Goiter	21 (30.43)	3	18	14	7
Autoimmune diseases	16 (23.18)	4	12	9	7
Graves' disease	12 (17.38)	4	8	7	5
Hashimoto's disorder	4 (5.79)	—	4	2	2
Thyroid carcinoma	32 (46.37)	9	23	17	15
Papillary	18 (26.07)	3	15	12	6
Follicular	7 (10.14)	2	5	2	5
Medullary	4 (5.79)	2	2	2	2
Anaplastic	3 (4.34)	2	1	1	2

disease, no one of them was taking immunosuppressive or immunomodulant drugs. Prior to pretherapeutic blood collection, written consent of the patients was taken. Serums were separated from all blood samples after centrifugation and were stored at −80°C until analysis. IL-8 and IFN-α were determined from the serum samples using commercially available enzyme immunoassay (EIA) kits from Immunotech (A Beckman Coulter Company, France) following the manufacturer's instructions. The detailed clinical and histopathological characteristics of all patients were noted from the case files maintained at Gujarat Cancer and Research Institute (Table 2).

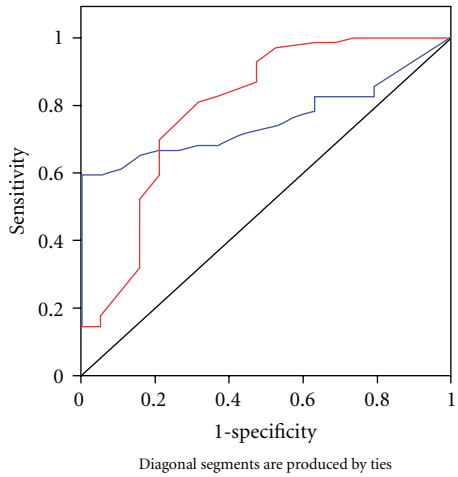
2.1. Statistical Analysis. The results were presented as mean ± standard error of mean (M ± S.E.). Mann-Whitney U test was performed to assess the differences in serum IL-8 and IFN-α levels between healthy individuals and patients with thyroid diseases. Receiver's operating characteristic (ROC) curves were also constructed to determine the discriminating efficacy of IL-8 and IFN-α between healthy individuals and patients with thyroid diseases. P values <.05 were considered statistically significant. Also, in thyroid cancer patients, the association between IL-8 and IFN-α levels and clinicopathological parameters was analyzed by Mann-Whitney U test, and the spearman's correlation was used to describe independent relationship between serum IL-8 and IFN-α levels.

3. Results and Discussion

Serum IL-8 and IFN-α levels were significantly elevated in all patients with thyroid disorders (goitre, autoimmune thyroid disorders, and thyroid carcinoma) as compared to healthy individuals. IL-8 and IFN-α levels in thyroid carcinoma patients with different histopathological subgroups and stages have also been compared to that of healthy individuals. Statistically significant higher levels of both these cytokines were found in patients with papillary and follicular carcinoma while, in medullary carcinoma patients, IFN-α and, in anaplastic carcinoma patients, IL-8 levels were found to be significantly increased as compared to healthy individuals. Early-stage thyroid carcinoma patients exhibited significant higher levels of IFN-α, but not of IL-8, while IL-8 and not IFN-α levels were found significantly elevated in advanced-stage thyroid carcinoma patients in comparison to healthy individuals (Tables 3 and 4).

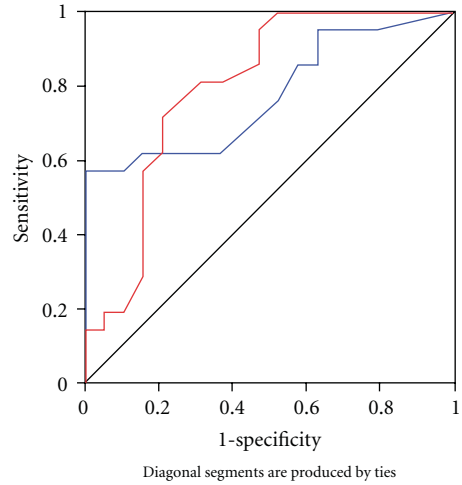
ROC curve (Figure 1(a)) indicates that both IL-8 and IFN-α exhibited a good discriminatory efficacy between healthy individuals and total patients with thyroid diseases (IL-8: AUC-0.756; IFN-α: AUC-0.795). Moreover, the ROC curves for both the cytokines between healthy individuals and individual groups of patients, that is, goiter, autoimmune disease, and thyroid cancer, revealed that both IL-8 as well as IFN-α showed good sensitivity and specificity to discriminate between healthy individuals and patients having different thyroid diseases (goiter- IL-8: AUC-0.774, IFN-α: AUC-0.797; autoimmune thyroid disease- IL-8: AUC-0.711, IFN-α: AUC-0.755, and thyroid cancer- IL-8: AUC-0.767, IFN-α: AUC-0.813) (Figures 1(b)–1(d)). Only IL-8 could significantly discriminate between early- and advanced-stage thyroid cancer patients (Figure 1(e)).

Similar to the present study, Limkov et al. have demonstrated statistically significant differences in IL-8 levels thyroid disease patients and reference normal group [30]. Bossowski and Urban and Siddiqi et al. have also observed significantly elevated levels of IL-8 in patients with Graves' disease and nodular goiter as compared to the respective healthy control groups [31, 32]. In contrast to these of studies, Krassas and colleagues found that IL-8 levels were not elevated in Graves' disease, toxic nodular goiter and Hashimoto's thyroiditis [33]. A study by Lee et al. has shown increased levels of cytokines including IL-8 and IL-10 in patients who frequently developed euthyroid sick syndromes followed by allogeneic bone marrow transplantation [34]. Antonelli et al. observed increased CXCL10 levels in autoimmune thyroiditis [35]. Also, they have demonstrated



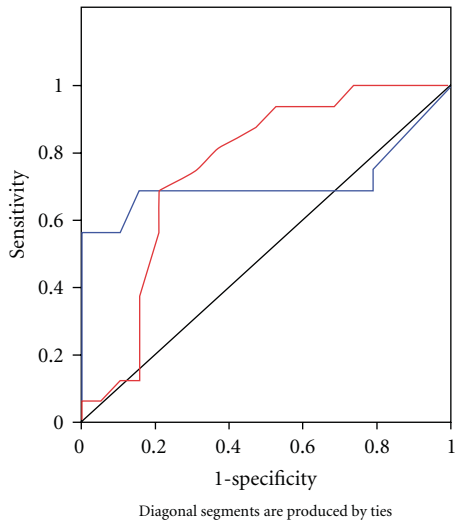
Area under the curve					
Test result variables	Area	Std. error	Asymptotic sig.	Asymptotic 95% confidence interval	
				Lower bound	Upper bound
IL-8	0.756	0.050	0.001	0.659	0.853
IFN-α	0.795	0.067	0.000	0.663	0.926

(a) Healthy individuals and total patients with thyroid disease



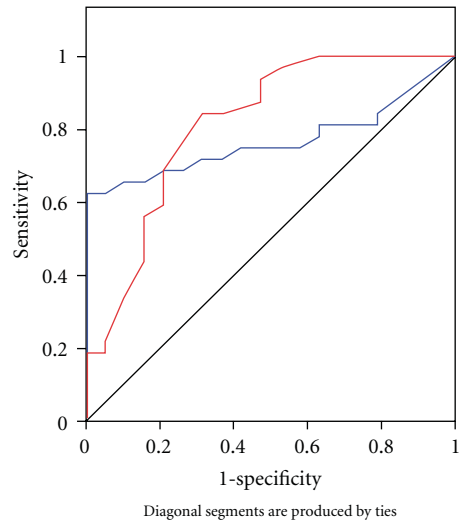
Area under the curve					
Test result variables	Area	Std. error	Asymptotic sig.	Asymptotic 95% confidence interval	
				Lower bound	Upper bound
IL-8	0.774	0.075	0.003	0.628	0.921
IFN-α	0.797	0.074	0.001	0.651	0.943

(b) Healthy individuals and patients with goiter



Area under the curve					
Test result variables	Area	Std. error	Asymptotic sig.	Asymptotic 95% confidence interval	
				Lower bound	Upper bound
IL-8	0.711	0.103	0.034	0.509	0.912
IFN-α	0.755	0.085	0.010	0.589	0.921

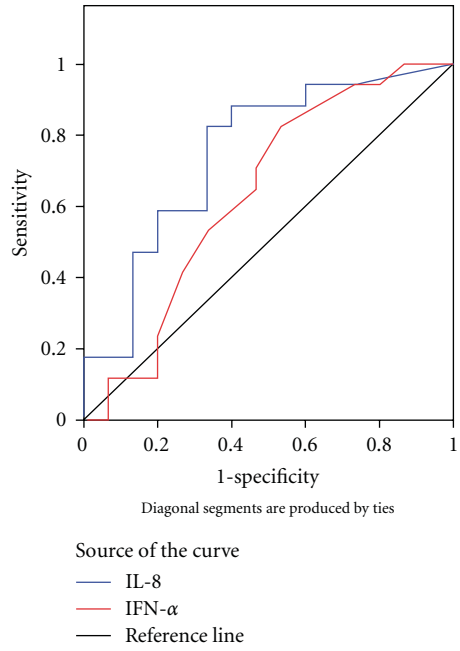
(c) Healthy individuals and patients with autoimmune thyroid disease



Area under the curve					
Test result variables	Area	Std. error	Asymptotic sig.	Asymptotic 95% confidence interval	
				Lower bound	Upper bound
IL-8	0.767	0.067	0.002	0.637	0.898
IFN-α	0.813	0.066	0.000	0.684	0.942

(d) Healthy individuals and thyroid cancer patients

Figure 1: Continued.



Area under the curve					
Test result variables	Area	Std. error	Asymptotic sig.	Asymptotic 95% confidence interval	
				Lower bound	Upper bound
IL-8	0.749	0.090	0.016	0.573	0.925
IFN-α	0.635	0.103	0.193	0.434	0.837

(e) Early stage and advanced stage thyroid carcinoma

Figure 1: ROC curves for IL-8 and IFN-α.

elevated circulating levels of CXCL10 and IFN-gamma in patients with Graves' disease, particularly in those with Graves' ophthalmopathy [36]. Increased IL-8 levels have also been observed in patients with other malignancies. In their study, Yi. Ren et al. also found that the level of serum IL-8 was markedly elevated in most patients with HCC as compared with healthy subjects [37]. In fact, elevated serum level of IL-8 was found to be a prognostic marker in soft tissue sarcoma [38], B-cell chronic lymphocytic leukemia [39], primary gastrointestinal non-Hodgkin's lymphoma [40], and malignant melanoma [41]. So, high serum levels of both the cytokines in thyroid carcinoma patients may be caused by an excessive production in tumor cells and subsequent release into the circulation.

The incidence of patients with thyroid diseases having higher levels of IL-8 and IFN-α than those of healthy individuals has been shown in Figures 2(a)–2(c). The levels of IL-8 $>$ 7.00 ng/ml (maximum level of IL-8 in healthy individuals) were found in 57.10% of patients with goitre, 56.30% patients having autoimmune diseases and in 62.50% thyroid carcinoma patients. While levels of IFN-α $>$ 4.00 ng/ml (maximum level in healthy individuals) were observed in only 14.30% patients with goitre, 6.30% patients with autoimmune diseases and in 18.80% thyroid carcinoma patients.

The incidence of thyroid carcinoma patients having higher levels of IL-8 as compared to that of healthy individuals increased with the increase in disease stage. That is, 40% of early-stage thyroid carcinoma patients as compared to 82.40% advanced-stage thyroid carcinoma patients had higher IL-8 levels than the maximum range found in healthy individuals.

Association of the serum IL-8 and IFN-α levels with different clinicopathological parameters have been studied by Mann-Whitney U test. It revealed that elevated serum IL-8 levels were significantly associated with advanced-disease stage (Figure 3). In fact, serum IL-8 levels were significantly increased in patients with advanced stage compared to those with early-stage disease (445.12 ± 214.30 versus 101.56 ± 53.89; $P = .016$).

Moreover, Spearman rank's correlation analysis revealed significant positive relationships between IL-8 levels and disease stage ($r = 0.437$, $P = .012$). IFN-α levels were not significantly associated nor did they exhibit any significant relationship with any of the clinicopathological parameters. Yi Ren et al. demonstrated that high serum IL-8 level correlated with large tumor volume and advanced tumor stage in patients with hepatocellular carcinoma [37]. Increased IL-8 expression has been found in various tumors and in some studies IL-8 serum and/or tissue levels correlate with

Table 2: Clinicopathological parameters of thyroid cancer patients.

Parameters	*N* (%)	Parameters	*N* (%)
Age		Multifocality	
<45 years	17 (53.10)	Present	14 (43.70)
≥45 years	15 (46.90)	Absent	18 (56.30)
Gender		Bilaterality	
Male	09 (28.10)	Unilateral	23 (71.90)
Female	23 (71.90)	Bilateral	09 (28.10)
Tumor size		Haemorrhagic area	
T1 + T2	16 (50.00)	Present	07 (21.90)
T3 + T4	16 (50.00)	Absent	25 (78.10)
Lymph node metastasis		Necrosis	
Present	18 (56.30)	Present	03 (9.40)
Absent	14 (43.70)	Absent	29 (90.60)
Distant metastasis		Calcification	
Present	21 (65.60)	Present	19 (59.40)
Absent	11 (34.30)	Absent	13 (40.60)
Stage		Sclerosis	
Early stage (Stage I & II)	15 (46.90)	Present	04 (12.50)
Advanced stage (Stage III & IV)	17 (53.10)	Absent	28 (87.50)
Lymphatic permeation		Extrathyroidal extension	
Present	04 (12.50)	Present	13 (40.60)
Absent	28 (87.50)	Absent	19 (59.40)
Vascular permeation		Fibrosis	
Present	08 (25.00)	Present	08 (25.00)
Absent	24 (75.00)	Absent	24 (75.00)
Capsular invasion		Inflammation	
Present	13 (40.60)	Present	14 (43.70)
Absent	19 (59.40)	Absent	18 (56.30)
Encapsulation		Differentiation	
Well-encapsulated	27 (84.40)	Well	22 (68.75)
Not encapsulated	05 (15.60)	Moderate/Poor	10 (31.25)

Table 3: Significance of IL-8 levels in patients with thyroid diseases as compared to healthy individuals.

Subjects	Mean ± S.E (pg/ml)	Median	Minimum	Maximum	*P* value
Healthy individuals	3.15 ± 0.58	2.97	0.00	7.00	
Total patients	231.67 ± 72.51				**.001**
Goitre	187.16 ± 93.88	8.41	0.00	1899.06	**.002**
Autoimmune disorders	185.30 ± 167.84	7.92	0.00	2700.00	**.034**
Graves' disease	20.21 ± 10.93				.085
Hashimoto disorder	680.57 ± 673.14				.097
Thyroid Carcinoma	284.08 ± 118.97	69.35	0.00	3200.00	**.001**
Papillary carcinoma	116.34 ± 44.36				**.006**
Follicular carcinoma	514.13 ± 295.64				**.030**
Medullary carcinoma	828.96 ± 790.79				.456
Anaplastic carcinoma	27.17 ± 19.50				**.003**
Early stage (Stage I & II)	101.56 ± 53.89				.286
Advanced stage (Stage III & IV)	445.12 ± 214.30				**<.001**

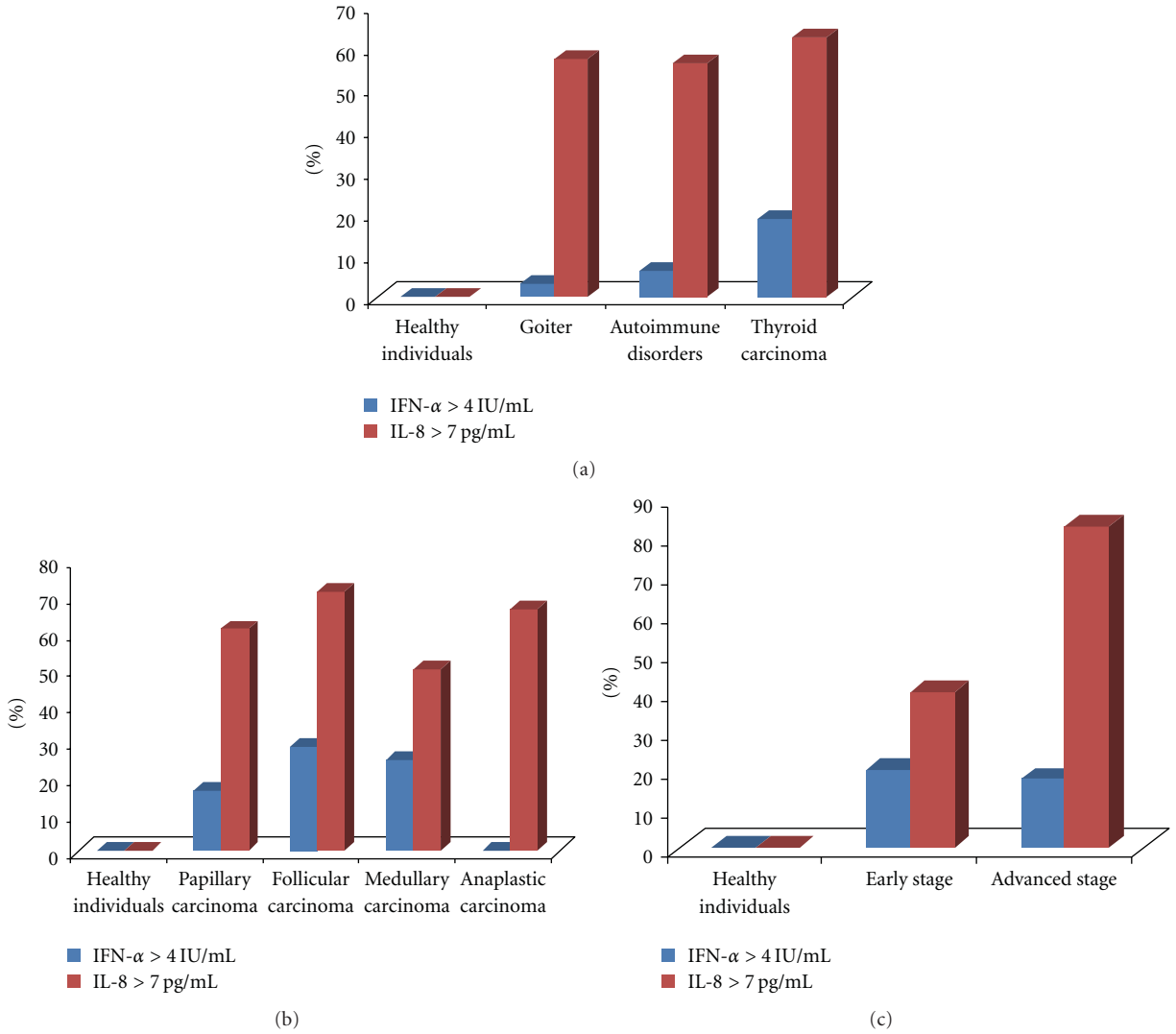


Figure 2: (a) Incidence of IL-8 and IFN-α levels in patients with thyroid diseases as compared to healthy individuals. (b) Incidence of IL-8 and IFN-α levels in thyroid carcinoma patients as compared to healthy individuals. (c) Incidence of IL-8 and IFN-α levels in early- and advanced-stage thyroid carcinoma patients as compared to healthy individuals.

Table 4: Significance of IFN-α levels in patients with thyroid diseases as compared to healthy individuals.

Subjects	Mean ± S.E (IU/ml)	Median	Minimum	Maximum	*P* value
Healthy individuals	2.41 ± 0.18	2.26	0.50	4.00	
Total patients	3.82 ± 0.50				**.002**
Goitre	3.40 ± 0.30	3.14	2.27	9.07	**.001**
Autoimmune disorders	2.97 ± 0.12	3.02	2.01	4.16	**.009**
Graves' disease	2.91 ± 0.15				**.028**
Hashimoto disorder	3.14 ± 0.21				.054
Thyroid carcinoma	4.51 ± 1.06	3.21	2.14	36.67	**<.001**
Papillary carcinoma	5.10 ± 1.87				**.003**
Follicular carcinoma	3.48 ± 0.31				**.007**
Medullary carcinoma	4.72 ± 1.29				**.006**
Anaplastic carcinoma	3.10 ± 0.27				.087
Early stage (Stage I & II)	5.37 ± 2.24				**.007**
Advanced stage (Stage III & IV)	3.76 ± 0.36				**<.001**

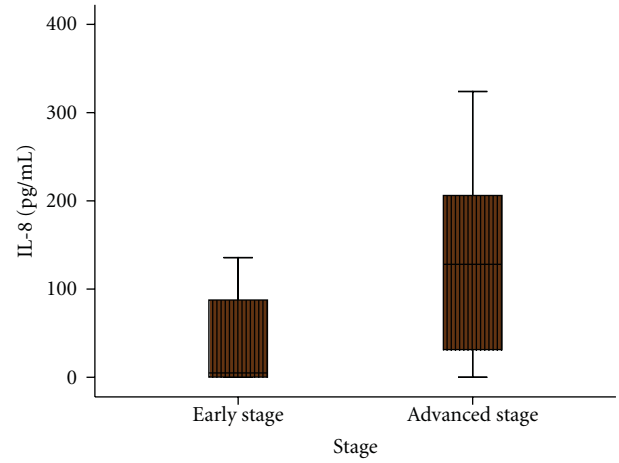
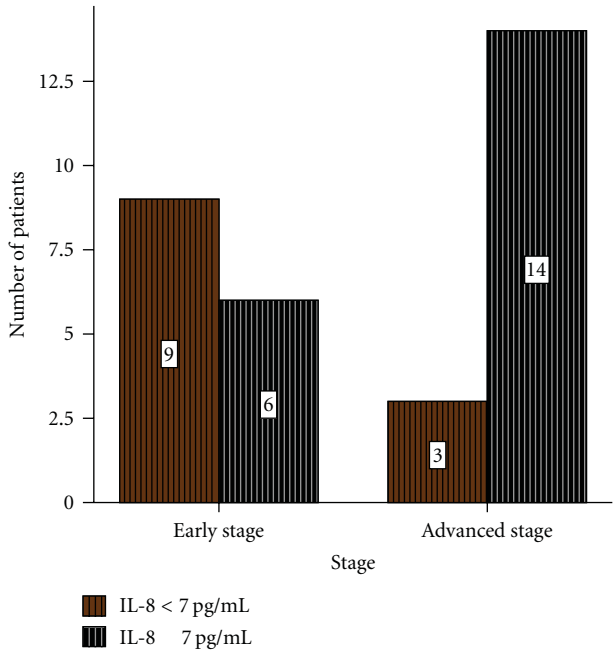


Figure 3: Association between serum IL-8 levels and histopathological stage of thyroid carcinoma patients.



Odds ratio	95% confidence interval		Chi square (χ^2)
	Lower	Upper	
7.000	1.386	35.345	0.017

Figure 4: Relative risk of IL-8 in early- and advanced-stage thyroid carcinoma patients.

tumor progression and metastasis [42–45]. So, this finding additionally supports the role of IL-8 in the progression of thyroid carcinoma.

The relative risk study indicated that 14/17 advanced-stage thyroid carcinoma patients and 6/15 early-stage thyroid carcinoma patients had elevated IL-8 levels (>7.00 pg/ml) (Figure 4), which indicates IL-8 as a risk factor in development of advanced-stage thyroid cancer.

4. Conclusion

Finally concluding, as both serum IL-8 and IFN-α levels were significantly higher, not only in thyroid cancer patients, but also in patients having goitre and autoimmune diseases, they can be used for differentiating such patients, having any disease of thyroid gland, from that of healthy individuals. Since such conditions often constitute prevalent pre-existing disease states in the pathogenesis of thyroid cancer, these results strongly suggest an association between IL-8 and IFN-α upregulation and development of thyroid carcinoma.

Moreover, IL-8 could efficiently discriminate between early- and advanced-stage disease. Also, the major findings demonstrate that IL-8 levels, were significantly positively related to the disease stage and the elevated IL-8 levels were predominantly associated with the advanced stage of the disease whereas IFN-α levels did not show any significant correlation or association with any of the histopathological parameters of thyroid carcinoma patients. Thus, IL-8 seems to have a role in thyroid cancer pathogenesis, and measurement of preoperative serum IL-8 levels might be more useful and feasible in the clinical setting to predict tumor stage where it may represent a putative target for innovative diagnostic and therapeutic strategies. However, further studies including more number of patients and evaluating the postoperative changes in serum IL-8 levels may provide additional predictive value on tumor recurrence and prognosis.

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