The c.63A>G polymorphism in the NKX2.5 gene is associated with thyroid hypoplasia in children with thyroid dysgenesis

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ABSTRACT

Objective: To search for genetic alteration in NKX2.5 gene in patients presenting both congenital heart disease (CHD) and TD. Subjects and methods: Individual phenotypes were carefully analyzed in 86 children with thyroid dysgenesis (TD) using thyroid function tests, scintigraphy, ultrasound and echocardiography. DNA was extracted and NKX2.5 gene coding region was amplified by polymerase chain reaction (PCR) and sequenced. Results: CHD were found in 8.1% of patients with TD. The mutation screening revealed two known polymorphisms in patients with isolated TD or TD associated with CHD. None of them are predicted to result in codon change in conserved domain. The c.63A>G polymorphism was detected in 54/86 patients (49 with isolated TD and 5 with TD combined with CHD). There was a significant association of c.63A>G polymorphism with hypoplasia (p < 0.036). The c.541G>A polymorphism was observed in only one patient with isolated thyroid hypoplasia. Conclusion: NKX2.5 mutations were not found. The c.63A>G polymorphism might be associated with thyroid hypoplasia.

Keywords
Thyroid dysgenesis; congenital hypothyroidism; congenital heart disease; NKX2.5

INTRODUCTION

Congenital hypothyroidism (CH) occurs in 1:3,500-1:4,000 newborns. In 80-85% of the cases, CH is due to thyroid dysgenesis (TD), which are developmental abnormalities of the thyroid gland including thyroid ectopy, thyroid agenesis or athyreosis, thyroid hypoplasia, and thyroid hemiagenesis (1). Several specific transcriptional factors, in view of their important role in thyroid organogenesis and thyroid specific gene expression, would be strong candidate genes for the etiology of TD; thus, the thyroid transcription factor 1 (TITF-1, also known as NKX2.1), forkhead box E1 (FOXE1) and the paired homeodomain factor PAX8 have been described as causes of human TD (1). Several loss of function mutations in NKX2.5 (OMIM 600584) have been described in patients with Congenital Heart Disease (CHD) and the most frequent one were atrial septal defect, ventricular septal defect and tetralogy of fallot (10,11). CHD has a higher frequency in children with CH than in the general population (11,12). Clinical interest in this gene have arisen from the identification of heterozygous mutations involved in the pathogenesis of human TD, including a mutational screening conducted in 241 patients with TD in Italy, allowing identification of three mutations in four patients (7).
In mouse, Nkx2.5 expression has been recently demonstrated in precursors of thyroidal cells in the pharyngeal floor at embryonic day 8.5 (E8.5), a period coincident with the appearance of Ttf-1, PAX8 and Pax8, but disappears around E12.5 (12). Therefore, once Nkx2.5 mRNA is present in the thyroid primordium at an early stage of development, it might be required in normal thyroid morphogenesis. Nkx2.5-/- embryos exhibited a smaller outgrowing bud of endodermal cells, indicating that Nkx2.5 is required as component of the genetic control of thyroid development (7). These observations propose a functional impact of NKX2.5 on genetic pathogenesis of TD and motivate us to address the question if genetic abnormalities of NKX2.5 could play an important role in patients presenting both CHD and TD or isolated TD. Our results indicate that no NKX2.5 mutations are not common in patients presenting TD, even in combination with CHD. However, the variant c.63A>G was significantly associated with hypoplasia.

**SUBJECTS AND METHODS**

**Subjects**

Between 2001 and 2013 we have identified 1,051 newborns with CH in the neonatal thyroid screening program of the State of Bahia, Brazil by dry blood spot TSH measurement collected after 24 hours of life from a heel prick (TSH cut-off 9 mU/L, Delphia Perkin-Elmer immunofluorimetric assay). Within 24 h of a positive screening result, the neonates had a history and a physical examination performed and a blood sample was taken for confirmation of diagnosis [serum TSH and total T4 (TT4)] employing chemoluminescent assays; reference intervals are 6-12 μg/dL for TT4 and 0.3-4 mU/L for TSH. After the confirmation of results, levothyroxine (L-T4) replacement treatment, 10-15 μg/kg/day, was started and L-T4 dosage was adjusted during infancy and childhood according to serum TSH and TT4. When the infants reach 3 years, they follow a protocol to determine if the CH is permanent or transient, consisting of serum thyroid function tests (TSH and TT4), thyroid 123I or 131I scanning, and thyroid ultrasound after 30 days of L-T4 therapy discontinuation. According to the results, children were divided into five groups: 1) ectopy, 2) agenesis, 3) hypoplasia, 4) hemiageneis and 5) normal eutopic thyroid gland or goiter. Eight hundred fifty one patients were confirmed for permanent CH, from which 710 have been followed in our institution.

We have selected 86 patients with TD for clinical and molecular studies. The cardiac phenotype was evaluated by history, review of medical records, physical examination, 12-lead electrocardiogram (EKG), and 2-dimension transthoracic echocardiography. Family history of CHD and thyroid disease was investigated by chart report (i.e., clinical testing of parents was not performed for the purpose of this study). Medical records of all patients were reviewed to determine whether any non-cardiac congenital malformations or other recognized genetic syndromes were present. Clinical studies were performed without knowledge of genotype. Written informed consent was obtained from the parents of all participants in accordance with protocols approved by the Federal University of Bahia.

**Genotype analysis**

Deoxyribonucleic acid was extracted from whole blood using standard techniques. The coding region of the NKX2.5 gene, including exon/intron boundaries, was amplified from genomic DNA by polymerase chain reaction (PCR). The exon 1 were amplified using two pair of primers: 1F 5´-CTTGTGCTCAGCGCTACCT-3´ and 1R 5´-CTCCTGGCCCTGAGTTTCTT-3´. The exon 2 were amplified by a total of 2 PCRs with the following two pair of primers derived from the flanking introns: 2A 5´-GCGCTCCGTAGGTCAAGC-3’, 2B 5´-TAGGGATTGAGGCCCAGC-3’, 2CF 5´-CACTCTGGAGCTGGTGG-3’ and 2BR 5´-CCCGAGAGTCAGGGA-3’. 100 ng of genomic DNA was amplified in a 25-μL volume containing: 40 ng of each oligonucleotide primers; 200 μmol/L each of deoxyadenosine triphosphate, and deoxythymidine triphosphate; 1.5 mM of MgSO4 and Taq polymerase. The reaction of exon 1 started with 5 minutes at 94C followed by 35 cycles of 30 seconds at 94C, 30 seconds at 56C or 60C, and 45 seconds at 72C and finished with a 10 minute extension period at 72C. For the exon 2, all reactions started with 2 minutes at 95C followed by 35 cycles of 45 seconds at 95C, 30 seconds at 59C or 60C, and 45 seconds at 72C and finished with a 10-minute extension period at 72C. DMSO (0.2 mL/20-.L reaction) was added to standard reagents for the exon 1 and for the first reaction of exon 2. PCR products were purified with PureLink Quick PCR Purification kit (Invitrogen, Germany) and sequenced using the ABI PRISM Dye
Terminator cycle sequencing Ready Reaction kit (PE Applied Biosystems) according to the manufacturer’s instructions. Bidirectional sequencing was performed by an automated sequencer (ABI3100 genetic analyzer). Sequences were analyzed with Bioedit Software and alterations were examined in context of the open reading frame. One hundred normal individuals were screened for the identified sequence alterations.

**Statistical analysis**

We used t test and Hardy-Weinberg test to estimate the differences in the frequency of NKX2.5 polymorphisms in our cohort and the general population and between each subgroup of TD. We built a two-way contingency table comparing the group of positive heart disease with each type of TD, thus we tested for dependence between each categorization and patients without CHD. The association of subtype of TD or presence of CHD in each subgroup and NKX2.5 polymorphisms was analyzed by $X^2$ (Fisher’s Exact Test).

**RESULTS**

**Clinical observations**

We found a high prevalence of CHD in patients with TD (7/86; 8.1%). Seventy nine infants presented with isolated TD (32 ectopy, 15 agenesis, 30 hypoplasia and 2 hemiagenesis). The overall female:male ratio was 2:1 (Table 1).

Five from seven patients with TD associated with CHD harbored the c.63A>G NKX2.5 polymorphism (Table 1). In family 1, the mother was diagnosed with autoimmune hypothyroidism. Her affected daughter (Patient A, Figure 1) was detected by neonatal screening, when the TSH level was 91.4 uIU/ML. The ultrasound showed an agenesis, but the level of serum Tg was 4.5 ng/mL and the thyroid scan showed an ectopic gland (Table 1, Figure 1). In family 2, a male patient (Patient B) was diagnosed lately and the ultrasound detected a hypoplastic thyroid gland (Table 1, Figure 1). In family 3 (Patient C, Figure 1), the neonatal TSH screening was 40 uIU/ML and a hypoplastic gland was confirmed by ultrasound. All the propositus from families 1-3 presented atrial septal defect on cardiac evaluation (Table 1, Figure 1). In Family 4, the proband (Patient D) had an absent thyroid gland by ultrasound associated with serum Tg levels of 17.3 ng/mL and an ectopic gland detected by thyroid scintigraphy. This patient had pulmonary stenosis (Table 1, Figure 1). The patient of family 5 (Patient E) was diagnosed with twenty-three days of birth and hypoplasia was observed by ultrasound (Figure 1, Table 1).

**Table 1.** Phenotype/Genotype summary in seven patients with TD associated with CHD

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Thyroid phenotype</th>
<th>Cardiac phenotype</th>
<th>Polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female</td>
<td>Ectopy</td>
<td>ASD</td>
<td>c.63A&gt;G</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>Hypoplasia</td>
<td>ASD</td>
<td>c.63A&gt;G</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>Hypoplasia</td>
<td>Lowen-Ganong-Levine Syndrome</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>Hypoplasia</td>
<td>ASD</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>Hypoplasia</td>
<td>ASD</td>
<td>c.63A&gt;G</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>Hypoplasia</td>
<td>AVB</td>
<td>c.63A&gt;G</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>Ectopy</td>
<td>PS</td>
<td>c.63A&gt;G</td>
</tr>
</tbody>
</table>

ASD: atrial septal defect; PS: pulmonary stenosis; AVB: atrio-ventricular block.

**Figure 1.** Pedigrees of the five families with patients harboring the c.63A>G polymorphism associated with TD and CHD. All family members were analyzed. Squares, men; circles, women; open symbols, clinically unaffected individuals; solid black symbols, affected by hypothyroidism with ectopy or hypoplasia; half black symbol represent a individual with hypothyroidism.

**Correlating NKX2.5 polymorphisms and clinical phenotype**

We did not find any NKX2.5 mutation in patients with both TD and CHD or isolated TD. Table 2 shows the polymorphisms found. The known c.63A>G NKX2.5 polymorphism was detected in heterozygosity in 33 (38.3%) of patients and in homozygosity in 21 (24.3%) of cases (Table 2) (Figure 2). Among patients positive
for the c.63A>G polymorphism, 49 had isolated TD (18 ectopy, 24 hypoplasia, 6 agenesis, 1 hemiagenesis) (Table 2). The polymorphism c.63A>G was associated with hypoplasia (p < 0.036).

A second polymorphism, c.541G>A was observed, in heterozygosity, in only 1 patient with isolated TD (Figure 2). A girl, diagnosed in neonatal screening (TSH 32.2 uIU/ML) presenting a hypoplastic thyroid gland.

DISCUSSION
Since NKX2.5 is one of the earliest transcription factors expressed in the thyroid lineage of developing vertebrate embryos and its targeted disruption results in perturbed morphogenesis, we hypothesized that it might be involved in TD etiology. The importance in human ontogenesis was further underscored by recent identification of patients with NKX2.5 mutation associated with TD (7). In this study of 86 infants with TD detected by Neonatal Screening Program for CH, in Brazil, we hypothesized the possible contribution of NKX2.5 gene to the pathogenesis of TD, firstly provided by Dentice and cols. (7).

In our screening for germeline inactivating mutation in the NKX2.5 gene, in a group of DT patients without a family history, we observed no significant variation but a positive association between the c.63A>G polymorphism and thyroid hypoplasia as phenotype.

This A → G polymorphism was previously detected in other studies and we have also found in normal subjects (13,14). Candidate gene mutations have been previously identified in familiar groups of CH, thus the patient’s selection may have played an important role because we analyzed patients selected from an entire

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Site</th>
<th>Polymorphism type</th>
<th>Allele frequency in patients</th>
<th>Allele Frequency in controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.63A&gt;G</td>
<td>TN domain</td>
<td>Silent</td>
<td>A/G 0.372</td>
<td>A/G 0.160</td>
</tr>
<tr>
<td>p.Glu21</td>
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<td></td>
<td>G/G 0.243</td>
<td>G/G 0.000</td>
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<tr>
<td>rs2277923</td>
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<td></td>
<td>A/A 0.385</td>
<td>A/A 0.840</td>
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<tr>
<td>c.541G&gt;A</td>
<td>Homeodomain</td>
<td>Silent</td>
<td>G/A 0.012</td>
<td>G/A 0.000</td>
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<tr>
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<td></td>
<td></td>
<td>A/A 0.000</td>
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<tr>
<td>rs72554028</td>
<td></td>
<td></td>
<td>G/G 0.988</td>
<td>G/G 1.000</td>
</tr>
</tbody>
</table>

Figure 2. Chromatograms showing the polymorphisms found in exon 2 of NKX2.5 gene. Top, Left: homozygous A-to-G transition at position 63 of codon 21 in the NKX2.5 gene; top midle: heterozygous A-to-G transition at position 63 of codon 21 in the NKX2.5 gene; top right: wildtype sequence; Bottom, left: wildtype sequence; Bottom, right: heterozygous G-to-A transition at position 541 of codon 181 in the NKX2.5 gene.
population, mostly sporadic cases. Our results indicate that the mutation rate of Nkx2.5 gene is rare in patients with TD, even in a phenotype-focused study from a different genetic background. In fact, a population-based study in a Czech cohort of 170 patients with CHD, including 15 with CHD, has not found mutations on Nkx2.5 gene (15). Similarly a Japanese study enrolling 102 patients, 37 with thyroid ectopy, did not find any mutations and confirms that Nkx2.5 mutations are rare (4). Indeed, a closer look at the Italian’s publication shows that some unaffected parents also carried the c.73C>T variant, suggesting that it might be not disease-causing but perhaps only disease-predisposing (7).

In Brazil, another study including 27 patients with TD without CHD, identified the same c.63A>G polymorphism in Nkx2.5 gene (20). We might consider that hypothetical mechanisms such as epigenetic or somatic changes could cause the inactivation of this gene (13,16). It is still discussed if somatic nature and mosaicism of Nkx2.5 mutations are major etiological pathway in CHD because the results obtained from fresh frozen diseased tissue sample are different from data obtained from formalin fixed archival tissues (13,16). However, unknown genes, but functionally similar in the same embryonic path, might be involved in the pathogenesis of TD associated or not with CHD, as for example, the report indicating that deficiency of the T-box transcription factor Tbx1 results in hemiagenesis and hypoplasia of the thyroid gland due to a failure of the embryonic thyroid to establish contact with vessels derived from the cardiac outflow tract at a critical step necessary for the proper guidance of bilateral growth and lobulation (2). Such genes may be either uniquely or differentially expressed and encode proteins including ion channels and signaling molecules. Indeed, the initial induction of follicular thyroid cells has been shown to be associated with factors secreted by endothelial cells and may involve input from epigenetic mechanical factors (2). Recent data revealed that Nkx2.5 was expressed in multipotent progenitors during cardiac development, suggesting role in regulation of the endocardial/endothelial fate in the developing heart and embryo, although the molecular mechanisms are unknown (17). Thyroid formation begins at approximately embryonic day (E) 20-22 in the human when progenitors cells migrate through the primitive streak to the more caudal side of the embryo to form the thyroid gland (18). Although a cooperative action of Nkx2.5 with other cellular factors could be essential for the maintenance of gene expression during thyroid embryogenesis, alternatively, loss of function of Nkx2.5 can lead to a limited gradual diminution of a downstream target genes during development, without interfere at its initial regulation. As Nkx2.5 is expressed so early at thyroid bud, it can potentially interact with other crucial transcription factors and modulates their activity post-translationally by changing its dimerization process.

In addition to the polymorphisms described in TD, many different heterozygous germline Nkx2.5 mutations have been identified in patients suffering from CHD (OMIM 600584). Those previously reported mutations associated with CHD are more primarily localized within the homeodomain. In this report, five of the patients positive for the c.63A>G polymorphism had a CHD phenotype. Although most published cases in CHD phenotype are in sequences affecting the homeodomain, there is no clear genotype-phenotype correlation. CHD of these patients with Nkx2.5 germline mutations were mainly of atrial septal defect with or without AV block, although there were reports of patients with tetralogy of Fallot, ventricular septal defect, double-outlet right ventricle, interrupted aortic arch, truncus arteriosus, L-transposition of the great arteries, hypoplastic left heart syndrome and aorta coarctation. So, the spectrum of Nkx2.5 mutations is diverse in terms of mutation type, position of the affected amino acid and its predicted impact on protein-protein interactions. In fact, experimental studies have shown that other portions of Nkx2.5, even far away from the homeodomain, are also functionally very important (21). The Nkx2.5 gene from diseased cardiac tissues of patients with complex cardiac malformations typically contains multiple mutations (14). As well, thyroid growth defect could be associated with single germline mutations or, alternatively, additional mutations arising in the hypoplastic gland could amplify the effect of the germlinal polymorphisms. Therefore, studies in which there is only examination of lymphocytic DNA may not reveal the molecular basis of TD.

Nkx2.5 appears to be an unlikely candidate gene for CHD associated with TD. The molecular mechanisms of TD with or without CHD are complex: while familial TD occurs at greater-than-random frequency, monozygotic (MZ) twins are discordant (19). We believe that germeline predisposing factors likely exist but
additional mechanisms are required to explain MZ twin discordance (somatic events, random mono-allelic expression even CNV – copy number variants).

However, it is known that, in the heart, NKX2.5 could have an essential role during the early thyroid morphogenesis, and might be implicated as a partner of the genetic circuit controlling thyroidal cell specification and migration and have pointed to the importance of its dosage in thyroid development (20). Importantly, NKX2.5 mutations are known to be central to the genesis of CHD and, in this case, might be necessary but not sufficient for TD. NKX2.5 binding elements has been identified in a number of expressed genes and many other tissues as in lingual muscle, spleen, stomach and in the lung (21).

It would be of interest to attempt to identify additional NKX2.5 downstream target genes and upstream signaling pathways for a more complete knowledge of its function during thyroid morphogenesis.

Acknowledgements: the authors are grateful for Ney Boa-Sorte, Paula Leite, Renata Arruti and Helena Pimentel support. We appreciate the cooperation of all patients, their families, and endocrinologists involved in their care.

Financial support: this work was supported in part by grants Fapesb (Fundação de Amparo à Pesquisa no Estado da Bahia) (AMTL and HER), and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) (HER). TLOC has financial support by Fiocruz.

Disclosure: no potential conflict of interest relevant to this article was reported.

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