FUNCTIONAL STUDIES OF *PAX8* GENE VARIANTS IN PATIENTS AFFECTED BY CONGENITAL HYPOTHYROIDISM WITH EUTOPIC THYROID GLAND

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INTRODUCTION

Thyroid dyshormonogenesis is a heterogeneous group of hereditary diseases produced by a total/partial blockage of the biochemical processes of thyroid-hormone synthesis and secretion. PAX8 is a transcription factor essential for thyroid morphogenesis and thyroid hormone synthesis. More than 50 *PAX8* variants were reported, but most not functionally tested. We identified 8 *PAX8* variants in 9 patients with congenital hypothyroidism (CH).

AIM

We aimed to determine if these *PAX8* variants are pathogenic with *in-vitro* functional studies.

PATIENTS

Patients: 3 girls and 6 boys with CH, eutopic thyroid gland and a detected ***PAX8* variant**. Catalan CH Neonatal Screening Programme (N=93).

Patient (Sex, Ethnicity)	Screening TSH	Thyroid funtion		Thyroid scintigraphy	Thyroid ultrasound	Diagnostic reevaluation		Final diagnosis	*PAX8* genetic variants (2) (zygosity)
		Age (d)	Hormone values (1)	Uptake	Thyroid size/localisation	Hormone values (1)	Perclorate test (%)		
CH-1 (F, Ca)	166	30	TSH=375; fT4 (NA); T4=2,2; TG=300	NA	Normal / normal	TSH=176; fT4=0,7	20	severe permanent CH	c.177C>A p.(Ser59Arg) (Het)
CH-2 (M, Ca)	15,9	48	TSH=42; fT4=1; TG (NA)	decreased	normal (S) / normal	TSH=231; fT4=0,16	0	severe permanent CH	**c.196_198delTAC p.(Tyr66del)** (Het)
CH-3 (F, Ca)	16	16	TSH=14; fT4=1,66; T4=16; TG=94,8	very decreased	Normal / normal	TSH=11,2 ; fT4=1,25	0	mild permanent CH	**c.208A>G p.(Ser70Gly)** (Het)
CH-4 (M, Ca)	185	15	TSH=359; fT4<0,3; TG (NA)	normal	Normal (S) / NA	TSH=50,6 ; fT4=0,92	0	mild permanent CH	**c.396_397delCCinsTT p.(Arg133Trp)** (Het)
CH-5 (M Ca)	20	13	TSH=5,3; fT4=1,4; TG=157	decreased	Normal / normal	TSH=15,4 ; fT4=1	20	mild permanent CH	c.397C>T p.(Arg133Trp)
CH-6 (M, Ca)	30	18	TSH=13,6; fT4=1,24; TG=121	no	Normal / normal	TSH=11,2 ; fT4=1,2	0	mild permanent CH	c.398G>A p.(Arg133Gln) (Het)
CH-7 (M, Ca)	110	17	TSH=375; fT4=0,3; TG (NA)	normal	NA / normal	TSH=8; fT4=1,5	NA	mild permanent CH	c.398G>A p.(Arg133Gln) (Het)
CH-8 (F, Af)	100	7	TSH=21,4; fT4=0,99; TG=493	normal, (normal morphology)	hipoplastic / normal	TSH=6,2; fT4=1,6	0	mild permanent CH	**c.1276+1G>A** (Hom)
CH-9 (M, Af)	200	15	TSH>750; fT4=0,22; TG=3247	normal	normal (S) /normal	NA	NA	NA	**c.1334C>T p.(Thr445Met)** (Het)

(1) Refence biochemical values (units): TSH<5 (mU/L); fT4=0,9-1,8 (ng/dL); T4=0,74-1,44 (µg/dL); TG=0-112 (ng/mL). (2) HGVS code (NM_003466.4; NP_003457.1). **In bold:** novel variant. F: female. M: male. Ca: Caucasian. Af: North African. d: days. NA: not analysed/not available data. (S): evaluated in scintigraphy.

METHODS

***In silico analysis*:**

- Alignment of human and 9-orthologue PAX8 peptide sequences (NCBI database) by Clustal Omega webtool (EMBL-EBI, Hinxton, UK).
- Construction of PAX8 domains from literature.
- *In silico* prediction studies with SpliceSiteFinder-like, MaxEntScan and NNSPLICE.

***In vitro* analysis:** Study of PAX8 transactivational activity on *TG* promoter

1. Production of PAX8 and *TG* promoter vector

Expression Vector **wt**-*PAX8*
Site-directed mutagenesis
Expression vector **mut**-*PAX8* (mutant)

***TG* promoter** (fused to luciferase gene)
TG Promoter /Luc *Reporter*

2. Study of PAX8 transactivation of *TG* promoter

Cell cultures (HEK293)

Experiments - Transfections

Day 0 – Cells seeded in well-plates
Day 1 – Vectors transiently co-transfected in cells, *6h*
A) Study of variants in homozygosis:
WT-PAX8 and 7 mut-PAX8 + TG-pGL3
A) B) Study of variants in heterozygosis:
WT-PAX8 and 5 mut-PAX8 + TG-pGL3

Incubation, *48h*
Day 3 – Cells collected

Dual Luciferase Reporter Assay:
Lysis of cells
Measurement of light emission
Data analysis

Statistical analysis

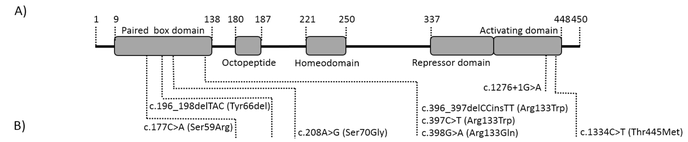
RESULTS

***In silico analysis*:**

Alignments: All 6 affected amino acids in the paired-box domain are well conserved within species, whereas the amino acid in the activating domain is less conserved.

***In silico* analysis of *PAX8* variants in our patient.**
(A) Transcription factor PAX8A (NP_003457.1), its functional domains and localization of genetic variants.
(B) **Alignment of PAX8 peptidic sequences** and localization of detected variants (Clustal Omega, EMBL).
Dotted lines indicate location of the *PAX8* variants in the peptide sequences whereas numbers indicate their position.

A)



B)

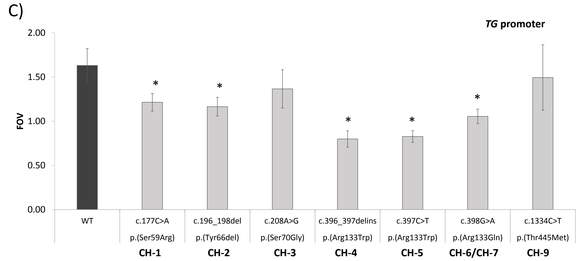
***In silico* prediction:**

In silico prediction studies (SpliceSiteFinder-like, MaxEntScan, NNSPLICE) of the splicing variant c.1276+1G>A predicted a loss of the canonical donor splicing position at intron IVS11 that may modify the peptide sequence.

***In vitro analysis*: Study PAX8 transactivation of *TG*-promoter**

C) Effect of the 7 exonic variants
D) Effect of the variants in heterozygosis: dominant-negative effect analysis ("patient mimicking")

C)

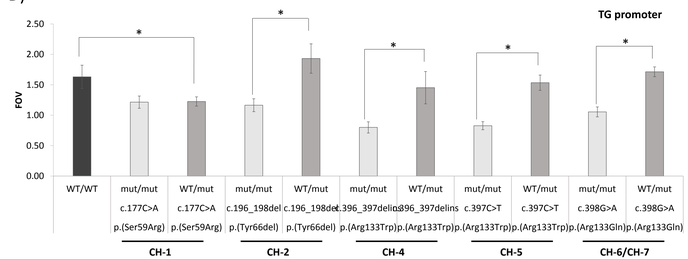


Five variants showed and impaired transactivational activity of *TG* promoter. Therefore, they were pathogenic and caused CH in 6 patients (CH-1, CH-2, CH-5, CH-5, CH-6 and CH-7).

Variants predicting the same amino acid change (c.396_397delCCinsTT and c.397C>T) gave similar results (patients CH-4 and CH-5).

C) *In vitro* analysis of PAX8 transactivation of *TG* promoter: PAX8-WT *versus* 7 PAX8 mutants.
Results expressed as fold-over vector (FOV) expressing the mean and SEM of 5-8 independent experiments (*P≤0.05, mutant *versus* WT).

D)



Variant in patient CH-1 was the only variant showing a dominant-negative effect.

D) *In vitro* analysis of effect of heterozygote variants.
Results expressed as fold-over vector (FOV) expressing the mean and SEM of 3-4 independent experiments (*P≤0.05).

CONCLUSIONS

Nine CH patients, with eutopic thyroid gland, presented *PAX8* candidate variants. *PAX8* functional studies have shown that six *PAX8* variants are deleterious. Our studies have proven their effectiveness in evaluating these variants.

REFERENCE

Camats *et al.*, Phenotypic variability of patients with *PAX8* variants presenting congenital hypothyroidism and eutopic thyroidJ Clin Endocrinol Metab 2021 Jan 1;106(1):e152-e170. doi: 10.1210/clinem/dgaa711.

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