Absence of mutations in *PAX8*, *NKX2.5*, and *TSH* receptor genes in patients with thyroid dysgenesis

Ausência de mutações nos genes PAX-8, NKX2.5 *e receptor de* TSH *em pacientes com disgenesia tireoidiana*

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ABSTRACT

Objectives: To precisely classify the various forms of TD, and then to screen for mutations in transcription factor genes active in thyroid development. **Subjects and methods:** Patients underwent ultrasound, thyroid scan, and serum thyroglobulin measurement to accurately diagnose the form of TD. DNA was extracted from peripheral leukocytes. The *PAX8,* and *NKX2.5* genes were evaluated in all patients, and *TSH* receptor (*TSHR*) gene in those with hypoplasia. **Results:** In 27 nonconsanguineous patients with TD, 13 were diagnosed with ectopia, 11 with hypoplasia, and 3 with athyreosis. No mutations were detected in any of the genes studied. **Conclusion:** Sporadic cases of TD are likely to be caused by epigenetic factors, rather than mutations in thyroid transcription factors or genes involved in thyroid development. Arq Bras Endocrinol Metab. 2012;56(3):173-7

Keywords

Thyroid dysgenesis; PAX8; NKX2.5; TSHR; congenital hypothyroidism; mutation

RESUMO

Objetivos: Classificar corretamente as várias formas de DT e depois rastrear por mutações em genes que participam no desenvolvimento da tireoide. **Sujeitos e métodos:** Os pacientes realizaram ultrassonografia, cintilografia e tireoglobulina sérica para o diagnóstico preciso de DT. DNA foi extraído de leucócitos periféricos. Os genes *PAX8* e *NKX2.5* foram estudados em todos os pacientes e o gene do receptor do *TSH* (*TSHR*) foi estudado na hipoplasia. **Resultados:** Avaliaram-se 27 pacientes sem consanguinidade com DT, dos quais 13 foram diagnosticados com ectopia, 11 com hipoplasia e 3 com atireose. Nenhuma mutação foi detectada nos genes estudados. **Conclusão:** Casos esporádicos de DT são provavelmente causados mais por fatores epigenéticos do que por mutações em fatores de transcrição ou genes envolvidos no desenvolvimento tireoidiano. Arq Bras Endocrinol Metab. 2012;56(3):173-7

Descritores

Disgenesia tireoidiana; PAX-8; NKX2.5; TSHR; hipotireoidismo congênito; mutação

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INTRODUCTION

Thyroid dysgenesis (TD) is the major cause of congenital and permanent hypothyroidism. Most cases are sporadic, affecting more females, and frequently associated to heart defects (1). Clinical presentation of TD includes athyreosis, the absence of thyroid tissue, hemiagenesis, the presence of only one thyroid lobe, hypoplasia, and ectopia (2,3). Transcription factors PAX8 and NKX2.5 are active in thyroid development (4), and are possible candidates for TD. Mutations in the TSH receptor (TSHR) cause hypothyroidism of variable severity via thyroid hypoplasia (5).

PAX8 is a member of the PAX family of transcription factors, active in several germline tissues in the human embryo (6). PAX8 is crucial to follicular development and thyroid hormone production induced by many genes.

Few patients with congenital hypothyroidism caused by ectopia or athyreosis have *PAX8* mutations inherited in an autosomal dominant fashion (7-9).

NKX2.5 is a homeobox-containing transcription factor essential to heart morphogenesis. In mouse embryos, *Nkx2.5* transcripts were observed in thyroid precursor cells in the pharyngeal floor. In later stages of development, *Nkx2.5* expression is limited to the thyroid primordium area. Mutations in *NKX2.5* have been described not only in patients with heart defects, but also in patients with thyroid ectopia or athyreosis without heart involvement (1,10).

Inactivating mutations in *TSHR* have been described in patients with congenital hypothyroidism and thyroid hypoplasia. Some of these patients had been diagnosed with athyreosis, but serum thyroglobulin was detectable, denoting thyroid tissue that was not visible by conventional imaging methods (11). The definition of hypoplasia is extremely difficult due to many variables that determine thyroid size in childhood, such as gender, age, height, body surface area, puberty, and iodine sufficiency. In prior studies, we used ultrasound in combination with thyroid scan and serum thyroglobulin levels to more precisely define primary congenital hypothyroidism (12).

In addition to defining the conditions of ectopia, athyreosis, and thyroid hypoplasia in patients with permanent and primary hypothyroidism using a combination of ultrasound, thyroid scan, and serum thyroglobulin levels, the aim of this study was to define candidate genes and search for mutations related to various clinical presentations of TD. Since *PAX8* and *NKX2.5* genes are involved in all steps of thyroid development (formation, migration, differentiation, and proliferation), these transcription factors were studied in all patients with TD. The *TSHR* gene was studied only in patients with thyroid hypoplasia, since this gene is involved only in the proliferation of thyroid cells.

SUBJECTS AND METHODS

Twenty-seven patients aged 3-19 years, diagnosed with primary congenital hypothyroidism were recruited in the outpatient clinic of the Association for Parents and Friends of Disabled Individuals (APAE), São Caetano, and referred to the Governmental Neonatal Screening Service to be studied at the Hospital das Clinicas – FMUSP (12). Informed consent was obtained from all parents, and the protocol was approved by the Ethics Committee of the Institution. Patients underwent color Doppler ultrasound (CD-US), combined serum thyroglobulin (TG) measurement, and thyroid scan with uptake of 99Tc Pertechnetate (^{99m}Tc) and radioactive iodine (^{131}I).

CD-US was performed using a Phillips scanner with a 7.5-12 MHZ transducer focusing on the thyroid gland and cervical region, from the mandible bone to the manubrium. Total thyroid volume was calculated as described elsewhere (13,14), and compared according to height, sex, age, and body surface area.

After a four-week washout with no levothyroxine treatment, total T3 and T4, free T4 (FT4), TSH, thyroglobulin (TG) and anti-TG antibody were measured in all patients by immunofluorometric assays (Autodelfia®, Wallac Oy, Turku, Finland). Patients with anti-TG antibodies were excluded from the study. A radionuclide scan was performed after the four- week levothyroxine washout and two weeks on a low iodine diet. Uptake of ^{131}I (5 µCi) was measured at 2 and 24 h after oral administration. Similar uptake measurements were carried out on the following day after intravenous injection of ^{99}Tc Pertechnetate (10 mCi).

Athyreosis diagnosis was determined when no thyroid was visualized by any of the imaging techniques used (CD--US and scan). Thyroid hypoplasia was diagnosed when total thyroid volume measured by CD-US was calculated to be less than 2 SD from the normal value for height, gender, chronological age, and body surface area. Ectopia was diagnosed when thyroid tissue was observed outside the normal bed. After patients were diagnosed and classified according to the various clinical presentations of TD, the *TSHR* gene was studied only in patients with thyroid hypoplasia. The *PAX8* and *NKX2.5* genes were studied in all patients.

Mutation screening by DNA sequencing

DNA was extracted from peripheral leukocytes (15). Coding regions and exon-intron boundaries of the candidate genes were amplified by PCR and sequenced using the ABI Prism 3130 *xl* (Applied Biosystems, Foster City, CA, USA), as previously described (1,7). DNA from 50 adults with normal thyroid function was used for comparison in gene sequencing.

RESULTS

We studied 27 nonconsanguineous patients (20 female) aged 3 to 19 years (median 4.6 years) with TD (Table 1). All patients presented high TSH levels after 4 weeks of levothyroxine suspension, confirming the diagnosis of primary congenital hypothyroidism. No patients had heart defects detected by echocardiogram. All had normal kidney function. None had respiratory insufficiency.

Table 1. Clinical and serum thyroglobulin levels from patients with thyroid dysgenesis

Diagnosis	Ectopia	Athyreosis	Hypoplasia
F/M*	12/1	2/1	5/6
Thyroglobulin (ng/mL)† (NR: 1.7-35)	4.5-123	< 1.0	4.0-65.2
PAX8	No mutation	No mutation	No mutation
NKX2.5	No mutation	No mutation	No mutation
TSHR	-	No mutation	No mutation

* F: female; M: male; † Minimal and maximal serum thyroglobulin levels; NR: normal reference values.

Twelve patients were diagnosed with ectopia, having thyroid tissue in the submandibular region observed on thyroid scan. One additional patient presented ectopia associated with left lobe hemiagenesia. Thyroglobulin levels of the 13 patients with ectopia ranged from 4.5 to 123 ng/mL (mean and SD = 46.2 ± 37.9 ng/mL, median 28.4 ng/mL) (Figure 1).

Eight patients were diagnosed with thyroid hypoplasia, having less than 2 SD of the normal thyroid volume, as assessed by CD-US. Thyroglobulin levels of these patients ranged from 6.8 to 65.2 ng/mL (mean and SD = 34.7 ± 18.6 ng/mL, median 35.2 ng/mL) (Figure 1).

Six patients were diagnosed with athyreosis using CD--US and thyroid scan. However, 3 of these 6 patients had measurable thyroglobulin levels of 4.0, 5.4, and 9.1 ng/mL. Therefore they were reclassified as having thyroid hypoplasia.

The coding regions and exon-intron boundaries of the *PAX8* and *NKX2.5* genes were fully sequenced in all 27 patients with TD. We identified no mutations. The single nucleotide polymorphism (SNP) rs2277923 (www.ncbi.nlm.nih.gov/snp) within the *NKX2.5* gene was detected in 93% of patients, but the differences in the allele and genotype frequencies between patients and controls were not statistically significant ($p > 0.05$).

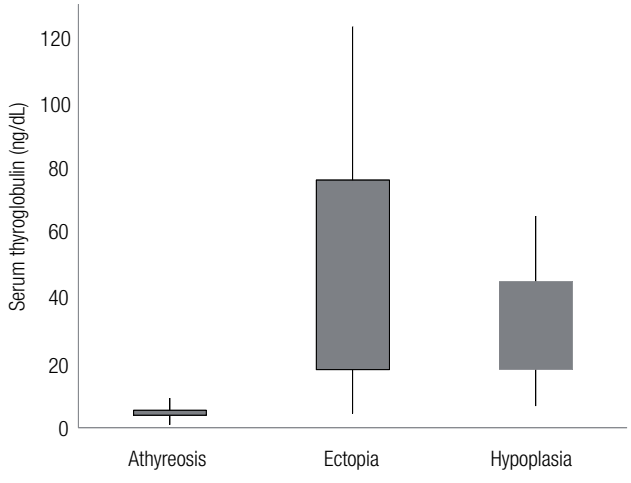

Figure 1. Serum thyroglobulin levels (ng/dL) comparison among thyroid dysgenesis.

The coding regions and exon-intron boundaries of the *TSHR* gene were fully sequenced in the 8 patients with thyroid hypoplasia and in the 3 patients with athyreosis who were reclassified as having thyroid hypoplasia due to detectable serum thyroglobulin. Similarly, no mutations were identified.

DISCUSSION

Thyroid dysgenesis (TD) comprises a broad spectrum of clinical presentations, including athyreosis, hemiagenesia, hypoplasia, and ectopia. All cause definitive hypothyroidism. It is possible that each subgroup of TD could be caused by a specific genetic modification (1). We therefore decided to better define each clinical presentation in order to more efficiently search for the involvement of candidate genes.

Kreisner and cols. suggested an initial approach with ultrasound to define congenital hypothyroidism (16). If the thyroid gland was absent, TD is the most probable diagnosis, with athyreosis or ectopia. If the thyroid gland was present at ultrasound, more exams are necessary to establish the etiology. Combined use of ultrasound and serum thyroglobulin measurement allows the differentiation between patients with ectopia, athyreosis, and thyroid hypoplasia. For example, we were able to reclassify three patients who had been misdiagnosed with athyreosis using only imaging methods. The presence of serum thyroglobulin in these patients revealed that their correct diagnosis was actually thyroid hypoplasia. Since thyroid scan is more difficult to perform, serum thyroglobulin measurement is an important tool in the differential diagnosis of TD, and should be routinely performed in cases of primary congenital hypothyroidism. Patients with undetectable serum thyroglobulin, in the absence of anti-thyroglobulin antibodies, certainly present athyreosis or thyroglobulin deficiency (dyshormonogenesis). In thyroglobulin deficiency, the presence of the thyroid, palpable or visible on the ultrasound, enables dyshormonogenesis diagnosis (12).

In this study, all 27 patients with TD were screened for mutations in the *PAX8* and *NKX2.5* genes, since these are transcription factors involved in thyroid embryogenesis. Mutations in these genes have been previously described in cases of athyreosis, ectopia, and hypoplasia (7,8,10,17-20). We found no mutations in the *PAX8* or *NKX2.5* ge-

nes in our cohort. The only variation we observed was SNP rs2277923 within the *NKX2.5* gene, which is a synonymous variation, and does not cause an amino acid change. However, we do not believe this SNP to be related to TD since the frequency of the SNP within patients and controls was not significantly different. In the patients with thyroid hypoplasia, we looked for mutations in the *TSHR* gene, but found none.

Our negative findings demonstrate the rare genetic etiology in sporadic cases of TD, at least in genes known to be involved in the formation and migration of follicular cells. Several previously studies in different ethnic populations with TD did not find mutations, either, particularly in sporadic cases (1,7,9,21-27) (Table 2). It is likely that other epigenetic factors determine TD, such as differential gene expressions or methylation (28,29). Considering the candidate genes for TD described above in a large number of negative patients with different genetic backgrounds studied so far, including those from our group, we can conclude that TD should be weakly correlated with inherited genetic defects. This finding make it necessary to carry out further molecular analyses, as the genes that are known to cause the disorder account for only very few cases.

Table 2. Frequency of described mutations in *PAX8*, *NKX2.5* and *TSHR* genes in thyroid dysgenesis cohorts

Author	*PAX8*	*NKX2.5*	*TSHR*	Reference
Al Taji and cols.	1/170 (0.6%)	0/15		21
Alves and cols.			0/90#	22
Camilot and cols.	0/15		3/16 (18.7%)	23-24
Cangul and cols.	0/120	0/120	6/120* (5%)	25
Esperante and cols.	0/60			26
Lanzerath and cols.	0/95			9
Macchia and cols.	3/120 (2.5%)			7
Mahjoubi and cols.	0/50			27
Ramos and cols.	1/35 (2.9%)	0/35	0/35	1
Total estimated frequency&	0.75%	0%	3.9%	

Only exon 10 TSHR was studied. * Only familial thyroid dysgenesis. & Based on total cohort from cited authors.

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REFERENCES

1. Ramos HE, Nesi-Franca S, Boldarine VT, Pereira RM, Chiamolera MI, Camacho CP, et al. Clinical and molecular analysis of thyroid hypoplasia: a population-based approach in southern Brazil. Thyroid. 2009;19(1):61-8.
2. Larsen PR, Davies TF, Schlumberger M, Hayan I. Thyroid physiology and diagnostic evaluation of patients with thyroid disorders. In: Larsen K, Melmed P, editors. Williams Textbook of Endocrinology. New York: Saunders; 2002. p. 331-73.
3. Dias VM, Campos AP, Chagas AJ, Silva RM. Congenital hypothyroidism: etiology. J Pediatr Endocrinol Metab. 2010;23(8):815-26.
4. Kopp P. Perspective: genetic defects in the etiology of congenital hypothyroidism. Endocrinology. 2002;143(6):2019-24.
5. De Felice M, Di Lauro R. Thyroid development and its disorders: genetics and molecular mechanisms. Endocr Rev. 2004;25(5):722-46.
6. Trueba SS, Auge J, Mattei G, Etchevers H, Martinovic J, Czernichow P, et al. PAX8, TITF1, and FOXE1 gene expression patterns during human development: new insights into human thyroid development and thyroid dysgenesis-associated malformations. J Clin Endocrinol Metab. 2005;90(1):455-62.
7. Macchia PE, Lapi P, Krude H, Pirro MT, Missero C, Chiovato L, et al. PAX8 mutations associated with congenital hypothyroidism caused by thyroid dysgenesis. Nat Genet. 1998;19(1):83-6.
8. Vilain C, Rydlewski C, Duprez L, Heinrichs C, Abramowicz M, Malvaux P, et al. Autosomal dominant transmission of congenital thyroid hypoplasia due to loss-of-function mutation of PAX8. J Clin Endocrinol Metab. 2001;86(1):234-8.
9. Lanzerath K, Bettendorf M, Haag C, Kneppo C, Schulze E, Grulich-Henn J. Screening for Pax8 mutations in patients with congenital hypothyroidism in South-West Germany. Horm Res. 2006;66(2):96-100.
10. Dentice M, Cordeddu V, Rosica A, Ferrara AM, Santarpia L, Salvatore D, et al. Missense mutation in the transcription factor NKX2-5: a novel molecular event in the pathogenesis of thyroid dysgenesis. J Clin Endocrinol Metab. 2006;91(4):1428-33.
11. Gagne N, Parma J, Deal C, Vassart G, Van Vliet G. Apparent congenital athyreosis contrasting with normal plasma thyroglobulin levels and associated with inactivating mutations in the thyrotropin receptor gene: are athyreosis and ectopic thyroid distinct entities? J Clin Endocrinol Metab. 1998;83(5):1771-5.
12. Beltrao CB, Juliano AG, Chammas MC, Watanabe T, Sapienza MT, Marui S. Etiology of congenital hypothyroidism using thyroglobulin and ultrasound combination. Endocr J. 2010;57(7):587-93.
13. Ueda D. Normal volume of the thyroid gland in children. J Clin Ultrasound. 1990;18(6):455-62.
14. Duarte GC, Tomimori EK, de Camargo RY, Catarino RM, Ferreira JE, Knobel M, et al. Excessive iodine intake and ultrasonographic thyroid abnormalities in schoolchildren. J Pediatr Endocrinol Metab. 2009;22(4):327-34.
15. Abrao MG, Billerbeck AE, Nishi MY, Marui S, Mendonca BB. [Standardization of DNA extraction with NaCl from oral mucosa cells: application in PROP1 gene study]. Arq Bras Endocrinol Metabol. 2005;49(6):978-82.
16. Kreisner E, Camargo-Neto E, Maia CR, Gross JL. Accuracy of ultrasonography to establish the diagnosis and aetiology of permanent primary congenital hypothyroidism. Clin Endocrinol (Oxf). 2003;59(3):361-5.

17. Bereket A, Liao XH, Turoglu T, Aribal E, Refetoff S. Analysis of the PAX8 gene in congenital hypothyroidism caused by different forms of thyroid dysgenesis in a father and daughter. J Pediatr Endocrinol Metab. 2004;17(7):1021-9.
18. Congdon T, Nguyen LQ, Nogueira CR, Habiby RL, Medeiros-Neto G, Kopp P. A novel mutation (Q40P) in PAX8 associated with congenital hypothyroidism and thyroid hypoplasia: evidence for phenotypic variability in mother and child. J Clin Endocrinol Metab. 2001;86(8):3962-7.
19. Meeus L, Gilbert B, Rydlewski C, Parma J, Roussie AL, Abramowicz M, et al. Characterization of a novel loss of function mutation of PAX8 in a familial case of congenital hypothyroidism with in-place, normal-sized thyroid. J Clin Endocrinol Metab. 2004;89(9):4285-91.
20. Tonacchera M, Banco ME, Montanelli L, Di Cosmo C, Agretti P, De Marco G, et al. Genetic analysis of the PAX8 gene in children with congenital hypothyroidism and dysgenetic or eutopic thyroid glands: identification of a novel sequence variant. Clin Endocrinol (Oxf). 2007;67(1):34-40.
21. Al Taji E, Biebermann H, Limanova Z, Hnikova O, Zikmund J, Dame C, et al. Screening for mutations in transcription factors in a Czech cohort of 170 patients with congenital and early-onset hypothyroidism: identification of a novel PAX8 mutation in dominantly inherited early-onset non-autoimmune hypothyroidism. Eur J Endocrinol. 2007;156(5):521-9.
22. Alves EA, Cruz CM, Pimentel CP, Ribeiro RC, Santos AK, Caldato MC, et al. High frequency of D727E polymorphisms in exon 10 of the TSHR gene in Brazilian patients with congenital hypothyroidism. J Pediatr Endocrinol Metab. 2010;23(12):1321-8.
23. Camilot M, Teofoli F, Gandini A, Franceschi R, Rapa A, Corrias A, et al. Thyrotropin receptor gene mutations and TSH resistance: variable expressivity in the heterozygotes. Clin Endocrinol (Oxf). 2005;63(2):146-51.
24. Camilot M, Teofoli F, Vincenzi M, Federici F, Perlini S, Tatò L. Implementation of a congenital hypothyroidism newborn screening procedure with mutation detection on genomic DNA extracted from blood spots: the experience of the Italian northeastern reference center. Genet Test. 2007;11(4):387-90.
25. Cangul H, Morgan NV, Forman JR, Saglam H, Aycan Z, Yakut T, et al. Novel TSHR mutations in consanguineous families with congenital nongoitrous hypothyroidism. Clin Endocrinol (Oxf). 2010;73(5):671-7.
26. Esperante SA, Rivolta CM, Miravalle L, Herzovich V, Iorcansky S, Baralle M, et al. Identification and characterization of four PAX8 rare sequence variants (p.T225M, p.L233L, p.G336S and p.A439A) in patients with congenital hypothyroidism and dysgenetic thyroid glands. Clin Endocrinol (Oxf). 2008;68(5):828-35.
27. Mahjoubi F, Mohammadi MM, Montazeri M, Aminii M, Hashemipour M. Mutations in the gene encoding paired box domain (PAX8) are not a frequent cause of congenital hypothyroidism (CH) in Iranian patients with thyroid dysgenesis. Arq Bras Endocrinol Metabol. 2010;54(6):555-9.
28. Vassart G, Dumont JE. Thyroid dysgenesis: multigenic or epigenetic ... or both? Endocrinology. 2005;146(12):5035-7.
29. Amendola E, De Luca P, Macchia PE, Terracciano D, Rosica A, Chiappetta G, et al. A mouse model demonstrates a multigenic origin of congenital hypothyroidism. Endocrinology. 2005;146(12):5038-47.