Molecular characterization of iodotyrosine dehalogenase deficiency in patients with hypothyroidism

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ABSTRACT

Context: The recent cloning of the human iodotyrosine deiodinase (*IYD*) gene enables the investigation of iodotyrosine dehalogenase deficiency, a form a primary hypothyroidism resulting from iodine wasting, at the molecular level.
Objective: In the current study, we identify the genetic basis of dehalogenase deficiency in a consanguineous family.
Results: Using HPLC tandem mass spectrometry, we developed a rapid, selective, and sensitive assay to detect 3-monoiodo-L-tyrosine (MIT) and 3,5-diodo-L-tyrosine (DIT) in urine and cell culture medium. Two subjects from a presumed dehalogenase deficient family showed elevated urinary MIT and DIT levels compared to 57 normal subjects without thyroid disease. Subsequent analysis of *IYD* revealed a homozygous missense mutation in exon 4 (c.658G>A p.Ala220Thr) that co-segregates with the clinical phenotype in the family. Functional characterization of the mutant iodotyrosine dehalogenase protein showed that the mutation completely abolishes dehalogenase enzymatic activity. One of the heterozygous carriers for the inactivating mutation recently presented with overt hypothyroidism indicating dominant inheritance with incomplete penetration. Screening of 100 control alleles identified one allele positive for this mutation suggesting that the c.658G>A nucleotide substitution might be a functional SNP.
Conclusion: This study describes a functional mutation within *IYD*, demonstrating the molecular basis of the iodine wasting form of congenital hypothyroidism. This familial genetic defect shows a dominant pattern of inheritance with incomplete penetration.

INTRODUCTION

The cascade of events leading to the production of T4 and T3 in the thyroid is well documented (1). A defect in this cascade may lead to insufficient thyroid hormone production and causative mutations in numerous genes explaining the molecular cause have been described (2-7). The concerted actions of TPO and DUOX2 result in the iodination of specific 3-monoiodo-L-tyrosine (MIT) and 3,5-diodo-L-tyrosine (DIT) within thyroglobulin. The subsequent coupling of MIT and DIT residues generate T4 and T3 that are secreted into the circulation. Upon degradation of thyroglobulin within the thyrocyte, non-coupled MIT and DIT are also released. Since these iodotyrosines contain the major amount of iodide within thyroglobulin, recycling of these compounds is essential to salvage the rare element iodide.
For decades the importance of this process for the maintenance of euthyroidism, especially in iodine deficient areas, has been well accepted (8), and patients with iodotyrosine dehalogenase deficiency (DD, OMIM 274800) were already described in the 19fifties based on chromatographic studies with radio-active labeled compounds and measurement of the enzymatic activity in goitrous thyroid gland (9;10). In general these patients will develop goiter when dietary iodide is limiting, are (compensated) hypothyroid, have a rapid and high uptake of radioiodine, and secrete higher than normal MIT and DIT in their urine (6). However, final diagnosis of these patients was not supported by molecular evidence because the causal gene had not been cloned.
We (11), and others (12;13) have recently identified the gene iodotyrosine deiodinase (*IYD*, also known as *Dehal1* or *C6orf71*) encoding the enzyme responsible for MIT and DIT deiodination. The IYD protein belongs to the NADH oxidase/flavin reductase superfamily, its sequence is highly conserved among mammals, and has a high level of similarity to bacterial NADH oxidase/flavin reductases (12). IYD is mainly expressed in the thyroid, but IYD mRNA is also detected in liver, kidney, and colon (11;13). The identification of IYD has provided detailed biochemical data on the in vitro deiodination of MIT and DIT. Recently the first homozygous mutations in the IYD gene have been reported in four hypothyroid patients included for genetic analysis because of biochemical or clinical features suggestive of DD (14).
In the current study we describe a rapid, highly selective and sensitive HPLC tandem mass spectrometry (HPLC-MS/MS) method for the detection of MIT and DIT that facilitates the detection of these metabolites in urine and enables structure-function analysis of *in vitro*-produced wild-type and mutant protein. Application of the assay identified 2 patients with congenital hypothyroidism that displayed markedly increased urinary DIT and MIT levels. Both individuals belong to a family with a putative DD and we identified a causal missense mutation within *IYD* that completely abolishes enzymatic activity. The phenotype displays dominant inheritance in a heterozygous sib who presented with non autoimmune goitrous hypothyroidism at the age of 15 years.

MATERIALS AND METHODS

MIT-DIT assay

Reagents

Analytical grade solvents were purchased from Merck, and MIT, DIT and 3-chloro-L-tyrosine (MCT) from Sigma. Other chemicals were of the highest purity available. Butanolic HCl (3 M) was prepared from butanol and acetyl chloride (4:1).

Internal standard (IS) and calibrators

MIT and DIT stock solutions (0.5 µM) and MCT (IS) stock solution (1.6 µM) were prepared in distilled water or pooled urine. Three calibrators sets were prepared by adding 60 µL of IS and a range of MIT and DIT stock solutions volumes to 100 µL of i) water, ii) pooled urine or iii) cell culture medium.

Sample preparation for assay validation:

To 100 µL of urine sample, 60 µL IS solution and 60 µL MIT and DIT stock solution (dissolved in either water or pooled urine) were added. After vortexing samples were evaporated to dryness (N_2, 40°C), derivatized with butanolic HCl (60°C, 15 min) to improve the signal-to-noise ratio followed by evaporation to dryness and subsequently reconstituted in 100 µL 2% acetonitrile in water. After determination of initial creatinine values by colorimetric Jaffe assay, all urine

samples were adjusted to a final creatinine concentration between 0.1 – 1 µM.

Sample measurements

To each 100 µL urine sample 60 µL IS solution was added. After vortexing samples were evaporated to dryness (N_2, 40°C), derivatized with butanolic HCl (60°C, 15 min) followed by evaporation to dryness and reconstitution in 100 µL 2% acetonitrile in water.

For measurements in cell culture medium 50 µL sample was added to 50 µL IS. After subsequent addition of 500 µl acetonitrile samples were centrifuged (10 min, 12.000xg), the supernatant was evaporated to dryness (N_2, 40°C) and derivatized with butanolic HCl (60°C, 15 min). Subsequently samples were evaporated to dryness and reconstituted in 100 µL 2% acetonitrile in water.

A 10 µL aliquot of the final sample was injected for HPLC-MS/MS analysis.

Instrumentation

HPLC: Chromatographic separation was achieved on a Surveyor modular HPLC System (Thermo Finnigan Corporation, San Jose, CA, USA) consisting of a thermostated autosampler, a low-flow quaternary MS pump and a 20 x 2 mm Supelguard Discovery C8 column (Sigma-Aldrich Chemie B.V. Zwijndrecht, Netherlands). Sample was eluted at a flow rate of 300 µL/min and a linear gradient of 5 min: start = 97% eluant A (0.1% formic acid in water), 3% eluant B (acetonitrile/water : 9/1); end = 100% eluant B; the system was hold on for 3 min with the initial eluant at a flow rate of 500 µl/min to equilibrate the column; column temperature was maintained at 20 °C.

Mass Spectrometry: MS/MS analyses were performed on a TSQ Quantum AM mass spectrometer (Thermo Finnigan Corporation, San Jose, CA, USA) using positive electrospray ionization (ESI). Nitrogen was used as sheath and drying gas; Argon was used as collision gas at a pressure of 0.20 Pa. The capillary voltage used was 3.5 kV. The source temperature was set at 85 °C; optimal cone voltage ranged 30 - 40 V for the analytes and IS. DIT, MIT and MCT were measured by multiple reaction monitoring (MRM) using transitions m/z 490 → m/z 388 for DIT, m/z 364 → m/z 135 for MIT and m/z 272 → m/z 135 for MCT with an optimal collision energy of 21, 42 and 42 eV respectively. Settings were optimized using a 0.1 µmol/L solution of DIT, MIT and MCT in water and urine.

Patients

The index patient (II-2 in Figure 3) comes from a highly consanguineous Belgian/Moroccan family. She came to medical attention in 1967 at the age of 5 years when she presented with delayed psychomotor development, stunted growth, and goiter. This phenotype was clearly family-related with several sibs and grandparents affected with a seemingly recessive mode of inheritance. At that time a loading test with intravenous administrated radiolabeled DIT showed excretion of 50-70% of the administered dose, in contrast to controls who secreted 15 to 20%. These data resulted in the diagnosis dehalogenase deficiency and was supported by the successful treatment of one of the subject with lugol (15). Patient III-2 was born with a goiter, and subsequently diagnosed with hypothyroidism due to dyshormonogenesis (TSH: 75 mU/L and T4: 60 nmol/L). She had prolonged neonatal jaundice due to cholestasis. A liver biopsy confirmed Byler disease and at the age of 3 she received a liver transplant. At that time, the family was approached for detailed testing of their thyroid status and informed consent for DNA testing was given. Plasma was collected for TSH, T4, fT4, T3, Thyroglobulin and TBG determination. Urine was collected to determine iodine, DIT and MIT excretion and peripheral blood cells were harvested for analysis of genomic DNA. Urinary MIT and DIT excretion was measured in 24 healthy adults from whom material was collected to determine iodine excretion in relation to food status (National Institute for Public Health and the Environment) and 23 healthy schoolchildren of 5 and 6 years old who anonymously contributed spot urine in order to determine a iodine excretion reference range (all with informed consent).

Mutation analysis of IYD

Primers (sequences available upon request) were designed and synthesized (Biolegio) to cover the 6 coding exons of *IYD*, based on both major *IYD* splice forms (13), by PCR amplification. Using AmpliTaq Gold (Applied Biosystems) under standard conditions PCR fragments were generated from patient blood DNA. Big Dye Terminator V3.1 (Applied Biosystems) nucleotide sequence analysis was

performed on the PCR fragments, and the resulting sequences (ABI3100/3730) were compared to the *IYD* reference genomic sequence (GenBank accession NT_025741) using CodonCode Aligner (CodonCode Corporation). cDNA reference nucleotide numbering according to GenBank NM_203395, and amino acid numbering according to Genbank NP_981932.

Plasmids

An *IYD* fragment encompassing nucleotide 138-1010 (GenBank NM_203395.1) was amplified from normal human thyroid cDNA and cloned into the vector pDONR/ZEO using Gateway Technology (Invitrogen). IYD-FLAG fragments were generated by PCR using primers 5'-GCCACCATGTATTTCCTGACTCCCATCT-3' and 5'-AGGCCTACTTATCGTCGTCATCCTTGTAATCCACTGTCACCATGATCTGGT-3, sub-cloned into pGEM-Teasy (Promega), and finally ligated into the CMVpromoter-based expression vector pCDNA3 (Invitrogen). Site-directed mutagenesis using the QuikChangeII protocol (Stratagene) was performed to mutate the alanine on position 220 (GenBank accession NP_981932) into a threonine or into a serine.

Cell culture and transfection

HEK-293 cells obtained from the American Type Culture Collection (CRL-1573) were maintained in DMEM supplemented with 10% Fetal Bovine Serum (BioWhitaker). One day before transfection, cells were seeded in a 6, 12 or 24-well culture dish. Transfection was performed with Fugene6 transfection reagent (Roche Applied Science) according to the manufacturer's instructions. Transfection efficiency was determined by co-transfection of pEGFP-C1 (Clontech). The percentage of fluorescent cells was determined using a FACSCalibur flow cytometer (BD Biosciences). IYD-FLAG protein expression was determined by Western blot analysis (FLAG M2 antibody, Stratagene) on cell lysates.

RESULTS

Validation HPLC-MS/MS assay

No sizeable background signal or mutual interferences were observed for MIT, DIT and MCT in the HPLC and MS assay (Figure 1). In urine signal responses for MIT and DIT decrease by respectively 1.1% (R=0.53) and 2.2% (R=0.63) per nmol/L creatinine. The limit of detection *(LOD)* was defined as the lowest signals detected with a signal-to-noise ratio (S/N) of 3. The LOD for MIT and DIT in water was 0.2 nmol/L. Depending on the extent of suppression LOD in urine was between 0.2-2 nmol/L, and. LOD ranged between 0.1-0.4 nmol/mmol creatinine for the corresponding creatinine range 0.5-20 mmol/L. 100 µL of urine sample was needed to determine MIT and DIT with a S/N > 3. Overall, in urine MIT could be determined with a c.v. < 7% (20 – 1000 nmol/L) and a c.v. < 15 % near LOD; DIT could be determined with a c.v. < 15 % (20- 1000 nmol/L) and a c.v. < 25 % near LOD. More details on the validation available on request.

MIT-DIT determination in patient urine samples

DIT and MIT levels in urine of individuals without thyroid disease were determined by HPLC-MS/MS. In addition, the index patient with a presumed DD and 4 of her children were included. The urinary MIT-DIT data are graphically presented in figure 2. The average values in the group of 57 individuals without any known thyroid disease are 3.2 ± 2.1 nmol/L MIT (range 1.0-9.8), and 0.6 ± 0.4 nmol/L DIT (range 0.2-2.7). For a sub-group of 24 adults without any known thyroid disease (average 2.6 ± 1.5 nmol/L MIT, 0.5 ± 0.1 nmol/L DIT) we could also calculate the daily MIT-DIT excretion: 1.4 ± 1.2 nmol MIT/day, 0.3 ± 0.1 nmol DIT/day. Two members of the DD-family (III-4 and II-2) showed increased levels of both DIT and MIT in their urine.

IYD mutation analysis in individuals with increased urinary MIT-DIT

For six members of the DD family, blood DNA was available for nucleotide sequence analysis of the entire coding area and the exon/intron boundaries of *IYD*. Index patient II-2 of the DD family, as well as her affected daughter (III-2), are homozygous for a missense nucleotide substitution in exon 4 of *IYD* (c.658G>A, p.Ala220Thr). Both had clinical symptoms of hypothyroidism and are

currently treated with T4 supplementation aimed at keeping TSH levels within the reference range. Four additional children were available for genetic analysis and they are heterozygous for the mutation. At the time of the DNA testing (2002) all were clinically euthyroid and did not have a palpable goiter, in spite of the clearly elevated serum thyroglobulin and urine MIT-DIT levels in III-4. In February 2008, patient III-4 came to medical attention with complaints of goiter, weight loss, fatigue, and lack of appetite. Thyroid function test showed increased TSH and TG, decreased T4 and T3 without any signs of thyroid autoimmunity. T4 therapy was initiated immediately.
MIT-DIT levels, *IYD* genotype, and clinical thyroid parameters of the investigated DD-family members are summarized in figure 3. The direct sequence analysis of 100 control alleles showed the presence of one *IYD* c.658A allele.

Functional analysis of IYD and the IYD A220T mutation

The A220T mutation falls within the enzymatic dehalogenase domain of IYD (11-13). To assess whether the IYD-A220T mutation has functional consequences, HEK293 cells were transfected with different CMVpromoter-IYD expression plasmids. IYD activity is measured by the decrease of substrate (MIT or DIT) in tissue culture medium as determined by HPLC-MS/MS. Expression of wild-type IYD-FLAG protein in a 24-well format shows a complete conversion of MIT within 43 hours after addition of the substrate. The conversion of DIT is slower, but after 62 hours over 80% of DIT is converted. IYD activity is not affected by the addition of the FLAG tag, and can be completely inhibited by the IYD inhibitor 3-nitro-L-tyrosine (16) (data not shown). The IYD-A220T mutant does not result in significant MIT or DIT conversion (figure 4A).
To exclude that the difference in dehalogenation kinetics between MIT and DIT is the result of differences in substrate membrane transport, the IYD enzymatic activity was also measured in lysates of transfected cells. As shown in figure 4B-C, also in lysates MIT is more rapidly converted than DIT upon addition of NADPH. Again, cells transfected with the mutant IYD-A220T do not display any dehalogenase activity.

The enzymatic dehalogenase domain of IYD is strongly conserved among vertebrates. Alignment shows that even in the most distantly related vertebrates to human, the zebrafish and pufferfish, there is a more that 77% identity at the amino acid level (figure 5). Alanine-220 is also highly conserved among the vertebrates listed. Only cattle and zebrafish have a variant serine at this position. Functional comparison of the alanine, threonine, and serine at position 220 shows that both the alanine and serine-containing IYD have full enzymatic activity, while cells transfected with IYD-A220T lack dehalogenase activity (figure 6A).
Western blot analysis indicates that the amount of IYD protein in cells transfected with IYD-A220T DNA is much less than in cells transfected with similar amounts of DNA containing either an alanine or serine at position 220 (figure 6A). To rule out that the lack of dehalogenase activity of the IYD-A220T mutant is completely the result of diminished protein production, cells were transfected with a decreased amount of IYD-FLAG plasmid. As shown in figure 6B, even when expressing substantial higher amounts of mutant protein compared to wild-type protein, the mutant IYD-A220T protein still does not display any enzymatic activity.

DISCUSSION

MIT and DIT determination using HPLC-MS/MS

In the 19fifties and sixties patients suffering from primary hypothyroidism often developed goiter requiring thyroidectomy and the availability of goitrous thyroid tissue made direct measurement of dehalogenase enzyme activity possible. In addition, the measurement of dehalogenase activity by measuring DIT conversion after in vivo application of radiolabeled DIT was used for diagnostic purposes, a method currently not advocated. Although progress has been made in the development of assays to detect DIT and MIT (17-20), most methods lack selectivity and/or are too laborious and time consuming to qualify as high throughput screening methods. One of the current diagnostic obstacles to identify DD patients at an early stage, is a proper tool to measure increased levels of DIT and MIT in urine or blood, one of the

hallmarks of DD (6). Iodotyrosine detection in urine by mass spectrometry will facilitate the neonatal detection of DD (14). This early detection followed-up by early treatment might prevent the mental retardation resulting from thyroid hormone deficiency in early life observed by us and others (14) in patients suffering from DD.
In this paper we describe a rapid, highly selective and sensitive HPLC-MS/MS assay to determine MIT and DIT levels in urine. In normal subjects urinary DIT levels of 1.23 nmol/24 h were determined by RIA (21), or 0.76 nmol/24 h as determined by a gas chromatographic mass spectrometric assay (22), which are levels in the same order of magnitude as our data of 0.3 ± 0.1 nmol DIT/day in adults without any known thyroid disease. In this group the daily MIT excretion is 1.4 ± 1.2 nmol MIT/day. As far as we know, MIT levels in urine have never been determined before.

Structure-function analysis of recombinant IYD protein

Although *in vitro* expression studies show that the IYD-A220T mutation results in reduced protein expression, the functional consequence of the alanine to threonine substitution is a complete abrogation of the enzymatic dehalogenase activity. This reduced *in vitro* protein expression has also been observed for an IYD-I116Y mutant that was reported, in contrast to 2 other mutations within the dehalogenase domain that apparently only affect the enzymatic activity (14). Strikingly, it is not so much the location of the mutation as the nature of the amino acid change since the in vitro production of the IYD-A220S is comparable to the wild type protein.
The alanine-220 residue is highly conserved in the vast majority of all currently known vertebrate IYD protein sequences (and also in more distantly related eukaryotes, data not shown). Cattle and zebrafish have a serine at this position, but we showed this has no effect on IYD enzymatic activity. Based on the structural homology of the iodotyrosine dehalogenases with the bacterial NADH oxidase/flavin reductases, alanine 220 might be involved in FMN binding (12) while according to the conserved domains database (23) the area surrounding position 220 is the putative dimer interface.
It remains to be shown whether the loss of enzymatic activity, reduced protein expression or a combination is responsible for the dehalogenation defect *in vivo*.

Phenotype-genotype correlation within a family with an inactivating IYD mutation

The index patient (II-2), who presented with clinical hypothyroidism (delayed psychomotor development, stunted growth, and goiter) at the age of 5 years, is homozygous for the inactivating IYD-A220T mutation. The hypothyroid/goitrous phenotype is familial and many family members suffer from mental retardation (15). We could speculate that this is due because of a chronic iodine deficiency caused by a dehalogenase defect that is not compensated by iodide ingestion in affected mother-sib pairs. Although subject II-2 receives thyroxin treatment, her low fT4 and high plasma TG suggest poor compliance explaining the increased MIT and DIT excretion in urine. The fact that although her fT4 is below the reference range, her T3 is above suggests an activated metabolic system adapted to low T4 levels, as can be seen in patients with e.g. a thyroglobulin synthesis defect (24).
The daughter (III-2) is also homozygous for the inactivating IYD mutation. She has no increased urinary MIT-DIT excretion, probably as a result of proper LT4 treatment aimed at maintaining serum TSH levels within the reference range. This is corroborated by her unsuppressed plasma TG levels. However, since she has received a transplanted liver from presumably a normal IYD individual, we cannot completely exclude that this liver contributes to the MIT and DIT deiodination, although at the mRNA level IYD expression in liver is very low (13).
Our pedigree analysis as depicted in Figure 3 is in line with the view that DD is a recessive disease (9;14;15;25;26). Interestingly, subject III-4 (who is heterozygous for the IYD-A220T mutation) where thyroid palpation in the past indicated a normal sized gland, recently came again to medical attention because of goiter and clinical and biochemical signs of hypothyroidism. On hindsight, this correlates with the increased serum TG and urinary MIT and DIT excretion; although at the time of these measurements there were no other signs of hypothyroidism. The reason for the development of goiter within a period of 4

months in this 14-year old boy is not known. It does however points to a possible dominant behavior of the genetic defect with variable penetrance since the other heterozygous sibs have no goiter, increased plasma TG or increased MIT or DIT excretion. Apart from the mutation in exon 4, analysis of 5 polymorphic positions covering IYD intron 1 to exon 5 all show heterozygosity, indicating that both alleles of *IYD* in subject III-4 are present (Supplementary Figure 1). Dominant inheritance of DD has been suggested in some families before (25). Although this might be explained by allele-specific *IYD* expression analysis of mRNA by RT-PCR in normal thyroid tissue did not demonstrate allele-specific *IYD* expression (supplementary Figure 2).

The occurrence of the mutant allele in the normal population (1 in 100) suggests *IYD* c.658G>A is a functional SNP. It is tempting to speculate that it may play a role in the clinical variable response to iodine deficiency. More investigations are essential to substantiate the role of heterozygous inactivating IYD mutations in the development of non-congenital goiter.

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REFERENCES

1. **Larsen PR, Davies TF, Schlumberger M-J, Hay ID** 2003 Thyroid Physiology and Diagnostic Evaluation of Patients with Thyroid Disorders. In: Larsen PR, Kronenberg HM, Melmed S, Polonsky KS, editors. Williams Textbook of Endocrinology. Philadelphia: Saunders; 331-373

2. **de Vijlder JJ** 2003 Primary congenital hypothyroidism: defects in iodine pathways. Eur J Endocrinol 149:247-256

3. **Park SM, Chatterjee VK** 2005 Genetics of congenital hypothyroidism. J Med Genet 42:379-389

4. **Moreno JC, de Vijlder JJ, Vulsma T, Ris-Stalpers C** 2003 Genetic basis of hypothyroidism: recent advances, gaps and strategies for future research. Trends Endocrinol Metab 14:318-326

5. **Zamproni I, Grasberger H, Cortinovis F, Vigone MC, Chiumello G, Mora S, Onigata K, Fugazzola L, Refetoff S, Persani L, Weber G** 2008 Biallelic inactivation of the dual oxidase maturation factor 2 (DUOXA2) gene as a novel cause of congenital hypothyroidism. J Clin Endocrinol Metab 93:605-610

6. **Knobel M, Medeiros-Neto G** 2003 An outline of inherited disorders of the thyroid hormone generating system. Thyroid 13:771-801

7. **Kopp P** 2002 Perspective: genetic defects in the etiology of congenital hypothyroidism. Endocrinology 143:2019-2024

8. **Roche J, Michel R, Michel O, Lissitzky S** 1952 Enzymatic dehalogenation of iodotyrosine by thyroid tissue; on its physiological role. Biochim Biophys Acta 9:161-169

9. **McGirr EM, Hutchison H, Clement WE** 1956 Sporadic non-endemic goitrous cretinism; identification and significance of monoiodotyrosine and diiodotyrosine in serum and urine. Lancet 271:906-908

10. **Stanbury JB, Kassenaar AA, Meijer JW, Terpstra J** 1955 The occurrence of mono- and di-iodotyrosine in the blood of a patient with congenital goiter. J Clin Endocrinol Metab 15:1216-1227

11. **Moreno JC, Keijser R, Aaraas S, de Vijlder JJ, Ris-Stalpers C** 2002 Cloning and characterization of a novel thyroidal gene encoding proteins with a conserved nitroreductase domain. Journal of Endocrinol Invest 25:23

12. **Friedman JE, Watson JA, Jr., Lam DW, Rokita SE** 2006 Iodotyrosine deiodinase is the first mammalian member of the NADH oxidase/flavin reductase superfamily. J Biol Chem 281:2812-2819

13. **Gnidehou S, Caillou B, Talbot M, Ohayon R, Kaniewski J, Noel-Hudson MS, Morand S, Agnangji D, Sezan A, Courtin F, Virion A, Dupuy C** 2004 Iodotyrosine dehalogenase 1 (DEHAL1) is a transmembrane protein involved in the recycling of iodide close to the thyroglobulin iodination site. FASEB J 18:1574-1576

14. **Moreno JC, Klootwijk W, van Toor H., Pinto G, D'Alessandro M, Leger A, Goudie D, Polak M, Gruters A, Visser TJ** 2008 Mutations in the iodotyrosine deiodinase gene and hypothyroidism. N Engl J Med 358:1811-1818

15. **de Maegd M, van Nevel C** 1970 Hypothyroidism caused by congenital familial defect of iodotyrosine deiodation. Acta Paediatr Belg 24:115-120

16. **Green WL** 1971 Effects of 3-nitro-L-tyrosine on thyroid function in the rat: an experimental model for the dehalogenase defect. J Clin Invest 50:2474-2484

17. **Doorn L, Jansen EH, Van Leeuwen FX** 1991 Comparison of high-performance liquid chromatographic detection methods for thyronine and tyrosine residues in toxicological studies of the thyroid. J Chromatogr 553:135-142

18. **Gika HG, Samanidou VF, Papadoyannis IN** 2005 Development of a validated HPLC method for the determination of iodotyrosines and iodothyronines in pharmaceuticals and biological samples using solid phase extraction. J Chromatogr B 814:163-172

19. **Kootstra PR, van den Broek HH, Hogendoorn EA, Goewie CE, de Vijlder JJ** 1988 Automated high-performance liquid chromatographic method for the determination of iodotyrosines and iodothyronines. J Chromatogr 458:175-183

20. **Michalke B, Schramel P, Witte H** 2000 Method developments for iodine speciation by reversed-phase liquid chromatography-ICP-mass spectrometry. Biol Trace Elem Res 78:67-79

21. **Meinhold H, Olbricht T, Schwartz-Porsche D** 1987 Turnover and urinary excretion of circulating diiodotyrosine. J Clin Endocrinol Metab 64:794-800

22. **Mohammed MN, Farmer M, Ramsden DB** 1983 3,5-Diiodotyrosine excretion in human urine in individuals of differing thyroidal status. Biomed Mass Spectrom 10:507-511

23. **Marchler-Bauer A, Anderson JB, Derbyshire MK, Weese-Scott C, Gonzales NR, Gwadz M, Hao L, He S, Hurwitz DI, Jackson JD, Ke Z, Krylov D, Lanczycki CJ, Liebert CA, Liu C, Lu F, Lu S, Marchler GH, Mullokandov M, Song JS, Thanki N, Yamashita RA, Yin JJ, Zhang D, Bryant SH** 2007 CDD: a conserved domain database for interactive domain family analysis. Nucleic Acids Res 35:D237-D240

24. **Kanou Y, Hishinuma A, Tsunekawa K, Seki K, Mizuno Y, Fujisawa H, Imai T, Miura Y, Nagasaka T, Yamada C, Ieiri T, Murakami M, Murata Y** 2007 Thyroglobulin gene mutations producing defective intracellular transport of thyroglobulin are associated with increased thyroidal type 2 iodothyronine deiodinase activity. J Clin Endocrinol Metab 92:1451-1457

25. **Codaccioni JL, Rinaldi JP, Bismuth J** 1978 The test of overloading L-diiodotyrosine (DIT) in the screening of iodotyrosine dehalogenase deficiency. Acta Endocrinol (Copenh) 87:95-105

26. **Ismail-Beigi F, Rahimifar M** 1977 A variant of iodotyrosine-dehalogenase deficiency. J Clin Endocrinol Metab 44:499-506

LEGENDS

Figure 1 MRM chromatogram.

MRM chromatogram of DIT (trace A), MIT (trace B) and MCT (trace C).

Figure 2 MIT and DIT concentrations in human urine samples.

MIT (A) and DIT (B) concentrations in human urine samples were determined by HPLC-MS/MS. Samples were from apparently healthy controls (No thyroid disease). In addition, 5 members of a family with dehalogenase defect (DD family) were included as a separate group.

Figure 3 Genotypic and phenotypic data of family with dehalogenase deficiency.

Top panel shows data from urine/serum/DNA from DD family members sampled in 2002. Bottom panel shows serum levels from patient III-4 sampled in February 2008.

Figure 4 The IYD mutation of Alanine-220 into a Threonine abolishes dehalogenase activity.

HEK293 cells (6-well format) were transfected with pCDNA3, IYD-FLAG or IYD-A220T-FLAG DNA. A: 12 hours post-transfection 0.5 μM MIT or 0.25 μM DIT was added to the cells (total volume 2ml). At the indicated time points 50 μl samples were taken and MIT-DIT concentrations were determined by HPLC-MS/MS. Percentage substrate converted is relative to cells transfected with pCDNA3. Values are averages from duplicate samples ± standard deviation. B: 2 days post-transfection, cells were harvested and lysed. The lysate was incubated for 3 hours in assay buffer with or without 0.1 mM NADPH (13) in the presence of either 100 nM MIT or 50 nM DIT. After the incubation a 50 μl sample was taken and MIT-DIT concentrations were determined by HPLC-MS/MS. Percentage substrate converted is relative to cells transfected with pCDNA3. Values are averages from duplicate samples ± standard deviation.

Figure 5 IYD is strongly conserved in vertebrates.

The iodotyrosine dehalogenase domain protein sequences of Rhesus monkey (Genbank XP_001099028), Chimpanzee (GenBank XP_527537), Orangutan (Swissprot Q5REW1), Dog (GenBank XP_533449), Mouse (GenBank NP_081667), Rat (Swissprot Q5BK17), Pig (Swissprot Q6TA49), Cattle (Genbank XP_869498), Frog (GenBank NP_001087329), Fowl (GenBank XP_419670), Opossum (GenBank XP_001381189), Zebrafish (GenBank XP_001335518), and Pufferfish (Genbank CR693638) were obtained by Blast search with the human iodotyrosine dehalogenase domain (GenBank NP_981932, amino acid 93-285). Multiple alignment was performed using ClustalW. Part of the iodotyrosine dehalogenase domain, that ranges from amino acid 193-242 is shown (amino acid numbering according to the human sequence). Identical amino acids are shown as dots. The shaded area indicates the amino acid corresponding to human Alanine 220. At the end of

each sequence the percentage of identical amino acids with the complete human iodotyrosine dehalogenase domain is depicted.

Figure 6 Threonine at position 220 results in decrease of protein production and inactivation of enzyme activity.

HEK-293 cells (A: 24-well format, B: 12-well format) were transfected with the indicated expression vectors. A: Twenty-two hours post-transfection MIT was added to a final concentration of 1 μM. Twenty-four hours later a 50 μl sample was taken for MIT determination. B: The amount of IYD-FLAG DNA used for transfection is 1/10 of the amount of IYD-A220T-FLAG. Seven hours post-transfection MIT was added to the 1 ml/well culture medium to a final concentration of 1 μM. Thirty-nine hours after the addition a 50 μl sample was taken for MIT determination. Percentage MIT converted is relative to cells transfected with pCDNA3.Values are mean ± standard deviation of triplicate samples. Transfection efficiencies as determined by co-transfection of pEGFP-C1 DNA and flow cytometry were in A: IYD-FLAG 32%, IYD-A220T-FLAG 27%, and IYD-A220S-FLAG 32%, and in B: IYD-FLAG 15%, and IYD-A220T-FLAG 24%. On the right side of the graph the corresponding Western blot signals are shown with the FLAG M2 antibody.

Figure 1

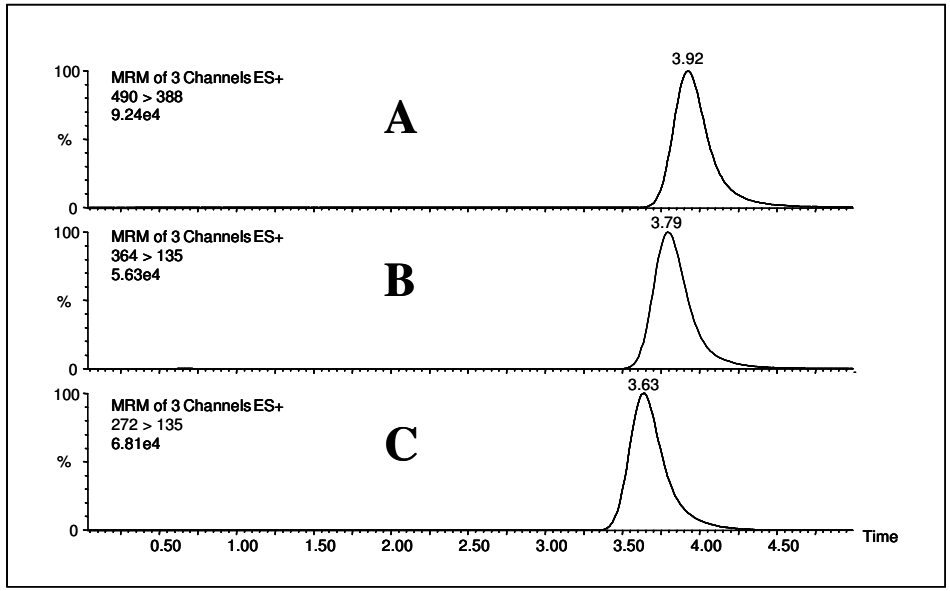

MRM of 3 Channels ES+
490 > 388
9.24e4
A
3.92
MRM of 3 Channels ES+
364 > 135
5.63e4
B
3.79
MRM of 3 Channels ES+
272 > 135
6.81e4
C
3.63
100
%
0
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
Time

Figure 2

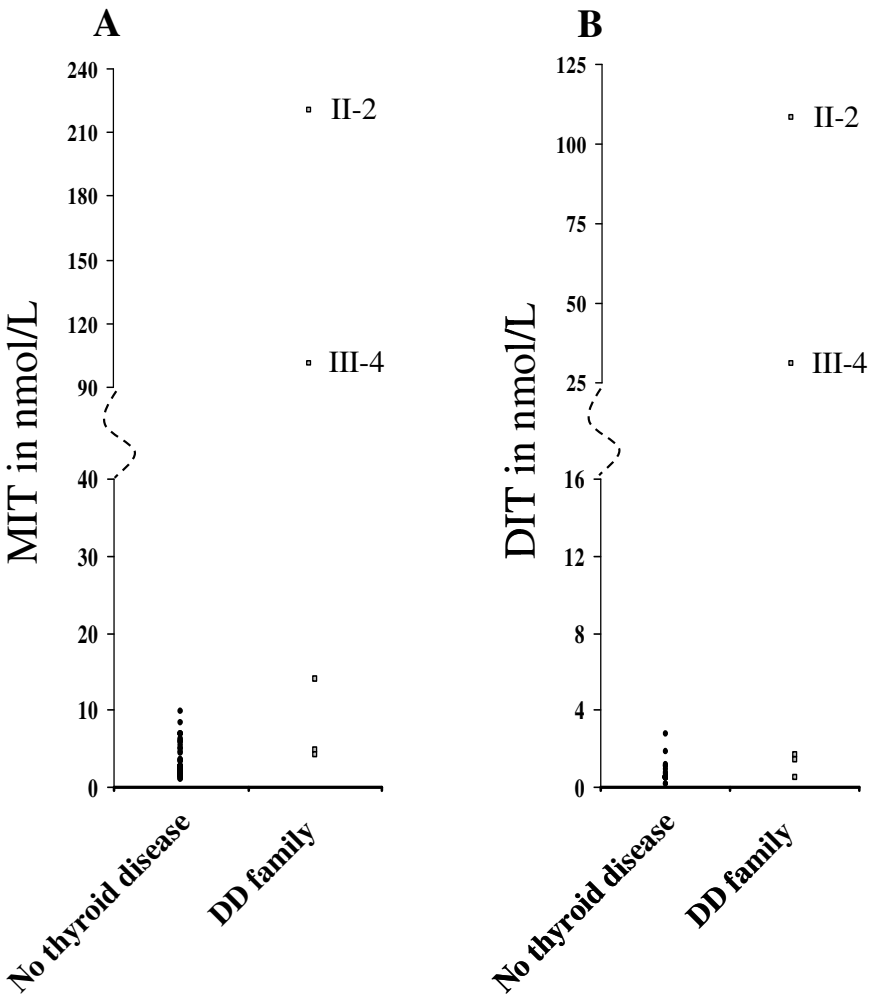
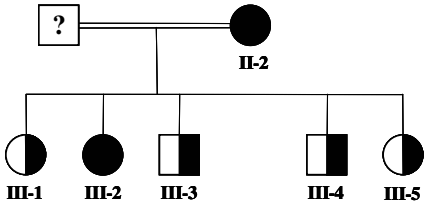

A
B
MIT in nmol/L
DIT in nmol/L
240
210
180
150
120
90
40
30
20
10
0
125
100
75
50
25
16
12
8
4
0
II-2
III-4
No thyroid disease
DD family

FIGURE 3



Year of birth		1988	1989	1990	1963	1993	1994	Reference value
Goiter		No	Yes	No	Yes	No	No	No
T4 treatment		No	Yes	No	Yes	No	No	No
TSH (mU/L)	SERUM	nd	0.6	2.1	1.2	0.5	1.1	0.4-4
T4 (nmol/L)	SERUM	nd	130	140	95	125	110	70-150
fT4 (pmol/L)	SERUM	nd	15.7	11.9	6.4	13.7	10.8	10-23
T3 (nmol/L)	SERUM	nd	1.90	2.60	2.95	2.25	2.60	1.30-2.70
TG (pmol/L)	SERUM	nd	<20	72	1040	110	52	5-60
TBG (nmol/L)	SERUM	nd	380	500	500	410	430	200-650
Iodine excretion (nmol/L)	URINE	nd	454	320	696	561	525	315-1575
MIT excretion (nmol/L)	URINE	nd	4.9	4.2	220.8	100.8	14.1	nd
DIT excretion (nmol/L)	URINE	nd	1.4	<1	108.2	31.2	1.7	nd
IYD genotype c.658G>A p.A220T	DNA	G/A	A/A	G/A	A/A	G/A	G/A	G/G

		Reference value
Goiter	Yes	No
TSH (mU/L)	>100	0.7-6.4
fT4 (ng/dl)	<0.3	0.8-1.9
fT3 (pg/ml)	2.60	1.45-4.50
TG (ng/ml)	1887	1-70
Anti-TG (U/ml)	<20	<40
Anti-TPO (U/ml)	<10	<35

Figure 4

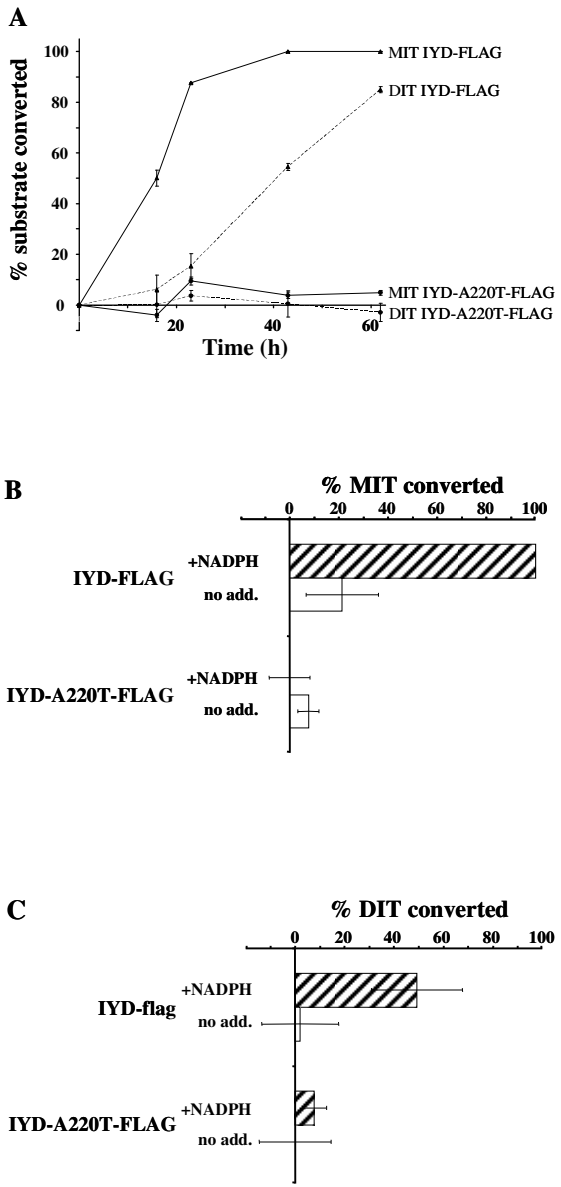

A
% substrate converted
100
80
60
40
20
0
20
40
60
Time (h)
MIT IYD-FLAG
DIT IYD-FLAG
MIT IYD-A220T-FLAG
DIT IYD-A220T-FLAG
B
% MIT converted
0
20
40
60
80
100
IYD-FLAG
+NADPH
no add.
IYD-A220T-FLAG
+NADPH
no add.
C
% DIT converted
0
20
40
60
80
100
IYD-flag
+NADPH
no add.
IYD-A220T-FLAG
+NADPH
no add.

Figure 5

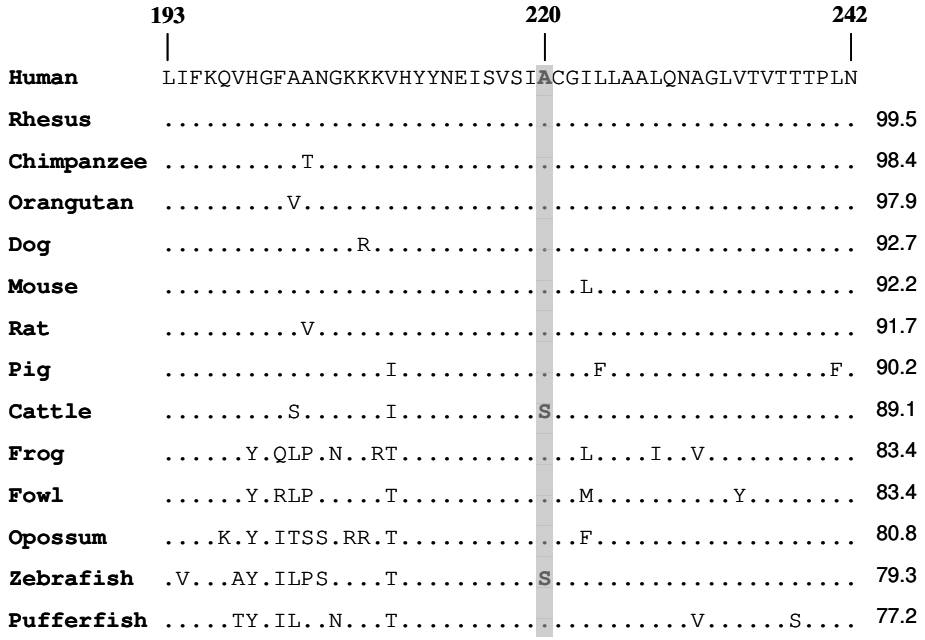
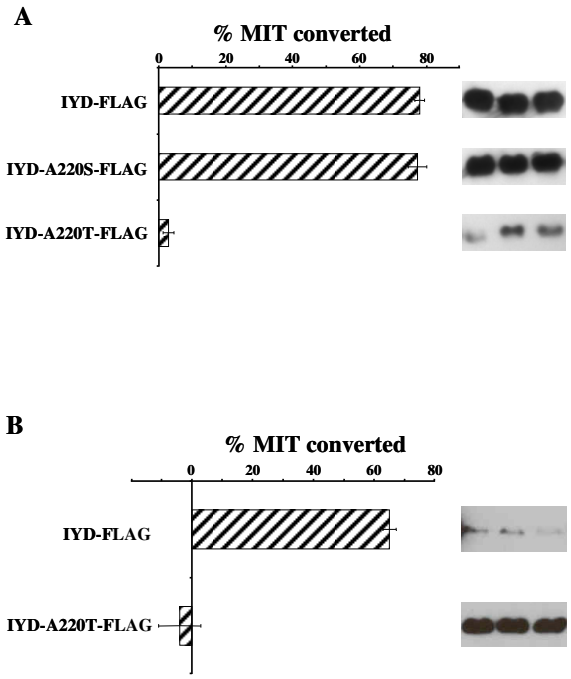

193
220
242
Human LIFKQVHGFAANGKKKVHYYNEISVSIACGILLAALQNAGLVTVTTTPLN
Rhesus 99.5
Chimpanzee .....T..... 98.4
Orangutan .....V..... 97.9
Dog .....R..... 92.7
Mouse .....L..... 92.2
Rat .....V..... 91.7
Pig .....I.....F.....F. 90.2
Cattle .....S.....I.....S..... 89.1
Frog .....Y.QLP.N..RT.....L....I..V..... 83.4
Fowl .....Y.RLP.....T.....M.....Y..... 83.4
Opossum ....K.Y.ITSS.RR.T.....F..... 80.8
Zebrafish .V...AY.ILPS....T.....S..... 79.3
Pufferfish .....TY.IL..N...T.....V.....S.... 77.2

Figure 6


A
% MIT converted
0
20
40
60
80
IYD-FLAG
IYD-A220S-FLAG
IYD-A220T-FLAG
B
% MIT converted
0
20
40
60
80
IYD-FLAG
IYD-A220T-FLAG