

**Introduction**

X-linked nephrogenic diabetes insipidus (NDI; MIM 304800) is a rare disorder mainly caused by mutations in the arginine vasopressin receptor (AVPR2) gene and characterized by the excretion of abnormally large volumes of diluted urine [1-3]. Identification of mutations in AVPR2 facilitates early diagnosis, which can prevent serious complications such as growth and mental retardation [4]. NDI is serious in newborns, in which recurrent episodes of dehydration can cause severe neurologic sequelae and even death [3-5]. Female carriers usually have mild symptoms, but affected male patients generally present with much more severe clinical symptoms, including dehydration and failure to thrive [3, 4]. Unless treated early, persistent severe dehydration can delay growth and development [3, 5, 6]. Hence, early diagnosis and treatment is critical [3, 4].

The AVPR2 gene responsible for congenital NDI has been mapped to chromosome Xq28 [7]. Ninety percent of congenital NDI mutations are X-chromosomal and are caused by loss-of-function mutations in AVPR2 [8]. In a minority of cases, germ-line mutations in the aquaporin-2 water channel have been detected, with either an autosomal recessive or autosomal dominant mode of inheritance [7, 9, 10].

AVPR2 consists of three exons, and encodes a 371-amino acid G protein-coupled receptor [4, 11]. On binding AVP the AVPR2 receptor activates Gs/adenylate cyclase which increases intracellular cyclic adenosine monophosphate (cAMP) [2, 12]. This initiates a phosphorylation cascade that promotes translocation of the water channel, aquaporin 2, to the apical membranes of the renal tubules [13]. Mutations of the AVPR2 gene result in receptor malfunction causing polyuria and eventually mental and/or growth retardation.

To date, examination of 250 families has led to
A novel AVPR2 mutation

The identification of 178 germ-line mutations in the AVPR2 gene [2, 12] (www.medicine.mcgill.ca/nephros). Of these, half are missense mutations, 27% are frameshift mutations caused by nucleotide deletions or insertions, and the remainder is other kinds of mutation [12, 14]. The functional characteristics of altered AVPR2 proteins have also been investigated [6, 15, 16]. Mutations of the AVPR2 gene result in receptor malfunction at different levels: improper trafficking, disturbance of receptor-ligand binding, and loss of receptor-G protein interaction [16]. We have genotyped the AVPR2 gene in a Korean NDI family and identified a novel frameshift mutation. By measuring cAMP accumulation and characterizing the mutant protein we show that the mutation interferes with proper receptor function, and is responsible for the AVP resistance in the NDI family.

Materials and methods

Patient profile

A 29-year-old male was referred to the endocrine division of Incheon St. Mary’s Hospital for investigation of his polyuria, polydipsia, and nocturia. He had been admitted with a diagnosis of upper respiratory infection. His elder brother was found to have similar symptoms, with polydipsia and nocturia. The pedigree of this family with congenital NDI is shown in Figure 1. The proband was 168 cm in height and 63 kg in weight, and there was no growth retardation. He had had no academic education beyond high school, and had not been diagnosed and treated for any disease before that time. He passed 7500 ml urine per day and the specific gravity of the urine was 1.000 and its osmolality 62 mOsm/kg. A simple abdominal radiogram and computed tomography revealed bilateral hydronephrosis, hydroureter and marked distension of the bladder (Figure 2). Blood analyses gave creatinine 1.1 mg/dL, sodium 140 mEq/L, potassium 4.0 mEq/L, calcium 9.4 mg/dL, phosphate 2.6 mg/dL, and osmolarity 305 mOsm/kg (normal reference range: 280-290 mOsm/kg). Serum ADH was 44.2 pg/mL (normal reference ranges: 1-13 pg/mL). NDI was diagnosed based on the clinical symptoms and laboratory findings, and on a failure of administration of 5 units of vasopressin to reduce urinary volume and to increase urine osmolality (Table 1). Urine volume was also not reduced by

Figure 1. Pedigree of the Korean family with X-linked congenital NDI. Arrow denotes the index patient, solid squares are affected males, solid circles within open circles are heterozygous females confirmed as carriers by DNA sequencing. Small superscript circles to the upper right of the symbols of the family members indicate those individuals whose DNA was available for analysis. Superscript circles with an asterisk in the middle are individuals with the confirmed mutation. Roman numerals refer to generations and Arabic numerals to individuals.
A novel AVPR2 mutation

Figure 2. Simple flat abdominal x-ray of the proband showing ileus and distension in the bladder (A). The abdominal computed tomograph also showing distension of the bladder (B), and hydronephrosis and hydroureter on both sides of the kidney (C).

Table 1. Response of the proband to water deprivation and ADH injection

<table>
<thead>
<tr>
<th>Hour</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>AVP SU</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bwt (Kg)</td>
<td>65.8</td>
<td>64.6</td>
<td>64.3</td>
<td>62.6</td>
<td>61.1</td>
<td>61.3</td>
<td>61.4</td>
<td>60.5</td>
<td></td>
</tr>
<tr>
<td>Urine volume (mL)</td>
<td>550</td>
<td>600</td>
<td>440</td>
<td>410</td>
<td>270</td>
<td>225</td>
<td>250</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td>Urine Osm (mOsm/L)</td>
<td>67</td>
<td>80</td>
<td>82</td>
<td>110</td>
<td>105</td>
<td>98</td>
<td>107</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>Serum Na (mEq/L)</td>
<td>138</td>
<td>139</td>
<td>142</td>
<td>144</td>
<td>145</td>
<td>148</td>
<td>146</td>
<td>146</td>
<td></td>
</tr>
<tr>
<td>Serum Osm (mOsm/L)</td>
<td>302</td>
<td>312</td>
<td>328</td>
<td>320</td>
<td>322</td>
<td>329</td>
<td>327</td>
<td>329</td>
<td></td>
</tr>
<tr>
<td>Plasma AVP (pg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>44.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AVP, arginine vasopressin (reference range : 1-13 pg/mL); Osm, osmolarity

hydrochlorothiazide or high dose desmopressin. All clinical laboratory and genetic investigations were conducted with the consent of the proband (Table 1) and selected family members (Table 2), according to the principles of the Declaration of Helsinki, and the research protocol was approved by the Ethics Committee of Incheon St. Mary's Hospital.

Sample preparation and AVPR2 gene analysis

Genomic DNA from the proband and relatives was extracted from peripheral blood leukocytes using a Flexi Gene DNA kit (Qiagen, Valencia, CA). Six overlapping primers were used to amplify the entire coding sequence and its flanking intronic sequences by PCR. The primer pairs are given in Table 3. The AVPR2 gene was amplified by standard PCR in 25 µM reaction mixtures containing 100 ng genomic DNA, one of the 6 primer pairs, 0.2 mM dNTPs, and 1U Tag polymerase (Qiagen, Hilden, Germany) in an automated thermal cycler (MWG Biotech, Ebersberg, Germany). The amplified products were analyzed for purity and size by electrophoresis on 2% agarose gels, and the purified PCR products were sequenced with an ABI3100 Prism automatic sequencer (Applied Biosystems, Fos-
A novel AVPR2 mutation

Table 2. Clinical parameters and mutational analysis of the NDI family

<table>
<thead>
<tr>
<th></th>
<th>II-3</th>
<th>III-2</th>
<th>IV-5</th>
<th>IV-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVPR2 genotype</td>
<td>302delC/-</td>
<td>302delC/-</td>
<td>302delC/-</td>
<td>302delC/-</td>
</tr>
<tr>
<td>Age (years) when test was done</td>
<td>66</td>
<td>31</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Serum Na (mEq/L)</td>
<td>140</td>
<td>139</td>
<td>136</td>
<td>135</td>
</tr>
<tr>
<td>Plasma AVP (pg/mL)</td>
<td>40.5</td>
<td>36.1</td>
<td>19.4</td>
<td>17.6</td>
</tr>
<tr>
<td>Serum osmolality (mOsm/L)</td>
<td>320</td>
<td>314</td>
<td>305</td>
<td>302</td>
</tr>
<tr>
<td>Polyuria/Nocturia</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
</tbody>
</table>

Table 3. Oligonucleotide primers used to amplify the 3 exons of AVPR2

<table>
<thead>
<tr>
<th>Primers cDNA 1</th>
<th>Exon nbr</th>
<th>Size (bp)</th>
<th>Tm (°C)</th>
<th>Upstream primer</th>
<th>Downstream primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVPR2 2-1</td>
<td>370</td>
<td>58</td>
<td>5'GGAGTCTTCGGGTGCTGC-3'</td>
<td>5'TGAAATGTCACACCACACTCT-3'</td>
<td></td>
</tr>
<tr>
<td>AVPR2 2-2</td>
<td>416</td>
<td>59</td>
<td>5'GCAGCACCCTCTCTCAACC-3'</td>
<td>5'GCCAGGAATGACATGATGAAGAGA-3'</td>
<td></td>
</tr>
<tr>
<td>AVPR2 2-3</td>
<td>441</td>
<td>60</td>
<td>5'GTGCGCTTTCAAGTCTGC-3'</td>
<td>5'GCCAGCTGATGAACTCTCC-3'</td>
<td></td>
</tr>
<tr>
<td>AVPR2 3'UTR</td>
<td>428</td>
<td>59</td>
<td>5'TCTGGAGACCCAACTTAGTCC-3'</td>
<td>5'ATACAGGCTGAGGATGGAG-3'</td>
<td></td>
</tr>
<tr>
<td>AVPR2 cloning</td>
<td>436</td>
<td>60</td>
<td>5'AGAAATGGGCAACAGCTG-3'</td>
<td>5'ACGCTTGGGAGGATGCGAC-3'</td>
<td></td>
</tr>
</tbody>
</table>

Tm, annealing temperature; nbr, number; UTR, untranslated region

The AVPR2 fragment tagged with Flag and this was introduced into a compatible site in the pcDNA3-myc vector [18] (kindly donated by Dr. Kwang-Hyun Baek [Cell and Gene Therapy Research Institute, Graduate School of Life Science and Biotechnology, Pochon CHA University, CHA General Hospital, Seoul, Korea]). The resulting AVPR2 wild type expression vector (pcDNA3-myc-AVPR2 wild-Flag) was mutated with a QuickChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) to obtain the expression vector for the mutant AVPR2 (pcDNA3-myc-AVPR2 mutant-Flag). Both expression vectors were sequenced directly by BigDye Terminator Cycle sequencing with an ABI3100 Prism automatic sequencer (Applied Biosystems).

Cell transfection and cAMP accumulation assay

COS-7 cells were purchased from the Korean cell-line bank (Cancer Research Institute, Seoul National University College of Medicine, Seoul, Korea) [19], grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum at 37°C in a humidified 5% CO₂ incubator and plated in 35 mm dishes for transfection. When 80–90% confluent, they were transfected with 2 mg of plasmid DNA (pcDNA3-myc-AVPR2 wild-Flag or pcDNA3-myc-AVPR2 mutant-Flag and

Poster City, CA). For allele-specific sequence analysis, the purified PCR products were subcloned with a pCR2.1-TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA) and the cloned inserts were analyzed by PCR and sequenced.

Construction of an expression vector for the AVPR2 mutant

To investigate the novel frameshift mutation (302delC), we first created an expression vector for the wild type gene, and then introduced the frameshift mutation into it. The wild type AVPR2 gene was amplified from vector RG000246 (Invitrogen) with an Expand High Fidelity PCR System (Roche, Boehringer Mannheim, Germany). A BamHI site and Kozak sequence were introduced into the upstream primer and a KpnI site into the downstream primer (Table 3). The resulting PCR product was first subcloned into pCR2.1-TOPO vector (Invitrogen) and then cloned between the EcoRI and KpnI sites of pcDNA3 vector with a C-terminal Flag tag [17] (kindly donated by Dr. Hyang-Sook Rhim [Research Institute of Molecular Genetics, Catholic Research Institute of Medical Science, The Catholic University of Korea, Seoul, Korea]) (Figure 3). The pcDNA3-AVPR2 wild-Flag construct was cut with BamHI and XhoI to release the AVPR2 fragment tagged with Flag and this was introduced into a compatible site in the pcDNA3-myc vector [18] (kindly donated by Dr. Kwang-Hyun Baek [Cell and Gene Therapy Research Institute, Graduate School of Life Science and Biotechnology, Pochon CHA University, CHA General Hospital, Seoul, Korea]). The resulting AVPR2 wild type expression vector (pcDNA3-myc-AVPR2 wild-Flag) was mutated with a QuickChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) to obtain the expression vector for the mutant AVPR2 (pcDNA3-myc-AVPR2 mutant-Flag). Both expression vectors were sequenced directly by BigDye Terminator Cycle sequencing with an ABI3100 Prism automatic sequencer (Applied Biosystems).
pcDNA3-myc-Flag or pcDNA3-Flag) complexed with 7.5 µl of lipofectamine 2000 (Invitrogen) in 250 µl of opti-MEM reduced serum medium (Invitrogen). Five hours after transfection, the cells were washed and the medium was replaced with DMEM containing 10% fetal bovine serum. Transfected cells were selected in DMEM containing 400 µg/ml of geneticin (Invitrogen) over 4 weeks. The geneticin-resistant transfectant populations were starved for another 24 hour before adding the indicated concentrations of AVP for 24 hours (Figure 5A). 0.1 N HCl was added and intracellular cAMP levels were determined with a BIOTRAK cAMP [125I] assay system (Amersham Pharmacia Biotech UK Limited, Little Chalfont, Buckinghamshire, UK). Data are presented as fold increases in cAMP over basal levels in the absence of AVP.

Western blotting and immunofluorescence analysis

To estimate cell surface expression of the receptors carrying N-terminal myc-AVPR2-tags, cells were washed twice with phosphate-buffered saline (PBS) and lysed in cell lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% NP-40). The lysates were centrifuged, and the supernatants were mixed with sample buffer (60 mM Tris-HCl, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 1% bromophenol blue), boiled for 10 min, separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and transferred to a PVDF membrane. Unbound sites were blocked with TBS (150 mM NaCl, 20 mM Tris, pH 7.4) containing 5% skim milk and 0.05% Tween 20. The blots were washed three times (5 min each time) with TBS containing 0.05% Tween 20 (TBST), and incubated for 1 h with a 1:30000 dilution of anti-c-myc polyclonal antibody (Bethyl Laboratories, Montgomery, TX) in TBST containing 1% BSA. After washing in TBST, the blots were incubated for 1 h with a 1:100000 dilution of peroxidase-conjugated goat polyclonal anti-rabbit antibody (Abcam, Cambridge Science Park, Cambridge, UK) in TBST. Signals were visualized with ECL Western blotting detection reagents (Amersham Bioscience, Arlington Heights, IL). β-actin was used to assess amounts of protein loaded. For immunofluorescence analysis, cells were grown on glass coverslips, washed twice with PBS and fixed for 20 min at room temperature with freshly prepared 4% paraformaldehyde in PBS. For permeabilization, the cells were treated with PBS containing 0.1% Triton X-100. After block-
A novel AVPR2 mutation

![Figure 4. The AVPR2 mutation in the Korean family with NDI. The 1 bp deletion (bp position 302: indicated by arrows) results in a shift of reading frame and a stop codon after 15 further amino acids.](image)

ing in PBS containing a 1:100 dilution of normal donkey serum, the cells were incubated at room temperature for 1 h with a 1:600 dilution of anti-c-myc antibody. Bound antibody was stained with a 1:200 dilution of rhodamine-conjugated AffiniPure donkey anti-rabbit IgG (Jackson Immuno Research Laboratories, West Grove, PA) and nuclei were stained with mounting medium containing DAPI (Vector, Burlingame, CA). Fluorescence images were obtained with a confocal laser microscope (LSM 510 Meta, Carl Zeiss, Germany).

**Results**

**Identification of a new AVPR2 mutation in the study subjects**

Genomic DNA was prepared from all available family members and the AVPR2 coding sequence was amplified by PCR. Analysis of the PCR fragments from the proband and his family members (II-3, III-2, IV-5 and IV-4) (Figure 1) revealed a novel 1-bp deletion (302delC). This mutation shifted the reading frame, which then encountered a stop codon after a further 15 residues (Figures 3 & 4). Mutational analysis also identified various family members as carriers (solid circles within open circles) (Figure 1) (Table 2).

**cAMP accumulation by transfectants harboring wild type and mutant AVPR2 expression vectors**

To further characterize the novel AVPR2 mutation, we constructed eukaryotic expression vectors for the wild-type and mutant AVPR2: pcDNA3-myc-AVPR2 wild-Flag and pcDNA3-myc-AVPR2 mutant-Flag, respectively (Figure 3) and expressed them in COS-7 cells. Intracellular cAMP accumulation in response to various concentration of AVP are shown in Figure 5A & B. Expression of the wild type construct resulted in an approximately 6.5-fold increase of cAMP over basal values, whereas expression of the mutant construct resulted in no significant cAMP production.

**Detection of tagged AVPR2 receptors in COS-7 cells**

We analyzed expression of the wild-type and mutant-AVPR2 receptor cDNA in COS-7 cells by Western blotting with anti-myc antibody (Figure 5C). In the case of the wild type receptor, only one specific band was formed, at approximately 74 kD. In contrast, the mutant produced a strong band at 25 kD presumably corresponding to the truncated myc-tagged mutant AVPR2 (which represent immature nonglycosylated receptor forms) [15, 20, 21].

To see whether the mutant AVPR2 was correctly transported to the cell surface, we performed confocal fluorescence microscopy using a polyclonal antibody directed against the N-terminal myc epitope of the AVPR2 proteins. Non-permeabilized COS-7 cells expressing the wild-type receptor showed intense staining of the plasma membrane, whereas cells expressing the mutant AVPR2 displayed only faint surface
A novel AVPR2 mutation

Figure 5. Functional characterization of the 302delC and wild type AVPR2 cDNA. A and B. Adenylyl cyclase activity was determined in COS-7 cells transfected simultaneously with pcDNA3-myc-AVPR2 wild-Flag or pcDNA3-myc-AVPR2 mutant-Flag vector and empty vector using 7.5 µl of lipofectamine 2000 in 250 µl of opti-MEM reduced serum medium. Each curve is representative of three independent experiments carried out in triplicate. C, Western blot of AVPR2 receptors. The blot was probed with rabbit antibody against the myc epitope. D, Immunofluorescence assay of the wild-type and mutant proteins. To detect the AVPR2 receptors, a polyclonal antibody directed against the N-terminal myc-tagged AVPR2 receptor was employed. Experiments were carried out with non-permeabilized (NP) and permeabilized (P) cells.

staining (Figure 5D). In contrast, immunostaining of permeabilized cells showed that the mutant receptor, like the wild-type, was present at a high level in the cell interior.

Discussion

Since the human AVPR2 gene was cloned in 1992 [22], about 178 distinct disease-causing
mutations of the gene have been identified in patients with X-linked congenital NDI [23, 24]. Most of the mutations so far described are scattered throughout exon 2. Because exon 2 is large, comprising 885 bp, it is a large target. In addition, these mutations cluster in the area of the transmembrane domains [2]. The AVPR2 receptor, which is composed of 371 amino acids, consists of seven transmembrane domains and belongs to the superfamily of G protein-coupled receptors [1, 22]. Elevated cAMP levels activate protein kinase A and initiate a phosphorylation cascade that promotes translocation of the water channel, aquaporin 2, to the apical membrane of renal tubules and collecting tubules. Mutations of the AVPR2 gene result in receptor malfunction leading to polyuria or nocturia.

In the present study, we identified a novel AVPR2 mutation, and confirmed by in vitro studies that the mutation is responsible for the congenital NDI. It is a frameshift mutation in the second transmembrane domain of exon 2 next to arginine 100.

Because functional evaluation of the deletion mutation could be valuable for identifying the receptor residues involved in specific receptor functions, we performed expression studies of the novel mutation. These showed that the mutant protein was not transported to the plasma membrane (Figure 5D) and hence the transfected COS-7 cells were unable to respond to arginine vasopressin by accumulating cAMP. We note that our results indicate that the cAMP accumulation assay is by far the most sensitive method of routinely screening AVPR2 mutations. Our Western blot analysis revealed the presence of four specific bands of approximately 25 kD in the mutant, suggesting that incorrect protein folding of the mutant AVPR2 cDNA in transfected leads to an increase in the intracellular accumulation of immature nonglycosylated receptors [15]. These bands were absent from pcDNA-Flag-transfected control cells.

The severity of the clinical symptoms of NDI varies, probably because of the heterogeneity of the mutations causing this disorder. Female carriers are usually phenotypically normal. Unless recognized and treated early, persistent severe dehydration in affected males may lead to growth and developmental delay or death. Most of the truncated receptor proteins found in NDI patients impair signal transduction [16, 25], and give rise to clinical symptoms such as polyuria and growth retardation. However, the proband in our study had normal stature and intelligence, suggesting that the 302delC gene product has some residual activity and that this results in only partial insensitivity to the antidiuretic hormone. Recent studies with systematically truncated AVPR2s have shown that a minimal length of 341 amino acids is required for proper receptor trafficking and maturation [26]. The 302delC mutation introduces a frameshift, resulting in premature chain termination and producing, in all likelihood, a protein of about 100 amino acids, not enough for proper receptor trafficking or maturation, and hence a clearly pathogenic mutation.

In summary, our proband carries a novel AVPR2 deletion mutation inherited from his carrier mother. Functional studies using an expression vector for the single-site AVPR2 deletion suggest that it is responsible for the congenital AVP insensitivity in this Korean family with NDI.

Acknowledgements

This work was supported by the Catholic Medical Center Research Foundation in the program year 2005.

Please address correspondence to: Dr. Je-Ho Han, Department of Internal Medicine, Incheon St. Mary’s Hospital, The Catholic University of Korea #665 Bupyong 6-dong, Bupyeong gu, Incheon, 403-720 Korea. E-mail: hjh60103@dreamwiz.com, Telephone: 82-32-510-5500, Fax: 82-32-510-5683.

References


A novel AVPR2 mutation


