The functional analysis of the variants of human asparagine synthetase

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Asparagine (Asn)

→ Asn is nonessential amino acid. Meal including much Asn.
→ Asn is necessary for cell proliferation.
→ Blain Blood Barrier do not pass Asn.

Asparagine synthetase (ASNS)

→ ASNS is only one enzyme which can create Asn.
→ ASNS is coded in ASNS gene (NM_133436.3).
→ ASNS works in whole body including brain.
→ Lack of ASNS does not cause any symptoms in the body.

Lack of ASNS causes serious problems in the brain.

Asparagine synthetase deficiency (ASNSD) (OMIM: 615574)

→ Autosomal recessive disease.
→ Ultra rare (reported only 55 families including 2 Japanese).
→ Symptom: Progressive microcephaly, cerebral atrophy, severe mental development delay, intractable seizure, etc.
→ Onset mechanism and pathophysiology are still unknown.
→ No characteristic laboratory findings.
(Amino acids in blood and spinal fluid do not show any specific changes)

→ Almost all cases were diagnosed by genetic examination. About 90% of reported variants were missense variant, but there are a few reports describing the functional evaluation of ASNS variants.
→ Evaluation method for variants of ASNS are needed.

Purpose of our study

• To establish in vitro methods to evaluate the pathogenicity of variants of ASNS
• To produce recombinant ASNS
• To evaluate enzyme activity of ASNS
**Methods**

**Study 1: Expression of recombinant human ASNS**

We performed protein expression using pFastBac1 vectors including human ASNS cDNAs (wild-type and four variants (p.L145S, p.L247W, p.V489D or p.W541Cfs*5)) in silkworm. Expression of recombinant ASNS was checked by immunoblot analysis using monoclonal antibody (anti-ASNS, anti-His tag).

**Study 2: Quantitative evaluation of ASNS enzymatic activity**

The enzymatic activity of each protein was calculated by subtracting the value of the amino acid concentration of the inactivated protein sample from the activated protein sample. All measurements were performed three times for each sample and the average value was recorded. Evaluation of enzymatic activity was performed at least three times for each of the five ASNS proteins.

**Study 3: Generation and cell proliferation assay of the ASNS deficient HEK293 cell line**

Whole cell lysates of ASNS deficient HEK293 cell line was checked by immunoblot analysis using monoclonal antibody (anti-ASNS, anti-β-actin).

**Study 4: Gene transduction of human ASNS variants into the ASNS deficient HEK293 cell line with lentiviruses and cell proliferation assay of transduced cells**

Whole cell lysates of ASNS deficient HEK293 cell line was checked by immunoblot analysis using monoclonal antibody (anti-ASNS, anti-β-actin). Cell proliferation assay were performed three times for each cell line.
Results

Study1: Expression of recombinant human ASNS

p.Leu145Ser and p.Val489Asp variants gave weaker bands compared with wild-type ASNS in the SDS-PAGE analysis. This result suggests that these ASNS proteins were less stable.

Study2: Quantitative evaluation of ASNS enzymatic activity

All variants of ASNS showed low enzymatic activity.

The same amount of asparagine and glutamate were produced by the enzymatic reaction of recombinant ASNS proteins.

The p.Leu247Trp variant had a slightly residual activity as in cases using ammonium acetate as an amino group donor.

Study3: Generation and cell proliferation assay of the ASNS deficient HEK293 cell line

The immunoblot analysis of whole cell lysates extracted from ASNS deficient HEK293 cell line showed the loss of ASNS.

The ASNS deficient HEK293 cells did not grow in medium without asparagine.
Study 4: Gene transduction of human ASNS variants into the ASNS deficient HEK293 cell line with lentiviruses and cell proliferation assay of transduced cells

Expression of wild-type ASNS gave good cell proliferation without asparagine in the medium. Expression of the p.Leu145Ser, p.Val489Asp or p.Trp541Cysfs*5 variants and mock (expressed GFP only) gave similar cell proliferation patterns as ASNS deficient HEK293 cells. Expression of the p.Leu247Trp variant gave similar cell proliferation pattern as ASNS deficient HEK293 cells expressing wild-type ASNS, except proliferation was slightly slower.

Leu145 is part of a hydrophobic cluster located in the sandwich-like α/β/α/β N-terminal domain and is part of the second β-sheet. The Leu145Ser mutation changed the side chain from a hydrophobic to a hydrophilic side chain. This change may disrupt packing between the β-sheets. The conformation of the first β-sheet in relation to the second β-sheet is postulated to be important and plays a key role in folding. The Leu145Ser variants of ASNS may destabilize the native conformation. Leu247 is in close proximity to the AMP- and aspartate-binding sites of the C-terminal domain. The Leu247Trp exchange alters the side chain volume and thus possibly affects a hydrophobic interaction with adjacent residue Phe362, which is near the catalytically crucial residue Glu365. The Leu247Trp variant of ASNS may reduce enzyme activity.

Val489 is part of a hydrophobic core in the C-terminal domain. The Val489Asp exchange also changes the side chain type from hydrophobic to hydrophilic. This amino acid exchange may facilitate an interaction with the unstructured C-terminal tail region that disrupts the structure of ASNS. The Val489Asp variants of ASNS may destabilize the native conformation.

Trp541 is located at the distal end of the C-terminal domain (residues 536–561). Residues 536–561 of ASNS are absolutely conserved among many species but the function of these residues is unknown.

Although the p.Leu247Trp variant had relatively high residual activity, especially in the cell proliferation assay, ASNSD patients with this variant had typical ASNSD symptoms. Human ASNS is hypothesized to exist as a homodimer in vivo. The formation of heterodimers is possible in compound heterozygous patients. Dominant positive or negative effects of a variant monomer(s) forming a heterooligomer over the other monomer(s) have been described in other inborn errors of metabolism. Consequently, the total amount of ASNS activity in vivo may be affected by the combination of variants in compound heterozygous patients.

Conclusion

We established evaluation methods for the pathogenicity of variants of ASNS.

These methods will contribute to the accurate diagnosis of ASNSD and the elucidation of its pathogenesis.

Take Home Message

If you find patient suspected ASNSD or novel ASNS gene variant please call our laboratory!

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