Clinical Case Reports

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CASE REPORT

Clinical whole exome sequencing from dried blood spot identifies novel genetic defect underlying asparagine synthetase deficiency

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Funding Information
No sources of funding were declared for this study.

Received: 18 May 2017; Revised: 11 September 2017; Accepted: 12 October 2017

Clinical Case Reports **2018; 6(1): 200–205**

doi: 10.1002/ccr3.1284

Key Clinical Message

We add two novel variants to the existing mutation spectrum of ASNS gene. Loss of ASNS function should be suspected in newborns presenting with congenital microcephaly, intellectual disability, progressive cerebral atrophy, and intractable seizures. Acquisition and sequencing of stored newborn blood spot can be a valuable option when no biological samples are available from a deceased child.

Keywords

Archived newborn blood spot, asparagine synthetase, asparagine synthetase deficiency, dried blood spot, whole exome sequencing.

Introduction

Loss of function of the ASNS gene has been reported to cause a rare and severe neurologic disorder. Ruzzo et al. reported the first characterization of ASNS deficiency (ASNSD) in four families presenting with congenital microcephaly, intellectual disability, progressive cerebral atrophy, intractable seizures, and recessive mutations in ASNS [1]. To date, ASNS deficiency has been reported in 16 cases evaluated for a range of brain abnormalities coupled with epileptic encephalopathy and global developmental delay. ASNS is expressed in most mammalian cells and encodes an enzyme that catalyzes the transfer of ammonia from glutamine to aspartic acid to form asparagine [2]. We report two novel compound heterozygous variants in ASNS using DNA isolated from archived newborn blood spots collected as part of a routine neonatal screening program.

Case

The proband was a 32-week neonate delivered following preterm labor who presented with clonic tremors, microcephaly, cerebellar hypoplasia, blindness, and seizures. Karyotype, microarray, and metabolic testing were all normal. MRI revealed increased extra-axial space, foreshortened frontal lobe, diffuse simplified gyral pattern more severe frontally, small, normal basal ganglia and thalami, reduced volume of white matter, mildly enlarged third and lateral ventricle, cavum septum pellucidum et vergae, thin corpus callosum, and mild brainstem hypoplasia due to flat base of the pons, and moderate cerebellar hypoplasia. The child had congenital microcephaly with low-sloping forehead, hypertonia, developmental delay, moderate sleep apnea, gastrointestinal reflux, epilepsy, and died at 15 months of age without a definitive diagnosis. Two years after the death of the child the

Table 1. Annotated list of variants in this and earlier reports.

Author	Case	Coordinates	Consequence	HGVS.c	HGVS.p	Zygosity	Inheritance	Existing_ Variation	ExAC_MAF	SIFT	PolyPhen
Present report	–	7:97484705-97484705	missense_variant	NM_001673.4:c.1097G>A	NP_001664.3:p.Gly366Glu	Heterozygous	ARCH	-	-	deleterious(0)	probably_ damaging(0.996)
Present report	–	7:97488213-97488213	missense_variant	NM_001673.4:c.728T>C	NP_001664.3:p.Val243Ala	Heterozygous	ARCH	rs148111963	G:8.237e-06	deleterious(0)	possibly_ damaging(0.454)
Alfadhel et al. [11]	Case 1	7:97483937-97483937	missense_variant	*NM_001178075.1:c. 1130A>G	NP_001171546.1:p.Tyr377Cys	Homozygous	AR	-	-	deleterious(0)	probably_ damaging(0.992)
	Case 2	7:97483937-97483937	missense_variant	*NM_001178075.1:c. 1130A>G	NP_001171546.1:p.Tyr377Cys	Homozygous	AR	-	-	deleterious(0)	probably_ damaging(0.992)
Seidahmed et al. [12]	Case 1	7:97483911-97483911	stop_gained	NM_001178076.1:c.970C>T	NP_001171547.1:p.Arg324Ter	Homozygous	AR	rs1140424	A:8.236e-06	-	-
	Case 2	7:97483937-97483937	missense_variant	NM_001178076.1:c.944A>G	NP_001171547.1:p.Tyr315Cys	Homozygous	AR	-	-	deleterious(0)	probably_ damaging(0.992)
Yamamoto et al. [14]	Case 1	7:97493624-97493624	missense_variant	*NM_001673.4:c.434T>C	NP_001664.3:p.Leu145Ser	Heterozygous	ARCH	-	-	deleterious(0)	possibly_ damaging(0.794)
	Case 1	7:97488201-97488201	missense_variant	*NM_001673.4:c.740T>G	NP_001664.3:p.Leu247Trp	Heterozygous	ARCH	-	-	deleterious(0)	probably_ damaging(1)
	Case 2	7:97482382-97482382	missense_variant	*NM_001673.4:c.1466T>A	NP_001664.3:p.Val489Asp	Heterozygous	ARCH	-	-	deleterious(0)	benign(0.318)
	Case 2	7:97481632-97481634	frameshift_variant	*NM_001673.4:c.1623_ 1624delGA	NP_001664.3:p. Trp541CysfsTer5	Heterozygous	ARCH	-	-	-	-
Sun et al. [13]	Case 1	7:97486013-97486013	missense_variant	*NM_001673.4:c.1019G>A	NP_001664.3:p.Arg340His	Homozygous	AR	-	-	deleterious(0.02)	benign(0.103)
	Case 2	7:97486013-97486013	missense_variant	*NM_001673.4:c.1019G>A	NP_001664.3:p.Arg340His	Homozygous	AR	-	-	deleterious(0.02)	benign(0.103)
Ruzzo et al. [1]	Case 1	7:97484718-97484718	missense_variant	NM_183356.3:c.1084T>G	NP_899199.2:p.Phe362Val	Homozygous	AR	rs398122973	C:8.236e-06	deleterious(0.01)	probably_ damaging(0.909)
	Case 2	7:97484718-97484718	missense_variant	NM_183356.3:c.1084T>G	NP_899199.2:p.Phe362Val	Homozygous	AR	rs398122973	C:8.236e-06	deleterious(0.01)	probably_ damaging(0.909)
	Case 3	7:97484718-97484718	missense_variant	NM_183356.3:c.1084T>G	NP_899199.2:p.Phe362Val	Homozygous	AR	rs398122973	C:8.236e-06	deleterious(0.01)	probably_ damaging(0.909)
	Case 5	7:97481609-97481609	missense_variant	NM_183356.3:c.1648C>T	NP_899199.2:p.Arg550Cys	Homozygous	AR	rs398122974	A:1.647e-05	deleterious(0.03)	benign(0.082)
	Case 6	7:97481609-97481609	missense_variant	NM_183356.3:c.1648C>T	NP_899199.2:p.Arg550Cys	Homozygous	AR	rs398122974	A:1.647e-05	deleterious(0.03)	benign(0.082)
	Case 7	7:97481609-97481609	missense_variant	NM_183356.3:c.1648C>T	NP_899199.2:p.Arg550Cys	Heterozygous	ARCH	rs398122974	A:1.647e-05	deleterious(0.03)	benign(0.082)
	Case 7	7:97498452-97498452	missense_variant	NM_183356.3:c.17C>A	NP_899199.2:p.Ala6Glu	Heterozygous	ARCH	rs398122975	A:1.647e-05	deleterious(0)	possibly_ damaging(0.84)
	Case 8	7:97481609-97481609	missense_variant	NM_183356.3:c.1648C>T	NP_899199.2:p.Arg550Cys	Heterozygous	ARCH	rs398122974	A:1.647e-05	deleterious(0.03)	benign(0.082)
	Case 8	7:97498452-97498452	missense_variant	NM_183356.3:c.17C>A	NP_899199.2:p.Ala6Glu	Heterozygous	ARCH	rs398122975	A:1.647e-05	deleterious(0)	possibly_ damaging(0.84)
	Case 9	7:97481609-97481609	missense_variant	NM_183356.3:c.1648C>T	NP_899199.2:p.Arg550Cys	Heterozygous	ARCH	rs398122974	A:1.647e-05	deleterious(0.03)	benign(0.082)
	Case 9	7:97498452-97498452	missense_variant	NM_183356.3:c.17C>A	NP_899199.2:p.Ala6Glu	Heterozygous	ARCH	rs398122975	A:1.647e-05	deleterious(0)	possibly_ damaging(0.84)

Author (First author of the report); Case (case number in the report); Coordinates (genomic coordinate of reported variation); Consequence (consequence of the genomic variation); HGVS.c (DNA change in HGVS nomenclature); HGVS.p (amino acid change in HGVS nomenclature); Zygosity (reported zygosity of the variation); Inheritance (AR – autosomal recessive, ARCH – autosomal recessive compound heterozygous); Existing_Variation (rsID if the variation is found in dbSNP); ExAC_MAF (minor allele frequency in Exome Aggregation Consortium database – reported in Allele: MAF format); SIFT (SIFT prediction and score); PolyPhen (PolyPhen prediction and score). Asterisk (*) before NM_ indicates that the original publication did not specify the transcript identifier and was inferred from the reported variation.

parents presented to our prenatal diagnosis center inquiring about risks to a future pregnancy. WES was suggested but no DNA or tissue specimen existed. Subsequently, the child's newborn screening blood spot filer paper was obtained from the New York State Department of Health lab for WES testing.

Methods

We received filter paper with one dried blood spot. Punch biopsies of the spots (3 mm) were placed directly in 20 μl of lysis solution from the Extract-N-Amp kit (Sigma-Aldrich, St. Louis, MO, USA). DNA was isolated following the manufactures protocol. Whole genome amplification was performed in duplicate using the Repli-g Amplification kit (Qiagen, Hilden, Germany). Peripheral blood drawn from both parents was obtained and DNA isolated using standard methods in the clinical laboratory. An exome library was prepared using SureSelectXT Human All Exon V5+UTR kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. Paired-end WES was performed on the HiSeq 2500 (Illumina, San Diego, CA, USA) to provide a mean sequence coverage of more than 100x with 98% of the target bases at least 20x coverage.

Data analysis

Data preprocessing was performed following the GATK best practices guidelines as follows

Raw reads were aligned to NCBI genome build 37 using Burrows-Wheeler Aligner (BWA) [3]. Picard tools were used to mark duplicate reads [4]. Base Quality Score Recalibration (BQSR) was performed using Genome Analysis Toolkit (GATK) [5]. Local realignment around indels was performed using GATK. Variant discovery was performed in two steps: Single-sample variant calls were discovered using GATK HaplotypeCaller. This was followed by jointly genotyping the single-sample gVCFs for the family along with gVCFs from 50 matched samples. Variant Quality Score Recalibration (VQSR) was performed using GATK. Truth sensitivity thresholds of 99.8 and 99.0 were used for SNVs and INDELs, respectively. Variant effects were predicted using SnpEff [6]. Additional annotations including variant frequencies in different populations, cross species conservation scores, functional prediction scores, variant disease associations, regulatory annotations, and gene ontology annotations were performed using BCFtools [7] and in-house software. Pedigree-aware variant categorization and functional prioritization were performed using software developed in-house.

Results

A dried blood spot (DBS) typically provides a very limited amount of starting material for DNA extraction and library preparation invariably requiring whole genome amplification (WGA) to achieve sufficient DNA for exome capture. Accordingly, to establish the quality of the DNA library derived from the DBS, we extensively analyzed sequencing quality (Table S1) compared with peripheral whole blood specimens. DBS and whole blood exome libraries showed comparable performance across all metrics evaluated. We achieved 264x mean target coverage for the proband while the parents were covered at 144x and 152x. There was a marginal increase in the number of bases covered outside the exome target region (off-target coverage) and AT dropout for the DBS library as compared to whole blood. Both differences are within acceptable ranges and expected to have little or no effect on the variant calling owing to (1) high mean target coverage providing adequate read depth in AT- or GC-rich target regions and (2) variant calling is performed only in the target regions eliminating erroneous off-target calls.

Variant prioritization workflow which includes filtering based on available phenotype information identified compound heterozygous (NM_001673.4:c.1097G>A and NM_001673.4:c.728T>C) variants in the asparagine synthetase domain of ASNS. The NM_001673.4:c.1097G>A variant was maternally inherited and the NM_001673.4:c.728T>C variant was paternally inherited. The amino acid changes NP_001664.3:p.Gly366Glu (NM_001673.4:c.1097G>A) and NP_001664.3:p.Val243Ala (NM_001673.4:c.728T>C) were predicted to be damaging/deleterious according to SIFT and Polyphen with CADD scores of 27.1 and 20.7, respectively. The NM_001673.4:c.1097G>A variant has not been reported in any large public data sets—1000 Genomes [8], ExAC [9], UK10K [10].

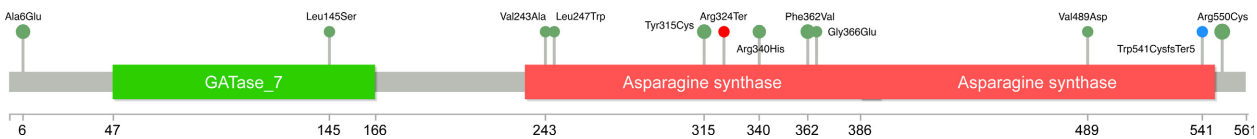

Figure 1. Location of reported ASNS mutations. Size of lollipop indicates the frequency of the reported mutation. Lollipop colors: Green=missense, Red=nonsense, Blue=frameshift. Uniprot/Swiss-prot accession P08243 was used for protein domain information..

Table 2. Summary of the phenotype in ASNSD cases.

	Case	Developmental delay	Microcephaly	Cortical blindness	Seizures	MRI	Plasma asparagine level
Present report	–	Y	Y	Y	Y	diffuse simplified gyral pattern, small, normal basal ganglia and thalami, reduced volume of white matter, mildly enlarged third and later ventricle, cavum septum pellucidum et vergae, thin corpus callosum and mild brainstem hypoplasia due to flat base of the pons, and moderate cerebellar hypoplasia	NA
Alfadhel et al. [11]	Case 1	Y	Y	N	Y	brain atrophy, simplified gryal pattern, delayed myelination	Low
	Case 2	Y	Y	N	Y	brain atrophy, simplified gryal pattern, delayed myelination	Low
Seidahmed et al. [12]	Case 1	Y	Y	Y	Y	cerebral atrophy, simplified gyral pattern and hypoplastic cerebellum and pons	Normal
	Case 2	Y	Y	Y	Y	smooth cortex with simplified gyral pattern, delayed myelination, dilatation of the ventricles, global brain atrophy and hypoplastic cerebellum and pons	Normal
Yamamoto et al. [14]	Case 1	Y	Y	N	Y	simplified gryal pattern, drastic reduction of cerebral volume with ventriculomegaly, enlarged subarachnoid space, thinned corpus callosum, delayed myelination, reduced pons volume	Normal
	Case 2	Y	Y	N	Y	cerebral atrophy, enlarged lateral ventricles, and hypoplasia of the brain- stem and cerebellum, simplified gyral pattern, prominent subarachnoid space, delayed myelination	Normal
Sun et al. [13]	Case 1	Y	N	N	N	mild ventriculomegaly, simplified gyral pattern, and hypoplasia of the pons and cerebellum and mild delay in myelination, cortical volume loss, corpus callosum thinning and bilateral caudate atrophy	NA
	Case 2	Y	Y	N	Y	simplified gyral pattern, mild ventriculomegaly with enlarged axial spaces, suggesting a moderate degree of atrophy and hypoplasia of the corpus callosum, pons and inferior cerebellum, myelination was age- appropriate.	Low
Ruzzo et al. [1]	Case 1	Y	Y	N	Y	decreased cerebral volume	NA
	Case 2	Y	Y	N	Y	decreased cerebral volume	Normal
	Case 3	Y	Y	N	Y	decreased cerebral volume	Normal
	Case 4	Y	Y	N	N	decreased cerebral volume, decreased size of pons, simplified gyri	NA
	Case 5	Y	Y	N	N	decreased cerebral volume, decreased size of pons, simplified gyri	Low
	Case 6	Y	Y	N	N	decreased cerebral volume, decreased size of pons, simplified gyri	NA
	Case 7	Y	NA	N	Y	decreased cerebral volume, decreased size of pons, simplified gyri	Low
	Case 8	Y	Y	N	Y	decreased cerebral volume, decreased size of pons, simplified gyri	Normal
	Case 9	Y	Y	N	Y	decreased cerebral volume, decreased size of pons, simplified gyri	NA

NA, data not available.

NM_001673.4:c.728T>C is a rare variant, reported at a minor allele frequency of 0.0002 and 0.000008 in 1000 Genomes and ExAC, respectively with no homozygous G/G genotype reported in either databases. Proband and parental genotypes were confirmed by Sanger sequencing. Considering the in-silico predictions, location in the protein, extreme rarity in the population, and functional studies of variants in the vicinity, both variants are expected to affect the function of asparagine synthetase domain which is crucial for the function of ASNS.

Discussion

Recessive mutations in the ASNS gene have been shown to cause a syndrome characterized by congenital microcephaly, intellectual disability, progressive cerebral atrophy, and intractable seizures. Asparagine synthetase enzyme catalyzes ammonia transfer from glutamine to aspartic acid via a beta-aspartyl-AMP intermediate. ASNS protein contains two domains-Glutamine amidotransferases class-II domain (amino acids 2-206) and asparagine synthetase domain (amino acids 228-538). The variants we report and all encephalopathy related previously reported pathogenic variants lie in the highly conserved asparagine synthetase domain. Ruzzo et al. studied nine children from four families presenting with similar phenotypes and reported two missense mutations-c.1084T>G (p.F362V; NM_183356) and c.1648C>T (p.R550C; NM_183356) in the asparagine synthetase domain that dramatically reduce ASNS protein abundance [1]. The authors concluded that accumulation of aspartate/glutamate secondary to ASNS depletion in the brain resulted in the neurologic impairment. One of the two mutations reported in that study, c.1084T>G (p.F362V; NM_183356), is four residues upstream of NP_001664.3:p.Gly366Glu seen in our patient. HEK293 cells expressing c.1084T>G (p.F362V; NM_183356) mutant allele showed dramatic reduction in protein abundance. Additionally, Ruzzo et al. reported a hypomorphic ASNS mouse knockout with structural brain abnormalities and deficits in learning/memory. Subsequently, eight more cases of ASNSD have been reported in the literature [11–14]. An annotated list of variants including our case and the reported cases in the literature is compiled in Table 1 and Figure 1.

Based on sequence similarity, several active binding sites have been identified for ASNS including amino acid 365 (one amino acid upstream of NP_001664.3:p.Gly366Glu) which is important for beta-aspartyl-AMP intermediate formation. Additionally, amino acids 363-364 have been identified as the protein region which binds nucleotide phosphates. Taken together, and based on available data, the novel NP_001664.3:p.Gly366Glu change we identified appears to be crucial for enzymatic activity.

The NM_001673.4:c.1097G>A variant has not been reported in any public database while one heterozygous NM_001673.4:c.728T>C variant has been reported in 60481 individuals available in ExAC. The extreme rarity of both variants indicates mutational intolerance and functional importance. Considering the known association of ASNS to neurologic phenotypes along with the extreme rarity of both, NM_001673.4:c.1097G>A and NM_001673.4:c.728T>C variants and their proximity to known functionally important locations, we conclude that the compound heterozygous missense variants we report are relevant and most likely the cause of the phenotype in this child.

Table 2 shows a summary of the common phenotypes described in the reported ASNSD cases. Based on the comparison of the reported phenotypic features of these cases, severe developmental delay, microcephaly, and seizures seem to be the hallmark of ASNSD in majority of reported cases. On the MRI, cerebellar hypoplasia, pontine hypoplasia, and simplified gyral pattern were reported in the majority of cases. Plasma asparagine levels do not seem to be a diagnostic marker for ASNSD.

It is questionable whether earlier knowledge of the causative alterations in asparagine synthesis would have changed the course of the disease in this child. Asparagine is required for normal brain development and is a nonessential amino acid in humans. Synthesized from oxaloacetate precursors using central metabolic pathway intermediates, Asparagine can alternatively be obtained by nutritional intake of various animal or plant products. Alrifai et al. [15] evaluated the effect of asparagine supplementation on mental status and seizures in one of the two individuals reported earlier [11]. After starting asparagine supplementation, the mental status improved slightly from the vegetative state to a minimally conscious state. However, on continuation of the treatment, the patient became irritable, developed sleep disturbance, and experienced worsening seizures leading to discontinuation of the treatment. Additional research and data are required to determine if such intervention could be effective in correcting the deficiency and reducing the detrimental effects of ASNS depletion in the brain. The knowledge of these mutations was valuable for this couple in planning future pregnancies with the availability of either preimplantation genetic diagnosis or prenatal testing.

Conclusions

We report novel compound heterozygous missense variants in asparagine synthetase gene as the likely cause of fatal asparagine synthetase deficiency in a child delivered

at 32 weeks of gestation. Acquisition and sequencing of stored newborn blood spot can be a valuable option when no samples are available from a deceased child.

Authorship

AA: Responsible for the primary analysis of the sequencing data and drafting the manuscript. ML-E: Contributed to DNA extraction. KB: Collected clinical notes and counseled the patient. JLG: Contributed to collection of clinical data. CE: Contributed to data analysis for sanger sequencing. VF: Contributed to libraries preparation and sequencing. RW: Evaluation and management of the patient as well as expert critiquing of the manuscript. VJ: Overall responsible for the sequencing, analysis, interpretation, and writing the manuscript.

Conflict of Interest

None declared

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Whole exome sequencing alignment metrics generated by Picard CollectHsMetrics.

