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

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Despite the high heritability of autism spectrum disorders (ASD), characterized by persistent deficits in social communication and interaction and restricted, repetitive patterns of behavior, interests or activities1, a genetic diagnosis can be established in only a minority of patients. Known genetic causes include chromosomal aberrations, such as the duplication of the 15q11-13 region, and monogenic causes, as in 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 established2,27. Here, we report ten 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-associated genes known to date.

Recent developments in next-generation sequencing (NGS), in particular whole-exome sequencing (WES), have substantially increased our insights into 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 cases8–11. 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 pathways2–7. This observation prompted the analysis of existing ASD cohorts with WES2,3,5,6,15. Although mutations were identified in patients with ASD, most mutations seem to be unique, and recurrently mutated genes are scarce16.

In an initial cohort of ten patients with intellectual disability, ASD and facial dysmorphisms, we identified a patient with a de novo mutation in the transcription factor–encoding gene ADNP using WES (Supplementary Fig. 1). De novo loss-of-function mutations in this gene had previously been identified in two patients by WES2 and targeted resequencing16 of patients with ASD. In those studies, however, the causal relationship did not reach locus-specific significance. On the basis of these initial findings and the association of ADNP with neuronal cell differentiation and maturation17, as well as the cognitive abnormalities observed in a mouse model18, we considered ADNP a strong candidate gene. We subsequently identified 3 mutations in ADNP in 240 patients from 3 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 4 more patients with mutations in this gene. In total, 10 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 nonsynonymous de novo variants. Neither did we find X-chromosomal, compound or homozygous variants in genes known to be associated with intellectual disability or ASD. Autism and comorbidity with mild to severe intellectual disability is a consistent feature in all patients (Table 2 and Supplementary Note). Other frequent findings include hypotonia, feeding problems in infancy and

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Table 1

| Patient ID | Disorder | Origin | Method | Mutations in genomic DNA (chr. 20) | Protein change | Inheritance | Notes |
|------------|----------|--------|--------|------------------------------------|----------------|-------------|-------|
| 111294     | ADNP     | Antwerp| WES    | g.49508752_49508755delTTTA c.2496_2499delTAAA p.Asp832Lysfs*80 Frameshift | |
| 22868140   | ADNP     | Antwerp| WES    | g.49509094G>C c.2157C>G p.Tyr719* Nonsense | |
| 22868140   | ADNP     | Antwerp| WES    | g.49509086_49509098delATTACGAGCAAT TGCTCGTAAG c.2153_2165delCTTAC GAGCAAAT TGCTCGTAAG p.Thr718Glyfs*12 Frameshift | |
| 3061-08D   | ADNP     | Stockholm| MIPs| g.49508443delG c.2808delC P.Tyr936* Frameshift | |
| 2376       | ADNP     | Troina | MIPs   | g.49508757_49508760delTTAA c.2491_2494delTTAA p.Lys831Ilefs*81 Frameshift | |

**Note:** All mutations are heterozygous frameshift or nonsense variants in the 3’ end of the last exon of ADNP and result in a premature termination codon (Table 1). None were present in the 1000 Genomes Project, in 1,728 MIP-sequenced unaffected siblings from 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 nonsense mutation encoding p.Gln361* upstream of all our mutations was reported in the 13,006 alleles of the Exome Sequencing Project (ESP). An inherited mutation encoding p.Gly1094Profs*5 was identified by MIP sequencing, but the reported frameshift affects the ninth amino acid from the C-terminal end of the protein and is 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 = 13.24686, one-sided Fisher’s exact test) in patients compared to the ESP and Simons controls. In addition to conducting the case-control analysis, we calculated locus-specific enrichment for truncating variation using a probabilistic model derived from human-chimpanzee fixed differences and sequence context as described. Under a de novo rate of 1.2 nonsynonymous coding variants per individual, we estimate the probability of detecting eight or more de novo truncating events in ADNP within our cohort as P = 2.65 × 10^-18 (binomial test).

The mutated gene, ADNP (chr. 20: 49,506,883–49,547,527, GRCh37/hg19), contains five exons, of which the last three are translated. The protein consists of 1,102 amino acids and contains nine zinc fingers and three other functional domains, including NAP, an 8-amino-acid neuroprotectant peptide (NAPVSIPQ). Administration of NAP ameliorated the short-term memory deficits in ApoE knockout mice, a model for Alzheimer’s disease. In Adnp-/- mice, NAP treatment restores learning and memory and reduces neurodegeneration. Further downstream, a DNA-binding homeobox domain is present, homologous to the HOX gene family homeobox domains. The HP1 protein binds to and mediates the histone H3 lysine 9 trimethylation post-translational modification. The homeobox domain and the HP1-binding motif are responsible for the transcription factor function of ADNP.

Almost the complete 1.6-kb sequence spanned by the mutations is conserved in mammals (PhyloP mean = 1.52, s.d. = 1.25) (ref. 26). All mutations result in the loss of at least the 166 last C-terminal amino acids. Strikingly, the identified mutations seem to cluster at specific positions. The 4-bp 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 4-bp deletion in patient 1. Additionally, the mutations observed in patients 5 and 10 fall within the 13-bp 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 architecture. 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) showed that the clustered 4-bp deletions of patients 1, 6 and 8 are located in the stem of the same short hairpin.
the excess ADNP mRNA in patients corresponds to the mRNA transcribed from the mutant allele. Because ADNP expression is under the control of an autoregulatory negative feedback loop33, the overall upregulation might be a consequence of the inability of the mutant protein to bind the ADNP promoter. This suggests deregulation of

we suggest that the underlying mechanism of the mutations may involve a DNA-repair defect following pausing of a replication fork at these hairpins.

Because no exon-exon boundary in the ADNP mRNA is present downstream from any of the mutations, nonsense-mediated RNA decay (NMD) is unlikely28–30. 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 ADNP18,23,31,32.

The total expression of ADNP mRNA was significantly ($P = 0.0101$) increased by 41% in patients 1, 2, 6 and 8 (Table 3 and Supplementary Fig. 3b). A single assay specific for the wild-type but not the mutant ADNP allele could be generated for patients 1, 6 and 8 to discriminate between wild-type and mutant mRNA expression. The expression of this ADNPwt amplicon was not different from controls, demonstrating that

**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         | M         | M         | M         | M         | M         | F         | M          |       |
| Developmental delay (motor) | +         | +         | +         | +         | +         | +         | +         | –         | +          | 9/10  |
| Developmental delay (speech) | +         | +         | +         | +         | +         | –         | +         | –         | +          | 8/9   |
| Intellectual disability | Mild     | Mild      | Mild      | Severe    | Severe    | Severe    | Mild      | Severe    | Severe     | 10/10 |
| ASD       | +         | +         | +         | +         | +         | +         | ±         | +         | ±          | 10/10 |
| ADHD      | –         | –         | –         | –         | –         | –         | –         | –         | –          | 2/9   |
| Hypotonia | +         | +         | +         | +         | +         | –         | +         | –         | –          | 5/8   |
| 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   |

±, mildly affected.

**Figure 1** Frontal facial photographs of patients. (a–f) Patients 1 (a), 2 (b), 4 (c), 5 (d), 6 (e) and 8 (f) at young ages. 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).
the negative feedback leading to increased expression of ADNP mRNA to restore homeostasis. Expression of ADNP2 (Supplementary Fig. 3c) was also significantly ($P = 0.0060$) upregulated in patients, which is in line with the reported high correlation between the expression of ADNP and ADNP2 (ref. 31). Of the other genes reported as differentially expressed in Adnp$^{−/−}$ (ref. 23) and Adnp$^{+/−}$ (ref. 18) mice (downregulated: Ccnc, Tmpo, Plagl2; upregulated: 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 of TP53, reported to be upregulated in HT29 cells incubated with ADNP antioligodeoxynucleotide23, was significantly ($P = 0.0003$) increased (Supplementary Fig. 3g), possibly as a result of augmented cellular stress due to an overall deregulation of genes under the 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 short hairpin RNA (shRNA) in P19 cells47. Furthermore, Adnp$^{−/−}$ mice are not viable owing to failure of neural-tube closure, whereas ADNP$^{+/−}$ mice show tautopathy, neuronal cell death and abnormalities in social behavior and cognitive functioning13,34. The severity of the phenotype in our cohort varies, but all patients show 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 of a correlation between the individual mutations and clinical presentation. The mutations in patients 6 and 8 are identical and differ only 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.

Table 3: Real-time quantitative expression analysis of mRNA from Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines of patients 1, 2, 6 and 8 compared to eight 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.
and studies on the role of ADNP in development may raise hope for treatment of these patients in the long term.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

The study was designed and the results were interpreted by A.T.V.-s., B.B.A.d.V., T.K., R.P.C., E.E.E., C.H., G.V., N.V.d.A. and R.F.K. Subject ascertainment and recruitment were carried out by A.T.V.-s., J.H.M.S.-H., C.L.M., M.H.W., B.B.A.d.V., T.K., C.R., J.v.d.E., N.V.d.A., A.N., G.A., M.B. and M.W. Sequencing, validation and genotyping were carried out and interpreted by C.H., L.R., G.V., H.M., K.T.W., P.B., R.P.C., L.E.L.M.V., M.F., K.T.W. and H.G.Y. The manuscript was drafted by C.H., G.V., N.V.d.A. and R.F.K. All authors contributed to the final version of the paper.

**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details are available in the online version of the paper.

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Sanger sequencing. Primers were designed using Primer3 (refs. 47, 48). PCR was performed using GO-Taq polymerase (Promega) on DNA from peripheral blood and on cDNA from lymphoblastoid cells, using standard protocol. Capillary electrophoresis sequencing (ABI 3130 genetic analyzer; Applied Biosystems) was performed using the ABI BigDye terminator V3.1 Cycle Sequencing Kit (Applied Biosystems), following standard protocol. Data was analyzed in CLC DNA Workbench (CLC Bio).

Whole-exome sequencing (WES). Patient 1 was detected in a family-based WES study (C.H., G.V., F.V.N., N.V.d.A. and R.F.K., unpublished data). Patient DNA was fragmented using Covaris M220 Focused-ultrasonicator, followed by TruSeq DNA Sample Preparation (Illumina), enrichment using the SeqCap EZ Human Exome Library v3.0 kit (NimbliGen, Roche), and sequencing on HiSeq 2000 (Illumina), all following standard protocols. Data analysis was performed using Galaxy (see URLs)49–51. Variants were filtered by VariantDB (see URLs) to exclude variants with (i) low quality, using thresholds based on correlation between NGS data and SNP-chip genotyping, (ii) intronic or intergenic location, except splice sites and (iii) inheritance from the parents. WES sequencing of patients 2, 3 and 4 was performed as described25,26. The mutation in patient 5 was identified in a family trio based study. WES was performed using Illumina technology, and sequence data was returned and analyzed 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 2,743 probands with intellectual disability and/or ASD. Patient 10 was included from a MIP-based screen of 2,446 patients with autism from the Simon Simplex Collection (SSC)15. The MIP screening and analysis was performed as previously described, and MIP probe sequences for ADNP are available16. Inheritance determination and validation were performed by Sanger sequencing.

High-resolution melting (HRM). We screened 192 control chromosomes for the presence of the mutations identified in the ten patients using HRM. Primers were designed using the HRMA Assay Design module of Beacon Designer 8.10 (Premier Biosoft). HRM was performed on a LightCycler 480 (Roche) with the LCGreen+ incorporating dye (Idaho Technology). Meltcurve analysis was performed by the Gene Scanning module of the LightCycler software. Samples with deviating curves were analyzed 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 earlier52. mRNA expression was examined by an optimized three-step real-time quantitative PCR assay following the protocol described before53. Besides ADNP itself, ADNP2 was included based on the reported correlation of expression in human brain tissue31. TMPO, CCNC and PLAGL2 were reported to be significantly downregulated in homozygous Adnp knockout mice embryos, whereas ABCF3 was reported to be upregulated in heterozygous Adnp knockout mice embryos18,23. Finally, TP53 is upregulated in HT29 cells incubated with ADNP antioligodeoxynucleotide32. YWHAZ and HPRT were selected as reference genes, according to geNorm calculations54. qPCR primers were selected from literature31,55, the RTPRimerDB56 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 5′ 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 between the patients compared to controls.