

Genetic analysis of TTF2 gene in congenital hypothyroid infants with thyroid dysgenesis

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ABSTRACT

Thyroid transcription factor 2 (TTF2) also known as FOXE1 is one of the candidate genes thought to have been involved in thyroid development. Impairment in this gene has been reported in a few cases of patients with congenital hypothyroidism resulting from thyroid dysgenesis (TD). In this study we analyzed the entire coding-region of TTF2 genes in 50 infants who were referred to the Endocrine and Metabolism Research Center of Isfahan University of Medical Sciences TD patients by direct sequencing. The analysis revealed a known polymorphism in ser 273 (TCC.TCT) in 74% unrelated patients. Furthermore, we found that the length of the alanine tract of TTF2 was 14 in some of our TD patients. This data may point to a role of the TTF2 polyA tract length in modulating genetic susceptibility to TD.

KEY WORDS: Thyroid transcription factor 2, Thyroid dysgenesis.

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INTRODUCTION

Congenital hypothyroidism (CH) is the most frequent neonatal endocrine disease that causes irreversible mental impairments if therapeutic intervention is delayed.¹ CH occurs with rate of about 1/3000-4000 newborns in North America and Europe.² However data through neonatal screening programs has revealed high incidence about 1/974 in Tehran and 1/357 in Isfahan

provinces of Iran.³⁻⁵ In about 85% of the cases, CH is caused by an alteration in the morphogenesis of the thyroid which is called thyroid dysgenesis, TD.⁶ These anomalies including thyroid (hemi) agenesis (the thyroid gland is absent so called 'athyrosis' and 'hemithyroidoea'), ectopic thyroid tissue (the thyroid gland is abnormally located in a sublingual position so called Thyroid ectopy) and thyroid hypoplasia (the thyroid gland is severely reduced in size resulted from remnants of thyroid tissue in the normal position).^{6,7} Some cases of dysgenesis are now discovered to be the result of mutations in at least three transcription factors (TTF1, TTF2 and PAX8).⁸ Thyroid transcription factor 2 (TTF2) also known as FOXE1 (Forkhead box E1), belongs to the forkhead gene family of transcription factors that regulates embryonic development and plays a crucial role in thyroid morphogenesis.^{9,10} The winged helix/forkhead gene family is characterized by a distinct conserved 100-amino acid DNA-binding motif called the 'forkhead domain'.¹¹ TTF2 is an intronless gene located on chromosome 9q22.

TTF2 gene encodes for a phosphoprotein contains a polyalanine tract with 11 to 19 alanine residues, 2 putative nuclear localization signals which flank

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the forkhead domain and unique C-terminal residues.^{3,6,10,12-14} This protein has a specific role in controlling the migration of thyroid follicular cell (TFC) precursors.¹⁵ TTF2 might act as a promoter-specific transcriptional repressor of TG and TPO gene in early stage of thyroid development.^{16,17} In current survey we aimed to screen the presence of mutations in TTF2 gene in patients with thyroid dysgenesis.

METHODOLOGY

Study Group: In all, 50 TD families including 48 families with one member affected and two families with two members affected were analyzed in this survey. Families were identified through neonatal screening program of CH in Endocrine and Metabolism Research Center of Isfahan University of Medical Sciences during August 2002 to April 2005. Our study group included 13 CH patients with ectopic and 37 patients with agenesis. Infants with the absence of thyroid in [Tc]-99m Thyroid Scintigraphy had significantly higher TSH value in comparison with those with ectopic TD (116.3_109.64 vs. 108.10_62.92 mIU/l, $P < 0.0001$) (Reference range 0.6–20 mIU/l).

The mean T4 level was 6.36_5.57 and 5.04_3 ug/dl in ectopic and athyrotic respectively (Reference range 6.5–15.0 ug/dl). Two patients with athyreosis suffered from ear disability. Affected individual did not manifest any clinical characterization of hypothyroidism like delay in growth and mental retardation as they had been treated soon after birth.

Sampling: Blood samples were collected from the patients and family members in EDTA supplemented vacutainers. Informed consent was obtained from parents or guardian of patients.

DNA Extraction: Genomic DNA from each individual was obtained using a QIAamp Blood Kit (QIAGEN) according to the manufacturer's instructions. The final concentration of extracted DNA was adjusted to a 50 ng/μl to develop the assay.

Mutational Analysis: Mutations in TTF2 were searched by the polymerase chain reaction (PCR)

followed by direct sequencing. Mutational analysis was performed for the single exon of TTF2 with primers flanking the entire coding region.

Two pairs of primer producing overlapped fragments were employed to amplify the entire coding region of TTF2 gene (Fig.1). Avoiding the formation of primer dimers and hairpins as well as low value for the Gibbs' free energy of 3' pentamer was confirmed by GeneRunner version 3.01 (copyright © hastings softwar Inc.) for selected primers.¹⁸ The primer sequences and the expected length for each amplicon were as follows:

F1: 5'-ctgagctctccgagaagg-3' and **R1:** 5'-cgcggggtagtagactggag-3' for amplifying the first segment with 754bp (nucleotides 552-1305, GenBank accession number NC_000009.11).

F2: 5'-cgcgctctatgcaggctac-3' and **R2:** 5'-gaacgtgtgaacagccgatg-3' for amplifying the last segment with 590bp (nucleotides 1242-1831, GenBank accession number NC_000009.11).

PCR reactions for both segments were prepared in a 25 μl total volume contain 0.4 μmol/l of each primer, 0.2 μmol/l of each dNTPs, 0.625 U Taq polymerase (Sinagen, Iran), 5mM MgCl₂, and 1 x PCR buffer (Sinagen, Iran), 200ng of genomic DNA, separately. Also 1 x Q solution (QIAGEN) was added to facilitate the amplification of GC rich regions in the first segment. PCR was performed in Thirty cycles contain 1 min at 94°C, 1 min at 68°C and 1 min at 72°C followed by a final extension 10 min at 72°C for one cycle using a Techne Thermal Cycler (PE Applied Biosystems, Foster City, CA, USA).

Direct DNA Sequencing: For direct sequencing, PCR products were purified using Roche's High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany). Both forward and reverse strands were sequenced by MWG Biotech AG, MWG Biotech AG.

Nomenclature: Nucleotide sequence refers to GenBank accession number NC_000009 for FOXE1, where A of the ATG, the initiator methionine codon, begins at 661. The polyalanine tract for this

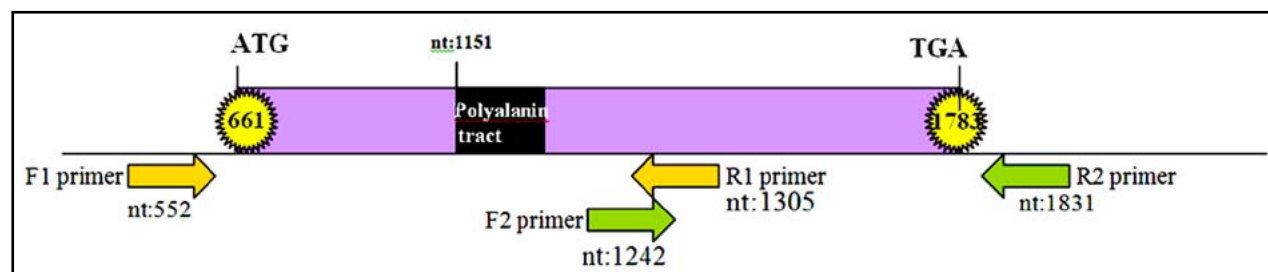


Fig.1: Schematic representation of the TTF-2 gene. The line is the non-coding region and the box is the single coding region. Dark box represents the poly Alanine tract. As are shown the coding region amplified with two pairs of primers producing overlapping fragments.

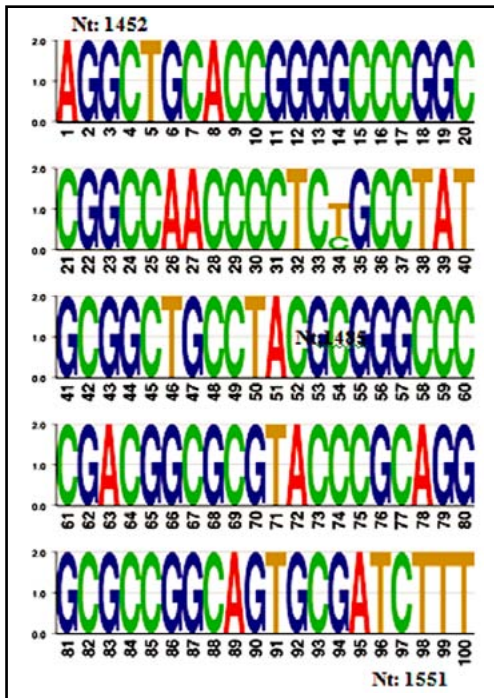


Fig.2: graphical representation nucleic acid multiple sequence alignment of 100 bp of coding region of TTF2. Each logo consists of stacks of symbols, one stack for each position in the sequence. The height of symbols within the stack indicates the relative frequency of each nucleic acid at that position. Existence of TCT in ser 273 (nt:1485) was revealed with more frequency than TCC. GENIO/logo - Sequence logos at Stuttgart/Germany. ExPASy Proteomics tools.

entry contains 16 repeats and spans nucleotides 1150–1197.

Homology Analysis: Nucleotide alignments were performed by tool of BLAST 2 SEQUENCES which use BLAST engine for global alignment of two given sequences available on <http://blast.ncbi.nlm.nih.gov/bl2seq/wblast2.cgi>. Nucleotide sequence refers to GenBank accession number NC_000009 was used as standard entry.

RESULTS

Based on the thyroid phenotype described in TTF-2 mutation carriers, we screened all patients with TD for mutations in the TTF-2 gene. Direct sequencing of coding region of TTF-2 did not revealed any mutation. However presence of a silent polymorphism in ser 273 (TCC.TCT) was detected in 74% unrelated patients (Fig.2). Interestingly the length of the alanine tract of TTF2 was 14 in all of our TD patients (Fig.3).

DISCUSSION

Thyroid dysgenesis is the most common cause of congenital hypothyroidism. Molecular studied has reveal that three genes encoding transcription fac-

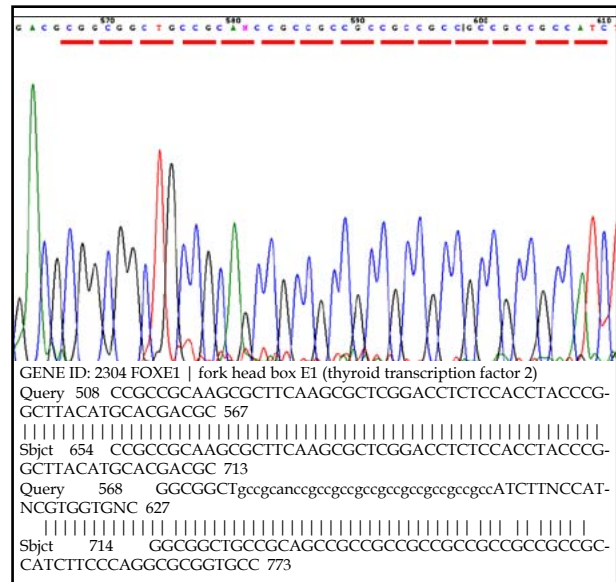


Fig.3: The alanine tract of TTF2 in one of our TD patients.

tors (TTF1, TTF2, and PAX8) are involved in thyroid gland development.⁸ However, in only few TD cases impairment of these genes has been reported.⁸ Clifton-Bligh (1998) identified homozygosity for a missense mutation at nucleotide 196 of TTF2 in two brothers with a syndromic thyroid agenesis.¹⁹ Castanet et al. (2002) described 2 male sibs with congenital hypothyroidism, athyreosis and incomplete syndromic phenotype. They were homozygous for a ser-to-asn (S57N) substitution in the forkhead DNA binding domain of TTF2. The mutant protein showed impaired DNA binding and partial loss of transcriptional function.¹⁸

Huebner (2004) diagnosed CH in a non-identical twin in association with cleft palate, curly hair and low-set posteriorly rotated ears. Screening of TTF2 shows a homozygous substitution of tryptophane by arginine at position 97 (W97R).¹³ Baris (2006) demonstrated an arginine to cysteine mutation (R102C) in TTF-2 gene in cases of syndromic CH without total athyreosis.²⁰

Incidence of CH in Isfahan, Iran, to be one case per 747 newborns which has been accounted about 10 times higher than that reported from America and Europe.⁴ In this study, we screened the TTF2 gene in an attempt to determine the underlying genetic cause of congenital hypothyroidism in TD patients from Isfahan, Iran based on thyroid dysgenesis phenotype. Direct sequencing of the entire coding region of the TTF-2 gene revealed only one sequence variation as a silent polymorphism (ser 273 (TCC.TCT)). This polymorphism has been reported previously by Paolo E. Macchia.¹⁴ We detected this polymorphism in 74% Iranian TD patients.

Our data confirm that the defects in the candidate gene "TTF2" are rare. This finding is well-suited with results of previous mutational analysis of TTF2 (21-23). Also, in recent years expansion of trinucleotide repeats encoding polyA tracts have been reported to be the cause of some disease through the alanine containing proteins as TTF2.²⁴ It is suggested that polyA disorders result in early developmental and cause malformation. It is interesting to know that the polymorphism of the polyA varies from 11 to 16 residues with the 14 as the most frequent allele in TD patients, while it is for example 12-19 repeats in premature ovarian failure.^{12,25}

Carre et al (2007) found that the presence of 16 alanines either at heterozygote state (i.e., 14/16 genotype) or at homozygote state (i.e., 16/16 genotype) significantly protects from the occurrence of TD in comparison with the 14/14 genotype. They suggested that the length of the alanine stretch within TTF2 modulates genetic susceptibility to TD.²⁶ Interestingly this survey revealed that the length of the alanine tract of TTF2 was 14 in all of our TD patients whose DNA was sequenced. Further investigation to see whether or not the length of the alanine stretch within TTF2 modulates genetic susceptibility to TD in our patients is strongly recommended. In summary, we reveal no mutation in TTF2 in our CH TD patients. This was not very surprising since despite the numerous cases of TD patients, only handful number of cases had mutations in TTF2 gene. We suggest detailed investigation of other genes involved in thyroid development. In Addition, genetic susceptibility to TD may lack the simple Mendelian pattern of inheritance as pointed by Castanet (2005).⁸ These observations can prove the role of epigenetic modifications in TD as suggested by Tonacchera.²³

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Note: The first two authors have equally contributed to this work.