Increased Detection Rate of Paired Box Domain Gene Mutations by Application of Multiplex Ligation-Dependent Probe Amplification Analysis in Patients with Primary Congenital Hypothyroidism and Thyroid Dysgenesis

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Financial support: The study was partially supported by the Fritz-Thyssen-Stiftung and MAIFOR
Disclosure: none

Context: The contribution of mutations in paired box domain (*PAX8*) gene in children with congenital hypothyroidism (CH) and thyroid dysgenesis (TD) still remains a subject of interest of researchers. While quantitative PCR and direct sequencing concentrate on single gene fragment analysis and identification of point mutations, Multiplex Ligation-Dependent Probe Amplification (MLPA) analysis might improve the detection rate of *PAX8* mutations in patients with congenital CH caused by TD.

Objective
The aim of the study was to determine if MLPA could improve the detection rate of *PAX8* gene mutations in patients with CH and TD.

Patients
The study included 45 children (32 girls, 13 boys) with CH associated with: thyroid ectopy (n=17), agenesis (n=9), hypoplasia (n=1), or thyroid dysgenesis of unknown cause (n=18). The study participants were born in south-eastern Poland in the years 1993-2009 and selected in neonatal mass screening for CH.
CH was confirmed, managed and followed-up by Outpatient Department of Endocrinology, University Children's Hospital of Cracow. In two of the 48 patients (4%) with TD, coexistent congenital diseases were detected.

Methods
Blood samples (2ml) were drawn from all the study participants. Subsequently, DNA was extracted from peripheral blood samples with the use of Master Pure DNA Purification Kit (Epicentre Biotechnologies). DNA samples were used in two types of genetic analysis for the presence of *PAX8* gene variants: Sanger sequencing method (promoter region and 12 exons with their exon-intron boundaries were sequenced) and MLPA technique (SALSA MLPA kit P319-A1 THYROID). Additionaly functional studies on HeLa cells were performed to assess the *in vitro* effect of two newly identified *PAX8* gene variants (p.E234K; p.P409S).

Results
In total, heterozygous PAX8 mutations were detected in 7 out of 45 (15.5%) patients with CH and TD [Table 1, Fig. 1]. Sanger sequencing method revealed PAX8 mutations in 5 out of 45 (11.1%) patients. In two of them (P1 and P2) new heterozygous substitutions (p.E234K and p.409S) with an amino-acid change in the coding sequence were detected. In the remaining three children (P3, P4, P5) a *PAX8* promoter region alteration at position -456C>T was revealed (1).Application of MLPA analysis allowed the identification of a heterozygous deletion of exon 7 of *PAX8* gene in two more patients (4.4%; P6, P7) [Fig. 2] (2). Functional studies showed that p.E234K variant seems to have no pathogenic effect, at least *in vitro*, whereas p.P409S variant might relate to diminished transactivation ability of the TG promoter at lower DNA doses. Both the mutated proteins exhibited normal DNA, binding to their promoter target sequences [Fig. 3, 4].

Table 1. Clinical and biochemical data in patients with detected *PAX8* variants

Patient/ gender	Etiology	TSH [mIU/L] fT4 [pmol/L]	*PAX8* variants
P1. Male	*Thyroid dysgenesis	49.5 no data	p.E234K
P2. Male	*Thyroid dysgenesis	no data	p.P409S
P3. Male	*Thyroid dysgenesis	>100 2.34	-456C>T promoter variant
P4. Female	*Thyroid dysgenesis	>100 2.34	-456C>T promoter variant
P5. Female	*Thyroid dysgensis	88 11.4	-456C>T promoter variant
P6. Female	Thyroid aplasia	96 6.97	Heterozygous del in exon 7
P.7 Female	Thyroid ectopy	>80 7.49	Heterozygous del in exon 7

Normal range: TSH 0.8-9.1 mIU/L, fT4 10-25 pmol/l
* lack of thyroid scintigraphy restricts defining an exact type of abnormality

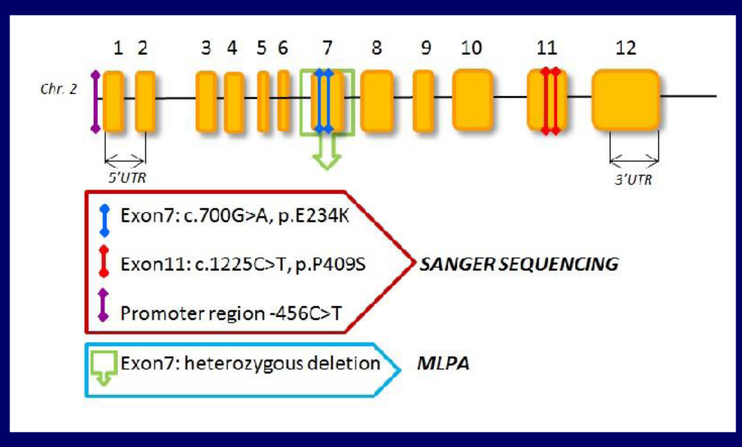

Fig 1. Detected variants of *PAX8* gene.

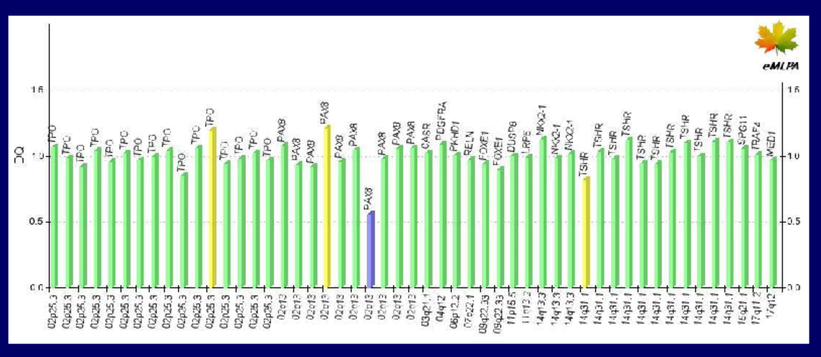

Fig 2. MLPA results: heterozygous deletion of exon 7 of *PAX8* gene (DQ=0.5)

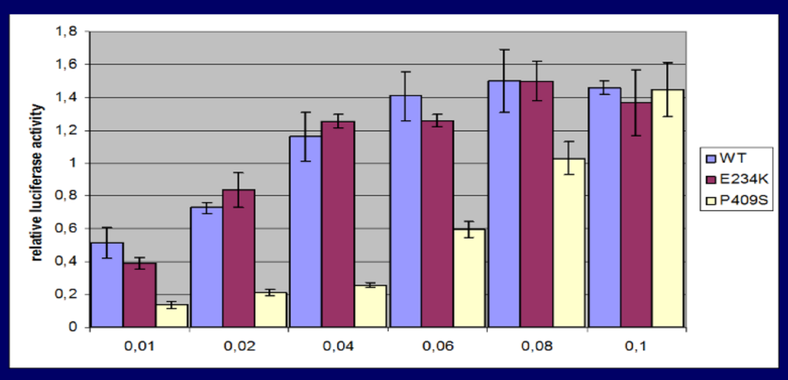

Fig. 3. Test of the ability of the *PAX8* WT and mutants to transactivate the TG promoter.

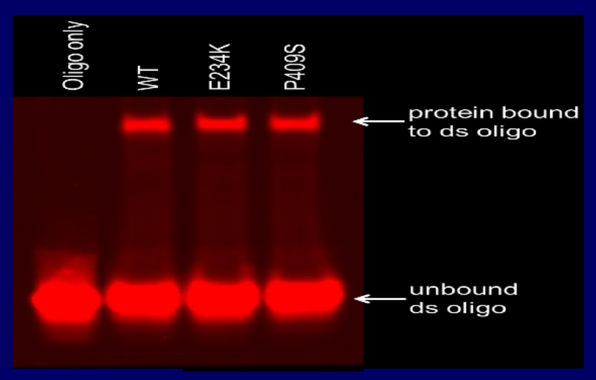

Fig. 4. Electro Mobility Shift Assay of the *PAX8* proteins.

Summary and conclusions
45 patients with congenital hypothyroidism and thyroid dysgenesis were screened for *PAX8* gene mutations by Sanger sequencing method and MLPA technique. In the study MLPA analysis increased *PAX8* mutation detection rate from 11.1% to 15.5%.
Application of MLPA analysis, in addition to direct sequencing, both improves and expands genetic analysis for CH and TD.

Acknowledgements
We appreciate greatly the cooperation of all patients and their families.

References
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ESPE 2015
131--P1
Thyroid
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DOI: 10.3252/pso.eu.54espe.2015
Poster presented at: 54th Annual Meeting 7-9 October ESPE 2015 BARCELONA European Society for Paediatric Endocrinology