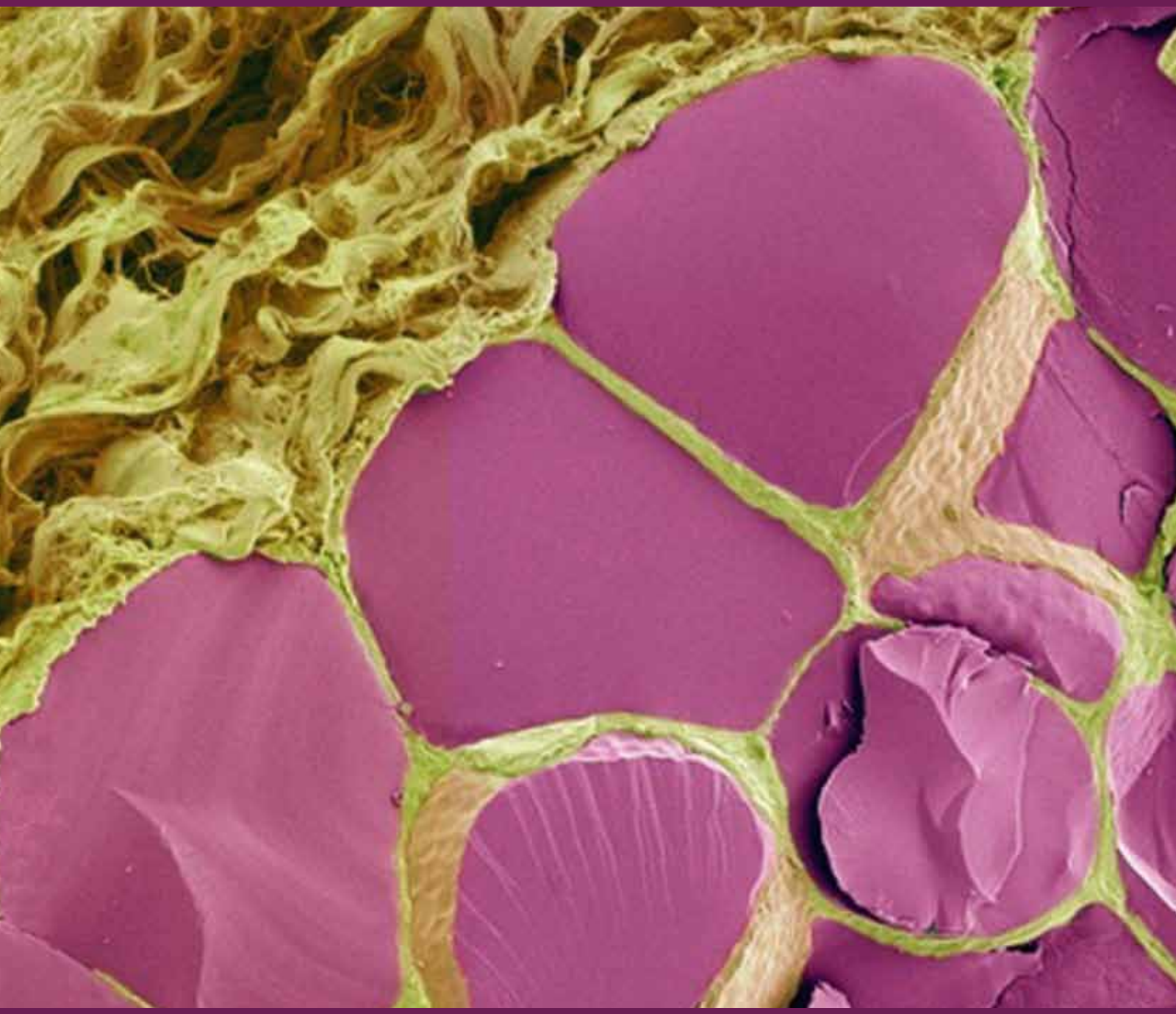
International Journal of Endocrinology

New Diagnostic and Therapeutic Tools for Thyroid Cancer

Guest Editors: Eleonore Fröhlich, Richard Wahl, Barbara Czarnocka, and Leonidas Duntas



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Editorial Board

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Editorial

New Diagnostic and Therapeutic Tools for Thyroid Cancer

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Thyroid cancer is the commonest endocrine malignancy but represents only 1% of all cancers. Although the prognosis of differentiated thyroid cancer is good, the survival time of anaplastic and of medullary thyroid cancer is still very short. More precise diagnosis with better stratification of patients and identification of risk patients may improve the prognosis of thyroid cancer. Treatment of thyroid cancer is multidisciplinary and involves endocrinologists, nuclear medicine specialists, and surgeons. The spectrum of contributions to this special issue in International Journal of Endocrinology is well reflecting this situation.

The contributions to this special issue, two reviews and three studies, report mainly on laboratory and imaging techniques to improve diagnosis in thyroid cancer. J. Hannallah et al. emphasize the multimodal approach in diagnosis and managing patients with poorly differentiated thyroid cancer in their review. The identification of poorly differentiated thyroid cancer has been complicated by the lack of clear criteria for diagnosis. A panel of histological, immunohistochemical, and genetic markers and clinical parameters is listed which are typical for this thyroid carcinoma entity. The value of current treatment possibilities is critically discussed. In the paper review by G. Treglia et al. the role of Fluorine-18-Fluorodeoxyglucose positron emission tomography in aggressive subtypes of different thyroid cancer types (Hürthle cell, anaplastic, poorly differentiated, more aggressive histological subtypes of differentiated thyroid cancer, and metastases) is discussed. FDG-PET-positivity in radio-iodine-refractory differentiated thyroid cancer appears to predict more aggressive tumor progression. Data on imaging of medullary thyroid carcinoma lesions using glucagon-like peptide 1 by [Lys40(Ahx-HYNIC-99mTc/EDDA)NH2]-exendin-4 are presented by D. Pach et al. Radioactively labeled GLP-1 analogues are successfully used in patients with insulinoma but the diagnostic value in medullary thyroid cancer is not clear. According to the first preliminary data, the analogues may possess a confirmatory role in lesions where inconsistent results are obtained with other imaging procedures. S. H. Hsieh et al. in their study identified male gender as prognostic parameter for higher recurrence in papillary thyroid cancer in stages II–IV. The study by B. C. Ahn et al., by contrast, deals with a very established clinical test, the measurement of serum thyroglobulin levels, for the identification of recurrent lesions in differentiated thyroid cancer. The importance of patients' antithyroglobulin levels in the calculation of thyroglobulin levels is addressed.

The identification of potentially aggressive subtypes of thyroid cancer and the early detection of recurrent lesions play key roles in the managing of the patient. By compiling these papers, we hope to add some knowledge with respect to screening and postoperative care particularly of the more aggressive thyroid cancer types.

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Review Article

The Role of Fluorine-18-Fluorodeoxyglucose Positron Emission Tomography in Aggressive Histological Subtypes of Thyroid Cancer: An Overview

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Aggressive histological subtypes of thyroid cancer are rare and have a poor prognosis. The most important aggressive subtypes of thyroid cancer are Hürthle cell carcinoma (HCTC) and anaplastic and poorly differentiated carcinoma (ATC and PDTC). The American Thyroid Association recently published guidelines for the management of patients with ATC, but no specific guidelines have been done about HCTC. We performed an overview of the literature about the role of Fluorine-18-Fluorodeoxyglucose positron emission tomography or positron emission tomography/computed tomography (FDG-PET or PET/CT) in aggressive histological subtypes of thyroid cancer. Only few original studies about the role of FDG-PET or PET/CT in HCTC, PDTC, and ATC have been published in the literature. FDG-PET or PET/CT seems to be useful in staging or followup of invasive and metastatic HCTC. FDG-PET or PET/CT should be used in patients with ATC in initial staging and in the followup after surgery to evaluate metastatic disease. Some authors suggest the use of FDG-PET/CT in staging of PDTC, but more studies are needed to define the diagnostic use of FDG-PET/CT in this setting. Limited experience suggests the usefulness of FDG-PET or PET/CT in patients with more aggressive histological subtypes of DTC. However, DTC presenting as radioiodine refractory and FDG-PET positive should be considered aggressive tumours with poor prognosis.

1. Introduction

Aggressive histologic subtypes of thyroid cancer are less frequent and have a worse prognosis than well-differentiated thyroid carcinoma (DTC). Most important aggressive subtypes of thyroid cancer are Hürthle cell carcinoma (HCTC) and anaplastic and poorly differentiated carcinoma (ATC and PDTC).

HCTC was firstly considered as subtypes of DTC. Now, it is included in aggressive histologic subtypes, because of its biological behaviour. ATC could be a de novo tumour or arising from dedifferentiation of DTC. During this process, thyroid cancer could be found in an intermediate differentiation pattern, classified as PDTC. Both ATC and PDTC have a poor prognosis and efficient diagnostic tools are needed to improve survival.

After those about DTC and medullary thyroid cancer (MTC) [1, 2], American Thyroid Association (ATA) recently published guidelines for the management of patients with ATC [3]. No specific guidelines have been done about HCTC.

Fluorine-18-Fluorodeoxyglucose (FDG), a glucose analogue, is the most used positron emission tomography (PET) tracer in oncology. Although FDG-PET and PET/CT have a moderate sensitivity for early-stage, well-differentiated thyroid malignancy [4], they are currently used in DTC, particularly in postthyroidectomy patients with high serum thyroglobulin (Tg) levels and a negative radioiodine whole-body scan, as prognostic tool in patients with metastases, for

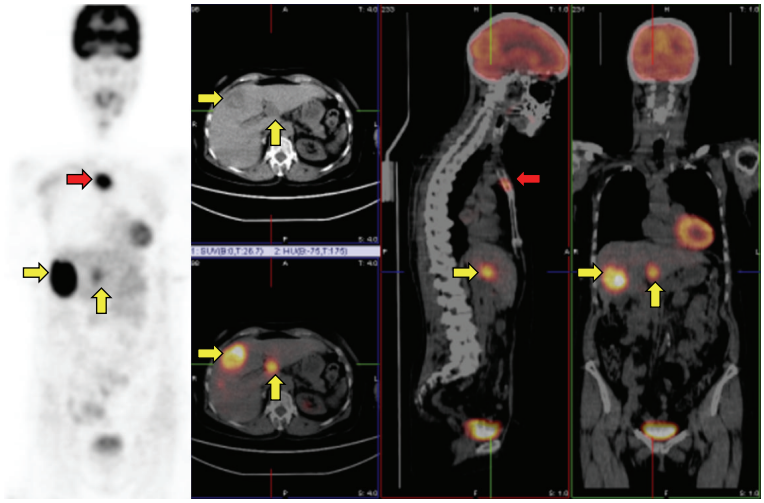

Figure 1: FDG-PET/CT in a 68-year-old female previously operated for HCTC showing the presence of sternal (red arrows) and liver metastases (yellow arrows).

the measurement of posttreatment response, and as selection tool in patients not eligible to radioiodine therapy [5–7]. FDG-PET or PET/CT is also used in recurrent MTC and in thyroid nodules with indeterminate or nondiagnostic fine needle aspiration biopsy (FNAB) [5].

During dedifferentiation process (from DTC to ATC), an inverse relationship between radioiodine (I-131) and FDG uptake in thyroid cancer cells was observed (the so-called flip-flop phenomenon) [8]. A recent study highlights that thyroid cancer dedifferentiation is characterized by glucose transporters (GLUT1) upregulation and reduced expression of sodium-iodide symporter (NIS) [9]. This is the rationale to propose FDG-PET or PET/CT as efficient diagnostic tools in ATC, PDTC, and other aggressive subtypes of thyroid cancer.

The aim of this paper is to perform an overview of the literature about the role of FDG-PET or PET/CT in aggressive histological subtypes of thyroid cancer.

2. FDG-PET in Hürthle Cell Thyroid Carcinoma (HCTC)

About 3.6% of thyroid cancers are HCTC [10]. Initially, HCTC was included in DTC group, but it has a different oncogenic expression and is now considered as different histological and clinic disease [11].

HCTC has a 10-year disease-free survival of 40% and mortality of 51% [12], worse than DTC. In fact, HCTC is associated with a high risk of distant and lymph nodal metastases having a worse prognosis compared to DTC.

Only few studies about the role of FDG-PET or PET/CT in HCTC have been published in the literature. Some authors suggested a good sensitivity of FDG-PET in HCTC [13, 14] and Hürthle cell adenoma [15]. Overall, HCTC seems to be unable to concentrate I-131, but it is an FDG-avid tumour.

Pryma et al. [16] studied 44 patients with HCTC. There were 24 positive and 20 negative FDG-PET scans giving a sensitivity of 95.8% and a specificity of 95%. FDG-PET demonstrated a good diagnostic accuracy in HCTC patients. Furthermore, a high FDG uptake was demonstrated to be a negative prognostic factor. These authors suggested that FDG-PET could be indicated in patients with HCTC in postoperative staging and as followup in patients with an increase of Tg or recurrent disease [16].

Lowe et al. [17] studied 14 FDG-PET scans in patients with HCTC. PET findings were positive in all but 1 of patients with known disease, with a sensitivity of 92%. Moreover, in 7 out of 14 PET scans, a disease not diagnosed by other techniques was demonstrated. In 7 patients, therapy was changed after FDG-PET. So, these authors concluded that FDG-PET improves staging and disease management in patients with HTCT [17].

Plotkin et al. [18] evaluated 17 HCTC with FDG-PET. In subgroup A, patients with an elevated Tg level were included ($n = 13$), and in 10 cases PET scans were true positive. In subgroup B, patients with a suspect morphologic imaging were included ($n = 4$), and PET scans were true negative in three cases. Only one false positive was found in each group. Overall, FDG-PET demonstrated a sensitivity of 92%, a specificity of 80%, a positive predictive value of 92%, a negative predictive value of 80%, and an accuracy of 89% in HCTC [18].

Overall, FDG-PET or PET/CT seems to be useful functional imaging methods in initial staging or restaging of HCTC (Figure 1), presenting high diagnostic accuracy in this setting (Table 1).

3. FDG-PET in Anaplastic Thyroid Carcinoma (ATC)

ATC is a rare and aggressive tumour, representing less than 5% of all thyroid carcinomas and originating by thyroid follicular cells (as DTC). ATC is often diagnosed in older patients and usually has a rapid growth and an extensive local invasion [21–23]. Three main histological subtypes of ATC are reported: spindle cell, pleomorphic giant cell, and

Table 1: Main findings about FDG-PET or PET/CT in Hürthle cell thyroid carcinoma, anaplastic thyroid carcinoma, and poorly differentiated thyroid carcinoma.

Type	Authors	Year	Device	Patients	Sensitivity	Specificity	Comments
HCTC	Pryma et al. [16]	2006	PET or PET/CT	44	95.8%	95%	FDG-PET has excellent diagnostic accuracy in HCTC, improving on CT and radioiodine scintigraphy. Intense FDG uptake is indicator of a poor prognosis. Patients with HCTC should undergo FDG-PET as part of their initial postoperative staging and periodically to screen for occult recurrence, particularly in patients with elevated serum thyroglobulin
	Lowe et al. [17]	2003	PET	12	91.6%	N.A.	HCTC demonstrates intense FDG uptake. PET improves disease detection and disease management in HCTC relative to anatomic or radioiodine imaging. FDG-PET should be recommended for the evaluation and clinical management of HCTC
	Plotkin et al. [18]	2002	PET	17	100%	60%	This study supports the efficiency of FDG-PET in the followup of HCTC
ATC	Grabellus et al. [9]	2012	PET/CT	4	100%	N.A.	ATC shows intense FDG uptake. FDG-PET/CT is an important imaging modality for ATC
	Poisson et al. [19]	2010	PET/CT	20	100%	N.A.	FDG-PET/CT appears to be the reference imaging modality for ATC at initial staging and seems promising in the early evaluation of treatment response and followup
	Bogsrud et al. [20]	2008	PET	16	100%	N.A.	FDG-PET may improve disease detection and have an impact on the management of patients with ATC relative to other imaging modalities
PDTC	Grabellus et al. [9]	2012	PET/CT	22	86.3%	N.A.	PDTC shows intermediate FDG uptake between DTC and ATC. FDG-PET/CT is an important imaging modality for PDTC

Legend: N.A.: not available; DTC: differentiated thyroid carcinoma; PDTC: poorly differentiated thyroid carcinoma; ATC: anaplastic thyroid carcinoma; HCTC: Hürthle cell thyroid carcinoma.

squamous cell subtype [3]. In over 70% of the patients the tumour infiltrates surrounding tissues, and median survival time is about 6–8 months [22]. In differential diagnosis, it is important to distinguish ATC and PDTC. In fact, proportion of ATC, PDTC, or DTC characterizing the thyroid tumour can change prognosis and clinical management [3].

This aggressive thyroid tumour is not able to uptake iodine and to produce Tg [19, 20, 24, 25]. Conversely, ATC has a high glucose metabolism and high FDG uptake [5, 9]. ATA recently published guidelines for management of patients with ATC [3]. ATA recommended FDG-PET and PET/CT in evaluating metastatic patients, especially bone lesions. Moreover, FDG-PET may be useful in distinguishing ATC from DTC metastases because of the higher FDG uptake of ATC. Other indications described about FDG-PET or PET/CT in ATC were resectability evaluation and followup, with a higher sensitivity than CT alone. FDG-PET is also recommended 3–6 months after therapy in patients with no disease or in persistent structural disease as a guide to therapy [3].

Few original studies have been published about the role of FDG-PET in ATC (Table 1).

Poisson et al. [19] studied 20 consecutive ATC patients with FDG-PET/CT for initial staging and during followup. Authors analysed progression on imaging followup (CT or PET/CT). Per lesion, organ, and patient analysis have been done. In univariate analysis, maximal standardised uptake value (SUVmax) and functional volume were a predictive factor for survival. Conversely, in bivariate analysis, only functional volume was a prognostic factor. Early evaluation of treatment has been done in 4 out of 11 patients in whom PET and CT were both registered. After treatment with combined radiotherapy and chemotherapy, a negative FDG-PET/CT scan confirmed a complete long-term remission. Finally, authors suggested the use of FDG-PET/CT in ATC during initial staging. Among other imaging modalities, only preoperative CT should be requested. FDG-PET/CT could be also recommended in both early and long-term followup and in the assessment of treatment response [19].

Bogsrud et al. [20] investigated the role of FDG-PET in the management of patients with ATC. PET data were compared with other diagnostic tools (CT, ultrasound, magnetic resonance imaging, bone scan, and histology) and with clinical follow-up. In all 16 patients included, PET records resulted true positive for primary tumours. In 50% of patients, PET data influenced the clinical management. These authors concluded that FDG-PET could improve disease staging changing the clinical management of patients with ATC [20].

Overall, FDG-PET or PET/CT should be used in patients with ATC in initial staging and in the followup after surgery to evaluate metastatic disease (Figure 2). In selected cases, these functional imaging methods may be helpful in directing treatment and in evaluating the efficacy of therapy. New

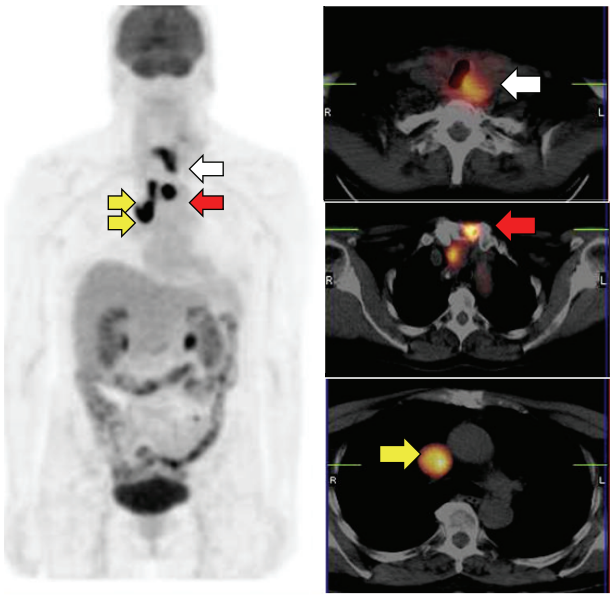

Figure 2: FDG-PET/CT in a 58-year-old female with ATC showing the presence of increased uptake in the thyroid tumour (white arrow) and sternal (red arrows) and mediastinal lymph nodal metastases (yellow arrows).

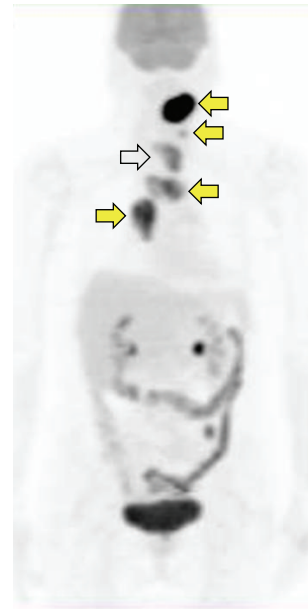

Figure 3: FDG-PET in a 48-year-old female with PDTC showing the presence of increased uptake in the thyroid tumour (white arrow) and multiple cervical and mediastinal lymph nodal metastases (yellow arrows).

studies are needed to investigate the role of FDG-PET or PET/CT in detecting the proportion of DTC, ATC, and PDTC in the same tumour.

4. FDG-PET in Poorly Differentiated Thyroid Carcinoma (PDTC)

PDTC is an intermediate histological subtype between DTC and ATC and may be a transition form. Conversely to ATC, PDTC preserves some markers of differentiation, such as Tg and thyroid transcription factor 1 (TTF1), and does not represent a de novo tumour [26]. In dedifferentiation process, PDTC and ATC present a growing number of chromosomal alteration, such as RAS, BRAF, TP53, and b-catenin mutations [3, 11]. Activation of the PTEN/PI3 kinase/Akt/mammalian target of rapamycin pattern and mutation of the AKT or PIK3CA genes are more common in PDTC and ATC than DTC [26–29]. These metabolic pathways could be related to the different FDG-PET pattern in different subtypes of thyroid cancer.

PDTC has an intermediate GLUT1 expression and FDG uptake between ATC and DTC, because of "flip-flop" phenomenon [9, 30]. More often PDTC is an FDG-PET positive tumour [5, 9]. An in vitro study suggested that thyrotropin (TSH) increases FDG uptake in PDTC cells; so, FDG-PET scans under TSH stimulation may be more efficient [31]. Surgery and radiotherapy could be indicated in treatment of PDTC but not radioiodine treatment, because of poor radioiodine uptake.

No studies analysed the role of FDG-PET in PDCT only. Some authors suggested the use of FDG-PET or PET/CT in staging patients with PDTC (Figure 3), especially in postthyroidectomy staging of high-risk patients [11]. Some authors studied the role of FDG-PET in all thyroid cancer subtypes but included only few cases of PDTC in their analysis, not sufficient to conclude that FDG-PET is efficient for this histological tumour type.

More preclinical and clinical studies are needed about FDG-PET or PET/CT in PDTC to demonstrate the clinical usefulness of FDG-PET in PDTC.

5. FDG-PET in More Aggressive Histological Subtypes of DTC

Limited experience exists about the role of FDG-PET or PET/CT in patients with more aggressive histological subtypes of DTC, including case reports or small case series in patients with tall cell [32], diffuse sclerosing [33–35], solid/trabecular [36] and insular variant [37] of DTC. These articles underlines that FDG-PET or PET/CT seem to be very useful tools for the staging and restaging of such tumours.

6. Noniodine Concentrating Metastases of DTC

Radioactive iodine-refractory (RAIR) FDG-PET positive thyroid carcinomas represent the major cause of deaths from thyroid carcinomas and are therefore the main focus of novel target therapies. Although the majority of primary thyroid carcinomas leading to RAIR FDG-PET positive metastatic disease are PDTC, DTC can also be responsible for RAIR disease. Histologic characterization of metastases/recurrence

in 70 RAIR FDG-PET positive thyroid carcinoma patients revealed that 47.1% had PDTC, 20% had tall-cell variant of papillary thyroid carcinoma, 22.9% had well-differentiated papillary thyroid carcinoma (including classic and follicular variants), 8.6% had HCTC, and 1.4% had ATC [30].

DTC presenting FDG uptake on PET scan and histological features such as necrosis should be considered aggressive differentiated cancers and FDG uptake in these tumours is highly prognostic for survival [38].

7. Conclusions

From this overview of the literature about the usefulness of FDG-PET or PET/CT in aggressive subtypes of thyroid tumours, we conclude the following:

(i) the role of FDG-PET or PET/CT in patients with HCTC is clear in initial staging or followup of invasive and metastatic tumours;

(ii) FDG-PET or PET/CT is recommended in staging, followup, and posttreatment restaging of ATC, especially in metastatic disease, as published in ATA guidelines;

(iii) further evaluations are needed to investigate the role of FDG-PET or PET/CT in PDTC, because of the difficulties connected to define the biological behaviour of this aggressive subtype of thyroid cancer;

(iv) limited experience suggests the usefulness of FDG-PET or PET/CT in patients with more aggressive histological subtypes of DTC;

(v) DTC presenting as RAIR and FDG-PET positive should be considered aggressive tumours with poor prognosis.

Conflict of Interests

The Authors declare no conflict of interests.

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Research Article

Glucagon-Like Peptide-1 Receptor Imaging with [Lys^{40}(Ahx-HYNIC-^{99m}Tc/EDDA)NH$_2$]-Exendin-4 for the Diagnosis of Recurrence or Dissemination of Medullary Thyroid Cancer: A Preliminary Report

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Introduction. Epidemiological studies on medullary thyroid cancer (MTC) have shown that neither a change in stage at diagnosis nor improvement in survival has occurred during the past 30 years. In patients with detectable serum calcitonin and no clinically apparent disease, a careful search for local recurrence, and nodal or distant metastases, should be performed. Conventional imaging modalities will not show any disease until basal serum calcitonin is at least 150 pg/mL. The objective of the study was to present the first experience with labelled glucagon-like peptide-1 (GLP-1) analogue [Lys^{40}(Ahx-HYNIC-^{99m}Tc/EDDA)NH$_2$]-exendin-4 in the visualisation of MTC in humans. *Material and Method*. Four patients aged 22–74 years (two with sporadic and two with MEN2 syndrome-related disseminated MTC) were enrolled in the study. In all patients, GLP-1 receptor imaging was performed. *Results*. High-quality images were obtained in all patients. All previously known MTC lesions have been confirmed in GLP-1 scintigraphy. Moreover, one additional liver lesion was detected in sporadic MTC male patient. *Conclusions*. GLP-1 receptor imaging with [Lys^{40}(Ahx-HYNIC-^{99m}Tc/EDDA)NH$_2$]-exendin-4 is able to detect MTC lesions. GLP-1 scintigraphy can serve as a confirmatory test in MTC patients, in whom other imaging procedures are inconsistent.

1. Introduction

Medullary thyroid cancer (MTC) is a neuroendocrine neoplasm arising from the parafollicular cells, or C cells, of the thyroid. It accounts for nearly 5–10 % of thyroid malignancies. In nearly all MTC cases, cancer cells secrete calcitonin, a specific and highly sensitive biomarker—its measurement plays an important role in diagnosis and postoperative followup of patients [1–3]. The majority of MTCs are sporadic, but up to 25% of all cases result from a germ-line activating mutation of the *RET* protooncogene [4, 5]. Hereditary MTCs occur in the setting of the multiple endocrine neoplasia (MEN) syndrome type 2 (2A or 2B) or as familial MTC (FMTC)—a variant of MEN2A syndrome. The most common form of hereditary MTC is MEN 2A (approximately 80–90% of patients with hereditary MTC). Overall, the prognosis for patients with MTC is good. The 10-year survival rate is 75–85%. Approximately half of the MTC patients present with disease limited to the thyroid gland with a 10-year survival rate of 95.6%. One-third of patients present with locally invasive tumour or clinically apparent spread to the regional lymph nodes. Patients with regional disease have a 5-year overall survival rate of 75.5%. Recurrent disease develops in approximately 50% of patients with MTC [1, 6]. Neck ultrasound should be performed as a part of the initial evaluation of each patient with newly diagnosed MTC. Fine-needle aspiration (FNA) cannot always distinguish MTC based on the appearance of tumor cells alone, so the diagnosis

is typically confirmed by immunostaining or by the measurement of calcitonin level in the washout fluid from FNA. This latter technique appears to be even more sensitive than cytology with immunohistochemistry [1].

The primary treatment for MTC is surgical resection. Total thyroidectomy with complete resection of central neck, paratracheal, and upper mediastinal lymph nodes is frequently needed. Currently, surgical excision is the only effective treatment for MTC. Patients who have clinically apparent disease are best treated with a minimum of total thyroidectomy and bilateral central neck dissection [7, 8]. Followup should start 2-3 months postoperatively by obtaining new baseline calcitonin levels. An undetectable basal serum calcitonin level is a strong predictor of complete remission. Patients with biochemical remission after initial treatment have only a 3% risk of recurrence during long-term followup [1, 2].

Calcitonin and stimulated calcitonin levels are very sensitive ways for detecting either residual or recurrent disease. When the postoperative calcitonin level is elevated, a careful search for metastases has to be performed prior to surgical exploration. Imaging techniques will not show any disease until basal serum calcitonin level exceeds 150 pg/mL. In patients with serum calcitonin lower than 150 pg/mL, localization of the disease should be focused on careful examination using neck ultrasound because such calcitonin levels are usually associated with locoregional disease. The optimal timing of this followup should be based on calcitonin and CEA (carcinoembryonic antigen) doubling times (DT), which are strongly correlated with disease progression [9–12].

There are some MTC patients in whom, despite of the elevated postoperative calcitonin levels and/or abnormal results of the pentagastrin test, there is no evidence of the disease in conventional imaging techniques. Prolonged delay in disease localization usually results in treatment failure even if the tumor recurrence/residue is finally detected. Molecular imaging techniques, based on the development of tracers which are taken up by MTC cells or are bound to MTC-specific receptors, could be applied in such group of patients. Therefore, besides the use of those well-known and commonly used radiotracers, such as labelled somatostatin analogues or mIBG, there are still clinical trials performed to find more specific and sensitive substances. Glucagon-like peptide 1 (GLP-1) labelled analogues have been considered as a promising tool for visualization of MTC. Physiologically GLP-1 (glucagon-like peptide-1) receptors have been found in organs like pancreas, blood vessels, stomach, or parafollicular C cells. Their expression is also observed in different types of neoplasms including MTC [13]. Both ^{111}In-labeled GLP-1 analogue ([Lys^{40}(Ahx-DTPA-^{111}In)NH$_2$]-exendin-4 and ^{68}Ga/^{99m}Tc labeled GLP-1 analogue exendin-4 were successfully used in patients with insulinoma [14–16]. ^{99m}Tc labelled GLP-1 analogue, may improve the quality of images and radiation safety for patients and the staff due to many procedural advantages related to the isotope physical properties.

The question of the management of patients with local recurrence and contraindications to surgical intervention or patients with dissemination of the disease has not been solved. Those patients are left with few therapeutic choices. Chemotherapy is of limited value. [17]. External beam radiation therapy (EBRT) may be used only to control local disease [7, 8]. Serum calcitonin and CEA concentrations do not normalize after EBRT, but long-term stabilization may be achieved. Patients with metastatic disease can have debilitating symptoms from calcitonin excess and therefore may benefit from medical treatment with somatostatin analogues. Since MTC cells express somatostatin receptors, a radionuclide-targeted therapy with labelled octreotide and its derivates is another therapeutic option [17, 18].

Molecular-targeted therapy is yet another therapeutic strategy in MTC. With the discovery of the *ret* protooncogene and its integral role in the pathogenesis of MTC, a new class of therapeutics—tyrosine kinase inhibitors—has been developed [17, 19, 20]. It is necessary to develop other alternative therapeutic strategies to control tumour growth, possibly through interference with various cellular signalling pathways [17, 19, 20].

The aim of the paper is to present the first experience of our department with the new radiopharmaceutical [Lys^{40}(Ahx-HYNIC-^{99m}Tc/EDDA)NH$_2$]-exendin-4 as a diagnostic tool in patients with suspected or confirmed recurrence or dissemination of MTC and to compare its performance with conventional imaging methods.

2. Material

Four patients (1 female, 3 males, aged 22–74 years) were enrolled in the study. In all of them, recurrence or dissemination of MTC was suspected, based on previous imaging results and elevated calcitonin levels. In all patients, neck ultrasound was performed with fine-needle aspiration biopsy of suspected lesions in 3 cases. In two patients, neck and chest computed tomography (CT) and in one case abdominal CT were also performed. All subjects underwent somatostatin receptor scintigraphy (SRS).

Patient 1 (J.S.). Patient with sporadic MTC underwent total thyroidectomy with complete lymph nodes resection in the central neck and paratracheal compartment in 2004. In 2009, based on results of CT and SRS, patient was diagnosed with liver metastases and qualified to the peptide receptor radionuclide therapy (PRRT). Patient received 13.32 GBq (360 mCi) of ^{90}Y-DOTA-TATE. Treatment led to the stabilization of the disease. GLP-1 receptor imaging was performed to compare results with standard imaging procedures (US, CT and SRS).

Patient 2 (S.S.). Patient with sporadic medullary cancer underwent total thyroidectomy with neck lymph nodes resection in 2003. In 2008, based on elevated calcitonin levels, neck ultrasound, fine-needle aspiration biopsy, neck and chest CT, and SRS dissemination to the neck and mediastinal lymph nodes were confirmed. Patient was disqualified from the surgery. Patient received 14.8 GBq (400 mCi) ^{90}Y-DOTA-TATE in 2008, which resulted in the stabilization of the disease. GLP-1 receptor imaging was performed to compare results with conventional imaging methods (US, CT, and SRS).

Table 1: Patients clinical data.

Initial	Age	Sex	Diagnosis	Genetic	CT	SRS	Diff. studies	GLP-1
J.S.	74	M	Dissem	Sporadic	+	+	US−	+
S.S.	70	M	Dissem	Sporadic	+	+	US+	+
K.G.	22	M	Recurr	MEN 2A	na	−	US+	+
Z.P.	60	F	Dissem	MEN 2B	+	−	US+	+

*F/M: female/male, −: negative result, +: positive result, na: not available, recur: recurrence, and dissem: dissemination.

This patient has also been diagnosed with chronic lymphocytic leukemia, diagnosed and operated for colon cancer in 2009, and metaplasia and dysplasia of the urinary bladder in 2010. In 2011 liver metastases from the colon cancer were diagnosed and patient was qualified to chemotherapy.

Patient 3 (K.G.). Patient with MEN 2A syndrome underwent total thyroidectomy with neck lymph node resection in 2001. In 2009, abnormal pentagastrin test results were observed, but imaging studies (which ones) did not detect any lesions. In 2010, patient underwent bilateral adrenalectomy due to pheochromocytoma. In 2011, hypoechoic lesion on the left side of the neck u was revealed by ultrasound, but the biopsy was negative. Thyroid scintigraphy with ^{99m}Tc and ^{131}I were positive, but SRS was negative. The GLP-1 receptor imaging was ordered to facilitate discrimination between the thyroid remnant and MTC recurrence.

Patient 4 (Z.P.). Patient with MEN 2B syndrome underwent total thyroidectomy with neck lymph node resection in 1990, followed by repeated surgery due to local recurrence in 1996. In 1993 patient underwent right adrenal and in 1997 left adrenal adrenalectomy due to pheochromocytoma. Based on chest CT, patient was diagnosed with lung metastases and local recurrence. SRS was negative. GLP-1 receptor imaging was ordered to confirm MTC recurrence in patient with discrepant results of other diagnostics images (positive CT, but negative SRS).

The detailed patient data are summarized in Table 1.

All patients gave their written informed consent to the local Medical College Ethics Committee which approved protocol.

3. Methods

3.1. GLP-1 Analogue Scintigraphy. Patients were on a liquid diet for 1 day before the beginning of the examination and fasted on the day of the tracer injection. Each of them was carefully checked for any adverse reactions. Due to natural activity of GLP-1 (stimulation of insulin secretion), blood pressure and glucose values were monitored before and after injection of the compound at several time points.

3.2. Preparation of [Lys^{40}(Ahx-HYNIC-^{99m}Tc/EDDA)NH$_2$]-Exendin-4. Technetium-99m labelled [Lys^{40}(Ahx-HYNIC/EDDA)NH$_2$]-exendin-4 was obtained from lyophilized kits prepared by the Institute of Atomic Energy, Radioisotope Center POLATOM. Exendin-4 (20 μg) was modified C-terminally with Lys^{40}-NH$_2$, where the lysine side chain was conjugated with Ahx-HYNIC (Ahx is aminohexanoic acid). Tricine and EDDA as coligands for ^{99m}Tc were added. The radiopharmaceutical preparation was carried out in the Nuclear Medicine Unit of the Endocrinology Department, Cracow University Hospital and was performed under aseptic conditions. Two-vial freeze-dried kits were used for radiolabelling with 0.3–1.5 mL pf ^{99}Mo/^{99m}Tc generator eluate (0.37–1.85 GBq) followed by 20 min incubation at 80°C. The TLC (thin layer chromatography) method was used for assessing the radiochemical purity of the compound. The mean injected activity was 740 MBq.

3.3. Imaging Technique. GLP-1 receptor imaging with the use of Lys^{40}(Ahx-HYNIC-^{99m}Tc/EDDA)NH$_2$]-exendin-4 was performed at the Nuclear Medicine Unit of the Endocrinology Department in the University Hospital in Cracow. At the beginning, images were acquired with a dual-head, large field of view E.CAM gamma camera with low-energy high-resolution (LEHR) collimators (Siemens Healthcare, 2000). From 2010 all examinations were performed with the use of Symbia TruePoint T16 hybrid system also with LEHR collimators (Siemens Healthcare, 2007).

SPECT studies were performed at 2 time points, between 3-4 h and 5-6 h after the injection of the tracer. The SPECT examinations were done with 128 × 128 matrix, 64 images, 30 sec per image, step and shoot mode, noncircular orbit and dual-energy window for scatter correction. The acquired data were reconstructed using OSEM Flash 3D iterative reconstruction method with 8 subsets and 10 iterations. After the installation of the new hybrid device in the unit, SPECT/CT studies were carried out in all next patients with the same settings for the SPECT part of the study.

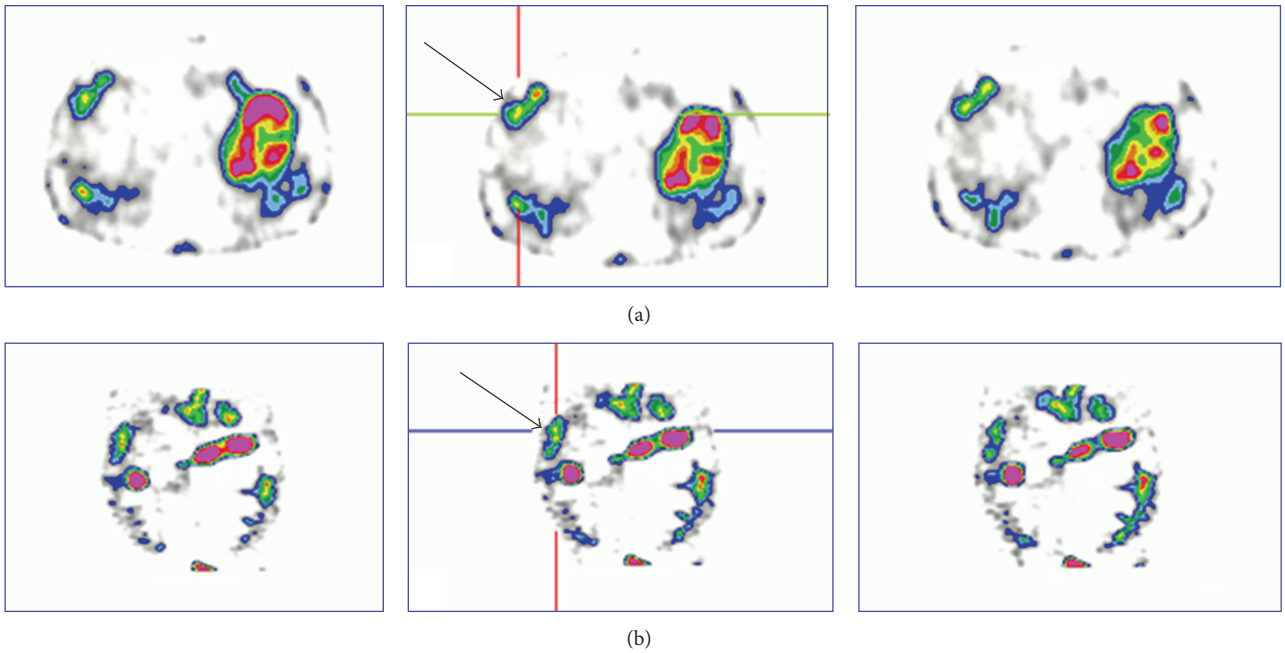
In all cases low-dose CT imaging was performed for the attenuation correction and a improved localization of pathological uptake of the tracer.

The obtained images were assessed by the experienced nuclear medicine specialist.

4. Results

The average radiochemical purity of the administered compound, prepared according to manufacturer's instruction and determined by TLC, was higher than 90%.

The quality of obtained Lys^{40}(Ahx-HYNIC-^{99m}Tc/EDDA)NH$_2$]-exendin-4 images was assessed by the nuclear medicine physician as very good.



(a)

(b)

Figure 1: The positive results of GLP-1 receptor imaging in a 74-year-old patient (J.S.) with sporadic MTC; pathological uptake of the tracer in liver metastases is visualized. (a) Axial slices and (b) coronal slices.

In all patients results of scintigraphy with [Lys^{40}(Ahx-HYNIC-^{99m}Tc/EDDA)NH$_2$]-exendin-4 corresponded to the results of previously performed imaging examinations.

In the first patient (J.S.) ^{99m}Tc-GLP-1 receptor scintigraphy revealed in homogenous liver uptake and focally increased tracer uptake at the location of previously confirmed liver metastases (Figure 1). Moreover, an additional liver lesion not seen on SRS, was detected. Patient 1 is still available for followup with stable disease after PRRT.

In the second patient (S.S.), ^{99m}Tc-GLP-1 receptor scintigraphy performed after PRRT revealed small focal uptake in the neck. The image was comparable with SRS findings.

In the patient K.G. (patient 3), ^{99m}Tc-GLP-1 receptor scintigraphy showed focal tracer uptake at the location of the ultrasonographically detected lesion on the left side of the neck. The patient was further qualified for the surgery.

In the patient Z.P. (patient 4), ^{99m}Tc-GLP-1 receptor scintigraphy revealed tracer uptake at the location of the neck and chest lesions seen on CT. However, patient was disqualified from surgery due to heart failure.

No adverse reactions were observed after tracer injection.

5. Discussion

MTC is still one of the most challenging endocrine cancers for both physicians and patients. In some MTC patients, despite of the elevated postoperative calcitonin levels and/or abnormal results of the pentagastrin test, there is no evidence of the disease in standard imaging procedures. Therefore searching for new targets for radioisotope diagnostics is warranted. ^{99m}Tc(V)-dimercaptosuccunic acid (DMSA) was considered by many authors the agent of choice in the postoperative work-up of MTC. Sensitivities ranging from 50% up to 85% have been reported in patients with primary and recurrent MTC using planar scans. SPECT has increased the sensitivity of lesion detection. Shahram reported that ^{99m}Tc(V)-DMSA had 91% sensitivity and 75% specificity for the detection of lung MTC compared to serum calcitonin as gold standard [21]. Another diagnostic modality is scintigraphy with ^{99m}Tc-MIBI. Overall sensitivity and specificity of this agent range from 36% to 89% and 89% to 100%, respectively [22]. Uğur et al. have compared the sensitivity of ^{99m}Tc-MIBI, ^{201}Tl, and ^{99m}Tc(V)-DMSA and shown them to be 47%, 19%, and 95%, respectively [23]. MIBG labelled with ^{123}I or ^{131}I, in spite of its high specificity (>95%), is of little clinical utility with a reported sensitivity of 30% [24]. Results from imaging with monoclonal antibodies including ^{123}I-, ^{131}I-, and ^{111}In-labelled CEA, both whole antibody and fragments, and ^{111}In-anticalcitonin antibody varied, ranging from 0% for anticalcitonin antibody to 78% for ^{131}I-anti-CEA antibody [25]. Results of somatostatin receptor scintigraphy (SRS) using an octreopeptides labeled with either ^{111}In-DTPA or ^{99m}Tc-EDDA/HYNIC in MTC patients reported in the literature have been also extremely variable. The overall sensitivity of ^{111}In-pentetreotide scintigraphy for the detection of MTC varies between 35 and 70% in different studies. Krenning et al. reported sensitivity of 65% in detecting MTC lesions by octreoscan, although the sensitivity was lower for liver metastases as a result of nonspecific hepatic uptake [26]. According to other authors, scintigraphy with ^{111}In-DTPA-octreotide has shown a sensitivity of 50–75%, that is higher than radiolabelled MIBG [27] and similar or slightly superior to ^{99m}Tc(V)-DMSA [28]. ^{18}FDG-PET was more sensitive especially in detecting cervical, supraclavicular, and

mediastinal lymph nodes, but failed to detect small lesions in the lungs and liver [29]. However, other studies have shown a lower sensitivity of ^{18}FDG-PET when compared with CT [30]. Data from the study by Ong and coworkers suggested that ^{18}FDG-PET is useful mainly in patients with calcitonin levels exceeding 1000 pg/mL (78% sensitivity), whereas it has limited value in patients with calcitonin levels below 500 pg/mL (20% sensitivity) [31]. Preliminary data suggest that ^{18}F-L-dihydroxyphenylalanine (L-DOPA) PET may provide a better lesion detection than ^{18}F-FDG for MTC lesions. Beheshti et al. observed that ^{18}F-DOPA correctly visualized 81% of MTC lesions compared to 58% detected with ^{18}F-FDG [32]. Hoegerle et al. reported an overall sensitivity of 63% for ^{18}F-DOPA PET in 11 patients with MTC, which was lower than that observed with CT/MRI (it should be stressed that authors used a stand-alone PET system and not a hybrid PET/CT system), but higher than those observed with ^{18}F-FDG and ^{111}In-DTPA-octreotide scan [33].

Above-mentioned diversity of sensitivity and specificity of different imaging modalities used in patients with suspicion of recurrence or dissemination of MTC stresses the necessity of searching for new more accurate diagnostics tools.

To the knowledge of the authors, this paper presents the first clinical experience with Lys^{40}(Ahx-HYNIC-^{99m}Tc/EDDA)NH$_2$]-exendin-4 in the detection of the recurrence or dissemination of MTC. The quality of obtained images was high; however, the image fusion was mandatory for proper diagnosis in all reported cases.

So far, the knowledge on GLP-1 application in MTC has emerged from experimental studies. The GLP-1 receptor protein expression was qualitatively and quantitatively investigated in many nonneoplastic and neoplastic human tissues with autoradiography method by Körner et al. [34]. They found GLP-1 receptor expression in 28% of medullary thyroid carcinomas examined and in 6% of normal human thyroid glands. GLP-1 receptor density of MTC cells was equal to 1,326 ± 264 dpm/mg tissue of receptor-positive cases. According to authors, medullary thyroid carcinomas exhibited a notable, but lower, GLP-1 receptor expression compared with, for example, pheochromocytomas. In recent paper by Gier et al. thyroids obtained from 12 individuals were examined for GLP-1 receptor protein by immunostaining [35]. GLP-1 receptor immunoreactivity was detected in approximately 10–30% of the tumor cells in five of the MTC cases. However, there was clear heterogeneity, with many calcitonin immunoreactive C cells being negative for GLP-1 receptor. The authors stated that these studies are in agreement with the work of Körner et al. [34].

Taking into account the GLP-1 receptor incidence and density in MTC, it seems that GLP-1 receptor imaging should not be used as the first-line diagnostic procedure in this group of patients. Nevertheless in case of patients with unclear or negative results of other imaging methods, but with clinical symptoms of MTC recurrence and/or elevated calcitonin concentration, this method gives the opportunity of localization of cancer tissue. Indeed, in our group of patients, the GLP-1 receptor imaging was carried out in two cases because of the discrepancy between results of performed diagnostic tests and resulted in confirmation MTC spread.

To sum up the GLP-1 receptor-expressing tumors, among others also MTC, are prospective candidates for in vivo targeting with Lys^{40}(Ahx-HYNIC-^{99m}Tc/EDDA)NH$_2$]-exendin-4.

6. Conclusions

Scintigraphy with Lys^{40}(Ahx-HYNIC-^{99m}Tc/EDDA)NH$_2$]-exendin-4 is able to detect the MTC lesions. It offers a new diagnostic tool to assess recurrence and staging of the disease in patients with MTC. GLP-1 receptor imaging should be considered as an alternative choice by clinicians especially in case of MTC patients in whom standard imaging techniques fail. However, further studies on the subject are needed.

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Research Article

Estimation of True Serum Thyroglobulin Concentration Using Simultaneous Measurement of Serum Antithyroglobulin Antibody

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We investigated the analytical interference of antithyroglobulin antibody (TgAb) to thyroglobulin (Tg) measurement and tried to convert measured Tg concentration to true Tg concentration using a mathematical equation which includes a concentration of TgAb. *Methods.* Tg was measured by immunoradiometric assay and TgAb by radioimmunoassy. Experimental samples were produced by mixing Tg and TgAb standard solutions or mixing patients' serum with high Tg or high TgAb. Mathematical equations for prediction of expected Tg concentration with measured Tg and TgAb concentrations were deduced. The Tg concentration calculated using the equations was compared with the expected Tg concentration. *Results.* Measured Tg concentrations of samples having high TgAb were significantly lower than their expected Tg concentration. Magnitude of TgAb interference with the Tg assay showed a positive correlation with concentration of TgAb. Mathematical equations for estimation of expected Tg concentration using measured Tg and TgAb concentrations were successfully deduced and the calculated Tg concentration showed excellent correlation with expected Tg concentration. *Conclusions.* A mathematic equation for estimation of true Tg concentration using measured Tg and TgAb concentration was deduced. Tg concentration calculated by use of the equation might be more valuable than measured Tg concentration in patients with differentiated thyroid cancer.

1. Introduction

Thyroglobulin (Tg), a glycoprotein synthesized in normal or malignant thyroid follicular cells, is an important marker for residual or recurrent differentiated thyroid cancer. Undetectable Tg is one of the criteria to establish the absence of a persistent tumor or recurrence in patients with differentiated thyroid cancer who have undergone total thyroidectomy and remnant ablation with radioiodine [1, 2]. Tg is the most sensitive marker for detecting recurrence of differentiated thyroid cancer; however, the presence of antithyroglobulin antibody (TgAb) interferes with measurement of Tg; therefore, development of Tg assays with limited or no interference by TgAb and development of methods for clearing of TgAb prior to measurement of Tg are warranted [1, 3, 4]. Until now, no TgAb-proof Tg assay (Tg assay without influence of TgAb) has been made available, and the presence of TgAb causes the concentration of measured Tg to be lower than that of the true concentration [4–6].

In patients with differentiated thyroid cancer who underwent curative treatment with total thyroidectomy followed by high-dose radioiodine ablation, the cut off value of Tg for performance of imaging studies for detection of persistent disease or recurrence is variable, according to the status of TSH and the concentration of measured TgAb [1]. Despite the lack of an international consensus regarding the appropriate Tg cut off value for residual or recurrent disease [7], almost all institutions or physicians have their own cut off value

Table 1: Expected Tg and TgAb concentrations of twenty experimental samples produced with standard solutions of Tg and TgAb. Values are expressed as Tg (ng/mL)-TgAb (U/mL).

2.0-10.0	2.0-30.0	2.0-100.0	2.0-300.0	2.0-1000.0
10.0-10.0	10.0-30.0	10.0-100.0	10.0-300.0	10.0-1000.0
50.0-10.0	50.0-30.0	50.0-100.0	50.0-300.0	50.0-1000.0
125.0-10.0	125.0-30.0	125.0-100.0	125.0-300.0	125.0-1000.0

Table 2: Expected Tg and TgAb concentrations of twenty experimental samples produced with patient serums. Values are expressed as Tg (ng/mL)-TgAb (U/mL).

4.7-3.5	4.7-5.2	4.7-18.8	4.7-643.0	4.7-930.0
18.5-3.5	18.5-5.2	18.5-18.8	18.5-643.0	18.5-930.0
111.0-3.5	111.0-5.2	111.0-18.8	111.0-643.0	111.0-930.0
246.0-3.5	246.0-5.2	246.0-18.8	246.0-643.0	246.0-930.0

for predicting persistent or recurrent disease according to TSH status (stimulated or not stimulated). Another factor to consider in interpretation of measured Tg value is the presence or absence of TgAb, the strongest serologic factor interfering in accuracy of available Tg assays [3, 8]. Measurement of TSH-stimulated Tg can result in failure to identify significant persistent or recurrent tumors in patients with TgAb. Influential magnitude of TgAb on measurement of Tg is known to show correlation with the concentration of measured TgAb [4]. In addition, it has been also known that Tg radioimmunoassay is less prone to the influence than other immunometric assays. Recently, Locsei et al. reported that decrease of measured Tg concentration by adding sheep TgAb from the electrochemiluninometric Tg assay and the magnitude of the influence was significant even in the reference range [9].

In this study, the authors assessed the influence of TgAb on measurement of Tg and developed a mathematical equation for estimation of true Tg concentration under various concentrations of TgAb using data from experiments that employed both standard solutions of Tg and TgAb measurement kits and patients' serum having high Tg or high TgAb.

2. Materials and Methods

2.1. Tg Measurements. Tg was measured by immunoradiometric assay (IRMA) using a commercial reagent set (Dynotest Tg-plus; Brahms Diagnostica, Berlin, Germany, detection limit; 0.08 ng/mL, measuring range; up to 250 ng/mL) according to the manufacturer's recommendations. The method described by the manufacturer is as follows. Standard solution or experimental serum (100 μL) is pipetted into test tubes coated with polyclonal TgAb. The tubes are then incubated for 18 hours at room temperature, and washed twice with 2 mL of washing solution. The tubes are turned upside down on blotting paper for at least 10 minutes. The tubes are again turned right side up, followed by addition of 200 μL of ^{125}I-labeled monoclonal TgAb. The tubes are incubated for 2-3 hours at room temperature with shaking (300–400 rpm), followed by washing twice with 2 mL of washing solution. The tubes are then turned upside down again on blotting paper for at least 10 minutes. Radioactivity of each tube is then measured. Concentration of Tg is obtained using a standard curve derived using the standard solutions.

2.2. TgAb Measurements. TgAb was measured by radioimmunoassay (RIA) using a commercial reagent set (Dynotest anti-Tgn; Brahms Diagnostica, Berlin, Germany, detection limit; 5.5 U/mL, measuring range; up to ~2000 U/mL) according to the manufacturer's recommendations. The method described by the manufacturer is as follows: standard solution or test serum (20 μL) is pipetted into test tubes coated with polyclonal anti-TgAb, followed by addition of 200 μL of ^{125}I-labeled Tg to the tubes. The tubes are incubated for 2 hours at room temperature with shaking (300–400 rpm), followed by washing three times with 2 mL of washing solution. The tubes are then turned upside down on blotting paper for at least 10 minutes. Radioactivity of each tube is then measured. Concentration of TgAb is obtained using a standard curve derived using standard solutions.

2.3. Preparation of Experimental Samples. Several concentrations of Tg standard solutions (4.0, 20.0, 100.0, and 250.0 ng/mL, Dynotest Tg-plus) and several concentrations of TgAb standard solutions (20.0, 60.0, 200.0, 600.0, and 2000.0 U/mL, Dynotest anti-Tgn) were prepared. In order to generate experimental samples containing various concentrations of Tg and TgAb, equal volumes of standard solutions were mixed (Table 1). Serum samples containing various concentrations of Tg (9.3, 37.6, 221.9, and 492.0 ng/mL) with a low level of TgAb (<20 U/mL) and serum samples containing various concentrations of TgAb (7.0, 10.4, 37.5, 1286.0, and 1860.0 U/mL) without Tg (<0.1 ng/mL) were collected. All the serum samples were obtained from patients with differentiated thyroid cancer. In order to generate experimental samples with various concentrations of Tg and TgAb, equal volumes of serum samples were also mixed (Table 2). In order to test reproducibility of measured Tg concentration for the experimental samples, triple samples were prepared for each concentration of every experimental sample produced using standard solutions or patients' serum.

2.4. Statistics and Deduction of Equations for Prediction of True Tg. Reproducibility of Tg measurement was tested. Influence of TgAb on measurement of Tg was analyzed and equations predicting expected (true) Tg concentration with measured Tg and TgAb concentration were deduced using the SAS program (version 9.22, SAS Institute Inc., Cary, NC, USA). $P < 0.05$ was considered significant.

3. Results

3.1. Reproducibility of Tg Measurement. Reproducibility of Tg measurement performed on triplicate samples of each concentration of experimental samples produced using either

Table 3: Decline of measured Tg value by TgAb in samples produced using Tg and TgAb standard solutions.

Expected Tg concentration (ng/mL)	Measured Tg concentration (ng/mL) under various TgAb concentrations				
	10.0 U/mL	30.0 U/mL	100.0 U/mL	300.0 U/mL	1000.0 U/mL
2.0	5.1 ± 0.1	4.0 ± 0.2	3.4 ± 0.1	2.6 ± 0.1	1.8 ± 0.1
10.0	13.0 ± 0.5	11.8 ± 0.4	10.9 ± 0.7	10.0 ± 0.3	6.2 ± 0.3
50.0	52.8 ± 1.8	47.1 ± 1.0	41.4 ± 1.1	40.3 ± 1.2	30.6 ± 0.5
125.0	134.1 ± 3.2	128.7 ± 2.0	90.0 ± 1.0	95.7 ± 2.5	75.5 ± 3.0

Values are expressed as mean ± SD.

standard solution or patients' serum was found to be excellent. Coefficient of variation for experimental samples produced using standard solutions was 4.21 ± 3.51% (0 ~ 14.82) (intraclass correlation coefficient = 0.998). Coefficient of variation for experimental samples produced using serum from patients was 2.83 ± 2.23% (0.87 ~ 11.21) (intraclass correlation coefficient = 0.999).

3.2. Influence of TgAb on Measurement of Tg Using Samples Produced from Standard Solutions. Measured Tg concentration showed a proportional decline with increase of TgAb concentration in every sample produced using standard solutions. Measured Tg concentrations in samples having the lowest concentration (10 U/mL) of TgAb were higher than the expected Tg concentrations of the samples. However, measured Tg concentrations in samples having high TgAb were lower than expected Tg concentrations (Table 3, Figure 1).

3.3. Influence of TgAb on Measurement of Tg Using Samples Produced from Patients' Serum. Measured Tg concentration showed a decline with increase of TgAb concentration in every sample produced using patients serum. Measured Tg concentrations for all samples were found to be lower than expected Tg concentrations (Table 4, Figure 2).

3.4. Equations for Prediction of Expected Tg Concentration. Data obtained with standard solution was used in deduction of an equation for prediction of expected Tg concentrations with measured Tg and TgAb concentrations using the SAS program.

$$\begin{aligned}\text{Calculated Tg (ng/mL)} &= -1.553 + 0.592 \left\{\text{measured Tg (ng/mL)} \times \sqrt{\log \text{TgAb (U/mL)}}\right\}.\end{aligned} \quad (1)$$

Calculated Tg concentrations were found to be more similar to expected Tg concentrations than measured Tg concentrations, and correlation between calculated Tg and expected Tg concentrations was found to be excellent ($r^2 = 0.9869$, $P < 0.0001$) (Figures 3 and 4).

In addition, data obtained with patient serum was used in deduction of an equation for prediction of expected Tg concentrations with measured Tg and TgAb concentrations using the SAS program.

$$\begin{aligned}\text{Calculated Tg (ng/mL)} &= 1.677 + 0.634 \text{ measured Tg (ng/mL)} \\ &\quad + 0.313 \text{ Measured Tg (ng/mL)} \\ &\quad \times \log \text{TgAb (U/mL)}.\end{aligned} \quad (2)$$

Calculated Tg concentrations were found to be more similar to expected Tg concentrations than measured Tg concentrations, and correlation between calculated Tg and expected Tg concentrations was found to be excellent ($r^2 = 0.9727$, $P < 0.0001$) (Figures 3 and 4).

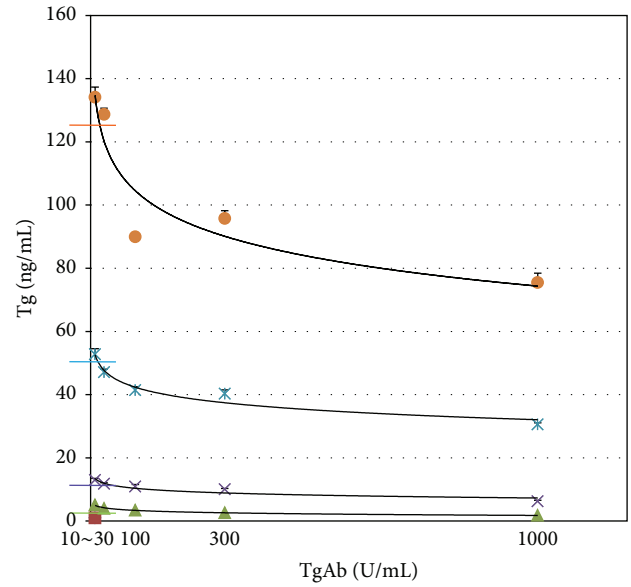

Figure 1: Measured Tg concentration showed a proportional decline according to increase of TgAb concentration in every sample produced using standard solutions. Transverse color bars represent expected Tg concentrations of each sample.

4. Discussion

Although the majority of patients with differentiated thyroid cancer are apparently rendered disease-free by initial treatment, approximately 15% experience persistent or

Table 4: Decline of measured Tg value by mixed TgAb in samples produced using patients' serum.

Expected Tg concentration (ng/mL)	Measured Tg concentration (ng/mL) under various TgAb concentrations				
	3.5 U/mL	5.2 U/mL	18.8 U/mL	643.0 U/mL	930.0 U/mL
4.7	4.5 ± 0.2	4.5 ± 0.2	3.4 ± 0.6	2.1 ± 0.1	2.0 ± 0.2
18.5	17.6 ± 0.3	18.7 ± 0.4	11.3 ± 0.4	9.1 ± 0.9	6.7 ± 0.6
111.0	102.3 ± 5.1	100.5 ± 5.1	56.0 ± 3.4	45.3 ± 0.4	39.3 ± 2.2
246.0	235.8 ± 6.2	234.8 ± 4.2	121.1 ± 1.9	99.4 ± 12.2	85.9 ± 5.2

Values are expressed as mean ± SD.

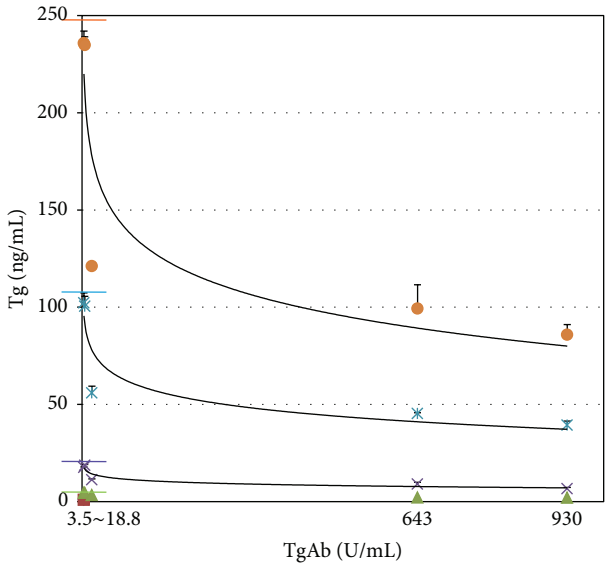

Figure 2: Measured Tg concentration in each sample produced using patients' serum showed a proportional decline according to an increase of TgAb concentration. Transverse color bars represent expected Tg concentrations of each sample.

recurrent cancer [10, 11]. Persistent disease or recurrence can be predicted by measurement of serum Tg, a sensitive and specific tumor marker for detection of differentiated thyroid cancer. Currently, the cut off value of 2 ng/mL under endogenous TSH or recombinant human TSH-stimulation is considered to represent significant risk [1, 10]. However, cut off values from 2 to 30 ng/mL, for example, 10 ng/mL, have also been applied in other clinical studies [7, 12].

Detectable TgAb is reported to be associated with persistence of an antigenic stimulus, and up to 40% of patients with differentiated thyroid cancer are positive for TgAb [13–15]. Some reports have suggested that persistence of TgAb positivity might suggest persistent or recurrent disease in some cases of differentiated thyroid cancer; however, other studies have reported no correlation between TgAb level and disease persistence [16, 17]. Therefore, the most important clinical issue with regard to high serum TgAb concentration is interference of the result of Tg assays with recurrence work up in patients with differentiated thyroid cancer [8, 14, 17, 18].

Endogenous TgAb is known to interfere with measurement of Tg in a method-dependent manner; therefore, prediction of Tg under a certain TgAb condition can be method-dependent as well [5]. Data found in the literature indicated that in the presence of TgAb, values of Tg determined by immunoradiometric assay are usually lower than real values, even if the concentrations of TgAb are very low [5, 9, 19]. In previous reports, we observed an erroneously low measured Tg value according to the presence of TgAb, and the degree showed positive correlation with concentration of TgAb [3, 4]. In the current study, influence of TgAb on the measurement of Tg was tested with experimental samples made by Tg and TgAb standard solutions or patients' serum. Two different equations which predict true Tg value were successfully deduced with the result from the tests, and the equation from the patients serum would be more appropriate for clinical application. According to findings from the current study, true Tg values in high concentrations of TgAb are more than twice the measured values. Serum with a true Tg value of 4.7 ng/mL can be measured as 2 ng/mL in samples containing a TgAb concentration of 1860 U/mL. It can be assumed that measured Tg value for a patient with a Tg of 4.7 ng/mL and a TgAb greater than 1860 U/mL might be a Tg of less than 2.0 ng/mL using the Tg assay. As a result, when applying a Tg cut off value of 2.0 ng/mL, the patient can be misclassified as low risk for recurrent or persistent disease. Serum with a true Tg value of 18.5 ng/mL can be measured as 9.1 ng/mL in samples containing a TgAb concentration of 1286 U/mL. It can also be assumed that measured Tg value for a patient with a Tg of 18.5 ng/mL and TgAb greater than 1286 U/mL might be a Tg of less than 10 ng/mL using the Tg assay. As a result, when applying a Tg cut off value of 10 ng/mL, the patient can be misclassified as low risk for recurrent or persistent disease.

Higher incidence of positive TgAb in patients with differentiated thyroid cancer, compared with the general population, has been reported. In addition, some patients have a high concentration of TgAb [14, 18]. Considering the results of the current study, some patients with a borderline Tg value can be misclassified into a low risk group and therefore would not undergo further diagnostic evaluation to detect recurrence or persistent disease. Management of disease can be delayed and prognosis of patients might be worse than that for patients diagnosed earlier with recurrence or persistent disease.

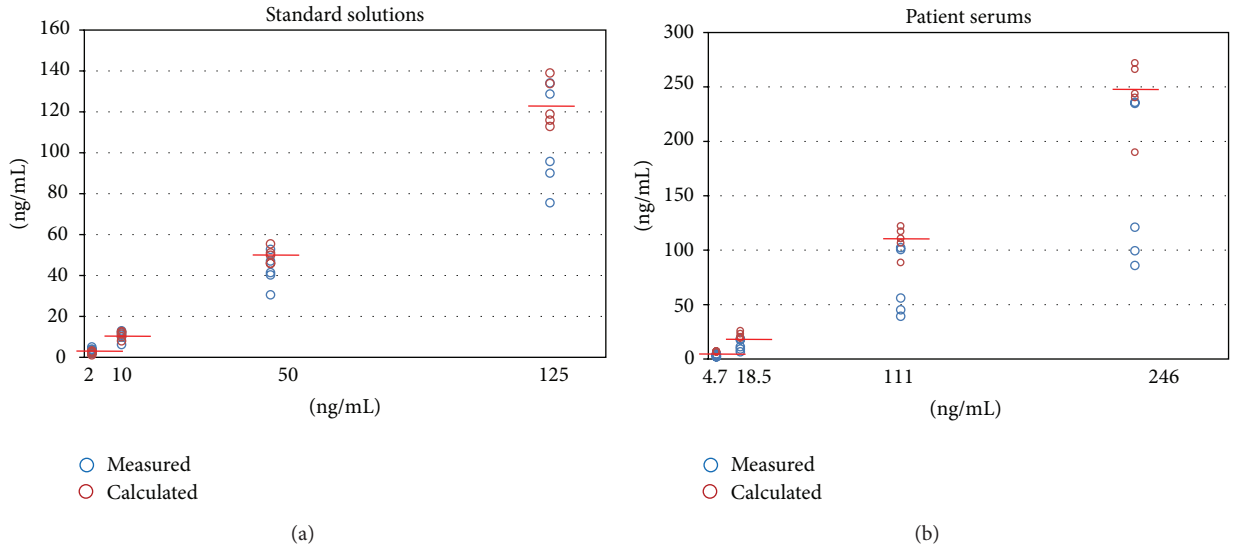

Figure 3: For each concentration of TgAb, the calculated Tg concentrations were more similar to expected Tg concentrations than measured Tg concentrations in samples from both standard solution and patients' serum.

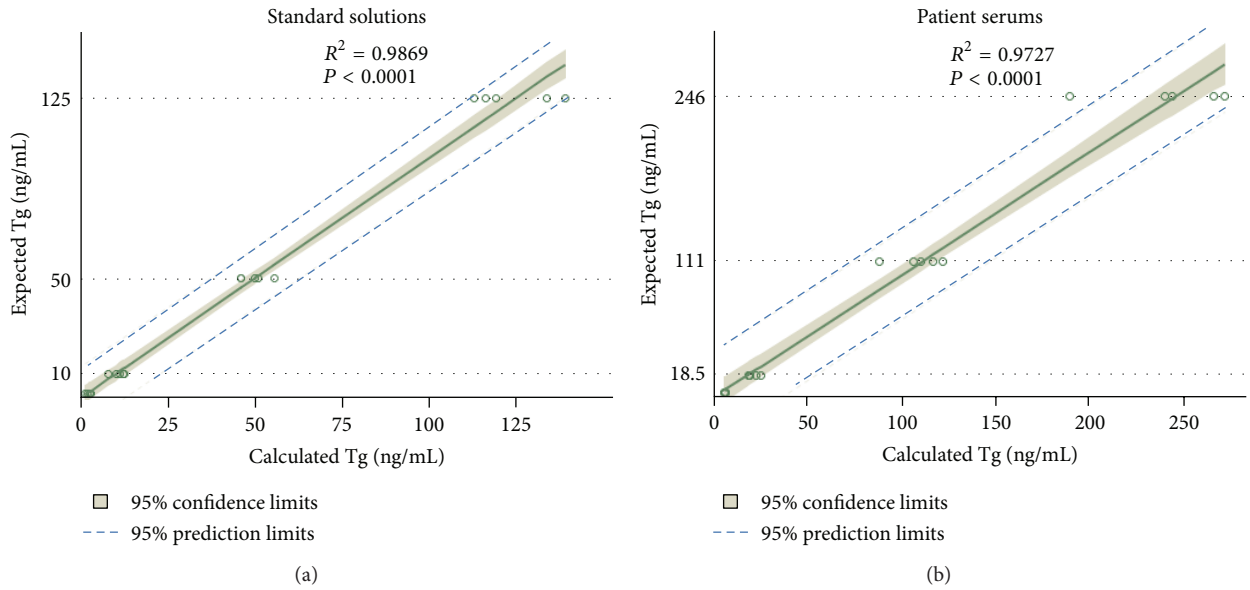

Figure 4: Correlations between the calculated Tg and expected Tg concentrations were found to be excellent in samples produced from both standard solution and patients' serum.

Consideration of TgAb when deciding on the clinical significance of Tg value has been basically by the presence or absence of TgAb only [1, 20]. It had been generally regarded that TgAb titer measured is below a clinical threshold will not be a significant influence on the Tg outcome; however, recent studies demonstrated that TgAb below the cut off can interfere the Tg outcome [19, 21]. Recently, Locsei et al. also reported that the measured Tg value of patients serum can be influenced by mixing sheep TgAb in the reference range of TgAb concentration and deduced an equation estimating true Tg concentration using TgAb concentration in the same sample [9]. They proved the general concept of TgAb influence on the Tg measurement; however, their equation cannot be generally applied to clinical practice due to difference between sheep TgAb and human TgAb. Verification of the TgAb influence using human TgAb, not sheep TgAb, is needed for that purpose.

In the current study, we used human TgAb from patients' serum for assessment of the influence of Tg to the Tg assay, and verified the same significant influence of human TgAb in reference range to the Tg assay. Results of this study demonstrated that concentration of human TgAb in the reference range also can result in a significantly lower measured Tg value, and a high concentration of TgAb can result in

the measured Tg value even lower; therefore, development of methods for use by clinicians in consideration of concomitant low or high concentration of TgAb for determination of the clinical significance of measured Tg values is a pressing issue. In contrary to experiment employing patients' serums, low concentration of TgAb incurred an overestimation of Tg in the experiment employing the standard solutions and elucidation of the cause was not performed in the current study.

Magnitude of the influence is known to not only depend on the class of assay methods, but also the type of Tg epitope recognized by patient's TgAb [16, 18, 22]. Therefore, development of an equation that can be applied to all assay methods and all patients might not be possible. In this study, there was a large deviation of many of the actual points from the curve fits on Figures 1 and 2, suggesting that the back calculation of the true Tg value according to the equation might give quite erroneous results in some patients. The deviation probably originates from the interpatient variability of influence magnitude related to heterogeneity of patients' TgAb. However, results of the current study demonstrated that the Tg value calculated by the equation is generally close to the true Tg value than the measured Tg value. Based on the results, the corrected Tg value by the equation might be more valuable than measured Tg value for predicting the presence or recurrence of a cancerous lesion in patients with differentiated thyroid cancer. However, in fact, clinical validation studies are needed for allowing physician to implement the approach in clinical laboratory practice.

This study has limitations. First, despite efforts to standardize thyroglobulin analytes across assay platforms, differences between platforms persist and can be related to genetic polymorphisms that introduce changes in protein primary structure, glycosylation pathways which could lead to variable protein processing, modification, or cross-linking [6, 8]. Result of TgAb assays was also known to be discordant by their epitope pattern, especially in patients without thyroiditis [23]. The equation would differ according to the assay platforms used for measurement of Tg and TgAb and it cannot be generalized. Therefore, institution's own equation has to be developed by the specific combination of Tg and TgAb assays used. Second, we did not evaluate the influence of Tg on measurement of TgAb. For estimation of true Tg value using measured Tg and TgAb values, the true TgAb value should be plugged into the equation. The influence of Tg on TgAb assay must also be considered [18]. Third, expected Tg and TgAb values might be inaccurate in samples produced using patients serum owing to presence of Tg in serum for TgAb and presence of TgAb in sera for Tg, albeit they are very low in titer. Fourth, in this study, we used only four concentrations of Tg and five concentrations of TgAb. Therefore, the equation formula for estimating true Tg concentration is not the most accurate one, and further studies are needed in order to develop the most accurate equation for estimation of true Tg concentration using measured Tg and TgAb concentrations.

In conclusion, findings from this study demonstrate a mathematic equation for prediction of true Tg concentration using measured Tg and TgAb concentrations. The true Tg concentration calculated by the equation might be more valuable than measured Tg value for predicting the presence of residual or recurrent cancerous lesions in patients with differentiated thyroid cancer.

Conflict of Interests

The authors have no conflict of interests.

Acknowledgments

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Review Article

Cellular Signaling Pathway Alterations and Potential Targeted Therapies for Medullary Thyroid Carcinoma

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Parafollicular C-cell-derived medullary thyroid cancer (MTC) comprises 3% to 4% of all thyroid cancers. While cytotoxic treatments have been shown to have limited efficacy, targeted molecular therapies that inhibit rearranged during transfection (RET) and other tyrosine kinase receptors that are mainly involved in angiogenesis have shown great promise in the treatment of metastatic or locally advanced MTC. Multi-tyrosine kinase inhibitors such as vandetanib, which is already approved for the treatment of progressive MTC, and cabozantinib have shown distinct advantages with regard to rates of disease response and control. However, these types of tyrosine kinase inhibitor compounds are able to concurrently block several types of targets, which limits the understanding of RET as a specific target. Moreover, important resistances to tyrosine kinase inhibitors can occur, which limit the long-term efficacy of these treatments. Deregulated cellular signaling pathways and genetic alterations in MTC, particularly the activation of the RAS/mammalian target of rapamycin (mTOR) cascades and RET crosstalk signaling, are now emerging as novel and potentially promising therapeutic treatments for aggressive MTC.

1. Introduction

Medullary thyroid carcinoma (MTC) is a rare neuroendocrine cancer that originates from thyroid parafollicular calcitonin-(CT-) producing cells. MTC accounts for approximately 4% of all thyroid malignancies; approximately 75% of these cases occur in the sporadic form, and 25% occur in the hereditary form [1–3]. MTC usually has a favorable prognosis, with a 10-year survival rate of 70%–80%, if it is diagnosed and treated at an early stage when the tumor is confined to the thyroid [4]. Unfortunately, most cases of MTC present at diagnosis with metastases to the local and regional lymph nodes and to distant organs, especially the lungs, liver, and bones [5]. Patients with metastatic MTC have a 10-year over-all survival rate of 40%, and metastasis is the main cause of death in patients with MTC [4, 6]. Locally advanced and distant metastatic diseases are incurable, as surgical resection and conventional radio- and cytotoxic chemotherapies are not effective against metastatic MTC [7, 8]. Clinical trials of various combinations of chemotherapeutic drugs have yielded unsatisfactory results [9, 10]. However, research over the last years has led to a good understanding of the genetic defects and altered molecular pathways that are associated with the development of MTC. Thus, multiple promising therapeutic agents that target these genetic alterations have been developed to treat progressive and advanced MTC. Activating mutations of the tyrosine kinase receptor (TKR) rearranged during transfection (*RET*) are believed to be the primary oncogenic event in a majority of MTC cases. This discovery has led to the development and introduction of targeted therapies, such as tyrosine kinase inhibitors (TKIs) that target RET. Several TKIs directed toward RET kinase have been tested *in vitro*, preclinical, and clinical studies with promising results. Unfortunately, these agents are not likely to be curative, as the longest duration of response observed was approximately 4 years, and the maintenance of agent-dependent effects may require continuous therapies [7], which are not without important side effects. The main reasons for the failure of these agents to cure MTC are the development of resistance

to TKIs that target the RET and other cell receptors and the activities of other signal transduction pathways that are involved in MTC tumorigenesis and progression but not directly targeted by TKIs. In recent years, the discovery of mechanisms of resistance to TKIs and of several other molecular events that contribute to MTC transformation and metastasis suggested that combinatory therapy may result in a more significant tumor growth inhibition. This has led to the development of novel compounds that have been used in several clinical trials, including TKIs that can target multiple TKRs simultaneously in addition to RET and agents that can target other altered signaling pathways. Other studies have demonstrated the potential for immunotherapy in combination with agents that target signal transduction pathways that are important for MTC growth [5]. Because the aim of these targeted therapies is to extend lifespan and increase the quality of life, it is very important to limit the toxicities of therapeutic agents, either alone or in combination. The possibility of testing these novel drugs *in vitro* (in primary thyroid cancer cells) and *in vivo* may help to improve the personalization of treatments [11].

2. Key Cellular Signaling Pathways and Alterations in MTC

2.1. RET Pathway. The role of the *RET* oncogene in the tumorigenesis of MTC has been characterized extensively [12]. The *RET* gene encodes a transmembrane tyrosine kinase that binds to glial cell line-derived neurotrophic factor (GDNF) family ligands [13]. RET signaling leads to the activation of the RAS/mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3′ kinase (PI3K)/Akt pathways and has key roles in cell growth, differentiation, and survival. Activating point mutations of the TKR *RET* have been reported in nearly all hereditary cases of MTC; some of these mutations are included in the MEN2A, familial MTC, or MEN2B syndromes in which there is a genotypic/phenotypic correlation between the type of *RET* mutation and clinical features. *RET* mutations are also found in 30%–50% of sporadic MTCs. Germline mutations in the *RET* proto-oncogene are responsible for hereditary MTC, while somatic *RET* mutations are responsible for sporadic MTC [14]. These data provide a strong rationale for targeting RET in selective cancer therapy. However, this paper will mainly focus on additional cellular signaling pathways other than RET responsible of MTC tumorigenesis and progression and potential targeted approaches for the treatment of advanced or metastatic MTC.

2.2. Additional Signaling Pathways That Accelerate MTC Progression. Although activating mutations of the TKR *RET* are believed to be the primary oncogenic event in the development of a majority of MTC cases, it is clear that RET cooperates with other signal transduction pathways to promote MTC tumorigenesis.

2.2.1. Tyrosine Kinase Receptors other than RET Are Implicated in MTC Tumorigenesis. In addition to RET, other kinase receptors may play a role in the development and progression of MTCs [15].

Similar to the RET receptor, the epidermal growth factor receptor (EGFR) is a TKR that is associated with the regulation of cell growth, proliferation, and apoptosis. Dimerization of the receptor following ligand binding results in transphosphorylation and the subsequent activation of several downstream signal pathways. EGFR has been shown to be frequently overexpressed in various types of thyroid carcinomas, including MTC, and to play a role in cancer development and progression [16]. In contrast, a recent report analyzing different MTC on tissue microarrays has demonstrated that only 20% of cases revealed moderate to strong reactivity for EGFR, whereas the majority of the cases revealed weak and very focal positivity [17]. A different study has shown that *EGFR*-activating mutations are rare in MTC. With respect to the numbers of *EGFR* gene copies in MTCs, the researchers did not detect amplifications but did find polysomes in 15% of the examined tumors [18]. Additionally, EGFR was activated in a subset of MTCs, which suggests that this subset of patients might benefit from drugs that target also EGFR. Recent findings have shown that the ligand-induced activation of EGFR can stimulate RET activation beyond signaling and growth stimulation [19]. Several EGFR inhibitors have been shown to markedly inhibit the growth of the MTC TT and MZ-CRC-1 cell lines. Because RET activation seems to be influenced by EGFR, a recent study investigated whether EGFR activation could be related to specific *RET* mutations in MTCs. The researchers found that tumors with the most aggressive *RET* mutations (in codons 883/918) exhibited reduced EGFR expression compared to other *RET* mutations. It could be speculated that the most aggressive *RET* mutations are less dependent on EGFR activation [18]. In fact, in the work by Croyle et al. [19], in which cell lines with *RET* mutations in codon 634 and codon 918 were compared, the effect of EGFR inhibition on the codon 918 mutated cell line appeared to be reduced, in agreement with the previous data. Because the activation status of EGFR seems to be related to RET activation, EGFR activation has been examined in *RET*-negative tumors [18]. However, no differences have been found in EGFR activation between RET-positive and -negative tumors, which likely indicates that other molecular mechanisms lead to RET activation, such as increased *RET* gene copy numbers, altered promoter activity, or increased transcription in the *RET* mutation-negative tumors. These data suggest that EGFR status determination in MTCs might be important but certainly deserves further investigations.

The vascular endothelial growth factor receptor (VEGFR) pathway is also important in the pathogenesis of MTC. There are three transmembrane receptors that mediate the angiogenic and lymphogenic effects of VEGF: VEGFR-1, VEGFR-2, and VEGFR-3. VEGFR-2 is thought to be implicated primarily in tumor growth and metastasis. Overexpression of VEGF and VEGFR-2 has been found in MTC compared to normal thyroid tissue [20]. The VEGF proteins (VEGF-A, B, C, and D), which are secreted by tumor cells, act as ligands for the VEGFR-2 receptors on endothelial cells and promote a signaling cascade through different pathways, such as PLC-γ-PKC-Raf-MEK-MAPK and PI3K-Akt, that stimulate cellular

proliferation, migration, and survival and induce neoangiogenesis [21]. Angiogenesis is an essential alteration in cell physiology that predisposes the development of malignancy and is fundamental in tumor growth and metastasis [22]. Similarly to EGFR, the overexpression of VEGFR-2 in MTC has been shown to correlate with metastasis [18].

Several multitargeting tyrosine kinase inhibitors that block VEGFR have shown promising clinical antitumor activity; unfortunately, in most thyroid carcinomas and other solid tumors, the antiangiogenic effects are often only transitory and really often may have late paradoxically protumorigenic effects. Additionally, it seems that a modest significant association has been observed between VEGFR-2 expression and *RET* mutation status in primary tumors [18].

The *MET* proto-oncogene codes for the TK receptor for the hepatocyte growth factor (HGF). The HGF-MET interaction activates signaling pathways that mediate cell adhesion and motility. MET hyperactivation reportedly correlates with the metastatic abilities of tumor cells [23]. MET and HGF coexpression has been observed in a subset of MTC tumors and is associated with multifocality in MTC [24], which makes this interaction a potentially important target. In one report, mutations in the *MET* RTK were detected in MTC [25]. Importantly, RET can induce the overexpression of c-MET in this type of thyroid tumor [26].

The fibroblast growth factor receptor 4 (FGFR4) has also been reported to be overexpressed in MTC cell lines. Inhibition of FGFR phosphorylation with the small molecule FGFR inhibitor PD173074 resulted in an arrest of cell proliferation and tumor growth [27]. Moreover, the dual inhibition of RET and FGFR combined with tyrosine kinase inhibitors resulted in greater suppressions of cell proliferation *in vitro* and tumor control *in vivo* than that which was achieved with either agent alone. These data highlight RET and FGFR4 as therapeutic targets and suggest a potential role for the use of combined tyrosine kinase inhibitors in the management of inoperable medullary thyroid cancers [28].

Finally, the platelet-derived growth factor receptor (PDGFR) also seems to play a role in differentiated thyroid cancer [29], although its role and function have not been fully investigated in MTC.

2.2.2. Other Signaling Pathways That Contribute to MTC Tumorigenesis. Several other signal transduction pathways have been implicated as contributors to MTC tumor growth, as illustrated in two recent studies [5, 30]. These pathways include RET interactions with pRB, p53, p18, and p27 as well as the phosphatidylinositol 3-kinase/AKT/mTOR and Ras/Raf/MEK/ERK pathways.

RET Interactions with Tumor Suppressor Genes

(a) pRB and p53. The tumor suppressor genes *RB1* (retinoblastoma; pRB protein) and *TP53* (p53 protein) are frequently mutated in human cancers, and it appears that, in cancer, both pathways must be inactivated to overcome senescence or apoptosis. There is extensive genetic evidence that the pRB and p53 pathways are involved in MTC in rodents. For example, *RB1*-deficient mice developed MTC [31]. Further, the loss of *TP53* further increased MTC formation in *RB1*-deficient mice [32, 33]. It has been shown that in mice, RET cooperates with the inactivation of pRB/p53 to cause experimental MTC. $pRB^{+/-}/p53^{+/-}$ mutant mice have been shown to acquire *RET* mutations that are analogous to activating germline mutations that are observed in human MEN2A and familial medullary thyroid carcinoma (FMTC). This suggests that murine MTC requires mutational dysregulations within both the *RET* and nuclear tumor suppressor gene pathways [34]. However, mouse models may not mimic human disease, and a systematic analysis of the genes in the *RB1* and *TP53* pathways in human samples will help to clarify their roles in human MTC formation. This information may be important for the development of novel targeted therapeutic approaches for MTC.

(b) p18 and p27. Thyroid tumors show low expression of the cyclin-dependent kinase inhibitor (CDKI) p27 (Kip1), and recent evidence demonstrates that p27 is downregulated by the active RET mutant, RET/PTC1, which is found in papillary thyroid carcinomas. These data implicate decreased p27 activity as an important event during thyroid tumorigenesis. However, $p27^{-/-}$ mice develop MEN-like tumors only in combination with the loss of p18 (Ink4c), another CDKI. This suggests that p18 and p27 are functional collaborators in the suppression of tumorigenesis, that the loss of both is critical to the development of MEN tumors, and that both p18 and p27 are regulated by RET [35].

PI3K-AKT-mTOR Pathway. The PI3K-AKT-mammalian target of the rapamycin (mTOR) cascade is important in tumorigenesis due to its ability to promote cell growth, proliferation, and survival. Several examples provide evidence to support a role for the activation of the PI3K/AKT/mTOR signaling cascade in medullary thyroid cancer [36–38].

Several mechanisms have been shown to be involved in the activation of PI3K signaling in medullary thyroid cancer.

A mutation of MEN2A (RET-MEN2A) has been shown to activate PI3K and its downstream effector, the serine/threonine kinase AKT/protein kinase B [35]. Previous studies have demonstrated that a mutation of Tyr-1062, which is the intracellular docking site for Shc and Enigma on RET, abolishes the RET-MEN2A transforming activity [39]. These studies further revealed that the mutation of Tyr-1062 abrogates the binding of the p85 regulatory subunit of PI3K to RET-MEN2A and subsequent stimulation of the PI3K/AKT pathway. Furthermore, retroviral transduction of rat fibroblasts with a dominant-interfering form of PI3K was shown to suppress RET-MEN2A-dependent transformation, whereas the overexpression of AKT enhanced RET-MEN2A oncogenic potential. In summary, these data are consistent with the notion that RET-mediated cell-transforming effects are critically dependent on the activation of the PI3K/AKT/mTOR pathway [36].

In cell lines, the PI3K-AKT/mTOR pathway has been shown to be important in the pathogenesis of MEN2B [40]. RET-MEN2B (*RET* M918T) is more effectively autophosphorylated at RET Y1062 than is RET-MEN2A, which

subsequently leads to increased constitutive activation of the Ras/mitogen-activated protein kinase (MAPK) and PI3K/AKT/mTOR signaling cascades [41].

Furthermore, previous data report other possible mechanisms of PI3K activation in thyroid carcinomas, such as the overexpression of RAI (ShcC/N-Shc), which is a substrate of RET oncoproteins [5, 42].

Finally, the loss of expression of the phosphatase and tensin homologue (*PTEN*) gene, a tumor suppressor gene, may have a role in the activation of PI3K/AKT in thyroid tumors. The absence of functional PTEN protein expression has been observed in various cancer cells and has led to the constitutive activation of downstream components of the PI3K pathway, including the Akt and mTOR kinases. Preclinical models showed that inactivation of these kinases is able to reverse the effects of PTEN loss [43]. These data raise the possibility that drugs that target either these kinases or PI3K itself might have significant therapeutic activity against PTEN-null cancers.

Mutations in major nodes of this signaling cascade have been observed in human cancers; these mutations include gain-of-function mutations and amplifications of the genes encoding PI3K and AKT [44, 45]. No systematic genetic analysis of PI3K pathway components has been reported in MTC. However, a recent study on a series of 49 MTCs has shown that the PI3K genes were not mutated, and that the activation of the PI3K pathway was significantly associated with the status of *RET* mutations [46]. In fact, using protein expression analysis, the same authors confirmed that the AKT/mTOR pathway was highly activated in MTC, especially in cases with germline *RET* mutations. Interestingly, this association was not dependent on the type of mutation (in either the codons of the juxta-membrane or of the tyrosine kinase portion of the receptors) but was dependent on the hereditary nature of the mutation. In contrast, medullary carcinomas with sporadic *RET* mutations or with wild-type RET were observed with heterogeneous expression of AKT/mTOR pathway molecules, which suggests the need for further elucidation of alternative activation mechanisms [46]. In a recent study, the PI3K/AKT/mTOR pathway was shown to be activated in MTC, particularly in the metastatic lymph nodes, and this pathway was shown to sustain malignant features of different MTC cell models [47]. Moreover, it has been shown that the selective inhibition of the mTOR pathway in a germline-RET-mutated MTC cell line can effectively decrease cell viability and block the phosphorylated status of mTOR signaling molecules, which confirms previously published *in vitro* data [48]. Another study used metformin, which is an antidiabetic agent that decreases the proliferation of cancer cells through the 5′-AMP-activated protein kinase-dependent inhibition of mTOR, in MTC cell lines to show that the growth-inhibitory effects on the cells were associated with the downregulation of both the mTOR/6SK and pERK signaling pathways [49]. Altogether, these results strongly suggest that mTOR might be a very efficacious target in patients with advanced or metastatic MTC.

Noteworthy, mTOR inhibitors are currently being used in clinical trials for the treatment of medullary thyroid carcinoma.

Ras-Raf-MEK-ERK Pathway. Unlike hereditary MTC, in which *RET* mutations are the critical events, in sporadic MTC, the genetic or molecular biomarkers have not been fully established. *Ras* is a frequently mutated oncogene in a broad spectrum of human tumors, including thyroid carcinoma, mainly in the follicular forms [50]. In this context, investigators aimed to determine whether mutations in the *Ras* oncogene could play a possible role in the carcinogenesis of sporadic MTC. The initial study analyzed 15 sporadic MTCs for mutations in known hot spots (codons 12, 13, and 61) of the *H-* and *K-Ras* oncogenes by a direct sequencing technique, although no *Ras* mutations were detected in any of the examined tumors [50]. A different study on 49 MTC confirmed that *Ras* mutations are a rare event in this type of tumor, regardless of *RET* mutational status [46]. By contrast, different studies have demonstrated the presence of *Ras* mutations in MTC. A mutational screening for *H-*, *K-*, *N-RAS*, and *BRAF* in 13 sporadic and inherited MTCs revealed one sporadic RET-negative MTC (stage III) with a mutation in the *H-Ras* codon 13 (G13R) [51]. Another study analyzed 188 hereditary and sporadic MTCs for *Ras* mutational status, revealing a low prevalence of mutations, which were confined only to RET-negative sporadic MTC [52]. Furthermore, recent studies have reported a quite high prevalence of *Ras* mutations in sporadic MTC, particularly in RET-negative MTC; 68% (17 of 25) of the RET-negative MTCs had mutations of *Ras* compared to only 2.5% (1/40) of the RET-positive MTCs [53]. These findings were confirmed by a different study that analyzed 17 sporadic MTCs by exome sequencing and found dominant and mutually exclusive oncogenic mutations in *RET* and *RAS* (*H-* and *K-Ras*) genes in 17 sporadic MTCs [54]. As expected, most *Ras* mutations corresponded to mutational hot spots in exons 2 and 3, although some mutations were also detected in exon 4. This study confirms that *RET* and *Ras* mutations are mutually exclusive, and that they are probably 2 different oncogenic driver events in MTC [55]. These results suggest that the activation of the proto-oncogenes *Ras* and *RET* represents alternative genetic events in sporadic MTC tumorigenesis, and that more sensitive sequencing techniques such as next generation sequencing are necessary to detect mutations. For decades, the *Ras* and *Raf* families of oncogenes have been known to be transforming genes. However, in many normal cultured cell types, the sustained expression of activated Ras or its downstream effector, Raf, can elicit cell cycle arrest or senescence. The Ras/Raf-mediated growth arrest has been proposed as a defense mechanism against the inappropriate activation of the Ras/Raf signal transduction pathway [56, 57]. According to this hypothesis, spontaneous mutations in *Ras* genes, which may occur stochastically in all cell types, would be innocuous for the organism because these mutations would lead to growth arrest or senescence. Hence, for carcinogenesis to occur in response to Ras/Raf activation, the growth arrest response must be inactivated. Thus, cell

transformation may involve additional events. The growth arrest response to Ras/Raf activation is not limited to normal cells. Several tumor cell lines that were derived from different tumor types, including medullary thyroid carcinoma, also experienced growth arrest, usually accompanied by differentiation or senescence [58–60]. These findings indicate that some tumors retain a capability for growth arrest in response to Ras/Raf activation. The mechanism by which Ras or Raf activation can induce growth arrest is not completely understood. Some investigators have reported that Ras or Raf activation could induce the expression and secretion of a protein that mediated differentiation and G1 cell cycle arrest in MTC cells. By protein purification and mass spectrometry, this protein has been identified as the leukemia inhibitory factor (LIF). STAT3 activation was necessary for LIF-mediated growth arrest and differentiation in MTC cells. In addition, the Ras/Raf/MEK/ERK pathway could also mediate growth arrest and differentiation by a second mechanism that is independent of LIF/JAK/STAT3. This novel autocrine-paracrine mechanism, which mediates crosstalk between the Ras/Raf/MEK/ERK and the JAK-STAT pathways, defines a novel mechanism of Ras/Raf-induced cell growth arrest [61, 62].

Raf-1 activation in human MTC TT cells resulted in the phosphorylation of GSK-3beta. The inactivation of GSK-3beta in TT cells by well-known GSK-3beta inhibitors, such as lithium chloride (LiCl) and SB216763, is associated with both growth suppression and a significant decrease in neuroendocrine markers, such as human achaete-scute complex-like 1 and chromogranin A. Growth inhibition by GSK-3beta inactivation was found to be associated with cell cycle arrest due to an increase in the levels of cyclin-dependent kinase inhibitors, such as p21, p27, and p15. Additionally, TT xenografts mice, treated with LiCl, showed a significant reduction in tumor volume compared with those that were treated with a control. Therefore, GSK-3beta is a key downstream target of the Raf-1 pathway in TT cells, and the inactivation of GSK-3beta alone is sufficient to inhibit the growth of TT cells both *in vitro* and *in vivo* [63].

β-Catenin Pathway. β-Catenin is a ubiquitously expressed multifunctional protein that plays an important role in cellular adhesion. A novel RET-β-Catenin signaling pathway was found to be a critical contributor to enhanced cell proliferation and tumor progression in thyroid cancer. Gujral et al. showed that RET could induce β-Catenin-mediated transcription, cell proliferation, and transformation *in vitro* and that β-Catenin nuclear localization and subsequent mediation of β-Catenin by RET are key secondary events in tumor growth and metastasis *in vivo* [64]. This novel interaction suggests a mechanism that may underlie the broad and early metastatic potential of MTC. These data suggest an unrecognized role for β-Catenin signaling that may have implications for tyrosine kinase-mediated tumorigenesis in multiple tumor types and provide another potential target for therapeutic agents. In support, a recent study performed on tissue microarray observed that WNT pathway proteins, including Wisp-1, Wisp-2, and β-Catenin, were actively expressed in MTC [17].

NF-κB Pathway. The nuclear factor kappa-B (NF-κB) proteins, a family of transcription factors that are found virtually in all cells, are known to play crucial roles in the growth of many human malignancies. The ability of NF-κB to target a large number of genes that regulate cell proliferation, differentiation, survival, and apoptosis provides clues towards its dysregulation during the processes of tumorigenesis, metastatic progression, and therapeutic resistance of tumors. NF-κB is constitutively active in MTC through the RET-induced phosphorylation, ubiquitination, and proteosomal degradation of inhibitors of NF-κB (IkB), which allows NF-κB to enter the nucleus and bind to DNA [65]. NF-κB is frequently activated in MTC, and the activation of RET by somatic or germline mutations may be responsible for NF-κB activation in these tumors [5]. These results suggest that the NF-κB pathway may be an important target for drug development in MTC.

Novel Protein Targets. A recent study examined the expression of proteins involved in angiogenesis, inflammation, apoptosis, cell cycle, cell-to-cell contact, and carcinogenesis using high-throughput tissue microarrays from 23 patients with MTC. These authors identified several novel potentially important protein targets such as COX-1/2, Bcl-2a, Gst-π, Gli-1, Gli-2, Gli-3, and Bmi-1 that may be therapeutically targeted in MTC. For example, COX-1 and COX-2, which are two inflammation-related factors, were significantly expressed in these cases, suggesting that nonsteroidal anti-inflammatory drugs may provide benefit in some patients with MTC. Then, the finding of antiapoptotic Bcl-2a and Gst-π overexpression in MTC suggests that Bcl-2a and Gst-π inhibitors might be a treatment option for patients with advanced or metastatic MTC. The same is applied to Gli-1, Gli-2, and Gli-3, members of Sonic Hedgehog Homolog (SHH) pathway, and Bmi-1, a cell-cycle marker that resulted overexpressed in these MTC samples [17]. The studies of these markers, particularly the members of the SHH, may improve our understanding of mechanism of resistance to current chemotherapeutic and/or TKI regimens and identify novel potential therapeutic approaches.

3. Potential Targeted Therapies for MTC

3.1. Tyrosine Kinase Receptor Inhibitors. Due to increased knowledge of the molecular pathogenesis of MTC, therapeutic agents that target specific altered pathways have been developed (Figure 1). Because the alterations of protein kinases and their pathways are involved in MTC development, several tyrosine kinase receptors inhibitors (TKIs) have been tested *in vitro*, preclinical, and clinical studies [66]. RET is certainly an attractive target for several types of tumors particularly for parafollicular C-cells-derived tumors, which are addicted to RET and its activity [66]. TKIs are small organic compounds that affect tyrosine kinase-dependent oncogenic pathways by competing with ATP-binding sites of the tyrosine kinase catalytic domains [67]. Occupation

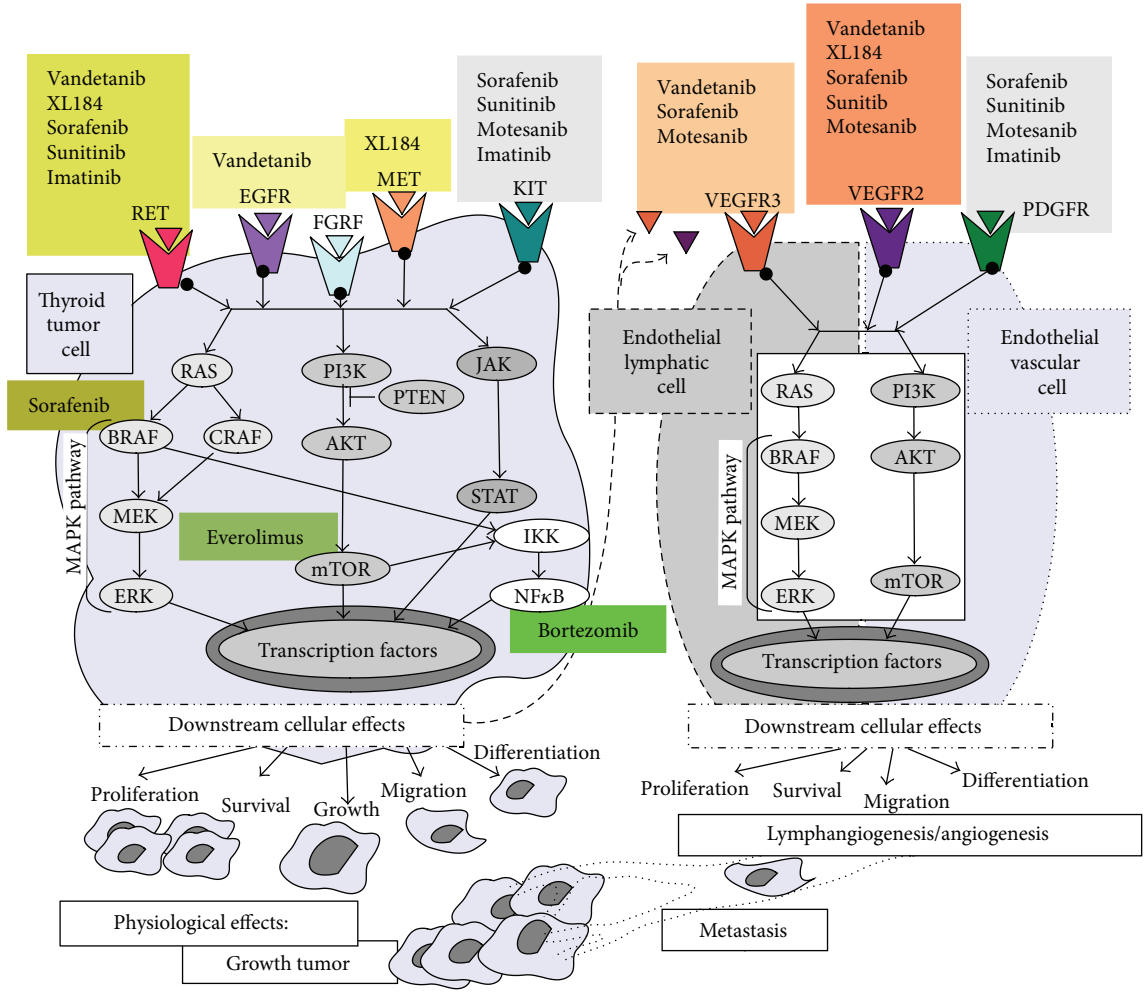

Figure 1: This figure schematically shows the tyrosine kinase receptors, such as RET, EGFR, FGFR, MET, KIT, VEGFR, PDGFR, and some of their downstream effectors. The two most important oncogenic signaling pathways are PI3K/AKT/mTOR and Ras/Raf/MEK/ERK. The activation of the pathways may transduce different transcription factors and induce tumor and endothelial cell proliferation, survival, migration, with subsequent tumor growth, lymphangiogenesis/angiogenesis, and metastasis. Moreover, the main tyrosine kinase inhibitors and their targets are shown.

of these sites inhibits autophosphorylation and activation of the tyrosine kinases and prevents the further activation of intracellular signaling pathways. TKIs can be specific to one or several homologous tyrosine kinases.

Several TKIs that are directed against RET kinase have been developed for the treatment of MTC, but there is no currently available tyrosine kinase inhibitor specific to RET. However, several multitargeted tyrosine kinase inhibitors have demonstrated significant activity against RET (Table 1), including vandetanib, sorafenib, motesanib, imatinib, and sunitinib [66, 68]. The inhibition of only one kinase receptor may induce the compensatory activation of other TKs and consequent resistance to treatment with TKIs [69, 70]. Therefore, the simultaneous inhibition of multiple activated TKs may be the best way to approach MTC [71–73]. TKIs that are currently undergoing testing in clinical trials are described as follows.

Vandetanib (ZD6474) is an oral TKI that targets RET, VEGFR-2, and -3, and at higher concentrations, EGFR [74]. Vandetanib selectively inhibits pathways that are critical for tumor growth and angiogenesis without leading to direct cytotoxic effects on tumor or endothelial cells [75]. Most of the mutant RET oncoproteins have demonstrated sensitivity to vandetanib, while mutations in which valine 804 is substituted by either leucine or methionine (as observed in some cases of MEN2A) rendered the RET kinase significantly resistant to vandetanib. This resistance is likely due to steric hindrance, as the Val804Gly mutation increased the sensitivity of RET to vandetanib [76]. When RET activity was inhibited, overstimulation of EGFR was able to partially compensate for RET through a partial rescue of MAPK pathway activation. The inhibition of EGFR by vandetanib has been shown to prevent this rescue of MAPK pathway activation. These data support the idea that the dual inhibition of RET and EGFR is important, as it may overcome the risk of an escape by MTC cells from a blockade of RET through the compensatory overstimulation of EGFR [69]. In addition, the expression of EGFR has been demonstrated in

Table 1: Main targeted therapies currently in use for the treatment of medullary thyroid cancer.

	Drug name	Key targets									IC_{50} RET [μM]*	http://www.clinicaltrials.gov/	
		RET	EGFR	VEGFRs	PDGFR	cKIT	FGFR	MET	BRAF	Other		Drugs in combination	Status identifier (phase)
Main TKIs	Vandetanib (ZD6474)	√	√	√	√						0.1		*Not yet recruiting*: NCT01661179 (1, 2) *Recruiting*: NCT00514046 (1, 2) NCT01496313 (4) *Active, not recruiting, has results*: NCT00410761 (3) NCT00358956 (2) NCT00098345 (2)
												Bortezomib	*Active, not recruiting*: NCT000923247 (1, 2) NCT01298323 (3)
												Docetaxel	*Withdrawn*: NCT000937417
	XL184 (Cabozantinib)	√		√		√		√		√	0.004		*Active, not recruiting*: NCT00704730 (3) NCT00215605 (1) *Available*: NCT01683110
	Sorafenib (BAY-43-9006)	√		√	√	√	√		√	√	0.0059–0.05		*Active, not recruiting*: NCT00390325 (2) *Unknown***: NCT006542387 (2)
	Sunitinib (SU11248)	√		√	√	√				√	0.22–1.3		*Active, not recruiting*: NCT00381641 (2) NCT00519896 (2) NCT00510640 (2)
	Motesanib (AMG-706)	√		√	√	√					0.05–0.09		*Completed*: NCT00121628 (2)
	Imatinib (STI571)				√	√				√	5–37	Xeloda, Dacarbazine	*Active, not recruiting*: NCT00354523 (1, 2)

Table 1: Continued.

	Drug name	Key targets									IC_{50} RET [μM]*	http://www.clinicaltrials.gov/	
		RET	EGFR	VEGFRs	PDGFR	cKIT	FGFR	MET	BRAF	Other		Drugs in combination	Status identifier (phase)
	Everolimus (RAD001)				mTOR							Pasireotide Pasireotide	*Recruiting*: NCT01625520 (2) NCT01270321 (2) NCT01164176 (2) *Unknown***: NCT0111865 (2)
Main Non-TKIs	Bortezomib (PS-341)				proteosome							Vandetanib	*Active, not recruiting*: NCT00923247 (1, 2)
	Pasireotide (SOM230)				sst1, 3, 5 (somatostatin receptors)							Everolimus Everolimus	*Recruiting*: NCT01625520 (2) NCT01270321 (2)

*IC_{50} RET [μM]: 50% inhibitory concentration.
**Status has not been verified in more than two years.

tumor-associated endothelial cells [77]. In this respect, anti-EGFR agents have been shown to block the proliferation and migration of endothelial cells. Therefore, the anti-EGFR activity of vandetanib might result in an additional direct antiangiogenetic mechanism. One phase II clinical trial showed the antitumor activity of vandetanib (300 mg/day) in patients with hereditary MTC. In this study, 20% of the patients showed a partial response to vandetanib (>30% reduction in tumor diameter), while an additional 53% of patients presented with disease stability at 24 weeks. Only 1 patient showed disease progression while receiving this agent. Of interest, this patient was not affected by any of two *RET* mutations correlated to vandetanib resistance in which valine 804 is substituted by leucine or methionine, but by Y791F *RET* germline mutation [78]. Another clinical trial, in which 19 patients with hereditary MTC were treated with vandetanib (100 mg/day), showed similar results [79]. Vandetanib is the only TKI approved for the treatment of symptomatic or progressive MTC in patients with unresectable locally advanced or metastatic disease [80]. The approval of vandetanib in April 2011 by the US Food and Drug Administration (FDA) was based on the results of the phase III clinical trial, "Study D4200C00058," in which 331 patients with unresectable locally advanced or metastatic MTC were randomly assigned to receive vandetanib (231) or a placebo (100). This study showed that the median progression-free survival duration (PFS) was 11 months longer in the group that received vandetanib than in the placebo control group and that 45% of the patients had an objective response rate (ORR). Common adverse events (any grade) occurred more frequently with vandetanib compared to the placebo and included diarrhea (56% versus 26%), rash (45% versus 11%), nausea (33% versus 16%), hypertension (32% versus 5%), and headache (26% versus 9%) [81]. The impact of overall survival of MTC patients treated with this compound is presently unknown.

XL184 (cabozantinib) is an oral selective inhibitor of RET, c-MET, and VEGFR-2. c-MET activation triggers tumor growth and angiogenesis. A phase I trial has shown clinical benefits of XL184 in patients with MTC [82]. These results have led to the expansion of an MTC-enriched patient cohort. The phase I trial results indicated that cabozantinib is active in patients with MTC, including those who harbor somatic *RET* mutations and are potentially at high risk for progression and death [83]. A global phase III pivotal study in MTC is ongoing (http://www.ClinicalTrials.gov/ number NCT00704730).

Sorafenib (BAY 43-9006)is a multikinase inhibitor that targets BRAF, VEGFR-2, VEGFR-3, KIT (a stem cell growth factor proto-oncogene involved in cell survival and differentiation), and PDGFR. This drug has been shown to strongly inhibit RET kinase activity *in vitro* [84]. A phase II clinical trial, in which sorafenib (400 mg/twice daily) was given to 21 patients with metastatic medullary carcinoma, reported that of the patients with sporadic MTC, 87.5% achieved disease stability and 6.3% demonstrated partial responses. The median PFS was 17.9 months. The treatment was prematurely terminated in MTC hereditary patients due to slow accrual [85]. In a similar trial, all 5 patients treated with sorafenib exhibited partial responses [86]. Recently, a study of sorafenib was conducted on advanced thyroid carcinoma patients, and partial responses were reported in six out of the 12 (50%) patients with MTC, although the small number of patients requires further prospective studies [87].

Sunitinib (SU011248) is a small molecule inhibitor that targets many of the same protein kinases as sorafenib, including VEGFR, PDGFR, KIT, and RET. In a phase II clinical trial, 33 patients with either well-differentiated thyroid carcinoma or MTC were treated with sunitinib. One patient had a complete response, 28% of the patients had partial responses, and 46% of the patients exhibited disease stability [88]. The intermediate results of the phase II THYSU study also showed the efficacy of sunitinib in advanced medullary thyroid carcinoma. The final results are yet to be released [89].

Motesanib (AMG 706) is a multikinase inhibitor that targets VEGFR receptors 1, 2, and 3, PDGFR, and KIT and exerts antiangiogenic and direct antitumor activities [90]. A phase II study performed in 91 patients with locally advanced or metastatic, progressive or symptomatic, MTC demonstrated that although the objective response rate was low, a significant proportion of the MTC patients (81%) achieved disease stability while receiving motesanib [91]. A recent study investigated the effects of motesanib on wild-type and mutant RET activity *in vitro* and on tumor xenograft growth in a mouse model of MTC. The results of this study demonstrated that motesanib inhibited thyroid tumor xenograft growth, predominantly through the inhibition of angiogenesis and possibly via the direct inhibition of VEGFR2 and RET, which were expressed in tumor cells. These data suggest that angiogenic pathways and specifically the VEGF pathway are still important for MTC cells [92].

Imatinib (STI571) is a TKI that inhibits RET, PDGFR, and KIT. A phase II trial in which imatinib was tested in metastatic MTC patients yielded disappointing results. The patients showed no objective responses; however, a minority of patients achieved disease stability [93].

Several TK inhibitors have been tested in clinical trials, but the most effective inhibitor and whether there is any specificity for particular *RET* mutations remain unknown. A recent study compared the effects of four TKIs (axitinib, sunitinib, vandetanib, and XL184) on cell proliferation, RET expression and autophosphorylation, and ERK activation in cell lines that express MEN2A (MTC-TT) and MEN2B (MZ-CRC-1) mutation. The findings showed that the inhibitors were specific for different mutations, with XL184 being the most potent inhibitor against the MEN2A mutation and vandetanib the most effective against the MEN2B mutation *in vitro*. No TK inhibitor was superior for all tested cell lines, which indicates that mutation-specific therapies could be beneficial in MTC treatment [94].

3.2. Other Emerging Therapies for Medullary Thyroid Cancer

3.2.1. Sensitization of Medullary Thyroid Carcinoma to Conventional Cytotoxic Treatments. Conventional chemotherapy has shown limited efficacy against metastatic MTC. One of the mechanisms for the resistance of MTC to chemotherapeutic drugs is multidrug resistance (MDR) [95]. MDR in cancer cells has been attributed to the overexpression of

several plasma membrane ATP-dependent efflux pumps, such as MDR-1 [96]. The enzyme cyclooxygenase (COX-2) has been shown to regulate MDR-1 expression in rat mesangial cells [97]. Furthermore, a study has shown that *in vitro* treatment of an MTC-derived cell line with rofecoxib, a COX-2 inhibitor, was able to sensitize MTC cells to doxorubicin [98]. A recent study, performed *in vitro*, has shown that celecoxib, another COX-2 inhibitor, was able to induce both MTC cell apoptosis and sensitization to vinorelbine, thus enhancing the chemotherapeutic effect of this drug [99]. A clinical trial, in which the *in vivo* activities of celecoxib were explored in MTC patients who cannot benefit from available treatments, would be desirable after accounting for the possible cardiovascular risks of this drug.

3.2.2. Drugs That Inhibit MTC Tumorigenesis Targets other than TKRs. Several other therapeutic agents are being investigated for their uses in the treatment of thyroid carcinomas, including MTC. These agents inhibit targets that are involved in development of MTC other than the tyrosine kinase receptors. We previously discussed that RAS operates in a complex signaling network with multiple activators and effectors, which allows them to regulate many cellular functions such as cell proliferation, differentiation, apoptosis, and senescence. Phosphatidylinositol 3-kinase (PI3K) is one of the main effector pathways of RAS, regulating cell growth, cell cycle entry, cell survival, cytoskeleton reorganization, and metabolism. The presence of *Ras* mutations in sporadic MTC and most importantly the frequent activation of the PI3K/AKT/mTOR pathway in several aggressive and metastatic MTCs strongly suggest that this cellular signaling pathway is a good candidate for targeted therapies against MTC. In fact, several *in vitro* evidences have demonstrated that the indirect blocking of this pathway, by PI3K inhibitors [100], or direct inhibition of mTOR [48] can mediate the induction of apoptosis and a decrease in cell viability in the MTC TT cell line. Currently, there are several clinical trials in which patients with radioiodine-refractory differentiated and medullary thyroid carcinomas were recruited to test the efficacy of everolimus (RAD001), a novel inhibitor of mTOR, in combination with other drugs (NCT01625520 and NCT01270321). Everolimus has demonstrated antitumor efficacy in various cancer types, including MTC. Recently, researchers have developed a liposomal form of everolimus and have demonstrated the anticancer efficacy of this formulation against TT cells [101]. Studies have shown that metformin can decrease the proliferation of cancer cells through 5′-AMP-activated protein kinase-(AMPK-) dependent inhibition of mTOR. Some researchers have reported growth inhibitory effects of metformin against MTC cell lines (TT and MZ-CRC-1), which were attributable to the downregulation of both mTOR/6SK and pERK signaling. The expression of the molecular targets of metformin in human MTC cells suggests that this drug may be potentially useful in the treatment of MTC [49].

PI3K-AKT-mTOR and RAF-MEK-ERK signaling have been shown to be important in the resistance of thyroid cancer cells to apoptosis and the promotion of tumor progression. In this context, targeted anti-VEGFR therapy or RAF inhibition may be ineffective if PI3K signaling remains intact. Therefore, two promising drugs, RAF265, a RAF inhibitor that is active against VEGFR2, and BEZ235, a PI3K inhibitor, were tested alone and in combination in preclinical MTC models that represented the key genotypes observed in refractory thyroid cancers. The study findings showed that a combination treatment with agents that inhibited both RAF and PI3K pathways strongly inhibited growth both *in vitro* and *in vivo*. In addition, the investigators showed for the first time that RAF265 potently inhibits the constitutively active RET^{C634W}, a form of the kinase that is observed in MEN2A [102].

Persistent RET activation, a frequent event in MTC, leads to the activation of the PI3K/AKT/mTOR, ERK/MAPK, and JAK/STAT3 pathways. Recently, the efficacy of the JAK1/2 inhibitor AZD1480 against the growth of thyroid cancer was tested *in vitro* and *in vivo* in thyroid cancer cell lines that expressed oncogenic RET. The findings showed that AZD1480 efficiently inhibited the growth and tumorigenesis of thyroid cancer cell lines that harbored oncogenic *RET* alterations, likely through the inhibition of PI3K-AKT signaling; this result supports the use of this inhibitor in patients with thyroid cancer, particularly in those with advanced MTC, for whom there are no effective therapeutic options [103].

Several MTCs display a rich but heterogeneous expression of somatostatin receptors (sst1–5) [104]. Thus, somatostatin (SRIF) peptide analogues in combination with TKIs may be a promising approach for the treatment of these tumors. The presence of different combinations of SRIF receptor (SSTR) subtypes in a given patient may explain the variable clinical response to SRIF analogues and may promote the search for more selective drugs with different affinities to the various receptor subtypes [105]. The currently available somatostatin analogues (octreotide and lanreotide) act preferentially through the somatostatin receptor subtype 2 (sst2). In MTC, these compounds have been reported to exert antisecretive effects on calcitonin but unfortunately are not reported to have antiproliferative effects. Pasireotide (SOM230) is a new somatostatin analogue that has a peculiar binding profile with high affinity for sst1, sst2, sst3, and sst5. Preliminary data from a phase II study of patients with metastatic carcinoma show that SOM230 is effective, and some clinical trials are exploring the efficacy of SOM230 alone or in combination with RAD001 in patients with MTC (NCT01625520 and NCT01270321).

The NF-κB pathway is also a potential target for drug development, and a number of compounds have been developed to inhibit this pathway at different levels in cancer cells. Studies have demonstrated that the proteosome inhibitor bortezomib exerted a promising antitumor effect in human MTC cells through the inhibition of IκB degradation, which led to the inactivation of the transcriptional factor NF-κB [106]. Patients are currently being recruited for a phase I/II trial to study the combination of vandetanib plus bortezomib (http://www.ClinicalTrials.gov/). Patients with MTC will participate in the phase II study [21].

3.2.3. Immunotherapy and Radioimmunotherapy. Immunization with tumor antigen-pulsed autologous dendritic cells (DCs) resulted in protective immunity and the rejection of various established human tumors. Specifically, vaccination immunotherapy with calcitonin and/or carcinoembryonic antigen (CEA) peptide-pulsed DCs was shown to result in the induction of a cellular, antigen-specific immune response in patients with MTC, which led to clinical responses in some patients. Therefore, for the first time, a potential DC vaccination therapy was developed for patients with metastatic MTC [107]. Another study, which was performed in a transgenic MTC mouse model, has confirmed this finding [108].

Papewalis et al. have reported on the *in vitro* findings of a vaccination trial in 5 MTC patients, who were treated with DCs that were generated using a new protocol, which consisted of granulocyte-macrophage colony-stimulating factor and interferon-alpha (IFN-DCs). These investigators demonstrated that immunization with IFN-DCs led to a tumor epitope-specific Th1 immune response in MTC patients [109]. Furthermore, a pilot trial of 10 patients has assessed the safety and efficacy, in terms of immune responses and clinical activities, of the DCs. In this study, DCs were injected into groin lymph nodes at 3-week intervals. Monitoring of the patients included serial measurements of calcitonin tumor markers, radiological imaging, and immunological *in vitro* tests, including T-cell interferon-gamma detection and cytotoxicity assays. DC vaccinations were determined to be well tolerated and safe. After a median followup of 11 months (range 7–26), 3 (30%) of the 10 patients exhibited disease stability, while 7 (70%) of the patients progressed during treatment. A combined treatment with different tumor cell lysate-pulsed DCs increased the likelihood of a calcitonin tumor marker response and should therefore be preferred over monotherapy with DCs pulsed with a single lysate [110].

Thus, immunotherapy may be a promising alternative therapy in combination with agents that target specific signal transduction pathways for aggressive forms of MTC that are resistant to classical therapies.

A significant antitumor effect was also observed with radioimmunotherapy using an anti-CEA ^{131}I-F6 monoclonal antibody in MTC-bearing nude mice. Nevertheless, no complete responses were observed. Similarly to chemotherapy, drugs that target the tumor microenvironment might improve the efficacy of radioimmunotherapy. This hypothesis was confirmed by a recent study in which pretreatment of mice grafted with the TT human medullary thyroid cancer cell line with antiangiogenic therapies was found to improve the efficacy of radioimmunotherapy with acceptable toxicity [111]. Another independent study, which was conducted in a mouse model of MTC, has found similar results with the angiogenesis inhibitor bevacizumab [112]. Future investigations will be performed to better understand how antiangiogenic agents enhance the efficacy of radioimmunotherapy.

3.2.4. Epigenetic Therapy. Epigenetic drugs are expected to target the two main mechanisms of epigenetic alterations, DNA methylation and acetylation, and are regarded with increasing interest by both endocrinologists and oncologists. Concluded trials of such drugs have shown that few patients with advanced thyroid cancer responded completely, which suggests that these treatments were effective at stabilizing progressive disease. Therefore, definitive results from clinical trials will clarify the true effectiveness of epigenetic drugs in these tumors. Epigenetic drugs, when used in combination with other target molecules, might significantly increase response rates to treatment in advanced thyroid cancer patients, either by relaxing the chromatin structure to make DNA more accessible to the effects of a DNA targeting drug or by acting synergistically with antimitotic drugs [113]. Thus, epigenetic therapy may be a promising novel approach for the treatment of some cases of MTC.

3.3. Potential Mechanisms of Resistance to Therapy in MTC. Tumor cells often devise strategies to bypass the effects of antineoplastic agents, and the selection of therapy-resistant clones is frequently the reason for treatment failure. One characteristic of endocrine cancer is a general resistance to conventional chemotherapies or radiotherapies that would normally lead to apoptosis of the cancer cells. MTC can develop resistances to cytotoxic drugs due to the expression of the multidrug resistance (MDR) 1 gene. Drugs that can oppose this mechanism of resistance to traditional chemotherapies may increase the sensitivity of MTC tumor cells to chemotherapy. For example, celecoxib, a COX-2 inhibitor, has been shown to potentiate the chemotherapeutic effect of vinorelbine in MTC [99]. Therefore, the synergistic actions of a cytotoxic drug and a compound that increases the sensitivity of MTC cells to such a drug could provide a treatment method for patients who cannot benefit from TKIs. However, the most promising results in patients with chemotherapy and radiotherapy-unresponsive MTC were obtained with TKIs. The inhibition of a single RTK may engage compensatory signaling that maintains cell growth. Multitargeted tyrosine kinase inhibitors, which inhibit multiple RTKs simultaneously, including RET, have been developed to bypass this potential resistance mechanism. One complication is that such inhibitors may not only be more effective for targeting receptors in tumor cells but may also exhibit greater toxicity. Thus, the challenge is to balance the increased efficacy of these inhibitors with the potential for a broader array of side effects [7]. Moreover, some MTC patients cannot take advantage of these therapies due to specific *RET* mutations that confer resistance to TKIs (e.g., RET V804 confers resistance to vandetanib) [76, 114]. Finally an important study reported the existence of cancer stem-like cells in MTC, which exhibit the features of self-renewal and of multiple lineage differentiation that is dependent on RET proto-oncogene receptor activity suggesting a potential mechanism of resistance to cytotoxic or TKIs agents [115].

4. Conclusions

Most cases of metastatic MTC are incurable due to resistance to conventional chemo- and radiotherapies. In recent decades, the identification of genetic defects and altered cellular signaling pathways involved in human MTC tumorigenesis have led to the development of targeted therapies,

of which the most important are tyrosine kinase inhibitors. However, the low rates of partial responses or complete responses and the short duration of responses in MTC patients who were observed while taking these drugs prompted researchers to develop new drugs and alternative therapies to be combined with multi-TKIs and to reduce potential cross-toxicity effects. To this end, a recent study has shown synergistic effects with a combination of sorafenib and the MEK inhibitor AZD6244 against a human MTC cell line. Concomitant use of a RAF inhibitor, RAF265, and a dual PI3K/mTOR inhibitor, BEZ-235, was another effective combinatorial therapy against thyroid cancer in xenograft mouse models [102]. The mTOR cascade is emerging probably as one of the most important deregulated pathways in advanced and metastatic MTC and certainly deserves further study. Targeting mTOR in combination might be efficacious in patients with this tumor types.

However, a complete overview of all signaling pathways, including their interactions, role of the activating gene mutations that contribute to MTC tumorigenesis, and mechanisms of intrinsic and acquired resistance to treatments, is required and will permit us to identify the best therapy for each patient. Novel combined or sequential therapies request a further step in the knowledge of the cellular signaling of this tumor. Finally, larger multi-institutional clinical trials to fully understand the clinical benefits of these therapies are warranted.

Conflict of Interests

The authors do not have any conflict of interests to disclose.

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Review Article

Comprehensive Literature Review: Recent Advances in Diagnosing and Managing Patients with Poorly Differentiated Thyroid Carcinoma

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Poorly differentiated thyroid carcinomas are a rare form of thyroid carcinomas; they display an intermediate behavior between well-differentiated and anaplastic thyroid carcinomas. PDTCs are more aggressive than the well-differentiated, but less aggressive than the undifferentiated or anaplastic, forms. No clinical features can accurately diagnose poorly differentiated thyroid carcinomas. Thus, the results of histocytology, immunohistochemistry, and molecular genetics tests aid in diagnosis. Given the aggressiveness of poorly differentiated thyroid carcinomas and the poor survival rates in patients who undergo surgery alone, a multimodality treatment approach is required. We conducted a comprehensive review of the current diagnostic and therapeutic tools in the management of patients with poorly differentiated thyroid carcinomas.

1. Introduction

Thyroid carcinomas are categorized along a continuum, usually based on the degree to which neoplastic parenchymal cells mimic the corresponding normal parenchymal cells, both in cellular morphology and functionality [1]. At one extreme, well-differentiated thyroid carcinomas (WDTCs), like the papillary and follicular thyroid forms (PTCs and FTCs), typically confer a favorable prognosis. However, at the opposite end of the spectrum, undifferentiated carcinomas, like anaplastic thyroid carcinomas (ATCs), are aggressive and rapidly fatal. Poorly differentiated thyroid carcinomas (PDTCs) exhibit a unique histologic architecture and morphologic changes that result in tumor behavior more aggressive than that of WDTCs but less aggressive than that of ATCs [2–4]. Although the literature collectively concurs on the clinical significance and existence of PDTCs the morphological cellular features for diagnosing PDTCs are still a topic of controversy.

The term "PDTC" was introduced and defined in the 1980s by Sakamoto et al. [5] and Carcangiu et al. [6]. Those two groups described similar neoplastic tumors, but with varying diagnostic criteria. This issue still holds true: some authors define PDTCs on the basis of unique histologic architectural growth patterns (insular, trabecular, or solid); other authors, on the basis of aggressive histologic behaviors (necrosis, increased mitotic rates, and vascular invasion). Recently, several studies have statistically validated that diagnosing PDTCs on the basis of biological behaviors (rather than on growth patterns) demonstrates greater clinical and prognostic significance [4, 7].

In light of the increased clinical significance of PDTCs and the lack of unifying diagnostic criteria, a consortium of experts met in Turin, Italy, in 2006, and proposed a homogenous set of diagnostic criteria for PDTCs [3]. Largely, they retrospectively established criteria based on both histologic architectural grade and cytomorphologic features of neoplastic thyroid cells. Establishing the criteria helped clarify and

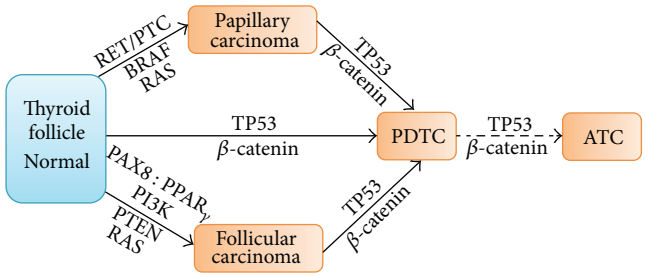


Figure 1: Poorly differentiated thyroid carcinomas (PDTCs) can develop de novo or from papillary or follicular carcinomas, through genetic alterations, possibly progressing into anaplastic thyroid carcinomas (ATCs).

reinforce the principles that PDTCs are a unique and separate pathologic process and that such patients require clinical care that is different from that for WDTC patients.

We conducted a comprehensive review of the current diagnostic and therapeutic tools in the management of poorly differentiated thyroid carcinomas.

2. Histocytology

Technically, PDTCs originate from either follicular or papillary epithelial cells and typically reveal a trabecular, insular, and/or solid (TIS) histomorphologic pattern [3]. PDTCs are classically characterized by the insular growth pattern, first described by Carcangiu et al., displaying large cellular nests with small round nuclei typically surrounded by a fibrovascular network [2, 4, 8–11]. These can be categorized as two major subtypes, the insular and insular-like carcinomas. Moreover, PDTCs can present as either a mixture of the three patterns or as one independent pattern. However, purely insular carcinomas are infrequent; insular-like carcinomas featuring trabecular or solid architecture are much more common [8, 12]. Most authors would agree that hematoxylin and eosin stain results solely demonstrating the aforementioned histomorphologic patterns are not enough to establish a diagnosis of PDTC; more morphologic features must be identified.

Many investigators have attempted to define PDTCs by cytology tests because of their increased clinical prognostic value. PDTCs are often cellular, with scant colloid, and lack both nuclear pleomorphism and high-grade atypia [8, 9]. Furthermore, mitoses, high nuclear/cytoplasmic (N/C) ratio, loss of cellular polarity, and hyperchromasia are common cytologic features of PDTCs [1, 8, 9]. In a statistical analysis of 32 cytomorphologic features in 40 histologically proven cases of PDTC, Bongiovanni et al. demonstrated that the following 4 cellular features from a fine-needle aspirate (FNA) were predictive of PDTC: an TIS growth pattern; a high N/C ratio; a single-cell pattern; and severe cellular crowding [8]. Moreover, in a clinicopathologic study of 58 patients, Hiltzik et al. demonstrated that necrotic and mitotic features are better able to stratify patients into different prognostic categories than growth patterns alone [7].

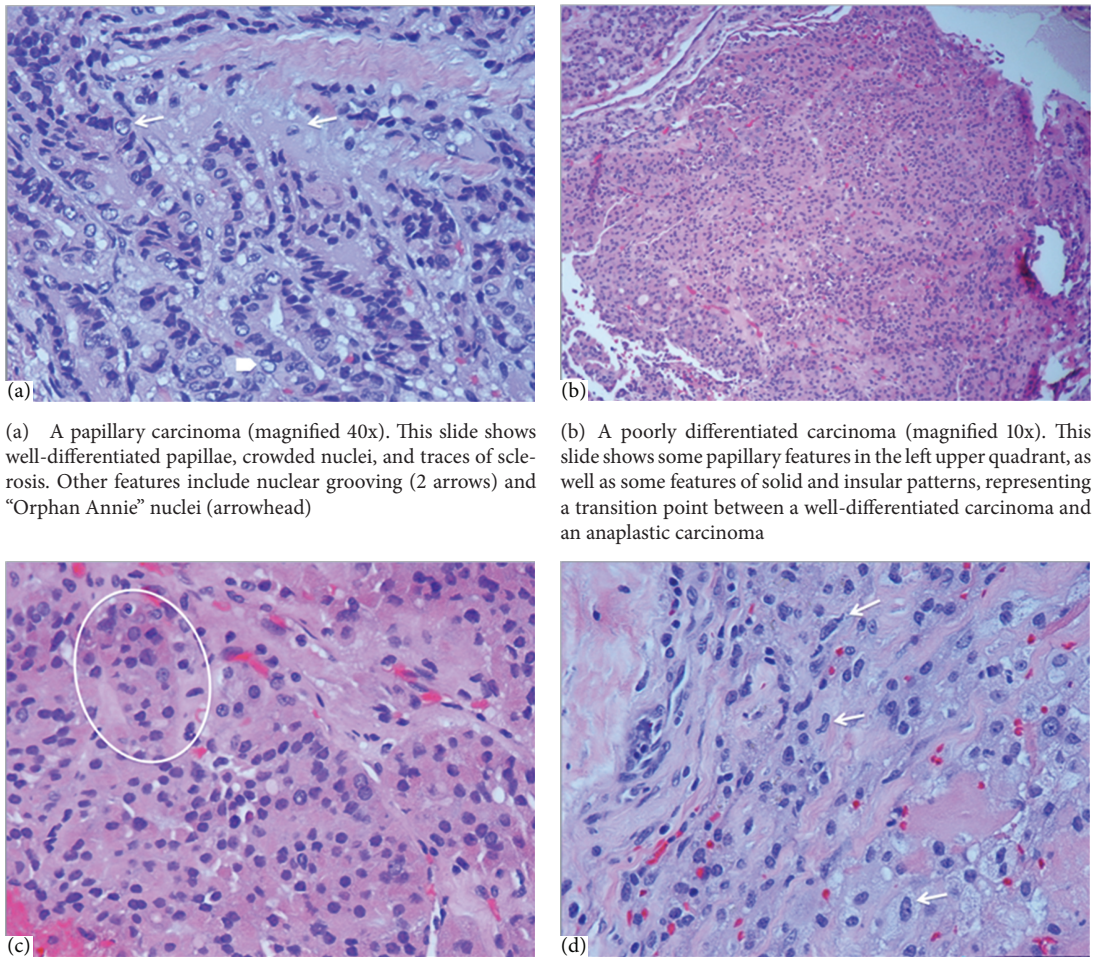
PDTCs can develop from WDTCs or de novo entities (Figure 1). Therefore, it is difficult to determine how much of an TIS pattern is required on an FNA sample to make the diagnosis of PDTC [3, 13]. Bongiovanni and Faquin found that WDTCs with a 10% or greater TIS component confer a more aggressive clinical course and a poorer prognosis than WDTCs without PDTC features [9]. Several studies have proposed parameters of architectural patterns of >10%, >40%, and >70% to establish the diagnosis of PDTC; however, a standard minimum cutoff point has yet to be determined or established [10, 14]. It is worth mentioning that Volante et al., in a study of 183 patients with TIS patterns, did not find a statistically significant difference in the overall survival rate of patients with only a minor TIS component (of 10% to 50%) versus patients with a well-represented TIS component (of 50% to 75%) or a virtually "pure" TIS component (>75%) [4]. Although survival rates were statistically insignificant, patients with a minor TIS architectural component had a trend towards a more favorable prognosis [4]. Moreover, in a 40-patient case series, Pulcrano et al. did not find a correlation between a particular PDTC architectural subtype and metastasis-free survival [15].

Although defining PDTCs on cytomorphologic features is less expensive, internationally feasible, and relatively reliable, immunohistochemical stains and molecular genetics are now playing an increased role in diagnosing PDTCs. So, given the limited resources of various medical institutions around the world, the 2006 Turin proposal remains, arguably, the most accepted unifying and encompassing criteria for diagnosing PDTCs. According to that proposal, PDTCs are defined by: (1) the presence of an TIS architecture (2) with at least one of the following features present: convoluted nuclei; mitotic activity greater than 3 per 10 high-power fields; or tumor coagulative necrosis; and (3) absence of the conventional nuclear features of papillary thyroid carcinoma [3] (Figure 2).

3. Immunohistochemistry

Immunohistochemical stains have been widely considered by many authors to increase the diagnostic predictability of PDTCs. No specific molecular marker or immunohistochemical stain is specific to the detection of PDTCs, yet test results can rule out other thyroid carcinomas [8, 13]. For instance, PDTCs are negatively immunoreactive to calcitonin, carcinoembryonic antigen (CEA), chromogranin, and synaptophysin; such results reliably rule out medullary thyroid carcinomas and neuroendocrine tumors [8, 9]. Similarly, PDTCs are not immunoreactive to hematopoietic cellular markers, such as B-lymphocyte antigen (CD19 and CD20) and plasma cell marker (CD138); such results help exclude lymphoproliferative disorder [8].

In a small case series, Bongiovanni et al. demonstrated that PDTC samples were strongly immunohistochemically reactive to thyroglobulin (Tg), thyroid transcript factor 1 (TTF-1), and a cytokeratin cocktail [13, 14]. To further differentiate PDTCs from other thyroid carcinomas, Patel et al. showed that ATCs are not immunoreactive to



(a) A papillary carcinoma (magnified 40x). This slide shows well-differentiated papillae, crowded nuclei, and traces of sclerosis. Other features include nuclear grooving (2 arrows) and "Orphan Annie" nuclei (arrowhead)

(b) A poorly differentiated carcinoma (magnified 10x). This slide shows some papillary features in the left upper quadrant, as well as some features of solid and insular patterns, representing a transition point between a well-differentiated carcinoma and an anaplastic carcinoma

(c) A poorly differentiated carcinoma (magnified 40x). This slide shows tumor cells with small, hyperchromatic nuclei, as well as increased mitotic activity and a nesting pattern (encircled)

(d) An anaplastic carcinoma (magnified 40x). This slide shows hyperchromatic nuclei with marked variations in size and shape (pleomorphism) (3 arrows); diffuse, destructive infiltration; and desmoplasia

Figure 2: These slides illustrate the various histologic features of well-differentiated thyroid carcinomas (WDTCs), poorly differentiated thyroid carcinomas (PDTCs), and anaplastic thyroid carcinomas (ATCs).

thyroglobulin [14]. PDTCs may also be weakly immunoreactive (<50% of cases) to HBME-1, galectin-3, CD44v6, and Bcl-2. However, such stains alone cannot definitively differentiate PDTCs from WDTCs [13, 16]. But the unique genetic behavior of the cadherin/catenin complex has improved the ability to differentiate PDTCs from WDTCs.

Cadherins aid in cell-to-cell adhesion; loss of adhesion correlates with an increased loss of differentiation in most carcinomas derived from epithelial cells [17]. Rocha et al. observed that WDTCs retained E-cadherin/catenin expression, whereas PDTCs displayed heterogeneous loss of E-cadherin and retained catenins [17]. Additionally, Rocha et al. concluded that the progressive loss of E-cadherins (rather than the previously assumed genetic mutation in catenins) is the more "crucial event" in determining the level of differentiation in thyroid carcinomas [17]. Nevertheless, according to many other studies, β-catenin mutations are significantly more specific for PDTCs—present, on average, in 25% of PDTC cases (range, 20% to 32%) [16, 18, 19].

Ki67 antigen is another immunohistochemical marker helpful in diagnosing PDTCs [15, 17]. A nuclear protein, Ki67, has an important role in cellular proliferation through ribosomal RNA transcription. Since PDTCs have a high mitotic activity, Ki67 antigen labeling can help to not only diagnose PDTCs but also to enumerate the extent of mitotic involvement [15, 20].

The *IMP3 protein* may also have value in diagnosing PDTCs as well as serving as a prognostic indicator [21]. Asioli et al. determined that IMP3 expression in PDTC patients is a negative prognostic indicator associated with an increased risk of death, lymph node metastasis, and distant metastasis [21]. Moreover, Asioli et al. stated that PDTCs are often associated with HBME-1, Ki67, and p53, but those markers are limited in their ability to specifically diagnose

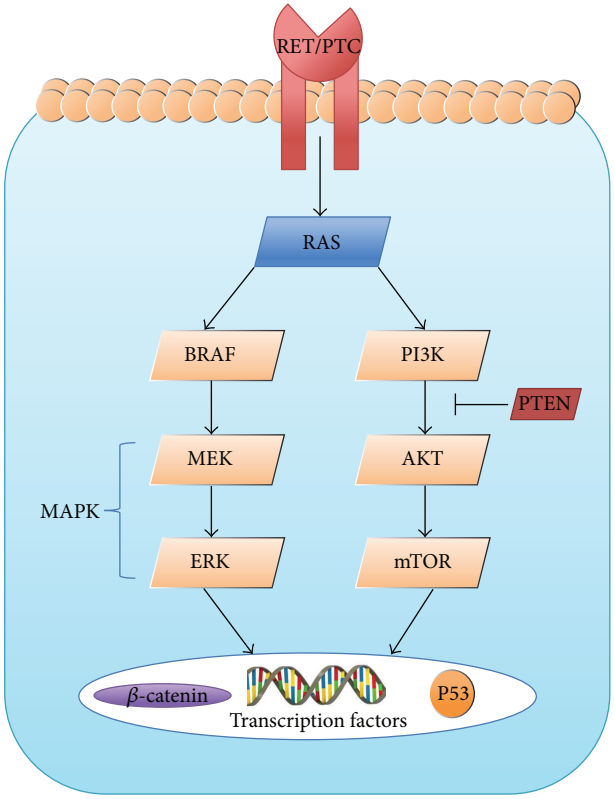


Figure 3: This diagram summarizes the signaling pathway in thyroid carcinomas.

PDTCs [21]. To date, IMP3 expression is one of the first novel immunohistochemical markers predictive of overall worse survival rates in PDTC patients [16, 21].

4. Molecular Genetics

Genetic alterations in thyroid follicular cells, such as point mutations and translocations, increase the mitogenesis and pathogenesis of thyroid carcinomas. Therefore, appreciating the molecular biology of PDTCs could help develop better-targeted therapeutic modalities and more accurate diagnoses. If left untreated, PDTCs (whether they arise de novo, or from PTCs or FTCs) could eventually progress by dedifferentiation to ATCs (Figure 2). Interestingly, according to the literature, those three pathways leading to PDTCs are invariably orchestrated by varying genetic alterations [16].

Genetic alterations of follicular cells that lead to carcinogenesis are caused by unopposed activation of either the mitogen-activated protein (MAP) kinase pathway or the phosphatidylinositol-3-kinase (PI3K)/AKT pathway [1]. Specifically, the MAP kinase pathway (encompassed by the MEK and ERK kinase cascade) is regulated by the RET, RAS, and BRAF genes. Point mutations in the BRAF and RAS genes or RET/PTC translocation can lead to unopposed cellular proliferation and to a carcinogenic environment via the MAP kinase pathway [1] (Figure 3).

BRAF gene alterations (commonly, valine-to-glutamate substitution at codon 600) are present in about 30% to 45% of patients with PTCs and, on average, in 15% (range, 12% to 47%) of patients with PDTCs [1, 16, 18]. Follicular carcinomas are not susceptible to BRAF mutation; therefore, PDTCs arising from FTCs must be BRAF-mutation-negative. The BRAF gene is a marker of adverse prognostic factors, including disease aggressiveness, tumor recurrence, lymph node or distant metastatic disease, and extrathyroidal extension [1, 18]. Interestingly, thyroid carcinomas with BRAF-mutations are also associated with a decreased capability to trap radioiodine (^{131}I) [18].

RAS gene alterations (commonly, point mutations at HRAS codon 12 or 61 and NRAS at codon 13) are present in about 40% to 50% of patients with FTCs and, on average, in 35% (range, 20% to 40%) of patients with PDTCs [1, 16, 18, 22]. Unlike the BRAF gene, which is specific to the activation of the MAP kinase pathway, RAS can activate both the MAP kinase pathway and the PI3K/AKT pathway. Specifically in patients with PDTCs, oncogenic RAS activation is a prevalent genetic alteration—and a marker of tumor dedifferentiation and adverse prognostic outcome [10, 18]. Of note, RAS mutations stimulate chromosomal instability and, thus, may predispose to tumor dedifferentiation, perhaps explaining the increased prevalence of mutant RAS in patients with ATCs [16, 18]. However, Nikiforov et al. noted that mutant RAS is unlikely to be solely capable of driving tumor dedifferentiation, given its high prevalence (45%) in patients with pure WDTC and in those with benign thyroid adenomas [16]. Conversely, Garcia-Rostanet al. stated that histologic dedifferentiation is not necessarily driven by BRAF or RAS mutations individually, but rather represents the cooperation of multiple genetic alterations that likely stimulate dedifferentiation [10].

TP53 gene alterations (point mutation at codon 273 leading to the inactivation of p53) are rarely associated with WDTCs; however, they are highly prevalent in patients with PDTCs (about 28%; range, 17% to 38%) and in patients with ATCs (64%; range, 20% to 88%) [18, 23, 24]. Termed the "molecular policeman" [1], p53 broadly has three principal functions that preferentially hinder, and possibly reverse, carcinogenic effects: quiescence, senescence, and apoptosis [1]. Interestingly, in histological samples of a tumor containing both WDTC and PDTC components, alterations in the p53 gene were circumscribed to the less differentiated component [24]. These findings suggest that, unlike the RAS and BRAF gene alterations, p53 mutations possess an exclusive function in triggering tumor dedifferentiation and evolution to PDTC and ATC [23, 24].

RET/PTC rearrangements (paracentric inversion of chromosome 10 or a reciprocal translocation between chromosomes 10 and 17), ultimately result in the unopposed activation of the MAP kinase pathway. Most commonly, RET gene rearrangements are exclusively expressed in patients with PTCs, usually those with a history of radiation exposure (e.g., from the 1986 nuclear power plant accident at Chernobyl) [1, 18, 23]. Such rearrangements are almost never expressed in patients with PDTCs [16, 18].

PAX8 : PPARγ rearrangements, (chromosomal translocation of (2;3)(q13;p25)) resulting in the fusion of the PAX8 gene and the peroxisome proliferator-activated receptor gene,

Table 1: Prevalence of genetic alterations in patients with various thyroid carcinomas.

Altered gene	PDTC	PTC	FTC	ATC
RET/PTC	0%	20%	0%	0%
TP53	20–30%	0%	0%	65–70%
BRAF	15%	45%	0%	20–25%
RAS	30–35%	10–15%	45%	50–55%
β-catenin	20–25%	0%	0%	65%
PAX8 : PPARγ	0%	0%	35%	0%

PDTC: poorly differentiated thyroid carcinoma.
PTC: papillary thyroid carcinoma.
FTC: follicular thyroid carcinoma.
ATC: anaplastic thyroid carcinoma.

are almost always associated only with follicular carcinomas [1, 18]. Such rearrangements are almost never expressed in patients with PDTCs [16, 18].

Table 1 summarizes the prevalence of genetic alterations in patients with various thyroid carcinomas.

5. Clinical Presentation

Of all thyroid carcinomas worldwide, PDTCs account for only 4% to 7% [17]. They are typically diagnosed in patients between the age of 55 and 63 years, a 2 : 1 female predominance [16]. Interestingly, a regional variance has been noted for instance, northern Italy (an endemic goiter region) has incidence rates as high as 15%, whereas the rates are much lower in the United States (1.8%) and Japan (<1%) [2, 16]. These discrepant rates suggest that the causes of PDTCs are multifactorial, including genetic, environmental, and dietary causes [19].

Consistent with their intermediate differentiation, PDTCs display clinical behaviors of intermittent biological aggressiveness. When diagnosed, PDTCs are typically already at an advanced stage of disease, with extrathyroidal extension and extensive local invasion [8, 16]. They have a propensity to metastasize to regional lymph nodes (50% to 85 %), and distantly (36% to 85%), most commonly to the lung (14% to 54%) and bones (18% to 33%) [2, 25]. Furthermore, the 5-, 10-, and 15-year survival rates are considerably lower in patients with PDTCs (50%, 34%, and 0%) than in patients with WDTCs (95%, 86%, and 81%) [2, 8, 14]. Several retrospective studies found that the following factors negatively correlate with survival rates: patient age >45 years, tumor size >4 cm, lack of radioactive iodine therapy postoperatively, cervical lymph node involvement, tumor necrosis, mitotic index >3 per 10 high-power fields, local recurrence, and distant metastasis at time of diagnosis [4, 10, 19, 26].

The initial workup typically includes an ultrasound examination (to evaluate the thyroid gland and cervical lymph node compartments) and FNA sampling (for cytologic analysis). In patients with suspected PDTCs, preoperative evaluation of vocal cord function is critical, in light of the high rates (50% to 75%) of extrathyroidal invasion [2]. The recurrent laryngeal nerve is frequently affected in patients with extrathyroidal disease. Randolph and Kamani found that vocal cord paralysis was the most reliable clinical marker (sensitivity, 76%; specificity, 100%) of invasive thyroid carcinomas [27]. In their study, preoperative laryngoscopy revealed that 70% of patients with invasive disease presented with vocal cord paralysis, as compared with only 0.3% of patients with noninvasive disease [27]. They noted that preoperative vocal cord paralysis involvement should be a presumptive diagnosis of invasive disease.

If extrathyroidal invasion, substernal extension, or distant metastasis is suspected, axial imaging with computed tomography or magnetic resonance imaging is important in operative planning. If extrathyroidal extension is suspected, further studies (including esophagography, esophagoscopy, and bronchoscopy) may be required to assess neighboring structures.

6. Treatment Modalities

Given the infrequency of PDTCs and the previously lack of standard diagnostic criteria, no standard guidelines currently exist for the management of PDTCs. However, most endocrine surgeons agree that the primary treatment is a total thyroidectomy, with lymph node dissection whenever feasible [14, 16, 19]. Postoperative options such as radioactive iodine (RAI) (^{131}I) ablation therapy, external beam radiation therapy (EBRT), and chemotherapy remain poorly established.

RAI (^{131}I) ablation therapy postoperatively is controversial. Sanders Jr. et al. recommended, in light of the potential for PDTC uptake and the lack of major side effects, considering ^{131}I therapy in all postoperative patients who underwent a complete resection [2]. PDTCs have demonstrated the capability to uptake ^{131}I in up to 80% to 85% of patients, yet studies have not statistically shown that ^{131}I prolongs 5-year survival [19]. Moreover, since over 15% of patients with PDTCs have an BRAF mutation, which is associated with a decreased capacity to trap radioiodine (^{131}I) [18], the value of RAI ablation in this population is limited.

EBRT is typically reserved for patients with aggressive forms of PDTCs or for patients whose resection was incomplete, with remnants of malignant disease retained in the neck postoperatively [2, 16, 19]. Extrapolating from poorly prognostic WDTC radiation studies, Sanders et al. recommended considering EBRT in patients with PDTCs who meet at least 1 of these criteria: (1) tumors >4 cm with minimal extrathyroidal extension without distant metastasis (i.e., extension to the sternothyroid muscle or perithyroid soft tissues); (2) extensive extrathyroidal extension of any tumor size (i.e., extending past the thyroid capsule into the subcutaneous soft tissues, larynx, trachea, esophagus, recurrent laryngeal nerve, or mediastinal vessels); and/or (3) regional lymph node metastasis [2, 28]. The utility of adjuvant EBRT is questionable: retrospective studies involving EBRT in patients with PDTCs showed no statistical improvement in overall survival rates [2, 19].

Chemotherapy has clinically been reserved for patients with inoperable PDTCs. It has some clinical practicality in

patients with ATCs. An intensive chemotherapy regimen can aid in locoregional control by improving resectability or by reducing disease progression [2, 16].

Chemoradiation is currently undergoing experimental review for any possible benefit in patients with PDTCs [19]. However, to date, survival rates in patients with PDTC have not improved after chemotherapy, either alone or in combination with radiotherapy.

Perhaps the most promising PDTC treatments will come from advancements in molecular inhibitors targeting the TP53 gene, the MAP kinase pathway, and/or the PI3K/AKT pathway [16, 18]. Novel therapeutic agents, such as sorafenib, vandetanib, sunitinib, and motesanib, are currently undergoing promising clinical trials. These agents can selectively target multiple kinase pathways like vascular endothelial growth factor (VEGF), which is important in angiogenesis. They also target RET, BRAF, and/or protein derived from the RET/PTC realignment [29]. In the near future, these molecular inhibitors might be administered (alone or in conjunction with other treatment modalities) to potentiate treatment options for patients with PDTC [18, 29].

7. Follow-Up Imaging

Traditionally, RAI imaging has been used to assess patients with PDTCs [14, 30]. Most WDTCs retain the capacity to take up iodine, so the use of RAI imaging is suitable in patients with WDTCs. In contrast, PDTCs have a reduced capacity to trap RAI [18]; so the use of RAI imaging in patients with PDTCs can falsely suggest a complete resection [2, 30].

The "flip-flop" theory postulates that as less differentiated malignant cells lose their ability to take up RAI, they increase their ability to take up fluorodeoxyglucose (FDG) [30]. Recent statistically sound studies have noted the value of FDG-positron emission tomography (PET) scans in localizing occult disease and in increasing the rates of early detection of recurrent or metastatic PDTCs [14, 30]. In a multivariate analysis, Wang et al. determined that the strongest single predictor of survival and prognosis was the volume of disease as detected by FDG-PET. FDG-avid tumors with volumes over 125 mL positively correlated with poorer short-term survival [30].

Furthermore, Ito et al. recommended regular postoperative examinations in patients diagnosed with PDTCs, not only to predict prognosis but also to allow for timely postoperative care [31].

8. Conclusion

A limitation of our review (as well as a clinical challenge) is the lack of an accepted, unified set of criteria for characterizing PDTCs. The 2006 Turin proposal, albeit criticized as overly restrictive by some investigators, at minimum offers a comprehensive, homogenous set of diagnostic criteria, paving the way for increasing our understanding of the pathogenesis and treatment of this disease. Perhaps with increased specificity in immunohistochemistry and molecular genetics tests, our definition of PDTCs will become more standardized and it can more readily be diagnosed. These tumors, though infrequent, do have distinct clinicopathologic features; pathologists and clinicians alike must know how to recognize and diagnose them. Clinical management and recurrence detection are still in their infancy for this disease, but advances in molecular genetics continue to promise a large role in the refinement of diagnosis, prognosis, and therapeutic modalities.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Clinical Study

Gender-Specific Variation in the Prognosis of Papillary Thyroid Cancer TNM Stages II to IV

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To investigate the correlation between gender and the clinical presentation of papillary thyroid cancer and the long-term followup results, 435 patients who underwent total or near-total thyroidectomy were enrolled in this study. Among these papillary thyroid cancer patients, 12.2% showed lymph node metastases and a higher incidence of male patients in the N1b group. There were 65 from 316 female (20.6%) and 49 from 120 male (40.8%) patients who had a postoperative disease progression. A total of 55 (12.6%) patients died of thyroid cancer. Male patients showed a higher thyroid cancer mortality than the females. Multiple regression analysis showed that male gender was an independent risk factor for cancer recurrence and mortality. Male patients with TNM stages II to IV of papillary thyroid cancer need to adopt aggressive surgical and postoperative ^{131}I therapy.

1. Introduction

Thyroid nodules and cancer have long been considered to occur predominantly among women [1]. On the other hand, male gender has been reported to be an important risk factor for the development of the subgroup of well-differentiated thyroid cancers, based on the occurrence of larger tumors [2], worse prognosis in follicular and Hürthle cell thyroid cancers [3], and lymph node invasion and local neck recurrence of papillary thyroid carcinomas [4–6]. In contrast, no gender-specific differences have been observed in surgically treatable Graves' disease and toxic thyroid nodules [7]. Thyroid cancer is associated with a wide range of prognoses with different histopathologic patterns. In addition, other factors, such as TNM stage, surgical method, and the application of postoperative adjuvant therapy, may also influence therapeutic outcomes. Even in papillary thyroid carcinoma, different histologic variants with variable presentation and prognoses after treatment have been reported [8].

This study aimed to determine the role of gender in the clinical presentation of papillary thyroid cancer and in the results of long-term followup schemes. To avoid bias due to the influence of age and the less aggressive nature of this cancer in women, papillary thyroid cancers at TNM stage I were excluded from the study. All enrolled patients underwent total thyroidectomy and postoperative remnant ablation. Long-term data were analyzed to identify trends in thyroid cancer progression in relation to gender and age groups.

2. Patients and Methods

All patients enrolled in this study underwent total or near-total thyroidectomy and postoperative ^{131}I treatment for remnant ablation in Chang Gung Memorial Hospital (CGMH) in Linkou, Taiwan. A total of 435 patients, consisting of 315 women (mean age 56.6 ± 8.9 years) and 120 men (mean age 57.9 ± 9.1 years), meeting the inclusion criteria during the treatment period between 1986 and 2009 were included in this study. All patients underwent regular followup until the end of 2011. A pathologic review was performed for all thyroid carcinomas using the World Health Organization (WHO) classification [9].

After thyroid surgery, thyroid remnant ablation was recommended 4–6 weeks after surgery for patients with papillary thyroid cancers, as in our previous study [10]. The ^{131}I ablation dose for most patients was 1.1 GBq (30 mCi). A whole-body scan (WBS) was performed 1 week after ^{131}I administration by using a dual-head gamma camera (Dual Genesys, ADAC, USA) equipped with a high-energy collimator. Cases in which the foci of ^{131}I uptake extended beyond the thyroid bed were classified as cases of persistent disease or metastases. These patients were given a higher therapeutic dose of 3.7–7.4 GBq (100–200 mCi) 3 to 6 months later. Hospital isolation was arranged for those who received doses exceeding 1.1 GBq, and a WBS was performed 2 weeks after administration of the higher therapeutic dose.

All patients were staged according to the UICC-TNM criteria (6th edition) [11]. Patients showing disease progression after the operation were classified into a residual cancer group or a relapse group. Fine-needle aspiration cytology (FNAC), ^{131}I WBS, or other noninvasive examination and elevated Tg levels were used to confirm the presence of local recurrence in the neck or distant metastases. The patients in the residual cancer group were diagnosed within a year of the first thyroidectomy, and those in the relapse group were diagnosed a year after the first thyroid surgery. At the end of 2010, patients were categorized as disease free if they showed negative results in the ^{131}I WBS, undetectable Tg levels without thyroxine treatment and a TSH level $\geq 30\,\mu$IU/mL, undetectable Tg antibody levels at the final followup, and no identifiable local or distant metastasis in the noninvasive examination. The study was carried out on humans in compliance with the Helsinki Declaration and following approval by ethics committee of the Institution Review Board in CGMH (reference number: 99-3565B).

All data are expressed as mean ± SE values. Univariate and multivariate analyses were performed to determine the significance of the various factors by using the Kaplan-Meier method and the log-rank test [12]. A P value < 0.05 was considered statistically significant. In addition, the survival rates were calculated using the Kaplan-Meier method and compared using the Breslow and Mantel-Cox tests.

3. Results

In the 435 subjects with papillary thyroid carcinoma, the mean age of the study population at enrollment was 57.0 ± 9.0 years. Male thyroid cancer patients were generally older than female patients, but no statistical difference was observed (57.9 ± 9.1 years versus 56.6 ± 8.9 years; $P = 0.1934$) (Table 1). Comparison of the mean tumor size in the two groups showed that tumors of a larger size were observed among males. In addition, the incidence of T3 and T4 based on the TNM classification was higher among males. Approximately 12.2% of the study population demonstrated pathologically proven lymph node metastases. There was a higher incidence of males in the N1b group, although no statistical significance was observed. Higher percentage of males was presented with distant metastases than females (10.8% versus 4.4%; $P = 0.0136$) at the time of diagnosis. Histological presentation at the time of thyroidectomy including TNM stage, multicentric pattern, postoperative Tg, and remnant ^{131}I uptake percentage showed no differences between the genders.

During the followup period, 49 (40.8%) male and 65 (20.6%) female patients had residual or relapsed. Of this group, 52.6% (60 of 114) were diagnosed within the first year after thyroidectomy. Higher percentage of residual groups was diagnosed in males (28 of 49; 57.1%) than in females (32 of 65; 49.2%). Fifty-four patients (33 males and 21 females) had relapsed one year after the operation. The mean duration of relapse after the operation was 4.3 ± 0.4 years. A significantly higher relapse frequency was observed among male patients as compared to females ($P = 0.0001$). Approximately 27.8% of enrolled patients were classified as disease free during followup, with the male group showing a lower frequency than the females, although without statistical significance ($P = 0.1267$). Multiple regression analysis of clinical factors for postoperative progression showed that male gender, postoperative Tg and TNM stage are independent risk factors (Table 2).

After the mean followup period of 7.2 ± 0.3 years, 55 (12.6%) patients had died of thyroid cancer. The male population showed a higher thyroid cancer mortality than the female population (24.2% versus 8.3%; $P = 0.0001$). Figure 1(a) shows the cancer-specific survival curves of the male, female, and total groups. The thyroid cancer-specific survival rates in the male, female, and total groups were 86.9%, 93.6%, and 92.3% at 5 years; 72.4%, 91.3%, and 86.1% at 10 years; and 47.2%, 71.2%, and 64.3% at 20 years, respectively. The recurrence-free rates for the male, female, and total groups are 58.9%, 81.5%, and 75.3% at 5 years; 50.1%, 73.9%, and 67.6% at 10 years; and 46.3%, 72.7%, and 65.2% at 20 years, respectively (Figure 1(b)). In addition, multiple regression analysis showed that male gender and TNM stage are independent risk factors in multiple regression analysis for thyroid cancer specific mortality (Table 3).

4. Discussion

The development of thyroid cancer involves multiple stages and genetic mutations, transforming normal follicular epithelial cells to differentiated malignant cancer cells [13]. During the processes of tumor initiation and progression, it has been reported that sex hormones may influence the rates of cancer cell proliferation, migration, or apoptotic change [14, 15].

According to prevalence studies, females have a higher incidence rate of thyroid nodules and surgical treatment than males [16, 17]. In contrast, the incidence of thyroid cancer in nodules is significantly higher in males. Because of differences in screening rates, gender-specific behavior, such as the greater tendency of men to seek medical attention later than females, and surgical methods, the therapeutic outcome varies between genders. [2, 6]. Previous study illustrated papillary thyroid cancer in females diagnosed at an earlier age than in males [18]. In contrast, there was no statistical difference of age between genders in this investigation. The main reason was the study exclude the patients in TNM stage I. Age has been used as an important prognostic factor in

Table 1: Clinical features of papillary thyroid carcinoma by gender.

	Female ($n = 315$)	Male ($n = 120$)	Total ($n = 435$)	P value
Age	56.6 ± 8.9	57.9 ± 9.1	57.0 ± 9.0	0.1934
TNM stage (II/III/IV)	90/60/165	27/27/66	117/87/231	
T1*	128 (31.0%)	26 (19.0%)	154 (28.0%)	
T2	102 (24.7%)	35 (25.5%)	137 (24.9%)	0.4023
T3	39 (9.4%)	20 (14.6%)	59 (10.7%)	
T4	144 (34.9%)	56 (40.9%)	200 (36.4)	
Tumor size (cm)	3.0 ± 0.1	3.7 ± 0.2	3.2 ± 0.1	0.0001
LN metastases	38 (12.1%)	15 (12.5%)	53 (12.2%)	
N1a	13 (41.9%)	3 (21.4%)	16 (35.6%)	0.1834
N1b	18 (58.1%)	11 (78.6%)	29 (64.4%)	
Soft-tissue invasion	154 (48.9%)	51 (42.5%)	205 (47.1%)	0.2328
Distant metastases	14 (4.4%)	13 (10.8%)	27 (6.2%)	0.0136
Multicentric	92 (29.2%)	30 (25.0%)	122 (28.0%)	0.3827
Aggressive histological patterns#	8 (2.5%)	7 (5.8%)	15 (3.4%)	0.1366
^{131}I dose accumulative dose (mCi)	152 ± 10.7	227 ± 27.9	173 ± 11.0	0.0023
Postoperative one month ^{131}I uptake (%)	6.2 ± 0.8	4.9 ± 0.7	5.9 ± 0.6	0.3018
Postoperative progression	65 (20.6%)	49 (40.8%)	114 (26.2%)	0.0001
Relapse/Residual	33/32	21/28	54/60	0.4023
Period of relapse from diagnosis (year)	4.4 ± 0.7	4.1 ± 0.6	4.3 ± 0.4	0.7132
Disease free	94 (29.8%)	27 (22.5%)	121 (27.8%)	0.1267
Followup period (yr)	7.3 ± 0.3	6.8 ± 0.5	7.2 ± 0.3	0.3501
Cancer mortality	26 (8.3%)	29 (24.2%)	55 (12.6%)	0.0001

T1*: all the patients with local invasion or distant metastases.
Aggressive histological patterns#: tall cell, insular pattern, column cell, and poorly differentiated thyroid cancers.

Table 2: Multiple regression analysis for factors associated with postoperative progression.

	SE	Standardized coefficient	t value	P value	95% confidence interval	
					Lower	Upper
Intercept	0.2438	−0.677	−2.724	0.007	−1.167	−0.186
Age	0.004	0.10	1.533	0.127	−0.002	0.012
Followup period	0.005	−0.049	−0.711	0.478	−0.014	0.006
Postoperative one month ^{131}I uptake	0.004	0.119	1.772	0.078	−0.001	0.014
Gender: female/male	0.068	0.251	3.672	0.000	0.115	0.383
Postoperative one month Tg	0.001	0.206	2.907	0.004	0.000	0.002
TNM stage	0.035	0.175	2.576	0.011	0.021	0.157

Dependent variable: postoperative progression (no/yes).

thyroid cancer, and this gender-specific variation thus makes survival analysis between the genders difficult. This study confirmed that based on a specific age group, TNM stage, and therapeutic modality, male gender is associated with a higher relapse rate and cancer-specific mortality.

Most patients with well-differentiated thyroid cancers have undergone long-term followup and treatment with good prognoses. It is important to minimize the risk of recurrence. Male gender has been reported to be an independent risk factor of cancer recurrence [5, 19]. In this study, consistent surgical and postoperative ^{131}I therapy for papillary thyroid cancer in the same institute was associated with a higher recurrence rate. The frequency of male patients in the N1b group was shown to be higher than that of female patients. The incidence of micrometastases in the central neck region in patients staged as N0 by preoperative and intraoperative was recently assessed in papillary thyroid carcinoma [20]. Male gender was shown to be associated with lymph node micrometastasis. In addition, using univariate analysis, a significant correlation was shown

Table 3: Multiple regression analysis for factors associated with cancer mortality.

	Standardized coefficient	SE	t value	P value	95% confidence interval	
					Lower	Upper
Intercept	−0.594	0.194	−3.059	0.003	−0.978	−0.211
Age	0.104	0.003	1.405	0.162	−0.002	0.009
Followup period	−0.023	0.004	−0.322	0.748	−0.009	0.0067
Postoperative one month ^{131}I uptake	0.055	0.003	0.784	0.434	−0.003	0.008
Gender: female/male	0.344	0.053	4.829	0.000	0.152	0.361
Postoperative one month Tg	−0.051	0.001	−0.688	0.434	0.000	0.001
TNM stage	0.140	0.027	1.98	0.049	0.002	0.107

Dependent variable: cancer mortality (yes/no).

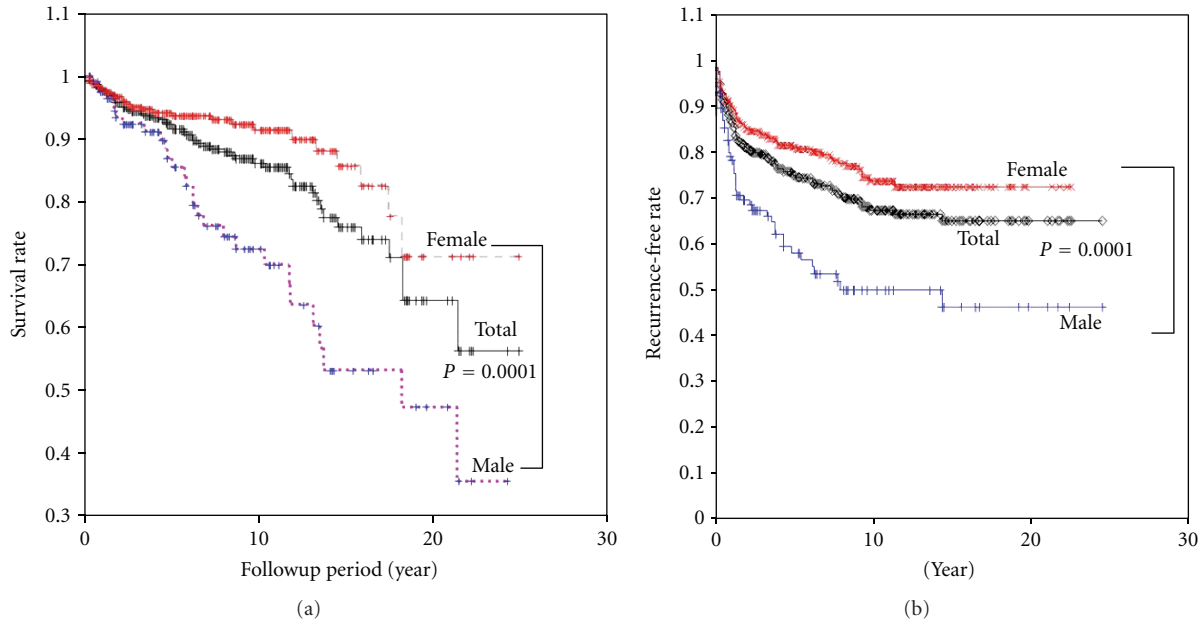

Figure 1: Thyroid cancer mortality (a) and recurrence-free survival (b) curves of the male, female, and total groups.

between male gender and lung metastasis in papillary thyroid carcinoma presenting with bilateral lateral cervical lymph node metastasis [21]. In this study, prophylactic lymph node dissection was not performed on any of the patients. The role of prophylactic lymph node dissection or sentinel node biopsy among male patients at TNM stages II to IV need to be further investigated [22].

Recently published guidelines concerning high-risk cases of papillary and follicular thyroid carcinomas do not consider gender as a risk factor [23, 24]. More aggressive postoperative modalities, such as higher doses of ^{131}I for thyroid remnant ablation and treatment for distant metastases, and closely monitored followup imaging after serum Tg level elevation need to be considered. Those with a histologic pattern of tall cell, insular pattern, and poorly differentiated thyroid cancer have a poorer prognosis, compared with those with classical, well-differentiated thyroid cancer [25, 26]. Our results illustrated that male patients had higher percentage of aggressive histologic patterns, otherwise no statistical difference. A number of limitations are also identified in this study. The data was selected from a single institution, and this is unlikely to represent the real prevalence of thyroid cancer in Taiwan. This study spanned more than 20 years, with different surgeons performing different surgical procedures. Similarly, the dose used in ^{131}I therapy for papillary thyroid cancer varied among endocrinologists. In conclusion, this study demonstrated higher mortality and recurrence rates in males with papillary thyroid cancer in TNM stages II to IV, suggesting the need for more aggressive surgical treatment and postoperative ^{131}I therapy for this specific group of patients.

Conflict of Interests

There is non-financial competing interest including political, personal, religious, ideological, academic, intellectual, commercial, or any other to declare in relation to this paper.

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