Thyroid Gene Expression in Familial Nonautoimmune Hyperthyroidism Shows Common Characteristics with Hyperfunctioning Autonomous Adenomas

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Context: Dominant activating mutations of the TSH receptor are the cause of familial nonautoimmune hyperthyroidism (FNAH) (inherited mutations affecting the whole gland since embryogenesis) and the majority of hyperfunctioning autonomous adenomas (AAs) (somatic mutations affecting only one cell later in the adulthood).

Objective: The objective of the study was defining the functional and molecular phenotypes of FNAH and comparing them with the ones of AA.

Design: Functional phenotypes were determined in vitro and molecular phenotypes by hybridization on microarray slides.

Patients: Nine patients with FNAH were investigated, six for functional in vitro study of the tissue and five for gene expression.

Results: Iodide metabolism, H₂O₂, cAMP, and inositol phosphate generation in FNAH slices stimulated or not with TSH were normal. The mitogenic response of cultured FNAH thyrocytes to TSH was normal but more sensitive to the hormone. Gene expression profiles of FNAH and AAs showed that among 474 genes significantly regulated in FNAH, 93% were similarly regulated in AAs. Besides, 783 genes were regulated only in AAs. Bioinformatic analysis pointed out common down-regulations of genes involved in immune response, cell/cell and cell/matrix adhesions, and apoptosis. Pathways up-regulated only in AAs mainly involve diverse biosyntheses. These results are consonant with the larger growth of AAs than FNAH tissues.

Conclusions: Whether hereditary or somatic after birth, activating mutations of the TSH receptor have the same qualitative consequences on the thyroid cell phenotype, but somatic mutations in AAs have a much stronger effect than FNAH mutations. Both are variants of one disease: genetic hyperthyroidism. (J Clin Endocrinol Metab 94: 2602–2609, 2009)
nant nonautoimmune hyperthyroidism). If it is a neomutation occurring early during the gestation, it is called sporadic congenital nonautoimmune hyperthyroidism (SCNAH) (2). In both cases, the whole gland is affected. If the mutation occurs later, generally in adulthood, autonomous activity of the receptor or of Gsα causes a clonal expansion of the mutated cell, leading to the very common hyperfunctioning autonomous adenomas (AAs) (3, 4). The rare cases of FNAH and SCNAH must be differentiated from the much more frequent and transient form of fetal and neonatal hyperthyroidism caused by transplacental passage of maternal stimulating TSHR autoantibodies. Most of the mutations found in SCNAH have also been found in AAs (about 88%). In contrast, the majority of mutations in FNAH are not observed in AA (about 24% in common) (Fig. 1). Twenty-eight different autosomal dominant activating germline mutations have been identified in FNAH and SCNAH, mostly located in the transmembrane regions (exon 10) of the receptor (5–28) (Fig. 1).

FNAH from 130 patients and 22 families present the following clinical characteristics (5, 6, 8, 11, 13, 14, 17, 20, 21, 24, 26–38): 1) autosomal dominant transmission, no sexual predomina-

cence (59 males and 71 females); 2) hyperthyroidism with a variable age of onset (even within a given family), with decreased plasma TSH levels and often elevated T3 and T4 serum levels and frequent relapses after partial thyroidectomy (11, 39); 3) goiter of variable size, either homogenous (51 cases on 67) or multinodular (15 cases on 67); and 4) absence of lymphocytic infiltrates. Total thyroid ablation alone or combined with radiiodine is the only treatment able to prevent relapses. None of the reported FNAH degenerated into malignant carcinomas (see supplemental information, Table 1, published as supplemental data on The Endocrine Society’s Journals Online Web site at http://jcem.endojournals.org). Apart from pathology, thyroid tissue from such patients has never been studied. In the present work, we describe the characteristics of the thyroids of nine cases of FNAH, eight with already known mutations (9, 11, 17, 18) and one with a never reported mutation; six were investigated for in vitro properties of their thyrocytes and five for gene expression. Primary cultures of thyroid cells and tissue slices from operated patients were studied with regard to signal transduction and gene expression profiles were obtained by microarrays and compared with those of a pool of four AAs. FNAH and AAs both result from TSHR mutations, which start to exert their effects in embryo or adult life, respectively, i.e. at stages when thyroid regulation is very different. Whereas TSH is mainly responsible for growth after birth, other, as-yet-undefined, pathways are responsible for growth in the embryo (40–42). One might there-

dore expect different phenotypes when comparing an inherited mutation affecting all thyrocytes since thyroid differentiation in
TABLE 1. TSHR mutation, age of the patient, TSH, and free T3 and free T4 levels before treatment for the nine FNAH patients (FNAH 1 to >9) investigated

<table>
<thead>
<tr>
<th>Samples</th>
<th>TSHR mutation</th>
<th>Age of patient (yr)</th>
<th>TSH levels (mU/liter)</th>
<th>FT3 levels (pmol/liter)</th>
<th>FT4 levels (pmol/liter)</th>
<th>Gene expression analysis</th>
<th>In vitro studies</th>
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<tr>
<td>FNAH1</td>
<td>Val509Ala</td>
<td>12</td>
<td>0.02</td>
<td>6.9</td>
<td>32</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>FNAH2</td>
<td>Cys672Tyr</td>
<td>54</td>
<td>0.006</td>
<td>15</td>
<td>46</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>FNAH3</td>
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<td>27</td>
<td>0.02</td>
<td>7.65</td>
<td>12</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>FNAH4</td>
<td>Val509Ala</td>
<td>32</td>
<td>0.04</td>
<td>6.7</td>
<td>51.7</td>
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<td>X</td>
</tr>
<tr>
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<td>12</td>
<td>N.D.</td>
<td>3.4</td>
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<td>X</td>
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<tr>
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<td>14.6</td>
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<td>9.5</td>
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<td>X</td>
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<td>33.3</td>
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<tr>
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<td>Val509Ala</td>
<td>9</td>
<td>0.03</td>
<td>22.8</td>
<td>57.3</td>
<td>X</td>
<td>X</td>
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<td>Normal values</td>
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<td>2.5–5.8</td>
<td>11–25</td>
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</table>

FNAH1, FNAH2, FNAH3, FNAH4, and FNAH5 were investigated for gene expression analysis. FNAH1, FNAH4, FNAH6, FNAH7, FNAH8, and FNAH9 were investigated for in vitro studies. N.D., Not defined.

the embryo with a somatic mutation affecting one cell after birth. Our study shows common characteristics in AA and FNAH and concludes that both are similar hyperfunctional minimal deviation tumors with a more severe phenotype for adenomas.

Materials and Methods

Tissue samples

For in vitro studies, tissues from six thyroids of the Nancy family were used (FNAH1, FNAH4, FNAH6, FNAH7, FNAH8, FNAH9) (see pedigree in supplemental information, Fig. 1). For gene expression studies, thyroid samples were obtained from five FNAH French patients (from Nancy [FNAH1 and FNAH4], Reims [FNAH2] (11), Lyon [FNAH3], and Angers [FNAH5]), Clinical data about FNAH patients, investigated before treatment, are summarized in Table 1. AAs were obtained from four different patients and pooled. To compare FNAH and AAs, we used the same reference pool of 23 normal thyroid tissues adjacent to different pathologies.

Tissues were immediately dissected, placed on ice, sliced or digested for incubation or snap frozen in liquid nitrogen, and stored at −80°C until RNA processing. Protocols have been approved by the ethics committees of the institutions. Diagnoses have been established on the basis of long-standing hyperthyroidism, absence of thyroid-stimulating antibodies in the serum and in some cases family history, and confirmed by DNA sequencing of the TSHR.

More detailed data on these familial cases of FNAH are summarized in supplemental information, Table 1.

Biochemical assays

For tissue slices studies, fresh tissues were sliced at room temperature with a Stadie-Riggs microtome (Arthur Thomas, Philadelphia, PA). The methods for cAMP, H2O2, and inositol phosphates measurements, iodide trapping, iodide organification (PB125I), preparation, primary cultures, and proliferation measurements are described in supplemental information, Text 1.

RNA purification, cDNA synthesis, labeling, and microarray hybridization

Total RNA was extracted from thyroid tissues using a Trizol reagent (Invitrogen, Carlsbad, CA), followed by purification on RNeasy columns (QIAGEN, Hilden, Germany). After amplification (using Amino Allyl MessageAmp II aRNA amplification kit; Ambion, Austin, TX), 8 µg aRNA were labeled, fragmented, and hybridized for 16–18 h onto human exonic evidence-based oligonucleotide 7-mer oligonucleotide microarrays, containing approximately 48,500 probes (representing exonic sequences, alternatively spliced exons, expressed sequence tag, and controls). All hybridizations were performed in duplicates with dye swap. The microarray slides were washed under stringent conditions and scanned using an GenePix 4000B scanner (Axon, Sunnyvale, CA).

Data filtering and analysis

Expression levels were quantified with GenePix Pro 5.0 (Axon). Data were normalized and expressed as the log2 ratio of fluorescence intensities of the sample and the reference, for each spot on the array and dye swapped duplicates were averaged. Spots with missing values for more than 25% of the samples were discarded. The subset of spots that varied from the baseline by at least 1.5-fold in at least four of the five FNAH samples (without any opposite regulation), called the FNAH list (supplemental information, Table 2), and those that varied from the baseline by at least 1.5-fold in the AA pool with no modulation in FNAH, called the AA list (supplemental information, Table 3), were selected for further analysis.

Validation of microarray gene expression data by quantitative real-time RT-PCR (qRT-PCR)

A number of modulated genes on the microarray slides were investigated using real-time RT-PCR (SYBR Green) (Eurogentec, Liege, Belgium) in the tissues used for microarray analysis. The following mRNA expressions were evaluated using, when possible, transexonic primers, designed with Primer Express software (Applied Biosystems, Foster City, CA); matrix metalloproteinase-1, gap junction protein-β2, laminin-γ2, caveolin 1 (CAV1), P-cadherin, regulator of G protein signaling 2, collagen, type XVII (COL17A1), and growth differentiation factor 15 (GDF15) (the sequences of the primers are provided in supplemental information, Table 4). qRT-PCRs were performed in triplicate for each gene on the five FNAH and three AAs. The data were normalized using tetradecapptide repeat domain 1 and neural precursor cell expressed developmentally down-regulated 8 (43, 44).

Results

In vitro studies on thyroid slices and on thyrocytes in primary culture

A first question was whether the functional phenotype in vitro of the FNAH thyroids reflects their in vivo hyperactivity. A comparison of various functional parameters was made between slices of thyroids from congenital hyperthyroid patients of the Nancy family and previously studied normal thyroids. The data were highly variable between individuals, which would mask the
most marked differences. Nevertheless, iodide trapping, as evaluated by the iodide trapping ratios, iodide binding to proteins, and its main controlling step, \( \text{H}_2\text{O}_2 \) generation, were not enhanced in hyperthyroid tissues. The two latter processes were significantly stimulated by TSH (supplemental information, Table 5). Obviously these measurements have been normalized to an equal amount of tissue and therefore do not reflect the larger size of FNAH thyroids.

We next investigated whether the main signal transduction cascades controlled by TSH, i.e. the cAMP cascade (as evaluated by cAMP accumulation) and the phosphatidyl inositol cascade (as evaluated by inositol phosphate (IP) generation) operate similarly in FNAH and normal tissues. Slices were treated with different concentrations of TSH, and both pathways were activated in normal and pathological tissues (Fig. 2, A and B). Whereas the stimulation of the cAMP cascade was monophasic but less sensitive to TSH, as already shown for normal tissues (45). The effects were qualitatively similar in both types of tissues, although the cAMP response was lower in FNAH tissue.

Because thyroids of FNAH patients are larger than normal thyroids, we next investigated whether their thyrocytes respond more to physiological mitogenic stimulants. Cell proliferation after TSH stimulation was assessed in primary cultures of thyrocytes prepared from FNAH tissues. As already reported for normal thyrocytes (46), TSH and forskolin in the presence of insulin at high concentration (5 \( \mu \text{g/ml} \)), which activates the IGF1 receptor, induced a marked increase in the proportion of cells entering into S phase, i.e. DNA synthesis. In the three cultures investigated, thyrocytes derived from FNAH appeared more sensitive to TSH than previously studied normal thyrocytes, with a response already at 0.03 mU/ml, which had never been found in normal thyrocytes (Fig. 2C). In addition, TSH as well as forskolin induced DNA synthesis, even in the absence of insulin or IGF-1, which had never been observed in normal thyrocytes (supplemental information, Fig. 2).

**Gene expression profiling of FNAH and AA**

Comparison of the molecular phenotypes of FNAH and AA thyroids can best be demonstrated by a comprehensive microarray analysis of gene expression in the two types of tissues. Gene expression profiles were investigated in five different FNAH and a pool of four AAs, compared with a common reference pool of 23 normal thyroid tissues. Tumor and reference RNA samples were cohybridized on human exonic evidence-based oligonucleotide microarrays, and differentially expressed genes were searched for, as described in Materials and Methods. We defined two lists of genes, which were analyzed in greater detail. First, we selected genes that were modulated in FNAH (FNAH list, supplemental information, Table 2). Among the 474 genes from this list, 432 (91\%) were regulated in the same way in AAs, 30 (6\%) were not regulated in AAs, and only two (0.4\%) were regulated in the opposite way: GDF15 and COL17A1, which were up-regulated in AAs and down-regulated in FNAH. Second, we looked for genes modulated in AAs but not in FNAH (AA list, supplemental information, Table 3): 783 genes filled this criterion. In addition, our gene expression data showed a large proportion of significantly down-regulated genes in FNAH and AAs, corresponding, respectively, to 96 and 77\% of the total number of regulated genes. Strikingly, among the down-regulated genes common to FNAH and AAs, 75.64\% were more down-regulated in AAs than FNAH.

**Validation of microarray data**

Given that FNAH does not contain adjacent tissue, we compared FNAH to a pool of 23 normal tissues, and to compare the two diseases, AAs were compared with the same pool rather than their normal but quiescent counterpart as in previous studies. It is therefore not surprising that the phenotype observed for AAs in this study is milder than in the previous studies. On the other hand, the confirmation of the majority of significant previous findings clearly shows that they truly reflect the pathology of autonomous adenomas.

The differential expression of several genes in the gene lists described above confirmed previously published data on AAs, including the large series of AAs studied by our group (4, 47–52), on thyroids of transgenic mice expressing the adenosine A2 receptor (Tg-A2aR) and developing huge goiters (53) and on primary cultures stimulated by TSH (43): NIS (48), Dio1 (4), ID3 (49), Dio2 (52), Fgfbp (4), TPO (4), MT1H (4), and PTPN4 (50) were up-regulated, whereas Cav1 (4, 47, 53), Cav2 (4, 47), IL8...
(43), RRAD (49), Dusp2 (4), Klf2 (4), Clu (4), Gadd45 (4), Nfk-bia (4), RGS2 (4, 43, 53), and Myc (48) were down-regulated.

To further validate our data, eight significantly modulated genes were investigated by real-time qRT-PCR. Experiments were performed on the five FNAH studied by microarrays and three independent AAs. Similar modulation patterns were found for the expression of seven genes comparing microarray analysis with qRT-PCR, thus validating the microarray data (Fig. 3). For one gene, COL17A1, although the qRT-PCR data for FNAH were consistent with the microarray results, the up-regulation observed by microarray for pooled autonomous adenomas was not confirmed by qRT-PCR.

**Analysis of our gene lists by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) categories using Database for Annotation, Visualization and Integrated Discovery software**

To assess the most representative biological activities altered in both FNAH and AA (FNAH list) and AAs only (AA list), we used the statistical methods from the Database for Annotation, Visualization and Integrated Discovery software (54, 55). Figure 4 shows the most represented altered pathways according to KEGG and supplemental information, Table 6, the most represented altered functions according to GO (GO level of 5, P < 0.05). As shown in Fig. 4, the main KEGG pathways altered in both AAs and FNAH, classified by increasing P values, involve immune cell infiltration and cell adhesion. The expressions of most of the genes involved in these pathways are down-regulated in the tumors (Fig. 4A). The main KEGG pathways altered only in AAs concern cell metabolism. Apart from genes involved in alanine and aspartate metabolism, all others are essentially up-regulated (Fig. 4B). In addition, analysis of supplemental Table 6 pointed out apoptosis among the most altered functions in FNAH and AAs, with 91% of the genes that are down-regulated.

**Discussion**

The investigation of both in vitro properties of FNAH thyroid slices and gene expression profiles in FNAH tissues leads to several major conclusions: 1) FNAH thyrocytes are not more functionally active than normal thyrocytes, but there are more of them; 2) the moderate increase in size of the thyroid of FNAH patients is not the result of an increased proliferation rate but rather of a decrease of apoptosis; and 3) the number of deregulated genes and their expression are milder but similar to autonomous adenomas, suggesting that FNAH and AAs are variants of a same disease: genetic hyperthyroidism.

Thyrocytes of FNAH thyroids do not appear more functionally active than normal tissue. There are no detected differences regarding iodide uptake and organification, $\text{H}_2\text{O}_2$ generation, cAMP and IP levels and their responses to TSH. There was only a mild effect on thyroid-specific gene expression (e.g. DUOX1, DUOX2) (data not shown). All these measurements are related to a similar amount of cells, suggesting that the hyperthyroidism of these patients reflects more the number of active cells, their goiter (28), than their level of activation. The situation is different in AA tissue, which takes up more iodide and secretes more hormone per gram than the normal tissue (56). The lower cAMP response to TSH in FNAH thyrocytes than normal thyrocytes, as already reported in AA-derived thyrocytes (57), might be explained by the expression of some phosphodiesterases (1A, 1B, 7B, 8A, 8B, 4D), negative feedbacks of the cAMP pathway, which are increased in AAs (43) and to a lesser extent in FNAH.

The thyroids of FNAH patients grow slowly all through life (28). Our data provide some explanation for that. FNAH thyrocytes in culture proliferated and responded to TSH almost as normal thyrocytes (46, 57); they do not overexpress Ki67 or proliferating cell nuclear antigen. This fits well with the absence of up-regulation of growth-related genes such as IGF1, their receptors, epithelial growth factor receptor, etc. Gene expression profiles showed that two types of modifications in gene expression can be correlated with the growth of the tissue: 1) the activation of the Wnt pathway in FNAH and AAs, with the up-regulation of activating proteins (low density lipoprotein receptor-related protein-8) and the down-regulation of inhibitory proteins (secreted frizzled related protein-2, SRY-box 15, Dickkopf-3), except one (secreted frizzled related protein-1), which is up-regulated (supplemental information, Fig. 3); 2) the down-regulation of CAV-1 mRNA expression, as in AAs, in...
in vitro TSH-stimulated thyrocytes and Tg-A2AR mice (4, 43, 53). Caveolins are considered as tumor suppressor genes, and data on CAV-1 suggested an antimitogenic role of this protein (47). The goiter formation in FNAH might also be explained by the initiation of DNA synthesis in FNAH cells in the absence of insulin and at low TSH concentrations, which is never observed in normal thyrocytes. In addition, a decreased rate of apoptosis, suggested by the down-regulation of several proapoptotic genes (91% of the genes in the GO apoptosis category), might also contribute to thyroid growth, as in AAs, in which a lower rate of apoptosis has directly been demonstrated (48). The down-regulation of several p53 pathway-related genes (supplemental information, Fig. 4) might also explain such a low apoptosis rate.

The analysis of KEGG and GO pathways pointed out another striking characteristic common to AAs and FNAH: the down-regulation of genes related to immune response and inflammation. Most (98.6%) of the genes present in the following categories were down-regulated: leukocyte transendothelial migration, cytokine-cytokine receptor integration, antigen processing and presentation, hematopoietic cell lineage, Toll-like receptor signaling pathway, and arachidonic acid metabolism (Fig. 4A). A down-regulation of prostaglandin, derived from arachidonic acid, had also been found in cultured dog thyroid cells after TSH stimulation (58). In line with these results, more than 50% of the significant GO categories related to the FNAH list with a P < 0.05 are involved in immune response and inflammation (supplemental information, Table 6). It is already known by pathologists that autonomous adenomas contain few lymphocytes and macrophages, explaining this down-regulation (4). This can now be extended to the thyroids of FNAH. Chronic stimulation of thyroid cells therefore rather reduces immune reactions, i.e., inflammation that is generally considered a contributing factor for carcinogenesis (59).

Genes involved in focal adhesion and cell adhesion categories which mediate cell/cell as well as cell/extracellular matrix (ECM) interactions (such as LAMA3, LAMC2, LAMB3, FN1, COL1A1) were also down-regulated in AAs and FNAH, which may explain the flaky aspect of the AA and FNAH tissues and is consistent with the observed looseness of both types of tissues.

Results obtained on in vitro-incubated tissues and gene expression in FNAH thyroids demonstrated a similar but milder phenotype as the one of AAs. The relatively low amount of gene expression changes observed, as in autonomous adenomas (4), fits well with the definition of a minimal deviation tumor, functioning like normal tissue but without control (3), and is supported by the great similarity of protein expression and phosphorylation in adenoma and quiescent tissue observed on two-dimensional gels (56). The thyroid of FNAH deviates from normal tissue with about 474 differentially expressed genes, and 93.25% of the genes regulated in FNAH were also regulated in the same way in AAs. Many genes regulated in AAs also varied in the same direction in FNAH, albeit nonsignificantly. There is therefore no support for the hypothesis that similar mechanisms operating in the whole thyroid in the embryo and some cells in adult life would lead to qualitatively different results. The same conclusion applies to the generation of thyroid nodules after in utero radiation exposure compared with childhood exposure (60).

Most of the modulated genes were down-regulated as in AAs (4, 50), which suggests a simplification of signal transduction in the cells (4). The fact that 783 genes were regulated only in AAs and that for the down-regulated genes in AAs and FNAH,
75.64% of them were more regulated in AAs than FNAH, suggests that the level of cAMP cascade activation achieved is higher in AAs than FNAH. This is confirmed by the higher functional activity and proliferation rate of the thyrocytes in AAs but not FNAH (48). In fact, the majority of the mutations affecting the TSHR are different in the two diseases, and it has been suggested that they are probably more aggressive in AAs than FNAH (5, 9, 24). For TSH receptor mutations described in FNAH, SCNAH, and AAs (Fig. 1), of the 21 mutations in FNAH, 16 are proper to this lesion, four are common to AAs, and one is common to FNAH, SCNAH, and AAs. Among the eight mutations found in SCNAH, seven are also described in AAs. One hypothesis is that a large part of the FNAH mutations are so mild that they would not cause an AA if sporadically expressed in thyrocytes and, conversely, that the strong activation encountered in AAs would be very toxic and lethal for embryonic thyroids and embryos and therefore would not be transmitted between generations.

Because adenomas are monoclonal (9), one can calculate that the generation of a 1 g nodule by one mutated cell would require a minimum of 30 divisions. The same amplification in a whole embryonic thyroid (1 mg) would lead to a gland of 1 ton. Presumably, adenomas stop growing at the end of the life span of their thyrocytes due to the shortening of their telomeres (61). FNAH thyroids with a much smaller amplification keep growing during adulthood. Also, the age of occurrence of hyperthyroidism in pediatric cases of FNAH and SCNAH differs: five of 10 at birth and five before 1 yr old for SCNAH, whereas only one at birth and two before 1 yr among 29 FNAH (38).

In conclusion, the present study shows that FNAH, AAs, and therefore SCNAH, with similar mutations as in autonomous adenomas, represent different forms of one disease: genetic hyperthyroidism. The characteristics of the three forms depend on the intensity of the constitutive activation of the TSHR/cAMP signaling cascade, the developmental stage at which the genetic defect occurs, and the cell population involved (Table 2).

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References

as cause of congenital hyperthyroidism. Exp Clin Endocrinol Diabetes 104(Suppl 4):124–128


