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Characterization of a novel variant in siblings with Asparagine Synthetase Deficiency

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ABSTRACT

Asparagine Synthetase Deficiency (ASD) is a recently described inborn error of metabolism caused by bi-allelic pathogenic variants in the asparagine synthetase (ASNS) gene. ASD typically presents congenitally with microcephaly and severe, often medically refractory, epilepsy. Development is generally severely affected at birth. Tone is abnormal with axial hypotonia and progressive appendicular spasticity. Hyperekplexia has been reported. Neuroimaging typically demonstrates gyral simplification, abnormal myelination, and progressive cerebral atrophy. The present report describes two siblings from consanguineous parents with a homozygous Arg49Gln variant associated with a milder form of ASD that is characterized by later onset of symptoms. Both siblings had a period of normal development before onset of seizures, and development regression. Primary fibroblast studies of the siblings and their parents document that homozygosity for Arg49Gln blocks cell growth in the absence of extracellular asparagine. Functional studies with these cells suggest no impact of the Arg49Gln variant on basal ASNS mRNA or protein levels, nor on regulation of the gene itself. Molecular modelling of the ASNS protein structure indicates that the Arg49Gln variant lies near the substrate binding site for glutamine. Collectively, the results suggest that the Arg49Gln variant affects the enzymatic function of ASNS. The clinical, cellular, and molecular observations from these siblings expand the known phenotypic spectrum of ASD.

1. Introduction

1.1. ASNS protein structure and enzyme activity

Asparagine synthetase (ASNS) catalyzes the synthesis of asparagine and glutamate from the substrates aspartate and glutamine [1,2]. The common name of the protein highlights its role in asparagine synthesis, but whether or not the enzyme activity impacts the cellular levels of one or more of the other three reactants has not been investigated. Particularly for neural tissue, the possible influence of ASNS activity on glutamate and aspartate levels must be a key point of interest. ASNS expression among tissues varies considerably, with much greater expression in the pancreas than most other tissues analyzed [3]. However, ASNS abundance in brain is similar to many other tissues in the body (http://www.proteinatlas.org/ENSG00000070669-ASNS/tissue). The human ASNS gene is located at chromosome 7 region 7q21.3 [4,5] and is 35 kb long with 13 exons [6]. ASNS expression is highly regulated in response to a wide variety of cell stresses, primarily by increased transcription [2]. Central among the genomic elements that control ASNS transcription is the C/EBP-ATF response element (CARE) within the promoter [7]. Protein limitation or an imbalanced dietary amino acid composition activate the ASNS gene through the GCN2-eIF2-ATF4 pathway culminating with ATF4 binding to the CARE. Endoplasmic reticulum stress also increases ASNS transcription through

Abbreviations: ASD, Asparagine Synthetase Deficiency; ASNase, Asparaginase; ASNS, asparagine synthetase; CARE, C/EBP-ATF response element; CSF, cerebral spinal fluid; EEG, electroencephalogram; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PDB, protein data base; SD, standard deviations

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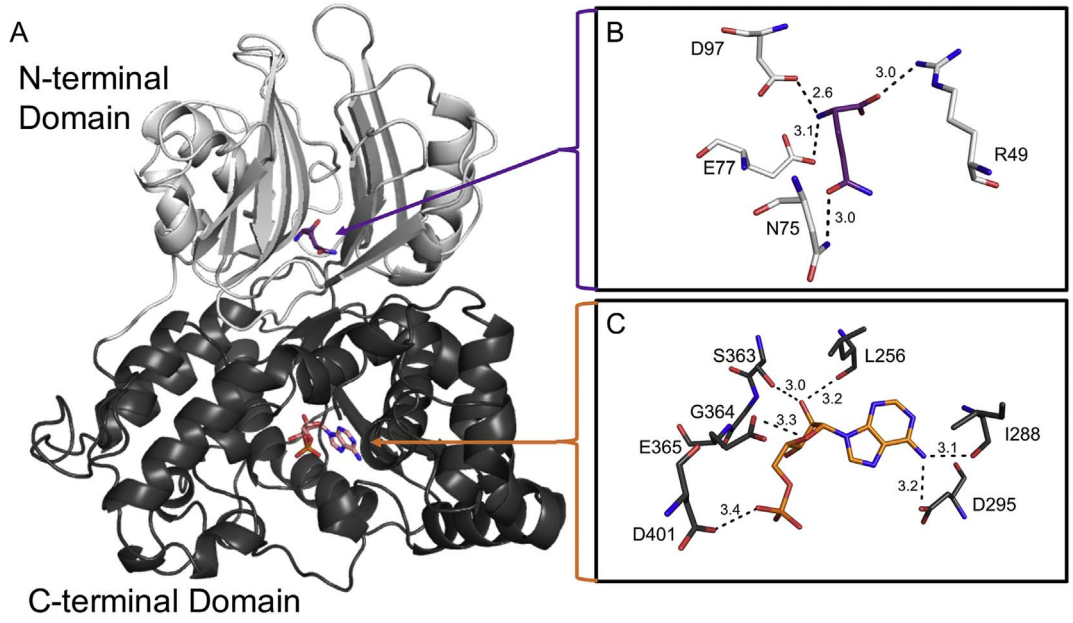

Fig. 1. Model of human ASNS protein structure. A human ASNS protein model was generated utilizing the crystal structure of *E. coli* asparagine synthetase B (PDB: 1CT9) as a template and then adapted to contain variants observed in patients with ASD, as described in the Methods section. (Panel A) N-terminal (light grey) and C-terminal (dark grey) domains with the substrate glutamine (purple) and the product AMP (orange) shown as sticks. (Panel B) Glutamine binding pocket illustrating glutamine hydrogen bonds. (Panel C) ATP/AMP binding pocket with AMP binding shown. Hydrogen bonds are represented as black dashes and the distance of each is shown in angstroms (Å). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the PERK-eIF2-ATF4 arm of the unfolded protein response (UPR) [8].

The human ASNS enzyme is a 65 kDa protein that has two primary domains, termed the N- and C- terminal domains (Fig. 1A). As there is no crystal structure of human ASNS reported in the protein data base (PDB), an *in silico* model based on the crystal structure of *E. coli* asparagine synthetase B (PDB: 1CT), which shares 40% sequence homology with human ASNS, is often used to highlight the main secondary structural features of the enzyme [9]. The N-terminal domain is composed of an antiparallel β-sheet core and contains the glutamine binding pocket. Glutamine interacts with residues Arg49, Asn77, Glu97, and Asp98 through hydrogen bonding (3.0, 3.0, 3.1, and 2.6 Å, respectively) (Fig. 1B). In contrast, the C-terminal domain consists of mainly alpha helices and binds ATP through hydrogen bonds with residues Leu256, Ile288, Asp295, Ser363, Gly364, Glu365, and Asp401 (3.2, 3.1, 3.2, 3.0, 2.8, 3.3, and 3.4 Å, respectively) (Fig. 1C). With the exception of Ile288, which is a valine in *E. coli* and *Drosophila melanogaster* (UniProt ID: P22106 and Q7KTW9, respectively), the ASNS residues in the glutamine and ATP binding pockets are conserved between species, emphasizing their importance in the function of the enzyme.

1.2. Mutations within the gene encoding ASNS cause Asparagine Synthetase Deficiency (ASD)

Asparagine Synthetase Deficiency (ASD) was first described in 2013 on the basis of four families with homozygosity or compound heterozygosity for missense mutations in ASNS [10]. The affected individuals shared the features of severe encephalopathy, congenital microcephaly, brain atrophy, early onset seizures, axial hypotonia, and severe appendicular spasticity [10]. These features can be considered characteristic of the syndrome and are shared by the subsequently reported patients. The vast majority of patients with ASD have missense mutations.

1.3. Functional cellular consequences of a novel variant in the ASNS enzyme

The clinical and functional effects of a homozygous ASNS variant, Arg49Gln, a novel variant shared by two siblings, are described within the current report. Functional studies on fibroblasts from the two affected siblings and their parents revealed that fibroblasts from the parents exhibited typical basal levels of ASNS mRNA or protein, and their ASNS genes each responded appropriately to transcriptional induction by amino acid deprivation. Fibroblasts from one of the two affected siblings expressed higher basal ASNS levels than the other three family members tested, but this difference was not reflected in a change in proliferation rate. Relative to either parent, proliferation of cells from both affected siblings was severely suppressed in the absence of asparagine. Collectively, the results indicate that the Arg49Gln variant blocks cell growth in the absence of sufficient extracellular asparagine, which suggests that Arg49Gln affects the enzymatic function of ASNS. Modelling the location of the variant within the ASNS protein indicates that the affected residue may disrupt protein structure in the substrate binding site for glutamine because of a loss of hydrogen bonding.

2. Materials and methods

2.1. Exome sequencing and targeted mutation analysis

Using genomic DNA, the Agilent Clinical Research Exome kit was used to target the exonic regions and flanking splice junctions of the genome. These regions were sequenced simultaneously by massive parallel (NextGen) sequencing on an Illumina HiSeq 2000 sequencing system with 100 bp paired-end reads. For targeted mutation analysis, the relevant portion of the gene of interest was PCR amplified and capillary sequencing was performed. For both exome sequencing and mutation analysis, bi-directional sequence was assembled, aligned to reference gene sequences based on human genome build GRCh37/UCSC hg19, and analyzed for sequence variants using a custom-developed analysis tool (Xome Analyzer). Capillary sequencing or another