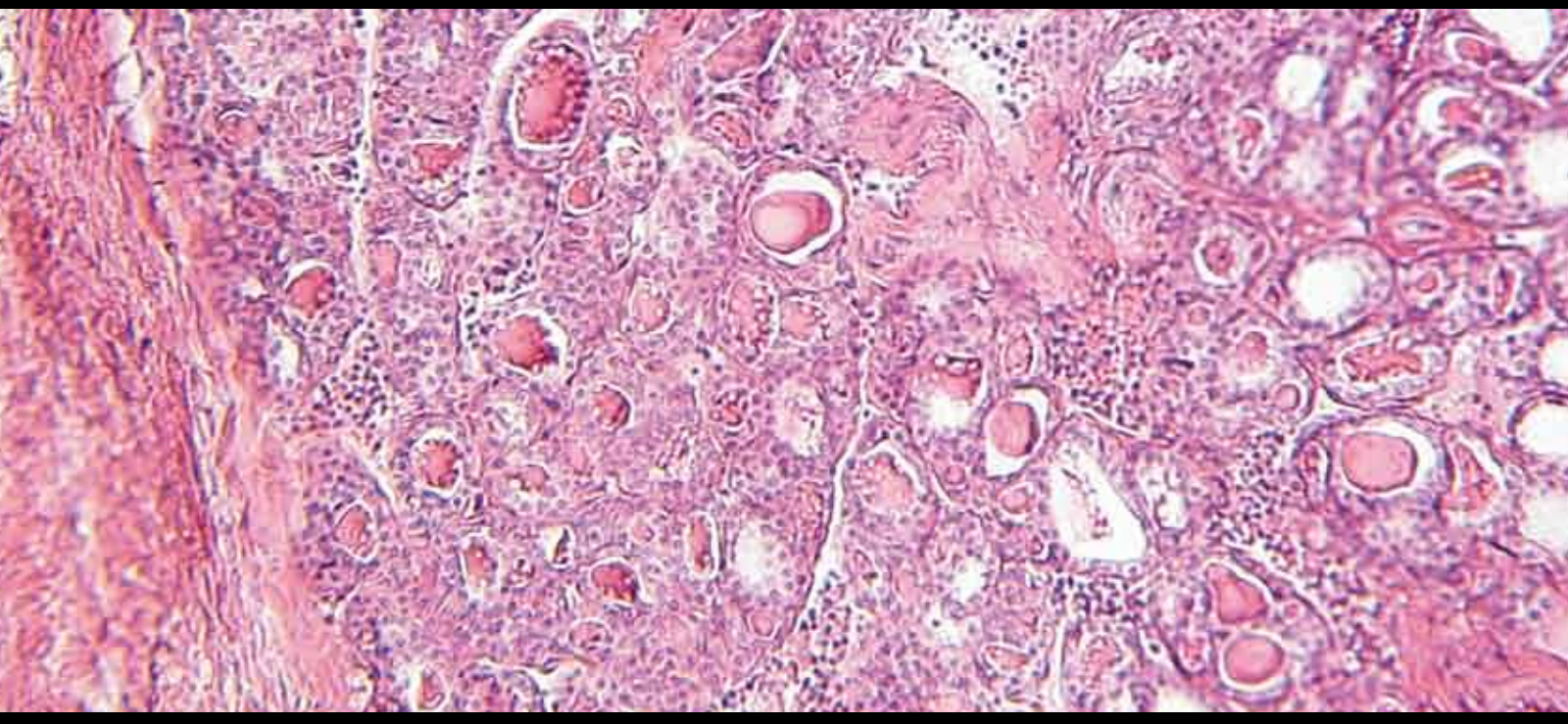
Journal of Thyroid Research

Thyroid Function and Growth Regulation under Normal and Abnormal Conditions

Guest Editors: Guillermo Juvenal, Daniel Christophe, Pierre Roger, and Mario Pisarev



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Editorial Board

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Editorial

Thyroid Function and Growth Regulation under Normal and Abnormal Conditions

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The thyroid gland exerts a major control on the physiology of the whole body, and it raises fundamental questions about molecular physiological processes. Moreover, an increased proliferation of thyroid cells is associated with several pathologies, and several mechanisms may be involved. Thyroid disorders are very common, affecting millions of people. These include hypothyroidism, hyperthyroidism, thyroid nodules, thyroid cancer, and so forth, but they are also associated with other nonthyroid disorders. This special issue is devoted to illustrate the particular richness of current investigations in the field of thyroid function and growth regulation under normal and abnormal conditions.

Although thyroid gland function is mainly under the control of pituitary TSH in normal conditions, other factors may also play a role in this process. Thyroid disease is more common in women than in men. Tania Weber Furlanetto and Ana Paula Santin reviewed the direct effects of estrogens on thyroid function and growth regulation in the paper titled "*Role of estrogen in thyroid function and growth regulation.*"

Thyroid cancer is the most common endocrine malignancy, and its incidence has significantly risen in the last decades in the world. The knowledge how thyroid cancer develops is expanding rapidly. The sequential acquisition of mutations which arise as a consequence of damage to the genome is required in order to transform a normal cell into a malignant one. The understanding of the process of thyroid carcinogenesis at the molecular level will improve not only the diagnostic but also the treatment of this pathology.

Ioannis Legakis and Konstantinos Syrigos describe the molecular events associated with the progression and dedifferentiation of thyroid carcinoma in the paper titled "*Recent advances in molecular diagnosis of thyroid cancer.*" Thyroid-specific transcription factors regulate thyroid-specific gene expression and organogenesis. Their possible role in thyroid cancer as well as in the maintenance and/or activity of stem cells is discussed by Shioko Kimura in the paper titled "*Thyroid-specific transcription factors and their roles in thyroid cancer.*" MicroRNAs (miRNAs) are short ribonucleic acid molecules (around 22 nucleotides) found in eukaryotic cells. miRNAs are posttranscriptional regulators that bind to complementary sequences on messenger RNA transcripts inducing the translational repression or messenger RNA degradation. Several miRNAs have been found to have links with some types of cancer. Francesca Marini, Ettore Luzi, and Maria Luisa Brandi reviewed the role of miRNAs in thyroid cancer development in the paper titled "*MicroRNA role in thyroid cancer development.*" Growth factors play a role in thyroid proliferation and function, while EGF acts as a mitogen for thyroid cells inhibiting also thyroid differentiation. TGF-β is a potent inhibitor of thyroid cell growth. However, in some transformed thyroid cells this inhibition is lost. The role of TGF-β and EGF on thyroid carcinogenesis and the crosstalk between these growth factors are discussed by Gabriella Mincione Maria Carmela Di Marcantonio, Chiara Tarantelli, Sonia D'Inzeo, Arianna Nicolussi, Francesco Nardi, Caterina Francesca Donini, and Anna Coppa in the paper titled "*EGF and TGF-β1 effects on thyroid function.*"

Grave's disease and Hashimoto's thyroiditis are the two main types of autoimmune thyroid disease. Occasionally they are also associated with other autoimmune diseases. Emina Kasumagic-Halilovic, Asja Prohic, Begler Begovic, and Nermina Ovcina-Kurtovic bring additional support to the existence of a significant association between vitiligo and thyroid autoimmunity in the paper titled "*Association between vitiligo and thyroid autoimmunity.*"

Although some authors have found an association between an abnormal thyroid condition and bipolar disorder, little is known about the implication of the hypothalamo-pituitary-thyroid in neuropsychological deficits. Subho Chakrabarti review the last findings on this topic including genetic and neuroimaging investigations (*Thyroid Functions and Bipolar Affective Disorder*).

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Review Article

Thyroid Functions and Bipolar Affective Disorder

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Accumulating evidence suggests that hypothalamo-pituitary-thyroid (HPT) axis dysfunction is relevant to the pathophysiology and clinical course of bipolar affective disorder. Hypothyroidism, either overt or more commonly subclinical, appears to the commonest abnormality found in bipolar disorder. The prevalence of thyroid dysfunction is also likely to be greater among patients with rapid cycling and other refractory forms of the disorder. Lithium-treatment has potent antithyroid effects and can induce hypothyroidism or exacerbate a preexisting hypothyroid state. Even minor perturbations of the HPT axis may affect the outcome of bipolar disorder, necessitating careful monitoring of thyroid functions of patients on treatment. Supplementation with high dose thyroxine can be considered in some patients with treatment-refractory bipolar disorder. Neurotransmitter, neuroimaging, and genetic studies have begun to provide clues, which could lead to an improved understanding of the thyroid-bipolar disorder connection, and more optimal ways of managing this potentially disabling condition.

1. Introduction

The association between thyroid functions and behavioural disturbances has been known for the last several hundred years. Although the effects of thyroid hormones on the developing brain were recognised long ago, recent advances in biotechnology have led to an improved understanding of the impact of thyroid functions on the adult, mature brain [1]. This development has been particularly helpful in elucidating the role of thyroid hormones in the pathophysiology of psychiatric disorders, especially mood disorders. The primary focus of interest has been on the connection between thyroid functions and depressive disorders. However, abnormalities of thyroid functions may also play an important role in the pathophysiology of bipolar affective disorder, but this area has received much less attention than it probably deserves.

This paper attempts to explore the links between thyroid hormone physiology and the presentation and pathogenesis of bipolar disorder. It briefly covers several areas of overlap, beginning with the association of bipolar disorders with thyroid disease among clinical and epidemiological populations, as well as the evidence of hypothalamo-pituitary-thyroid (HPT) axis abnormalities among patients with bipolar disorder. Rapid cycling and other refractory forms of bipolar disorder have been particularly highlighted, since the prevalence of thyroid dysfunction appears to be greater in such forms of the disorder. The research relating to the widespread and potent antithyroid effects of lithium carbonate, the drug most commonly used for treating bipolar disorder, has been summarised next. The role of thyroid hormones in the treatment of bipolar disorder is also reviewed. Although the evidence supporting the use of adjunctive thyroid hormone treatment of bipolar disorder is somewhat meagre, such strategies may be useful in a subset of patients with chronic and refractory forms of bipolar disorder. Despite rapid strides made in uncovering cellular and molecular mechanisms of actions of thyroid hormones, the specific neurobiological processes that underlie the modulatory effect of thyroid hormones in mood disorders are far from clear. Animal studies have provided considerable data on the reciprocal interactions between thyroid hormones and neurotransmitter systems believed to play a role in genesis of mood disorders [2]. These studies provide the basis for several hypotheses (included in this paper), which propose that the modulatory effects of thyroid hormones on mood are mediated by their actions on different neurotransmitter systems. A brief mention has also been made of genetic and neuroimaging investigations that are beginning to attract considerable attention, since they can offer vital clues to the

link between thyroid dysfunction and bipolar disorder. The paper ends with a discussion of the pertinent methodological issues and suggestions for future research, which can enhance our understanding of the thyroid-bipolar disorder link.

2. The HPT Axis

The organization and regulation of the HPT system has been extensively reviewed elsewhere [1–8]. Hence, only the relevant aspects are described here. The thyroid gland is the largest endocrine organ in the human body. The thyroid regulates cellular activity by releasing two different hormones, the prohormone thyroxine (T4) and the biologically active triiodothyronine (T3). The HPT system has a hierarchical structure similar to that of the hypothalamo-pituitary adrenal axis, with the thyrotropin-releasing hormone (TRH) as the hypothalamic master hormone. TRH is released from nerve endings in the median eminence; from here it enters the anterior pituitary through the portal system. In the pituitary, TRH induces synthesis and release of thyrotropin or the thyroid-stimulating hormone (TSH), from thyrotrophs. TSH enters the circulation and acts on the thyroid gland causing release of T3 and T4.

All T4 comes from the thyroid, but, under usual circumstances, only about 20% of T3 is derived from the gland. The remaining 80% comes from the removal of iodine from the T4 molecule by enzymes called deiodinases. Type-II deiodinase converts T4 to T3. This enzyme is located mostly in glial cells of various regions of the brain, principally the cortical areas and the anterior pituitary. The activity of type II deiodinase is primarily responsible for regulating brain T3 concentrations. The actions of thyroid hormones at the cellular level are initiated by the intracellular binding of T3 to nuclear thyroid hormone receptors. These receptors are widely distributed in the adult brain, with higher densities in phylogenetically younger brain regions (e.g., amygdala and hippocampus), and lower densities in the brain stem and cerebellum. The entry of T3 into the cell is mediated by two plasma membrane carriers, the monocarboxylate transporter and the organic anion-transporting polypeptide. After the coupling of T3 to nuclear receptors, the transcriptionally active complex binds to thyroid hormone-responsive elements located on thyroid hormone-responsive genes. This binding produces conformational changes in thyroid hormone responsive genes, which initiates a sequence of transcription of messenger ribonucleic acid, increased gene expression, and synthesis of proteins. Although the mechanisms of thyroid hormone effects on the brain are not fully known, they probably include genomic actions, an effect on neurotransmission directly at the synapse, and modulation of neurotransmitter systems and intracellular signalling pathways.

The HPT axis is regulated by several complex feedback mechanisms at all levels. Unbound or free T3 and T4 feed back at the level of the hypothalamus to inhibit TRH release, and at the anterior pituitary level to inhibit TSH release. Different neurotransmitters and hormones either promote or inhibit release of TRH and TSH. The HPT axis is also regulated by stress-responsive elements, which influence TRH levels, and by the circadian system's influence on TSH. At the level of brain, additional mechanisms such as circulating levels of T3 and T4, intracellular transport, and deiodinase activity regulate local concentrations of thyroid hormones. Consequently, levels of T3 within the brain are tightly controlled within narrow limits, even under adverse conditions [1–8].

3. Thyroid Disease and Bipolar Disorder

Neuropsychiatric symptoms, such as mood disturbances and cognitive impairment, are very common among patients with thyroid disorders.

Hyperthyroidism or thyrotoxicosis is usually associated with symptoms such as anxiety, depression, mood lability, and insomnia in a majority of the patients. However, overt psychiatric disorder is rare and occurs in only about 10% of the patients [1, 5]. Manic episodes have been known to occur in patients with hyperthyroidism, but are quite unusual [9]. Occasionally, patients with late-onset mania are detected to have hyperthyroidism, which requires to be treated to achieve full recovery [10]. Nevertheless, patients who develop a true manic episode while thyrotoxic, frequently have an underlying mood disorder, or a family history of mood disorder [11, 12]. Manic episodes can also result from the relatively uncommon phenomenon of lithium carbonate-associated thyrotoxicosis [9, 13]. Lithium may induce thyrotoxicosis by several mechanisms including triggering of the autoimmune process with resultant thyroiditis, abnormal iodine kinetics, that is, overflow of thyroid hormone after expansion of the intrathyroid iodine pool, Jod-Basedow-like phenomenon, direct toxicity to thyroid follicles resulting in release of thyroglobulin, and coincidental Graves' disease and hyperthyroidism [14–16].

Psychiatric symptoms in hyperthyroidism, such as anxiety or mania, appear to be mediated by beta-adrenergic hyperactivity. Accordingly, psychiatric symptoms and psychiatric disorders secondary to hyperthyroidism should be first treated by restoring the euthyroid state. Additional treatment with beta-adrenergic antagonists is also helpful. Antimanic agents are required only when symptoms fail to respond to these measures [8].

The most common psychiatric symptoms related to hypothyroidism are depression and cognitive dysfunction [1, 3, 8]. Only a few instances of mania or hypomania associated with hypothyroidism have been reported in the literature [17]. Underlying mechanisms are less clear; they could include dysregulation of CNS catecholamine receptor sensitivity, associated thyroditis and thyrotoxicosis, or a disruption of circadian rhythms [18]. A retrospective review based on 18 patients described an organic affective syndrome-manic type occurring shortly after the initiation of thyroid replacement in hypothyroid patients [19]. Patients experiencing mania were predominantly female, often had concurrent psychotic symptoms, frequently had a personal or familial history of psychiatric disorder, and had received more than 150 mcg/day of thyroxine. The authors suggested that rapid administration of thyroxine could abruptly augment catecholamine receptor sensitivity, thereby

precipitating a hypercatecholaminergic state and subsequent manic symptoms. Similar instances of T3-induced mania in patients with bipolar depression have also been reported [20]. It has been speculated that thyroid hormone-catecholamine receptor interactions might underlie these T3-associated clinical manifestations as well [20].

Even though thyroid disorders are associated with psychiatric symptoms in clinical populations, existence of a similar association in general population is less certain. On one hand, are the reports of a positive association between thyroid disease and mood disorders in some community studies. For example, a group of investigators at Copenhagen conducted prospective cohort studies utilising historical data from Danish case registers to determine the association between thyroid and affective disorders [21–23]. In separate reports, it was demonstrated that patients hospitalised with bipolar disorder tended to be at a greater risk of re-admission with hyperthyroidism than controls [21], while patients hospitalised with hyperthyroidism were at greater risk of readmission with depressive disorder or bipolar disorder than controls [22]. Finally, patients hospitalized with hypothyroidism also had a greater risk of readmission with depression or bipolar disorder, than control patients [23]. These reports thus provided strong epidemiological support for a link between thyroid disease and mood disorders, including bipolar disorder. Further evidence for this association came from two other studies. The first such study was based on analysis of a series of insurance claims for inpatient hospitalisation, physician office visits, and laboratory testing [24]. These data were used to estimate the risk of having a comorbid condition among patients with bipolar disorder. In this study, the risk of hypothyroidism among bipolar patients was twice that of those with no mental health disorders. Another multicentric study from France included 1090 patients with bipolar I disorder, 9% of whom had rapid cycling bipolar disorder (RCBD). Examination of comorbid medical conditions revealed that among the various physical disorders, only thyroid disorders were associated with rapid cycling [25]. On the other hand, quite a few other investigations of medical comorbidity among patients with bipolar disorder have not found a significant increase in the prevalence of thyroid disorders [26–28].

In conclusion, even though both hyperthyroidism and hypothyroidism are associated with changes in mood, overt bipolar disorder is uncommon in thyroid dysfunction. Moreover, data from community-based samples, in contrast to clinical samples, provide conflicting results regarding the association between thyroid diseases and bipolar disorder.

4. HPT Axis Dysfunction in Bipolar Disorder

Although HPT axis dysfunction appears to be equally relevant for the pathophysiology of bipolar disorder, as it is for depressive disorders, this subject has received far less attention from researchers. However, there is now growing evidence of all manner of thyroid abnormalities in patients with bipolar disorder, which often far exceed those found among patients with unipolar depression [1, 3, 5, 29, 30]. As discussed subsequently, thyroid dysfunction is particularly common in patients with the rapid cycling variant of bipolar disorder. However, the antithyroid action of mood-stabilisers, particularly lithium carbonate, frequently confounds the findings among patients with bipolar disorder. Accordingly, there is some uncertainty about the true extent of HPT abnormalities in bipolar disorder and the proportion of HPT dysfunction that can be attributed to lithium-treatment [1, 5, 29].

Overt hyperthyroidism is uncommon in bipolar disorder; its prevalence is no greater than 2% across different studies [13, 15, 31]. Much of this has been attributed to lithium [32], which can induce thyrotoxicosis by autoimmune mechanisms or thyroiditis [14–16]. A transient elevation of T4 or free T4 levels has often been noted among patients with mania shortly after hospitalization [33–36]. These levels gradually normalize after a few weeks of treatment, as patients achieve remission. There is some suggestion that elevated T4 levels following hospitalisation are positively associated with severity of symptoms, and that the rate of fall in these levels is linked to a better outcome [34, 36, 37]. However, this finding is not specific to mania, as transient mild elevations of free and total T4 ("euthyroid hyperthyroxinemia") have been commonly noted in acutely admitted psychiatric patients, including those with depression. This indicates that such elevations are more likely to be nonspecific effects of the stress of hospitalisation [3, 38]. Currently, the most diagnostically sensitive tests to detect thyroid dysfunction are the ultra-sensitive immunoradiometric assays of serum TSH [39]. However, prior to the development of highly specific and sensitive TSH assays, the TSH response to an intravenous dose of TSH was the most widely used test for detecting HPT dysfunction. The response is exaggerated in hypothyroidism and blunted in hyperthyroidism. A blunted TSH response occurs in 25–30% of patients with unipolar major depression [40]. However, blunted TSH responses to TRH may be far more common among patients with bipolar disorder, including those with mania [41, 42], bipolar depression [43, 44], and rapid cycling disorder [45]. Moreover, the severity of mood symptoms and milder fluctuations in these symptoms has been found to correlate with blunted TSH responses to TRH [46]. On the other hand, many patients with bipolar disorder may show an exaggerated response of TSH to TRH [47]. This is often associated with elevated basal serum TSH levels; approximately 20% of the patients have levels above the upper normal reference range [48, 49]. Exaggerated TSH responses, along with elevated basal levels of TSH, have also been noted among patients with rapid cycling and are consistent with the high prevalence of subclinical hypothyroidism often found in this condition [3, 29]. Gyulai et al. [50] found that patients with RCBD did not differ from controls on any of thyroid function tests prior to treatment with lithium. However, after 4 weeks of lithium-treatment, exaggerated TSH responses to TRH were significantly more common among such patients. They, thus, proposed that RCBD is associated with a latent hypofunction of the HPT system, which becomes manifest with lithium treatment. Given lithium's antithyroid actions, it is not surprising that an exaggerated TSH response

to TRH stimulation is extremely common and has been reported in 50–100% of lithium-treated patients [51]. Then again, evidence of overt or subclinical hypothyroidism, including raised antibody titres, has often been found among patients with bipolar disorder, prior to treatment with lithium [31, 52, 53]. Accordingly, it appears that, at least in a subgroup of patients with bipolar disorder, treatment with lithium, rather than inducing hypothyroidism, actually exacerbates a preexisting (overt) HPT dysfunction [32].

In summary, HPT axis abnormalities are quite common among patients with bipolar disorder. However, there are several concerns regarding the specificity of these abnormalities, and the effect of lithium in inducing HPT dysfunction in bipolar disorder.

5. HPT Axis Dysfunction in Rapid Cycling Bipolar Disorder and Mixed Affective States

Rapid cycling usually affects about 9 to 20% of all patients with bipolar disorder [25, 54–56]. This subpopulation is characterized by more severe morbidity and a refractory clinical course. More women, than men, suffer from rapid cycling [54, 55].

Of all the potential risk factors for rapid cycling, hypothyroidism has received the most attention. All categories of HPT axis dysfunctions have been reported in RCBD. These have included overt hypothyroidism [29, 57–59], elevated TSH levels [58, 60–62], exaggerated TSH responses to TRH [62], elevated antibody titres [63], and antidepressant-induced rapid cycling [41, 58]. However, methodological problems such as retrospective designs, lack of controls, predominance of female subjects, and varying definitions of hypothyroidism have all hindered any consistent conclusions from these data [50]. Moreover, a number of other studies have been unable to document this association [45, 52, 53, 64–68], promoting considerable scepticism about the presence of HPT axis abnormalities in RCBD [54, 55, 68, 69]. More pertinently, many of the studies reporting a positive association have included patients being treated with lithium. Lithium treatment clearly contributes to the development of hypothyroidism among patients with rapid cycling [50, 70]. In this regard, the study by Gyulai et al. [50] is of some significance. Their contention that RCBD is associated with a latent hypofunction of the HPT system, which becomes manifest with short-term lithium challenge, remains a possibility. (The wide ranging anti-thyroid effects of lithium are described in the next section.) A latent hypofunction of the thyroid axis in RCBD may also explain why high doses of T4 added to the established treatment with lithium and other psychotropic drugs can reverse the rapid cycling pattern [1, 5].

Mixed affective states have also been associated with reduced thyroid functioning in certain studies. In a study of first-episode manic and mixed types of bipolar disorder, 33% of the patients in mixed episodes had elevated TSH levels, in comparison with 7% of patients experiencing pure mania [71]. In similar vein, Chang et al. [72] found significantly lower T4 levels and elevated TSH levels in patients with mixed mania, compared with those with pure mania. These differences were not associated with exposure to lithium. Others have reported that patients with mixed states have a higher rate of positive anti-thyroid antibody titres, than other unipolar or bipolar subgroups, apparently unrelated to lithium treatment [73, 74]. However, not all studies have been able to confirm the association of overt or subclinical thyroid dysfunction with mixed manic episodes [75, 76]. Thus, the question of HPT dysfunction in mixed affective states remains an unresolved one [69].

To conclude, the prevalence of HPT dysfunction is very high among patients with RCBD. Despite concerns about methodology, contrary findings and confounding effects of lithium-treatment, the existence of a latent thyroid dysfunction in RCBD, which is exacerbated by lithium, remains a possibility. In contrast, the evidence linking HPT dysfunction and mixed affective states is inadequate and inconsistent.

6. Lithium and HPT Axis Dysfunction

The anti-thyroid effects of lithium carbonate are well documented [51, 77, 78]. The mechanisms by which lithium can cause hypothyroidism are complex. Lithium is concentrated by the thyroid gland and inhibits thyroidal iodine uptake. It also inhibits iodotyrosine coupling, alters thyroglobulin structure, inhibits thyroid hormone secretion [51], and interferes with the deiodination of T4 to T3 by inhibiting type-II deiodinase in the brain [79]. Lithium may evoke an exaggerated TSH response to TRH [51]. The drug may have an immunostimulant effect, either by inducing, or by exacerbating a preexisting autoimmune disease [32, 80]. Additionally, lithium alters cellular responsiveness to thyroxine, and influences thyroid hormone receptor gene expression [81]. Inhibition of thyroid hormone release, a process mediated by cyclic adenosine monophosphate, appears to be the critical mechanism in the development of lithium-induced hypothyroidism [32]. Compensatory mechanisms may operate to prevent the development of hypothyroidism or goitre in the majority of patients with lithium-induced impairments in thyroxine secretion. However, when additional risk factors such as iodine deficiency, preexisting autoimmunity, or genetic vulnerability are present, such compensatory mechanisms fail and hypothyroidism eventually ensues [32].

Rates of overt hypothyroidism vary from 0 to 47% (average of about 10%) among patients on long-term treatment with lithium [32, 80, 82, 83]. Differences in study design, definitions of hypothyroidism, age, gender, and geographical origin of patients, are often responsible for such wide variations in rates. Nevertheless, both the incidence and prevalence of overt hypothyroidism is significantly higher among patients on lithium, compared to general population figures [32]. The average duration of lithium therapy before the diagnosis of hypothyroidism is around 18 months [83], though there are a few reports of hypothyroidism occurring within the first few months of lithium-treatment [84, 85]. Female gender, middle age (>50 years), preexisting autoimmunity, and family history of thyroid diseases are established

risk factors for lithium-induced hypothyroidism [82, 83, 86, 87]. An even larger number of patients appear to develop subclinical hypothyroidism. Low or normal T4 levels and elevated TSH levels are reported among 5 to 35% (average of about 25%) patients on lithium [88], while exaggerated TSH responses are found among 50%, or more, of such patients [51, 89]. Cross-sectional studies of lithium-induced goitre reveal a prevalence of 0 to 60% [51]; prevalence estimates are much higher (30–59%) when more sensitive ultrasonographic scans are used to detect increases in thyroid volumes [32, 87, 90]. Several studies of patients on lithium have found an elevated rate of anti-thyroid antibodies, ranging from about 8 to 49% in such patients; these rates were significantly higher than those among control patients or the general population [84, 89, 91–100]. An almost equal number of studies have failed to find an association between elevated antibody titres and exposure to lithium [32, 53, 73, 74, 101–104]. Studies are similarly inconsistent as to whether thyroid antibodies are elevated in bipolar disorder, unrelated to lithium-treatment; reported rates range from 0 to 43% among patients with bipolar disorder not on lithium. Some controlled comparisons have reported a higher prevalence of thyroid antibodies in bipolar disorder [73, 74, 105], especially in depressed and mixed states [73, 74], and RCBD [63]. In the largest study of this kind, Kupka et al. [53] found thyroperoxidase antibodies in 64 of 226 (28%) outpatients with bipolar disorder, a rate higher than general population subjects and patients with other psychiatric disorders. The presence of anti-thyroid antibodies among patients with bipolar disorder was associated with thyroid failure, but not with age, gender, mood state, rapid cycling, or lithium exposure [53]. However, several other controlled studies have not been able to find a higher prevalence of raised antibody tires in bipolar disorder, unrelated to lithium treatment [64, 103, 106].

To summarise, anti-thyroid actions of lithium are diverse and complex. Rates of overt and subclinical hypothyroidism, goitre, and raised antibody titres all appear to be significantly higher among patients on lithium, compared to the general population and nonbipolar controls. However, prospective studies have shown that elevated rates of thyroid hypofunction continue to decline with continued lithium treatment. Consequently, the prevalence of lithium-induced hypothyroidism begins to approximate rates among general population, after several years of lithium treatment, even in the absence of thyroid supplementation [32, 88]. The development of hypothyroidism is, thus, not a contraindication to continuing lithium. Thyroxine supplementation is recommended instead, even though there is considerable disagreement about when T4 should be added. Nevertheless, overt hypothyroidism, significantly enlarged thyroid volumes, clear evidence of subclinical pathology, and the presence of rapid cycling or treatment-resistance are unequivocal indications for T4 supplementation. Baseline tests of thyroid functions and size, and similar annual examinations are essential for all patients on lithium. Closer monitoring is recommended when risk factors such as female gender, middle age, autoimmunity, or positive family history are present [3, 32, 88, 107, 108].

7. HPT Axis Dysfunction and Outcome of Bipolar Disorder

Regardless of the controversies about the nature and extent of HPT axis dysfunction in bipolar disorder, there is substantial evidence that even minor perturbations of thyroid function play a significant role in the clinical course, treatment response, and outcome of bipolar disorder. For example, studies have shown that among patients with bipolar depression, a relatively elevated free T4 index in men was associated with a faster response to antidepressants and a shorter length of hospital-stay [109], while lower free T4 values and higher TSH values were significantly associated with a poorer response during the initial phase of treatment [110]. A similar relationship between T3 and T4 levels and short-term outcome of mania has also been demonstrated [33, 36]. Moreover, the long-term efficacy of lithium prophylaxis also seems to be determined by alterations in the HPT axis. Higher T3 levels were found to predict better response to lithium, and lesser likelihood of depressive recurrences during the first few years of lithium treatment in a couple of studies [111, 112]. Additionally, Frye et al. [85] reported that a lower mean serum level of free T4 was associated with more affective episodes and greater severity of depression during the first year of lithium-treatment. More recently, a retrospective analysis has shown that lithium-treated subjects who required an intervention for a depressive episode had significantly increased mean TSH levels, in comparison to lithium-treated subjects who did not require any intervention for depression [113].

In conclusion, several HPT axis abnormalities, which may have an important bearing on outcome, have been documented during acute-phase treatment of bipolar disorder. Similar findings during maintenance-phase treatment with lithium are consistent with the well-known anti-thyroid effects of lithium. Therefore, lithium-induced changes in thyroid function, even within the normal range, are detrimental to its prophylactic efficacy, especially with regard to depressive symptoms [85, 110, 113]. The presence of HPT dysfunction during lithium-treatment further underlines the need for regular monitoring of thyroid functions and rapid correction of any abnormalities that arise during such treatment. It may also explain why T4 supplementation can enhance treatment-response in some patients with refractory mood disorders on lithium treatment.

8. Thyroid Hormone Supplementation in Bipolar Disorders

The use of synthetic thyroid hormones T3 and T4 as supplementary agents in affective illness has a long history, with the first reports appearing in the late 1960s [1, 48]. However, the bulk of the studies have been carried out among patients with depression, where mostly T3, and occasionally T4, have been used to accelerate or augment antidepressant treatment. Among patients with bipolar disorder, supraphysiological doses of T4 have been used to supplement prophylactic efficacy of mood stabilizing treatments and

to augment antidepressant treatment in patients with treatment-refractory bipolar depression.

Stancer and Persad [114] were the first to report the effects of supraphysiological doses of T4 used as the sole prophylactic agent in RCBD. Such treatment was only partially successful, with cessation of cycling in five of the eight women included in their study, but not in the two men. This study was followed by case reports which suggested that addition of supraphysiological doses of T4 to mood stabilizing treatments was more likely to prevent rapid cycling [115]. Bauer and Whybrow [116] conducted the first open-label trial of adjunctive supraphysiological doses of T4 in 11 patients with treatment-refractory RCBD. Adjunctive treatment with T4 reduced the severity of manic and the depressive phases in both amplitude and frequency, and even led to complete remission in some patients. Of the four patients who subsequently underwent single- or double-blind placebo substitution, three relapsed. In responders, supranormal circulating levels of free thyroxine were necessary to induce a clinical response. Side effects were minimal, and there were no signs or symptoms of thyrotoxicosis. Subsequently, other open-label studies found adjunctive treatment with supraphysiological doses of L-T4 to be effective in the maintenance treatment of patients with severe rapid cycling or resistant bipolar disorder, who did not respond to standard measures [117, 118]. Thyroxine was used in doses of 250–500 mcg/day in these studies; the goal was to achieve TSH suppression by increasing free T4 levels by $\geq$50% of pretreatment levels. Despite concerns about adverse effects, the treatment was rated favourably by recipients and was well tolerated [119]. There was little evidence of cardiovascular side effects [116]. Moreover, the risk of bone demineralisation was not increased among women, even after several years of treatment [120–122].

In a separate set of open trials, supraphysiological doses of T4 were used to augment antidepressant treatment among treatment-resistant patients with bipolar depression [122–124]. Augmentation of antidepressants with high dose T4 had a beneficial effect on depressive symptoms in this group of refractory patients as well. The treatment was well tolerated, the rise in T3 and T4 levels was minimal, and no complications were reported [124, 125]. This pattern of response was significantly different from healthy controls administered thyroxine [125]. Two of the more recent studies have attempted thyroid hormone augmentation of patients with refractory bipolar depression using slightly different strategies. Łojko et al. [126] found addition of moderate doses of T4 (100 mcg/day) to be a successful augmentation strategy in female patients with bipolar depression, who had had an unsatisfactory response to serotonergic antidepressants. Another retrospective chart review of 125 patients with treatment-resistant bipolar depression showed augmentation with high dose T3 to be highly effective, though there were some concerns about adverse effects of this treatment [127].

The mechanisms underlying successful treatment with adjunctive T4 are as yet unclear. Earlier, it was suggested that adjunctive T4 counteracts the effects of subclinical hypothyroidism on neuronal adaptation [4, 5]. However, contrary to this notion, most patients who responded had normal thyroid functions [123]. This has led to several alternative hypotheses, such as correction of peripheral resistance to thyroid hormones, correction of isolated CNS hypothyroidism, and positive modulation of cathecholaminergic systems by T4, being responsible for this beneficial effect [123].

To summarise, there is some evidence favouring the usefulness of T4 supplementation of mood stabilising treatments in a subset of patients with chronic and refractory forms of bipolar disorder. However, such evidence is still meagre. There are no randomised controlled trials, and the total number of patients included in existing studies is too small. Therefore, this strategy can only be considered as a treatment of last resort in patients who have failed to respond to all other measures.

When augmentation is attempted, thyroxine is usually started at 50–100 mcg/day and increased by 25–50 mcg per week, to a maximum of 500 mcg per day. Response to treatment is usually evident within the first 2 weeks. Treatment is continued in responders for a few months. In nonresponders, T4 is tapered off gradually, since abrupt discontinuation can result in iatrogenic hypothyroidism. Most side effects can be avoided to a great extent by gradually building up the dose, adjusting it carefully, and monitoring the patient closely. Special precautions are required in those with endocrine or cardiovascular disorders. Administration during pregnancy is not recommended. A careful lookout should also be kept for the drugs being abused for their weight reduction effects [1, 3–5, 8].

9. HPT Axis Dysfunction and Bipolar Disorder: Underlying Neurobiological Mechanisms

The mechanisms, by which thyroid dysfunction produces mood symptoms, as well as those involved in amelioration of mood symptoms by thyroid hormones, remain to be more fully elaborated and understood. However, studies involving neurotransmitter functions, genetics, and neuroimaging have uncovered some of the cellular and molecular processes, which may explain the link between HPT axis dysfunction and mood disorders.

9.1. Neurotransmitter Systems. The role of several neurotransmitter systems including norepinephrine (NE), serotonin(5-HT), dopamine (DA), and gamma aminobutyric acid (GABA) in the pathogenesis of mood disorders is now reasonably well established [128–130]. Interactions between thyroid hormones and these neurotransmitter systems may not only account for the psychiatric symptoms accompanying thyroid disease, but also for the HPT dysfunction in mood disorders, and the therapeutic actions of thyroid hormones in mood disorders [1, 2, 5, 48, 49]. There are several similarities between the HPT and neurotransmitter systems, which endorse the possibility of mutual interactions. Firstly, because of their common biosynthetic precursor tyrosine, thyroid hormones (especially T3) are structurally similar to NE and DA [131]. Moreover, both systems are present in

key brain regions. Thyroid hormone receptors are widely distributed in the brain; many of the limbic system structures where these receptors are present have been implicated in the pathogenesis of mood disorders. The neurotransmitter systems originate in the brainstem and extend through the midbrain into the limbic regions and the cortex. They regulate mood by modulating the activity of these brain areas [2, 5]. Finally, components of both systems appear to coexist at the tissue level. Immunohistochemical mapping studies have shown that T3 is concentrated in the nuclei and projection sites of central noradrenergic systems [132], while the thyroid gland exhibits GABA transport mechanisms, as well as enzyme activities for GABA synthesis and degradation [133]. This suggests that thyroid hormones could act as neurotransmitters and neuromodulators by themselves; alternatively, their mood-regulatory properties could be mediated by interactions with the principal neurotransmitter systems.

The interactions between thyroid and neurotransmitter systems are often complex and reciprocal. Effects of neurotransmitter systems on TRH and TSH are better characterised. NE stimulates both TRH and TSH release, while 5-HT, DA, and GABA inhibit their release [134, 135]. On the other hand, evidence about the effect of thyroid hormones on neurotransmitters is mostly derived from animal studies. Such evidence principally consists of altered responsiveness of NE, 5-HT, DA, and GABA systems in the adult/mature brain, resulting from experimentally induced hypothyroid or hyperthyroid states [2, 5, 133, 136, 137]. In addition, thyroid hormones also appear to have important effects on intracellular signal transduction mechanisms, such as G proteins, adenylate cyclase, and phosphoinositide-based signalling pathways in the adult brain [2, 5]. Apart from these interactions in the mature brain, thyroid-neurotransmitter interactions also play a significant role in the developing brain. Indeed, the actions of thyroid hormones on neurotransmitter systems appear to be more pronounced in neonatal animals [2, 138], thus, underlining the important effects of thyroid hormones on formation and organization of neurotransmitter systems in the developing brain [139–141].

The hypothesis that interactions between thyroid and neurotransmitter systems may have a causal role in the pathophysiology of mood disorders was originally proposed by Whybrow and Prange [142]. They suggested that the antidepressant properties of T3 could be explained by its augmentation of postsynaptic beta-adrenergic activity. Hypothyroidism was, thus, believed to cause depression by producing a functional decrease in noradrenergic transmission. The obverse of this would be mania caused by a hyperadrenergic state. The reports of mania following rapid administration of thyroid hormones described earlier [19, 20] seem to support this possibility. The noradrenergic hypothesis has since been modified to include the modulating influence of thyroid hormones on other neurotransmitters. Research data, primarily from animal studies, indicate similar effects of thyroid hormones on the serotonin system. Augmentation of serotonergic transmission by thyroid hormones results from a combination of a reduction of the sensitivity of 5-HT 1A autoreceptors in the raphe nuclei and an increase in 5-HT 2 receptor density and sensitivity in the cortex [2]. Additionally, neuroendocrine challenge studies in hypothyroid patients have shown reduced 5-HT responsiveness, which is reversible with thyroid replacement therapy [2, 137]. Abnormalities of the 5-HT systems have also occasionally been found among patients with depression with documented HPT axis dysfunction [2, 137]. This has led to the speculation that the serotonin system may be involved in the mood-modulating effects of thyroid hormones among patients with mood disorders [2], and that serotonin deficiency could account for several of the HPT axis abnormalities observed in depression [48]. On similar lines, it has also been suggested that disorders of dopaminergic and GABAergic neurotransmission could account for the psychiatric manifestations of thyroid dysfunction [137, 140], but, the evidence for such suppositions is insufficient. Moreover, it is apparent that much of the evidence on thyroid-neurotransmitter interactions is currently based on animal studies. Studies among humans are scarce [137]; the few that have involved patients with mood disorders have been limited to those with depression [2]. Thus, though thyroid-neurotransmitter interactions seem to play a role in the pathogenesis and treatment of mood disorders, the specific interactions underlying modulatory effects of thyroid hormones among patients with bipolar disorder, are yet to be clearly elucidated.

9.2. Neuroimaging Investigations. Newer findings from neuroimaging studies have suggested that HPT axis dysfunction may be more fundamentally related to the aetiopathogenesis of bipolar disorder. In a PET study of hypothyroid patients undergoing thyroid hormone replacement, reduction of the behavioural complaints during therapy was associated with a restoration of metabolic activity in brain areas that were integral to the regulation of affect and cognition [143]. Similarly, in another PET study of untreated Graves' disease, thyrotoxicosis and attendant psychological symptoms were associated with regional metabolic changes of limbic structures that mediate affect [144]. These findings have been complemented by neuroimaging investigations of patients with bipolar disorder. In a seminal PET study of medication-free, treatment-resistant patients with primarily RCBD, serum TSH levels were inversely related to both global and regional cerebral blood flow, and cerebral glucose metabolism [145]. These results suggested that relationships between thyroid and cerebral activity could not only explain HPT axis contributions to the genesis of bipolar disorders, but, could also account for the therapeutic effects of thyroid hormones in bipolar disorders. In another study, ten women with bipolar depression underwent PET, before and after seven weeks of adjunctive treatment with supraphysiological doses of L-T4 [123]. The authors found that patients with bipolar depression had abnormal uptake in prefrontal and limbic brain areas, in structures integral to affect regulation, which have been specifically implicated in bipolar disorder. Administration of thyroxine appeared to improve mood by affecting circuits involving the very same areas. The role of autoimmunity in development of cerebral perfusion abnormalities in patients with thyroid disease is still

unclear. However, SPECT studies of asymptomatic, euthyroid patients with autoimmune (Hashimoto's) thyroiditis had earlier revealed a high prevalence of mild brain perfusion abnormalities [146, 147]. More recently, cortical perfusion asymmetry (particularly between frontal lobes) was found in a SPECT study of a patient with bipolar disorder and Hashimoto's thyroiditis, leading the authors to hypothesize that abnormalities in cortical blood flow might represent a pathogenic link between thyroid autoimmunity and bipolar disorders [148].

9.3. Genetic Investigations. One of the key recent developments in this area has been the research evidence suggesting that HPT abnormalities may be a potential endophenotypes for bipolar disorder. Vonk et al. [149] compared the prevalence of thyroperoxidase antibodies among 22 monozygotic twins and 29 dizygotic twins with bipolar disorder, with 35 healthy control twins. Antibody titres were positive in 27% of the twins with bipolar disorder, compared to only 16% in healthy control twins. The authors proposed that autoimmune thyroiditis (with raised antibody titres as markers) could be an endophenotype for bipolar disorder and could be related to the genetic vulnerability to develop bipolar disorder. In another study, a significantly higher prevalence of thyroperoxidase antibody titres was predominantly found in daughters of parents with bipolar disorder, compared to the female high school and young adult comparisons [150]. Therefore, children of parents with bipolar disorder were found to be more vulnerable to develop thyroid autoimmunity, independently of their vulnerability to develop psychiatric disorders. Coincidentally, recent studies have found HPT abnormalities among children with severe affective, behavioural, and cognitive impairments, who could be a part of the broad behavioural phenotype of bipolar disorder [151].

Additionally, a few recent studies utilising genetic variant analysis have also attempted to elucidate elements of HPT axis dysfunction underlying thyroid-mood disorder interactions [7]. For example, in a case-control association study of Chinese patients, genetic variations of the type II deiodinase gene were associated with bipolar disorder [152]. Moreover, animal studies have shown that genetic mechanisms are involved in regulation of striatal physiology by T3; this could explain the beneficial effects of thyroid hormones in mood disorders [153]. Genetic mechanisms have also been invoked to explain lithium-induced hypothyroidism [154]. Although the research is still at a preliminary stage, these findings suggest that genetic investigations are more likely to eventually unravel the link between thyroid dysfunction and bipolar disorder.

10. Methodological Issues

Despite the impressive advances made in research on HPT axis dysfunction in abnormal mood states, including bipolar disorder, there are quite a few methodological hurdles that are yet to be overcome. One of the principal areas of concern relates to the variability and inconsistency of the nature of HPT axis abnormalities documented among patients with these disorders. Much of this stems from inadequate sample sizes, diagnostic heterogeneity, lack of proper controls for confounding factors, and improper standardization of thyroid function tests [5]. Moreover, given the unique organization of brain thyroid systems, peripheral measures of thyroid function may not adequately characterise central thyroid metabolism [1]. A clearer understanding of the role of HPT axis dysfunction in bipolar disorder is unlikely to emerge if these aspects of study-designs are not addressed. Additionally, the bulk of research on neurobiological mechanisms underlying the thyroid-mood disorder link has been conducted among animals. Studies among mood disordered subjects are very few and limited to those with depression. The methods employed to assess CNS neurotransmitter function have also varied considerably. Therefore, more methodologically sound studies among clinical subjects are required to assess potential interactions between these neurochemical systems in the CNS and thyroid functions [2, 137].

11. Conclusions and Future Directions

There is now more or less incontrovertible evidence that, apart from their developmental effects on the CNS, thyroid hormones have major effects on the metabolic activity of the mature brain. Mood disorders are intimately associated with suboptimal thyroid function. Although comparatively less investigated, increasing evidence has shown that HPT axis dysfunction is relevant to the aetiopathogenesis, course, treatment, and outcome of bipolar disorder. Hypothyroidism either overt or more commonly subclinical appears to the commonest abnormality found among patients with bipolar disorder. It is also likely that the prevalence of thyroid dysfunction is greater in patients with rapid cycling and more refractory forms of the disorder. Lithium has potent anti-thyroid effects and can induce hypothyroidism among patients on this treatment; alternatively, it can exacerbate a preexisting hypothyroid state. Even minor perturbations of the HPT axis in the normal range have the potential to affect the outcome of bipolar disorder. Awareness of this fact is required among clinicians, and patients should be carefully monitored and managed for HPT axis dysfunction. Supplementation with high dose T4 can be considered in some patients, refractory to standard measures of treatment. Genetic, neuroimaging, and neurotransmitter studies are providing newer insights into the complex interactions between HPT function and bipolar disorder.

Although current research, especially preclinical, research has provided strong leads, the precise cellular and molecular mechanisms underlying the role of thyroid hormones in pathophysiology and treatment of mood disorders are still to be delineated. Future attempts need to fill this gap by focusing on translational studies, which can successfully extend preclinical findings to the clinical realm of bipolar disorder, in the true spirit of "bench-to-bedside" research. Additionally, the clinical component of future research needs to identify those patients with bipolar disorders who are most likely to benefit from therapeutic manipulations of the HPT axis, for example, by focusing on genetic markers. Together,

these two strands of research can not only enhance our understanding of the thyroid-bipolar disorder connection, but also lead to more optimal ways of managing this potentially disabling condition.

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Review Article

EGF and TGF-β1 Effects on Thyroid Function

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Normal epithelial thyroid cells in culture are inhibited by TGF-β1. Instead, transformed thyroid cell lines are frequently resistant to its growth inhibitory effect. Loss of TGF-β responsiveness could be due to a reduced expression of TGF-β receptors, as shown in transformed rat thyroid cell lines and in human thyroid tumors, or to alterations of other genes controlling TGF-β signal transduction pathway. However, in thyroid neoplasia, a complex pattern of alterations occurring during transformation and progression has been identified. Functionally, TGF-β1 acts as a tumor suppressor in the early stage of transformation or as a tumor promoter in advanced cancer. This peculiar pleiotropic behaviour of TGF-β may result from cross-talk with signalling pathways mediated by other growth factors, among which EGF-like ligands play an important role. This paper reports evidences on TGF-β1 and EGF systems in thyroid tumors and on the cross-talk between these growth factors in thyroid cancer.

1. Introduction

Thyroid gland homeostasis is maintained through a fine regulation of thyrocyte growth and differentiation. This regulation occurs through complex interactions between thyroid-stimulating hormone (TSH) and other growth factors and cytokines [1]. Evidence supports the role of transforming growth factor-beta 1 (TGF-β1) and epidermal growth factor- (EGF-) like ligands in the regulation of thyroid proliferation and differentiation, given the numerous information focused on mechanism of signal transduction and cross-talk. It is emerging the concept that the basis behind the pleiotropic nature of TGF-β in the context of cell-type and tumorigenesis derived probably from differences in the mechanism of such cross-talk.

The TGF-β superfamily, which includes TGF-β_s, activins, and bone morphogenetic proteins (BMPs), is a multi-functional dimeric proteins family that regulates growth, differentiation and extracellular matrix production in many different cell types [2]. In epithelial cells, TGF-β acts as a tumor suppressor by inhibiting cell growth or by regulating cellular differentiation or apoptosis.

EGF is the prototype of a large family of peptides that consists of about a dozen members and has an essential role in embryonic development as well as in inducing cell growth. Mutations in the kinase domain of EGFR/ErbB1 (epidermal growth factor receptor) and ErbB2 are responsible of ligand-independent activation of cytoplasmic signal transducers that regulate motility, adhesion, protection from apoptosis, and transformation [3].

TGF-β and EGF are physiological regulators of thyroid cell differentiation and proliferation. TGF-β is normally expressed and secreted by thyrocytes, acting as a potent inhibitor of thyroid cell growth [4]. EGF, instead, acts as a strong mitogen for follicular thyroid cells [5]. Any alterations of these two factors or their signalling pathways

may play an important role in the stepwise transition towards malignancy, including the ability to become, at least partially, resistant to growth inhibition, to proliferate without dependence on growth factors, to replicate without limit, to invade, and to metastasize. TGF-β1 appears to have a dual effect in tumorigenesis. It can act as a tumor suppressor in the pretumor stage, and as a tumor promoter in late stage of tumorigenesis. It is likely that during tumorigenesis, as a result of genetic and/or epigenetic changes, the balance between those opposing functions of TGF-β1 changes resulting in a switch to tumor promotion; however, the precise mechanism for this switch remains to be clarified [6]. In this paper we focused our attention on the role of EGF and TGF-β in the control of proliferation and differentiation of thyroid cells.

2. Thyroid Cell Regulation by Growth Factors

Physiological regulation of thyroid cell growth and function involves a complex network of factors that act through endocrine, paracrine, or autocrine mechanisms. The proliferation and differentiation of thyroid epithelial cells are under the control of a positive systemic signal, TSH, and a negative locally produced signal, TGF-β. The main function of the thyroid is the formation, storage, and secretion of thyroid hormones tightly controlled by TSH, but requiring insulin/insulin-like growth factor I (IGF-I) [7]. The steps leading to the thyroid hormone formation include thyroglobulin (TG) synthesis and transport to the lumen of thyroid follicles, the iodide uptake by the sodium iodide symporter (NIS), iodination of TG, and coupling of TG iodotyrosine residues by the thyroperoxidase (TPO). In rat thyroid cells, the expression of the TG, TPO, NIS, and TSH receptor (TSHR) is under the control of thyroid-restricted transcription factors, such as thyroid transcription factor-1 (TTF-1) [8], that plays the most important role in the expression of all the genes, Pax-8, and TTF-2 [9, 10].

TSH, through the activation of its receptor, has been shown to stimulate more than one signal transduction pathway, the main of which being the adenylcyclase/cAMP (cyclic adenosine monophosphate) pathway. cAMP seems to account for the mitogenic effects of TSH in human thyroid cells, mediated by the activation of cAMP-dependent protein kinases [11]. TSH-induced cAMP also appears to play a central role in iodide uptake and metabolism in the dog follicular cells [12], in *TG* and *TPO* gene expression in rat follicular cells [13]. Although TSH is the major regulator of thyroid growth and functions, it has been shown that a number of growth factors affect the proliferation and function of thyroid epithelial cells. In fact, TSH effects can be potentiated by several growth factors such as insulin and IGF-I in rat thyroid cells in culture [14]. Insulin or IGF-I synergizes with TSH to induce thyroid cell growth and to maintain specialized cell functions [11]. There is evidence that *TSHR* and *TG* gene expression are regulated by insulin/IGF-I as well as TSH in a rat thyroid cell line (FRTL-5) [15, 16]. Moreover, an important regulator of thyroid growth that stimulates proliferation *in vitro* includes EGF.

3. Epidermal Growth Factor-Related Ligands and Their Receptors

EGF is the prototype of a large family of peptides structurally related by possessing an EGF-like domain that consists of 6 cysteine residues capable of forming three disulfide-bonded intramolecular loops. These ligands are expressed in the extracellular domain of transmembrane proteins and are generated by regulated proteolysis to yield growth factors that contain 49–85 amino acids. The components of the EGF-like growth factors family are functionally related on the basis of binding to the members of the tyrosine kinase ErbB family (EGFR/ErbB1, ErbB2, ErbB3, and ErbB4) and are divided into three groups: the first includes EGF, transforming growth factor-alpha (TGF-α) and amphiregulin, which all bind specifically to EGFR/ErbB1. A second group includes betacellulin, heparin-binding EGF (HB-EGF), and epiregulin binding to both EGFR/ErbB1 and ErbB4, while the third group comprises the neuregulin family, differentiated by their binding to ErbB3 and ErbB4 (NRG1 and NRG2) or only to ErbB4 (NRG3 and NRG4) [17]. All four human receptors share four extracellular domains with high structural homology, a single transmembrane spanning helix, and a cytoplasmic portion that contains a conserved but not equally functional tyrosine kinase domain. Only the EGFR/ErbB1 and ErbB4 are fully functional in terms of ligand binding and kinase activity. ErbB2 fails to bind any of the known ErbB ligands but contributes its potent kinase activity to all possible heterodimers. ErbB3 has an impaired kinase activity and relies on the kinase activity of its heterodimerization partners for activation. Heterodimers of ErbB2 and ErbB3 are the most potent ErbB pair in mitogenic signalling [17–19].

After ligand binding, ErbB receptors achieve activation by forming homo- or heterodimeric receptor complexes. The dimerization of ErbB receptors represents the most important mechanism that drives transformation. Dimeric receptors become catalytically active and are able to phosphorylate the cytoplasmic receptor domain that serves as docking site for a variety of signalling molecules whose recruitment leads to the activation of intracellular pathways controlling diverse genetic programs. The two major signalling pathways activated by ErbB receptors are the mitogen-activated protein kinases (MAPKs) pathway which stimulates proliferation, and Phosphatidylinositol 3-kinases/AKT (PI3K-AKT) pathway which promotes cell survival (Figure 1). The specific combination of ErbB receptors in the dimer defines the downstream signalling network as well as the intensity and the duration of the stimulation. Indeed, heterodimers that involve ErbB3 stimulates the activation of PI3K pathway [20].

Amplification, overexpression and gene mutation of EGFR/ErbB1 and ErbB2 have been found in various human cancers [17, 21]. EGFR/ErbB1 is overexpressed in bladder, breast, head and neck, kidney, nonsmall cell lung, and prostate cancers [22]. Three truncated forms of the EGFR/ErbB1 have been described [23] among which the EGFRvIII (variant III) lacks the majority of the ectodomain and does not bind EGF. This variant is the most common in glioblastoma multiforme and also occurs in lung, breast,

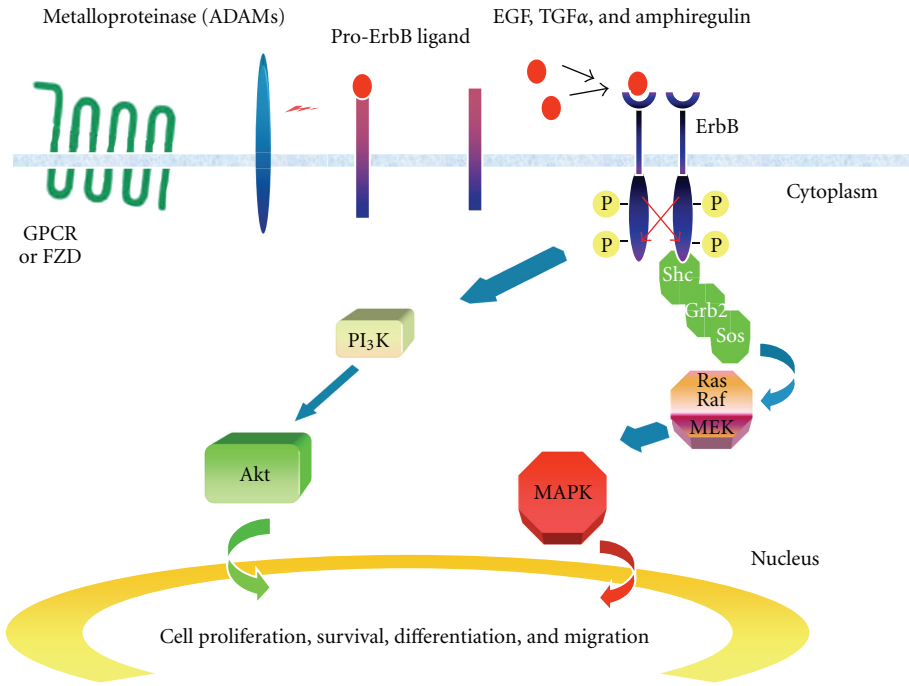

Figure 1: Mechanisms of action of ErbB receptor. In tumor cells, ErbB receptor tyrosine kinases are activated by autocrine or paracrine production of EGF family ligands. Autocrine ligand production results from the activation of G-protein coupled receptors (GPCRs) or frizzled (FZD) receptor which causes the metalloproteinases-mediated cleavage and release of pro-EGF-related ligands (ectodomain shedding). Binding of ligands to the extracellular domain of ErbB receptors leads to receptor dimerization, autophosphorylation, and activation of several downstream signalling pathways. In particular, tyrosine-phosphorylated ErbB receptors bind the adaptor proteins Shc and Grb2 leading to Sos recruitment and Ras/MAPK pathway activation. The PI3K/Akt pathway is stimulated through recruitment of the p85 adaptor subunit of PI3K to the receptor.

ovarian, and prostate cancer [24]. ErbB2 protein is overexpressed in breast cancer due to gene amplification in 15%–30% of invasive ductal breast cancers and overexpression correlates with poor prognosis and disease progression [25]. A number of studies have shown mutations in the kinase domain of EGFR/ErbB1 and ErbB2 [3, 21]. Intragenic somatic mutations in the *ErbB2* gene were reported in 5% of nonsmall cell lung cancer, 5% of gastric carcinomas, 3% of colorectal carcinomas, and 5% of breast carcinomas [26–28]. Accumulating evidence has suggested that also ErbB3 plays a critical role in cancer. Overexpression of ErbB3 often accompanies EGFR/ErbB1 or ErbB2 overexpression and has been frequently detected in a variety of cancers, including those of the breast [29], colon [30], stomach [31], ovary [32], and pancreas [33]. In ErbB2-driven cancers, ErbB3 functions as an intimate signalling partner that promotes the transforming potency of ErbB2, usually by activating the PI3K/AKT pathway [34]. ErbB4 receptor is made in at least four different full-length isoforms as a consequence of alternative mRNA splicing [35]. It has both oncogenic and tumor suppressive functions. Supporting a role in promoting growth, overexpression of ErbB4 enhances growth of human breast cancer cells [36] and transforms mouse mammary epithelial cells to form tumors *in vitro* and *in vivo* [37]. Supporting a suppressive role for mammary tumor growth activation of ErbB4 in breast cancer cells has been associated with cell-cycle arrest, differentiation, and apoptosis *in vitro* [38].

4. Epidermal Growth Factor-Related Ligands and Their Receptors in Thyroid

EGF is synthesized by thyroid gland and is able to induce thyroid cell proliferation in several species together with the loss of thyroid specific functions [5]. Moreover, EGF enhances the migration and invasiveness of papillary thyroid cancer [5, 39–41]. Accordingly, *in vitro* growth was inhibited when either neutralizing anti-TGF-α or anti-EGFR/ErbB1 antibodies were applied to thyroid carcinoma cell lines [42]. A correlation between the staining intensity of EGF and recurrence has been found statistically significant in a set of human papillary thyroid tumors, indicating EGF as a predictor of papillary thyroid carcinoma aggressiveness [43]. Follicular epithelial thyroid cells expressed weakly TGF-α protein, whereas 66% of hyperplastic thyroid nodules, 100% of thyroid adenocarcinomas, and all cases of papillary, follicular, and medullary carcinomas displayed intense staining for TGF-α. A parallel pattern of staining was observed for the EGFR/ErbB1 in these tissues, suggesting the potential for an *in vivo* autocrine loop [44]. Papillary carcinomas and their lymph node metastases coexpressed EGFR/ErbB1 and EbB2 mRNA transcripts at a higher level than in normal thyroid tissues [45]. Moreover, in the same histotype of cancer, it has also been demonstrated the coexpression of TGF-α and EGFR/ErbB1 mRNA transcripts at higher levels than in non-neoplastic thyroid tissues [46].

Rat thyroid epithelial cells transformed with K-ras oncogene were found to express high levels of ErbB4 receptor and Neuregulin-1 (HRG/NDF/NRG1) ligand compared to rat thyroid epithelial control cells. Treatment of K-ras transformed thyroid cells with neutralizing antibody against NRG1 reduced by 50% cell proliferation, demonstrating the presence of an active NRG1 protein secreted in the supernatant by the cells. These data indicate that in K-ras rat thyroid epithelial cells, the growth factor NRG1 signals through the heterodimer ErbB2/ErbB4 receptors in an autocrine fashion [47]. Nevertheless, in human papillary carcinomas the protein overexpression and nuclear localization of the NRG1 precursor isoform compared to normal thyroid tissues was not associated with the expression of ErbB receptors, while the expression of neuregulin-β3 (Hrgβ3) was significantly correlated to ErbB3 protein expression, indicating this receptor as the cognate one [48].

EGFR/ErbB1 was higher expressed in anaplastic and papillary thyroid cancers than in normal thyroid tissues. In particular, Bergstrom et al. [49] have shown that EGFR/ErbB1 was expressed in all six anaplastic thyroid carcinomas examined and it was constitutive phosphorylated in 3 of the 6 cell lines tested. Song [50] has demonstrated that EGFR/ErbB1 was expressed in 90% of papillary carcinomas analyzed. Moreover, the role of EGFR/ErbB1 in stimulating the growth of thyroid tumors has been highlighted by the capability of Gefitinib, a small molecule inhibitor of EGFR/ErbB1, to reduce the growth of papillary, follicular, and anaplastic thyroid cancer cells [51, 52]. By immunohistochemical analyses, cytoplasmic immunopositivity of EGFR/ErbB1 was observed in papillary carcinomas, while no nuclear positivity for EGF and EGFR/ErbB1 was demonstrated. A study by Akslen et al. significantly associated cytoplasmatic EGFR/ErbB1 with the increased risk of recurrent tumor. Nuclear positivity for EGF and EGFR/ErbB1 was demonstrated to be a feature of both follicular adenomas and follicular carcinomas [53, 54].

The role of the ErbB2 proto-oncogene in thyroid carcinoma has been controversially discussed. It has been reported that high levels of ErbB2 mRNA expression correlated with lymph node metastasis in papillary carcinoma [45]. Studies by Utrilla et al. [55] and Haugen et al. [56] showed that there are marked differences in the pattern of ErbB2 immunoreactivity depending on the tumor type. The investigators demonstrated positivity for papillary carcinomas and negativity for follicular adenomas and follicular carcinomas, but they showed controversial results about medullary carcinomas. Moreover, ErbB2 was not detected in papillary carcinomas by immunohistochemistry [57]. Some studies investigated ErbB2 correlation with prognosis. Sugg et al. [58] found ErbB2 staining correlation with degree of differentiation, while Gumurdulu et al. [59] demonstrated that further investigations on ErbB2 role in thyroid tumors are required for determination of prognosis. Other studies have associated cytoplasmatic reactivity with patient's sex in tumor [60] or with development of metastasis [61].

Few studies analyzed all ErbB family members and their implication in thyroid tumors. Wiseman et al. [62] showed that EGFR/ErbB1, ErbB2, ErbB3, and ErbB4 were expressed in 76%, 2%, 57%, and 73% of differentiated thyroid carcinomas analyzed (90 cases of papillary and 6 cases of follicular carcinomas examined), respectively. Moreover, EGFR/ErbB1 and ErbB3 showed significantly increased expression, while ErbB4 showed significantly decreased expression in these tumors compared with benign thyroid lesions. ErbB3 expression correlated with the presence of lymph node metastases, tumor type, and higher N stage; the expression of ErbB4 correlated with lower T stage. Kato et al. [63] demonstrated that the transcription level of *ErbB2* and *ErbB3* genes was increased in papillary carcinomas compared to normal tissues, suggesting that the expression of an ErbB2-ErbB3 heterodimer may correlate with the aggressiveness of a thyroid tumor. Interestingly, coexisting protein overexpression of EGFR/ErbB1, ErbB2, ErbB3, and ErbB4 was demonstrated in 64% of papillary thyroid carcinomas providing numerous possibilities for functional receptor interactions [64].

5. TGF-β Effects on Thyroid Cell Physiology

TGF-β is synthesized as an inactive precursor that can be activated by different proteases produced by thyrocytes. Its expression is upregulated during TSH-induced thyroid hyperplasia in rats, suggesting that an increased local expression of TGF-β1, during thyroid hyperplasia, may contribute to the temporal stabilization of goiter size [65]. TGF-β signalling is propagated via cell surface serine/threonine kinases, TGF-β type I receptor (TβRI) and TGF-β type II receptor (TβRII). Both receptors are expressed on thyrocytes at equimolar amount [66] and, upon ligand binding by type II receptor, TβRI is recruited to an heteromeric complex and phosphorylated by TβRII, thus activating its serine/threonine kinase in order to phosphorylate the transcription factors R-Smad$_s$ (Smad2 and Smad3). The phosphorylated Smad2 or Smad3 associate with the common partner Co-Smad, Smad4, forming complexes that accumulate in the nucleus, where they regulate target genes expression either by interacting with other transcription factors and coactivators or corepressors or by directly binding to defined sequences in the promoter [67]. In contrast to R-Smad$_s$ and Co-Smad, the Inhibitory Smad$_s$ (I-Smad$_s$), including Smad6 and Smad7, bind to TβRI and compete with R-Smad$_s$ for activation by TβRI resulting in the inhibition of TGF-β signalling [68, 69]. A Smad ubiquitin regulatory factor 1 (Smurf1), being a HECT-type E3 (Homologous to the E6-AP C Terminus) ubiquitin ligase, interacts with inhibitory Smad7 and induces cytoplasmic localization of Smad7. Smurf1, then, associates with TβRI and enhances the turnover of this receptor [70] (Figure 2).

TGF-β is the important negative regulator of thyrocyte: it antagonizes the mitogenic effects of the main growth factors in cultured cells of human [65, 71], dog [72], pig [73], and rat origin [4, 74]. TGF-β delays progression during the mid–late G1 phase by directly controlling cyclin D1-3 levels and preventing the relocalization of p27/kip1 inhibitor from cyclin E/cdk2 to cyclin D3/cdk4 complexes [72, 74]. TGF-β has been shown to downregulate the expression of thyroid-specific genes in the majority of species. The iodide

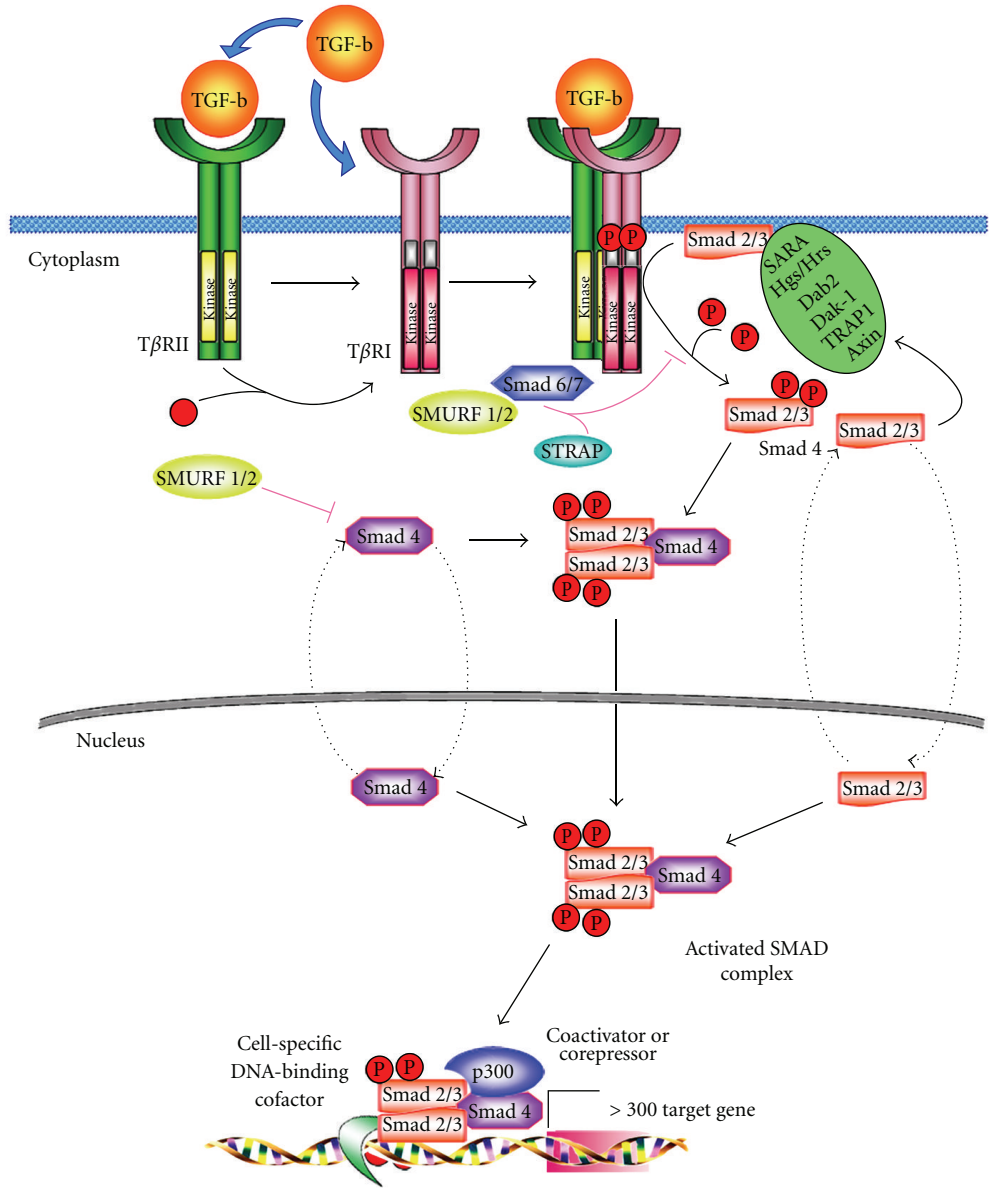

Figure 2: Intracellular signal transduction of TGF-β signalling. TGF-β ligand binds to TβRI and TβRII to constitute active heterodimers that possess serin-threonin kinase activity. Activated TβRI phosphorylates Smad2 and Smad3, which in turn form a complex with Smad4. Smad2 or Smad3/Smad4 complex translocates into the nucleus where interacts with other DNA-binding transcription factors, coactivators and corepressors, regulating the transcription of target genes.

trapping is inhibited in rat thyroid cells by blocking the protein kinase A (PKA) pathway, but not the PKA-induced DNA synthesis [75]. Moreover, we have demonstrated that the overexpression of ErbB2 in FRTL-5 cells, responsible of the resistance to the inhibitory action of TGF-β on cell proliferation, does not affect the inhibition of iodide uptake caused by TGF-β [66]. This effect was likely due in part to the inhibition of the expression of NIS mRNA and its protein [76, 77] and in part to the inhibition of the expression and activity of the Na+/I- ATPase, an enzyme that plays a key role in iodide uptake [78]. Similar responses have also been observed in porcine cell cultures, where TGF-β inhibits iodide uptake and metabolism, cAMP formation, and T4 release [41]. In human thyroid primary cultures, TGF-β inhibits most effects of cAMP on gene expression [79]. The rat thyroid cell lines represent a good model to study the TGF-β action in regulating the TG expression respect to porcine thyroid follicular cells where, TGF-β has no effect [80]. In the FRTL-5 rat thyroid epithelial cell line, the addition of TGF-β inhibits Pax8 mRNA causing a decreased formation of Pax8/DNA complexes, both with or without the addition of TSH that is responsible of the TGFβ1-induced suppression of *TG* gene expression [81]. In the same cells, it has been demonstrated that the inhibition of TG biosynthesis and TSHR expression by TGF-β1 could be counterbalanced by blocking the nuclear translocation of

Smad2 and Smad4 [82]. Instead, Smad3 has a key role in the reduction of NIS expression, because its physical interaction with Pax8 in turn diminishes Pax8 binding to DNA sequence involved in the regulation of NIS [83].

In porcine thyroid cells cultured in suspension, TGF-β counteracts TSH positive effect on folliculogenesis causing an inversion of cell polarity [84]. The ability of TGF-β to modulate cytoskeleton organization and extracellular matrix protein distribution has been demonstrated not only in porcine thyroid cells, where it stimulates the expression of plasminogen activator inhibitor-1 (PAI-1), clusterin, trombospondin-1 [80, 85, 86], but also in rat thyroid cells [87]. Finally, in the same cellular model TGF-β1 inhibits the major histocompatibility complex (MHC) class I by regulating two elements at the transcription start site of the flanking region and increases the downstream regulatory element (DRE) binding of an ubiquitously expressed Y-box protein, termed TSEP-1 (TSHR suppressor element-binding Protein-1), an important suppressor of the TSHR and of MHC class I and class II expression [88]. TGF-β1 stimulates monocyte chemoattractant Protein-1 (MCP-1) and colony-stimulating factor (CSF) [89], as well as endothelin and its receptors in human thyroid follicular cells [90].

Therefore, it can be concluded that TGF-β exerts an important effect on thyroid cells in all the species tested, inhibiting the proliferation and function and modulating extracellular matrix (ECM) formation.

6. Role of TGF-β in Thyroid Cancer

The inhibition of cellular proliferation is one of the primary action of the TGF-β signalling. This factor is involved in the regulation of other numerous cellular functions as embryogenesis, differentiation, apoptosis, angiogenesis, immunosuppression, and wound-healing process [91]. Given the multifunctional role of TGF-β, any aberration of its normal signalling cascade may have wide-ranging pathologic consequences. Yet, paradoxically, TGF-β also modulates processes such as cell invasion and microenvironment modification that cancer cells may exploit to their advantage. Consequently, the output of a TGF-β response is highly contextual throughout development, across different tissues, and also in cancer [92].

Thyroid cancer incidence has significantly increased during the past decades [93], and it has become one of the ten leading cancer types in females being more frequent than ovarian, urinary, bladder, or pancreas cancer [94]. Although the majority of thyroid cancers have an excellent prognosis, there are a small percentage of cases that show an extensive local invasion and distant metastases, which frequently do not respond to standard treatments and have a worsened prognosis. The genetic basis for the initiation and development of the common type of thyroid cancer, papillary thyroid carcinoma (PTC), is well characterized. It has been demonstrated that the activation of oncogenes like RAS, BRAF, RET/PTC, and PI3K/AKT plays an important role in thyroid tumorigenesis [95]. However, it is also interesting to underscore the differences among the tumors arising from the different mutations. Studies *in vitro* and *in vivo* have clearly shown that other oncoproteins, like EGF and TGF-β, exert their own oncogenic drive, conferring a distinct biological behaviour on thyroid tumors. The homoeostasis of growth in differentiated epithelia reflects a critical balance between the promotion and suppression of cell division. The thyroid gland is among the most common sites of epithelial hyperplasia, affecting up to 15% of the adult population, typically presenting as "sporadic" or multinodular nontoxic Goiter (MNTG), the hyperplastic gland usually contains well-defined nodules of varying size, surrounded by a normal epithelium. A wide range of studies has revealed evidence for an involvement of several autocrine growth stimulators and their receptors in the progression of MNTG. Prominent among these is TGF-β, and a lack of response to TGF-β inhibitory action in thyroid cells may be responsible for some cases of MNTG in human cells [71]. In rats, thyroid hyperplasia induced by iodide deficiency and goitrogen is accompanied by an increase in TGF-β1 expression and the arrest of goiter growth after 4 weeks. This surprising result is thought to reflect a critical role of TGF-β1 in stabilizing goiter mass [65, 96]. This would suggest that the increase in TGF-β1 levels observed during goiter induction might be a mechanism to counteract the goitrogenic effect of endogenous TSH [65, 97]. Instead, in a group of patients with either papillary adenomas ($n = 14$) or carcinomas ($n = 14$), not significant changes in blood levels of TGF-β1 have been observed compared to normal controls [98].

Increased expression of TGF-β, NFkB (nuclear factor of κB), and CDC42, compared to the normal thyroid tissue, has been demonstrated in a group of human papillary thyroid carcinomas, analyzed by oligonucleotide microarray of microscopically dissected intratumoral samples from central and invasive regions. These data together with reduced levels of mRNAs encoding proteins involved in cell-cell adhesion and communication and an overexpression of vimentin strongly support the hypothesis that the TGF-β, responsible of epithelial mesenchymal transition (EMT) induction, increases the tumor invasiveness in papillary thyroid carcinomas [95].

Perturbations of TGF-β signalling are central to tumorigenesis and tumor progression. TβRII is commonly inactivated through mutation and loss of heterozygosity (LOH) in several types of carcinoma [99]. Lazzereschi et al. [100], have obtained similar results in a series of human thyroid tumors, from benign lesions (adenomas) to neoplastic lesions of increasing aggressiveness (papillary and follicular carcinomas) up to the extremely aggressive anaplastic tumors. Northern blot analyses demonstrated a statistically significant reduction (about 2-3-fold less) of TβRII expression in papillary thyroid carcinomas in comparison with the respective healthy tissues. Immunostaining of the formalin-fixed sections with specific anti-TβRII antibodies substantiates these data, clearly demonstrating that the greatest reduction in TβRII immunoreactivity was found in the highly malignant, undifferentiated anaplastic thyroid tumors. A comparative study performed in different types of epithelial thyroid carcinomas of patients from different regions of the world demonstrated a strong decrease of TβRII

expression in follicular cancer of patients from China, Japan, and USA (50%, 55%, and 90% respectively). In papillary thyroid carcinoma TβRII was decreased in 75%, 77% and 96% of patients from Japan, China, and USA, respectively. Finally, in undifferentiated cancer, the reduction of TβRII expression was observable in 83% of patients from USA and in 100% of patients from China [101].

Inactivating mutations in Smad2 and 4 are frequently found in some cancers [102]. In a group of 20 follicular thyroid neoplasms, classified as 11 adenomas and 9 minimally invasive follicular carcinomas, according to current pathological criteria, Smad2 expression investigated by immunohistochemistry has been lost, while TβRII expression was lost in 78%. These data indicate that the downregulation of TβRII remains the major consistent abnormality in thyroid carcinomas that may be used to differentiate minimally invasive carcinomas from adenomas, while the downregulation of Smad2 could be another mechanism by which carcinomas become independent from TGFβ–mediated growth inhibition [103].

The tumor suppressor role of TβRII has been demonstrated *in vitro* in a model of K-ras transformed rat thyroid cells, where the overexpression of TβRII induces not only a partial reversion of malignant phenotype restoring the sensitivity to TGF-β, but also a significant reduction in spontaneous and lung artificial metastases when transplanted in athymic nude mice [104]. In this model, the overexpression of TβRII is essential to reduce the invasive potential and to modulate the adhesive and migratory cell behaviours by controlling the integrin functions rather than integrin receptor expression [105]. The inhibitory action of TGF-β1 on cellular migration, invasion, and adhesion is present in a set of human PTC and follicular thyroid carcinoma (FTC) cell lines, while inhibition of TGFβ-induced cell growth is maintained only in FTC cell lines [106]. Other authors demonstrated that in human papillary thyroid carcinoma cell line TPC-1, these effects are probably due to the lower level of Smads 2, 3, and 4 associated to an increase in Smad7 expression [107]. Otherwise, the altered expression of TGF-β pathway proteins not always is responsible of resistance to the TGF-β action. A human anaplastic carcinoma cell line, despite the activity of receptors signalling, Smad2 phosphorylation and nuclear translocation of Smad2/4 complexes, is strongly resistant to the TGF-β action, suggesting that other signalling mechanisms might be related to the escape from TGF-β sensitivity [108].

Sensitivity to TGF-β is impaired in thyroid tumors and escape from TGF-β action is actively selected during thyroid tumor development. Destruction of the TGF-β signalling at the level of Smad genes is common in human carcinomas, the deficiencies of Smad4 have been hypothesized to underlie TGF-β resistance of tumor cells and to strongly accelerate the malignant progression of neoplastic lesions initiated by other oncogenic stimuli [92]. Lazzereschi et al. [109] showed, for the first time, the mutational and the expression status of Smad4 in a consistent number of thyroid tumors of different histotypes, demonstrating the high frequency of Smad4 abnormalities (27%) in thyroid tumors, comparable only with Smad4 mutation frequency in tumors arising from the gastrointestinal tract, both sporadic and inherited. The high frequency of alterations in Smad4 sequence led the authors to propose that these changes may constitute a nearly and frequent event in thyroid tumors natural history. Smad4 inactivation in tumors is generally a late event linked to progression to overt carcinoma. In human papillary thyroid cell lines, TPC-1 and BCPAP, it has been recently demonstrated a strong reduction in the level of SMAD4 protein, which is responsible for an alteration of TGF-β signalling and for some of the TGF-β-mediated biological effects. The overexpression of Smad4, restoring TGF-β signal transduction, determines a significant increase of antiproliferative response to TGF-β, reduces the invasive behaviour of these cells as well as is responsible of a significant increase of E-cadherin expression, indicating that the level of SMAD4 is a critical regulator of these processes. To remark the important role of SMAD4 in thyroid carcinogenesis contributes also the finding obtained by immunohistochemistry that 7 out of 23 (30%) PTC tumor samples, including 1 case of follicular variant of PTC_s, present a weak and focal intensity of SMAD4 staining compared to normal tissue from the opposite lobe [110].

The stability of TβRI represents an important regulatory mechanism for TGF-β signalling both in cell culture studies and *in vivo* models. TGF-β receptors are ubiquitinated and degraded through the action of several cooperating protein complexes containing E3 ligases as well as other important regulators of protein degradation. The I-$Smad_s$ regulate many of these complexes, orchestrating both ubiquitination and de-ubiquitination [111]. The levels of Smurf1 and Smad7 are overexpressed in the anaplastic thyroid carcinoma cell line [112], and an increase of SMAD7 expression has been found in a group of papillary and follicular carcinomas with respect to benign pathologies, indicating SMAD7 as another SMAD involved in thyroid tumorigenesis [113].

It is known that TGF-β's role in human cancer appears both complex and context depended. Depending on the tumor type and the stage of tumor progression, it can exercite strong tumor suppressive or tumor-promoting functions. More recently, it has been demonstrated that also in thyroid cells, as well as in the skin tumors, or in metastatic colon cancer [114], TGF-β can acts as tumor-promoting factor. The expression of BRAFV660E, in normal rat thyrocytes and in fifty cases of human PTC determines a reduction of NIS expression and an increase of TGF-β secretion, suggesting an hyperactivation of TGF-β signalling, responsible of the protumorigenic activity [115].

The aberrant microRNA (miR) expression, involved in the cell growth suppressive function, has been demonstrated in a large number of follicular thyroid neoplasias [116]. More recently, a new important function of TGF-β, involving the regulation of expression of miR levels (miR-200 and miR-30), has been discovered in human anaplastic thyroid carcinoma (ATC). ATCs represent a more aggressive type of thyroid cancer arising from mesenchymal de-/transdifferentiation of epithelial thyroid cells that rapidly invade the adjacent tissue. The main function of miR-200

and miR-30 is to negatively regulate the EMT process in follicular cells. The low levels of miR-200 and miR-30 in ATCs respect to that observed in thyroid normal tissues, in PTCs or in FTCs, strongly suggest that the invasive potential of ATC is due to enhancement of EMT process. In addition, it has been demonstrated that the reduction of miR-200 and miR-30 in these carcinomas is caused by a strong activation of TGF-β signalling due to an upregulation of TβRI and Smad2. Therefore, the authors not only propose a novel molecular panel to identify ATCs, but also they suggest the inhibition of TGF-β signalling represent a new likely approach for the treatment of these carcinomas [117].

7. Growth Factors Cross-Talk in Thyroid: Role of TGF-β and EGF Systems in the Regulation of Thyroid Growth

Complex and apparently redundant interactions between hormones and growth factors regulate thyroid cell proliferation and differentiation. However, information about the cross-talk between different growth factors that regulate thyroid cell growth is limited. Ariga et al. [118] studied the signalling pathway through which the synergistic actions between IGF-I and TSH are mediated in FRTL-5 cells. Also, TGF-β1 and IGF-I appear to interact and have opposite effects on the growth of rat thyroid cells [75]. In particular, TGF-β1 attenuates IGF-I-stimulated MAPK phosphorylation through inhibition of IRS-1 (insulin receptor substrate-1) tyrosine phosphorylation, IRS-1/Grb2/Sos complex formation and CrkII tyrosine phosphorylation thus leading to the suppression of FRTL-5 cell growth [119]. ErbB ligands and ErbB receptors provide a complex and multilayered network of signalling that is deregulated in many human tumors. However, several are the causes and mechanisms of uncontrolled signalling by ErbB receptors suggesting that differences exist within the ErbB family in the mechanism of regulation but also in the cross-talk with other growth factors.

TGF-β is involved in two opposing activities: it is able to function as a growth inhibitor at early stages of carcinogenesis and as a growth promoter at later stages of neoplasia when tumor cells that have developed the capability to bypass the tumor inhibitor function of TGF-β, paradoxically, may use it for tumor progression by means of multiple mechanisms. Overexpression of TGF-β ligands has been reported in most cancers [120] and correlates with markers of a more metastatic phenotype and/or a poor patient outcome. This dual role of TGF-β is believed to result from molecular cross-talk with a complex network of signalling pathways involving either direct effects on tumor cells or paracrine effects on other cells [121]. Several reports provided evidence that TGF-β can collaborate with EGF/ErbB receptors system. Indeed, it has been shown that Smads proteins may cross-talk with mitogenic growth-factor signalling through receptor tyrosine kinase-induced MEK/MAPK protein kinases both in a synergistic (MEK1-induced Smad2 phosphorylation) [122] or an antagonistic interplay (MAPK-induced Smad1 phosphorylation) [123, 124]. In tumors, ErbB receptors and their ligands promote growth and confer apoptosis resistance, thus overcoming TGF-β1 growth inhibition and apoptotic effect. Indeed, in rat hepatocytes an autocrine loop of TGF-β in cells that have undergone EMT, induces an upregulation of EGFR/ErbB1 ligands by promoting their shedding through the activation of ADAM17 (a disintegrin and metalloprotease 17) and thus allowing some cells to escape from TGFβ-induced pro-apoptotic effect [125, 126]. A further mechanistic insight on the conversion of the function of TGF-β from tumor suppressor to tumor promoter has been provided by the evidence that the EGF signalling pathway may enhance TGF-β responses. EGF increases the stability of TβRII thus preventing full loss of TβRII expression in late stage of cancers, and thereby permits some of the direct oncogenic behaviour of TGF-β during tumor progression [127]. Uttamsingh et al. [128] showed that in conjunction with EGF, TGF-β1 helps to augment migration, invasion and anchorage-independent growth of intestinal epithelial cells, in agreement with reports indicating that activation of TGF-β signalling promotes pulmonary metastasis of mammary tumors in neu transgenic mice [129]. Moreover, the elevated and prolonged activation of ERK/MAPK and its requirement for EGF and TGFβ1-induced EMT and migration/invasion of intestinal cells is in agreement with that between ErbB2 and TGF-β1 in mammary epithelial cells [130]. In fibroblasts, TGF-β induces the upregulation of $ErbB_s$ ligands and activation of cognate receptors via the canonical Smad pathway, thus allowing the induction of fibroblast cell morphologic transformation and anchorage-independent growth [131].

Contrasting to this *plethora* of data on cross-talk between EGF and TGF-β1 signals, there are very few results in the literature relating the interconnection between EGF and TGF-β signalling pathways in thyroid cancer cells.

Rat thyroid epithelial cells overexpressing the ErbB2 proto-oncogene are not transformed *in vitro* but no longer depend on TSH for cell growth and become resistant to the growth inhibitory effects of TGF-β1, thus suggesting that ErbB2 proto-oncogene, when overexpressed, is able to interfere and cross-talk with growth factors that control in a positive and negative manner the thyroid cell proliferation [132]. Using collagen gel-cultured porcine follicles, Nilsson et al. [133] demonstrated that the morphoregulatory effects of EGF are highly influenced by TGF-β1. In particular, TGF-β1 inhibits EGF-induced thyrocytes proliferation, but it synergizes with EGF in the stimulation of cell migration. This latter effect could be due to the known role of TGF-β1 to regulate the cell-matrix interactions, by stimulating the synthesis of extracellular matrix components, inhibiting proteases and inducing changes of integrin synthesis. However, contrasting to these results, TGF-β1 inhibited both EGF-induced mitogenesis and motogenesis in rabbit corneal epithelial cells [134] indicating that the modulation by TGF-β1 of EGF responses differs among epithelial cell types.

Moreover, in a follicular thyroid cancer cell line lacking endogenous TSHR, EGF, and TGF-β have been shown to enhance VEGF (vascular endothelial growth factor) secretion. Since the loss of the TSHR is characteristic of anaplastic thyroid cancer, which usually exhibits significantly

increased VEGF expression and a high degree of angiogenesis compared with differentiated thyroid cancer, the finding of VEGF stimulation by EGF and TGF-β, highlights the important role of these growth factors in thyroid tumor progression and aggressiveness [135]. Preliminary data from our laboratory demonstrate that cotreatment with EGF and TGF-β1 results in opposite effects in human thyroid cancer cell lines. Indeed, TGF-β1 inhibits EGF-mediated migration in invasion/wound healing assay, while a synergistic effect between TGF-β1 and EGF is observed in anchorage-independent growth assay. These findings demonstrate that cell invasion and anchorage-independent growth capability involve different factors and molecular mechanisms (Mincione G. et al., unpublished results).

8. Conclusions

The findings described in this paper support the hypothesis that a network formed by the EGF/ErbBs system and TGF-β pathway is involved in the pathogenesis and progression of thyroid tumors. Further understanding of the complexity of cross-talk between these pathways in thyroid disease related to gain of function of ErbB, inactivation of growth suppression function, or activation of tumor promoter activity of TGF-β1 will offer a broader spectrum of points of intervention and will lead to continued advances in thyroid cancer treatment.

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Review Article

MicroRNA Role in Thyroid Cancer Development

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MicroRNAs (miRNAs) are endogenous noncoding RNAs that negatively regulate gene expression by binding the 3′ noncoding region of the messenger RNA targets inducing their cleavage or blocking the protein translation. They play important roles in multiple biological and metabolic processes, including developmental timing, signal transduction, and cell maintenance and differentiation. Their deregulation can predispose to diseases and cancer. miRNA expression has been demonstrated to be deregulated in many types of human tumors, including thyroid cancers, and could be responsible for tumor initiation and progression. In this paper we reviewed the available data on miRNA deregulation in different thyroid tumors and describe the putative role of miRNA in thyroid cancer development.

1. Introduction

MicroRNAs (miRNAs) are endogenous single-stranded noncoding RNAs of about 22 nucleotides which suppress gene expression by selectively binding to the complementary 3′ untranslated region (3′-UTR) of messenger RNAs (mRNAs) through base-pairing. They play important roles in multiple biological and metabolic processes, such as cell differentiation, proliferation, and survival. Presently, miRNAs are considered one of the most important regulators of gene expression at posttranscriptional level, and it has been estimated that over one third of all human genes could be targeted by miRNAs [1]. Moreover, miRNAs are strongly conserved even among different species, strengthening the hypothesis of their important roles in many essential biological processes.

Recent studies have also supported a role of miRNAs in the initiation and progression of human malignancies. The analysis of global miRNA expression in cancer patients showed different patterns of miRNA overexpression or downregulation in cancer versus normal tissues [2] in several human tumors, such as colorectal neoplasia [3], B cell chronic lymphocytic leukaemia [4, 5], B cell lymphoma [6], lung cancer [7], breast cancer [8], and glioblastoma [9, 10]. The involvement of miRNAs in human cancer is probably due to the fact that >50% of miRNA genes are located at chromosomal fragile sites or common break point sites or within regions of deletion or amplification that are generally altered in human tumors [11]. The deregulation of miRNA expression is suspected to be an important regulator of tumor development and progression in several human tissues. The overexpression of specific miRNAs could lead to the repression of tumor suppressor gene expression, and conversely the downregulation of specific miRNAs could result in an increase of oncogene expression; both these situations induce subsequent malignant effects on cell proliferation, differentiation, and apoptosis that lead to tumor growth and progress.

Experimental evidence demonstrated that the majority of miRNAs present lower expression levels in tumors compared to normal tissues, independent of the cell type. Global miRNA expression is higher in normal tissues compared to their tumoral counterparts or to cancer cell lines. In addition, poorly differentiated tumors present a lower global level of miRNA expression compared to more differentiated tumors [12]. All these data are consistent with the hypothesis that a higher global miRNA expression is associated with cellular differentiation. The reduction of global miRNA expression may reduce cell differentiation that is the hallmark of all human cancers.

2. miRNA Transcription, Maturation, and Mechanisms of Action

miRNAs are transcribed as long, poly-adenylated, and capped primary transcripts (pri-miRNAs) that are cleaved, at nuclear level, to ~60–70 nucleotide hairpin-shaped intermediates (pre-miRNAs) by the nuclear RNase III Drosha [13]. Following nuclear processing by Drosha, pre-miRNAs are exported to the cytoplasm by the nuclear transport receptor exportin-5 [14]. At cytoplasmic level pre-miRNAs are processed into ~22 nucleotide miRNA duplexes by the cytoplasmic RNase III Dicer [15]. These double-stranded products are unwound by a still unidentified helicase and incorporated as single-stranded RNAs (guide strand) into a ribonucleoprotein complex, known as the RNA-induced silencing complex (RISC) [16]. Which of the two RNA strands is incorporated in the RISC is determined by the stability of the base pairs at the 5′ end of the duplex; the other strand is degraded. The incorporate guide strand leads the RISC to the complementary sequence in the 3′-UTR of target mRNA, negatively regulating gene expression at the posttranscriptional level by targeting the 3′-UTR region of the mRNAs. Nucleotides 2–8 (referred to as "seed") of the mature miRNAs are evolutionary conserved and result in being crucial in determining target specificity. miRNAs can downregulate gene expression by two distinct post-transcriptional mechanisms: mRNA cleavage or translational repression. The choice of mechanism is determined only by the identity of the mRNA target and the degree of miRNA-mRNA complementarity: miRNA will specify induce the cleavage of mRNA if the target has sufficient complementarity to the miRNA itself, or it will repress translation if the mRNA does not have sufficient complementarity [17, 18] (Figure 1). In the first case miRNA may act as small interfering RNAs (siRNAs) and it cleaves mRNA targets between the nucleotides pairing to positions 10 and 11 of the miRNA [19, 20]; after the cleavage of the target the miRNA remains intact and can guide the recognition and destruction of other mRNAs [17]. Conversely, the reduced complementarity between miRNA and its target mRNA generally creates mismatches and bulges in the central region of the miRNA-mRNA duplex (at positions 10–12 of the mature miRNA sequence) that prevent the siRNA-like cleavage of target mRNA. miRNA-mediated translational repression can be regulated both at initiation level or at postinitiation level of the translation process. In the initiation block the RISC complex inhibits translation by interfering with eIEF4F-cap recognition and 40S small ribosomal subunit recruit or by preventing the assembly of the 60S subunit to form the 80S ribosomal complex. In the postinitial block RISC may inhibit ribosome elongation, inducing ribosome drop-off or facilitating proteolysis of the nascent polypeptides [21].

Recently, it has been hypothesized a role of miRNAs in the upregulation of protein translation. A recent study [22] showed that miR-369-3 linked the AU-rich elements (AREs) of the TNFα mRNA to upregulate translation, through direct base pairing between miR-369-3 "seed" region and complementary ARE regions, under cell cycle arrest conditions. The authors hypothesized that miRNAs may switch between translation repression or activation based on cell cycle status: in proliferating cells they repress translation while under cell cycle arrest they promote translation.

Another study [23] indicated a possible role of miRNAs in positive activation of translation. The authors found that miR-10a interacted with the 5′ untranslated region (5′-UTR) of the mRNAs encoding ribosomal proteins to enhance their translation. Moreover, miR-10a resulted in being capable of both translation repression through 3′-UTR binding or of translation promotion via 5′-UTR binding of different mRNA targets. These data suggest that the same miRNA may exert different effects depending on the site of interaction with its targets.

3. Identification of miRNA Targets

Identification of miRNA targets and of their specific interaction sites is fundamental in the comprehension of miRNA roles in the regulation of biological processes as well as in the development of human malignancies.

Some algorithms, such as miRanda (http://www.microrna.org/microrna/home.do), TargetScan (http://www.targetscan.org/), or PicTar (http://pictar.mdc-berlin.de/), have been developed for the computational prediction of miRNA targets. All these bioinformatic predictions are primarily based, and limited, on conserved interactions involving the miRNA "seed" region and the 3′-UTRs of target mRNAs. A limitation of these prediction programs is that they are not able to reveal novel aspects of miRNA target recognition. False-positive predictions can be eliminated by experimental validation studies but false-negative predictions remain often unsolved.

Experimental approaches to miRNA target identification have mainly focused on analyses of both transcriptome and proteome expression arrays in cells in which a single miRNA has been overexpressed or inhibited. Effects on endogenous target protein levels serve as a good indicator to validate the miRNA-target interaction. However, this approach suffers from a limitation: in fact when a miRNA is introduced, by transfection, in high concentrations into a cell, this may effect the observed effect on target mRNA and generate false-positive results, since the level of translation repression is strongly dependent not only on miRNA and mRNA target complementarity but also on both the amount of target mRNA and the amount of available miRNA in the cell [24].

A direct evidence that a miRNA binds a specific target mRNA can be obtained by formaldehyde cross-linking of the miRNA to its targets [22] or by 4-thiouridine-modified miRNAs [23], both these techniques allow the subsequent mapping of the exact site of binding using primer extension.

4. miRNA Deregulation in Thyroid Tumors

Thyroid tumors represent a good model for studying multistep cancer development in epithelial cells as they comprise a range of lesions with different degrees of malignancy: from benign differentiated and noninvasive adenomas to malignant undifferentiated anaplastic invasive carcinomas.

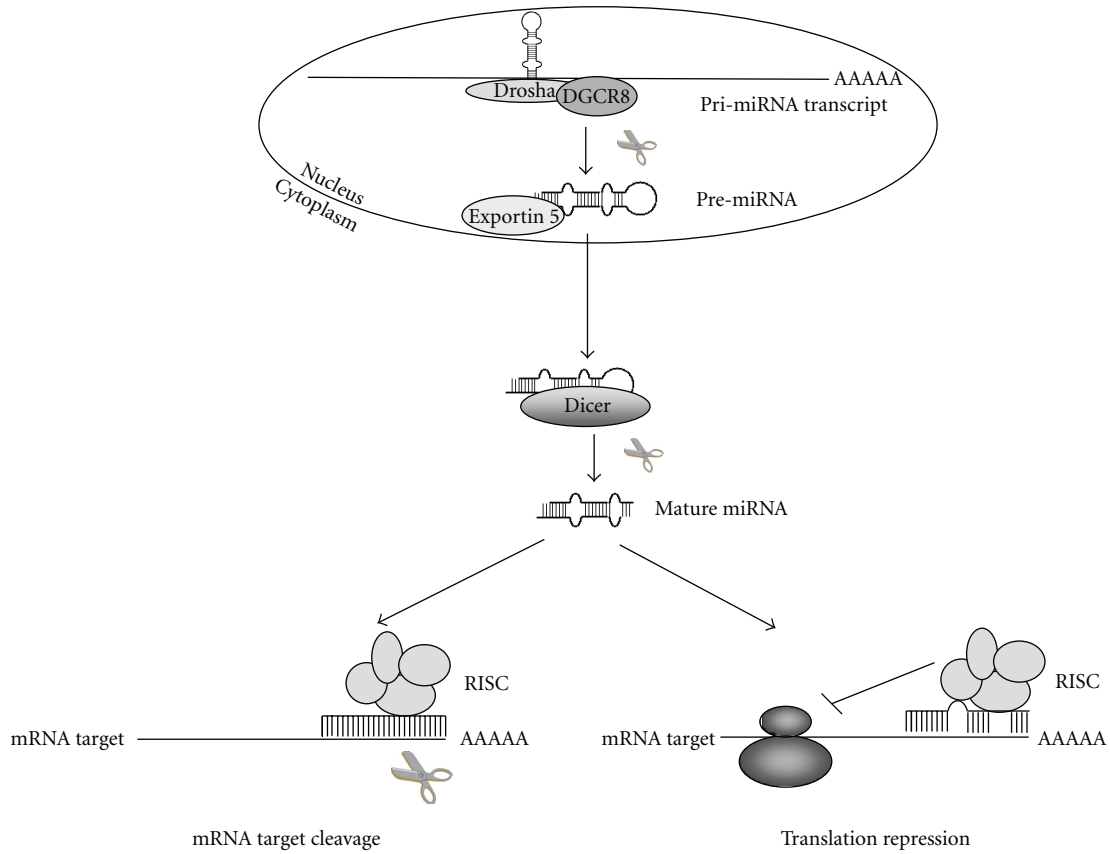

Figure 1: Model for miRNA biogenesis and functional mechanisms. miRNA genes are transcribed by RNA polymerase II (pol II) into primary transcripts (pri-miRNAs) that are cleaved by the Drosha-DGCR8 complex to 60–70 nt pre-miRNAs. Pre-miRNAs are then transported to the cytoplasm by exportin-5 and there processed by the endonuclease Dicer to generate a double-stranded mature miRNA of about 21–23 nt. After a strand selection/separation process, the mature miRNA is incorporated into the RISC complex while the other strand is degraded. RISC complex will recognize and mediate cleavage or repression of specific mRNAs.

The two most common thyroid tumors are papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC), accounting, respectively, for about 80% and 15% of all cases, both well differentiated and originating from thyroid follicular cells. PTCs are often multifocal, characterized by classical papillary architecture, and they usually metastasize to the regional lymph nodes. FTCs are often unifocal, encapsulated, and they usually metastasize via the vascular system to lungs and bones. Both these tumors may progress to poorly differentiated carcinoma or to completely nondifferentiated anaplastic carcinoma. Anaplastic thyroid carcinomas (ATCs) are very rare thyroid cancers (2–5% of all cases), extremely undifferentiated and highly aggressive. Medullary thyroid carcinoma (MTC) is a rare thyroid tumor, accounting for less than 5% of all thyroid cancers, that originates from the thyroid intrafollicular C cells.

Several independent studies have analyzed miRNA expression in numerous and different types of thyroid tumors, evidencing a miRNA deregulation in cancer tissues compared to their normal counterparts [25–38]; in thyroid tumors 32% of all known human miRNAs resulted in being upregulated and 38% to be downregulated with more than a 2-fold change as compared to normal tissues [39]. Moreover, the miRNA expression profile presents a significant variability between different kinds of thyroid cancers, even if they originate from the same type of thyroid cells [25, 39]. C-cell-derived MTC has a miRNA expression profile significantly different from those of thyroid tumors originating from follicular cells; but also papillary carcinomas, conventional follicular adenomas and carcinomas, and oncocytic follicular adenomas and carcinomas, all originating from follicular cells, show different and specific miRNA expression profiles. At the moment the exact biological roles of miRNAs in thyroid carcinogenesis remain to be fully elucidated but it seems reasonable that the distinctive pattern of miRNA expression in thyroid tumors compared to normal thyroid tissue may be useful in diagnosis and/or therapy of thyroid neoplasia and that different miRNA expression patterns in different types of thyroid tumors could be useful tools for their classification.

However, the majority of miRNA profiling studies do not provide an estimate of miRNA abundance in normal thyroid tissues and in thyroid tumors. Today, it is recognized that only the most abundantly expressed miRNAs are able to occupy a substantial fraction of their mRNA targets, affecting their translation. The magnitude of translation

repression is strongly dependent on the number of miRNA-RISC complexes with respect to the amount of target mRNA molecules. It is hypothesizable that, among all the misregulated miRNAs, only those which are abundantly overexpressed or strongly downregulated are involved in thyroid tumorigenesis. The most abundantly expressed miRNAs in human healthy thyroid gland are listed in Table 1; data are derived from http://www.mirz.unibas.ch/cloningprofiles/ using the "visualization of miRNA expression profiles" tool.

Table 1: Most abundantly expressed miRNAs in human healthy thyroid gland. The table reports the most abundantly expressed miRNAs in human normal thyroid gland; data are derived from http://www.mirz.unibas.ch/cloningprofiles/. The tool screened a total of 768 human miRNAs. Only miRNAs with an abundance value over 3.0 have been reported in the table.

miRNA	Abundance value in thyroid gland
let-7b	56.0
let-7a	52.0
miR-143	47.0
miR-126	39.0
let-7i	32.5
let-7c	29.5
miR-125b	29.0
miR-16	27.0
miR-200c	24.5
miR-26a	23.6666666
let-7f	21.5
miR-23b	16.0
miR-24	14.0
miR-99a	13.0
miR-29a	12.0
miR-30d	12.0
miR-451	12.0
miR-23a	11.0
miR-15a	9.0
miR-27b	9.0
miR-30c	9.0
miR-21	8.0
miR-27a	8.0
miR-30a	8.0
miR-100	8.0
miR-191	8.0
let-7e	7.0
let-7g	6.0
miR-99b	6.0
miR-125a-5p	6.0
miR-145	6.0
miR-195	5.0
let-7d	4.0
miR-25	4.0
miR-206	4.0
miR-10b	3.0
miR-22	3.0
miR-138	3.0
miR-152	3.0
miR-423-3p	3.0

5. The Role of MicroRNAs in Papillary Thyroid Carcinoma

Some studies analyzed the miRNA expression profile in PTCs [26–32] (Table 2).

Comparing global miRNA expression in human PTCs versus unaffected thyroid tissue He et al. [26] individuated a set of five miRNAs (miR-146, miR-221, miR-222, miR-21, and miR-181a) that were significantly overexpressed in PTCs compared to the adjacent normal tissue. Particularly, three of them, miR-146, miR-221, and miR-222, showed 11- to 19-fold higher level in tumor tissues. The probe sequence of miR-146 used in the miRNA array chip was designed for miR-146a isoform, located on human chromosome 5. Recently a second isoform, named miR-146b, has been described on human chromosome 10. These two miRNAs differ only for two nucleotides in the sequence of their mature forms. Using primers for premature forms of miR-146a and miR-146b, no expression of miR-146a was detected in thyroid tissues by RT-PCR analysis, while a significant overexpression of miR-146b was found in PTC tumor samples as confirmed also by Northern blot for mature miR-146b. Deregulation of miR-146b, miR-221, and miR-222 in the thyroid may be a crucial component of PTC initiation and development. The putative target of these miRNAs was suspected to be KIT, a tyrosine kinase receptor that plays an important role in cell growth and differentiation, acting as an oncogene in many cancers [41, 42]. Nevertheless, neoplastic transformation has been shown to be associated with either overexpression or downregulation of c-KIT in different tissues [43–45]. In PTC tissues in which miR-146b, miR-221, and miR-222 were strongly overexpressed there was a downregulation of KIT transcript and KIT protein. In 50% of cases the reduced expression of KIT was associated with germline single-nucleotide changes in both the two recognition sites of KIT for miR-221 and miR-222 (3′-UTR region of KIT) and for miR-146b (exon 18 of KIT). In conclusion, the upregulation of these five specific miRNAs, and particularly of miR-146b, miR-221, and miR-222, and the subsequent downregulation of KIT seem to be involved in PTC pathogenesis, and sequence changes in miRNA target genes can contribute to their regulation.

c-KIT is frequently expressed in benign thyroid adenomas and goiters, while its expression results in being lowered in about 60% of FTCs and completely absent in PTCs and ATCs. Moreover, the absence of c-KIT expression has been demonstrated also in metastases from primary thyroid tumors, indicating that the modulation of this tyrosine kinase receptor is not dependent on the thyroid microenvironment but is associated to the transformed malignant cell phenotype [46]. To date, the biological significance of loss of c-KIT in thyroid tumors is not elucidated. Surprisingly, the depletion of c-KIT expression in thyroid tumors in contrast with the gain of function of other tyrosine kinase

Table 2: Studies of deregulation of miRNA expression profile in thyroid tumors. The table reports a list of published studies on the deregulation of miRNA expression profile in different kinds of thyroid tumors. Type of analyzed thyroid samples, used analysis methods and individuated upregulated or downregulated miRNAs in thyroid tumors are depicted in the table.

Thyroid tumor type	Analyzed samples	Methods	Tumor upregulated miRNAs	Tumor downregulated miRNAs	Reference
PTC	20 fresh PTC tissues versus 20 normal adjacent normal thyroid tissues	Global miRNA microarray, quantitative RT-PCR and Northern blots	miR-146b, miR-221, and miR-222	—	He et al. [26]
PTC	30 fresh PTC tissues versus 10 normal thyroid tissues	Global miRNA microarray, quantitative RT-PCR, and Northern blots	miR-181b, miR-221, and miR-222	—	Pallante et al. [27]
PTC	20 formalin-fixed paraffin-embedded PTC tissues versus 20 formalin-fixed paraffin-embedded multinodular goiter	Global miRNA microarray and quantitative RT-PCR	miR-21, miR-31, miR-34a, miR-172, miR-181a, miR-181b, miR-213, miR-221, miR-222, miR-223, and miR-224	miR-19b-1,2, miR-30a-5p, miR-30c, miR-130b, miR-145sh, miR-218, miR-292-as, miR-300, and miR-345	Tetzlaff et al. [28]
PTC versus non-PTC	84 formalin-fixed paraffin-embedded tissues and 40 *ex vivo* aspirate specimens of PTC and non-PTC tumors (follicular adenoma, follicular carcinoma hyperplastic nodules)	Quantitative RT-PCR	miR-146b, miR-221, and miR-222	—	Chen et al. [29]
PTC	28 *BRAF* mutated PTC tissues versus 26 *BRAF* wild-type PTC tissues	Quantitative RT-PCR	No difference between mutated and nonmutated PTC	No difference between mutated and nonmutated PTC	Sheu et al. [31]
PTC	100 PTC tissues versus 16 paired normal thyroid control	Quantitative RT-PCR	miR-146b, miR-221, and miR-222	—	Chou et al. [32]
PTC	2 PTC cell lines bearing a *RET* mutation versus normal thyroid cell lines	Global miRNA microarray	miR-34a, miR-96, miR-99a, miR-100, miR-125b, miR-128b, miR-130b, miR-139, miR-141, miR-142-3p, miR-146, miR-148, miR-185, miR-200a, miR-200b, miR-211, miR-213, miR-216, and let-7d	miR-15a, miR-34c, miR-107, miR-127, miR-135b, miR-145, miR-149, miR-154, miR-181a, miR-218, miR-299, miR-302b, miR-302c, miR-323, and miR-370	Cahill et al. [40]

Table 2: Continued.

Thyroid tumor type	Analyzed samples	Methods	Tumor upregulated miRNAs	Tumor downregulated miRNAs	Reference
FTC	22 FTC samples versus 20 FA and 4 normal control thyroid tissues	Global miRNA microarrayand quantitative RT-PCR	miR-192, miR-197, miR-328, and miR-346	—	Weber et al. [33]
FTC PTC, other thyroid tumor variants	A 60-fresh-thyroid tumor and normal samples (23 PTCs, 9 FTCs, 8 FAs, 4 ATCs, 4 poorly differentiated carcinomas, 2 MTCs, 5 hyperplastic nodules, and 5 normal thyroid tissues) and 62 fine-needle aspiration samples	Quantitative RT-PCR	PTC: miR-31, miR-122a, miR-146b, miR-155, miR-187, miR-205, miR-221, miR-222, and miR-224 Conventional FA: miR-190, miR-205, miR-210, miR-224, miR-328, miR-339, and miR-342 Oncocytic FA: miR-31, miR-183, miR-203, miR-221, miR-224, and miR-339 Conventional FTC: miR-146b, miR-155, miR-187, miR-221, miR-222, and miR-224 Oncocytic FTC: miR-183, miR-187, miR-197, miR-221, miR-222, and miR-339 Poorly differentiated carcinomas: miR-129, miR-146b, miR-183, miR-187, miR-221, miR-222, and miR-339 ATC: miR-137, miR-155, miR-187, miR-205, miR-214, miR-221, miR-222, and miR-224 MTC: miR-9, miR-10a, miR-124a, miR-127, miR-129, miR-137, miR-154, miR-224, miR-323, and miR-370	—	Nikiforova et al. [25]
ATC	ATC tissues versus normal thyroid tissues	Global miRNA microarray, quantitative RT-PCR, Northern blots, and in situ hybridization	—	miR-26a, miR-30a-5p, miR-30d, and miR-125b	Visone et al. [34]
ATC	10 ATC and 5 FTC cell lines, 3 ATC and 8 PTC tissues versus normal thyroid samples	Quantitative RT-PCR	miR-21, miR-146b, miR-221, and miR-222	miR-26a, miR-138, miR-219, and miR-345	Mitomo et al. [38]
ATC	ATC cell lines and ATC cancer lesions versus normal thyroid tissues	Global miRNA microarray, quantitative RT-PCR, and Northern blots	miR-17-3p, miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92-1	—	Takakura et al. [37]
ATC	ATC tissues versus PTC and FTC tissues	Global miRNA microarray and quantitative RT-PCR	—	miR-30 and miR-200	Braun et al. [35]

receptors such as c-RET and c-MET or of oncogenes such as *c-RAS*, suggesting that different tyrosine kinase receptor signaling pathways may exert opposite biological effects in a given cell type, alternatively controlling mitogenesis or cell differentiation. It is hypothesizable that, in thyroid tissue, the c-KIT signaling pathway may control some aspects of the thyrocyte differentiation rather than cell proliferation.

Pallante et al. [27] analyzed the global miRNA expression profile in 30 human PTCs versus 10 normal thyroid tissues using a microarray chip containing 368 human precursor and mature miRNA oligonucleotide probes, accounting for 245 human and mouse miRNA genes and including all the three miR-181 human isoforms (miR-181a, miR-181b, and miR-181c). The analysis revealed an altered miRNA expression profile that distinguished PTCs from normal thyroid tissues: five miRNAs (miR-221, miR-222, miR-213, miR-220, and miR-181b) were overexpressed in the neoplastic tissues. However, a recent work by Chiang et al. [47] strongly suggested that miR-220 is not a miRNA, and miR-213 is not recognized as a miRNA in the miRNA database (http://www.mirbase.org/). The study of Pallante et al. found that miR-221, miR-222, and miR-181b resulted in being abundantly expressed in PTCs, while their expression was only weakly detectable in the healthy thyroid tissues, with the overexpression of miR-221 reaching up to 70-fold increase. overexpression of miR-181b has been also confirmed by quantitative RT-PCR analysis using a couple of primers specifically designed for precursor of the miR-181b isoform to exclude a false-positive result for miR-181b, due to possible cross-hybridization of probes for the miR-181a and miR-181c isoforms in the microarray chip. The authors also demonstrated that miR-221 and miR-222 downregulated the level of c-KIT in PTCs confirming data previously published [41]. In addition, a human thyroid carcinoma cell line transfected with a vector over-expressing miR-221 showed a significant increase of cell growth, and, conversely, the inhibition of miR-221 function by antisense oligonucleotide transfection in the same cell line resulted in a significant reduction of cell growth [27]. The results of these functional studies, together with the previously reported expression data, suggest a critical role of miR-221 in thyroid carcinoma cell growth and, thus, probably in the process of papillary thyroid carcinogenesis.

A study of Tetzlaff et al. [28] analyzed global miRNA expression profile by microarray chip (including known miRNAs from human, mouse, rat, and predicted/candidate human miRNAs) in a series of PTC formalin-fixed paraffin-embedded (FFPE) samples versus benign proliferative multinodular goiters (MNGs). The analysis revealed a set of 13 upregulated miRNAs and a set of 26 downregulated miRNAs. Misregulation has been validated by real-time RT-PCR, in an independent series of tumoral tissues, only for miR-21, miR-31, miR-221, and miR-222, with miR-221 and miR-222 showing a strong upregulation in PTCs compared to independent set of MNGs. Data from this study confirmed miR-221 and miR-222 to be altered in PTCs, as previously described in fresh tissue analyses [26, 27] and, mostly, supported the possibility to use FFPE tumor tissues to analyze miRNA expression when fresh tissues are not available or to perform retrospective studies. The author demonstrated the possibility to extract sufficient miRNA from FFPE tissues using a miRNA labeling method for total RNA extracts (All Total Nucleid Acid Isolation SyStem, Ambion) that bypassed the need for miRNA enrichment and reduced the amount of starting samples, followed by an ammonium acetate/ethanol precipitation.

A recent study by Chen et al. [29] analyzed the expression of a selected set of miRNAs by quantitative RT-PCR in PTCs, non-PTC lesions (follicular adenoma, follicular carcinoma hyperplastic nodules), and normal thyroid tissue. They evidenced an overexpression of miR-146b, miR-221, and miR-222 in PTCs compared to both non-PTC group and healthy controls, but with miR-221 and miR-222 presenting a substantial overlap between different tumor groups. The authors concluded that the expression analysis of miR-221 and miR-222 cannot be resolving in distinguishing PTCs from other tumoral lesions. Conversely, miR-146b resulted in being consistently and specifically overexpressed in classical PTCs, suggesting this miRNA as a possible diagnostic tool to identify PTCs from other thyroid tumors. Putative targets of miR-146b are the nuclear factor-κB (*NF-κB*), the interleukin-1 receptor-associated kinase 1 (*IRAK1*), and the tumor necrosis factor receptor-associated factor 6 (*TRAF6*), whose role in thyroid carcinogenesis has not yet been elucidated.

Mutations of the *BRAF* gene are implicated in the pathogenesis of PTC through the constitutive activation of the MAPK pathway; classical PTCs are often *BRAF* mutation positive while follicular PTCs are almost always *BRAF* mutation negative [48]. Chen et al. [29] found also that miR146b overexpression is common both in classical and follicular variants of PTCs, independently by the *BRAF* mutation status, suggesting this overexpression as a late event in the PTC progression and probably important for the definition of a complete carcinoma phenotype. This result has been confirmed also by Sheu et al. [31] who analyzed, in a series of 221 PTCs, if the *BRAF V600E* mutational status was correlated to the miRNA expression profile. They found no difference in the expression of five miRNAs (miR-146b, miR-181b, miR-21, miR-221, and miR-222) between *BRAF* mutated PTCs and wild-type PTCs. In addition, this study confirmed the overexpression of miR-146b, miR-221, and miR-222 as a useful tool to diagnostic PTCs.

More recently, Chou et al. [32] measured the expression of miR-146b, miR-221, and miR-222 in 100 cases of PTCs finding that their expression levels were associated with extra-thyroidal invasion. In particular miR-146b resulted in being highly expressed in tumors with high risk features and with the *BRAF V600E* mutation.

Results from all these studies agreed with the fact that miR-221 and miR-222 are both overexpressed in PTCs compared to normal thyroid tissue. Functional analysis on miR-221 function in human PTC-derived cell lines indicated a direct role of this miRNA in PTC carcinogenesis. A vector-induced miR-221 overexpression in these cell lines resulted in a higher number of cell colonies compared to negative control transfected cells; conversely, the block of miR-221 function by antisense oligonucleotide caused a significant reduction in cell proliferation [27]. A subsequent

study investigated which pathways or molecular targets were regulated by miR-221 and miR-222 [49]. Some bioinformatic programs suggested the *CDKN1B* (*p27^{kip1}*) gene, an important regulator of cell cycle that inhibits the initiation of the S phase, as the putative target of miR-221 and miR-222. This study demonstrated that miR-221 and miR-222 negatively regulate the p27^{kip1} protein levels in Hela cells and thyroid carcinoma cells by binding to two specific target sites in the 3′-UTR of the *p27^{kip1}* gene. Transfection of TPC-1, thyroid papillary carcinoma cell line, with vectors for the overexpression of miR-221 and miR-222 resulted in decreased p27^{kip1} protein levels. Conversely, inhibition of miR-221 and miR-222 expression by specific 2′-O-Me-221 and 2′-O-Me-222 antisense oligonucleotides increased the p27^{kip1} protein levels. In both transfection experiments no significant variation of p27^{kip1} mRNA expression was observed. Results from this study strongly suggested that the overexpression of miR-221 and miR-222 in tumor thyroid cells could be responsible for the posttranscriptional negative regulation of p27^{kip1} protein expression, inducing cells to enter the S phase of cell cycle and, thus, increasing cell growth.

The *RET* oncogene mutations occur in about 43% of PTCs [50], constitutively activating the MAPK signal and promoting carcinogenesis. These mutations are responsible for the deregulation of thyroid cell proliferation and differentiation and for cell tumoral transformation. Cahill et al. [40] investigated miRNA expression in two human PTC cell lines bearing a *RET* mutation, compared to normal thyroid cell lines, finding that 21 miRNAs were significantly overexpressed (miR-34a, miR-96, miR-99a, miR-100, miR-125b, miR-128b, miR-130b, miR-139, miR-141, miR-142-3p, miR-146, miR-148, miR-185, miR-200a, miR-200b, miR-211, miR-213, miR-216 and let-7d) and 14 miRNAs were downregulated (miR-15a, miR-34c, miR-107, miR-127, miR-135b, miR-145, miR-149, miR-154, miR-181a, miR-218, miR-299, miR-302b, miR-302c, miR-323, and miR-370) in tumor cell lines when compared to normal thyroid. These differentially expressed miRNAs potentially regulate genes involved in thyroid functions, and their deregulation could be implicated in thyroid carcinoma progression.

A functional study of Ricarte-Filho et al. [36] investigated the involvement of the let-7f miRNA, recently associated to RAS protein level reduction in lung tumor, in PTC development. The authors found that, in thyroid *RET*-mutated cell lines, the enhanced expression of *RET* oncogene reduced the expression of let-7f. The stable transfection of *RET*-mutated TPC-1 cell line with vector for the overexpression of let-7f inhibited the MAPK activation and reduced cell proliferation. In particular, let-7f increases the expression of thyroid cell differentiation markers such as the TITF1 transcription factor, a key factor in normal thyroid development, and, thus, let-7f is fundamental for the correct regulation of thyroid cell growth and differentiation and the reduced expression of let-7f in *RET*-mutated thyroid cells is responsible for cell dedifferentiation during PTC malignant progression. Interestingly, let-7f, together with the miRNA let-7 family, is one of the most expressed miRNAs in healthy thyroid gland (Table 1), further suggesting its crucial role in normal thyroid development and functionality. All these data suggested let-7f acting as a tumor suppressor and indicated this miRNA as a potential therapeutic agent in patients with PTCs bearing a *RET* mutation.

6. The Role of MicroRNAs in Follicular Thyroid Carcinoma

Only two studies have analyzed the miRNA expression alteration in FTCs [25, 33] (Table 2).

In 2006 Weber et al. [33] investigated if miRNAs are differentially expressed between human follicular thyroid carcinomas (FTCs) and follicular adenomas (FAs) testing two high-density miRNA expression arrays on 23 FTCs versus 20 FA samples and 4 normal thyroid controls. Four miRNAs (miR-192, miR-197, miR-328, and miR-346) resulted in being overexpressed in FTCs compared to FAs. None of these miRNAs have previously been associated with other thyroid neoplasia and appear to be specific for FTC phenotype. Two of them, miR-197 and miR-346, have been validated also by quantitative real-time RT-PCR that confirmed their significant overexpression in carcinomas compared to adenomas and healthy tissue. These two miRNAs may participate in the transformation of follicular tumors from benign to malignant status, and they and their target genes may provide novel molecular markers to differentiate malignant (FTCs) from benign (FAs) follicular thyroid neoplasia. The effects of these two deregulated miRNAs have been functionally investigated using two human thyroid cancer cell lines, FTC133 and K5, a human papillary thyroid cancer cell line, NPA87, and a human embryonic kidney cell line, HEK293T as control. The induction of miR-197 and miR-346 overexpression induced cell proliferation *in vitro*, whereas their inhibition led to cell growth arrest in both FTC133 and K5 cell lines, but not in NPA87 cell line, confirming that the deregulation of miR-197 and miR-346 is a marker of FTC phenotype but not of PTC phenotype. *In silico* analyses indicated putative targets of miR-197 and miR-346 that have been validated also by *in vitro* functional analyses. *EFEMP2* (fibulin 4), a protein involved in stabilization and organization of extracellular matrix structures that exerts tumor suppressor functions [51, 52], is inhibited by miR-346 overexpression. Activin A receptor type 1 (*ACVR1*), a potent inhibitor of cell growth in several human cell types including thyroid epithelium [53], and tetraspanin 3 (*TSPAN3*), whose exact biological role in tumors is still unknown, are both downexpressed as a consequence of miR-197 overexpression. Moreover, the authors performed functional studies also on miR-221 and miR-222 demonstrating that they do not have a role in FTC tumorigenesis.

More recently, Nikiforov et al. [25] compared miRNA expression profiles of principal types of thyroid cancers, finding a distinctive expression pattern associated to follicular thyroid tumors: miR-155, miR-187, miR-221, miR-222, and miR-224 resulted in being highly overexpressed in conventional FTCs, while miR-183, miR-187, miR-197, miR-221, miR-222, and miR-339 were overexpressed in the FTC oncocytic variants.

7. The Role of MicroRNAs in Anaplastic Thyroid Carcinoma

Few studies have analyzed the miRNA expression profiles in ATCs [34, 35, 37, 38] (Table 2).

Visone et al. [34] analyzed the miRNA expression of ATCs using a miRNA microarray chip, finding a significant down-expression of miR-26a, miR-30a-5p, miR-30d, and miR-125b in ATCs compared to normal thyroid samples. These data have been further validated by quantitative RT-PCR, Northern blot analyses, and *in situ* hybridization. Induced overexpression of miR-26a and miR-125b in two human ATC-derived cell lines resulted in cell growth inhibition, suggesting a role of these two miRNAs in negative cell cycle regulation and that their downregulation could be involved in thyroid tumorigenesis. No effect on cell proliferation was observed after induction of miR-30d and miR-30a-5p overexpression in the same ATC-derived cell lines. miR-26a influences cell cycle progression by negatively regulating the expression of *EZH2* oncogene, an epigenetic gene silencer involved in neoplastic development. Recently, Sander et al. [54] suggested miR-26a acting as a potential tumor suppressor in MYC-induced tumors, since overexpression of miR-26a in murine lymphoma cell lines reduced cell proliferation by increasing the percentage of cells in G1 phase. miR-125b resulted in being deregulated in several human tumors, suggesting a role of miR-125b in human carcinogenesis [55–57]. An upregulation of miR-125b expression reduced the proliferation of the CD133-positive glioma cells and arrested the cell cycle at the G1/S transition level [58], through the downregulation of CDK6 and CDC25A, respectively, a cyclin-dependent kinase positively regulating the transition from the G0/G1 phase to the S phase of the cell cycle and a positive regulator of G1/S transition by dephosphorylation and activation of cyclin-CDK complexes. Both CDK6 and CDC25A have been previously reported to modulate the G1/S transition in human embryonic stem cells [59]. Recently, Liang et al. [60] demonstrated that miR-125b suppresses hepatocarcinoma cell proliferation both *in vitro* and *in vivo* and that this miRNA increases the expression of p21Cip1/Waf1, arresting cell cycle at the G1 phase.

Another study [38] examined the expression of miRNAs in ATC-derived cell lines versus PTC-derived cell lines as well as ATC tissue samples versus PTC tissue samples, finding that miR-21, miR-146b, miR-221, and miR-222 were overexpressed, while miR-26a, miR-138, miR-219, and miR-345 were downregulated in ATC cell lines and tissues. Moreover, since miR-138 resulted in being the unique miRNA that showed a different expression profile between ATCs and other follicular cell-derived thyroid tumors, the authors investigated its putative role in ATC tumorigenesis, demonstrating that miR-138 could directly target the human telomerase reverse transcriptase (*hTERT*) gene at posttranscriptional level. hTERT is a catalytic subunit of telomerase that results in being overexpressed in primary ATCs compared to PTCs and associated with cell dedifferentiation and increased metastatic potential [61]. The upregulation of hTERT, subsequent to miR-138 downregulation, could be responsible for malignant progression of well-differentiated PTCs toward undifferentiated ATCs. Since miR-138 downregulation seemed to be strongly associated with the ATC phenotype, this miRNA could be useful as a diagnostic tool for ATC recognition and it may contribute to the development of novel treatment strategy for ATCs.

Takakura et al. [37] reported a group of seven miRNAs (miR-17-3p, miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92-1), compressively named as miR-17-92 cluster, to be overexpressed in ATC cell lines and ATC cancer lesions compared to adjacent thyroid normal tissues. To investigate functional role of these deregulated miRNAs in ATC tumorigenesis, human ATC cell lines have been induced to silence these miRNAs expression by transfection with specific miRNA antisenses. Inhibition of miR-17-3p expression totally suppressed cell growth and induced apoptosis by the strong activation of caspases 3 and 9. Suppression of miR17-5p or miR-19a caused a strong reduction of cell proliferation, not associated with apoptosis or caspase activation. Moreover, inhibition of miR17-5p, but not of miR-19a, was also responsible for cell senescence. miR-17-5p and miR-19a target, respectively, retinoblastoma 1 (*RB1*) and phosphatase and tensin homolog (*PTEN*) genes as confirmed by the fact that the expression of RB1 and PTEN proteins was increased in cells transfected with miR-17-5p and miR-19a inhibitors. Mutations of *PTEN* gene are associated with Cowden syndrome characterized by breast and thyroid tumors. *PTEN* acts as a tumor suppressor, negatively regulating cell growth by the repression of the cyclin-dependent kinase inhibitor $p27^{kip1}$ expression. A reduced expression of PTEN protein is associated with thyroid cancer development [62, 63], and PTEN inactivation is associated with undifferentiated malignant ATCs, rather than other thyroid tumor types [64]. Therefore, the overexpression of miR-19a (and of its isoform miR-19b) might be responsible for *PTEN* posttranscriptional downregulation and subsequent increased cell growth in ATCs. In addition, downregulation of tumor suppressor activity of *RB1* by overexpression of miR-17-5p could further contribute to increased tumor cell proliferation. Selective inhibitors of miR-17-3p, miR-17-5p, and miR-19a demonstrated to significantly reduce cell growth in ATC-derived cell lines, and inhibitor of miR-17-3p also induced cell death. Therefore, these inhibitors could represent valid therapeutic approaches for the treatment of ATCs.

Very recently, Braun et al. [35] identified two miRNAs, miR-30 and miR-200, which were significantly downexpressed in ATCs compared to PTCs and FTCs. overexpression of these two miRNAs in mesenchymal ATC-derived cell lines reduced their invasive and metastatic potential, negatively regulating the expression of the mesenchymal-epithelial transition (MET) proteins. Conversely, inhibition of endogenous miR-200 expression was sufficient to induce the inverse process, the epithelial-mesenchymal transition (EMT) responsible for tumor cell invasiveness and metastatic potential. miR-200 targets the *ZEB1* and *ZEB2*, both repressors of the E-cadherin (*CDH1*) gene expression. Park et al. [65] demonstrated, in 60 human cell lines conserved at the Nation Cancer Institute, a significant association between miR-200 expression and E-cadherin-vimentin ratio. Induced overexpression of miR-200 caused the upregulation of

E-cadherin in tumor cell lines and reduced their motility and invasiveness. Conversely, the inhibition of miR-200 reduced E-cadherin expression, increased expression of vimentin, and induced EMT. All these data suggested miR-200 as a key factor of the epithelial phenotype in cancer cells that could be used as therapeutic agent to reduce the rates of invasive and metastatic thyroid carcinomas.

8. The Role of MicroRNAs in Medullary Thyroid Carcinoma

Only one study [25] has analyzed the miRNA expression in MTCs compared to other thyroid tumors and normal thyroid tissue, finding a set of 10 specific miRNAs (miR-9, miR-10a, miR-124a, miR-127, miR-129, miR-137, miR-154, miR-224, miR-323, and miR-370) upregulated in this tumor type. No functional studies have been yet performed.

9. Conclusion and Future Perspectives

miRNAs are powerful key regulators of gene expression in many fundamental cellular processes such as proliferation, differentiation, and apoptosis. Deregulation of miRNA expression was observed in the initiation, development, and malignant progression of numerous human tumors [2–10]. miRNA expression profiles resulted in being different not only between tumors and healthy tissues but also between different histopathological lesions of the same tissue, between tumors at different stages of malignancy, and between primary tumors and metastases. Therefore, miRNA expression profiles may become useful novel biomarkers for tumor diagnostic and histological characterization. Recent findings suggested that miRNA expression profiles could enable classifying poorly characterized human tumors that can not be accurately classified only by the classical mRNA expression patterns [66]. Moreover, since differences in miRNA expression are, in some cases, associated with the prognosis, analysis of miRNA expression profiles could help also in the therapeutic management of patients.

Very recent data supported the possibility to use circulating miRNAs (plasma, serum, urine, or other body fluids) as a novel class of biomarkers to diagnose tumors, as expression patterns of circulating miRNAs are different between normal tissues and cancers and peculiar miRNA expression profiles are specifically associated with certain types of tumors [67]. In addition, circulating miRNAs present the advantage to be stable molecules with a great resistance to RNase activity that can be easily dosed by noninvasive techniques.

Moreover, since miRNAs regulate cancer cell proliferation, differentiation, apoptosis, and invasiveness, miRNAs and their biological targets could be potential targets of therapeutic genetic strategies in human tumors to interfere with cancer initiation and progression. miRNAs are possible targets for a RNA-based therapy both by positively modulating the expression of specific miRNAs *in vivo* using expression vectors [68] and/or by inhibiting miRNA expression by transfecting specific 2′-O-methyl-modified antisense RNA (antagomirs) [69].

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Review Article

Role of Estrogen in Thyroid Function and Growth Regulation

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Thyroid diseases are more prevalent in women, particularly between puberty and menopause. It is wellknown that estrogen (E) has indirect effects on the thyroid economy. Direct effects of this steroid hormone on thyroid cells have been described more recently; so, the aim of the present paper was to review the evidences of these effects on thyroid function and growth regulation, and its mechanisms. The expression and ratios of the two E receptors, α and β, that mediate the genomic effects of E on normal and abnormal thyroid tissue were also reviewed, as well as nongenomic, distinct molecular pathways. Several evidences support the hypothesis that E has a direct role in thyroid follicular cells; understanding its influence on the growth and function of the thyroid in normal and abnormal conditions can potentially provide new targets for the treatment of thyroid diseases.

1. Introduction

Thyroid diseases are more prevalent in women particularly between puberty and menopause [1], and women are more susceptible to the goitrogenic effect of iodine deficiency [2]. Carcinomas of the thyroid are three-times more frequent in women than in men, and the peak rates occur earlier in women [3]. These epidemiological data suggest a role of estrogen in the pathogenesis of thyroid diseases.

Estrogen has a well-known indirect effect on thyroid economy, increasing the thyroxine binding globulin [4], and the need for thyroid hormone in hypothyroid women [5]. Direct effects of estrogen on thyroid cells have been described more recently [6], so the aim of the present paper was to review the evidences of these effects on thyroid function and growth regulation, and its mechanisms.

2. Estrogen and Its Receptors

17-β-estradiol (E2) is a lipophilic hormone with low-molecular weight that occurs naturally. Cellular signaling of estrogen is mediated classically upon the binding on two soluble intracellular nuclear receptors, estrogen receptor (ER) alpha, and ER beta [7]. The isoform β is smaller than the isoform α, and the DNA-binding domains of both subtypes are highly conserved. After binding of E2, ER forms a stable dimer that interacts with specific sequences called estrogen response elements (EREs) to initiate the transcription of target genes. Ligand-bound ERs can also interact with other transcription factors complexes and influence transcription of genes that do not harbor EREs. Third and fourth mechanisms of ERs regulatory actions are, respectively, non-genomic and the ligand independent pathway. A variety of rapid signaling events such as activation of kinases and phosphatases and increases in ion fluxes across membranes has been described. These and other aspects of signaling and targets of ERs have been reviewed recently [7].

Recently, a transmembrane intracellular nonclassical ER mediating rapid cell signaling was described, a G protein-coupled receptor (GPCR), named GPR30 [8].

2.1. Expression of ERs in Human Thyroid Tissue. Classically, the presence of ER is fundamental for a direct action of estrogen in a given cell. ER has been described in both neoplastic and nonneoplastic human thyroid tissues, but the results are discordant. Immunohistochemical assays,

Table 1: Estrogen receptor (ER) in human normal thyroid, and benign and malignant thyroid diseases.

Study	Method	Normal	All benign lesions	All neoplastic lesions	All carcinoma	Benign lesions		Carcinoma			
						Adenoma	Goiter	Papillary	Follicular	Medullary	Anaplastic
Tavangar et al. [10]; 2007	IHC					8/37	31/130	37/119	2/18	0/35	0/12
Arain et al. [11]; 2003	IHC	0/25				0/9	0/8	0/19	0/10	0/4	
Lewy-Trenda et al. [12]; 1998	IHC					2/19	0/20	4/8	3/5		0/4
Valle et al. [13]; 1998	RT-PCR	28/33				12/12	6/7	26/26	1/1	1/1	1/1
Bonacci et al. [14]; 1996	DCC	26/38	11/28		7/20						
Jaklic et al. [15]; 1995	IHC					0/1	0/5	0/4		0/1	
Colomer et al. [16]; 1996	IHC									1/7	
Inoue et al. [17]; 1993	IHC							24/74			
Inoue et al. [18]; 1993	IHC							18/70			
Yane et al. [19]; 1994	RT-PCR			5/27							
Yane et al. [20]; 1993	IHC	0/10			2/19	2/12	0/7				
Hiasa et al. [21]; 1993	IHC					44/130	23/39	19/115	7/23		0/6
Diaz et al. [22]; 1991	IHC					20/30		23/30	11/20		
Mizukami et al. [23]; 1991	IHC		8/18			4/8		47/62			
Takeichi et al. [24]; 1991	IHC							11/12			1/6
Hong et al. [25]; 1991	IHC							1/27	1/20		
Miki et al. [26]; 1990	DCC	0/14	12/46		7/23	5/11	2/12	6/20	0/1		1/1
Haruta et al. [27]; 1990	IHC							30/52			0/12
Chaudhuri et al. [28]; 1989	SDG	3/8				7/9	5/23	8/8		0/6	
Money et al. [29]; 1989	IHC		20/22								
Clark et al. [30]; 1985	SDG			14/15							
Hampl [15]; 1985	RBA	0/8			0/5						
Molteni et al. [37]; 1981	SDG	0/2						2/4			

Data are shown as number of ER-positive samples/total number of samples. IHC: immunohistochemical assay; DCC: dextran-coated charcoal assay; RT-PCR: reverse transcriptase-polymerase chain reaction technique; SDG: sucrose density gradient assay; RBA: radioligand binding assay.

Table 2: Estrogen receptors (ER) α and β in human normal thyroid, and benign and malignant thyroid diseases, by immunohistochemistry (IHC).

Study	Isoform	All benign	All carcinoma	Benign lesions		Carcinoma			
				Adenoma	Goiter	Papillary	Follicular	Medullary	Anaplastic
Vaiman et al. [31]; 2010	ERα			0/34	0/150	0/90	0/6	0/4	0/5
	ERβ			30/34	126/150	60/90	4/6	3/4	3/5
Winters et al. [32]; 2010	ERα					1/1			
Vannucchi et al. [33]; 2010	ERα		12/38						
Cho et al. [34]; 2007	ERα							10/11	
	ERβ							8/11	
Bléchet et al. [35]; 2007	ERα							0/28	
	ERβ							26/28	
Ceresini et al. [36]; 2006	ERα				0/17	0/17			
	ERβ				17/17	14/17			

Data are shown as number of ER-positive samples/total number of samples.

with monoclonal antibodies, are the most commonly used methods for establishing receptor status. As may be seen in Table 1, some studies have found ER-positivity in normal and abnormal thyroid tissue while others have not detected ER protein in any tissue studied. This discrepancy could be due to methodological issues; the development of monoclonal antibodies against ER with high sensitivity and specificity, and others factors such as tissue fixation, tissue processing, interpretation of immunohistochemistry, and *cutoffs* for positive results, could have contributed to the sensitivity of the techniques employed [9].

2.2. Expression of ERα and ERβ in Human Thyroid Tissue. ER expression in human thyroid was first reported in 1981 [37]. ERα was first described in 1973 [38], and ERβ was identified in 1996 [39], so only from this moment on it was possible to evaluate the relationship between isoforms of ERs in thyroid tissue. An important role of different patterns of distribution and expression of subtypes ERs in thyroid carcinoma has been proposed: estrogen binding to ERα would promote cell proliferation and growth, and, in contrast, ERβ would promote apoptotic actions and other suppressive functions in thyroid tumors, as reviewed by Chen et al. [40]. Then, ERα : ERβ ratio could have a role in the pathophysiology of thyroid cancer [40], similar to that postulated for breast cancer [41].

In differentiated thyroid follicular tumors, the expression of ERα has been associated with well-differentiated tumors and reduced incidence of disease recurrence [54]. ERα protein [55] and ERα mRNA [19, 56] are expressed in normal and neoplastic follicular cells of the thyroid. Also, the expression of ERα and ERβ was detected in human medullary thyroid cancer [34] with an increased ratio of ERα/ERβ, suggesting a possible role in tumor growth and progression. A few studies evaluated ERα and ERβ expression in normal and abnormal thyroid tissue, as shown in Table 2.

The effects of the agonists of ERα and ERβ, respectively, propyl-pyrazole-triol (PPT) and diarylpropionitrile (DPN), in the proliferation of thyroid cancer cell lines has been studied: PPT had a stimulatory effect, while inhibition of proliferation and DNA fragmentation were observed after DPN [45]. In the same study, small interference ribonucleic acid (siRNA) blocking ERα or ERβ demonstrated that knockdown of the ERα attenuated E2-mediated B-cell lymphoma 2 (Bcl-2) expression, an important antiapoptotic protein, while knockdown of the ERβ enhanced E2-induced Bcl-2 expression [45].

2.3. Expression of GPR30 in Thyroid Cells Lines. Growing evidence suggests that estrogens are also able to exert non-genomic events mediated by GPR30 [8]. Vivacqua and colleagues analyzed the effects of E2 and the phytoestrogen genistein in human follicular thyroid carcinoma cell lines, WRO and FRO, and ARO, a human anaplastic thyroid carcinoma cell line [46]. Both hormones stimulated *in vitro* proliferation of these cell lines through the GPR30 and mitogen-activated protein kinase signaling cascade [46]. In other human benign and malignant thyroid tissue, the expression of GPR30 has not been studied.

3. Response to E2 Stimulation *In Vitro*

3.1. Proliferation. Several studies described proliferation of thyroid cells induced by E2, as shown in Table 3. Some of the most commonly used assays are incorporation of bromodeoxyuridine (BrdU) [6], 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) [45, 47, 50, 57], [(3)H]-thymidine incorporation [48, 52, 53], and trypan blue solution [43]. Cotreatment with ICI182780, fulvestrant, an antagonist of E2 by inhibition and degradation of ER [58], significantly attenuated these proliferative effects.

Based in these studies, E2 increases proliferation of thyroid cells.

3.2. ER-Dependent Effects on Thyroid Differentiation Proteins. Few studies evaluated E2 effect on gene transcription of

Table 3: E2 effects on thyroid protein expression, function, and proliferation *in vitro*.

Study	Thyroid cells	Presence of ERα/ERβ	Erα expression	Erβ expression	Proliferation	Nis expression	Iodide uptake	TG mRNA
Kumar et al. [42]; 2010	NPA87	ERα+/ERβ+			↑			
	KAT5	ERα+/ERβ+			↑			
	WRO	ERα+/ERβ+			↑			
Rajoria et al. [43]; 2010	BCPAP	ERα+/ERβ+			↑			
	Nthy-3-1	ERα+/ERβ+			↑			
Zeng et al. [44]; 2008	KAT5	ERα+/ERβ+	↑	0	↑			
	FRO	ERα+/ERβ+	0	↑	↓			
Zeng et al. [45]; 2007	KAT5	ERα+/ERβ+	↑	0	↑			
	FRO	ERα+/ERβ+	↑	↑	↑			
	ARO	ERα+/Erβ+	↑	0	↑			
Vivacqua et al. [46]; 2006	WRO	ERα+/ERβ−			↑			
	FRO	ERα+/ERβ−			↑			
	ARO	ERα−/ERβ−			↑			
Lee et al. [47]; 2005	KAT5				↑			
Banu et al. [48]; 2001	NPA87	ER+		↑	↑			
	WRO	ER+			↓			
Manole et al. [6]; 2001	HTC-TSHr	ERα+/ERβ+	↑	↑	↑			
	Goiter	ERα+/ERβ+	↑	↑	↑			
	XTC 133				↑			
Furlanetto et al. [49]; 2001	FRTL-5				↑		↓	
Furlanetto et al. [50]; 1999	FRTL-5	ERα+			↑	↓		
Nagy et al. [51]; 1999*	Mng				↑			
	Ca				↑			
	Ade				↓			
Del Senno et al. [52]; 1989**	N				↑			↑
	Ade				↑			↑
	Ca				0			0
Yang et al. [53]; 1988	TT				↑			

Estrogen receptor (ER) +: presence of expression, –: absence of expression; NPA87, KAT5, and BCPAP: human papillary thyroid carcinoma cell lines; WRO and FRO: human follicular thyroid carcinoma cell lines; Nthy-3-1: human normal transformed thyroid cell line; ARO: human anaplastic thyroid carcinoma cell line; HTC-TSHr: human thyroid carcinoma cell line lacking endogenous TSH receptor; XTC-133: thyroid cancer cell line of Hurthle cell origin; FRTL-5: Fischer rat thyroid cell line. Mng: multinodular goiter; Ca: carcinoma; Ade: adenoma; N: normal thyroid; TT: human medullary thyroid carcinoma cell line; ↑: increase, ↓: decrease, and 0: no effect, after E2 exposure. *: thyroid tissue obtained in surgical resection, under organotypic culture conditions for 48 hours; **: suspension cultures of thyroid follicles.

differentiation proteins in thyroid cells. In Fischer rat derived thyroid cell line, FRTL-5, E2 treatment decreased the sodium-iodide symporter (NIS) gene expression [50], and the iodide uptake [49]. E2 increased the thyroglobulin gene expression in suspension cultures of human thyroid follicles of adenoma and carcinoma [52]. These data are shown in Table 3. The opposite effects of E2 on the NIS gene expression and iodide uptake, in FRTL-5 cells, and the thyroglobulin gene expression, in suspension culture of thyroid cells, could be due to the different systems studied; it cannot be excluded that estradiol affects these genes by different intracellular pathways. These results, together with the increase in cell growth caused by estrogen, could implicate this hormone in the pathogenesis of goiter and thyroid carcinoma; nevertheless, as just one study evaluated the effect of estrogen on thyroid differentiated proteins in human thyroid tissue, more studies should be done to better understand the role of estrogen in thyroid differentiated protein expression.

3.3. Non-Genomic Effects of E2. Some of the actions of E2 in the proliferation of thyroid cells are mediated by the activation of signal transducing pathways, as shown in

Table 4: Non-genomic estrogen effects on thyroid cells.

Study	Cells	GPR30	MAPK	PI3k	Cyclin D1	*c-fos*	BcL-2	Bax
Kumar et al. [42]; 2010	NPA87	−	↑	↑				
	KAT5	−	↑	↑	↑			
	WRO	+	↑	↑	↑			
Zeng et al. [45]; 2007	KAT5						↑	↓
	FRO						↑	↓
	WRO						↑	↓
Vivacqua et al. [46]; 2006	WRO	+	↑		↑	↑		
	FRO	+	↑		↑	↑		
	ARO	+	↑		↑	↑		
Manole et al. [6]; 2001	HTC-TSHr		↑		↑			
	Goiter		↑		↑			
	XTC 133		↑		↑			

NPA87 and KAT5: human papillary thyroid carcinoma cell lines; WRO and FRO: human follicular thyroid carcinoma cell lines; HTC-TSHr: human thyroid carcinoma cell line lacking endogenous TSH receptor; XTC-133: thyroid cancer cell line of Hurthle cell origin; Goiter: primary culture of human thyroid cells isolated from goiter nodules. (+): presence of expression; (−) absence of expression; (↑): increase, (↓): decrease, and (0): no effects, after E2 exposure.

Table 4. E2 can induce activation of phosphatidylinositol 3-kinase (PI3K) [42] and phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in follicular thyroid carcinoma cells, mainly due to interaction via membrane-associated ER [42, 45, 46]. PI3K and Erk1/2 signaling may play a critical role in preventing apoptosis and inducing cell cycle progression by induction of key genes expression [59].

Expression of early response genes and regulatory genes of the cell cycle are necessary for proliferation of cells. As E2 has been demonstrated to stimulate the growth of thyroid cells, it is important to study the expression of key cell-cycle genes such as cyclin D1 after stimulation with E2. Cyclin D1 regulates the cell progression cycle facilitating G_1 to S phase transition and also has an estrogen-responsive regulatory region [60], that is likely different from the canonical EREs. Overexpression of cyclin D1 in thyroid malignancies has been reported [61–65], moreover, its expression has been associated with an aggressive behavior in papillary thyroid microcarcinomas, because over 90% of the metastasizing microcarcinomas expressed cyclin D1 [66].

E2 significantly increased the expression of cyclin D1 in a human thyroid carcinoma cell line lacking endogenous TSH receptor (HTC-TSHr cells), and in a thyroid cancer cell line of Hurthle cell origin (XTC-133), which was abolished by PD.098059 that blocked G0/G1 to S phases [6]. E2 upregulated cyclins A and D1, as well as the proto-oncogene *c-fos*, in WRO, FRO, and ARO cells [46]. Cyclin D1 was also shown to be upregulated by E2 in KAT5, a papillary thyroid cancer cell line, and WRO cells [42].

Together, these results are very compelling, pointing to an ability of E2 to regulate genes mediating cell cycle progression in thyroid cells, and potentially contributing to the pathogenesis of thyroid cancer or thyroid hyperplasia.

4. Conclusions

There are evidences that estrogen may have direct actions in human thyroid cells by ER-dependent mechanisms or not, modulating proliferation, and function. Different patterns of distribution, expression, and ratios of ERα and ERβ may have a role in thyroid cancer cells proliferation, as well as in the outcome of thyroid cancer. Studying estrogen effects on thyroid cells is a potential tool to better understand the pathogenesis of thyroid diseases, and to develop targets to its treatment. Further studies on the influence of E2 on the growth and function of the thyroid are needed, preferably in primary culture of normal and abnormal human thyroid cells.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Association between Vitiligo and Thyroid Autoimmunity

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Background. Vitiligo is a common skin disorder characterized by macular depigmentation of the skin. The etiopathogenesis of the disease is still unclear, but there is evidence that autoimmunity and endocrine disfunction may be involved. *Objective*. The aim of this study was to determine whether vitiligo is statistically associated with thyroid autoimmunity. *Method*. In a prospective case-control study, we compared the frequency of thyroid autoantibodies (thyroglobulin antibody, anti-Tg and thyroid peroxidase antibody, and anti-TPO) in 33 patients with vitiligo and in 33 healthy volunteers. Thyroid autoantibodies and thyroid hormones (thyroxine (T4), triiodothyronine (T3), and thyroid stimulating hormone (TSH) were measured in all subjects. *Results*. Thyroid functional abnormalities were found in 6 (18.18%) patients. Anti-Tg and anti-TPO were positive in 9 (27.27%) and 8 (24.24%) patients, respectively. In control group, only one subject (3.03%) had abnormalities in thyroid hormonal status, and two subjects had positive thyroid autoantibodies. Compared with the control group, the frequency of both anti-Tg and anti-TPO was significantly higher in those with vitiligo ($P < .05$). *Conclusion*. This study shows a significant association between vitiligo and thyroid autoimmunity, and that tests to detect thyroid autoantibodies are relevant in patients with vitiligo.

1. Introduction

Vitiligo is one of disorders of melanin pigmentation that affects approximately 0.5–2% of the population [1]. It is characterized by macular depigmentation of varying sizes or shapes with a tendency to progress. Depending on the extent of the lesions, vitiligo can be classified into two main categories: generalized and localized. Although the pathogenesis of vitiligo is not yet fully understood, the autoimmune hypothesis is the most commonly accepted. This theory is supported by the clinical association of vitiligo with autoimmune disorders, the frequent detection of circulating autoantibodies to surface and cytoplasmatic antigens of melanocytes [2, 3]. Furthermore, there are findings of activated T cells in the periphery of actively progressing lesions in some vitiligo patients [4]. Thyroid functional disorders and autoimmune thyroid diseases have been reported in association with vitiligo, and it seems that the incidence of clinical and subclinical thyroid involvement is more common in vitiligo patients than healthy subjects [5, 6].

The aim of this study was to determine whether vitiligo is statistically significantly associated with thyroid autoimmunity.

2. Patients and Methods

The study included 33 patients with vitiligo, 19 female and 14 male, median age 42.39 (±13.66) years. Of them, there were 14 (42.4%) patients with generalized vitiligo and 19 (57.6%) patients with localized form of disease. A detailed history and examination were taken in all study subjects, including patients age, age at onset, duration of disease, associated diseases, history of thyroid disorders, and the extent and severity of disease. The diagnosis of vitiligo was made on clinical grounds. Skin biopsy was performed in selected cases. The control group consisted of 33 volunteers, 19 female, and 14 male, median age 40.33 (±14.78) years. Blood samples were taken and a physical examination and thyroid sonography was performed. All subjects gave their informed consent in accordance with the requirements of the institutional Ethichs Commitettee.

Table 1: Demographic data of patients (Vitiligo group) and volunteers (Control group).

	Vitiligo group *n* (%)	Control group *n* (%)	*P*
Men, *n* (%)	19 (58)	19 (58)	
Women, *n* (%)	14 (42)	14 (42)	
Age range, years	16–64	17–69	
Age, mean years (SD)	42.39 (13.66)	40.33 (14.78)	.558

Table 2: Clinical characteristics of patients with vitiligo.

Mean age of onset (SD) (year)	37.74 (12.45)
Age of onset range (year)	14–58
Mean duration (SD) (month)	55.85 (66.24)
Duration Range (month)	2–252
Type of vitiligo *n*, (%)	
Generalized 14 (42)	
Localized 19 (58)	

Thyroid autoantibodies (thyroglobulin antibody, anti-Tg and thyroid peroxidase antibody, anti-TPO) and thyroid hormones (thyroxine (T4), triiodthyronine (T3), and thyroid stimulating hormone (TSH) were measured in all subjects. Total T4 (normal range: 70–180 nmol/L) and total T3 (normal range 1.3–3.3 nmol/L) were measured by use of radioimmunoassay (RIA); TSH (normal range: 0.3–4.2 mL U/L) was determined by use of immunoradiometric assay (IRMA) (BRAHMS Aktiengesellschaft, Hennigsdorf, Germany). Serum levels of anti-Tg (threshold value: 115 IU/mL) and anti-TPO (borderline value: 34 IU/mL) were measured by use of electrochemiluminescence immunoassay (ECLIA) according to standard protocols (COBAS, Roche Diagnostics GmbH, Germany). The upper limit of autoantibody was determined by the laboratory.

Statistical comparisons were performed using χ^2 test. Data were considered statistically significant at $P < .05$.

3. Results

We performed a cross-sectional study in 33 consecutive patients with vitiligo and 33 age- and sex-matched controls. Demographic data of patients and controls are shown in Table 1. The mean (SD) age of the patient and control groups was 42.39 (±13.66) and 40.33 (±14.78), respectively ($P = .558$). The duration of vitiligo ranged from 2 to 252 months. Fourteen patients had generalized, and nineteen patients had localized vitiligo (Table 2). A family history of thyroid diseases was recorded in 2 (6.06%) patients. Thyroid functional abnormalities were found in 6 (18.18%) patients. In the control group only one (3.03%) subject had abnormalities in hormonal status. Hypoechogenic thyroid tissue was seen in 2 (6.06%) patients. Goitre was diagnosed in 4 (12.12%) patients with generalized vitiligo, from which 3 (9.09%) of them had elevated levels of thyroid autoantibodies and 2 (6.06%) had hormonal abnormalities. The ultrasound examination of the thyroid gland in control group was interpreted as normal in 32 (96.96%), and 1 (3.03%) volunteers had small simple goiter.

In patients with vitiligo anti-Tg titers were ranging from 11 to 1012 IU/mL and anti-TPO antibody titers from 6 to 457 IU/mL. In control group anti-Tg titers were ranging from 10 to 153 IU/mL, and anti-TPO antibody titers from 5.1 to 129 IU/mL. Anti-Tg antibody in 9 (27.27%) patients, anti-TPO antibody in 8 (24.24%) and both anti-Tg and anti-TPO antibodies in 6 (18.18%) were higher than the normal antibody titres. In the control group, one subject (3.03%) had positive anti-Tg and one volunteer (3.03%) had positive anti-TPO. The frequency of thyroid autoantibodies was significantly higher in vitiligo patients than in control group (Table 3). Statistically significant difference was also found in values of anti-Tg and anti-TPO between patients with generalized and patients with localized vitiligo ($P < .05$).

A Chi-square test for independence (with Yates Continuity Correction) indicated significant association between higher values of anti-Tg (values more than 115 IU/ml) and vitiligo, $\chi^2\ (1, n = 66) = 5.775, P = .0163$.

A Chi-square test for independence (with Yates Continuity Correction) indicated significant association between higher values of anti-TPO (values more than 34 IU/ml) and vitiligo, $\chi^2\ (1, n = 66) = 4.632, P = .0314$.

4. Discussion

Vitiligo is an ancient disease that was known to Egyptians even in the pre-Christian time [7]. Despite its long history, our knowledge is actually limited. A number of genetic and environmental factors have been implicated in the etiology of vitiligo, but the mechanism of initiation of melanocyte destruction and progression of disease is not yet clear [8].

Vitiligo has been reported in association with numerous endocrine disorders. One of the main associations is with thyroid abnormalities. It was already in 1941, when Robert suggested that vitiligo might be connected with an increased activity of the thyroid gland [9]. He noted a distinct rise of the basal metabolism in 10 out 20 vitiligo patients tested. Several authors reported a significantly increased prevalence of autoimmune thyroid disease in vitiligo patients; the rate of positivity of thyroid autoantibodies varied from 2.2% [10] to 50% [11]. In addition, there is also a study reporting a significantly increased prevalence of vitiligo in patients with autoimmune thyroid disease compared to patients with nonautoimmune thyroid disease [5, 6].

In accordance to previous studies, we also demonstrated that antithyroid autoantibodies were significantly increased in vitiligo patients in comparison to healthy subjects. We detected elevated anti-Tg in 9 (27.27%) and elevated anti-TPO in 8 (24.24%) of patients with vitiligo. Usually about 10% of general population has positive antithyroid antibodies; in this study the prevalence of autoantibodies in control group is much lower than expected. The difference it may partly be attributed to genetic factors. Compared with the control group, the frequency of both anti-Tg and anti-TPO antibodies was significantly higher

Table 3: Frequency of thyroid autoantibodies in the study group.

	Anti-Tg (threshold value 115 IU/mL)		Anti-TPO (threshold value 34 IU/mL)	
Group	Negative *n* (%)	Positive *n* (%)	Negative *n* (%)	Positive *n* (%)
Vitiligo	24 (73)	9 (27)	25 (76)	8 (24)
Control	32 (97)	1 (3)	32 (97)	1 (3)
Total	56 (85)	10 (15)	57 (86)	9 (14)
Difference *n* (%)	8 (24)		7 (21)	
χ^2, P	$\chi^2 = 5.775, P = .0163$		$\chi^2 = 4.632, P = .0314$	

in those with vitiligo. Our results are consistent with a clinical study performed by Sedighe and Gholamhossein [12]. They analyzed antithyroid antibodies in 109 Iranian patients with vitiligo and found that anti-TPO and anti-Tg antibody were positive in 40 (36.7%) and 35 (32.1%) cases, respectively. Daneshpazhooh and colleagues measured only the serum level of anti-TPO antibody and reported significantly high levels in vitiligo patients compared to healthy controls [13]. In study that was carried out in India, the anti-TPO antibody was positive in 31.4% cases [14]. Our findings showed that the frequency of anti-TPO was more significant than anti-Tg. This antibody, historically referred to as the antimicrosomal antibody, is established as a sensitive tool for the detection of early subclinical autoimmune thyroid diseases and identification of at-risk cases for autoimmune thyroid diseases [15]. Nordyke et al. reported that anti-TPO antibody tends to have more correlation with thyroid dysfunction than does the anti-Tg antibody [16].

Vitiligo frequently precedes the thyroid involvement, thus screening vitiligo patients for thyroid antibody seems plausible [17].

5. Conclusion

The study revealed a significant association between vitiligo and thyroid autoimmunity and showed the tests used to detect thyroid autoantibodies to be relevant in patients with vitiligo. Vitiligo offers many benefits as a model for the study of autoimmunity, in that it can be used to identify the contributing roles of immunogenetics and endocrine factors in the initiation and propagation of autoimmune disease.

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Review Article

Thyroid-Specific Transcription Factors and Their Roles in Thyroid Cancer

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Homeodomain, forkhead domain, and paired domain-containing transcription factors play a major role in development, tissue-specific gene expression, and tissue homeostasis in organs where they are expressed. Recently, their roles in stem cell and cancer biology are emerging. In the thyroid, NKX2-1, FOXE1, and PAX8 transcription factors are responsible for thyroid organogenesis and expression of thyroid-specific genes critical for thyroid hormone synthesis. In contrast to their known roles in gene regulation, thyroid development and homeostasis, their involvement in stem cell, and/or cancer biology are still elusive. In order to further understand the nature of thyroid cancer, it is critical to determine their roles in thyroid cancer.

1. Introduction

Tissue-specific transcription factors play a pivotal role in regulating expression of tissue-specific genes, thereby controlling the function, homeostasis, and differentiation of tissue where they are expressed. Their altered expression due to gene mutation, deletion, amplification, and/or epigenetic modification, and/or posttranslational modification can change the cell fate and perturb metabolism and differentiation status, leading to various clinical conditions. Since both cell proliferation and differentiation are involved in the process of normal and cancer development, it is not surprising that genes critical for development play an important role in oncogenesis. Transcription factors, containing the homeobox [1, 2], forkhead domain (FOX) [3], and paired domain (PAX) [4], that are among those expressed tissue-specifically that play a critical role in tissue homeostasis and development, can also have roles in carcinogenesis. Thyroid is an organ in which the homeodomain, forkhead domain, and paired domain-containing transcription factors all play major roles in tissue-specific gene expression and thyroid development. The current view on the roles of thyroid-specific transcription factors in thyroid cancer will be summarized below.

2. Thyroid-Specific Transcription Factors

The three distinct thyroid-specific transcription factors are critical for the function of thyroid: NKX2-1 (also called TTF1, TITF1, T/EBP, or NKX2.1) [5, 6], FOXE1 (also called TTF2 or TITF2) [7], and PAX8 [8] (Table 1). They are members of the homeodomain, forkhead box, and paired box family of transcription factors, respectively, and regulate genes encoding thyroglobulin, thyroid peroxidase, thyrotropin receptor, and sodium/iodide symporter, proteins critical for thyroid hormone synthesis [5–7, 9–14]. They are also essential for thyroid development [15–17]; *Nkx2-1*-null mice are born without the thyroid (agenesis) [16], while *Pax8*-null mice are severely hypothyroidism with rudimental thyroid remnant [17]. *Foxe1*-null mice have either agenesis or thyroid ectopy [15]. These transcription factors are responsible for the athyreosis, hypothyroidism, and/or ectopic thyroid, which provide crucial clues to their roles in thyroid dysgenesis in humans [18]. In addition to thyroid, NKX2-1 is expressed in lung primordium and ventral forebrain [16], PAX8 in developing kidney [8], and FOXE1 in the floor of the foregut and the craniopharyngeal ectoderm including Rathke's pouch during development [15]. At later stages, FOXE1 is expressed in the secondary palate, definitive

Table 1: Thyroid-specific transcription factors and thyroid cancers.

	Thyroid phenotype in null mice	Gene requirement	Expression in thyroid cancer	Common variants/Gene mutations for thyroid cancer predisposition	Other specifics
NKX2-1	Athyreosis	Proliferation and survival of thyroid follicular cells and C cells	Level correlates with the degree of differentiation	14q13.3: PTC, FTC	Gene mutation increases thyroid cell proliferation
				A339V: PTC	Lineage-specific oncogene amplified in lung cancer
PAX8	Athyreosis, rudimental thyroid remains	Proliferation and survival of thyroid follicular cells	Correlation between expression level and differentiation not clear	PAX8/PPARγ fusion protein: FTC	Expression found in cancers of other tissues such as kidney, Müllerian system, and ovary
FOXE1	Athyreosis or ectopia	Migration of thyroid primordium	Level correlates with the degree of differentiation	rs965513 on 9q22.33: PTC, FTC, radiation-induced PTC	LOH at D9S180 on 9q22.3: frequently found in skin SCC
				rs1867277 (−283G > A): PTC	16 Ala variant: associated with SCC

PTC: papillary thyroid carcinoma, FTC: follicular thyroid carcinoma, SCC: squamous cell carcinoma, and LOH: loss of heterozygosity.

choanae, whiskers, and hair follicles [19]. Some structures derived from these areas are also defective in respective null mice. Thus, *Nkx2-1*-null mice also have severely hypoplastic lung, defective hypothalamus, and pituitary agenesis [16]. *Foxe1*-null mice have cleft palate [15]. Similar to the defects found in the *Nkx2-1*-null mice and sometimes more manifested in humans, various mutations in the *NKX2-1* gene result in the Brain-Thyroid-Lung syndrome, which is characterized by benign hereditary chorea, congenital hypothyroidism, and respiratory diseases [20–22]. Mutations in the *FOXE1* gene are responsible for syndromic congenital hypothyroidism dysgenesis, cleft plate, and spiky hair [22–24].

2.1. NKX2-1

2.1.1. NKX2-1 and Cancer. Due to the nature of tissue-specific expression, NKX2-1 is expressed in human thyroid and lung cancers [25–28]. In particular, NKX2-1 is highly expressed in human lung adenocarcinomas and small cell carcinomas (~60–90%) [25, 26, 29]. NKX2-1 has been widely used as a marker for the diagnosis of primary and metastatic lung cancer [30] and as a prognostic indicator for survival [26, 31, 32]. In fact, *NKX2-1* is a lineage-specific oncogene amplified in lung cancers and the survival of a subset of adenocarcinoma cells depends on the sustained expression of NKX2-1 [33–35]. However, no mutations in the *NKX2-1* gene are described in any adenocarcinomas examined in these studies. Patients with adenocarcinomas that lack NKX2-1 expression or have NKX2-1 expression accompanied by *NKX2-1* gene amplification tend to have a significantly worse prognosis than patients with NKX2-1 expression and no *NKX2-1* gene amplification [32].

In contrast to the expression in lung, NKX2-1 is expressed at lower levels in malignant thyroid as compared to normal thyroid [36]. The level of expression is significantly correlated with the progressive dedifferentiation and increase of malignancy of thyroid tumors [27]. Thus, the expression is generally found in the order of follicular thyroid adenoma > follicular thyroid carcinoma > papillary thyroid carcinoma > medullary thyroid carcinoma > anaplastic thyroid carcinoma [27, 37–39]. These studies use immunohistochemical analysis of primary thyroid tissues, and low or no expression of NKX2-1 is found in anaplastic thyroid carcinomas. Using RT-PCR, NKX2-1 expression is reported in some anaplastic thyroid carcinoma-derived cell lines [40, 41]. The latter studies present different results for the expression of NKX2-1 within the same cell line, suggesting the controversial nature of NKX2-1 expression. In order to explain the loss of NKX2-1 expression in most of undifferentiated thyroid carcinomas and cell lines, epigenetic silencing of the *NKX2-1* gene through DNA hypermethylation and histone H3 modification has been suggested [40]. Further studies are required to obtain clear understanding of the relationships in between expression of NKX2-1, differentiation status of tissues and primary carcinomas versus cell lines, and the mechanisms underlying the loss of NKX2-1 expression in malignancy.

A genome-wide association study (GWAS) revealed the predisposition of common variants on 9q22.33 and 14q13.3 to both papillary and follicular thyroid cancers. The gene nearest to the 9q22.33 is *FOXE1*, and among the genes located at the 14q13.3 locus is *NKX2-1* [42], suggesting

potential roles for these two thyroid-specific transcription factors in thyroid cancers. A germline mutation of *NKX2-1* gene leads to a mutant NKX2-1 protein (A339V) that has impaired transactivation of thyroid-specific genes such as thyroglobulin, thyrotropin receptor, and PAX8, while the expression is associated with the increased cell proliferation, thyrotropin-independent growth, and enhanced activation of survival signaling molecules such as Stat3 and Akt as compared to wild-type protein [43]. A population study demonstrated that the *NKX2-1* A339V mutant contributes to predisposition of maltinodular goiter and/or papillary thyroid carcinomas and to the pathogenesis of papillary thyroid carcinomas [43].

2.1.2. Nkx2-1 Thyroid-Specific Conditional Knockout Mouse as a Model to Study Thyroid Carcinogenesis. *Nkx2-1(fl/fl);TPO-Cre* thyroid-specific conditional knockout mouse provides an animal model to study the role of NKX2-1 in adult thyroid, which circumvents the problem of immediate neonatal lethality of *Nkx2-1*-null mouse [44]. In the *Nkx2-1(fl/fl);TPO-Cre* mouse, the recombination of *Nkx2-1* floxed gene occurs at the rate of ~50%, resulting in *Nkx2-1* thyroid-specific conditional hypomorphic mouse [45]. These mice exhibit either atrophic/degenerative thyroids with frequent presence of adenomas and extremely high TSH levels, or thyroids with reduced numbers of extremely dilated follicles having more number of follicular cells than usual within a follicle. The atrophic/degenerative thyroid mostly consists of atrophic/degenerative follicles, in which many follicular cells frequently have lost NKX2-1 expression, suggesting that the loss of NKX2-1 may be the cause of atrophic/degenerative follicular cells [45]. These findings further suggest that NKX2-1 is required for the maintenance of ordered architecture and function of the differentiated thyroid [45].

In chemical carcinogenesis bioassays using the genotoxic mutagen N-bis(2-hydroxypropyl)-nitrosamine (DHPN) followed by sulfadimethoxine (SDM) as a promoter, the *Nkx2-1(fl/fl);TPO-Cre* mice developed significantly higher incidence of adenomas as compared with wild-type or *Nkx2-1*-heterozygous mice [46]. In contrast, with the non-genotoxic carcinogen amitrole (3-amino-1,2,4-triazole), all three genotype groups of mice developed adenomas at similar incidence. Surprisingly, no gene mutation was identified in any adenoma-developed thyroids. The increased incidence of adenomas in the *Nkx2-1(fl/fl);TPO-Cre* mice after genotoxic carcinogen exposure may be partially explained by more than a twofold higher cell proliferation rate found in these mouse thyroids as compared to those of wild-type or *Nkx2-1*-heterozygous mice. These results may be analogous to human exposure to genotoxic mutagens or radiation, which could cause somatic mutation of *NKX2-1* gene → inactivation of *NKX2-1* gene → degeneration of thyroid follicular cells → increased cell proliferation → augmentation of the damage occurred in DNA, and/or chromosomes by genotoxic mutagens or radiation exposure, ultimately leading to cancer [46].

2.2. FOXE1 and Cancer. The human *FOEX1* gene is located on chromosome 9q22.3 [47]. The loss of heterozygosity of marker D9S180 from this chromosomal area is frequently observed in squamous cell carcinomas of skin, suggesting the presence of tumor suppressor gene in this genomic region [48]. The common variant rs965513 on 9q22.33 contributes to an increased risk of papillary and follicular thyroid cancer [42]. Further, a high incidence of *FOXE1* gene promoter methylation is found in cutaneous squamous cell carinomas (SCC) [49], pancreatic cancers [50], and breast cancers [51]. FOXE1 protein has a polyalanine tract starting at the 13th amino acid residue from the end of the forkhead domain, which stretches from 12 to 17 residues with the 14 alanine stretch at the highest frequency [47]. The less common variant (allele 16) is associated with SCC, suggesting that the more common variant (allele 14) may be protective against developing SCC [52].

Similar to NKX2-1, FOXE1 expression is found in various thyroid cancers [38, 53]. The level of expression correlates with their differentiation status as seen with NKX2-1, and anaplastic thyroid carcinoma has very little expression of FOXE1 [38, 53]. The candidate gene association study revealed that the variant rs1867277 (−283G > A) located in the *FOXE1* 5′ UTR is associated with papillary thyroid cancer susceptibility through recruitment of USF1/USF2 transcription factors to the −283A allele, which affects gene expression [54]. FOXE1 is required for thyroid cell precursors to migrate into the underlying mesenchyme from the thyroid bud [15, 55]. Although the exact mechanism for the enhanced transcription of *FOXE1* gene leading to increased susceptibility to papillary thyroid cancer remains unknown, the enhanced expression of FOXE1 in thyroid carcinomas could be related to a motile advantage of malignant thyroid cells [54].

Radiation exposure causes papillary thyroid cancer as revealed by various studies after the Chernobyl accident [56]. Genome-wide association studies (GWAS) employing Belarusian patients and control subjects demonstrated that the variant rs965513 on 9q22.33 is significantly associated with the radiation-induced papillary thyroid cancer [57]. This variant was identified together with NKX2-1, as those having the strongest rink to papillary and follicular thyroid cancers [42]. Although *Foxe1* thyroid conditional null mice are currently not available, they would be a useful model to understand the role of FOXE1 in the pathogenesis of thyroid cancer.

2.3. PAX8

2.3.1. PAX8 and Cancer. PAX8 is a crucial transcription factor for organogenesis of the thyroid, kidney, and Müllerian system [8, 58]. PAX8 is expressed in normal as well as neoplastic renal tissues, and in Wilms' tumors [58, 59]. PAX8 is a useful marker for Müllerian carcinomas [60] and ovarian cancer [61, 62] and can be used to distinguish ovarian serous tumors from malignant mesothelioma [61–64] or from other metastatic tumors such as breast and colon [63, 64].

PAX8 is expressed in various thyroid cancers; however, the pattern of expression is somewhat controversial;

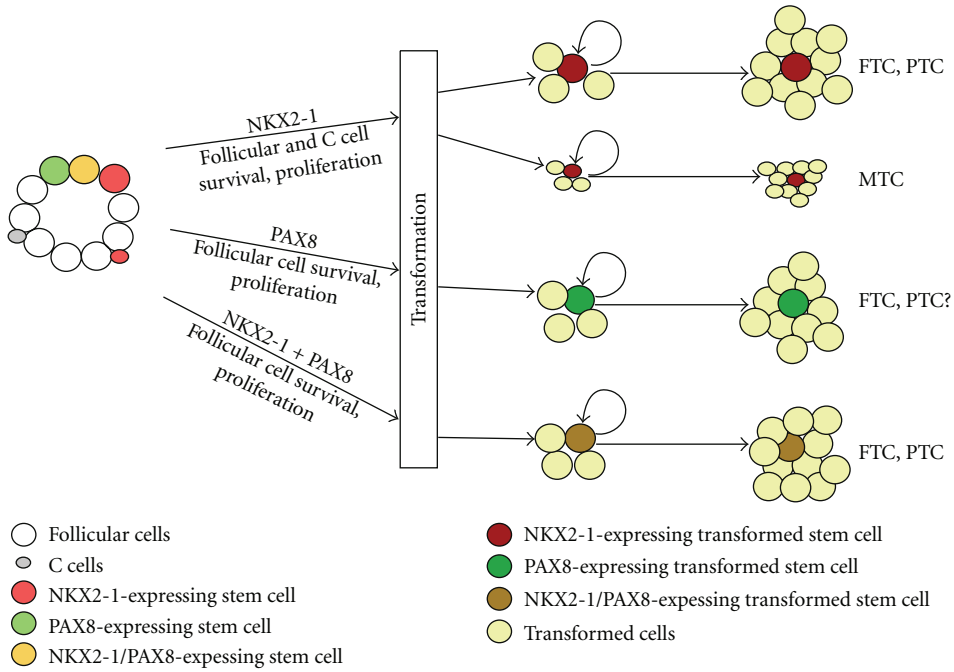

Figure 1: Possible involvement of NKX2-1 and/or PAX8 in the maintenance and/or activity of stem cells of the thyroid. Transformation of NKX2-1 and/or PAX8-expressing stem cells leads to thyroid cancer. Transformed NKX2-1 and/or PAX8-expressing stem cells self-renew and proliferate to produce cancer. Upon becoming tumorigenic, most of transformed cells may lose NKX2-1 and/or PAX8 expression. FTC: follicular thyroid carcinoma, PTC: papillary thyroid carcinoma, and MTC: medullary thyroid carcinoma.

one study showed that the nuclear PAX8 staining is correlated with the thyroid differentiation phenotype as seen with NKX2-1 and FOXE1 [27], while others demonstrated that PAX8 is a useful marker for the diagnosis of anaplastic carcinomas [38]. More studies are required to determine the expression pattern and the role of PAX8 in thyroid cancers, including the use of *Pax8* thyroid-conditional null mice.

2.3.2. PAX8/PPARγ Fusion Oncogene in Thyroid Cancer. The chromosomal translocation of the 2q13-qter region to 3p25 results in an in-frame fusion protein (PPFP) between most of the coding sequence of PAX8 and the entire translated reading-frame of the nuclear receptor-family member peroxisome proliferator-activated receptor gamma (PPARγ) ([65] reviewed in [66, 67]). The PPFP has several different PAX8 breakpoints while the PPARγ breakpoint seems to be constant [65, 67, 68]. This fusion protein is more prevalently expressed in follicular thyroid carcinomas (36%, reviewed in [69]); however, follicular adenomas (11%), follicular variant of papillary carcinoma (16%), and Hürthle cell carcinoma (2%) also express PPFP [68–70]. PPFP has been proposed to be an early follicular thyroid carcinoma-specific oncogene [65, 71]. Several *in vitro* studies demonstrated that PPFP has oncogenic activity such as increased cell cycle transition, reduced apoptosis, and enhanced growth [71], which is partly due to PPFP's dominant negative activity to suppress wild-type transcriptional activities of PPARγ [65, 71, 72], the suggested tumor suppressor [73, 74]. PPFP can also work as a dominant negative inhibitor of wild-type PPARγ *in vivo* [75]. Further studies are required to establish the mechanisms for the PPFP-mediated tumorigenesis.

3. Thyroid-Specific Transcription Factors, Cancer, and Stem Cells

Normal embryogenesis is believed to share many of the same pathways as neoplasia, such as Wnt/β-catenin, Hedgehog, and Notch pathways. These signaling pathways are also involved in the maintenance and/or activity of stem cells, while their dysregulation plays a role in tumorigenesis (reviewed in [76–80]). It is increasingly recognized that homeobox proteins including PAX proteins play a critical role in stem cell maintenance [4, 81]. PAX3 or PAX7 is essential for generating the cell pool of muscle progenitors from which satellite cells derive [82]. Overexpression of PAX3 and 7 is frequently found in pediatric soft-tissue malignant tumor rhabdomyosarcomas [83, 84]. PAX6 is essential for maintenance of the multipotency of retinal progenitor cells [85]. On the other hand, *HOX* genes are expressed in hematopoietic cells in a stage- and lineage-specific manner, and are implicated in leukemogenesis [81]; for instance, HOXA10 is a critical regulator for haematopoietic stem cells, and erythroid and megakaryocyte development [86], while HOXA9 is required for normal hematopoietic stem cell function [87]. The involvements of other homeobox genes in the maintenance of stem cells are described in various tissues including brain [88] and kidney [89]. In the prostate, NKX3-1, another member of the *NKX* gene family, is required for stem cell maintenance [90]. The targeted deletion of *Pten*,

a tumor suppressor gene in castration-resistant NKX3-1-expressing cells, results in rapid carcinoma formation after androgen-mediated regeneration [90].

The three transcription factors, NKX2-1, FOXE1, and PAX8, are critical for normal embryogenesis and appear to play a role in tumorigenesis in various tissues where they are expressed, including the thyroid. By analogy to other homeodomain/PAX proteins, it is likely that NKX2-1 and PAX8 may be involved in the maintenance and/or activity of stem cells in the thyroid, dysregulation of which may lead to thyroid cancer (Figure 1). Currently, it is not clear whether FOX transcription factors are involved in stem cell maintenance/activity [3]. Knockout mouse studies demonstrated that in the absence of NKX2-1, primordium cells to both thyroid follicular and C cells disintegrate during thyroid organogenesis [16, 91], while PAX8 is required for the survival of follicular cells [17]. It would be interesting to determine whether NKX2-1 and/or PAX8-expressing stem/progenitor cells exist that can rapidly form cancers upon targeted disruption of a tumor suppressor gene in cell pools, similar to that seen with NKX3-1. In this regard, NKX2-1 in lung cancers may be more analogous to this scenario since NKX2-1 is a lineage-specific oncogene and is required for survival of a subset of adenocarcinoma cells [33–35].

4. Conclusions

It appears that most transcription factors, if not all, that are critical for developmental process are involved in the maintenance and/or activity of stem cells, whose dysregulation results in cancers. Currently, it is entirely unknown whether and/or how the thyroid-specific transcription factors NKX2-1, FOXE1, and PAX8 are related to stem/progenitor cells of the thyroid that may lead to cancer when dysregulated. Identification/characterization of thyroid stem/progenitor cells, their relation to the expression of NKX2-1, FOXE1, and/or PAX8, and more detailed characterization of various thyroid cancers and/or cancer cells, particularly in relation to the expression of these transcription factors, are urgently required in order to better understand the roles of NKX2-1, FOXE1, and PAX8 in thyroid cancer.

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Review Article

Recent Advances in Molecular Diagnosis of Thyroid Cancer

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Recent molecular studies have described a number of abnormalities associated with the progression and dedifferentiation of thyroid carcinoma. These distinct molecular events are often associated with specific stages of tumor development. In particular, remarkable advances have occurred in several major biological areas of thyroid cancer, including the molecular alterations for the loss of radioiodine avidity of thyroid cancer, the pathogenic role of the MAP kinase and PI3K/Akt pathways and their related genetic alterations, and the aberrant methylation of functionally important genes in thyroid tumorigenesis and pathogenesis. Recognition of these features is crucial to the management of patients with thyroid cancer. Novel treatments are being designed based on our enhanced understanding of this disease process.

1. Introduction

The incidence of thyroid cancer, the most common endocrine malignancy, has been rising gradually over the past decade. The major histological types of the follicular cell-derived thyroid cancer are papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), and anaplastic thyroid cancer (ATC) [1–4]. Benign thyroid adenoma (BTA) is a common endocrine tumor. On the other hand, medullary thyroid cancer arises from the parafollicular or C cells and is not of follicular cell origin. For that reason, it is not presented in this paper.

Accumulating evidence indicates that follicular cell-derived thyroid carcinomas constitute a biological continuum progressing from the highly curable well-differentiated thyroid carcinoma (WDTC) to the often fatal undifferentiated or anaplastic thyroid carcinoma (ATC) [5, 6]. Poorly differentiated thyroid carcinoma (PDTC) and aggressive variants of WDTC, such as tall cell and columnar cell, frequently serve as intermediates in this progression model.

Clinical, epidemiologic, and pathologic evidence supports the concept of stepwise progression and dedifferentiation [7]. For example, the gradual loss of papillary and follicular growth patterns and the simultaneous increase in a solid growth pattern, with increased mitoses, necrosis, and nuclear pleomorphism, are often observed in aggressive thyroid carcinomas [8, 9]. A majority of these tumors exhibit residual foci of differentiated thyroid carcinoma.

There are also several diagnostic challenges that are often encountered in the clinical management of thyroid cancer. One is the diagnostic dilemma associated with "indeterminate cytology" on the widely used fine needle aspiration biopsy (FNAB) in the evaluation of thyroid nodules. For example, in the United States, about 300,000 cases of thyroid nodules, which are mostly BTA, are diagnosed annually [10]. Moreover, 20–30% of these FNAB cases show "indeterminate" cytological findings, a pattern that has been reported to remain essentially unchanged over the last two decades [11, 12]. These patients currently virtually all pursue thyroid surgery to definitely reveal the nature of the nodules although vast majority of them will surgically prove to have benign nodules.

Careful risk stratification is a key step in the decision making for appropriate surgical and medical managements of patients with thyroid cancer. This risk evaluation is conventionally based on clinicopathological factors, which

are often unreliable and are mostly unavailable prior to thyroid surgery.

Most thyroid cancer patients have an excellent outcome, and standard treatment usually consists of total thyroidectomy, often with lymph node resection, thyroid hormone suppressive therapy, and in more advanced staged disease, radioactive iodine (I-131) for either remnant ablation or therapeutic treatment [13–15]. These standard therapies are dependent on the tumor exhibiting a differentiated phenotype similar to normal thyrocytes consisting of responsiveness to the growth factor TSH via the presence of the TSH receptor and expression of the sodium-iodide symporter (NIS) [16, 17]. Surveillance for these patients typically consists of combination of anatomical imaging such as neck ultrasound [18, 19], radioiodine whole body scans, and serum measurement of the thyroid-specific protein thyroglobulin with antithyroglobulin antibody levels [20, 21].

On the other hand, thyroid cancer patients with recurrent or metastatic disease can have mortality rates approaching 50% [22]. Dedifferentiation of thyroid cancer may consist of loss of expression of the TSH receptor, NIS, and loss of thyroglobulin production. In the process of a tumor losing NIS expression, the clinician loses the ability to use radioiodine for monitoring and treatment. However, this subset of tumors frequently become visible with 18F-fluorodeoxyglucose positron emission tomography scans (FDG-PET) [23]. Clinically, these FDG-PET positive, noniodine avid tumors have limited treatment options which may include observation, additional surgery, external beam radiation, conventional chemotherapy like the US FDA-approved agent doxorubicin, and clinical trials.

The recent introduction of targeted therapeutic agents that have multiple targets, including the receptor tyrosine kinases, nonreceptor tyrosine kinases, and serine-threonine kinases, has shown much promise in trials for thyroid cancer patients with advanced disease [24].

The current goal of molecular medicine is to be able to profile each patient's tumor in order to determine which treatments will achieve the maximal response with minimal side effects. There have been significant conceptual and technical advances in elucidating areas of tumor biology such as genetic and/or epigenetic regulation, but a unifying theory for thyroid carcinogenesis is lacking. This review aims to provide a framework to understand the rationale of how selected research developments may segregate tumors into different therapeutic regimens.

2. Chromosomal Rearrangements

One of the earliest genetic changes identified in papillary thyroid cancer was chromosomal alterations involving the proto-oncogene RET (rearranged during transfection) [25]. The RET (rearranged during transfection) proto-oncogene is a 21-exon gene located on the proximal long arm of chromosome 10 that encodes a tyrosine kinase receptor. It is involved in the regulation of growth, survival, differentiation, and migration of cells of neural crest origin. It is not normally expressed in the follicular cell [26]. The unique spatial proximity of translocation-prone gene loci, which may be preferentially occurring in thyrocytes in their mitotic interphase, favors RET gene rearrangements [27, 28]. This may help explain why RET rearrangements are specific for thyroid tumors [29, 30].

Although more than 10 rearrangements have been described, RET/PTC1, RET/PTC2, and RET/PTC3 account for most of the rearrangements found in PTC [31, 32]. In each of these rearrangements, the upstream (5′) component of a "housekeeping" (or ubiquitously expressed) gene drives the expression of the tyrosine kinase domain of RET. Expression of the RET/PTC chimeric proteins is facilitated by the heterologous promoters provided by the fused genes and results in constitutive, ligand-independent activation of RET receptor tyrosine kinase in papillary cancer cells [33–35].

Clinically, RET/PTC variants are often found in radiation-associated PTC. The increase in pediatric thyroid cancers following the Chernobyl nuclear plant explosion in 1986 resulted in two groups of thyroid cancer. The first was an early appearing and aggressive solid variant of PTC which contained the RET/PTC3 rearrangement while a later onset of PTC, with a more classical phenotype and clinical course, in the Chernobyl survivors contained RET/PTC1 [36].

Both transgenic mouse models and in vitro cellular work have shown these fusion proteins as capable of initiating PTC [37, 38]. RET/PTC rearrangements are less commonly found in undifferentiated thyroid cancers, suggesting that these tumors may be managed with conventional treatment [39]. Moreover, the utility of RET/PTC identification from FNA of thyroid nodules for diagnosis of PTC is beginning to be used [40].

There is evidence to support the belief that RET/PTC rearrangements represent early genetic changes leading to the development of PTC [41]. In approximately 20% of sporadic PTCs, RET/PTC rearrangements have been found [42]. Moreover, it has been also found in adenomas and other benign lesions of the thyroid [43, 44]. However, since it is present in most tumor cells, it is reasonable to consider it specific for PTCs [45].

Several studies have shown that RET/PTC rearrangements are associated with PTC that lacks evidence of progression to PDTC or ATC [46]. A recent study from Santoro et al. [47] showed that less than 10% of PDTCs were positive for RET/PTC rearrangements. They concluded that PTCs with RET/PTC rearrangements have a relatively low potential for progression to PDTC or ATC.

Recently, compounds have been identified that exhibit significant inhibitory activity on RET kinase [48]. In particular, the recent success in the treatment of chronic myelogenous leukemia with imatinib mesylate, an inhibitor of constitutively activated ABL kinase, has generated considerable interest in developing therapeutic protein kinase inhibitors. There are multiple drugs in trial with activity against RET, most notably ZD6474-Vandetanib and Bay 43-9006-Sorafenib, but these agents also target other tyrosine kinase receptors [49]. In particular, Sorafenib has now been shown in two phase II clinical trials to have achieved a partial response rate of 15 and 23% in patients [50]. Due to Vandetanib's ability to block RET signaling activity, it is primarily being used for medullary thyroid cancer patients;

however, there is an ongoing phase II trial including DTC patients [51].

3. RAS Mutations

The RAS (an abbreviation of RAt sarcoma) family of oncogenes regulates two important signaling pathways in thyroid 3 cancer, the mitogen-activated protein kinase/extracellular signal-regulated kinase (RAS/Raf/MEK/ERK) and the phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathways. Ras mutations occur in both benign and malignant thyroid tumors, with variable frequency in ATCs [52].

Three RAS genes, H-RAS, K-RAS, and N-RAS, synthesize a family of 21-kDa proteins that play an important role in tumorigenesis [53]. The RAS proteins exist in two different forms: an inactive form that is bound to guanosine diphosphate (GDP) and an active form that exhibits guanosine triphosphatase (GTPase) activity. Their function is to convey signals originating from tyrosine kinase membrane receptors to a cascade of mitogen-activated protein kinases (MAPKs). This activates the transcription of target genes involved in cell proliferation, survival, and apoptosis [54]. Oncogenic RAS activation results from point mutations, affecting the GTP-binding domain (codons 12 or 13) in exon 1 or the GTPase domain (codon 61) in exon 2, which fix the protein in the activated state and thus resulting in chronic stimulation of downstream targets, genomic instability, additional mutations, and malignant transformation [55]. The RAS mutations are among the most common mutations found in transformed cells. Mutations in all three cellular RAS genes have been identified in benign and malignant thyroid tumors. They seem to be common in follicular carcinoma, PDTC, and ATC and occur less frequently in PTC [56, 57]. The role of oncogenic RAS in thyroid tumor progression is unclear.

Some studies have shown a similar prevalence of RAS mutations in benign and malignant thyroid neoplasms, suggesting that RAS activation may represent an early event [58]. Other studies have shown that RAS mutations, specifically mutations at codon 61 of N-RAS, are involved with tumor progression and aggressive clinical behavior [59, 60]. Transgenic mice with thyroid-specific mutant RAS expression develop thyroid hyperplasia and carcinoma [61]. A recent study by Garcia-Rostan et al. [62] demonstrated that the presence of RAS mutations predicted a poor outcome for WDTC independent of tumor stage.

Furthermore, they found that PDTC and ATC often harbor multiple RAS mutations. These mutations probably represent an intermediate event in the progression of thyroid carcinoma.

4. BRAF Mutation and MAP Kinase Signaling Pathway in Thyroid Cancer

The evolutionarily conserved mitogen-activated protein kinase (MAPK) signaling pathway allows a cell to respond to external stimuli such as hormones and growth factors that interact with various receptors, including tyrosine kinase receptors like RET and G-protein-coupled receptors like the TSH receptor. In thyroid cancer, RET/PTC rearrangement is a common activator of the MAP kinase pathway [63]. Activating Ras mutations, which can activate the MAP kinase pathway, are also common in thyroid cancer [64].

BRAF mutation is a major cause of aberrant activation of the MAP kinase pathway in human cancers [65]. Among the three known Raf kinases, A-Raf, B-Raf (BRAF), and C-Raf, BRAF is the most potent activator of the MAP kinase pathway [66]. The T1799A point BRAF mutation accounts for more than 90% of the more than 40 mutations identified in the BRAF gene [63]. This mutation causes a V600E amino acid change in the BRAF protein, resulting in constitutive and oncogenic activation of the BRAF kinase [67, 68].

Discovery and characterization of the T1799A BRAF mutation in thyroid cancer represent one of the most exciting advances in the molecular biology of thyroid cancer in recent years [69, 70]. In fact, this mutation is the most common known genetic alteration in thyroid cancer. A few other activated BRAF mutants are only rarely found in thyroid cancer. These include the BRAF K601E [71], AKAP9-BRAF [66], BRAF V600E+K601del [72, 73], BRAF V599ins [74], and V600D+FGLAT601-605ins, which result from an insertion of 18 nucleotides at nucleotide T1799 [73]. Thus, the T1799A mutation is the most common and virtually the only BRAF mutation identified in thyroid cancer, commonly referred to as "BRAF mutation."

Previous studies have showed that BRAF mutation was not a germline mutation in familial nonmedullary thyroid cancers [75, 76] and, as a somatic genetic alteration, occurs exclusively in PTC and PTC-derived ATC, with an average prevalence of about 45% in the former and 25% in the latter; it does not occur in FTC or other types of thyroid tumors [66, 68]. Transgenic mouse model [73] with cell line and xenograft tumor studies [77, 78] demonstrated the tumorigenic ability of the BRAF mutation and its requirement to maintain cancer cell growth and proliferation.

Numerous clinical studies demonstrated an association of BRAF mutation with aggressive clinicopathological outcomes, including tumor invasion, metastasis, and recurrence of PTC [66, 68]. Moreover, it was demonstrated an interesting association of BRAF mutation with loss of radioiodine avidity in recurrent PTC and its failure to be cured [79]. This is consistent with research data of BRAF mutant-promoted silencing of thyroid iodide-handling genes and the reversal of this process by silencing the expression of BRAF mutant in thyroid cells. Additionally, several studies demonstrated a close association of BRAF mutation with dedifferentiation of PTC as reflected by decreased expression of thyroid-specific genes in PTC, including NIS [80, 81], TPO [77–84], pendrin [84], and Tg [80]. Therefore, BRAF mutation is a novel powerful molecular prognostic marker for poorer prognosis of thyroid cancer.

5. PI3K/Akt Signaling Pathway in Thyroid Cancer

Like the MAP kinase pathway, the phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway (PI3K pathway) plays a fundamental role in the regulation of cell growth,

proliferation, and survival, and in human tumorigenesis [85, 86]. Among the several classes of PI3Ks, class I is the best characterized and is composed of heterodimers of a regulatory subunit, particularly p85, and one of the several p110 catalytic subunits. The α-type (PIK3CA) and β-type (PIK3CB) p110 subunits are widely expressed in different tissues, whereas other types of p110 subunits are only expressed in limited tissues.

There are three types of Akts: Akt-1, Akt-2, and Akt-3. Activated Akt phosphorylates downstream protein effectors and amplifies the signaling cascade, promoting cell proliferation and inhibiting apoptosis. Signaling of the PI3K/Akt pathway is antagonized by the tumor suppressor gene PTEN product, PTEN, which is a phosphatase that dephosphorylates PIP3, hence terminating the signaling of the PI3K/Akt pathway [87].

Previous studies showed common activation of the PI3K signaling in thyroid cancers [88]. The three isoforms of Akt, Akt-1, and Akt-2 were the most abundant and important in thyroid cancer. It was reported that genomic copy gain and amplification of the PIK3CA occur in thyroid tumors, particularly FTC and ATC [89–91]. Moreover, PIK3CA mutation is particularly common in ATC and is relatively uncommon but can occur in differentiated thyroid cancer [88–91]. A number of genetic alterations in the PI3K pathway, including PIKCA mutation and amplification, Ras mutation, and PTEN mutation are found in a relatively high prevalence, particularly in FTC and ATC tumors [90, 91]. Coexistence of some of these genetic alterations and their coexistence with BRAF mutation were more frequently seen in aggressive thyroid cancers, particularly ATC [90]. Interestingly, genetic alterations that could activate both the MAP kinase and PI3K pathways were found in most (81%) ATCs.

These data provide the strongest genetic evidence for an extensive role of dual involvement of the MAP kinase and PI3K pathways in the pathogenesis of ATC, supporting a recent hypothesis that targeting multiple signaling pathways, particularly the MAP kinase and PI3K/Akt pathways, may be an effective and necessary therapeutic strategy for thyroid cancer.

6. PAX8-PPARγ Rearrangement

The PAX8 gene encodes a transcription factor essential for the genesis of thyroid follicular cell lineages and regulation of thyroid-specific gene expression. The peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear hormone receptor superfamily that includes thyroid hormone, retinoic acid, and androgen and estrogen receptors [92]. The PAX8-PPARγ rearrangement leads to in-frame fusion of exon 7, 8, or 9 of PAX8 on 2q13 with exon 1 of PPARγ on 3p25 [93].

The exact mechanism by which this rearrangement imparts a carcinogenic phenotype is not fully understood. It appears as though the PAX8-PPARγ chimeric protein inactivates the wild-type PPARγ, which is a putative tumor suppressor [93, 94].

As with RAS mutations, PAX8-PPARγ rearrangement has also been shown to be involved in the development of thyroid follicular carcinoma. The PAX8-PPARγ rearrangement is found in follicular thyroid carcinoma and in the follicular variant of PTC, where it occurs in approximately 33% of all tumors [95, 96]. The rearrangement has also been shown to occur in follicular adenomas and is not specific for carcinoma [95].

The role of this rearrangement in the progression and dedifferentiation of follicular thyroid cancer to PDTC and ATC has not been well defined.

7. p53 Inactivation

The p53 gene encodes a nuclear transcription factor that plays a central role in the regulation of cell cycle, DNA repair, and apoptosis [97]. As the guardian of the genome, p53 is overexpressed after cellular exposure to DNA-damaging agents and causes transient cell cycle arrest, presumably to allow for DNA repair [98].

However, if the damage is severe, it initiates apoptosis to prevent replication of the flawed cell. Cells with impaired p53 function are likely to accumulate genetic damage and are at a selective advantage for clonal expansion. Alterations in the p53 tumor suppressor gene by inactivating point mutations, usually involving exons 5–8, or by deletion result in progressive genome destabilization, additional mutations, and propagation of malignant clones. This represents the most frequent genetic damage in human cancer, usually occurring as a late tumorigenic event.

Among thyroid tumors, p53 mutations are generally restricted to PDTC and ATC [99, 100]. Point mutations of p53 occur in approximately 60% of ATC and in 25% of PDTC [99–101]. Moreover, in tumors with both well-differentiated and anaplastic components, p53 mutations were present only in the anaplastic component [102–104]. These findings are consistent with the hypothesis that p53 inactivation likely serves as a second hit, triggering tumor dedifferentiation and progression to PDTC and ATC.

Experimental studies have shown that loss of p53 results in progressive dedifferentiation of thyroid tumors. Transgenic mice with thyroid-specific RET/PTC rearrangements developed PTC, but when crossed with $p53^{-/-}$ mice, the progeny succumbed to rapidly growing PDTC and ATC [105, 106]. Conversely, the recovery of wild-type p53 in cultured ATC cells resulted in the re-expression of thyroid-specific genes and the reability to respond to thyroid-stimulating hormone [107, 108].

It is unlikely that p53 mutation is an initiating event in PDTC or ATC; it is likely a late event that contributes to the evolution of the transformed phenotype.

8. Epigenetic Regulation in Thyroid Cancer

Recognizing that DNA is associated with histone proteins to form a condensed structure known as chromatin, research is now investigating how modifications in chromatin structure may contribute to carcinogenesis. Epigenetic modifications refer to heritable alterations of the DNA structure, histones, and/or in nucleosome remodeling, resulting in altered gene

expression [109]. Epigenetic changes have been described in thyroid cancer, most notably the altered DNA methylation patterns in the CpG islands of promoters of genes important in normal thyrocyte function such as the sodium-iodide symporter and the TSH receptor [110, 111]. Increased promoter methylation by DNA methyltransferases (DNMTs) leads to gene silencing and further dedifferentiation of the thyroid tumor. DNMT inhibitors such as 5′-aza-deoxycytidine are being evaluated as "redifferentiation" agents, thereby allowing tumors to again become more responsive to conventional therapy such as radioactive iodine [112]. Identification of specific methylation patterns may also allow stratifying tumors that may no longer be responsive to thyroid hormone suppressive therapy and I-131.

Research on how posttranslational modification of histones may influence cancer has recently seen tremendous growth. The nucleosome, or basic structural unit of chromatin, consists of 147 bp of DNA wrapped around an octamer of four core histone proteins (H2A, H2B, H3, and H4) [113]. Histone modifications include methylation, acetylation, phosphorylation, and ubiquitination and may act in concert with DNA promoter methylation to modulate gene silencing [114]. Epigenetic drug targets may play a more central role in cancer treatment in the future.

9. Conclusions

Remarkable advances have occurred in recent years in understanding the molecular biology of thyroid cancer.

This is reflected in several major biological areas of thyroid cancer, including the molecular alterations for the loss of radioiodine avidity of thyroid cancer, the pathogenic role of the MAP kinase and PI3K/Akt pathways and their related genetic alterations, and the aberrant methylation of functionally important genes in thyroid tumorigenesis and pathogenesis. These exciting advances in molecular biology shine great promises on the development of novel molecular-based strategies to effectively tackle these diagnostic, prognostic, and therapeutic obstacles of thyroid cancer.

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