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A Novel Mutation in the Sodium/Iodide Symporter Gene in the Largest Family with Iodide Transport Defect*

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ABSTRACT

We previously reported nine children with an autosomally recessive form of congenital hypothyroidism due to an iodide transport defect in a large Hutterite family with extensive consanguinity living in central Canada. Since the original report, we have diagnosed congenital hypothyroidism by newborn TSH screening in 9 additional children from the family. We performed direct sequencing of the PCR products of each NIS (sodium/iodide symporter) gene exon with flanking introns amplified from genomic DNA extracted from peripheral blood cells of the patients. We identified a novel NIS gene mutation, G395R (Gly^{395}→Arg; GGA→AGA), in 10 patients examined in the present study. All of the parents tested were heterozygous for the mutation, suggesting that the patients were homozygous. The mutation was located in the 10th transmembrane helix. Expression experiments by transfection of the mutant NIS complimentary DNA into COS-7 cells showed no perchlorate-sensitive iodide uptake, confirming that the mutation is the direct cause of the iodide transport defect in these patients. A patient who showed an intermediate saliva/serum technetium ratio (14.0; normal, ≧20) and was considered to have a partial or less severe defect in the previous report (IX-24) did not have a NIS gene mutation. It is now possible to use gene diagnostics of this unique NIS mutation to identify patients with congenital hypothyroidism due to an iodide transport defect in this family and to determine the carrier state of potential parents for genetic counseling and arranging rapid and early diagnosis of their infants. (*J Clin Endocrinol Metab* **84:** 3248–3253, 1999)

THE IODIDE transport defect (ITD; Online Mendelian Inheritance in Man, OMIM 274400) is a disorder caused by the inability to actively transport iodide into thyrocytes, which is the first and rate-limiting step in the synthesis of iodide-containing thyroid hormones and is mediated by the Na^+/I^- symporter (NIS). Forty-two cases with ITD from 25 families have been reported to date (1–21).

We (17, 20) and others (15, 21) have identified a homozygous, missense, and loss of function mutation of the NIS gene, T354P (Thr^{354}→Pro), in nine Japanese patients. We also found other missense and loss of function mutations of NIS [G543E (Gly^{543}→Glu) homozygous mutation and a compound heterozygous mutation of T354P/G93R (Gly^{93}→Arg)] in three Japanese patients with ITD (19). In addition, Pohlenz *et al.* reported a homozygous nonsense mutation C272X (Cys^{272}→stop) in a Brazilian kindred (16) and a compound heterozygous mutation of Q267E (Gln^{267}→Glu)/deletion (67 bp) in a patient of Mexican origin (18). Thus, NIS mutations have been detected in only two patients with ITD outside Japan; the clinical features of such ITD patients with NIS mutation have not been well characterized.

We reported nine patients with congenital hypothyroidism in a Hutterite family living in central Canada (9). Five of these patients were confirmed to have complete ITD. Since this previous report (9), we have found nine additional infants with congenital hypothyroidism in the family by neonatal screening. In the present study, we identified a novel, missense, and loss of function germline mutation of the NIS gene in the patients from this largest ITD family reported to date. We also describe here the clinical features of the patients and discuss the genotype-phenotype relationship and clinical significance of gene diagnostics in this family.

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Subjects and Methods

Patients

The pedigree of the Hutterite family with ITD is shown in Fig. 1. We constructed the pedigree by direct interviews in the present study, and it includes a part of the pedigree from the previous report (9). The generation of the patients is designated VI, which corresponded to generation IX in the previous pedigree (9). Individuals with congenital hypothyroidism are shown by *closed symbols*. The geographic and cultural isolation of this religious group living in rural colonies has led to a remarkable degree of consanguinity. Most of the patients were born from a consanguineous marriage. The clinical features and biochemical results of neonatal screening of the patients in this family are summarized in Table 1. Some of the data from the seven patients described in the previous report (9) are duplicated for ease of comparison and comprehension.

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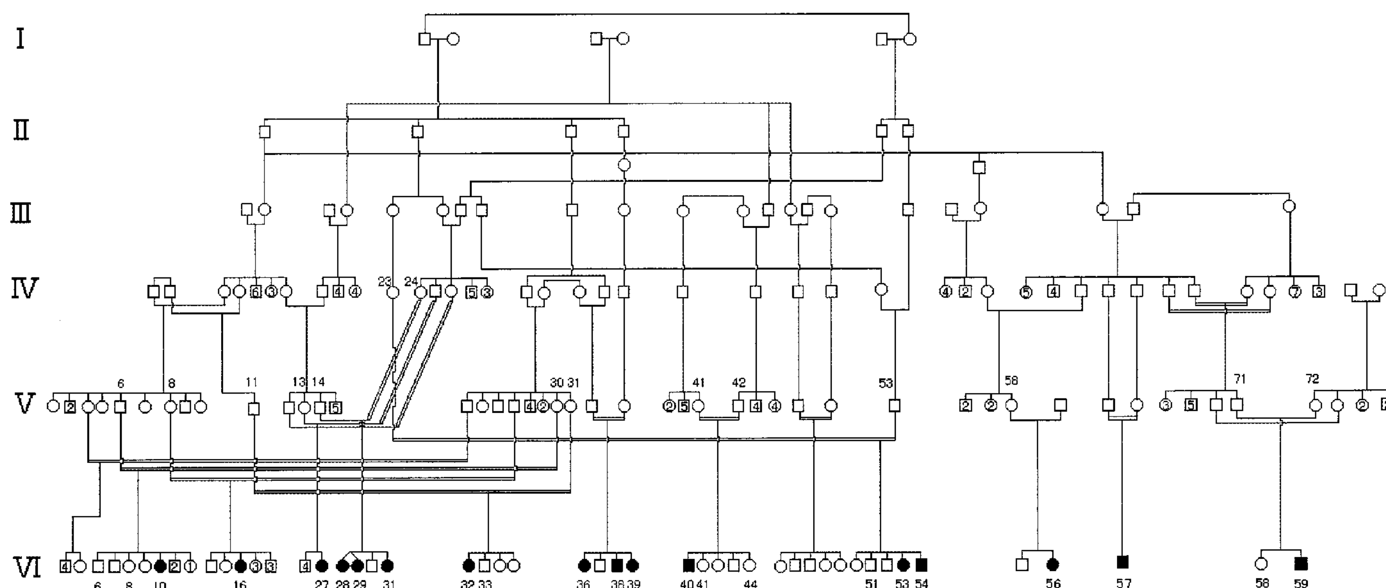

FIG. 1. Pedigree of the Hutterite family with iodide transport defect. This was constructed by direct interviews in the present study. Individuals are counted from *left* to *right* in each generation, but not all numbers are indicated. The generation of the patients is designated VI, which corresponds to generation IX in the previous pedigree (9). Individuals with congenital hypothyroidism are shown by *closed symbols*. *Numbers within open symbols* show the number of unaffected siblings. VI-28 and VI-29 are identical twins. Consanguineous marriages are shown by *double lines* joining parents. Dead family members are not struck out with *oblique lines*.

TABLE 1. Summary of clinical features of the patients with congenital hypothyroidism from the Hutterite family

Case number (Generation VI)	10	16	27	28	29	31	32	36	38	39	40	53	54	56	57	59
Old case number		IX20	IX13	IX14	IX15	IX17					IX24	IX28				
Sex	F	F	F	F	F	F	F	F	M	F	M	F	M	F	F	M
Year of birth	1986	1982	1971	1969	1969	1973	1988	1987	1990	1993	1984	1983	1988	1994	1997	1997
TSH (mU/L; normal, 0.3–6.0)	>50	>50	74	>50	>50	139	>50	>50	>324	>100	>50	>50	165	207	150	>100
T_4 (nmol/L; normal, 75–400)	21	8	17	18	4	0		29	<20	*5*[a]	18	34	23		*1.5*[a]	*<3*[a]
T_3 (nmol/L; normal, 0.9–4.5)	1.2							1.3	1.9							
Goiter	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
^{123}I thyroidal uptake (%)[b]		<1	<1	<1	<1	2										
Saliva/serum ^{123}I ratio[c]		2.8	1.4	0.2	1.8	5.2										
$^{99m}TcO_4$-thyroidal uptake	No							No								
$^{99m}TcO_4$-salivary uptake	No							No								
Saliva/serum $^{99m}TcO_4$ ratio[d]		0.1	0.3	0.1	0.1	0.2					14.0					
Treatment started (days)	8	7	112	35	35	42	10	11	7	7	7	7	3	6	7	10
Heterozygotes in family[e]	FMS	M	M	FM	FM	FM	FMB				MS	FMB	FMB	M		FMS
NIS mutation[f]	M/M	M/M	M/M	M/M	M/M	M/M	M/M				W/W	M/M	M/M	M/M		

[a] Free T_4 (pmol/L; normal range, 20.8–54.4).
[b] 24 h uptake, normal range, 7–30%.
[c] Normal, above 20.
[d] Normal, above 20.
[e] F, father; M, mother; S, sister; B, brother.
[f] M/M, G395R/G395R homozygote; W/W, wild-type/wild-type homozygote.

Genomic DNA extraction, PCR of exons of NIS DNA, and direct sequencing

Genomic DNA was extracted as previously described (17, 20) from peripheral blood cells of the patients and their family members with their informed consents. Each exon was amplified by PCR with a pair of primers derived from the flanking introns. Exons 3 and 4, 6 and 7, 9 and 10, and 11 and 12 were coamplified with intervening introns. The locations of all intronic primers were at least 22 nucleotides distant from the exon(s) to be amplified. Nucleotide sequences of all exons, from nucleotide −37 to +1952 in NIS complimentary DNA (cDNA) covering the full-length coding region, and those of all exon-intron boundaries containing at least 15 nucleotides in introns (GenBank accession no. AF049198-AF049220), were determined in both orientations by direct sequencing with a GeneScan DNA sequencer 373A (Perkin Elmer Corp., Foster City, CA) (19).

Detection of the G395R (Gly^{395}→Arg; GGA→AGA) mutation by AlwI digestion

Exons 9 and 10 were coamplified with the intervening intron 9 using primers derived from intron 8 (5′-GATGGTGTGGACGGTCTCTCCAT-3′) and intron 10 (5′-AAGGTGCCCCCACCTCTCAGGA-3′). In the wild-type (WT) allele, there is an *Alw*I site (GGATCN_4↓) in exon 10, which produces fragments of 267 and 103 bp. When the G395R mutation (GGATC→AGATC) exists, the 370-bp PCR product remains undigested.

Construction of expression vectors, transfection, and iodide uptake assay

WT human NIS cDNA construct was obtained by TA cloning of the full-length (nucleotide −59 to +1975) human NIS cDNA in the pCR3.1

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vector (Invitrogen, San Diego, CA) under control of the cytomegalovirus promoter (17, 19). Mutant construct G395R was generated by site-directed mutagenesis. COS-7 cells were transfected with 25 μg mutant or WT NIS DNA or control vector DNA (pCR3-CAT, Invitrogen) by electroporation. To mimic the family members who had heterozygous G395R mutation, 12.5 μg each of two kinds of constructs were used to transfect COS-7 cells. To monitor transfection efficiencies, 0.1 μg pSVGH was cotransfected with mutant or WT NIS plasmid cDNA or control vector. Cells were aliquoted into 24-well plates ($\sim 10^5$ cells/well). Forty-eight hours after transfection, the medium was taken for RIA of human GH concentration, and assays of iodide uptake were performed as previously described (17, 19). Iodide uptake was determined by incubating cells with 500 μL HBSS incubation buffer (HBSS containing 0.5% BSA and 10 mmol/L HEPES-NaOH, pH 7.4) with about 0.1 μCi carrier-free $Na^{125}I$ and 10 μmol/L NaI to give a specific activity of approximately 20 mCi/mmol at 37 C for 2 min. After finishing the incubation, cells were washed twice on wet ice with ice-cold 2 mL HBSS incubation buffer as quickly as possible (<15 s). Cells were solubilized with 1 mL 0.1 mol/L NaOH, 0.1% (wt/v) SDS, and 2% Na_2CO_3 and subjected to protein concentration determination by the Bradford method (22) using BSA as standard and to counting of radioactivity by a γ-counter. Some wells of cells were trypsinized for cell number counting. Data for iodide uptake are expressed as picomoles per min/mg cell protein.

Results

Clinical summary

Previously, we described nine children with congenital hypothyroidism in a Hutterite family with extensive consanguinity living in central Canada (9). Five of the patients who showed negligible ^{123}I uptake and low saliva/serum iodide and technetium ratios were diagnosed as having definite and complete ITD (Table 1) (9).

Since then, we have identified 9 additional children with congenital hypothyroidism by neonatal screening in this family, bringing the total number of patients to 18. The pedigree shown in Fig. 1 indicates only relationships that were confirmed by direct interviews in the present study. Upper generations that were not directly confirmed this time but were described in the figure of the 1985 paper (9) were eliminated from Fig. 1. The 2 patients included in the previous pedigree (9) who were not available for further characterization even in the original study (VIII-27 and VIII-29) were excluded from Fig. 1.

No patients showed clinical hypothyroidism at the time of diagnosis. T_4 treatment for most of patients was started in the neonatal period and successfully continued; we had no chance to administrate large amounts of iodide to these patients.

NIS mutation

Initially, we sequenced all exons and flanking introns of NIS genomic DNA in patients VI-27 and VI-31 who had been characterized most extensively and were diagnosed definitively as having ITD. In both of these patients, a novel and homozygous nucleotide change of G→A at nucleotide +1530 in exon 10 resulting in a change of Gly^{395} to Arg (GGA→AGA) in the 10th transmembrane domain was identified (Fig. 2). No other nucleotide changes were found in the coding region or exon-intron boundaries. This mutation resulted in disappearance of the *Alw*I site ($GGATCN_4\downarrow$) between nucleotides +1538 and +1539. In the WT allele, *Alw* I digestion produced fragments of 267 and 103 bp from the PCR product containing exons 9 and 10. When the G395R mutation (GGATC→AGATC) was present, the 370-bp PCR product remained undigested. *Alw*I digestion confirmed the G395R mutation and revealed the presence of the G395R homozygous mutation in the 10 patients (Fig. 3): VI-10, VI-16, VI-27, VI-28, VI-29, VI-31, VI-32, VI-53, VI-54 and VI-56. The parents of patients VI-10 (V-6 and V-30); VI-28, VI-29, and VI-31 (V-14 and IV-24); VI-32 (V-11 and V-31); and VI-53 and

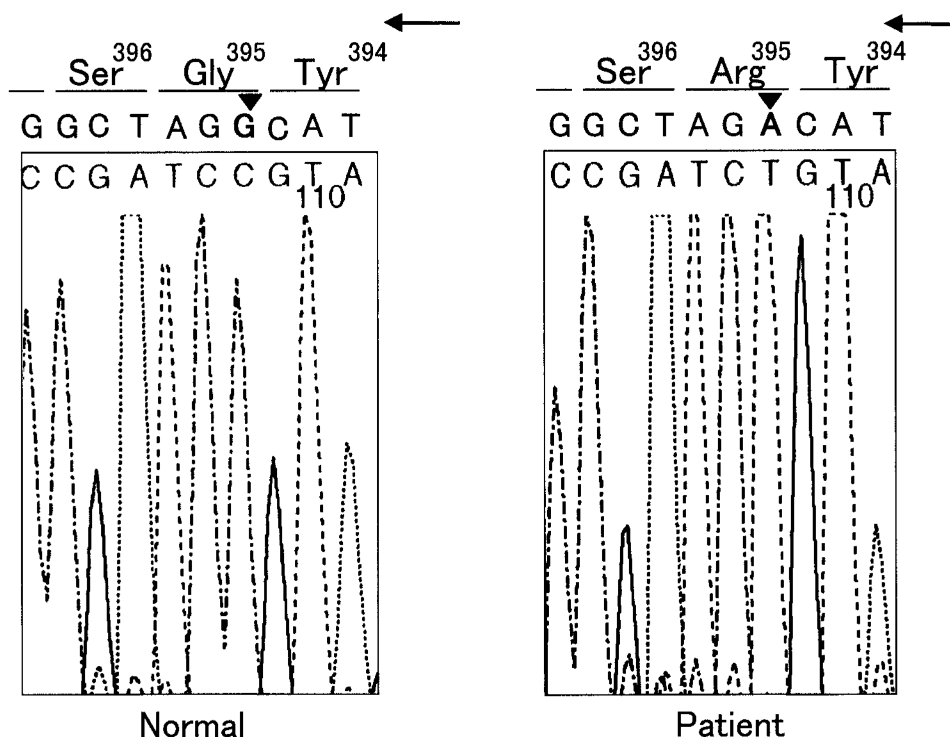

FIG. 2. Sequences around codon 395 in a normal subject and patient VI-27. Antisense sequences are shown. Gly^{395} (GGA) was substituted with Arg (AGA) in the patient. Patient VI-31 showed the same results.

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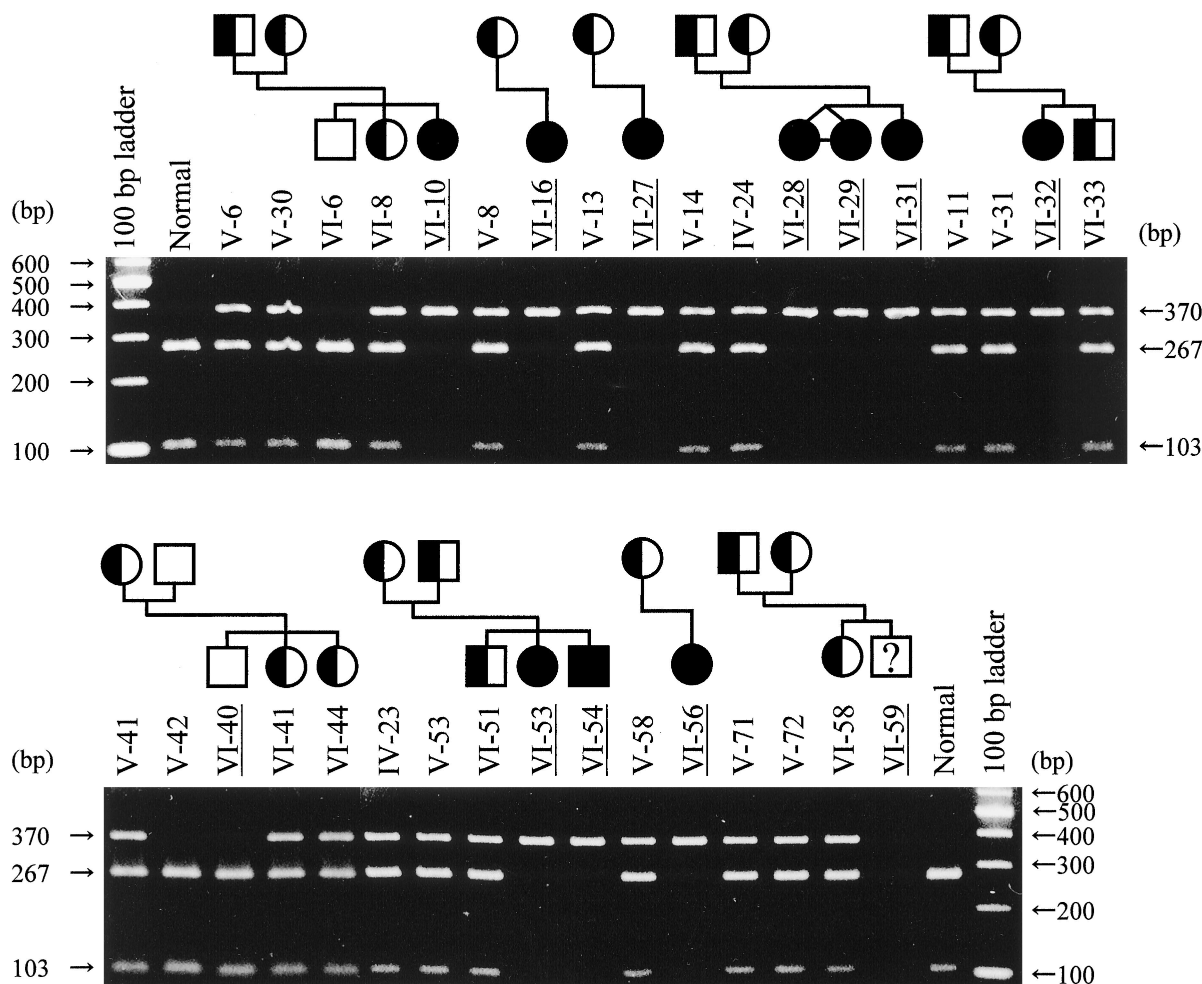

FIG. 3. *Alw*I digestion of PCR products containing exons 9 and 10 of the NIS gene from members of the Hutterite family. *Alw*I digestion produced 267- and 103-bp fragments in the WT allele, whereas the mutant allele of G395R remained undigested (370 bp). The numbers of individuals are given in Fig. 1. Patients with congenital hypothyroidism are *underlined*. VI-28 and VI-29 were identical twins. *Closed circles/squares*, homozygotes for G395R mutation; *semiclosed circles/squares*, heterozygotes for G395R mutation; and *open circles/squares*, homozygotes for WT NIS gene.

VI-54 (IV-23 and V-53) were heterozygous, confirming that these patients were homozygous for G395R. Mothers of patients VI-16 (V-8), VI-27 (V-13), and VI-56 (V-58) were heterozygous for G395R, but no blood samples were obtained from their fathers.

No DNA samples were obtained from patient VI-59 because he was too young, but his parents (V-71 and V-72) were heterozygous for G395R, suggesting that the patient was likely to be homozygous for the mutation.

Brothers and sisters of patients with homozygous G395R mutation, *i.e.* the brother of patients VI-53 and VI-54 (VI-51), the brother of patient VI-32 (VI-33), the sister of patient VI-10 (VI-8), and the sister of patient VI-59 (VI-58), were shown to be heterozygous for the G395R mutation. None of the individuals heterozygous for the G395R mutation had thyroid disorders.

No blood samples were obtained from the families of patients VI-36, VI-38, and VI-39 or from patient VI-57, who was very young.

Patient VI-40, who was described as IX-24 in the original report (9), did not have the G395R mutation. Full-length sequencing was performed, but no NIS mutation was found in the coding region or exon-intron boundaries. The saliva/serum $^{99m}TcO_4^-$ ratio (normal, 20 or higher) of this patient was 14.0, which was much higher than those of the other patients with ratios lower than 1.0 (Table 1). Further, he showed a small amount of early uptake of $^{99m}TcO_4^-$ by the salivary glands, which disappeared by 30 min. Therefore, in the original study, he was considered to have a less severe or partial defect (9).

Including the finding of the NIS mutation in the 10 patients in this family, the total number of ITD patients with identified NIS mutation(s) worldwide has become 24. NIS mutations of the 20 cases of the 24 were identified in our laboratory.

Expression experiments

Expression experiments by transfection of the mutant (G395R) NIS cDNA into COS-7 cells showed no perchlorate-

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sensitive iodide uptake (Fig. 4), confirming that the mutation is the direct cause of the ITD in these patients. Cells cotransfected with WT/G395R, mimicking the heterozygous state in unaffected family members, had approximately half the level of iodide uptake activity as that observed in cells transfected with 25 μg WT NIS DNA, but a level similar to that in cells transfected with 12.5 μg each of WT NIS and control vector, pCR3-CAT DNAs. These results suggested that the G395R mutant NIS protein does not interfere with the function of WT NIS (no dominant negative effect) similar to other NIS mutants, G93R, T354P, and G543E, and iodide uptake activity in transfected cells is correlated with the level of WT NIS expression. Cotransfection with pSVGH and measurement of the GH concentration in the culture medium showed no differences in transfection efficiencies among the transfectants.

Discussion

We identified a novel and homozygous NIS germline mutation, G395R, in 10 patients with congenital hypothyroidism in a Hutterite family. G395R was confirmed to be the direct cause of the disease in these patients by functional assays showing that the G395R mutant NIS had no iodide uptake activity in transfected COS-7 cells. Analyses by immunoblotting and immunocytochemistry suggested that G395R mutant NIS protein was properly synthesized and targeted to plasma membrane of transfected COS-7 cells such as WT and T354P mutant (20) NIS proteins (data not shown).

Patients VI-16, VI-27, VI-28, VI-29, and VI-31, who only had alleles with the G395R mutation, in whom ^{123}I thyroidal uptake, and ^{123}I and $^{99m}TcO_4^-$ saliva/serum ratios were very low, were definitively diagnosed as ITD. Patient VI-10, who had no $^{99m}TcO_4^-$ uptake in the thyroid or salivary glands, and patients VI-32, VI-53, VI-54, and VI-56, who only had

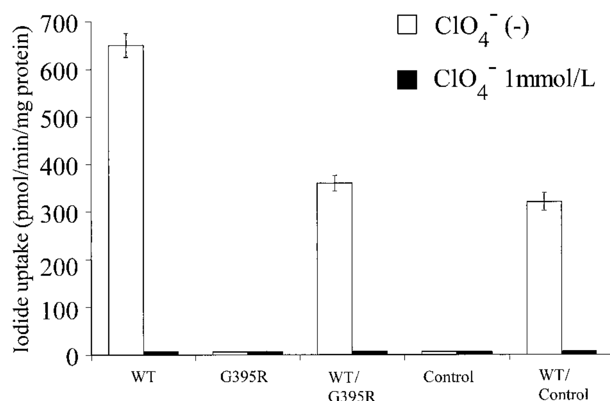

FIG. 4. Iodide uptake activity in COS-7 cells transfected with WT or mutant (G395R) NIS cDNA or control vector, pCR3-CAT (Control). WT/G395R and WT/Control indicate cells cotransfected with half (12.5 μg) the usual amount (25 μg) of two kinds of DNA constructs. Forty-eight hours after transfection, iodide uptake was determined by incubating cells with 500 μL HBSS containing 0.5% BSA and 10 mmol/L HEPES-NaOH, pH 7.4, with approximately 0.1 μCi carrier-free Na^{125}I and 10 μmol/L NaI at 37 C for 2 min. After finishing the incubation, cells were washed twice with ice-cold 2 mL HBSS incubation buffer as quickly as possible and were solubilized and subjected to counting of radioactivity by a γ-counter. Nonspecific transport or binding of iodide in the presence of 1 mmol/L ClO_4^- is shown in *black columns*. The *error bars* show the SEMs (n = 6).

G395R mutant NIS alleles but who had never undergone thyroidal scanning or further clinical studies, were presumed to have ITD. Further, the congenitally hypothyroid patient VI-59, whose genotype was unknown but whose parents were heterozygous for G395R, was likely to have ITD caused by homozygous G395R NIS mutation.

The hypothyroidism in patient VI-40 without a NIS mutation was considered to be due to a cause other than partial or less severe ITD. However, the limited availability of clinical data for the patient did not allow us to reach any definite conclusion.

It was notable that ITD patients in this family were predominantly female; the male/female ratio of the patients shown in Fig. 1 was 4:12. Further, of the 10 patients with homozygous G395R mutation, 9 were female. In ITD patients with another NIS mutation(s), the ratio was 7:7 (15–21). Whether the female predominance in the family was due to chance or to a specific cause(s) is unknown.

Previously, NIS was suggested to be composed of 12 transmembrane domains with both amino- and carboxyl-termini inside the plasma membrane (22, 23). However, by characterization of potential asparagine-linked glycosylation sites, NIS protein is now considered to have 13 transmembrane helices (24). The highly hydrophobic region comprising amino acid residues 389–410 located between the previously proposed transmembrane domains 9 and 10 forms a new transmembrane helix (24). Therefore, G395R involves a residue located in the 10th transmembrane domain of NIS.

Gly^{395} is conserved between human and rat NIS, and amino acids in the 10th transmembrane helix (residues 389–410) are identical in 21 of 22 between the two homologues (22, 23). This Gly is conserved in sodium/glucose cotransporter 1 from various species, but not in human sodium/myo-inositol cotransporter, rabbit sodium/nucleoside cotransporter, or pig sodium/neutral amino acid cotransporter (Swiss-Prot).

Four kinds of NIS missense mutations causing ITD, G93R, T354P, G395R, and G543E, all of which were identified in our laboratory, are located in the transmembrane domains. Three of these mutations involve a conserved Gly residue that is a destabilizer in an α-helix, suggesting possible structural and/or functional importance. However, we must await detailed three-dimensional characterization by mutagenesis, modeling, and purification before discussing the roles of the involved residues.

It is noteworthy that no ITD patients in this Hutterite family had or developed goiter, in contrast to many previously reported patients with ITD who had diffuse (sometimes huge) or nodular goiter. This might be a specific characteristics of the G395R mutation. A more likely explanation is the early diagnosis. Most of these patients were diagnosed by neonatal screening and were treated with T_4 at 3–14 days of age. For the older patients, treatment was started at 1–4 months of age, much earlier than that in other patients with ITD. We speculate that T_4 treatment from the early neonatal period may prevent the development not only of cretinism but also of goiter. There have been no other reports of patients with ITD diagnosed as having congenital hypothyroidism by neonatal screening and treated from the neonatal period. Neonatal screening for hypothyroidism was started

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in 1977 in Manitoba as the first provincewide screening program in Canada. The average age of treatment of patients with congenital hypothyroidism in Manitoba is 10 days.

The description by Fujiwara *et al.* (21) that ITD is usually found by neonatal screening as primary hypothyroidism seems incorrect. With the exception of ITD patients in this Hutterite family, few reported ITD patients have undergone neonatal screening for cretinism. Most were diagnosed as having transient hyperthyrotropinemia (case b_2 in Ref. 19) or as normal (case 6 in Ref. 20 and case b_1 in Ref. 19). Although only case 1 in Ref. 21 among the ITD cases reported by Fujiwara *et al.* showed high TSH value by neonatal screening, her thyroid function normalized at 4 weeks of age, and T_4 treatment was started at 3 months of age. The reason why Japanese ITD patients do not show very high TSH levels in the neonatal period may be because of high iodide intake.

It is interesting that Fujiwara *et al.* (21) speculated that the thyroid-stimulating effect of TSH may be enhanced by low intrathyroidal iodide concentration. Extremely high TSH for a short period of time in early life might greatly enhance the initiation of somatic mutations or focal hyperplasia in multiple thyroid follicular cells and result in multinodular goiter at a young age.

It is now possible to use gene diagnostics rather than uptake of isotopes in the thyroid and salivary glands and saliva/serum iodide or technetium ratios to identify this unique NIS mutation in infants born with congenital hypothyroidism in this family. Gene diagnostics can also be used to determine the carrier state of potential parents for genetic counseling and to arrange rapid and early diagnosis by selective cord blood TSH screening and/or direct analysis of G395R mutation by *Alw*I digestion, which can be performed within 3 h.

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