Department of Paediatrics
3rd Faculty of Medicine, Charles University, Prague

Academic Dissertation

PATHOPHYSIOLOGY OF PRIMARY CONGENITAL AND EARLY-ONSET NON-AUTOIMMUNE HYPOTHYROIDISM

MUDr. Eva Al Taji

Prague 2009

This academic dissertation was conceived in the frame of postgraduate PhD study in biomedicine, branch "Human Physiology and Pathophysiology" at 3rd Faculty of Medicine, Charles University, Prague.

This work was carried out during years 2001 - 2008 in a close cooperation of the Department of Paediatrics, 3rd Faculty of Medicine, Charles University in Prague with the Institute of Experimental Paediatric Endocrinology, University Children´s Hospital Charité in Berlin.

Author
MUDr. Eva Al Taji
Department of Paediatrics - 3rd Faculty of Medicine, Charles University
Šrobárova 50, 100 34 Prague 10
tel.+420 2 6716 2561, e-mail: evataji@seznam.cz

Dissertation supervisor
Prof. MUDr. Jan Lebl, CSc.
Department of Paediatrics - 2nd Faculty of Medicine, Charles University
V Úvalu 84, 150 06 Prague 5
tel. +420 2 2443 2000, e-mail: jan.lebl@lfmotol.cuni.cz

Dissertation co-supervisor
Assoc. Prof. Dr. Heiko Krude
Institute of Experimental Paediatric Endocrinology
University Children´s Hospital Charité
Augustenburger Platz 1, 13353 Berlin, Germany
tel.+49 30 450 666 039, e-mail: heiko.krude@charite.de

Opponents

To be publicly discussed by permission of the Academic Council of "Human Physiology and Pathophysiology"of Charles University in Prague:

PREFACE

This dissertation focused on genetically determined disorders of thyroid development, growth and function, leading to primary congenital or more rare early-onset non-autoimmune hypothyroidism. When I started this research in 2001, general knowledge of molecular events involved in thyroid development and function was very limited and world-wide only individual patients with monogenic forms of congenital hypothyroidism had been reported. Prior beginning of our study, no systematic population-based phenotype-focused mutation screening had been performed.

I provided myself all the proper steps of this extensive study from general organisation through collecting clinical data and creating a nation-wide DNA bank up to laboratory procedures. I had a unique opportunity to learn all necessary laboratory methods and to perform molecular-genetic studies in almost 200 Czech patients in the Institute of Experimental Paediatric Endocrinology in Berlin. Selected group of four patients with clinical diagnosis of Pendred syndrome was subsequently molecular-genetically studied by Dr. K. Banghová in a close cooperation with me.

Most of the results of this comprehensive study have been already published in peer-reviewed international medical journals, last manuscript is currently under peer-reviewing.

This thesis is based on the following articles:

1. Al Taji E, Biebermann H, Límanová Z, Hníková O, Zikmund J, Dame C, Grüters A, Lebl J, Krude H. Screening for mutations in transcription factors in a Czech cohort of 170 patients with congenital and early-onset hypothyroidism: identification of a novel PAX8 mutation in dominantly inherited early-onset non-autoimmune hypothyroidism. Eur J Endocrinol 2007; 156: 521-529. IF 3.239.
2. Banghová K, Al Taji E, Cinek O, Novotná D, Zapletalová J, Hníková O, Lebl J. Pendred syndrome among patients with congenital hypothyroidism detected by neonatal screening: identification of two novel *PDS/SLC26A4* gene mutations. Eur J Pediatr 2008; 167: 777-783. IF 1.277.
3. Banghová K, Cinek O, Al Taji E, Zapletalová J, Vidura R, Lebl J. Thyroidectomy in a patient with multinodular dyshormonogenetic goitre - a case of Pendred syndrome confirmed by finding mutations in the *PDS/SLC26A4* gene. J Pediatr Endocrinol Metab 2008; 21: 1179-1184. IF 0.858.

4. Al Taji E, Biebermann H, Ambrugger P, Venháčová J, Pomahačová R, Lebl J, Hníková O, Grüters A, Krude H. Goitrous congenital hypothyroidism: low mutation rate of TPO mutations in Czech children.
   Submitted for publication (J Pediatr Endocrinol Metab).

ACKNOWLEDGEMENTS

I am truly grateful to my supervisor *Professor Dr. Jan Lebl*. I wish to thank him for his expert guidance, encouragement and realistic approach. He was always available for help and advice, stimulating my further efforts and showing enormous patience - not only by reading through numerous manuscripts. He helped me in my research activities as well as to find my way in clinical medicine, encouriging and supporting my interest in paediatric endocrinology and showing me the beauty and mystery of this field of medicine.

With a great respect, I express my gratitude and admiration to *Professor Dr. Olga Hníková*, who introduced nation-wide neonatal screening of congenital hypothyrodism in the Czech Republic, and who shared with me her huge lifelong experience with the care of patients with congenital hypothyroidism.

This work could not be realized without a long-standing, close and fruitful cooperation with *Professor Dr. Annette Grüters*, a great doctor, excellent scientist and wonderful woman, and her team from the Institute of Experimental Paediatric Endocrinology at the University Children´s Hospital Charité in Berlin. Spending more than a half year of an extremely intensive laboratory work in a friedly and inspirative atmosphere in their Paediatric Endocrinology Research Laboratory was an unforgettable life experience for me. I wish to warmly thank especially *Professor Dr. Heiko Krude*, head of the laboratory, who kindly agreed to take over the status as a co-supervisor of my postgraduate studies, and who introduced me to the amazing world of molecular-genetics of congenital hypothyrodism, and especially of the regulation of thyroid development, and filled me with a deep enthusiasm for this topic. I appreciate very friendly, supportive and practical attitude of *Assoc. Professor Dr. Heike Biebermann* who guided me in laboratory work and learned me a lot from laboratory techniques. In their lab, I met many other wonderful workers and friends, namely *Nele Haufs*, *Petra Ambrugger*, *Ingeborg Gläser*.

I am very grateful to *Assoc. Professor Dr. Zdenka Límanová* from 3rd Medical Clinic, 1st Faculty of Medicine, Charles University who, taking care of adult patients from

our unique PAX8 family, far-sightedly clinically diagnosed the youngest member in presymptomatic period and subsequently offered these patients to our molecular-genetic study. I would like to thank also to *Dr. Jaroslav Zikmund* from our department, who provided careful ultrasound examinations of numerous patients and his cooperation was always effective and fluent. Many thanks to *Ing. Petr Kračmar* and the whole staff of neonatal screening laboratory in Prague for providing me screening data and to *Mrs. Eva Mattušová* for a kind general assistance. I wish to thank *Assoc. Professor Dr. Felix Votava*, current head of our department, for allowing me to extend my activities to daily work with endocrine patients in our outpatient clinic.

I have to mention a very intensive recent cooperation with my younger colleague *Dr. Karolína Banghová* who completed molecular-genetic studies in four patients that had been previously clinically diagnosed as Pendred syndrome by myself.

I deeply appreciate an outstanding cooperation of all Czech patients, their families and endocrinologists involved in this nation-wide study: in alphabetic order namely *Dr. M. Finková* (Prague*), Dr. V. Janštová* (Ostrava), *Dr. J. Klabochová* (Plzeň), *Dr. E. Nováková* (České Budějovice), *Dr. D. Novotná* (Brno), *Dr. R. Pomahačová* (Plzeň), *Dr. J. Škvor* (Ústí nad Labem), *Dr. H. Vávrová* (Vsetín), *Dr. J. Venháčová* (Olomouc), *Assoc. Prof. Dr. J. Zapletalová* (Olomouc).

I wish to thank *Prof. Guy van Vliet* from Montreal and *Prof. Herwig Frisch* from Vienna for many interesting discussions in last years.

Finally, my warmest thanks to my dear *parents* for their love, never-ending support, great understanding and sharing with me all life difficulties. They also gave me time to work by taking loving care of my dearest *daughter Hanadi*, who has fulfilled my life with joy and hope.

Support of the study

Czech Ministry of Education (grant MSM 0021620814)

German Academic Exchange Service (DAAD short research term scholarship)

German Research Council (Sonderforschungs Bereich No. 577 - A10)

Charles University in Prague (grant GAUK 2008/2007)

Prague, February 2009 MUDr. Eva Al Taji

CONTENTS **Page**

1. SUMMARY

Background: Thyroid dysgenesis (TD) and thyroid dyshormonogenesis clinically manifest as permanent primary congenital hypothyroidism (CH) and only rarely as non-congenital, postnatal non-autoimmune hypothyroidism. As basic molecular events underlying the regulation of thyroid development, growth and function were clarified in the last decade, molecular pathogenesis of TD and dyshormonogenesis has been intensively studied. Candidate genes for TD and dyshormonogenesis had been described and their mutations were subsequently detected in several patients with non-syndromic and syndromic CH. Nevertheless, no systematic population-based phenotype-focused molecular genetic analysis had been performed and concerning TD, the data regarded only a few individual patients.

Aim: The aim of this extensive study was to identify monogenic forms of TD and dyshormonogenesis in a population-based cohort of Czech patients mostly with CH. Systematic mutation screening was based on a detailed clinical information and phenotype description, and thus focused on clinically defined subgroups of patients matching the phenotypes of already known candidate gene mutations.

Patients: One hundred and ninety-three Czech paediatric, adolescent and young adult patients (130 girls, 63 boys) with primary permanent CH or early-onset non-autoimmune hypothyroidism were included in the study. Among 190 patients with CH, 168 patients had non-goitre phenotype and 22 patients manifested with goitrous CH. Thyroid enlargement was observed also in one of 3 patients with early postnatal hypothyroidism.

Methods: Clinical data were retrospectively analysed via phenotypical questionnaires. All 170 patients with non-goitre congenital and early-onset primary hypothyroidism were screened by single stranded conformation polymorphism (SSCP) for *PAX8* mutations. Twenty-two patients with goitrous CH were screened by SSCP for *TPO* mutations. In addition to that, in probands with syndromic hypothyroidism coding regions of following genes were directly sequenced: *NKX2.5*, *NKX2.1/TTF1*, *FOXE1/TTF2*, *PAX8* and *HEX* genes in patients with non-goitre CH associated with structural congenital malformations of other organs and the *PDS/SLC26A4* gene in patients with goitrous congenital or postnatal

hypothyroidism associated with sensorineural hearing loss. The R52P PAX8 mutation was functionally characterized *in vitro* by DNA binding analysis.

Design: Study 1 focused on the role of transcription factor PAX8 in thyroid development and early postnatal thyroid growth and on the role of transcription factors PAX8, NKX2.1/TTF1, FOXE1/TTF2, NKX2.5, and HEX in pathogenesis of associated congenital malformations in patients with non-goitre CH. In **Study 2**, regarding the genetic background of thyroid dyshormonogenesis, firstly 22 patients with goitrous CH were analyzed for *TPO* mutations. Thereafter, four patients with goitrous hypothyroidism (3 patients with CH, 1 patient with early postnatal hypothyroidism) associated with sensorineural hearing loss, and thus with clinical diagnosis of Pendred syndrome, were studied.

Results: The mutation detection rate in known candidate genes for CH is very low even in a population-based cohort and phenotype-focused screening study. In **Study 1**, searching for mutations revealed a novel *PAX8* gene mutation R52P, dominantly inherited in three members of a three-generation pedigree with non-congenital, early-onset non-goitre non-autoimmune hypothyroidism with the postnatal regression of the thyroid size and function. R52P PAX8 mutation leads to a substitution of a highly conserved residue of the DNA-binding domain and results in the loss of function of the mutant protein as confirmed by functional studies. In **Study 2**, two patients each carrying 2 *TPO* mutations (among them so far unpublished mutations c.740delA in exon 7 and c.2134 C >T in exon 12) and 3 other patients with a single *TPO* mutation were identified among patients with goitrous CH. Clinical diagnosis of Pendred syndrome was genetically confirmed in 2 patients with CH and 1 patient with postnatal hypothyroidism associated with sensorineural hearing loss.

Conclusions: According to our knowledge, we present one of the largest population-based cohorts of patients with CH systematically screened for mutations in candidate genes. The very low frequency of genetic defects in transcription factors in children affected by non-goitre CH, even in a phenotype-focused screening study, suggests the pathogenetic role of either non-classical genetic mechanisms like epigenetic or somatic defects or the involvement of so far unknown genes. Identification of a novel *PAX8* mutation in a particular new phenotype of non-congenital hypothyroidism indicates the key function of PAX8 in the postnatal thyroid growth and function. In our non-dyshormonogenesis-enriched cohort, the detection mutation rate in the *TPO* gene in the subgroup of patients

displaying a goitrous phenotype was much lower than previously reported in highly preselected patient cohorts. Our results also indicate the rarity of Pendred syndrome as a cause of CH, however this diagnosis should be taken into consideration in children with goitrous congenital or postnatal non-autoimmune hypothyroidism and sensorineural hearing loss.

2. INTRODUCTION

2.1. Background of the study

2.1.1. Congenital hypothyroidism in the era of newborn screening

Congenital hypothyroidism (CH), as the most frequent inborn endocrine disorder occuring with a frequency of **1 in 3,000 - 4,000 newborns**, is one of the most common preventable causes of mental retardation (Toublanc 1992, La Franchi 1999). L-thyroxine replacement therapy can prevent the severe neurological, mental, and motor consequences if started as soon as possible within the first trimester of life (Klein 1972, Oppenheimer and Schwartz 1997, Chan and Kilby 2000). Nevertheless, most of affected newborns lack specific clinical features since the deficiency of fetal thyroid hormones is partially compensated by a transplacentar passage of maternal thyroxine (Vulsma 1989). Since only about 5% can be diagnosed by a physical examination after birth, **screening newborns** for CH was added to neonatal screening programmes several decades ago (overviewed by Dussault 1999).

Nation-wide neonatal CH screening was introduced by Prof. Dr. Olga Hníková in our country in 1985 (Hníková and Kračmar 1989). Using a dried blood spot from a heel prick, initially it was based on the assessment of total thyroxine (tT_4) by RIA. Since 1996 thyrotropin (TSH) levels have been measured by DELFIA (cut off level 15 mU/l). Between 1994 and 1996 both methods were used in different screening centres (Prague and Brno). The central screening laboratory at the Department of Paediatrics, 3rd Faculty of Medicine in Prague provides the examination of TSH for all the children born in the Czech region.

World-wide, introducing neonatal screening for CH enabled to establish the diagnosis in most of patients in a clinically presymptomatic stadium and to initiate the substitution treatment in newborn age (Delange 1997, Working Group on Neonatal Screening of ESPE 1999). Therefore, psychomotor development and long-term outcome is favourable in most of patients (Grüters 2002). Screening programmes also facilitated to gather large series of patients and to follow epidemiological and pathogenetic aspects of CH.

2.1.2. Epidemiology of congenital hypothyroidism

CH is a frequent condition with a consistent frequency of 1 in 3,000 - 4,000 newborns (Toublanc 1992, La Franchi 1999). However, lower prevalence has been observed in Afro-Americans (1: 10,000 - 1: 30,000), and higher frequency in Hispanics (1:

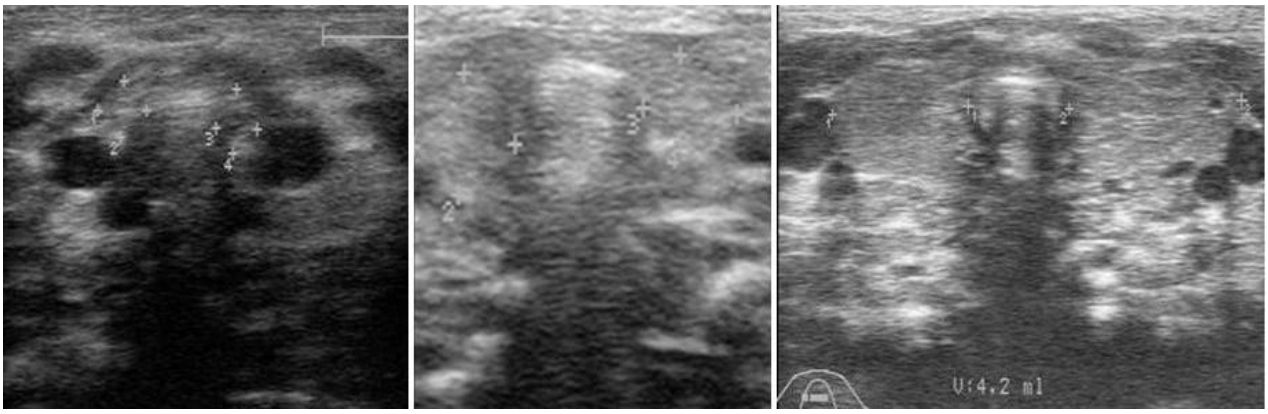
2,000-1: 3,000) (Toublanc 1992, Klett 1997, Roberts 1997). CH is more frequently diagnosed in Down syndrome (Roberts 1997). The risk of CH is also higher in multiple pregnancies,with the low concordance rate among twins (Olivieri 2007). Females are affected twice more often than males (Roberts 1997, Devos 1999).

2.1.3. Classification and aetiology of congenital hypothyroidism

A lot has been discovered about CH since the development of newborn screening programmes. Nowadays, speaking about CH, we usually mean **non-endemic** or so called "**sporadic**" **CH**. **Endemic CH**, that neurological form used to be called "endemic cretenism", is a different disease due to severe maternal and fetal iodine deficiency (for overview see Delange 2001).

Primary (peripheral) permanent CH is the most frequent cause of CH. In 75-80% of the cases, CH is caused by the impaired thyroid development that leads to developmental anomalies of the thyroid gland - **thyroid dysgenesis (TD)** (athyreosis, thyroid hypoplasia or rudiment, ectopy, hemithyroid, cystic malformation) - OMIM 218700 (Grüters 1997, La Franchi 1999, Devos 1999, Park and Chatterjee 2005). In the remaining 20%, CH results from **dyshormonogenesis -** OMIM 274400-274900. That is caused by defects in biochemical mechanisms responsible for the thyroid hormone biosynthesis, and it is associated with congenital, neonatal or postnatal goiter or normal-sized and normally structured thyroid gland (Grüters 1994, Grüters 1997) (Figure 1, Chart 1).

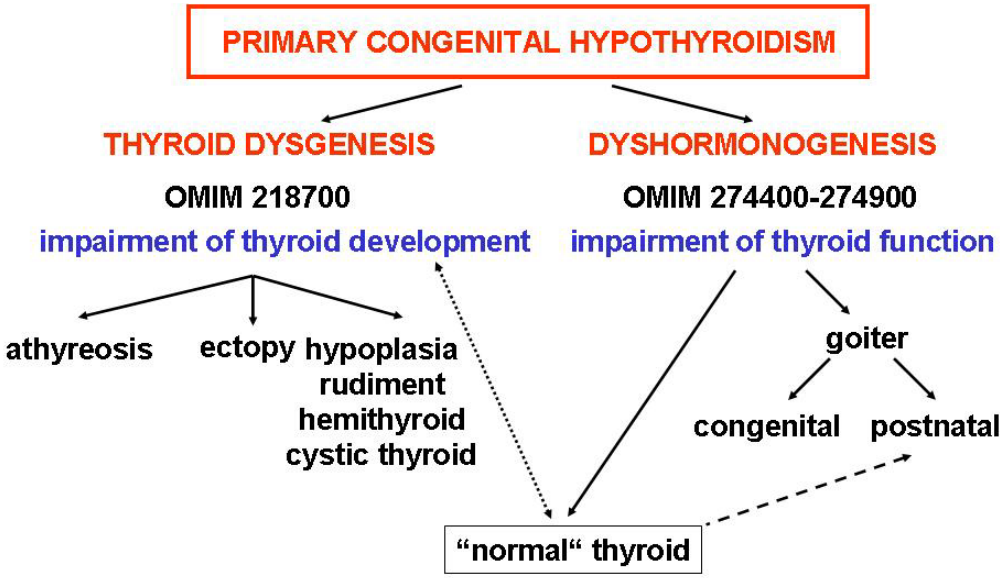
Figure 1 - Transversal ultrasound imaging of thyroid gland in newborns with CH



Left: thyroid dysgenesis - rudiment (hyperechogenic remnants of thyroid tissue), middle: thyroid gland of a normal size and structure, right: thyroid dyshormonogenesis - large neonatal goiter (the courtesy of Dr. J. Zikmund).

Despite the fact, that primary CH is clasically subdivided into TD and dyshormonogenesis based on the thyroid morphology at diagnosis, in some cases the diagnosis has to be reevaluated in the course of life. These cases of CH with initially "normal thyroid" diagnosed as CH due to thyroid dyshormonogenesis, demonstrate postnatal thyroid growth impairment leading to hypoplastic or even rudimental thyroid gland showing dysgenetic features (Meeus 2004) (Chart 1). In respect of this new knowledge, the attention is paid to non-autoimmune **early postnatal thyroid growth impairment**. Another important remark is that milder forms of TD can be asymptomatic at birth (Léger 2002, Maiorana 2003). All these special situations will be discussed in more detail later on.

Chart 1 - Aetiology of primary congenital hypothyroidism



In fact, CH can be caused by a disorder on any level of the hypothalamic-pituitary-thyroid axis (for overview of CH classification see Table 1). However, **central CH** represents less than 5% of CH cases (Foley 2001). Rare cases of isolated **secondary** (pituitary) **CH** (OMIM 275100) due to **isolated deficiency of TSH** are caused by homozygous mutations of the gene encoding for **TSH β- subunit** (chromozome 1p13) (OMIM 188540). They manifest either as mild CH or as a severe form of CH (Doeker 1998). Although levels of TSH are usually low or undetectable, they can be detected as normal or even higher in presence of circulating biologically inactive but immunoreactive TSH-like protein (Bonomi 2001). **TRH receptor** (TRHR) defects (OMIM 188545) due to

Table 1 - Classification of congenital hypothyroidism

Permanent congenital hypothyroidism			**Genetic forms** **- mutations of genes encoding for:**
Peripheral - primary	**dysgenesis** - thyroid developmental defects - incidence 1: 4,500	- athyreosis, hypoplasia, rudiment, ectopy, hemithyroid, cystic malformation	- TSHR, transcription factors NKX2.1, PAX8, FOXE1, NKX2.5
	dyshormonogenesis - thyroid hormone synthesis defects - incidence 1: 30,000	- iodide trapping defects - oxidation, organification defects - abnormal TG - deiodinase defect	- NIS, pendrin - TPO, THOX2, pendrin - TG - DEHAL
Central incidence 1: 25,000 - 1: 100,000	**pituitary** - secondary	- isolated TSH deficiency - TSH and PRL deficiency - pituitary deficiency	- β TSH - TRHR - POU1F1, PROP1, LHX3
	hypothalamic - tertiary	- TRH deficiency	
Transient congenital hypothyroidism incidence 1: 25,000 - 1: 100,000			THOX2 (heterozygous mutations)

Adapted according to Al Taji E. Molecular pathogenesis of congenital hypothyroidism. In: Lebl J., Zapletalová J., Kolouškova S. Paediatric endocrinology. Prague, Galén 2004, 307-321. CH - congenital hypothyroidism, DEHAL - dehalogenase, NIS - natrium-iodide symporter, T4 - thyroxine, TG - thyroglobulin, THOX - thyroid oxidase, TPO - thyroid peroxidase, TRH - thyroliberin, TSH - thyrotropin, TRHR - thyrotropin releasing hormone receptor, TSHR- thyrotropin receptor.

its gene mutations (chromozome 8q23) lead to the pituitary incapacity for binding hypothalamic TRH and consequently to the autosomal recessive **combined TSH and PRL deficiency** (Collu 1997). Defects of transcription factors regulating the pituitary development (e.g. HESX1, POU1F1, PROP1, LHX3) result in the **combined pituitary hormone deficiency**, including TSH deficiency. Symptoms of CH can dominate already in newborn age in POU1F1 and LHX3 defects (Blankenstein 2001). **Tertiary** (hypothalamic) forms of **CH - TRH deficiency** (OMIM 275120) are extremely rare (Niimi 1982).

2.1.4. Pathogenesis of primary congenital hypothyroidism (CH)

2.1.4.1. Molecular pathogenesis of primary CH

Recently, a progress has been made in understanding the pathogenesis of congenital thyroid disorders on molecular level. While dyshormonogenesis has been recognized to be a genetic disease with autosomal recessive inheritance and many various mutations in several candidate genes have been reported, molecular pathogenesis of TD is less clear (for overview see e.g. Macchia 1999, Macchia 2000, Krude 2000, Grüters 2004, Park and Chatterjee 2005).

In thyroid **dyshormonogenesis**, mutations of genes encoding for proteins responsible for thyroid hormone biosynthesis as **thyroid peroxidase** (TPO), thyroid oxidase (THOX), **natrium-iodide symporter** (NIS), **pendrin** (PDS), **thyroglobulin** (TG), **and dehalogenase** (DEHAL) are inherited in autosomal recessive fashion. With the exception of Pendred syndrome (accompanied by congenital sensorineural deafness), they are not associated with the involvement of other organs (for overview see e.g. Gillam and Kopp 2001, De Vijlder 2003).

In **TD**, firstly loss-of-function mutations of the **TSH-receptor** gene were described (Sunthornthepvarakul 1995, Abramowicz 1997, Biebermann 1997, overviewed by Szkudlinski 2002). With the discovery of transcription factors, which play a key role in prenatal thyroid development, there was an interest whether mutations in the genes coding for these factors might play a role in TD. The **transcription factors NKX2.1 (TTF1)**, **FOXE1 (TTF2)**, **PAX8**, **NKX2.5**, and **HEX** are essential for the thyroid morphogenesis and differentiation but also for the development of other organs. Therefore, their defects may affect not only thyroid development but also other organs (pulmonary and neurological impairment, brain and pituitary abnormalities in *NKX2.1* mutations; cleft lip, palate and epiglottis in *FOXE1* mutations, renal malformation in *PAX8* mutations, heart malformations in *NKX2.5* mutations) (for overview see e.g. Gillam and Kopp 2001, De

Felice and Di Lauro 2004, Polak 2004). However, genes known so far represent probably only a part of all genes involved in the thyroid development, differentiation and function. The others remain to be discovered (Pauws 2000, Moreno 2003, Castanet 2005, Grasberger 2005).

Currently, except of these **monogenic forms** of primary CH due to TD, other genetic mechanisms as **polygenic trasmission** are investigated (Amendola 2005). The discordancy of monozygotic twins for TD offers the possibility of non-hereditary **postzygotic mechanisms** as somatic mutations (Perry 2002, Olivieri 2007).

2.1.4.2. Non-genetic forms of primary CH

Environmental factors may be involved especially in **transient forms** of CH. Mild maternal **iodine deficiency** (Klett 1997), peripartal maternal, fetal or newborn **iodine excess** (Wolff-Chaikoff effect described by Wolff in 1949), or thyreostatic drugs in mother lead to transient neonatal thyroid disorders (Köhler 2000). In maternal autoimmune thyroid disease, neonatal CH can be caused by the transplacental passage of maternal thyroid **antibodies** that block TSH receptor (TRBAb, TRIAb) (Brown 1996, Evans 2004). These cases connected with maternal thyroid disease are again mostly transient, however, the possibility of the impairment of thyroid development leading to TD and permanent CH is not excluded (Blizzard 1960, Sutherland 1960).

The impact of other enviromental factors on prenatal thyroid development and/or neonatal thyroid function, except of rare situations of maternal exposure to radioiodine, has not been clarified so far. Results of population studies following seasonal variations in the incidence of TD and thus connection with prenatal infections are controversial (Miyai 2005).

2.1.4.3. Heredity of primary CH

Only few systematic population-based molecular genetic studies are available in patients with CH so far. Regarding primary permanent CH due to thyroid **dyshormonogenesis**, molecular-genetic studies confirmed previous clinical observations (Stanbury and Hedge 1950, Medeiros-Neto 1982, Medeiros-Neto 1993) of the **autosomal recessive** manner of inheritance (for overview see e.g. Gillam and Kopp 2001, de Vijlder 2003). An interesting exception is autosomal dominant transient CH due to THOX2 mutations (Moreno 2002).

Heredity of **TD** is less clear. It has been considered as a sporadic disease so far. Nevertheless, recent studies of large cohorts of patients demonstrated that the familial occurrence is more frequent than expected (Castanet 2001, Léger 2002). **Monogenic forms** of TD have been intensively investigated and it has become apparent that TD, at least in a subset of patients, is an inherited disorder. Whilst transmission of TSHR defects is **autosomal recessive,** defects of transcription factors are inherited mostly in **autosomal dominant** fashion. **De novo** mutations must be also taken into consideration (De Felice and Di Lauro 2004, Park and Chatterjee 2005).

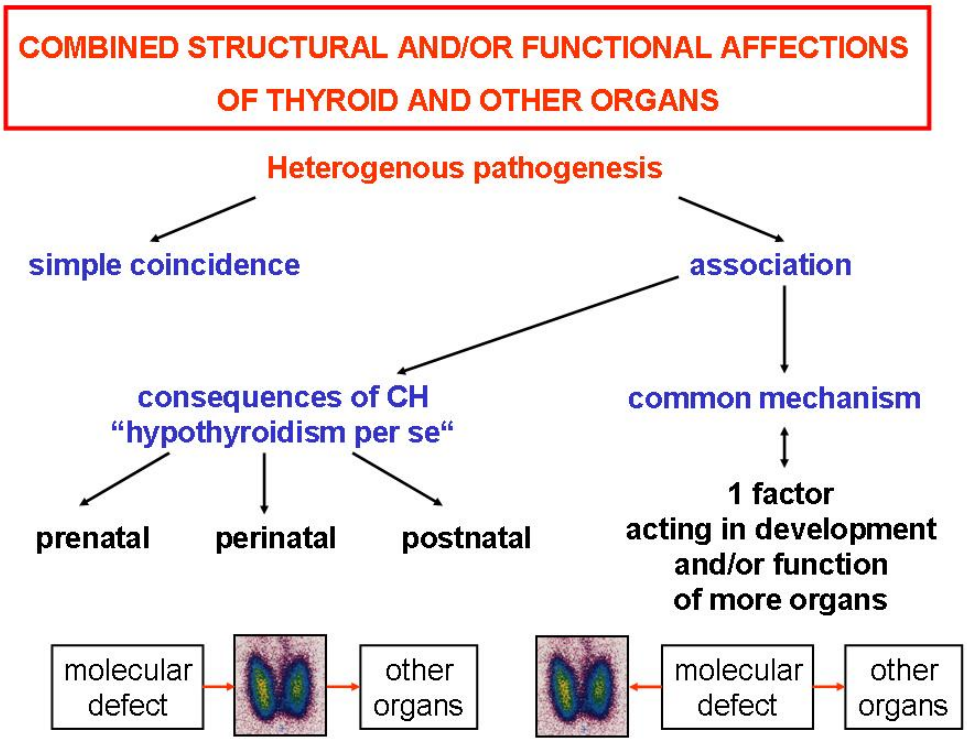
2.1.4.4. Congenital malformations and associated affections in CH children

It has soon become obvious that children with CH have higher prevalence of **associated congenital malformations** (CMs) (Fernhoff 1987, Lazarus and Hughes 1988, Siebner 1992, Majeed-Saidan 1993, Roberts 1997, Devos 1999, Olivieri 2002). Published frequencies of CMs in children with CH range from **2.4%** (Chanoine 1986) to **20-25%** (Fernhoff 1987). The risk of associated CMs is usually given **2.2** (Roberts 1997) to **5 times** (Siebner 1992) higher in comparison to the normal population. Overall, **sensorineural hearing impairment** is the most frequent affection in children with CH (La Franchi 1999). Significantly higher incidence of structural CMs is associated with TD (Devos 1999). Among them, **cardiovascular anomalies** are the most frequent, followed by musculoskeletal anomalies, malformations of central nervous system and gastrointenstinal tract, cleft lip and palate (Olivieri 2002). In a number of cases, multiple CMs are observed. In the presence of associated CMs, the term **syndromic CH** is used. The current view of pathogenesis of associated CMs is summarized in Chart 2.

For many years **hypothyroidism per se** was considered to be the main pathogenetic mechanism of a higher occurrence of CMs accompanying CH. The severity of hypothyroidism is usually evaluated according to initial T_4 levels, thyroid morphology and bone age at diagnosis (La Franchi 1999). It was proposed that severe hypothyroidism can lead not only to the postnatal sequelae if untreated but can cause also prenatal and perinatal impairment. Hypothyroidism per se plays the main role in the pathogenesis of so-called “classical” CMs accompanying CH as e.g. hernias, dislocation of hip. The influence of severe CH on CNS and auditory system in prenatal life is also considered (Rovet 1987, Tillotson 1994). However, even severe hypothyroidism probably will not have impact on the occurrence of structural CMs of other organ systems. Therefore, other mechanisms involved in the pathogenesis of **combined affection of the thyroid and other organs**

must exist. It is of a high importance that a higher occurrence of CMs was observed mostly in children with TD (Devos 1999, Olivieri 2002, Grüters 2004). Therefore, defects in molecular mechanisms leading to impaired thyroid development may also result in defective development of other organs and tissues. So far, defects of the genes encoding **transcription factors** have been already identified in several patients with combined affection of thyroid and other organs (*NKX2.1* in pulmonary and neurological affection, *FOXE1* in cleft lip, *NKX2.5* in heart anomalies, *PAX8* in renal malformations).

Chart 2 - Pathogenesis of associated affections in CH patients



2. 2. General aims of the present study

We performed first systematic **molecular-genetic study** in a population-based cohort of Czech children, adolescents and young adults affected by **CH** and **early postnatal non-autoimmune hypothyroidism,** based on a detailed **phenotypical description** and focused on clinically defined subgroups of patients matching the phenotypes of already known candidate gene mutations.

The study was proposed to:

- identify monogenic forms of TD and dyshormonogenesis
- join running efforts of other working groups in clarifying the role of transcription factors in the pathogenesis of TD

- discover mechanisms responsible for syndromic forms of non-goitre and goitre CH
- describe in our population the distribution of *TPO* mutations previously detected in other groups of European patients with CH due to thyroid dyshormonogenesis
- identify molecular-genetic processes responsible for early postnatal non-autoimmune hypothyroidism.

We expected results valuable for physicians, patients and their families as well as for general biomedical knowledge, with further **implications for clinical practice and research:**

- clarifying pathogenic processes in the regulation of the thyroid development, growth and function on molecular level in each patient and in general
- extending the spectrum of known mutations and their functional consequences, thus understanding more exactly the function of genes involved in the development and function of the thyroid gland and genotype-phenotype correlation
- identifying sporadic and genetic hereditary forms of CH, establishing heredity pattern in certain families
- predicting additional defects of other organs
- gathering information necessary for genetic counselling, prenatal and early postnatal presymptomatic diagnostics, and strategy of a long-life treatment and long-term follow-up.

2.3. Patient cohort

2.3.1. Collection of patients

Paediatric endocrinologists all over the country were requested to gather **blood samples** and **phenotypic data** from patients with permanent primary CH and a rare variant of early-onset permanent primary non-autoimmune hypothyroidism. Establishing **nation-wide DNA bank** of properly phenotypically characterized individuals enabled to provide general characterization of the Czech cohort of these patients and subsequent molecular-genetic analysis based on phenotypical characterization of individual patients.

2.3.2. Ethical aspects

The study was approved by the institutional ethics committe of 3rd Faculty of Medicine, Charles University, Prague. Informed consent was obtained from all subjects and/or their parents.

2. 4. General methodology

2.4.1. Clinical evaluation and phenotypical characterization

Data were referred by paediatric endocrinologists from 8 major paediatric endocrine clinics throughout the country. Detailed **clinical questionnaires** focussed especially on T_4/TSH screening levels, hormone levels (fT_4 or tT_4, TSH) and if available also TG and thyroid autoantibodies (antiTG, antiTPO, anti TSHR) levels before the initiation of L-thyroxine substitution treatment as well as on thyroid morphology before and during treatment. The classification of thyroid morphology was based mostly on ultrasound studies as ^{99}Tc scintigraphy was provided only in a small number of patients in the early beginning of the screening programme. Clinical symptoms of hypothyroidism present at the time of diagnosis (prolonged jaundice, feeding difficulties, somnolence, constipation, muscular hypotony, umbilical hernia, large tongue, hoarse cry, palpable small fontanelle) (Hníková 1989) as well as further mental and motor development, occurence of additional congenital malformations (with a special attention towards cardiovascular system) or functional affections (with a special attention towards hearing impairment) were carefully followed. The occurence of associated malformations was noted from medical records or referring physicians. Moreover, screening kidney ultrasound was performed in 57 children with TD (hitherto unknown renal malformations were described in 3 of them). Since 2002, otoacoustic emissions (OAE) have been obligatory tested in all patients with CH till three months of age (Věstník MZ 2002). Parental consanguinity, family history of disorders of thyroid morphology and function were also included to the questionnaire.

According to these **retrospectively analyzed data** acquired via phenotypical questionnaires, probands were subdivided into several groups. Based on thyroid morphology evaluated together with thyroid hormone levels prior to treatment, patients were classified as **CH due to TD, CH with apparently normal thyroid gland, CH due to thyroid dyshormonogenesis** and **non-CH early-onset non-autoimmune hypothyroidism**. Further, they were classified according to the presence of congenital malformations as **syndromic** and **non-syndromic** forms of thyroid disease (Chart 3).

2.4.2. Laboratory procedures

Genomic DNA (deoxyribonucleic acid) was extracted from peripheral blood lymphocytes using a standardized salting-out method (Miller 1988). To perform systematic screening for mutations in the coding regions of the candidate genes involved in thyroid development and function, the appropriate regions of genes were amplified by **PCR** (polymerase chain reaction).

Two standard **PCR-based molecular-genetic mutation screening techniques - SSCP** (single stranded conformation polymorphism) and **RFLP** (restriction fragment length polymorphism), were used. **SSCP** (Orita 1989), a gel-based screening method for detection of unknown point mutations, uses differences in electrophoretic mobilities of wild type and mutant nucleic acids. Under certain conditions, single-stranded nucleic acids form secondary structure in solution. The secondary structure depends on the base composition and may be altered by a single nucleotide substitution, causing differences in electrophoretic mobility under nondenaturing conditions. Fragments for detection can be labeled radioactively, we used silver staining. **RFLP** is a gel-based screening method for detection of known point mutations. A fragment of DNA amplified by PCR is cut at a specific sequence by enzyme called type II restriction endonuclease. If this site is altered, the enzyme will no longer cut and this is detected by agarose gel electrophoresis, which separates DNA by size and charge.

All abnormities detected by screening methods were confirmed by **direct sequencing**. We performed direct dye-terminator cycle sequencing, with a commercially available kit from PE Applied Biosystems. Fluorescently labeled terminator nucleotides (one colour for each nucleotide) are bound to sequence products at each round of amplification. These products are run on a polyacrylamide gel and the dye-terminators are read by a laser detector.

Once a sequence alteration was detected and confirmed by bi-directional direct sequencing, the prediction that it is a **mutation** had to be proved. It can be supported by several arguments: eg. the sequence alteration cannot be demonstrated in DNA isolated from leukocytes of healthy controls (normal subjects from a randomly selected population), the alteration cosegregates with the appropriate phenotype in the family and cannot be identified in unaffected family members. Another important argument is the changing of a highly conserved amino-acid or generally, expected effect on the protein structure with its negative impact on the protein function and stability. However, the final confirmation of the deleterious effect of the mutation bring **functional studies** *in vitro*.

2. 5. General design of the project

Classification of patients as (1) CH due to TD, (2) CH with apparently normal thyroid, (3) CH due to dyshormonogenesis, and (4) non-CH early-onset non-autoimmune hypothyroidism and further as (a) syndromic and (b) non-syndromic with detailed clinical description enabled to provide **phenotype-focused molecular-genetic study** (see Chart 3). Therefore, the whole project was subdivided into following parts:

Study 1 (see Chapter 3): **Thyroid transcription factors**

In this comprehensive study, we focused on the **role of transcription factors in thyroid development and early postnatal thyroid growth and in pathogenesis of associated congenital malformations in patients with non-goitre CH.**

A large group of 170 patients with non-goitre CH and early postnatal non-goitre non-autoimmune hypothyroidism was tested for mutations in transcription factor PAX8. Among these patients, 20 patients with associated structural CMs of other organ systems were directly sequenced for mutations in transcription factors NKX2.1/TTF1, FOXE1/TTF2, PAX8, NKX2.5, and HEX according to their phenotypes.

Results of the "Study 1" were published as an original article ***"Screening for mutations in transcription factors in a Czech cohort of 170 patients with congenital and early-onset hypothyroidism: identification of a novel PAX8 mutation in dominantly inherited early-onset non autoimmune hypothyroidism"***.

Study 2 (see Chapter 4): **Genetic background of thyroid dyshormonogenesis**

Part 1: Screening for *TPO* mutations

Twenty-two patients with goitrous CH were screened for *TPO* mutations in exons where mutations have been identified most frequently in previous studies in other European populations. Results were summarized in the manuscript (submitted for publication) ***"Goitrous congenital hypothyroidism: low mutation rate of TPO mutations in Czech children"***.

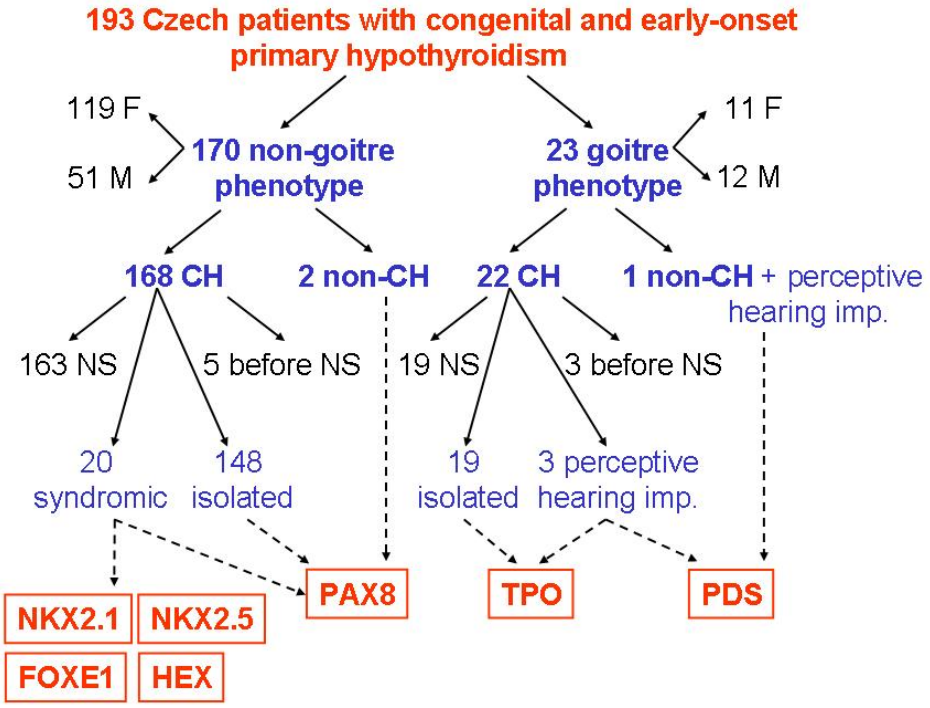
Part 2: Pendrin and its role in pathogenesis of congenital and non-congenital goitrous hypothyroidism

Three patients with CH and clinical diagnosis of Pendred syndrome were studied.

These results were published as an original article ***"Pendred syndrome among patients with congenital hypothyroidism detected by neonatal screening: identification of two novel PDS/SLC26A4 gene mutations"***.

The case report of a female patient with goitrous non-congenital non-autoimmune hypothyroidism and sensorineural hearing loss was referred as an article ***"Thyroidectomy in a patient with multinodular dyshormonogenetic goitre - a case of Pendred syndrome confirmed by finding mutations in the PDS/SLC26A4 gene".***

Chart 3 - Characterization of the cohort and design of the study



CH - congenital hypothyroidism, F - female, imp. - impairment, M - male, NS - neonatal screening.

3. STUDY 1: THYROID TRANSCRIPTION FACTORS

3.1. Introduction

3.1.1. Role of transcription factors in thyroid development and growth

Thyroid organogenesis involves the dorso-caudal migration of a median endodermal bud that originates from the posterior region of the pharyngeal floor.The thyroid primordium migrates to the area located between the fourth pharyngeal pouches and eventually fuses with them. As the thyroid gland develops from two distinct embryonic lineage, it is composed of two different hormone-producing cell types that have distinct embryonic origins: thyroid follicular cells (TFCs) of endodermal origin derived from the floor of the foregut produce thyroxine and parafollicular C-cells of neural crest origin arosen from the cells within the ultimobranchial body produce calcitonin (overviewed by De Felice and Di Lauro 2004).

Thyroid development is a **complex process** of specification - induction, evagination, migration, bifurcation, differentiation, proliferation, and survival during embryogenesis, followed by fetal period of growth and initiation of function. It is precisously controlled by a regulatory network of several **transcription factors** (Damante 2001, Parlato 2004) (Figure 2). Transcription factors are the chief regulators of gene expression, therefore playing a critical role in the control of cell differentiation. These proteins recognize downstream target genes through interaction with specific DNA sequences and modulate gene expression by acting either on the basal transcriptional machinery or on chromatin structure.

As firstly demonstrated **in mice**, the expression of transcription factors **Pax8** (Plachov 1990), **Nkx2.1/Titf1** (Lazzaro 1991), **Foxe1/Titf2** (Zannini 1997), **Hhex** (Thomas 1998), and **Nkx2.5** (Dentice 2006) begins in the early phases of the thyroid development in thyroid cell precursors, reflectig a crucial role of these transcription factors in thyroid morphogenesis and organogenesis (see Table 2). In addition, these transcription factors are expressed during the development of other organs (see Table 2). Therefore, transcription factors gene mutations may lead to various disorders of thyroid development but also other organs can be involved (see Table 2).

None of these transcription factors is expressed solely in the thyroid, but their combination is **unique** to this endocrine gland (Damante 2001). Moreover, *Pax8, Nkx2.1*, *Foxe1*, and *Hhex* are not simply coexpressed in the thyroid, but they are linked in

Table 2 - Expression of transcription factors regulating thyroid development and phenotypes of knock-out mice

Part 1

Transcription factor	Expression in mice embryos	Phenotype of knock-out mice	
		homozygotes	heterozygotes
Nkx2.1/Titf1 (T/ebp)	thyroid (E10.5) 4th pharyngeal pouch lung anlage forebrain, diencephalon, neurohypophysis *(Lazzaro 1991)*	born dead no thyroid (no TFCs, no C-cells) normal parathyroid no lung parenchyma defects of ventral region of forebrain missing pituitary *(Kimura 1996)*	normal phenotype *(Kimura 1996)* normal thyroid elevated TSH, normal T_4 poor coordination *(Pohlenz 2002)*
Pax8	thyroid (E10.5) midbrain-hindbrain boundary myelencephalon spinal cord kidneys but not ureteric bud *(Plachov 1990)*	hypoplastic thyroid (no TFCs, only C-cells) normal kidney normal CNS *(Mansouri 1998, Bouchard 2000)*	healthy, fertile more frequently elevated TSH *(Mansouri 1998)*

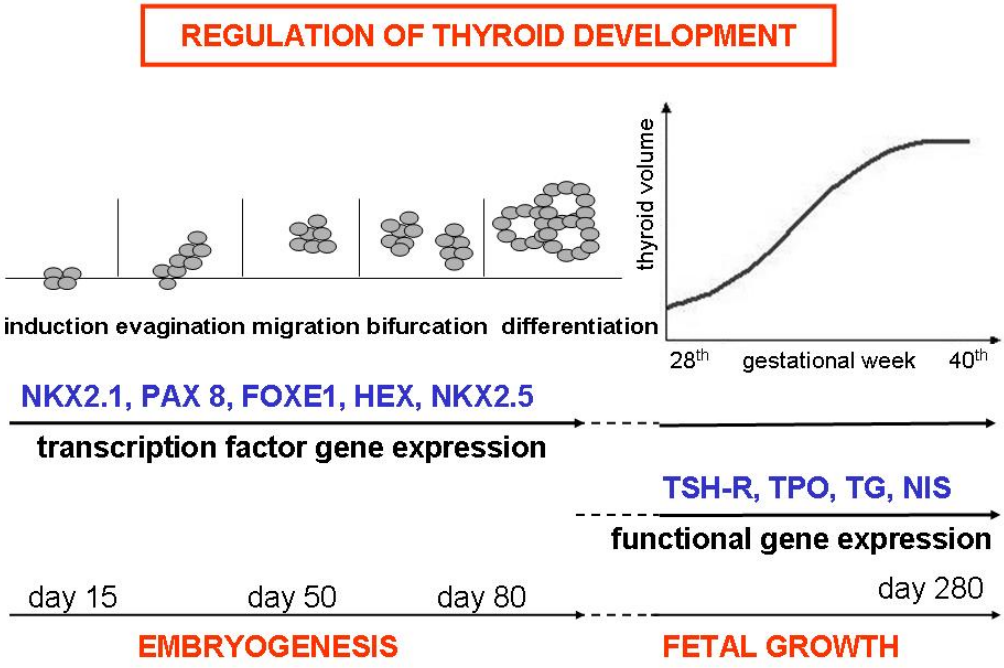
Part 2

Transcription factor	Expression in mice embryos	Phenotype of knock-out mice	
		homozygotes	heterozygotes
Foxe1/Titf2	thyroid (E8.5, turned off between E13-15) endodermal lining of the foregut anterior pituitary, hair follicles *(Zannini 1997, Dathan 2002)*	thyroid agenesis, thyroid ectopy cleft palate *(De Felice 1998)*	not described
Nkx2.5	pharyngel floor (portion of which evaginates thyroid E8 - 8.5) developing heart *(Lints 1993)*	thyroid? – not studied no malformations of pharynx cardiac development arrested prior looping *(Lyons 1995)* smaller thyroid bud *(Dentice 2006)*	thyroid not described
Hhex	thyroid primordium (E9.5) hepatic anlage lungs forebrain *(Keng 1998, Thomas 1998)*	arrested thyroid development liver defects defects of rostral forebrain *(Martinez Barbera 2000)*	not described

E - day of embryonal development

an **integrated regulatory network**, each of them controlling the presence of other members of the network (Parlato 2004, Puppin 2004) and also directly interacting and cooperating in their function (Di Palma 2003). The expression of thyroid trascription factors continues through the prenatal and postnatal life, as they are essential not only for the **early thyroid development** but also for the **functional differentiation of TFCs**, their proliferation and survival, and thus for the **maintenance of thyroid differentiated state** (Damante 2001). Transcription factors, and among them *Pax8* as a master gene for the regulation of the thyroid differentiated phenotype (Pasca di Magliano 2000), regulate the expression of TSHR, TG (Fabbro 1998), TPO (Zannini 1992) and NIS (Ohno 1999). The early steps of thyroid differentiation are **independent of TSHR signaling** (OMIM603372), which regulates proliferation and functioning in later steps of prenatal thyroid development and within postnatal life (Postiglione 2002, De Felice 2004).

Figure 2 - Regulation of human thyroid development



3.1.2. Role of transcription factors in pathogenesis of thyroid dysgenesis and associated congenital malformations

Further insights into mechanisms responsible for TD and associated CMs were enabled by animal models of homozygous **mice knock-out for Nkx2.1/Titf1**/ (Kimura 1996), **Foxe1/Titf2** (De Felice 1998), **Pax8** (Mansouri 1998), **Hhex** (Martinez Barbera 2000), and **Nkx2.5** (Dentice 2006) that display a spectrum of thyroid developmental

anomalies and, except of Pax8$^{-/-}$ mice, major malformations of other organs corresponding to the extrathyroidal expression patterns of appropriate transcription factors (see Table 2).

Recently, timing of events during thyroid development and expression of transcription factors **PAX8**, **NKX2.1/TTF2**, **FOXE1/TTF2** have been preciously studied at different stages of **human** embryonic and fetal development (Sura-Trueba 2005) (Table 3). Although temporo-spatial human expression patterns of these transcription factors are similar to those in mouse, they show some differences (Table 3). This new insight into the role of transcription factors PAX8, NKX2.1/TTF1, and FOXE1/TTF2 in human embryogenesis also confirms previous explanations regarding the malformations associated with TD in patients carrying *PAX8*, *NKX2.1*, *FOXE1* gene mutations (Table 4).

Twelve years ago, first mutation screening of the human ***NKX2.1/TTF1*** gene (OMIM 600635) in non-syndromic CH failed to identify any mutations (Perna 1997, Lapi 1997, Hishinuma 1998). As heterozygous deletions encompassing the *TITF1* locus were reported in two children with respiratory, neurological and thyroid impairment (Devriendt 1998, Iwatani 2000), *NKX2.1/TTF1* was later studied in patients with complex neurological, respiratory and thyroid symptoms. Indeed, mutations were identified in several patients with this **syndromic form of CH** (Krude 2002, Pohlenz 2002, Willemsen 2005).

Similarly, the role of ***FOXE1*** (OMIM 602617) in non-syndromic CH was not confirmed (Hishinuma 2001). *FOXE1* mutations were detected only in rare cases of strictly-defined Bamforth-Lazarus syndrome (OMIM 241850) (Bamforth 1989, Clifton-Bligh 1998, Castanet 2002, Baris 2006). Surprisingly, no mutations were found also in patients with CH and cleft palate (Tonacchera 2004).

Particularly interesting is ***NKX2.5*** (OMIM 600584) that is expressed both in the developing thyroid and heart (Lints 1993) and which defects are known to be responsible for various congenital heart defects (Schott 1998, Benson 1999). As cardiac CMs are the most frequent structural defects in CH patients, *NKX2.5* causative role in the association of CH and cardiac malformations has to be more investigated - only one patient with combined thyroid and heart affection due to *NKX2.5* mutations has been described so far (Dentice 2006).

Several familial and sporadic cases of non-syndromic non-goitre CH (Macchia 1998, Vilain 2001, Komatsu 2001, Congdon 2001, Meeus 2004, de Sanctis 2004, Grasberger 2005) and rare cases of TD associated with renal malformations (Krude 1998, Meeus 2004) were explained by the defect in ***PAX8*** (OMIM 167415). *PAX8* mutations are

Table 3 - Expression of transcription factors regulating human thyroid development (according to Sura-Trueba 2005)

Developmental stage	Transcription factors		
	FOXE1/ TTF2	**PAX8**	**NKX2.1/TTF1**
CS 14 (32 d):		median thyroid anlage 4th pharyngeal pouch ventral part of otic vesicle midbrain-hindbrain boundary mesonephros	median thyroid primordium not 4th pharyngeal pouch
CS 15 (33 d)	thyroid primordium thymus oropharyngeal epithelium	thyroid anlage, thyroglossal duct ultimobranchial body midbrain-hindbrain boundary lateral part of spinal cord metanefric blastema, ureteric bud	median thyroid primordium lung bud forebrain - ventral forebrain, diencephalon, telencephalon
C19 (48 d)		thyroid ventral part of myelencephalon dorsal part of cerebellum anlage kidney mesenchym, collecting system	thyroid hypothalamic floor, infundibulum, telencephalon (basal ggl.) primary bronchi epitelia

CS - Carnegie staging

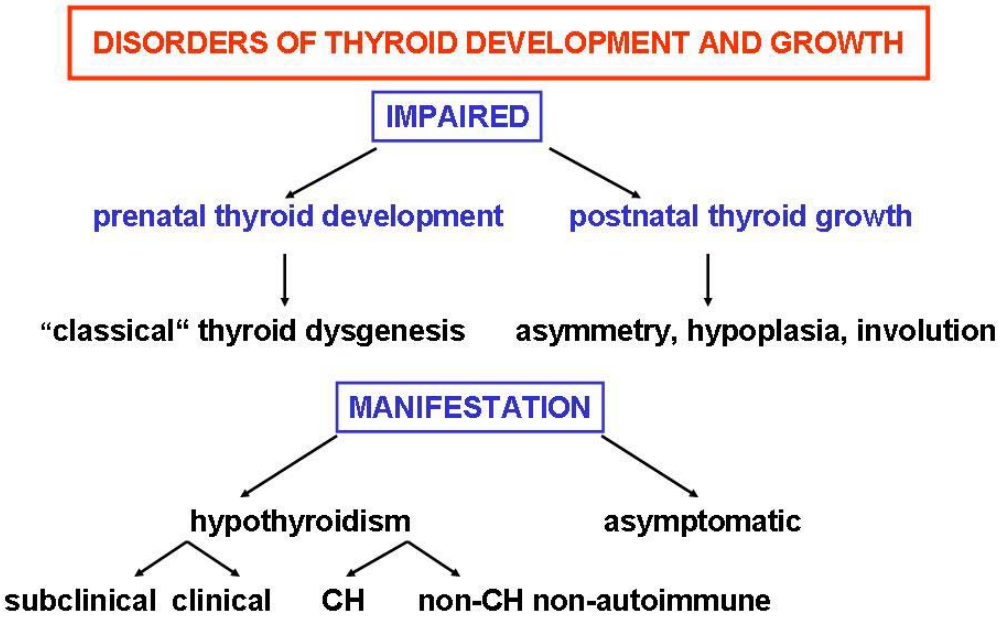
Table 4. - Transcription factors regulating thyroid development and human phenotypes of their defects

Transcription factor	Protein family	Chromosome	OMIM	Inheritance	Human phenotypes	
					thyroid	other organs
NKX2.1/ TTF1	homeodomain transcription factor	14q13	600635	autosomal dominant	agenesis, hypoplasia, normal severe to moderate CH hyperthyrotropinemia	choreoathetosis mental retardation respiratory distress pulmonary infections
PAX8	paired domain transcription factor	2q12-14	167415	autosomal dominant	hypoplasia, hemithyroid, rudiment, ectopy, normal severe to mild CH hyperthyrotropinemia asymptomatic	none or renal developmental defects (unilateral kidney agenesis)
FOXE1/TTF2	forkhead domain transcription factor	9q22	602617	autosomal recessive	agenesis, hypoplasia severe CH	Bamforth syndrome: CH + cleft palate, bifid epiglottis, choanal atresia, spiky hair
NKX2.5	homeodomain transcription factor	5q34	600584	autosomal dominant	agenesis, ectopy CH	none or mild cardiac defects (PFO, MV insufficiency)

characterized by a **wide phenotypical variability**, also within one family (Congdon 2001, de Sanctis 2004Meeus 2004): variable degree of TD manifested as CH, hyperthyrotropinemia or even asymptomatic, or CH with in-place thyroid of normal size at birth, showing postnatal thyroid involution, or less frequently completely asymptomatic phenotype (Chart 4).

Among other transcription factors, ***HHEX*** **(OMIM 604420)** is another candidate gene for TD, but no systematic screening studies in CH patients have been performed till now.

Chart 4 - Classification and manifestation of thyroid development and growth disorders



Hence, in a part of patients, TD (OMIM218700) can be transmitted as a **Mendelian monogenic disease** due to de novo or inherited mutations of genes encoding for thyroid transcription factors, following autosomal dominant (*NKX2.1*, *PAX8*, *NKX2.5*) or autosomal recessive (*FOXE1*) mode of inheritance. The pathogenetic mechanism of autosomal dominant transmission of thyroid transcription factor defects can be explained by **haploid insufficiency** - in the presense of a heterozygous loss-of-function mutation, the amount of a product synthesized by the single wild-type allele is not sufficient for the protein function. As this quantitative phenomen typically occurs in genes whose amount of protein products are critical, haploinsufficiency is often the mechanism by which transcription factor defects cause the disease (Damante 1998, Seidman and Seidman 2002).

Phenotypic effects of haploinsufficiency have a variable expressivity, and therefore could be very sensitive to the genetic background.

Except of transcription factors, another monogenic - autosomal recessive type of CH with variable degree of thyroid hypoplasia is represented by loss-of-function mutations of the **TSHR** (OMIM 275200, chromosome 14q31) (Sunthornthepvarakul 1995, Abramowicz 1997, Biebermann 1997, molecular genetics of TSHR overviewed e.g. by Russo 1997).

However, in most of patients the underlying genetic defect and manner of inheritance remains still unclarified. Recent studies described **2% of familial** occurrence of **CH due to TD** (15 fold more frequent than would be expected from chance alone) (Castanet 2001) and increased frequency of asymptomatic thyroid development anomalies in first-degree relatives of patients with CH due to TD (Léger 2002). Further evidence in support of a genetic basis of TD is provided by a **higher prevalence of non-thyroidal congenital anomalies (**Fernhoff 1987, Lazarus and Hughes 1988, Siebner 1992, Majeed-Saidan 1993, Roberts 1997, Grüters 1997, Devos 1999, Olivieri 2002). Other factors indicating the role of genetic background in pathogenesis of TD are more frequent chromosomal defects in TD patients, significant female predominance (Grüters 1997, Roberts 1997, Devos 1999, Castanet 2001), or racial differences in the incidence (Klett 1997, Roberts 1997).

Pressing questions concerning **genetic basis of TD and non-goitrous CH** enforced us to perform a comprehensive **phenotype-focused molecular-genetic study** and to look in more detail at the cohort of 170 Czech Caucasian paediatric and adolescent patients with permanent primary non-goitre congenital or early-onset hypothyroidism. Using several genetic techniques, five different genes encoding for thyroid transcription factors were investigated according to their known spectrum of defects and based on a detailed phenotype description, with the aim to get a more deep insight into the molecular genetic mechanisms in the pathogenesis of **permanent primary non-goitre congenital or early-onset hypothyroidism**.

3. 2. Study results: Thyroid transcription factors

Results of this study were published as an original article

Al Taji E, Biebermann H, Límanová Z, Hníková O, Zikmund J, Dame C, Grüters A, Lebl J, Krude H. ***Screening for mutations in transcription factors in a Czech cohort of 170 patients with congenital and early-onset hypothyroidism: identification of a novel PAX8 mutation in dominantly inherited early-onset non-autoimmune hypothyroidism.***
Eur J Endocrinol 2007, 156 (5): 521-529. IF 3.239.

This chapter is identical to the published version of the article.

This article has been already citated:

1. Esperante SA, Rivolta CM, Miravalle L, Herzovich V, Iorcansky S, Baralle M, Targovnik HM. Identification and characterization of four PAX8 rare sequence variants (p.T225M, p.L233L, p.G336S and p.A439A) in patients with congenital hypothyroidism and dysgenetic thyroid glands. Clin Endocrinol 2008; 68: 828-835.
2. Banghová K, Al Taji E, Cinek O, Novotná D, Zapletalová J, Hníková O, Lebl J. Pendred syndrome among patients with congenital hypothyroidism detected by neonatal screening: identification of two novel *PDS/SLC26A4* gene mutations. Eur J Pediatr 2008; 167: 777-783.
3. Kumar J, Gordillo R, Kaskel FJ, Druschel CM, Woroniecki RP. Increased prevalence of renal and urinary tract anomalies in children with congenital hypothyroidism. J Pediatr 2009; 154: 263-266.
4. Ramos HE, Nesi-Franca S, Boldarine VT, Pereira RM, Chiamolera MI, Camacho CP, Graf H, de Lacerda L, Carvalho GA, Maciel RMB. Clinical and molecular analysis of thyroid hypoplasia - a population-based approach in Southern Brazil. Thyroid 2009; 19: 61-68.

SCREENING FOR MUTATIONS IN TRANSCRIPTION FACTORS IN A CZECH COHORT OF 170 PATIENTS WITH CONGENITAL AND EARLY-ONSET HYPOTHYROIDISM: IDENTIFICATION OF A NOVEL PAX8 MUTATION IN DOMINANTLY INHERITED EARLY-ONSET NON-AUTOIMMUNE HYPOTHYROIDISM

Eva Al Taji[1], Heike Biebermann[2], Zdeňka Límanová[3], Olga Hníková[1], Jaroslav Zikmund[1], Christof Dame[4], Annette Grüters[2], Jan Lebl [5] and Heiko Krude[2]

[1] Department of Paediatrics, 3rd Faculty of Medicine, Charles University, Prague, Czech Republic

[2] Institute of Experimental Paediatric Endocrinology, University Children´s Hospital Charité, Berlin, Germany

[3] 3rd Medical Clinic, 1st Faculty of Medicine, Charles University, Prague, Czech Republic

[4] Department of Neonatology, Campus Virchow-Klinikum, University Children´s Hospital Charité, Berlin, Germany

[5] Department of Paediatrics, 2nd Faculty of Medicine, Charles University, Prague, Czech Republic

Short title: Novel *PAX8* mutation in non-congenital hypothyroidism

Word count: abstract 246, text 3,641

ABSTRACT

Objective: Mutations in *NKX2.1*, *NKX2.5*, *FOXE1* and *PAX8* genes, encoding for transcription factors involved in the development of the thyroid gland, have been identified in a minority of patients with syndromic and non-syndromic congenital hypothyroidism (CH).

Design: In a phenotype-selected cohort of 170 Czech paediatric and adolescent patients with non-goitre CH, including thyroid dysgenesis (TD), or non-goitre early-onset hypothyroidism *PAX8*, *NKX2.1*, *NKX2.5*, *FOXE1* and *HHEX* genes were analysed for mutations.

Methods: *NKX2.1*, *NKX2.5*, *FOXE1* and *HHEX* genes were directly sequenced in patients with syndromic CH. *PAX8* mutational screening was performed in all 170 patients by single stranded conformation polymorphism, followed by direct sequencing of samples with abnormal findings. The R52P PAX8 mutation was functionally characterized by DNA binding studies.

Results: We identified a novel PAX8 mutation R52P, dominantly inherited in a three-generation pedigree and leading to non-congenital, early-onset, non-goitre, non-autoimmune hypothyroidism with gradual postnatal regression of the thyroid size and function. The R52P PAX8 mutation results in the substitution of a highly conserved residue of the DNA-binding domain with a loss-of-function effect.

Conclusions: The very low frequency of genetic defects in a population-based cohort of children affected by non-goitre congenital and early-onset hypothyroidism, even in a phenotype-focussed screening study, suggests the pathogenetic role of either non-classic genetic mechanisms or the involvement of genes unknown so far. Identification of a novel *PAX8* mutation in particular variant of non-congenital early-onset hypothyroidism indicates a key function of PAX8 in the postnatal growth and functional maintenance of the thyroid gland.

Key words: congenital hypothyrodism, thyroid dysgenesis, transcription factors

INTRODUCTION

Primary congenital hypothyroidism (CH) is the most frequent inborn endocrine disorder (1). It can be caused by impaired thyroid development leading to a variable degree of thyroid dysgenesis (TD) (OMIM 218700) or by dyshormonogenesis (OMIM 274400-274900) when any step of thyroid hormone biosynthesis can be affected. While dyshormonogenesis has been recognized as a genetic disorder mostly with autosomal recessive inheritance due to mutations in genes critical for thyroid hormone synthesis, the molecular pathogenesis of TD representing about 80% of CH cases (2) is still unsolved (reviewed by (3)). In general, TD might be considered as a non-genetic condition due to its mainly sporadic occurrence and only 2% of familial cases (4). In addition, a significant female predominance (5) as well as the discordance of monozygotic twins (6) argues against a classic Mendelian inheritance of TD. However, in few cases a recessive inheritance of thyroid hypoplasia and CH was described due to mutations in the thyroid-stimulating hormone (TSH)-receptor, which proves in general the potential of genetic alterations in thyroid development. Apart from mutations in the TSH-receptor which is expressed during later steps of thyroid development, transcription factors expressed during early steps of thyroid budding and migration are other likely candidate genes for TD, in particular Nkx2.1/Ttf1 (7), Foxe1/Ttf2 (8), Pax8 (9) and Hhex (10). TD in homozygous knock-out mice for *Nkx2.1/Titf1* (11), *Foxe1/Titf2* (12), *Pax8* (13) and *Hhex* (14) revealed the critical role of these genes for normal thyroid development. Interestingly, except in $Pax8^{-/-}$ mice, the rest of the null mutations had major malformations of other organs representing the extrathyroidal expression pattern of the respective transcription factors.

Based on the phenotype of the knock-out mice, mutations of *FOXE1* (15-17) and *NKX2.1* (18) were identified in several patients with syndromic forms of CH. While patients with *FOXE1* mutations are affected by cleft palate in addition to TD (Bamforth-Lazarus syndrome (19), OMIM 241850), patients with *NKX2.1* mutations suffer from choreoathetosis. Mutations of *FOXE1* (OMIM 602617) and *NKX2.1* (OMIM 600635) in non-syndromic CH have not been reported yet (e.g. 20). More recently, mutations in *NKX2.5* (OMIM 600584), that is expressed both in the developing thyroid and heart (21) and which was found to be mutated in various congenital heart defects (e.g. 22), were described in four patients with CH and TD, of whom one was also affected by heart defect (23). Most mutations in patients with TD were found in the *PAX8* gene (OMIM 167415) so far with some familial dominant or sporadic cases of mainly non-syndromic non-goitre CH (24-31).

Although PAX8 is also expressed during renal development, knock-out mice do not manifest an overt kidney phenotype and an additional renal malformation has been described only in two patients (28, 31). Mutations in the human *HHEX* gene (OMIM 604420) have not been identified yet.

Taken together, these data imply that some genetic defects in transcription factors expressed during early steps of thyroid development can be present in a given cohort of patients with TD, besides the majority of cases with a non-mendelian genetic defect. To describe the incidence of these mutations, especially in the case of thyroid transcription factors within a population-based context, we have screened a cohort of 170 patients mostly diagnosed by the Czech nation-wide neonatal screening for CH. The screening was focussed on those phenotypes that had been described before in other large studies investigating each candidate gene separately. In our phenotype-based mutation screening we investigated *PAX8* in all patients with non-goitre congenital or early-onset hypothyroidism as well as *NKX2.1*, *NKX2.5*, *FOXE1* and *HHEX* in several particular patients with CH and associated malformations. We have identified a novel loss-of-function mutation of the *PAX8* gene (R52P) causing non-congenital early-onset hypothyroidism dominantly inherited in three generations of one family.

PATIENTS AND METHODS

Patients

One hundred and seventy Czech Caucasian paediatric and adolescent patients with permanent primary non-autoimmune non-goitre hypothyroidism diagnosed in infancy or early childhood were included in this study. 163 probands were identified within the frame of the Czech nation-wide neonatal screening programme for CH in the period from 1985 to 2002 (the assessment of tT_4 by RIA in 1985 - 1995, since 1996 TSH levels measured by DELPHIA on dry blood spots from a heel prick, TSH cut off 15 mIU/l). Except of six children with compensated hypothyroidism, all cases were characterized by high TSH and low fT_4 and/or tT_4 levels. Five patients were born before routine neonatal screening for CH was established. Two patients had negative screening results, but developed early-onset non-goitre hypothyroidism. With regard to the thyroid morphology determined before the initiation of T_4 treatment, the cohort was composed as shown in Table 1. The classification

is primarily based on ultrasound studies, since ^{99}Tc scintigraphy was provided only in a small number of patients in the early phase of the screening programme.

One hundred and sixty-five patients were unrelated individuals. Additionally, the study cohort included one pair of dizygotic male twins and one pair of dizygotic female twins with its sibling (whose mother had adult-onset hypothyroidism) (Figure 1A and B). Other 22 individuals had a positive family history of variable thyroid disorders: one boy with early-onset hypothyroidism and hypothyroid mother and grandmother, three girls with TD and CH in older brothers, one boy with TD and CH in father, one boy with hemithyroid and euthyroid mother with hemithyroid, two boys and four girls with TD and mothers with adult-onset hypothyroidism, and 10 girls with TD and hypothyroidism in grandparents or siblings of parents (Figure 1C-E).

The occurrence of associated malformations (Table 2) was obtained from medical records or referring physicians. Moreover, screening for kidney malformation by ultrasound was performed in 57 children with TD (hitherto unknown renal malformations were described in three of them).

The study was approved by the institutional ethics committee of the 3rd Faculty of Medicine, Charles University, Prague. Informed consent has been obtained from all subjects or their parents.

Methods

DNA-extraction

Gene analysis was performed with genomic DNA extracted from leukocytes taken from peripheral blood using a modified salting out method (32).

PCR-conditions

PCR reactions were performed using the Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany). PCRs were run in a Gene Amp PCR system 9700 cycler (PE Applied Biosystems, Foster City, CA, USA). Negative controls were always included. PCR products mixed with a loading dye were run on a 1-1.5% agarose gel (Ultra Pure Agarose Gel, Life Technologies, Paisley, Scotland, UK), stained with ethidium bromide and visualized under UV light (all primers and PCR conditions are available upon request).

Direct sequencing

For direct sequencing, PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing reactions were prepared using appropriate primers and the DNA sequencing Kit - Big Dye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems, Warrington, UK) in a 10-µl volume under standard conditions. Sequencing was performed in both directions on ABI PRISM 377 sequencer (PE Applied Biosystems, Foster City, CA, USA). Sequence variations were nomenclatured and numbered according to Ref. (33).

Single stranded conformation polymorphism (SSCP)

Two microlitres of a PCR product were mixed with 14 µl of a formamide stop solution (95% formamide, 10 mM NaOH, 0.1% bromphenol blue, 0.1% xylene cyanol, 10% DMSO), denatured for 5 min and immediately cooled on ice. PCR products were loaded onto a denaturating formamide gel (2.5 ml 95% formamide, 2.5 ml 2 x MDE, 250 µl 10 x TBE, 15 µl TEMED, 80 µl 10% APS) and run on a non-denaturating 1 x MDE gel (1.75 ml 10 x TBE, 17.5 ml 2 x MDE, 14 µl TEMED, 140 µl 10% APS, 16 ml H_2O) using Mutation Detection Enhancement gel solution (BioWhittaker Molecular Applications, Rockland, ME, USA). Electrophoresis was performed in 0.5 x TBE at 2 W constant power at a room temperature for 20-30 hours and at 4 C for 50-60 hours. SSCP bands were detected with the silver staining method using 0.1% silver nitrate according to the standardized protocol, and gels were dried in vacuum. Samples showing an abnormal mobility pattern within the matrix as compared with the wild type control were submitted to the direct sequencing.

Synthesis of wild-type PAX8 and mutant PAX8 R52P proteins

For functional characterization, human PAX8 cDNA was cloned into the ClaI and EcoRI cloning sites of the pCS2+ expression vector (constructed by Dave Turner). Mutant R52P PAX8 was obtained by standard mutagenesis procedure. Wild-type and mutant proteins for shift assay were generated using the *in vitro* cell-free transcription/translation (TnT system, Promega, Mannheim, Germany).

Electrophoretic mobility shift assay (EMSA)

EMSA was prepared as described previously (34). 4 µg of human wild-type PAX8 or mutant PAX8 R52P proteins were incubated with 1 µg BSA, 0.1 µg herring sperm

DNA, and 0.5 μg poly[dI-dC] in the binding buffer (10 mmol/l TrisHCl pH 7.5, 1 mmol/l EDTA, 4% Ficoll 1 mmol/l dithiothreitol and 2 mmol/l PMSF). In the competition experiment, a 800-fold excess of cold wild-type competitor was added. Reactions were incubated for 20 min at 30 C. Then, 100 fmol of an end-radiolabeled 24 bp double-stranded oligonucleotide containing the rat PAX8 binding site, located at –72 nt to –66 nt relative to the transcription start site of the thyroglobulin gene (35), has been added to the reaction and incubated for additional 45 min at 30 C. Immediately after incubation, samples were loaded onto a 5% polyacrylamide gel in 0.5x TBE buffer (pH 8.3). Electrophoresis was performed at 34 mA at 4 C for approximately 2.5 hours. After drying of the gel, complex formation was visualized by autoradiography overnight. The following double-stranded oligonucleotide was used in the experiment: 5′-CACTGCCCAGTCAAGTGTTCTTGA-3′ (only the non-coding strand is shown and the consensus PAX8 binding site is underlined; National Center for Biotechnology Information [NCBI] accession no. X06162) (29).

RESULTS

The aim of the study was to describe the prevalence of transcription factor gene mutations in TD patients based on previously described phenotypes. Those patients with associated malformations already described as a part of the phenotype in *NKX2.1*-, *FOXE1*- and *NKX2.5*-gene mutation carriers were primarily analysed for mutations in these respective candidate genes. One patient with a central nervous system midline defect was screened for *HHEX* gene mutations based on the knock-out mice phenotype although no mutation has been found in TD up to now. In addition all patients were screened for mutations in the *PAX8* gene, since most of the patients with identified *PAX8* gene mutations so far had isolated TD.

NKX2.5, *NKX2.1*, *FOXE1* and *HHEX* genes

Fifteen patients with heart defects were investigated for *NKX2.5* mutations. A male patient with CH due to TD (eutopic hypoplastic thyroid gland) associated with the neonatal respiratory distress and perinatal asphyxia, severe neurological impairment (central muscular hypotony, movement disorder, paroxysms, psychomotor retardation) and congenital hydronephrosis was analysed for *NKX2.1* mutations. A female patient with CH due to TD (eutopic hypoplastic thyroid gland), associated with multiple congenital

anomalies, including cleft palate, and severe psychomotor retardation, was tested for *FOXE1*. One female patient with mild forms of cerebral (cavum septum pelucidum) and thyroid dysgenesis (mild hypoplasia) was investigated for *HHEX* mutations, since the complex phenotype of the *Hhex*$^{-/-}$ mouse includes a brain midline structure defect in addition to TD.

No significant sequence variations were observed in all four genes. Among 15 patients with CH and associated congenital cardiac malformations, two known sequence variations of the *NKX2.5* gene were found: c.63A>G (p.E21E) in 7 heterozygotes and 1 homozygote and c.*61T>G in 8 heterozygotes and 4 homozygotes. Direct sequencing of the *FOXE1* revealed the heterozygous polymorphisms c.819 C>T (p.S273S) and c.510C>A (p.A170A) in 14 residues alanine stretch.

***PAX8* gene**

Based on the thyroid phenotype described so far in *PAX8* mutation carriers, we screened all patients with CH due to TD for mutations in the *PAX8* gene as well as all patients with congenital or early-onset non-goitre hypothyroidism, including familial cases with dominant inheritance. Exon 2 with the ATG initiation codon and exons 3-4 encoding for the DNA-binding paired domain were screened by SSCP in all 170 individuals. In 34 of those, suffering either from renal malformations or with positive family history, all coding exons 2-12 were screened by SSCP. In addition exons 3-4 have been also sequenced.

In a boy with unsuspicious neonatal screening for CH, but early postnatal regression of the thyroid size and function, we identified a novel heterozygous missense mutation in exon 3. The mutation changes a G in position 155 into a C (numbering starts with the adenine nucleotide at the ATG initiation codon in exon 2) (Figure 2B), leading to an amino-acid exchange of a highly conserved arginine to prolin at codon 52 (R52P) in the DNA-binding domain of PAX8. The same mutation was detected in his hypothyroid mother and maternal grandmother, both treated for early-onset non-autoimmune hypothyroidism. Neither siblings of mother nor maternal great grandmother carried the mutation. The mutation has not been documented in any of 100 chromosomes of 50 subjects randomly selected from Czech Caucasian non-CH paediatric patients.

Since the R52P mutation cosegregates with a hypothyroid phenotype in a three-generation pedigree (Figure 2A), affects a highly conserved amino-acid and is absent from normal DNA samples, we hypothesised a loss-of-function effect. After cloning of the mutation into the wild type PAX8 expression vector and production of wild type and R52P

mutant PAX8 proteins in a transcription/translation system, we found a complete loss of binding of the PAX8 R52P mutant to the thyroglobulin promoter oligonucleotide C, that indicates a loss-of-function effect of the mutation (Figure 3).

Detailed clinical presentation of R52P-PAX8-gene mutation carriers

The index patient was born at term (birth weight 3500 g, length 51 cm) as the first son and only child of non-consanguineous parents after an apparently normal pregnancy (mother was treated with combined T_4 and T_3 substitution for non-autoimmune permanent hypothyroidism) in 1999. Neonatal screening for CH was negative (TSH 9.49 mIU/l, cut off 15 mIU/l), and therefore no further detailed thyroid functional data are available. The diagnosis of hypothyroidism was established when he was still asymptomatic at the age of 18 months due to the positive family history of non-autoimmune permanent hypothyroidism in his mother and grandmother. TSH was remarkably elevated (183.9 mIU/l, normal range 0.25-5 mIU/l), fT_4 and fT_3 were low (fT_4 7.19 pmol/l, normal range 10-26 pmol/l, fT_3 3.32 pmol/l, normal range 4.2-8.1 pmol/l). His body length was 87 cm. Ultrasound examination showed a eutopic thyroid gland of a normal structure, but at a lower end of a normal size (total volume 1 ml, own normative data for this age 1.39±0.31 ml). Subsequently, under T_4 substitution, the thyroid gland did not grow properly and even got reduced in its size. The tissue structure changed into the hyperechogenic fibrous rudiment (total volume 0.45 ml in 3 years, own normative data 1.82±0.53 ml) (Figure 2C). At the age of 3 years, blood samples taken after 4 weeks of the withdrawal of treatment confirmed permanent hypothyroidism (TSH 100 mIU/l, fT_4 4.74 pmol/l) with low thyroglobulin levels, relative to elevated TSH (57 μg/l to 33 μg/l, normal range 30-85 μg/l). His psychomotor and physical development is normal. His mother, born in 1974 before the initiation of screening programme for CH, was diagnosed at the age of 6 months due to the diagnosis of permanent hypothyroidism of her mother, born in 1953 and diagnosed at the age of 3 years because of her short stature and obesity. No thyroid imaging study was performed prior to treatment, but recent ultrasound examinations showed a eutopic rudimental thyroid gland in mother and grandmother. Antithyroid antibodies were not detectable in all three carriers of the PAX8 R52P mutation. There were no detectable abnormalities of the structure or function of the kidneys.

DISCUSSION

To our knowledge, this investigation of 170 patients, mostly diagnosed by the Czech nation-wide neonatal screening for CH, represents one of the largest cohorts systematically screened for defects in thyroid transcription factors so far.

However, despite the fact that we focussed the analysis of *NKX2.5*, *NKX2.1* and *FOXE1* genes on TD patients with associated malformations or complications that have been previously reported, such as movement defects (*NKX2.1)*, heart defects (*NKX2.5)* or cleft palate (*FOXE1)*, we did not find any mutations in these particular subgroups of patients. Our data confirm that the mutation rate of the three candidate genes *NKX2.1*, *NKX2.5* and *FOXE1* is very low, even in a phenotype-focussed study. Other mechanisms, such as epigenetic or somatic changes that are not inherited, could cause the inactivation of these three respective candidate genes. Alternatively, unknown genes, functionally similar to *NKX2.1*, *NKX2.5* and *FOXE1*, might be involved in the pathogenesis of these particular cases with associated malformations.

Searching for *PAX8* gene mutations in this large cohort of 170 Czech patients only revealed one novel loss-of-function mutation leading to non-congenital, non-autoimmune, but early-onset hypothyroidism dominantly inherited in three generations (Figure 2). All three affected members of the index family were diagnosed as heterozygous carriers of a novel R52P mutation in the DNA-binding paired domain (Figure 4C-D). The PAX8 paired domain is a 128 amino-acid DNA-binding domain highly conserved in the human PAX protein family, showing sequence similarity to the Drosophila paired protein (36). It consists of two structurally independent subdomains, each containing a helix-turn-helix motif joined by a linker region (37) (Figure 4D). Except of two mutations (24, 29), all *PAX8* mutations published so far (24-28, 30) are located within the N-terminal subdomain (Figure 4C and D). At the structural level, these mutations either affect residues directly contacting DNA at the protein-DNA interface or residues involved in the folding and stability of the protein. At the functional level, most of heterozygous mutations located in the N-terminal subdomain are known to reduce binding affinity to a specific DNA sequence (24, 25, 27, 28).

The alignment of Drosophila and nine human PAX proteins paired domains (Figure 4B) indicates that the arginine at codon 52 of the N-terminal part of the PAX8 paired domain is highly conserved in PAX proteins 1- 3, 5, 7- 9. It is also a homolog of the arginine residue at position 44 of Drosophila paired protein, located between the second

and third α-helix (37). It interacts with the loop between two strands of the β sheet, and stabilizes the docking of the β turn together with other protein-protein and protein-DNA contacts making a critical base that allows contacts in the minor groove of the DNA (37). Taken together, these structural and evolutional considerations suggest that the R52P mutation leads to loss of DNA binding confirmed by our DNA binding studies (Figure 3).

The phenotypical expression of the R52P PAX8 mutation seems to be unique because the index patient was shown to be normal in the newborn screening for CH, but later on at the age of 18 months diagnosed with severe hypothyroidism. Obviously, the R52P mutation carrier, identified in our study, would have been missed in all other cohorts screened so far for *PAX8* gene mutations and focussed solely on CH patients identified in screening programmes. However, the mild phenotype in the index patient of one previously described familial case of *PAX8* gene mutation (24), characterized by a borderline elevated screening TSH and normal T_4 in the newborn period, but aggravating hypothyroidism during the first month of life, resembles the findings in our R52P mutation index case. Taken together, the findings in the R52P mutation carriers in our study and the familial case presented before (24) argue that PAX8 might not play only a role during early thyroid organogenesis, but also participate in the postnatal maintenance of thyroid function. Thus, our findings suggest that even if the results of neonatal screening for CH are negative, and thyroid gland is correctly developed and apparently normal, *PAX8* gene deficiency can cause hypothyroidism in the early postnatal life due to the gradual growth and functional impairment. Therefore the search for *PAX8* gene mutations might be extended to those rare patients with early-onset hypothyroidism who were negative in CH screening.

The exact molecular mechanism responsible for the insufficient postnatal growth and functional regression of the thyroid gland in PAX8 deficiency is still unclear. However, Pax8 is essential for the formation of thyroid follicular structures (13) and has a fundamental role in the initiation of the thyroid cell differentiation and in the maintenance of the differentiated state (38). Thereby PAX8 deficiency may also result in postnatal dysregulation of the proliferation of thyroid cells and follicles or alternatively in a reduced survival due to an increased apoptotic degradation of thyroid follicular structures. Thus, identification of the new R52P PAX8 mutation leading to a hitherto not described dominantly inherited form of hypothyroidism with an early non-congenital-non-autoimmune onset implies an additional role of PAX8 in the postnatal maintenance of thyroid function.

Overall our study confirms the very low prevalence of mutations in the known thyroid transcription factor genes in TD. Taking into account the rare occurrence of TSH-receptor gene mutations described before, it seems obvious that even the search in a phenotype-focussed strategy will not reveal a significant number of additional genetic defects in the known candidate genes for TD. More efforts to identify non-classical genetic defects like an epigenetic silencing of critical candidate genes in TD are mandatory to unravel the pathogenesis of TD.

ACKNOWLEDGEMENTS

This study was supported by Research Project MSM 0021620814 of the Czech Ministry of Education and „Sonderforschungs Bereich SFB" Nr. 577-A10 of the German Research Council. C. D. is supported by the Deutsche Forschungsgemeinschaft (Da 484/2-1). E.A.T. was a recipient of the DAAD (Deutscher Akademischer Austauschdienst) short research-term scholarship. The authors are grateful to Nele Haufs and Nathalie Renault for the cooperation in designing the primers, Ingeborg Gläser, Sabine Jyrch, Helena Francová and Eva Mattušová for excellent technical assistance, and Petr Kračmar (Central Laboratory for CH Neonatal Screening, Prague) for providing screening data. We appreciate an outstanding cooperation of all Czech patients, their families and endocrinologists involved in this nation-wide study (especially M. Finková, V. Janštová, J. Klabochová, E. Nováková, D. Novotná, R. Pomahačová, J. Škvor, H. Vávrová, J. Venháčová, J. Zapletalová).

REFERENCES

1. Toublanc JE. Comparison of epidemiological data on congenital hypothyroidism in Europe with those of other parts of the world. *Hormone Research* 1992 **38** 230-235.
2. Grüters A, Finke B, Krude H & Meinhold H. Etiological grouping of permanent congenital hypothyroidism with a thyroid gland in situ. *Hormone Research* 1994 **41** 3-9.
3. De Felice M & Di Lauro R. Thyroid development and its disorders: genetics and molecular mechanisms. *Endocrine Reviews* 2004 **25** 722-746.
4. Castanet M, Lyonnet S, Bonaïti-Pellié C, Polak M, Czernichow P & Léger J. Familial forms of thyroid dysgenesis among infants with congenital hypothyroidism. *New England Journal of Medicine* 2000 **343** 441-442.
5. Devos H, Rodd C, Gagné N, Laframboise R & Van Vliet G. A search for the possible molecular mechanisms of thyroid dysgenesis: sex ratios and associated malformations. *Journal of Clinical Endocrinology and Metabolism* 1999 **84** 2502-2506.
6. Perry R, Heinrichs C, Bourdoux P, Khoury K, Szöts F, Dussault JH, Vassart G & Van Vliet G. Discordance of monozygotic twins for thyroid dysgenesis: implications for screening and for molecular pathophysiology. *Journal of Clinical Endocrinology and Metabolism* 2002 **87** 4072-4077.
7. Lazzaro D, Price M, De Felice M & Di Lauro R. The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and restricted regions of the foetal brain. *Development* 1991 **113** 1093-1104.
8. Zannini M, Avantaggiato V, Biffali E, Arnone M, Sato K, Pischetola M, Taylor BA, Phillips SJ, Simeone A & Di Lauro R. TTF-2, a new forkhead protein, shows a temporal expression in the developing thyroid which is consistent with a role in controlling the onset of differentiation. *EMBO Journal* 1997 **16** 3185-3197.
9. Plachov D, Chowdhury K, Walther C, Simon D, Guenet JL & Gruss P. Pax-8, a murine paired box gene expressed in the developing excretory system and thyroid gland. *Development* 1990 **110** 643-651.
10. Thomas PQ, Brown A & Beddington R. Hex: a homeobox gene revealing peri-implantation assymmetry in the mouse embryo and an early transient marker of endothelial cell precursors. *Development* 1998 **125** 85-94.

11. Kimura S, Hara Y, Pineau T, Fernandez-Salguero P, Fox CH, Ward JM & Gonzalez FJ. The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes & Development* 1996 **10** 60-69.
12. De Felice M, Ovitt C, Biffali E, Rodriguez-Mallon A, Arra C, Anastassiadis K, Macchia PE, Mattei MG, Mariano A, Schöler H, Macchia V & Di Lauro R. A mouse model for hereditary thyroid dysgenesis and cleft palate. *Nature Genetics* 1998 **19** 395-398.
13. Mansouri A, Chowdhury K & Gruss P. Follicular cells of the thyroid gland require Pax8 gene function. *Nature Genetics* 1998 **19** 87-90.
14. Martinez Barbera JP, Clements M, Thomas P, Rodriguez T, Meloy D, Kioussis D & Beddington RSP. The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation. *Development* 2000 **127** 2433-2445.
15. Clifton-Bligh RJ, Wentworth JM, Heinz P, Crisp MS, John R, Lazarus JH, Ludgate M & Chatterjee VK. Mutation of the gene encoding human TTF-2 associated with thyroid agenesis, cleft palate and choanal atresia. *Nature Genetics* 1998 **19** 399-401.
16. Castanet M, Park SM, Smith A, Bost M, Léger J, Lyonnet S, Pelet A, Czernichow P, Chatterjee K & Polak M. A novel loss-of-function mutation in TTF-2 is associated with congenital hypothyroidism, thyroid agenesis and cleft palate. *Human Molecular Genetics* 2002 **11** 2051-2059.
17. Baris I, Arisoy AE, Smith A, Agostini M, Mitchell CS, Park SM, Halefoglu AM, Zengin E, Chatterjee VK, Battaloglu E. A novel missense mutation in human TTF-2 (FKHL15) gene associated with congenital hypothyroidism but not athyreosis. *Journal of Clinical Endocrinology and Metabolism* 2006 **91** 4183-4187.
18. Krude H, Schütz B, Biebermann H, von Moers A, Schnabel D, Neitzel H, Tönnies H, Weise D, Lafferty A, Schwarz S, De Felice M, von Deimling A, van Landeghem F, Di Lauro R & Grüters A. Choreoathetosis, hypothyroidism, and pulmonary alterations due to human NKX2-1 haploinsufficiency. *Journal of Clinical Investigation* 2002 **109** 475-480.
19. Bamforth JS, Hughes IA, Lazarus JH, Weaver CM & Harper PS. Congenital hypothyroidism, spiky hair, and cleft palate. *Journal of Medical Genetics* 1989 **26** 49-60.

20. Lapi P, Macchia PE, Chiovato L, Biffali E, Moschini L, Larizza D, Baserga M, Pinchera A, Fenzi GF & Di Lauro R. Mutation in the gene encoding thyroid transcription factor-1 (TTF-1) are not a frequent cause of congenital hypothyroidism (CH) with thyroid dysgenesis. *Thyroid* 1997 **7** 383-387.
21. Lints TJ, Parson LM, Hartley L, Lyons I & Harvey RP. Nkx2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development* 1993 **119** 419-431.
22. Benson DW, Silberbach GM, Kavanaugh-McHugh A, Cottrill C, Zhang Y, Riggs S, Smalls O, Johnson MC, Watson MS, Seidman JG, Seidman CE, Plowden J & Kugler JD. Mutations in the cardiac transcription factor NKX2.5 affect diverse cardiac developmental pathways. *Journal of Clinical Investigation* 1999 **104** 1567-1573
23. Dentice M, Cordeddu V, Rosica A, Ferrara AM, Santarpia L, Salvatore D, Chiovato L, Perri A, Moschini L, Fazzini C, Olivieri A, Costa P, Stoppioni V, Baserga M, De Felice M, Sorcini M, Fenzi G, Di Lauro R, Tartaglia M & Macchia PE. Missense mutation in the transcription factor NKX2-5: a novel molecular event in the pathogenesis of thyroid dysgenesis. *Journal of Clinical Endocrinology and Metabolism* 2006 **91** 1428-1433
24. Macchia PE, Lapi P, Krude H, Pirro MT, Missero C, Chiovato L, Souabni A, Baserga M, Tassi V, Pinchera A, Fenzi G, Grüters A, Busslinger M & Di Lauro R. PAX8 mutations associated with congenital hypothyroidism caused by thyroid dysgenesis. *Nature Genetics* 1998 **19** 83-86.
25. Vilain C, Rydlewski C, Duprez L, Heinrichs C, Abramowicz M, Malvaux P, Renneboog B, Parma J, Costagliola S & Vassart G. Autosomal dominant transmission of congenital thyroid hypoplasia due to loss-of-function mutation of PAX8. *Journal of Clinical Endocrinology and Metabolism* 2001 **86** 234-238.
26. Komatsu M, Takahashi T, Takahashi I, Nakamura M, Takahashi I & Takada G. Thyroid dysgenesis caused by PAX8 mutation: the hypermutability with CpG dinucleotides at codon 31. *Journal of Pediatrics* 2001 **139** 597-599.
27. Congdon T, Nguyen LQ, Nogueira CR, Habiby RL, Medeiros-Neto G & Kopp P. A novel mutation (Q40P) in PAX8 associated with congenital hypothyroidism and thyroid hypoplasia: evidence for phenotypic variability in mother and child. *Journal of Clinical Endocrinology and Metabolism* 2001 **86** 3962-3967.
28. Meeus L, Gilbert B, Rydlewski C, Parma J, Roussie AL, Abramowicz M, Vilain C,

Christophe D, Costagliola S & Vassart G. Characterization of a novel loss of function mutation of PAX8 in a familial case of congenital hypothyroidism with in-place, normal-sized thyroid. *Journal of Clinical Endocrinology and Metabolism* 2004 **89** 4285-4291.

29. de Sanctis L, Corrias A, Romagnolo D, Di Palma T, Biava A, Borgarello G, Gianino P, Silvestro L, Zannini M & Dianzani I. Familial PAX8 small deletion (c.989-992delACCC) associated with extreme phenotype variability. *Journal of Clinical Endocrinology and Metabolism* 2004 **89** 5669-5674.
30. Grasberger H, Ringkananont U, Lefrancois P, Abramowicz M, Vassart G & Refetoff S. Thyroid transcription factor 1 rescues PAX8/p300 synergism impaired by a natural PAX8 paired domain mutation with dominant negative activity. *Molecular Endocrinology* 2005 **19** 1779-1791.
31. Krude H, Macchia PE, Di Lauro R & Grüters A. PAX-8 mutations associated with congenital hypothyroidism caused by thyroid dysgenesis (abstract). *Hormone Research* 1998 **50** (Suppl 3) 17.
32. Miller SA, Dykes DD & Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research* 1988 **16** 1215.
33. den Dunnen JT & Antonarakis E. Nomenclature for the description of human sequence variations. *Human Genetics* 2001 **109** 121-124.
34. Dame C, Sola MC, Lim KC, Leach KM, Fandrey J, Ma Y, Knöpfle G, Engel JD & Bungert J. Hepatic erythropoietin gene regulation by GATA-4. *Journal of Biological Chemistry* 2004 **279** 2955-2961.
35. Musti AM, Avvedimento EV, Polistina C, Ursini VM, Obici S, Nitsch L, Cocozza S & Di Lauro R. The complete structure of the rat thyroglobulin gene. *Proceedings of the National Academy of Sciences of the U.S.A*. 1986 **83** 323-327.
36. Bopp D, Burri M, Baumgartner S, Frigerio G & Noll M. Conservation of a large protein domain in the segmentation gene paired and in functionally related genes of Drosophila. *Cell* 1986 **47** 1033-1040.
37. Xu W, Rould MA, Jun S, Desplan C & Pabo CO. Crystal structure of a paired domain-DNA complex at 2.5A resolution reveals structural basis for Pax developmental mutations. *Cell* 1995 **80** 639-650.
38. Pasca di Magliano M, Di Lauro R & Zannini M. Pax8 has a key role in thyroid cell differentiation. *Proceedings of the National Academy of Sciences of the U.S.A*. 2000 **97** 13144-13149.

TABLES

Table 1

Thyroid morphology and sex ratios in the examined group of patients

Thyroid dysgenesis[a]					**Normal thyroid**[b]	**N.A.**[c]
athyreosis	ectopy	hemithyroid	hypoplasia	rudiment		
26	8	1	36	39		
110					**39**	**21**
F/M=2.5					F/M=1.4	F/M=4.2

Total 170 (F/M=2.3)

Thyroid morphology was described according to thyroid imaging studies (ultrasonography or scintigraphy) performed before the beginning of T_4 treatment.

[a] The distribution of various forms of TD and low number of ectopies can be attributed to the image technique used - in most of patients the description was based solely on ultrasound imaging. Scintigraphy was performed just in the early beginning of CH screening and it was done only in a minority of patients (all cases of ectopy were diagnosed by scintigraphy). According to ultrasound findings, a eutopic thyroid gland with a size under normal age and sex limits but with normal structure was described as "hypoplasia". The term "rudiment" was used if only eutopic remnants of thyroid tissue with a high degree of fibrous rearrangement were visible by ultrasound (corresponding to hyperechogenic ultrasound signals).

[b] In the group of patients with a eutopic thyroid gland of a normal size and structure, gradual postnatal regression of the thyroid size and function developed in four patients with initially compensated hypothyroidism and in one patient with negative screening results.

[c] Thyroid imaging before the beginning of T_4 treatment was not done or results are not available. No palpable goitre was observed in physical examination but the thyroid morphology was not further characterized.

N.A. - not available, F/M - female to male ratio.

Table 2

Associated cardiac and renal malformations in the examined group of patients

Associated malformations (n=19)					
cardiac (n=12)		**renal** (n=4)		**renal + cardiac** (n=3)	
PDA	1	polycystosis	1	congenital hydronephrosis	
PFO	3			+ASD+VSD [d]	1
PDA+PFO	1	unilateral agenesis	1		
PDA+ASD	1			horse-shoe kidney+PFO	1
VSD	2	congenital			
AVC[a]	1	hydronephrosis[c]	1	mild renal abnormalities+PFO	1
MVP	1				
SPA[b]	2	cortical cyst	1		
NKX2.5		***PAX8***		***PAX8 + NKX2.5***	

[a] Karyotype 47, XY, +21, [b] karyotype 46, XY, 15p+ in 1 patient, [c] male patient with CH (TD) associated with the neonatal respiratory distress and perinatal asphyxia, severe neurological impairment and congenital hydronephrosis (analysed also for *NKX2.1*),

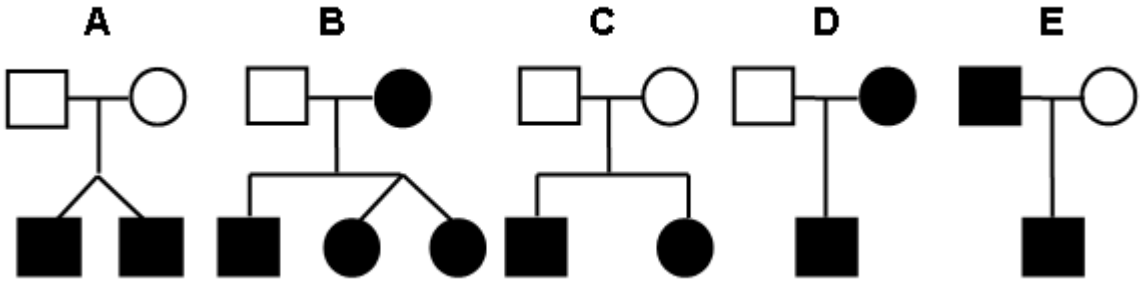
[d] female patient with CH (TD), multiple congenital anomalies (renal and cardiac malformations, cleft palate) and severe psychomotor retardation (analysed also for *FOXE1)*. Investigated candidate genes were proposed due to the spectrum of associated congenital malformations, moreover all patients were screened for *PAX8* mutations due to their non-goitre phenotype. One female patient with mild forms of cerebral and thyroid dysgenesis investigated for *HHEX* was not included to the table.

ASD - atrial septal defect, AVC - atrioventricular channel, PFO - patent foramen ovale, MVP - mitral valve prolaps, PDA - patent ductus arteriosus, SPA - stenotic pulmonary artery, VSD - ventricular septal defect.

FIGURES

Figure 1

Selected familial cases of congenital hypothyroidism and/or thyroid dysgenesis



A Dizygotic male twins: CH diagnosed by neonatal screening (TSH 33.5 mIU/l and 23 mIU/l), mild thyroid hypoplasia. Parents euthyroid.

B Dizygotic female twins: CH diagnosed by neonatal screening (TSH 125.6 mIU/l, tT_4 55.2 nmol/l and TSH 127.5 mIU/l, tT_4 25 nmol/l), normal thyroid. Older brother: CH diagnosed by neonatal screening (TSH 116 mIU/l, tT_4 23 nmol/l), severe thyroid hypoplasia. Mother: mild adult-onset hypothyroidism, normal thyroid.

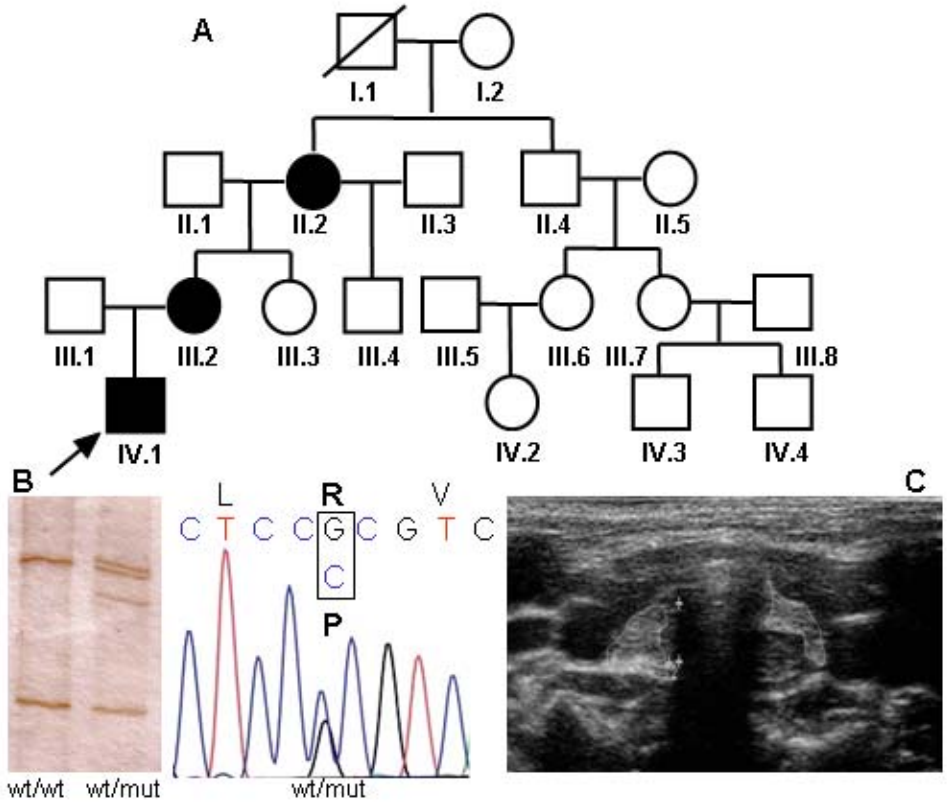
C Female patient: CH diagnosed by neonatal screening (TSH 192.8 mIU/l, tT_4 47 nmol/l), mild thyroid hypoplasia. Older brother: CH diagnosed by neonatal screening (TSH 150 mIU/l, tT_4 36 nmol/l), thyroid morphology not known. Parents euthyroid.

D Male patient: compensated CH diagnosed by neonatal screening (TSH 21 mIU/l, tT_4 159 nmol/l), hemithyroid (severely hypoplastic left thyroid lobe), gradually decreasing thyroid function, substitution treatment since 4 months of age. Afterwards mother diagnosed with asymptomatic hypoplastic right thyroid lobe.

E Male patient: compensated CH diagnosed by neonatal screening (TSH 43 mIU/l, fT_4 36.5 pmol/l), thyroid hypoplasia. Father treated with T_4 first 3 years of life, clinical data not available. Normal ranges: fT_4 10-26 pmol/l, tT_4 60-160 nmol/l, TSH 0.25-5 mIU/l, neonatal screening TSH cut off 15 mIU/l. Thyroid morphology was assessed by ultrasound prior to substitution treatment.

Figure 2

Identification of the R52P PAX8 mutation in a three-generation pedigree



A Pedigree: I.1 - dead of cardiac failure in 62 years, no obvious thyroid abnormalities, I.2 and III.4 - normal thyroid morphology and function, R52P mutation not present, II.4 - refused further investigation, III.3 - eutopic thyroid gland, asymptomatic thyroid nodule, R52P mutation not present, II.2, III.2, IV.1 - early-onset non-goitre non-autoimmune hypothyroidism, heterozygous carriers of the R52P PAX8 mutation.

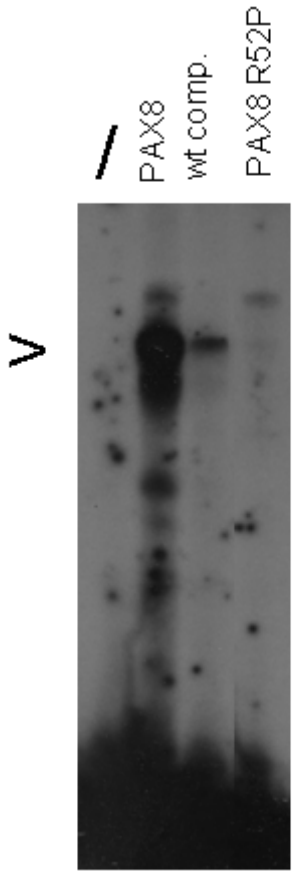
B Identification of the mutation: SSCP of exon 3 (left) (1x MDE gel, room temperature, power 2W, run time 20 hours) - abnormal bands in the index patient compared with the wild type control, part of the direct sequencing of exon 3 (right): heterozygous c.155G >C transition leading to the amino-acid exchange p.R52P.

C Thyroid rudiment (transversal ultrasound imaging) in the index patient at the age of 3 years. SSCP - single stranded conformation polymorphism, wt - wild type allele, mut - allele carrying mutation.

Figure 3

DNA binding analysis

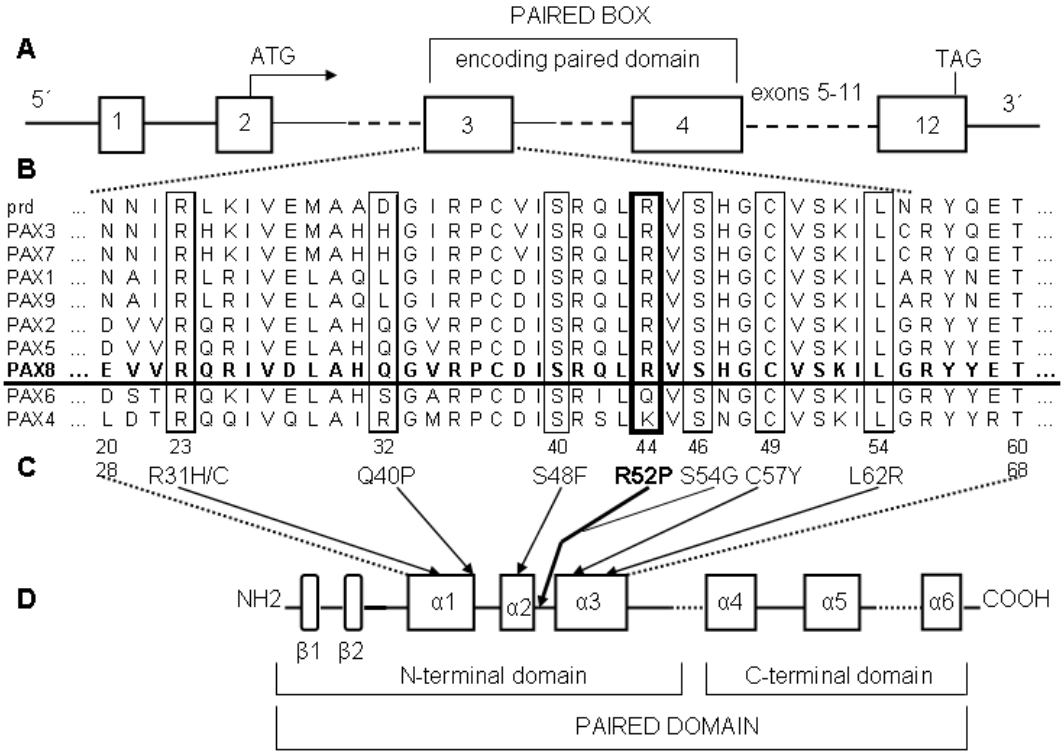
.



In vitro translated wild-type (wt) PAX8 or mutant PAX8 R52P protein binding to an oligonucleotide probe corresponding to the thyroglobulin promoter. The specific complex was identified by competition with unlabeled wild-type oligonucleotide (>). The novel PAX8 R52P mutation results in a loss of binding to the thyroglobulin promoter element.

Figure 4

Position of R52P in the PAX8 paired domain



A Partial schematic presentation of human *PAX8* gene.

B Alignment of Drosophila paired domain (prd) and nine human PAX paired domains (sequences composing α1- α3 helices) according to the sequence similarity and common structural features of PAX proteins (37). Amino-acids affected by mutations in human PAX8 and corresponding amino-acids of other PAX proteins are boxed.

C PAX8 mutations in the N-terminal subdomain of the paired domain detected so far (numbering corresponding to human PAX8 numbering): R31H (24), R31C (26), Q40P (27), S48F (30), R52P (present study), S54G (28), C57Y (25), L62R (24), numbering corresponding to Drosophila paired protein above.

D Schematic presentation of the secondary structure of the paired domain (37) with marked positions of PAX8 mutations: the N-terminal part (PAI subdomain) composed of a β sheet (β1 and β2 strand), a type II β turn (thickened line), three α helices and a C-terminal tail, connected with a linker to the C-terminal subdomain (RED subdomain) containing three α helices.

4. STUDY 2: GENETIC BACKGROUND OF THYROID DYSHORMONOGENESIS

4.1. Introduction

4.1.1. Thyroid hormone biosynthesis

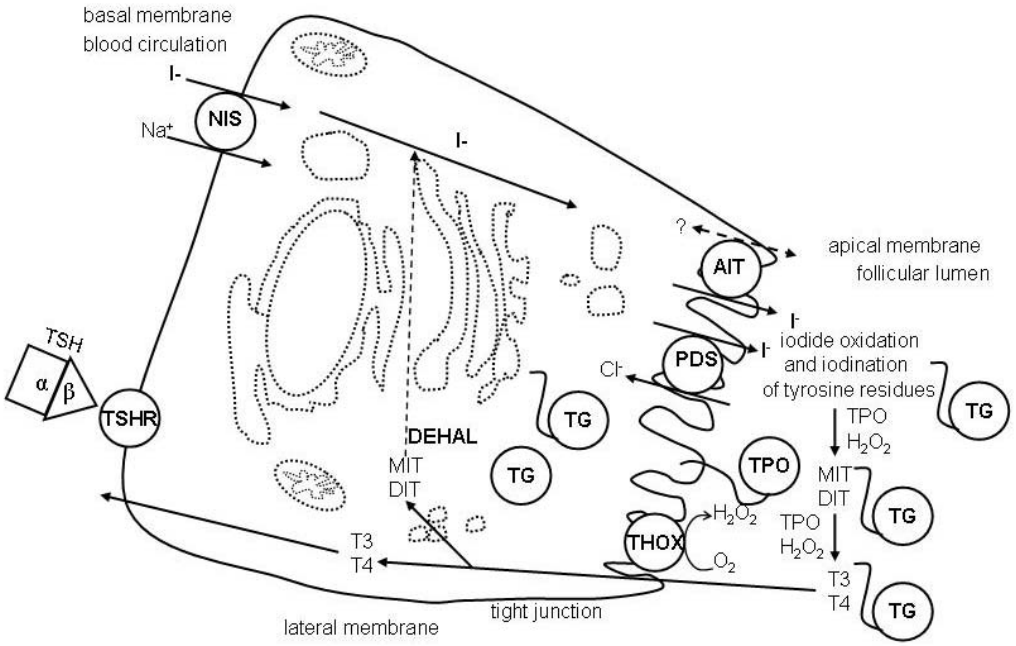
Thyroid hormone biosynthesis is a rather sophisticated process including several biochemical steps. Iodide uptake at the basolateral membrane is arranged by the perchlorate-sensitive sodium-iodide symporter (**NIS**) (for review see Dohan 2003). Efflux of iodide at the apical membrane of TFCs is mediated by the anion exchanger **pendrin** (**SLC26A4**) (Yoshida 2002) and by the apical iodide transporter (**AIT**) (Rodriguez 2002). On the luminal side of the apical membrane, the membrane bound enzyme thyroid peroxidase (**TPO**) (reviewed by McLachlan 1992) oxides iodide and subsequently iodinates tyrosine residues in the intrafollicular thyroglobulin (**TG**). The oxidation of iodide and its covalent binding to tyrosyl residues in the protein matrix of TG is known as the iodide **organification.** This process depends on four factors: TPO activity, generation of peroxide (H_2O_2), availability of substrates - iodide and the protein matrix - TG, and a normal spatial organization of these components at the apical membrane of the follicular cell (Medeiros-Neto 1993). **TG** is the key element in thyroid hormone synthesis and storage. Hydrogen peroxide, as an essential cofactor, is produced by recently described two NADPH oxidases - **THOX1** and **THOX2** (also known as DUOX) (De Deken 2000). Subsequently, the iodinated tyrosines (**MIT** and **DIT**) are coupled to form T_4 or T_3, a reaction that is also catalyzed by TPO and that is called **coupling**. Then, after entering the follicular cell, TG is hydrolyzed, and T_4 and T_3 secreted into the blood at the basolateral membrane. The iodotyrosines MIT and DIT are **deiodinated** by an intrathyroidal dehalogenase (**DEHAL**) (Gnidehou 2004) and recycled for further hormone synthesis (Figure 3).

4.1.2. Molecular pathogenesis of thyroid dyshormonogenesis

Defects in any of these steps required for thyroid hormone production lead to **thyroid dyshormonogenesis** which typically manifests as **permanent CH** and **goitre**. Dyshormonogenesis cases compose a minor part of patients with CH. Nevertheless, these inborn errors of thyroid hormone synthesis can manifest also as **transient CH** or **postnatal non-autoimmune goitrous hypothyroidism** (Chart 5). In recent years, progress has been achieved in the clarification of thyroid dyshormonogenesis at the molecular level. Various

defects caused by inactivating mutations in several candidate genes encoding for iodide transporters, thyroid enzymes or TG, mostly with autosomal recessive inheritance, have been reported so far (for overview see e.g. de Vijlder 1997, Gillam and Kopp 2001, de Vijlder 2003, Park and Chatterjee 2005).

Figure 3 - Thyroid follicular cell



Adapted according to Al Taji E et al. About Roman whose thyroid gland grew too much. In: Lebl J., Macek M. Case histories in molecular genetics. Prague, Galén 2006, 96-98.

AIT - apical iodide transporter, DEHAL - dehalogenase, NIS - natrium-iodide symporter, PDS - pendrin, TG - tyreoglobulin, THOX - thyroid oxidase, TPO - thyroid peroxidase, TSHR - thyrotropin receptor.

4.1.2.1. Defects of iodide transport

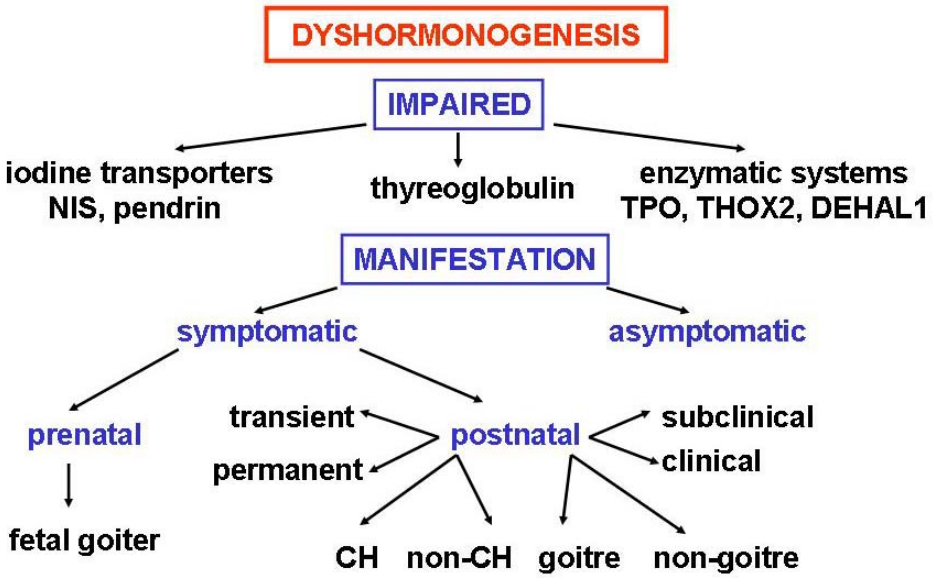
Defects of iodide transport afftect the transport of iodide from blood circulation into TFCs or from TFCs to the follicular lumen.

NIS (**SLC5A5**, gene map locus 19p13.2-p12, OMIM 601843, overviewed by Shen 2001, Dohan 2003, Riesco-Eizaguirre 2006) actively transports iodide across the basolateral plasma membrane of the TFCs. Except of thyroid, it is expressed in other organs concentrating iodide (stomach, salivary glands). It also regulates placental iodide transport from maternal to fetal side and iodide transport in lacting mammary gland (Lacroix 2001).

Mutations of *NIS*, following the autosomal recessive mode of inheritance, lead to iodide trapping or accumulation defects (**ITD**, OMIM 274400) and manifest as congenital

or early postnatal goitrous hypothyroidism. Timing of manifestation is also influenced by dietetary iodide intake. Most patients with ITD demonstrate little or no uptake of radioiodine and a decreased saliva/serum radioiodine ratio (Fujiwara 1997, Szinnai 2006).

Chart 5 - Classification and manifestation of thyroid dyshormonogenesis



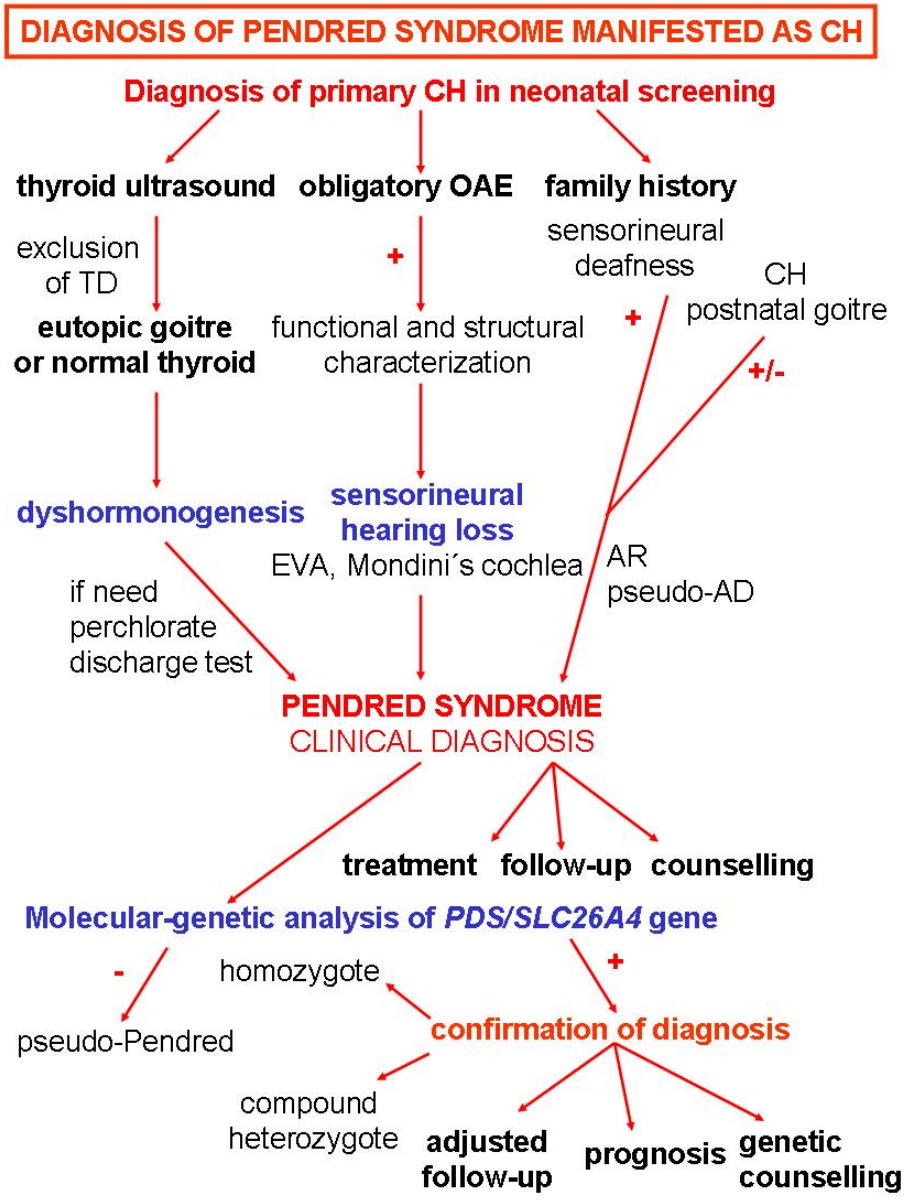
Pendrin (**SLC26A4,** gene map locus 7q31, OMIM 605646, SLC26 gene family overviewed by Mount 2004) is an anion transporter expressed in several organs. In the **thyroid gland**, pendrin is localized at the apical pole of thyrocytes and it is responsible for the iodide efflux from thyrocytes into the colloid in the follicular lumen where iodide is organificated (Yoshida 2002). The **extrathyroidal expression** was shown in the inner ear, kidney, placenta and mammary gland (Lacroix 2001, Royaux 2003).

Carriers of *PDS* mutations display **variable phenotypical features** with the autosomal recessive manner of the inheritance: combined thyroid and hearing affection (Pendred syndrome - OMIM 274600), nonsyndromic autosomal recessive neurosensory deafness (DFNB4 - OMIM 600791) or isolated enlarged vestibular aqueduct (EVA - OMIM 603545). The most typical manifestation of *PDS* mutations (Everett 1997, Coyle 1998, Fugazzola 2000, Campbell 2001, Taylor 2002) - **Pendred syndrome**, was clinically firstly described by Vaughan Pendred (Pendred 1896). It is traditionally defined by the triad of sensorineural congenital deafness, goiter, and a partially positive perchlorate test (the partial discharge of radioiodine after the administration of perchlorate indicates that the gland has also impaired ability to organify iodide) (reviewed by Kopp 1999). The new updated redefinition describes Pendred syndrome as the autosomal recessive disease with

sensorineural hearing impairment that can be accompanied by thyroid dysfunction (Fugazzola 2000). The thyroid affection is usually manifested as euthyroid or hypothyroid goitre in the second decade of life. In a minority of patients, dyshormonogenesis is present at birth, and the disease is diagnosed in the frame of the nation-wide neonatal screening for congenital hypothyroidism (Chart 6).

The role of recently identified **AIT** (**SLC5A8**, OMIM 608044) (Rodriguez 2002) in thyroid dyshormonogenesis has to be studied.

Chart 6 - Diagnosis of Pendred syndrome



Adapted according to Banghová K, Al Taji E, Lebl J. Pendrin and its role in pathogenesis of congenital hypothyroidism. DMEV 2006; 9 (2): 80-84. AD - autosomal dominant, AR - autosomal recessive, CH - congenital hypothyroidism, EVA - enlarged vestibular aqueduct, TD - thyroid dysgenesis.

4.1.2.2. Defects of iodide organification and coupling

Iodide organification defects (defects in the oxidation of iodide, iodination and iodothyronine synthesis, OMIM 274500, 274700) can be partial (**PIOD**) or total (**TIOD**), depending on the degree to which iodide can be organified.

This form of dyshormonogenesis is most frequntly caused by **TPO** defects (*TPO* gene map locus 2p25, OMIM 606765). Generally, TPO defects are among the most frequent causes of inborn abnormalities of thyroid hormone synthesis. Quantitative deficiency of TPO is characterized by partial or complete absence of the TPO activity. Less frequent qualitative abnormalities of the TPO enzyme include defective TPO heme-binding site resulting in defective binding of the prosthetic group, the existence of a putative TPO inhibitor, possible abnormalities in the intracellular location of TPO, and abnormality in the ability of TPO to bind substrate (Medeiros-Neto 1993). Mutations in the *TPO* gene have been reported in numerous patients with PIOD or TIOD (e.g. Abramowicz 1992, Bikker 1995, Bakker 2000, Kotani 2003). Most typical clinical manifestation includes CH, goiter, autosomal recessive inheritance pattern, consanguinity, and positive perchlorate discharge test (significant discharge of radioiodine after the administration to perchlorate).

The peroxide generating system at the apical membrane of TFCs includes two NADPH oxidases **THOX1** (**DUOX1**, OMIM 606758**)** and **THOX 2** (DUOX2, OMIM 606759, gene map locus 15q15.3, De Deken 2000). Defects in H_2O_2 synthesis could be explained by finding mutations in *THOX2* (OMIM 607200) (Moreno 2002, Varela 2006, Moreno 2007). Interestingly, patients with homozygous mutations suffered from permanent CH, whereas heterozygous carriers demonstrated transient CH. Thus monoallelic THOX2 mutations represent the first genetically determined form of transient hypothyroidism and confirm that also transient hypothyroidism can be caused by inborn dyshormonogenesis.

4.1.2.3. Defects of thyroglobulin

TG (gene map locus 8q24.2-q24.3, OMIM 188450) is a large glycoprotein that serves both as the matrix for thyroid hormone synthesis and as a store for the inactive thyroid hormones.

Defects of TG synthesis or degradation (OMIM 274900) due to autosomal recessive mutations (summarized by Vono-Toniolo 2005) were reported in several cases of **congenital goitrous hypothyroidism** (Ieri 1991, Targovnik 1993), in patients with fetal

goiter (Medeiros-Neto 1997, Caron 2003), and even in a case of metastatic follicular thyroid carcinoma arising form congenital goiter (Alzahrani 2006). Despite the fact that the role of TG in the pathogenesis of familial non-goitrous CH has been also suggested (Ahlbom 2002), the gene is so large (Mendive 2001) that no extensive studies have been performed so far.

4.1.2.4. Defects in iodine recycling

Patients lacking the ability to deiodinate MIT and DIT, losing iodine in urine and developing goitres had been described many years before the molecular basis of iodotyrosine dehalogenase deficiency (**IDD**, OMIM 274800) was identified (Moreno 2003, Gnidehou 2004) and first ***DEHAL1*** mutation carriers diagnosed (Moreno 2008).

4.2. Study results (part 1): Screening for *TPO* mutations

Results of this study were summarized in the manuscript

Al Taji E , Biebermann H, Ambrugger P, Venháčová J, Pomahačová R, LeblJ, Hníková O, Grüters A, Krude H. ***Goitrous congenital hypothyroidism: low mutation rate of TPO mutations in Czech children***. Submitted for publication (J Pediatr Endocrinol Metab).

GOITROUS CONGENITAL HYPOTHYROIDISM: LOW MUTATION RATE OF TPO MUTATIONS IN CZECH CHILDREN

Eva Al Taji[1], Heike Biebermann[2], Petra Ambrugger[2], Jitřenka Venháčová[3], Renata Pomahačová[4], Jan Lebl [5], Olga Hníková[1], Annette Grüters[2] and Heiko Krude[2]

[1] Department of Paediatrics, 3rd Faculty of Medicine, Charles University, Prague, Czech Republic

[2] Institute of Experimental Paediatric Endocrinology, University Children´s Hospital Charité, Berlin, Germany

[3] Department of Paediatrics, Faculty of Medicine, Palacky University, Olomouc, Czech Republic

[4] Department of Paediatrics, Faculty of Medicine, Charles University, Plzeň, Czech Republic

[5] Department of Paediatrics, 2nd Faculty of Medicine, Charles University, Prague, Czech Republic

Short title: Novel TPO mutations

Word count: abstract 202, text 3,086

ABSTRACT

Objective: Mutations in the thyroid peroxidase (*TPO*) gene have been most frequently described molecular defects in goitrous congenital hypothyroidism (CH) so far.

Design: In a population-based, non-dyshormonogenesis enriched cohort of 190 Czech patients with permanent primary CH, twenty-two patients demonstrated goitrous CH. These patients were screened for *TPO* mutations in exons which have been reported as most frequently affected in a large number of European patients till now.

Methods: *TPO* mutation screening in exons 2, 8, 9, 10, and 14 was performed by single-stranded conformation polymorphism (SSCP) and restriction enzyme analysis, followed by direct sequencing of samples with abnormal findings. If only one significant sequence variation was identified within the tested exons, all remaining *TPO* exons were directly sequenced.

Results: We identified two patients each carrying two *TPO* mutations (among them two so far unpublished mutations c.740delA in exon 7 and c.2134 C >T in exon 12) and three other patients with a single *TPO* mutation.

Conclusions: In this population-based phenotype-focused study, patients displaying a goitrous phenotype represented only a minority (11.6%) of CH patients. In these subgroup, the detection mutation rate in the *TPO* gene was low (22.7%) and much lower than in previously reported patient cohorts which where mostly preselected.

Key words: congenital hypothyroidism, dyshormonogenesis, goitre, thyroid peroxidase.

INTRODUCTION

Congenital hypothyroidism (CH) associated either with fetal and neonatal goitre or with postnatal thyroid enlargement is caused by thyroid dyshormonogenesis when one of the mechanisms required for thyroid hormone biosynthesis is impaired. Despite the fact, that thyroid dyshormonogenesis causes only a minority part of CH cases, the underlying pathogenetic mechanisms are much better described and more clear than pathogenesis of incomparably more frequent thyroid dysgenesis (TD). A great deal of pathogenesis of thyroid dyshormonogenesis was clarified even on molecular level in last two decades. Gradually, with the identification of various mutations in candidate genes encoding for proteins responsible for thyroid hormone production, genetically determined defects of iodide transporters - natrium-iodide symporter (NIS, Fujiwara 1997) and pendrin (Everett 1997), defects in enzymatic systems responsible for iodide organification - thyroid peroxidase (TPO, Bikker 1995) and thyroid oxidase type 2 (THOX2, Moreno 2002), and recycling - dehalogenase (DEHAL, Moreno 2008), as well as defects in thyroglobulin synthesis (TG, Targovnik 1993) have been described. Therefore, the absolute majority of CH cases due to thyroid dyshormonogenesis can be recognized as an inborn, monogenic Mendelian disease, mostly following autosomal recessive mode of inheritance (genetic defects in thyroid hormonogenesis I-VI, OMIM 274400-274900, 607200) (reviewed e.g. by de Vijlder 2003, Park 2004).

TPO defects (OMIM 274500, 274700) are most frequently reported underlying pathogenetic mechanism in goitrous CH. They are caused by inactivating mutations in the *TPO* gene (OMIM 606765) with autosomal recessive trait of inheritance. TPO is a membrane-bound enzyme, a glycosylated hemoprotein localized on the luminal side of the apical membrane of the thyroid follicular cell. It is s a key enzyme of thyroid hormone biosynthesis as it catalyzes the iodide organification process (oxidation of iodide and its covalent binding to tyrosine residues) as well as subsequent iodotyrosines coupling. The *TPO* gene (human chromozome 2p25) contains 17 exons, with the initiation ATG codon in the second exon. Full-length TPO1 consists of 933 amino acids which organization into a large extracellular domain, a transmembrane domain and a short intracytoplasmic tail shows a striking homology with human myeloperoxidase (Kimura 1987, Kimura 1989). A large extracellular N-terminal part with histidine-containing regions encoded by exons 8, 9, and 10, binds a haem group and represents a catalytic centre of the enzyme, essential for enzyme activity.

World-wide, mutations of the *TPO* gene have been reported in numerous patients with CH due to total organification defect (e.g. Bikker 1994, Bakker 2000) and in several patients with partial organification defects (e.g. Kotani 2003). In this study, we present the first mutation screening in the *TPO* gene in Czech patients with goitrous CH.

PATIENTS AND METHODS

Patients

Twenty-two Czech Caucasians with permanent primary goitrous CH (Table 1), selected among 190 Czech children and adolescents with CH on the base of retrospectively analyzed phenotypical characteristics, particularly thyroid morphology (Al Taji 2007), were studied.

Nineteen probands were diagnosed by the Czech nation-wide neonatal screening programme for CH in years 1985-2002 (assessment of total T_4 levels by RIA in 1985-1995, TSH by DELPHIA since 1996 on dry blood spots from a heel prick, TSH cut off 15 mIU/l) (Table 1, groups A, B, C). Three patients were born before the introducing of neonatal CH screening and they were diagnosed due to clinical symptoms of hypothyroidism and early postnatal goitre development (Table 1, group D). TSH levels were high and free T_4 and/or total T_4 levels were low in all the cases at the diagnosis. The progression of thyroid enlargement (cystic nodular goitres with volume up to 60 ml) despite the substitution treatment deserved strumectomy in 2 male patients in adolescence.

Patients were unrelated individuals originating from 22 unconsanguineous families. Three probands had a positive family history of thyroid disorders in a first-degree relative (older brother with CH, mother with euthyroid goiter, mother with post-partum hypothyroidism).

The study was approved by the institutional ethics committee of the 3rd Faculty of Medicine, Charles University, Prague. Informed consent was given by all patients and/or their parents.

Methods

DNA extraction

The genomic DNA was extracted from peripheral blood leukocytes with a standard method (Miller 1988).

PCR reaction

The PCR was performed in 50-µl reactions in 30 cycles (Gene Amp PCR system 9700 cycler, PE Applied Biosystems, Foster City, CA, USA) with the Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany). One cycle consisted of 1 min denaturation at 95 C, 1 min annealing at a specific temperature and 1 min elongation at 68 C. Primers and annealing temperatures are available upon request.

Mutation screening methods

Restriction enzyme analysis was performed as a rapid screening method to detect previously described mutations in exons 2 (Bikker 1994), 8 (Abramowicz 1992) and 9 (Ambrugger 2001) with *BsrI*, *NaeI* and *MspI* enzymes respectively, following the conditions recommended by the manufacturer (New England BioLabs, Ipswich, MA, USA). Digested PCR fragments were separated on a 2% agarose gel, stained with ethidium bromide and analyzed under UV light.

Single stranded conformational polymorphism (SSCP) analysis of exons 2, 8, 9, 10, 14 was provided according to the standardized protocol as we described before (Al Taji 2007). Samples showing differences in comparison with wild type controls were submitted to the direct sequencing.

Direct sequencing

Purified PCR products (QIAquick PCR Purification Kit, Qiagen, Hilden, Germany) were prepared using the DNA sequencing Kit - Big Dye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems, Warrington, UK) and they were directly sequenced in both directions on an ABI PRISM 377 sequencer (PE Applied Biosystems, Foster City, CA, USA). If only one significant sequence variation was identified within tested exons, all remaining exons were directly sequenced.

Sequence variations were nomenclatured as generally recommended (den Dunnen 2001), but usual nucleotide numbering for *TPO* mutations was followed (Kimura 1989).

RESULTS

In the frame of a population-based, phenotype focused study of 190 patients with permanent primary CH, we performed *TPO* mutation screening in 22 CH patients selected

on the basis of their goitrous phenotype. We focused on exons 8, 9, 10, 14 and 2, where mutations had been described most frequently in other European populations (Figure 1, Table 2, Bikker 1995, Bikker 1996, Bakker 2000, Grüters 1996, Ambrugger 2001, Avbelj 2007). Surprisingly, except of same-sense sequence variations and other previously described polymorphisms, the detection rate of mutations in the exons, which had been most frequently affected in previous studies, was extremely low. We were able to detect significant sequence variations of the *TPO* gene on both alleles only in two male patients with goitrous CH: c.[740delA] + [2512delT], c.[1276_1277insGGCC] + [2134 C >T]. In additional 3 children with severe CH diagnosed by neonatal screening, the mutation could be identified only on one allele: c.[1276_1277insGGCC] + [?], c.[808G>A] + [?], c.[2485G>A] + [?].

Case reports

Patient 1

Patient 1 was born in early 1985, closely before neonatal screening for CH was established as nation-wide. He seemed to be a healthy male newborn (birth weight 3900g, length 50cm, anterior fontanel 5 cm by 3 cm, posterior fontanel closed). Nevertheless, poor appetite, feeding problems, sleepiness and constipation appeared shortly after the neonatal period, followed by the failure in thrive. Muscle hypotony, umbilical hernia, macroglosia, hoarse voice and above all palpable goiter became evident. His bone age was delayed. The diagnosis of primary CH was laboratory confirmed with low total T_4 (11.9 nmol/l, normal range 60-160 nmol/l) and remarkably elevated TSH (247 mIU/l, normal range 0.15-3.2 mIU/l) at the age of 2.5 months. Substitution treatment with L-thyroxine was immediately initiated. Palpable goiter dimished within the first year of his life, later ultrasound examination showed eutopic thyroid gland of a normal size and structure. Mild psychomotor retardation and delayed speech development were gradually normalized and he reached 94 and 112 points in IQ testing in 5 and 10 years of his age, respectively.

Patient 1 was identified as a carrier of two heterozygous point deletions in the *TPO* gene (Figure 2): novel c.740delA in exon 7 and already known c.2512delT in exon 14 (Bakker 2000). Both mutations lead to a frameshift causing early termination signal in exons 7 and 14 (p.N217fsX230, p.C808fsX831). DNA samples of patient´s biological parents were not available for testing since he was adopted shortly after birth. Therefore also the occurrence of thyroid disease in his biological family remains unknown.

Patient 2

Patient 2 was born in 2000 as the first child of healthy unconsanguineous parents after a normal pregnancy in term by a caesarian section due to fetal distress. Apgar scores were 7-9-10, he was shortly resuscitated. Physical examination (birth weight 3710g, birth length 53cm, anterior fontanel 4 cm by 4 cm) revealed palpable neonatal goitre and opened posterior fontanel (sized 1 cm by 1 cm). TSH was elevated (50 mIU/l, screening cut off 15 mIU/l) and free T_4 was low (4.5 pmol/l, normal range 10-26 pmol/l). Thyroglobulin was as high as 327 µg/l (normal range 30-85 µg/l), urinary iodine was normal (148 µg/l, normal population range 80-300 µg/l) at diagnosis. Ultrasound examination prior to treatment showed a large eutopic goitre (volume 10.4 ml, own normative data for sex and age 0.57±0.18ml). L-thyroxine substitution was started as soon as 5th day of his life and led to a gradual decrease of thyroid volume to a normal size. Further psychomotor and physical development was normal and unremarkable. There is no family history of thyroid disorders in his family. The parents did not agree with a family genetic testing.

Two heterozygous mutations in the *TPO* gene were detected in patient 2 (Figure 3): previously frequently described frameshift mutation c.1276_1277insGGCC (or 1273_1276dupGGCC) in exon 8 (p.R396fsX472) and so far unpublished nonsense mutation c.2134 C >T in exon 12 (p.Q682X).

DISCUSSION

We found only 11.6% of goitrous cases within the population-based, non-dyshormonogenesis enriched, non-inbred CH cohort. These twenty-two Czech Caucasians were screened for mutations in the *TPO* gene which defects have been reported as the most frequent cause of CH due to thyroid dyshormonogenesis so far. The predominant majority of previously detected mutations in large series of patients from the Netherlands (Bikker 1995, Bikker 1996, Bakker 2000), Germany (Grüters 1996, Ambrugger 2001), and other European countries (e.g. Avbelj 2007) affected exons 8, 9, 10, encoding for the catalytic centre of the enzyme, and exon 14 and 2 (Figure 1, Table 2). Unexpectedly, the occurrence of such *TPO* mutations is much lower among Czech patients with goitrous CH compared to these previous reports. Most surprisingly even the frequency of GGCC duplication in exon 8, which is overall most frequently reported *TPO* mutation (e.g. Bakker 2000, Avbelj 2007) is very rare among our patients (mutant allele frequency 2/44). It can be

hypothesised that a low detection rate of *TPO* mutations in our study could be caused by an untypically different distribution of mutations within the *TPO* gene, not affecting the catalytic centre of the enzyme, as a consequence of a founder effect (analogously as ref. Niu 2002). However, a low detection rate of *TPO* mutations - only in 22.7% of goitrous CH cases (5 in 22, including single *TPO* mutation carriers), just can reflect reality and thus the role of other candidate genes in goitrous CH cases as e.g. defects of thyroglobulin synthesis must be taken into consideration. In contrary to previous *TPO* mutation screening results in preselected study groups, we assume the occurrence of *TPO* defects as few as 2.6% in our population-based cohort of 190 CH patients. We conclude that TPO defects are quite rare in a non-preselected, non-inbred CH population.

We identified six different *TPO* gene mutations, two of them being unpublished so far.

In patient 1, frameshift mutations in exons 14 and 7 were detected. The mutation c. 2512delT in exon 14 was firstly reported by Bakker (Bakker 2000). It leads to early termination signal in exon 14 (p.C808fsX831). It has been expected to have a similar effect as another frameshift mutation in exon 14 (c.2505_2511 insC), which functional characterization showed enzymatically totally inactive TPO (Bikker 1997). It was concluded that this region of the TPO protein is important not only for the insertion to the membrane but also for proper folding of the enzyme (Bikker 1997). Later on, c.2512delT was functionally tested itself and it was demonstrated that the truncated TPO protein, which lost the part of the transmembrane domain coded by exon 15, could not translocate onto the cell surface (Kotani 2001). Generally, mutations in exon 14 have been reported among the most frequent alterations of the *TPO* gene so far, noteworthly also in a patient with thyroid follicular adenoma (Kotani 1999) and in a newborn with metastatic thyroid follicular cancer (Medeiros-Neto 1998). The second mutation in patient 1 was detected in exon 7. On the opposite, exon 7 of the *TPO* gene is only rarely affected. According to our knowledge, only two mutations have been published previously: a missense mutation c.808G>A (p.D240N) leading to the loss of TPO activity (Kotani 1999) and a nonsense mutation c.793C>T (p.Q235X, Pfarr 2006) that was not functionally studied. A novel point mutation c.740delA in exon 7 detected in our patient creates an early termination signal in exon 7 (p.N217fsX230). Thus, it can be expected that such a truncated mutant TPO protein, completely lacking the catalytic centre of the enzyme, will be functionally inactive. Interestingly, the mutation distribution in exons 7 and 14 in our patient corresponds to the distribution in a patient with CH due to total iodide organification

deficiency (TIOD) who developed goitre with thyroid follicular adenoma (Kotani 1999). TPO enzyme activity was undetectable in the microsomal fractions of thyroid tissues in this patient. All together, it is evident that the mutant TPO protein due to the two frameshift mutations identified in our patient 1 would lack the enzyme activity, that is consistent with the phenotypical expression.

Patient 2 was diagnosed as a carrier of 2 mutations: frequently described GGCC duplication in exon 8 and a novel nonsense mutation c.2134 C >T in exon 12. The former mutation leads to a frameshift introducing an early termination signal in exon 9. Premature stop codon in such a critical region for the function of the enzyme must lead to an inactive TPO. GGCC duplication (firstly referred by Abramowicz 1992) is actually the most frequently reported defect of the *TPO* gene (e.g. Bakker 2000, Avbelj 2007). Again in opposite, the occurrence of mutations in exon 12 is much more rare (Bakker 2000, Calaciura 2002, Fugazzola 2003). The mutant TPO protein p.Q682X can be compared with previously described mutant protein p.F718X identified in a Dutch patient with TIOD (Bakker 2000). Similarly, p. Q682X will result in a truncated enzyme missing the membrane-spanning domain which is not likely to insert the membrane properly. Therefore, it can be concluded that the expected deleterious effect of the novel mutation is causally related to the phenotype of patient 2, compatible with TIOD, and both mutations of the *TPO* gene detected in patient 2 together had to result in a loss of the TPO enzyme activity.

Only a single *TPO* mutation was identified in a certain number of patients in several screening studies (Grüters 1996, Medeiros-Neto 1998, Bakker 2000, Wu 2002, Rivolta 2003, Fugazzola 2003, Nascimento 2003, Avbelj 2007). Avbelj et al. identified single allele mutations even in 65% of patients (13 in 20 patients carrying *TPO* mutations, Avbelj 2007). A single *TPO* mutation was found in 60% of *TPO* mutation carriers (3 in 5 patients) also in the present study. One patient carried a well-known GGCC duplication in exon 8, another patient already described c.2485G>A in exon 14. This substitution p.E799K was firstly described and functionally characterized by Bikker (Bikker 1995, 1997). In Bikker´s functional study, the cellular distribution of this mutant was comparable with wild-type, but TPO was enzymatically inactive probably as a result of its unproper folding (Bikker 1997). Our third patient was identified to carry already known c. 808G>A in exon 7 (p. D240N) (Kotani 1999).

So far, the association of CH phenotype with a single *TPO* mutation has been clearly explained only in single cases by the mechanism of a maternal isodisomy for

chromosome 2p (Bakker 2001), deletion of the paternal TPO gene at chromosome 2p25 (Kotani 2001), and monoallelic expression of mutant thyroid peroxidase allele (Fugazzola 2003). The possibility of alterations in the regulatory regions of the gene, intronic mutations, or functional consequences of a single *TPO* mutation associated either with *TPO* polymorphisms or with the second mutation affecting another candidate gene for thyroid dyshormonogenesis should be taken into consideration.

We performed the first *TPO* mutation screening in Czech patients with goitrous CH within a large population-based phenotype-focused study. The mutation detection rate in the *TPO* gene was extremely low (mutant allele frequency 7/44) and the molecular pathogenesis of dyshormonogenesis could be clearly clarified only in two patients (9%). The identification of two novel mutations expands the spectrum of known mutations in this gene. Identifying new goitrous patients carrying a single *TPO* mutation confirms non-coincidental and causal relationship with still unexplained underlying mechanism.

The establishing of molecular genetic diagnosis in goitrous CH cases is of a high importance also from the clinical point of view. Undesirable proliferation of thyroid tissue and a large multinodular goitre deserving thyroidectomy was described in several patients with *TPO* mutations (e.g. Abramowicz 1992, Bikker 1996, Fugazzola 2003). Futhermore, the development of solitary or multiple follicular adenoma was reported (Kotani 1999, Pfarr 2006). Most noteworthly, metastatic thyroid carcinoma arose from congenital goiter in a carrier of the *TPO* defect (Medeireos-Neto 1998). Therefore, regular ultrasound investigations of the thyroid gland are highly recommended in patients with *TPO* defects. Prolonged elevations of TSH must be consistently avoided during thyroid hormone replacement therapy - the stimulation by TSH can result in the proliferation of the thyroid tissue. Despite the fact, that *TPO* mutation rate even in goitrous CH patients is not necessarily as high as previously published, *TPO* mutation screening should be optimally offered to all patients with goitrous CH. *TPO* mutation carriers and their families should be informed not only regarding the heredity of the disease and undergo genetic counselling but they should be educated about the natural course of the disease, the necessity to keep long-time euthyroid status and the potential risks of noncompliance.

ACKNOWLEDGEMENTS

This work was supported by Research Project MSM 0021620814 of the Czech Ministry of Education, ”Sonderforschungs Bereich SFB” Nr. 577-A10 of the German Research Council, and Deutscher Akademischer Austauschdienst. Authors would like to thank J. Zikmund for ultrasound examinations, P. Kračmar for providing screening data and I. Gläser and E. Mattušová for technical assistance. All patients, their families and cooperating endocrinologists (V. Janštová, J. Klabochová, E. Nováková, D. Novotná, J. Škvor, H. Vávrová, J. Zapletalová) are deeply acknowledged.

REFERENCES

1. Abramowicz MJ, Targovnik HM, Varela V, Cochaux P, Krawiec L, Pisarev MA, Prapato EVE, Juvenal G, Chester HA, Vassart G. Identification of a mutation in the coding sequence of the human thyroid peroxidase gene causing congenital goiter. J Clin Invest 1992; 90: 1200-1204.
2. Al Taji E, Biebermann H, Límanová Z, Hníková O, Zikmund J, Dame C, Grüters A, Lebl J, Krude H. Screening for mutations in transcription factors in a Czech cohort of 170 patients with congenital and early-onset hypothyroidism: identification of a novel PAX8 mutation in dominantly inherited early-onset non-autoimmune hypothyroidism. Eur J Endocrinol 2007; 156: 521-529.
3. Ambrugger P, Stoeva I, Biebermann H, Torresani T, Leitner C, Grüters A. Novel mutations of the thyroid peroxidase gene in patients with permanent congenital hypothyroidism. Eur J Endocrinol 2001; 145: 19-24.
4. Avbelj M, Tahirovic H, Debeljak M, Kusekova M, Toromanovic A, Krzisnik C, Battelino T. High prevalence of thyroid peroxidase gene mutations in patients with thyroid dyshormonogenesis. Eur J Endocrinol 2007; 156: 511-519.
5. Bakker B, Bikker H, Hennekam RCM, Lommen EJP, Schipper MGJ, Vulsma T, De Vijlder JJM. Maternal isodisomy for chromosome 2p causing severe congenital hypothyroidism. J Clin Endocrinol Metab 2001; 86: 1164-1168.
6. Bakker B, Bikker H, Vulsma T, de Randamie JSE, Wiedijk BM, de Vijlder JJM. Two decades of screening for congenital hypothyroidism in the Netherlands: TPO gene mutations in total iodide organification defects (an update). J Clin Endocrinol Metab 2000; 85: 3708-3712.
7. Bikker H, Baas F, de Vijlder JJM. Molecular analysis of mutated thyroid peroxidase detected in patients with total iodide organification defect. J Clin Endocrinol Metab 1997; 82: 649-653.
8. Bikker H, den Hartog MT, Baas F, Gons MH, Vulsma T, de Vijlder JJM. A 20-basepair duplication in the human thyroid peroxidase gene results in a total iodide organification defect and congenital hypothyroidism. J Clin Endocrinol Metab 1994; 79: 248-252.
9. Bikker H, Vulsma T, Baas V, de Vijlder JJM. Identification of five novel inactivating mutations in the human thyroid peroxidase gene by denaturating gradient gel electrophoresis. Human Mutation 1995; 6: 9-16.

10. Bikker H, Waelkens JJJ, Bravenboer B, de Vijlder JJM. Congenital hypothyroidism caused by a premature termination signal in exon 10 of the human thyroid peroxidase gene. J Clin Endocrinol Metab 1996; 81: 2076-2079.
11. Börgel K, Pohlenz J, Holzgreve W, Bramswig H. Intrauterinne therapy of goitrous hypothyroidism in a boy with a new compound heterozygous mutation (Y453D and C800R) in the thyroid peroxidase gene. A long-term follow-up. Am J Obstet Gynecol 2005; 193: 857-858.
12. Calaciura F, Miscio G, Coco A, Leonardi D, Cisternino C, Regalbuto C, Bozzali M, Maiorana R, Ranieri A, Carta A, Buscema M, Trischitta V, Sava L, Tassi V. Genetic of specific phenotypes of congenital hypothyroidism: a population-based approach. Thyroid 2002; 12: 945-951.
13. den Dunnen JT, Antonarakis E. Nomenclature for the description of human sequence variations. Hum Genet 2001; 109: 121-124.
14. De Vijlder JJM. Primary congenital hypothyroidism: defects in iodine pathways. Eur J Endocrinol 2003; 149: 247-256.
15. Everett LA, Glaser B, Beck JC, Idol JR, Buchs A, Heyman M, Adawi F, Hazani E, Nassir E, Baxevanis AD, Sheffield VC, Green ED. Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS). Nat Genet 1997; 17: 411-422.
16. Fugazzola L, Cerutti N, Mannavola D, Vannucchi G, Fallini C, Persani L, Beck-Peccoz P. Monoallelic expression of mutant thyroid peroxidase allele causing total iodide organification defect. J Clin Endocrinol Metab 2003; 88: 3264-3271.
17. Fugazzola L, Mannavola D, Vigone MC, Cirello V, Weber G, Beck-Peccoz P, Persani L. Total iodide organification defect: clinical and molecular characterization of an Italian family. Thyroid 2005; 15: 1085-1088.
18. Fujiwara H, Tatsumi K, Miki K, Harada T, Miyai K, Takai S, Amino N. Congenital hypothyroidism caused by a mutation in the Na^+/I^- symporter. Nat Genet 1997; 16: 124-125.
19. Grüters A, Köhler B, Wolf A, Söling A, de Vijlder L, Krude H, Biebermann H. Screening for mutations of the human thyroid peroxidase gene in patients with congenital hypothyroidism. Exp Clin Endocrinol Diabetes 1996; 104 (Suppl 4): 121-123.

20. Kimura S, Hong YS, Kotani T, Othaki S, Kikkawa F. Structure of the human thyroid peroxidase gene: comparison and relationship to the human myeloperoxidase gene. Biochemistry 1989; 28: 4481-4489.
21. Kimura S, Kotani T, McBride OW, Umeki K, Hirai K, Nakayama T, Ohtaki S. Human thyroid peroxidase: complete cDNA and protein sequence, chromosome mapping, and identification of two alternatively spliced mRNAs. Proc Nat Acad Sci 1987; 84: 5555-5559.
22. Kotani T, Umeki K, Kawano J, Suganuma T, Hishinuma A, Ieri T, Harada S. Partial iodide organification defect caused by a novel mutation of the thyroid peroxidase gene in three siblings. Clin Endocrinol (Oxf) 2003; 59: 198-206.
23. Kotani T, Umeki K, Yamamoto I, Maesaka H, Tachibana K, Ohtaki S. A novel mutation in the human thyroid peroxidase gene resulting in a total iodide organification defect. J Endocrinol 1999; 160: 267-273.
24. Kotani T, Umeki K, Yamamoto I, Ohtaki S, Adachi M, Tachibana K. Iodide organification defects resulting from cosegregation of mutated and null thyroid peroxidase alleles. Mol Cell Endocrinol 2001; 182: 61-68.
25. Medeiros-Neto G, Gil-Da-Costa MJ, Santos CLS, Medina AM, Costa E Silva J, Tsou RM, Sobrinho-Simoes M. Metastatic thyroid carcinoma arising from congenital goiter due to mutation in the thyroperoxidase gene. J Clin Endocrinol Metab 1998; 83: 4162-4166.
26. Miller SA. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988; 85:1215.
27. Moreno JC, Bikker H, Kempers MJ, van Trotsenburt AS, Baas F, de Vijlder JJ, Vulsma T, Ris-Stalpers C. Inactivating mutations in the gene encoding for thyroid oxidase 2 (THOX2) and congenital hypothyroidism. N Eng J Med 2002; 347: 95-102.
28. Moreno JC, Klootwijk W, van Toor H, Pinto G, D´Alessandro M, Leger A, Goudie D, Polak M, Grüters A, Visser TJ. Mutations in the iodotyrosine deiodinase gene and hypothyroidism. N Eng J Med 2008; 358: 1811-1818.
29. Nascimento AC, Guedes DR, Santos CS, Knobel M, Rubio IG, Medeiros-Neto G. Thyroperoxidase gene mutations in congenital goitrous hypothyroidism with total and partial iodide organification defect. Thyroid 2003; 13: 1145-1151.
30. Niu DM, Hwang B, Chu YK, Liao CJ, Wang PL, Lin CY. High prevalence of a novel mutation (2268 insT) of the thyroid peroxidase gene in Taiwanese patients

with total iodide organification defect, and evidence for a founder effect. J Clin Endocrinol Metab 2002; 87: 4208-4212.

31. Pannain S, Weiss RE, Jackson CE, Dian D, Beck JC, Sheffield VC, Cox N, Refetoff S. Two different mutations in the thyroid peroxidase gene of a large inbred Amish kindred: power and limits of homozygosity mapping. J Clin Endocrinol Metab 1999; 84: 1061-1071.
32. Park SM, Chatterjee VKK. Genetics of congenital hypothyroidism. J Med Genet 2005; 42: 379-389.
33. Pfarr N, Borck G, Turk A, Napiontek U, Keilmann A, Muller-Forell W, Kopp P, Pohlenz J. Goitrous congenital hypothyroidism and hearing impairment associated with mutations in the TPO and SLC26A4 genes. J Clin Endocrinol Metab 2006; 91: 2678-2681.
34. Pfarr N, Musholt TJ, Musholt PB, Brzezinska R, Pohlenz J. Congenital primary hypothyroidism with subsequent adenomatous goiter in a Turkish patient caused by a homozygous 10-bp deletion in the thyroid peroxidase (TPO) gene. Clin Endocrinol 2006; 64: 514-518.
35. Rivolta CM, Esperante SA, Gruneiro-Papendieck L, Chiesa A, Moya CM, Domene S, Varela V, Targovnik HM. Five novel inactivating mutations in the thyroid peroxidase gene responsible for congenital goiter and iodide organification defect. Hum Mut 2003; 22: 259.
36. Rivolta CM, Louis-Tisserand M, Varela V, Gruneiro-Papendieck L, Chiesa A, González-Sarmiento R, Targovnik HM. Two compound heterozygous mutations (c.215delA/c.2422T→C and c.387delC/c.1159G→A) in the thyroid peroxidase gene responsible for congenital goitre and iodide organificaton defect. Clin Endocrinol 2007; 67: 238-246.
37. Rodriguez C, Jorge P, Soares JP, Santos I, Salomao R, Madeira M, Vaz Osório R, Santos R. Mutation screening of the thyroid peroxidase gene in a cohort of 55 Portuguese patients with congenital hypothyroidism. Eur J Endocrinol 2005; 152: 193-198.
38. Santos CLS, Bikker H, Rego KGM, Nascimento AC, Tambascia M, de Vijlder JJM, Medeiros-Neto G. A novel mutation in the TPO gene in goitrous hypothyroid patients with iodide organification defect. Clin Endocrinol 1999; 51: 165-172.
39. Tajima T, Tsubaki J, Fujieda K. Two novel mutations in the tyroid peroxidase gene with goitrous hypothyroidism. Endocrine J 2005; 52: 643-645.

40. Targovnik HM, Medeiros-Neto G, Varela V, Cochaux P, Wajchenberg BL, Vassart G. A nonsense mutation causes human hereditary congenital goiter with preferential production of a 171 nucleotide deleted thyroglobulin RNA messenger. J Clin Endocrinol Metab 1993; 77: 210-215.
41. Tenenbaum-Rakover Y, Mamanasiri S, Ris-Stalpers C, German A, Sack J, Allon-Shalev S, Pohlenz J, Refetoff S. Clinical and genetic characteristics of congenital hypothyroidism due to mutations in the thyroid peroxidase (TPO) gene in Israelis. Clin Endocrinol 2007; 66: 695-702.
42. Umeki K, Kotani T, Kawano J, Suganuma T, Yamamoto I, Aratake Y, Furujo M, Ichiba Y. Two novel missense mutations in the thyroid peroxidase gene, R665W and G771R, result in a localization defect and cause congenital hypothyroidism. Eur J Endocrinol 2002; 146: 491-498.
43. Umeki K, Yamamoto I, Yukizane S, Kotani T. Congenital hypothyroidism caused by a unique peroxidase allele containing two mutations, C1708T and C2737T. J Pediatr Endocrinol Metab 2004; 17: 231-234.
44. Wu JY, Shu SG, Yang CF, Lee CC, Tsai FJ. Mutation analysis of thyroid peroxidase gene in Chinese patients with total iodide organification defect: identification of five novel mutations. J Endocrinol 2002; 172: 627-635.

TABLES

Table 1

Description of the patient cohort

Sex	Goitre				Total
	A	**B**	**C**	**D**	
♀	2	5	1	2	10
♂	2	7	2*	1	12
Total	4	12	3	3	**22 (F/M 0.8)**

Patients in groups A, B, C were diagnosed by neonatal CH screening. Their thyroid volumes were measured by ultrasound and compared to own normative data for sex and age. Patients in group D were born before neonatal CH screening and presented with palpable goiter at diagnosis. A - congenital goiter, persisting or recurrent despite substitution treatment, B - congenital goiter, subsequent normalization during substitution treatment, C - early postnatal goitre development despite substitution treatment (* large goitres in adolescence deserving strumectomy), D - postnatal goiter development in patients born before CH screening.

Table 2

Overview of *TPO* mutations reported so far

Ex	Mutation	Effect	Ref.	Ex	Mutation	Effect	Ref.
2	20 bp dup	frameshift	***8, 9, 3***	**10**	1708C>T	R540X	***9***, 43, 41
3	247G>C	A53P	30	**10**	1780 C>A	L564I	29
4	305delA	frameshift	36	**10**	1808_13del	D574_L575del	22
4	439G>C	splice site	***6***	**10**	1858 G>A	G590S	***9***
5	477delC	frameshift	35, ***17***	**10**	+1 intron 10 G>A	splice site	***6***
5	481T>C	S131P	***37***	**11**	2033G>A	R648Q	31
6	613C>T	R175X	***4***	**11**	2068C>G	Q660E	38, 29, ***37***
7	740delA	frameshift	*	**11**	2083C>T	R665W	42
7	793C>T	Q235X	***33***	**11**	2084G>A	R665A	29
7	808G>A	D240N	23	**11**	2089G>A	G667S	***4***
8	933delC	frameshift	44	**12**	2134 C>T	Q682X	*
8	965C>T	S292F	41	**12**	2149G>T	E687X	***12***
8	1010A>C	N307T	35	**12**	2167C>T	R693W	***6, 16***
8	1066G>A	A326T	***6***	**12**	2243_2244 delTT	frameshift	***6***
8	1222G>A	E378K	39	**13**	2333del T	frameshift	30
8	1242G>T	E384D	29	**13**	2358 insT	frameshift	44, 30
8	1249G>A	G387R	36	**13**	2401G>A	G771R	42
8	1277ins GGCC (1273_1276 dupGGCC)	frameshift	1, ***9***, ***19***, ***6***, ***12***, 29, 35, ***37***, ***17, 4, ***	**13**	2476 G>T	D796Y	44
8	1364A>G	N425S	***37***	**14**	2485 G>A	E799K	***9***, 31, ***4***
8	1387G>A	V433M	35	**14**		C800R	***11***
8	1425delC	frameshift	***6***	**14**	2503delC	frameshift	44
9	1429 A>T	I447F	***7***	**14**	2505_2511 insC	frameshift	***9***, 25, 23, 29
9	1447T>G	Y453D	***9***, ***33***, ***11***	**14**	2512T>C	C808R	35, ***17***
9	1463 T>C	L458P	***3***	**14**	2512delT	frameshift	***6***, ***5***, 24, ***37***, ***4***, *
9	1519 _1539del	A477 _N483del	***4***	**14**	2602 T>A	C838S	***37***
9	1562G>A	R491H	***3***	**14**	2605G>A	V839I	***12***
9	1567G>A	G493S	44, ***37***,41	**15**	2669G>A	G860R	***4***
9	1586C>T	P499L	35	**16**	2737 C>T	P883S	43
9	1671G>T	W527C	***6***	**16**	10bp del intron15-exon 16	splice site	39
9	1687G>T	G533C	22	**16**	2812del10bp	frameshift	34
9	9+1G>T	splice site	29	**16**	2838 G>A	splice site	***37***

Nucleotide numbering for *TPO* mutations according to Kimura 1989 was followed. For numbering according to Antonarakis 2001 (beginning with the A of the ATG start codon) subtract 90 nucleotides. European patients are expressed in bold/italic.

Ex - exon, Ref. - references, * - present study

FIGURES

Figure 1

Mutations identified within the *TPO* gene

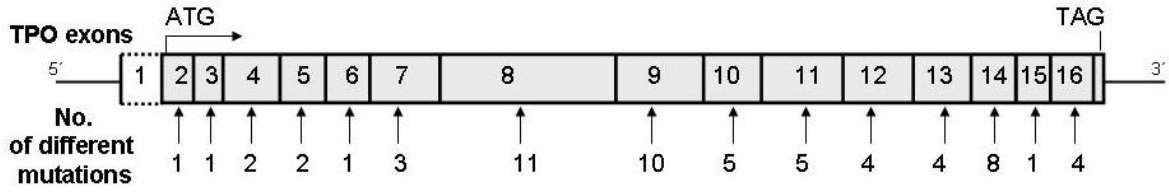
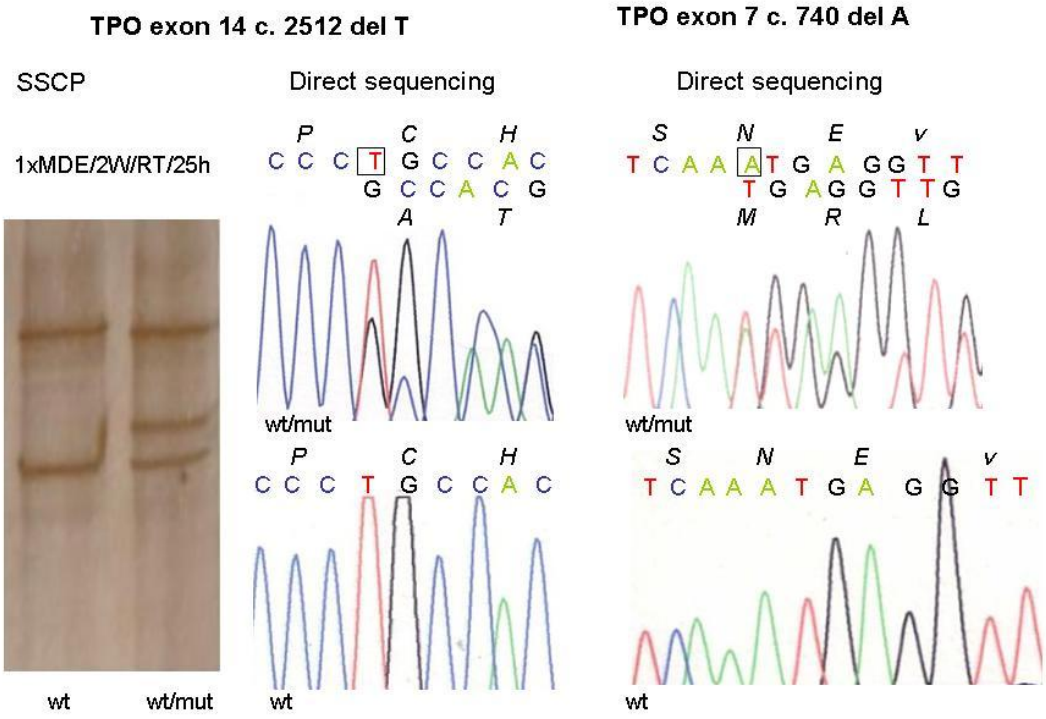


Figure 2

Mutations detected in patient 1



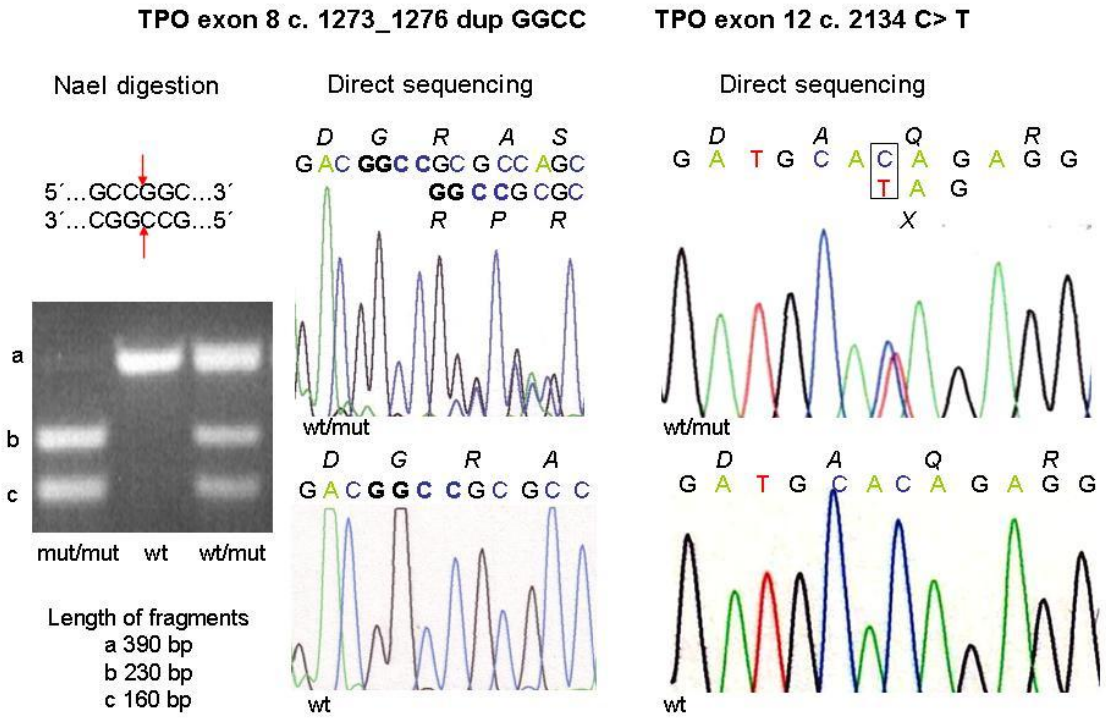
Left side: Heterozygous point mutation c.2512 del T in exon 14 leading to a frameshift. Mutation screening was performed by SSCP, the mutation was identified by direct sequencing (see heterozygous state compared to wild type control).

Right side: Heterozygous point mutation c.740 del A in exon 7 leading to a frameshift. As no mutation was detected by SSCP in exons 2, 8, 9, 10, all remaining exons were directly sequenced and the mutation was found in exon 7 (see heterozygous state compared to wild type control).

Wt - wild type, mut - mutation, SSCP - single stranded conformation polymorphism.

Figure 3

Mutations detected in patient 2



Left side: Heterozygous mutation c.1273_1276 dup GGCC in exon 8 leading to a frameshift. Mutation screening was performed by NaeI digestion, the mutation was confirmed by direct sequencing (see heterozygous state compared to wild type control). Restriction endonuclease digestion with NaeI was performed in a 30 µl reaction mixture containing 8 µl H_2O, 3µl NE Buffer 10x conc., 2 µl NaeI 10,000 U/ml (New England BioLabs, Ipswich, MA, USA), and 17 µl of PCR product, being incubated at 37°C for 12 hours.

Right side: Heterozygous nonsense mutation c.2134 C>T in exon 12 leading to a stop codon. As no mutation was detected by SSCP in exons 2, 8, 9, 10, all remaining exons were directly sequenced and the mutation was found in exon 12 (see heterozygous state compared to wild type control).

Wt - wild type, mut - mutation, SSCP - single stranded conformation polymorphism.

4.3. Study results (part 2): Pendrin and its role in pathogenesis of congenital and non-congenital goitrous hypothyroidism

Results of this study were published as an original article

Banghová K, Al Taji E, Cinek O, Novotná D, Zapletalová J, Hníková O, Lebl J.
Pendred syndrome among patients with congenital hypothyroidism detected by neonatal screening: identification of two novel PDS/SLC26A4 gene mutations.
Eur J Pediatr 2008, 167 (7): 777-783. IF 1.277.

and as a case report

Banghová K, Cinek O, Al Taji E, Zapletalová J, Vidura R, Lebl J. ***Thyroidectomy in a patient with multinodular dyshormonogenetic goitre - a case of Pendred syndrome confirmed by finding mutations in the PDS/SLC26A4 gene.***
J Pediatr Endocrinol Metab 2008, 21 (12): 1179-1184. IF 0.858.

This chapter is identical to the published version of the articles.

PENDRED SYNDROME AMONG PATIENTS WITH CONGENITAL HYPOTHYROIDISM DETECTED BY NEONATAL SCREENING: IDENTIFICATION OF TWO NOVEL *PDS/SLC26A4* MUTATIONS

Karolína Banghová[1], Eva Al Taji[2], Ondřej Cinek[1], Dana Novotná[3], Radka Pourová[1], Jiřina Zapletalová[4], Olga Hníková[2] and Jan Lebl[1]

[1] Department of Paediatrics, 2nd Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

[2] Department of Paediatrics, 3rd Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

[3] 2nd Department of Paediatrics, University Hospital, Brno, Czech Republic

[4] Department of Paediatrics, Faculty of Medicine, Palacky University, Olomouc, Czech Republic

Short title: Pendred syndrome in congenital hypothyroidism

Word count: abstract 248, text 3,065

ABSTRACT

Pendred syndrome is an autosomal recessive disorder characterised by sensorineural hearing loss and thyroid dyshormonogenesis. It is caused by mutations in *PDS/SLC26A4* gene (OMIM 605646) encoding for pendrin. Hypothyroidism in Pendred syndrome can be - although rarely - present from birth and therefore diagnosed by neonatal screening. The aim of our study was to identify patients with Pendred syndrome among a historical cohort of patients with congenital hypothyroidism (CH) identified by neonatal screening, and to find their mutations in the *PDS/SLC26A4* gene. We investigated 197 Czech Caucasian children with CH detected by the neonatal screening between years 1985 and 2005. The clinical diagnosis of Pendred syndrome was based on the laboratory and sonographic signs of thyroid dyshormonogenesis in association with sensorineural hearing loss. In subjects clinically diagnosed as Pendred syndrome, we sequenced all exons and exon-intron boundaries of the *PDS/SLC26A4* gene. Hearing loss was present in 10/197 children with screening-detected CH. Of these, three fulfilled the diagnostic criteria of Pendred syndrome. Two patients were compound heterozygotes for *PDS/SLC26A4* mutations: patient 1 carried c.2089+1G>A / c.3G>C, patient 2 carried p.Tyr530His / p.Val422Asp. Two of the four identified mutations were novel (c.3G>C in patient 1 and p.Val422Asp in patient 2). The third patient was free of mutations in the *PDS/SLC26A4* gene, representing a phenocopy. In conclusion, our results indicate the rarity of Pendred syndrome as a cause of CH. The identification of two novel mutations expands the spectrum of mutations in the *PDS/SLC26A4* gene and emphasizes their marked allelic heterogeneity.

Keywords: congenital hypothyroidism, PDS, SLC26A4, pendrin, sensorineural hearing loss.

INTRODUCTION

Pendred syndrome (OMIM 274600) is an autosomal recessive disease, characterised by functional impairment of the thyroid gland due to thyroid dyshormonogenesis, sensorineural hearing loss and developmental malformations of the inner ear [23]. It is caused by mutations in the gene encoding pendrin, a transmembrane anion transporter that is highly expressed in the thyroid and in the inner ear [12]. The gene, being referred as *PDS* or *SLC26A4* (member 26 of the Solute Linked Carrier family A4, OMIM 605646), consists of 21 exons [12] and has been mapped to chromosome 7q31 [9, 29].

In the thyroid, pendrin is expressed at the apical surface of thyrocytes [25]. It acts as a chloride-iodide exchanger transporting iodide from the cell to the colloid in the follicular lumen, where iodide is organified [27]. Loss of pendrin function results in defective iodide organification that induces thyroid overgrowth and goitre in most affected individuals. However, an overt hypothyroidism develops only in about one third of subjects with Pendred syndrome [21].

In the inner ear, pendrin is probably involved in the maintenance of the endolymph homeostasis in the membranous labyrinth [24]. Deafness or milder forms of hearing impairment develop pre- or perilingually and are frequently associated with radiologically detectable structural malformations of the inner ear - enlarged vestibular aqueduct (EVA) and Mondini's cochlea [19].

Whilst sensorineural hearing loss and structural malformations of the inner ear are consistent features of Pendred syndrome, the expression of thyroid impairment is highly variable [13, 22]. In a minority of patients dyshormonogenesis is present at birth and is diagnosed by neonatal screening for congenital hypothyroidism (CH) [14, 21]. Nevertheless, very little is known about the proportion of patients with Pendred syndrome among children with CH. To our knowledge, only one study has been designed to assess the incidence of Pendred syndrome in a cohort of subjects with CH. However, it was conducted before genetic testing became available, and therefore the diagnosis was based solely on the clinical symptoms [8].

Our study therefore aimed to ascertain patients carrying mutations in the *PDS/SLC26A4* gene among subjects with CH detected by neonatal screening. We conducted this study with two main goals: a) to investigate the *PDS/SLC26A4* gene as one candidate gene for CH and b) to establish how frequently Pendred syndrome causes

hearing impairment in our patients with CH. A retrospective cohort of patients with screening-detected CH was evaluated regarding the occurrence of hearing impairment during their childhood. Patients with the combination of CH due to thyroid dyshormonogenesis and sensorineural hearing loss were tested for mutations in *PDS/SLC26A4* gene.

SUBJECTS AND METHODS

Historical cohort of patients with CH

Previously, a large group of patients with CH was collected, thoroughly characterized and analysed for mutations in several candidate genes [2]. In this work, among children diagnosed by CH screening between 1985-2005, a group of 197 phenotypically characterized patients was revisited with the intention to identify patients with Pendred syndrome. Retrospectively analyzed data were referred by paediatric endocrinologists from eight major paediatric endocrine clinics throughout the country. Phenotypic characteristics included medical and family history, thyroid hormone levels and TSH levels before initiation of treatment with L-thyroxine, results of thyroid ultrasound at diagnosis, and the occurrence of hearing impairment. In patients with any kind of hearing loss, information on its type, grade, and age of onset was obtained.

In the Czech Republic, neonatal screening for CH is provided in two screening laboratory centres. It uses dried blood spots taken from a heel prick on the fifth day after birth. From 1985 until 1995, it was based on the assessment of total thyroxine (tT4) by RIA. Since 1996 thyrotropin (TSH) levels have been measured by DELPHIA (cut off level 15 mU/l). Between 1994 and 1996 both methods were used.

It is noteworthy that since 2002 every child with CH has been compulsorily screened for deafness with otoacoustic emissions testing (OAE). Before 2002, only children with clinically suspected hearing loss were investigated using OAE, auditory brainstem response (ABR), or pure tone audiometry, depending on the child's age. Hearing loss was classified as absent (loss of 0-20 dB), mild (20-40 dB), moderate (40-60 dB), severe (60-80dB), or profound (>80 dB).

Molecular studies

The testing was approved by the ethics committee of the Third Faculty of Medicine, Charles University, Prague. The subjects or their legal guardians gave their written informed consent. DNA was obtained using standard methods from blood, or from saliva samples collected into the Oragene DNA Self-Collection Kits (DNA Genotec Inc, Ontario, Canada). All 21 exons of the *PDS/SLC26A4* gene and their flanking intron-exon junctions were amplified and directly sequenced using primers and PCR conditions as described by Borck et al. [7] with further modifications. All mutations were confirmed by bi-directional sequencing.

Further examination of subjects with mutations and their families

Available relatives of probands with *PDS/SLC26A4* mutations were tested for the presence of the respective mutation. In subjects carrying mutations in the *PDS/SLC26A4* gene, the presence of enlarged vestibular aqueduct was investigated by high-resolution CT of temporal bones in coronal and axial planes (1 mm-contiguous sections). The vestibular aqueduct was considered enlarged when its diameter at the midpoint between the common crus and the external aperture was ≥1.5 mm on thin CT sections [32].

RESULTS

Ten individuals with combined thyroid and hearing impairment were identified among the 197 patients with screening-detected CH. Three of them fulfilled clinical criteria of Pendred syndrome - thyroid dyshormonogenesis (CH with normal or enlarged thyroid volume) and sensorineural hearing loss. The characteristics of the patients are listed in Table 1. In the remaining seven patients CH developed due to thyroid dysgenesis, therefore the diagnosis of Pendred syndrome was very unlikely. Moreover, in four of these seven children, the hearing loss was not sensorineural but conductive.

The three subjects (two females, one male) with CH and clinically defined Pendred syndrome were analysed for mutations in *PDS/SLC26A4* gene. All three were born to unrelated parents of Czech Caucasian origin. Sequence analysis of 21 *PDS/SLC26A4* exons showed mutations in two of the three subjects. A total of four mutations were identified, two of them not previously reported. Additionally, an intronic variant was found in one

patient. Mutations were classified according to the nomenclature recommendations, with the A of the ATG of the initiator Met codon denoted as +1 [3].

Patient 1

A 12 year-old-female was found to be a compound heterozygote for one already published mutation in the splice site of intron 18 (c.2089+1G>A = c.IVS18+1G>A) [6] and for a novel mutation in the start codon of the gene in exon 2 (c.3G>C, Fig.1).

She was diagnosed as subclinically hypothyroid at birth in the frame of the CH–TSH screening. The screening level of TSH was elevated to 71 mU/L (cut off 15 mIU/l) although the tT_4 level was normal (114 nmol/l, normal range 77-154). The thyroid ultrasound at diagnosis revealed a diffuse goitre (thyroid volume 2.5 ml, own normative data for age and sex 0.55 ± 0.14 ml). L-thyroxine administration was started on the 14th day of life. Under T_4 substitution the goitre remained unchanged, despite euthyroid status. Sensorineural hearing loss was first recognised at the age of 4 years. At 12 years, she suffered from severe hearing loss of 70 dB in both ears and used binaural hearing aids. The high-resolution CT of the temporal bones revealed an enlargement of the vestibular aqueduct bilaterally.

The proband's 10-year-old sister was found to carry identical mutations as the index patient both in intron 18 and exon 2 (pedigree of the family, Figure 2). She is deaf-mute due to profound sensorineural hearing loss of 120 dB in the right and 110 dB in the left ear, diagnosed prelingually. She also wears hearing aids. Her thyroid status was first examined at the age of 10 years. The fT_4 and TSH levels were normal (fT_4 15.38 pmol/l, normal range 10.5-27, TSH 2.8 mIU/l, normal range 0.34-5.5). The thyroid ultrasound revealed a mild diffuse goitre (thyroid volume 11 ml, upper normal range of own normative data for age and sex 9 ml). Bilateral enlargement of the vestibular aqueduct was found on high-resolution CT scan.

Their mother was found to be a heterozygous carrier of the splice site mutation in intron 18. Although she was originally reported to be deaf, further investigation revealed just a mild conductive hearing loss of the left ear, probably due to several episodes of otitis media during her childhood. Her thyroid function tests and ultrasound were normal (fT_4 17.03 pmol/l, TSH 0.872 mIU/l, thyroid volume 14.4 ml). A younger brother of the two sisters inherited the maternal intronic mutation in a heterozygous pattern. The father, a potential carrier of the mutation in exon 2, was not available for the molecular study. He is reported to be clinically unaffected. Parents are unrelated.

Patient 2

A 17-year-old male was found to have one allele of his *PDS/SLC26A4* gene harbouring a previously described transversion c.1588T>C in exon 14 resulting in a substitution of tyrosine for histidine in position 530 (p.Tyr530His)[6, 10]. The second allele is a novel missense mutation in exon 11 - c.1265T>A - leading to substitution of valine for aspartate in codon 422 (p.Val422Asp, Fig.1). Additionally, a thus far unreported intronic variant deep in the intron 9 (c.IVS9-139T>C) was found.

His thyroid disease was diagnosed at birth. The screening level of tT_4 was 9 nmol/l and his TSH was elevated to 38 mU/l. A diffuse thyroid enlargement of 2 ml (own normative data for age and sex 0.55 ± 0.18 ml) was found at birth by ultrasound. Substitution therapy was started on day 15. The follow-up evaluation during therapy revealed a euthyroid state leading to a decrease of his thyroid volume to normal by the age of ten years. The boy suffered from a prelingual, profound bilateral hearing loss, first recognised at the age of 1.5 years using the ABR method. It revealed a loss of 110 dB in the left and 100 dB in the right ear. Further audiometric investigation showed no further deterioration. He uses binaural hearing aids. The high-resolution CT of temporal bones was not performed. As the child was adopted by new parents soon after birth, family history is not known.

Patient 3

In a 14-year-old female, sequencing of the *PDS/SLC26A4* gene revealed no mutations. She presented with CH (screening level of tT_4 15 nmol/l, TSH 150 mIU/l). The first ultrasound revealed thyroid volume in the upper normal range (0.73 ml, own normative data for age and sex 0.55 ± 0.14 ml). L-thyroxine administration was started at the age of 17 days. Further follow-up examination revealed decreasing thyroid volume. A moderate nonprogressive hearing loss of 50 dB bilaterally was recognised at five years of age. No hearing loss was recorded in any of her family members. Her paternal aunt was reported to have a goitre. In an attempt to disclose the reason for the hearing loss, direct sequencing of the *GJB2/connexin26* gene was performed in the index patient but revealed no mutations.

DISCUSSION

We here report a study on genetically confirmed Pendred syndrome with thyroid disease manifested as CH. Collecting a cohort of 197 children with CH diagnosed by neonatal screening over the last 21 years in the Czech Republic gave us a unique opportunity to a) investigate the proportion of children who developed hearing loss, b) to find out in how many children the association of hearing loss and CH is caused by Pendred syndrome and c) to establish the frequency of mutations in the *PDS/SLC26A4* gene as one of the candidate genes for CH in our patients.

Mutations in *PDS/SLC26A4* as a cause of deafness in CH

Making a retrospective investigation we found that among 197 children with CH, ten developed hearing loss later in life. Deafness is known to be one of the frequently associated noticeable features of congenital thyroid disease [11]. It has been estimated that hereditary hearing loss with hypothyroidism accounts for 1-10% of all hereditary deafness [5]. This is reflected in clinical guidelines that recommend that every child with CH is screened for hearing loss with the otoacoustic emissions method (OAE) until the age of 3 months. Most cases of deafness in CH are secondary, caused by the lack of thyroid hormones that are necessary for the normal development of the peripheral and central auditory system [17]. This is in agreement with histological findings in CH animals, where an immature development of the organ of Corti including hair cells and tectorial membrane has been observed [4, 31]. However, sensorineural hearing loss developing in children with CH due to dyshormonogenesis may be also a manifestation of Pendred syndrome. A detailed clinical evaluation can be very helpful in recognizing these cases. In our study, of ten children with CH and hearing loss two were identified to have hearing loss due to Pendred syndrome. One child who fulfilled the clinical criteria but was without mutations in the *PDS/SLC26A4* gene was concluded to be a phenocopy and the cause of the association of sensorineural hearing loss and thyroid disease remained unclear. Because congenital deafness is relatively common, affecting 1 of 2,000 newborns [20], and more than 50 different genes have been found to be involved [30], the origin of hearing loss in this case may be on account of mutations in other deafness genes and the association with CH may be coincidental. For this reason we additionally tested this patient for mutations in the *GJB2/connexin26* gene, which is the most common cause of congenital sensorineural deafness [28], but did not found any mutations.

Mutations in PDS/SLC26A4 as a cause of CH

The second aspect of our study was the identification of mutations in the *PDS/SLC26A4* gene as one of the candidate genes for CH due to thyroid dyshormonogenesis. CH with goitre or a normal sized thyroid gland is a heterogeneous group accounting for approximately 20% of all CH cases. Until now, several candidate genes associated with thyroid dyshormonogenesis have been identified including those for thyroid peroxidase (*TPO*), thyroglobulin (*TG*), sodium-iodide symporter (*NIS*), pendrin and more recently thyroid oxidase 2 (*THOX2*) [15]. Although the exact proportion has not been assessed, defects in the *TPO* gene have been estimated to be the most prevalent cause of thyroid dyshormonogenesis [1, 16], probably followed by mutations in *TG*. Defects in the remaining candidate genes including *PDS* seem to be less frequent. This notion is supported by the fact that we identified only 2/197 cases of CH (1.0%, CI95%: 0.31% - 3.6%), caused by mutations in the *PDS/SLC26A4* gene.

Manifestation of thyroid disease in Pendred syndrome

Finally, an additional goal of our study was to determine how many cases of Pendred syndrome can be diagnosed right after birth as they manifest as CH. As previously described in several studies, the thyroid disease in Pendred syndrome is phenotypically highly variable. It can develop at any age from birth to adulthood, showing the highest occurrence during the second decade of life (70%) [21]. However, single cases of Pendred syndrome manifested as CH have been previously reported [6, 7, 18, 21]. To our best knowledge, just one study was designed to asses the incidence of Pendred syndrome among children with screening-detected CH. Among 213 children with CH, Coakley et al. [8] reported five patients with the clinical diagnosis of Pendred syndrome, representing an incidence of 1 in 153,000 births. As this study was conducted before genetic testing became available, the diagnosis was based solely on clinical symptoms and therefore some of these subjects might represent phenocopies. Finding only two cases of true Pendred syndrome in our group of CH patients indicates that the thyroid disease in Pendred syndrome indeed can be diagnosed by neonatal screening, but the manifestation in the newborn age is rare. Also, screening for CH alone is not sufficient to identify the disorder, leading to significant underascertainment.

Heterogeneity of mutations in PDS

After the identifications of the *PDS/SLC26A4* gene in 1997 [12], more than 100 different mutations have been identified, scattered all over the gene, of which the majority are missense substitutions [6, 10, 18]. Several mutations have also been functionally characterized showing a loss-of-function effect, e.g. on the base of an impaired iodide transport capacity of the mutant protein [26]. However, clear genotype-phenotype correlations have not been found. The present study reports the clinical picture of two patients, each carrying one already known and one novel mutation in the *PDS/SLC26A4* gene. Both novel mutations can be expected to result in the impairment of pendrin function. In particular, the c.3G>C mutation affecting the start codon of the gene may activate another cryptic start codon located at base position 61, which makes it likely that the protein will be truncated of the first 20 amino acids. The other so far unreported mutation is a transversion T>C in position 1588 leading to a substitution of valine for aspartate in codon 422 (p.Val422Asp). According to the model of the gene proposed by Everett et al. [12], this mutation lies in the extracellular loop between the ninth and the tenth transmembrane domain of pendrin, where other pathogenetic mutations have been already identified [6, 10]. The alignment of the *PDS/SLC26A4* sequence and two highly homologous human genes, *DRA* (down-regulated in adenoma) and *DTD* (diastrophic dysplasia) [12], also indicates that the mutation affects a conserved amino acid. Moreover, the affection of the protein function can be also predicted since the mutation changes an uncharged and neutral amino acid (valine) for a highly acidic and negatively charged amino acid (aspartate).

CONCLUSIONS

Pendred syndrome appears to be a rare cause of CH. However, this diagnosis should be taken into consideration whenever a child with CH due to dyshormonogenesis develops sensorineural hearing loss, and these individuals should undergo molecular-genetic analysis of the *PDS/SLC26A4* gene.

ACKNOWLEDGEMENTS

We wish to thank Prof. J. Pohlenz and colleagues at the Hospital of the Johannes Gutenberg University, Mainz, Germany for providing us with the currently used primer sequences and PCR conditions. We are grateful to L. Cerny, M. Finkova, R. Havlik, V. Janstova, J. Klabochova, E. Novakova, R. Pomahacova, M. Skacha, J. Skvor, H. Vavrova, J. Venhacova and J. Zikmund for their assistance in clinical evaluations. K. Rakova is acknowledged for expert technical assistance. We also thank Prof. C. J. H. Kelnar and Prof. G. Van Vliet for a critical review of the manuscript. The cooperation of the patients and their families is greatly appreciated.

The study was supported by grants of the Czech Ministry of Education (MSM 0021620814) and Charles University in Prague (GAUK 2008/2007)

REFERENCES

1. Abramowicz MJ, Targovnik HM, Varela V, Cochaux P, Krawiec L, Pisarev MA, Propato FV, Juvenal G, Chester HA, Vassart G (1992) Identification of a mutation in the coding sequence of the human thyroid peroxidase gene causing congenital goiter. J Clin Invest 90: 1200-1204
2. Al Taji E, Biebermann H, Limanova Z, Hnikova O, Zikmund J, Dame C, Grueters A, Lebl J, Krude H (2007) Screening for mutations in transcription factors in a Czech cohort of 170 patients with congenital and early-onset hypothyroidism: identification of a novel PAX8 mutation in dominantly inherited early-onset non-autoimmune hypothyroidism. Eur J Endocrinol 156: 521-529
3. Antonarakis SE (1998) Recommendations for a nomenclature system for human gene mutations. Nomenclature Working Group. Hum Mutat 11: 1-3
4. Bargman GJ, Gardner LI (1967) Otic lesions and congenital hypothyroidism in the developing chick. J Clin Invest 46: 1828-1839
5. Batsakis JG, Nishiyama RH (1962) Deafness with sporadic goiter. Pendred's syndrome. Arch Otolaryngol 76: 401-406
6. Blons H, Feldmann D, Duval V, Messaz O, Denoyelle F, Loundon N, Sergout-Allaoui A, Houang M, Duriez F, Lacombe D, Delobel B, Leman J, Catros H, Journel H, Drouin-Garraud V, Obstoy MF, Toutain A, Oden S, Toublanc JE, Couderc R, Petit C, Garabedian EN, Marlin S (2004) Screening of SLC26A4 (PDS) gene in Pendred's syndrome: a large spectrum of mutations in France and phenotypic heterogeneity. Clin Genet 66: 333-340
7. Borck G, Roth C, Martine U, Wildhardt G, Pohlenz J (2003) Mutations in the PDS gene in German families with Pendred's syndrome: V138F is a founder mutation. J Clin Endocrinol Metab 88: 2916-2921
8. Coakley JC, Keir EH, Connelly JF (1992) The association of thyroid dyshormonogenesis and deafness (Pendred syndrome): experience of the Victorian Neonatal Thyroid Screening Programme. J Paediatr Child Health 28: 398-401
9. Coyle B, Coffey R, Armour JA, Gausden E, Hochberg Z, Grossman A, Britton K, Pembrey M, Reardon W, Trembath R (1996) Pendred syndrome (goitre and sensorineural hearing loss) maps to chromosome 7 in the region containing the nonsyndromic deafness gene DFNB4. Nat Genet 12: 421-423

10. Coyle B, Reardon W, Herbrick JA, Tsui LC, Gausden E, Lee J, Coffey R, Grueters A, Grossman A, Phelps PD, Luxon L, Kendall-Taylor P, Scherer SW, Trembath RC (1998) Molecular analysis of the PDS gene in Pendred syndrome. Hum Mol Genet 7: 1105-1112
11. Debruyne F, Vanderschueren-Lodeweyckx M, Bastijns P (1983) Hearing in congenital hypothyroidism. Audiology 22: 404-409
12. Everett LA, Glaser B, Beck JC, Idol JR, Buchs A, Heyman M, Adawi F, Hazani E, Nassir E, Baxevanis AD, Sheffield VC, Green ED (1997) Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS). Nat Genet 17: 411-422
13. Fraser GR (1965) Association of congenital deafness with goitre (Pendred's syndrome) a study of 207 families. Ann Hum Genet 28: 201-249
14. Gaudino R, Garel C, Czernichow P, Leger J (2005) Proportion of various types of thyroid disorders among newborns with congenital hypothyroidism and normally located gland: a regional cohort study. Clin Endocrinol (Oxf) 62: 444-448
15. Gillam MP, Kopp P (2001) Genetic defects in thyroid hormone synthesis. Curr Opin Pediatr 13: 364-372
16. Mangklabruks A, Billerbeck AE, Wajchenberg B, Knobel M, Cox NJ, DeGroot LJ, Medeiros-Neto G (1991) Genetic linkage studies of thyroid peroxidase (TPO) gene in families with TPO deficiency. J Clin Endocrinol Metab 72: 471-476
17. Meyerhoff WL (1979) Hypothyroidism and the ear: electrophysiological, morphological, and chemical considerations. Laryngoscope 89: 1-25
18. Napiontek U, Borck G, Muller-Forell W, Pfarr N, Bohnert A, Keilmann A, Pohlenz J (2004) Intrafamilial variability of the deafness and goiter phenotype in Pendred syndrome caused by a T416P mutation in the SLC26A4 gene. J Clin Endocrinol Metab 89: 5347-5351
19. Phelps PD, Coffey RA, Trembath RC, Luxon LM, Grossman AB, Britton KE, Kendall-Taylor P, Graham JM, Cadge BC, Stephens SG, Pembrey ME, Reardon W (1998) Radiological malformations of the ear in Pendred syndrome. Clin Radiol 53: 268-273
20. Reardon W (1992) Genetic deafness. J Med Genet 29: 521-526
21. Reardon W, Coffey R, Chowdhury T, Grossman A, Jan H, Britton K, Kendall-Taylor P, Trembath R (1999) Prevalence, age of onset, and natural history of thyroid disease in Pendred syndrome. J Med Genet 36: 595-598

22. Reardon W, Coffey R, Phelps PD, Luxon LM, Stephens D, Kendall-Taylor P, Britton KE, Grossman A, Trembath R (1997) Pendred syndrome--100 years of underascertainment? QJM 90: 443-447
23. Reardon W, Trembath RC (1996) Pendred syndrome. J Med Genet 33: 1037-1040
24. Royaux IE, Belyantseva IA, Wu T, Kachar B, Everett LA, Marcus DC, Green ED (2003) Localization and functional studies of pendrin in the mouse inner ear provide insight about the etiology of deafness in pendred syndrome. J Assoc Res Otolaryngol 4: 394-404
25. Royaux IE, Suzuki K, Mori A, Katoh R, Everett LA, Kohn LD, Green ED (2000) Pendrin, the protein encoded by the Pendred syndrome gene (PDS), is an apical porter of iodide in the thyroid and is regulated by thyroglobulin in FRTL-5 cells. Endocrinology 141: 839-845
26. Scott DA, Wang R, Kreman TM, Andrews M, McDonald JM, Bishop JR, Smith RJ, Karniski LP, Sheffield VC (2000) Functional differences of the PDS gene product are associated with phenotypic variation in patients with Pendred syndrome and non-syndromic hearing loss (DFNB4). Hum Mol Genet 9: 1709-1715
27. Scott DA, Wang R, Kreman TM, Sheffield VC, Karniski LP (1999) The Pendred syndrome gene encodes a chloride-iodide transport protein. Nat Genet 21: 440-443.
28. Seeman P, Malikova M, Raskova D, Bendova O, Groh D, Kubalkova M, Sakmaryova I, Seemanova E, Kabelka Z (2004) Spectrum and frequencies of mutations in the GJB2 (Cx26) gene among 156 Czech patients with pre-lingual deafness. Clin Genet 66: 152-157
29. Sheffield VC, Kraiem Z, Beck JC, Nishimura D, Stone EM, Salameh M, Sadeh O, Glaser B (1996) Pendred syndrome maps to chromosome 7q21-34 and is caused by an intrinsic defect in thyroid iodine organification. Nat Genet 12: 424-426
30. Steel KP, Bussoli TJ (1999) Deafness genes: expressions of surprise. Trends Genet 15: 207-211
31. Uziel A, Gabrion J, Ohresser M, Legrand C (1981) Effects of hypothyroidism on the structural development of the organ of Corti in the rat. Acta Otolaryngol 92: 469-480
32. Valvassori GE, Clemis JD (1978) The large vestibular aqueduct syndrome. Laryngoscope 88: 723-728

TABLES

Table 1

Summary of clinical and molecular features of the three patients with clinical diagnosis of Pendred syndrome

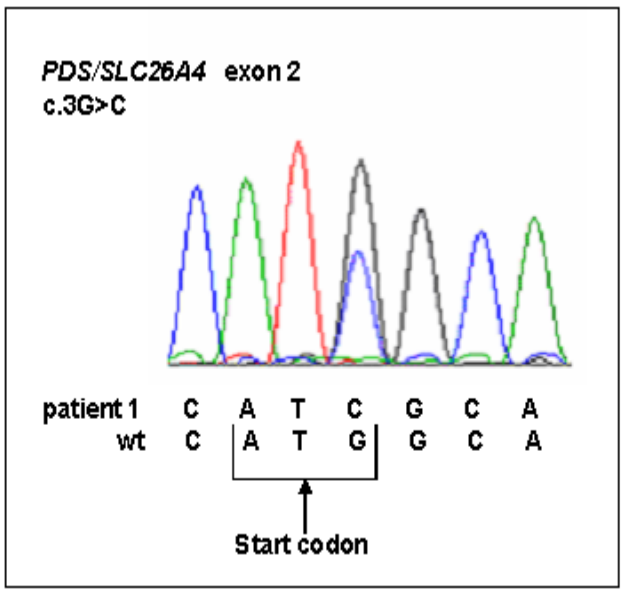
Clinical and molecular features	Patient number		
	1	**2**	**3**
Age at investigation	12 years	17 years	14 years
Gender	F	M	F
Thyroid hormone status (screening result)	subclinical hypothyroidism TSH 71 mIU/l tT_4 114 nmol/l	hypothyroidism TSH 38 mIU/l tT_4 9 nmol/l	hypothyroidism TSH 150 mIU/l tT_4 15 nmol/l
Thyroid ultrasound after birth	goitre v = 2.5 ml	goitre v = 2 ml	normal v = 0.73 ml
Onset of hypothyroidism	congenital	congenital	congenital
Type and degree of hearing impairment	sensorineural profound	sensorineural profound	sensorineural moderate
Onset of hearing impairment	4 years	1.5 years	5 years
HR-CT of temporal bones	enlarged vestibular aqueduct bilaterally	not performed	not performed
Allele 1	**c.2089+1G>A** (c.IVS18+1G>A)	**p.Val422Asp** (c.1265T>A)	wt
Allele 2	**c.3G>C**	**p.Tyr530His** (c.1588T>C)	wt

v- volume, wt - wild type

FIGURES

Figure 1

Sequence analysis of the two novel *PDS/SLC26A4* mutations described in patient 1 (c.3G>C) and patient 2 (p.Val422Asp)



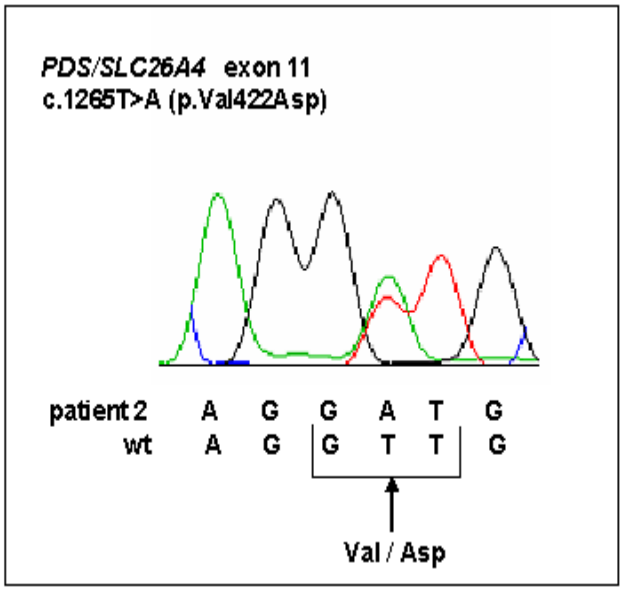
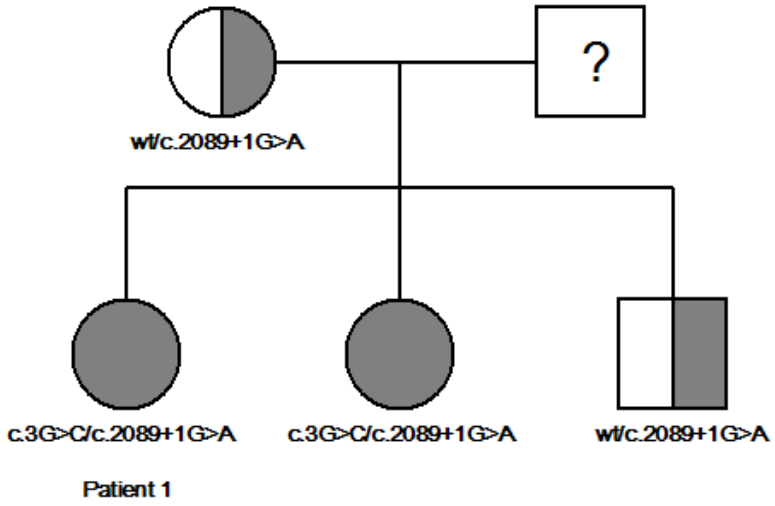


Figure 2

Family pedigree of patient 1



The two sisters inherited identical mutations in both exon 2 and intron 18. The younger brother and the mother were found to be heterozygote carriers of the intronic mutation. The father, a potential carrier of the exonic mutation, was not available for the genetic investigation.

THYROIDECTOMY IN A PATIENT WITH MULTIDONULAR DYSHORMONOGENETIC GOITRE - A CASE OF PENDRED SYNDROME CONFIRMED BY FINDING MUTATIONS IN THE *PDS/SLC26A4* GENE

Karolína Banghová[1], Ondřej Cinek[1], Eva Al Taji[2], Jiřina Zapletalová[3], Radan Vidura[4] and Jan Lebl[1]

[1] Department of Paediatrics, 2nd Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

[2] Department of Paediatrics, 3rd Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

[3] Department of Paediatrics, Faculty of Medicine, Palacky University, Olomouc, Czech Republic

[4] Department of Otorhinolaryngology, Faculty of Medicine, Palacky University, Olomouc, Czech Republic

Short title: Thyroidectomy in a patient with dyshormonogenetic goitre

Word count: abstract 138, text 2,003

ABSTRACT

We report a young woman with genetically confirmed Pendred syndrome and discuss the current therapeutic strategies of dyshormonogenetic goitre. A small diffuse thyroid enlargement developed during infancy and although substitution therapy with L-thyroxine was adequate, it progressed and underwent multinodular transformation. Cervical ultrasound at the age of 22 years demonstrated three solid nodules and fine-needle aspiration biopsy showed a finding typical of follicular adenoma. It is known that dyshormonogenetic goitres have a tendency to grow despite appropriate treatment with L-thyroxine. Management of a patient with Pendred syndrome requires careful follow-up and regular imaging of the thyroid. Although the therapeutic approach to dyshormonogenetic goitres is still controversial, in our patient we chose total thyroidectomy as the most advantageous method to prevent the development of malignancies that may arise more frequently from dyshormonogenetic goitres than from goitres of other aetiologies.

Keywords: hypothyroidism, goitre, thyroid surgery, Pendred syndrome, *SLC26A4,* dyshormonogenesis,

INTRODUCTION

Pendred syndrome (OMIM 274600) is characterized by the classical phenotype of dyshormonogenetic goitre and sensorineural deafness (1). Additional clinical features that can be radiologically diagnosed are structural malformations of the inner ear (2) - enlarged vestibular aqueduct and/or Mondini cochlea - which are not specific for Pendred syndrome and can also be associated with other types of hearing loss. The disease shows an autosomal recessive pattern of inheritance and is caused by mutations in the *PDS* (Pendred syndrome) gene (also known as *SLC26A4* - member 4 of the solute carrier family 26; OMIM 605646). The gene is located on chromosome 7q31 (3) and encodes for pendrin, a highly hydrophobic transmembrane glycoproteine, expressed predominantly in the thyroid gland, inner ear and kidney (3).

In the thyroid gland, pendrin is located at the apical surface of the thyroid cell and functions as a chloride-iodide exchanger (4). It transports iodide from the cell to the colloid in the follicular lumen, where iodide is bound on tyrosine residues of thyroglobulin (5). The loss of pendrin function results in an organification defect and leads to thyroidal dyshormonogenesis which in the majority of cases manifests as a euthyroid or hypothyroid goitre (6, 7). The defect may be partially compensated via iodide intake and the hypothyroidism is more severe if iodide intake is low.

Dyshormonogenetic goiters are an important clinical challenge as they often undergo nodular transformation, probably due to prolonged stimulation by thyrotropin. Especially in patients with inadequate treatment, the development of malignancies is a feared complication and surgery often becomes necessary. Moreover, other studies have shown that the occurrence of carcinomas is higher in goiters developing due to dyshormonogenesis than in those of other aetiology (8). Particularly in Pendred syndrome, cases with very aggressive metastatic follicular carcinomas have repeatedly been reported (9-11).

We describe a girl with sensorineural hearing loss and dyshormonogenetic goitre in whom the diagnosis of Pendred syndrome was confirmed by identification of mutations in both alleles of the *PDS* gene. Although the therapy of the thyroid disease was adequate and the patient showed very good compliance, she developed a multinodular goitre during adolescence. Although we acknowledge that the therapy of nodular thyroid disease remains a controversial issue, a total thyroidectomy was performed at the age of 22 years.

CASE REPORT

The now 22 year-old female was born as the only child of non-consanguineous parents who originate from the Czech Republic. The parents have normal hearing, no signs of hypothyroidism and no goitre. The pregnancy and perinatal history was unremarkable. She was born at term with a birth weight of 2,700 g and a length of 47 cm. In spite of otherwise normal neuropsychological development, she had not developed speech up to the age of 2 years. Additionally, her parents realised that she did not respond appropriately to sounds. An audiometric investigation during her 3rd year of life revealed bilateral moderate hearing loss that was classified as sensorineural. At the age of 3 years she was provided with binaural hearing aids and her further psychic and somatic development was normal.

Thyroid disease was diagnosed at the age of 7 years when the girl presented with a swelling of the neck. Thyroid function tests revealed mild clinical hypothyroidism with a decreased level of free thyroxine (fT_4; 6.53 pmol/l, normal range 10.5-27) and elevated thyrotropin (TSH; 14.8 mIU/l, normal range 0.34-5.5). Anti-thyroperoxidase and anti-thyroglobulin antibodies were negative. Thyroid utrasound showed a small diffuse goitre. Substitution therapy with L-thyroxine was started immediately and the patient soon achieved a euthyroid state and showed good compliance.

The clinical diagnosis of Pendred syndrome was established based on the association of goitre and sensorineural hearing loss, but at that time no genetic testing was available. Almost 15 years later the patient was referred to our department for molecular genetic analysis of the *PDS* gene that confirmed the clinical diagnosis. Direct sequencing of all 21 exons and intron-exon boundaries was performed using primers and PCR conditions as described by Borck et al. (12) with further modifications. Two known mutations were found in compound heterozygous state (Fig. 1). The first mutation is a common transversion A>C at position 1246 of the cDNA sequence (c.1246A>C). It is located in exon 10 and predicts an amino acid change from threonine to proline in position 416 of the pendrin protein (p.Thr416Pro). The second mutation is also a missense mutation, located in exon 14 - c.1589A>C - and leads to substitution of tyrosine for serine in codon 530 (p.Tyr530Ser). The father of the patient was found to be a heterozygous carrier of the mutation in exon 14. The mother carries the second mutation in exon 10, also in a heterozygous state.

Although the patient regularly attended follow-ups and thyroid function tests were always within the normal range, the goitre progressed during adolescence and underwent

multinodular transformation (Fig. 2). Thyroid ultrasound at the age of 22 years showed two solid isoreflective nodules (7 and 11 mm in diameter) in the middle to upper part of the right thyroid lobe, and one larger nodule (20 x 14 x 9 mm) in the left thyroid lobe (Fig. 3). Fine-needle aspiration biopsy from the larger nodule revealed benign follicular cells and microfollicular formations leading to the diagnosis of a follicular adenoma. Thyroid hormone levels at that time were again normal (fT_4 18.7 pmol/l, TSH 2.99 mIU/l) under T_4 replacement therapy. The patient did not complain about any neck discomfort or swallowing difficulties but the goitre started to become a cosmetic problem. Subsequently a total thyroidectomy (Fig. 2) was performed and histological examination showed that the nodules have rather characteristic features of benign hyperplastic lesions than of true neoplasms. After the thyroidectomy no complications occurred; the girl is doing well on the full substitution therapy.

In contrast to the thyroid disease, the hearing loss has showed only a slight deterioration until the present time. The latest audiometry revealed moderate to severe bilateral sensorineural hearing loss - the threshold mounted up to a mean of 65 dB in the right and 70 dB in the left ear. Additionally a high-resolution computer tomography (HR-CT) scan of the inner ear was carried out and documented a bilaterally enlarged vestibular aqueduct of 4.6 mm (Fig. 4).

DISCUSSION

Pendred syndrome with its involvement of two different systems - the auditory and the endocrine - and its diagnostic and therapeutic complexity, requires continuous interdisciplinary management with the cooperation of endocrinologists, otologists, geneticists and sometimes surgeons. For the endocrinological section it is important that the disease is one of the genetic causes of congenital hypothyroidism based on dyshormonogenesis (13). However, the diagnostic difficulty lies in the very high phenotypic variability, especially of the thyroid involvement of the disease. Apart from cases in which the thyroid dysfunction does not develop at all, in the majority of patients it presents in the form of a euthyroid or hypothyroid goitre which most often occurs in the second decade of life (6, 7).

Dyshormonogenetic goitre is a rare type of goitre, being observed in 1 of 30,000 births. Apart from *PDS*, other genes have also been shown to be involved in its

pathogenesis, including those for thyroidal peroxidase (*TPO*), thyroglobulin (*TG*), sodium iodide symporter (*NIS*) and more recently thyroid oxidase 2 (*THOX2*) (14).

We report here a patient with dyshormonogenetic goitre due to mutations in the *PDS* gene. In this case a small diffuse hypothyroid goitre manifested at a relatively early age, progressed during subsequent years and finally underwent nodular transformation. This course is observed frequently in dyshormonogenetic goiters, although more often if compliance is poor or the treatment inadequate, and is thought to be a consequence of a long-term stimulation by elevated levels of thyrotropin (8). Interestingly, our patient showed good cooperation and a high TSH was never documented during regular follow-up.

One of the most serious complications of goitres is the development of malignancies. As known, continuously replicating cells under TSH stimulation may undergo epigenetic changes and assume autonomy of growth that can proceed into the arising of an invasive thyroid carcinoma. Hence, a significantly higher occurrence of malignant tumors has been found in goitres of patients with congenital hypothyroidism due to dyshormonogenesis (17%) (8) than in multinodular goitres of other aetiologies (6-8%) (15). In a study of 109 thyroidectomized patients with dyshormonogenesis, Medeiros-Neto et al. (8) found 19 with follicular carcinomas of which many displayed aggressive behaviour.

Furthermore, many cases of patients with Pendred syndrome and thyroid malignancy have been reported in the literature. Camargo *et al.* (9) studied an inbred pedigree with Pendred syndrome in which the index patient - a 53 year-old female - developed a follicular carcinoma with areas of anaplastic transformations and lung metastasis. Watanabe *et al.* (10) described a women with an undifferentiated carcinoma that invaded the trachea and metastasised to the lungs and bones and Abs *et al.* (11) followed a case of 54 year-old man who expressed a metastasising thyroid carcinoma in the remnant tissue after subtotal thyroidectomy.

In view of these facts the optimal strategy in such cases is still not unknown. Numerous authors suggest that the primary therapy should be conservative, based on the fact that thyroxine given in a dose sufficient to cause TSH suppression can lead to a complete regression of the goitre (8). This has already been observed in several cases, although it seems that there is less regression if the goitre is nodular. Therefore it is important to identify and treat the dyshormonogenetic goitre early, before it undergoes nodular transformation, since at that stage complete regression is most likely and surgery

may be avoided (16). Nevertheless, it is still contentious how to proceed if the goitre does not respond to the conservative treatment, as in our patient, and the thyroid enlargement progresses despite good adjustment of the substitution therapy. When is the right moment for the patient to undergo an operation and what is the optimal surgical procedure?

The current indications for a thyroidectomy of a multinodular goitre are based on the occurrence of compression-induced symptoms, suspected malignancy or cosmesis (17). In our patient, there were no signs of tracheal compression, and fine-needle aspiration biopsy, which is according to the majority the most efficient method to uncover malignancies, revealed no malignant cells. However, a negative biopsy does not exclude with certainty the possibility of a carcinoma, especially in multinodular goitre where the error in sampling the correct area is large (18). In addition, our patient started to complain about the cosmetic aspect of the goiter, which supported us in our decision to move towards a surgical solution.

In accordance with the literature and having regard for the fact that dyshormonogenetic goiters have a higher recurrence rate (8), we chose total thyroidectomy as the most advantageous surgical method for our patient. An increasing number of reports recommend total or near total thyroidectomy as the procedure of choice for the management of multinodular thyroid disease, although this issue remains controversial, and others still propose less radical treatment in the form of limited thyroid resection (17, 19). However, for dyshormonogenetic goitres we see a clear advantage of the total thyroidectomy to avoid the regrowth of remnant tissue and the possibility of malign transformation, and thus to reduce the risk of re-operation, which is associated with increased morbidity.

In conclusion, dyshormonogenetic goitres can progress and become nodular even though substitution therapy is adequate and there are normal levels of TSH. According to the literature, nodules in dyshormonogenetic goitre more often become malignant than nodules of other aetiology. Therefore regular follow-up and thyroid imaging by an endocrinologist is mandatory in patients with Pendred syndrome, and total thyroidectomy can be the best method for particular patients in which the thyroid dysfunction shows progression despite previous long-term conservative therapy.

ACKNOWLEDGEMENTS

The study was supported by Charles University in Prague (grant GAUK 2008/2007) and by the Czech Ministry of Education (grant MSM 0021620814). We express our thanks to Prof. J. Pohlenz and his colleagues from Hospital of the Johannes Gutenberg University in Mainz, Germany, for providing us with the currently used primer sequences and PCR conditions.

REFERENCES

1. Reardon W, Trembath RC. Pendred syndrome. J Med Genet 1996; 33: 1037-1040.
2. Phelps PD, Coffey RA, Trembath RC, Luxon LM, Grossman B, Britton KE, Kendall-
   Taylor P, Graham JM, Cadge BC, Stephens SG, Pembrey ME, Reardon W. Radiological malformations of the ear in Pendred syndrome. Clin Radiol 1998; 53: 268-273.
3. Everett LA, Glaser B, Beck JC, Idol JR, Buchs A, Heyman M, Adawi F, Hazani E, Nassir E, Baxevanis D, Sheffield VC, Green ED. Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS). Nat Genet 1997; 17: 411-422.
4. Scott DA, Wang R, Kreman TM, Sheffield VC, Karniski LP. The Pendred syndrome gene encodes a chloride-iodide transport protein. Nat Genet 1999; 21: 440-443.
5. Royaux IE, Suzuki K, Mori A, Katoh R, Everett LA, Kohn LD, Green ED. Pendrin, the protein encoded by the Pendred syndrome gene (PDS), is an apical porter of iodide in the thyroid and is regulated by thyroglobulin in FRTL-5 cells. Endocrinology 2000; 141: 839-845.
6. Fraser GR. Association of congenital Deafness with goitre (Pendred's Syndrome) a study of 207 families. Ann Hum Genet 1965; 28: 201-249.
7. Reardon W, Coffey R, Chowdhury T, Grossman A, Jan H, Britton K, Kendall-Taylor P, Trembath R. Prevalence, age of onset, and natural history of thyroid disease in Pendred syndrome. J Med Genet 1999; 36: 595-598.
8. Medeiros-Neto G, Stanbury JB. Thyroid malignancy and dyshormonogenetic goiter. In: Medeiros-Neto G, Stanbury JB, eds. Inherited Disorders of the Thyroid system. Boca Raton, FL: CRC Press, 1994; 909-916.
9. Camargo R, Limbert E, Gillam M, Henriques MM, Fernandes C, Catarino L, Soares J, Alves VA, Kopp P, Medeiros-Neto G. Aggressive metastatic follicular thyroid carcinoma with anaplastic transformation arising from a long-standing goiter in a patient with Pendred's syndrome. Thyroid 2001; 11: 981-988.
10. Watanabe I. Dyshormonogenesis - with special reference to its relationship with thyroid adenoma. Horumon To Rinsho 1983; 31: 627-636.

11. Abs R, Verhelst J, Schoofs E, De Somer E. Hyperfunctioning metastatic follicular thyroid carcinoma in Pendred's syndrome. Cancer 1991; 67: 2191-2193.
12. Borck G, Roth C, Martine U, Wildhardt G, Pohlenz J. Mutations in the PDS gene in German families with Pendred's syndrome: V138F is a founder mutation. J Clin Endocrinol Metab 2003; 88: 2916-2921.
13. Banghova K, Al Taji E, Cinek O, Novotna D, Pourova R, Zapletalova J, Hnikova O, Lebl J. Pendred syndrome among patients with congenital hypothyroidism detected by neonatal screening: identification of two novel PDS/SLC26A4 mutations. Eur J Pediatr 2008; 167: 777-783.
14. Gillam MP, Kopp P. Genetic defects in thyroid hormone synthesis. Curr Opin Pediatr 2001; 13: 364-372.
15. Pelizzo MR, Bernante P, Toniato A, Fassina A. Frequency of thyroid carcinoma in a recent series of 539 consecutive thyroidectomies for multinodular goiter. Tumori 1997; 83: 653-655.
16. Vittal S, Chandrasekaran M, Kumar KB, Sucharitha V, Jeevaratinam R. Dyshormonogenetic goitre. J R Coll Surg Edinb 1993; 38: 205-207.
17. Ozbas S, Kocak S, Aydintug S, Cakmak A, Demirkiran MA, Wishart GC. Comparison of the complications of subtotal, near total and total thyroidectomy in the surgical management of multinodular goitre. Endocr J 2005; 52: 199-205.
18. Tollin SR, Mery GM, Jelveh N, Fallon EF, Mikhail M, Blumenfeld W, Perlmutter S. The use of fine-needle aspiration biopsy under ultrasound guidance to assess the risk of malignancy in patients with a multinodular goiter. Thyroid 2000; 10: 235-241.
19. Gough I R, Wilkinson D. Total thyroidectomy for management of thyroid disease. World J Surg 2000; 24: 962-965.

FIGURES

Figure 1

Sequence analysis of the two PDS/SLC26A4 mutations identified in our patient.

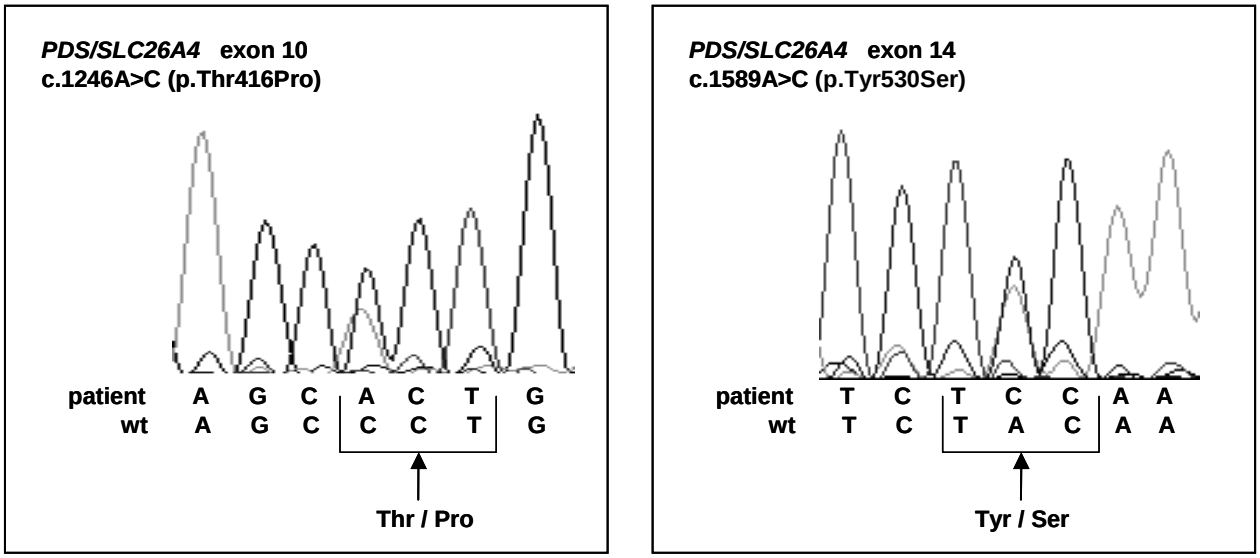


Figure 2

Left: The 22 years old female with the multinodular goitre.

Right: Multinodular goitre after total thyroidectomy**.**

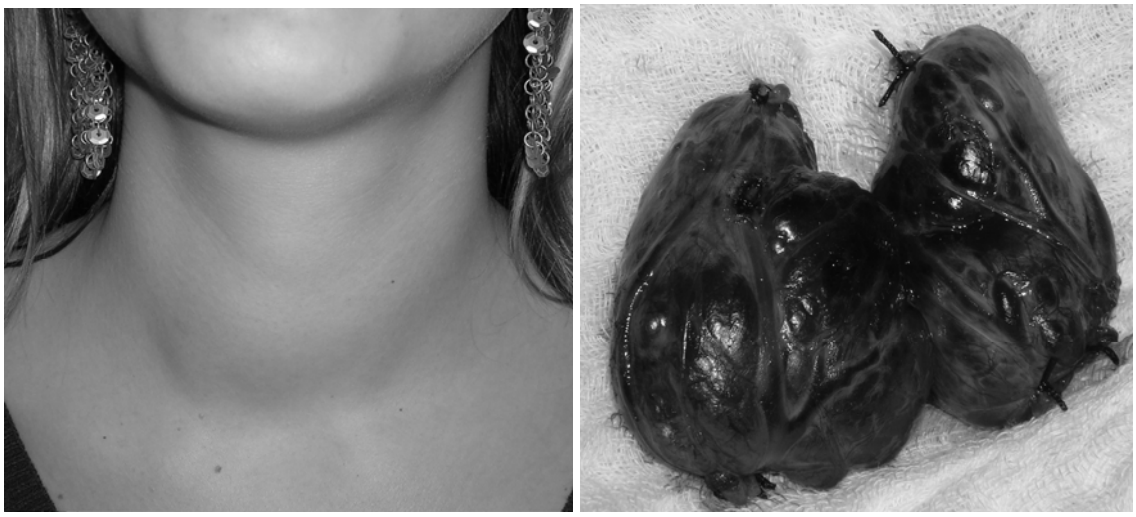
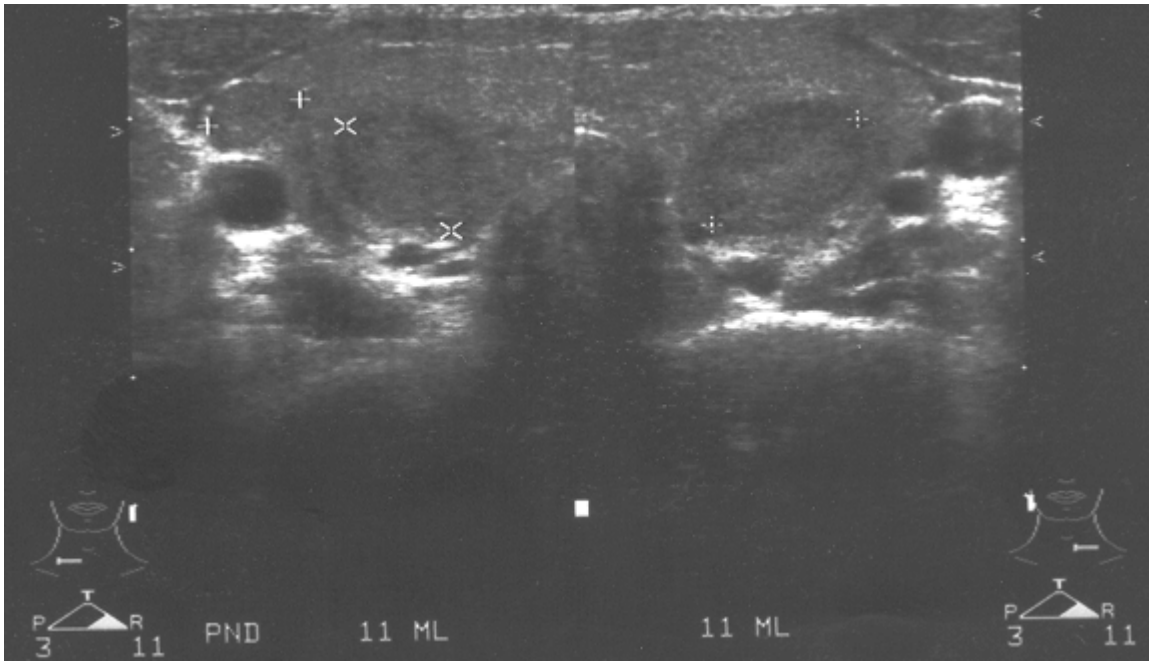


Figure 3

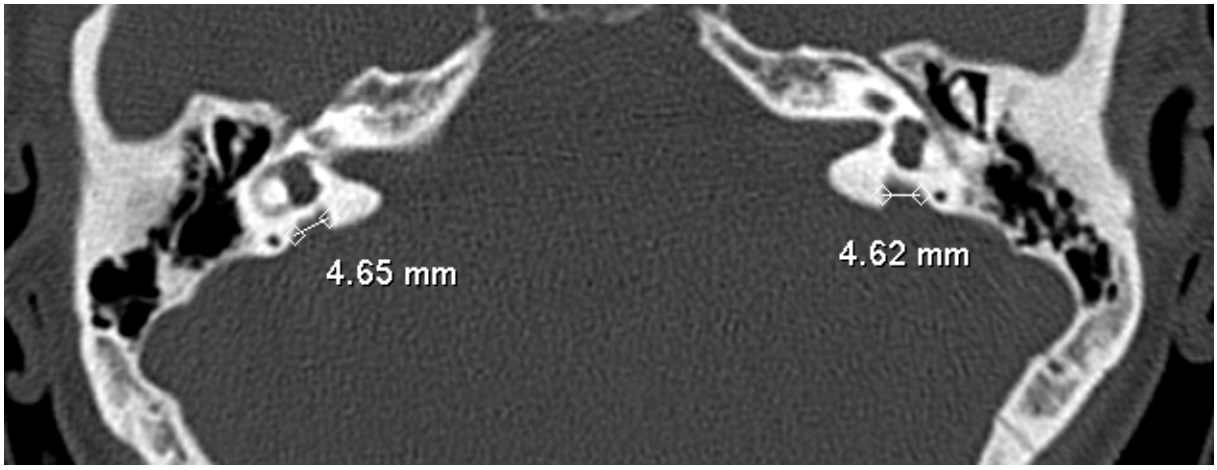
Thyroid ultrasound at the age of 22 years



Two well-circumscribed solid isoreflective nodules (7 and 11 mm in diameter) in the right thyroid lobe and one bigger nodule (20 x 14 x 9 mm) in the left thyroid lobe.

Figure 4

HR-CT scan of the inner ear showing bilateral enlarged vestibular aqueduct



5. CLOSING REMARKS

Implications of current knowledge of molecular pathogenesis of thyroid dysgenesis and dyshormonogenesis for further research and clinical practise

In recent years, endocrinologists have begun to understand human thyroid disease in terms of aberrations of the molecular pathways which govern normal thyroid development and function. However, we are still awaiting molecular explanation of many processes in the thyroid gland and we are still a long way from the detailed molecular and genetic understanding of the regulation of the thyroid development and growth.

TD in the era of newborn screening has to be considered a hereditary disorder, but neither the mode of inheritance, nor the modulation of inheritance by environmental factors have been clarified so far (Grüters 2002). Although most of TD cases appear sporadically, recurring **familial TD** - suggesting a genetically determined disorder - have been repeatedly described (Macchia 1998, Castanet 2000, Castanet 2001, Léger 2002). Moreover, the real familial occurrence may be underestimated. Firstly, affected children from previous generations were usually not fit enough to reproduce due to untreated hypothyroidism. Secondly, it was demonstrated that a single genetic defect can lead to heterogeneous phenotypes even within one family, ranging from major forms of TD with CH to asymptomatic TD which can remain undiagnosed (Léger 2002).

So far, molecular genetic analyses have been focussed on TD as a **monogenic disease**, caused by inherited or de novo mutations in genes encoding for TSHR or transcription factors PAX8, NKX2.1/TTF1, FOXE1/TTF2, NKX2.5. The low frequency of mutations detected in these genes in patients with TD could have several explanations. The mutation screening studies were limited to the coding regions thus excluding potential disease causing mutations in **non-coding regions** that might affect regulatory regions or result in aberrant splicing. Obviously, so far **unknown genes** are involved in the pathogenesis of TD. Novel genes could be either downstream target genes of the known executor genes, or may sit atop a molecular cascade and may be involved in the initiation of thyroid morphogenesis (Castanet 2005, Grasberger 2005). Particularly interesting is a novel regulator of thyroid development Shh (sonic hedhegog) which indirectly governs the symmetric bilobation of the thyroid during late organogenesis and which defects may be responsible for thyroid ectopy and hemiagenesis (Fagman 2004). As the classical

description of thyroid development is currently being revised, the way to identify novel genes goes through detailed studying mechanisms involved in the thyroid development and growth regulation. In near future, we can expect new concepts of pathogenesis of TD reflecting the current knowledge of thyroid inductive signals arising from embryonic vessels (Fagman 2006) and the involvement of vessels in thyroid relocalisation (Alt 2006). Similarly, **studying CMs** associated with CH and underlying molecular mechanisms critically contributes to the understanding of the pathogenesis of CH, as genes responsible for the development of other affected organs become candidate genes for TD.

It is supposed that **TD** is a **genetically heterogeneous disease**. Recently proposed concept of the **multigenic origin** of TD, with several interacting genes participating to produce the phenotype and being influenced by specific genetic background (by so called modifier genes), as having been demonstrated in double heterozygous Ttf1$^{+/-}$ Pax8$^{+/-}$mice (Amendola 2005), has to be considered also in humans**.** Such a pathogenic mechanism would explain why most TD patients do not display a clear Mendelian transmission and would support the observation of higher incidence of thyroid conditions in families with at least one case of TD. The incomplete penetrance and variable expressivity is also consistent with a multigenic mechanism.

Beside the germline hereditary mechanisms described above, studies of monozygotic twins discordant for TD have indicated the possibility of **nonheritable postzygotic stochastic events** e.g. early somatic mutations or epigenetic phenomena (Perry 2002, Olivieri 2007).

It is necessary to point out that except for "classical" TD diagnosed by neonatal screening with clearly elevated TSH and low peripheral thyroid hormones, the impairment of prenatal thyroid development can present as a thyroid size just at the lower normal limit with normal peripheral thyroid hormone levels but high TSH. These findings indicate that the limited thyroid capacity is compensated by TSH hyperstimulation. Elevated levels of circulating TG may suggest a defect in thyroid follicular organization. Moreover, the **impaired thyroid growth** may manifest only **postnatally** after the "normally" developed foetal thyroid gland underwent early postnatal involution or did not catch up the normal postnatal growth. In these cases, the results of neonatal screening can be either positive (but thyroid is still morphologically "normal" and the case is misdiagnosed as dyshormonogenesis) (Meeus 2004), or negative, as described in our study. Our findings confirm and extend previously published data that heterozygous *PAX8* mutations may play a causative role in the alteration of thyroid function also in the absence of congenital TD.

PAX8 defects can be compatible with still normal prenatal development, however may lead to the impaired thyroid function due to ineffective regulation of the *TPO* gene expression by *PAX8* (Pasca di Magliano 2000). As clearly demonstrated, it can be followed by consequent postnatal thyroid involution. It is important that in spite of negative results of neonatal screening for CH the regression of thyroid tissue and function can develop already within the first months or years of life that are so critical in the respect of the need of the adequate quantity of thyroid hormones. Even if such cases are probably not so frequent, they are of a high clinical importance, because negative screening results followed by early postnatal thyroid impairment may lead to prolonged, undiagnosed and thus untreated hypothyroidism with all its negative consequences.

The exact molecular mechanism responsible for the insufficient postnal growth and even regression of the thyroid tissue in *PAX8* defects is not elucidated. This transcription factor is essential for the formation of TFCs (Mansouri 1998) and it has a fundamental role as in the initiation of thyroid cell differentiation as in the maintenance of the differentiated state that it is necessary for the regular thyroid cell proliferation (Pasca di Magliano 2000). Deduced from the well characterized crucial role of *PAX8* both in developmental and functional thyroid regulatory networks, the dysregulation of the proliferation, differentiation and survival of TFCs or an increased apoptosis of undifferentiated cells may be the key mechanisms responsible for the postnatal thyroid growth impairment in *PAX8* mutation carriers. Based on these observations, the updated classification of disorders of thyroid development comprises not only classical congenital TD (or congenital thyroid developmental anomalies) but also postnatal thyroid growth impairment (Chart 4).

Molecular pathogenesis and phenotypical variability of **thyroid dyshormonogenesis** seems to be a much better clarified field. It has been shown that thyroid dyshormonogenesis can manifest not solely as CH (permanent or rarely transient) but also as postnatal non-autoimmune euthyroid or hypothyroid goitre (Chart 5). Such a phenotypic variability was demonstrated as well in our studies regarding iodide transporter pendrin. However, several unsolved tasks still remain e.g. the explanation of the association of CH due to dyshormonogenesis and only a single *TPO* mutation, as it was observed and discussed also in our *TPO* study. Thyroid dyshormonogenesis is considered to be a monogenic disease but the possible association of a single *TPO* mutation with e.g. various polymorphisms leading to phenotypical expression opens the question of a

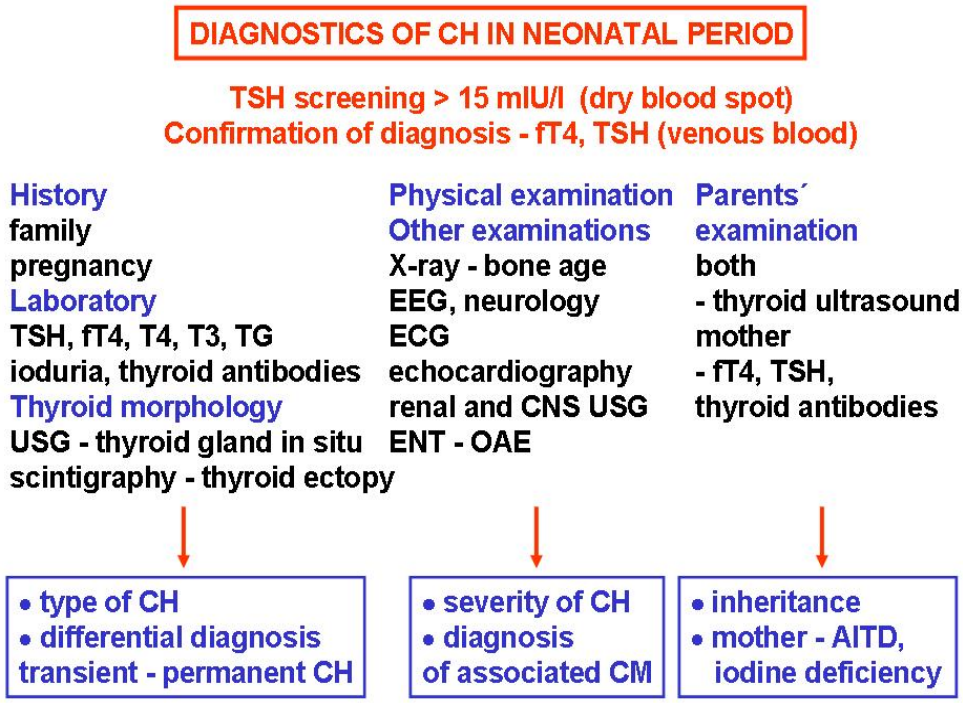
polygenic transmission. Another not completely answered question is the relation of thyroid dyshormonogenesis to the risk of thyroid cancer. Agressive follicular thyroid carcinomas with a tendency to early distant metastases were reported in patients with genetically confirmed TPO (Medeiros-Neto 1998), TG (Alzahrani 2006), and pendrin (Camargo 2001) defects. The causative role of constant and prolonged TSH stimulation is frequently mentioned, nevertheless responsible particular molecular mechanisms have not been described till now.

Thus we can assume, that molecular genetic studies of CH bring implications not only for further scientific research but they have significant **impact on clinical practise** . Complete up-to-date knowledge of molecular pathogenesis of TD and dyshormonogenesis is necessary and mandatory for correct and **up-to-date diagnostic work-up** and **long-term follow-up** of children with CH, for predicting **long-term outcome**, and last but not least for adequate and individual **counselling and support** which are central aspects of the care of patients with CH and their families (Grüters 2002). Molecular genetic studies may help to explain the less favourable outcome present despite appropriate treatment in 5-10% of the patients, or higher occurrence of associated CMs and permanent functional impairments in children with CH. As mentioned before, the current hypothesis suggests one common molecular defect, genetically determined, that leads to the combined structural or functional impairment of several organs (Chart 2). Such a model can apply not only to defects of transcription factors leading to combined structural impairment of thyroid and other organs, but also to defects of some other proteins involved either in the regulation of the thyroid development, or in the thyroid function as e.g. pendrin.

To summarize, the current knowledge of the pathogenesis of CH underlines the necessity of a **comprehensive care and approach** to paediatric and adolescent patients with CH and to their families. Our data supported by other studies underline the importance of a careful and complete investigation of every newborn with CH and the necessity of an active search for associated CMs, with a special attention to the cardiovascular system, and for the hearing impairment (Chart 7). Then, diagnosis of syndromic forms of CH must be considered in all cases of combined affection of the thyroid and other organs. Regular, long-term follow-up cannot be focused just on the thyroid itself (Chart 8) and it should be individual according to the type of CH and the occurrence of associated affections.

Chart 7

Diagnostic work-up in a newborn with CH

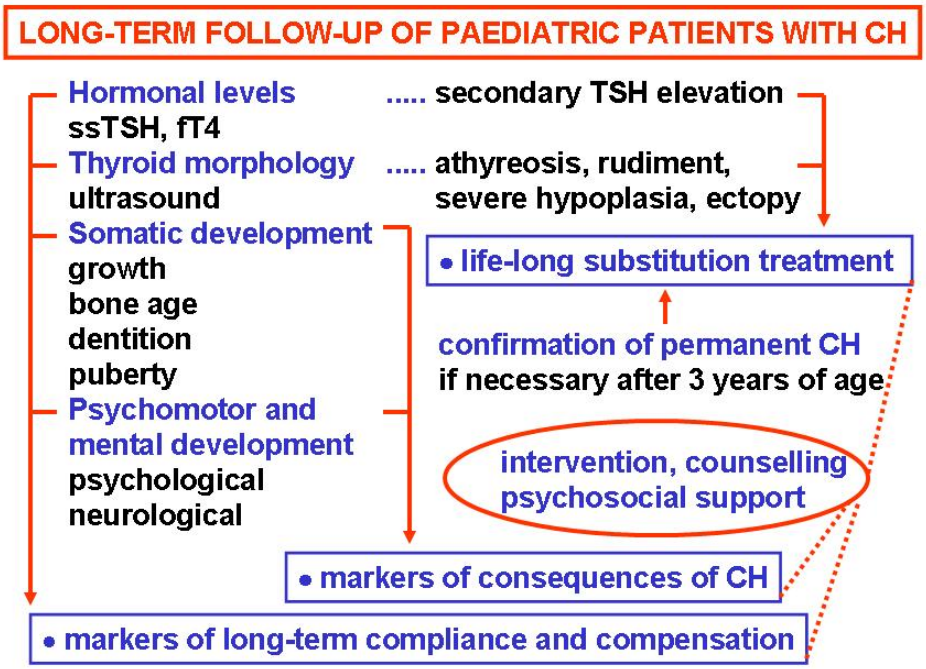


Adapted according to Al Taji E. Congenital thyroid disorders. In: Kreze A, Langer P, Klimeš I, Stárka L, Payer J, Michálek J. General and clinical endocrinology. Bratislava, Academic Electronic 2004; 298-310.

AITD - autoimmune thyroid disease, CNS - central nervous system, ECG - electrocardiography, EEG - electroencephalography, ENT- ear, nose, throat examination, OAE - otoacoustic emission, USG - ultrasonography.

Chart 8

Long-term follow-up of children and adolescents with CH



Adapted according to Al Taji E. Congenital thyroid disorders. In: Kreze A, Langer P, Klimeš I, Stárka L, Payer J, Michálek J. General and clinical endocrinology. Bratislava, Academic Electronic 2004; 298-310.

6. APPENDICES

6.1. Appendix 1 - Selected laboratory procedures

6.1.1. PCR

Equipment

cycler Gene Amp PCR system 9700 (PE Applied Biosystem, Foster City, CA, USA)

Sequencing Grade Solution dNTP´s 100 mM solution (Amersham Pharmacia Biotech Inc, USA)

polymerase Expand High Fidelity PCR System 3.5 U/µl, buffer Expand High Fidelity, buffer 10x conc. with $MgCl_2$ (Roche Diagnostics, Mannheim, Germany)

polymerase AmpliTaq DNA Polymerase 250 Units 5U/µl, buffer 10x conc. PCR Buffer 15mM $MgCl_2$ (Roche Diagnostics, Mannheim, Germany)

Technique

PCR reactions were performed using a 50-µl PCR mixture:

0.5 µl Expand HiFi enzyme mix 3.5 U/µl

5 µl Expand HF Buffer II 10 x conc. with 15 mM $MgCl_2$

0.5-1.5 µl 10 mM dNTP

1 µl 50 µM primer F

1 µl 50 µM primer R

2 µl DNA

in case of need additionally 10 µl 5 M betaine

filled up with H_2O to the total volume

PCR conditions were established in general as follows:

initial denaturation at 95 C for 5 min

30 cycles of

- denaturation at 95 C for 1 min
- annealing at a specific temperature for 1-4 min
- (depending on the melting temperature of the primer)
- elongation at 68 C for 1-2 min

ended by a prolonge elongation at 68 C for 6-7 min

6.1.2. Electrophoresis

Equipment

apparatus Biometra (Göttingen, Germany)

1 Kb DNA Ladder (Invitrogen Life Technologies, U.S.A)

Ultra pure agarose electrophoresis grade gel (Life Technologies, Paisley, Scotland)

Technique

2 µl of PCR product were mixed with 4 µl of dye, loaded and run on 1% agarose gels, stained with ethidium bromide, and visualized with UV light.

Agarose gel loading dye

50 mg bromphenol

50 mg xylene cyanol

15 ml H_2O

+ add 5 ml glycerol

Ethidium Bromide

1 tbl

add 8 ml H_2O

1kB DNA ladder

4 µl 2M Tris pH 8.3

2 µl 50 mM EDTA

2 µl 5M NaCl

328 µl bidistilled H_2O

24 µl DNA marker

keep in 65 C for 10 min

add 40 µl loading dye

or

336 µl 5xTBE

24 µl DNA marker

keep in 65 C for 10 min

add 40 µl loading dye

6.1.3. Direct sequencing

PCR purification equipment

QIAquick PCR Purification Kit (Qiagen, Hilden, Germany)

3M NaAc (Fulka, Germany)

Sequence PCR equipment

Big Dye RR Terminator Cycle Sequencing Kit - Terminator Premix (PE Applied Biosystems)

DNA Sequencing Kit - Big Dye Terminator Cycle - Sequencing Ready Reaction with Ampli TaqDNA polymerase (Applied Biosystem, Warrington, UK)

ABI PRISM 377 Sequencer (PE Applied Biosystems, Foster City, Ca, USA)

6.1.4. SSCP

Solutions

Top silan

100 µl Silan A (methacryloxypropyltrimethoxysilane adhesive promoter)

45 µl 100% acetic acid glacial

25 ml 100% ethanol

1x MDE gel

1.75 ml 10x conc. TBE

17.5 ml 2x conc. MDE

16.0 ml H_2O

14.0 µl TEMED

140 µl 10% APS

Top gel

2.5 ml formamide

2.5 ml 2x conc. MDE

250 µl 10x konc. TBE

15.0 µl TEMED

80.0 µl 10% APS

10x TBE

80 ml 0.5M EDTA pH 8

216 g Tris Base

110g boric acid

ad H_2O up to 200 ml

SSCP loading dye

95% formamide	=	750 µl 100% formamide
10mM NaOH		10 µl 5M NaOH
0.1% bromphenol blue		5 mg BPB
0.1% xylene cyanol		5 mg XCi
10% DMSO		0.5 ml DMSO + 230 µl H_2O

Sample preparation

2 µl PCR product

14 µl SSCP loading dye

in 95 C for 5-10 min, cooled on ice

Quick silvering method

- *fixation* (2 times for 3-5 min)

50 ml 100% ethanol

2.5 ml 100% acetic acid

filled up to 500 ml with distilled H_2O

- *silvering* (for 10 min)

0.3 g silver nitrate AgNO3

250 ml distilled H_2O

- *washing* (2 times for 10 s)

- *developing*

4g NaOH solved in 250 ml distilled H_2O

50 µl of solution of 0.1g natrium thiosulphate disolved in 10 ml distilled H_2O

after 10-15 min add 1 ml 37% formaldehyd

- *stopping* **(**for 5min)

10% acetic acid

6.2. Appedix 2 - Specific PCR primers and annealing conditions for investigated genes

6.2.1. Genes encoding for transcription factors

Gene	Exon	Primers	Annealing
PAX8	exon 2	2F 5′-GGATGCAGGCATCGAATCTCATCG-3′	68 C/1 min *
		2R 5′-CGAGATCCAACCACCCGAGCGC-3′	
	exon 3	3F 5′-CATAGCTAATCCCCACCC-3′	60 C/1 min
		3R 5′-CCTGCGGTGAATTTCGTG-3′	
	exon 4	4F 5′-ATTGGGTAATTCTTTGGGATTC-3′	60 C/1 min
		4R 5′-CCAGGCCTTTCTTGTCTCTT-3′	
		(both ref.Macchia et al. 1998)	
	exon 5	5F 5′-AGGGGTGTCAAAAAGGCGACTG-3′	60 C/1 min
		5R 5′-TGGGTATGCTGAAGGGGAGGTG-3′	
		(both ref. Macchia et al. 1998)	
	exon 6	6F 5′-AGAGTCACCCAGGGCTGTGAG-3′	60 C/1 min
		6R 5′-GCAGAGCCCCTACAAAGTCC-3′	
		(ref. Macchia et al. 1998)	
	exon 7	7F 5′-CCTCTACTTTGGCCTAGAG-3′	60 C/1 min
		7R 5′-CACAGGCTCATTTGGAGAAT-3′	
		(ref.Macchia et al. 1998)	
	exon 8	8F 5′-GTCTCTGTGCGCTGACTTCT-3′	60 C/1 min
		8R 5′-CACACCTTCCGCCTGAC-3′	
		(both ref. Macchia et al. 1998)	
	exon 9	9F 5′-CCTCCCCGCCATCTCACACC-3′	64 C/1 min
		9R 5′-TCCCACCCGCCGCCATAG-3′	
		(both ref. Macchia et al. 1998)	
	exon 10	10F 5′-GCCCCCATGGTCCAACTGAC-3′	60 C/1 min
		10R 5′-TGCCTCTGCTCCTTGTGTCCAC-3′	
		(both ref. Macchia et al. 1998)	
	exon 11	11F 5′-GATGCCCTTCACCTCACAGGCC-3′	64 C/1 min
		11R 5′-CCACCACTCCATCCATCCTGCC-3′	
	exon 12	12F 5′-TTTTCCTTTCTGACCAGCAGTG-3′	64 C/1 min

		12R 5′-ATTCCTTTGTGTGACTCTCTGG-3′	
NKX2.5	exon1+2	F 5′-GTCCCGCCTCTCCTGCCCCTTGTG-3′ R 5′-GCGTGCCCGAGCTCAGTCCCAGTT -3′ (both ref. Benson et al. 1999) [a] sequence primers [b]: F 5′-TGGGCGCTCCAGGCAGGACACAGT-3′ F 5′-GACTCTGGAGCTGGTGGGGCTGCC-3′ R 5′-CCTCCTGGCCCTGAGTTTCTTGGG-3′	68 C/2 min
NKX2.1	exon1-3	F 5′-CGGGAGGCAGTCGATCCCTACTCAG-3′ R 5′-AGGGCCGGCCCGGCGTCCTCTCACC-3′ (ref. Krude et al. 2002) [a]	68 C/4 min
FOXE1	exon	F 5′-TGGAGAGGACCAGCCTCAGGTCGC-3′ R 5′-AGATTGCGGGGAACGTGTGAACAG-3′ sequence primers [b]: F 5′-AGACCACGGACGCTGAG-3′ F 5′-AAGCGCCCCCTGCAGCGCGGGAAG-3′ F 5′-TCGAGAGCGGCAGCTTCCTGC-3′ R5′- CGGGTAGGTGGAGAGGTCCGA-3′ R 5′-GCGGCAAAGATCGCACTGCCG-3′	68 C/2 min*
HHEX	exon 1 exon 2+3 exon 4	F 5′- GAGGCCGGCGGAGCCTATCGCCGG-3′ R 5′-GCCACAGCCGTCTACCGCCTGCCC-3′ F 5′-GGTTGGGTGGCCTCCTGGCCTACC-3′ R 5′-TGATATAGGAACCCCATTAGGTGC-3′ F 5′-TTATGTTCATGACTGGTTCTCATC-3′ R 5′-ACAACCTAAGAGCAGTACATAAAC-3′	68 C/2 min * 68 C/2 min 57 C/2 min *

[a] Primers were designed to amplify the whole coding region in a single PCR.

[b] Additional sequence primers were designed to get a complete sequence of the coding region.

* PCR mixture contained betaine.

6. 2. 2. *TPO* gene

Exon	Primers	Annealing temperatures
2	F 5′-GTTCCCATGGCCTTGTCAGT-3′ R 5′-CTCAGGAGCTACCATTATGCC-3′	65 C*
3	F 5′-GAACTGTCATTGCGCTTTGAC-3′ R 5′-CACACGTGTGTGGATGTCAGG-3′	62 C*
4	F 5′-GATACCATAGACAAATAATC-3′ R 5′-CAAAGTCAAGGTGTCCTCCT-3′	53 C*
5	F 5′-GAATTCATGGTTTCCTATTT-3′ R 5′-GATCCAACTTTCACGAGAAG-3′	55 C*
6	F 5′-GGAACTCACTGCTTCTGTG-3′ R 5′-CAATCTTAGTCCAGGATAG-3′	59 C*
7	F 5′-AGGTCATCTTTCTGCTACCA-3′ R 5′-CTACCCCTGGGAATAGGACA-3′ F 5′-GTCCTCCTATGTCCTGACCAATG-3′ R 5′-CTGCTACCCCTGGGAATAGGAC-3′	61 C*
8	607F 5′-GGCCTCGAACTTCCAGAGTCTTACAA-3′ 888F 5′-TGGACCAGTGCCGAAGGGCTGCTC-3′ 1278R 5′-CCACGATGCTGCGCACCCACACGA-3′	65/63 C*
9	F 5′-CTGTCAAGGAAGATGCTCTTCCACAC-3′ R 5′-AAAGAAGGGAGAGTTCATGGGGACCA-3′	63 C*
10	F 5′-TGTTTCTCTAGAACTGAGCCAAGAGC-3′ R 5′-CCTCTAAATCAGTCTCTCTAGCAGCAGGT-3′	64 C
11	F 5′-GGGCTGAACAAAAGTTCAGTT-3′ R 5′-TTCACAACCAACGCAGACCAC-3′	61 C*
12	F 5′-AGCACACAGCTGTGGGCAGC-3′ R 5′-AGCTCCTGGGGAAGATAAGC-3′	61 C*
13	F 5′-CTCGTAGTTTGACTACATGTC-3′ R 5′-GCATTGTTAACTTATATCGG-3′	58 C*
14	F 5′-GTGTGTGGCCTTGTGTGTCT-3′ R 5′-GATGGTGATTGACAGTTGCCT-3′	61 C*

	F 5′-CAGAGAGAAGCACCTCCCAGAAC-3′ R 5′-CAGTTGCCTAACTTGAACACAC-3′	
15	F 5′-GGAAGGTTCTTCTAACCAGG-3′ R 5′-GAGATTGCTTTGTGTCTAAG-3′	58 C*
16	F 5′-CTTGCCGTCGCTCGTGCCGTGCTCTC-3′ R 5′-GTCCAACCACTGCGGTGGCAGCTGGA-3′	62 C*
17	F 5′-GAATTCAGACGTTATTAATGTTTGT-3′ R 5′-GATTTTGGGAACATGAAGCTGTTCT-3′	61 C*

* PCR mixture contained betaine. Annealing 1 min.

6.3. Appendix 3 - Screening for *PAX8* SNP in intron 2 (additional study)

***PAX8* SNP** (single nucleotide polymorphisms) **c.25+24T>C** (c.IVS2+24T>C) was examined in 168 patients with non-goiter CH as a simple marker excluding a deletion of one *PAX8* allele or of the appropriate region of the *PAX8* gene if present in a heterozygous state. Restriction enzyme analysis of a selected PCR product was performed with Dpn II in conditions recommended by the manufacturer (New England BioLabs, Ipswich, MA, USA). Digest products were separated on a 3% cooled agarose, stained with ethidium bromide and analyzed under UV light. Results of restriction enzyme analysis were confirmed by SSCP. 77 heterozygous carriers of *PAX8* SNP c.25+24T>C were identified- *PAX8* gene deletion was excluded in 45.8% of patients with non-goitre CH.

PAX8 intron 2 polymorphism c.25+24T>C (c.IVS2+24T>C)

PCR product 235 bp = 55bp of intron 1 + exon 2 (100bp) + 80bp of intron 2, PCR primers underlined.
Ezyme digestion with DpnII - cutting GATC.

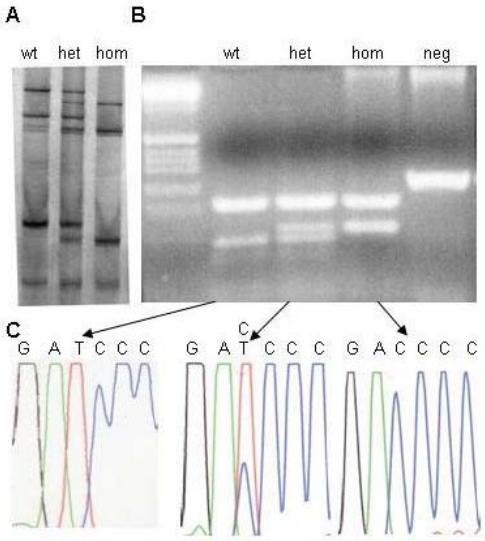
ggatgcaggcatcgaatctcatcgcatctcatgcccttctcctgggtttgtgcag

GGCAGCGGCAGGCGCGGCCCGGACCTACGGGAGGAAGCCCCGAGCCCTCGGCGGGCTG

CGAGCGACTCCCCGGCG**ATG**CCTCACAACTCCATCA↓GATCTG

gtaagaacgcggtgtggtcag↓ga**t/c**ccccgagcccccgcgccgctgagcgtccgtgcg

tgcgctcgggtggttg↓gatctcg



PAX8 SNP 25+24T>C (IVS2+24T>C)

A SSCP screening (1x MDE gel, room temperature, power 2W, run time 24 hours) showing of the wild type and heterozygous or homozygous carriers of SNP.

B Electrophoresis of DpnII digest products on a 3% cooled agarose gel comparing the wild type (the length of digestion products: 149 bp, 52 bp, 27 bp, 7 bp), heterozygote (149 bp, 79 bp, 52 bp, 27 bp, 7bp), homozygote (149 bp, 79 bp, 7 bp) and negative control (235 bp). 27 bp and 7 bp bands are not visible.

C Parts of the direct sequencing of the wild type, heterozygote and homozygote confirming results of screening methods.

(wt - wild type, het - heterozygote, hom - homozygote, neg - negative control)

7. SUMMARY - SOUHRN

Východisko: V uplynulém desetiletí se podařilo objasnit na molekulární úrovni některé základní pochody regulace vývoje, růstu a funkce štítné žlázy. Do popředí se tak dostala problematika molekulární patogeneze tyreoidální dysgeneze a dyshormonogeneze, které se u většiny pacientů manifestují pod obrazem kongenitální hypotyreózy (KH), jen vzácně jako časně postnatálně nastupující neautoimunitní hypotyreóza. Po objevení kandidátních genů byly jejich mutace detekovány u několika pacientů s nesyndromickou i syndromickou vrozenou hypotyreózou; nicméně chyběly fenotypicky zaměřené molekulárně-genetické studie větších souborů neselektovaných pacientů. Zejména u dysgeneze štítné žlázy poznatky vycházely z pozitivních výsledků analýz jen u několika jednotlivých pacientů.

Cíle: Cílem této rozsáhlé studie bylo identifikovat monogenní formy dysgeneze a dyshormonogeneze štítné žlázy ve skupině českých pacientů převážně s KH. Příslušné geny byly analyzovány na základě podrobných klinických informací a fenotypické charakteristiky, tedy se zaměřením na jednotlivé klinicky definované skupiny pacientů s fenotypy odpovídajícími dříve identifikovaným genovým defektům.

Soubor: Do studie bylo zahrnuto 193 českých dětí, dospívajících a mladých dospělých (130 dívek, 63 chlapců) s primární permanentní KH nebo časně nastupující neautoimunitní hypotyreózou. Ve skupině 190 pacientů s KH byla pouze u 22 pacientů pozorována struma. Struma byla popsána také u jednoho ze tří pacientů s postnatální časnou hypotyreózou.

Metodika: Klinická data byla zpětně získávána prostřednictvím fenotypických dotazníků. Všech 170 pacientů s vrozenou a časně nastupující primární hypotyreózou bez strumy bylo vyšetřeno metodou SSCP na mutace v genu *PAX8*. U 22 pacientů s KH a strumou byl analyzován gen *TPO*. U jedinců se syndromickou hypotyreózou byly navíc vyšetřovány i geny *NKX2.5*, *NKX2.1/TTF1*, *FOXE1/TTF2* a *HEX* u případů KH bez strumy sdružené se strukturální vrozenou vývojovou vadou dalšího orgánu a *PDS/SLC26A4* u pacientů se strumou a percepční poruchou sluchu.

Strategie: První studie byla zaměřena na úlohu transkripčního faktoru PAX8 pro vývoj a časný postnatální růst štítné žlázy a dále na roli transkripčních faktorů PAX8, NKX2.1/TTF1, FOXE1/TTF2, NKX2.5 a HEX v patogenezi sdružených vrozených

vývojových vad u pacientů s KH bez strumy. V rámci druhé studie , zabývající se genetickým pozadím dyshormonogeneze, byl u 22 pacientů s KH a strumou analyzován gen *TPO*. Gen *PDS/SLC26A4* byl analyzován u 4 pacientů se strumou (3 pacienti s KH, 1 pacient s postnatální neautoimunitní hypotyreózou) provázenou percepční poruchou sluchu, a tedy s klinickou diagnózou Pendredova syndromu.

Výsledky: Přestože byla studie fenotypicky zaměřena, záchyt mutací ve známých kandidátních genech pro KH byl velmi nízký. V genu *PAX8* byla identifikována nová mutace R52P, autozomálně dominantně dědičná ve 3 generacích, která se projevila u 3 členů rodiny jako časně postnatálně nastupující neautoimunitní hypotyreóza bez strumy s postupnou regresí velikosti a funkce štítné žlázy. Tato mutace vede k záměně evolučně konzervované aminokyseliny v oblasti domény vázající DNA s následnou ztrátou funkce, jak bylo potvrzeno funkční studií. Ve skupině pacientů se strumou byly detekovány 2 mutace *TPO* genu u 2 pacientů (z toho 2 dosud nepublikované mutace c.740delA v exonu 7 a c.2134 C >T v exonu 12), u 3 dalších pacientů byla detekována pouze jedna mutace. Klinická diagnóza Pendredova syndromu byla potvrzena molekulárně-genetickým vyšetřením u 2 jedinců s KH a jedné dívky s postnatální neautoimunitní strumou.

Závěr: Práce předkládá populačně založenou studii velkého souboru pacientů s vrozenou a časnou neautoimunitní hypotyreózou. Přestože byla molekulárně-genetická analýza zaměřena na specifické fenotypy, ukázala velmi nízkou frekvenci defektů v genech pro transkripční faktory u dětí s hypotyreózou bez strumy.To pokazuje na možnou úlohu dalších genetických mechanismů jako epigenetických nebo somatických změn, popř. roli zatím neznámých genů. Identifikace dosud nepublikované mutace *PAX8* u nově popsaného fenotypu časné postnatální neautoimunitní hypotyreózy svědčí pro klíčovou roli transkripčního faktoru PAX8 v postnatálním růstu štítné žlázy a v zachování její funkce. Počet detekovaných mutací v *TPO* genu u pacientů se strumou byl mnohem nižší, než bylo popsáno v přechozích studiích u preselektovaných skupin pacientů. Naše výsledky také ukázaly, že Pendredův syndrom je vzácnou příčinou KH. Diagnóza Pendredova syndromu by však měla být zvažována u dětí s KH se strumo, která je provázena senzorineurální poruchou sluchu.

8. REFERENCES

1. Abramowicz MJ, Dupres I, Parma J, VassartG, Heinrichs C. Familial congenital hypothyroidism due to inactivating mutations of the TSHR receptor gene causing profound thyroid hypoplasia. J Clin Invest 1997; 99: 3018-3024.
2. Abramowicz MJ, Targovnik HM, Varela V, Cochaux P, Krawiec L, Pisarev MA, Prapato EVE, Juvenal G, Chester HA, Vassart G. Identification of a mutation in the coding sequence of the human thyroid peroxidase gene causing congenital goiter. J Clin Invest 1992; 90: 1200-1204.
3. Abs R, Verhelst J, Schoofs E, De Somer E. Hyperfunctioning metastatic follicular thyroid carcinoma in Pendred's syndrome. Cancer 1991; 67: 2191-2193.
4. Ahlbom BE, Yaqoob M, Gustavsson P, Abbas GH, Annerén G, Larsson A, Wadelius C. Linkage analysis identifies the thyroglobulin gene region as a major locus for familial congenital hypothyroidism. Hum Genet 2002; 110:145-147
5. Alt B, Elsalini OA, Schrumpf P, Haufs N, Lawson N, Schwabe GC, Mundlos S, Grüters A, Krude H, Rohr KB. Arteries define the position of the thyroid gland during its developmental relocalisation. Development 2006; 133: 3797-3804.
6. Alzahrani AS, Baitei EY, Zou M, Shi Y. Metastatic follicular thyroid carcinoma arising from congenital goiter as a result of a novel splice donor site mutation in the thyroglobulin gene. J Clin Endocrinol Metab 2006; 91: 740-746.
7. Ambrugger P, Stoeva I, Biebermann H, Torresani T, Leitner C, Grüters A. Novel mutations of the thyroid peroxidase gene in patients with permanent congenital hypothyroidism. Eur J Endocrinol 2001; 145: 19-24.
8. Amendola E, De Luca P, Macchia PE, Terraciano D, Rosica A, Chiappetta G, Kimura S, Mansouri A, Affuso A, Arra C, Macchia V, Di Lauro R, De Felice M. A mouse model demonstrates a multigenic origin of congenital hypothyroidism. Endocrinology 2005; 146: 5038-5047.
9. Antonarakis SE, the Nomenclature Working Group. Recommendations for a nomenclature system for human gene mutations. Hum Mutat 1998; 11: 1-3.
10. Avbelj M, Tahirovic H, Debeljak M, Kusekova M, Toromanovic A, Krzisnik C, Battelino T. High prevalence of thyroid peroxidase gene mutations in patients with thyroid dyshormonogenesis. Eur J Endocrinol 2007; 156: 511-519.
11. Bakker B, Bikker H, Hennekam RC, De Vijlder J. Maternal isodisomy for chromosome 2p causing severe congenital hypothyroidism. J Clin Endocrinol Metab 2001; 86: 1164-1168.
12. Bakker B, Bikker H, Vulsma T, de Randamie JSE, Wiedijk BM, de Vijlder JJM. Two decades of screening for congenital hypothyroidism in the Netherlands: TPO gene mutations in total iodide organification defects (an update). J Clin Endocrinol Metab 2000; 85: 3708-3712.
13. Bamforth JS, Hughes IA, Lazarus JH, Weaver CM, Harper PS. Congenital hypothyroidism, spiky hair, and cleft palate. J Med Genet 1989; 26: 49-60.
14. Bargman GJ, Gardner LI. Otic lesions and congenital hypothyroidism in the developing chick. J Clin Invest 1967; 46: 1828-1839.
15. Baris I, Arisoy AE, Smith A, Agostini M, Mitchell CS, Park SM, Halefoglu AM, Zengin E, Chatterjee VK, Battaloglu E. A novel missense mutation in human TTF-2 (FKHL15) gene associated with congenital hypothyroidism but not athyreosis. J Clin Endocrinol Metab 2006; 91: 4183-4187.
16. Batsakis JG, Nishiyama RH. Deafness with sporadic goiter. Pendred's syndrome. Arch Otolaryngol 1962; 76: 401-406.

17. Benson DW, Silberbach GM, Kavanaugh-McHugh A, Cottrill C, Zhang Y, Riggs S, Smalls O, Johnson MC, Watson MS, Seidman JG, Seidman CE, Plowden J, Kugler JD. Mutations in the cardiac transcription factor NKX2.5 affect diverse cardiac developmental pathways. J Clin Invest 1999; 104: 1567-1573.
18. Biebermann H, Schoneberg T, Krude H, Schultz G, Gudermann T, Grüters A. Mutations of the human thyrotropin receptor gene causing thyroid hypoplasia and persistent congenital hypothyroidism. J Clin Endocrinol Metab 1997; 82: 3471-3480.
19. Bikker H, Baas F, de Vijlder JJM. Molecular analysis of mutated thyroid peroxidase detected in patients with total iodide organification defect. J Clin Endocrinol Metab 1997; 82: 649-653.
20. Bikker H, den Hartog MT, Baas F, Gons MH, Vulsma T, de Vijlder JJM. A 20-basepair duplication in the human thyroid peroxidase gene results in a total iodide organification defect and congenital hypothyroidism. J Clin Endocrinol Metab 1994; 79: 248-252.
21. Bikker H, Vulsma T, Baas V, de Vijlder JJM. Identification of five novel inactivating mutations in the human thyroid peroxidase gene by denaturating gradient gel electrophoresis. Human Mutation 1995; 6: 9-16.
22. Bikker H, Waelkens JJJ, Bravenboer B, de Vijlder JJM. Congenital hypothyroidism caused by a premature termination signal in exon 10 of the human thyroid peroxidase gene. J Clin Endocrinol Metab 1996; 81: 2076-2079.
23. Blankenstein O, Muhlenberg R, Kom C, Wuller S, Pfaffle R, Heimann G. A new C-terminal located mutation (V272ter) in the PIT-1 gene manifesting with severe congenital hypothyroidism. Horm Res 2001; 56: 81-86.
24. Blizzard RM, Chandler RW, Landing BH, Pettit MD, West CD. Maternal autoimmunization to thyroid as a probable cause of athyreotic cretenism. N Eng J Med 1960; 263: 327-336.
25. Blons H, Feldmann D, Duval V, Messaz O, Denoyelle F, Loundon N, Sergout-Allaoui A, Houang M, Duriez F, Lacombe D, Delobel B, Leman J, Catros H, Journel H, Drouin-Garraud V, Obstoy MF, Toutain A, Oden S, Toublanc JE, Couderc R, Petit C, Garabedian EN, Marlin S. Screening of SLC26A4 (PDS) gene in Pendred's syndrome: a large spectrum of mutations in France and phenotypic heterogeneity. Clin Genet 2004; 66: 333-340.
26. Bonomi M, Proverbio MC, Weber G, Chiumello G, Beck-Peccoz P, Persani L. Hyperplastic pituitary gland, high serum glycoprotein hormone α-subunit, and variable circulating thyrotropin (TSH) levels as hallmark of central hypothyroidism due to mutations of the TSHβ gene. J Clin Endocrinol Metab 2001; 86: 1600-1604.
27. Bopp D, Burri M, Baumgartner S, Frigerio G, Noll M. Conservation of a large protein domain in the segmentation gene paired and in functionally related genes of Drosophila. Cell 1986;, 47: 1033-1040.
28. Borck G, Roth C, Martiné U, Wildhardt G, Pohlenz J. Mutations in the PDS gene in German families with Pendred's syndrome: V138F is a founder mutation. J Clin Endocrinol Metab 2003; 88: 2916-2921.
29. Börgel K, Pohlenz J, Holzgreve W, Bramswig H. Intrauterinne therapy of goitrous hypothyroidism in a boy with a new compound heterozygous mutation (Y453D and C800R) in the thyroid peroxidase gene. A long-term follow-up. Am J Obstet Gynecol 2005; 193: 857-858.
30. Bouchard M, Souabni A, Mandler M, Neubüser A, Busslinger M. Nephric lineage specification by Pax2 and Pax8. Genes Dev 2002; 16: 2958-2970.
31. Brown RS, Bellisario RL, Botero D, Fournier L, Abrams CAL, Cowger ML, David R, Fort P, Richman RA. Incidence of transient congenital hypothyroidism in maternal

thyrotropin receptor-blocking antibodies in over one million babies. J Clin Endocrinol Metab 1996; 81: 1147-1151.
32. Calaciura F, Miscio G, Coco A, Leonardi D, Cisternino C, Regalbuto C, Bozzali M, Maiorana R, Ranieri A, Carta A, Buscema M, Trischitta V, Sava L, Tassi V. Genetic of specific phenotypes of congenital hypothyroidism: a population-based approach. Thyroid 2002; 12: 945-951.
33. Camargo R, Limbert E, Gillam M, Henriques MM, Fernandes C, Catarino L, Soares J, Alves VA, Kopp P, Medeiros-Neto G. Aggressive metastatic follicular thyroid carcinoma with anaplastic transformation arising from a long-standing goiter in a patient with Pendred's syndrome. Thyroid 2001; 11: 981-988.
34. Campbell C, Cucci RA, Prasad S, Green GE, Edeal JB, Galer CE, Karniski LP, Sheffield VC, Smith RJH. Pendred syndrome, DFNB4, and PDS/SLC26A4 identification of eight novel mutations and possible genotype-phenotype correlations. Hum Mutat 2001; 17: 403-411.
35. Caron P, Moya CM, Malet D, Gutnisky VJ, Chabardes B, Rivolta CM, Targovnik HM. Compound heterozygous mutations in the thyroglobulin gene (1143delC and 6725G→A R2223H) resulting in fetal goitrous hypothyroidism. J Clin Endocrinol Metab 2003; 88: 3546-3553.
36. Castanet M, Lyonnet S, Bonaïti-Pellié C, Polak M, Czernichow P, Léger J. Familial foms of thyroid dysgenesis among infants with congenital hypothyroidism. N Eng J Med 2000; 343: 441-442.
37. Castanet M, Park SM, Smith A, Bost M, Léger J, Lyonnet S, Pelet A, Czernichow P, Chatterjee K, Polak M. A novel loss-of-function mutation in TTF-2 is associated with congenital hypothyroidism, thyroid agenesis and cleft palate. Hum Mol Genet 2002; 11: 2051-2059.
38. Castanet M, Polak M, Bonaiti-Pellié C, Lyonnet S, Czernichow P, Léger J. Nineteen years of national screening for congenital hypothyroidism: familial cases with thyroid dysgenesis suggest the involvement of genetic factors. J Clin Endocrinol Metab 2001; 86:2009-2014
39. Castanet M, Sura-Trueba S, Chauty A, Carré A, de Roux N, Heath S, Léger J, Lyonnet S, Czernichow P, Polak M. Linkage and mutational analysis of familial thyroid dysgenesis demonstrate genetic heterogeneity implicating novel genes. Eur J Hum Genet 2005; 13: 232-239.
40. Chan S, Kilby MD. Thyroid hormone and central nervous system development. J Endocrinol 2000; 165: 1-8.
41. Chanoine JP, Bourdoux P, Delange F. Congenital anomalies associated with hypothyroidism. Arch Dis Child 1986; 61: 1147.
42. Clifton-Bligh RJ, Wentworth JM, Heinz P, Crisp MS, John R, Lazarus JH, Ludgate M, Chatterjee VK. Mutation of the gene encoding human TTF-2 associated with thyroid agenesis, cleft palate and choanal atresia. Nat Genet 1998; 19: 399-401.
43. Coakley JC, Keir EH, Connelly JF. The association of thyroid dyshormonogenesis and deafness (Pendred syndrome): experience of the Victorian Neonatal Thyroid Screening Programme. J Paediatr Child Health 1992; 28: 398-401.
44. Collu R, Tang J, Castagné J, Lagacé G, Masson N, Huot C, Deal C, Delvin E, Faccenda E, Eidne KA, Van Vliet G. A novel mechanism for isolated central hypothyrodism: inactivating mutations in the thyrotropin-releasing hormone receptor gene. J Clin Endocrinol Metab 1997; 82: 1361-1365.
45. Congdon T, Nguyen LQ, Nogueira CR, Habiby RL, Medeiros-Neto G, Kopp P. A novel mutation (Q40P) in PAX8 associated with congental hypothyroidism and

thyroid hypoplasia: evidence for phenotypic variability in mother and child. J Clin Endocrinol Metab 2001; 86: 3962-3967.
46. Coyle B, Coffey R, Armour JA, Gausden E, Hochberg Z, Grossman A, Britton K, Pembrey M, Reardon W, Trembath RC. Pendred syndrome (goitre and sensorineural hearing loss) maps to chromosome 7 in the region containing the nonsyndromic deafness gene DFNB4. Nat Genet 1996; 12: 421-423.
47. Coyle B, Reardon W, Herbrick JA, Tsui LC, Gausden E, Lee J, Coffey R, Grüters A, Grosmann A, Phelps PD, Luxon L, Kendall-Taylor P, Scherer SW, Trembath RC. Molecular analysis of the PDS gene in Pendred syndrome (sensorineural hearing loss and goitre). Hum Mol Genet 1998; 7: 1105 -1112.
48. Damante G. Thyroid defects due to Pax8 gene mutations. Eur J Endocrinol 1998; 139: 563-566.
49. Damante G, Tell G, Di Lauro R. A unique combination of transcription factors controls differentiation of thyroid cells. Prog Nucleic Acid Res Mol Biol 2001; 66: 307-356.
50. Dame C, Sola MC, Lim KC, Leach KM, Fandrey J, Ma Y, Knöpfle G, Engel JD, Bungert J. Hepatic erythropoietin gene regulation by GATA-4. J Biolog Chem 2004; 279: 2955-2961.
51. Dathan N, Parlato R, Rosica A, De Felice M, Di Lauro R, Distribution of the titf2/foxe1 gene product is consistent with an important role in the development of foregut endoderm, palate, and hair. Dev Dyn 2002; 224: 450-456.
52. Debruyne F, Vanderschueren-Lodeweyckx M, Bastijns P. Hearing in congenital hypothyroidism. Audiology 1983; 22: 404-409.
53. De Deken X, Wang D, Many MC, Costagliola S, Libert F, Vassart G, Dumont JE, Miot F. Cloning of two human thyroid cDNAs encoding new members of the NADPH oxidase family. J Biol Chem 2000; 23277-23233.
54. De Felice M, Di Lauro R. Thyroid development and its disorders: genetics and molecular mechanisms. Endocrine Reviews 2004; 25:722-746.
55. De Felice M, Ovitt C, Biffali E, Rodriguez-Mallon A, Arra C, Anastassiadis K, Macchia PE, Mattei MG, Mariano A, Schöler H, Macchia V, Di Lauro R. A mouse model for hereditary thyroid dysgenesis and cleft palate. Nat Genet 1998; 19: 395-398.
56. De Felice M, Postiglione MP, Di Lauro R. Tshr signaling in development and differentiation of the thyroid gland: insights from mouse models and human diseases. Endocrinology 2004; 145: 4062-4067.
57. Delange F. Neonatal screening for congenital hypothyroidism: Results and perspectives. Horm Res 1997; 48: 51.
58. Delange F. Iodine deficiency as a cause of brain damage. Postgrad Med J 2001; 77: 217-220.
59. den Dunnen JT, Antonarakis E. Nomenclature for the description of human sequence variations. Hum Genet 2001; 109: 121-124.
60. Dentice M, Cordeddu V, Rosica A, Ferrara AM, Santarpia L, Salvatore D, Chiovato L, Perri A, Moschini L, Fazzini C, Olivieri A, Costa P, Stoppioni V, Baserga M, De Felice M, Sorcini M, Fenzi G, Di Lauro R, Tartaglia M, Macchia PE. Missense mutation in the transcription factor NKX2.5: a novel molecular event in the pathogenesis of thyroid dysgenesis. J Clin Endocrin Metab 2006; 91: 1428-1433.
61. De Sanctis L, Corrias A, Romagnolo D, Di Palma T, Biava A, Borgarello G, Gianino P, Silvestro L, Zannini M, Dianzani I. Familial PAX8 small deletion (c.989-992delACCC) associated with extreme phenotype variability. J Clin Endocrinol Metab 2004; 79: 5669-5674.

62. De Vijlder JJM. Primary congenital hypothyroidism: defects in iodine pathways. Eur J Endocrinol 2003; 149: 247-256.
63. De Vijlder JJM, Ris-Stalpers C, Vulsma T. Inborn errors of thyroid hormone biosynthesis. Exp Clin Endcrinol Diabetes 1997; 105 (Suppl. 4): 32-36.
64. Devos H, Rodd C, Gagné N, Laframboise R, Van Vliet G. A search for the possible molecular mechanisms of thyroid dysgenesis: sex ratios and associated malformations. J Clin Endocrinol Metab 1999; 84: 2502-2506.
65. Devriendt K, Vanhole CH, Matthijs G, De Zegher F. Deletion of thyroid transcription factor-1 gene in an infant with neonatal thyroid dysfunction and respiratory failure. New Engl J Med 1998; 338: 1317-1318.
66. Di Palma T, Nitsch R, Mascia A, Nitsch L, Di Lauro R, Zannini MS. The paired domain-containing factor Pax8 and the homeodomain-containing factor TTF-1 directly interact and synergistically activate transcription. J Biol Chem 2003; 278: 3395-3402.
67. Doeker BM, Pfäffle RW, Pohlenz J, Andler W. Congenital central hypothyroidism due to a homozygous mutation in the thyrotropin β-subunit gene follows an autosomal recessive inheritance. J Clin Endocrinol Metab 1998; 83: 1762-1765.
68. Dohan O, De la Vieja A, Paroder V, Riedel C, Artani M, Reed M, Ginter CS, Carrasco N. The sodium/iodide symporter (NIS): characterization, regulation and medical significance. Endocrine Reviews 2003; 24: 48-77.
69. Dussault JH. The anecdotal history of screening for congenital hypothyroidism. J Clin Endocrinol Metab 1999; 84: 4332-4334.
70. Evans C, Jordan NJ, Owens G, Bradley D, Ludgate M, John R. Potent thyrotrophin receptor-blocking antibodies: a cause of transient congenital hypothyroidism and delayed thyroid development. Eur J Endocrinol 2004; 150: 265-268.
71. Everett LA, Glaser B, Beck JC, Idol JR, Buchs A, Heyman M, Adawi F, Hazani E, Nassir E, Baxevanis A, Sheffield VC, Green ED. Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS). Nat Genet 1997; 17: 411-422.
72. Fabbro D, Pellizzari L, Mercuri F, Tell G, Damante G. Pax-8 protein levels regulate thyroglobulin gene expression. J Mol Endocrinol 1998; 21: 347-354.
73. Fagman H, Andersson L, Nilsson M. The developing mouse thyroid: embryonic vessel contacts and parenchymal growth pattern during specification, budding, migration, and lobulation. Developmental Dynamics 2006, 235: 444-455.
74. Fagman H, Grände M, Gritli-Linde A, Nilsson M. Genetic deletion of Sonic Hedgehog causes hemiagenesis and ectopic development of the thyroid in mouse. Am J Pathol 2004, 164: 1865-1872.
75. Fernhoff PM, Brown AL, Elsas L. Congenital hypothyroidism and increased risk of neonatal morbidity results in delayed treatment. Lancet 1987; i: 490-491.
76. Foley TP. Pediatric thyroid disorder. In: Cooper DS. Medical management of thyroid disease. New York, USA, Marcel Dekke 2001; 313-344.
77. Fraser GR. Association of congenital deafness with goitre (Pendred's Syndrome) - a study of 207 families. Ann Hum Genet 1965; 28: 201-249.
78. Fugazzola L, Cerutti N, Mannavola D, Vannucchi G, Fallini C, Persani L, Beck-Peccoz P. Monoallelic expression of mutant thyroid peroxidase allele causing total iodide organification defect. J Clin Endocrinol Metab 2003; 88: 3264-3271.
79. Fugazzola L, Mannavola D, Cerutti N, Maghnie M, Pagella F, Bianchi P, Weber G, Persani L, Beck-Peccoz P. Molecular analysis of the Pendred´s syndrome gene and magnetic resonance imaging studies of the inner ear are essential for the diagnosis of true Pendred´s syndrome. J Clin Endocrinol Metab 2000; 85: 2469-2475.

80. Fugazzola L, Mannavola D, Vigone MC, Cirello V, Weber G, Beck-Peccoz P, Persani L. Total iodide organification defect: clinical and molecular characterization of an Italian family. Thyroid 2005; 15: 1085-1088.
81. Fujiwara H, Tatsumi K, Miki K, Harada T, Miyai K, Takai S, Amino N. Congenital hypothyroidism caused by a mutation in the Na^+/I^- symporter. Nat Genet 1997; 16: 124-125.
82. Gaudino R, Garel C, Czernichow P, Leger J. Proportion of various types of thyroid disorders among newborns with congenital hypothyroidism and normally located gland: a regional cohort study. Clin Endocrinol (Oxf) 2005; 62: 444-448.
83. Gillam MP, Kopp P. Genetic defects in thyroid hormone synthesis. Curr Opin Pediatr 2001; 13: 364-372.
84. Gillam MP, Kopp P. Genetic regulation of thyroid development. Curr Opin Pediatr 2001; 13: 358-363.
85. Gnidehou S, Caillou B, Talbot M, Ohayon R, Kaniewski J, Noël-Hudson MS, Morand S, Agnangji D, Sezan A, Courtin F, Virion A, Dupuy C. Iodotyrosine dehalogenase 1 (DEHAL1) is a transmembrane protein involved in the recycling of iodide close to the thyroglobulin iodination site. FASEB J 2004; 18: 1574-1576.
86. Gough IR, Wilkinson D. Total thyroidectomy for management of thyroid disease. World J Surg 2000; 24: 962-965.
87. Grasberger H, Mimouni-Bloch A, Vantyghem MC, van Vliet G, Abramowicz M, Metzger DL, Abdullatif H, Rydlewski C, Macchia PE, Scherberg NH, van Snad J, Mimouni M, Weiss RE, Vassart G, Refetoff S. Autosomal dominant resistance to thyrotropin as a distinct entity in five multigenerational kindreds: clinical characterization and exclusion of candidate loci. J Clin Endocrinol Metab 2005; 90: 4025-4034.
88. Grasberger H, Ringkananont U, Lefrancois P, Abramowicz M, Vassart G, Refetoff S. Thyroid transcription factor 1 rescues PAX8/p300 synergism impaired by a natural PAX8 paired domain mutation with dominant negative activity. Mol Endocrinol 2005; 19: 1779-1791.
89. Grasberger H, Vaxillaire M, Pannain S, Beck JC, Mimouni-Bloch A, Vatin V, Vassart G, Froguel P, Refetoff S. Identification of a locus for nongoitrous congenital hypothyroidism on chromosome 15q25.3-26.1. Hum Genet 2005; 118: 348-355.
90. Grüters A, Finke R, Krude H, Meinhold H. Etiological grouping of permanent congenital hypothyroidism with a thyroid gland in situ. Horm Res 1994; 41:3-9. PAX
91. Grüters A, Jenner A, Krude H. Long-term consequences of congenital hypothyroidism in the era of screening programmes. Best Pract Res Clin Endocrinol Metab 2002; 16: 369-382.
92. Grüters A, Köhler B, Wolf A, Söling A, de Vijlder L, Krude H, Biebermann H. Screening for mutations of the human thyroid peroxidase gene in patients with congenital hypothyroidism. Exp Clin Endocrinol Diabetes 1996; 104 (Suppl 4): 121-123.
93. Grüters A, Krude H, Biebermann H. Molecular genetic defects in congenital hypothyroidism. Eur J Endocrinol 2004; 151: U39-U44.
94. Grüters A, Liesenkötter KP, Zapico M, Jenner A, Dütting C, Pfeiffer E, Lehmkuhl U. Results of the screening program for congenital hypothyrodism in Berlin (1978-1995). Exp Clin Endocrinol Diabetes 1997; 105 (Suppl 4): 28-31.
95. Hishinuma A, Kuribayashi T, Kanno Y, Onigata K, Nagashima K, Ieri T. Sequence analysis of thyroid transcription factor-1 gene reveals absence of mutations in patients wit thyroid dysgenesis but presence of polymorphism in the 5´flanking region and intron. Endocr J 1998; 45: 563-567.

96. Hishinuma A, Ohyama Y, Kuribayashi T, Nagakubo N, Namatame T, Shibayama K, Arisaka O, Matsuura N, Ieri T. Polymorphism of the polyalanine tract of thyroid transcription factor-2 gene in patients with thyroid dysgenesis. Eur J Endocrinol 2001; 143: 385-389.
97. Hníková O. Poruchy funkce štítné žlázy u novorozence. In: Hníková O, Kračmar P. Kongenitální hypotyreóza. Praha, Avicenum 1989; 31-67.
98. Hníková O, Kračmar P. Screening novorozenců na tyreopatie. In: Hníková O, Kračmar P. Kongenitální hypotyreóza. Praha, Avicenum 1989; 75-88.
99. Ieri T, Cochaux P, Targovnik HM, Suzuki K, Shimoda S, Perret J, Vassart G. A 3´splice site mutation in the thyroglobulin gene responsible for congenital goiter with hypothyroidism. J Clin Invest 199; 88: 1901-1905.
100. Iwatani N, Mabe H, Devriendt K, Kodama M, Müke T. Deletion of NKX2.1 gene encoding thyroid transcription factor-1 in two siblings with hypothyroidism and respiratory failure. J Pediatr 2000; 137: 272-276.
101. Keng VW, Fujimori KE, Myint Z, Tamamaki N, Nojyo Y, Noguchi T. Expression of Hex mRNA in early murine postimplantation embryo development. FEBS 1998; 426: 183-186.
102. Kimura S, Hara Y, Pineau T, Fernandez-Salguero P, Fox CH, Ward JM, Gonzalez FJ. The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. Genes Dev 1996; 10: 60-69.
103. Kimura S, Hong YS, Kotani T, Othaki S, Kikkawa F. Structure of the human thyroid peroxidase gene: comparison and relationship to the human myeloperoxidase gene. Biochemistry 1989; 28: 4481-4489.
104. Kimura S, Kotani T, McBride OW, Umeki K, Hirai K, Nakayama T, Ohtaki S. Human thyroid peroxidase: complete cDNA and protein sequence, chromosome mapping, and identification of two alternatively spliced mRNAs. Proc Nat Acad Sci 1987; 84: 5555-5559.
105. Klein AH, Meltzer S, Kenny FM. Improved prognosis in congenital hypothyroidism treated before age three months. J Pediatr 1972; 81: 912-915.
106. Klett M. Epidemiology of congenital hypothyroidism. Exp Clin Endocrinol Diabetes 1997; 105 (Suppl 4): 19- 23.
107. Köhler B, Schnabel D, Biebermann H, Grüters A. Transient congenital hypothyroidism and hyperthyrotropinemia: normal thyroid function and physical development at the ages of 6-14 years. J Clin Endocrinol Metab 1996; 81: 1563-1567.
108. Komatsu M, Takahashi T, Takahashi I, Nakamura M, Takahashi I, Takada G. Thyroid dysgenesis caused by PAX8 mutation: the hypermutability with CpG dinucleotides at codon 31. J Pediatr 2001; 139:597-599.
109. Kopp P. Pendred´s syndrome: Identification of the genetic defect a century after its recognition. Thyroid 1999; 9: 65-69.
110. Kotani T, Umeki K, Kawano J, Suganuma T, Hishinuma A, Ieri T, Harada S. Partial iodide organification defect caused by a novel mutation of the thyroid peroxidase gene in three siblings. Clin Endocrinol (Oxf) 2003; 59: 198-206.
111. Kotani T, Umeki K, Yamamoto I, Maesaka H, Tachibana K, Ohtaki S. A novel mutation in the human thyroid peroxidase gene resulting in a total iodide organification defect. J Endocrinol 1999; 160: 267-273.
112. Kotani T, Umeki K, Yamamoto I, Ohtaki S, Adachi M, Tachibana K. Iodide organification defects resulting from cosegregation of mutated and null thyroid peroxidase alleles. Mol Cell Endocrinol 2001; 182: 61-68.

113. Krude H, Biebermann H, Schnabel D, Ambrugger P, Grüters A. Molecular pathogenesis of neonatal hypothyroidism. Horm Res 2000; 53 (Suppl 1): 12-18.
114. Krude H, Macchia PE, Di Lauro R, Grüters A. PAX-8 mutations associated with congenital hypothyroidism caused by thyroid dysgenesis (abstract). Horm Res 1998; 50 (Supp l3): 17.
115. Krude H, Schütz B, Biebermann H, von Moers A, Schnabel D, Neitzel H, Tönnies H, Weise D, Lafferty A, Schwarz S, De Felice M, von Deimling A, van Landeghem F, Di Lauro R, Grüters A. Choreoathetosis, hypothyroidism, and pulmonary alterations due to human NKX2-1 haploinsufficiency. J Clin Invest 2002; 109: 475-480.
116. Lacroix L, Mian C, Caillou B, Talbot M, Filetti S, Schlumberger M, Bidart JM. Na/I symporter and Pendred syndrome gene and protein expressions in human extra-thyroidal tissues. Eur J Endocrinol 2001; 144: 297-302.
117. LaFranchi S. Congenital hypothyroidism: etiologies, diagnosis, and management. Thyroid 1999; 9: 735-740.
118. Lapi P, Macchia PE, Chiovato L, Biffali E, Moschini L, Larizza D, Baserga M, Pinchera A, Fenzi G, Di Lauro R. Mutations in the gene encoding thyroid transcription factor-1 (TTF-1) are not a frequent cause of congenital hypothyroidism (CH) with thyroid dysgenesis. Thyroid 1997; 7: 383-387.
119. Lazarus JH, Hughes IA. Congenital abnormalities and congenital hypothyroidism. Lancet 1988; 2: 52.
120. Lazzaro D, Price M, De Felice M, Di Lauro R.The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and restricted regions of the foetal brain. Development 1991; 113: 1093-1104.
121. Léger J, Marinovic D, Garel C, Bonaiti-Pellié C, Polak M, Czernichow P. Thyroid developmental anomalies in first degree relatives of children with congenital hypothyroidism. J Clin Endocrinol Metab 2002; 87: 575-580.
122. Lints TJ, Parson LM, Hartley L, Lyons I, Harvey RP. Nkx2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. Development 1993; 119: 419-431.
123. Lyons I, Parsons LM, Hartley L, Li R, Andrews JE, Robb L, Harvey RP. Myogenic and morphogeneic defects in the heart tubes of murine embryos lacking the homeobox gene Nkx2-5. Gnes Dev 1995; 9: 1654-1666.
124. Macchia PE. Recent advances in understanding the molecular basis of primary congenital hypothyroidism. Mol Med Tod 2000; 6: 36-42.
125. Macchia PE, De Felice M, Di Lauro R. Molecular genetics of congenital hypothyroidism. Cur Opin Genet Dev 1999; 9: 289-294.
126. Macchia PE, Lapi P, Krude H, Pirro MT, Missero C, Chiovato L, Souabni A, Baserga M, Tassi V, Pinchera A, Fenzi G, Grüters A, Busslinger M, Di Lauro R. PAX8 mutations associated with congenital hypothyroidism caused by thyroid dysgenesis. Nat Genet 1998; 19: 83-86.
127. Maiorana R, Carta A, Floriddia G, Leonardi D, Buscema M, Sava L, Calaciura F, Vigneri R. Thyroid hemiagenesis: prevalence in normal children and effect on thyroid function. J Clin Endocrinol Metab 2003; 88: 1534-1536.
128. Majeed-Saidan MA, Joyce B, Khan M, Hamam HD. Congenital Hypothyroidism: The Riyadh Military Hospital Experiences. Clin Endocrinol 1993; 38: 191-195.
129. Mangklabruks A, Billerbeck AE, Wajchenberg B, Knobel M, Cox NJ, DeGroot LJ, Medeiros-Neto G. Genetic linkage studies of thyroid peroxidase (TPO) gene in families with TPO deficiency. J Clin Endocrinol Metab 1991; 72: 471-476.

130. Mansouri A, Chowdhury K, Gruss P. Follicular cells of the thyroid gland require Pax8 gene function. Nat Genet 1998; 19: 87-90.
131. Martinez Barbera JP, Clements M, Thomas P, Rodriguez T, Meloy D, Kioussis D, Beddington RSP. The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation. Development 2000; 127: 2433-2445.
132. McLachlan SM, Rapoport B. The molecular biology of thyroid peroxidase: cloning, espression and role as autoantigen in autoimmune thyroid disease. Endocrine Reviews 1992; 13: 192-206.
133. Medeiros-Neto GA, Billerbeck AEC, Wajchenberg BL, Targovnik HM. Defective organification of iodide causing hereditary goitrous hypothyroidism. Thyroid 1993; 3: 143-159.
134. Medeiros-Neto G, Bunduki V, Tomimori E, Gomes S, Knobel M, Martin RT, Zugaib M. Prenatal diagnosis and treatment of dyshormonogenetic fetal goiter due to defective thyroglobulin synthesis. J Clin Endocrinol Metab 1997; 82: 4239-4242.
135. Medeiros-Neto G, Gil-Da-Costa MJ, Santos CLS, Medina AM, Costa E Silva J, Tsou RM, Sobrinho-Simões M. Metastatic thyroid carcinoma arising from congenital goiter due to mutation in the thyroperoxidase gene. J Clin Endocrinol Metab 1998; 83: 4162-4166.
136. Medeiros-Neto G, Okamura K, Cavaliere H, Taurog A, Knobel M, Bisi H, Kallas WG, Mattar E. Familial thyroid peroxidase defect. Clin Endocrinol 1982; 17: 1-14.
137. Medeiros-Neto G, Stanbury JB. Thyroid malignancy and dyshormonogenetic goiter. In: Medeiros-Neto G, Stanbury JB, eds. Inherited disorders of the thyroid system. Boca Raton, FL: CRC Press, 1994; 909-916.
138. Meeus L, Gilbert B, Rydlewski C, Parma J, Roussie AL, Abramowicz M, Vilain C, Christophe D, Costagliola S, Vassart G. Characterization of a novel loss of function mutation of PAX8 in a familial case of congenital hypothyroidism with in-place, normal-sized thyroid. J Clin Endocrinol Metab 2004; 89: 4285-4291.
139. Mendive FM, Rivolta CM, Moya CM, Vassart G, Targovnik H. Genomic organization of the human thyroglobulin gene: the complete intron-exon structure. Eur J Endocrinol 2001; 145: 485-496.
140. Meyerhoff WL. Hypothyroidism and the ear: electrophysiological, morphological, and chemical considerations. Laryngoscope 1979; 89: 1-25.
141. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acid Res 1988; 85: 1215.
142. Miyai K, Inaoka K, Miyagi T. Further studies on episodic occurrence of congenital dysgenetic hypothyroidism in Osaka, Japan. Endocr J 2005; 52: 599-603.
143. Moreno JC. Identification of novel genes involved in congenital hypothyroidism using serial analysis of gene expression. Horm Res 2003; 60 (Suppl 3): 96-102.
144. Moreno JC, Bikker H, Kempers MJ, Van Trotsenburg AS, Baas F, De Vijlder JJ, Vulsma T, Ris-Stalplers C. Inactivating mutations in the gene for thyroid oxidase 2 (THOX2) and congenital hypothyroidism. N Eng J Med 2002; 347: 95-102.
145. Moreno JC, Klootwijk W, van Toor H, Pinto G, D´Alessandro M, Leger A, Goudie D, Polak M, Grüters A, Visser TJ. Mutations in the iodotyrosine deiodinase gene and hypothyroidism. N Engl J Med 2008; 358: 1811-1818.
146. Moreno JC, Visser TJ. New phenotypes in thyroid dyshormonogenesis: hypothyroidism due to DUOX2 mutations. Endocr Dev 2007; 10: 99-117.
147. Mount DB, Romero MF. The SLC26 gene family of multifunctional anion exchangers. Eur J Physiol 2004; 447: 710-721.

148. Musti AM, Avvedimento EV, Polistina C, Ursini VM, Obici S, Nitsch L, Cocozza S, Di Lauro R. The complete structure of the rat thyroglobulin gene. Proc Natl Acad Sci 1986; 83: 323-327.
149. Napiontek U, Borck G, Müller-Forell W, Pfarr N, Bohnert A, Keilmann A, Pohlenz J. Intrafamilial variability of the deafness and goiter phenotype in Pendred syndrome caused by a T416P mutation in the SLC26A4 gene. J Clin Endocrinol Metab 2004; 89: 5347-5351.
150. Nascimento AC, Guedes DR, Santos CS, Knobel M, Rubio IG, Medeiros-Neto G. Thyroperoxidase gene mutations in congenital goitrous hypothyroidism with total and partial iodide organification defect. Thyroid 2003; 13: 1145-1151.
151. Niimi H, Inomata H, Sasaki N, Nakajima H. Congenital isolated thyrotrophin releasing hormone deficiency. Arch Dis Child 1982; 57: 877-878.
152. Niu DM, Hwang B, Chu YK, Liao CJ, Wang PL, Lin CY. High prevalence of a novel mutation (2268 insT) of the thyroid peroxidase gene in Taiwanese patients with total iodide organification defect, and evidence for a founder effect. J Clin Endocrinol Metab 2002; 87: 4208-4212.
153. Ohno M, Zannini MS, Levy O, Carrasco N, Di Lauro R. The paired-domain transcription factor Pax8 binds to the upstream enhancer of the rat sodium/iodide symporter gene and partecipates in both thyroid-specific and cyclic-AMP-dependent transcription. Mol Cell Biol 1999; 19: 2051-2060.
154. Olivieri A, Medda E, De Angelis S, Valensise H, De Felice M, Fazzini C, Cascino I, Cordeddu V, Sorcini M, Stazi MA, and the Study Group for Congenital Hypothyroidism. High risk of congenital hypothyroidism in multiple pregnancies. J Clin Endocrinol Metab 2007; 93: 3141-3147.
155. Olivieri A, Stazi MA, Mastroiacovo P, Fazzini C, Medda E, Spagnolo A, De Angelis S, Grandolfo ME, Taruscio D, Cordeddu V, Sorcini M and the study group for congenital hypothyroidism. A population-based study on the frequency of additional congenital malformations in infants with congenital hypothyroidism: data from the italian registry for congenital hypothyroidism (1991-1998). J Clin Endocrinol Metab 2002; 87: 557-562.
156. Oppenheimer JH, Schwartz HL. Molecular basis of thyroid hormone-dependent brain development. Endocrine Reviews 1997; 18: 462-475.
157. Orita M, Suzuki Y, Sekiya T, Hayashi K. Rapid and sensitive detection of point mutations and SNA polymorphisms using the polymerase chain reaction. Genomics 1989; 5: 874-879.
158. Ozbas S, Kocak S, Aydintug S, Cakmak A, Demirkiran MA, Wishart GC. Comparison of the complications of subtotal, near total and total thyroidectomy in the surgical management of multinodular goitre. Endocr J 2005; 52: 199-205.
159. Pannain S, Weiss RE, Jackson CE, Dian D, Beck JC, Sheffield VC, Cox N, Refetoff S. Two different mutations in the thyroid peroxidase gene of a large inbred Amish kindred: power and limits of homozygosity mapping. J Clin Endocrinol Metab 1999; 84: 1061-1071.
160. Park SM, Chatterjee VKK. Genetics of congenital hypothyroidism. J Med Genet 2005; 42: 379-389.
161. Parlato R, Rosica A, Rodriguez-Mallon A, Affuso A, Postiglione MP, Arra C, Mansouri A, Kimura S, Di Lauro R, De Felice M. An integrated regulatory network controlling survival and migration in thyroid organogenesis. Dev Biol 2004; 276:464-475
162. Pasca di Magliano M, Di Lauro R, Zannini M. Pax8 has a key role in thyroid cell differentiation. Proc Natl Acad Sci 2000; 97: 13144-13149.

163. Pauws E, Moreno JC, Tijssen M, Baas F, De Vijlder JJM. Serial analysis of gene expression as a tool to assess the human thyroid expression profile and to identify novel thyroidal genes. J Clin Endocrinol Metab 2000; 85: 1923-1927.
164. Pelizzo MR, Bernante P, Toniato A, Fassina A. Frequency of thyroid carcinoma in a recent series of 539 consecutive thyroidectomies for multinodular goiter. Tumori 1997; 83: 653-655.
165. Pendred V. Deaf mutism and goitre. Lancet 1896; II: 532
166. Perna MG, Civitareale D, De Filippis V, Sacco M, Cisternino C, Tassi V. Absence of mutations in the gene encoding thyroid transcription factor-1 (TTF-1) in patients with thyroid dysgenesis. Thyroid 1997; 7: 377-381.
167. Perry R, Heinrichs C, Bourdoux P, Khoury K, Szöts F, Dussault JH, Vassart G, Van Vliet G. Discordance of monozygotic twins for thyroid dysgenesis: implications for screening and for molecular pathophysiology. J Clin Endocrinol Metab 2002; 87: 4072-4077.
168. Pfarr N, Borck G, Turk A, Napiontek U, Keilmann A, Muller-Forell W, Kopp P, Pohlenz J. Goitrous congenital hypothyroidism and hearing impairment associated with mutations in the TPO and SLC26A4 genes. J Clin Endocrinol Metab 2006; 91: 2678-2681.
169. Pfarr N, Musholt TJ, Musholt PB, Brzezinska R, Pohlenz J. Congenital primary hypothyroidism with subsequent adenomatous goiter in a Turkish patient caused by a homozygous 10-bp deletion in the thyroid peroxidase (TPO) gene. Clin Endocrinol 2006; 64: 514-518.
170. Phelps PD, Coffey RA, Trembath RC, Luxon LM, Grossman B, Britton KE, Kendall-Taylor P, Graham JM, Cadge BC, Stephens SG, Pembrey ME, Reardon W. Radiological malformations of the ear in Pendred syndrome. Clin Radiol 1998; 53: 268-273.
171. Plachov D, Chowdhury K, Walther C, Simon D, Guenet JL, Gruss P. Pax-8, a murine paired box gene expressed in the developing excretory system and thyroid gland. Development 1990; 110: 643-651.
172. Pohlenz J, Dumitrescu A, Zundel D, Martiné U, Schönberger W, Koo E, Weiss RE, Cohen RN, Kimura S, Refetoff S. Partial deficiency of Thyroid transcription factor 1 produces predominantly neurological defects in humans and mice. J Clin Invest 2002; 109: 469-473.
173. Polak M, Sura-Trueba S, Chauty A, Szinnai G, Carre A, Castanet M. Molecular mechanisms of thyroid dysgenesis. Horm Res 2004; 62 (Suppl 3): 14-21.
174. Postiglione MP, Parlato R, Rodriguez-Mallon A, Rosica A, Mithbaokar P, Maresca M, Marians RC, Davies TF, Zannini MS, De Felice M, Di Lauro R. Role of the thyroid-stimulating hormone receptor signaling in development and differentiation of the thyroid gland. Proc Natl Acad Sci USA 2002; 99: 15462-15467.
175. Puppin C, Presta I, D´Elia AV, Tell G, Arturi F, Russo D, Filetti S, Damante G. Functional interaction among thyroid-specific transcription factors: Pax8 regulates the acitivity of Hex promoter. Mol Cell Endocrinol 2004; 214: 117-125.
176. Reardon W. Genetic deafness. J Med Genet 1992; 29: 521-526.
177. Reardon W, Coffey R, Chowdhury T, Grossman A, Jan H, Britton K, Kendall-Taylor P, Trembath R. Prevalence, age of onset, and natural history of thyroid disease in Pendred syndrome. J Med Genet 1999; 36: 595-598.
178. Reardon W, Coffey R, Phelps PD, Luxon LM, Stephens D, Kendall-Taylor P, Britton KE, Grossman A, Trembath R. Pendred syndrome - 100 years of underascertainment? Quart J Med 1997; 90: 443-447.
179. Reardon W, Trembath RC. Pendred syndrome. J Med Genet 1996; 33: 1037-1040.

180. Riesco-Eizaguirre G, Santisteban P. A perspective view of sodium iodide symporter research and its clinical implications. Eur J Endocrinol 2006; 155: 495-512.
181. Rivolta CM, Esperante SA, Gruneiro-Papendieck L, Chiesa A, Moya CM, Domene S, Varela V, Targovnik HM. Five novel inactivating mutations in the thyroid peroxidase gene responsible for congenital goiter and iodide organification defect. Hum Mut 2003, 22: 259.
182. Rivolta CM, Louis-Tisserand M, Varela V, Gruneiro-Papendieck L, Chiesa A, González-Sarmiento R, Targovnik HM. Two compound heterozygous mutations (c.215delA/c.2422T→C and c.387delC/c.1159G→A) in the thyroid peroxidase gene responsible for congenital goitre and iodide organificaton defect. Clin Endocrinol 2007; 67: 238-246.
183. Roberts HE, Moore CA, Fernhoff PM, Brown AL, Khoury MJ. Population study of congenital hypothyroidism and associated birth defect, Atlanta, 1979-1992. Am J Med Genet 1997; 71: 29-32.
184. Rodriguez C, Jorge P, Soares JP, Santos I, Salomao R, Madeira M, Vaz Osório R, Santos R. Mutation screening of the thyroid peroxidase gene in a cohort of 55 Portuguese patients with congenital hypothyroidism. Eur J Endocrinol 2005; 152: 193-198.
185. Rodriguez AM, Perron B, Lacroix L. Identification and characterization of a putative human iodide transporter located at the apical membrane of thyrocytes. J Clin Endocrinol Metab 2002; 87: 3356-3361.
186. Rovet JF, Ehrlich RM, Sorbara DL. Intellectual outcome in children with fetal hypothyroidism. J Pediatr 1987; 700-704.
187. Royaux IE, Belyantseva IA, Wu T, Kachar B, Everett LA, Marcus DC, Green ED. Localization and functional studies of pendrin in the mouse inner ear provide insight about the etiology of deafness in Pendred syndrome. J Assoc Res Otolaryngol 2003; 4: 394-404.
188. Royaux IE, Suzuki K, Mori A, Katoh R, Everett LA, Kohn LD, Green ED. Pendrin, the protein encoded by the Pendred syndrome gene (PDS), is an apical porter of iodide in the thyroid and is regulated by thyroglobulin in FRTL-5 cells. Endocrinology 2000; 141: 839-845.
189. Russo D, Arturi F, Chiefari E, Filetti S. Molecular insights into TSH receptor abnormality and thyroid disease. J Endocrinol Invest 1997; 20: 36-47.
190. Santos CLS, Bikker H, Rego KGM, Nascimento AC, Tambascia M, de Vijlder JJM, Medeiros-Neto G. A novel mutation in the TPO gene in goitrous hypothyroid patients with iodide organification defect. Clin Endocrinol 1999; 51: 165-172.
191. Schott JJ, Benson DW, Basson CT, Pease W, Silberbach GM, Moak JP, Maron BJ, Seidman CE, Seidman JG. Congenital heart disease caused by mutations in the transcription factor NKX2-5. Science 1998; 281: 108-111.
192. Scott DA, Wang R, Kreman TM, Andrews M, McDonald JM, Bishop JR, Smith RJH, Karniski LP, Sheffield VC. Functional differences of the PDS gene product are associated with phenotypic variation in patients with Pendred syndrome and non-syndromic hearing loss (DFNB4). Hum Mol Genet 2000; 9: 1709-1715.
193. Scott DA, Wang R, Kreman TM, Sheffield VC, Karniski LP. The Pendred syndrome gene encodes a chloride-iodide transport protein. Nat Genet 1999; 21: 440-443.
194. Seeman P, Malíková M, Rašková D, Bendová O, Groh D, Kubálková M, Sakmaryová I, Seemanová E, Kabelka Z. Spectrum and frequencies of mutations in the GBJ2 (Cx26) gene among 156 Czech patients with pre-lingual deafness. Clin Genet 2004; 66: 152-157.

195. Seidman JG, Seidman C. Transcription factor haploinsufficiency: when half a loaf is not enough. J Clin Invest 2002; 109: 451-455.
196. Sheffield VC, Kraiem Z, Beck JC, Nishimura D, Stone EM, Salameh M, Sadeh O, Glaser B. Pendred syndrome maps to chromosome 7q21-34 and is caused by an intrinsic defect in thyroid iodine organification. Nat Genet 1996; 12: 424-426.
197. Shen DHY, Kloos RT, Mazzaferri EL, Jhiang SM. Sodium iodide symporter in health and disease. Thyroid 2001; 11: 415-424.
198. Siebner R, Merlob P, Kaiserman I, Sack J. Congenital anomalies concomitant with persistent primary congenital hypothyroidism. Am J Med Genet 1992; 44: 57-60.
199. Stanbury JB, Hedge AN. A study of a family with goitrous cretins. J Clin Endocrinol Metab 1950; 10: 1471-1484.
200. Steel KP, Bussoli TJ. Deafness genes: expressions of surprise. Trends Genet 1999; 15: 207-211.
201. Sunthornthepvarakul T, Gottschalk M, Hayashi Y, Refetoff S. Resistence to thyrotropin caused by mutations in the thyrotropin-receptor gene. N Eng J Med 1995; 332:155-160.
202. Sura-Trueba S, Augé J, Mattei G, Etchevers H, Martinovic J, Czernichow P, Vekemans M, Polak M, Attié-Bitach T. PAX8, TITF1, and FOXE1 gene expression patterns during human development: new insights into human thyroid development and thyroid dysgenesis-associated malformations. J Clin Endocrinol Metab 2005; 90: 455-462.
203. Sutherland JM, Esselborn VM, Burket RL, Skillman TB, Benson JT. Familial nongoitrous cretenism apparently due to maternal antithyroid antibody. N Eng J Med 1960; 263: 336-341.
204. Szkudlinski MW, Fremont V, Ronin C, Weintraub BD. Thyroid-stimulating hormone and thyroid-stimulating hormone receptor structure-function relationships. Physiol Rev 2002; 82: 473-502.
205. Szinnai G, Kosugi S, Derrien C, Lucidarme N, David V, Czernichow P, Polak M. Extending the clinical heterogeneity of iodide transport defect (ITD): a novel mutation R124H of the sodium/iodide symporter (NIS) gene and review of genotype-phenotype correlations of ITD. J Clin Endocrinol Metab 2006; 91: 1199-1204.
206. Tajima T, Tsubaki J, Fujieda K. Two novel mutations in the tyroid peroxidase gene with goitrous hypothyroidism. Endocrine J 2005; 52: 643-645.
207. Targovnik HM, Medeiros-Neto G, Varela V, Cochaux P, Wajchenberg BL, Vassart G. A nonsense mutation causes human hereditary congenital goiter with preferential production of a 171 nucleotide deleted thyroglobulin RNA messenger. J Clin Endocrinol Metab 1993; 77: 210-215.
208. Taylor JP, Metcalfe RA, Watson PF, Weetman AP, Trembath RC. Mutations of the PDS gene, encoding pendrin, are associated with protein mislocalization and loss of iodide efflux: implications for thyroid dysfunction in Pendred syndrome. J Clin Endocrinol Metab 2002; 87: 1778-1784.
209. Tenenbaum-Rakover Y, Mamanasiri S, Ris-Stalpers C, German A, Sack J, Allon-Shalev S, Pohlenz J, Refetoff S. Clinical and genetic characteristics of congenital hypothyroidism due to mutations in the thyroid peroxidase (TPO) gene in Israelis. Clin Endocrinol 2007; 66: 695-702.
210. Thomas PQ, Brown A, Beddington R. Hex: a homeobox gene revealing peri-implantation assymmetry in the mouse embryo and an early transient marker of endothelial cell precursors. Development 1998; 125: 85-95.

211. Tillotson SL, Fuggle PW, Smith I, Ades AE, Grant DB. Relation between biochemical severity and intelligence in early treated congenital hypothyroidism: a treshold effect. Br Med J 1994; 309: 440-445.
212. Tollin SR, Mery GM, Jelveh N, Fallon EF, Mikhail M, Blumenfeld W, Perlmutter S. The use of fine-needle aspiration biopsy under ultrasound guidance to assess the risk of malignancy in patients with a multinodular goiter. Thyroid 2000; 10: 235-241.
213. Tonacchera M, Banco M, Lapi P, Di Cosmo C, Perri A, Montanelli L, Moschini L, Gatti G, Gandini D, Massei A, Agretti P, De Marco G, Vitti P, Chiovato L, Pinchera A. Genetic analysis of TTF-2 gene in children with congenital hypothyroidism and cleft palate, congenital hypothyroidism, or isolated cleft palate. Thyroid 2004; 14: 584-588.
214. Toublanc JE. Comparison of epidemiological data on congenital hypothyroidism in Europe with those of other parts of the world. Horm Res 1992; 38: 230-235.
215. Umeki K, Yamamoto I, Yukizane S, Kotani T. Congenital hypothyroidism caused by a unique peroxidase allele containing two mutations, C1708T and C2737T. J Pediatr Endocrinol Metab 2004; 17: 231-234.
216. Uziel A, Gabrion J, Ohresser M, Legrand C. Effects of hypothyroidism on the structural development of the organ of Corti in the rat. Acta Otolaryngol 1981; 92: 469-480.
217. Valvassori GE, Clemis JD. The large vestibular aqueduct syndrome. Laryngoscope 1978; 88: 723-728.
218. Varela V, Rivolta CM, Esperante SA, Gruneiro-Papendieck L, Chiesa A, Targovnik HM. Three mutations (p.Q36H, p.G418fsX482, and g.IVS19-2A> C) in the dual oxidase 2 gene responsible for congenital goiter and iodide organification defect. Clin Chem 2006; 52:182-191
219. Věstník MZ ČR. Metodický návod zajištění celoplošného novorozeneckého laboratorního screeningu a následné péče, oddíl 2-kongenitální hypotyreóza. 2002; 9: 4 -11.
220. Vilain C, Rydlewski C, Duprez L, Heinrichs C, Abramowicz M, Malvaux P, Renneboog B, Parma J, Costagliola S, Vassart G. Autosomal dominant transmission of congenital thyroid hypoplasia due to loss-of-function mutation of PAX8. J Clin Endocrinol Metab 2001; 86: 234-238.
221. Vittal S, Chandrasekaran M, Kumar KB, Sucharitha V, Jeevaratinam R. Dyshormonogenetic goitre. J R Coll Surg Edinb 1993; 38: 205-207.
222. Vono-Toniolo J, Rivolta CM, Targovnik HM, Medeiros-Neto G, Kopp P. Naturally occurring mutations in the thyroglobulin gene. Thyroid 2005; 15: 1021-1033.
223. Vulsma T, Gons MH, De Vijlder JJ. Maternal-fetal transfer of thyroxine in congenital hypothyroidism due to a total organification defect or thyroid agenesis. New Eng J Med 1989; 321: 13-16.
224. Watanabe I. Dyshormonogenesis-with special reference to its relationship with thyroid adenoma. Horumon To Rinsho 1983; 31: 627-636.
225. Willemsen M, Breedveld GJ, Wouda S, Otten BJ, Yntema JL, Lammens M, de Vries B. Brain-thyroid-lung syndrome: a patient with a severe multi-system disorder due to a de novo mutation in the thyroid transcription factor 1 gene. Eur J Pediatr 2005; 164: 28 -30.
226. Wolff J, Chaikoff IL, Goldberg D, Meier JR. The temporary nature of the inhibitory action of excess iodine on organic iodine synthesis in the normal thyroid. Endocrinology 1949; 45: 504-513.

227. Working Group on Neonatal Screening of the European Society for Pediatric Endocrinology (ESPE). Revised guidelines for neonatal screening programmes for primary congenital hypothyroidism. Horm Res 1999; 52: 49-52.
228. Wu JY, Shu SG, Yang CF, Lee CC, Tsai FJ. Mutation analysis of thyroid peroxidase gene in Chinese patients with total iodide organification defect: identification of five novel mutations. J Endocrinol 2002; 172: 627-635.
229. Xu W, Rould MA, Jun S, Desplan C, Pabo CO. Crystal structure of a paired domain-DNA complex at 2.5A resolution reveals structural basis for Pax developmental mutations. Cell 1995; 80:639-650
230. Yoshida A, Taniguchi S, Hisatome I, Royaux IE, Green ED, Kohn LD, Suzuki K. Pendrin is an iodide-specific apical porter responsible for iodide efflux from thyroid cells. J Clin Endorinol Metab 2002; 87: 3356-3361.
231. Zannini M, Avantaggiato V, Biffali E, Arnone M, Sato K, Pischetola M, Taylor BA, Phillips SJ, Simeone A, Di Lauro R. TTF-2, a new forkhead protein, shows a temporal expression in the developing thyroid which is consistent with a role in controlling the onset of differentiation. EMBO J 1997; 16: 3185-3197.
232. Zannini M, Francis-Lang H, Plachov D, Di Lauro R. Pax8, a paired domain-containing protein, binds to a sequence overlapping the recognition site of a homeodomain and activates transcription from two thyroid-specific promoters. Mol Cel Biol 1992; 12: 4230-4241.

9. LIST OF ABBREVIATIONS

Abbreviations chemicals:

APS	ammoniumperoxidesulphate
DMSO	dimethylsulphoxide
dNTP	deoxynucleotides triphosphate
EDTA	ethylenediaminetetracetic acid
NaAC	sodium acetate anhydrous
TEMED	tetramethylendiamine

Abbreviations:

AIT	apical iodide transporter
AITD	autoimmune thyroid disease
AD	autosomal dominant
AR	autosomal recessive
CH	congenital hypothyroidism
CM	congenital malformation
CNS	central nervous system
CT	computer tomography
DELPHIA	delayed fluorescence-immunoassay
DEHAL	dehalogenase
DHPLC	denaturating high pressure liquid chromatography
DNA	deoxyribonucleic acid
ECG	electrocardiography
EEG	electroencephalography
EMSA	electrophoretic mobility shift assay
ENT	ear, nose, throat department
EVA	enlarged vestibular aqueduct
F	female
F/M	female to male ratio
fT_4	free thyroxine
IDD	iodotyrosine dehalogenase deficiency
ITD	iodide transportation defect
L-thyroxine	levothyroxine

M	male
MDE	mutation detection enhancement
MUT	allele carrying mutation
N.A.	not available
NIS	natrium-iodide symporter
NS	neonatal screening
OAE	otoacoustic emission
OMIM	Online Mendelian Inheritance in Man
PCR	polymerase change reaction
PDS	pendrin
PIOD	partial iodide organication defect
PRL	prolactin
RFLP	restriction fragment length polymorphism
RIA	radio-immunoassay
SLC	solute carrier family
SNP	single nucleotide polymorphism
SSCP	single stranded conformation polymorphism
TD	thyroid dysgenesis
TFC	thyroid follicular cell
TG	thyroglobulin
THOX	thyroid oxidase
TIOD	total iodide organification defect
TPO	thyroid peroxidase
TRBAb	thyroid receptor blocking antibodies
TRH	thyrotropin-releasing hormone
TRHR	thyrotropin-releasing hormone receptor
TRIAb	thyroid receptor inhibiting antibodies
tT_4	total thyroxine
TSH	thyrotropin, thyroid stimulating hormone
TSHR	thyrotropin receptor
USG	ultrasonography
WT	wild type allele

10. LIST OF PUBLICATIONS

10. 1. Original articles related to the thesis published in peer-reviewed journals

1. Al Taji E, Biebermann H, Límanová Z, Hníková O, Zikmund J, Dame C, Grüters A, Lebl J, Krude H. Screening for mutations in transcription factors in a Czech cohort of 170 patients with congenital and early-onset hypothyroidism: identification of a novel PAX8 mutation in dominantly inherited early-onset non-autoimmune hypothyroidism. European Journal of Endocrinology 2007; 156 (5): 521-529. IF 3.239.
2. Banghová K, Al Taji E, Cinek O, Novotná D, Zapletalová J, Hníková O, Lebl J. Pendred syndrome among patients with congenital hypothyroidism detected by neonatal screening: identification of two novel *PDS/SLC26A4* gene mutations. European Journal of Pediatrics 2008; 167 (7): 777-783. IF 1.277.
3. Banghová K, Cinek O, Al Taji E, Zapletalová J, Vidura R, Lebl J. Thyroidectomy in a patient with multinodular dyshormonogenetic goitre - a case of Pendred syndrome confirmed by finding mutations in the *PDS/SLC26A4* gene. Journal of Pediatric Endocrinology and Metabolism 2008; 21 (12): 1179-1184. IF 0.858.

10. 2. Other original articles published in peer-reviewed journals

Hennies HC, Rauch A, Seifert W, Schumi C, Moser E, Al Taji E, Tariverdian G, Chrzanowska KH, Krajewska-Walasek M, Rajab A, Giugliani R, Neumann TE, Eckl KM, Karbasiyan M, Reis A, Horn D. Allelic heterogeneity in the COH1 gene explains clinical variability in Cohen syndrome. American Journal of Human Genetics 2004; 75 (1): 138-145. IF 11.092.

10. 3. Original articles currently under peer-reviewing

Al Taji E , Biebermann H, Ambrugger P, Venháčová J, Pomahačová R, Lebl J, Hníková O, Grüters A, Krude H. Goitrous congenital hypothyroidism: low mutation rate of TPO mutations in Czech children. Submitted for publication in Journal of Pediatric Endocrinology and Metabolism.

10. 4. Review articles covering the topic of PhD. studies in other medical journals

1. Al Taji E, Zahradníková M, Lebl J. Molecular pathogenesis of congenital hypothyroidism. Czech and Slovak Paediatrics 2001; 56 (11): 636-643.

2. Al Taji E, Lebl J. Thyroid dysgenesis: Current knowledge of the role of transcription factors in the pathogenesis of congenital hypothyroidism. Diabetology, metabolism, endocrinology, nutrition 2002; 5 (3): 163-168.
3. Banghová K, Al Taji E, Lebl J. Pendrin and its role in pathogenesis of congenital hypothyroidism. Diabetology, metabolism, endocrinology, nutrition 2006; 9 (2): 80-84.
4. Banghová K, Al Taji E, Novotná D, Zapletalová J, Hníková O, Čáp J, Klabochová J, Kúseková M, Lebl J. Pendred syndrome among patients with hypothyroidism: genetic diagnosis, phenotypic variability and occurrence of phenocopies. Journal of Czech Physicians (Čas lék čes) 2008; 147 (12): 616-622.

10. 5. Book chapters in monographies covering the topic of PhD. studies

1. Al Taji E, Hníková O, Lebl J. Congenital hypothyroidism in a pair of siblings - a gene defect? In: Lebl J, Šnajderová M, Novotná D. Case histories in paediatric endocrinology. Prague, Galén 2001; 84-85.
2. Al Taji E. Molecular pathogenesis of congenital hypothyroidism. In: Lebl J, Zapletalová J, Koloušková S. Paediatric endocrinology. Prague, Galén 2004; 307-321.
3. Al Taji E. Congenital thyroid disorders. In: Kreze A, Langer P, Klimeš I, Stárka L, Payer J, Michálek J. General and clinical endocrinology. Bratislava, Academic Electronic 2004; 298-310.
4. Al Taji E, Venháčová J, Biebermann H, Lebl J. About Roman whose thyroid gland grew too much. In : Lebl J, Macek M. Case histories in molecular genetics. Prague, Galén 2006; 96-98.
5. Al Taji E, Límanová Z, Hníková O, Krude H, Lebl J. About Matyas whose thyroid gland stopped growing. In: Lebl J, Macek M. Case histories in molecular genetics. Prague, Galén 2006; 93-95.

10.6. Abstracts of oral presentations covering the topic of PhD. studies

International congresses and meetings

1. Al Taji E, Krude H, Biebermann H, Ambrugger P, Renault N, Lebl J, Grüters A. Congenital hypothyroidism in Czech population: first screening for mutations in candidate genes. In: Proceedings of 18th AESF, Berlin, EnForCé 2002; unp. 18.

Arbeitstagung Experimentelle Schilddrusenforschung, Berlin, Germany, December 2002.

2. Al Taji E, Biebermann H, Ambrugger P, Haufs N, Límanová Z, Hníková O, Lebl J, Grüters A, Krude H. Screening for mutations in congenital hypothyroidism in Czech population: low mutation rate and identification of a new PAX8 mutation. Hormone Research 2003; 60 (suppl 2): 17- 18. IF 2.015. 42nd Annual Meeting of the European Society for Paediatric Endocrinology, Lubljana, Slovenia, September 2003.
3. Al Taji E, Biebermann H, Límanová Z, Hníková O, Lebl J, Grüters A, Krude H. Hypothyroidism in three generations. Slovenska Pediatrija 2003; 10 (3): 178-179. 10th Workshop of the Middle European Society for Paeditric Endocrinology, Köszeg, Hungary, November 2003.
4. Al Taji E, Krude H, Banghová K, Zikmund J, Hníková O, Lebl J. Associated congenital malformations in children with congenital hypothyroidism: implications for clinical practice and research. Slovenska Pediatrija 2006; 13 (3): 148. 13th Workshop of the Middle European Society for Paediatric Endocrinology, Portorož, Slovenia, October 2006.

National congresses and meetings

1. Al Taji E, Hníková O, Lebl J. Congenital hypothyroidism in a pair of siblings - a gene defect? In: Proceedings "Case histories in paediatric endocrinology I", Working group of Paediatric Endocrinology, Brno 2001; unp. Paediatric endocrinology meeting - 2nd meeting of Working group of Paediatric Endocrinology of Czech Paediatric Society, Brno, March 2001.
2. Al Taji E, Hníková O, Lebl J. Molecular pathogenesis of congenital hypothyroidism. Diabetology, metabolism, endocrinology, nutrition 2001; 4 (suppl 3): 40. 24th Endocrinology congress with international partecipation, Hradec Králové, September 2001.
3. Al Taji E, Hníková O., Lebl J. Molecular pathogenesis of congenital hypothyroidism. Journal of Czech Physicians 2002; 141 (23): 749. Czech medical society meeting, Prague, February 2002.
4. Al Taji E, Hníková O, Lebl J. Congenital hypothyroidism: Molecular-genetic point of view. Czech and Slovak Paediatrics 2002; 57 (8): 467. Paediatric endocrinology meeting - 3rd meeting of Working group of Paediatric Endocrinology of Czech Paediatric Society, Prague, March 2002.

5. Al Taji E, Venháčová J, Krude H, Biebermann H, Ambrugger P, Lebl J, Grüters A. Congenital hypothyroidism due to dyshormonogenesis: New diagnostic tools. In: Proceedings "Case histories in paediatric endocrinology II", Working group of Paediatric Endocrinology, Olomouc 2003; unp. Paediatric endocrinology meeting - 4th meeting of Working group of Paediatric Endocrinology of Czech Paediatric Society, Olomouc, March 2003.
6. Al Taji E, Krude H, Límanová Z, Hníková O, Lebl J, Grüters A. Congenital hypothyroidism in the Czech population: First screening in candidate genes and identification of a novel PAX8 mutation. Diabetology, metabolism, endocrinology, nutrition 2003; 6 (suppl 2): 28. 26th Endocrinology congress with international partecipation, Liberec, October 2003.
7. Al Taji E, Hníková O, Lebl J. Molecular genetic aspects of pathogenesis of paediatric thyroid disorders. In: Proceedings "50 years of Czech paediatric endocrinology", České Budějovice, Leština 2004; unp. Paediatric endocrinology meeting - 5th meeting of Working group of Paediatric Endocrinology of Czech Paediatric Society, Jindřichův Hradec, January 2004.
8. Al Taji E, Banghová K, Zikmund J, Hníková O, Lebl J. Congenital malformations in children with congenital hypothyroidism: implications for research and clinical practice. Czech and Slovak Paediatrics 2006; 61 (5): 246. 7th Czech paediatric congress with international partecipation, Prague, June 2006.
9. Al Taji E. Genetics of thyroid dysgenesis. In: Proceedings of 30th Endocrinology congress with international partecipation, Nucleus, Hradec Králové 2007; 13-14. 30th Endocrinology congress with international partecipation, Špindlerův Mlýn, October 2007.

10. 7. Abstracts as a co-autohor covering the topic of PhD. studies

International congresses and meetings

1. Banghová K, Al Taji E, Novotná D, Zapletalová J, Hníková O, Cinek O, Lebl J. Pendred syndrome: sensorineural hearing loss and goitre. Slovenska Pediatrija 2006; 13 (3): 152. 13th Workshop of the Middle European Society for Paeditric Endocrinology, Portorož, Slovenia, October 2006.
2. Banghová K, Al Taji E, Cinek O, Novotná D, Zapletalová J, Hníková O, Lebl J. Pendred syndrome among patients with congenital hypothyroidism detected by neonatal screening: identification of two novel PDS/SLC26A4 mutations. Hormone

Research 2007; 68 (suppl 1): 105. IF 2.015. 46th Annual Meeting of the European Society for Pediatric Endocrinology, Helsinki, Finland, June 2007.

3. Hníková O, Zikmund J, Votava F, Al Taji E, Kračmar P, Vinohradská H, Finková M, Horká J. Preventive approaches to endocrinopathies in the Czech Republic. Hormone Research 2007; 68 (suppl 1): 182. IF 2.015. 46th Annual Meeting of the European Society for Pediatric Endocrinology, Helsinki, Finland, June 2007.
4. Banghová K, Al Taji E, Cinek O, Novotná D, Pourová R, Zapletalová J, Hníková O, Lebl J. Pendred syndrome among patients with congenital hypothyroidism detected by neonatal screening. In: Proceedings of 16th Meeting of Paediatric Research of Central European Countries, Innsbruck 2007; 25.
5. Banghová K, Al Taji E, Cinek O, Novotná D, Zapletalová J, Hníková O, Čáp J, Klabochová J, Kúseková M, Lebl J. Pendred syndrome among patients with congenital or postnatal non-autoimmune hypothyroidism. In: Proceedings of 14th MESPE meeting, unp. 14th Annual Meeting of the Middle European Society for Paediatric Endocrinology, Bratislava, Slovakia, November 2007.

National congresses and meetings

1. Lebl J, Průhová Š, Al Taji E, Vosáhlo J, Čiháková D. How transcription factors support understanding endocrine diseases in our patients. Diabetology, metabolism, endocrinology, nutrition 2003; 6 (suppl 2): 26. 26th Endocrinology congress with international partecipation, Liberec, October 2003.
2. Lebl J, Průhová Š, Al Taji E, Vosáhlo J. Embryonic and foetal development of endocrine glands. In: Proceedings "50 years of Czech paediatric endocrinology", České Budějovice, Leština 2004; unp. Paediatric endocrinology meeting - 5th meeting of Working group of Paediatric Endocrinology of Czech Paediatric Society, Jindřichův Hradec, January 2004.
3. Banghová K, Al Taji E, Hníková O, Zapletalová J, Novotná D, Lebl J. Pendrin in pathogenesis of congenital hypothyrodism: molecular genetic diagnostics of Pendred syndrome. In: Proceedings of 7th meeting of Working group of Paediatric Endocrinology of Czech Paediatric Society, Plzeň, Hillary 2006; 24. Paediatric endocrinology meeting - 7th meeting of Working group of Paediatric Endocrinology of Czech Paediatric Society, Chodová Planá, March 2006.
4. Banghová K, Al Taji E, Novotná D, Zapletalová J, Hníková O, Lebl J. Pendred syndrome in differential diagnostics of hearing impairment in congenital

hypothyroidism. Czech and Slovak Paediatrics 2006; 61 (5): 2486. 7th Czech paediatric congress with international partecipation, Prague, June 2006.

5. Banghová K, Al Taji E, Cinek O, Zapletalová J, Lebl J. Why Dasa could not hear and had to undergo thyroidectomy. In: Proceedings of 8th meeting of Working group of Paediatric Endocrinology of Czech Paediatric Society, Ostrava 2007; 41. Paediatric endocrinology meeting - 8th meeting of Working group of Paediatric Endocrinology of Czech Paediatric Society, Ostrava, March 2007.
6. Banghová K, Al Taji E, Cinek O, Novotná D, Zapletalová J, Hníková O, Lebl J. Pendred syndrome in patients with congenital hypothyroidism diagnosed by neonatal screening: identification of two novel mutations in *PDS/SLC26A4* gene. In: Proceedings of 30th Endocrinology congress with international partecipation, Nucleus, Hradec Králové 2007; 49. 30th Endocrinology congress with international partecipation, Špindlerův Mlýn, October 2007.

10.8. Other professional activities related to PhD. studies

Research fellowships

CEEPUS (Central-European Exchange Programme for University Studies) **scholarship**
10/2001 - 12/2001
Department of Paediatrics, Endocrine Unit, University of Vienna, Austria
Topic: Paediatric endocrinology, under the supervision of Prof. Dr. Herwig Frisch.

DAAD (German Academic Exchange Service) **short-research term scholarship**
8/2002 - 12/2002
Institute of Experimental Paediatric Endocrinology, University Children Hospital Charité, Humboldt University, Berlin, Germany
and as a **guest researcher** (grant of the German Research Council)
1/2003 - 2/2003
Institute of Human Genetics, Campus Virchow-Klinikum, Humboldt University, Berlin
Topic: Molecular genetics of congenital hypothyroidism and screening for mutations in candidate genes, under the supervision of Prof. Dr. Annette Grüters and Prof. Dr. Heiko Krude.

Postgraduate education in paediatric endocrinology organized by ESPE

(European Society for Paediatric Endocrinology):

9/2003 17th ESPE Summer School of Pediatric Endocrinology, Bled, Slovenia

2/2007 ESPE Winter School of Paediatric Endocrinology, Prague, Czech Republic

4/2008 2nd ESPE Advanced Seminar in Developmental Endocrinology: Thyroid development and its disorders, Paris, France

Other selected postgraduate education in biomecidine

2002 Science writing workshop

2002 Basics of DNA diagnostics

2007 Endocrinology workshop

2007 Methods and organization of scientific work

2008 Ultrasound diagnostics of thyroid diseases

Grants

Grant of the Czech Ministry of Education MSM 0021620814 - active participant

Grant of Charles University in Prague GAUK 2008/2007 - co-researcher

Memberships in professional organizations and societies:

National:

Czech Medical Association of J. E. Purkyne

Czech Society of Paediatrics

Czech Society of Diabetology

Czech Society of Endocrinology

International:

Middle European Society of Paediatric Endocrinology (MESPE)

European Society of Paediatric Endocrinology (ESPE) - applied

Professional awards

"Prize for the best publication of Czech authors in the field of paediatric endocrinology published in peer- reviewed international journal in 2007" - awarded in 9th meeting of Working group of Paediatric Endocrinology of Czech Paediatric Society, January 2008.

11. ATTACHMENTS - Full texts of selected publications in Czech language

Book chapters in monographies covering the topic of PhD. studies:

1. Al Taji E, Hníková O, Lebl J. Congenital hypothyroidism in a pair of siblings - a gene defect? In: Lebl J., Šnajderová M., Novotná D. Case histories in paediatric endocrinology. Prague, Galén 2001; 84-85.
2. Al Taji E. Molecular pathogenesis of congenital hypothyroidism. In: Lebl J., Zapletalová J., Koloušková S. Paediatric endocrinology. Prague, Galén 2004, 307-321.
3. Al Taji E: Congenital thyroid disorders. In: Kreze A., Langer P., Klimeš I., Stárka L., Payer J., Michálek J. General and clinical endocrinology. Bratislava, Academic Electronic 2004; 298-310.
4. Al Taji E, Límanová Z, Hníková O, Krude H, Lebl J. About Matyas whose thyroid gland stopped growing. In: Lebl J., Macek M. Case histories in molecular genetics. Prague, Galén 2006; 93-95.
5. Al Taji E, Venháčová J, Biebermann H, Lebl J. About Roman whose thyroid gland grew too much. In : Lebl J., Macek M. Case histories in molecular genetics. Prague, Galén 2006; 96-98.

Review articles in Czech medical journals covering the topic of PhD. studies

1. Al Taji E, Zahradníková M, Lebl J. Molecular pathogenesis of congenital hypothyroidism. Czech and Slovak Paediatrics 2001; 56 (11): 636-643.
2. Al Taji E, Lebl J. Thyroid dysgenesis: Current knowledge of the role of transcription factors in the pathogenesis of congenital hypothyroidism. Diabetology, metabolism, endocrinology, nutrition 2002; 5 (3): 163-168.
3. Banghová K., Al Taji E., Lebl J. Pendrin and its role in pathogenesis of congenital hypothyroidism. Diabetology, metabolism, endocrinology, nutrition 2006; 9 (2): 80-84.
4. Banghová K, Al Taji E, Novotná D., Zapletalová J., Hníková O., Čáp J., Klabochová J., Kúseková M., Lebl J. Pendred syndrome among patients with hypothyroidism: genetic diagnosiss, phenotypic variability and occurrence of phenocopies. Journal of Czech Physicians 2008; 147 (12): 616-622.

Declaration

I declare the present academic dissertation was conceived on my own. Only literature sources mentioned above were used. I agree this academic dissertation can be used for study purposes.

Prague, February 2009 MUDr. Eva Al Taji