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Identification of PENDRIN (*SLC26A4*) mutations in patients with congenital hypothyroidism and "apparent" thyroid dysgenesis

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Context: Congenital hypothyroidism (CH), the most frequent endocrine congenital disease, can occur either based on a thyroid hormone biosynthesis defect or can predominantly be due to thyroid dysgenesis. However, a genetic cause could so far only be identified in less than 10% of patients with a thyroid dysgenesis.

Objectives: Exome-sequencing was used for the first time to find additional genetic defects in thyroid dysgenesis.

Patients and Methods: In a consanguineous family with thyroid dysgenesis exome-sequencing was applied and findings were further validated by Sanger sequencing in a cohort of 94 patients with thyroid dysgenesis.

Results: By exome-sequencing we identified a homozygous missense mutation (p.Leu597Ser) in the *SLC26A4* gene of a patient with hypoplastic thyroid tissue, who was otherwise healthy. In the cohort of patients with thyroid dysgenesis we observed a second case with a homozygous missense mutation (p.Gln413Arg) in the *SLC26A4* gene, who was additionally affected by severe hearing problems. Both mutations were previously described as loss-of-function mutations in patients with Pendred syndrome and nonsyndromic EVA (enlarged vestibular aqueduct).

Conclusion: We identified unexpectedly *SLC26A4* mutations, that were hitherto diagnosed in thyroid dyshormonogenesis patients, now for the first time in patients with structural thyroid defects. This result resembles the historic description of thyroid atrophy in patients with the so-called myxedematous form of cretinism following severe iodine deficiency. Most likely, the thyroid defect of the two homozygous *SLC26A4* gene mutation carriers represents a kind of secondary thyroid "atrophy", rather than a primary defect of thyroid development in the sense of thyroid "agenesis". Our study extends the variable clinical spectrum of patients with *SLC26A4* mutations and points out the necessity to analyze the *SLC26A4* gene in patients with "apparent" thyroid dysgenesis in addition to the known candidate genes *TSHR, PAX8, NKX2.1, NKX2.5* and *FOXE1*.

Congenital hypothyroidism (CH) is the most frequent congenital endocrine disease with an incidence of 1:3500–4000 (1). Only in the Afro-American population a lower incidence of 1:25000 has been observed. Apart from iodine deficiency, which in some geographic regions is still one major cause for endemic hypothyroidism of

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neonates, the sporadic cases are grouped into the two categories: dyshormonogenesis and thyroid dysgenesis. In 15%–20% of cases "dyshormonogenesis" in a morphologically normal or enlarged gland (goiter) results in hypothyroidism by a defect of thyroid hormone production. Recessive mutations in the genes for *TPO*, *NIS*, *TG*, *DUOX2* and *SLC26A4* (pendrin) are the major causes of dyshormonogenesis. Most CH cases (approx. 80%–85%) are affected by a structural defect of the thyroid, which can occur – based on radiological examinations by scintigraphy or ultrasound - as the complete absence of thyroid tissue ("thyroid agenesis"), thyroid hypoplasia or thyroid ectopy. It has been discussed whether molecular defects in early embryonic development of the thyroid gland may lead to the occurrence of such structural thyroid defects. During the last ten years several genes were identified in mouse models that play a crucial role for thyroid organogenesis, eg, *PAX8, TSHR, NKX2.1, NKX2.5, FOXE1* (2). However, so far a mutation in one of these candidate genes has been identified in only 3% of patients with thyroid dysgenesis. Moreover a recent report has shown only few noninformative chromosomal aberrations in a cohort of thyroid dysgenesis patients by comparative-genome-hybridization-array (Array-CGH) (3), excluding CNV as a frequent cause of thyroid dysgenesis. Together all efforts failed in more than 90% of CH patients with a structural defect of the thyroid to unravel the molecular defect. Therefore we decided to apply exome-sequencing in informative patients from consanguineous families with thyroid dysgenesis. By this approach we aimed to unravel, mutations in novel genes and to elucidate further mechanisms in the pathogenesis of congenital hypothyroidism.

Materials and Methods

Patients

All clinical investigations and genetic analyses were performed according to the guidelines of the Declaration of Helsinki and with written consent of the families. The study was approved by the Ethical Committee of the Charité-Universitätsmedizin Berlin (EA2/131/11).

Exome-sequencing was performed in a patient with thyroid dysgenesis with consanguinity of the parents, diagnosed in Istanbul, Turkey. The index patient carrying a homozygous loss of function *SLC26A4* mutation was diagnosed at the age of 40 days. The thyroid function, analyzed due to prolonged jaundice, revealed a TSH level of $>$ 75 mU/L (normal range: 1,5–4,5 mU/l) and T4 levels of 0,45 μg/dl (normal range: 4,5–12,5 μg/dl) (Figure 1A). Thyroid ultrasound showed 22 mm and 19 mm structures on both sides of the trachea at the age of 7 years and heterogeneous structures with cystic malformations in follow-up ultrasound examinations (Figure 1B). L-thyroxine supplementation was started at day 40 after birth and the cognitive development of the patient is mildly delayed so far. No hearing problem has been recognized and a hearing test was performed at the age of 18 years, which was normal. No uptake was detected on thyroid scintigraphy at the age of 12 years with a TSH of 218 mU/L (normal range: 1,5–4,5 mU/l), T 4 of 1,93 μg/dl (normal range: 5,1–14,1 μg/dl) and f T4 of 0,23 ng/dL (0,93–1,7 ng/dL). The father of this index patient had a papillary thyroid carcinoma and polycystic kidney disease. No further diseases were found in the family history.

A cohort of 94 patients (64 females, 30 males) with thyroid dysgenesis, diagnosed primarily in the Berlin newborn screening program, including 37 individuals with thyroid agenesis, 52 patients with thyroid hypoplasia and 5 patients with an ectopic thyroid, were screened for *SLC26A4* mutations. In all patients of this cohort mutations in known candidate genes for thyroid dysgenesis (*TSHR, PAX8* and *NKX2.1*) have already been excluded.

DNA extraction and mutation screening

DNA was extracted from 0,5 ml EDTA blood samples according to standard protocols (Qiagen, Hilden, Germany). For *SLC26A4* mutation screening, all exons were amplified by PCR and subsequently sequenced using the ABI 3130 xl automatic sequencer (Applied Biosystems, Carlsbad, CA, USA). The primer sequences are available on request.

Exome sequencing and bioinformatic methods

A blood sample of the index patient of the Turkish family was subjected to exome-sequencing. Genomic DNA was isolated from the blood sample using standard methods. Two micrograms of genomic DNA were enriched using the Agilent Human All Exon V4 kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's protocol. Whole-exome libraries were sequenced on an Illumina HiSeq 2000 system for 1×101 cycles following the manufacturer's instructions (Illumina, San Diego, CA, USA). All raw sequencing reads were mapped onto UCSC hg19 using BWA (4) 0.5.9-r169, mappings were converted into BAM file format using samtools 0.1.18. Initial mappings were postprocessed using GATK 1.6 (5) following their 'best practices V3'. In brief, reads were realigned around sites of known INDELs. Then, likely PCR duplicates were detected using Picard 1.48. Finally, raw base quality scores were empirically recalibrated. SNPs and INDELs were identified using the UnifiedGenotyper from GATK. Variants were classified as novel or known variants according to dbSNP 135 (6). Functional consequences of each variant were annotated using snpEff 2.0.5d20 for UCSC hg19 RefSeq genes and ENSEMBL 68 human gene models (7, 8). The potential deleterious effect was evaluated using PolyPhen 2, SIFT, PhyloP (9), MutationTaster (10), GERP++ (11), LRT and OMIM, if available. Based on the pedigree, variants were filtered for an autosomal-recessive inheritance pattern.

Results

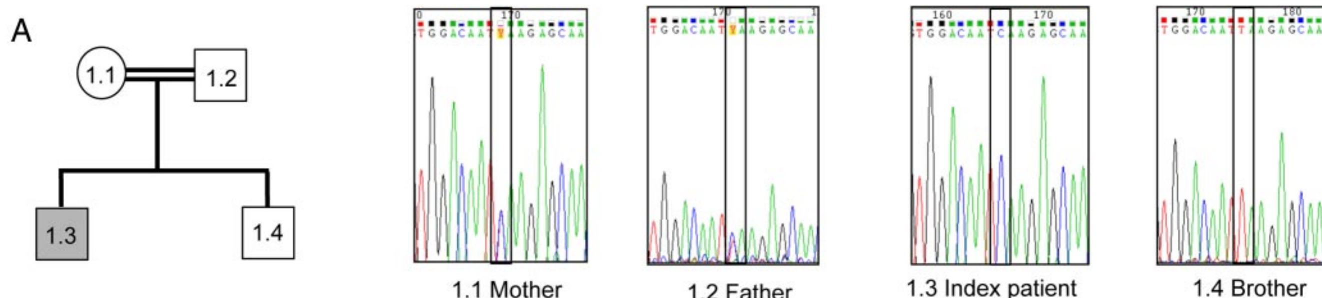
Exome-Sequencing Results

The index patient of one consanguineous family from Turkey was subjected to whole exome sequencing in the expectation to identify a new gene for thyroid dysgenesis.

As documented by ultrasound at an age of 7 years as well as scintigrayphy at an age of 12 years he was diagnosed to have a hypoplastic thyroid gland. In total, exome sequencing obtained 93.4 million single-end 101 bp reads per sample, of which 98.5% could be mapped onto the human genome. After removing duplicated reads, which possibly derived from PCR artifacts, 31.4 million unique reads were mapped to the targeted protein coding regions, resulting in an average of 65.0x coverage within the targeted coding region.

Using GATK, we detected 23,251 SNPs and INDELs in the exome of the index patient, of which 97.7% are known variants deposited in dbSNP 135. Neither the *TSHR*, nor the *PAX8* gene had any variants below 5% minor allele frequency within its coding regions in the affected patient. Given the pedigree, we searched for deleterious mutations in the affected patient with an autosomal-recessive inheritance pattern, not present in the unaffected sibling. From a list of 6,302 variants with minor allele frequency below 5%, 1,993 are in a homozygous state. Among these rare homozygous variants, 760 have the potential to alter protein structure, whereas only 17 are predicted to be damaging by at least two variant effect assessment methods (Supplement Table 1).

Thereby we detected a homozygous c.1790T>C missense mutation in the *SLC26A4* gene leading to an amino acid change p.Leu597Ser. The mutated alleles were inherited from the parents, which were both heterozygous for the c.1790T>C mutation. The unaffected brother was carrying the wildtype alleles. We validated the mutations by Sanger sequencing of the affected and nonaffected family members (Figure 1A). The mutation is localized in the C terminal intracellular part of pendrin, according to the 12 transmembrane domains model (Figure 2). In previous mutation screenings of the *SLC26A4* gene in cohorts of hearing loss patients, this mutation has been described in



Family members	Thyroid hormone levels	additional diseases
1.1 Mother	TSH 3,53 mU/l T4 9,64 µg/dl	none
1.2 Father	TSH 5,46 mU/l T4 9,33 µg/dl	Papillary thyroid carcinoma Polycystic kidney disease
1.3 Index patient	TSH > 75 mU/l T4 0,45 µg/dl	Thyroid hypoplasia
1.4 Brother	TSH 2,11 mU/l T4 8,28 µg/dl	none

B

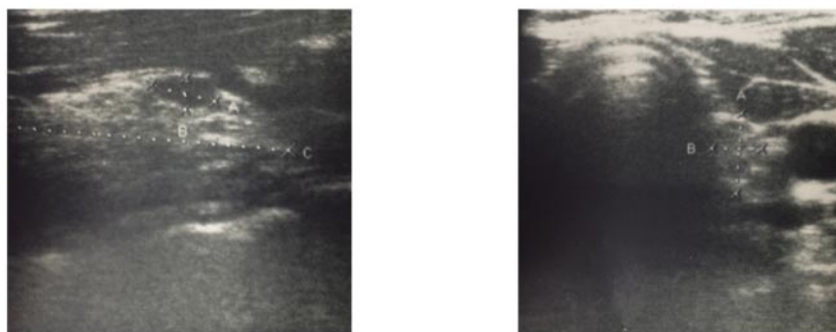


Figure 1. A) Pedigree, genotype and phenotype of the family of the patient with the homozygous p.Leu597Ser mutation. B) Example of the ultrasound of the hypoplastic thyroid gland.

Table 1. This table gives an overview about the observed *SLC26A4* genetic variants and the phenotypes in our cohort of patients with thyroid dysgenesis.

Patient No.	SLC26A4 genotype	Patient phenotype
1	p.Leu597Ser (homozygous)	Thyroid hypoplasia
2	p.Gln413Arg (homozygous)	Thyroid hypoplasia Deafness
3	p.Leu597Ser (heterozygous)	Thyroid hypoplasia (1 ml)
4	p.Leu597Ser (heterozygous)	Athyroisis
5	p.Gly334Val (heterozygous) Exon15 – 26 T>C (heterozygous)	Athyrosis
6	Exon 15 – 5 G>A (heterozygous)	Thyroid hypoplasia (1.7 ml)

19 individuals (12–18). Functional characterization of the p.Leu597Ser mutant in Xenopus oocytes revealed an impaired protein function with a reduced Cl^-/HCO_3^-, Cl^-/Cl^- and Cl^-/I^- exchange (16). After identifying the *SLC26A4* mutation, we re-evaluated the patients' thyroid by ultrasound and performed a hearing test. The ultrasound confirmed the thyroid hypoplasia and the hearing test was normal.

SLC26A4 variants in a cohort of 94 patients with thyroid dysgenesis

Based on the unexpected identification of the *SLC26A4* missense mutation in a patient with thyroid dysgenesis by exome-sequencing, we screened for *SLC26A4* mutations in a cohort of 94 patients with thyroid dysgenesis who had already been examined for known thyroid dysgenesis candidate genes and CNVs (3). We identified a second patient affected by a homozygous *SLC26A4* missense mutation (c.1238A>G) leading to an amino acid exchange p.Gln413Arg. The patient was affected by severe congenital hypothyroidism, thyroid hypoplasia, deafness and mental retardation (Figure 3). He was diagnosed in the newborn screening program and at an age of 15 years a hypoplastic thyroid gland with a volume of 2,1 ml (normal range: 4,4 ml ± 1,4) (19) was diagnosed by ultrasound. The mutation is located at a highly conserved position in the fifth extracellular loop of pendrin (Figure 2). The p.Gln413Arg mutation has already been described as a compound heterozygous variant together with a heterozygous p.Val138Phe mutation in a patient with bilaterally enlarged vestibular aqueducts and hearing loss in combination (20).

In the same cohort sequencing of the *SLC26A4* gene revealed additional heterozygous variants in four patients (Table 1). In two patients the same mutation leading to an amino acid exchange of Leucine at postion 597 to Serine was found while in a further patient position 334 was

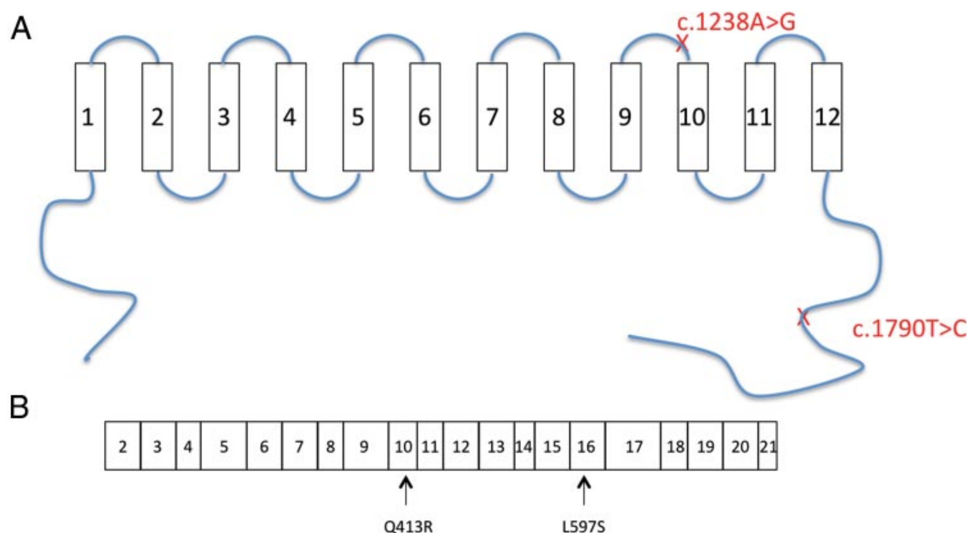


Figure 2. A) Localization of the observed gene mutations in the two affected patients with thyroid dysgenesis based on the 12 transmembrane model (http://healthcare.uiowa.edu/labs/pendredandbor/domains) and (38). B) Localization of the mutations within the *SLC26A4* gene locus.

changed from Glycine to Valine. In this patient the p.Gly334Val mutation was compound heterozygous with a variant in a non coding variant in Exon 15 (-26T>C). The fourth patient was found to harbor a heterozygous noncoding variant in Exon 15 (-5G>A).

Discussion

Congenital hypothyroidism due to thyroid dysgenesis is a rare disease, which usually occurs sporadically. So far, in only 5% of patients with thyroid dysgenesis a mutation could be detected in the known candidate genes *PAX8*, *TSHR*, *FOXE1*, *NKX2.5* and *NKX2.1* (2). In 10% of the patients, different chromosomal copy number variants have been described recently, without conclusive candidate genes within the affected genomic regions. Therefore, at least in 90% of the patients the pathogenesis of thyroid dysgenesis remains unclear. In order to further identify genetic defects in thyroid dysgenesis we performed a sequencing study of all coding exons in a consanguineous family from Turkey. Surprisingly, we identified a homozygous *SLC26A4* mutation in one patient of consanguineous parents. So far the gene *SLC26A4* was identified to be affected in Pendred syndrome, which includes the combination of deafness and goiter (21). It encodes for the anion transporter pendrin, which exchanges I^-, Cl^-, and $HCO3^-$. However, the detailed function and potency of this ion transporter is a matter of debate and the published data is controversial (22, 23). Pendrin is expressed in the apical membrane of thyrocytes (24) where it leads to an iodine transport into the follicular lumen. Moreover, pendrin is expressed in the kidney (25) and in the inner ear where it maintains and stabilizes ionic homeostasis in the endolymph (26). *SLC26A4* mutation leads to the development of a variable clinical spectrum of hearing loss due to inner ear malformation as an enlarged vestibular aqueduct (EVA) or Mondini Cochlea (27) associated with goiter and in some cases congenital hypothyroidism (28). No clear genotype-phenotype correlation has been observed yet and, based on the broad spectrum of clinical manifestation, epigenetic or environmental modifiers have been postulated (14, 29). Impaired thyroid function is postulated to be due to reduced iodine organification leading to the development of an enlarged gland with and without altered

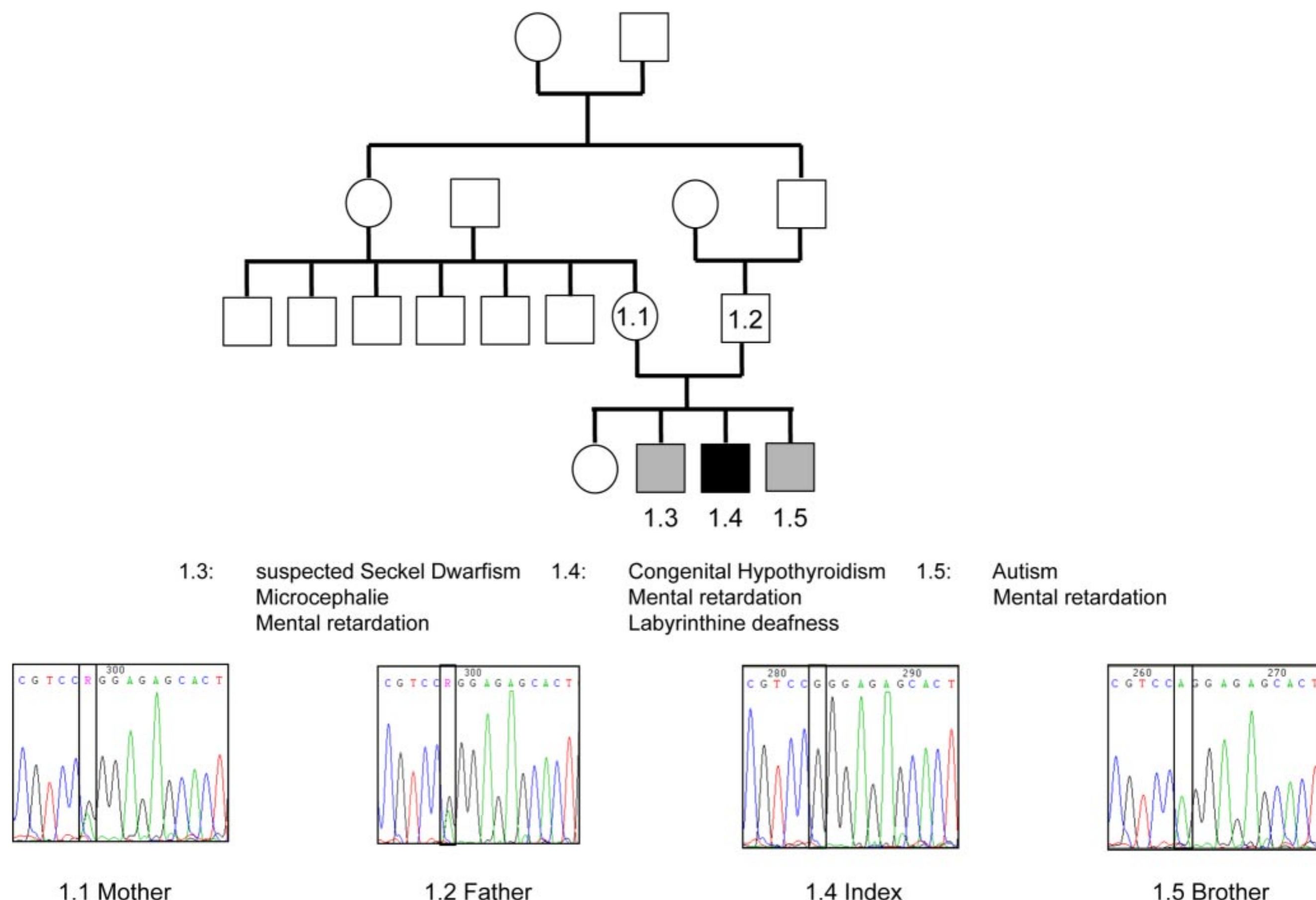


Figure 3. Pedigree of the consanguineous family from the patient with the homozygous p.Gln413Arg mutation. One brother is suffering from Seckel Dwarfism, microcephaly and mental retardation. The younger brother is mentally retarded and has autism. The genetic cause for these phenotypes is so far unknown.

thyroid hormone production. This is supported by the observation using the "Perchlorate Discharge Test", in which the initial scintigraphy shows normal radioiodine uptake but an increased radioiodine wash-out is observed after perchlorate administration in patients with Pendred syndrome.

To test the so far unrecognized role of *SLC26A4* as an additional candidate gene not only for Pendred Syndrome but also for thyroid dysgenesis, we sequenced all coding exons of *SLC26A4* in a cohort of 94 patients with thyroid dysgenesis and identified a second patient with thyroid hypoplasia to be affected by a homozygous *SLC26A4* mutation (p.Gln413Arg). This patient was diagnosed in a newborn screening program with severe congenital hypothyroidism, but despite early and sufficient treatment he developed mental retardation and was suffering from deafness. While mental retardation was also found in two other children of the consanguineous family and seems not to be a consequence of the *SLC26A4* mutation, deafness can now be attributed to the identified homozygous pendrin defect and in accordance to the first identified patients with a *SLC26A4* mutation and thyroid hypoplasia it seems likely that also in this second patient thyroid hypoplasia results very surprisingly from pendrin insufficiency.

Both diagnosed homozygous *SLC26A4* mutations have been already identified in patients with Pendred syndrome and EVA. Moreover, the p.Leu597Ser mutation has been functionally characterized showing a reduced transporter capacity in vitro (16). A functional characterization of the p.Gln413Arg variant is so far missing. Being localized in a conserved gene position within the fifth extracellular loop this mutation may lead to either structural changes and thereby impaired transport function or reduced binding capacities.

The finding, that we identified in three out of six patients the p.Leu597Ser mutation, either homozygous in the first patient and heterozygous in further two patients, is surprising given the number of > 50 different mutations found so far in Pendred Syndrome patients. Except for one patient being compound heterozygous, all other patients described so far to carry the p.Leu597Ser variant are heterozygous. All of these patients had moderate to severe hearing problems. One patient was additionally affected with mild hypothyroidism and in another patient a goiter was observed. This heterozygous p.Leu597Ser variant was also found in single healthy control individuals (16, 29), which suggests that the p.Leu597Ser might reflect a common variant without functional relevance. However the minor allele frequency (MAF) has been annotated to be < 0.01 (http://www.ensembl.org/Homo sapiens/Variation) and in in vitro studies documented an impaired function of the p.Leu597Ser gene product. Therefore one might speculate, that especially the heterozygous p.Leu597Ser mutation can cause a functional defect in concert with potential additional modifying genetic alterations leading to a variable penetrance,

Together, our data argue for the first time, that in addition to dyshormonogenesis *SLC26A4* mutations can cause the absence or hypoplasia of functional thyroid tissue. How can this unexpected structural defect be explained by loss of pendrin function? A primary defect during thyroid embryogeneses due to loss of *SLC26A4* function seems unlikely, because the *SLC26A4* gene is only expressed in later fetal stages of the thyroid development - when the thyroid anlage has reached its final position in the front part of the neck - and has already started to differentiate into a follicular structure (30).

One potential explanation evolves from the critical role of pendrin for the transport of iodine into the thyroid follicle and is related to historic reports about severe and untreated iodine deficiency. A variety of former studies from areas of very severe iodine deficiency - like Switzerland, China and the Democratic Republic of Congo (former Zaire) - report about different forms of cretinism either myxedematous or neurologic, which result from a postnatal or fetal iodine deficiency, respectively (31). The postnatal myxedematous form was found to be associated with a high rate of thyroid atrophy (31–34) that was described histologically as fibrosis and loss of thyroid epithelial tissue (31). The mechanism for thyroid tissue loss due to postnatal sustained severe iodine deficiency has not been resolved so far; the hypothesis of a secondary autoimmune response with thyroid growth inhibiting antibodies could be refuted (35). There were also speculations about the role of elevated TSH levels mediating an increased intracellular oxidative stress via increased levels of O_2^- and H_2O_2 (36, 37) and about the role of free radicals, which might possibly lead to an atrophic thyroid gland. Since the rate of thyroid atrophy is variable in the different cohorts of patients with myxedematous cretinism from different geographic regions and it has been shown that within each cohort some patients develop a goiter instead of an atrophy, most likely additional genetic factors contribute to the final thyroid outcome in endemic severe postnatal iodine deficiency.

In analogy to these reports of thyroid atrophy in environmental iodine deficiency, our new observation of thyroid dysgenesis in two patients with *SLC26A4* mutations argue, that also genetic forms of intra-thyroideal iodine deficiency might cause a variable structural defect of thyroid tissue. In general environmental deficiency and genetic defects of iodine transport result both in the lack of sufficient iodine in the thyroid follicle. The finding of thy-

roid hypoplasia and atrophy in both conditions implies, that sufficient iodine in the follicle is critical to maintain the proper thyroid structure. Irrespective of the exact molecular mechanism – which now needs to be determined - this structural influence of iodine seems to be relevant only in the postnatal period, because the adequate substitution with iodine after birth can prevent in the "neurological" form of fetal cretinism and the thyroid atrophy as described in the postnatal prolonged iodine deficient "myxedomatous" form of cretinism.

Unfortunately we do not have a documented longitudinal course of thyroid imaging in the two patients, which we have identified. However, the available ultrasound images of the thyroid in both patients argue, that at least at an age of 7 years the structural integrity was affected. Because pendrin is located at the apical membrane supplying iodine transport from the thyroid cell into the follicular lumen and the transporter NIS, which is intact in this patient and which is expressed at the baso-lateral membrane to support iodine accumulation into the thyroid cell, a defect of pendrin is expected to allow a positive uptake in a scintigraphy. Our finding of no uptake at an age of 12 years in the first patient therefore argue that already a structural defect of the thyroid cells developed before that time point that interfere with iodine accumulation into the thyroid cell. This potential explanation is supported by the finding in thyroid tissue of a patient with Pendred syndrome, in which a zone of destroyed follicles has been observed histologically (37). In this case the authors could demonstrate different cellular responses to pendrin deficiency including a state of compensation by upregulation of another transporter CLC-5 as well as a state of only partial compensation of intracellular thyroglobulin-iodination with increased oxidative stress and apoptosis (activated Caspase-6) that can result in the destruction of thyroid tissue. A loss of adequate iodine supply in the follicular lumen seems therefore to result in a state of compensating intracellular iodination that can result in H_2O_2 based cellular damage (37).

Based on these considerations one might speculate that beside environmental iodine deficiency and pendrin deficiency a defect of the NIS transporter can in addition result in a thyroid structural defect too. So far to our knowledge no such screening for NIS mutations in thyroid dysgenesis patients has been performed yet.

In conclusion, we diagnosed - using an exome-sequencing approach - the first *SLC26A4* mutations in patients with a structural thyroid defect from consanguineous families. The structural defects of the ear in pendrin deficiency together with our observation of a thyroid defect in the *SLC26A4* mutation carrier point to a more general theme: disturbed transport function in a given functional system might lead to a secondary loss of structural integrity and maintenance. In terms of the thyroid gland, this implies that the loss of pendrin function most likely leads to a secondary loss of normal thyroid follicles in the sense of a thyroid "atrophy" and does not cause a primary thyroid developmental defect as thyroid "dysgenesis" or "agenesis". However, both variants - atrophy and agenesis - are difficult to discriminate in given radiological studies like ultrasound and scintigraphy. This shows the need for longitudinal imaging studies in patients with an early diagnosis of pendrin deficiency to document the accumulating loss of functional thyroid tissue in the case of intrafollicular iodine deficiency. In addition in some patients with thyroid dysgenesis we need to reconsider thyroid atrophy, which leads to a longer list of potential candidate genes, now including *SLC26A4* and potentially also NIS.

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