Expanding the Clinical and Mutation Spectrum of PRUNE Syndrome Within the Context of Microtubule Dynamics

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Abstract:

PRUNE syndrome, a neurodevelopmental disorder characterized by global developmental delay, microcephaly, hypotonia, and various other pathogenic phenotypes (OMIM#617481) is a rare autosomal recessive neurodevelopmental disease recently identified in homozygous and compound heterozygous variants of the *PRUNE1* gene. PRUNE is a member of the DHH phosphoesterase family that mediates cellular motility through interaction with GSK-3β and subsequent downregulation of the Wnt/β-catenin signaling pathway. This study presents an additional case of PRUNE syndrome, as identified in two similarly affected siblings from a non-consanguineous Indian family. Both patients present with severe developmental delay, primary microcephaly, and spastic quadriparesis. Whole exome sequencing and Sanger sequencing identified a novel homozygous splice acceptor PRUNE1 variant (c.119-2A>G) among the two siblings. These subjects follow the course of a combined neuromuscular and neurodegenerative disease that can be traced back to abnormalities in neuronal migration due to cytoskeletal and microtubule malformation. In addition to the growing literature identifying PRUNE as fundamental for normal cortical development in humans, this study elucidates a possible mechanism for PRUNE neuropathology through its downstream effects on the Wnt/ β-catenin signaling pathway in the context of microtubule dynamics. Finally, this study explores the prospect of this novel splice variant as a founder mutation within the Indian population, proposing future implications to the field of population genetics.

Introduction:

A. *Introduction to Neurodevelopment*

Human brain development is a dynamic process that begins in the third gestational week and extending throughout early adulthood, although plasticity is observed throughout life. The final product of neurodevelopment is a product of genome encoded instructions and environmental input, both of which drive the structural and functional aspects of the human brain (Stiles and Jernigan, 2010). Gene-by-environment interaction studies help to identify how environment can shape biological processes that alter brain development and function. Genetic studies provide important clues about the cell biology and biochemical processes required to establish the basic structural features of the brain and the complex cognitive functions they facilitate (Karaca et al., 2016). Human genetic studies, investigating the genetic causes of neural developmental disorders and syndromes, have been a very effective strategy to identify the genes and genetic components required for normal brain development and associated neuropathology. Neuronal development can be summarized as three major steps: neurogenesis, neuronal migration, and postmigrational cortical organization and circuit formation (Karaca et al., 2016), (Stiles and Jernigan, 2010). The aggregative effects of molecular events occurring at the cellular level lead to the gross morphological changes observed in the developing prenantal neural system. The adult brain is estimated to have 60 trillion neuronal connections, which make up the neural circuitry in our brain. Initially, the level of connectivity throughout the human brain is far greater than that found in adulthood. These connections are later pruned back by competitive processes experienced by the organism; this concept is the foundation of the idea of neural plasticity, further emphasizing the dynamic nature of neurodevelopment (Stiles and Jernigan, 2010).

Neurogenesis and Differentiation of Neural progenitor cells:

Neural stem cells are among the three primary stem cell lines that develop during fetal gastrulation, and are denoted neural progenitor cells (NPCs). NPCs eventually differentiate into different cell types and give rise to all of the cells that make up the brain and central nervous system. Neurogenesis is the process by which neural stem and progenitor cells generate neurons. Neurogenesis for neurons of the central nervous system occurs in the neural tube, and is followed by neuronal migration, differentiation, dendrite and axon formation, synaptogenesis, and the establishment of neuronal connectivity/synapses (Stiles and Jernigan, 2010). Early on in embryonic development, most neuroepithelial progenitor cells undergo symmetric division in the ventricular zone (VZ) which increases the founder cell (radial glia) population. At the onset of neurogenesis, asymmetric neurogenic divisions in the VZ yields self-renewal of radial glial cells and produces neurons destined for different layers in the cortical plate (Stiles and Jernigan, 2010). Another mechanism of neuron generation involves the production of intermediate progenitor cells from glial cells. These intermediate progenitors migrate to the embryonic subventricular zone (SVZ) where they go on to produce neurons through symmetric division. The purpose of this two-step mechanism is to create a population of neurons of a particular subtype, which will ultimately occupy the same cortical layer. The mechanism through which the developing brain creates neuronal diversity is a result of coordinated changes in gene expression coupled to a pattern of asymmetric neuroblast divisions. Neuronal precursor cells sequentially express a series of distinct transcription factors. The change in expression defines a temporal window in which the neuroblast can create a sublineage of a particular identity, which then go on to perform distinct functions within the nervous system (Kriegstein et al., 2006).

Neuronal migration:

Neurons are produced as stem cells at embryonic day 42 (E42) in the walls of the brain ventricles. After their final mitosis in the ventricular zone, most neuroblasts migrate substantial distances in order to reach their final location. These stem cells must migrate to different parts of the brain where they make connections with other neurons, thus establishing the beginnings of neural pathways. Neuronal migration causes the formation of different proliferative zones, creating distinct lineages and genetic programs where adjacent neurons interact with one another. Different kinds of neurons arrive at a particular location at a specific time during development, creating neural circuits in a specific order. This migration occurs during the early fetal period of embryonic brain development, which begins at GW8 and lasts until midgestation. This is a critical period in the development of the neocortex, as this is when the cortical neurons migrate to their positions in the neocortex and are beginning to form rudimentary networks with other neurons. The majority of neurons are produced in the VZ and migrate radially towards the center of the brain out to the neocortex. The earliest produced neurons employ a method of migration known as somal translocation, in which it moves out of the VZ and into the embryonic cortex. (Cooper, 2013) (Stiles and Jernigan, 2010). The final location of a postmitotic nerve cell is therefore critical for proper functioning of the brain, because neural function depends on precise connections made by neurons and their targets. Neurons of the central nervous system remain within the limits of neural tube, whereas neurons of the peripheral nervous system come from the neural crest and have travelled long distances through several embryonic environments. Neurons of the cerebral cortex, cerebellum, hippocampus, and spinal cord are guided to their final destinations by crawling along radial glia cells, which are glial cells that at as cellular guides.

Neurons bound for the peripheral nervous system, on the other hand, are guided along distinct migratory pathways by specialized adhesion molecules in the extracellular matrix, or by molecules on cell surfaces in the embryonic periphery. Neuronal migration is thus a dynamic process that involves signaling across different cells in order to occur properly, and provides a means of constraining cell-cell signaling to specific times and places (Purves D et al., 2001).

Gene expression during neuronal migration:

Neuronal migration depends critically on the sequential activation of genes in accordance with various molecular events and complex cell-cell interactions. Recent studies have revealed the intricacy of this process, which has been subdivided into several distinct, but closely related steps. These steps can be summarized as exit from the cell cycle, detachment from the ventricular surface, establishment of neuronal identity, establishment of cell polarity, nuclear and somatic translocation, and ending with the cessation of migration at the correct final position (Rakic et al., 2007). A few of the transcription factors known to participate in these processes are Doublecortin (DCX) and Filamin-A (FLNa), which serves as a scaffolding protein for the complex mitogen-activated protein kinase (MAPK) signaling pathway during neuronal migration. Each of these steps relies on the coordination of specific reciprocal signaling interactions, and is thus susceptible to serious complications if any one of these signaling partners is misregulated (Rakic et al., 2007).

B. Genetic defects that lead to problems in early brain development

Genes, the heritable sequences that underlie protein production in the cell, play a fundamental role in the process of brain development. Particular gene products and the signaling cascades

they participate in work together to drive the complex process of neurodevelopment. (Stiles and Jernigan, 2010). Genetic mutations lead to defects in the integrity or the amount of the protein products that are made, and can thus have serious consequences on any stage of brain development. There are several different types of mutations that a gene can undergo, with differing degrees in the severity of their impact. Some examples are missense mutations, in which a change in one nucleotide causes an amino acid to be switched for another, and frameshift mutations, in which the insertion or deletion of an mRNA nucleotide causes a shift in reading frame (Hershberg, 2015).

Splice variants are mutations that occur in the cell due to a process called alternative splicing. Splicing is a eukaryotic post-transcriptional modification that occurs in the nucleus, in which the introns (non-coding regions) of a nascent mRNA molecule are excised and the exons (coding regions) are ligated together. This mechanism is carried out by the spliceosome, a dynamic ribonucleoprotein machinery that cycles through seven distinct states as it catalyzes the pre-mRNA splicing reaction (Jankowsky, 2011). The two steps involved in the splicing reaction are called branching and exon ligation; they proceed via an S_N2-type transesterification mechanism, mediated by two magnesium ions (Mg^{2+}) called M1 and M2 (Steitz and Steitz, 1993). Splicing is a necessary component for the production of mature mRNA, as it creates a continuous sequence of exons that is ready to be translated into a properly functioning protein. Alternative splicing is a process by which various functionally distinct mRNA transcripts are produced from a single gene through the selective exclusion/retention of different exons/introns from the pre-mRNA (Chow et al., 1977). Weaker splicing signals at alternative splice sites, shorter exon length, or higher sequence conservation surrounding orthologous alternative exons influence the spliceosome in dictating which exons are ultimately included in the mature mRNA (Zheng et al.,

2005). This process is fundamental to many processes within the biological system, as the increased flexibility in gene expression is manipulated is processes such as cellular differentiation and organism development. However, alternative splicing is also the molecular basis of many human diseases, including Mediterranean anemia, type II diabetes, Alzheimer's disease, Duchenne–Aran disease, retinitis pigmentosa, and cancer (Li and Yuan, 2017). Improperly spliced mRNA can have deleterious effects on the protein product's structure and/or function, and pathogenic splice variants are frequently identified in patients suffering from genetic diseases.

Brain abnormalities with distinct genetic contributions:

Malformations of cortical development (MCDs) encompass a wide range of structural brain abnormalities, resulting from the disruption of one or more of the key steps involved in neurogenesis. MCDs are the underlying cause of many neurodevelopmental disorders, including epilepsy, developmental delay, intellectual disability, autism, and schizophrenia (Juric-Sekhar et al., 2019). The majority of MCDs can be attributed to genetic mutations, which result in a disruption of the encoded proteins and the complex signaling pathways that dictate corticogenesis (Barkovich et al., 2012). MCDs can be classified as disorders of neuronal or glial proliferation or apoptosis, disorders of cell migration, disorders of postmigrational development, and malformations caused by metabolic disorders, peroxisomal disorders, or sublobar dysplasia (Hong et al., 2017) (Barkovich et al., 2012). The genetic etiologies of many MCDs are incorporated into their classifications, as many of them have been linked to mutations in genes involved in cellular activities such as cell-cycle regulation and apoptosis, cytoskeletal structure and function, basement membrane function, neuronal migration, and intracellular metabolic activities (Juric-Sekhar et al., 2019).

Microtubules (MTs) are a feature of cellular cytoskeletons that are of particular importance, because of their key role in the process of neuronal migration. Microtubules surround the nucleus of the cell, while actin filaments localize to the cell periphery in close association with the plasma membrane (Rivas and Hatten, 1995). Neuronal migration is highly dependent on the activity of microtubules, as they are responsible for the dynamic remodeling of the cytoskeleton and nuclear translocation (Wu et al., 2014). During migration, neurons project a cell extension called the leading process, and the microtubule organizing center (MTOC) is positioned between the leading process and the nucleus of the cell. In the leading process, the positive ends of microtubules are uniformly oriented toward the growing tip, and the negative ends face the nucleus (Rakic et al., 1996) Different signaling proteins interact with one another mediate this process of cytoskeletal rearrangement. Proteins such as LIS1 and CLIP-170 are thought to promote growth of polarized microtubules. DCX and MAPs are thought to stabilize and bundle microtubules in the leading process. (Bielas and Gleeson, 2004). Microtubules navigate through intracellular space by alternating between periods of growth and shrinkage. Upon cell polarization, proteins localized at the plus end of microtubules are bound by microtubule anchors at the plasma membrane, tethering the microtubules to distinct subcellular regions of the cell membrane (Schuyler and Pellman, 2001) (Gundersen, 2002). Examples of these proteins are CLIP-170 and LIS1, which are thought to localize at the plus end of microtubules, promoting microtubule growth and bundling (Coquelle et al., 2002) (Bielas and Gleeson, 2004). Genes involved in microtubule formation include *TUBA1A*, *TUBB2B*, *TUBB3*, and *TUBG1*, and mutations in these genes have been identified in cases of primary microcephaly, lissencephaly , microlissencephaly, pachygyria, and polymicrogyria (Bahi-Buisson et al., 2014). Additionally, microtubule-associated proteins (MAPs) are critical modulators of cytoskeletal organization and

dynamics, and are necessary for proper microtubule function. Examples of MAPs include LIS1, DCX, DYNC1H, and KIF5C, and mutations in these proteins have been associated with severe microcephaly, diverse lissencephalies, and subcortical band heterotopia (SBH)(Di Donato et al., 2017).

The *Lissencephaly1* (*LIS1*) gene codes for a microtubule binding protein necessary for cortical migration, whose mutation leads to the MCD lissencephaly (Reiner et al., 1993). Lissencephaly (LIS) is a disorder of neuronal migration and proliferation that causes the absence of gyri (agyria) or excessively wide gyri (pachygyria). Other phenotypes associated with LIS are thickening and disorganization of the cortical plate, misplaced or ectopic neurons in the subcortical white matter, and smaller brain size with enlarged ventricles (Allanson et al., 1998). Clinical symptoms include feeding problems, delayed motor milestones, mental retardation, or seizures (usually before 6 months) (Barkovich et al., 2012). Most classic LIS is caused by mutations in genes encoding cytoskeletal proteins, such as *LIS1*. The *LIS1* gene (17p.13.3) encodes a 45 kDA protein with dual functions as both a regulatory subunit of platelet-activating factor acetylhydrolase 1B and a component of the neuronal cytoskeleton that interacts with microtubule-associated proteins, especially dynein, and is thus important for cell division and migration. It is known to affect the posterior regions of the brain more severely, specifically the hippocampus (Juric-Sekhar et al., 2019) (Bielas and Gleeson, 2004). LIS1 is also implicated in neuronal migration through its positive regulation of cytoplasmic dynein, a motor protein that drives a variety of cargoes towards the minus ends of microtubules (Allan 2011). LIS1 colocalizes with components of the dynein and dynactin complexes; its underexpression leads to the diffuse perinuclear localization of the dynein intermediate chain and the concentration of microtubules to the center of the cell, effectively disrupting cell migration (Smith et al., 2000)

(Bielas and Gleeson, 2004). *DCX* (doublecortin) is a related gene of interest, as it causes an X-linked form of LIS in males, or SBH (double cortex) in females. DCX is a microtubule-associated protein that is expressed in IPs and new postmitotic neurons. Most cases of classic LIS are associated with mutations in LIS1, while most cases of SBH are associated with DCX mutations. DCX mutations are thought to affect the anterior regions of the brain more severely. (Juric-Sekhar et al., 2019). LIS1 and DCX are thus critical mediators of neuronal migratory processes through their regulation of the microtubule cytoskeleton, and their absence/dysfunction underlie the pathology of cytoskeletal-associated MCDs. Analysis of lissencephaly provides a concrete example of the physical neurological manifestations that result from disrupted microtubule dynamics as a result of genetic mutations, suggesting that similar structural brain abnormalities could be a result of microtubule defects as well.

B. PRUNE1

Literature Review:

This report describes a rare autosomal recessive disorder called PRUNE syndrome which results from defects in the PRUNE1 gene (OMIM #617413). To date, there are 12 different PRUNE mutations described in the literature, affecting a total of 48 individuals. In 2015, Karaca et al. identified bi-allelic PRUNE1 mutations in five children from four families with developmental brain abnormalities, including microcephaly, brain atrophy, developmental disability, seizures, and spastic quadriparesis. An identical homozygous variant was identified in two apparently unrelated families from nearby villages in Turkey (c.G136A, p.D106N). Both subjects presented with microcephaly, frontotemporal cortical atrophy, and cerebellar atrophy. A founder effect was suspected to play a role in the etiology of the mutation, due to the proximity of the two villages and the high rates of consanguineous marriages among this population. Additionally, an 18-

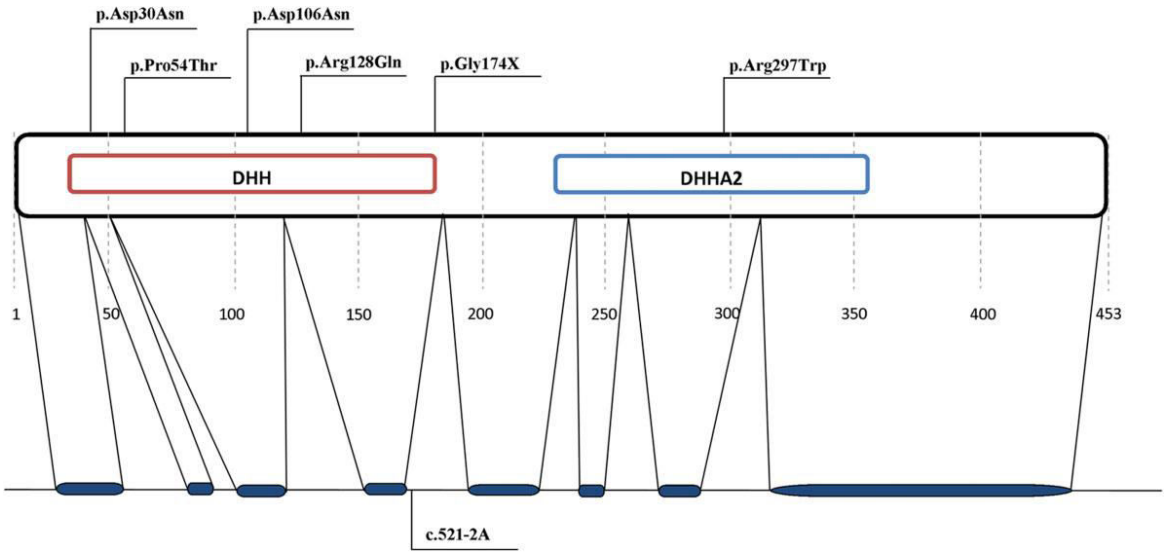
month-old child from a consanguineous Saudi Arabian family was identified as homozygous for a rare variant (cG88A, p.D30N). The patient presented with cerebral and cerebellar atrophy, microcephaly, seizures, and severe developmental delay. Two siblings from a fourth non-consanguineous family from the US were identified with a compound heterozygous mutation (c.G383A, p.R128Q and c.G520T, p.G174X). Both probands presented with developmental delay, regression, seizures, and microcephaly marked by cerebral and cerebellar atrophy. (Karaca et al., 2015). Shortly after, Zollo et al. identified PRUNE mutations in 13 individuals in extended families from Iran and Oman, along with two smaller families from India and Italy. All of the identified PRUNE variants had coding mutations, specifically c.88G4A (p.Asp30Asn), c.160C4A (p.Pro54Thr), c.316G4A (p.Asp106Asn), and c.889C4T (p.Arg297Trp) that would be identified in additional cases in the future. These individuals presented with overlapping clinical features, such as microcephaly, profound developmental delay, spastic quadriparesis, etc. (Zollo et al., 2017). Karakaya et al reported an additional affected individual from a healthy consanguineous Turkish family, presenting with profound global developmental delay, congenital hypotonia, secondary microcephaly, and respiratory insufficiency. Trio-whole genome sequencing identified a homozygous frameshift variant, c.874_875insA (p.H292Qfs*3) in *PRUNE1* as the primary candidate mutation (Karakaya et al., 2017). Additionally, Alfadhel et al presented a case of two unrelated Saudi Arabian girls with typical representations of PRUNE syndrome, presenting with profound developmental delay, progressive microcephaly, and dysmorphic features. Interestingly, both patients were identified as having the same homozygous missense mutation (c.383G>A, p. Arg128Gln), suggesting a founder effect at play within the Saudi population (Alfadhel et al., 2017). Iacomino et al reported an additional case of a homozygous PRUNE mutation in an Italian child from a non-consanguineous family, presenting

with typical PRUNE syndrome phenotypes, along with spinal muscular atrophy not seen in previously reported cases. The proband was identified with a homozygous missense mutation (c.316G>A, p.D106N) (Iacomino et al., 2018). Imagawa et al identified 3 PRUNE mutations in one Caucasian and three Japanese families. One recurrent missense mutation (p.Asp106Asn) had been previously reported in Turkish and Italian families, while the other two mutations (Leu18Serfs*8 and p.Cys180*) were novel. This report was unique in that the PRUNE patients showed atypical NMIHBA phenotypes and did not display progressive microcephaly. Furthermore, the Caucasian patient in this report presented with significant macrocephaly, which has not been described in any other PRUNE cases. This report elucidated the variability in the physical manifestations of PRUNE mutations, depending on the type of variant the individual is affected by (Imagawa et al., 2018). Costain et al. and Hartley et al. were the first to identify a biallelic splice acceptor variant (c.521-2A>G) affecting 10 individuals within the Oji-Cree population, indicating a founder effect present within the population due to the mutation's abnormally high frequency (Costain et al., 2017) (Hartley et al., 2018). This case report describes a novel splice acceptor variant (c.119-2A>G) identified within an Indian family that has not yet been described in the literature, further elucidating the mechanistic implications PRUNE protein has on neurodevelopment and corticogenesis.

PRUNE1:

PRUNE1 is a gene of particular significance as it is highly expressed in human fetal brain and has been previously correlated with neurodevelopmental pathology. This gene encodes prune protein (h-prune), a member of the DHH phosphoesterase (PDE) family and has been shown to be highly expressed during early neurogenesis. It functions in cell proliferation, migration, and motility (Reymond et al., 1998). Overexpression of h-prune in cultured cells has been shown to

promote cellular motility, and inhibition of PDE activity by a PDE inhibitor has been shown to inhibit h-prune-induced motility. Phosphoesterase enzymes hydrolyze phosphoester bonds across a broad range of key substrates found within the body, including CTP, TTP, GTP, DNA, and RNA, catalyzing a reaction that produces both mono and diphosphate nucleotides. Inhibition of PDE activity by dipyramidole suppresses cell motility (D'Angelo et al., 2004). Prune is thought to mediate cellular motility both by PDE activity and through interaction with GSK-3β (Kobayashi et al., 2006). PRUNE1 forms a homodimer via the C-terminal cortexillin homology (CHD) domain. The CHD domain also includes binding sites for NM23-H1 and GSK-3β, whose interactions are associated with enhancement of PRUNE1 activity and cell migration. Interestingly, all of the previously described missense mutations retain normal CHD domain and may lead to gain-or-loss of function in PRUNE 1 (Middelhaufe et al., 2007). This DHH phosphoesterase interacts with GSK-3β and proteins involved in cytoskeletal organization, including microtubules (Reymond et al., 1999). Prune's phosphoesterase activity functions to inactivate GSK-3β, effectively inducing changes in GSK-3β downstream signaling pathways. Proper h-prune formation and function is thus essential for proper microtubule regulation in cells.



Alfadhel et al., 2017

Figure 1: Depiction of the *PRUNE1* gene, which consists of 8 exons. PRUNE protein is composed of 453 amino acids, comprising 2 domains: DHH (20-172) and DHHA2 (215-358). The majority of described mutations have been found in the DHH domain, with only one mutation identified thus far in the DHHA2 region. Several recently identified PRUNE mutations are missing from the above figure.

The enzyme GSK-3 is a serine/threonine kinase glycogen synthase kinase that regulates various signaling pathways including glycogen synthesis, protein synthesis, gene expression, subcellular localization, of proteins, and protein degradation. Specifically, GSK-3 has been shown to be a potent regulator of neurodevelopmental processes. In mammals, GSK-3 comes in two forms, either GSK-3alpha or GSK3-β. GSK3-β is of interest to us because it interacts with h-prune. GSK-3β and h-prune cooperatively regulate the disassembly of focal adhesions to regulate cell migration (Kobayashi et al., 2006). The kinase activity of GSK-3β is required for the interaction of GSK-3β with h-prune. Amino acid region 333 to 453 of h-prune was necessary and sufficient for the complex formation with GSK-3β in intact cells (Kobayashi et al., 2005). Knockdown of GSK-3β or h-prune delayed the disassembly of paxillin (protein expressed at focal adhesions;

adheres cells to extracellular matrix). The tyrosine phosphorylation (and subsequent activation) of focal adhesion kinase (FAK) and the activation of Rac were suppressed in GSK-3 or h-prune knocked-down cells. As discussed above, cell migration is a crucial step in neurodevelopment that involves protrusion and adhesion at the cell front and contraction and detachment at the rear (Kobayashi et al., 2005) (D'angelo et al., 2004).

Wnt Signaling Pathway:

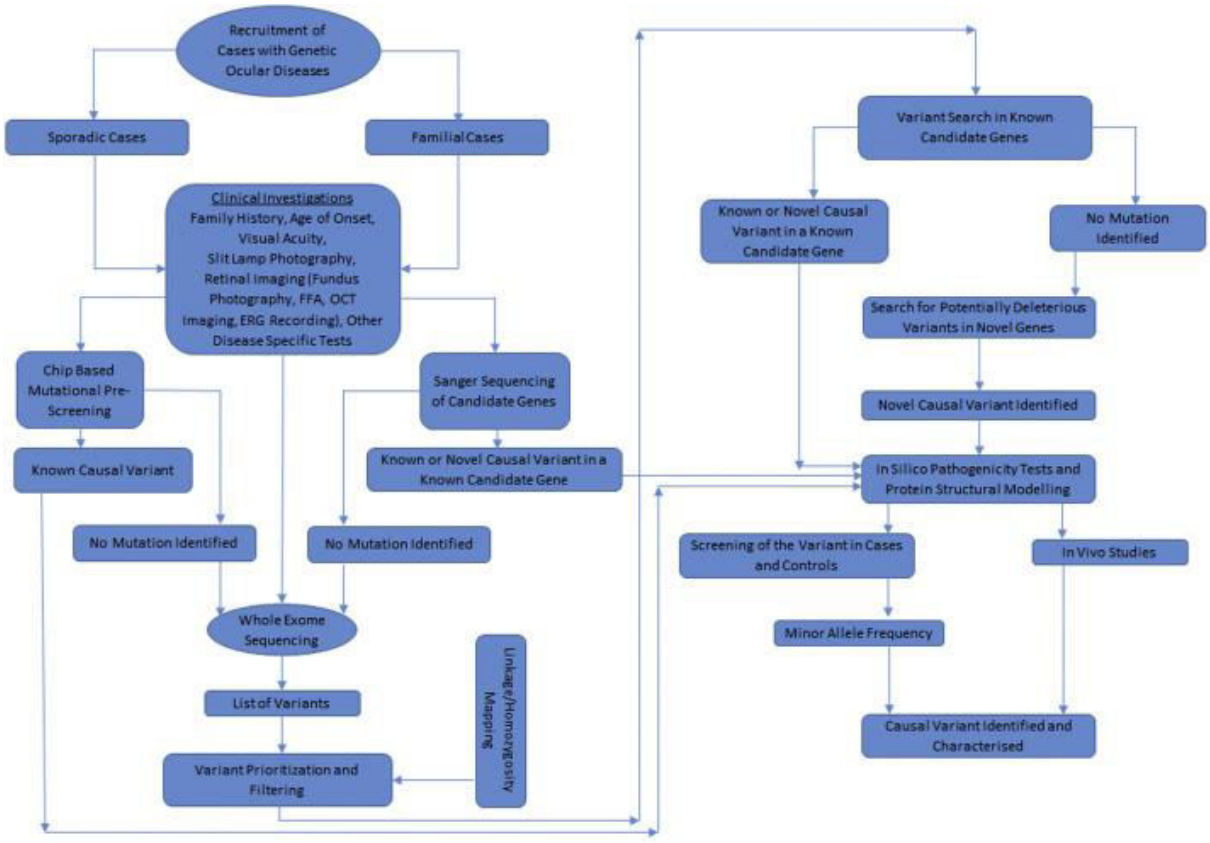
The canonical Wnt (**Wnt/β-catenin**) signaling pathway is involved in many biological processes, such as cell fate determination, cell proliferation, and stem cell maintenance. For example, activation of the Wnt/β-catenin pathway facilitates synaptic plasticity through stimulation of the voltage gated ion channels that mitigate CAMK and CREM mediated transcription (Caracci et al., 2016). Dysregulation of this pathway is observed in many cancers and hereditary syndromes. Wnt signaling is regulated by a series of steps catalyzed by a dedicated cytoplasmic destruction complex (APC complex), which is made up of the proteins Axin, adenomatous polyposis coli (APC), glycogen synthase kinase-3 alpha/beta (GSK-3), and casein kinase-1 (CKI) (Clevers, 2006). Gsk-3β functions within the APC complex to phosphorylate the Wnt downstream effector molecule β -catenin. Phosphorylation of β -catenin by GSK-3β initiates its degradation within the cytoplasm, which is a key step in Wnt signaling regulation (Yost et al., 1996). Inhibition of the APC complex or Gsk-3β activity results in protein stabilization of β-catenin, allowing it to translocate to the nucleus and induce the transcriptional activation of genes involved in cell growth, invasion, stem cell phenotype, and metastasis, via binding to TCF/LEF transcription factors (Li et al., 2012). TCF/LEF activation leads to increased secretion of Wnt3a, a paracrine activator of the Wnt signaling pathway (Carotenuto et al., 2014). Interestingly, research has demonstrated that h-prune plays a role in activating canonical WNT/β-catenin signaling by

promoting sequestration of Gsk-3β inside multivesicular bodies (MVBs). Sequestering of Gsk-3β inside MVBs physically separates it from its many cytosolic substrates, which is an essential step in the WNT/β-catenin signaling pathway (Taelmen et al., 2010). Thus, GSK-3β is a potent inhibitory regulator of the Wnt/β-catenin signaling pathway, further emphasizing the significance the h-prune/ GSK-3β interaction has on cell proliferation. This, in turn, identifies h-prune as an early diagnostic marker for lung cancer (Carotenuto et al., 2014).

Whole Exome Sequencing (WES):

Whole-exome sequencing (WES) is a diagnostic tool utilized in clinical genetics that provides an accurate, high-resolution sequence of the patient's genome, as a means of elucidating the genomic contribution to the pathogenesis of a disease (Mackley et al., 2017). Traditional methods that attempt to identify and characterize pathogenic genes are based upon functional/candidate gene cloning. Modern, high-throughput methods, such as genome-wide linkage search and genome-wide association studies (GWAS) have also been used in familial and sporadic cases of genetic diseases. These techniques are limited, however, in that they can only reveal the genetic locus associated with the disease, relying on known variants that only explain a fraction of the mechanism of disease inheritance. These limitations lead to an inability to identify the novel and rare causal variants that could account for a much larger fraction of disease heritability. By turning to DNA sequencing instead, geneticists can screen the entire genome or targeted genomic region and identify all potential genetic mutations within an individual. This provides a comprehensive analysis of the genetic makeup of the patient, allowing clinicians to investigate all of the potential genetic variations (and thus the biochemical implications) that could be contributing to the pathogenesis of a disease. Because the genomic

data gathered through these tests are specific to each person, DNA sequencing methods also offer personalized disease-risk profiling, paving the way for direct therapeutic strategies based on pharmacogenetics. Several sequencing methods have been developed over the past decades, but whole exome sequencing has now become the favored method for pathogenic gene detection. Exome sequencing involves targeted enrichment and sequencing of the complete protein coding region, which actually accounts for less than 2% of the entire genome. Figure2 provides an elementary depiction of mutation identification using WES.



Gupta et al., 2017

Figure 2: Basic work flow of mutation detection in diseases using WES. The study subjects recruited for the human genetics study consist of familial and/or sporadic cases. Prior to thorough clinical analysis of the phenotypic data, chip-based mutational pre-screening or targeted Sanger sequencing can be utilized to identify causal variants. If not, WES can be used to identify an extensive list of variants present within the genome. The list can be categorized based upon various criteria, such as mode of inheritance, nature of variants, or genome-wide linkage searches. Novel causal variants can be searched in known candidate

genes. If known candidate genes do not reveal pathogenic variants, novel genes can be screened for deleterious variants. Once a candidate mutation is identified, it can be further characterized using in silico analysis, validation in sporadic cases and controls, and functional studies.

WES has gained overwhelming popularity due to its efficacy in pathogenic gene discovery. Exome sequencing is particularly effective because 85% of diseases causing mutations with high penetrance are found within the protein coding region of a gene (Majewski et al., 2001) (Botstein and Risch, 2003). The success of WES analysis is contingent on additional factors as well, such as the nature and location of the causal variant, penetrance, coverage efficacy, and data analysis. Through its personalized and accurate genome analysis, WES has been at the forefront of major advancements in genetic discoveries, and has been successful in identifying genetic defects in 60-80% of Mendelian diseases (Gilissen et al., 2012). Thus, WES was the analytical strategy employed in this study to identify the pathogenic *PRUNE1* variant present in this case.

Materials and Methods:

Patient recruitment was facilitated through a US-Indo grant by the main physician responsible for this case. Blood samples were obtained from each of the patients. DNA was extracted from the blood using standard techniques DNA samples were submitted to the University of Washington Center for Mendelian Genomics (UW-CMG) where library construction, WES, and analysis were performed. Briefly, sequencing libraries were generated for each DNA sample in an automated, 96-well plate format (PerkinElmer Janus II). Sample libraries were constructed from 1 μg of genomic DNA, which underwent a series of shotgun library construction steps including acoustic sonication (Covaris), end-repair, A-tailing ligation of unique sequencing adaptors, and polymerase chain reaction amplification. Sample libraries were hybridized to the

Nimblegen SeqCap EZ v2.0 target (~36.5 Mb) in multiplex for a period of 72 h. Captured DNA was then purified, polymerase chain reaction amplified, and normalized for sequencing. Captured DNA was sequenced on Illumina HiSeq machines using paired-end sequencing. Raw sequence data in FASTQ format was aligned to the human genome reference hg19 using the Burrows–Wheeler Aligner algorithm for the generation of BAM files. The quality of each sample was assessed for coverage (80% of sequenced target with ≥20 × coverage and 90% of target with ≥8 × coverage) and transition/transversion ratios. Additionally, samples were quality controlled to confirm sex using PLINK (v1.90b2m) software, and estimations of kinship were corroborated with pedigrees using KING v.1.4.0 software.

Variant Detection, Interpretation, and Validation

Variants and indels were detected and genotyped using HaplotypeCaller from Genome Analysis Toolkit with hard filtering parameters. A multisample VCF was generated for all samples following Genome Analysis Toolkit best practices. The multisample VCF was annotated using the Variant Effect Predictor (VEP) tool v.83 within GEMINI (http://uswest.ensembl.org/info/docs/tools/vep/index.html). Copy-number variants (CNVs) were detected from exome sequence data using the program CoNIFER (Moccia et al., 2018). GEMINI v.0.19.1 was used to filter variants and indels detected in affected individuals. Variants were excluded if allele frequency in reference populations (ExAC, National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project (ESP) 6500, or 1000 Genomes) exceeded 0.005 or exceeded 0.05 in the UW-CMG internal database. Variant impact determined by Variant Effect Predictor was also utilized to exclude variants and indels with an impact severity of “low” (e.g., intergenic, intronic, synonymous, 5’UTR, and 3’ UTR variants). Variant prioritization was based on the following parameters: variant impact, presence in a disease-

associated gene, conservation of impacted nucleotide by genomic evolutionary rate profiling (GERP), or predicted deleteriousness (combined annotation dependent depletion score (CADD) >15). Interpretation of detected CNVs was based on size, frequency in the UW-CMG internal database, and overlap with known disease-associated regions. In addition, CNVs were filtered based on phenotypic overlap according to the American College of Medical Genetics and Genomics guidelines for postnatal CNV calling (Moccia et al., 2018).

Sanger sequencing was used to validate rare, protein-altering variants. Variant segregation was also used to confirm inheritance pattern. Analysis was performed according to American College of Medical Genetics (ACMG) guidelines in preparation for future publication.

Results:

The results of these experiments identified PRUNE1 gene as responsible for the pathogenic phenotypes found in two similarly affected siblings from a non-consanguineous Indian family. WES was performed on 2 individuals (subjects I and II), and Sanger sequencing was used to validate rare, protein-altering variants. The WES analysis identified biallelic PRUNE1 ((NM_021222.1) variants in each of the subjects. Analysis of the WES data identified a novel homozygous splice acceptor variant (c.119-2A>G) in subjects I and II.

Clinical Description

Subject I:

Subject I is a five year old male child born to non-consanguineous parents of Indian origin. He was first brought to the clinic with the chief complaint of global developmental delay since birth. He was born by full term normal vaginal delivery with no antenatal or neonatal complication. On examination his body weight, height and head circumference are 9.5 kg, 88 am, 45 cm (-3.5 SD). At one-year of age MRI showed delayed myelination in subcortical area and mildly hypoplastic

corpus callosum. At the age of 5 years Subject I did not attain any neck holding, or nor could he say bisyllables. He attained social smile and cooing at 4 years of age. He is not yet toilet trained. According to the parents, there is persistent posturing of the limbs, suggestive of dystonia. There is spasticity of the upper and lower limbs. There is no history of seizures, or any abnormal movements. He is having apparently normal vision and hearing. His final diagnosis is microcephaly with spastic quadriparesis.

On examination:

Weight: 9.5 kg

Height: 88 cm

Head circumference: 45 cm (-3.5 SD)

Eyes: normal

CNS: there is persistent posturing of the upper limbs and lower limbs suggestive of dystonia.

Tone: spasticity in the upper and lower limb

DTR: knee jerk and ankle jerk is brisk.

No abnormal movements

Investigations:

Outside investigations:

Fundus: normal

NCCT head: normal

Urine for IEM: negative

Lactate: normal

Ammonia: normal

TMS/GCMS : normal

MRI at 1 year of age: delayed myelination in subcortical area. Corpus callosum is mildly hypoplastic

Karyotype: 46, XY

MLPA for common microdeletion: normal

Figure 3: Photographs of Subject I at age 5. Subject I presents with microcephaly, global developmental delay, persistent stiffness and posturing of the limbs, and inability to hold his neck up.

Subject II

Subject II is a similarly affected elder female sibling of Subject I. She is seven years old and was born by full-term normal vaginal delivery with an average birth weight. Subject II also presents with global developmental delay and microcephaly. At the age of 7 years she did not attain any neck holding, and presents with persistent posturing of the body similar to Subject I. Her final diagnosis is microcephaly with spastic quadriparesis.

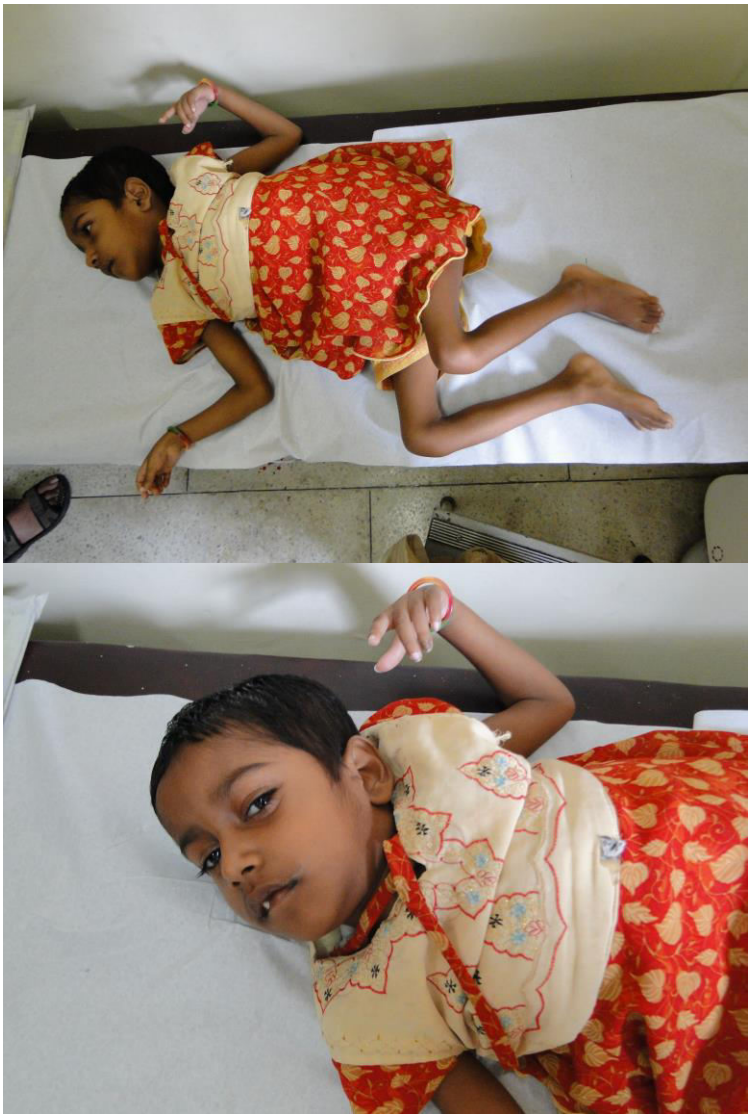


Figure 4: Photographs of Subject II at age 7. Subject II presents with microcephaly, global developmental delay, persistent posturing of the limbs and inability to hold her neck up.

Clinical report provided by Sanjay Gandhi Pst Graduate Institute of Medical Sciences Lucknow-226014 Department of Medical Genetics
Consultant: Prof. S. R. Phadke M.D (Pediatrics); D.M (Medical Genetics).

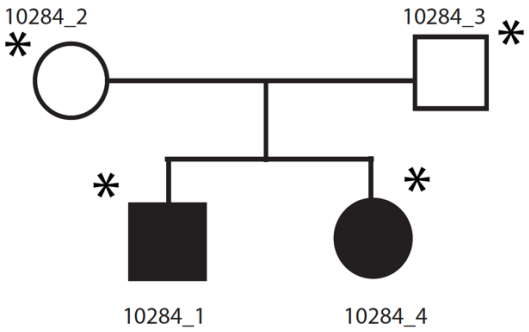


Figure 5: Family pedigree and PRUNE1 mutation. Both parents were carriers of autosomal recessive PRUNE1 mutation, depicted by the white shapes marked with an asterisk. Parents had two children, both of whom inherited the novel PRUNE splice variant. Both children developed PRUNE syndrome with similar phenotypes, depicted as black squares marked with an asterisk.

Exome Sequencing:

Results of exome sequencing identified a biallelic novel slice acceptor (c.119-2A>G) varaint in both subjects I and II. This variant has not yet been identified in any other PRUNE syndrome patients.

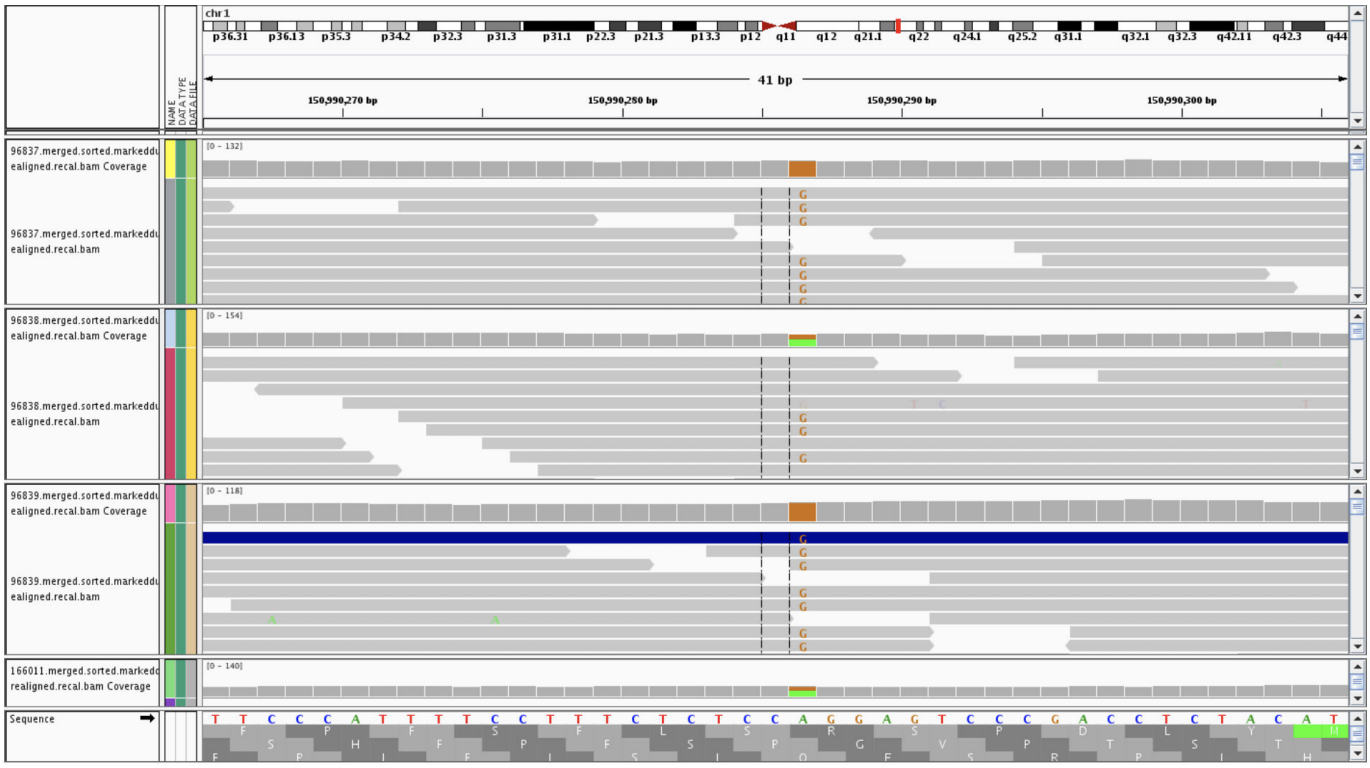


Figure 6: Results of WES sequencing of both parents and both children. Splice acceptor mutation is depicted in the above image, and can be seen in half the reads of the parents (meaning they are heterozygous) and all the reads for the affected individuals (meaning they are homozygous).

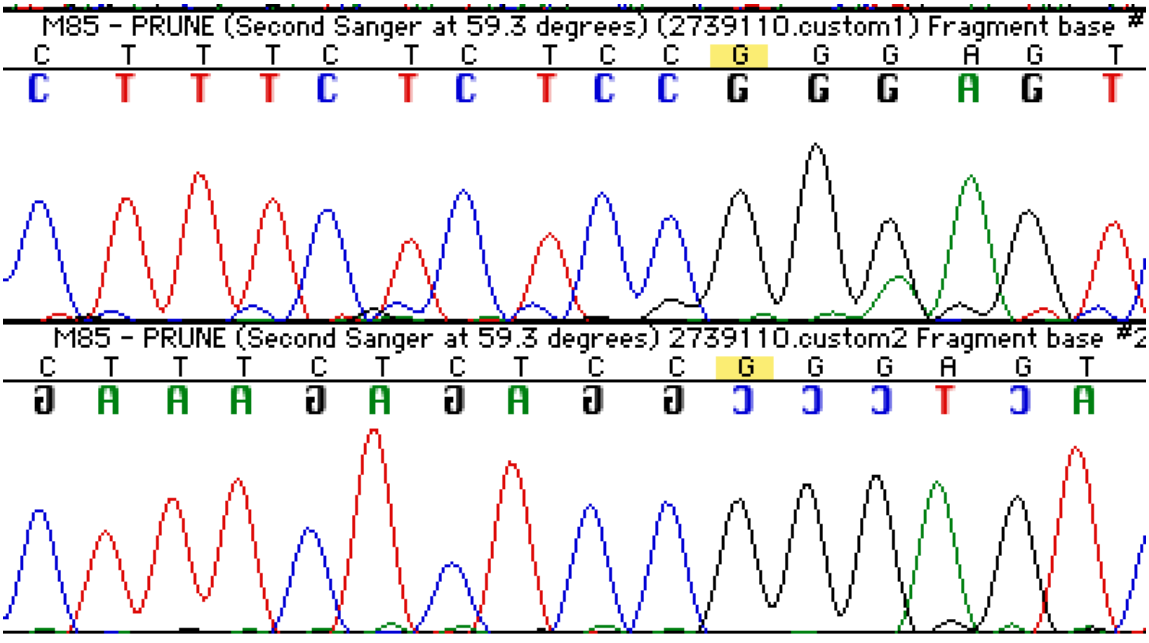


Figure 7a: Sanger sequencing confirmation and segregation analysis of c.119-2A>G variant in the *PRUNE1* gene (NM_0121222) in Subject I. Confirmed by Sanger sequencing which shows individual alleles.

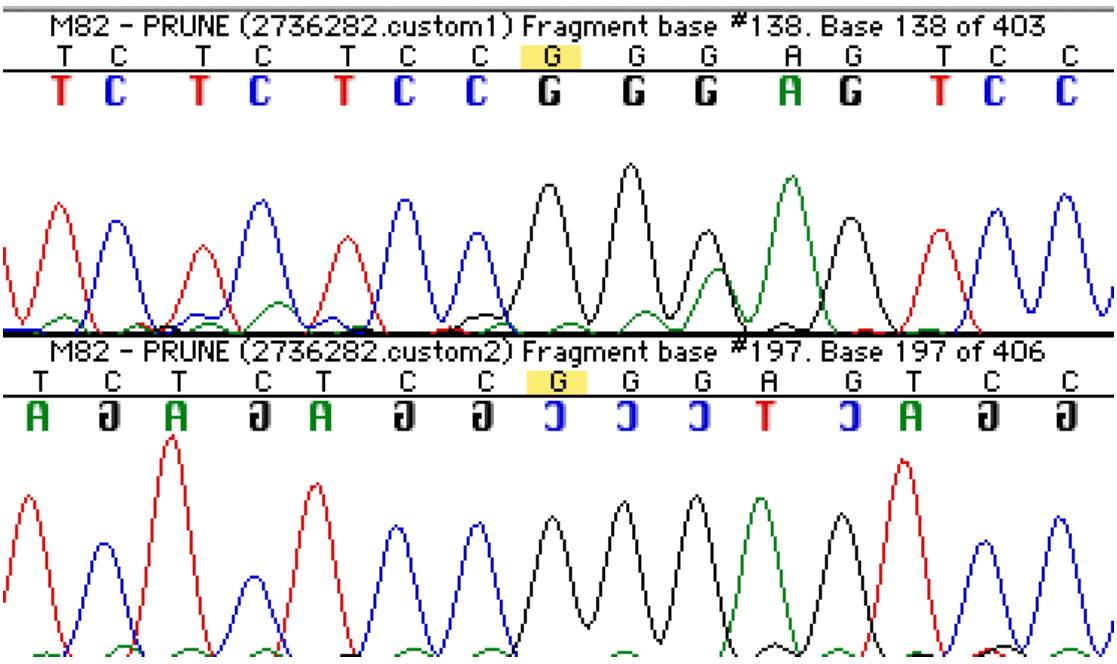


Figure 7b: Sanger sequencing confirmation and segregation analysis of c.119-2A>G variant in the *PRUNE1* gene (NM_0121222) in Subject II.

MRI Results:

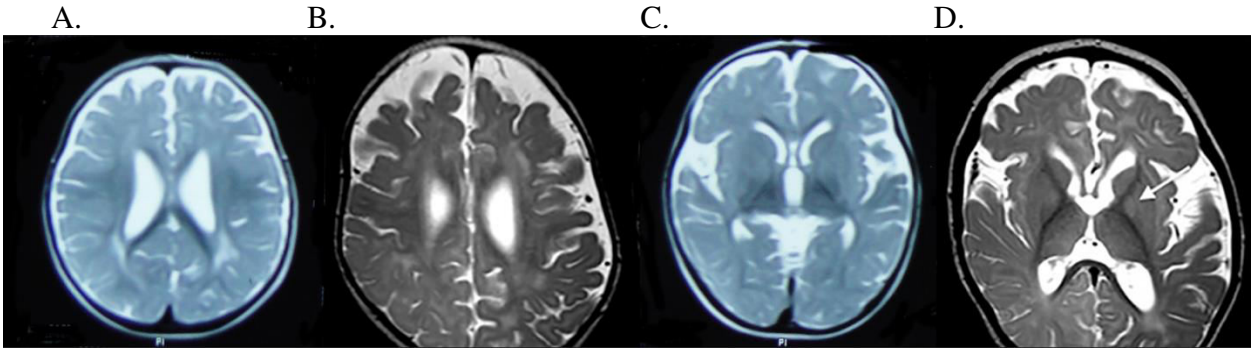


Figure 8: MRI of Subject I compared to splice variant previously described by *Costain et al*. Figures A and C belong to Subject I and figures B and D are taken from the previously described individual. Both individuals present with hypoplastic corpus callosum, delayed myelination, cortical atrophy, and small cerebellum. Cerebral atrophy is evident through the abnormally large volume of gray matter (dark area),

as well as the enlarged ventricles. Delayed myelination can be seen in the blurred boundary between the cortex and the subcortical white matter.

Discussion:

To date, 12 different mutations in 48 individuals total have been reported in PRUNE among various nationalities, such as Turkey, Italy, USA, Saudi Arabia, Oman, Ojibwee-Cree (Canada), Iran, India, and Lebanon. 11 of these mutations have are coding mutations/amino acid substitutions, and 1 is a splicing mutation. 8 of these mutations are biallelic/homozygous, and 3 are compound heterozygous. Previous PRUNE cases report similar phenotypes across the board, however certain phenotypes tend to be associated with particular mutations. Additionally, there are slight inconsistencies within the literature about the prevalence of certain phenotypes, as certain studies did not provide information regarding each symptom. Developmental delay/disability has the highest degree of penetrance in PRUNE patients, and is seen in 100% of reported cases. Microcephaly is another phenotype characteristic of PRUNE mutations, and has been reported in (18/43) (42%) of previously known cases (Table 1). Seizures were found in (31/43) (72%) of cases. Spastic quadriparesis, a condition of stiff, jerky movements as a result of hypotonia in the muscles, is found in 17/34 (50%) of cases. Similar malformations in brain structure are present across patients, as depicted in their MRI scans. Cerebral atrophy, which is the loss of neurons and the connections between them, affects 29/42 (69%) of patients. Cerebellar atrophy/degeneration, which is the loss of cerebellar neurons that coordinate balance and voluntary movements, is found in 18/40 (45%) of patients. Hypoplastic corpus callous is present in 11/33 (33%) of cases. Hypomyelination is present in 19/30 (63%) of cases. These phenotypes reveal the physical manifestations of PRUNE interactions, as the loss of neurons

observed in cortical/cerebellar atrophy and loss of white matter can be attributed to migrational defects.

TABLE 1 Clinical features of 48 patients with *PRUNE1* mutations

Study Cohort #	Karaca (2015) 5	Costain (2017) 1	Zollo (2017) 13	Karakaya (2017) 1	Alfadhel (2017) 2	Iacomino (2018) 1	Imagawa (2018) 4	Alhaddad (2018) 12	This study 9	Total 48	Percentage
Clinical features											
Profound developmental disability	5/5	1/1	13/13	1/1	2/2	1/1	4/4	11/11	9/9	47/47	100%
Hypotonia	2/2	1/1	13/13	1/1	2/2	1/1	4/4	9/12	9/9	42/45	93%
Eventual spasticity	2/2	0/1	12/13	1/1	2/2	0/1	4/4	10/12	6/9	37/45	82%
Primary microcephaly[a]	5/5	0/1	13/13	0/1	0/2	Na	0/4	0/8	0/9	18/43	42%
Secondary microcephaly[a]	0/5	0/1	0/13	1/1	2/2	Na	0/4	2/7	1/9	6/42	14%
Seizures	Na	1/1	6/13	1/1	0/2	1/1	4/4	11/12	7/9	31/43	72%
Swallowing dysfunction	Na	1/1	Na	1/1	0/2	1/1	Na	11/11	9/9	23/25	92%
Visual impairment[b]	Na	1/1	2/4	0/1	0/2	1/1	1/4	5/12	2/9	12/34	35%
Contracture	Na	1/1	7/8	0/1	0/2	1/1	Na	2/12	6/9	17/34	50%
Hypoventilation	Na	1/1	Na	1/1	0/2	1/1	Na	3/12	7/9	13/26	50%
MRI imaging											
Hypomyelination	2/2	0/1	9/10	1/1	1/2	1/1	3/4	Na	2/9	19/30	63%
Cerebral atrophy	5/5	0/1	3/10	1/1	2/2	1/1	4/4	7/9	6/9	29/42	69%
Cerebellar atrophy	5/5	0/1	2/10	1/1	0/2	1/1	3/4	6/7	0/9	18/40	45%
Thin corpus callosum	5/5	0/1	2/10	1/1	0/2	0/1	2/4	Na	1/9	11/33	33%
Peripheral neuromuscular findings											
Neurogenic EMG/NCV	Na	1/1	1/1	1/1	Na	1/1	Na	5/6	2/6	11/16	69%
Abnormal muscle biopsy	Na	Na	Na	Na	Na	1/1	Na	1/1	6/7	7/8	88%
Molecular findings											
Mutations	c.88G>A (p.D30N) c.316G>A (p.D106N) c.383G>A (p.R128Q) c.520G>T (p.G174X)	c.521-2A>G (IVS4-2A>G)	c.88G>A (p.D30N) c.160C>A (p.P54T) c.88G>A (p.D106N) c.889C>T (p.R297W)	c.874_875insA (p.H292Qfs*3)	c.383G>A (p.R128Q)	c.316G>A (p.D106N)	c50dup (p.L18Sfs*8) c.316G>A (p.D106N) c.540T>A (p.C180X)	c.316G>A (p.D106N) c.515T>C (p.L172P) Ex2-8 Del[c]	c.521-2A>G (IVS4-2A>G)	12	

Hartley et al., 2019 American Journal of Medical Genetics

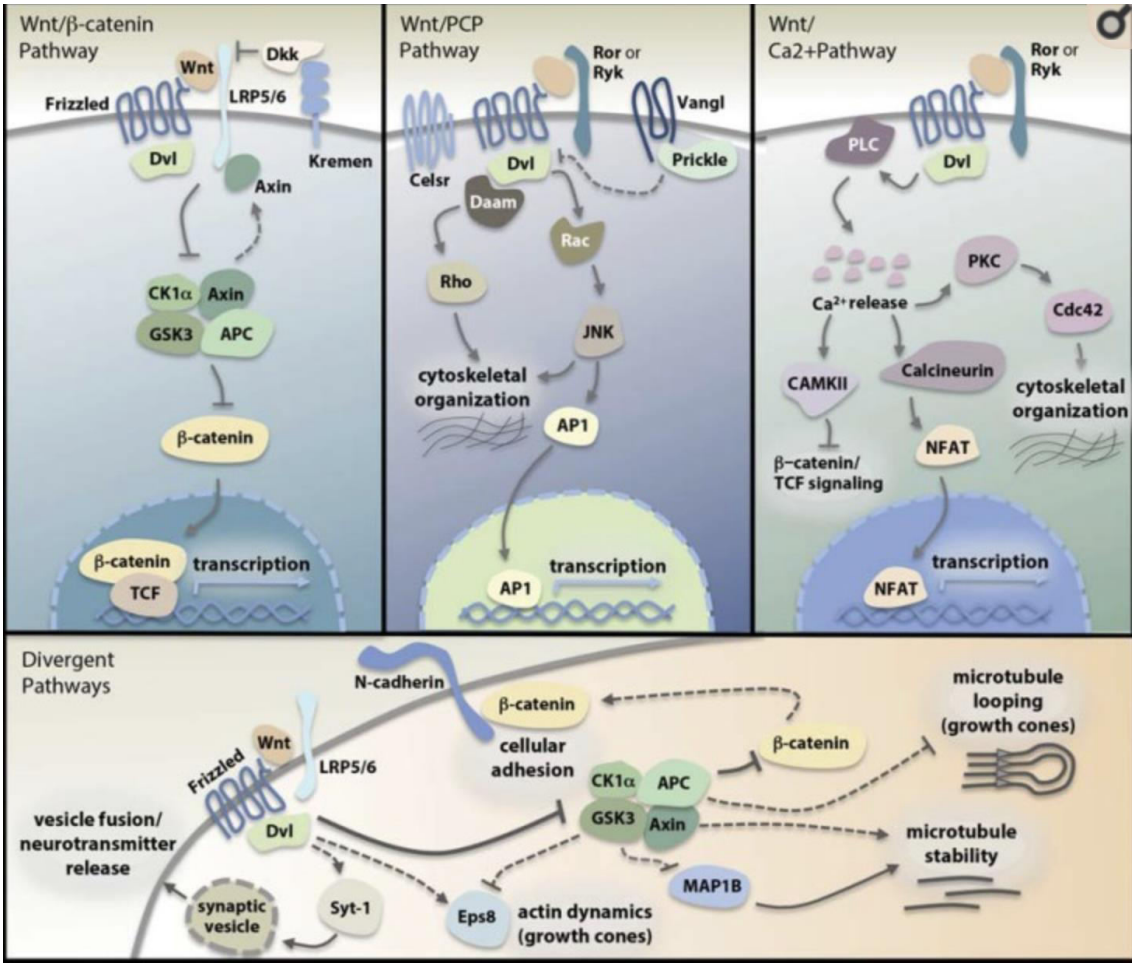
Table 1: Comprehensive table listing the various phenotypes found in PRUNE syndrome along with their respective penetrance.

Case Report:

The novel case presented in this study is consistent with the diagnostic criteria of PRUNE syndrome, and also provides an expansion of the clinical features associated with this gene by introducing a novel phenotype not seen in other patients. Subjects I and II both presented with global developmental delay, a phenotype found in all identified PRUNE patients. They present with microcephaly, spastic quadriparesis, and no history of seizures. Their MRIs show similar structural brain abnormalities as previously described patients, including cerebral atrophy,

delayed myelination, and hypoplastic corpus callosum. Comparison of Subject I to the splice variant identified by *Costain et al* demonstrates nearly identical neurological phenotypes among the two splice variants. Previously described literature showed discrepancies in the prevalence and severity of neurological phenotypes across different coding mutations. The striking degree of consistency between the two splice site variants could potentially be attributed to their identities as splice site acceptors. Further research studying the differences in the molecular pathology between splice site variants and coding variants of PRUNE1 using human IPS cells could further elucidate the mechanistic pathology of different PRUNE mutations. Additionally, this case expands the clinical spectrum associated with PRUNE through the induction of a novel phenotype present in both patients. Both probands present with persistent posturing of the limbs, and could not lift or hold their necks up at 5 and 7 years of age. Inability to sustain the weight of the head at these ages is indicative of severe neuromuscular defects, which can be attributed to abnormalities involved in neurogenesis and neuronal migration.

H-prune and GSK-3β regulate cell migration by modulating focal adhesions (Kobayashi et al., 2006). PRUNE phosphoesterase activity functions to bind and inactivate GSK-3β, inducing downregulation of downstream GSK-3β signaling pathways. GSK-3β is a key effector molecule in the Wnt/β-catenin signaling pathway. GSK-3β functions within the APC complex to phosphorylate and inactivate β -catenin, targeting it for degradation (Yost et al., 1996).



Mulligan et al., 2017

Figure 9: Schematic summary of Wnt pathways.

The Wnt signaling pathway can be thought of as a network of interconnected biochemical cascades, as Wnt receptor binding may simultaneously activate several cross-regulated intracellular pathways, inducing a coordinated change in cellular state (Mulligan et al., 2017). Wnt signaling inadvertently impacts microtubule organizations, as dissociation of the destruction complex not only reduces phosphorylation of β-catenin but also phosphorylation of microtubule associated protein 1B (MAP1B). This leads to posttranslational changes in microtubule organization and stability, which is critical for various cellular functions (Lucas et al., 1998). As described above, microtubule organization is a critical component in the neuronal migratory

process, and defects in microtubule organization or stability can have adverse impacts on neuronal migration, leading to severe neurodevelopmental pathologies. Disruption of cytoskeletal remodeling causes the neuro-progenitor cells born within the Ventricular Zone (VZ) to stay in place, as they are unable to migrate to their respective cortical layers. This leads to the clustering of neurons inside the ventricles, and the development of brains without gyri and sulci, as evident in pathologies such as lissencephaly (Juric-Sekhar et al., 2019). Thus, understanding Prune regulation of Wnt signaling in the context of microtubule dynamics and neuronal migration proposes a mechanism of PRUNE syndrome pathogenesis not yet described in the literature.

Further implications:

Most of the variants observed in this gene are coding mutations that cause a single amino acid missense substitution. Aside from this case, there is only one other splice site variant described in the literature (c.521-2A<G) (Costain et al., 2017) (Hartley et al., 2018). Interestingly, the homozygous c.521-2A<G splice acceptor variant was found in patients from Oji-Cree ethnic origin, belonging to the Manitoba province of Canada. This mutation is likely present at a high frequency in this population due to the founder effect. The founder effect is a phenomenon described in population genetics that results when a new colony is started by a few members of a population. The small population size causes the colony to have reduced genetic variation from their parent population, as well as non-random sampling of the genes in the original population. The lack of genetic diversity within the population increases its risk for inheritance of recessive diseases. Within the Cree population, there is an overrepresentation of multiple autosomal recessive conditions, due to the geographic isolation of their communities. The Oji-Cree people live in small, remote communities across central Manitoba and north-western Ontario (Hartley at

al., 2019). Comprehensive analysis of the various mutations in PRUNE1 raises interesting questions regarding the pathogenicity of PRUNE variants. Interestingly, there are only a few variants in PRUNE1 that lead to PRUNE syndrome, despite the gene's high degree of tolerability. The variant identified in our study may be also be a founder mutation in the Indian population. Founder mutations, such as NDUFV1, have been previously identified as responsible for the development of neurodevelopmental disorders within the Indian population (Srivastava et al., 2018). The results of this study suggest that this novel splice variant is a founder mutation in the Indian population, further supporting the notion that the splice acceptor in the Cree population is a founder mutation as well. Autosomal recessive diseases are particularity easy to track, and they are the most definitive way of locating founder mutations within populations. In the past, large consanguineous families and small populations have been helpful in the identification pathogenic genes linked to neurodevelopmental disorders. These conclusions support the initiative to perform whole exome sequencing in diverse populations, as a means of identifying other founder variants that could be the source of varying pathogenicity around the world.

Acknowledgements

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Medical genomics research performed in diverse population facilitates a better understanding of the genetic basis of developmental disorders, with regional implications for community genetics. Autosomal recessive mitochondrial complex I deficiency (MCID) accounts for a constellation of clinical features, including encephalopathies, myopathies, and Leigh Syndrome. Using whole-exome sequencing, we identified biallelic missense variants in NDUFV1 that encodes the 51-kD subunit of complex I (NADH dehydrogenase) NDUFV1. Mapping the variants on published crystal structures of mitochondrial complex I demonstrate that the novel c.1118T > C (p.(Phe373Ser)) variant is predicted to diminish the affinity of the active pocket of NDUFV1 for FMN that correlates to an early onset of debilitating MCID symptoms. The c.1156C > T (p.(Arg386Cys)) variant is predicted to alter electron shuttling required for energy production and correlate to a disease onset in childhood. NDUFV1 c.1156C > T (p.(Arg386Cys)) represents a founder variant in South Asian populations that have value in prioritizing this variant in a population-specific manner for genetic diagnostic evaluation. In conclusion, our results demonstrate the advantage of analyzing population-

specific sequences to understand the disease pathophysiology and prevalence of inherited risk variants in the underrepresented populations.

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