

# Mutations in the gene encoding paired box domain (PAX8) are not a frequent cause of congenital hypothyroidism (CH) in Iranian patients with thyroid dysgenesis

As mutações no gene PAX8 não constituem uma causa frequente de hipotireoidismo congênito em pacientes iranianos com disgenesia tireoidiana

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## ABSTRACT

**Objective:** Congenital hypothyroidism (CH) may be caused by defects in the thyroid or in one of the stages in the synthesis of thyroid hormones. Thyroid dysgenesis may be associated with mutation in the paired box transcription factor 8 (*PAX8*) gene. We attempted to screen *PAX8* gene mutation in 50 CH patients with thyroid dysgenesis. **Subjects and methods:** The patients were classified in two groups as agenesis and ectopic based on biochemical and para clinical tests. By employing PCR, Single Strand Conformation Polymorphism (SSCP) and sequencing, exons 3 to 12 of *PAX8* gene with their exon-intron boundaries were studied. **Results:** No mutation was found in these patients in any of the exons. **Conclusion:** Our results, once again, indicate that the *PAX8* mutation rate is very low and can only explain a minority of the cases. Therefore, it is highly needed to further investigate the genes controlling development and function of thyroid. *Arq Bras Endocrinol Metab.* 2010;54(6):555-9

## Keywords

Congenital hypothyroidism; thyroid dysgenesis; *PAX8*; gene mutation

## RESUMO

**Objetivo:** O hipotireoidismo congênito (HC) pode ser causado por defeitos na formação da tireoide ou em uma das etapas da síntese dos hormônios tireoidianos. A disgenesia da tireoide pode ser associada a mutações no fator de transcrição *PAX8*. Neste estudo, foram rastreadas mutações no gene *PAX8* em 50 pacientes com CH com disgenesia da tireoide. **Sujeitos e métodos:** Os pacientes foram classificados em dois grupos, com agenésia ou com ectopia, segundo os testes bioquímicos e paraclínicos. Foram empregadas as técnicas de SSCP (Single Strand Conformation Polymorphism) e sequenciamento para analisar os éxons 3 a 12 do gene *PAX8* e suas bordas éxon-intron. **Resultados:** Nenhuma mutação foi encontrada nesses pacientes, em qualquer um dos éxons. **Conclusão:** Nossos resultados, mais uma vez, indicam que a taxa de mutação *PAX8* é muito baixa e só pode explicar a minoria dos casos. Portanto, é altamente necessário investigar outros genes que controlam o desenvolvimento e as funções tireoideanas. *Arq Bras Endocrinol Metab.* 2010;54(6):555-9

## Descritores

Hipotireoidismo congênito; disgenesia da tireoide; *PAX8*; mutação genética

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## INTRODUCTION

Permanent congenital hypothyroidism is a common disease with the prevalence of 1 of 3,000-4,000 newborns worldwide (1). This prevalence has been represented to be much higher in Iran compared to the world wide report (1 in 914 and 1 in 748 newborns in Tehran and Isfahan, respectively) (1,2).

Congenital hypothyroidism can be caused by either a defect in thyroid development (thyroid dysgenesis, in 85% of cases) or inborn errors of thyroid hormone biosynthesis (dyshormonogenesis in 15% of CH patients). The main group of CH patients with thyroid dysgenesis is also divided in 3 groups with different rates of occurrence. Thyroid dysgenesis can be due to agenesis (in 40% of cases), ectopic (in 40% of cases) or hypoplasia (in 5% of cases) (3).

In a few patients with thyroid dysgenesis, some mutations in transcription factors, such as transcription factor-1 (*TTF1*) (4-9); transcription factor-2 (*TTF2*) (10-12); paired box transcription factor-8 (*PAX8*) (1,13-21); or TSH receptor genes have been reported (22-23).

*PAX8* is a member of a family of transcription factors characterized with recognizing specific DNA sequence through highly conserved 128- amino acids, called paired box domain (7,24). It has a key role in mammalian embryogenesis and it is expressed in adult thyroid as a transcription factor for TPO, TG and NIS (2-27). The *PAX8* gene is located on chromosome 2q12-q14 and contains a 4-kb transcript sequence divided into 12 exons (28-30).

To date, 15 mutations have been identified in 5 exons of *PAX8* (1,13-21). The exon number 3 and 4 which make the paired box domain are the hotspot with 9 mutations recognized. Exon 7 with 3 and exon 9 and 12, each with 1 mutation recognized. In Italy 8 mutations out of 14 have been found (1,13,17,18,20-21), 3 in French (13,15-16,18-19) and 1 in each Japanese (15), German (19) and American patients with thyroid dysgenesis (14).

In the current study, we aimed to screen the presence of mutations in the *PAX8* gene in patients with thyroid dysgenesis employing PCR-SSCP and sequencing.

## SUBJECTS AND METHODS

### Patients

One hundred fifty samples, including 50 CH patients caused by thyroid dysgenesis (13 ectopic, 47 agenesis)

and their parents were collected from Isfahan Endocrine and Metabolism Research

Center of Isfahan University. On 7th-28th days of birth, neonates were considered as hypothyroid if T4 was < 6.5 ug/dL and TSH was > 10 mIU/L.

The study was approved by the Research and Ethics Committee of National Institute of Genetic Engineering and Biotechnology, Iran (NIGEB).

Written informed consents were obtained from the parents of the patients.

### PCR SSCP analysis

SSCP was used to screen the presence of mutations in each of the exons of *PAX8* gene (exons 3-12). DIAtom DNA Prep 100 kit (Isogen Laboratory, Russia) was used to extract Genomic DNA from peripheral blood. Exons 3 to 12 with their flanking intronic regions were amplified in a 25 µL reaction using some of the primers shown in table 1. PCR was performed in 25 µL, using 200 ng of genomic DNA, 0.2 µM of each deoxy (d)-NTP (dATP, dCTP, dTTP, dGTP), 1.5 mM MgCl<sub>2</sub>, 1X buffer, 1.5 U Taq polymerase (Cynagen, Tehran, Iran) and 5 pmol of each forward and reverse primer. Samples were denatured at 94 °C for 3 minutes followed by 35 cycles of amplification. Each cycle consisted of denaturation at 95 °C for 40 seconds, primer specific annealing temperature for 40 seconds, and primer extension at 72 °C for 50 seconds. After the last cycle, the samples were incubated for an additional 6 minutes at 72 °C to ensure that the final extension step was complete. The amplified products were analyzed in 2% agarose gel.

PCR products were diluted 1 time in a buffer containing 95% formamide, 100 mM NaOH, 0.25% xylene cyanol, and 0.25% bromophenol blue and denatured at 95 °C for 10 minutes and cooled down on ice and loaded on non-denaturing gel containing 8% acrylamide/bis-acrylamide (39:1), 5% glycerol and 0.5X TBE. The gel was electrophoresed at 4 °C with constant power of 200W for 20 h. DNA was visualized by silver staining.

### Direct DNA sequencing

Samples showing aberrant pattern and few samples of normal pattern in SSCP analysis were directly sequenced. The results were analyzed using the Chromas and Sequencher and Nucleotide BLAST software programs.

**Table 1.** List of primers with their specific size and annealing temperature

Exon no	Forward primer	Reverse prime	Annealing temperature	Size of PCR product	Ref. n <sup>o</sup>
3	5'-CATAGCTAATCCCACCCAAAC-3'	5'-GCCTGCGGTGAATTCGT-3'	57 °C	259 bp	13
4	5'-ATTGGGTAATTCTTTGGGATTC-3'	5'-CCAGGCCCTTTGTCTCTT-3'	55 °C	314 bp	13
5	5'-AGGGGTGTCAAAAAGCGGACTG-3'	5'-TGGGTATGCTGGGGAGGTG-3'	61 °C	241 bp	13
6	5'-TCTCCCTCTCCCCACTG-3'	5'-GCAGAGCCCTACAAAGTCC-3'	58 °C	255 bp	13
7	5'-GAGCATGAATGATAGTCCC-3'	5'-CACAGGCTCATTGGAGAAT-3'	58 °C	304 bp	13
8	5'-GTCTCTGTGCGCTGACTTCT-3'	5'-CACACCTCCGCCTGAC-3'	58 °C	232 bp	13
9	5'-CCTCCCCGCCATCTCACACC-3'	5'-TCCACCCGCCCATAG-3'	61 °C	290 bp	13
10	5'-CCCCCATGGTCCAACACTGAC-3'	5'-CCTCTTGCTCCTTGTGTCCCAC-3'	62 °C	220 bp	Self communication with Dr. Sebastian Esperante
11	5'-TGATTGATGCCCTTCACCTCA-3'	5'-AGGTAACCTTTGACCCACCTT-3'	59 °C	200 bp	
12	5'-AAAGTGCAGCAGATGCAGGGAA-3'	5'-CGCAATGCTGGACTTTGTGGTTA-3'	63 °C	274 bp	

## RESULTS

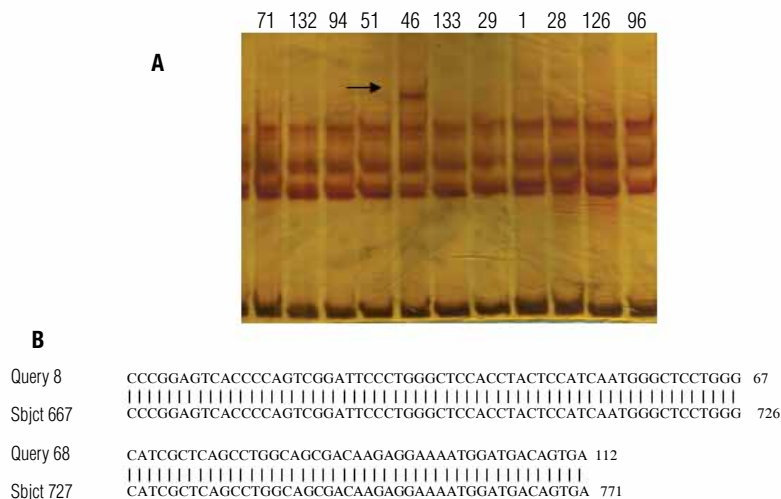
### SSCP analysis and direct sequencing of *PAX8*

Exons 3 to 12 of *PAX8* from all 50 unrelated CH patients were successfully amplified with PCR and screened by SSCP followed by direct sequencing of suspicious ones and a few samples with normal patterns. Our study group included 13 CH patients with ectopic and 37 patients with agenesis. Fourteen samples for exon 3, fifteen samples for exon 4, two for exon 5, seven for exon 6, eleven for exon 7, five for exon 8, twelve for exon 9, six for exon 10, six for exon 11 and five for exon 12 were selected to be sent for sequencing. SSCP analysis of some PCR products showed different banding patterns (an example is illustrated in Figure 1).

The resulting sequences were compared with the sequences of the most complete and the longest *PAX8* mRNA (GenBank accession n<sup>o</sup> NM\_003466). No mutation or polymorphism was found in any of the samples.

## DISCUSSION

In this study, the entire coding regions of *PAX8* of 50 unrelated CH patients were analyzed by SSCP followed by direct sequencing. The patients were composed by two groups (ectopic and agenesis). CH patients with ectopic and agenesis were 13 (26%) and 32 (74%), respectively. No mutation in *PAX8* gene was found among any of these patients.



**Figure 1.** SSCP analysis and direct sequencing. A) An example of aberrant band found in SSCP analysis of one of the exons (in this case exon n<sup>o</sup> 6). The arrowhead indicates the abnormal band in patient 46. B) The direct sequencing of the same aberrant sample (exon n<sup>o</sup> 6 patient 46) showed that it matches the wild type 100%.

In only 5% of CH patients a mutation in one of the transcription factors (*TTF1*, *TTF2*, *PAX8*) or the TSH receptor was found to be the cause of thyroid dysgenesis. This means that there is almost 1% of chance for each gene. This indicates that the defect in the *TTF1*/*TTF2*/TSH receptor or even other unknown gene(s) can be responsible for thyroid dysgenesis in this group of patients.

Reviewing literature revealed that 60% of the *PAX8* mutation in this gene were found in Italian patients (1,17,20-21), 20% in French (13,16-18), almost 7% in US (14), 7% In Germany (19) and 7% in Japan (15).

Only 9 out of 302 (3%) CH patients studied in Italy had a mutation in the *PAX8* gene. In France 5% (3/57), in US 25% (1/4) and in Germany 0.6% (1/170) had mutation in *PAX8*.

Sixty four percent (9/14) of the reported *PAX8* mutations were found in the paired box domain causing reduction of the DNA binding activity of this gene (1,13-21). About 21.4% (3/14) of the *PAX8* mutations were in exon 7 that codes a residual paired type homeodomain in C-terminal region, which is necessary for transcriptional activity of *PAX8* (17,21). One mutation is found in exon 9 known to have an antagonistic role for the activating domain which can influence the normal thyroid development or transcriptional control of several thyroid specific genes not tested yet (28). A mutation found in exon 12 seems to be synonymous with no change of any amino acids.

Interestingly, although, only 5% of the CH patients with thyroid dysgenesis have thyroid hyperplasia (1) but, most of the mutation found in *PAX8* (64%) are from this group of patients.

Among 14 mutations recognized in *PAX8*, 10 mutations are inactivating (71%) and all of them are located in the paired box domain except one which is in exon 7 (30,31). Two mutations (14%) are synonymous which are showing the wild type or comparable protein as the wild type. These two mutations are located in C-terminal region and it is possible to influence the normal thyroid development or the transcriptional control of several thyroid specific genes. Two mutations (14%) are nonsynonymous substitutions that might have inhibitory role on an unknown particular function.

This result, once again, indicates the very low rate mutation in *PAX8* gene in CH patients

The result presented in this report shows that no mutation in *PAX8* gene is responsible for the thyroid

dysgenesis in this cohort of Iranian CH patients. All these data, once again, highlights the need for further study in higher level to find the cause of CH in other genes.

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