A Novel Peculiar Mutation in the Sodium/Iodide Symporter Gene in Spanish Siblings with Iodide Transport Defect

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Previously, we reported two Spanish siblings with congenital hypothyroidism due to total failure of iodide transport. These were the only cases reported to date who received long-term iodide treatment over 10 yr. We examined the sodium/iodide symporter (NIS) gene of these patients. A large deletion was observed by long and accurate PCR using primers derived from introns 2 and 7 of the NIS gene. PCR-direct sequencing revealed a deletion of 6192 bases spanning from exon 3 to intron 7 and an inverted insertion of a 431-base fragment spanning from exon 5 to intron 5 of the NIS gene. The patients were homozygous for the mutation, and their mother was heterozygous. In the mutant, deletion of exons 3–7 was suggested by analysis using programs to predict exon/intron organization, resulting in an in-frame 182-amino acid deletion from Met142 in the fourth transmembrane domain to Gln323 in the fourth exoplasmic loop. The mutant showed no iodide uptake activity when transfected into COS-7 cells, confirming that the mutation was the direct cause of the iodide transport defect in these patients. Further, the mutant NIS protein was synthesized, but not properly expressed, on the cell surface, but was mostly accumulated in the cytoplasm, suggesting impaired targeting to the plasma membrane. (J Clin Endocrinol Metab 87: 3830–3836, 2002)

Subjects and Methods

Patients

The patients discussed in the present study were described previously (12).

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

Genomic DNA was extracted as described previously (17, 20) from peripheral blood cells of the patients and their family members with their informed consent. All procedures were performed in accordance with our institutional guidelines. 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 (Fig. 1). The locations of all intronic primers were at least 15 nucleotides distant from the exon(s) to be amplified. Nucleotide sequences of all exons, from nucleotide −37 to +1952 in NIS 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, Foster City, CA) (19).

Long and accurate (LA)-PCR encompassing exons 3–7 and intervening introns

LA-PCR was performed with a primer derived from the 5′ portion of intron 2 (5′-CTTCACCTAGCCCGGCCCCATCTCAGACAGA-3′) and a reverse primer derived from the 3′ portion of intron 7 (5′-GAGGGACCAGGGGACATCTATGCTCTGATG-3′) to amplify a fragment of ap-
proximately 6.6 kb. Five hundred nanograms of template genomic DNA, 10 pmol of each primer, 2 nmol of each dNTP, and 2.5 U LA Taq polymerase (Takara, Tokyo, Japan) were incorporated in 50 μl GC buffer I, the final Mg²⁺ concentration of which was 2.5 mM. The reaction mixtures were first incubated at 94°C for 60 sec, then subjected to 30 cycles of 20 sec at 98°C and 1200 sec at 68°C.

Sequencing the short approximately 800-bp LA-PCR fragment obtained from the ITD patients

In addition to the primers used for the LA-PCR reaction, two kinds of primers were used for sequencing the fragment of approximately 800 bp obtained by LA-PCR from the ITD patients. One was on the boundary of intron 2 and exon 3 (5’-CACACTCTGCTACAGATGC-3’), and the other reverse primer was on the 5’ end of intron 7 (5’-TGCTATTAGTGGTACACAGATC-3’).

Construction of expression vectors, transfection, and iodide uptake assay

Wild-type (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 vector (Invitrogen, San Diego, CA) under control of the cytomegalovirus promoter (17, 19). Mutant construct Δ (142–323) was generated by site-directed mutagenesis (17). COS-7 cells were transfected with 25 μg WT or mutant NIS DNA or with control vector DNA (pCR3-CAT, Invitrogen) by electroporation. To mimic the family members who had the heterozygous Δ (142–323) mutation, a half amount of each of the two kinds of constructs was used to transfect COS-7 cells. To monitor transfection efficiencies, 0.1 μg pSVGH was cotransfected with Δ or WT NIS DNA or with control vector DNA. After finishing the incubation, cells were washed twice on wet ice with 2 ml ice-cold Hanks’ balanced salt solution incubation buffer as quickly as possible (<15 sec). Cells were solubilized with 1 ml 0.1 n NaOH, 0.1% (w/v) sodium dodecyl sulfate, and 2% Na₂CO₃, the protein concentration was determined by the method of Bradford (22) using BSA as a standard, and radioactivity was counted using a γ-counter. Some wells were trypsinized to allow counting of cell number. Iodide uptake is expressed as picomoles per minute per milligrams of cell protein.

Immunocytochemical staining of transfected cells

Transfection with wild-type and mutant NIS constructs was performed as described above. Forty-eight hours after transfection, cells were harvested by pipetting with 1.0 mM EDTA in PBS and collected by centrifugation with small pieces of 1% agarose gel. Cells incorporated into the agarose gel were fixed with formalin and embedded in paraffin. The blocks were used for immunostaining as described previously for thyroid tissue specimens using an antibody against C-terminal NIS peptide (1:4000) (20).

Results

Clinical summary

The two Spanish sibling cases were reported in 1987 (12). Patient A and patient B in the original report correspond to II-1 and II-3 in the present report (Fig. 2), respectively. Briefly, the male patient (II-1) was born in 1958, and his sister (II-3) was born in 1964. Their parents were not consanguineous. II-1 had goiter as a baby and had been treated with thyroid extract, but had severe mental retardation, probably due to the delay of initiation and insufficiency of treatment. The diagnosis of congenital hypothyroidism of patient II-3 was made at birth, and her treatment was started immediately, which is why she did not develop goiter or significant mental activity of about 20 mCi/mmol at 37°C for 2 min. After finishing the incubation, cells were washed twice on wet ice with 2 ml ice-cold Hanks’ balanced salt solution incubation buffer as quickly as possible (<15 sec). Cells were solubilized with 1 ml 0.1 n NaOH, 0.1% (w/v) sodium dodecyl sulfate, and 2% Na₂CO₃, the protein concentration was determined by the method of Bradford (22) using BSA as a standard, and radioactivity was counted using a γ-counter. Some wells were trypsinized to allow counting of cell number. Iodide uptake is expressed as picomoles per minute per milligrams of cell protein.

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retardation. Twenty-four-hour $^{131}$I thyroidal uptake was 1% (normal, 15–45%) for the two patients, and $^{131}$I saliva/plasma ratios were 1.19 and 0.96, respectively (normal, >10), confirming the clinical diagnosis of iodide transport defect. Treatment with iodide (18 mg/d) was initiated in 1973 and continued to 1987 for II-1 and to 1985 (discontinued due to pregnancy) for II-3. The patients remained euthyroid over the 10 yr of iodide treatment, and TSH suppression never occurred. The TSH peak response to TRH was always in the normal range (4–20 mU/liter for II-1 and 11–30 mU/liter for II-3) (12).

Their mother had nontoxic multinodular goiter and was receiving suppressive therapy with $\text{L}^{-}\text{T}_{4}$. She was euthyroid without medication and showed normal 24-h $^{131}$I thyroidal uptake (57%) and $^{131}$I saliva/plasma ratio (36.9).

The father (I-1) of the two patients was dead when we started the genetic study. Other family members included in the genetic study (II-4, III-1, III-2, III-3, III-4, and III-5) and the brother (II-2) of the patients, from whom a blood sample was unavailable, were euthyroid and had no goiter.

**NIS mutation**

For direct sequencing of all exons and flanking introns of NIS genomic DNA, we initially performed PCR amplification of each exon individually or in combination. No amplified band was obtained from the genomic DNA of patient II-1 for exons 3 and 4, 5, or 6 and 7 (Fig. 1), whereas bands of 397, 282, and 483 bp were obtained from normal subjects, respectively (data not shown). Patient II-3 showed the same results as II-1 (data not shown).

Therefore, we speculated that they had a large deletion involving these exons. LA-PCR was performed using primers derived from intron 2 and intron 7. Normal subjects yielded the expected approximately 6.6-kb PCR product. However, patients II-1 and II-3 showed a band of approximately 800 bp (Fig. 2). The mother (I-2) and sister (II-4) of the two patients and two children (III-4 and III-5) of patient II-3 yielded both approximately 6.6- and 800-bp bands, suggesting that they were heterozygous for the deletion.

To examine the location of the deletion, sequencing of the approximately 800-bp short fragment from patient II-1 was performed. PCR-direct sequencing revealed a deletion of 6,192 bases spanning from exon 3 to intron 7 and an insertion of a 431-base fragment of unknown sequence (Fig. 3). BLAST search revealed that the fragment showed 100% homology to a portion of cosmid clone R31408 (GenBank accession no. AC005796). This was an inverted insertion of a fragment spanning from exon 5 to intron 5 of the NIS gene. Fifty-four normal subjects yielded the same approximately 6.6-kb band only by the LA-PCR reaction, but the smaller approximately 800-bp band was not obtained (data not shown).

As thyroid specimens were unavailable, we used a program to predict exon/intron organization. The nucleotide sequence from 10,001–40,000 containing the entire NIS gene and the corresponding sequence identified in the patient were analyzed using BCM gene finder (http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html). Compared with the exon/intron organization obtained for the wild-type sequence, deletion of exons 3–7 in the mutant was predicted, resulting in an in-frame 182-amino acid deletion; from Met$^{142}$ in the fourth transmembrane domain to Glu$^{323}$ in the fourth exoplasmic loop (Fig. 4). Other programs (GENESCAN; http://genes.mit.edu/GENESCAN.html, MZEF Gene Finder; http://argorn.cshl.org/genefinder/) gave the same results. Direct sequenc-
ing of exons 1, 2, and 8–15 from patient II-1 exhibited normal sequence. The approximately 800-bp short fragment from patient II-3 had the identical deletion/inverted insertion.

Expression experiments

Expression experiments by transfection of the mutant Δ (142–323) NIS cDNA into COS-7 cells showed no perchlorate-sensitive iodide uptake (Fig. 5), confirming that the mutation was the direct cause of the ITD in these patients. Cells cotransfected with WT/Δ (142–323), mimicking the heterozygous state in unaffected family members, showed approximately half the iodide uptake activity observed in cells transfected with 25 μg WT NIS DNA, but uptake was similar to that in cells transfected with half the amount of the two kinds of DNA constructs, WT NIS and control vector (pCR3-CAT) DNAs. These results suggested that the Δ (142–323) mutant NIS protein does not interfere with the function of WT NIS (no dominant negative effect), similar to other NIS mutants, G93R, T354P, G543E, and G395R, and iodide uptake activity in transfected cells was correlated with the level of WT NIS expression. Cotransfection with pSVGH and measurement of GH concentration in the culture medium showed no differences in transfection efficiencies among the transfectants. Immunostaining of cells transfected with the deletion construct revealed that the mutant NIS protein was synthesized and accumulated in the cytoplasm, but was not properly expressed on the cell surface (Fig. 6). This may have been due to the disrupted membrane topology and/or three-dimensional structure suggested by the location of the deletion (Fig. 4).

Discussion

We identified a novel and homozygous NIS germline mutation, 6192-bp deletion and 431-bp inverted insertion, in two sibling cases with total iodide transport in a Spanish family. This mutation was confirmed to be the direct cause of the disease in these patients by functional assays showing that the mutant NIS had no iodide uptake activity in transfected COS-7 cells. These two siblings were the only ITD cases reported to date with long-term iodide therapy over 10 yr (12).

As the patients’ mother (I-2) and the children (III-4 and III-5) of patient II-3 were heterozygous for the mutation, the patients were likely to be homozygous for the mutation. However, the possibility that the patients were hemizygotes for the mutation of the NIS gene could not be excluded. Patients II-1 and II-3, who had only alleles with the mutation, in whom 131I thyroidal uptake and 131I saliva/plasma ratios were very low, were definitively diagnosed as having ITD. Their mother, I-2, who had diffuse goiter and normal TSH...
level, $^{131}$I uptake, and saliva/plasma radioiodide ratio, was heterozygous for the mutation, but was not considered to have partial ITD, confirming the recessive nature of ITD in these patients. Her goiter was probably due to another etiology, because other heterozygous subjects (III-4 and III-5) had no goiter.

Most of the mutations of the NIS gene found in patients with ITD are point mutations; T354P, G93R, G543E, C272X, V59E, Q267E, and G395R. One exception was a deletion of 67 nucleotides in NIS cDNA due to a cryptic 3' splice site produced by a single nucleotide substitution (18). Therefore, the present mutation was the first caused by a mechanism other than a single nucleotide substitution. However, how this peculiar mutation was formed is unclear. What is known is that sequences of the four nucleotides before the insertion and the two nucleotides after the insertion are the same as reversed sequences around the insertion. Therefore, the length of the insertion could be from 431–437 bp.

Deletion of exons 3–7 suggested by gene structure analysis programs resulted in an in-frame 182-amino acid deletion from Met142 in the fourth transmembrane domain to Gln323 in the fourth exoplasmic loop (Fig. 4). A large intramolecular deletion involving a highly hydrophobic membrane protein with multiple transmembrane helixes can be expected to result in disruption of three-dimensional structure and proper membrane topology.

Among NIS mutants identified in patients with ITD, the T354P mutant was shown to be properly expressed on the plasma membrane in patients’ thyrocytes (17, 20), but Q267E and S515X mutant NIS proteins were retained in cytoplasm in transfected COS cells (25). However, an apparent genotype-phenotype corelationship has not been established. Storage of a large amount of mutant protein can be cytotoxic and can induce endoplasmic reticulum stress (26) that might account in part for severe hypothyroidism in some patients.
In general, diffuse (sometimes huge) or nodular goiter develops in patients with iodide transport defect even under thyroid hormone therapy. However, it is noteworthy that the goiter disappeared in case II-1 and never developed in case II-3 during long-term iodide therapy for over 10 yr, although suppression of TSH never occurred, and thyroid hormone levels maintained normal. The thyroid-stimulating effect of the normal range of TSH may be enhanced by low intrathyroidal iodide concentration, as speculated by Gilboa et al. (3) and Fujiwara et al. (21). This would explain the difference in goiter development between the present cases with long-term iodide therapy and others treated with thyroid hormones. Alternatively, this specific phenotype might be due to the specific genotype.

Including the finding of the NIS mutation in the 2 patients in this family, the total number of IDT patients with an identified NIS mutation(s) worldwide has reached 30, and NIS mutations of 22 of these 30 cases were identified in our laboratory.

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Fig. 6. Immunostaining of mutant transfecant with anti-NIS antibody. COS-7 cells transfected with WT (A and C) and mutant (B) NIS expression vector. C (Absorbed). The anti-NIS antibody was preabsorbed before use for immunostaining by incubation with the antigen peptide for 60 min at 37 C (20).
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