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Original Article

Functional characterization of a large deletion in *AVPR2* gene causing severe nephrogenic diabetes insipidus in a Turkish patient

Emel Saglar[1], Beril Erdem[1], Ferhat Deniz[2], Hatice Mergen[1]

[1]*Department of Biology, Faculty of Science, Hacettepe University, Beytepe, Ankara 06800, Turkey;* [2]*Department of Endocrinology and Metabolism, GATA Haydarpasa Teaching Hospital, Istanbul 34668, Turkey*

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Abstract: Objective: The main purpose of this study is to characterize functionally a large deletion in arginine vazopression type-2 receptor (*AVPR2*) gene. Changes in *AVPR2* gene mostly lead to a rare hereditary polyuric disease, X-linked nephrogenic diabetes insipidus (NDI). The disease is characterized by the extreme production of urine and the patients have problems with concentrating the urine in response to the antidiuretic hormone vasopressin. In our previous study we have identified a novel 388 bp deletion in the *AVPR2* gene in a patient with NDI and in his family. Methods: For functional analyze studies, identified deletion was re-created by PCR based site-directed mutagenesis and restriction fragment replacement strategy based on DNA sequence and expressed in COS7 cells. We performed total and surface ELISA assay and cAMP assay for assessing the ability of transfected cells to produce cAMP in response to stimulation with dDAVP. Fluorescence staining was performed for determining the cell trafficking of the mutant protein. Results: Results of functional characterization of 388 bp deletion have revealed that mutant V2R did not show any expression on the cell surface compared to the wild type receptor while showed reduced cellular expression in total (31.93%±8.8) compared to the wild type receptor. cAMP accumulation assay results are supported the ELISA results of the mutant receptor protein. Conclusions: In conclusion, our results will provide valuable information about the *AVPR2* trafficking and function of the mutant protein and we believe that our study will contribute to shedding light on mechanisms of molecular pathology of *AVPR2* deletions.

Keywords: *AVPR2*, NDI, deletion, cAMP, ELISA

Introduction

Nephrogenic diabetes insipidus (NDI) is characterized by inability of the kidney to concentrate urine in response to arginine vasopressin (AVP) and the disease symptoms are polyuria, hypoosmolar urine, and hypernatremia [1]. Patients with NDI have great difficulty in concentration of urine and this situation leads to severe liquid-balance impairment [2, 3]. X-linked NDI (OMIM 304800) is a form of Diabetes Insipidus (DI), and results from the mutations in the arginine-vasopressin receptor 2 (*AVPR2*) gene (NM_000054) [4]. 90% of NDI cases are inherited in an X-linked manner [5]. X-linked NDI symptoms usually appear at birth. Male patients are affected whereas heterozygous females show various degrees of polyuria and polydipsia due to skewed X-chromosome inactivation, which is preferential methylation of the normal allele of the *AVPR2* gene [6]. Depends on the severity of the disease, growth failure, mental retardation, fever, vomiting and poor weight gain were reported symptoms for the children possessing the X-linked NDI [1, 7].

AVPR2 gene is located on chromosome Xq28 and consists of three exons and two small introns. The gene encodes a 371-amino acid G protein-coupled receptor (GPCRs) with seven transmembrane, four extracellular and four cytoplasmic domains [8, 9]. The receptor is activated after binding of AVP, and allosteric structural rearrangements occurs after this binding. The AVP binding sites of *AVPR2* are within the transmembrane domains II–IV of *AVPR2* (residues 88-96, 119-127, 284-291, and 311-317) [1]. Severity of the disease are varied depends

on the location of the mutations within the *AVPR2* gene. Several disease-causing mutations within the *AVPR2* gene have been characterized functionally and these studies revealed different types of mutant receptors, which result in receptor malfunction at different levels or defective intracellular trafficking or reduced receptor transcription leading to unstable mRNA [10-12].

In vitro expression studies are important for determining the functional consequences of the *AVPR2* mutations [13]. In the present study, we functionally analyzed the 388 bp deletion in exon 2 of the *AVPR2* gene based on DNA sequence, which was firstly identified in our previous study [6]. 388 bp deletion results in the absence of the three transmembrane domains, two extracellular domains, and one cytoplasmic domain. In our previous study we have reported that this mutation is a de novo mutation for the mother of the proband patient and we did the bioinformatics and comparative genomics analyses the deletion via considering the DNA level damage [6]. The aim of this study was to characterize the 388 bp deletion functionally and compare the clinical data of the NDI patient with the results from the in vitro studies.

Materials and methods

Study subject and DNA sequencing of the AVPR2 gene

A twenty-five years old male NDI patient was admitted to Gulhane Military Medical Academy, Department of Endocrinology and Metabolism in 2012 and he has under control for treatment. He has two sisters and consanguineous parents. Detailed information about the family of the patient were described in our previous study [6]. Patient had a severe history of urination more than twenty liter/day (22 L/day), fatique, polydipsia since infancy. After treatment, his serum natrium level was 146 mmol/L, his glycemia level was 76 mg/dL, his potassium level was 3.1 mmol/L, his blood urea level was 18 mg/dL, his creatinine level was 0,94 mg/dL. His urinary tract ultrasonography was normal. Urine specific gravity was 1001, urine osmolality was as low as 72 mOsm/kg and these values were stable at the end of the water deprivation-desmopressin test (78 mOsm/kg). All clinical, laboratory and genetic investigations were conducted according to Declaration of Helsinki principles.

Genomic DNA of the patient was isolated from peripheral venous blood and spectrophotometrically quantified. The entire *AVPR2* gene was amplified by polymerase chain reaction. The primer sets, amplification and sequencing conditions were described in our previous study [6].

Construction of mutant AVPR2

The 388 bp deletion were introduced into the pLV2R and pLV2R-EGFP (Dr. Angela Schulz, Leipzig University, Faculty of Medicine, Institute of Biochemistry) which are human V2R expression plasmid and human V2R expression plasmid tagged with EGFP, respectively via PCR based site-directed mutagenesis and restriction fragment replacement strategy. For immunological studies, both wild type and mutant *AVPR2*s were N- and C-terminal epitope-tagged (N-terminal: Hemagglutinin (HA)-tag; C-terminal: FLAG-tag) to allow receptor detection. For fluorescence studies, the mutant and wild type *AVPR2*s were tagged with EGFP sequence to be tracked. Wild type and mutant *AVPR2* constructs were verified by DNA sequencing.

Cell culture and transfection

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. To achieve functional assays, COS-7 cells were transiently transfected with TurboFect (Thermo Scientific™TurboFect™ Transfection Reagent). Transfection efficiency was controlled using with EGFP-transfected cells. For cAMP accumulation assay, 96-well plates (2×10^4 cells/well) were used and cells were transfected with a total amount of 200 ng DNA and 0.4 µl TurboFect per well. In addition, for sandwich ELISA and cell surface ELISA, 6-well plate (7×10^5 cells/well) and 48-well plate (4×10^5 cells/well) were used, respectively. In both 6-well plate and 48-well plate, cells were transiently transfected with a total amount of 6 µg DNA and 10 µl TurboFect, and 700 ng DNA and 0.7 µl TurboFect, respectively. Also for western blotting and fluorescence studies, 48-well plates (4×10^5 cells/well) were used and transfection

protocol was the same which was used for cell surface ELISA experiments.

AVP stimulation and ALPHA screen™ cAMP assay

72 h after transfection, cells were washed with in DMEM without serum, fenol red and antibiotics which contained 1 mM 3-isobutyl-1-methylxanthine (IBMX) and then they were stimulated in the presence of different AVP concentrations (10 μM, 1 μM, 100 nM, 10 nM, 1 nM, 100 pM) ([Arg8]-vasopressin acetate salt, Sigma-Aldrich, Seelze, Germany) for 1 h at 37°C. Reactions were terminated by aspiration of the medium and addition of 25 μl ice-cold lysis buffer containing 1 mM IBMX. 5 μl lysate from each well were transferred to a 384-well plate. After that, acceptor and donor beads were added and cAMP quantity measurement was done with non-radioactive cAMP assay based on AlphaScreen technology (Perkin Elmer Life Science, Inc. Boston, MA) according to the manufacturer's protocol. The data of cAMP accumulations were analyzed via GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA).

Sandwich ELISA and cell surface ELISA studies

Cell surface expression of both wild type and mutant *AVPR2*s, which were tagged with N-terminal HA, were estimated by cell surface ELISA. In brief, 72 h after transfection, cells fixed with 4% formaldehyde in phosphate-buffered saline (PBS) and incubated with peroxidase-labelled monoclonal anti-HA antibody (3F10; Roche Applied Science, Mannheim, Germany; 1:1000 in DMEM with 10% FBS). Unbound antibodies were removed and substrate reaction was performed with H_2O_2 and *o*-phenylenediamine (2.5 mmol/L each in 0.1 mol/L phosphate/citrate buffer, pH 5.0). After 15-30 min, the enzyme reaction was stopped by addition of 1 mol/L HCl and color development was measured biochromatically at 492 nm using ELISA reader (EZ Read 400 Microplate Reader, Biochrom). In addition, measurement of the total expression of full-length double-tagged *AVPR2*s (N-terminal HA-tag, C-terminal FLAG-tag) was done with sandwich ELISA. Microtitre plates were coated with polyclonal anti-FLAG antibody (M2; Sigma-Aldrich) and COS-7 cell lysates (72 h after transfection) were added. Followed by washing with PBS-T (PBS containing 0.05% Tween-20), plates incubated for 1 h at room temperature with peroxidase-labelled monoclonal anti-HA antibody. Substrate reaction and measurement of color development were done as described above.

Immunoblot analysis

Total protein was extracted from COS7 cells using TRItidy G™ (A4051, PanReacAppliChem). Briefly, 72 h after transfection, cells in 12 well-plate were homogenized in 500 μL TRIdity G, mixed well and transferred to Eppendorf tube. 100 μL chloroform was added and the mixture was centrifuged at 1200 g for 15 min at 4°C. The RNA phase was removed and 150 μL ethanol were added, then incubated 5 minutes in room temperature (RT) and centrifuged again at 2000 g for 5 min at 4°C. The supernatants were transferred to new tubes. Isopropanol were added, then incubated 10 minutes in RT and centrifuged at 1200 g for 10 min at 4°C. The protein pellet were washed in 1 ml of 300 mM guanidine hydrochloride and incubated in 20 minutes in RT and then centrifuged at 7500 g for 5 min at 4°C. The protein pellet were dried and solved in 1% SDS. The amounts of the protein samples were measured in Quawell Q5000 UV Spectrophotometer (Quawell Technology, Inc).

30 μg of the samples were subjected to Mini-PROTEAN® TGX™ (Bio-Rad Laboratories, Inc) precast gel using the 4 X Laemmli buffer (Bio-Rad Laboratories, Inc) after denaturation at 65°C for 5 minutes. Electrophoresis was run 30 minutes at 200 Volt. After electrophoresis proteins were transferred to PVDF membrane in a Trans-Blot® Turbo™ Transfer Sistem (Bio-Rad Laboratories, Inc) according to the manufacturer protocol. The blots were blocked in 5% skim milk diluted in PBST (1 X PBS containing 0.05% Tween-20) for 1 hour at RT. The blots were washed three times for 10 minutes in PBST. Thereafter, blots were incubated with monoclonal peroxidase anti-HA (3F10; Roche Applied Science, Mannheim, Germany) in 5% skim milk for 2 hours at RT. The blots were washed three times for 10 minutes in PBST again and then incubated in enhanced chemiluminescence Clarity Western ECL Substrate (Bio-Rad Laboratories, Inc.) for 1 minute, and visualized with ChemiDoc™ imaging system (Bio-Rad Laboratories, Inc.).

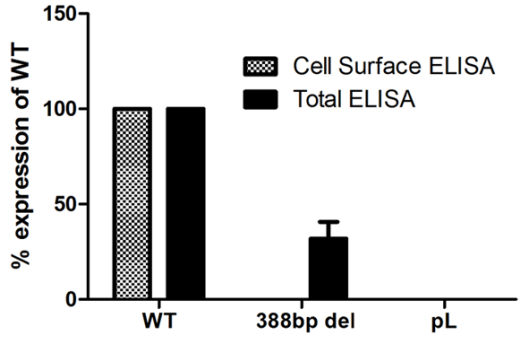

Figure 1. Results of cell surface ELISA assay and total ELISA assay for receptor expression.

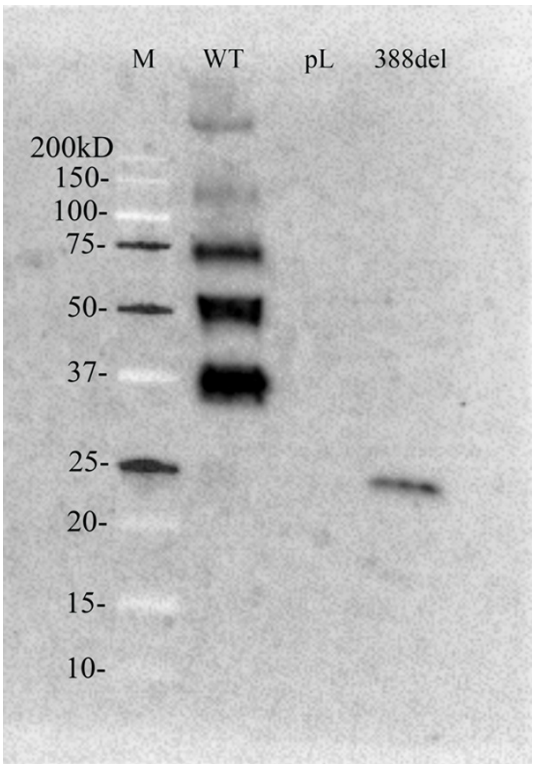

Figure 2. Immunoblot analyse for wild type *AVPR2* and mutant *AVPR2*. (M: Marker, WT: Wild Type, pL: Vector without coding *AVPR2* sequence, 388 del: Mutant protein).

Organelle visualization

Transfected COS-7 cells were fixed with 4% paraformaldehyde/PBS for 10 minutes and then washed with PBS three times. Cells in 24 well plates were treated for 30 minutes at 37°C with 1 μM ER-Tracker™Red (BODIPY® TR Glibenclamide) (Thermo Fisher Scientific) and treated for 30 minutes at 4°C with 5 μM Golgi-Tracker Red (BODIPY® TR Ceramide complexed to BSA) (Thermo Fisher Scientific) for visualization of endoplasmic reticulum (ER) and Golgi, respectively according to manufacturer protocol. After the incubation cells were incubated 10 minutes on ice with 250 μL 1 X PBST (0.05% Tween-20 in 1 X PBS) for DAPI dyeing. Cells were washed in 1 X PBS, treated for 5 minutes with DAPI (Sigma) dye and washed with 1 X PBS. Thereafter, cells were viewed at fluorescence microscope (EVOS FLoid Cell Imaging Station, Thermo fisher SCIENTIFIC, USA).

Results

Functional characterization of mutant construct

For functional characterization, 388 bp deletion were introduced into human *AVPR2* sequence and both wild type and mutant *AVPR2* constructs were expressed in COS-7 cells. The efficiency of the transient expression of V2R in COS-7 cells was examined by transfection of the EGFP plasmid. After stimulation with different AVP concentrations of the cells according to the wild type, COS-7 cells expressing the mutant receptor, showed no detectable cAMP formation.

To understand the expression of mutant *AVPR2*, total ELISA and cell surface ELISA experiments were performed. That kind of large deletion in *AVPR2* did not show any expression on the cell surface compared to the wild type receptor. In addition to result of cell surface expression level, the truncated receptor showed reduced cellular expression in total (31.93%±8.8) compared to the wild type receptor. The results of cell surface ELISA assay and total ELISA assay for receptor expression are shown in **Figure 1**.

According to the western blot analysis, our results are compatible with our previous study, in which theoretical molecular weights of the wild type protein were predicted as about 40,279.09 Da whereas the mutant protein were predicted as about 27,607.16 Da. The western blot analysis results were shown in **Figure 2**.

We performed fluorescence experiments with the mutant-EGFP and wild-type *AVPR2* constructs on COS-7 cells to analyze trafficking of the mutant protein. Our results suggest that the mutant protein most probably retains in ER

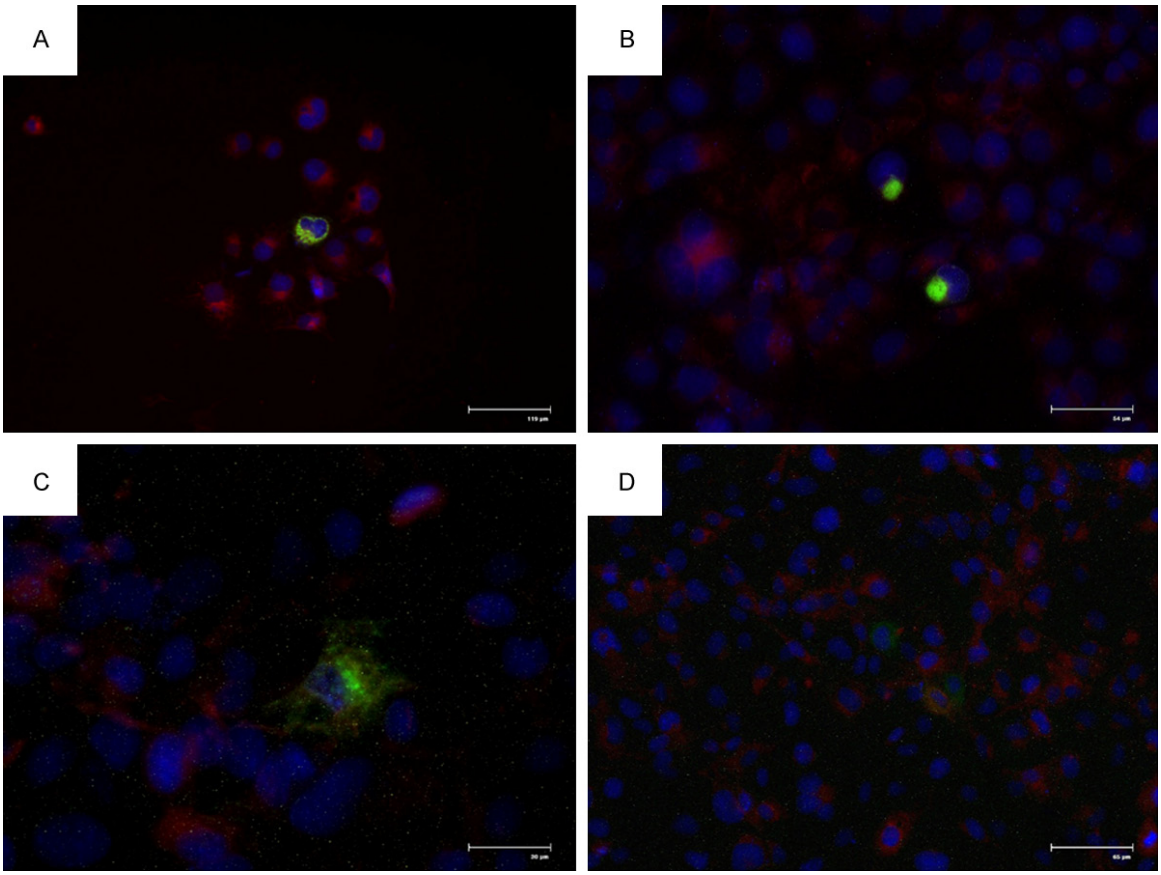

Figure 3. Organelle visualization for both wild type and mutant protein: A. ER-Tracker™Red (BODIPY® TR Glibenclamide) for wild type protein. B. ER-Tracker™Red (BODIPY® TR Glibenclamide) for mutant protein. C. Golgi-Tracker Red (BODIPY® TR Ceramide complexed to BSA) for wild type protein. D. Golgi-Tracker Red (BODIPY® TR Ceramide-complexed to BSA) for mutant protein.

as a result of inappropriate folding of the deleted protein. We compared the wild type receptor with the mutant one for both ER and Golgi apparatus. The figures were shown in **Figure 3**. As expected, cells expressing the wild-type *AVPR2* intensive staining of the cell surface while the mutant *AVPR2* were seen as accumulated. Our results suggest that full intracellular retention of the mutant *AVPR2* was most likely due to improper folding.

Discussion

The genetic analysis for the NDI patients is a standard in diagnosis. Especially, nowadays in vitro characterization of the mutant proteins is highly important for both treatment and genetic counseling of families [14, 15]. The results of the functional characterization studies of *AVPR2* gene mutations in NDI patients will provide the opportunity to evaluate the molecular pathology of the disease when it is thought together with the clinical data of the NDI patients.

We described a Turkish male patient with NDI due to a 388 bp deletion in exon 2 of the *AVPR2* gene, which was reported as a novel deletion mutation in our previous study, and we determined that this deletion mutation was a *de novo* mutation for the mother of the proband patient [6]. We also did several bioinformatics analyses based on DNA sequence and comparative genomics analyses of the deletion and we made some predictions on mRNA structure about mutated *AVPR2* in that study [6]. In the present study we functionally characterized the deletion based on DNA sequence and compared the results with clinical features of the proband patient and bioinformatics studies. Functional characterization of 388 bp deletion in exon 2 of the *AVPR2* gene showed us, mutant receptor most probably is misfolded due to the large deletion. We estimated that the mutant

receptor protein is retained in endoplasmic reticulum (ER) and subsequently targeted for proteosomal degradation, since the ER is the organelle that responsible for the cellular quality control of the proper folding of the proteins [16, 17]. Our results supported that idea because we can see the mutant receptor is partially expressed in the cell but we did not detect any expression on the cell surface (**Figures 1** and **3**). Results of cAMP accumulation assay show concordance with the ELISA results. Truncated receptor could not be stimulated with the highest AVP concentration compared to the wild type receptor because of the retention problems. We concluded that, that kind of large deletion has a really dramatic affect on the receptor assembly and function processes. That's why we can see the loss of receptor function from our ELISA and cAMP accumulation assay results. In addition the organelle staining results confirms the other functional analyze studies of the mutant receptor.

According to the HGMD data, in *AVPR2* gene, there are more than 16 gross deletions [18]. Some of these mutations are just in the borders of *AVPR2* gene and some of them are abnormally deletions that consist other gene regions close to *AVPR2* gene [19-24]. However, functional analyze of such a large deletion in *AVPR2* gene are not mention mostly in the literature. Therefore, our study is one of the first functional characterization studies of such a large deletion. In conclusion, we believe that our results provide valuable information about the *AVPR2* trafficking and function of the mutant protein and will contribute to shedding light on mechanisms of molecular pathology of *AVPR2* deletions.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hatice Mergen, Department of Biology, Faculty of Science, Hacettepe University, Beytepe, Ankara 06800, Turkey. E-mail: mergen@hacettepe.edu.tr

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