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2 *In vitro* functional analysis of four variants of human asparagine synthetase

3

4 **Authors' names and institutional affiliations:**

5 *Hideki Matsumoto[1], Nana Kawashima[2], Takahiro Yamamoto[1,3], Mina Nakama[4], Hiroki*

6 *Otsuka[1], Yasuhiko Ago[1], Hideo Sasai[1,4], Kazuo Kubota[1,3,4], Michio Ozeki[1], Norio*

7 *Kawamoto[1], Yukihiro Esaka[2], Hidenori Ohnishi[1,3,4]*

8 [1]Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, Japan

9 [2]Laboratory of Pharmaceutical Analytical Chemistry, Gifu Pharmaceutical University,

10 Gifu, Japan

11 [3]Educational Support Center for Pediatric Home-Based Medical Care, Graduate School

12 of Medicine, Gifu University, Gifu, Japan

13 [4]Clinical Genetics Center, Gifu University Hospital, Gifu, Japan

14

15 **Name, email address and full postal address, including postal (ZIP) code, of the**

16 **author who will be dealing with correspondence and proofs:** Hideo Sasai,

17 sasai@gifu-u.ac.jp, Department of Pediatrics, Graduate School of Medicine, Gifu

18 University, Yanagido 1-1, Gifu 501-1194, Japan

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28

29   **Summary**

30   The loss-of-function variants of the human asparagine synthetase (*ASNS*) gene cause

31   asparagine synthetase deficiency (ASNSD). Diagnosis of ASNSD requires genetic tests

32   because a specific biochemical diagnostic for ASNSD is not available. There are a few

33   reports describing the functional evaluation of ASNS variants. Therefore, *in vitro*

34   methods to evaluate the detected variants in patients are needed. In this report, five types

35   of human ASNS proteins (wild-type and our reported four variants: p.Leu145Ser,

36   p.Leu247Trp, p.Val489Asp, p.Trp541Cysfs*5) were expressed in silkworm using a

37   baculoviral expression system. An enzymatic activity assay of ASNS was performed, and

38   the concentration of asparagine by ninhydrin and HPLC methods using the purified

39   recombinant proteins was measured. We established *ASNS* deficient HEK293 cells using

40   the CRISPR/Cas9 method, and evaluated the growth of cells without asparagine after

41   transduction of ASNS variants with a lentiviral expression system. The four ASNS

42   variants displayed significantly low enzymatic activity. The *ASNS* deficient HEK293

43   cells transduced with wild-type ASNS grew without asparagine, whereas cells transduced

44   with the variants did not grow or showed significantly slower growth than cells

45   transduced with wild-type ASNS. Herein, we established a method for evaluating the

46   enzymatic activity of the recombinant human ASNS variants. The results of the

3

47 cell-based assay corroborated the results of the enzymatic activity. These methods should

48 enable the evaluation of the pathogenicity of ASNS variants.

49

50 **Synopsis:** We established *in vitro* assays to evaluate the pathogenicity of asparagine

51 synthetase (*ASNS*) gene variants.

52

5

70   **Details of ethics approval:** This study was carried out under approval by the ethics

71   review committee of Gifu University Medical Research (Protocol numbers 3–14 and

72   3–30).

73   **A list of approximately six keywords:** *ASNS*, asparagine synthetase, asparagine

74   synthetase deficiency, enzyme assay, HPLC fluorescence detection, lentiviral expression

75   system.

76

77   **1. INTRODUCTION**

78   Asparagine synthetase (ASNS) is the only enzyme that biosynthesizes asparagine in

79   humans. ASNS produces asparagine, glutamate and AMP from aspartate, glutamine (or

80   ammonia) and ATP.[1] The *ASNS* gene (NM_133436.3), which encodes ASNS, is

81   expressed in whole body tissue, and gene expression is particularly strong in neurons.[2]

82   Asparagine synthetase deficiency (ASNSD) (OMIM: 615574), first described by Ruzzo

83   *et al.*[3] in 2013, is an autosomal recessive disease caused by biallelic variants of the *ASNS*

84   gene, resulting in congenital microcephaly, intractable seizures and severe psychomotor

85   developmental delays. The specific pathogenesis of ASNSD remains unknown[4]; however,

86   ASNSD may reduce proliferation of neural cells in the central nervous system under

87   asparagine-free conditions.

88   Currently, more than 50 cases of ASNSD have been reported (Figure S1), and

89   several crucial problems in the diagnosis and treatment of ASNSD exist. Experiments

90   with fibroblasts established from ASNSD patients[3,5,6] and the report of an ASNSD mouse

91   model[3] suggest that a decrease in ASNS expression or reduced enzymatic activity may

92   play a key role in the pathogenesis of ASNSD. However, it is difficult to quantitatively

93   assess the enzymatic activity of ASNS *in vivo*. The concentrations of amino acids in

94   serum and spinal fluid were not consistently altered in ASNSD patients,[4] and there are no

95   biochemical diagnostic methods for ASNSD currently available.

96   ASNSD is generally diagnosed by the detection of biallelic *ASNS* variants and

97   ASNS enzymatic activity is indirectly assessed by using fibroblasts taken from patients;

98   however, most reported ASNSD cases have been diagnosed based on clinical phenotypes

99   and by genetic variants evaluated using *in silico* prediction. Furthermore, because most of

100   the genetic variants identified in ASNSD patients are missense variants, it is difficult to

101   confirm the pathogenicity of these variants. Therefore, there are some cases that are

102   difficult to diagnose solely by genetic analysis because of the lack of accumulated

103   information on functional analysis of ASNS variants.

104   In this study, to resolve these issues we established a new method to express

105   recombinant ASNS proteins and quantitative assessment methods to measure the

7

106   enzymatic activity of ASNS. In addition, ASNSD model cells and the lentivirus

107   transduction system were established to confirm the pathogenicity of the *ASNS* gene

108   variants.

109

110   **2. MATERIALS AND METHODS**

111   **2.1 Vector preparation**

112   The cDNA of the *ASNS* gene (accession number: NM_133436.3, position 507–2192)

113   with a 3' terminal 6×His-tag was chemically synthesized and cloned into the pFastBac1

114   vector (Invitrogen, Carlsbad, CA, USA). Four different expression constructs were

115   created using site-directed mutagenesis (four variants of ASNS: p.Leu145Ser (c.434T>C),

116   p.Leu247Trp (c.740T>G), p.Val489Asp (c.1466T>A), p.Trp541Cysfs*5

117   (c.1623_1624del)). For the p.Trp541Cysfs*5 variant expression construct, the 6×His-tag

118   was added just before the new termination codon generated by the frameshift mutation.

119   The cDNA of the *ASNS* gene was cloned into the pLVSIN-EF1α-AcGFP-N1

120   vector (Takara Bio Inc., Otsu, Japan). Vectors of the four ASNS variants were created as

121   described above. For the p.Trp541Cysfs*5 variant expression construct, the new

122   termination codon generated by the frameshift mutation was skipped.

123

124 **2.2 Cell culture**

125 All cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2. Dulbecco's

126 modified Eagle's medium (DMEM) (FUJIFILM Wako Pure Chemical Corporation,

127 Osaka, Japan; Cat. No. 044–29765) with 10% heat-inactivated fetal bovine serum,

128 penicillin (100 units/mL) and streptomycin (100 μg/mL) was used. Sterilized asparagine

129 (final concentration of 500 μM) was added to DMEM as needed because DMEM does not

130 contain asparagine.

131

132 **2.3 Visible protein detection**

133 Protein samples were loaded onto a 10% XV PANTERA gel (D.R.C. Co., Tokyo, Japan;

134 CAT No. NXV–225P). The gel was stained with Coomassie Brilliant Blue (CBB; final

135 concentration 0.1% CBB-R250, 45% methanol, 10% acetic acid) when required. We used

136 anti-ASNS (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. A6485), anti-His-tag

137 (FUJIFILM Wako Pure Chemical Corporation; Cat. No. 010-23181), anti-GFP (Clontech

138 Laboratories, Inc., Mountain View, CA, USA; Cat. No. 632380) and anti-β-actin

139 (FUJIFILM Wako Pure Chemical Corporation; Cat. No. 010-27841) antibodies, as well

140 as iBlot™ 2 Dry Blotting System (Invitrogen, Waltham, MA, USA; Cat. No. IB21001),

141 iBlot™ 2 Transfer Stacks and PVDF (Invitrogen; Cat. No. IB24001) for immunoblotting.

142 The signals were visualized using a light capture system (ATTO, Tokyo, Japan; Cat. No.

143 AE6970CP).

144

145 **2.4 Expression of recombinant human ASNS proteins by silkworms**

146 We used modified methods based on previous reports.[7,8] The donor plasmids of the

147 pFastBac1 vectors containing the human *ASNS* gene were transformed into *Escherichia*

148 *coli* BmDH10Bac. BmNPV bacmid DNA was purified from BmDH10Bac cells by using

149 the Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany; Cat. No. 12163). Then, One

150 microgram BmNPV bacmid DNA and 1 µL Cellfectin II reagent (Invitrogen; Cat. No.

151 10362100) suspended in Grace insect cell medium were injected into 5th instars *Bombyx*

152 *mori* silkworm larvae. The silkworms were reared in a 25 °C incubator with food changed

153 every 24 h. After 6 to 8 days following BmNPV bacmid DNA injection, the fatty body

154 was recovered from the recombinant BmNPV-infected larvae and phosphate-buffered

155 saline (PBS) and sodium thiosulfate (final concentration 0.5% w/v) were added

156 immediately. Fluid and fatty body recovered from the larvae were centrifuged at 4 °C and

157 20,000 $\times g$ for 10 min. The supernatant was discarded and the precipitant dissolved in 2

158 mL lysis buffer (50 mM HEPES, 150 mM NaCl, 10 mM imidazole, 10 mM aspartate, 15

159 mM glutamine, 1 mM DTT, 5 mM ATP·nH$_2$O, cOmplete™ Protease Inhibitor Cocktail,

160   pH 8.0) and homogenized sufficiently by using a Dounce tissue grinder. After

161   centrifugation at 4 °C and 20,000 $\times g$ for 60 min, the supernatant was passed through 1.2,

162   0.45 and 0.22 μm filters, placed in dialysis tubing (MWCO 50,000) and dialyzed against

163   1 L lysis buffer for 8 to 12 h. The dialysis step was carried out twice. After dialysis, the

164   recombinant ASNS protein was separated from other cellular proteins and material by

165   Ni-NTA affinity chromatography (GE Healthcare Bio-Sciences AB., Uppsala, Sweden;

166   Cat. No. 17–5318–02) using a lysis buffer and elute buffer (50 mM HEPES, 150 mM

167   NaCl, 500 mM imidazole, 10 mM aspartate, 15 mM glutamine, 1 mM DTT, 5 mM

168   ATP·nH_2O, cOmplete™ Protease Inhibitor Cocktail, pH 8.0). Ten milliliters of the elute

169   buffer containing the purified recombinant ASNS protein was centrifuged and

170   concentrated at 4 °C and 3000 $\times g$ for 40 min using Amicon™ Ultra-15 Centrifugal Filter

171   Units (30,000 MWCO) (Millipore, Billerica, MA, USA; Cat. No. UFC903024). The

172   concentration of recombinant ASNS was measured by the Lowry protein assay. Protein

173   expression was performed at least three times each for the five ASNS constructs (i.e.,

174   wild-type and four variants).

175

176   **2.5 Quantitative evaluation of ASNS enzymatic activity**

177   We prepared four samples per protein. Sixty-four micrograms of recombinant ASNS was

178   added to 150 μL ASNS enzyme assay mix A (100 mM EPPS, 150 mM NaCl, 10 mM

179    $MgCl_2 \cdot 6H_2O$, 1 mM DTT, 10 mM glutamine, 10 mM aspartate, 5 mM $ATP \cdot nH_2O$, pH

180   8.0) or ASNS enzyme assay mix B (100 mM EPPS, 150 mM NaCl, 10 mM $MgCl_2 \cdot 6H_2O$,

181   1 mM DTT, 50 mM ammonium acetate, 10 mM aspartate, 5 mM $ATP \cdot nH_2O$, pH 8.0).

182   After adding the protein, one sample was boiled immediately for 5 min (inactivated

183   protein sample), and another sample was incubated at 37 °C for 60 min and then boiled

184   for 5 min (activated protein sample). The samples were examined by the following two

185   methods.

186   The asparagine concentration was measured by the modified method established

187   by Sheng et al.[9] The samples were centrifuged and 110 μL of the supernatant was added

188   to 990 μL of 0.05 % ninhydrin (dissolved in 100% ethanol). This sample was incubated at

189   37 °C for 180 min. The absorbance at 340 nm was measured by using an Hitachi U-2910

190   spectrophotometer (Hitachi High-Technologies Corp. Tokyo, Japan).

191   The asparagine and glutamate concentrations were also measured by the

192   modified HPLC fluorescence detection method.[10] After the enzyme reaction, samples

193   were centrifuged at 4 °C and 20,000 $\times g$ for 60 min with a 3 K molecular weight cut-off

194   filter. The filtered liquid was diluted to an appropriate concentration and 140 μL of the

195   diluted solution was mixed with 10 μL 50 mM KCN (dissolved in borate buffer, pH 9.5)

196   and 50 μL 4 mM NDA (dissolved in 100% methanol) and manually injected into the

197   HPLC system. The HPLC system consisted of two PU-980 pumps (JASCO Corp., Tokyo,

198   Japan), a FP-2025 Plus fluorescence detector (JASCO Corp.), a CO-965 column oven

199   (JASCO Corp.) equipped with a Model 7125 syringe-loading sample indicator (Reodyne,

200   Berkeley, CA, USA) and a reversed-phase TSKgel ODS-100V column (Tosoh

201   Corporation Tokyo, Japan, 3 μm, 4.6 × 150 mm). The column oven was 40 °C, the flow

202   rate was 0.4 mL per min, the excitation wavelength was 420 nm, the fluorescence

203   wavelength was 490 nm and the measurement was performed using an eluent of 0.2 M

204   acetate buffer (pH 4.6)/Acetonitrile -Plus- (KANTO CHEMICAL CO., INC., Tokyo,

205   Japan; Cat. No. 01031-1B) at a ratio of 70/30. Next, 140 μL of various amino acid

206   solutions (glutamine, glutamate, asparagine, aspartate) of known concentrations were

207   mixed with 10 μL 50 mM KCN and 50 μL 0.4 mM NDA, derivatized for 20 min and 20

208   μL of the mixture was taken and measured using the same HPLC method described above

209   to measure the concentration of each amino acid in the sample using the absolute

210   calibration curve method. The calibration curves were linear across the examined

211   dynamic range of 3.5 to 600 nM ($n = 6$, $r^2 = 0.998$) for asparagine and 15 to 600 nM ($n =$

212   6, $r^2 = 0.990$) for glutamate.

213   The enzymatic activity of each protein was calculated by subtracting the value of

214   the amino acid concentration of the inactivated protein sample from the activated protein

215   sample. All measurements were performed three times for each sample and the average

216   value was recorded. Evaluation of enzymatic activity was performed at least three times

217   for each of the five ASNS proteins.

218

219   **2.6 Generation of an *ASNS* deficient HEK293 cell line**

220   We created the *ASNS* deficient HEK293 cell line by using the CRISPR/Cas9 method.

221   Initially, pX330-U6-Chimeric_BB-CBH-hSpCas9 plasmid (Addgene, Cambridge, MA,

222   USA; Cat No. 42230) with the target sequence (5'-GGATATTTCTTCACTCGAAT-3')

223   was prepared. A double-strand DNA break point (DSB) was placed between c.187 and

224   c.188, which is located in *ASNS* exon 4. HEK293 cells (Japanese Collection of Research

225   Bioresources, Osaka, Japan) processed after the plasmid nucleofection with

226   Nucleofector™ II (Lonza Biosciences, Cologne, Germany; Cat. No. AAD-1001N) and

227   Nucleofector™ kit V (Amaxa, Cologne, Germany; Cat. No. VCA-1003) were performed

228   limited dilution, and then selected *ASNS* deficient cells. The loss of ASNS protein

229   expression for these cells was confirmed by immunoblotting analysis.

230

231 **2.7 Gene transduction of human ASNS into the *ASNS* deficient HEK293 cell line**

232 **using lentiviruses**

233 Using the Lenti-X 293T cell line (Takara Bio Inc.; Cat. No. Z2180N) and the

234 pLVSIN-EF1α-AcGFP-N1 vector (five types of ASNS vectors and the empty

235 pLVSIN-EF1α-AcGFP-N1 vector), we transduced various ASNS vectors into *ASNS*

236 deficient HEK293 cells. The Lenti-X 293T cell line was spread on 9 cm plates in diameter

237 with 5.0×10^6 cells per plate and cultured in DMEM for 24 h. The

238 pLVSIN-EF1α-AcGFP-N1 vector (5.5 μg) was added to 7 μL Lentiviral Mix High Titer

239 Packaging Mix (Takara Bio Inc.; Cat. No. 6194), 1500 μL serum free DMEM and 45 μL

240 Trans IT-293 Transfection Reagent (Takara Bio Inc.; Cat. No. MIR2704). After 15 min,

241 this mixture was added to Lenti-X 293T cells and incubated at 37 °C. After 24 h, the

242 medium was exchanged to DMEM. After a further 48 h, the culture medium was

243 collected and passed through a 0.45 μm filter (this solution contains recombinant

244 lentiviruses). Six different recombinant lentiviral solutions were prepared using the five

245 types of ASNS vectors and the pLVSIN-EF1α-AcGFP-N1 vector. *ASNS* deficient

246 HEK293 cells were spread into 6-well plates with 2.0×10^5 cells per well. The

247 recombinant lentiviral solution was diluted with DMEM to give solutions with initial

248 concentrations of 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64. The medium was discarded from each

249   well, and 2 mL of the recombinant lentiviral solutions were added to each well. Polybrene

250   was added to the medium to a final concentration of 4 μg/mL. After 48 h, puromycin was

251   added to a final concentration of 2.5 μg/mL. To select stably GFP-only or C-terminal

252   GFP-fused ASNS expressing cells, repeated exchange of the medium and the addition of

253   puromycin (final concentration of 2.5 μg/mL) were performed every 48 h. ASNS

254   expression in these cells was evaluated by immunoblotting using whole cell lysates.

255

256   **2.8 Cell proliferation assay**

257   HEK293 cells were spread into 6-well plates with 1.0×10^5 cells per well. Twenty-four

258   hours after spreading, the medium was exchanged to DMEM (asparagine free) (day 0).

259   The medium was exchanged every 24 h and cell counts were measured 24, 48 and 72 h

260   after the first medium change. Next, the same cell proliferation assay was performed

261   using DMEM (with asparagine). These measurements were performed three times for

262   each cell line.

263

264   **2.9 Statistical analysis**

265   Statistical analysis was performed by using Prism 9 (GraphPad Software, LLC., San

266   Diego, CA, USA). The statistical significance of the differences was determined by

267   one-way ANOVA, Welch's t-test or Student's t-test. Statistical significance was assigned

268   to be $P < 0.05$.

269

270   **3. RESULTS**

271   **3.1 Expression of recombinant human ASNS**

272   The purified recombinant ASNS proteins were visualized by a CBB-stained SDS-PAGE

273   gel (Figure 1A). The predicted molecular weights of the C-terminal 6×His-tagged ASNS

274   wild-type and p.Trp541Cysfs*5 variant were approximately 64.8 and 63.3 kDa,

275   respectively. Compared with wild-type ASNS, the p.Leu145Ser and p.Val489Asp

276   variants gave weaker bands in the SDS-PAGE analysis, suggesting that these ASNS

277   proteins were less stable. The results of immunoblot analysis with the anti-ASNS and

278   anti-His-tag antibodies are shown in Figures 1B and 1C, respectively. Recombinant

279   His-tagged ASNS were expressed as designed.

280

281   **3.2 Quantitative evaluation of ASNS enzymatic activity**

282   Using the recombinant ASNS proteins and glutamine as an amino group donor, the

283   concentration of asparagine produced by the enzymatic reaction and measured by the

284   ninhydrin method is shown in Figure 2A. The four variants showed significantly low

17

285 enzymatic activity when compared with that of the wild-type protein. All variants except

286 for the p.Trp541Cysfs*5 variant showed slight residual activity. The results using

287 ammonium acetate as an amino group donor are shown in Figure 2B. A clear difference in

288 enzymatic activity between the wild-type ASNS and the four variants was observed;

289 however, the p.Leu247Trp variant yielded a slightly different result (the residual activity

290 was stronger if using ammonium acetate as an amino group donor) when compared with

291 the results in Figure 2A.

292   The asparagine and glutamate concentrations of the same specimens used in

293 Figure 2A measured by the HPLC fluorescence detection method are presented in Figures

294 2C and 2D, respectively. The results are similar to those of Figure 2A. The same amount

295 of asparagine and glutamate were produced by the enzymatic reaction of recombinant

296 ASNS proteins.

297   Notably, the enzymatic activity of all recombinant ASNS proteins decreased

298 rapidly after purification and was essentially absent 3 to 4 days after purification (Figure

299 S2A,B,C). Therefore, all recombinant proteins were used for enzymatic activity assays

300 one day after purification.

301

302 **3.3 Generation and cell proliferation assay of the *ASNS* deficient HEK293 cell line**

18

303   The immunoblot analysis of whole cell lysates extracted from HEK293 cells and *ASNS*

304   deficient HEK293 cells with the anti-ASNS and anti-β actin antibodies are shown in

305   Figures 3A and 3B, respectively. The DNA sequence derived from the *ASNS* deficient

306   HEK293 cells showed termination just after the designed DSB, or termination 15 amino

307   acids after the DSB (data not shown), and the subcloned sequences did not reveal the

308   normal *ASNS* sequence. The results of the cell proliferation assay (with or without

309   asparagine) of *ASNS* deficient HEK293 cells are shown in Figure 3C. The *ASNS* deficient

310   HEK293 cells did not grow in medium without asparagine and did not survive.

311

312   **3.4 Gene transduction of human ASNS variants into the *ASNS* deficient HEK293**

313   **cell line with lentiviruses and cell proliferation assay of transduced cells**

314   Immunoblot analysis of the *ASNS* deficient HEK293 cells transduced with empty

315   (isolated AcGFP protein expression), ASNS wild-type or variants with AcGFP proteins

316   expressing lentiviruses with anti-GFP antibodies and anti-β actin antibodies are shown in

317   Figure 4A and 4B, respectively. The predicted molecular weight of AcGFP is 26.9 kDa.

318   Recombinant AcGFP-fused ASNS were expressed as designed.

319   The results of the cell proliferation assay (with or without asparagine) using

320   stably transfectant *ASNS* deficient HEK293 cell lines of AcGFP, or AcGFP-fused

321 wild-type or variants of ASNS are shown in Figure 4C. Expression of wild-type ASNS

322 gave good cell proliferation with or without asparagine in the medium. However, when

323 the p.Leu145Ser, p.Val489Asp or p.Trp541Cysfs*5 variants were expressed, cell

324 proliferation was similar to *ASNS* deficient HEK293 cells. In contrast, when the

325 p.Leu247Trp variant was expressed, the cell proliferation pattern was similar to the

326 proliferation profile of HEK293 cells expressing wild-type ASNS, except proliferation

327 was slightly slower.

328

329 **4. DISCUSSION**

330 ASNSD is a rare and intractable inborn error of metabolism. A diagnostic method and

331 effective therapy for this disease are not available. To overcome these issues, we showed

332 three important findings in this study: 1) we established a novel expression method of

333 biologically active recombinant ASNS; 2) all variants found in Japanese ASNSD patients

334 showed reduced ASNS enzymatic activity; and 3) the proliferation of *ASNS* deficient

335 cells was recovered without the addition of asparagine by transducing wild-type ASNS

336 into cells using lentiviruses.

337   We established an expression protocol by using silkworms to produce

338 recombinant ASNS. Although human ASNS has been produced previously by using

339 *Escherichia coli*,[11] yeast,[12] and Sf9 insect cells,[13] it has proven difficult to synthesize a

340 large amount of recombinant ASNS that retains enzymatic activity. We established a

341 simple and easy method to express active recombinant ASNS. Therefore, this method

342 may be suitable for use in future enzyme replacement therapy of ASNSD if a method to

343 maintain the stability of the produced recombinant ASNS can be found. Furthermore, as

344 we also expressed ASNS variants successfully, this method should facilitate the

345 evaluation of newly identified genetic variants of *ASNS*.

346   In previous reports, all Japanese ASNSD patients are compound heterozygote

347 cases, and all variants found in ASNSD patients reported from Japan[14] show reduced

348 ASNS activity; however, none of the variants in both alleles resulted in a complete loss of

349 enzymatic activity. The p.Leu145Ser and p.Val489Asp variants displayed reduced

350 enzyme stability and activity, and the protein expression level of these variants was low.

351 The p.Leu247Trp and p.Trp541Cysfs*5 variants showed significantly lower enzymatic

352 activity when compared with that of wild-type ASNS, although the p.Leu247Trp variant

353 had a relatively high residual activity. These results suggest that cells from ASNSD

354 patients have residual ASNS activity. Currently, most reported variants of the *ASNS* gene

355 are missense variants (Figure S1), with only a large deletion reported in one case,[15]

356 indicating that the complete loss of enzymatic activity on both alleles of *ASNS* may cause

357   embryonic lethality. The genotype-phenotype correlation of ASNSD had not been

358   elucidated. Our method should facilitate the evaluation of how residual activity of ASNS

359   produces different clinical manifestations. There are potential limitations with

360   interpretation of data for the recombinant ASNS proteins used in this study. Recombinant

361   ASNS proteins were expressed with a C-terminal His-tag. This affinity tag facilitates easy

362   and rapid purification of the protein to avoid inactivation of the enzyme when lengthy

363   purification protocols are required for ASNS purification without affinity tags. However,

364   the His-tag may affect enzymatic activity when compared with the activity of the enzyme

365   without this tag, i.e., native state. Nonetheless, to compare the enzymatic activity of

366   wild-type ASNS and variants these His-tagged ASNS proteins were suitable for

367   straightforward determination of the pathogenicity of the variants.

368   Although the p.Leu247Trp variant had relatively high residual activity,

369   especially in the cell proliferation assay, ASNSD patients with this variant had typical

370   ASNSD symptoms.[14] Human ASNS is hypothesized to exist as a homodimer *in vivo*.[13,16]

371   The formation of heterodimers is possible in compound heterozygous patients. Dominant

372   positive or negative effects of a variant monomer(s) forming a heterooligomer over the

373   other monomer(s) have been described in other inborn errors of metabolism.[17-19]

374   Consequently, the total amount of ASNS activity *in vivo* may be affected by the

375   combination of variants in compound heterozygous patients. Clarification of this issue

376   requires a novel co-expression method to evaluate the pathogenicity of compound

377   heterozygous patients and is a subject for future study.

378   The crystal structure of human ASNS (PDB ID: 6GQ3) was elucidated by Zhu *et*

379   *al.* in 2019.[13] ASNS comprises two major domains: residues 1–203 form the glutaminase

380   domain, whereas the synthetase domain consists of residues 204–561. The synthetase

381   domain is highly conserved in among species.[13] Structural changes or changes in activity

382   to the four variants analyzed in this study have been discussed in our previous report

383   based on the crystal structure of *Escherichia coli* asparagine synthetase B (PDB ID:

384   1CT9).[14] Leu145 is part of a hydrophobic cluster located in the sandwich-like α/β/β/α

385   N-terminal domain and is part of the second β-sheet.[13,14] Leu247 is in close proximity to

386   the AMP- and aspartate-binding sites of the C-terminal domain.[13,14] Val489 is part of a

387   hydrophobic core in the C-terminal domain.[13,14] Trp541 is located at the distal end of the

388   C-terminal domain (residues 536–561). Residues 536–561 of ASNS are absolutely

389   conserved among many species but the function of these residues is unknown.[13,14] The

390   Leu145Ser mutation changed the side chain from a hydrophobic to a hydrophilic side

391   chain. This change may disrupt packing between the β-sheets. The conformation of the

392   first β-sheet in relation to the second β-sheet is postulated to be important and plays a key

393 role in folding. The Leu247Trp exchange alters the side chain volume and thus possibly

394 affects a hydrophobic interaction with adjacent residue Phe362, which is near the

395 catalytically crucial residue Glu365. The Val489Asp exchange also changes the side

396 chain type from hydrophobic to hydrophilic. This amino acid exchange may facilitate an

397 interaction with the unstructured C-terminal tail region that disrupts the structure of

398 ASNS. Therefore, Leu145Ser and Val489Asp variants of ASNS may destabilize the

399 native conformation, whereas the Leu247Trp variant of ASNS may reduce enzyme

400 activity.

401   Finally, we successfully established a cell disease model of ASNSD (*ASNS*

402 deficient HEK293 cells) by using the CRISPR/Cas9 method, and recovered the

403 expression of ASNS and cell growth using the lentiviral expression system. Because cells

404 without ASNS activity cannot survive in asparagine-free medium, the results herein

405 suggest that future gene therapy of ASNSD is feasible. Studies have examined the

406 practical application of gene therapy for unsettled inborn errors of metabolism.[20,21] For

407 example, gene therapy using adeno-associated viral vectors for aromatic L-amino acid

408 decarboxylase deficiency that causes severe damage of the central nervous system from a

409 very early stage of life similar to ASNSD have been reported to have dramatic effects.[20]

410 Thus, the practical application of gene therapy to treat ASNSD is also anticipated in the

411 future. In addition, ASNSD already shows symptoms such as microcephaly at birth,[4] and

412 cases of fetal diagnosis have been reported.[22] Early diagnosis, early intervention and fetal

413 treatment may be necessary. We evaluated the enzymatic activity of reported variants of

414 ASNS using a lentivirus, *ASNS* deficient HEK293 cells and recombinant ASNS

415 expressed in silkworms. Our experiments succeeded in doubly confirming the evaluation

416 of the enzymatic activity of ASNS variants.

417

418 **5. CONCLUSION**

419 We established a method for evaluating the enzymatic activity of human ASNS variants

420 with purified recombinant ASNS produced by silkworms. The results of the cell-based

421 assay were congruous with the enzymatic activity results. These methods should enable

422 the evaluation of the pathogenicity of ASNS variants.

423

436

437   **CONFLICT OF INTEREST**

438   The authors declare no potential conflicts of interest with respect to the research,

439   authorship and/or publication of this article.

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441   **ORCID**

442   *Hideki Matsumoto: https://orcid.org/0000-0002-5538-0739*

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503

504 **Figure legends**

505 **FIGURE 1** Protein expression of recombinant human ASNS. A, SDS-PAGE analysis of

506 recombinant human ASNS. B, Immunoblot of recombinant human ASNS with the

507 anti-ASNS antibody. C, Immunoblot of recombinant human ASNS with the anti-His-tag

508 antibody

509

510 **FIGURE 2** Concentration of asparagine after the enzymatic reaction using recombinant

511 human ASNS. A, Concentration of asparagine measured by the ninhydrin method using

512 64 μg of recombinant human ASNS and glutamine as the substrate for the enzymatic

513 reaction. B, Concentration of asparagine measured by the ninhydrin method using 64 μg

514 of recombinant human ASNS and ammonium acetate as the substrate for the enzymatic

515 reaction. C, Concentration of asparagine measured by the HPLC fluorescence detection

516 method using 64 μg of recombinant human ASNS and glutamine as the substrate for the

517 enzymatic reaction. D, Concentration of glutamate measured by the HPLC fluorescence

518 detection method using 64 μg of recombinant human ASNS and glutamine as the

519 substrate for the enzymatic reaction. The bars in all graphs are standard errors

520

521 **FIGURE 3** Generation and cell proliferation assay of *ASNS* deficient HEK293 cells. A,

522 Immunoblot of whole cell lysates of HEK293 cells and *ASNS* deficient HEK293 cells

523 with the anti-ASNS antibody. B, Immunoblot of whole cell lysates of HEK293 cells and

524 *ASNS* deficient HEK293 cells with the anti-β actin antibody. C, Cell counts of *ASNS*

525 deficient HEK293 cells after incubation with or without asparagine. The bars in the

526 graphs are standard errors

527

528 **FIGURE 4** Transduction of AcGFP-fused ASNS to *ASNS* deficient HEK293 cells using

529 the lentiviral expression system. A, Immunoblot of whole cell lysates of *ASNS* deficient

530 HEK293 cells transduced with AcGFP or AcGFP-fused wild-type or variants of ASNS

531 with the anti-GFP antibody. B, Immunoblot of whole cell lysates of *ASNS* deficient

532 HEK293 cells transduced with AcGFP or AcGFP-fused wild-type or variants of ASNS

533 with the anti-β actin antibody. C, Cell counts of *ASNS* deficient HEK293 cells transduced

534 with AcGFP or AcGFP-fused wild-type or variants of ASNS after incubation with or

535 without asparagine. The bars in the graphs are standard errors

536

537 **Supporting Information:**

538   Additional supporting information may be found online in the Supplemental

539   Information section at the end of this article.