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Mutational and bioinformatics analysis of the *NKX2.1* gene in a cohort of Iranian pediatric patients with congenital hypothyroidism (CH)

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ABSTRACT

Congenital hypothyroidism (CH) occurs with a relatively alarming prevalence in infants, and if not diagnosed and treated in time, it can have devastating consequences for the development of the nervous system. CH is associated with genetic changes in several genes that encode transcription factors responsible for thyroid development, including mutations in the NK2 homeobox 1 (*NKX2.1*) gene, which encodes the thyroid transcription factor-1 (TTF-1). Although CH is frequently observed in pediatric populations, there is still a limited understanding of the genetic factors and molecular mechanisms contributing to this disease. The sequence of the *NKX2.1* gene was investigated in 75 pediatric patients with CH by polymerase chain reaction (PCR), single-stranded conformation polymorphism (SSCP), and direct DNA sequencing. Four missense heterozygous variations were identified in exon 3 of the *NKX2.1* gene, including three novel missense variations, namely c.708A>G, p.Gln202Arg; c.713T>G, p.Tyr204Asp; c.833T>G, p.Tyr244Asp, and a previously reported variant rs781133468 (c.772C>G, p.His223Gln). Importantly, these variations occur in highly conserved residues of the TTF-1 DNA-binding domain and

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were predicted by bioinformatics analysis to alter the protein structure, with a probable alteration in the protein function. These results indicate that nucleotide changes in the *NKX2.1* gene may contribute to CH pathogenesis.

KEYWORDS

Congenital Hypothyroidism, *NKX2.1* gene, *In-silico* analysis, Missense variation

INTRODUCTION

Congenital hypothyroidism (CH) is the most common congenital endocrine disorder in neonates and children with a global incidence of 1 in 3,000–4,000. Adequate thyroid hormone levels in the bloodstream are essential for the normal growth and development of the nervous system in children. Consequently, CH is associated with irreversible central nervous system and mental health problems, as well as poor growth of untreated children with CH. Girls are more likely to be affected than boys (female to male ratios are 2:1) [1–3]. Early diagnosis methods for CH are still necessary to establish more efficient treatments of CH, which is an important public health issue worldwide, including developed countries [4].

CH can be classified either as primary or as central hypothyroidism, and it is an autosomal recessive disorder. But in a significant percentage of patients, CH is sporadic and non-hereditary and caused by *de novo* developmental defects in the thyroid gland (primary congenital hypothyroidism). Hereditary forms of CH with decreased thyroid hormone synthesis are either due to a defective thyroid gland development (thyroid dysgenesis) present in 80% of patients or to a failure in thyroid hormone synthesis (thyroid dyshormonogenesis) present in 20% of the patients [5, 6]. Thyroid dysgenesis (TD; OMIM 218700), which refers to the most common cause of congenital hypothyroidism and abnormal structural malformations in the thyroid, is subcategorized into: (i) thyroid ectopy, an abnormally located and small thyroid gland; (ii) thyroid athyreosis, complete lack of the thyroid gland in imaging studies; and (iii) hypoplasia, a smaller gland of thyroid tissue but in a normal position. Dyshormonogenesis (OMIM 274400–274900) refers to the failure of thyroid hormone synthesis by a structurally normal thyroid gland [7].

In recent years, the overall incidence of CH has increased, including the transient form of CH in children, which is a consequence of thyroid hormone deficiency due to low thyroxine and elevated thyrotropin expression. Temporary CH may show symptoms of mild dyshormonogenesis during the first months of life due to insufficient production of thyroxine (T4) [8, 9]. Transient CH usually resolves itself in the first few months of infancy because of an increase in thyroxine production, but some of these children may also be treated for CH in infancy with levothyroxine (a manufactured form of the thyroid hormone thyroxine). However, after treatment endogenous hormonal levels are normalized, and by the age of 3, these children no longer need medication.

CH is caused by genetic alterations in one or more genes coding for transcription factors responsible for hormone biosynthesis, as well as thyroid differentiation, migration, and growth [10]. The genes encoding for the thyroid-stimulating hormone receptor (TSH receptor) and the transcription factors PAX8 (Paired box 8), NKX2.1/TTF-1 (NK2 homeobox 1), NKX2.5 (NK2 homeobox 5), FOXE/TTF-2 (Forkhead box E1), GLIS3 (GLIS family zinc finger 3), and HHEX

(haematopoietically expressed homeobox) are all expressed during the early steps of thyroid differentiation and migration, and mutations in these genes are likely to contribute to CH in humans [11–13]. Among these transcription factors, NKX2.1/TTF-1, encoded by the *NKX2.1* gene located on the chromosome 14q13, is one of the main transcription factors associated with CH [14]. To date, more than a hundred mutations of the *NKX2.1* gene have been described, most of which are point mutations and approximately 30% of them are large deletions [15–17]. The *NKX2.1* gene, composed of 3,836 nucleotides, can give rise to two types of mRNA from the gene and two protein isoforms. The *NKX2.1* gene has three exons and encodes a transcription factor consisting of two transactivation domains located at the N-terminal and C-terminal regions of the protein and a DNA-binding homeodomain in between [16]. The NKX2.1 DNA-binding homeodomain, which plays a crucial role in target-gene recognition and binding, is encoded by exon 3 of the *NKX2.1* gene. During embryonic organogenesis and development, *NKX2.1* is expressed in the thyroid, the lung, the forebrain, the basal ganglia, and especially in the hypothalamus [18]. Heterozygous *NKX2.1* mutations have been associated with CH and a broad phenotypic spectrum including a variable combination of lung, thyroid, and neurological defects. [19]. This transcription factor plays an important role in the regulation of crucial genes important during thyroid development, such as TSHR (Thyroid Stimulating Hormone Receptor), TG (Thyroglobulin), and TPO (Thyroid peroxidase) [20]. To assess the prevalence of *NKX2.1* mutations in pediatric patients with congenital hypothyroidism, a mutation screening was performed in a group of 75 patients with CH. The diagnosis of CH was based on characteristic phenotypic criteria previously described in other studies [21, 22]. However, it is very difficult to describe hypothyroidism because of the many variables that affect thyroid function in childhood, including age, sex, body mass, iodized diet, height, and puberty [23].

MATERIALS AND METHODS

Patients

Seventy-five unrelated patients (54 females and 21 males) aged 3–11 years (Mean ± SD = 5.68 ± 2.7), diagnosed with primary CH by an expert pediatric endocrinologist were enrolled from both the Metabolism and Endocrinology Clinic and the Department of Pediatrics of Shahid Sadoughi Medical Hospital, Yazd, Iran. All patients were evaluated by detection of high TSH (TSH >10 mU/L) and low free T4 (fT4) levels (fT4 < 9.0 pmol L^{-1}) (Table 1). Thyroid hypoplasia was assessed by measurement of total thyroid volume and related to height, sex,

Table 1. Clinical phenotypes in the CH patients and control group

Medical records	Patients (n = 75)	Controls (n = 69)
Age in sampling time	3–11 years (Mean ± SD = 5.68)	3.5–12.5 (Mean ± SD = 6.91)
Female/Male	54/21	49/20
Weight in birth time (g)	2,100–3,800	3,000–4,100
TG (μg L^{-1})	0.05–9.66	3.5–56
TSH (mU/L)	9.61–48	0.7–5.6
fT4 (pmol L^{-1})	0.7–6.5	9.0–19

TSH: thyroid stimulating hormone; fT4: free tetra-iodothyronine; TG: thyroglobulin.

age, and body surface area. Among the patients of the group, medical records showed that 15 cases had a positive family history of thyroid disorders: A 4.5-year-old girl with early-onset hypothyroidism had two brothers aged 3.5 and 6 with a positive history of CH in the older brother, one girl with three brothers and 8 girls with TD and hypothyroidism in siblings of their parents. The Ethics Committee of Yazd University approved the study protocol, and informed consent had been obtained from the parents of all selected CH patients (IR.Yazd.REC.1399.077).

DNA extraction

Molecular analysis and determination of the *NKX2.1* gene variations were performed on wgenomic DNA extracted from the patients' peripheral blood samples using the standard salting-out method [24]. The quantity of extracted DNA was measured and evaluated by a NanoDrop '2000' spectrophotometer.

DNA amplification

Evaluation of point mutations and nucleotide variations in the genomic region of *NKX2.1* (chromosome 14, NG_013365.1) was performed on PCR-amplified fragments spanning the entire coding region and exon-intron boundaries with three pairs of forward and reverse primers designed using *Primer3 design* online software (Primer3, Design Program, Canada) (Table 2). DNA amplification was achieved in a Techne Prime thermal cycler (Techne, Staffordshire, UK). PCR reactions were performed in a final volume of 25 µL containing 200 ng of genomic DNA, 10 pmol of each forward and reverse primer, 1.8 mM MgCl2, 100 µM of each dNTPs, 2.5 µL 10× PCR buffer (Qiagen), and 0.5 U Taq polymerase (Qiagen). For amplification of all of the exons of the *NKX2.1* gene, touchdown PCR programs were run with an initial denaturation step of 7 min at 95 °C, 5 cycles of 95 °C for 30 s, 68-61 °C for 50 s, and extended at 72 °C for 1 min. Subsequent 30 cycling reactions consisted of denaturation at 95 °C for 30 s, annealing at 65.5 °C for 35 s, extension at 72 °C for 1–2 min (depending on PCR length products), and final extension at 72 °C for 7 min. Amplification of PCR products was confirmed by 1–1.5% agarose gel electrophoresis separation of PCR samples, staining with ethidium bromide, and visualization by gel documentation systems from Bio-Rad.

Single-stranded conformation polymorphism (SSCP)

Amplified PCR products were screened by SSCP from all CH patients and healthy controls. Briefly, 10 µl of PCR products were first mixed with 7 µl of SSCP loading solution

Table 2. Primer sequences, nucleotide positions, and lengths of PCR products for the amplification of 3 exons in the *NKX2.1* gene

Primers	Exons	Nucleotide positions	sequences	Product sizes
NKX2.1.F1	1	−21 to −2	5′- GGCTCAGCGCAGCGAAGCCC-3′	286bp
NKX2.1.R1		243 to 265	5′-AACCACTTTCCAATTCGGTCGG-3′	
NKX2.1. F2	2	844 to 863	5′-CTCTTCCTTCCTCCTCCAGC-3′	425bp
NKX2.1. R2		1249 to1269	5′-ATGGGGGCGGCCTCACTTAC-3′	
NKX2.1. F3	3	2197 to 2219	5′-CGTTTGTCGCTTACAGTCTCCC-3′	760bp
NKX2.1. R3		2935 to 2957	5′-CTCACCAGGTCCGACCGTATAG-3′	

(95% formamide/10 mM NaOH/45% sucrose/0.2% xylene cyanol FF). Next, the DNA fragments were denatured for 10 min at 95 °C and immediately cooled on an ice bath for 5 min. DNA fragments were loaded and separated on 7% polyacrylamide gel (49:1 acrylamide to bisacrylamide) (Qiagen, Germany) during 16–18 h at 110 V, at room temperature. SSCP bands on the gel were visualized with the silver staining method according to the standardized protocol [25]. Fragments showing different migration mobility patterns within the gel when compared with the healthy control samples were directly sequenced for nucleotide change identification. Sequences were analyzed by the MEGA5 software and the chromas program. Nucleotide variations were compared with the mutation database of the *NKX2.1* gene (The Human Gene Mutation Database: http://www.hgmd.cf.ac.uk/).

Bioinformatics analysis for the pathogenicity prediction of the mutations

The sequences were compared with the NCBI (National Centre for Biotechnology Information) database and BLAST program (http://www.ncbi.nlm.nih.gov/blast/). The results of several Prediction softwares and databases were analyzed for the identification of the possible functional effects of the pathogenic and deleterious nonsynonymous nucleotide variations. SIFT (http://sift.jcvi.org/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), I-Mutant 2.0 (http://gpcr2.biocomp.unibo.it/I-Mutant2.0), PhD-SNP (https://snps.biofold.org/phd-snp), Mutation Taster (http://www.mutationtaster.org/), PROVEAN (http://provean.jcvi.org), PredictSNP (https://loschmidt.chemi.muni.cz/predictsnp/), Ensembl genome browser (https://asia.ensembl.org/index.html), PMut (https://www.bsc.es/medicahead/scientific-outputs/pmut), MutPred2 (http://mutpred.mutdb.org/), NetSurfP 2.0 (http://www.cbs.dtu.dk/services/NetSurfP/), PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/), and RaptorX software and databases were used for the prediction of the damaging functional effects in secondary structure alterations in the mutant protein and protein stability changes upon single point mutations. Evolutionary conservation analysis and identification of functional regions in proteins were performed using the ConSurf server (https://consurf.tau.ac.il/). Protein domains were evaluated from UniProt and InterPro databases (https://www.uniprot.org/ and http://www.ebi.ac.uk/interpro/). Furthermore, the ExPASy (Expert Protein Analysis System) Prot-scale tool (http://web.expasy.org/protocol) was used for the prediction of hydrophobicity or hydrophilicity scales in the protein. Finally, I-TASSER (https://zhanglab.dcmb.med.umich.edu/I-TASSER/) was used for protein structure modeling and protein PyMol software (https://www.pymol.org/), and HEX 8.0.0 protein-ligand docking software used for visualization of the 3D shapes and the identification of binding changes in the protein and between the protein and other complex molecules. In this software, the three-dimensional structure along with the number and length of bonds in normal and mutant proteins were investigated.

Table 3. Sequence variations observed in the coding regions of the *NKX2.1* gene

Mutation	Genomic variation	Coding change	Amino acid change	Exon	Homo/ Hetero	Novel/ SNP	Frequency in patients
Missense	g.2354A>G	c.708A>G	p.Gln202Arg	3	Hetero	Novel	11/75
Missense	g.2359T>G	c.713T>G	p.Tyr204Asp	3	Hetero	Novel	4/75
Missense	g.2418C>G	c.772C>G	p.His223Gln	3	Hetero	rs781133468	18/75
Missense	g.2479T>G	c.833T>G	p.Tyr244Asp	3	Hetero	Novel	5/75

Statistics analysis

Genetic polymorphism differences in coding exons of the *NKX2.1* gene were compared between patients and healthy controls through a Fisher Exact test. A *P*-value of <0.05 was considered to specify a statistically significant difference.

RESULTS

SSCP analysis and direct sequencing of the entire coding sequence of the *NKX2.1* gene (comprising exons 1–3 of isoform 1) in our patients (54 females and 21 males) with congenital hypothyroidism revealed four missense and heterozygous alterations in the nucleotide sequence (Table 3, Fig. 1). Eleven patients (14.7%) showed c.708A>G, p.Gln202Arg substitution, four patients (5.3%) presented c.713T>G, p.Tyr204Asp variation, eighteen patients (24%) showed

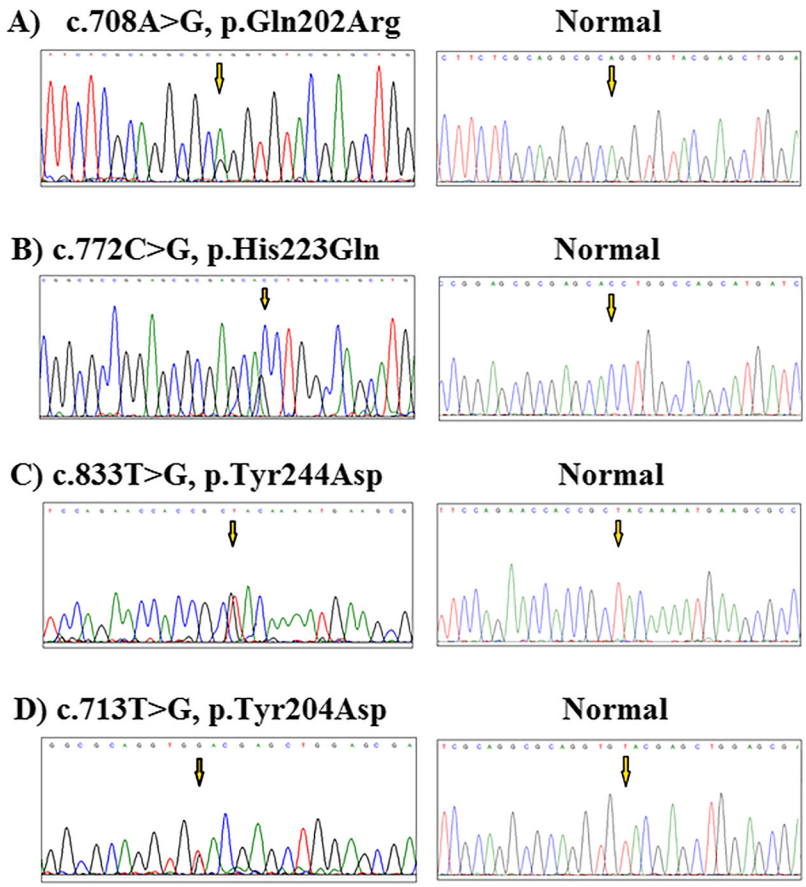


Fig. 1. Sequencing results of four heterozygous missense variations in CH patients are compared to sequencing results of control/normal samples

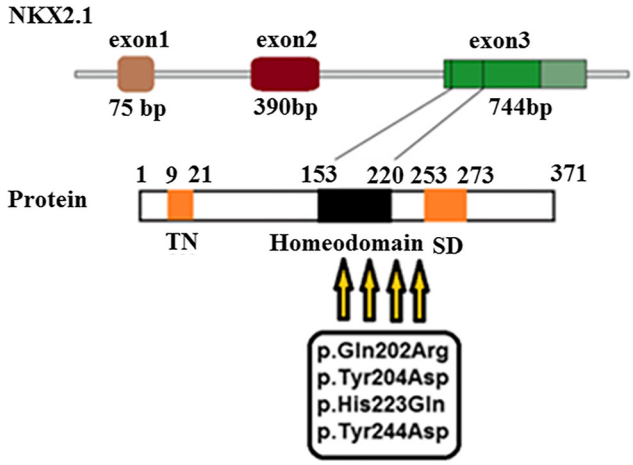


Fig. 2. Amino acid positions of four heterozygous missense mutations in exon 3 of *NKX2.1* gene are indicated. A simple map of the functional homeodomain of the human NKX2.1 protein is shown

rs781133468 variation (c.772C>G, p.His223Gln), a known single nucleotide polymorphism (SNP), and five patients (6.7%) showed a substitution (c.833T>G, p.Tyr244Asp), but compound heterozygotes were not observed in any of our patients. All of these heterozygous variations occurred in the sequence of exon 3 of the *NKX2.1* gene (Fig. 2), and three of them are novel missense variations that have not been reported in any SNP database. Sequence analysis of exons 1 and 2 of the *NKX2.1* gene in our samples did not reveal any genetic alteration, and SSCP results showed similar migration patterns of patient and control samples. Out of a total of 75 patients studied, 20 cases from different families had positive family histories of thyroid

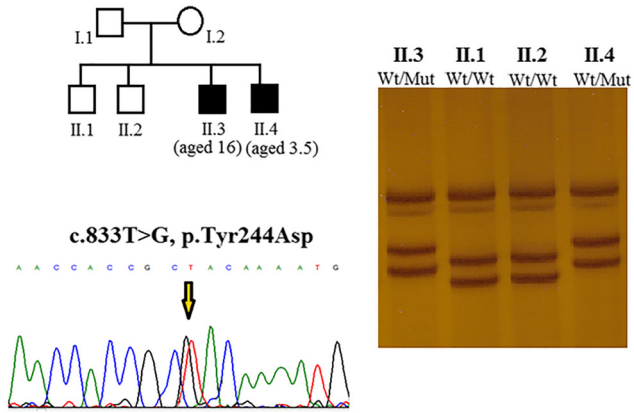


Fig. 3. Sequencing and SSCP results for the Tyr244Asp mutation in a 3.5-year-old boy with CH and an older brother (aged 16). SSCP bands showed different migratory patterns in these affected brothers. Also, SSCP results showed that the other two brothers have normal alleles

Table 4. Results for prediction of missense mutations by computational tools

Server Mutation	SIFT* / Score	Polyphen-2/ Score	Polyphen-2 Sensitivity/ Specificity	Provean/ Score	Panther Preservation time** /result/ pdel***
p.Gln202Arg	Affected/0.00	Probably damaging/0.981	0.75/0.96	Deleterious/-3.809	1037/Probably damaging/0.85
p.Tyr204Asp	Affected/0.01	Probably damaging/0.999	0.14/0.99	Deleterious/-9.501	456/Probably damaging/0.57
p.His223Gln	Tolerated/0.33	Probably damaging/0.997	0.41/0.98	Deleterious/-7.600	1037/Probably damaging/0.85
p.Tyr244Asp	Affected/0.01	Probably damaging/0.999	0.14/0.99	Deleterious/-8.776	1037/Probably damaging/0.85

*SIFT Seq Rep for all mutations: 1.00.
**Million year preservation time.
***pdel: Probability of deleterious effect

disorders and hypothyroidism, but a total of 10 parents with affected children were sampled and the rest of the parents, for personal reasons, rejected further research. Among those parents who are clinically asymptomatic, four mothers were heterozygous for the rs781133468 variation (c.772C>G, p.His223Gln) in the *NKX2.1* gene. However, statistical analysis showed that only the three novel nucleotide variations in the present study had significant differences between the patient and control groups (OR (95% CI = 5.297 (1.188–23.619)), and *P*-value = 0.016) and the rs781133468 variation showed no statistically significant difference (*P*-value = 0.85).

A 3.5-year-old boy with CH associated with a movement disorder, paroxysms, and psychomotor retardation had an older brother (aged 16), who showed the same heterozygous

Table 5. Summary of results for prediction of effective missense mutations by functional prediction tools and hydrophobicity programs

Missense variants	PMut	Predict SNP	Conservation by ConSurf	I-Mutatnt2.0/ DDG	ERIS	ExPASy/ Protscale
p.Gln202Arg	Disease	Deleterious	9 (completely conserved)	Decrease/-1.04	Destabilizing	Decrease Hydrophobicity/-0.111
p.Tyr204Asp	Disease	Deleterious	8 (Highly conserved)	Decrease/-0.24	Destabilizing	Decrease Hydrophobicity/-0.245
p.His223Gln	Disease	Neutral	9 (completely conserved)	Decrease/-0.48	Destabilizing	Decrease Hydrophobicity/-0.033
p.Tyr244Asp	Disease	Deleterious	9 (completely conserved)	Decrease/-0.53	Destabilizing	Decrease Hydrophobicity/-0.245

variation (c.833T>G, p.Tyr244Asp). SSCP results showed different migratory patterns in these affected brothers, although the sequence analysis showed that they carried the same variation. Thyroid ultrasound examination for the older brother showed normal morphology. In addition, their unaffected parents refused to participate in the study, whereas SSCP and the results of

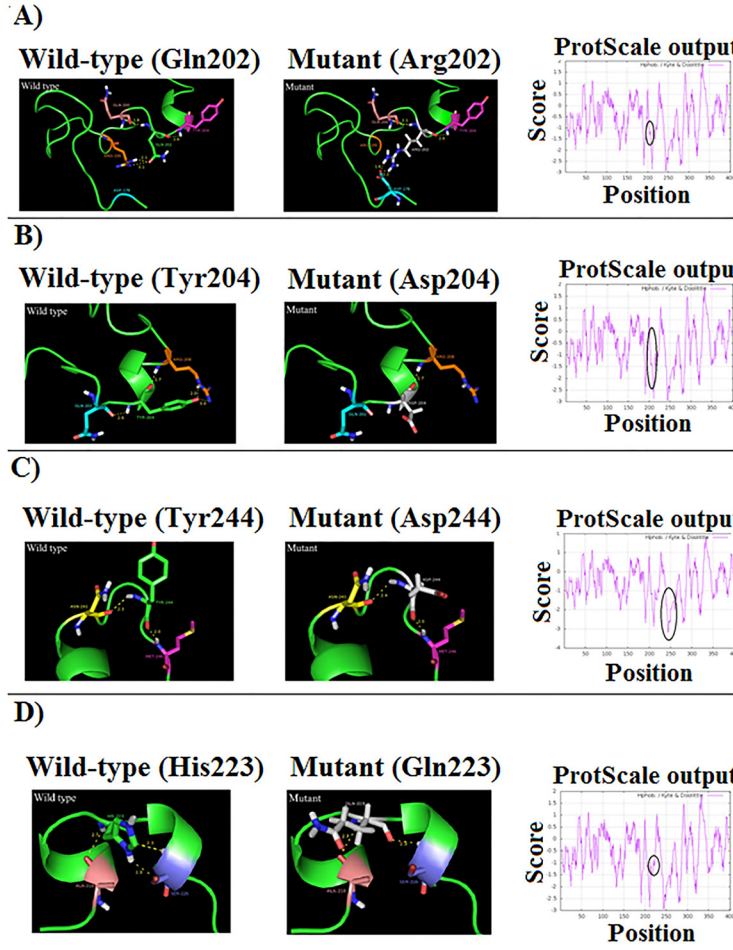


Fig. 4. Three-dimensional structure model prediction and the plots of hydrophobicity changes are shown for normal and mutant variants (p.Gln202Arg, p.Tyr204Asp, p.His223Gln, and p.Tyr244Asp) of NKX2.1 by PyMol. A) In the normal protein, Gln202 connects with Arg195 (2.1Å and 3.1Å), Gln200 (1.8Å), and Tyr204 (2.6Å). In the mutant protein, Arg202 connects with Asp178 (1.8Å and 1.2Å), Gln200 (2.1Å), and Tyr204 (2.6Å). B) In the normal protein, Tyr204 connects with Gln202 (2.6Å), and Arg208 (1.7Å, 2.0Å, and 3.0Å), and in the mutant protein, Asp204 connects only with Arg208 (1.7Å). C) In the normal protein, His223 connects with Ala218 (2.7Å) and Ser226 (2.3Å), and in the mutant protein, Gln223 connects with Ala218 (2.7Å) and Ser226 (2.3Å). D) In the normal protein, Tyr244 connects with Asn241 (2.3Å) and Met246 (2.0 Å), and in the mutant protein, Asp244 connects with Asn241 (2.4Å) and Met246 (2.0 Å)

sequencing analysis showed that the other two brothers have normal alleles (Fig. 3). None of the three novel missense variations were observed in 69 healthy controls, whereas the rs781133468 variation (c.772C>G, p.His223Gln) was detected in 11 additional CH patients and in 13 of the 69 controls individuals tested (7.24%).

To infer a potential pathogenic effect of the four missense variations, we predicted whether these amino acid substitutions were deleterious or damaging for the structure, stability, and function of the protein using several prediction tools such as PolyPhen-2, SIFT, I-Mutant, Psipred, ERIS, Protscale, PROVEAN, PredictSNP, PMut, PANTHER, and Expasy software and online programs (Tables 4 and 5). Interestingly, the three novel missense mutations that we identified (p.Gln202Arg, p.Tyr204Asp, and p.Tyr244Asp) occur in highly conserved amino acid sequences of NKX2.1/TTF-1, and are predicted to provoke alterations in protein structure and function. Therefore, the three novel NKX2.1/TTF-1 variants are likely to present deleterious functions with pathogenic consequences. In addition, these missense variations would certainly result in a change of the polarity of the protein, due to the replacement of a neutral amino acid with a hydrophilic amino acid (p.Gln202Arg), and a hydrophobic amino acid with a hydrophilic amino acid (p.Tyr204Asp, and/or p.Tyr244Asp). The plot created by the ExPASy tool presented noticeable changes according to the Hphob/Kyte & Doolittle scale in protein hydrophobicity for p.Gln202Arg (−3.500 to −4.500), and for p.Tyr204Asp and p.Tyr244Asp (−1.300 to −3.500) when compared with the wild-type protein (Fig. 4).

To predict protein structure, the protein sequence was loaded in the Swiss model (a protein structure homology-modeling server) based on sequence homology. Next, to study the effect of mutations on the structure of the protein, modeling was performed in I-TASSER based on the amino acid sequence. The server proposed five models for the protein structure, with a C-score for each of these models. A higher C-score indicates the higher accuracy of the model predicted by the server. Therefore, this model was used to study the effect of observed nucleotide variations on protein structure (Fig. 5).

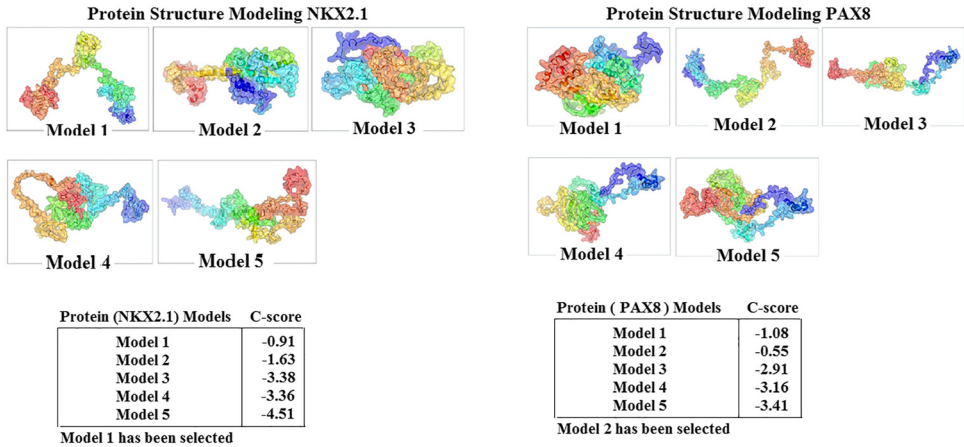


Fig. 5. Protein structure modeling for NKX2.1 and PAX8 by I-TASSER. The server proposed five models for the protein structure. A higher C-score was used to study the effect of variations on protein structure

DISCUSSION

The *NKX2.1* gene is composed of three exons and produces two different isoforms of the protein: a 371-amino-acid peptide encoded by the last 2 exons (translated from the first ATG in exon 2) and a 401-amino-acid peptide including all 3 exons. The isoform with 371 amino acids is expressed at a higher level in the lungs, whereas the isoform with 401 amino acids is expressed in the thyroid and the brain [26]. The *NKX2.1* gene is about 4 kb long (3836bp), is located on chromosome 14 (13.3q14) and encodes for a transcription factor with a homeodomain encompassing amino acids 153–220. Mutations in this region have been shown to affect the regulation of transcriptional activity of the target genes [27]. During development, NKX2.1 controls the expression of several important genes for thyroid formation, such as those coding for thyroglobulin, thyroperoxidase, the thyrotropin receptor, and the sodium/iodide symporter [12, 28]. Clinical phenotypes have been associated with genomic alterations in the NKX2.1 gene locus, such as the deletion of one of the alleles on chromosome 14 (14q13 - q13.3) that was shown to be linked to disease in a single case, as well as in a sibling with hyperthyroidism, microcephaly, and respiratory distress [29]. However, in these studies the neurological defects detected in the patients were suggested to be due to the disruption or deletion of neighboring genes located in this chromosomal region [5]. Heterozygous mutations in this gene encoding a transcription factor are introduced as risk factors in congenital hypothyroidism. Indeed, most mutations are thought to result in a clinical phenotype due to gene haploinsufficiency or by reduction by half of the NKX2.1 protein levels, but a few variants have been described to be dominant negative [16, 30].

NKX2.1 mutations may appear as autosomal dominant inheritance with variable expression and penetrance in patients with CH, but evidence suggests that most mutations occur *de novo* without hereditary origin [31].

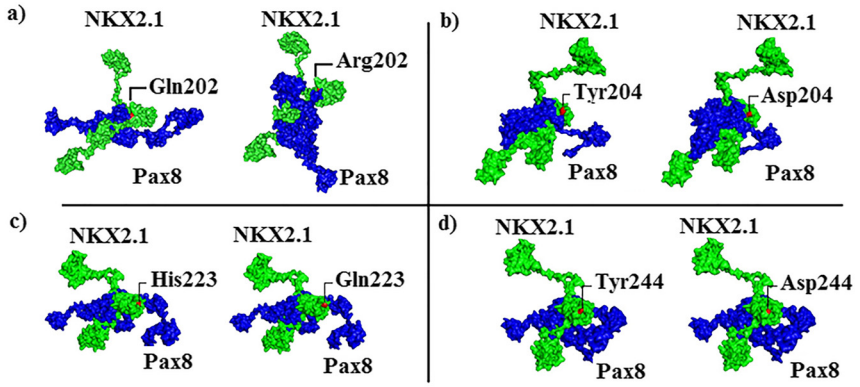
In this study, we identified four heterozygous missense variations and amino acid substitutions in *NKX2.1* in seventy-five pediatric patients with congenital hypothyroidism. Three of the missense variations, p.Gln202Arg, p.Tyr204Asp, and p.Tyr244Asp have not yet been described and recorded. To our knowledge, the present study was the first investigation to identify *NKX2.1* gene mutations in Iranian pediatric patients with congenital hypothyroidism. The nucleotide variations p.Gln202Arg and p.Tyr204Asp, located in the DNA-binding paired domain, were present in 38 patients out of 75. Importantly, the NKX2.1/TTF-1 DNA-binding domain is highly conserved in vertebrates, inferring the importance of this domain along with evolution. Homozygous mutations in this gene have not been reported in humans, which means the affected fetuses may not survive, perhaps due to the lethal effect of these mutations as seen in *NKX2.1* knockout mice [30, 32]. Indeed, mice lacking both *NKX2.1* alleles die around 10–11 days after birth, and display a complex phenotype, including no observable embryonic thyroid bud, whereas heterozygous mice show no abnormalities of thyroid development. Together, these studies have suggested that NKX2.1 plays an important role in thyroid progenitor cell survival [18, 29].

Using *in-silico* assays, we predicted that these missense variations would alter the polarity and three-dimensional structure of the protein (Fig. 4). Also, our results in HEX6.1 docking software for two proteins NKX2.1 and PAX8 showed that the Gln202Arg and Tyr204Asp mutations in the NKX2.1 protein would alter its interaction with PAX8, a member of the pair box family of transcription factors which is critical for thyroid development and involved in CH

when mutated [33]. Also, the comparison of the amount of interaction energy between the two proteins NKX2.1 and PAX8 in normal and mutant types showed that the Gln202Arg and Tyr204Asp mutations have the most extensive changes in interaction energy among the other missense variations; therefore, the heterozygous Gln202Arg and Tyr204Asp missense variations probably affect the transcriptional regulatory properties of NKX2.1, which is thoroughly associated with thyroid development (Fig. 6).

Moreover, bioinformatics analyses at the functional level indicated that heterozygous variations p.Gln202Arg and p.Tyr204Asp located in the homeodomain are likely to reduce binding affinity to a specific DNA sequence, and were predicted to have deleterious impacts on the protein function. Therefore, these predicted structural changes in mutants and evolutional conservation of the residues are strong indicators that the nucleotide variations identified in our patients may impair proper DNA binding of NKX2.1/TTF-1 to its target genes, or interfere with the interaction of partner transcription factors also necessary for correct thyroid development, such as PAX8. Consequently, these variations might have important effects during early thyroid organogenesis, and also in the postnatal functional impairment of the thyroid. It would be of interest to conduct future functional studies to confirm this hypothesis. Since the frequency of all the variants identified in this study is rather low in the control cases, and because their minor allele frequency (MAF) in the general populations is extremely low, according to the 1000-Genomes project and Genome aggregation database (gnomAD), it can be suggested that the novel nucleotide variations identified in the present study are likely to contribute to CH etiology.

Also, according to pathogenic and benign criteria in the ACMG guideline (Standards and guidelines for the interpretation of sequence variants) [34], three heterozygous variations



e)

	Wild-type protein	Gln202Arg	Tyr204Asp	His223Gln	Tyr244Asp
Lowest energy	-1757.1	-1622.1	-1677.6	-1757.4	-1757.1

Fig. 6. **a-d)** Docking of two proteins NKX2.1 (green chain) and PAX8 (Blue chain) present interactions of protein NKX2.1 in natural form and mutated forms with 4 observed missense variations. **e)** Comparison of the amount of interaction energy between two proteins NKX2-1 and PAX8 in normal and mutated forms

p.Gln202Arg, p.Tyr204Asp, and p.Tyr244Asp were classified as pathogenic variants: The prevalence of the variants in affected individuals are significantly increased compared to the prevalence in controls (PS4), variants were located in a well-established functional domain (PM1), they were absent from controls (PM2), were assumed de novo, but without confirmation of paternity and maternity (PM6), and multiple lines of computational evidence support deleterious effects on the gene (PP3). Also, the rs781133468 variation (c.772C>G, p.His223Gln) was classified as benign variation with supporting evidence: It was observed in healthy adult individuals (BS2).

In our study, two novel missense variations p.Gln202Arg and p.Tyr204Asp were observed in 15 children with mild to severe impairment of thyroid function (congenital hypothyroidism), neurological symptoms, but not neonatal respiratory distress previously known as *NKX2.1*-related disorders. These *NKX2.1*-related disorders have a highly variable phenotypic range, ranging from defects in a specific tissue to the complex involvement of many organs such as the thyroid, brain, and lungs. These novel mutations were not observed in parents, despite a clear history of walking delays in some of their families. The two other missense variations (His223Gln and Tyr244Asp) reported here were observed in pediatric patients characterized by mild clinical presentation. No respiratory distress at birth and no respiratory symptoms were detected during childhood. According to previous studies, this observation is not surprising, because they also show an absence of genotype-phenotype correlation in *NKX2.1*-related disorders, probably due to the pathogenic role of genetic mutations and environmental causes which might involve haploinsufficiency, [16, 35] which should be further investigated.

Several *NKX2.1* mutations have been reported upstream or downstream of the DNA binding domain that are associated with high phenotypic variability, and in the majority of patients, haploinsufficiency has been implicated as a mechanism responsible for these different manifestations, as also suggested for the *NKX2.1* heterozygous knockout mice [36, 37]. In a study performed by Moya et al., a new frameshift mutation (825delC) in the *NKX2.1* gene was reported in two sisters affected by choreoathetosis and hypothyroidism, but without respiratory distress [15]. Doyle et al. conducted a study on 85 patients with congenital hypothyroidism from neonatal screening programs for the presence of mutated *NKX2.1*. They identified a splicing mutation at the 3′ splice consensus of intron 2 of the *NKX2.1* gene in two affected children, mother, and maternal grandmother. Next, they extended the *NKX2.1* mutational analysis to other patients with congenital hypothyroidism and found more unrelated patients with various *NKX2.1* mutations. Also, parental screening for *NKX2.1* mutations in families was negative. Such an observation of *de novo* mutations supports an autosomal dominant mode of transmission in the *NKX2.1* gene [38]. Gillett et al. found a novel missense mutation (c.621C > G, p.Ile207Met) in exon 3 of *NKX2.1* gene in a full-term neonate associated with hypothyroidism and lethal respiratory failure [39]. Also, a different substitution of this same amino acid residue, p.Ile207Phe, had been reported previously by Maquet et al. in a term girl with lethal respiratory failure and mild primary hypothyroidism [40]. Monti et al. performed a genetic analysis of the *NKX2.1* gene in a neonate without respiratory distress, but with mild thyroid dysfunction and delayed independent walking. They identified a novel *NKX2.1* nonsense mutation (Tyr130X) and a single base exchange in intron 2 (c.463 + 41C > T) [16]. In several studies, Trp143X, Ser175X, and Cys117X nonsense mutations were presented in CH cases with different clinical manifestations (chorea, hypothyroidism, hypotonia, development delay, and recurrent pulmonary infections, and respiratory distress) [30, 36, 41]. Thorwarth et al. performed a comprehensive genotyping of

NKX2.1 mutations in patients with thyroid dysfunction, neurological deficit, and pulmonary disease and identified six frameshift mutations due to insertion/deletion mutations, seven nonsense mutations, and one missense mutation (p.Thr233Arg). They developed a homology structure model and concluded that missense mutations within the homeobox domain interfered with DNA binding [17]. Also, Veneziano and colleagues studied the *NKX2.1* gene in 160 CH patients. After sequencing exons 1, 2, and 3 of this gene, they found a new mutation in exon 3 in patients with a variety of symptoms, including mental retardation and chorea, cerebellar ataxia, and congenital hypothyroidism. This mutation was heterozygous and resulted in the conversion of nucleotide A to T at position 727. This heterozygous mutation altered the amino acid lysine at position 211 to stop codon (p.Lys211X). The amino acid lysine in this protein is one of the highly conserved amino acids in the homeodomain of this protein [42]. In other studies, a high rate of de novo mutations was also detected in this gene [37]. The high rate of new mutations and their incomplete penetration have significant clinical diagnostic and genetic counseling implications. Nettore et al. identified a novel heterozygous deletion (c.493delC) in the *NKX2.1* gene in a family with five affected patients in two generations with hypothyroidism, benign hereditary chorea, and respiratory distress. This frameshift mutation is responsible for producing a truncated protein with a premature stop codon at position 196. Even though the autosomal dominant transmission of hereditary CH caused by mutations of *NKX2.1* has been confirmed, however, thyroid dysfunction was not studied in these patients [37]. Our results also suggest that screening for *NKX2.1* mutations should be carefully considered for pediatric patients with congenital hypothyroidism, and patients who have received proper thyroid hormone therapy. In addition, identifying *NKX2.1* defects can be a noticeable risk factor in children with congenital hypothyroidism.

CONCLUSION

NKX2.1 plays an important role in the early steps of thyroid differentiation and migration and is probably a candidate gene for CH in both humans and mice, therefore we propose that the *NKX2.1* gene is a good candidate for further research of CH and experimental conclusions in the improvement of therapeutic strategies. These genetic approaches to detect mutations in the *NKX2.1* gene in pediatric patients with congenital hypothyroidism provide a great opportunity for physicians for obtaining diagnostic, prognostic, and therapeutic indicators as well as for offering genetic counseling in the affected families. However, additional research is needed to better understand the complexities of the genotype-phenotype correlation between the *NKX2.1* mutations and congenital hypothyroidism.

Consent to participate: All patients (or parents) had provided informed consent for this study.

Author contributions: M.M.H. and M.K. conceived the study, participated in its design, and drafted the initial manuscript. S.A.M., A.R.E., and F.T. collected the data. J.B. drafted the paper and approved the final version. M.K., M.M.H., and M.O. performed statistics and interpretation of data and drafted the last version of the manuscript. M.M.H., R.C., and F.G. coordinated data interpretation, participated in the design of the study, and helped to draft the manuscript. All authors read and approved the final manuscript.

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Conflicts of interest: The authors declare that there is no conflict of interest.

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