EXPERIMENTAL STUDY

Identification of a novel mutation in the arginine vasopressin-neurophysin II gene affecting the sixth intrachain disulfide bridge of the neurophysin II moiety

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Abstract

Objective: Most mutations of the arginine vasopressin-neurophysin II (AVP-NP_{II}) gene cause autosomal dominant familial neurohypophyseal diabetes insipidus (adFNDI). Such mutations are predicted to alter the three-dimensional structure of the prohormone, which accumulates in the cell body, ultimately leading to neuronal degeneration and hormonal deficit. In this study we describe the case of a 26-year-old female reporting a long-lasting history of polyuria/polydipsia. The father of the patient was affected by diabetes insipidus and was under desmopressin treatment until the time of his death. Nevertheless, the patient had never been subjected to endocrine evaluation.

Design and methods: Clinical and genetic studies were performed. An 8-h fluid deprivation test plus desmopressin challenge and a 5% saline solution test were performed, in order to confirm the diagnosis. DNA was extracted from peripheral blood lymphocytes and subjected to direct sequencing of the entire coding region of the AVP-NP_{II} gene.

Results and conclusions: Clinical assessment of the patient confirmed the diagnosis of neurohypophyseal diabetes insipidus. Desmopressin treatment was started, which effectively reversed the polyuria/polydipsia syndrome. Genetic analysis revealed a novel mutation (1665T > A) in exon 2 of the AVP-NP_{II} gene, disrupting one of the disulfide bonds present in the NP_{II} moiety which play a fundamental role in determining the proper folding of the molecule. In summary, in the present study we have described a novel mutation of the AVP-NP_{II} gene, which is consistent with the misfolding/toxicity hypothesis underlying the pathogenesis of adFNDI.

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Introduction

Autosomal dominant familial neurohypophyseal diabetes insipidus (adFNDI) is a rare disorder characterized by polyuria accompanied by polydipsia, and which is due to a deficient secretion of the antidiuretic hormone arginine vasopressin (AVP) (1, 2). In most cases the disease is not present at birth but develops in early childhood. Autopsy studies have demonstrated a selective degeneration of the AVP-producing magnocellular neurons in the supraoptic and paraventricular nuclei of the hypothalamus (3–6). Fifty-four different mutations in the AVP-neurophysin II (AVP-NP_{II}) gene (GenBank accession number M11166), linked to adFNDI, have been identified so far. The majority of the known mutations are located in the coding region of the NP_{II} moiety (i.e. 7–9), whereas only a few mutations localize in the part of the gene encoding the signal peptide (7). Only recently, one mutation affecting the AVP moiety has been described (10). Mutations affecting the AVP-NP_{II} gene are predicted

to determine cytotoxic accumulation of the prohormone in the supraoptic and paraventricular nuclei, ultimately leading to neuronal damage. The expression of mutations causing adFNDI in cell models supports this hypothesis, because the mutant AVP prohormone was constantly found to be retained in the endoplasmic reticulum (11–17). More recently, these observations have been confirmed in transgenic animals expressing a mutated AVP-NP_{II} gene (18–20).

The NP_{II} moiety contains seven disulfide bridges, which are covalent bonds formed by the oxidation of two cysteine residues and are essential in determining the three-dimensional structure of the molecule. In particular, disulfide bonds occur between codons 41–85, 44–58, 52–75, 59–65, 92–104, 98–116 and 105–110 of the prepro-AVP-NP_{II} sequence. The formation of disulfide bonds occurs during the folding of proteins in the endoplasmic reticulum and their function is to stabilize the structure of proteins. Mutations involving cysteine residues which form disulfide bonds in the NP_{II} protein have been described (one in

the third bond, one in the fourth, three in the fifth, four in the sixth and three in the seventh) (16, 21–28). These mutations are predicted to destabilize the folded protein and allow the formation of abnormal disulfide bridges with some of the other cysteine residues. We describe here the clinical and molecular features of a patient with neurohypophyseal diabetes insipidus, in which a novel mutation (1665T > A), determining a substitution of cysteine at position 98 of the prepro-AVP-NP_{II} by serine, was identified by direct sequencing.

Subjects and methods

Subjects

Studies were performed on the affected patient, as well as on her son, her sister and two paternal aunts who did not report symptoms suggestive for diabetes insipidus. No other living relative of the patient was reported to have polyuria/polydipsia.

Clinical procedures

A 26-year-old woman was referred to our unit by her family doctor, because of a long-lasting history of polyuria/polydipsia. Her father, dead at the age of 43 secondary to an acute myocardial infarction, was affected by neurohypophyseal diabetes insipidus and was under desmopressin treatment. Nevertheless, the patient had never been evaluated before in relationship to the polyuria/polydipsia history together with the familial anamnesis of diabetes insipidus. However, the mother remembered her daughter crying a lot as an infant, and the patient reported that, to her memory, she had always been affected by polyuria/polydipsia. An 8-h fluid deprivation test followed by 2 µg desmopressin i.m. (DDAVP, Minirin; Ferring Pharmaceuticals Ltd, North York, Ontario, Canada) was performed, as well as a challenge with 5% saline solution. Plasma levels of prolactin (PRL), growth hormone (GH), insulin-like growth factor-I (IGF-I), follicle-stimulating hormone (FSH), luteinizing hormone (LH), adrenocorticotropin (ACTH), cortisol, thyrotropin (TSH), free thyroxine (fT₄), and free triiodothyronine (fT₃) were determined. A computed tomography (CT) scan of the brain was performed.

Genetic analysis

Genomic DNA was extracted from peripheral blood lymphocytes using a standard proteinase K and phenol–chloroform method. The coding region of the AVP-NP_{II} gene and the exon–intron boundaries were amplified by PCR using the primers and the conditions reported in Table 1, after a previous denaturation step at 96 °C for 5 min.

The PCR reactions were performed in a total volume of 100 µL, using 1 µg genomic DNA, 10 µM of each primer, 10 µM of dNTPs, PCR buffer (10 mM Tris–HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatin) and 2U Taq polymerase. Because of the high guanine–cytosine (GC) content of exon 3, 10% DMSO was added to the PCR buffer. Amplicons were purified using the QIAquick purification kit (Qiagen, Crawley, W Sussex, UK) and direct sequencing was carried out using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems (ABI) Warrington, Cheshire, UK), according to the manufacturer's instructions. Sequencing reactions were purified using the DyeEx 2.0 Spin kit (Qiagen) and samples were run on an ABI 310 capillary sequencer (Applied Biosystems).

Modeling

Three-dimensional modeling of the wild-type and the mutated NP_{II} protein was performed by SWISS-MODEL, a fully automated protein structure homology-modeling server (<http://www.expasy.org/spdbv/>) (29).

Results

Clinical studies

The results of the fluid deprivation test and the desmopressin test, shown in Table 2, unequivocally indicated that the patient was affected by neurohypophyseal diabetes insipidus. In Fig. 1 the inverse relationship between the urinary volume and the urine osmolality during the fluid deprivation test and after desmopressin administration is shown. In addition, the diagnosis was further confirmed by i.v. infusion of hypertonic (5%) saline solution (0.06 ml/kg/min) (Table 3). The test was stopped after 45 min (a total volume of 170 ml

Table 1 PCR conditions and primers sequences for the AVP gene. All the PCR reactions were preceded by a denaturation step at 96 °C for 5 sec.

Exon	PCR conditions		Primers sequence (5' → 3')
Exon 1	96 °C for 30 sec } 62 °C for 30 sec } 72 °C for 30 sec }	35 cycles	CACCAAGCAGTGCTGCATAC CTCTTTCTAGCCCTGACC
Exon 2	96 °C for 30 sec } 65 °C for 30 sec } 72 °C for 45 sec }	35 cycles	CTCGCTGCGTTCCCCTCCAACCCCTCGACTC CCCCAGCCCCAGGCCCGCCCCGCGCGC
Exon 3	96 °C for 30 sec } 60 °C for 30 sec } 72 °C for 30 sec }	35 cycles	CCGTGCTCACACGTCCTC CATTGGCGGAGGTTTATTGT

Table 2 Results of the fluid deprivation test plus DDAVP test.

Time (h)	Weight (kg)	Plasma osmolality (mosm/kg)	Time (h)	Urine		Urine osmolality (mosm/kg)
				Volume (ml/h)	Density	
0700	63	279	2400–0700	270	1004	103
0800	62.5	277	0700–0800	310	1003	84
0900	62.1	—	0800–0900	310	1002	83
1000	61.8	286	0900–1000	310	1001	87
1100	61.5	288	1000–1100	310	1001	98
1200	61	316	1100–1200	255	1002	135
1300	60.8	290	1200–1300	155	1003	171
1400	60.7	289	1300–1400	114	1003	220
1500	60.6	287	1400–1500	127	1002	196
DDAVP injection						
1800	61.9	284	1500–1800	8.6	1014	441
2000	62.6	281	1800–2000	52.5	1010	476
0300	62.8	276	2000–0300	20.85	1012	563

was administered), because of vomiting. However, the marked increase in plasma osmolality together with unmeasurably low AVP levels fully confirmed the diagnosis. Plasma levels of PRL, GH, IGF-I, FSH, LH, ACTH, cortisol, TSH, fT₄ and fT₃ were within the normal range and a CT scan of the brain did not reveal any pathological findings. DDAVP treatment was started and polyuria/polydipsia were satisfactorily controlled using 0.2 mg orally twice a day.

Genetic analysis

The pedigree of the family is shown in Fig. 2. Sequence analysis of the entire AVP-NP_{II} coding region in the patient revealed the presence of a heterozygous transversion mutation of a thymidine to adenine at

nucleotide 1665 (1665T > A) in exon 2 (Fig. 3A). This mutation alters codon 98 of the prepro-AVP-NP_{II} sequence, corresponding to codon 67 of the NP_{II} moiety, from TGC to AGC, substituting serine for cysteine (C98S). This mutation disrupts the disulfide bond normally linking C98 with C116. No other mutation was detected in exon 2, or in exon 1 or 3. Because of the previous death of the father of the patient, who was the only member of the family known to be affected by diabetes insipidus, further sequencing was restricted to unaffected relatives (the 1-year-old son of the patient, her sister and two paternal aunts). In no case was the 1665T > A mutation (Fig. 3B) or any other mutation in the coding region of the AVP-NP_{II} gene detected. The effect of the C98S mutation on the predicted

Figure 1 Relationship between diuresis and urinary osmolality during the fluid deprivation test plus DDAVP test.

Table 3 Results of the saline hypertonic infusion test.

Time (h)	Weight (kg)	Plasma		
		BP (mmHg)	Osmolality (mosm/kg)	AVP (ng/l)
0	54	110/80	280	<1
+30 min	62.5	—	299	<1
+45 min	62.1	115/70	299	<1

BP, blood pressure.

Figure 2 Family pedigree. The arrow indicates the carrier of the 1665T > A mutation. The black symbols indicate the family members affected by diabetes insipidus. **Figure 3** Automated sequencing of the AVP-NP11 gene. A portion of exon 2, in which the novel mutation was identified, is represented. (A) The 1665T > A mutation in one allele of the AVP-NP11 gene of the affected patient is indicated by the arrow and (B) the nucleotide substitution was not present in the son of the patient (arrow).

structure of the NP11 protein was evaluated by the SWISS-MODEL server (see section on Modeling). A schematic representation of the three-dimensional structure of the mutated vs the wild-type protein is shown in Fig. 4A and B respectively.

Discussion

This study describes a case of neurohypophyseal diabetes insipidus due to a novel mutation in the

Figure 4 Three-dimensional structure of the (A) mutated and (B) the wild-type NP11, as derived by the SWISS-MODEL server (29) (see also www.expasy.org/spdbv/).

AVP-NP_{II} gene. Although the father of the patient was dead by the time she came to our attention at the age of 26 years, the genetic transmission of the disease is strongly suggested by the fact that he was also affected by diabetes insipidus and was treated with desmopressin throughout his life. Based on the long-lasting history of polyuria/polydipsia of the patient, clinical assessment to evaluate the AVP reserve was performed; this confirmed the diagnosis of diabetes insipidus. Genetic analysis revealed that the patient was heterozygous for the missense mutation 1665T > A encoding the amino acid substitution C98S. This newly described mutation is located in exon 2 of the AVP-NP_{II} gene, which encodes part of the NP_{II} moiety. It affects the sixth of the seven disulfide bonds present in the NP_{II} protein, linking C98 to C116. Two other mutations involving C98 have been described previously. In one case a stop codon was introduced, determining the formation of a truncated protein (24), whereas in the other case the missense mutation 1665T > G determined the amino acid substitution C98G (25). Two other missense mutations affecting the C116 residue, thus disrupting the same disulfide bond, have been described (28). Furthermore, eight mutations involving the other disulfide bonds in the NP_{II} protein have been described (16, 21–23, 26, 27). Therefore, in a total of 55 (included the novel mutation we have described) different mutations in the AVP-NP_{II} gene described so far, almost one-fourth affected one of the seven disulfide bonds present in NP_{II}; these are important in determining its tertiary structure. If only the mutations affecting the NP_{II} moiety are taken into account (a total of 45), the proportion of those affecting cysteine residues which form disulfide bonds rises close to 30%.

This pattern of mutations is very interesting, because it suggests that abnormal folding and self-association of the AVP-NP_{II} precursor may be a common pathogenic mechanism in adFNDI. The misfolding hypothesis predicts a series of abnormalities that could explain the main clinical features of the disease. Similarly to other newly synthesized proteins destined for further processing and secretion (30, 31), pro-AVP-NP_{II} must fold and self-associate correctly in the endoplasmic reticulum before it can be directed to the Golgi apparatus and secretory vesicles for further processing and storage (32). The misfolded protein is retained in the endoplasmic reticulum, thus accumulating and eventually destroying the cell (11–17). Alternatively, a structurally abnormal protein may bind to chaperones (33, 34) and undergo proteolytic degradation (35). The progressive cell loss in adFNDI is in agreement with the dominance of the mutation as a consequence of a dominant negative mechanism, with the delayed onset of the clinical signs of the AVP deficiency, characteristic of these patients, as well as with the autopsy finding of degeneration of magnocellular neurons (3–6).

The accumulation of a structurally abnormal pro-AVP-NP_{II} in the endoplasmic reticulum has been beautifully demonstrated in the past few years in animal models of adFNDI (18–20). Among the four transgene models of naturally occurring human mutations causing adFNDI generated so far, the C98stop mutation was targeted in three cases (18–20), whereas in the remaining case the AVP signal sequence was mutated [A(-1)T] (20). The C98stop mutation determined an early onset of diabetes insipidus and a markedly worse phenotype in mice than the [A(-1)T] mutation (20), similarly to that which occurs in humans. The mutated protein accumulated in the endoplasmic reticulum as autophagic vesicles targeted for lysosomal degradation (18, 19). This phenomenon appears to be substantiated by the observed overexpression of immunoglobulin heavy chain binding protein (BiP), a member of the heat-shock proteins 70 (HSP70) family of molecular chaperones, which binds avidly to misfolded proteins accumulated in the endoplasmic reticulum (20). Eventually, the autophagic vesicles might also trap the wild-type protein by aberrant disulfide bond interactions with the mutant protein. The 'autophagy hypothesis' does not exclude the possibility that neuronal atrophy might ensue with the progression of time. In fact, loss of AVP-producing neurons was found in mutant animals (20). Besides the C98stop mutation, the C98G substitution was also found to determine a very early onset of diabetes insipidus in an affected family (25), thus further suggesting that the C98 plays a critical role in the proper pro-AVP-NP_{II} folding and that substitutions at this amino acid residue may be particularly deleterious and lead to rapid accumulation of misprocessed precursor. In the case we have described here the exact age of onset of the disease of the patient could not be ascertained, because of the poor compliance of the patient as well as of her relatives. Surprisingly enough, considering the presence of diabetes insipidus in her family, she came to medical attention only at the age of 26. Nevertheless, as far as she could remember, she had 'always' been affected by polyuria and polydipsia. In addition, her mother well remembered her daughter crying a lot when she was an infant, possibly suggesting a dehydration status. Therefore, in agreement with previous observations, apparently also in this case was a mutation involving the C98 of the prepro-AVP-NP_{II} molecule associated with a precocious onset of diabetes insipidus.

In conclusion, in the present study we have described a case of FNDI due to a novel missense mutation disrupting the sixth disulfide bond in the AVP-NP_{II} precursor protein. This mutation enlarges the group of genetic abnormalities in the AVP-NP_{II} gene involving a cysteine residue and underlines the fundamental role of disulfide bonds in maintaining the structural and functional integrity of the molecule.

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