Original Article

Mutations of Dual Oxidase 2 (DUOX2) Gene among patients with Permanent and Transient Congenital Hypothyroidism

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ABSTRACT

Objective: The prevalence of congenital hypothyroidism (CH) is high in Isfahan, Iran. In addition, it has different etiologies compared with other countries. The rate of parental consanguinity is also high in the city. Moreover, DUOX2 gene is effective in transient CH and permanent CH due to dyshormonogenesis. Therefore, the aim of this research was to investigate the mutations of DUOX2 gene in patients with transient CH and permanent CH due to dyshormonogenesis.

Methodology: In this descriptive, prospective study, patients diagnosed with transient and permanent CH due to dyshormonogenesis during CH screening program were selected. Venous blood samples were obtained to determine the 3 mutations (Q36H, R376W, and D506N) of DUOX2 gene using polymerase chain reaction (PCR) method by specific primers and complementary methods such as restriction fragment length polymorphism (RFLP) and single-strand conformation polymorphism (SSCP).

Results: In this study, 25 patients with transient CH and 33 subjects with permanent CH due to dyshormonogenesis were studied. In addition, 30 children were studied as the control group. We did not find any mutations of the 3 mentioned mutations of DUOX2 gene.

Conclusion: Considering the findings of the current study, further studies with other methods are required to evaluate other gene mutations such as pendrin, sodium-iodide symporter (NIS) and thyroglobulin.

KEY WORDS: Congenital Hypothyroidism, Dual Oxidase 2 (DUOX2) gene, Permanent, Transient.

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INTRODUCTION

Congenital hypothyroidism (CH), with a prevalence of 1/3000-4000 live births, is the most common endocrine disorder and preventable cause of childhood mental retardation.[1,2] CH is considered as a multifactorial disease and many genetical, environmental and autoimmune factors contribute in its pathogenesis.[3-5] It could be presented in transient or permanent forms. In the transient form, the need for thyroid replacement therapy would be temporary, i.e. for the first 1-3 years, while in the permanent form, treatment would be continued lifelong.[6] The causes of transient CH are excessive or lack of iodine intake and transplacental migration of antibodies and antithyroid drugs.[6] Permanent CH in 85% of cases is associated with a spectrum of thyroid gland developmental defects (dysgenesis) due to abnormal differentiation, migration, or growth of the gland. In 10-20% of cases, it results from defects in one of the multiple steps of thyroid hormone synthesis or defects in the receptor of

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thyroid hormone (dyshormonogenesis) which is a heritable disorder represented by goiter in some cases.[6]

Thyroid dysgenesis is a sporadic disorder whose pathogenesis is still unknown. On the contrary, several molecular studies have found different genes, including thyroid stimulating hormone receptor (TSHR), sodium-iodide symporter (NIS), thyroglobulin (TG), thyroid peroxidase (TPO) and pendrin, to be involved in thyroid dyshormonogenesis. Recently, dual oxidase 2 (DUOX2) has been identified as a new gene involved in the etiology of dyshormonogenesis.[7-11]

Hydrogen peroxide (H2O2) is considered as an essential compound for thyroid hormone formation and has an important role in the initial step of triiodothyronine (T3) and thyroxine (T4) synthesis in the follicular lumen of thyrocytes. This biochemical requirement of H2O2 for thyroid hormone production has been known as H2O2-generating system. DUOX2, a reduced nicotinamide adenine dinucleotide (NAD) phosphate: O2 oxidoreductase flavoprotein located at the apical plasma membrane of thyrocytes, is a component of the thyroid H2O2-generating system. It is a 1548-aminoacid polypeptide, including a 26-amino-acid signal peptide. Its gene is located on chromosome 15 and consists of 33 exons encoding an mRNA with 6376 nucleotides. This polypeptide is involved in the Ca2+/reduced nicotinamide adenine dinucleotide phosphate-dependent H2O2 generation. H2O2 is used by TPO to catalyze both the iodination of tyrosine residues and the coupling of iodotyrosine residues of TG. DUOX2 is considered as the principal element in generating the H2O2 needed for TPO function.[12-17]

Previous studies have indicated that a defect in the H2O2-generating system causes congenital hypothyroidism. They have suggested that biallelic mutations of DUOX2 cause permanent congenital hypothyroidism and that monoallelic mutations cause transient congenital hypothyroidism.[18,19] However, some studies have reported transient CH cases, despite biallelic mutations. In addition, familial cases of DUOX2 have also been reported.[18]

The prevalence of CH is high in Isfahan. Moreover, it has different etiologies compared to other countries. The prevalence of transient CH and also the rate of permanent CH due to dyshormonogenesis are high according to CH screening results.[20] On the other hand, high rate of parental consanguinity can be effective on CH incidence.[21] Moreover, DUOX2 gene is involved in transient CH and permanent CH due to dyshormonogenesis. Therefore, the aim of this research was to investigate the mutations of DUOX2 gene in patients with transient CH and permanent CH due to dyshormonogenesis in Isfahan, Iran.

METHODOLOGY

In this descriptive, prospective study, patients diagnosed with transient CH or permanent CH due to dyshormonogenesis in Isfahan Endocrine and Metabolism Research Center, during CH screening program (2002-2009) in Isfahan were selected. CH screening program was initiated in 2002 and continued until 2005 when the nationwide CH screening program was implemented.

Newborns with abnormal screening results were re-examined and those with abnormal T4 and TSH levels on their second measurement (TSH > 10 mIU/l and T4 < 6.5 µg/dl) were diagnosed as CH patients and received treatment and regular follow-up. Hypothyroid neonates underwent treatment at a dose of 10-15 µg/kg/day as soon as the diagnosis was confirmed. The TSH and T4 levels were monitored during the follow-up.

Permanent and transient cases of CH were determined at the age of three years old by measuring TSH and T4 concentrations four weeks after withdrawal of L-T4 therapy. Patients with elevated TSH levels (TSH > 10 mIU/l) and decreased T4 levels (T_4 < 6.5 µg/dl) were considered as permanent CH sufferers. The etiology of CH was determined by thyroid scan and/or ultrasound before treatment in neonatal period or at the age of 3 years old after confirming the permanency of CH. Patients with thyroid gland of normal size were considered to have dyshormonogenesis.[20]

The protocol was approved by the Institutional Review Board and Medical Ethics Committee of Isfahan University of Medical Sciences. Written consents were obtained from the parents of CH patients.

The selected CH patients were examined by a pediatrician and the demographic characteristics and screening findings regarding the level of TSH and T4, parental consanguinity and the etiology of CH were recorded using a questionnaire.

Venous blood samples were obtained from the selected patients. The samples were transferred to the Department of Genetics, Isfahan University of Medical Sciences for molecular analysis and determining the 3 mutations (Q36H, R376W, and D506N) of DUOX2 gene using the polymerase chain reaction (PCR) method by specific primers and complementary methods such as restriction fragment length

Table-I: Demographic characteristics of patients with transient and permanent CH due to dyshormonogenesis and the control group.

	Patients with transient CH	*Patient with permanent CH due to dyshormonogenesis*	*Control group*
Age (months)	68.2 ± 25.5	64.6 ± 23.7	69.3 ± 27.2
Sex (male/female)	18/7	15/18	19/11
Parental consanguinity (%)	69%	62%	39%
TSH (mIU/l)			
-primary	25.5 ± 19.7	47.3 ±46.1	4.5 ± 2.7
-after treatment interruption	3.9 ± 2.0	36.5 ± 30.7	-
T4 (µg/dl)			
-primary	5.7 ± 3.5	6.2 ± 3.5	11.6 ± 3.9
-after treatment interruption	8.1 ± 1.9	7.1 ± 2.9	-

polymorphism (RFLP) and single-strand conformation polymorphism (SSCP).

Genetic study: DNA was extracted from peripheral blood by the QIAamp DNA Blood Mini Kit (Qiagen, Germany). Real-time PCR and melting curve analysis were performed in the Corbett Rotor-Gene 6000 instrument (Corbett Research, Sydney, Australia).

Primers were designed by Beacon Designer 7.91 to flank the coding regions (Premier Biosoft International, USA) and synthesized by TIB MolBiol (Germany). The primers included Q36H forward primer (5′-GGGAGGGGTAGCTGGGAGC-3′) and reverse primer (5′- CCGCTCAGGGC-CTTTCGC-3′), R376W forward primer (5′-TCC-CTCACCACATCCTTTGTTCTCA-3′) and reverse primer (5′- TGTTGCTTTTCCCAGCCTGTGTG-3′), and D506N forward primer (5′-CATGGGGAC-CCTGGACCC) and reverse primer (5′- GTGTG-GTGGGCTGACTGGG-3′). The final optimal reaction conditions were empirically determined. The reaction mixture used was Type-it HRM Kit (Qiagen, Germany).

The amplification mixture of a total volume of 25 µL included 12.5 µL of high resolution melt (HRM) PCR master mix, 1.75 µL of 10 µM primer mix, 2 µL of genomic DNA as template and 8.25 µL of RNase-free water. The PCR cycling conditions are reported in Tables II-IV. The HRM analysis was performed by instrument software, which allows clustering the samples into groups on the basis of difference plots obtained by analyzing the differences in melting curve shapes.

One sample from each group with less than 90% confidence was confirmed by sequencing PCR products in the 3730xl Genetic Analyzer (Bioneer, South Korea).

Statistical Analysis: The obtained data was analyzed using the $SPSS_{13}$ (SPSS Inc., Chicago, IL, U.S.A.).

RESULTS

In this study, 25 patients with transient CH and 33 cases of permanent CH due to dyshormonogenesis were studied. Thirty children were studied as the control group. Demographic characteristics of the studied population are presented in Table-I. The difference plot showed the approximately same shapes detected in the same amplicons (Fig. 1-3). Sequencing analysis did not show different results. We did not find any of the three mentioned DUOX2 gene mutations (Q36H, R376W, and D506N).

DISCUSSION

Similar to other etiological investigations during the CH screening in Isfahan, this study evaluated the role of three mutations of DUOX2 (Q36H, R376W, and D506N) in transient CH patients and permanent

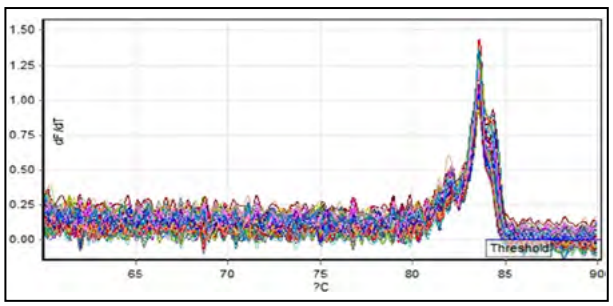

Fig.1: Sequencing analysis of the Q36h mutation.

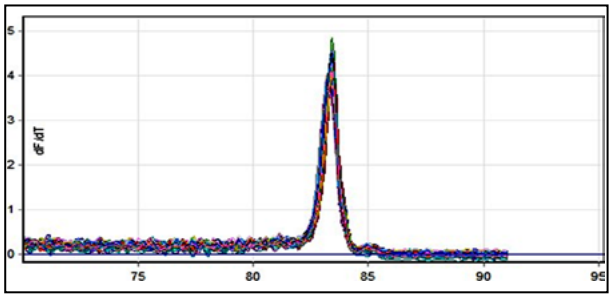

Fig.2: Sequencing analysis of R376W mutation.

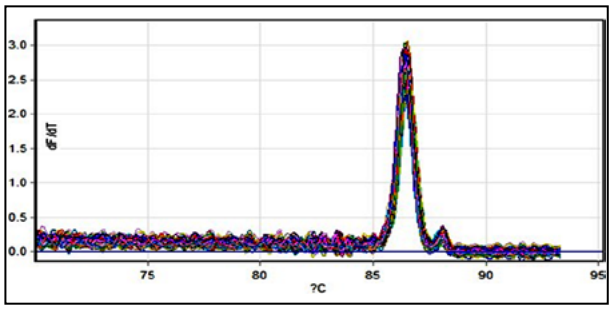

Fig.3: Sequencing analysis of the D506N mutation.

CH patients due to dyshormonogenesis. According to our results, the studied population, including both transient and permanent CH patients, did not have any of the mentioned mutations.

Many human and animal studies have suggested the important role of DUOX2 gene in the synthesis of thyroid hormone and the etiology of CH.[22] The mentioned association have mostly been recognized by the neonatal screening test for CH since it is difficult to diagnose adult cases of DUOX2 mutation. The role of DUOX2 gene mutation in CH was reported by Moreno et al. in 2002 for the first time.[18] Since then, several studies indicated different mutations of DUOX2 gene to be responsible for transient and permanent CH. Overall, 20 mutations have been reported from which 11 are located in the first long intracellular loop, 8 in the extracellular peroxidase-like domain and one in the second intracellular loop. The types of identified mutations include missense, nonsense, frame shift and splice-site mutations.[23]

It has been proposed that biallelic and monoallelic mutations of the gene cause permanent and transient CH, respectively. However, some studies have also reported biallelic mutations of DUOX2 gene among transient CH cases, which suggests the presence of an alternative mechanism for producing H2O2 in complete defect of DUOX2 gene. Familial cases of CH with heterozygous mutations of DUOX2 have also been reported.[18,19]

In addition, an identical DUOX2 mutation could be present with a high intra- and inter-familial phenotypic variability. Vigone et al have reported that two siblings with the same identical DUOX2 mutation had different clinical phenotypes.[24] This variability of the DUOX2 phenotype could be explained by some hypotheses such as the existence of other H202 generating systems, the different requirements for thyroid hormones synthesis according to age, the ethnicity and the intake of iodine.[24,25] Genetic studies have showed some factors as DUOX2 maturation factors which enhance the endoplasmic reticulum (ER)-to-Golgi transition, maturation and surface expression of functional DUOX2 in a heterologous cell system. In this regard, three natural missense mutations including Q36H, R376W, and D506N have been reported. They were also evaluated in the current study. These mutations cause partial (D506N) or complete (Q36H and R376W) loss of H2O2 generating system activity which consequently results in iodine organification defect. The defects caused by these mutations are made by trafficking defects of the mutant proteins resulting in complete retention in the ER due to Q36H and R376W mutations or reduced plasma membrane translocation due to D506N mutation.[26]

Considering the key role of the mentioned mutations in the synthesis of thyroid hormone and iodine organification process, we investigated the role of these three mutations of the peroxidase-like domain. The mentioned three mutations have been identified during the CH screening programs. For the first time, the mutation of R376W, Q36H and D506N were reported by Vigone et al in 2005,[24] Varela et al in 2006,[27] and Pfarr et al in 2006,[28] respectively. Afterwards, Grasberger et al performed a functional study and analyzed the three mutations in Cos-7

Table-II: The PCR cycling conditions for determining the Q36H mutation.

Cycle	*Cycle Point*
Hold @ 95°c, 6 min 0 secs	
Cycling (45 repeats)	Step 1 @ 95°c, hold 12 secs Step 2 @ 55°c, hold 31 secs Step 3 @ 72°c, hold 16 secs,
Melt (60-90°c), hold secs on the 1st step, hold 2 secs on next steps, Melt A([HRM]	

Table-III: The PCR cycling conditions for determining the R376W mutation.

Cycle	*Cycle Point*
Hold @ 95°c, 5 min 0 secs	
Cycling (45 repeats)	Step 1 @ 95°c, hold 11 secs Step 2 @ 59°c, hold 24 secs Step 3 @ 72°c, hold 15 secs,
Melt (70-95°c), hold secs on the 1st step, hold 2 secs on next steps, Melt A([HRM]	

Table-IV: The PCR cycling conditions for determining the D506N mutation.

Cycle	*Cycle Point*
Hold @ 95°c, 5 min 0 secs	
Cycling (45 repeats)	Step 1 @ 95°c, hold 11 secs Step 2 @ 59°c, hold 24 secs Step 3 @ 72°c, hold 15 secs,
Melt (70-95°c), hold secs on the 1st step, hold 2 secs on next steps, Melt A([HRM]	

cells. According to their findings, due to complete inhibition of the transition of DUOX from the ER to the plasma membrane by Q36H and R376W mutations or partial (D506N) inhibition of H2O2-generating system activity by these mutations, these DUOX2 mutants were retained within the ER. They suggested that post-transitional processing of the peroxidase-like domain had an important role in the exit of DUOX2 from ER.[26]

We did not find any mutation of the mentioned three mutations (Q36H, R376W, and D506N) of DUOX2 gene among either group of transient or permanent CH patients in the current study. The most important explanation for this finding could be the ethnical variation in the genes involved in thyroid hormonogenesis as reported by other studies.[29] Mutations in other genes involved in the pathogenesis of transient and permanent CH due to dyshormonogenesis, such as NIS gene, pendrin gene (PDS) and thyroglobulin gene, may have had a role in our studied population. However, such hypothesis needs further investigations.

On the other hand, previous studies have indicated that some factors such as age, ethnicity, and iodine intake could explain the different phenotype variations of an identical DUOX2 gene mutation. The mentioned factors could also have a role in different mutations of thyroid hormone synthesis. The sample size was enough for the purpose of the study and so it could not be considered as a limiting factor in this field.

Our finding could also be explained by different factors related to the method of the genetic study. In the current study, only the exonic parts of the genes were analyzed. However, mutations in intronic sequences or in the promoter region and unexamined regulatory regions of the three studied genes could have been the causes of thyroid dyshormonogenesis or transient CH in these patients.[30]

Another explanation is that although the method used in this study has a high sensitivity, it is highly dependent on laboratory conditions. Therefore, some mutations with slight impacts on changes in single-strand confirmation could not be detected by this method. Thus, some existing mutations might have remained unidentified with SSCP.[31]

The limitation of this research was that we did not determine iodine organification defects in the studied patients due to insufficient facilities to perform perchlorate discharge test. In addition, it is necessary to examine other identified mutations of DUOX2 gene in the studied population.

In conclusion, the results of this study indicated that the mutations of the peroxidase-like domain of DUOX2 did not have a role in the pathogenesis of transient and permanent CH due to dyshormonogenesis in this population. However, for more conclusive results, further studies using another screening method besides SSCP, and screening of intronic mutations and other identified DUOX2 gene mutations on the other sites of the gene are recommended. Investigation of other gene mutations responsible for thyroid dyshormonogenesis and transient CH such as pendrin, NIS and thyroglobulin is recommended, too.

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Mohammad Hassan Tajaddini, Shaghayegh Haghjooy; Design, literature search, experimental and laboratory studies, data collection, manuscript preparation, editing and review.

Awat Feizi; Design, literature search, data analysis, statistical analysis, manuscript preparation, editing and review.

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