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A SWI/SNF related autism syndrome caused by *de novo* mutations in *ADNP*

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Author contributions

The study was designed and the results were interpreted by ATVvS, BBAdV, TK, BPC, EEE, CH, GV, NVdA, RFK. Subject ascertainment and recruitment were carried out by ATVvS, JHMSH, CLM, MHW, BBAdV, TK, CR, JvdE, NVdA, AN, GA, MB, MW. Sequencing, validation and genotyping were carried out and interpreted by CH, LR, GV, HM, KTW, PB, BPC, LELMV, MF, KTW and HGY. The manuscript was drafted by CH, GV, NVdA and RFK. All authors contributed to the final version of the paper.

Competing financial interests

EEE is on the scientific advisory boards for Pacific Biosciences, Inc., SynapDx Corp., and DNAnexus, Inc.

URLs

Clinical information: http://www.adnpgene.com
Galaxy pipeline: http://biominavm-galaxy.biomina.be/galaxy/u/geert-vandeweyer/w/adnp
VariantDB: http://www.biomina.be/app/variantdb
Realtime PCR design tool: http://biomina.be/apps/qpcr-primers
Exome Variant Server: http://evs.gs.washington.edu/EVS/

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Abstract

Despite a high heritability, a genetic diagnosis can only be established in a minority of patients with autism spectrum disorder (ASD), characterized by persistent deficits in social communication and interaction and restricted, repetitive patterns of behavior, interests or activities[1]. Known genetic causes include chromosomal aberrations, such as the duplication of the 15q11-13 region, and monogenic causes, such as the Rett and Fragile X syndromes. The genetic heterogeneity within ASD is striking, with even the most frequent causes responsible for only 1% of cases at the most. Even with the recent developments in next generation sequencing, for the large majority of cases no molecular diagnosis can be established [2-7]. Here, we report 10 patients with ASD and other shared clinical characteristics, including intellectual disability and facial dysmorphisms caused by a mutation in *ADNP*, a transcription factor involved in the SWI/SNF remodeling complex. We estimate this gene to be mutated in at least 0.17% of ASD cases, making it one of the most frequent ASD genes known to date.

Recent developments in next-generation sequencing (NGS), in particular whole-exome sequencing (WES), have substantially increased our insights in the genetic causes of neurodevelopmental disorders. By trio analysis of patients with intellectual disability, a causal *de novo* mutation can be identified in 16-50% of cases[8-11]. Interestingly, intellectual disability shows a high comorbidity with ASD, which is present in up to 40% of intellectual disability cases and may be caused by defects in the same genes or pathways[12-14]. This observation prompted the analysis of existing ASD cohorts with WES[2,3,5,6,15]. Although mutations were identified in a significant percentage of ASD patients, most mutations seem to be unique and recurrently mutated genes are scarce[16].

In an initial cohort of 10 patients with intellectual disability, ASD and facial dysmorphisms, we identified a patient with a *de novo* mutation in the transcription factor *ADNP* using WES (Supplementary Fig. 1). *De novo* loss of function mutations in this gene had previously been identified in two patients by WES[2] and targeted resequencing[16] of patients with ASD. In those studies however, causal relationship did not reach locus-specific significance. Based on these initial findings and the association of *ADNP* with neuronal cell differentiation and maturation[17], as well as the cognitive abnormalities observed in a mouse model[18], we considered *ADNP* a strong candidate gene. We subsequently identified three mutations in *ADNP* in 240 patients from three independent WES studies (Table 1). Next, we targeted *ADNP* using molecular inversion probes (MIPs) or high resolution melt curve analysis (HRM) in a cohort of 2,891 patients with syndromic ASD and identified four more patients with mutations in this gene. In total, ten mutations were found in 5,776 patients. For nine patients the parents were available for testing and in each case the mutation appeared *de novo* (Table 1). We found no additional non-synonymous *de novo* variants. Neither did we find X-chromosomal, compound or homozygous variants in known intellectual disability/ASD genes. Autism and comorbidity with mild to severe intellectual disability is a

consistent feature in all patients (Table 2, Supplementary Note). Other frequent findings include hypotonia, feeding problems in infancy and congenital heart defects. A seizure disorder was noted in two patients. Additional neuropsychiatric features are relatively common, including attention deficit/hyperactivity disorder, anxiety disorder and obsessive compulsive behavior. Dysmorphic features include a prominent forehead, high hairline, eversion or notch of the eyelid, broad nasal bridge, thin upper lip and smooth/long philtrum (Figure 1).

All mutations are heterozygous frameshift or nonsense variants in the C-terminal part of the last exon of *ADNP* and result in a premature termination codon (Table 1). None were present in the 1,000 Genomes Project[19], in 1,728 MIP sequenced unaffected siblings form the Simons Simplex Collection, or in 192 HRM analyzed chromosomes from healthy Belgian controls. Putative truncating mutations for *ADNP* are in fact rare. Only one p.Q361* nonsense mutation upstream of all our mutations was reported in the 13,006 alleles of the Exome Sequencing Project (ESP). An inherited p.Gly1094Profs*5 mutation was identified by MIP sequencing[16] but the reported frameshift is the ninth amino acid from the C-terminal end of the protein and not associated with any protein domains. Typically, variations that close to the end of a protein are unlikely to affect function. The frequency of truncating mutations in *ADNP* is significantly higher (p: 0.001852, odds ratio 13.24668, one-sided Fisher's exact test) in patients compared to the ESP and Simons Siblings controls. In addition to the case-control analysis, we calculated a locus-specific enrichment for truncating variation using a probabilistic model derived from human-chimpanzee fixed difference and sequence context as described[16]. Under a *de novo* rate of 1.2 non-synonymous coding variants per individual, we estimate the probability of detecting eight or more *de novo* truncating events in *ADNP* within our cohort as p = 2.65e-18 (binomial test).

The mutated gene, *ADNP* (chr20:49506883-49547527, GRCh37/hg19), contains five exons, of which the last three are translated (Figure 2). The protein consists of 1,102 amino acids and contains nine zinc fingers and three other functional domains, including NAP, an eight amino acid neuroprotectant peptide (NAPVSIPQ)[20,21]. Administration of NAP ameliorated the short-term memory deficits in *ApoE* knockout mice, a model for Alzheimer's disease[22]. In *ADNP*$^{+/-}$ mice, NAP treatment restores learning and memory and reduces neurodegeneration[18]. Further downstream a DNA binding homeobox domain is present, homologous to the *HOX* gene family homeobox domains (InterPro, EBI). A P*V*L motif, which can bind the HP1 protein is located just downstream of the homeobox domain. The HP1 protein binds to and mediates H3 lysine 9 trimethylation histone posstranslational modification[23-25]. The homeobox domain and the HP1-binding motif are responsible for the transcription factor function of *ADNP*.

Almost the complete 1.6Kb sequence spanned by the mutations is conserved in mammals (PhyloP M=1.52, s=1.25)[26]. All mutations result in the loss of at least the 166 last C-terminal amino acids. Strikingly, the identified mutations appear to cluster at specific positions. The 4bp *de novo* deletions in both patient 6 and 8 are identical even though these patients are unrelated and were born and live in different countries. This mutation is separated by only one nucleotide from the 4bp deletion in patient 1. Additonally, the mutations observed in patients 5 and 10 fall within the 13bp deletion in patient 4. Clustering

of *de novo*, rare variants is suggestive of a mutation predisposition mechanism, potentially as a result of a particular local genomic architecure. We found no evidence for the presence of simple or tandem repeats in this region. Mfold analysis (web server for nucleic acid folding and hybridization prediction)[27] showed that the clustered 4bp deletions of patients 1, 6 and 8 are located in the stem of the same short hairpin (Supplementary Fig. 2). Hence, we suggest that the underlying mechanism of the mutations may involve a DNA repair defect following pausing of a replication fork at these hairpins.

Since no exon-exon boundary in the *ADNP* mRNA is present downstream from any of the mutations, nonsense mediated RNA decay (NMD) is unlikely[28-30]. Indeed, the mutations were present in the cDNA generated from lymphoblastoid cell lines of patients 1, 2, 6 and 8. To quantify the impact of truncating mutations on the expression of *ADNP*, we performed expression analysis. Also included in the expression analysis is a set of selected genes previously shown to interact or to be coregulated with *ADNP*[18,23,31,32]. The total level of *ADNP* mRNA was significantly increased by 41% in patients 1, 2, 6 and 8 (Supplementary Fig. 3b, Table 3). A single assay specific for the wildtype but not the mutant *ADNP* allele could be generated for patients 1, 6 and 8 to discriminate between wildtype and mutant mRNA expression. The expression of this *ADNPwt* amplicon was not different from controls, demonstrating that the excess *ADNP* mRNA in patients corresponds to the mRNA transcribed from the mutant allele. Since *ADNP* expression is under control of an autoregulatory negative feedback loop[33], the overall upregulation might be a consequence of the inability of the mutant protein to bind the *ADNP* promoter. This suggests a deregulation of the negative feedback leading to increased levels of *ADNP* mRNA in order to restore homeostasis. Expression of *ADNP2* (Supplementary Fig. 3c) was also significantly upregulated in patients, which is in line with the reported high correlation between the expression levels of *ADNP* and *ADNP2*[31]. Of the other genes reported as differentially expressed in *Adnp*$^{-/-}$[23] and *Adnp*$^{+/-}$[18] mice (down: *CCNC, TMPO, PLAGL2*; up: *ABCF3*), only *PLAGL2* was found to be differentially regulated in our patients (Supplementary Fig. 3e). This may be the consequence of differences in tissue and developmental stage between the knockout mice and the human cell lines. Expression levels of *TP53*, reported upregulated in HT29 cells incubated with *ADNP* antioligodeoxynucleotide[32], were significantly increased (Supplementary Fig. 3g), possibly as a result of augmented cellular stress due to an overall deregulation of genes under transcriptional control of *ADNP*.

ADNP has multiple cellular functions that seem compatible with the clinical presentation of our patients. A role in neuronal cell differentiation and maturation was suggested after observing a substantial decrease in the number and size of embryoid bodies and the number of neurites after knockdown of *ADNP* with shRNA in P19 cells[17]. Furthermore, *Adnp*$^{-/-}$ mice are not viable due to failure of the neural tube closure, while *ADNP*$^{+/-}$ mice show tauopathy, neuronal cell death and abnormalities in social behaviour and cognitive functioning[18,34]. The severity of the phenotype in our cohort varies, but all patients exhibit various degrees of ASD and all are intellectually disabled. Dysmorphic features vary from patient to patient, but a prominent forehead, broad nasal bridge, thin upper lip and smooth philtrum are frequently present. Cardiac, brain and behavioral abnormalities are more frequent in our patients than in the general population. At the moment, there are no

indications for a correlation between the individual mutations and the clinical presentation. The mutations in patients 6 and 8 are identical and differ only by a single amino acid from the mutation in patient 1. Yet, these three patients do not share more clinical characteristics amongst each other than with other patients. However, at this moment it is not possible to draw firm conclusions on a possible genotype:phenotype correlation due to the small sample size. No patients with a pure deletion of *ADNP* have been reported. In our own databases and in DECIPHER[35], five deletions with sizes 313Kb-3.31Mb, taking away 5-23 genes including (part of) *ADNP*, have been described. In the four cases where the parents were tested, the deletion was *de novo*. The patients with *ADNP* deletions all share some clinical characteristics with the patients reported here, carrying truncating mutations (Supplementary Fig. 4, Supplementary Table 1).

The C-terminal part of *ADNP* directly interacts with *ARID1A*, *SMARCA4* and *SMARCC2*, three essential components of the BAF complexes, the functional eukaryotic equivalent of the SWI/SNF complex in yeast that is involved in the regulation of gene expression[36]. These ATP-dependent chromatin remodelling complexes consist of 15 subunits, including one of both ATPase core subunits *SMARCA4* or *SMARCA2*[37]. A switch of complex composition is essential for initiation of post-mitotic activity-dependent dendritic outgrowth and axonal development. This transition of neural progenitor cells to mature neurons occurs in all neurons and highlights the fundamental role of BAF complexes in neural development[38]. Interestingly, mutations in patients with intellectual disability have been reported in six components of these complexes (*SMARCB1*, *SMARCA4*, *SMARCA2*, *SMARCE1*, *ARID1A* and *ARID1B*). The phenotype associated with mutations in these genes ranges from non-syndromic intellectual disability with hypotonia and speech delay to recognizable syndromes such as the Coffin-Siris syndrome and Nicolaides-Baraitser syndrome. These disorders are sometimes refered to as “SWI/SNF-related intellectual disability syndromes”[39-41]. It has been proposed that the syndromic features might be explained by the role of BAF complexes in developmental processes in the affected tissues[42]. It is believed that most reported mutations in these genes have a dominant negative effect on the functioning of the BAF complex as a whole[43-46]. As we were able to detect mutant RNA in our patients, we hypothesize that the mutant *ADNP* protein competes with the wildtype protein in aberrant interaction with the BAF complex. Wildtype *ADNP* directly binds target genomic regions and mediates the recruitment of the BAF complex through the C-terminal end. Hence, it can be hypothesised that the mutant protein with an altered C-terminal structure will hamper the recruitment of the BAF complex, while it still occupies DNA binding sites. This will lead to a diminished functionality of the complex and ultimately to deregulation of several cellular processes.

In summary, we identified a recurrent SWI/SNF-related ASD syndrome, caused by mutations in *ADNP*. These findings expand the phenotypic spectrum of SWI/SNF-related disorders, several of which are caused by mutations in direct interaction partners of *ADNP*. Mutations in *ADNP* may explain the etiology of 0.17% of patients with ASD (95% binomial confidence interval: 0.083% - 0.32%) and thus constitute one of the most frequent known causes of autism. Our findings will increase the diagnostic yield in this population and

studies on the role of *ADNP* in development may raise hope for treatment of these patients in the long term.

Methods

Patients

Patients were selected for inclusion in this study from different cohorts tested on either family-based WES, targeted resequencing or high-resolution melting analysis (Table 1). Clinical evaluation was performed by at least one expert clinical geneticist. Written informed consent for inclusion in the study was obtained for all patients and consent for the publication of photographs was obtained for patients 1, 2, 4, 5, 6 and 8.

Sanger sequencing

Primers were designed using Primer3[47,48]. PCR was performed using GOTaq polymerase (Promega, Madison, WI, USA) on DNA from peripheral blood and on cDNA from lymphoblastoid cells, using standard protocol. Capillary electrophoresis sequencing (ABI 3130 genetic analyzer; Applied Biosystems, Carlsbad, CA, USA) was performed using the ABI BigDye terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA), following standard protocol. Data was analysed in CLC DNA Workbench (CLC Bio, Aarhus, Denmark).

Whole-exome sequencing (WES)

Patient 1 was detected in a family-based WES study (unpublished data (CH, GV, Filip Van Nieuwerburg, NVdA, RFK)). Patient DNA was fragmented using Covaris® M220 Focused-ultrasonicator™ (Covaris, MA, USA), followed by TruSeq DNA Sample Preparation (Illumina Inc, San Diego, CA, USA), enrichment using the SeqCap EZ Human Exome Library v3.0 kit (NimbleGen, Roche, Penzberg, Germany), and sequencing on HiSeq 2000 (Illumina Inc, San Diego, CA, USA), all following standard protocols. Data analysis was performed using Galaxy (see URLs)[49-51]. Variants were filtered by VariantDB (see URLs, manuscript in preparation) to exclude variants with (1) low quality, using thresholds based on correlation between NGS data and SNP-chip genotyping; (2) intronic or intergenic location, except splice sites; and (3) inheritance from the parents. WES sequencing of patients 2, 3 and 4 was performed as described[2,8]. The mutation in patient 5 was identified in a family trio based study. WES was performed using Illumina technology (Illumina Inc, San Diego, CA, USA), and sequence data was returned and analysed using software supplied from Oxford Gene Technology. Presence of reported (*de novo*) mutations were confirmed by an independent technique such as Sanger sequencing. Raw sequence data will be uploaded in The European Genome-phenome Archive (EMBL-EBI) database.

Molecular inversion probes (MIPs)

Patients 7, 8 and 9 were discovered from a MIP based screen of 2743 probands with intellectual disability and/or ASD). Patient 10 was included from a MIP based screen of 2446 autism patients from the Simon Simplex Collection (SSC)[16]. The MIP screening and analysis was performed as previously described, and MIP probe sequences for *ADNP* are

available in O'Roak *et al*, 2012[16]. Inheritance determination and validation were performed by Sanger sequencing.

High-resolution melting (HRM)

192 control chromosomes were screened for the presence of the mutations identified in the 10 patients using HRM. Primers were designed using the HRMA Assay Design module of Beacon Designer™ 8.10 (Premier Biosoft, CA, USA). HRM was performed on a LightCycler 480 (Roche, Penzberg, Germany) with the LCGreen$^+$ incorporating dye (Idaho Technology Inc., Salt Lake City, UT, USA). Meltcurve analysis performed by the Gene Scanning module of the LightCycler software. Samples with deviating curves were analysed by Sanger sequencing. The mutation in patient 6 was identified using the same protocol, as part of the cohort of 148 probands with idiopathic ASD, for which microarray analysis did not reveal any abnormalities.

Real-time quantitative PCR

RNA isolation, cDNA synthesis and quality control were performed as described earlier[52]. mRNA expression was examined by an optimized three-step real-time quantitative PCR assay following the protocol described before[53]. Besides *ADNP* itself, *ADNP2* was included based on the reported correlation of expression in human brain tissue[31]. *TMPO*, *CCNC* and *PLAGL2* were reported to be significantly downregulated in homozygous *Adnp* knockout mice embryos, while *ABCF3* was reported to be upregulated in heterozygous *Adnp* knockout mice embryos[18,23]. Finally, *TP53* is upregulated in HT29 cells incubated with ADNP antioligodeoxynucleotide[32]. *YWHAZ* and *HPRT* were selected as reference genes, according to geNorm calculations[54]. qPCR primers were selected from literature[31,55], the RTPrimerDB[56] or designed using an in-house automated pipeline (see URLs), conforming to requirements of intron-spanning location, no SNP content, no dimer formation at the 3' end of the primers, and low amplicon folding, with no folding in primer binding sites. The amplification efficiency of the different primers was assessed and confirmed to be above 1.85. Primer sequences are available on request. Expression values of two cDNA syntheses originating from two different RNA isolations per patient were compared to the values obtained from eight control individuals. Statistical testing was performed using linear mixed models in order to investigate significant differences in expression levels of the patients compared to controls.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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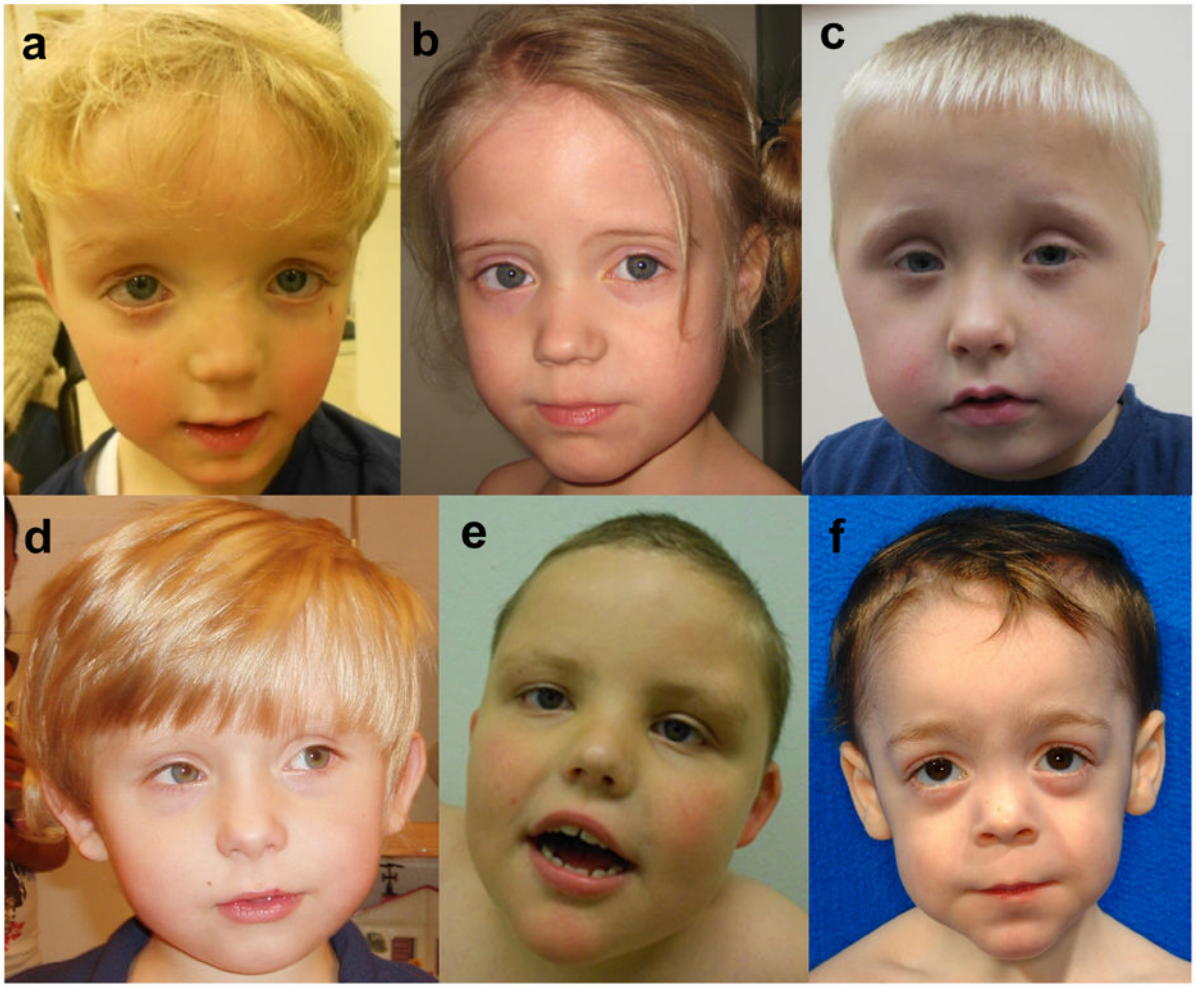

Figure 1.

Frontal facial photographs of patient 1 (**a**),2 (**b**), 4 (**c**), 5 (**d**), 6 (**e**) and 8 (**f**) at young age. Note the clinical similarities, including a prominent forehead, a thin upper lip and a broad nasal bridge. Consent for the publication of photographs was obtained for these patients (1, 2, 4, 5, 6 and 8).

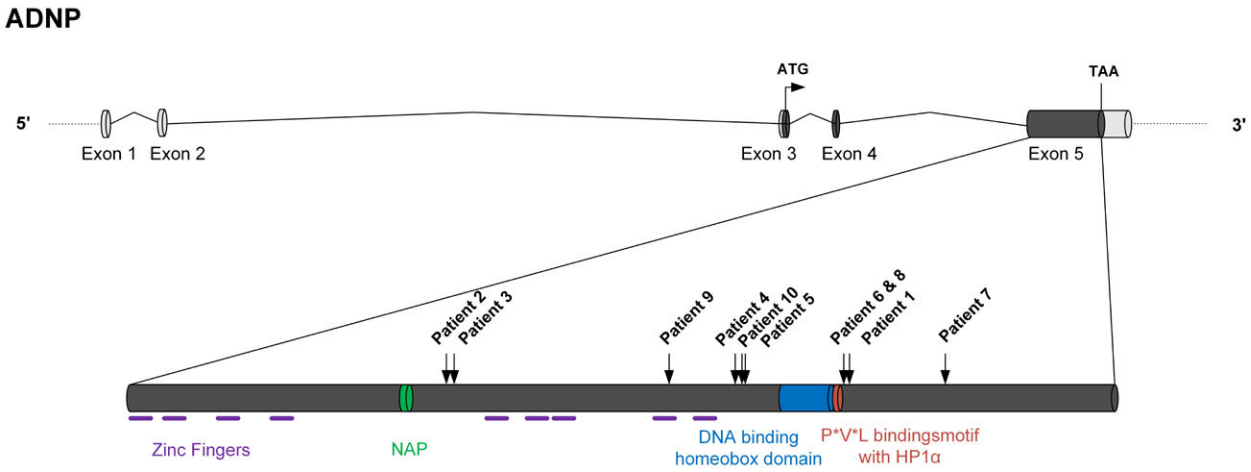

Figure 2.

Schematic overview of the *ADNP* gene structure and functional domains. Identified mutations and corresponding patients are indicated by black arrows.

Table 1

Summary of mutations, detection methods and cohorts compositions for the reported patients. All genomic coordinates relate to genome build GRCh37. WES: Whole Exome Sequencing, HRM: High Resolution Melting, MIPs: Molecular Inversion Probes

Patient	Patient ID	Origin	Screening method	Cohort composition	Cohort size	mutation in genomic DNA (chr20)	mutation in cDNA (NM_015339.2)	Protein	Mutation Type	Inheritance
1	111294	Antwerp	WES	Moderate to severe intellectual disability and/or autism + dysmorphic features	10	g.49508752_49508755delTTTA	c.2496_2499delTAAA	p.Asp832Lysfs*80	Frameshift	*de novo*
2	11-08612	Nijmegen	WES	Non-syndromic severe intellectual disability	100	g.49510040G>T	c.1211C>A	p.Ser404*	Nonsense	*de novo*
3	12130.p1	Seattle	WES[2,16]	ASD from the Simon Simplex Collection	189	g.49510028_49510029delTT	c.1222_1223delAA	p.Lys408Valfs*31	Frameshift	*de novo*
4	1050237	Westmead	WES	Non-syndromic severe intellectual disability	95	g.49509086_49509098delATTTGCTCGTAAG	c.2153_2165delCTTACGAGCAAAT	p.Thr718Glyfs*12	Frameshift	*de novo*
5	3061-08D	Stockholm	WES	Moderate to severe intellectual disability and/or autism + dysmorphic features	45	g.49509094G>C	c.2157C>G	p.Tyr719*	Nonsense	*de novo*
6	122793	Antwerp	HRM	Autism	148	g.49508757_49508760delTTAA	c.2491_2494delTTAA	p.Lys831Ilefs*81	Frameshift	*de novo*
7	07-06960	Nijmegen	MIPs	Intellectual disability and/or autism	2743*	g.49508443delG	c.2808delC	P.Tyr936*	Frameshift	*de novo*
8	2376	Troina	MIPs	Intellectual disability and/or autism	2743*	g.49508757_49508760delTTAA	c.2491_2494delTTAA	p.Lys831Ilefs*81	Frameshift	*de novo*
9	2533	Troina	MIPs	Intellectual disability and/or autism	2743*	g.49509321G>A	c.1930C>T	p.644Arg*	Nonsense	parents not available
10	13545.p1	Seattle	MIPs[16]	ASD from the Simon Simplex Collection	2446	g.49509094_49509095insT	c.2156_2157insA	p.Tyr719*	Frameshift	*de novo*

* patients 7, 8 and 9 from the same cohort

Table 2

Clinical characteristics of the patients with ADNP mutations

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Total
Sex	M	F	F	M	M	M	F	M	F	M	
Developmental delay (motor)	+	+	+	+	+	+	+	+	-	+	9/10
Developmental delay (speech)	+	+		+	+	+	+	+	-	+	8/9
Intellectual Disability	mild	mild	mild	severe	severe	severe	mild	severe	mild	severe	10/10
ASD	+	+	+	+	+	+	±	+	±	+	10/10
ADHD	-	-	+	-	-	+	-	-	-		2/9
Hypotonia	+	+	+	+	+	+	-	+	-		7/9
Growth retardation / Short stature	+	-		+	+	-	-	+	+		5/8
Feeding problems	+	+	+	+	-		-	-		+	5/8
Recurrent infections	+	+		-	+	-	-	+	+		5/8
Congenital heart defect	+	+	-	-		-		+	-	-	3/8
Hyperlaxity	+	+		+	+	+	-	-	+		6/8
Obesity		-		-	+	+	-	±	+		4/7
Hypermetropia		+		+	+		+	+	+		6/6
Seizures		+		-	-	+	-	-	-		2/7
Behavior		+	+	-	+		-	+	+		5/7
Insensitivity to pain				-	+	+		-	-		2/5
MRI brain abnormality	+	+	-	+	+	-		+	-	-	5/9
Prominent forehead	+	+		-	+	-	+	+	-		5/8
High hairline	+	+		-	+	+	+	+	+		7/8
Eversion/notch eyelid	+	+		-	-		-	+	-		3/7
Hypertelorism	+	-		-	-	-	-	-	-		1/8
Broad nasal bridge	+	+		+	-	+	-	+	+		6/8
Short nose	-	-		-	-	+	-	-	+		2/8
Thin upper lip		+		+	+	+	+	+	-		6/7
Hand abnormalities	+	+	+	+	-		-	+	+		6/8
Constipation		-		+	+		-	-	-		2/6

Table 3

Realtime quantitative expression analysis of mRNA from EBV transformed lymphoblastoid cell lines of patients 1, 2, 6 and 8 compared to 8 control samples.

Gene	Relative Expression (%)	S.E.M.	P-value	Significance
ABCF3	94.03	14.31	0.6507	
ADNP	141.67	13.4	0.0101	*
ADNP2	148.52	17.58	0.0060	**
ADNPwt	74.28	4.14	0.0729	
CCNC	87.98	6.72	0.2857	
PLAGL2	153.49	21.4	0.0040	**
TMPO	80.26	11.24	0.2462	
TP53	164.81	6.17	0.0003	***

* : $p < 0.05$,

** : $p < 0.01$,

*** :$p<0.001$, according to Linear Mixed Models.