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RESEARCH ARTICLE

Common Variants Near ZIC1 and ZIC4 in Autopsy-Confirmed Multiple System Atrophy

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ABSTRACT: Background: Multiple System Atrophy is a rare neurodegenerative disease with alpha-synuclein aggregation in glial cytoplasmic inclusions and either predominant olivopontocerebellar atrophy or striatonigral degeneration, leading to dysautonomia, parkinsonism, and cerebellar ataxia. One prior genome-wide association study in mainly clinically diagnosed patients with Multiple System Atrophy failed to identify genetic variants predisposing for the disease.

Objective: Since the clinical diagnosis of Multiple System Atrophy yields a high rate of misdiagnosis when compared to the neuropathological gold standard, we studied only autopsy-confirmed cases.

Methods: We studied common genetic variations in Multiple System Atrophy cases (N = 731) and controls (N = 2898).

Results: The most strongly disease-associated markers were rs16859966 on chromosome 3, rs7013955 on chromosome 8, and rs116607983 on chromosome 4 with *P*-values below 5×10^{-6} , all of which were supported by at least one additional genotyped and several imputed single nucleotide polymorphisms. The genes closest to the

chromosome 3 locus are *ZIC1* and *ZIC4* encoding the zinc finger proteins of cerebellum 1 and 4 (*ZIC1* and *ZIC4*).

Interpretation: Since mutations of *ZIC1* and *ZIC4* and paraneoplastic autoantibodies directed against *ZIC4* are associated with severe cerebellar dysfunction, we conducted immunohistochemical analyses in brain tissue of the frontal cortex and the cerebellum from 24 Multiple System Atrophy patients. Strong immunohistochemical expression of *ZIC4* was detected in a subset of neurons of the dentate nucleus in all healthy controls and in patients with striatonigral degeneration, whereas *ZIC4*-immunoreactive neurons were significantly reduced in patients with olivopontocerebellar atrophy. These findings point to a potential *ZIC4*-mediated vulnerability of neurons in Multiple System Atrophy. © 2022 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society

Key Words: multiple system atrophy; genome-wide association study; autopsy-confirmed; *ZIC1*; *ZIC4*

Multiple system atrophy (MSA) is a rapidly progressive rare neurodegenerative disease presenting with variable combinations of dysautonomia, parkinsonism, and cerebellar ataxia.¹ Two forms of MSA can be clinically distinguished, characterized by either predominant parkinsonism or predominant cerebellar symptoms.² Its estimated prevalence is 3.4–4.9 cases per 100,000 individuals in the general population, and 7.8 cases per 100,000 in persons older than 40 years.³ The mean survival time from disease onset is 6–10 years.^{4,5} Currently, only limited symptomatic treatments and no disease-modifying therapies are available.⁶

The typical symptoms of MSA are caused by the progressive degeneration of neurons in different brain regions, particularly in the substantia nigra, striatum, inferior olivary nucleus, pons, and cerebellum, but also other parts of the central nervous systems, emphasizing the multisystem character of MSA.^{2,7} The histological hallmarks in brains of patients with MSA are glial cytoplasmic inclusions (Papp–Lantos bodies) in oligodendrocytes containing aggregated and misfolded α -synuclein.⁸ Neuropathologically, two subtypes can be distinguished, one with predominant olivopontocerebellar atrophy (OPCA), the other with mainly striatonigral degeneration (SND).^{9,10} In addition, a mixed phenotype displaying features of both OPCA and SND is found in the brains of some patients.^{9,10}

The pathogenesis of MSA is unclear. MSA is considered a sporadic disease.¹¹ Epidemiological studies have investigated the influence of environmental factors in MSA, including exposure to farming-related factors (pesticides,

solvents, mycotoxins, dust, fuels, oils, fertilizers, animals) and certain lifestyles (consumption of well water, rural living, diet, and physical activity).^{12–14} Apart from a marginal effect of pesticides, no other environmental factors have been convincingly associated with an increased risk for development of MSA.^{12–14}

Hypothesis-driven candidate gene studies have been inconsistent with respect to variants that might be associated with MSA. Associations of MSA with the genes *COQ2*, *SNCA*, *MAPT*, and *PRNP* have been discussed.^{15–20} One prior genome-wide association study (GWAS) did not identify hits of statistical significance at a genome-wide level, despite the analysis of 918 cases and 3864 controls.²¹ This GWAS had mainly included clinically diagnosed MSA cases. It needs to be stressed that clinical diagnosis is frequently not accurate in MSA. For example, a recent clinicopathological study demonstrated a false-positive diagnosis at autopsy in 38% of patients with clinically diagnosed MSA.²²

To avoid inclusion of misdiagnosed patients in the GWAS described in this study, we included only autopsy-confirmed cases and appropriate ethnicity-matched controls.

Subjects and Methods

Patient Recruitment

Ethical approval had been obtained from all responsible ethics committees. All participants had given written consent.

Neuropathologists at each recruitment site (Table 1) based the definite neuropathological diagnosis of MSA on histopathological criteria, taking into account glial cytoplasmic inclusions immunoreactive for α -synuclein in characteristic anatomical distribution as a defining feature of MSA.²³ Age, sex, disease history (including disease onset and duration), and neuropathological findings were recorded in a standardized manner for all cases.

Controls were ethnically matched to cases and either derived from biobanks KORA-gen²⁴ or popGen²⁵ (Europe sites) or from a North American site (Alzheimer's Disease Genetics Consortium).²⁶ The Alzheimer's Disease Genetics Consortium assembled and genotyped DNA from subjects enrolled in the 29 NIA-Alzheimer's Disease Centers located across the United States. For this study, the Alzheimer's Disease Genetics Consortium provided a subset of mostly clinical, cognitively normal controls. Patients and controls were of North-Western European and African American ancestry.

DNA Extraction

We isolated DNA from 30 mg frozen cerebellar cortex using QIAamp DNA Mini Kit (Qiagen, Venlo, the Netherlands). DNA extraction was performed at German Center for Neurodegenerative Diseases (DZNE, Munich, Germany). DNA was stored at -80°C until use. DNA concentration was measured using a NanoDrop Spectrophotometer. DNA quality was determined by gel electrophoresis.

Genotyping

All samples were genotyped on Infinium Global Screening Arrays (Illumina, San Diego, CA, USA). The cases were genotyped at the Institute of Clinical Molecular Biology, Kiel University, Germany. The samples were genotyped in one batch on array version 2.0 for cases and version 1.0 for controls. Genotypes were called using Illumina Genome studio according to the manufacturer's instructions using in-house cluster files.

Quality Control and Imputation

We used PLINK (v. 1.9) [1] and R (v. 3.6.3)²⁷ for all analyses. Only variants successfully genotyped in both the patient and the control populations were included in the subsequent analyses. Variants with multicharacter allele codes, insertions, deletions, duplicated markers, and all A/T and G/C variants were excluded. We excluded all samples discordant between reported and genotypic sex. Missing sex was imputed, and samples with ambiguously imputed sex were discarded. After a first step of filtering out samples and variants with call rate of less than 85%, we excluded variants with an individual call rate of less than 98% in a second filtering step. Next, we removed variants with a minor allele frequency <0.01 , a significant deviation from Hardy-Weinberg equilibrium

($P < 1 \times 10^{-6}$) in controls, or informative missingness ($P < 1 \times 10^{-5}$). Subsequently, we excluded individuals with a variant call rate of $<98\%$ or an outlying heterozygosity rate (mean ± 3 standard deviations). We used a pruned dataset containing only markers in low linkage-disequilibrium regions (pairwise $r^2 < 0.2$) to test for duplicated individuals and cryptic relatedness (Pihat > 0.125) using pairwise genome-wide estimates of the proportion of identity by descent. For each detected sample pair we excluded the individual with a lower call rate. Ethnical outliers were identified by a principal-component analysis (PCA) together with the publically available 1000 Genomes data with known ethnicities.²⁸ Because the study population has genetically a mainly European ancestry, as ascertained by the PCA, we determined a European center and excluded samples more than 1.5 times the maximal European Euclidean distance away from this center. After a first association analysis of genotyped single nucleotide polymorphisms (SNPs) only, we inspected visually the cluster plots of all variants with a P value $< 1 \times 10^{-5}$ and discarded variants without adequate cluster separation. Imputation was carried out on the quality-assured dataset using the TOPMed Imputation Server, which employs Eagle2 for phasing and minimac4 for the imputation of genotypes.^{29,30} The most likely genotype is used in downstream analyses. Variants were again filtered for minor allele frequency and deviation from Hardy-Weinberg equilibrium in controls with the same thresholds as before. In addition, SNPs with an imputation quality score $R^2 < 0.7$ were excluded, leaving 8,131,900 variants for analyses. As a final step of the quality-control procedure, we used the R package PCAmatchR to ethnically match cases to controls with a 1:4 ratio to overcome possible difficulties with population stratification, leading to 3240 individuals for the analyses.³¹

Association Analysis

We used logistic regression to test the additive genetic model of each marker for association with disease status. Following scree plot analysis, we incorporated the first two dimensions of the PCA and sex as covariates. We used a genome-wide significance threshold of $P < 5 \times 10^{-8}$ and the threshold of $P < 5 \times 10^{-6}$ for suggestive association. Conditional analyses, including, in turn, each SNP with a suggestive association as additional covariate, were conducted to identify adjacent independent signals. Furthermore, we tested for clumps of correlated SNPs, ie, to assess how many independent loci had been associated, and determined the number of variants supporting the lead SNP at each locus, ie, variants with P values less than the clumping threshold of 5×10^{-5} are in linkage disequilibrium ($r^2 \geq 0.4$) and not farther than 250 kb away from the respective SNP. Visualization of the results was carried out with R and LocusZoom³² for regional plots. Variant positions in

TABLE 1 Recruitment centers and brain bank sources

| City, Country | Source | MSA cases |
|---------------------------|---|-----------|
| Zürich, Switzerland | Institute of Neuropathology, University Hospital of Zurich, Zurich, Switzerland | 1 |
| Göttingen, Germany | University Medical Center Göttingen, Department of Neurology and Paracelsus-Elena-Klinik, 34,128 Kassel, Germany | 2 |
| San Francisco, CA, USA | University of California, San Francisco, CA, USA | 2 |
| Vancouver, BC, Canada | University of British Columbia, Department of Pathology and Laboratory Medicine | 2 |
| New York, NY, USA | Mount Sinai NBTR | 3 |
| Atlanta, GA, USA | Emory University, Department of Neurology & Pathology | 4 |
| Los Angeles, CA, USA | The Human Brain and Spinal Fluid Resource Center | 4 |
| Stockholm, Sweden | Department of Neurology, Karolinska University Hospital, Stockholm, Sweden | 4 |
| Vienna, Austria | Institute of Neurology, Medical University of Vienna | 6 |
| Newcastle upon Tyne, UK | Newcastle Brain Tissue Resource, Newcastle University, Campus for Ageing and Vitality, Newcastle upon Tyne NE4 5PL, UK | 7 |
| Chicago, IL, USA | University of Chicago, Department of Neurology | 8 |
| Indiana, IN, USA | Indiana University School of Medicine | 8 |
| San Diego, CA, USA | San Diego Shiley-Marcos AD Research Center, University of California | 8 |
| Tübingen, Germany | Department of Neuropathology, University Hospital of Tübingen, Tübingen, Germany | 8 |
| Madrid, Spain | Centro de Biología Molecular “Severo Ochoa,” c/Nicolás Cabrera, 1, Universidad Autónoma de Madrid, Cantoblanco, Madrid, Spain | 10 |
| Seattle, WA, USA | Department of Pathology, University of Washington, Seattle, WA, USA | 10 |
| Prague, Czech Republic | Department of Pathology and Molecular Medicine, Thomayer University Hospital, Prague | 12 |
| Sydney, NSW, Australia | Brain and Mind Centre, Sydney Medical School, The University of Sydney, Sydney, NSW, Australia | 12 |
| Arizona, AZ, USA | Banner Sun Health Research Institute | 13 |
| Parkville, VIC, Australia | Australian Brain Bank Network, Howard Florey Laboratories, The Florey Institute of Neuroscience and Mental Health | 13 |
| Dallas, TX, USA | Alzheimer’s Disease Center, University of Texas Southwestern Medical Center, Dallas, Texas, USA | 15 |
| Rosthern, SK, Canada | Saskatoon Health Region/University of Saskatchewan, Rosthern; and Movement Disorders | 17 |
| Paris, France | Raymond Escourolle Neuropathology Department, Groupe Hospitalier Pitié-Salpêtrière, Paris, France | 20 |
| London, UK | Imperial College London | 22 |
| Baltimore, MD, USA | Johns Hopkins Medical Institution Brain Resource Center, MD, USA | 24 |
| London, UK | MRC London Neurodegenerative Diseases Brain Bank, Institute of Psychiatry, Psychology and Neuroscience, King’s College | 26 |
| Munich, Germany | Neurobiobank Munich, Center for Neuropathology and Prion Research, Ludwig-Maximilians University | 29 |
| Boston, MA, USA | Massachusetts General Hospital | 30 |

(Continues)

TABLE 1 Continued

| City, Country | Source | MSA cases |
|----------------------------|--|------------|
| Barcelona, Spain | Neurological Tissue Bank of the Biobanc-Hospital Clinic-IDIBAPS | 34 |
| Amsterdam, the Netherlands | Alzheimer Center | 36 |
| Ann Arbor, MI, USA | University of Michigan, Department of Pathology, University of Michigan Medical School, Ann Arbor, MI, USA | 37 |
| Miami, FL, USA | UM Brain Endowment Bank, an NIH NeuroBioBank | 45 |
| Philadelphia, PA, USA | The Penn FTD Center – University of Pennsylvania, USA | 54 |
| Jacksonville, FL, USA | Department of Neuroscience, Mayo Clinic, Jacksonville | 205 |
| Total | | 731 |

MSA, multiple system atrophy.

this article are reported on human genome version 38 (GRCh38/hg38).

Immunohistochemistry on MSA Patients' Brain

Formalin-fixed and paraffin-embedded (FFPE) tissues from patients with MSA and controls without neurological or psychiatric diseases were obtained from the Neurobiobank Munich (Germany). All autopsy cases of the Neurobiobank Munich were collected on the basis of an informed consent according to the guidelines of the ethics commission of the Ludwig-Maximilians-University (Munich, Germany; #345-13). MSA cases had been diagnosed according to established histopathological diagnostic criteria.^{10,23}

For ZIC4 immunohistochemistry, 5- μ m-thick sections of FFPE tissues of the frontal cortex and the cerebellar hemisphere, including the dentate nucleus, were prepared. After deparaffinization, heat-induced epitope retrieval was performed in Tris/EDTA, pH 9, at 95°C for 30 minutes. For blocking of endogenous peroxidase and unspecific protein binding, the sections were incubated with 5% H₂O₂ in methanol for 20 minutes and I-Block reagent (Applied Biosystems, Waltham, MA, USA) for 15 minutes. Subsequently, ZIC4 primary antibody (rabbit, polyclonal; Merck/Sigma-Aldrich, Darmstadt, Germany) was applied overnight at 4°C at a dilution of 1:100. Signal detection was performed using the DCS ChromoLine DAB kit (DCS, Hamburg, Germany) according to the manufacturer's instructions. Sections were counterstained for 1 minute with Mayer's hemalum solution (Waldeck, Münster, Germany).

To determine the fractions of ZIC4-positive neurons of all neurons in the dentate nucleus, we scanned stained slides using a slide scanner (Axio Scan. Z1; Zeiss, Oberkochen, Germany) and visualized using the free ZEN lite software (v. 3.3; Zeiss). For statistical evaluation of the data, Student *t* test was used, and statistical significance was defined as $P < 0.05$.

Results

Patient Sample

From the initial sample of 731 cases, 13 cases had to be excluded because of insufficient tissue quality. After thorough quality control and filtering, 648 cases and 2592 controls covering 8,131,900 variants were included in the association analysis (Fig. 1). The number of excluded samples and variants in each step of the quality-control procedure is shown in Tables S1 and S2.

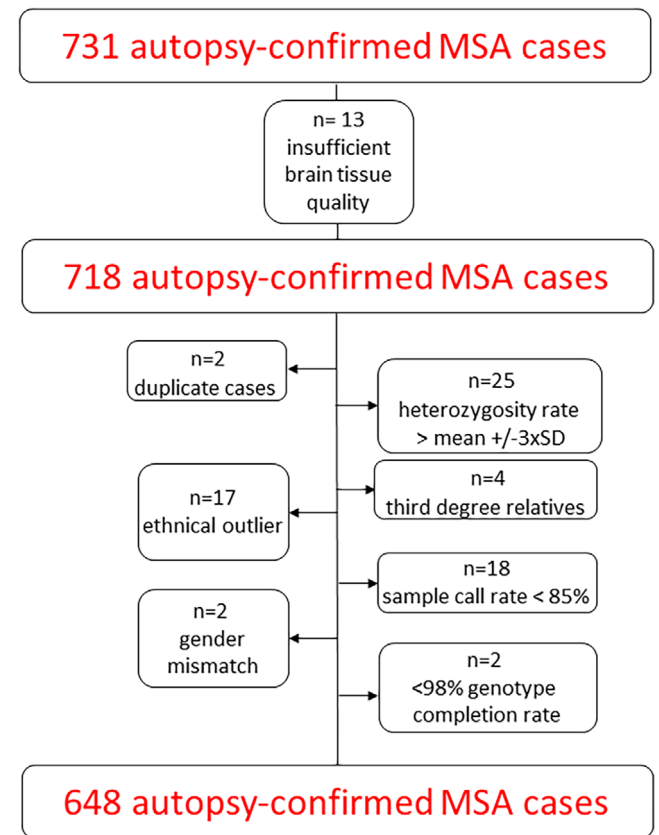


FIG. 1. Flowchart sample quality control. SD, standard deviation. [Color figure can be viewed at wileyonlinelibrary.com]

Association Results

We performed logistic regression incorporating sex and determined the first two dimensions of PCA as covariates using the scree plot method. The genomic

inflation factor of $\lambda = 1.01$ (unimputed $\lambda = 1.01$; Fig. S1) indicates that no significant population stratification was present (Fig. 2A). We did not identify any disease-associated variants with a P value less than the

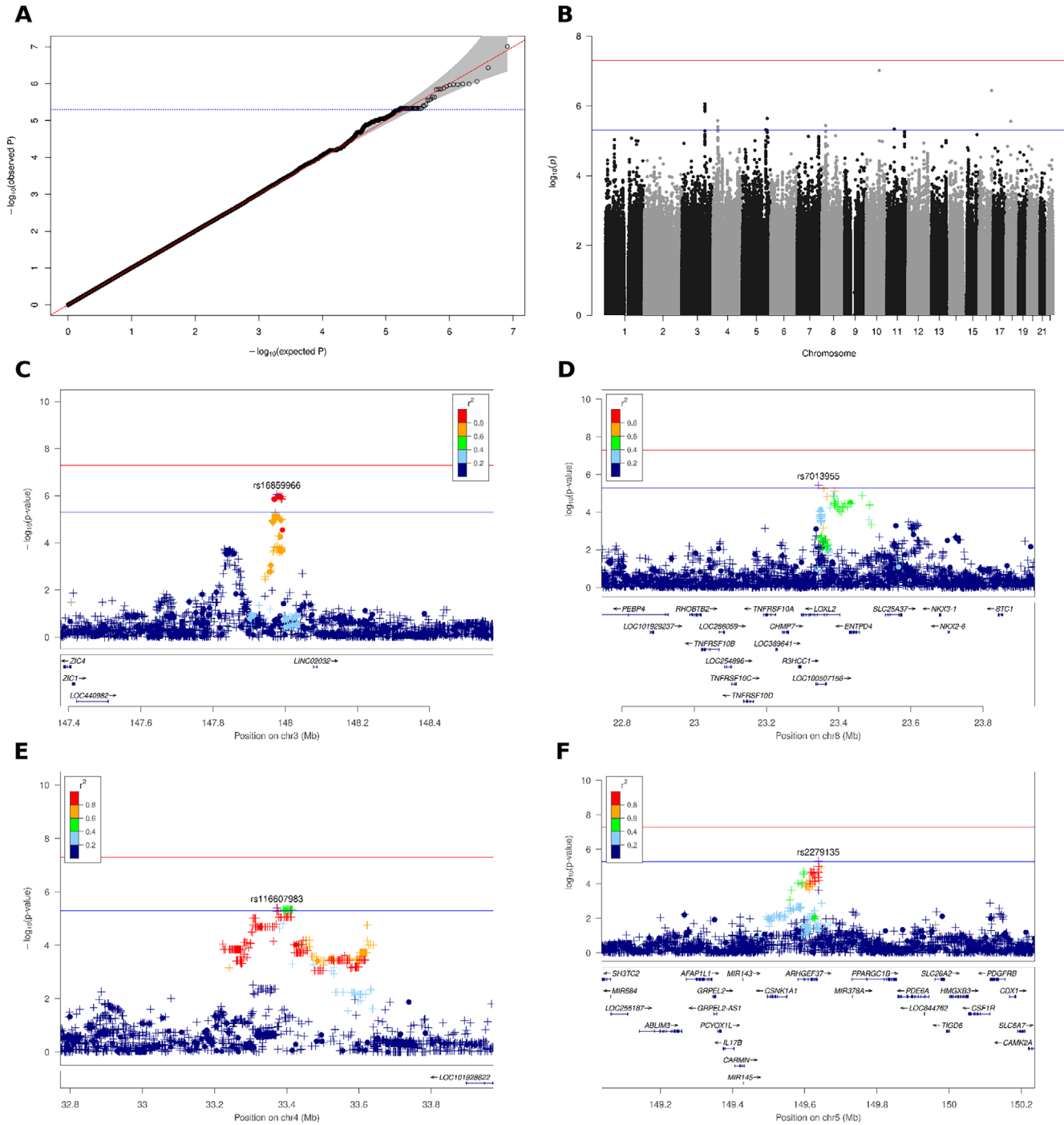


FIG. 2. Association plots for multiple system atrophy (MSA). (A) QQ (quantile-quantile) plot based on 8,109,760 variants after imputation. (B) Manhattan plot showing $-\log_{10} P$ values from logistic regression on imputed variants with sex and two principal components as covariates plotted against their chromosomal position. The red and blue lines indicate the genome-wide significance threshold of 5×10^{-8} and threshold for suggestive associations of 5×10^{-6} , respectively. (C) Regional plot for the association between MSA and variants on chromosome 3 in the genomic region from 147.4 to 148.6 Mb. A circle represents a genotyped variant and a plus symbol an imputed variant. The r^2 metric displays the pairwise linkage-disequilibrium (LD) between the leading and the respective variant. The bottom part shows gene positions. (D) Regional plot for associations on chromosome 8 in the genomic region from 22.7 to 23.9 Mb. (E) Regional plot for associations on chromosome 4 in the genomic region from 32.8 to 34.0 Mb. (F) Regional plot for associations on chromosome 5 in the genomic region from 149.0 to 150.2 Mb. [Color figure can be viewed at wileyonlinelibrary.com]

genome-wide significance threshold of $P < 5 \times 10^{-8}$, but suggestive associations with $P < 5 \times 10^{-6}$ at 10 different loci (Fig. 2B) with the leading SNP at each locus shown in Table 2. Conditional analyses, including, in turn, any SNP with $P < 5 \times 10^{-6}$, excluded the presence of multiple independent signals at each locus. All variants with suggestive associations are listed in Table S3. The most noteworthy hits were rs16859966 on chromosome 3 ($P = 8.6 \times 10^{-7}$; odds ratio [OR], 1.58; 95% confidence interval [CI]: 1.32–1.89), rs7013955 on chromosome 8 ($P = 3.7 \times 10^{-6}$; OR, 1.8; 95% CI: 1.40–2.31), and rs116607983 on chromosome 4 ($P = 4.0 \times 10^{-6}$; OR, 2.93; 95% CI: 1.86–4.63), which were supported by at least one additional genotype, as well as several imputed SNPs with P values less than the clumping threshold of 5×10^{-5} as discovered in the clumping analysis (Table 2). The genes closest to the chromosome 3 locus are the Long Intergenic Non-Protein Coding RNA 2032 (*LINC02032*) approximately 100 kb downstream and the zinc-finger proteins of cerebellum 1 and 4 genes (*ZIC1*, *ZIC4*), located roughly 600 kb upstream (Fig. 2E). The top SNP rs7013955 on chromosome 8 maps to the lysyl oxidase-like 2 gene (*LOXL2*; Fig. 2D). The association signal around SNP rs116607983 on chromosome 4 is located in a region devoid of protein-coding genes approximately 2000 kb to either side (Fig. 2E). A fourth locus on chromosome 5 (rs2279135) was also supported by multiple clumped SNPs, but all SNPs, including the lead SNP, were imputed (Table 2). Several variants clumped at the chromosome 5 locus were located in the *ARHGEF37* gene,

coding for Rho Guanine Nucleotide Exchange Factor 37 (Fig. 2F). None of the identified SNPs is an expression quantitative trait locus in brain tissues according to the Genotype Tissue Expression project.³³ At four of the six remaining loci with variants exhibiting suggestive associations, at most two supporting SNPs were present, which were all imputed; in the other two loci, no supporting SNPs could be found in the clumping analysis (Table 2, Fig. S2). We did not investigate these loci further because it is unlikely that they represent valid associations. No significant associations with Bonferroni-adjusted P values were detected with previously reported Parkinson's disease associations from a meta-analysis of 17 datasets from a Parkinson's disease GWAS (Table S4).³⁴

ZIC4 Immunohistochemistry on MSA Patients' Brain

ZIC4 and *ZIC1* are known to play a critical role in the embryonal development of the cerebellum. Heterozygous deletions comprising the *ZIC1* and *ZIC4* locus have been associated with the Dandy–Walker malformation, a rare congenital condition characterized by a hypoplastic cerebellar vermis and an enlarged fourth ventricle.^{35,36} In mice, deletions of *ZIC1* and *ZIC4* lead to a striking phenotype similar to the Dandy–Walker malformation with cerebellar hypoplasia and foliation defects.^{35,36} In addition, paraneoplastic autoantibodies against *ZIC4* protein are linked to severe cerebellar dysfunction and degeneration.^{37,38}

TABLE 2 Top SNPs at each locus with $P < 5 \times 10^{-6}$

| CHR | dbSNP ID | BP | Minor allele | MAF | | OR (95% CI) | P | IM/GT | No. of SNPs in Clump | | |
|-----|-------------|-------------|--------------|-------|----------|------------------|---------|-------|----------------------|----|----|
| | | | | Cases | Controls | | | | Total | GT | IM |
| 10 | rs4933352 | 85,280,795 | G | 0.42 | 0.52 | 0.71 (0.62–0.80) | 9.7E–08 | IM | 2 | 0 | 2 |
| 16 | rs79418449 | 80,515,374 | C | 0.04 | 0.02 | 2.54 (1.77–3.63) | 3.7E–07 | GT | 1 | 1 | 0 |
| 3 | rs16859966 | 147,976,678 | G | 0.17 | 0.12 | 1.58 (1.32–1.89) | 8.6E–07 | IM | 45 | 24 | 21 |
| 5 | rs114019803 | 159,559,041 | T | 0.02 | 0.01 | 3.36 (2.03–5.56) | 2.3E–06 | IM | 3 | 0 | 3 |
| 4 | rs933953 | 31,356,173 | C | 0.25 | 0.32 | 0.71 (0.62–0.82) | 2.6E–06 | IM | 1 | 0 | 1 |
| 18 | rs116914137 | 30,589,500 | A | 0.05 | 0.02 | 2.17 (1.57–3.00) | 2.8E–06 | IM | 3 | 0 | 3 |
| 8 | rs7013955 | 23,343,590 | A | 0.08 | 0.05 | 1.80 (1.40–2.31) | 3.7E–06 | IM | 20 | 1 | 19 |
| 4 | rs116607983 | 33,372,461 | A | 0.03 | 0.01 | 2.93 (1.86–4.63) | 4.0E–06 | IM | 88 | 3 | 85 |
| 11 | rs141819348 | 47,698,235 | T | 0.05 | 0.03 | 2.10 (1.53–2.88) | 4.6E–06 | IM | 3 | 0 | 3 |
| 5 | rs2279135 | 149,637,742 | C | 0.32 | 0.27 | 1.39 (1.21–1.60) | 4.8E–06 | IM | 24 | 0 | 24 |

Results from an association analysis with logistic regression including sex and the first two dimensions of principal-component analysis (PCA) as covariates in 648 cases with MSA and 2898 controls. For an OR < 1, the minor allele has a protective effect, whereas an OR > 1 indicates that the minor allele is associated with an increased risk for development of the disease. Only the leading SNP at each locus with a suggestive association between the disease status and a variant is reported. Table S3 lists all suggestive associations.

SNP, single-nucleotide polymorphism; CHR, chromosome; dbSNP, database of single-nucleotide polymorphism; BP, base-pair coordinates according to human reference genome GRCh38; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval; IM, imputed; GT, genotyped.

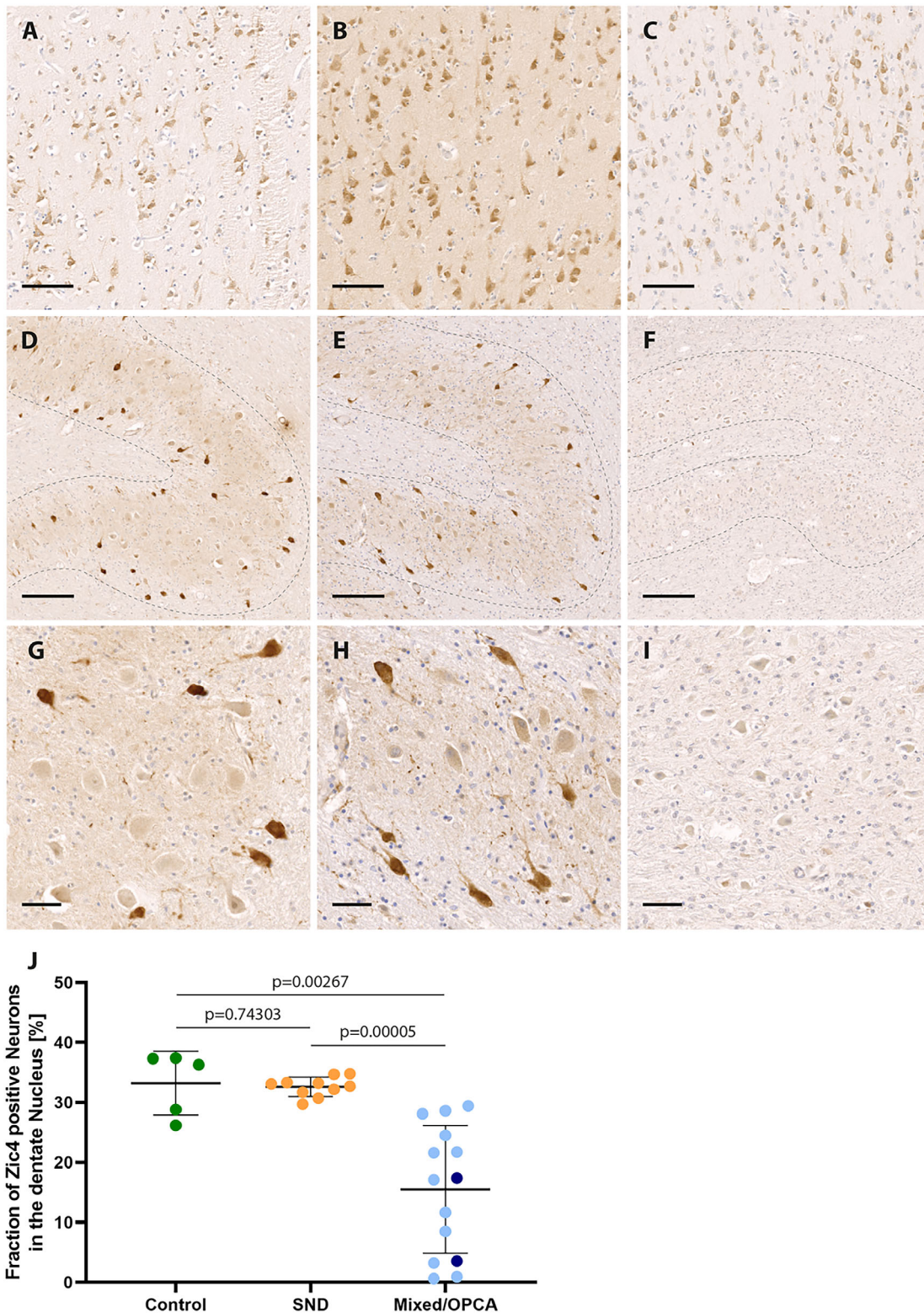


FIG. 3. ZIC4 immunohistochemical staining of multiple system atrophy (MSA) patients and control brains. Representative ZIC4 immunohistochemical stainings of different brain regions (antibodies binding specifically to antigens in biological tissues, eg, brain tissue) of a control without neurodegenerative disease (**A, D, G**) and two MSA patients with striatonigral degeneration (SND) (**B, E, H**) and mixed subtype (**C, F, I**), respectively. (**A–C**) Nuclear and cytoplasmic expression of ZIC4 (brown staining) was detected in a comparable manner in the frontal cortex of healthy controls and patients with MSA. In the cerebellar dentate nucleus (dotted lines in **D–I**) of healthy controls and patients with SND, a constant subset of neurons stained strongly positive for ZIC4, whereas in patients with olivopontocerebellar atrophy (OPCA) or mixed subtype, only weak staining could be observed, and the number of ZIC4-positive neurons was clearly reduced (**D–I**, with higher magnification in **G–I**). (**J**) Quantification of ZIC4-immunoreactive neurons in relation to the total number of neurons of the dentate nucleus depicted on the entire slide showed significantly reduced fractions of ZIC4-immunoreactive neurons in patients with either mixed subtype (light blue) or OPCA (dark blue) compared with SND or controls without neurodegenerative disease, while no difference was seen between patients with SND and healthy controls. Scale bars: 100 μ m (**A–C**), 200 μ m (**D–F**), 50 μ m (**G–I**). [Color figure can be viewed at wileyonlinelibrary.com]

Because cerebellar degeneration and corresponding symptoms are also a central hallmark of MSA, we decided to follow up on a potential role of ZIC4 in MSA patient brains by performing immunohistochemical stainings. For ZIC1, no primary antibody was appropriately sensitive and specific on human tissue in our hands. Thus, FFPE tissues of the cerebellum and, for comparison, the frontal cortex of patients with MSA ($n = 10$ SND, $n = 14$ OPCA/mixed phenotype) and healthy controls ($n = 5$) were stained with antibodies raised against ZIC4.

Nuclear and cytoplasmic staining of frontal cortex neurons was observed in all brains examined without differences between healthy controls and patients with MSA (Fig. 3A–C). In the cerebellar dentate nucleus, we found strong expression of ZIC4 in a subset of neurons in healthy controls, as well as patients with MSA with predominant SND (Fig. 3D,E,G,H). In contrast, patients with MSA with mixed subtype or OPCA showed reduced numbers of ZIC4-positive neurons, which were furthermore only weakly stained (Fig. 3F,I). Quantification of the proportions of ZIC4-positive neurons among the total number of dentate nucleus neurons depicted relatively constant proportions in healthy controls and patients with MSA-SND ($33.2\% \pm 0.0\%$ vs $32.6\% \pm 0.0\%$), whereas in patients with MSA-OPCA or MSA-mixed phenotype, we found significantly lower percentages of ZIC4-positive neurons ($15.5\% \pm 0.1\%$) (Fig. 3J).

Discussion

As part of the study, brain banks were contacted worldwide, and all available white MSA brains were included. As in the prior GWAS with 918 predominantly clinically diagnosed MSA patients, our current GWAS of 648 patients with autopsy-confirmed MSA did not identify disease-associated common variants less than the genome-wide significance threshold. Previously, hypothesis-driven candidate gene studies found inconsistent results for genetic variants and genes potentially associated with MSA. An association of MSA with genetic variants in *COQ2*, *SNCA*, *MAPT*, and *PRNP* had been discussed.^{16–20,39} However, these genes have not been convincingly confirmed in other candidate gene studies and have not been associated in a previous MSA GWAS.²¹ This preceding GWAS analyzed 918 mostly clinical cases and 3864 controls. Overall, this GWAS did not identify any genome-wide significant hits. Because our prior GWAS of 219 patients with autopsy-confirmed corticobasal degeneration did identify significant disease-associated common variants, our current findings strongly suggest that the genetic contribution to disease risk is smaller in MSA.⁴⁰

Nevertheless, our study demonstrates several suggestive associations at different loci, which may provide relevant hypotheses for follow-up investigations into the pathogenesis of MSA.

Specifically, we identified a variant on chromosome 3 (rs16859966; $P = 8.6 \times 10^{-7}$; OR, 1.58; 95% CI: 1.32–1.89) located upstream of *ZIC1* and *ZIC4*. *ZIC1* and *ZIC4* are located in close genomic proximity to each other and encode transcription factors highly expressed in different brain areas.^{41,42}

Proper function of these proteins is critical for the development of the CNS, particularly the cerebellum.³⁶ Although no effect of rs16859966 on *ZIC1* or *ZIC4* expression is recorded in the Genotype Tissue Expression database, rare genetic variants or deletions in *ZIC1* or *ZIC4* result in congenital cerebellar defects.^{35,36,43} A heterozygous deletion of *ZIC1* and *ZIC4* causes the Dandy–Walker malformation, a developmental disorder of the cerebellum.^{35,44} Remarkably, two recent epigenomic analyses in brain tissue of MSA point to *ZIC4*.^{45,46} Moreover, paraneoplastic autoantibodies against ZIC4 induce cerebellar degeneration.³⁸ Due to the pronounced cerebellar degeneration in MSA, we followed up on a possible role of ZIC4 in MSA.

Although we could detect a relatively constant proportion of approximately one-third ZIC4-positive neurons among all neurons in the cerebellar dentate nucleus in healthy controls and patients with MSA-SND, cases with MSA-OPCA or the mixed MSA phenotype showed significantly lower fractions of ZIC4-positive neurons. This finding suggests that ZIC4 may be involved in the neurodegeneration in MSA. The involvement of *ZIC4* mutations in the Dandy–Walker cerebellar malformation and the paraneoplastic ZIC4 autoantibody-associated cerebellar degeneration could suggest a pathomechanism in MSA, by which altered *ZIC4* expression could increase neuronal vulnerability. Further analyses of a potential functional interaction of α -synuclein and ZIC4 are currently ongoing.

Explorative analysis of PD-related associations identified by GWAS yielded no significant association in MSA when adjusting for multiple testing. However, for unadjusted P values, five SNPs reached a significance threshold of $P < 0.05$, which might be interesting to study further.

This study has a major limitation. Typically, a GWAS is conceptualized as a two-stage design with a discovery stage and a replication stage and supposedly achieving “genome-wide significance” in the discovery stage. The P values in the replication stage should remain significant after Bonferroni correction. Due to the limited number of autopsy-confirmed MSA cases worldwide, we could not conduct a two-stage procedure, let alone a further independent replication. In view of the aforementioned diagnostic uncertainty in clinical cases, a replication in predominantly clinically diagnosed MSA cases did not seem desirable.

Therefore, we strongly encourage bringing MSA cases to autopsy and conducting a further independent replication study to confirm or refute the hypotheses provided by our study. ■

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.