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### Authors
Valente, E M
Brancat, F
Silhavy, J L
et al.



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# *AHI1* gene mutations cause specific forms of Joubert Syndrome Related Disorders

Enza Maria Valente, MD, PhD,[1]* Francesco Brancati, MD,[1,2,3] Jennifer L. Silhavy, MS,[4] Marco Castori, MD,[1,3] Sarah E. Marsh, MS,[4] Giuseppe Barrano, BSc,[1,3] Enrico Bertini, MD,[5] Eugen Boltshauser, MD,[6] Maha S. Zaki, MD, PhD, [7] Alice Abdel-Aleem, MD, PhD,[8] Ghada M.H. Abdel-Salam, MD, PhD,[7] Emanuele Bellacchio, PhD,[1] Roberta Battini, MD,[9] Robert P.Cruse, DO, [10] William B. Dobyns MD, [11] Kalpathy S. Krishnamoorthy, MD, [12] Clotilde Lagier-Tourenne, PhD, [13] Alex Magee, MD, [14] Ignacio Pascual-Castroviejo MD, [15] Carmelo D. Salpietro, MD,[16] Dean Sarco, MD,[17] the International JSRD Study Group, Bruno Dallapiccola, MD,[1,3] Joseph G. Gleeson, MD [4]*

*to whom correspondence should be addressed. E-mails: e.valente@css-mendel.it (EMV) and jogleeson@ucsd.edu (JGG).

**Authors' affiliations:**

[1]IRCCS CSS, Mendel Institute, viale Regina Margherita 261, 00198 Rome, Italy, tel. +39 (06) 4416 0503

[2]Department of Biological Sciences, G. D'Annunzio University, Via dei Vestini 31, 66013 Chieti, Italy, tel. +39 0871 3551

[3]Department of Experimental Medicine and Pathology, La Sapienza University, viale Regina Elena 324, 00187 Rome, Italy, tel. +39 (06) 4416 0501

[4]Laboratory of Neurogenetics, Department of Neurosciences, University of California, San Diego, Leichtag 332, 9500 Gilman Drive, La Jolla, CA 92093-0691, USA, tel. +1 (858) 822 3535

[5]Molecular Medicine Unit, Department of Laboratory Medicine, Bambino Gesu' Hospital IRCCS, Piazza S. Onofrio, 4, 00165 Rome, Italy, tel. +39 (06) 6859 2105

[6]Department of Neurology, Children's University Hospital, Steinwiesstrasse 75, 8032 Zurich, Switzerland, tel. +41 (1) 266 7330

[7]Department of Clinical Genetics and [8]Department of Molecular Genetics, National Research Center, El-Tahrir Street, Dokki, Cairo, Egypt, fax +11 (20) 2359 8710

[9]Division of Child Neurology and Psychiatry, University of Pisa Stella Maris Scientific Institute, Via dei Giacinti 2, 56018 Calambrone, Pisa, Italy, tel. +39 (050) 886 227

[10]Clinical Care Center, Texas Children's Hospital, 6701 Fannin, Houston TX 77030, USA, tel. +1 (832) 822 3961

[11]Department of Human Genetics, Division of Biological Sciences, University of Chicago, CLCS 31993C (MC 0077), Chicago, IL 60637, USA, tel. +1 (773) 834 3597

[12]Pediatric Neurology Unit, Massachusetts General Hospital, 55 Fruit St, Boston, MA 02114, USA, tel. +1 (617) 726 3877

[13]Institut de Genetique et de Biologie Moleculaire et Cellulaire (IGBMC), 1 rue Laurent Fries, BP10142 67404 Illkirch cedex, France, tel. +33 (3) 8865 3399

[14]Regional Genetics Service, Belfast City Hospital, Lisburn Road, BT9 7AB Belfast, Northern Ireland, tel. +44(0)28 9026 3873

[15]Pediatric Neurology, Hospital Universitario La Paz, Paseo de la Castellana 261, 28046 Madrid, Spain.

[16]Operative Unit of Pediatric Genetics and Immunology, Department of Medical and Surgical Pediatric Sciences, University of Messina, viale Gazzi, 98100 Messina, Italy, tel. +39 (090) 221 3114

[17]Department of Neurology, Children's Hospital, Harvard Medical School, 300 Longwood Avenue, Boston, MA 02115, USA, tel. +1 (617) 355 6388

**Corresponding author:**

Dr. Joseph G. Gleeson
University of California-San Diego
Leichtag 3A16
9500 Gilman Drive
La Jolla, CA 92093-0691
tel. +1 (858) 822 3535
fax. +1 (858) 822 1021
e-mail: jogleeson@ucsd.edu

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**Summary**

Objective: Joubert syndrome (JS) is a recessively inherited developmental brain disorder with several identified causative chromosomal loci. It is characterized by hypo/aplasia of the cerebellar vermis and a particular midbrain-hindbrain "molar-tooth" sign (MTS), a finding shared by a group of "Joubert syndrome related disorders" (JSRD), with wide phenotypic variability. The frequency of mutations in the first positionally cloned gene, *AHI1*, is unknown.

Methods: We performed mutation for the *AHI1* gene among a cohort of 137 families with JSRD and radiographically proven MTS

Results: We identified 15 deleterious mutations in 10 with pure JS or JS plus retinal and/or additional central nervous system abnormalities. Mutations among families with JSRD including kidney or liver involvement were not detected. Transheterozygous mutations were identified in the majority of those without history of consanguinity. Most mutations were truncating or splicing errors, with only one missense mutation in the highly conserved WD40 repeat domain that led to disease of similar severity.

Interpretation: *AHI1* mutations are a frequent cause of disease in patients with specific forms of JSRD.

## Introduction

Joubert syndrome (JS) is an autosomal recessive disorder presenting with hypotonia, ataxia, developmental delay, breathing abnormalities in the neonatal period and oculomotor apraxia.[1] The key neuroradiological hallmark of JS is a complex malformation of the hindbrain/midbrain junction characterized by cerebellar vermis hypo/aplasia, thick and mal-oriented superior cerebellar peduncles and abnormally deep interpeduncular fossa. On axial MRI sections at the ponto-mesencephalic level, this malformation gives rise to a peculiar appearance resembling a molar tooth, the so called “molar tooth sign” (MTS).[2] After its original description, the MTS has been identified in a number of conditions termed “Joubert Syndrome Related Disorders” (JSRDs), displaying the neurological features of JS associated with involvement of several other organs such as the eye, kidney and liver.[3,4] Moreover, the MTS can be associated with other central nervous system malformations such as corpus callosum abnormalities, polymicrogyria, hydrocephalus and encephalo-meningocele.[5] At least eight distinct JSRDs have been described so far. Nevertheless, despite numerous classification efforts, their nosology still remains problematical due to significant overlap between syndromes, the presence of incomplete phenotypes and clinical variability even within families.[5] The resulting difficulties in distinguishing distinct JSRDs on a pure clinical basis have warranted the attempt to reclassify these syndromes on a molecular basis. To date, three loci (JBTS1, MIM[#213300]; JBTS2, MIM[%608091]; JBTS3, MIM[#608629]) have been implicated in JSRDs and deletions of the NPHP1 gene (MIM[*607100]), usually responsible for isolated juvenile nephronophthisis, have been rarely found in JSRD patients with kidney involvement.[6-11] We have recently evaluated

the phenotypes associated with the known genetic causes of JSRD to define clinical and radiographic hallmarks for each condition. Genotype-phenotype correlates appeared to be striking, with JBTS1 and JBTS3 showing features restricted to the central nervous system (CNS) and the retina, and JBTS2 being associated with multiorgan involvement (specifically frequent retinopathy, progressive renal cystic disease, polydactyly and dysmorphisms) and extreme phenotypic variability.[12] However these correlates are hampered by the paucity of linked families and larger numbers of patients are needed to define the phenotypic spectrum associated with each locus. Recently, mutations in the *AHI1* gene (MIM[*608894]) were identified in ten patients from six families linked to the JBTS3 locus.[13,14] The clinical spectrum in these and in two other JBTS3-linked families (not proven to have *AHI1* mutations) [9] included pure JS and phenotypes associated with either retinal involvement and/or cerebral cortical abnormalities. *AHI1* encodes Jouberin, an 1196-amino acid protein containing a coiled-coil region, seven WD40 repeats and one Sarcoma homology 3 (SH3) domain. Its role in cerebellar and cortical development is unknown, but the domain structure suggests involvement in intracellular signaling pathways.[13]

## Subjects and methods

### *Subjects*

In order to define *AHI1* mutation frequency within the JSRD spectrum and to establish genotype-phenotype correlates, we searched for *AHI1* mutations in 137 families with at least one affected child with neuroradiologically proven MTS. These patients were ascertained from among 29 different countries representing four continents (Europe, North and South America, Africa and Asia) according to approved institutional human subjects protocols. Patients underwent a detailed diagnostic protocol, including a standardized clinical questionnaire, careful assessment of eye, kidney and liver function and expert evaluation of brain MRI by one of the co-authors to identify additional CNS malformations other than the MTS. Clinical information was comprehensive for all patients with mutations reported here, although it was not possible to evaluate all items in mutation-negative patients because of limited access to medical testing. The clinical spectrum in our cohort encompassed all known JSRD phenotypes including pure JS, JS with variable association of retinal, renal and other CNS abnormalities, and rarer JSRDs such as COACH syndrome (MIM[216360]) and Oro-Facio-Digital Syndrome type VI (OFD6 or Varadi-Papp syndrome, MIM[%277170]).[4,5]

### *Methods*

In this screening, we adopted a three-step strategy for mutation analysis of the *AHI1* gene. First we performed a Denaturing High-Performance Liquid Chromatography (DHPLC)-based analysis on DNA from one of each proband's parent (usually the mother), followed by direct sequencing of all abnormal profiles. In a second step, all

mutations identified in the parents were interrogated in the affected relatives and, in case of homozygosity, in the other parent as well. In a third step, patients with heterozygous mutations underwent complete gene DHPLC/sequencing to identify the second mutation. The strategy proposed here was chosen to overcome two major problems, namely the scarcity of DNA from affected children and the limit of DHPLC technique in identifying homozygous mutations. Although this strategy would miss *de novo* mutations in patients, we considered that this occurrence is extremely rare in autosomal recessive conditions such as JSRDs.[15]

Primers were designed in order to amplify all exons and exon-intron junctions by PCR, which were then performed in a final volume of 25μl containing 50ng genomic DNA in a standard AmpliTaq Gold DNA polymerase reaction (Applied Biosystems, Foster City, CA) and standard cycle conditions (annealing 58°C for all except exon 29, which was 61°C). Fragments were analyzed on a DHPLC based WAVE DNA Fragment Analysis System (Transgenomic, Crewe, UK). The start concentrations of buffer B and the oven temperature for optimal heteroduplex separation were determined by using the WAVEmaker software version 4.1.40 (Transgenomic, Crewe, UK) and manually optimized to ensure optimal covering of the whole amplicon including splice sites (Supplemental Table 1). When distinct melting domains were identified in the same amplicon, DHPLC analysis was performed using two or more temperatures. Any peaks falling below 2 mV were considered not reliable and re-analyzed. Samples with abnormal elution profiles identified and corresponding proband samples underwent direct bi-directional sequencing using the Big Dye Terminator Chemistry and an ABI Prism 3100 (Applied Biosystems, Foster City, CA).

**Results**

We identified 15 mutations in the *AHI1* gene in 11 patients from 10 families (Table 2, Fig. 1). In three consanguineous families, affected individuals were homozygous for the detected mutation, while in six families probands were compound heterozygous for two distinct mutations. In the remaining family (MK23), only one mutation could be identified in the proband despite complete sequencing of the whole coding region and splicing junctions.

All identified mutations were novel, and only one (C1267T substitution resulting in the nonsense mutation Q423X) recurred in two unrelated families. Both of these probands contained a different second site mutation, although we did not exclude an ancestral haplotype accounting for the common mutation. Twelve mutations were truncating (7 small insertions or deletions causing frameshift and premature protein truncation and 5 nonsense mutations), one was missense and two were splice-site mutations.

All truncating mutations but one were predicted to generate proteins lacking completely the SH3 domain and all or part of the WD40 repeats. In the fs1103X mutant protein (family MTI229), the mutation predicts a frameshift from amino acid 1088, thus disrupting the last twenty amino acids of the SH3 domain.

The only identified missense mutation changes the highly evolutionary conserved arginine in position 723 to a glutamine. This mutation, located within the WD40 domain, was modeled using ModBase (at http://alto.compbio.ucsf.edu/modbase-cgi/index.cgi) with PDB 1got-B as a homologous WD40 template. R723 forms a salt-bridge with aspartic acid in position 719 (D719) forcing the aspartic acid side chain towards the arginine and contributing to the formation of the protein turn around the negatively

charged residue (Fig. 1). This salt-bridging is predicted to be disrupted following replacement of the positively charged arginine by the uncharged glutamine in the R723Q mutant.

Although no cDNA was available to confirm the pathogenic effect of the two splicing mutations, computer modeling of splicing consistently predicted the abolition of the natural donor sites of exons 12 and 14 respectively, with the replacement of the consensus +1 G residue. Moreover, these two splicing mutations and the missense mutation R723Q were not represented in the SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) and were not detected in 200 control chromosomes from diverse geographical populations. Finally, these mutations segregated with the disease: both sets of parent carry one mutation and an unaffected sibling in both families is not transheterozygous for these alleles. Therefore, each of these mutations likely results in deleterious effects on protein function.

We also identified several polymorphisms and heterozygous silent and missense changes in the tested parents (Table 3). None of the exonic changes segregated in the affected offspring. Additional intronic polymorphisms were identified that either did not disrupt consensus splice sites, did not create cryptic splice donor or acceptors, did not segregate with the phenotype in unaffected offspring, or were identified in patients with known deleterious mutations. The significance of these exonic and intronic polymorphisms is at present unclear.

## Discussion

Our data raise to twenty the number of distinct mutations identified in the *AHI1* gene, of which two (Q423X and V443D) recurred in two unrelated families.[13,14] Most identified mutations cluster within exons 7 to 16, with the sole exception of a frameshift-causing mutation in exon 25 (Fig. 1). Overall, truncating mutations are the most frequent type (80%) and abolish completely or in part the two critical domains (SH3 and WD40) of the Jouberin protein. These mutations may cause nonsense mediated mRNA decay or may act by disrupting specific protein-protein interactions. In fact, SH3 and WD40 domains are believed to be involved in the assembly of specific protein complexes and in regulatory functions resulting from multiple protein interactions.[16,17] Interestingly, the only identified missense mutation (R723Q) changes a highly evolutionary conserved amino acid within the WD40 domain, with predicted dramatic effects on its structure and function.

In only one patient was a single deleterious heterozygous mutation identified (MK23). In this patient, the second mutation could be a large genomic rearrangement such as a deletion or insertion, or it could reside in the promoter region or within an intron, thus not being detectable by the direct sequencing method used here.

Clinical analysis of the eleven patients with mutations strongly suggests that *AHI1* is associated with a phenotype characterized by pure JS or JS with retinal abnormalities and / or other supratentorial CNS malformations (Table 2). In fact, six patients had JS plus retinopathy (ranging from retinitis pigmentosa with mild visual reduction to Leber congenital amaurosis - LCA), two had JS plus other CNS abnormalities (cavum septum pellucidum, occasionally found in healthy individuals), or white matter abnormalities

(leading to a diagnosis of multiple sclerosis), and two had JS plus both retinal abnormalities and other CNS malformations (abnormal midline in occipital region with interdigitating gyri and mega cisterna magna, corpus callosum abnormality and frontal atrophy)(Fig. 2).

One of the patients with JS plus LCA (MTI-112) also suffered from seizures, which is not typical for JS. The MRI did not demonstrate associated cerebral cortical polymicrogyria such as has been reported in association with *AHI1* mutations[13]. The polymicrogyria previously reported was associated most strongly with a V443D mutation, one that was not encountered in the current study, suggesting possible further genotype-phenotype correlations. Exons 8 and 10 were sites of mutations in both the previous study and the current study, but even mutations within these closely spaced exons are associated with differences in phenotype, perhaps due to subtle effects on protein function, or genetic modifying factors.

The overall frequency of *AHI1* mutations in our cohort of 137 probands is 7.3%. Considering all tested patients with a similar phenotype (JS ± retinopathy ± other CNS abnormalities, n=79 cases), the mutation frequency in this subset raises to 12.7%. The phenotypic group of JS plus retinopathy showed the highest mutation frequency, with five mutated out of 23 tested probands (21.7%). Various forms of retinopathy can be present, ranging from retinitis pigmentosa with mild visual reduction to the more severe form of LCA, characterized by early blindness. Conversely, we found no mutations in 37 patients with kidney involvement (either renal cysts or nephronophthisis), in five patients with liver involvement and in seven patients with OFD6 syndrome. This strong phenotypic correlation is consistent among patients of different nationality and ethnicity.

However, it is important to consider that our cohort was ascertained based upon the presence of the MTS brain malformation, so it is possible that more expanded testing of patients with rare phenotypes may uncover *AHI1* mutations. Evidence for this possibility is suggested by the finding of rare mutations in NPHP1 in some but not all mutation screens of patients with JS.[10,11,18] No correlate was evident between the type of mutation (truncating / missense / splicing) or the exon involved and the phenotypes observed.

This is the first large-scale screening of the *AHI1* gene in patients with a neuroradiologically proven MTS and a wide range of JSRD phenotypes. Our data demonstrate that *AHI1* mutations are a frequent cause of JS with retinal involvement and / or other CNS abnormalities, while their role in causing JS with other organ involvement (such as kidney and liver) or OFD6 remains to be proven.

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## Appendix

The members of the International JSRD Study Group are as follows:

Richard Leventer (Parkville, Australia); Padraic Grattan-Smith (Sydney, Australia); Andreas Janecke (Innsbruck, Austria); Marc D'Hooghe (Brugge, Belgium); Rudy Van Coster (Ghent, Belgium); Karin Dias, Carla Moco, Ana Moreira (Porto Alegre, Brazil); Chong Ae Kim (Sao Paulo, Brazil); Gustavo Maegawa (Toronto, Canada); Itxaso Marti, Susana Quijano-Roy (Garches, France); Alain Verloes (Paris, France); Renaud Touraine (St. Etienne, France); Michel Koenig, Jean Messer (Strasbourg, France); Heike Philippi (Mainz, Germany); Sofia Kitsiou Tzeli (Athens, Greece); Saevar Halldorsson (Reykjavik, Iceland); Shubha R. Phadke (Lucknow, India); Bernard Stuart (Dublin, Ireland); Dorit Lev (Holon, Israel); Bruria Ben-Zeev (Ramat-Gan, Israel); Rita Fischetto, Mattia Gentile (Bari, Italy); Lucio Giordano (Brescia, Italy); Loredana Boccone (Cagliari, Italy); Martino Ruggieri (Catania, Italy); Stefania Bigoni (Ferrara, Italy); Maria Alide Donati, Elena Procopio (Florence, Italy); Gianluca Caridi, , Francesca Faravelli, Gianmarco Ghiggeri (Genoa, Italy); Silvana Briuglia, Gaetano Tortorella (Messina, Italy); Stefano D'Arrigo, Chiara Pantaleoni, Daria Riva, Graziella Uziel (Milan, Italy); Stefania Bova, Elisa Fazzi, Sabrina Signorini (Pavia, Italy); Maria Roberta Cilio, Marilù Di Sabato, Francesco Emma, Vincenzo Leuzzi, Pasquale Parisi (Rome, Italy); Alessandro Simonati (Verona, Italy); Mirjam M. de Jong (Groningen, The Netherlands); Matloob Azam (Islamabad, Pakistan); Berta Rodriguez (La Coruna, Spain); Hulya Kayserili (Istanbul, Turkey); Lihadh Al Gazali, Laszlo Sztriha (Al Ain, UAE); David Nicholl (Birmingham, UK); C. Geoffrey Woods (Cambridge, UK); Raoul Hennekam (London, UK); Saunder Bernes (Mesa, Arizona, US); Henry Sanchez (Fremont, California, US);

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## Legends to figures

### Figure 1

Schematics of the *AHI1* gene (A) encoding the Jouberin protein (B). Mutations identified in this paper are reported above the two panels, while previously described mutations[13,14] are shown below. Most mutations cluster within exons 7 to 16 and all are truncating mutations except the two missense changes V443D and R723Q (underlined in the figure). Panel C shows the high conservation of the R723 residue among *AHI1* homologues. Panel D shows the structure of the amino acid region 556-909, a WD repeat domain formed by several WD repeats and characterized by a propeller-like shape. The domain is represented as a blue ribbon with the repeat containing amino acid R723 highlighted in white. Panel E shows a detailed view of this latter feature. The mutated amino acid R723 and the highly conserved amino acids D719 and W725 are shown as azure, red and yellow sticks respectively (see text for details). CC: coiled coil region.

### Figure 2

MRI of the probands from nine of the ten families with *AHI1* mutations. Upper row: axial sections at the pontine or ponto-mesencephalic level demonstrating the molar tooth sign. Lower row: parasagittal sections showing thickening and malorientation of superior cerebellar peduncles and vermis hypoplasia. For MTI-152, asterisk shows mega cisterna magna. For MTI-208, arrowheads show frontal cortical atrophy and arrow shows thin corpus callosum (see Table 1). For COR63, insert (at the level of the corona radiata) shows white matter lesions (arrows).

## Table 1

Clinical findings of patients with *AHI1* mutations

| **Family** | MTI-229 | MTI-105 | MTI-107 | MTI-155 | MTI-152 | MTI-208 | MTI-112 | COR11 | MK23 | COR63 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Characteristics** | | | | | | | | | | |
| n. of affecteds (sex) | 2 (F, M) | 1 (M) | 1 (F) | 1 (M) | 1 (F) | 1 (M) | 1 (M) | 1 (M) | 1 (M) | 1 (M) |
| Consanguinity | y | n | n | n | n | n | n | y | n | n |
| Country of origin | Egypt | USA African American | Spain | Ireland | USA Mixed European | USA Mixed European | USA Mixed European | Italy | Luxembourg | Switzerland |
| Nucleotide changes: | 3263-3264delGG homoz | C1765T homoz | 903insA IVS14+1G>A | C1267T 1417delA | IVS12+1G>A C2212T | C662G 1898insGG | 911insC C1267T | G2168 A homoz | C1500A not found | 623insAAGA 1899insT |
| Exons: | 25 | 13 | 8, 14 | 10, 11 | 12, 16 | 7, 14 | 8, 10 | 16 | 12, n.a. | 7, 14 |
| Protein mutations: | fs1103X homoz | R589X homoz | fs309X splice mut | Q423X fs509X | splice mut R738X | S221X fs648X | fs309X Q423X | R723Q homoz | Y500X not found | fs228X fs640X |
| Age at examination | 12yr, 9yr | 1yr | 10yr | 6mo | 18yr | 2yr | 1,5yr | 16y | 5y | 30y |
| **Neurological signs:** | | | | | | | | | | |
| - hypotonia/ataxia | + | + | + | + | + | + | + | + | + | + |
| - developmental delay | + | + | + | + | + | + | + | + | + | + |
| - mental retardation | + | + | + | + | + | + | + | + | + | - |
| - OMA | + | + | n.a. | + | - | + | + | + | + | + |
| - breathing abn | - | + | + | + | + | + | + | + | - | - |
| - other abn | - | - | - | - | - | - | seizures | - | - | spasticity |
| **Ocular signs:** | | | | | | | | | | |
| - retinopathy | RP | RP | - | - | LCA | LCA | LCA | RP | RP | - |
| - other abn | Ny | - | - | - | Ny | - | Ny | - | - | Ny |
| **Renal signs:** | | | | | | | | | | |
| - NPH/UCD | - | - | - | - | - | - | - | - | - | - |
| - other abn | - | - | - | - | - | - | - | - | - | - |
| **Other organs:** | | | | | | | | | | |
| - Liver abn | - | - | - | - | - | - | - | - | - | - |
| - Polydactyly | - | - | - | - | - | - | - | - | - | - |
| - Cleft lip/palate | - | - | - | - | - | - | - | - | - | - |
| - Other abn | - | - | - | - | - | Mc | - | - | - | - |
| **MRI features:** | | | | | | | | | | |
| -CVH/MTS | + | + | + | + | + | + | + | + | + | + |
| - polymicrogyria | - | - | - | - | - | - | - | - | - | - |
| - other abn | - | - | - | CSP | +# | +* | - | - | - | WMA** |

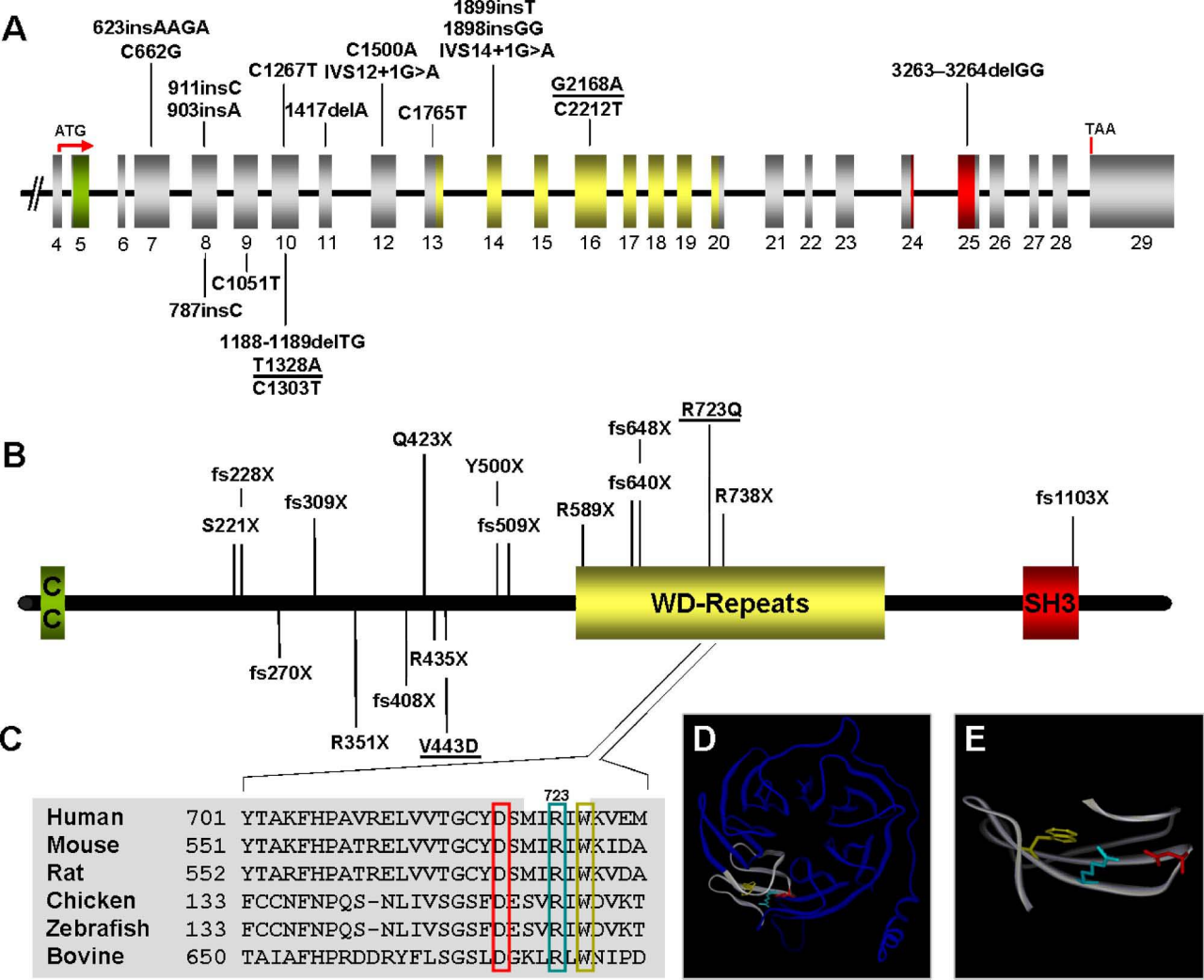
Legend: + Present; – Absent when specifically interrogated; abn-abnormalities; CSP-cavum septum pellucidum; CVH-cerebellar vermis hypoplasia; LCA-Leber Congenital amaurosis; Mc-macrocephaly; MR-mental retardation; MTS-molar tooth sign; Ny-nystagmus; NPH/UCD-Nephronophthisis/Urine Concentration Defect; OMA-Oculomotor Apraxia; RD-Retinal Dystrophy; RP-retinitis pigmentosa; WMA-white matter abnormalities; # abnormal midline in occipital region with interdigitating gyri and mega cisterna magna; *corpus callosum abnormality and frontal atrophy; **oligoclonal bands in cerebrospinal fluid. The two siblings from family MTI-229 showed an identical phenotype, so are listed with a single entry.

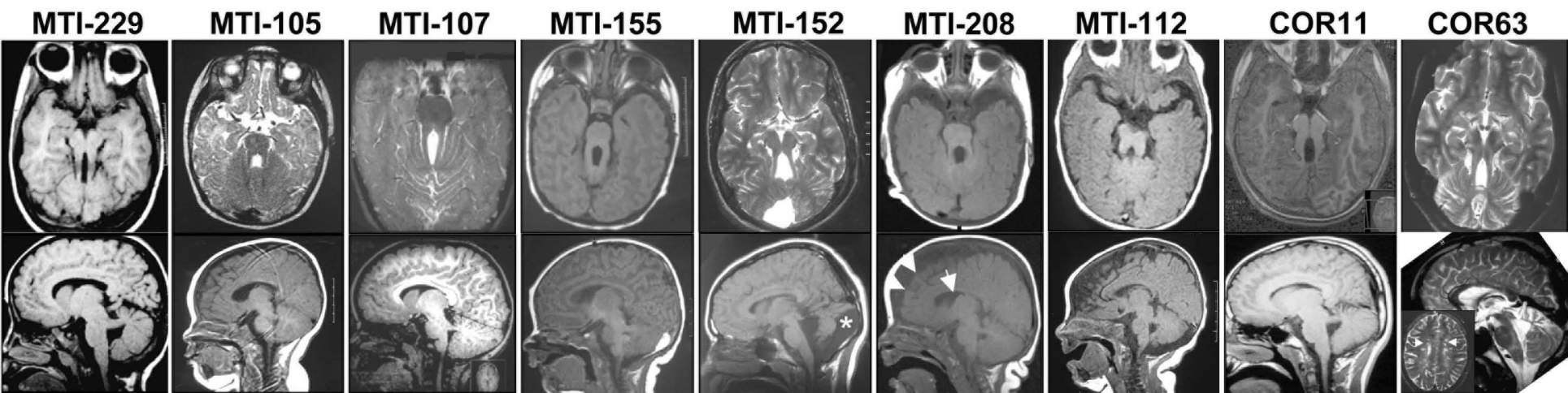
## Table 2

Polymorphisms and variants observed in the *AHI1* gene.

| Nucleotide variation | Amino acid change | Exon / Intron | Allele frequency (n=274 chromosomes) |
|---|---|---|---|
| -49T>C | --- | 5'UTR | 1 |
| IVS4+115C>A | --- | intron 4 | common |
| 146T>A | I49N | exon 6 | 1* |
| IVS8-12insT | --- | intron 8 | 1# |
| IVS12-92T>A | --- | intron 12 | 1 |
| 1643G>A | R548H | exon 13 | 2* |
| IVS13-14C>T | --- | intron 13 | common |
| IVS15+1G>T | --- | intron 15 | 1* |
| 2223T>C | D741D | exon 16 | 2* |
| 2282C>T | S761L | exon 17 | 1* |
| IVS17-16T>C | --- | intron 17 | 1 |
| 2382A>G | K794K | exon 18 | 1* |
| 2488C>T | R830W | exon 18 | 2* |
| 2505G>A | R835R | exon 19 | 1* |
| 2567C>G | T856S | exon 19 | 1* |
| IVS19-6A>G | --- | intron 19 | common |
| IVS20+59A>C | --- | intron 20 | 1 |
| 2798A>G | Y933C | exon 21 | 1* |
| IVS21-16A>G/T | --- | intron 21 | common |
| 3015A>G | S1005S | exon 23 | common* |
| IVS23-22delTCAC | --- | intron 23 | 2 |
| 3282A>T | I1094I | exon 24A | 1* |
| 3368C>T | S1123F | exon 26 | 1* |
| 3418C>T | P1140S | exon 26 | 1* |
| IVS26+13G>A | --- | intron 26 | common |
| IVS26+78delT | --- | intron 26 | 1 |
| IVS26-6delCTT | --- | intron 26 | 1* |
| IVS28-28C>G | --- | intron 28 | 1 |
| IVS28-27delGT | --- | intron 29 | 1 |
| +28G>C | --- | 3'UTR | common |

common: allele frequency greater than 2%; *variation found in the parent but not segregating in affected offspring; # variation found in a parent carrying another deleterious mutation.


A
623insAAGA
C662G
911insC
903insA
C1267T
1417delA
C1500A
IVS12+1G>A
C1765T
1899insT
1898insGG
IVS14+1G>A
G2168A
C2212T
3263–3264delGG
ATG
TAA
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
C1051T
787insC
1188-1189delTG
T1328A
C1303T
B
Q423X
R723Q
fs648X
Y500X
fs228X
fs640X
fs309X
R589X
R738X
S221X
fs509X
fs1103X
CC
WD-Repeats
SH3
fs270X
R435X
fs408X
R351X
V443D
C
723
Human 701 YTAKFHPAVRELVVTGCYDSMIRIWKVEM
Mouse 551 YTAKFHPATRELVVTGCYDSMIRIWKIDA
Rat 552 YTARFHPATRELVVTGCYDSMIRIWKVDA
Chicken 133 FCCNFNPQS-NLIVSGSFDESVRIWDVKT
Zebrafish 133 FCCNFNPQS-NLIVSGSFDESVRIWDVKT
Bovine 650 TAIAFHPRDDRYFLSGSLDGKLRLWNIPD
D
E


MTI-229
MTI-105
MTI-107
MTI-155
MTI-152
MTI-208
MTI-112
COR11
COR63