HHS Public Access

Author manuscript

Eur J Neurol. Author manuscript; available in PMC 2021 February 01.

Published in final edited form as:

Eur J Neurol. 2020 February ; 27(2): 334–342. doi:10.1111/ene.14082.

[*]**Correspondence to** Dr Viorica Chelban, v.chelban@ucl.ac.uk, UCL Institute of Neurology, Queen Square, London WC1N 3BG, UK. Tel: +442034484249, Dr. Namik Kaya, nkaya@kfshrc.edu.sa, King Faisal Specialist Hospital and Research Centre, P.O. Box 3354, Riyadh 11211, Saudi Arabia. Tel: +9661464727239612; Fax:+966 4427858.

[a,b]Equal Contributions

Financial Disclosures of all authors

Viorica Chelban -**None**
Maysoon Alsagob -**None**
Katja Kloth -**None**
Adela Chirita-Emandi -**None**
Jana Vandrovcova -**None**
Indran Davagnanam -**None**
Somayeh Bakhtiari -**None**
Moeenaldeen D. AlSayed -**None**
Zuhair Rahbeeni -**None**
Hamad AlZaidan -**None**
Nancy T. Malintan -**None**
Jessika Johannsen -**None**
Stephanie Efthymiou -**None**
Eloïse Tribollet -**None**
Kshitij Mankad -**None**
Saif A. Al-Shahrani -**None**
Mai AlShammariv -**None**
Stanislav Groppa-**None**
Nourelhoda A. Haridy -**None**
Laila AlQuait -**None**
Alya Qari -**None**
Rozeena Huma -**None**
Rawan Almass -**None**
Faten B Almutairi -**None**
Khushnooda Ramzan -**None**
Ibrahim A. Alorainy -**None**
Muddathir H. Hamad -**None**
Mustafa A. Salih -**None**
Faiqa Imtiaz -**None**
Maria Puiu -**None**
Michael C Kruer -**None**
Tatjana Bierhals -**None**
Nicholas W. Wood -**None**
Dilek Colak -**None**
Henry Houlden -**None**
Namik Kaya -**None**

Author roles:

(1) Research Project: A. Conception, B. Organization, C. Execution; (2) Statistical Analysis: A. Design, B. Execution, C. Review and Critique; (3) Manuscript Preparation: A. Writing of the First Draft, B. Review and Critique.

Viorica Chelban 1A, 1B, 1C, 2A, 2B, 3A
Maysoon Alsagob 1C, 2A, 2B, 3A
Katja Kloth 1C, 3B
Adela Chirita-Emandi 1C, 3B
Jana Vandrovcova 1C, 2B, 3B
Indran Davagnanam 1C, 3B
Somayeh Bakhtiari 1C, 3B
Moeenaldeen D. AlSayed 1C, 3B
Zuhair Rahbeeni 1C, 3B
Hamad AlZaidan 1C, 3B
Nancy T. Malintan 1C, 3B
Jessika Johannsen 1C, 3B
Stephanie Efthymiou 1C, 3B
Eloïse Tribollet 1C, 3B
Kshitij Mankad 1C, 3B
Saif A. Al-Shahrani 1C, 3B
Mai AlShammariv 1C, 3B

Genetic and phenotypic characterization of *NKX6-2*-related spastic ataxia and hypomyelination

Viorica Chelban, MD[1,2,*,a], **Maysoon Alsagob, MSc**[#3,a], **Katja Kloth, MD**[4], **Adela Chirita-Emandi, PhD**[5], **Jana Vandrovcova, PhD**[1], **Reza Maroofian, PhD**[6], **Indran Davagnanam, MD**[7], **Somayeh Bakhtiari, PhD**[8,9], **Moeenaldeen D. AlSayed, MD**[10,b], **Zuhair Rahbeeni, MD**[10,b], **Hamad AlZaidan, MD**[10,b], **Nancy T. Malintan, PhD**[11], **Jessika Johannsen, MD**[12], **Stephanie Efthymiou, MSc**[1], **Ehsan Ghayoor Karimiani, PhD**[6], **Kshitij Mankad, MD**[13], **Saif A. Al-Shahrani, BSN**[10], **Mehran Beiraghi Toosi, MD**[14], **Mai AlShammari, MD**[3], **Stanislav Groppa, PhD**[2], **Nourelhoda A. Haridy, MD**[1,15], **Laila AlQuait, BSc**[3], **Alya Qari, MSc**[10], **Rozeena Huma, MD**[10], **Mustafa A. Salih, MD**[16], **Rawan Almass, BSc**[3], **Faten B Almutairi, BSc**[3], **Muddathir H. Hamad, MD**[16], **Ibrahim A. Alorainy, MD**[17], **Khushnooda Ramzan, PhD**[3], **Faiqa Imtiaz, PhD**[3], **Maria Puiu, PhD**[5], **Michael C Kruer, MD**[8,9], **Tatjana Bierhals, MD**[4], **Nicholas W. Wood, PhD**[1], **Dilek Colak, PhD**[18], **Henry Houlden, PhD**[1], **Namik Kaya, PhD**[3,*]

[1]Department of Neuromuscular Diseases, University College London Institute of Neurology

[2]Department of Neurology and Neurosurgery, Institute of Emergency Medicine, Chisinau, Republic of Moldova.

[3]Department of Genetics, Epidemiology and Scientific Computing, KFSHRC, Riyadh, 11211, KSA

[4]Institute of Human Genetics, University Hospital Hamburg-Eppendorf, Germany

[5]Genetics Department, University “Victor Babes”, Timisoara, Romania

[6]Genetics Research Centre, Molecular and Clinical Sciences Institute, St George’s University of London

[7]Brain Repair & Rehabilitation, University College London Institute of Neurology

[8]Barrow Neurological Institute, Phoenix Children’s Hospital

---

Stanislav Groppa 1C, 3B
Nourelhoda A. Haridy 1C, 3B
Laila AlQuait 1C, 3B
Alya Qari 1C, 3B
Rozeena Huma 1C, 3B
Rawan Almass 1C, 3B
Faten B Almutairi 1C, 3B
Khushnooda Ramzan 1C, 3B
Ibrahim A. Alorainy 1C, 3B
Muddathir H. Hamad 1C, 3B
Mustafa A. Salih 1C, 3B
Faiqa Imtiaz 1C, 3B
Maria Puiu 3B
Michael C Kruer 1C, 3B
Tatjana Bierhals 1C, 3B
Nicholas W. Wood 1A, 1C, 3B
Dilek Colak 3B
Henry Houlden 1A, 1B, 1C, 3B
Namik Kaya 1A, 1B, 1C, 3B

All authors declare no conflict of interest.

[9]Departments of Child Health, Cellular & Molecular Medicine, and Neurology University of Arizona College of Medicine Phoenix

[10]Medical Genetics, Epidemiology and Scientific Computing, KFSHRC, Riyadh, 11211, KSA

[11]Clinical and Experimental Epilepsy, University College London Institute of Neurology

[12]Department of Paediatrics, University Hospital Hamburg-Eppendorf, Germany

[13]Great Ormond Street Hospitals, London.

[14]Department of paediatric diseases, Faculty of medicine, Mashhad University of Medical Sciences, Mashhad, Iran.

[15]Department of Neurology and Psychiatry, Assiut University Hospital Egypt

[16]Pediatric Neurology, Epidemiology and Scientific Computing, KFSHRC, Riyadh, 11211, KSA

[17]Radiology & Medical Imaging, Epidemiology and Scientific Computing, KFSHRC, Riyadh, 11211, KSA

[18]Department of Biostatistics, Epidemiology and Scientific Computing, KFSHRC, Riyadh, 11211, KSA

[#] These authors contributed equally to this work.

Abstract

Background: Hypomyelinating leukodystrophies are a heterogeneous group of genetic disorders with a wide spectrum of phenotypes and a high rate of genetically unsolved cases. Bi-allelic mutations in *NKX6–2* were recently linked to spastic-ataxia 8 with hypomyelinating leukodystrophy (SPAX8).

Methods: Using a combination of homozygosity mapping, exome sequencing, detailed clinical and neuroimaging assessment we describe a series of new *NKX6–2* mutations in a multicentre setting. We then combined all reported *NKX6–2* mutations, combining all reported *NKX6–2* mutations, analyzing spectrum of *NKX6–2*-related disease.

Results: We identified 11 new cases from 8 families of different ethnic backgrounds carrying compound heterozygous and homozygous pathogenic variants in *NKX6–2,* evidencing a high *NKX6–2* mutation burden in the hypomyelinating leukodystrophy disease spectrum. Our data reveals a phenotype spectrum with neonatal onset, global psychomotor delay and worse prognosis at the severe end and a childhood onset with mainly motor phenotype at the milder end. We describe the phenotypic and neuroimaging expression in *NKX6–2* and show that phenotypes with epilepsy in the absence of overt hypomyelination and diffuse hypomyelination without seizures can occur.

Conclusions: We show that *NKX6–2* mutations should be considered in patients with autosomal recessive, very early onset of nystagmus, cerebellar ataxia with hypotonia that rapidly progresses to spasticity, particularly when associated with neuroimaging signs of hypomyelination. Therefore, we recommend that *NXK6–2* should be included in hypomyelinating leukodystrophy and spastic-ataxia diagnostic panels.

Keywords

spastic ataxia 8; SPAX8; NKX6–2; hypomyelination; leukodystrophy

INTRODUCTION

Hypomyelinating leukodystrophies are a heterogeneous group of genetic disorders with a wide spectrum of phenotypes. Given that myelination is a highly regulated process these disorders usually result from genetic abnormalities. However, the majority of individuals with hypomyelinating disorders have no genetic diagnosis [1].

Hypomyelination can result from dysfunctions in myelin generation or maintenance pathways including mutations in the myelin proteins (*PLP1*), protein translation (*POLR3A, POLR3B, POLR1C*) and gap junction proteins linking astrocytes and oligodendrocytes (*GJC2*).

We have recently described a new phenotype associated with bi-allelic mutations in *NKX6–2* leading to spastic-ataxia 8 (SPAX8), autosomal recessive, with hypomyelinating leukodystrophy (OMIM: 617560) [2]. The reported *NKX6–2* mutations were bi-allelic truncating or located in the highly conserved homeobox domain. Clinically, they presented with early onset spastic-ataxia or hypotonia progressing to severe spasticity within a few months and were associated with hypomyelination [2].

Here, we present a large, ethnically diverse cohort, describing comprehensively an expanding clinical and neuroimaging syndrome and a large genotypic spectrum of *NKX6–2*-related disease.

METHODS

The study included affected individuals with spastic-ataxia and hypomyelination from unrelated families of different ethnic backgrounds. Families were recruited under Institutional Review Board/ethics-approved research protocols (UCLH: 04/N034) with informed consent. For comprehensive genotype-phenotype analyses we included all reported genetically diagnosed *NKX6–2* mutations. Extended methods are in Supplementary S1.

RESULTS

Genotype spectrum in *NKX6–2*-related disease

In this study we identified 11 new cases from 8 families (Figure 1) carrying pathogenic variants in *NKX6–2*. Eight distinct mutations were found including 4 were novel variants (Figure 2A). One was present in gnomAD with very low allele frequency in heterozygous state (MAF 0.0001170) but absent as homozygous (c.541C>G) (Figure 2B), and three were known pathogenic variants. The c.196delC identified in family IV and V was present in a shared homozygous region (Figure 2C). All missense variants were located in conserved amino acid positions (Figure 2D).

We extended our analysis to include 33 individuals from 21 families carrying *NKX6–2* mutations identified in this study and previously reported [2–5] (Table 1, Supplementary S2–S3). So far, 13 distinct *NKX6–2* variants have been linked to SPAX8 disease. Several mutations (c.121A>T, c.487C>G, c.196delC) were reported in multiple families. The c.196delC and c.487C>G were identified in 5 families each, all originally from the Middle East. Haplotype analysis from three families confirmed that c.196delC was a founder mutation. However, the c.487C>G carriers didn't share same haplotype, the mutation arising recurrently [5]. The c.121A>T was identified in three families of Indian origin. Haplotype analysis data from two of these families confirmed a founder effect [2].

With one exception (p.Arg101Ser), all missense mutations affected the Homeobox domain. To establish the deleterious effect of p.Arg101Ser variant we performed RT-PCR and WB. Control RT-PCR for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) confirmed the presence of cDNA in all samples. In the patient, *NKX6–2* cDNA was severely reduced compared to controls (Figure 3A). Immunoblot analysis confirmed significant reduction in NKX6–2 protein levels in the patient compared to controls (Figure 3B–C, Supplementary 4).

The majority of reported cases (81.8%, 27/33) presented in the first year of life, half of these (14/27) as neonates. We analyzed whether the mutation type influenced the age of onset. Sixteen cases carried two truncating alleles, 13 had bi-allelic missense mutations and four had compound heterozygous variants including one truncating mutation. Although an earlier mean onset age was observed in the group harboring 2 truncating alleles, versus the group with 2 missense alleles (7.3 months vs 8.3 months), the difference was not statistically significant (p=0.78).

Phenotype spectrum in *NKX6–2*-related disease

Assessment of the age of onset and disease severity revealed two ends of an expanding phenotype spectrum of *NKX6–2* mutations.

Neonatal and very early onset—Neonatal onset was associated with higher rate of severe global psychomotor disability when compared with onset after 1 month old (p=0.05) (Figure 4A). Twenty cases with information on motor milestones showed very severe motor deficit; all children failing to achieve independent ambulation and 70% (14/20) failing to achieve head control. Furthermore, 90% of children with disease onset before one year never achieved verbal milestones/meaningful speech.

Complex medical needs included severe dysphagia requiring gastrostomy (6/33 cases), congenital heart disease (1/33), respiratory failure (2/33) leading to death, undescended testicles (1/33), severe dental and/or gum abnormalities (6/33 cases), inguinal hernia (1/33). Survival in this group was shorter with 12.1% (4/33) of children dying in their first five years.

Childhood onset—Childhood onset with predominantly motor delay and complex spastic-ataxia was identified in seven individuals in this study and those previously reported [2]. Case F1-III:1 has the least severe motor phenotype in our series (Supplementary S5). Case F8-II:1 has a phenotype resembling the previously reported cases with the c.121A>T

mutation-severe spastic-ataxia but relatively preserved cognition [2]. The seven cases all achieved ambulation, though required walking aids (walking frame) 1 to 3 years into the disease and wheelchair 3 to 8 years later. Features including head titubation and severe dystonia in the previously reported patients who achieved adulthood, suggest that they may be related to disease progression rather than genotype.

Complex spastic-ataxia and developmental delay—The most common symptom at onset was nystagmus (25/33 cases) (Figure 4B) described as horizontal gaze-evoked, in the majority of cases. Other ocular manifestations were square wave jerks, hypometric saccades, impaired smooth pursuit and reduced visual acuities (4 cases). Limitation of bilateral, lateral gaze eye movements was seen later in three cases that reached adulthood.

Spasticity with brisk reflexes in the upper and lower limbs, and upgoing plantar responses were present in all cases. Axial hypotonia was reported at presentation in 9 patients, all with onset of disease before 1 year of age associated with upper and lower limb spasticity soon after presentation. Cervical and/or limb dystonia was present in around ~ 40% of cases where information was available. Examination of the peripheral nervous system was normal in all cases and normal nerve conduction studies were reported in two cases that reached adulthood.

Seizures were present in four (12.5%) patients harbouring *NKX6–2* mutations. Case F1-III:1 presented primary tonic progressing to secondarily generalised seizure at six years. Electroencephalography at the age of 13 showed loss of age-based background activity and absent anterior-posterior gradient of background activity with focal tonic seizure presenting clinically as hemifacial seizures. There was intermittent frequency slowing, most pronounced at frontal electrodes and multifocal epileptic discharges (sharp waves and sharp-slow-waves) pronounced over the right hemisphere and several focal tonic seizures. The seizures were multi-drug resistant. No details regarding seizure phenomenology are available in the other three cases [3, 5].

Cognitive function varied greatly between patients. Interestingly, two cases reported here (F1-III:1 and F8-II:1) and four previously reported [2] (total 6/33) had normal cognitive development for their age. Case F8-II:1 and the four previously reported cases with normal cognitive development carry the homozygous nonsense mutation p.Lys41* while F1-III:1 carries two missense compound heterozygous variants (p.Arg101Ser; p.Leu181Val). However, severe cognitive impairment with arrested speech development was present in 66% of all children with bi-allelic *NKX6–2* pathogenic variants and in all reported children with neonatal onset. We acknowledge that in most cases, an accurate cognitive function assessment was difficult due to severe motor impairment and/or developmental language delay.

Other features present in SPAX8 patients included strabismus (4/33 patients), scoliosis (6/33), neck or/and limb dystonia (9/23), contractures (4/33 patients), dysmorphism (2/33), hip dislocation (2/33) and single cases of hearing impairment and hirsutism.

Neuroimaging spectrum in *NKX6–2*-related disease

The key neuroimaging feature in the majority of cases was a hypomyelinating leukodystrophy. Magnetic resonance images (MRI) (Supplementary S6) showed signal abnormality with atrophy noted supratentorially within the thalami, and the globus pallidi. Infratentorially, there was notable involvement of the pons with signal abnormality involving the transverse pontine fibres with relative expansion to the entire pons. This contrasted with the orthogonal orientated fibres of the corticospinal tracts of relatively normal signal, providing a distinctive prominent appearance of the mid-pons on the axial T2-weighted sequences. Furthermore, signal change was noted in the cerebellar hemispheres, particularly involving the subcortical white matter (WM) and the dentate nuclei. Cerebellar volume was relatively increased in very young patients, likely related to the underlying WM changes, and demonstrated mild atrophic change over time. Not all children developed cerebellar atrophy (Supplementary S7).

Interestingly, in case F1-III:1 with two missense compound heterozygous mutations neuroimaging findings were milder compared to cases carrying homozygous truncation mutations (Figure 4C). Marked cerebellar atrophy was a key finding in this case. Furthermore, two other paediatric *NKX6–2*-related cases were reported previously without overt hypomyelination [4]. Thinning of the corpus calosum was present in 6/33 patients. A longitudinal MRI study in F7-II:3 at 4 and 8 years old shows progressive thinning of corpus callosum and cerebellar atrophy, associated with WM abnormality sparing the U fibers (Figure 4D).

DISCUSSION

Recently we described the first cases of *NKX6–2* mutations leading to hypomyelination and spastic-ataxia phenotype in humans [2]. Given the rarity of hypomyelinating disorders and the absence of an unbiased cohort to screen, it is difficult to estimate the frequency of *NKX6–2* mutations. However, genetic analysis of the UCL leukodystophies cohort identified 10 cases of hypomyelination with pathogenic mutations in *PLP1* (four families), single cases of *TUBB4A*, *POLR3A/B* and *CLCN2* [6]. Here we present six additional cases (three families) of hypomyelination due to *NKX6–2* mutations from the same research group suggesting a more significant burden of *NKX6–2* mutations.

We expanded the phenotypic spectrum and showed that *NKX6–2* mutations lead to a neonatal onset at the severe end and childhood onset at the milder end. Interestingly, the compound heterozygous missense variants c.301C>A (p.Arg101Ser) and c.541C>G (p.Leu181Val) led to a significant reduction (>70%) of the NKX6–2 protein. It is possible that the small amount of NKX6–2 protein identified by western blot provided a degree of myelination leading to a less severe clinical picture. This individual and the three cases previously published [5] presented with multi-drug resistant epilepsy. In focal cortical dysplasia, a common cause of drug-resistant epilepsy, hypomyelination abnormality was confirmed in numerous histopathological epilepsy surgery specimen studies [7]. Focal dysplasia is currently linked to abnormalities in the differentiation of glial cells from their progenitors and their migration to the cortical place [8], a process regulated by transcription factors including *NKX6–2* [9].

Additional clinical features associated with *NKX6–2* mutations-cervical and/or limb dystonia, congenital abnormalities (congenital heart disease, undescended testes), severe dental and/or gum abnormalities -reflect the developmental role of *NKX6–2* as a member of the homeobox gene family [10]. These genes are involved in the development, specifying geographical orientation of the body by directing the formation of limbs and organs [11]. Some homeobox genes such as *PAX6* [12], *OTX2* [13], and *MEOX1* [14] were involved in a variety of developmental and neurological disorders with brain abnormalities.

Clinical and neuroimaging findings in *NKX6–2*-related disease reflect the involvement of WM and pyramidal tracts (spasticity, brisk reflexes, upgoing plantars), cerebellum (truncal and limb ataxia, nystagmus), and bulbar function (dysarthria, dysphagia). Complications related to these clinical manifestations led to significant impairment in vital functions including swallowing (although dysphagia was not routinely assessed in all cases, severe dysphagia requiring gastrostomy was reported in several cases), respiration and functionally disabling spasticity, similarly to other myelin-related diseases [15]. Early screening and recognition of these disease-related complications are important aspects during the clinical assessments of these patients.

Furthermore, our study expands the MRI spectrum of *NKX6–2* mutations from diffuse hypomyelination to focal T2-weighted hyperintensity and parenchymal volume loss. Recently, other hypomyelinating leukodystrophy genes such as *TUBB4A* and *POLR3A* have been shown to have distinct phenotypes and neuroimaging spectrum, ranging from spastic paraplegia to spastic-ataxia without overt hypomyelination to hypomyelinating leukodystrophies resulting from different mutations [16, 17]. We identified a similar pattern in *NKX6–2* with cases presenting clinically with spastic-ataxia in the absence of hypomyelination and a reduction of NKX6–2 protein levels (case F1-III:1) compared to extended hypomyelination in cases with truncating mutations resulting in no expression of NKX6–2 protein as previously reported [2]. Therefore, we highlight the importance of molecular diagnosis with additional functional work and confirmatory evidence.

In conclusion, we show that the phenotypic and neuroimaging expression in *NKX6–2* mutations can range from a complex, neonatal onset at the severe end, a childhood onset at the milder end of the spectrum and that phenotypes with epilepsy in the absence of overt hypomyelination, and diffuse hypomyelination without seizures, can occur. We recommend that *NXK6–2* should be included in hypomyelinating leukodystrophy and spastic-ataxia diagnostic panels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

The authors would like to thank the patients and their families for their essential help with this work. We are grateful to The Spastic Paraplegia Foundation and The UK HSP Society. This study was supported by the Medical Research Council (MRC UK MR/J004758/1, G0802760, G1001253), The Wellcome Trust in equipment and strategic award (Synaptopathies) funding (WT093205MA and WT104033/Z/14/Z), Ataxia UK, British Neurological Surveillance Unit (BNSU), the National Institute for Health Research (NIHR). We also thank the

Sequencing and Genotyping Core Facilities at KFSHRC for their technical help. N.K is supported by KACST Grant (14-MED2007–20) and KFSHRC seed grant (RAC#2120022). I.D receives support from the NIHR UCL/UCLH Biomedical Research Centre. Supported in part by Doris Duke Clinical Scientist Development Award 2014112 MCK and NIH NINDS NS083739 (MCK). M.A.S. was supported by the Deanship of Scientific Research, King Saud University, Riyadh, Saudi Arabia through the research group project number RGP-VPP-301.

References:

1. Vanderver A., et al., Whole exome sequencing in patients with white matter abnormalities. Ann Neurol, 2016 79(6): p. 1031–1037. [PubMed: 27159321]
2. Chelban V., et al., Mutations in NKX6–2 Cause Progressive Spastic Ataxia and Hypomyelination. Am J Hum Genet, 2017 100(6): p. 969–977. [PubMed: 28575651]
3. Dorboz I., et al., Biallelic mutations in the homeodomain of NKX6–2 underlie a severe hypomyelinating leukodystrophy. Brain, 2017 140(10): p. 2550–2556. [PubMed: 28969374]
4. Anazi S., et al., Expanding the genetic heterogeneity of intellectual disability. Hum Genet, 2017 136(11–12): p. 1419–1429. [PubMed: 28940097]
5. Baldi C., et al., Expanding the clinical and genetic spectra of NKX6–2-related disorder. Clin Genet, 2018.
6. Lynch DS, et al., Clinical and genetic characterization of leukoencephalopathies in adults. Brain, 2017 140(5): p. 1204–1211. [PubMed: 28334938]
7. Blumcke I., et al., The clinicopathologic spectrum of focal cortical dysplasias: a consensus classification proposed by an ad hoc Task Force of the ILAE Diagnostic Methods Commission. Epilepsia, 2011 52(1): p. 158–74. [PubMed: 21219302]
8. Cepeda C., et al., Epileptogenesis in pediatric cortical dysplasia: the dysmature cerebral developmental hypothesis. Epilepsy Behav, 2006 9(2): p. 219–35. [PubMed: 16875879]
9. Briscoe J., et al., A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell, 2000 101(4): p. 435–45. [PubMed: 10830170]
10. Zhong YF and Holland PW, The dynamics of vertebrate homeobox gene evolution: gain and loss of genes in mouse and human lineages. BMC Evol Biol, 2011 11: p. 169. [PubMed: 21679462]
11. Pearson JC, Lemons D., and McGinnis W., Modulating Hox gene functions during animal body patterning. Nat Rev Genet, 2005 6(12): p. 893–904. [PubMed: 16341070]
12. Azuma N., et al., Mutations of the PAX6 gene detected in patients with a variety of optic-nerve malformations. Am J Hum Genet, 2003 72(6): p. 1565–70. [PubMed: 12721955]
13. Ragge NK, et al., Heterozygous mutations of OTX2 cause severe ocular malformations. Am J Hum Genet, 2005 76(6): p. 1008–22. [PubMed: 15846561]
14. Mohamed JY, et al., Mutations in MEOX1, encoding mesenchyme homeobox 1, cause Klippel-Feil anomaly. Am J Hum Genet, 2013 92(1): p. 157–61. [PubMed: 23290072]
15. Van Haren K., et al., Consensus statement on preventive and symptomatic care of leukodystrophy patients. Mol Genet Metab, 2015 114(4): p. 516–26. [PubMed: 25577286]
16. Curiel J., et al., TUBB4A mutations result in specific neuronal and oligodendrocytic defects that closely match clinically distinct phenotypes. Hum Mol Genet, 2017 26(22): p. 4506–4518. [PubMed: 28973395]
17. La Piana R., et al., Diffuse hypomyelination is not obligate for POLR3-related disorders. Neurology, 2016 86(17): p. 1622–6. [PubMed: 27029625]

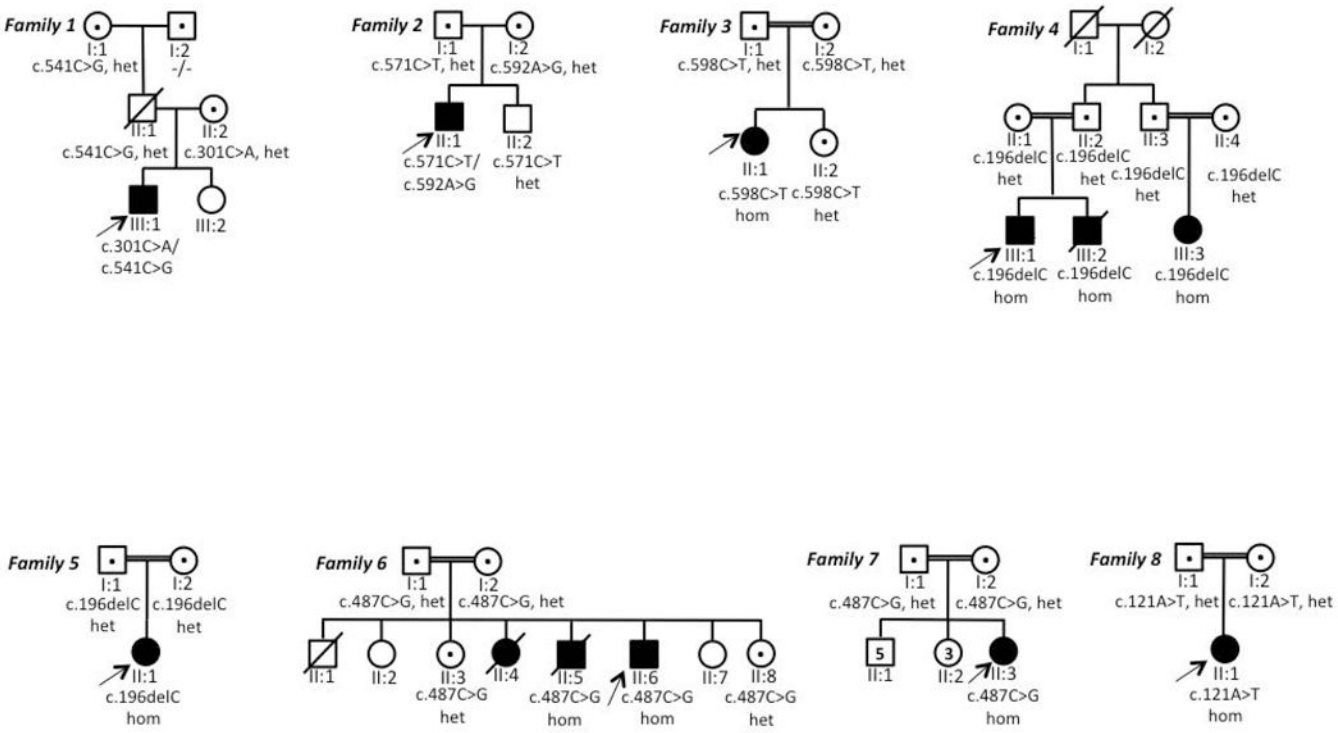

Figure 1. Family trees in all new families reported in this study.

het=heterozygous; hom= homozygous; the individuals tested in this study are indicated with a dot.

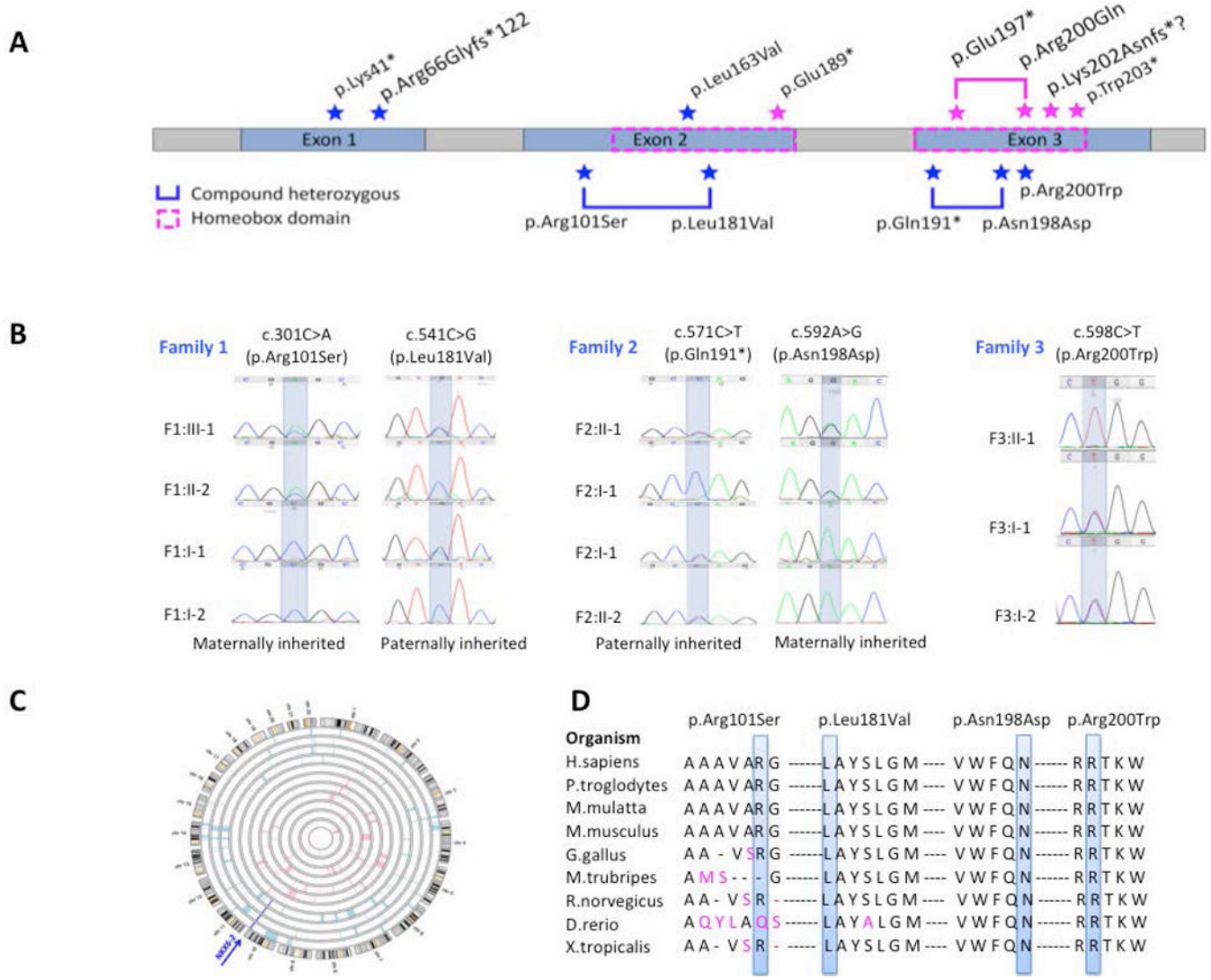

Figure 2. Mutation spectrum of *NKX6–2*–related disease.

A. *NKX6–2* gene with all the mutations identified. All known and novel mutations identified in our cohort are labeled in blue star; all mutations previously reported are labeled in magenta star and plotted on top of the gene.

B. Sanger sequencing confirmation with segregation analysis for novel *NKX6–2* variants reported in this study.

C. Homozygozity mapping in family IV and V identified a homozygous region on chromosome 10, shared by affected individuals and containing the pathogenic homozygous variant c.196delC in *NKX6–2*.

D. Conservation across species of each novel missense mutation reported in this study.

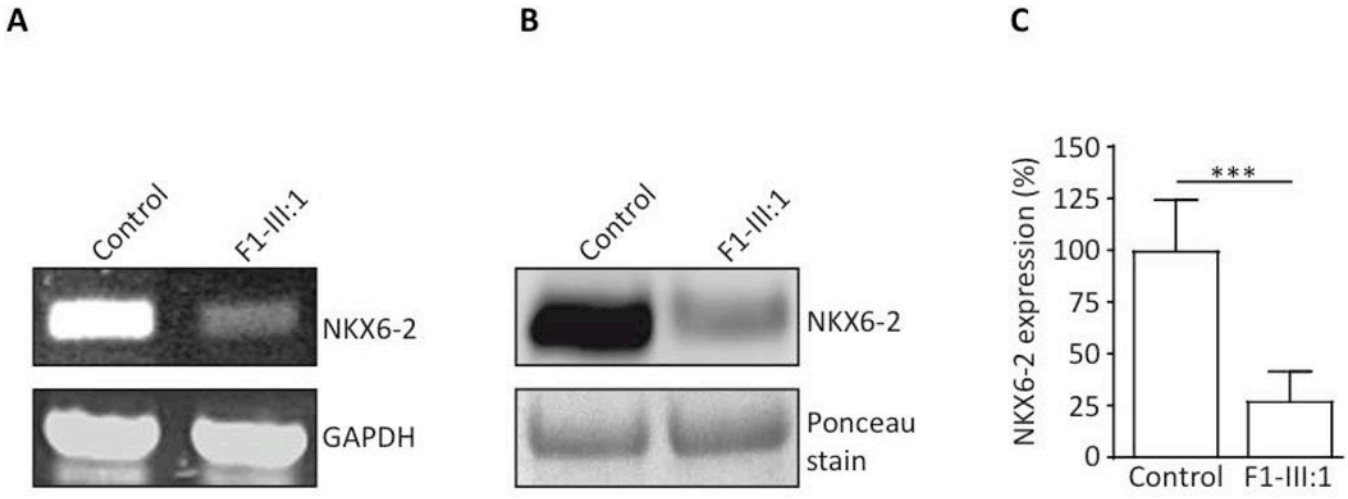

Figure 3. Functional analyses and pathogenicity of variants identified in Family 1.

A. Reverse transcription PCR in compound heterozygous missense case F1-III:1 showed absent or severely reduced *NKX6–2* compared to control (CTR); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as a loading control.

B. Reduced NKX6–2 protein levels confirmed by Western blot in individual F1-III:1. Total protein lysate extracted from human fibroblasts assessed by SDS-PAGE and analysed by western blotting using anti-NKX6–2 antibody (left panel).

C. Densitometry analysis shows significant reduction in NKX6–2 protein levels in fibroblasts harbouring the NKX6–2 missense mutation compared to control cells. *** $p < 0.01$, replicate values, mean and SD are shown; one-way ANOVA with Bonferroni post-hoc test.

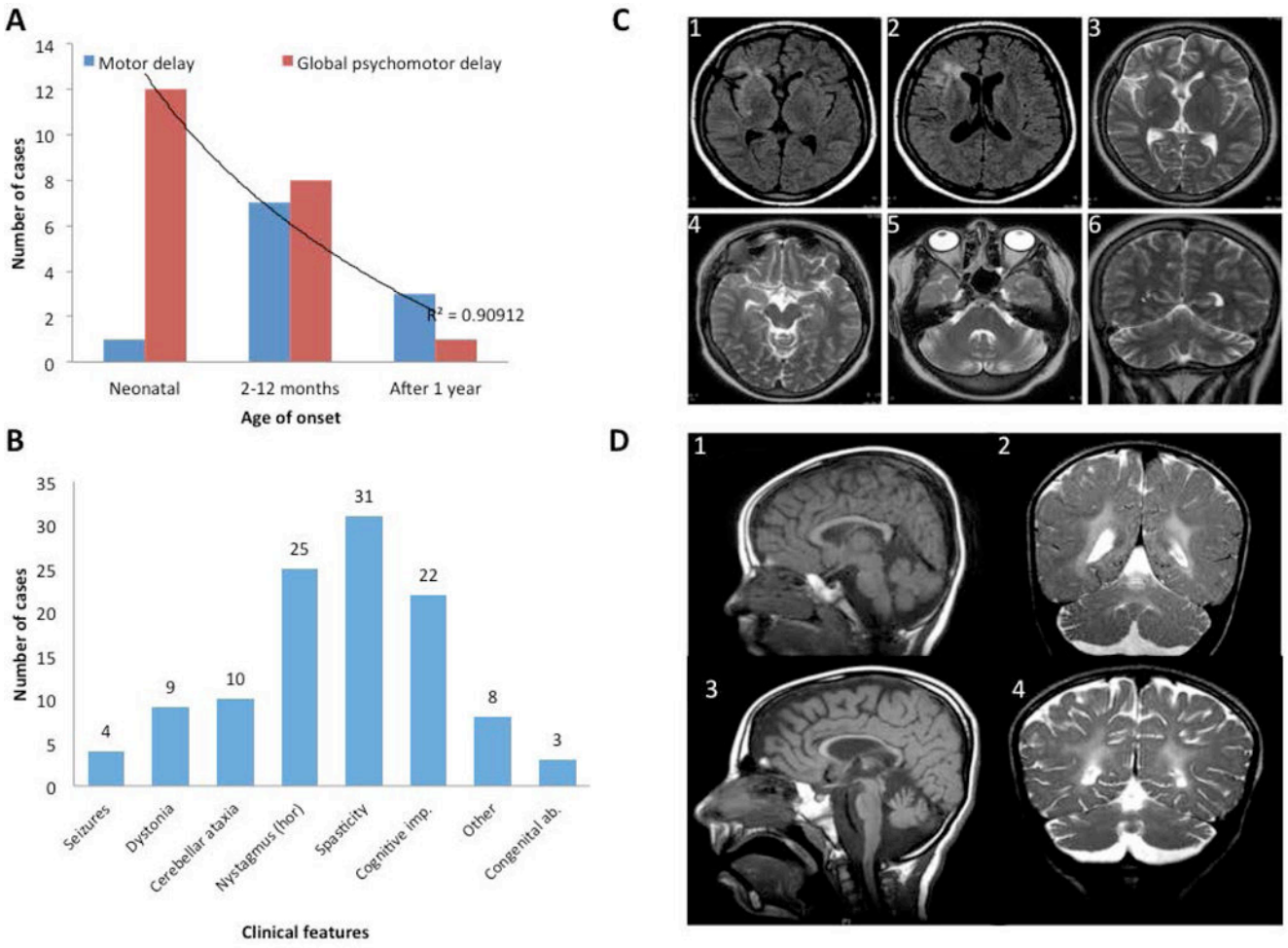

Figure 4. Genetype-phenotype correlation and neuroimaging spectrum of *NKX6–2* mutations.

A. The neonatal onset group has statistically significant higher frequency of global psychomotor developmental delay (red) compared with the other 2 groups (onset from 2 months -1 year and onset after 1 year). Childhood onset is associated with predominantly motor delay (blue). P=0.05, r2=0.9). n.s=not significant.

B. Clinical features associated with *NKX6–2* mutations (the horizontal axis), the number of cases on the vertical axis (total n=33). Hor =horizontal, gaze evoked nystagmus.

C. FLAIR and T2 weighted MRI acquisitions from case F1-III:1 exhibiting T2 hyperintense signal change in periventricular WM surrounding the frontal horn of the right lateral ventricle, and frontal and temporal opercular and subinsular WM T2 weighted hyperintense signal change associated with a degree of cortical volume loss. Note the normal signal intensity of the globi pallidi, thalami, and external capsules, mesencephalon and pons. There is disproportionate cerebellar volume loss with mild T2-weighted hyperintense signal change in the peri-dentate WM.

D. Longitudinal MRI in case F7-II:3 at ages of 4 years (D1, D2) and 8 ½ years (D3, D4)(D1, D3-mid-sagittal T1-weighted; D2, D4-coronal T2-weighted) showing progressive thinning of the corpus callosum and cerebellar atrophy associated with WM abnormality sparing the

U fibers (2,4). There is progressive enlargement of the cortical sulci and extra axial CSF spaces indicating underlying global brain atrophy.

Table 1.

Variant description of all *NXK6–2* mutations reported to date.

Zygosity	c.DNA change	Amino acid change	Type of mutation	Novel/ Known	ACMG score	ACMG classification	Onset	Phenotype	Additional signs reported	Hypomyelination	Cerebellar atrophy	Reference
Compound heterozygous	c.301C>A	p.Arg101Ser	Missense	Novel	PM2, PM3, PP1, PP3	Likely pathogenic	Childhood	Predominantly motor delay	Seizures	Mild	Yes	This study
	c.541C>G	p.Leu181Val	Missense	Known (rs369901030) (MAF= 0.0001170)	PM1, PM3, PP1, PP3	Likely pathogenic						This study
Compound heterozygous	c.571C>T	p.Gln191*	Nonsense	Novel	PVS1, PM1, PM2, PM3, PP1	Pathogenic	Neonatal	Severe global psychomotor delay	No	Diffuse, severe	No	This study
	c.592A>G	p.Asn198Asp	Missense	Novel	PM1, PM2, PM3, PP1	Likely pathogenic						This study
Homozygous	c.598C>T	p.Arg200Trp	Missense	Novel	PS1, PM1, PM2, PM3, PP1	Pathogenic	Neonatal	Severe global psychomotor delay	No	Diffuse, severe	Yes	This study
Homozygous	c.121A>T	p.Lys41*	Nonsense	Known	PVS1, PS3, PM2, PM3, PP1	Pathogenic	Childhood	Predominantly motor delay	Dystonia. Limitation of eye movements	Diffuse, severe	Yes	This study, Chelban et al[1]
Homozygous	c.196delC	p.Arg66Glyfs*122	Frameshift	Known	PVS1, PM2, PM3, PP1	Pathogenic	Neonatal	Severe global psychomotor delay	Limitation of eye movements, hearing impairment. Gastrostomy for severe dysphagia. Scoliosis	Diffuse, severe	No	This study, Anazi et al[2], Baldi et al[3]
Homozygous	c.487C>G	p.Leu163Val	Missense	Known	PM1, PM2, PM3, PP1	Likely pathogenic	Neonatal	Severe global psychomotor delay	Seizures. Gastrostomy for severe dysphagia.	Variable. 2 cases reported with no hypomyelination	Yes	This study, Chelban et al[1],

Zygosity	c.DNA change	Amino acid change	Type of mutation	Novel/ Known	ACMG score	ACMG classification	Onset	Phenotype	Additional signs reported	Hypomyelination	Cerebellar atrophy	Reference
												Baldi et al[3]
Homozygous	c.565G>T	p.Glu189*	Nonsense	Known	PVS1, PM1, PM2, PM3, PP1	Pathogenic	Neonatal	Severe global psychomotor delay	Severe dystonia	Diffuse, severe	Yes	Dorboz et al[4]
Compound heterozygous	c.589C>T	p.Gln197*	Nonsense	Known	PVS1, PM1, PM2, PM3, PP1	Pathogenic	Neonatal	Severe global psychomotor delay	Swallowing difficulties. Poor visual acuity	Diffuse, severe	No	Dorboz et al[4]
	c.599G>A	p.Arg200Gln	Missense	Known	PM1, PM2, PM3, PP1	Likely pathogenic						
Homozygous	c. 606delinsTA	p.Lys202Asnfs?1	Frameshift	Known	PVS1, PM1, PM2, PM3, PP1	Pathogenic	Neonatal	Severe global psychomotor delay	Severe dystonia	Diffuse, severe	Yes	Dorboz et al[4]
Homozygous	c.608G>A	p.Trp203*	Nonsense	Known	PVS1, PM1, PM2, PM3, PP1	Pathogenic	Neonatal	Severe global psychomotor delay	Seizures	Diffuse, severe	No	Baldi et al[3]

AA =amino acid; Comp. het. = compound heterozygous; Homoz.=homozygous;

1. Chelban V, Patel N, Vandrovcova J, et al. Mutations in NKX6–2 Cause Progressive Spastic Ataxia and Hypomyelination. *American journal of human genetics.* 2017;100(6):969–977.

2. Anazi S, Maddirevula S, Salpietro V, et al. Expanding the genetic heterogeneity of intellectual disability. *Human genetics.* 2017;136(11–12):1419–1429.

3. Baldi C, Bertoli-Avella AM, Al-Sannaa N, et al. Expanding the clinical and genetic spectra of NKX6–2-related disorder. *Clinical genetics.* 2018.

4. Dorboz I, Aiello C, Simons C, et al. Biallelic mutations in the homeodomain of NKX6–2 underlie a severe hypomyelinating leukodystrophy. *Brain : a journal of neurology.* 2017;140(10):2550–2556.