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Brief communication

A deep intronic mutation of c.1166-285 T > G in *SLC46A1* is shared by four unrelated Japanese patients with hereditary folate malabsorption (HFM)

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ABSTRACT

Hereditary folate malabsorption (HFM) is an autosomal recessive disease caused by mutations in *SLC46A1* encoding the proton-coupled folate transporter (PCFT). HFM patients present with various clinical features including megaloblastic anemia, thrombocytopenia, combined immunodeficiency and neurodevelopmental disorders. In this study, we report the same deep intronic mutation of c.1166-285 T > G shared by four unrelated Japanese patients with HFM. This mutation was shown to generate a cryptic splice donor site for a 168-bp insertion of intron 3 sequences, leading to premature termination in the middle of this insertion. This mutation could be a founder mutation in the Japanese population, but also could be a hot-spot and could be present in undiagnosed HFM patients worldwide because of the difficulty to detect this mutation.

1. Introduction

Hereditary folate malabsorption (HFM) is an autosomal recessive disease caused by mutations in *SLC46A1* encoding the proton-coupled folate transporter (PCFT) [1–3]. Folate deficiency due to the defects of folate uptake in the intestine and choroid plexus results in various clinical features including megaloblastic anemia, thrombocytopenia, combined immunodeficiency and neurodevelopmental disorders [3–5]. The diagnosis of HFM is based on undetectable levels of serum folate, characteristic clinical features, and genetic studies. So far, 25 *SLC46A1* mutations among 35 patients have been reported, most of which are sporadic [1,2,4,6–20]. One of the challenges is how to detect splicing defects caused by deep intronic mutations because significant PCFT expression is limited to intestine and choroid plexus. However, at the same time, it is critical to diagnose HFM early and accurately because patients with HFM need early parenteral folate replacement which is quite different from the treatment strategy for severe combined immunodeficiency diseases (SCID): hematopoietic stem cell transplantation (HSCT).

We previously reported clinical features of an HFM patient (Patient 1) with compound heterozygous mutations of c.566G > T, p.G189 V and a deep intronic mutation of c.1166-285 T > G [8]. In this study,

Abbreviation: HFM, hereditary folate malabsorption; PCFT, proton-coupled folate transporter

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Table 1
Patients' characteristics at onset and genotypes.

Patient	1	2	3	4
Sex	Female	Female	Male	Female
Onset	3 m	3 m	3 m	8 m
Failure to thrive	(+)	(+)	(+)	(−)
Megaloblastic anemia	(+)	(+)	(+)	(+)
White blood cell (x 10^9/l) (normal: 5.0–19.5)	6.1	4.7	5.1	7.1
Neutrophil (x 10^9/l) (normal: 1–9)	1.6	1.0	1.5	1.7
Lymphocyte (x 10^9/l) (normal: 2.5–6.5)	3.5	3.6	3.3	4.1
T-cell (%) (normal: 48–75)	87.3	N.A.	55	N.A.
B-cell (%) (normal: 14–39)	15.5	N.A.	44	N.A.
NK-cell (%) (normal: 2–14)	6.9	N.A.	Undetectable	N.A.
(CD4 + CD45RA+)/CD4+ (%) (normal: 78.5–91.9)	31.2	N.A.	N.A.	N.A.
Hemoglobin (g/dl) (normal: 9.5–13.5)	5.9	2.7	3.2	8.9
Platelets (x 10^9/l) (normal: 150–300)	17	33	18	450
IgG (mg/dl) (normal: 176–581 (3 m), 217–904 (7–9 m))	97	25	184	329
IgA (mg/dl) (normal: 4.6–46.0 (3 m), 11–90 (7–9 m))	14	3.1	8	3
IgM (mg/dl) (normal: 24–89 (3 m), 34–129 (7–9 m))	22	2.8	8	111
Serum folate (ng/ml) (normal: 1.8–9.0)	Undetectable	Undetectable	Undetectable	Undetectable
CSF folate (nM/l) (normal: 89–136)	N.A.	N.A.	N.A.	Undetectable
Lymphocyte proliferation with PHA (Stimulation Index (normal: 70.3–194.9))	5529 cpm (4.8)	N.A.	32,320 cpm (12.5)	N.A.
Variant	c.1166-285 T > G, p.G389Afs*20 (Father) c.566G > T, p.G189 V (Mother)	c.1166-285 T > G, p.G389Afs*20 (Father and Mother)	c.1166-285 T > G, p.G389Afs*20 (Father) c.954C > G, p.S318R (Mother)	c.1166-285 T > G, p.G389Afs*20 (Mother) c.1174 T > G, p.F392 V (Father)

CSF, cerebrospinal fluid; PHA, phytohemagglutinin; m, month; N.A., not analyzed.

we show the mutation of c.1166-285 T > G causes a non-leaky 168-bp insertion of intron 3 sequences and premature termination, and leads to absent PCFT expression and impaired folate transport activity. We further studied three unrelated HFM patients and demonstrated that all of them had the same deep intronic mutation of c.1166-285 T > G as homozygous in Patient 2 and heterozygous in Patients 3 and 4.

2. Materials and methods

This study was conducted in accordance with the Declaration of Helsinki and the national ethical guidelines, and was approved by the Ethics Committees of Hokkaido University Faculty of Medicine and Graduate School of Medicine (017-0409).

2.1. Patients

All of the present patients with HFM are sporadic, unrelated, and were born to nonconsanguineous Japanese healthy parents. Clinical characteristics of each patient at onset were shown in Table 1.

Patient 1: She was previously reported [8]. Briefly, she developed persistent cough and failure to thrive at the age of 3 months. Laboratory examination showed anemia, thrombocytopenia, hypogammaglobulinemia and undetectable levels of serum folate. Bone marrow aspiration revealed megaloblastic anemia. Further studies indicated *Pneumocystis jirovecii* pneumonia. Parenteral folinic acid administration normalized the laboratory data.

Patient 2: She developed recurrent vomiting and failure to thrive at 3 months of age. Laboratory examination revealed anemia, thrombocytopenia, hypogammaglobulinemia and undetectable levels of serum folate. Bone marrow aspiration revealed megaloblastic anemia. Parenteral folinic acid administration normalized the laboratory data.

Patient 3: He was born healthy at 35 weeks and 4 days with normal complete blood count. However, at 2 months of age, he developed failure to thrive and respiratory failure due to pneumonia with pleural fluid. He was admitted to a pediatric intensive care unit and received mechanical ventilation. Laboratory examination showed undetectable levels of serum folate, lower limit of normal range of IgG on admission, and he soon developed pancytopenia. Bone marrow aspiration revealed megaloblastic anemia. Although intravenous folic acid of 15 mg daily resolved pancytopenia within 2 weeks, mechanical ventilation was needed for a while due to refractory respiratory failure caused by diffuse alveolar hemorrhage. His condition gradually improved, and intravenous folic acid dosage was decreased to 7.5 mg daily.

Growth and development were normalized after parenteral folate replacement in Patients 1, 2 and 3.

Patient 4: She presented at 8 months of age with seizure and pneumonia, although she hadn't presented with failure to thrive or neurodevelopmental delay until then. Laboratory examination showed

anemia and undetectable levels of serum folate, but didn't show hypogammaglobulinemia or thrombocytopenia. Bone marrow aspiration revealed megaloblastic anemia. Oral folic acid administration, intravenous immunoglobulin based on reduced IgG2 levels of 0.61 g/l, antibiotics and antiepileptic drugs were initiated. She subsequently recovered from pneumonia and anemia in a month. However, although serum folate level was elevated to 10.6 ng/ml after 10 mg/day of oral folic acid administration, no more neurological development was observed and cerebral fluid folate concentration was still undetectable. Therefore, oral folate was switched to oral folinic acid of 50 mg/day which resulted in increased cerebral fluid folate concentration of 8.4 nM/l. She had no more seizures and started to show some improvement of neurological development under 70 mg/day of oral folinic acid administration, although it was not fully evaluated because of short-term observation.

2.2. DNA and total RNA isolation, PCR and RT-PCR, TOPO-TA cloning and sequence analysis

These procedures were described elsewhere [21]. Primer sequences for PCR, RT-PCR and sequence analysis of *SLC46A1* were as follows; Exon 1, Forward, 5′-CCGCCGGACATTTAAGGAG-3′; Reverse, 5′-CAGT TACCCGCCACTACCAT-3′; Exon 2, Upper half, Forward, 5′-GATTGTG GAACCCAGAGTGAG-3′; Reverse, 5′-ACCAAAGCAGAAAGCTGCATA-3′; Exon 2, Lower half, Forward, 5′-TATGCAGCTTTCTGCTTTGGT −3′; Reverse, 5′-CAAAGACACAGGAATGAACCAC-3′; Exon 3, Forward, 5′-AGACAAGGGCAGTCTCCATTC-3′; Reverse, 5′-ATGTGTTTGTTTTGG CCACTT-3′; Exon 4, Forward, 5′-GGTGAGGAAGGGGAGACATAG-3′; Reverse, 5′-AAGACACCCAGAGGGTAAGGA-3′; Exon 5, Forward, 5′-GGAGGAGGTTCAGGAGAGCTA-3′; Reverse, 5′-CCTAGACAGAGGCT GGGTCA-3′; Primers to detect c.1166-285 T > G mutation, Forward, 5′-TTTCCCAGCACTTTCTCTGTG-3′; Reverse, 5′-CTATGTCTCCCCTTCC TCACC-3′; Primers to detect the transcript with 168-bp insertion, Forward, 5′-GATGCCTGGGTAGCTGAGAT-3′; Reverse, 5′-GTTCAGAGTGG CTGGGTAGAG-3′; Primers to amplify 168-bp positive or negative products, Forward, 5′-CCTTCTGGCTGCTAGCTTTG-3′; 168-bp positive Reverse (posiR), 5′-ATGAAAGCTTGATGGAAATGG-3′; 168-bp negative Reverse (negaR), 5′-AGCACCCTGCTCTGTCTCTCT-3′; Primers to amplify upper half of SLC46A1, FLF, 5′-GTCCCAGGCAGCGAGTC-3′; Reverse, 5′-CCAGAAGGGGTTGGCATAAC-3′; Primers to amplify lower half of SLC46A1, Forward, 5′-AGGCCCTAGTGTCCGTTTTT-3′; FLR, 5′-CTCCAGTTGCTTGGTGTTCA-3′; Primers to clone into a TOPO-TA vector, *Eco*RI-FLF, 5′-GAATTCATGGAGGGGAGC-3′; BamHI-FLR, 5′-GGATCCTCAGGGGCTCTG-3′.

2.3. Expression vector constructs

As full-length (FL) *SLC46A1* cDNA was not specifically amplified, we first amplified upper and lower half of *SLC46A1* cDNA separately and cloned into TOPO-TA cloning vectors. We then digested each with *Eco*RI and *Pvu*II, ligated each other at PvuII site and PCR-amplified the FL with EcoRI-FLF and BamHI-FLR primers. We then cloned the ligated cDNA into pcDNA3-HisC (Life Technologies, Carlsbad, CA). Mutagenesis was performed with PrimeSTAR Mutagenesis Basal Kit (Takara Bio Inc., Japan) following the manufacturer's protocol.

2.4. Transient transfection of plasmids expressing PCFT fusion-protein in HeLa cells and Western blot analysis

We transiently co-transfected with pcDNA3-HisC-SLC46A1 wild-type (Wt) or one of the variants and pEGFP-C2 construct (Takara Bio Inc.) as a transfection marker at a weight ratio of 5:1 into HeLa cells using the TransIT-LT1 reagent (Mirus, Madison, WI) according to the manufacturer's protocol. We harvested each transfectant 48 h after transfection. To study the membranous PCFT expression, we obtained whole cell lysates as previously described [22]. Then we proceeded to Western blot analyses of PCFT expression firstly with anti-Xpress antibody (Invitrogen, Carlsbad, CA) corresponding to N-terminal Xpress fusion protein derived from pcDNA3-HisC-PCFT constructs to evaluate truncated PCFT expression. The membrane was then stripped and reprobed with rabbit anti-PCFT antibody (Abcam, ab25134, Cambridge, United Kingdom) or anti-actin antibody (Sigma, St Louis, MO). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibodies (BioLegend, San Diego, CA) were used at 1:2000 dilution. The blots were then visualized by ECL Select Western Blotting Detection Reagent (GE Healthcare, UK). We performed this experiment twice.

2.5. MTX uptake study in HeLa cells ectopically expressing PCFT-Wt or one of the variants

To evaluate the folate transport function of each PCFT variant, we analyzed the uptake of FITC-labelled MTX (MTX-FITC)-positive cells by flow cytometry in HeLa cells ectopically expressing PCFT-Wt or one of the PCFT variants. For the ectopic PCFT expression, we transiently co-transfected into HeLa cells with pcDNA3-HisC-SLC46A1 Wt or one of the variants and pCMV-CD20 expression construct as a transfection marker at a weight ratio of 5:1 using the TransIT-LT1 reagent (Mirus, Madison, WI) according to the manufacturer's protocol. For MTX-FITC uptake, we followed the protocol described previously [23]. Briefly, the cells were washed twice with HEPES-buffered saline (HBS) (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM dextrose [pH 7.4]) 48 h after transfection. The buffer was then removed and MTX-FITC uptake was initiated by the addition of 0.5 ml of MES-buffered saline (MBS) (20 mM MES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM dextrose [pH 5.5]) containing 0.5 μM MTX-FITC (Life Technologies: M1198MP) at 37 °C for 2 min. Uptake was terminated by adding 5 ml of ice-cold HBS. After the cells were washed 3 times with ice-cold HBS, they were stained with CD20-PE (BD: 80225 Franklin Lakes, NJ) at 4 °C for 30 min, and were washed 3 times. We gated CD20-positive cells and calculated the proportion of MTX-positive cells by flow cytometry. Data from triplicated independent experiments were shown as the mean ± SD.

3. Results

3.1. Identification of homozygous or compound heterozygous variants of SLC46A1 in four patients with HFM

In the previous study [8], direct DNA sequence analysis of each exon and exon-intron boundary revealed a heterozygous base change resulting in amino acid change (c.566G > T, p.G189 V) in Patient 1 and her mother. As HFM is an autosomal recessive disorder, we performed RT-PCR to see if there is a splicing defect from the other allele. Amplification of an additional band in RT-PCR was observed in Patient 1 and her father (Fig. 1A), which was demonstrated to have a 168-bp insertion of the intron 3 sequences resulting in premature termination in the middle of the insertion (Fig. 1B). Studies of intron 3 DNA sequences in Patient 1 and her father revealed a base change at the end of the 168-bp insertion (c.1166-285 T > G) which could generate a cryptic splice donor site for the insertion. Splice Site Prediction by Neural Network (http://www.fruitfly.org/seq_tools/splice.html) also predicted that the c.1166-285 T > G base change generates a splice donor site with a score of 0.91. To determine if the transcript with this 168-bp insertion is not a transcriptional variant and if c.1166-285 T > G is not a leaky splice-site mutation, we separately amplified the 168-bp positive or negative RT-PCR products with posiR and negaR reverse primers, respectively. Sequence analysis of each product demonstrated that the 168-bp negative RT-PCR products only had the c.566G > T signal from her mother, and the 168-bp positive RT-PCR products only had the wild-type signal of c.566G from her father. Therefore, the 168-bp positive transcript was exclusively derived from her father's allele and the 168-bp negative transcript was exclusively

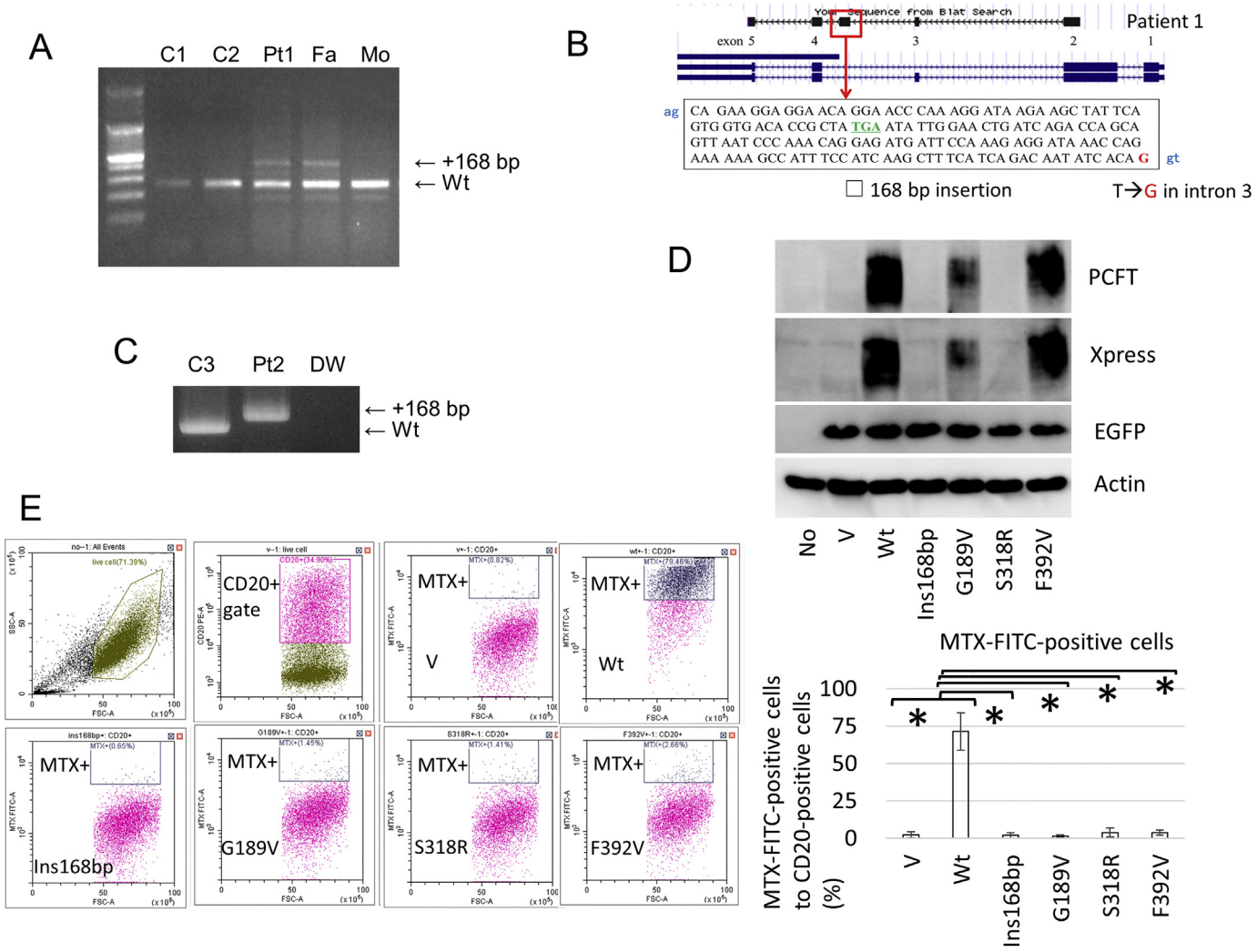

Fig. 1. Four patients with HFM had compound heterozygous or homozygous mutations in *SLC46A1*, variable PCFT expression, and mostly absent uptake of MTX-FITC.

(A) Agarose gel electrophoresis of *SLC46A1* RT-PCR products of Patient 1 and her parents was shown. C1, Control 1; C2, Control 2; Pt1, Patient 1; Fa, Father; Mo, Mother; DW, Distilled water; Wt, wild-type.

(B) Schematic representation of the 168-bp insertion of the intron 3 sequences is shown.

(C) Agarose gel electrophoresis of *SLC46A1* RT-PCR products was shown for Patient 2. C3, Control 3; Pt2, Patient 2.

(D) Results of Western blot analysis of ectopic PCFT expression of Wt and each variant were shown. No, no transfection; V, empty vector.

(E) The uptake of MTX-FITC in HeLa cells expressing PCFT-Wt or one of the variants was measured by flow cytometry. Raw data of the uptake of MTX-FITC in HeLa cells expressing PCFT-Wt or one of the variants and the ratio of MTX-FITC positive cells to CD20-positive cells were shown. Data from triplicated independent experiments were shown as the mean ± SD. The *p*-values were calculated with Welch's *t*-test. $*p < .005$.

derived from her mother's allele, indicating 168-bp insertion is specific to c.1166-285 T > G and this is not causing a leaky splicing.

The deep intronic mutation of c.1166-285 T > G was not present in the Integrative Japanese Genome Variation Database by Tohoku Medical Megabank Organization (https://ijgvd.megabank.tohoku.ac.jp) which is based on the 3500 individuals to construct Japanese whole-genome reference panel. This mutation was not present either in The International Genome Sample Resource (http://www.internationalgenome.org/1000-genomes-project-publications) which is based on 1000 international genomes.

We further studied three additional patients with clinical features consistent with HFM and undetectable levels of serum folate at onset. Direct DNA sequence analysis of each exon and exon-intron boundary revealed no variants in Patient 2, a heterozygous mutation of c.954C > G, p.S318R reported previously [2] in Patient 3, and a heterozygous variant (c.1174 T > G, p.F392 V) in Patient 4. As HFM is an autosomal recessive disorder, we further studied intronic sequence and found that the same deep intronic mutation of c.1166-285 T > G was present as homozygous in Patient 2 and heterozygous in Patients 3 and 4. RT-PCR study demonstrated only the transcript with the 168-bp insertion in Patient 2 with homozygous mutations of c.1166-285 T > G, showing again that c.1166-285 T > G is a non-leaky splice-site mutation (Fig. 1C). Together with sequence analysis of their parents, these results revealed all of the patients had compound heterozygous or homozygous mutations or variants inherited from their parents as shown in Table 1.

There are multiple software tools which predict if an amino acid substitution has an impact on the biological function of a protein. Thus, we evaluated impact of the amino acid changes observed in three unrelated patients with HFM. All of the amino acid changes of p.G189 V, p.S318R, and p.F392 V were predicted to be affecting protein function with SIFT, probably damaging with Polyphen-2 and disease causing with Mutation Taster. The variant of p.F392 V observed in Patient 4 with delayed onset HFM had a lower score of 50 with Grantham score.

3.2. Variable expression of PCFT variants transiently transfected in HeLa cells

To study the impact of each variant on protein expression, we transiently co-transfected HeLa cells with pcDNA3-HisC constructs expressing PCFT Wt, G189 V, S318R, F392 V or 168-bp insertion (Ins168bp) variants and pEGFP-C2 construct as a transfection marker. Western blot analysis of PCFT expression with anti-Xpress and anti-PCFT antibodies reproducibly demonstrated almost no expression of p.S318R and Ins168bp mutants, while reduced but detectable expression of p.G189 V variant and almost normal expression of F392 V variant (Fig. 1D). As for Ins168bp, truncated PCFT expression was not observed with these antibodies, either (data not shown).

3.3. MTX-FITC uptake was mostly absent in HeLa cells ectopically expressing each PCFT variant

Folate transport function was evaluated by MTX-FITC uptake in HeLa cells after transient transfection of Wt or each PCFT variant. All of the variants were demonstrated to have mostly absent MTX-FITC uptake without significant difference between the variants (Fig. 1E). These findings indicated all of the variants detected in our patients are loss-of-function mutations.

4. Discussion

In this study, we demonstrated that the deep intronic mutation of c.1166-285 T > G in *SLC46A1* intron 3 is responsible for HFM in four unrelated Japanese patients. This is a non-leaky splice-site mutation resulting in a 168-bp insertion and premature termination, and was associated with the absence of PCFT expression and folate transport function. As this mutation has not been reported elsewhere, it could be a founder mutation specific to the Japanese population. It is also possible that it is a hot spot mutation spreading worldwide but it has not been detected because of the difficulty to amplify *SLC46A1* RT-PCR products mostly due to low expression of *SLC46A1* mRNA in blood cells. Genome database search with Tohoku Medical Megabank Organization and The International Genome Sample Resource showed this mutation was not present in alleles from 3500 Japanese and 1000 international individuals, respectively. Accumulation of genomic data will elucidate the frequency of this mutation. In any case, we emphasize the necessities to cover this intronic DNA sequence in addition to exon-intron boundaries, which might lead to early and accurate diagnosis of HFM.

So far, three missense mutations of p.G147R, p.R376Q and p.P425R have been reported to be associated with residual transport activity upon transfection into HeLa cells null for constitutive PCFT expression [3,17,24]. However, ectopic expression of G147R and P425R was discrepantly absent, and residual activity of R376Q was not demonstrated with [^{3}H]-MTX or [^{3}H]-folate but only with [^{3}H]-pemetrexed, and even more importantly, none of these patients were associated with milder clinical phenotype. Patient 4 in this study developed seizure and pneumonia since the age of 8 months, which prompted us to think the patient may have delayed-onset HFM associated with hypomorphic mutation. However, the residual folate transport function of the F392 V mutant observed in Patient 4 was not detected by the uptake study with MTX-FITC (Fig. 1E), indicating F392 V is not a hypomorphic mutation.

Either folic acid or folinic acid were chosen for the folate replacement therapy depending on the hospitals in this study. Folinic acid is a metabolically active form of folate, and is recommended to be used in patients with severe side effects of MTX therapy [25]. As HFM patients could present with critical conditions and show undetectable levels of serum folate at onset, folinic acid should be recommended in this situation. Patient 3 developed diffuse alveolar hemorrhage requiring mechanical ventilation during parenteral folic acid replacement therapy, raising questions about the choice of folate. Although the mechanisms of diffuse alveolar hemorrhage are unknown, this should be the kept in mind as one of the critical complications of HFM.

5. Conclusions

We found four unrelated patients of HFM shared the same deep intronic mutation of c.1166-285 T > G, which could be a founder mutation in the Japanese population or could be a hot-spot.

Disclosure

The authors have no financial conflicts of interest.

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