Gene Variants in *NKX2-1* Do Not Represent a Major Etiological Factor of Primary Congenital Hypothyroidism in Mexican Population

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J Pediatr Genet 2019;8:41–46.

Abstract

Congenital hypothyroidism (CH), attributable to thyroid dysgenesis (TD), has an unusually high prevalence in Mexican population but the causes are unknown. *NKX2-1*, as a candidate gene, was subjected to automated Sanger sequencing in 122 unrelated Mexican patients with CH/TD. Although this study includes the largest number of TD-related CH patients in whom *NKX2-1* has been analyzed, no pathogenic variants were detected; only three benign polymorphic changes were identified. These results suggest that *NKX2-1* is not a major contributor to the etiology of CH or its high prevalence in Mexicans. Our work identifies misannotations of *NKX2-1* variants in three previous published reports.

Keywords

- Mexican population
- *NKX2-1* gene
- thyroid dysgenesis

Introduction

The worldwide prevalence of congenital hypothyroidism (CH) is two to three cases per 10,000 live births, but in some populations, such as the Mexican one, it can be up to 7.3 per 10,000 live births.¹ Most primary and permanent cases of CH (80–85%) are attributable to forms of thyroid dysgenesis (TD), including ectopic thyroid (50–60%), thyroid agenesis (20–30%), and thyroid hypoplasia (5%). The etiology of CH is unknown in most patients, although reports of a family history of CH (2%), familial cases of extrathyroidal congenital anomalies, a 1.4 female/male ratio, and the presence of asymptomatic

thyroid defects in first-degree relatives collectively suggest that there is a genetic component to this disease.²

The *NKX2-5* (NK2 homeobox 5), *NKX2-1* (NK2 homeobox 1), *FOXE1* (forkhead box E1), and *PAX8* (paired box 8) genes encode transcription factors that play key roles in the embryonic development of the thyroid gland. Germline loss-of-function variants of the latter three genes have been documented in syndromic forms of TD, and they are thus considered candidate genes for CH development. However, for the four mentioned genes, the cumulative frequency of such loss-of-function variants in nonsyndromic TD-related CH cases ranges from 0 to 5%.^{2–4}

NKX2-1 (14q13.3, MIM*600635) encodes two isoforms of the homeobox protein, Nkx-2.1, which is critical for the embryonic development of the lung and certain areas of

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the forebrain, and for the maintenance of thyroid follicular cells. Seven previous studies^{3–9} failed to clearly demonstrate the presence of pathogenic variants of *NKX2-1* in 440 non-syndromic CH patients (► **Table 1**), suggesting that this gene does not play a major role in the etiology of TD-related CH. However, we lack information from other non-Caucasian and non-Asian populations, such as the Mexican one. Thus, we were unable to rule out the possibility that pathogenic variants of the *NKX2-1* gene could be a causal factor for this congenital defect in Mexico, where the prevalence of CH is relatively high.¹ In the present work, we explored whether germline pathogenic variants of *NKX2-1* account for a proportion of TD-related CH in patients of Mexican descent.

Materials and Methods

A total of 122 unrelated Mexican patients diagnosed with nonsyndromic CH due to TD were included. Diagnoses were based on the thyroid profile and thyroid ^{99m}Tc-scintigraphy ($n = 92$) or ultrasound ($n = 30$), which revealed ectopic thyroid ($n = 56$), thyroid hypoplasia ($n = 14$), and thyroid agenesis ($n = 52$). The mean age of patients was 48.7 days, and 93 (76.2%) were female. Genomic DNA samples were obtained from peripheral blood leukocytes by the saline precipitation method (Puregene kit; Gentra Systems, Minneapolis, Minnesota, United States). Polymerase chain reaction (PCR) amplification and direct bidirectional automated sequencing were applied to the three coding exons and the intron–exon boundaries of *NKX2-1* (NM_001079668.2 and NG_013365.1 RefSeqGene; PCR conditions and primer sequences are available upon request). The c.-85G > T (rs2076751) variant was tested for Hardy–Weinberg equilibrium (HWE) using Fisher's exact test, and p -values were calculated with the DeFinetti software (<http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>). We compared the results from cases and 88 healthy and ethnically related controls (176 *NKX2-1* alleles) to identify whether there was an association between this variant and the CH trait. Informed consent was obtained from parents of all individuals included in this study, which was approved by the Research and Ethics Committee of the National Institute of Pediatrics (008/2007), Mexico.

Results

Patient's clinical manifestations documented in order of frequency were jaundice (64%), umbilical hernia (54%), wide anterior fontanel (43%), constipation (43%), hoarse crying (37%), macroglossia (33%), hypoactivity (33%), dry skin (32%), typical facies (23%), edema (18%), hypotonia (18%), perioral cyanosis (10%), hypothermia (7%), and respiratory distress (5%).

No germline pathogenic variants of the *NKX2-1* gene were detected in the 122 unrelated Mexican CH patients analyzed in the present work. Instead, we identified three polymorphic variants. The first, c.-85G > T (rs2076751) located in the 5'UTR had allelic frequencies of 0.63 for the G allele and 0.37 for the T allele; the genotype distributions of G/G, T/T, and G/T were $n = 49, 55$, and 18, respectively, for cases and $n = 40, 35$, and 13, respectively, for controls. Both groups showed HWE, as assessed by Fisher's exact test ($p > 0.24$), and there was no

statistically significant difference between them ($\chi^2 = 0.29$, $p = 0.592$, odds ratio = 1.090). The other two identified polymorphic variants were rare variants previously recorded in the Exome Aggregation Consortium (ExAC, <http://exac.broadinstitute.org/>) database. The c.306G > A or p.(Ala102 =) variant (rs769642515, ExAC Variant: 14:36988347 C/T, minor allelic frequency: 0.001%) was found in a 20 days old heterozygous female in whom her newborn screening suggested the CH diagnosis, which was further confirmed by serum thyroid profile demonstrating high thyroid-stimulating hormone (TSH) 75 IU/mL (ref 0.40–4.0 IU/mL), low free thyroxine (FT4) 0.75 ng/dL (ref 0.8–1.9 ng/dL), and ectopic thyroid gland documented by ^{99m}Tc-scintigraphy. Her clinical phenotype at age of 4 months, when she started treatment, was characterized by jaundice, typical facies, wide anterior fontanel, umbilical hernia, constipation, hoarse crying, hypoactivity, and hypotonia. The c.805_813del or p.(Gly269_Gly271del) variant (rs747378330, ExAC Variant: 14:36986875 TGCCCCGCC/T, minor allelic frequency: 0.029%) was found in a 2 months old heterozygous male patient, whose newborn screening and serum thyroid profile demonstrated high TSH 75 IU/mL (ref 0.40–4.0 IU/mL), low FT4 0.75 ng/dL (ref 0.8–1.9 ng/dL), and thyroid hypoplasia on ultrasound. He presented jaundice and umbilical hernia and started treatment in his first month of life.

Discussion

The focus of this study was to explore if germline pathogenic variants of *NKX2-1* account for a proportion of TD-related CH in patients of Mexican descent. About the three polymorphic variants detected, the first one, c.-85G > T (rs2076751), was previously described as a polymorphic variant in 11 Japanese controls.⁵

Approximately 638 variants have been identified throughout the sequence of *NKX2-1* (<http://gnomad.broadinstitute.org/gene/ENSG00000136352>), but none has yet been subjected to a comprehensive genetic association study in CH. Hishinuma et al.⁵ described three polymorphic *NKX2-1* variants located at intronic regions (rs2076735 and rs2076736) and in the 5' UTR (rs2076751) of CH patients of Japanese descent ($n = 9$). (► **Table 1**). Minor alleles of these variants were also identified in 23, 10, and 28%, respectively, of healthy controls, prompting the authors to conclude that there was no association with CH.⁵ However, the small sample size of the previous work makes it difficult to definitively discard any association. Here, although our molecular strategy could not identify the deeply intronic rs2076735 and rs2076736 variants in our CH patients, the identification of rs2076751 genotypes allowed us to fully examine, whether differences in allelic frequencies between the cases and the healthy subjects suggested a possible association with CH. We did not find any significant difference between them ($p = 0.592$), and thus discard the possibility of such an association in our population.

The other two identified rare variants enlisted in ExAC, c.306G > A (rs769642515) and the c.805_813del (rs747378330) were found at very low allelic frequencies

Table 1 Summary of the molecular studies performed on *NKX2-1* gene in patients with nonsyndromic CH

Patients included	Thyroid phenotype	Inclusion criteria	Methodology	Variants identified in <i>NKX2-1</i>	Conclusion	Reference
15	9 agenesis 4 ectopic 2 hypoplasia	Diagnosed by NBS (Rome, Italy) based on high TSH levels, low T3 and T4, neck echotomography, and ^{99m}Tc -scintigraphy	PCR-SSCP of <i>NKX2-1</i> gene	None	Mutations in <i>NKX2-1</i> may be extremely rare events in the pathogenesis of CH/TD	Perna et al 1997 ⁷
61	22 agenesis 18 ectopic 1 hypoplasia 20 with thyroid phenotype not indicated	Diagnosed by NBS (Italian) based on low T4, high TSH, and thyroid ^{99m}Tc -scintigraphy (41 patients) and ultrasonography (some patients)	PCR-SSCP and direct Sanger sequencing of <i>NKX2-1</i> gene	None	Absence of mutation indicates that <i>NKX2-1</i> is not a frequent cause of CH	Lapi et al 1997 ⁸
9	3 agenesis 5 ectopic 1 hypoplasia	Diagnosed based on thyroid ultrasound and serum concentrations of T4, T3, and TSH (Japan)	RNase cleavage assay and direct Sanger sequencing of all <i>NKX2-1</i> coding and noncoding regions	rs2076735 (c.463 + 470G > A) ^a rs2076736 (c.464–98C > A) ^a rs2076751 (c.85G > T) ^a	The three reported changes were considered to be benign polymorphisms	Hishinuma et al 1998 ⁵
102	51 non-TD 51 TD (37 ectopic, 6 agenesis, 8 hypoplasia)	Permanent primary CH patients (Kanagawa, Japan) diagnosed by NBS. The method used for thyroid imaging was not specified	Direct Sanger sequencing (<i>NKX2-5</i> , <i>PAX8</i> , <i>NKX2-1</i> , and <i>FOXE1</i>) and MLPA (<i>PAX8</i> , <i>NKX2-1</i> , and <i>FOXE1</i>)	Novel variant c.178C > T. Originally reported as p.(His60Trp) or “H60W” instead of p.(His60Tyr) or “H60Y” ^b	“H60W” exhibited a transactivating capacity comparable to that of wild-type <i>NKX2-1</i> , suggesting that it is a benign variant ^c	Narumi et al 2010 ⁶
43	16 ectopic 9 agenesis 1 hypoplasia 9 absent/rudimentary thyroid tissue 8 thyroid phenotype not indicated	Permanent primary CH patients (Cracow, Poland) diagnosed by NBS and confirmed based on thyroid profile, ultrasound ($n = 43$), and ^{99m}Tc -scintigraphy ($n = 26$)	MLPA for <i>PAX8</i> , <i>FOXE1</i> , <i>NKX2-1</i> , <i>TSHR</i> , and <i>TPO</i>	None	The MLPA technique increased the detection of monoallelic deletions in <i>PAX8</i> , <i>TSHR</i> , and <i>FOXE1</i> (11%) among sporadic CH/TD patients, but no identified any change in <i>NKX2-1</i>	Kumorowicz-Czoch et al 2015 ⁴
100	Agenesis	Diagnosed by NBS (Shandong, China) based on high TSH levels and low T4, ^{99m}Tc -scintigraphy and ultrasonography	NGS for <i>NKX2-1</i> , <i>FOXE1</i> , <i>NKX2-5</i> , <i>PAX8</i> , and <i>TSHR</i>	c.131G > A or p.(Gly44Asp) ^b	NGS increased the detection of pathogenic variants in the <i>TSHR</i> gene among sporadic CH/TD patients. No clinical or zygosity data was provided for the p.(Gly44Asp) patient, and <i>in silico</i> analysis was inconclusive for this change (VUS). The other four cited <i>NKX2-1</i> variants actually	Wang et al 2017 ⁹

(Continued)

Table 1 (Continued)

Patients included	Thyroid phenotype	Inclusion criteria	Methodology	Variants identified in <i>NKX2-1</i>	Conclusion	Reference
110	Not evaluated in most included cases Only 10 patients with an unspecified TD type	Permanent primary CH patients (Fujian, Anhui, Jiangsu, and Shanghai provinces, China) diagnosed by NBS and confirmed based on thyroid profile	NGS for <i>NKX2-1</i> and other 20 CH-related loci	rs200560568 [c.1054G > A or p.(Gly352Ser)] ^a	correspond to the <i>TTF1</i> gene (MIM *600777, NM_007344.3) NGS showed that 51.82% (57/110) of CH cases are related to dysmorphogenesis, where biallelic <i>DUOX2</i> pathogenic variants account for the most common genetic defect (37.27%, 41/110). The p.(Gly352Ser) only described in East Asian population at MAF of 0.32% and classified as VUS, was identified in heterozygous state in one TD case, as in one normal thyroid gland CH patient (1.8%, 2/110), as well as in two healthy parents	Sun et al 2018 ³
122	56 ectopic 14 hypoplasia 52 agenesis	Permanent primary CH patients (Mexico) diagnosed based on thyroid profile, thyroid ^{99m} Tc scintigraphy, or ultrasound	Direct Sanger sequencing of all <i>NKX2-1</i> coding regions and intron-exon boundaries	rs2076751 (c.-85G > T) ^a rs769642515 (c.306G > A) ^a rs747378330 (c.805_813del) ^a	The absence of germline <i>NKX2-1</i> pathogenic variants and lack of an association between rs2076751 and the CH/TD trait suggest that <i>NKX2-1</i> is not a major genetic contributor to the development or high prevalence of nonsyndromic TD-related CH in the Mexican population	This study

Abbreviations: CH, congenital hypothyroidism; MAF, minor allelic frequency; MLPA, multiplex ligation-dependent probe amplification; NBS, newborn screening; NGS, next-generation sequencing; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism; T3, triiodothyronine; T4, thyroxine; TD, thyroid dysgenesis; TSH, thyroid-stimulating hormone; VUS, variant of uncertain significance.
^aAccording to the *NKX2-1* reference sequence, NM_001079668.2 (isoform 1 of three coding exons).
^bAccording to the *NKX2-1* reference sequence, NM_003317.3 (isoform 2 of two coding exons).
^cNo in vitro assay was performed with the correct p.(His60Tyr) mutant *NKX2-1* construct.⁶

in cases (each one in one patient). Hence, it would not be possible to establish associations and we did not need to search them in controls. Although the c.805_813del (rs747378330) variant is enlisted in ExAC, we do not know of any detailed clinical information regarding a linked phenotype. However, in-frame deletions/insertions in a repetitive region without a known function are generally assumed to introduce benign changes (Genetic Variant Interpretation Tool, http://www.medschool.umaryland.edu/Genetic_Variant_Interpretation_Tool1.html). Consistent with this assumption, our heterozygous p.(Gly269_Gly271del) patient lacked any clinical data suggesting the syndromic CH form conditioned by *NKX2-1* haploinsufficiency due to heterozygous genotypes for null alleles (brain-thyroid-lung syndrome, MIM#610978). At present, this 5-year-old patient has adequate endocrine control, with normal weight, height, and psychomotor development. These data could support the benignity of the c.805_813del or p.(Gly269_Gly271del) variant. However, future work should examine whether this variant contributes to the genetic susceptibility for developing nonsyndromic CH due to TD, as suggested for polyalanine stretch lengths in *FOXE1*.¹⁰

The absence of pathogenic genetic variants in *NKX2-1*, along with the previously documented 1% of Mexican CH-TD patients bearing pathogenic variants in *PAX8* gene,¹¹ supports that other loci might be implicated in the high prevalence of CH in our population. Although the present work has the limitation of only performing *NKX2-1* analysis, to our best of knowledge, it included the largest number of CH patients with a corroborated TD phenotype (►Table 1). Despite that the results of one recent next-generation sequencing (NGS) approach were obtained in a lesser number of analyzed TD patients ($n = 10/110$) than ours, one variant of unknown significance for *NKX2-1* was identified in two of nonsyndromic CH Chinese patients³ (►Table 1), which suggest that the genetic etiology of CH may differ among populations including ours. To document a possible major CH genetic etiology factor for this public health concern in Mexico, other TD-related genes (i.e., *TSHR*, *NKX2-5*, and *FOXE1*) are currently under analysis in our patients.

In our literature review, we identified that Narumi et al⁶ reported a novel variant, designed as p.His60Trp, that had a transactivating capacity comparable to that of wild-type *NKX2-1*, suggesting that it is a benign variant. Although, according to the genetic code, we observe that the variant was wrongly described, so the authors did not perform the in vitro assay with the correct NM_003317.3:c.178C > T or p.(His60Tyr) mutant *NKX2-1* construct (►Table 1). On the other hand, Wang et al⁹ found the novel NM_003317.3:c.131G > A or p.(Gly44Asp) variant in one of 100 Chinese patients with thyroid agenesis (►Table 1) that was not identified in our patients. Although the encoded change lies outside the homeobox domain (a.a. 194–247) of *Nkx-2.1*, *in silico* analyses performed also by us, using MutationTaster, SIFT, and PolyPhen programs, revealed contradictory results. Moreover, the report lacked clinical and *NKX2-1* zygosity descriptions for this patient. Hence, we suggest that at this time the p.(Gly44Asp) could be considered a

variant of uncertain significance. Also, it is important to emphasize that this previous report included four additional missense variants in *NKX2-1* [rs3739915, rs3739916, rs145664933, and the novel c.1357G > C or p.(Glu453Gln)] which actually correspond to a different gene (*TTF1*, encoding the transcription-termination factor, RNA polymerase I, NM_007344.3).⁹ The same situation was noted in the work published by Long et al,¹² where the authors report four missense variants (three novel: c.1598C > T, c.515A > G, c.269G > A, and rs765553668) that also not correspond to *NKX2-1*. We consider relevant to highlight this confusion related to legacy *NKX2-1* gene names (i.e., *TTF1*, *BCH*; *BHC*; *NK-2*; *TEBP*; *TTF1*; *NKX2A*; *NMTC1*; *T/EBP*; *TITF1*; *TTF-1* or *NKX2.1*, <https://www.ncbi.nlm.nih.gov/gene/7080>) and its official gene symbol established by the HUGO Gene Nomenclature Committee (https://www.genenames.org/data/gene-symbol-report/#!/hgnc_id/HGNC:11825) to prevent misannotations in future work. Unfortunately, according to the genetic variant nomenclature and provided rs ID, these previous findings bar us from further discussion in the context of our population.

Conclusions

To our knowledge, this study includes the largest number of TD-related CH patients in whom a molecular analysis of *NKX2-1* has been performed (►Table 1). Our failure to identify any pathogenic change in *NKX2-1* in this sample of Mexican TD-related CH patients agrees with the previous findings in Polish,⁴ Japanese,^{5,6} Italian,^{7,8} and Chinese^{3,9} populations. Collectively, these results suggest that germline pathogenic variants of *NKX2-1* are not a major genetic contributor to the etiology of nonsyndromic TD-related CH or its high prevalence in the Mexican population.

Conflict of Interest

None declared.

Acknowledgments

This research was funded by the National Institute of Pediatrics (Recursos Fiscales 2014–2016, Programa E022 Investigación y Desarrollo Tecnológico en Salud, Ciudad de México) and Fondo Sectorial de Investigación en Salud y Seguridad Social SS/IMSS/ISSSTE-CONACyT (69491 and 233654). SVI is grateful to CONACyT for a scholarship (No. 290590).

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