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Molecular Pathogenesis of Euthyroid and Toxic Multinodular Goiter

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The purpose of this review is to summarize current knowledge of the etiology of euthyroid and toxic multinodular goiter (MNG) with respect to the epidemiology, clinical characteristics, and molecular pathology.

In reconstructing the line of events from early thyroid hyperplasia to MNG we will argue the predominant neoplastic character of nodular structures, the nature of known somatic mutations, and the importance of mutagenesis. Furthermore, we outline direct and indirect consequences of these somatic mutations for thyroid pathophysiology and summarize information concerning a possible genetic background of euthyroid goiter.

Finally, we discuss uncertainties and open questions in differential diagnosis and therapy of euthyroid and toxic MNG. (*Endocrine Reviews* 26: 504–524, 2005)

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Abbreviations: AFTN, Autonomously functioning thyroid nodule; BRAF, v-raf murine sarcoma viral oncogene homolog B1; CTN, cold thyroid nodule; FNAC, fine-needle aspiration cytology; IGFBP, IGF binding protein; MNG, multinodular goiter; NIS, Na^+/I^- symporter; PDS, pendrin gene; Smad, mothers against decapentaplegic homolog; TA, toxic adenoma; TG, thyroglobulin; THOX, thyroid-specific oxidase; TMNG, toxic MNG; TPO, thyroid peroxidase; TRAB, TSHR antibodies; TSHR, TSH receptor.

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I. Definition and Epidemiology

BENIGN NODULAR THYROID disease constitutes a heterogenous thyroid disorder, which is highly prevalent in iodine-deficient areas. On a very general basis, it can be divided into solitary nodular and multinodular thyroid disease. Histologically, benign thyroid nodules are distinguished as 1) encapsulated lesions (true adenomas) or adenomatous nodules, which lack a capsule; and 2) by morphological criteria according to the World Health Organization (WHO) classification (1). On functional grounds, nodules are classified as either "cold," "normal," or "hot" depending on whether they show decreased, normal, or increased uptake on scintiscan. Approximately 85% of all nodules are cold, 10% are normal, and 5% are hot (2, 3), although the prevalence may vary geographically with the ambient iodine supply. In contrast to solitary nodular thyroid disease, which has a more uniform clinical, pathological, and molecular picture, euthyroid multinodular goiter (MNG) and toxic multinodular goiter (TMNG) are a mixed group of nodular entities, *i.e.*, one usually finds a combination of hyperfunctional, hypofunctional, or normally functioning thyroid lesions within the same thyroid gland. The overall balance of functional properties of individual thyroid nodules within a MNG ultimately determines the functional status in the individual patient, which may be euthyroidism (normal TSH and free thyroid hormone levels), subclinical hyperthyroidism (low or suppressed TSH and normal free thyroid hormone levels), or overt hyperthyroidism (suppressed TSH and elevated free thyroid hormone levels). The term MNG is applied to the first scenario, whereas TMNG refers to the latter situations. It is important to emphasize that this functional picture is not stationary, but patients with TMNG usually have a history of long-standing MNG (4). Moreover, the status of TSH suppression in TMNG does not only imply clinical consequences for the patient but, importantly, it also indicates that a critical level of thyroid autonomy, *i.e.*, inde-

pendence of TSH, the physiological regulator of thyroid function and growth (5, 6), has been reached. Constitutive activation of the cAMP signaling pathway is widely accepted as the biochemical driving force of thyroid autonomy, as suggested by the presence of somatic activating TSH receptor (TSHR) mutations in scintigraphically nonsuppressible foci in euthyroid goiters in iodine-deficient areas; the presence of somatic TSHR mutations and, less frequently, $G_s\alpha$ protein mutations in macroscopic toxic thyroid nodules both in solitary nodules and multinodular disease; the phenotype of patients with activating germline TSHR mutations; and a number of animal models of thyroid autonomy (reviewed in Refs. 7–10).

Iodine deficiency is by far the best studied epidemiological risk factor for nodular thyroid disease. The prevalence of nodular thyroid disease (as well as goiter) is inversely correlated with the population's iodine intake (11, 12). This has formerly been assessed clinically by palpation, nowadays considered highly inaccurate (13–15), but is also clearly documented by thyroid ultrasonography. Based on ultrasound investigation, a frequency of thyroid nodular disease as high as 30–40% (in women) and 20–30% (in men) of the adult population has been reported in iodine-deficient areas. Furthermore, even minor differences in the ambient iodine supply may be reflected in the different prevalence of thyroid abnormalities; Knudsen *et al.* (16) found a difference in goiter prevalence (15% in mild and 22.6% in moderate deficiency) and nodule size (increased in the moderate iodine deficiency group). The prevalence of thyroid nodules seems to increase with age (4, 17, 18). In a borderline iodine deficiency area, MNG was present in 23% of the studied population of 2656 Danish people aged 41 to 71 yr and increased with age in women (from 20 to 46%) as well as men (from 7 to 23%) (3). In contrast, the relation between age and thyroid volume is less coherent, whereby in iodine-deficient areas (except for severe deficiency), thyroid enlargement peaks around 40 yr with no tendency for further increase (19). Interestingly, similar observations have been made in an iodine-sufficient area. In the 20-yr follow-up of the Whickham Survey, the frequency of goiter decreased with age (goiter prevalence initially, 23% women and 5% men; at 20-yr follow-up of the same patients, 10% women and 2% men) (20).

Thyroid nodules are found with higher frequency in enlarged thyroid glands, although all clinicians will agree that they may also be present in an otherwise normal thyroid gland (4, 18, 21). The correlation between iodine supply and prevalence of nodular thyroid disease can similarly apply to TMNG. The high frequency of thyroid autonomy, which accounts for up to 60% of cases of thyrotoxicosis in iodine-deficient areas is largely due to TMNG (~50%, solitary toxic nodules ~10%) (12, 22). Prevalence of thyroid autonomy correlates with increased thyroid nodularity and increases with age (4, 22). In contrast, thyroid autonomy is rare (3–10% of cases of thyrotoxicosis) in regions with sufficient iodine supply (22, 23). Correction of iodine deficiency in a population results in decrease of thyroid autonomy as demonstrated by the impressive 73% reduction in prevalence of TMNG only 15 yr after the doubling of iodine content of salt in Switzerland (12, 24). Although goiter and euthyroid and toxic nodular thyroid disease share the common and important epidemiology of iodine deficiency, it needs to be stressed that most epidemiological conclusions are derived from cross-sectional studies. Thyroid nodules (and goiter) also occur in individuals without exposure to iodine deficiency, and not all individuals in an iodine-deficient region develop a goiter. Moreover, there is a strong clustering of goiter in families (see *Section VIII*).

Screening has been performed for other "environmental factors" (19). Smoking has been proposed as a risk factor for goiter (25), and nodules were also found with higher prevalence in goiters of smokers compared with nonsmokers. The impact of smoking on thyroid disease could be due to increased thiocyanate levels in smokers exerting a competitive inhibitory effect on iodide uptake and organification (19, 26). The association is more pronounced in iodine deficiency (26). Radiation is another environmental risk factor not only for thyroid malignancy but also for benign nodular thyroid disease. An increased prevalence of nodular disease has been associated with exposure to radionuclear fallouts and therapeutic external radiation, and this theory is being discussed by some authors to explain occupational exposure to low-level radiation (27–31). Furthermore, several studies suggest that thyroid volume is also significantly correlated with body weight and body mass index. In agreement with this, a recent study has shown that in obese women, weight loss of more than 10% may result in a significant decrease in thyroid volume (32).

Nodular disease is more frequent (5- to 15-fold) (33, 34) in women, but the reasons for this are poorly understood. Thus, at present, one can only speculate as to a genetic susceptibility for thyroid disease (for details, see *Section VIII*) and/or a direct impact of steroid hormones. In fact, a growth-promoting effect of estrogen has been described *in vitro* in rat FRTL-5 cells and thyroid cancer cell lines and has been proposed as a possible contributing, constitutional effect of gender (35, 36). In addition, 17β-estradiol has been suggested to amplify growth factor-induced signaling in normal thyroid and thyroid tumors (36). Interestingly, the use of oral contraceptives, which antagonize the physiological hormonal cycle, has been reported to be associated with a decrease in goiter (but not nodules, although this may represent an age artifact of the studied population). On the other hand, pregnancy-related thyroid enlargement was clearly related to iodine deficiency (19), and in one German study (37), increased MNG prevalence with parity was only observed in those women who had not taken iodine supplementation during an earlier pregnancy.

In summary, the development of nodular disease is influenced by multiple environmental components interacting with constitutional parameters of gender and age. However, whether these factors actually result in goiter or nodular thyroid disease is a different matter, ultimately decided by the genetic background of the individual patient (see *Section VIII*).

II. Clinical Aspects of MNG and TMNG

Clinical features in a patient with MNG can be attributed to thyroid enlargement and thyrotoxicosis in the case of

TMNG. Thus, a patient may present with a lump or disfigurement of the neck, intolerance of tight necklaces, or increase in collar size. Dysphagia or breathing difficulties due to local esophageal or tracheal compression may be apparent, especially with large goiters (33). In addition to cosmetic aspects and compression signs, the daily challenge is to identify very rare thyroid malignancy in very frequent nodular thyroid disease, and strategies to approach that goal have been reviewed in detail elsewhere (38–41).

Alternatively, patients may present with symptoms suggestive of hyperthyroidism, the clinical presentation of which varies considerably with age. In a series of 84 French patients with overt hyperthyroidism, classical signs of thyrotoxicosis, *e.g.*, nervousness, weight loss despite increased appetite, palpitations, tremor, and heat intolerance were more frequently observed in younger patients (≤50 yr) (42), whereas atrial fibrillation and anorexia dominated in the older age group (≥70 yr). In addition, subclinical hyperthyroidism, defined by low or suppressed TSH with normal free T_4 and free T_3 levels is more commonly observed in older patients with TMNG (43). In fact, the incidental finding of low or suppressed TSH levels on routine investigation in iodine-deficient regions for other conditions is frequently a first indicator for presence of thyroid autonomy (4). Subclinical hyperthyroidism is more than just a low TSH status, because it is associated with increased prevalence of atrial fibrillation and bone density loss (43). In addition, an increased cardiovascular mortality rate in patients with low serum TSH levels has been described in a 10-yr cohort-study in the United Kingdom (44). The management of euthyroid and toxic multinodular thyroid disease has recently been extensively reviewed by Hegedüs *et al.* (33).

A. Differential diagnosis

Very rarely, TMNG occurs as an autosomal, dominantly inherited disease caused by activating germline mutations in the TSHR gene (45). A positive family history of recurrent hyperthyroidism and goiter with absence of typical diagnostic features of Graves' disease, persistent neonatal thyrotoxicosis, and relapsing nonautoimmune thyrotoxicosis in childhood are highly suggestive of the condition. So far, more than 150 patients (10 families and 11 children with sporadic occurrence of TSHR germline mutations) have been reported in the literature (http://www.uni-leipzig.de/innere/TSHR). Thyroid ablation is advocated as the first-line treatment (surgery and/or radioiodine) to prevent relapses. Molecular analysis for germline TSHR mutations offers the possibility for family screening, preclinical diagnosis, and genetic counseling (7, 46).

In iodine-deficient areas, the distinction between thyroid autonomy and Graves' disease can be complicated by absence of extrathyroidal signs of autoimmune thyroid disease and "atypical" diagnostic findings. In this regard, several possibilities may be encountered. First is the erroneous classification of Graves' disease as TMNG due to presence of thyroid nodules observed in 10–15% of Graves' disease patients or a patchy scintiscan appearance compatible with TMNG (47, 48). Second is the failure to detect TSHR antibodies (TRAB) in Graves' disease using less sensitive assays. This is illustrated by the detection of TRAB with highly sensitive second-generation assays and/or bioassays in up to 56% of patients with scintiscan appearance of TMNG and up to 22% of patients with diffuse uptake and absence of eye disease and negative TRAB results in older assays (erroneously classified as "diffuse thyroid autonomy") (47–49). Third is confusion of familial occurrence of (autoimmune) hyperthyroidism with hereditary thyroid autonomy, which might clinically masquerade as Graves' disease. In this scenario, absence of TRAB is highly suggestive of familial nonautoimmune hyperthyroidism due to a constitutively activating TSHR germline mutation (46).

III. Natural Course of MNG and TMNG

A. Nodule growth

From the epidemiological data discussed above, one might expect an inherent progressive course of nodular thyroid disease. Studies aimed at accurate assessment of the nodules by ultrasonography differ in terms of follow-up period, definition of growth (increase in volume or nodule diameter), type of thyroid lesion (solid, cystic), and the background in which they are conducted (*e.g.*, environmental factors, specialized thyroid clinic). Moreover, the interobserver variability of long-term studies of nodule volumes is not known. With these caveats in mind, the following observations have been reported: in iodine-sufficient areas, nodule "growth" has been reported in 35% of U.S. patients over a follow-up period of 4.9 to 5.6 yr (50). In another U.S. study, nodule growth (>15% increase in volume) was observed with similar frequency over a highly variable follow-up period (1 month to 5 yr) (51). On long-term follow-up over 15 yr in an area of iodine sufficiency, only one third of benign nodules showed growth as assessed by palpation and ultrasonography compared with the majority of nodules, which remained unchanged or even showed a decrease in size (52, 53). In the German setting, for which the iodine deficit has been calculated at 30% of the recommended intake (54), a mean 3-yr follow-up of 109 consecutive patients showed a steady and significant (>30% volume) increase in nodular size in 50% of patients (55). In a Danish study (3), only four (8%) of 45 cold nodules in an area of borderline iodine deficiency showed a change in size (>5 mm in diameter), of which only one nodule actually increased and three nodules shrank over a follow-up period of 2 yr (Table 1). The conclusion, which is suggested by these data, is that both in an iodine-deficient and -sufficient setting a variable portion, but most likely not all, nodules will grow, and the speed of growth is highly heterogeneous. Thus, identification of nodules with an increased growth potential is a challenge. This may also be relevant to therapeutic management. In fact, one could speculate that discrepant results reported in various treatment studies may actually reflect this heterogeneity of proliferation (and/or the potential to taper it down by treatment) rather than an evidence-based treatment effect of iodine *vs.* iodine plus levothyroxine or levothyroxine alone. Furthermore, results of currently available studies (3, 52, 53, 55) do not allow conclusions as to whether nodule growth is associated with an increased risk of thyroid malignancy, and thus

TABLE 1. Natural course of benign thyroid nodules in studies in iodine-deficient and iodine-sufficient areas with more than 2 yr follow-up

Study	Country	No. of patients	Location	Assessment	Follow-up	Definition of growth	Growth (%)	Comment
Papini *et al.* (56)	Italy	41	Multicenter study	10 MHz ultrasound	5 yr	>12% Increase in volume	56	Placebo group in L-T_4 study
Brander *et al.* (50)	Finland	34	Cohort study	7.5 MHz ultrasound	4.9–5.6 yr	Not defined	35	Investigation also of lesions < 10 mm
Alexander *et al.* (51)	USA	268	Thyroid nodule clinic	5–15 MHz ultrasound	1 month to 5 yr	>15% Increase in volume	39	Growth predominantly in solid nodules
Kuma *et al.* (53)	Japan	134	Hospital	Palpation and 7.5 MHz ultrasound	9–11 yr	Not defined	21	>40% (80% of cystic nodules) of nodules decreased or disappeared
Quadbeck *et al.* (55)	Germany	109	Endocrine clinic	7.5 MHz ultrasound	3–12 yr	>30% Increase in volume	50	No correlation with nodule function, age, and gender
Knudsen *et al.* (3)	Denmark	45	Cohort study	7.5 MHz ultrasound	2 yr	>5-mm change in diameter	2	10% Decreased only cold nodules studied

the question arises as to the benefits of nodule volume reduction. In the authors' opinion, therapy, if efficient, is possibly better aimed at the primary prevention of the evolution of novel/further thyroid nodules in predisposed patients (56, 57), with the long-term prospective goal to reduce ablative thyroid treatment for cosmetic reasons, compression symptoms, and, importantly, thyroid malignancy. However, the proof of principle for any of these suggestions is still awaited.

B. Thyroid function

Transition from euthyroidism to hyperthyroidism in a patient with multinodular thyroid disease is a more relevant clinical issue than growth. We know that hyperthyroidism in TMNG develops insidiously and that TMNG is usually preceded by a long-standing MNG. In fact, autonomous areas have been described in up to 40% of euthyroid goiters in iodine-deficient regions (58). The most accurate epidemiological data on evolution of hyperthyroidism have been published for solitary toxic adenoma (TA), and most of these aspects can possibly also apply to MNG. The natural course is slow. An overall 4.1% annual incidence of thyrotoxicosis was observed in a group of 375 untreated euthyroid patients with TA in Germany, who were followed for a mean period of 53 months (59). In two longitudinal studies an incidence of 9–10% of overt thyrotoxicosis has been reported in patients with euthyroid MNG over a mean follow-up period of up to 12.2 yr (60, 61). There is a correlation between nodule size and development of hyperthyroidism: in an American study (23), 93.5% of patients with overt hyperthyroidism had TA greater than 3 cm in size, and patients with a euthyroid TA of greater than 3 cm size carried a 20% risk of developing hyperthyroidism during a 6-yr follow-up period as opposed to a 2–5% risk of patients with nodules less than 2.5 cm in size. Similarly, in areas with iodine deficiency, an autonomous volume of 16 ml has been determined to be critical for clinical manifestation of hyperthyroidism (62). In TMNG the extent of thyroid nodularity (and hence the autonomous volume) is related to the prevalence of low or suppressed TSH levels, and both parameters are correlated with age (4). A sudden stimulation of thyroid function resulting in clinical manifestation as thyroid autonomy can be induced by the administration of excessive amounts of iodine, *e.g.*, in the form of contrast media widely used for angiography and computed tomography scans or by iodine-containing drugs, *e.g.*, amiodarone (63). In the European Study Group of Hyperthyroidism, a high proportion of iodine contamination was observed ranging from 18% in Graves' disease to 54% in nonautoimmune hyperthyroidism (64). Severity of iodine deficiency, autonomous thyroid cell mass, the quantity of administered iodine, and older age have been proposed as risk factors for the development of iodine-induced hyperthyroidism (64).

IV. Clonal Origin of Thyroid Nodules

Studies that address the clonal expansion of a tumor have provided a valuable tool to decide the nature or etiology of a focal growth event to be either neoplasia or hyperplasia. Although hyperplasia is a reversible outcome of an external trophic stimulus (*e.g.*, iodine deficiency in the thyroid), neoplasia results from an intracellular defect (*i.e.*, genetic alteration) and is irreversible (this definition of neoplasia in not equivalent to malignancy). For the thyroid gland, a critical review of the early work on clonal analysis was given by Thomas *et al.* (65). Later, heterozygous polymorphisms in X chromosome-linked markers (66) have been extensively used to demonstrate a predominant clonal origin of tumor tissues, including the thyroid gland (67–71). Despite increasing technical concern (reviewed in Ref. 72), clonal analysis is still a frequently used tool in tumor biology often seen as an intermediate step in pursuing the ultimate goal, which is the detection of the molecular cause of neoplastic growth, namely mutations in the genomic DNA. Recent results of clonal analysis after PCR amplification of X-linked markers from microdissected tumors need to be interpreted with caution (73). The thyroid develops from a number of progenitor cells that migrate from the floor of the primitive pharynx called the median thyroid anlage (74). In females each progenitor shows a defined pattern of inactivation for most genes on one of the two X chromosomes that is conferred to progeny (75). Proliferation of these progenitors forms a cluster of cells (the thyroid patch) that share the same pattern of X chromosome inactivation. If a sample for clonal analysis (*e.g.*, from a microdissected tumor) lies entirely within such

a patch/cluster, an identical pattern of X chromosome inactivation, which implies monoclonality, is not a reliable marker for neoplasia (72, 76). Vice versa, without microdissection the distinction of monoclonal origin of samples from true neoplasia could be concealed by contamination with blood, connective tissue, and surrounding healthy tissue. In both cases, a histochemical analysis of clonal origin that allows the examination of the clonal architecture would be very helpful. However, available techniques cannot be applied in general, because the tissue under investigation has to meet several requirements (73, 77). Nevertheless, histology-based clonal analysis (73, 77) indicates a patch size in the thyroid gland that is much smaller than patch sizes determined with PCR using paraffin-embedded tissue sections (78). Moreover, in line with the histology-based data, our own study using the PCR approach on microdissected thyroid follicles demonstrates a polyclonal origin in about 25% of single follicles (K. Krohn, unpublished observations). If a hyperplastic lesion is likely to arise from a single patch, then clonal analysis with the current methodology would have a strong bias toward showing monoclonality for this lesion. It is therefore crucial to consider the possible conditions, assuming that a hyperplastic nodule could arise from a single thyroid patch. This decision depends mainly on the extent of thyroid patch size and the growth potential of a hyperplastic lesion. If the thyroid patch size is small, a higher growth potential would be necessary to allow a hyperplastic lesion to develop from a few follicles into a macroscopically detectable thyroid nodule. Because data that could determine this growth potential *in vivo* are not directly available, we would instead like to consider data that show the extent of thyroid hyperplasia after goitrogenic stimulation in animal models. These data suggest a rather low growth potential because thyroid enlargement under extrinsic goitrogenic stimulation (*e.g.*, iodine deficiency or extended TSH stimulation) is rarely higher than 3- to 5-fold (4, 79–81). In contrast, intrinsic or intracellular growth stimulation caused by genetic manipulation in transgenic mice leads in some cases to increases of thyroid mass in the range of 100-fold (10, 82, 83). If this difference also applies to focal stimulation, it is very unlikely that a hyperplastic thyroid lesion (caused by extrinsic stimuli) that originates only from a single patch would reach the cell mass of a normal thyroid nodule. Therefore, it is more likely that a macroscopically detectable thyroid hyperplastic nodule originates from more than one patch. If so, this nodule should be detectable as polyclonal, if a large part of the respective tissue is studied for X chromosome inactivation. As a result, studies of clonal analysis in our group used DNA extracted from the entire nodular tissue. This approach very likely reduces the strong bias toward showing monoclonality for a hyperplastic lesion.

Our investigations of the clonal origin of autonomously functioning thyroid nodules (AFTNs) and solitary cold thyroid nodules (CTNs) (both adenomas and adenomatous nodules) used a PCR approach to amplify the X-linked human androgen receptor from genomic DNA of female patients (84–86). After thorough screening for somatic mutations in these thyroid nodules (for details, see *Section V*), we could demonstrate that thyroid nodules with a somatic mutation are predominantly of clonal origin (84–86). This is not surprising, because it is in full agreement with the widely accepted paradigm in tumor biology that neoplasia originates from a single mutated cell (87). Moreover, we were especially interested in data that would elucidate the etiology of mutation-negative nodules. Interestingly, more than 50% of mutation-negative cases from female patients show a monoclonal origin when tested for X chromosome inactivation (84–86). This could indicate a neoplastic process with a mutation in a gene other than the TSHR, the $G_s\alpha$ protein, or the ras family of oncogenes. Moreover, our finding of an overall frequency for the monoclonal origin of thyroid nodules at about 60–70% agrees with a number of other studies (67, 68, 70, 71) and further underscores that thyroid nodules predominantly result from a neoplastic process with somatic mutations as the starting point (8).

V. Hot Thyroid Nodules

A. Signal transduction of hot thyroid nodules with and without TSHR mutations

Both growth and function of the thyroid are controlled by TSH (5). Although the activation of the TSHR preferentially leads to stimulation of the adenylyl cyclase via the $G_s\alpha$ protein, at higher TSH concentrations an activation of the phospholipase C cascade by $G_q\alpha$ has also been shown (88, 89). Moreover, there is evidence that the TSHR may be coupled to other members of the G protein family (88, 90). However, experimental data are frequently focused on the cAMP branch of TSH signaling. Early work by Pisarev *et al.* (91) demonstrated that cAMP elevation causes goiter. Also, in the thyroid gland and cultured thyroid epithelial cells as well as other endocrine tissues, it is widely accepted that cAMP stimulates proliferation (92–95). More recently, transgenic models were studied to further understand TSHR signaling in more detail: chronic *in vivo* stimulation of the cAMP cascade stimulates epithelial cell proliferation *in vivo* (82, 96, 97). A dominant, negative cAMP response element binding protein blocks signaling downstream of cAMP and causes severe growth retardation and primary hypothyroidism (98). TSH/TSHR signaling generally controls iodine metabolism but only affects growth in the adult thyroid gland and not during embryonic development (99, 100).

Somatic point mutations that constitutively activate the TSHR were first identified by Parma *et al.* (101) in hyperfunctioning thyroid adenomas. However, in different studies, the prevalence of TSHR and $G_s\alpha$ mutations in AFTNs has been reported to vary from 8 to 82% and 8 to 75%, respectively (101–112). These studies differ in the extent of mutation detection and the screening methods. Comparisons with respect to the obvious differences between the studies have been performed elsewhere (8, 113, 114). A comprehensive study of our group using the more sensitive denaturing gradient gel electrophoresis (115–117) revealed a frequency of 57% TSHR mutations and 3% $G_s\alpha$ mutations in 75 consecutive AFTNs (86). These results raise the question of the molecular etiology of TSHR and $G_s\alpha$ mutation-negative nodules. A possible answer is given by clonal analysis of these AFTNs that demonstrate a predominant clonal origin of thyroid nodules and imply a neoplastic process driven by ge-

netic alteration (for details, see *Section IV*). In a recent study (118), we found that AFTNs without a TSHR mutation show an increased expression of the tumor suppressor protein p53-binding protein 2, which interacts with p53 and specifically enhances p53-induced apoptosis but not cell cycle arrest (119). From this finding, one could speculate that increased expression of this gene could increase apoptosis in AFTNs without a TSHR mutation and thus have a negative effect on the growth of the tumor. However, data on apoptosis in AFTNs do not allow a comparison with respect to the TSHR mutation status (120, 121). Furthermore, the AFTNs without a TSHR mutation differ from the nodules harboring a TSHR mutation in their increased expression of two genes that are involved in the signal transduction of G protein-coupled receptors RGS 6 and G protein-coupled receptor kinase 2. Members of the RGS family have been shown to modulate the function of G proteins by activating the intrinsic GTPase activity of the α-subunits (122), whereas G protein-coupled receptor kinases play a role in the receptor desensitization (123). In general, a higher expression of these genes would rather restrict cAMP accumulation in AFTNs and could have a negative effect on functional autonomy. However, further experiments have to explore the importance of these genes in the etiology of AFTNs without TSHR mutations.

AFTNs with TSHR mutations lack a clear genotype/phenotype correlation (113). A similar finding is evident for germline TSHR mutations (124). Variable phenotypes associated with the same TSHR mutation could be the result of influences on signaling downstream of the TSHR. This modulation could have a number of targets, such as G protein coupling, receptor desensitization, and internalization or cross talk with other signaling cascades. Although our knowledge concerning these targets is far from complete, recent findings are very promising. First, the TSHR itself could be the subject of regulatory mechanisms that contribute to the etiology of AFTNs and the clinical phenotype. Voigt *et al.* (125) showed that β-arrestins interact with the TSHR and are able to desensitize the receptor. Increased expression of β-arrestin 2 in AFTNs could cause desensitization of the TSHR and thereby down-regulate constitutive activation. A similar result could be caused by increased TSHR internalization due to increased expression of G protein-coupled receptor kinases in AFTNs (118, 126). In addition to the direct effects on the TSHR protein, altered interaction with G proteins would be the next downstream level where modulation interferes with constitutive activation. For example, the mutated TSHR could show a shift of the coupling specificity for G proteins (127). Such a shift could allow a more efficient activation of other downstream cascades (*e.g.*, JAK/STAT pathway) through protein kinase C in addition to cAMP and inositol phosphate (128, 129). Gene expression analysis in AFTNs with TSHR mutations in comparison with AFTNs without a TSHR mutation would support this hypothesis, demonstrating an increased expression of JAK 1, protein kinase Cβ1, and ζ mRNA (118). Evidence for other cascades (*e.g.*, RAS/RAF/MEK/ERK/MAP pathway) to play a role in constitutive TSHR signaling in AFTNs is currently missing.

In addition to the intracellular signaling network connected to the TSHR, the extracellular action of different growth factors enhances the complexity of the signal flux into the thyroid cell. Growth factors such as IGF-I, epidermal growth factor, TGF-β, and fibroblast growth factor stimulate growth and dedifferentiation of thyroid epithelial cells (130, 131). Studies that have been focused on insulin and IGF show a permissive effect of insulin and IGF-I on TSH signaling (132–136) and a cooperative interaction of TSH and insulin/IGF-I (137). Signal modulation of the TSHR that would define the etiology of AFTNs and the clinical phenotype could therefore take part at a number of stages and very likely involves genetic/epigenetic, sex-related, and or environmental factors.

B. Secondary/indirect effects of activating TSHR mutations

Because constitutively activating TSHR mutations disturb the coordinated signal transduction network of the thyroid drastically, subsequent changes in the signal transduction network can be expected. These alterations based on the constitutive activation of the TSHR signaling can be described as indirect or secondary effects of the activating TSHR mutations. The use of the microarray technique offers the advantage of a highly parallel analysis of gene expression to analyze changes between AFTNs and CTNs compared with surrounding tissue. This approach also allows the evaluation of which genes and groups of genes are most frequently affected in the molecular etiology of thyroid nodules, so that we may possibly deduce a molecular defect from the expression pattern. In a recent study using the Affymetrix GeneChip technology (Affymetrix UK Ltd., High Wycombe, United Kingdom), we could show a distinctly changed pattern of gene expression of the TGF-β signaling pathway between AFTNs with and without TSHR mutations and their normal surrounding tissue (Fig. 1) (118). The type III TGF-β receptor, mothers against decapentaplegic homolog (Smad) 1, 3, and 4, as well as p300, a transcriptional coactivator, showed a decreased expression in AFTNs, whereas the inhibitory Smad 6 and 7 showed an increased expression in AFTNs. These findings suggest inactivation of TGF-β signaling in AFTNs due to constitutively activated TSHR (*e.g.*, resulting from TSHR mutations). This assumption is supported by the findings of Gärtner *et al.* (138), who showed a decreased expression of TGF-β1 mRNA after TSH stimulation of thyrocytes. Because TGF-β1 has been shown to inhibit iodine uptake, iodine organification, and thyroglobulin (TG) expression (139, 140), as well as cell proliferation in different cell culture systems (141–144), these and our novel findings suggest that inactivation of TGF-β signaling is a major prerequisite for increased proliferation in AFTNs (120, 145).

Eggo *et al.* (146) have shown that enhanced production of IGF binding proteins (IGFBPs) is correlated with inhibition of thyroid function, whereas the TSH-cAMP signaling is capable of inhibiting IGFBP production. Moreover, recent studies (118, 147) reveal a significantly decreased expression of IGF-II and IGFBP-5 and -6 in AFTNs in comparison with their normal surrounding tissue. Taken together, the decreased expression of the IGFBPs and of IGF-II in AFTNs are most likely secondary effects of the increased TSHR-cAMP signaling in AFTNs.

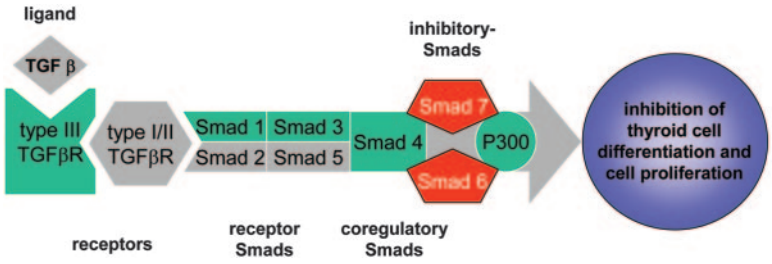

FIG. 1. Differential mRNA expression in components of TGF-β signaling in AFTNs (118). Gene expression analysis revealed a significantly decreased expression (*green boxes*) of the type III TGF-β receptor, Smad 1, 3, and 4 and the adenoviral early region 1A-associated protein p300 in AFTNs in comparison with their normal surrounding tissue, whereas the inhibitory Smad 6 and 7 are characterized by a significantly increased expression (*red boxes*). These findings argue for an inactivation of the TGF-β signaling cascade in AFTNs, which is normally responsible for inhibition of iodine uptake, iodine organification, TG expression, and cell proliferation in thyroid cells.

VI. CTNs

With a frequency of about 85%, CTNs constitute the most abundant thyroid nodular lesion (for detailed definition and epidemiology, see *Section I*). The term "cold" indicates that this thyroid lesion shows reduced uptake on scintiscan. Because histological diagnosis is typically used to exclude thyroid cancer, many investigations of thyroid nodules only refer to the histological diagnosis of thyroid adenoma. This histological entity should not be confounded with the scintigraphically characterized entity cold nodule, which like AFTNs or "warm nodules" (for the distinction, see *Section I*) can histologically appear as thyroid adenomas or adenomatous nodules according to the WHO classification (1). In this review, we will focus on benign neoplastic lesions, because substantial information concerning genetic events and molecular mechanism is available in the literature on human cancer, particularly thyroid carcinoma, which very likely also applies to benign neoplasia of thyroid follicular cells. In contrast, focal hyperplasia is not very well explained on the molecular level and has been discussed in detail elsewhere as the cause of thyroid tumors (148, 149). As detailed in *Section IV*, a monoclonal origin has been detected for the majority of thyroid nodules, which implies nodular development from a single mutated thyroid cell.

Hypotheses in studies that aim to understand the molecular or genetic causes of human cancer in general (150, 151) or AFTN (8) and thyroid carcinomas (152) in detail have often also been applied to studies of CTNs [*e.g.*, ras mutations (153, 154); for review, see Wynford-Thomas (155)]. In contrast to thyroid carcinomas, where a number of genes have been implicated in the pathogenesis of these lesions (152, 156), and in contrast to AFTN where constitutively activating TSHR mutations are very prevalent genetic events (7, 8), knowledge concerning the molecular etiology of CTNs is limited.

A. Iodide transport and metabolism

With reference to their functional status (*i.e.*, reduced iodine uptake), failure in the iodide transport system or failure of the organic binding of iodide has been detected as a functional aberration of CTNs long before the molecular components of iodine metabolism were known (for review, see Ref. 157). Later, a decreased expression of the Na^+/I^- symporter (NIS) in thyroid carcinoma and benign CTNs suggested the molecular mechanism for the failure of the iodide transport (reviewed in Refs. 158–160). Although the extent of decrease in NIS mRNA expression of CTNs varies in different studies (160–163), reduction is in many cases very likely the result of hypermethylation in the NIS promoter (160). Moreover, *in vitro* studies suggest that reduced NIS mRNA expression could be caused by constitutive activation of RET or RAS genes (164–166). However, reduced NIS mRNA expression does not necessarily lead to reduced NIS protein expression (Fig. 2) (160). In contrast to other thyroid disorders with congenital iodide transport defects (for review, see Refs. 167 and 168), no NIS gene mutation that would render this protein nonfunctional was detectable in CTNs (160). Therefore, the recently identified defective cell membrane targeting of the NIS protein is a more likely molecular mechanism that could account for the failure of the iodine uptake in CTNs (158, 160, 169). However, the ultimate cause of this defect is currently unknown.

Compared with iodine transport, the organic binding of iodine is a multistep process with a number of protein components that still awaits final characterization (170). mRNA expression of enzymatic components [*e.g.*, thyroid peroxidase (TPO) or flavoproteins] and the substrate of iodination (TG) have been quantified in CTNs without significant dif-

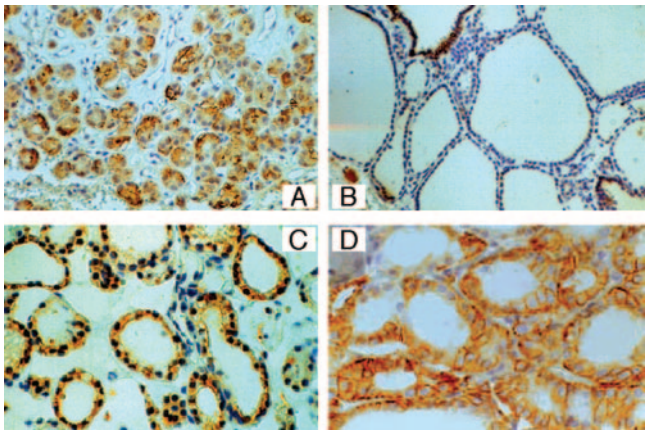

FIG. 2. Immunohistochemical analysis of NIS expression in thyroid nodules and surrounding tissues (160). A, CTN 65: nodule tissue; predominant intracytoplasmic NIS staining, only partial cell membrane immunoreactivity. B, CTN 65: surrounding tissue; human NIS protein immunoreactivity is very low and heterogeneous. C, CTN 13: nodule tissue, mostly intracytoplasmic immunoreactivity. D, Toxic thyroid nodule; NIS cell membrane immunoreactivity is predominant.

ferences to normal follicular tissue (163, 171). TPO, TG, and thyroid-specific oxidases (THOX) have been successfully screened for molecular defects, especially in congenital hypothyroidism (172). Although CTNs could be considered a form of focal hypothyroidism, somatic mutations in enzymes that catalyze organic binding of iodine would need to exert a growth advantage on the affected cell to cause the development of a thyroid nodule. At least in the case of inactivating mutations in the TPO or THOX genes, growth advantage could result from a lack of enzyme activity that would reduce not only thyroid hormone synthesis but also follicular iodide trapping in organic iodocompounds. Because these compounds have been shown to inhibit thyroid epithelial cell proliferation (133, 173, 174), reduced synthesis could have a proliferative effect. Therefore, somatic TPO or THOX mutations could be a molecular cause of CTN. However, mutations in the TPO gene could not be detected (175), and an ongoing screening for mutations in the THOX genes is also negative thus far (K. Krohn, R. Paschke, and C. Ris-Stalpers, unpublished observations).

B. Signaling proteins

In addition to a failure in metabolic proteins that might explain the development of CTNs on the molecular level, pathological changes of signaling molecules might reprogram the growth stimulus and lead to clonal expansion of thyroid epithelial cells. Although not a particular subject of this review, much can be learned from findings in thyroid carcinoma. In this regard, genetic changes (*i.e.*, point mutations) that cause constitutive activation of the RAS/RAF/MEK/ERK/MAP pathway have been suggested as a key mechanism during tumor initiation or progression in thyroid follicular cells (for review, see Ref. 155). So far, the only known molecular event that evidently causes such activation in thyroid carcinomas and CTNs is a mutation in one of the small RAS oncogenes (153). Recently, BRAF mutations, first detected in melanomas and with lower frequency in other cancers (176), have been detected in thyroid papillary carcinomas (177). They can also activate this pathway and might therefore also cause benign follicular lesions. Strikingly, both in colorectal and thyroid cancers, v-raf murine sarcoma viral oncogene homolog B1 (BRAF) mutations occur only in tumors that do not carry mutations in a RAS gene. In a recent study of 40 cold thyroid adenoma and adenomatous nodules, we detected ras mutations in only a single case (85). Moreover, in the same set of CTNs we did not detect point mutations in the mutational hot spots of the BRAF gene (178). This is in line with the lack of BRAF mutations in benign follicular adenoma in other studies (177, 179, 180). So far, only one study (181) detected a single BRAF mutation in a set of 51 follicular adenomas. Instead of RAS and BRAF mutations, there could be other molecular events that could constitutively activate the RAS/RAF/MEK/ERK/MAP pathway. Such candidate molecules include other members of the RAF gene family, like RAF-1, or downstream genes like ERK or MAPKs. However, mutations in these genes have not been reported in benign thyroid lesion thus far. Furthermore, molecular events that lead to activation of other cascades that exert synergy with MAPK signaling (*e.g.*, cAMP signaling) or inactivation of independent cascades that restrict proliferation (*e.g.*, TGF-β signaling) could explain CTNs. In addition to the TSHR, several G protein isoforms like $G_{i2\alpha}$ (182) or G_q, G_{11} (183), and some candidate genes mediating downstream cAMP signaling like Epac and Rap1 (184) have been screened in CTNs for mutations. However, only a single somatic mutation in the $G_{i2\alpha}$ gene (182) was found in follicular adenomas.

C. Results of gene expression studies by arrays

Currently, expression profiling of signaling proteins using microarray methodology is a promising approach that may contribute to further understanding the molecular events that lead to the development of AFTNs (147, 185) or thyroid carcinoma (186–188). Functional characteristics of CTNs suggest that mechanisms initiating growth but not leading to hyperfunction need to be defined. As far as future screenings for genetic defects are concerned, expression profiling could describe the molecular mechanism and rule out a number of possible targets (*e.g.*, because they are not expressed) or unmask alternative candidates. Moreover, as demonstrated for AFTNs, results of expression profiling might shift attention to other signaling cascades (for details on AFTNs, see *Section V*). For differentially expressed genes within these cascades, a sequencing approach might then be warranted. In addition, knowledge of the molecular signature of CTNs and benign thyroid tumors in general could be very helpful to define differences between benign and malignant thyroid disease with diagnostic or therapeutic relevance.

Recently, our group investigated 588 genes by cDNA expression arrays in three AFTNs, three CTNs, and corresponding normal surrounding tissue. In general, changes in the expression of several signal-transducing components were detected. Although this seems to reflect a disturbed signaling system, the results of that limited study did not allow us to identify specific signal transduction cascades that might be involved in nodular development (147). To gain a higher resolution, we compared gene expression for approximately 10,000 full-length genes between CTNs and their corresponding normal surrounding tissue (307). Regulation of gene expression in CTNs was most consistent for a group of several histone mRNAs. Increased expression of these histone mRNAs and of cell cycle-associated genes like cyclin D1, cyclin H/cyclin dependent kinase 7, and cyclin B most likely reflect a molecular setup for an increased proliferation in CTNs (189). In line with the low prevalence of ras mutations in CTNs (85), we find a reduced expression of ras-MAPK cascade-associated genes, which might suggest a minor importance of this signaling cascade.

D. Chromosomal aberrations

Loss of heterozygosity, microsatellite instability, and, more recently, gene rearrangements and chromosomal translocations as different forms of chromosomal aberration are considered important steps in carcinogenesis and have been investigated as potential markers to discern benign from malignant nodular disease. Findings of chromosomal aberrations and microsatellite instability in benign thyroid tu-

mors, although sometimes sporadic, suggest that there is a difference in the extent of these DNA changes (190, 191). Alternatively, these results could stem from errors in histological characterization (192).

Gene rearrangements unique to thyroid adenomas have recently been the focus of study (reviewed in Ref. 193). These studies led to the identification of the thyroid adenoma associated gene that encodes a death receptor-interacting protein (194).

Although also reported for thyroid follicular carcinoma (195), our finding of loss of heterozygosity at the TPO locus is characteristic for some CTNs (~15%) but rather points to defects in a gene near TPO on the short arm of chromosome 2 (175). Moreover, after identification in a significant portion of follicular carcinomas (196), Pax-8/peroxisome proliferator-activated receptor γ gene rearrangement have also been reported for CTNs (197) but seem to be a rare finding (198–200) or attributable to histological misclassification of the thyroid nodules (192). Although the frequency of each of these DNA aberrations is rather low, these chromosomal changes need to be considered together in the further elucidation of the molecular etiology of CTNs.

VII. Multinodular Goiter

MNG refers to an enlargement of the thyroid with deformation of the normal parenchymal structure by the presence of nodules. These nodules vary considerably in size, morphology, and function (for detailed definition and epidemiology, see *Section I*). In areas without endemic goiter, MNG is often referred to as sporadic nontoxic goiter. MNG usually develops in an already enlarged thyroid independent of the cause of hyperplasia (for review, see Ref. 149). Over time (sometimes decades), many MNGs enlarge further, and some develop subclinical hyperthyroidism and subsequently present as TMNG (4, 201). The main epidemiological determinants outlined in detail in *Section I* for the development of MNG and TMNG are iodine deficiency (22), age, sex, and duration of goiter in iodine-deficient (4, 17, 18) and also in iodine-sufficient areas (for review, see Ref. 202). It is widely accepted that the basis for the development of nodular structures is an early stimulus that causes enlargement of the thyroid. However, clinical manifestations of MNG might only appear after a long period of time (sometimes up to 30+ years). In general, development of MNG proceeds in two phases: global activation of thyroid epithelial cell proliferation (*e.g.*, as the result of iodine deficiency or other goitrogenic stimuli) leading to goiter, and a focal increase of thyroid epithelial cell proliferation causing thyroid nodules. So far, the most common stimulus for local proliferation is somatic mutations (see *Sections V and VI*).

A. Mutagenesis as the cause of nodular transformation

From animal models of hyperplasia caused by iodine depletion (79, 203, 204) we learn that, in addition to an increase in functional activity, a tremendous increase in thyroid cell number occurs. These two events very likely orchestrate a burst of mutation events. Although the enzymatic setup awaits further characterization (171), it is known that thyroid hormone synthesis goes along with increased H_2O_2 production and free radical formation (205), which may damage genomic DNA and cause mutations (206). As a consequence, the spontaneous mutation rate in the thyroid is almost 10 times higher than in other organs (*e.g.*, compared with liver) (K. Krohn, unpublished observations). Together with a higher spontaneous mutation rate, a higher replication rate will more often prevent mutation repair and increase the mutagenic load of the thyroid, thereby also randomly affecting genes crucial for thyrocyte physiology. Mutations that confer a growth advantage (*e.g.*, TSHR or $G_s\alpha$ protein mutations) very likely initiate focal growth. Hence, AFTNs are likely to develop from small cell clones that contain advantageous mutations as shown for the TSHR in hot microscopic regions of euthyroid goiters (9).

B. Etiology

Epidemiological studies, animal models, and molecular/genetic data outline a general theory of nodular transformation. Based on the identification of somatic mutations and the predominant clonal origin of AFTNs, we propose the following sequence of events that could lead to thyroid nodular transformation in three steps (Fig. 3). In the first step, iodine deficiency, nutritional goitrogens, or autoimmunity cause diffuse thyroid hyperplasia. Then, at this stage of thyroid hyperplasia-increased proliferation together with a possible DNA damage due to H_2O_2 action causes a higher mutational load with a higher number of cells bearing a mutation. Some of these spontaneous mutations confer constitutive activation of the cAMP cascade (*e.g.*, TSH-R and $G_s\alpha$ mutations) that stimulate growth and function. Finally, in a proliferating thyroid growth factor expression (IGF-I, TGF-β1, or epidermal growth factor) is increased. As a result of growth factor costimulation, all cells divide and form small clones. After increased growth factor expression ceases, small clones with activating mutations will further proliferate if they can achieve self-stimulation. They could thus form small foci, which will develop into thyroid nodules. This mechanism could explain AFTNs by advantageous mutations that both initiate growth and function of the affected thyroid cells as well as CTNs by mutations that stimulate proliferation only (*e.g.*, ras mutations or other mutations in the RAS/RAF/MEK/ERK/MAP cascade). Moreover, nodular transformation of thyroid tissue due to TSH-secreting pituitary adenomas (207), nodular transformation of thyroid tissue in Graves' disease (208), and in goiters of patients with acromegaly (209) could follow a similar mechanism, because thyroid pathology in these patients is characterized by early thyroid hyperplasia.

As an alternative to the increase of cell mass and as illustrated by those individuals who do not develop a goiter when exposed to iodine deficiency, the thyroid might also adapt to iodine deficiency without extended hyperplasia (210). Although the mechanism that allows this adaptation is poorly understood, preliminary data from a mouse model suggest an increase of mRNA expression of TSHR, NIS, and TPO in response to iodine deficiency, which might be a sign for increased iodine turnover in the thyroid cell in iodine deficiency (K. Krohn, unpublished observations).

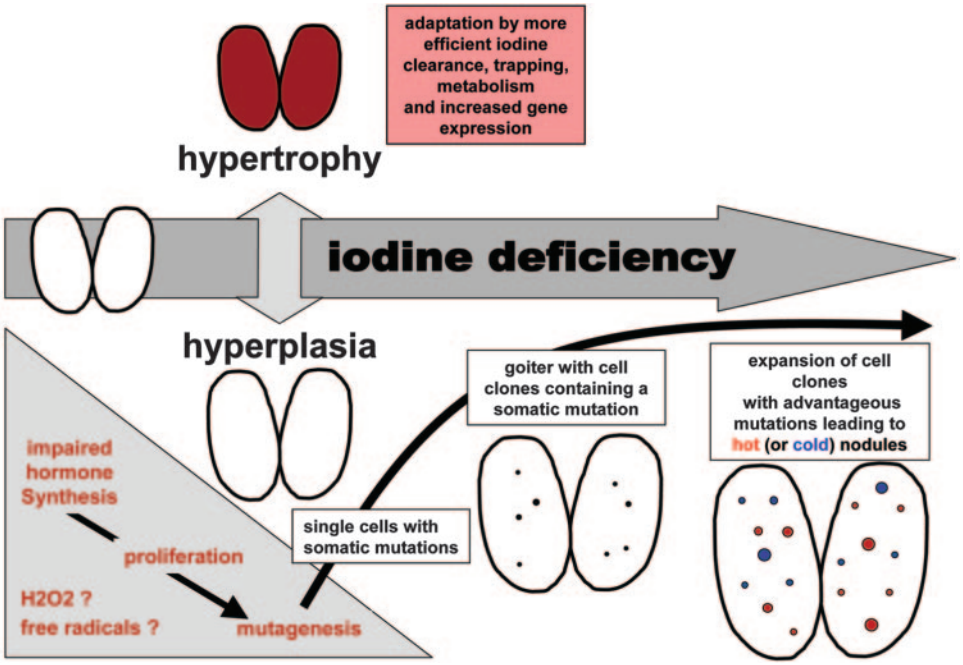

FIG. 3. Hypothesis for thyroid nodular transformation. The starting point for the development of MNG is hyperplasia induced by goitrogenic stimuli (*e.g.*, iodine deficiency). Iodine deficiency increases mutagenesis directly (production of H_2O_2/free radicals) or indirectly (proliferation and increased number of cell divisions). Subsequently, hyperplasia forms cell clones. Some of them contain somatic mutations of the TSH-R, leading to AFTNs (*red dots*), or contain mutations that lead to dedifferentiation and therefore CTNs or cold adenoma (*blue dots*).

VIII. Pathogenesis and Genetic Etiology of Euthyroid Goiter

Considering recent advances in the methodology of genetic analysis, the genetic etiology of goiter is underinvestigated. This applies especially to studies that target the genetic basis for euthyroid and toxic MNG. As outlined in the preceding section, the development of nodular goiter is very likely a continuous process that starts with thyroid hyperplasia and simple goiter. Therefore, defects in genes that play an important role in thyroid physiology and hormone synthesis (see *Section VIII.B*) could be genetic factors that predispose for the mechanisms that lead to MNG. Such defects likely lead to dyshormonogenesis as an immediate response and might not directly explain nodular transformation of the thyroid. In this section, we therefore also consider genetic studies that concern other forms of goiter.

A. Family and twin studies

Although lack of iodine is the most prevalent factor for simple goiter as well as endemic goiter, other causes are likely. Familial clustering of goiters and the female predominance of goiters are the two major arguments suggesting a genetic background for euthyroid goiters. Family and twin pair studies in endemic and nonendemic areas clearly demonstrated a genetic predisposition for goiter development. Within a Greek region, endemic goiter affects some families more than others (211). This could provide evidence for a genetic etiology, although environmental factors that differ between families must also be considered (212). The familial aggregation of goiters in Greece was confirmed in a subsequent study (213). The progeny of affected persons were more often affected by goiter than were the descendants of unaffected subjects. Likewise, other euthyroid goiter family studies in Greece, Slovakia, and Africa also lead to the conclusion that a genetic predisposition is present in the affected individuals (211, 214, 215). In addition, more rapidly growing goiters in a subgroup of schoolchildren (15–20%), despite iodine supplementation (214) and differences in thyroid volume of adolescent siblings with sufficient iodine intake in Slovakia (216), also support a genetic influence on thyroid growth. Moreover, studies in Greek populations (217) have shown the persistence of endemic goiters in certain regions despite iodine supplementation. Familial occurrence of euthyroid goiter in an iodine-replete region in Sweden was reported for 41% of the patients with goiter, with an even higher frequency of familial occurrence in those individuals with prepubertal development of the goiter (218). Although family studies are a reliable method to determine goiters in many family members over several generations, it is impossible to definitively conclude whether the members shared the same susceptible genetic makeup or the same environment. Therefore, twin studies are more informative in demonstrating a genetic component in the etiology of goiter. Several investigations have provided evidence that there is a predisposing genetic background for goiter in twins. In endemic as well as nonendemic areas, female monozygotic twins [80% (213, 219) and 42% (220), respectively] have a higher concordance rate for goiter than female dizygotic twins [40–50% (213, 219) and 13% (220)]. Twins of the same sex are supposed to share the same family environment. Therefore, the increased concordance was attributed to greater genetic similarity characterizing the monozygotic twins. Contribution of genetic susceptibility to the development of goiter was calculated to be 39% in endemic regions (219). Moreover, a study of 5479 monozygotic and dizygotic twins (220) performed by path analyses (structural equation modeling) suggests that the genetic predisposition to develop goiter is 82%, with 18% according to individual environmental factors in a nonendemic area. However, the previously reported twin studies show the importance of both hereditary and environmental factors (33).

B. Candidate loci

Because of their important role in thyroid physiology and hormone synthesis, TG, TPO, NIS, the pendrin gene (PDS), and TSHR are major candidate genes for familial euthyroid goiters.

Studies of hypothyroid goiters have identified several genetic defects in TG and TPO (172). Congenital goiter and hypothyroidism caused by qualitative and quantitative defects of the TG gene were described by Medeiros-Neto *et al.* (221). Other studies have also shown a link between the TG gene and congenital goiter and hypothyroidism (222–229). Furthermore, an inherited abnormality in TG synthesis leading to a lower content of TG in the thyroid gland was reported by Yoshida *et al.* (230), and a single amino acid substitution in the TG protein ($Leu^{2366}Pro$) causes endoplasmic reticulum storage disease as determined in the cog/cog mouse (231). Although TG was postulated to be a major candidate gene for euthyroid simple goiter, only one genetic variation associated with euthyroid goiter has been identified in the TG gene up to date. Corral *et al.* (232) found a G→T substitution at position 2610 of the TG cDNA. This resulted in replacement of histidine for glutamine at codon 870. This sequence alteration was located within exon 10 of the TG gene and was present in 25 of 26 members of three families affected by euthyroid goiter. However, Perez-Centeno *et al.* (233) found the same point mutation in TG exon 10 gene in only one of 36 patients with endemic euthyroid goiter. Hishinuma *et al.* (226, 229) found two novel cysteine substitutions in TG, which caused defects in the intracellular transport of TG in patients with a variant type of adenomatous euthyroid goiter. Gonzalez-Sarmiento *et al.* (234) identified a large heterozygous deletion within the TG gene in a study of 50 cases affected with nonendemic goiter. The deletion involved the promotor region and the exons 1 to 11 of the TG gene and was associated with euthyroid goiter.

Mutations responsible for dyshormonogenesis have also been described in the TPO gene. TPO catalyzes the oxidation of iodide to an iodination species that forms iodotyrosines and iodothyronines. Defects of TPO synthesis caused by a heterogenous spectrum of TPO mutations (235–240) have been reported to result in reduced TPO activity in combination with total iodide organification defect. Hagen *et al.* (241) described an intelligent, euthyroid child with goiter. Together with her affected sister she showed no iodide peroxidation or thyroxine iodination activity. Likewise, a euthyroid woman with a recurrent goiter and partial iodide discharge was described by Pommier *et al.* (242). She had normal iodide peroxidation but deficient TG iodination. However, these are the only two examples for TPO mutation resulting in euthyroid goiters. Most of the previously reported homozygous or compound heterozygous mutations in the TPO gene lead to goiter and hypothyroidism (236, 243, 244).

Since the cloning and molecular characterization of the human NIS gene (245), several defects in this gene have been detected in patients with different phenotypes of thyroid diseases (246). A heterozygous T354P mutation results in congenital hypothyroidism and goiter (247–251). However, two studies (252, 253) reported that the homozygous T354P mutation is associated with euthyroidism and goiter. Moreover, other allelic variants producing deletion, missense, or truncation of the NIS protein have been described (254, 255).

Mutations in the PDS gene cause Pendred syndrome, which is characterized by congenital sensorineural hearing loss combined with goiter. In Pendred syndrome the thyroid enlargement typically begins in childhood and can vary between and within families (256, 257). Reported goiter sizes vary from small nodules to large MNG (258). Almost all affected individuals are clinically and biochemically euthyroid. Positive perchlorate tests suggest that the PDS defects impair the organification of iodide. Different PDS mutations, each segregating with the disease in the families in which they occurred, have been identified (259–263). Most of them are loss-of-function mutations that directly cause thyroid disease in Pendred syndrome. Therefore, the PDS gene is a candidate gene for development of euthyroid goiter.

Furthermore, the TSHR on chromosome 14q31 could be a candidate gene for euthyroid goiter according to its central role for thyroid function and growth. TSHR germline mutations have been found in rare cases of euthyroid familial goiter (7) (see also *Section I.A*). Moreover, a germline genetic variation in codon 727 of the TSHR gene (Asp→Glu) (264) has been associated with TMNG in iodine-deficient areas (265). However, the wild-type TSHR response to TSH was very low in this study, and this *in vitro* finding was not confirmed in another study (266, 267). Moreover, the putative predisposition for TMNG was based on functional analysis of the TSHR codon 727 variation, which revealed a higher cAMP response compared with the wild-type receptor. However, this *in vitro* finding was not confirmed in a recent study (266). Recently, Peeters *et al.* (268) reported that the homozygous C variant (coding for aspartic acid) of the D727E polymorphism was associated with a lower serum TSH level in 156 healthy blood donors. Although this finding supports a possible functional relevance of this polymorphism, the role of a lower TSH level in the development of MNG is unknown.

C. Linkage analysis

Over the past years, linkage analyses became a reliable method to identify novel susceptibility loci for both Mendelian and complex diseases in large families or affected sibling pairs by using genetic markers for microsatellite DNA or repetitive sequences in the entire human genome. Several different susceptible areas have been discovered in large families with non-Mendelian transmission of euthyroid familial goiter. The multinodular goiter 1 (MNG-1) locus on chromosome 14q31 was first reported by Bignell *et al.* (269) as the result of a genomewide linkage analysis of a large Canadian family with 18 patients affected with MNG. A maximum two point LOD score of 3.8 at D14S1030 and a multipoint LOD score of 4.88, defined by D14S1062 and D14S267, was calculated. In another study, a family with recurrent euthyroid goiters was investigated for linkage to the same candidate region (270). According to a dominant pattern of inheritance with full penetrance, indication for linkage was obtained by a maximal two-point LOD score of 1.5 at marker D14S1030 at the MNG-1 locus. Moreover, a maximum multipoint LOD score of 1.49 was obtained for the

region between the TSHR and the MNG-1 candidate loci. The haplotype cosegregation of microsatellite markers confirmed the entire chromosomal segment between both loci on chromosome 14q31 as a positional candidate region for nontoxic goiter. Although the TSHR was a first-line candidate gene, sequence analysis of the TSHR alone revealed several previously reported polymorphisms. In the study by Bignell *et al.* (269), the TSHR was previously clearly excluded as a candidate gene. Another study reported the analysis of a three-generation, Italian pedigree, including 10 affected females and two affected males (271). An X-linked dominant pattern of inheritance was observed. The investigation of 18 markers spaced at 10-cM intervals on the X chromosome revealed evidence for linkage at marker DXS1226 with a significant LOD score of 4.73. These findings led to the conclusion that defects in the Xp22 region caused thyroid disease in this family. Moreover, the haplotype inspection reduced the critical interval to 9.6 cM between the markers DXS1052 and DXS8039.

In view of the different susceptibility loci for euthyroid goiter, a heterogenous mode of inheritance for euthyroid goiter is very likely. Linkage analysis for the thyroid candidate genes has been performed in four German and one Slovakian family (272). The candidate genes were also analyzed, assuming different recombination fractions for the microsatellite markers in two-point and multipoint analysis. Linkage analysis results of this study were not significant enough to definitely exclude or confirm linkage to the investigated candidate genes TG, TPO, and NIS. To date, there is no evidence for or against susceptibility of the investigated candidate genes for euthyroid familial goiter. Because linkage to MNG-1 (14q31) was previously reported in two families (269, 270) and Xp22 in a single family (271), the four German families and one Slovakian family were also investigated to test a more general validity of these candidate regions (272). However, the absence of a correlation of inheritance patterns for the investigated markers in the families and the nonsignificant LOD scores determined according to the Lander-Kruglyak guide suggested a lower probability for MNG-1 and Xp22 as major monogenic causes for the etiology of euthyroid goiter. Moreover, a very weak indication for linkage to PDS and Xp22 was identified in two families (272). Furthermore, the nonsignificant LOD scores calculated in this study suggest that the strongest genetic locus detectable by linkage is unknown to date and that it is probable that different candidate genes or loci cause euthyroid goiter in different families. In conclusion, these studies gave further indications for genetic heterogeneity of euthyroid familial goiters.

To discover novel and more general candidate regions or genes, we performed a genomewide scan to detect susceptibility loci that predispose for euthyroid goiter using 450 microsatellite markers in 18 Danish, German, and Slovakian families, comprising 79 affected and 68 unaffected family members (273). Assuming genetic heterogeneity and a dominant pattern of inheritance, four novel candidate loci on chromosomes 2q, 3p, 7q, and 8p were identified. Four families showed linkage to the 3p locus, whereas the loci 2q, 7q, and 8p each showed linkage in one family. The haplotype inspection delimited a critical interval of 16 cM on 3p (Fig. 4).

Within this interval the thyroid hormone receptor β is mapped. Our mutation screen also included the two thyroid hormone receptor interactor genes 6 and 12 on 7q and 2q in addition to the thyroid hormone receptor β gene (273). However, sequencing of all these candidate genes revealed no germline mutations that would cosegregate with the goiter in the affected families. In conclusion, these genetic studies confirm that genetic heterogeneity is likely to explain the identification of different candidate loci such as MNG-1 (269, 270) and Xp22 (271) in several families.

Most cases of familial goiter present an autosomal dominant pattern of inheritance. However, for the majority of euthyroid goiter cases, a multifactorial genesis with complex interactions of environmental factors such as iodine deficiency, cigarette smoking, age, sex, certain drug use, or emotional stress on a genetic background is more likely. Loci from linkage analysis or association studies could provide important genetic risk factors in a more complex genetic background.

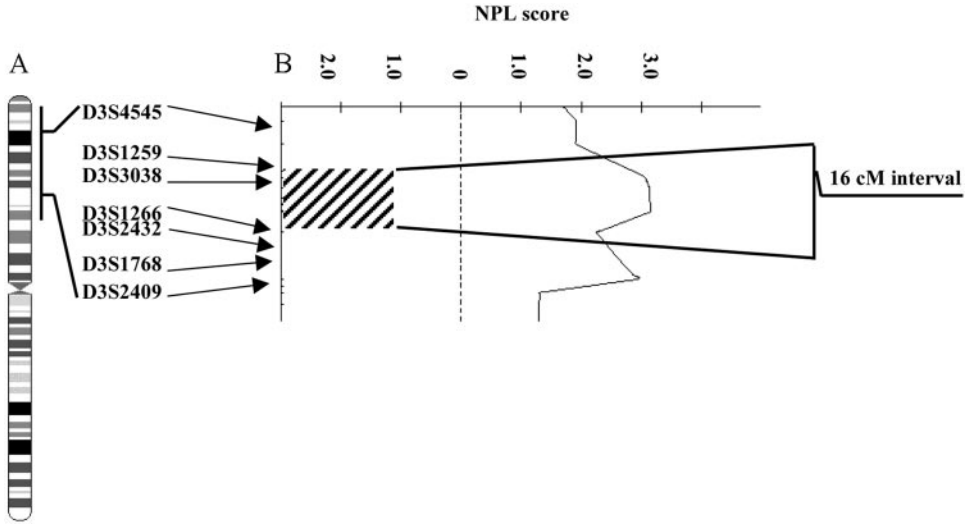

FIG. 4. The figure shows the mapping strategy for susceptible chromosomal regions responsible for euthyroid goiter at chromosome 3p. A, Highly polymorphic microsatellite markers on 3p, used to decide linkage of euthyroid goiter to this locus. B, The location of the peak nonparametric linkage (NPL) score, which indicates linkage of euthyroid goiter.

IX. Perspectives

A. Therapeutic implications

It is still poorly understood how genes interact with different environmental factors (33). Therefore, we have actually no ideal treatment for euthyroid benign (multi-)nodular goiter. Most clinical trials (56, 57, 274–281) investigated the efficacy of levothyroxine to suppress TSH and to arrest further growth or reduce the size of thyroid nodules. T_4 Suppressive therapy is given with the hope that nodules might decrease in size. However, these studies reported contradictory results concerning the investigated endpoint (*i.e.*, reducing the size of thyroid nodules). Moreover, the benefit of arresting growth or reducing the size of a thyroid nodule has not been conclusively answered because there are controversial reports on a possible correlation between thyroid nodule size and the development of thyroid epithelial cell carcinomas (52, 282–284).

Moreover, a decrease in serum TSH is related to increasing nodularity and size of the thyroid (4). Cross-sectional studies provide no evidence that the stimulation of thyroid growth or thyroid function through serum TSH is responsible for thyroid nodule growth (285, 286) because patients with benign CTNs did not exhibit elevated TSH levels in comparison with controls (56, 57, 274, 277–279).

Furthermore, TSH suppression may lead to hyperthyroidism, reduced bone density, and atrial fibrillation (287, 288), and levothyroxine therapy can lower the intrathyroidal iodine content (289–291). The pathophysiological rationale for levothyroxine therapy of thyroid nodules with the aim of reducing their volume is therefore questionable. Moreover, there is uncertainty about predictors of response like clonality (8, 84), growth (52), size at time of diagnosis (282–284), or cell-rich nodules (292).

Because thyroid nodules, thyroid autonomy, and thyroid cancer were more often detected in iodine-deficient areas than in iodine-sufficient areas (2, 18, 33, 293), areawide iodine supplementation became the first choice in thyroid nodule prevention (24). Although iodine supplementation is an adequate therapy for nodular goiter (291, 294), this option is often ignored. Possible benefits of treating or preventing a thyroid nodule (growth) could be the avoidance of thyroid nodules/goiter-associated symptoms (hoarseness, pain, hyperthyroidism, hypothyroidism) and, more rarely, prevention of thyroid malignancy, prevention of surgical intervention, and its related risks (295). In addition, this could lead to reduction of costs for common surgical interventions and postoperative pharmacotherapy (296). Therefore, the benefit of treating or preventing thyroid nodules is more likely prevention of clinical disease rather than reduction of nonclinical disease (295). In the authors' view, future studies should include patient-relevant outcomes such as thyroid cancer incidence, health-related quality of life, and costs. Multicenter studies are needed to investigate whether thyroid nodule growth is associated with an increased frequency of thyroid malignancies.

B. Diagnostic implications

Evaluation of patients with nodular thyroid disease is directed at two aspects: exclusion of thyroid malignancy and definition of the functional and, if possible, pathomorphological character of the nodule to stratify the best treatment approach.

Diagnosis of thyroid malignancy is ultimately based on the histological examination but can be strongly suggested clinically, *e.g.*, by the presence of a rapid growing nodule, cervical lymph nodes, sudden onset of hoarseness, and almost established on the basis of a malignant fine-needle aspiration cytology (FNAC) of the thyroid nodule (38, 39, 41). However, despite this clear-cut approach, the ultimate challenge in past and present thyroidology remains the identification of generally very rare thyroid cancer among the highly prevalent condition of nodular thyroid disease. This is not the only reason for concern of the affected patients and a daily task for all doctors dealing with thyroid disease, but it increasingly poses an economic problem in times of limited health care budgets.

Hence, many studies and reviews have been dedicated to the resolution of this problem: there is agreement that both ultrasonography and thyroid scintiscan add little to nothing to the clarification of the benign or malignant nature of a nodule, with the exception that "hot" nodules, *i.e.*, AFTNs, very rarely represent malignancy (38, 39, 41). Furthermore, it is widely acknowledged that FNAC represents the most sensitive and specific means for preoperative diagnosis of thyroid malignancy (40, 297). The drawback remains that FNAC is only reliable if performed and analyzed by an expert thyroid team (40, 41). In addition, it will be impossible to perform FNAC in all patients with nodular thyroid disease, which in countries with iodine deficiency may affect up to 30+% of the adult population (18). Guidelines have therefore defined a nodule size of at least 10 to 15 mm and/or hypofunctionality as indications for performing FNAC (38, 39, 41). However, it is unclear how to proceed in case of the much more frequent MNGs, which probably harbor the same malignancy risk as solitary lesions (2); a pragmatic approach here may be to perform FNAC of the prominent cold nodule (33) or to recommend thyroid surgery in cases of diagnostic uncertainty. The same pragmatic, but not evidence-guided, approach is to perform surgery in patients with risk constellations, *e.g.*, past history of radiation, family history of thyroid cancer.

Furthermore, even in an ideal setting, *e.g.*, in a thyroid specialty clinic, FNAC may be nondiagnostic or suspicious in more than 20% of cases. What are the options then? If FNAC has been nondiagnostic it needs to be repeated (298–300). "A number of at least six clusters of thyrocytes on each of at least two slides prepared from separate aspirates," has been proposed by Hamburger (301) as a quality criterion for a diagnostic thyroid FNAC. Recently, however, Oertel (292) has critically discussed that adequacy of the specimen cannot be based solely on the cell count. In case of suspicious FNAC results, which represent 10–20% of all FNACs (41), markers could be helpful, particularly to discriminate follicular adenoma from follicular carcinoma from follicular variant of papillary carcinoma and to distinguish Hürthle cell adenoma and cancer, *etc.* (302). However, since much but still too little is known about the molecular etiology of the "common" thyroid nodule, it is even more difficult to define a marker of benignity *vs.* malignancy, although many candidates have

been screened and diagnosed. Some of the markers seem promising, *e.g.*, galectin-3, thyroperoxidase (*MoAb47*), PAX-8/peroxisome proliferator-activated receptor γ rearrangements, and BRAF mutation (152, 303–305), but none of them have made their way into routine diagnostics yet. In contrast, novel technologies, microarray and proteomics methodologies in particular, will almost certainly contribute to changing this rather pessimistic state-of-the-art situation. Through these methods, which are increasingly applied in research labs everywhere, we have the ability for the first time to perform genome- and proteomewide screening studies, and despite all drawbacks they may in fact be ideally suited to identify useful markers similarly to the *in vivo* situation. The methods are also performed in "cross-sectional" studies. Contribution will also come from the many genomewide linkage studies, which have contributed to identifying a number of disease "genes" in the past (306). One example is familial euthyroid goiter, discussed in *Section VIII*.

Which diagnostic markers would then be needed, other than the important cytological differentiation of the above-discussed entities of thyroid pathology?

It would be ideal to have markers that indicate which nodules, in the long term, may turn into thyroid malignancy. More realistically, it could be feasible to define markers that correlate with augmented nodule growth or that correlate with ongoing thyroid dedifferentiation. Clarification of the molecular characteristics and application of markers that define increased nodule growth and dedifferentiation will allow formation of nodule subgroups. This would be the basis for a therapeutic strategy study to determine whether iodide supplementation, combination of levothyroxine and iodine, no therapy, ethanol injection, radioiodine, or surgery are indicated for which nodule subgroup.

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