A New Krüppel-Like Factor 1 Mutation (c.947G > A or p.C316Y) in Humans Causes β-Thalassemia Minor

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Abstract

Here we describe a Japanese patient with mild β-thalassemia (β-thal) with an intact β-globin gene but a new missense mutation of c.947G > A or p.C316Y in the erythroid Krüppel-Like Factor (KLF1) gene which is strongly associated with the expression of the β-globin gene. The association of the KLF1 mutation with β-thal, is here described. The p.C316Y mutation occurred at one of the cysteines that constitute the second zinc finger motif of KLF1, and would have changed the zinc finger conformation to impair the DNA binding properties or the promoter function of the β-globin gene. Our expression study found that the mutant KLF1 gene had a markedly negative effect on the β-globin gene expression, or 70.0% of that of its normal counterpart. A presumed heterozygous state, or equimolar presence of the mutant and normal KLF1s reduced the expression rate to 70.0% of the normal alone. This degree of the decrease may explain the very mild phenotype of the patient’s β-thal. Furthermore, the patient’s whole-exome analysis using next-generation sequencing revealed that the β-thal defect is caused by only this KLF1 gene mutation. The Hb A2 and Hb F levels that are frequently elevated in KLF1 mutations were elevated by 4.1 and 1.3%, respectively, in this case. The contribution to their elevation by KLF1: p.C316Y is uncertain.

Introduction

β-Thalassemia (β-thal) is one of the most prevalent congenital hemolytic disorders in the world. It occurs because of impaired production of β-globin mutating the β-globin gene; mostly these are point or frameshift mutations of a few base deletions/insertions. Mutation of the distal and proximal CACCC boxes within the β-globin gene promoter cause β-thal. The mutations that occur in the CACCC box affect the binding and responsiveness to erythroid Krüppel-like factor (EKLF, KLF1) (1), which is essential for expression of the β-globin gene. Furthermore, a number of KLF1 mutations have been reported (2). The KLF1 has three C2H2 zinc finger (ZF) domains that bind to the CACCC box and increase the expression of the β-globin gene (Figure 1). Moreover, KLF1 works one of the key regulators of the γ- to β-globin gene switching (3), and its mutation gives rise to increase in the level of Hb F and Hb A₂ (4–6). One of the mutations causes a congenital dyserythropoietic anemia (CDA) type (7) and hemolytic anemia. Thus, KLF1 mutations exert diverse functional effects.

In this study we describe a Japanese patient suspected of carrying a mild β-thal. However, no mutations were found from 1 kb upstream to 1 kb downstream of the β-globin gene that includes the promoter region, all exons and introns and the 5′ and 3′ non coding regions. In addition, no β-globin gene deletion was found. Therefore, we focused on the KLF1 that was known to cause β-thal in a KO model mouse (8). In addition, it was suspected that some of the undefined β-thalassemias have abnormally unlinked to the β-globin gene (9). As expected, the mutation of the KLF1: c.947G > A coding for KLF1: p.C316Y was discovered in our patient. This has as yet not been reported in the literature, and we studied the association of the KLF1: p.C316Y with β-globin gene expression in humans by analysis of transient transfection assay. We used the next-generation sequencing (NGS) analysis to see whether other mutations relevant to hemoglobinopathies were present anywhere in the patient’s whole exons.
Figure 1. The structures of the KLF1 gene and its protein product, KLF1. The solid boxes show the exons of the gene. Three ZF domains are located at the N-terminal side of the KLF1 where target DNA binds. The arrows indicate the place of the primers used for this analysis. Their numbers correspond to those in Table 1. The new mutation reported here, p.C316Y, is indicated by the vertical arrow at the second ZF.

Table 1. Primers for the KLF1 polymerase chain reaction.

<table>
<thead>
<tr>
<th>n</th>
<th>Primers</th>
<th>Primer Sequences (5’→3’)</th>
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<tr>
<td>1</td>
<td>EKLFex1-D</td>
<td>CTG CTG GGG TGT CTG ATA ATG TTT G</td>
</tr>
<tr>
<td>2</td>
<td>EKLF-D2</td>
<td>GCC CAG GCT ACC TTC GGT TTT CAT T</td>
</tr>
<tr>
<td>3</td>
<td>EKLF-D2-U</td>
<td>GTG GAC ACC TGA TCG GTT T</td>
</tr>
<tr>
<td>4</td>
<td>EKLF-D3</td>
<td>CGG GCC CCG GGT ACA CCG GTT GCA G</td>
</tr>
<tr>
<td>5</td>
<td>EKLF-D-SgfI</td>
<td>ATA TCC GCC GCC GGC GAG TCT G</td>
</tr>
<tr>
<td>6</td>
<td>EKLFex1-U1</td>
<td>CTT CAT CGT GTC ACA CCC GTC TAC T</td>
</tr>
<tr>
<td>7</td>
<td>EKLFex2-U</td>
<td>CTG CAT CTG GTC ACA CCC CTT TAC T</td>
</tr>
<tr>
<td>8</td>
<td>EKLFex3-D</td>
<td>CTG AAC TGG GTG GGA AAA GAG AGG A</td>
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<td>9</td>
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<td>GGACAG ACA AGT GGC GCT TAT GGC TT</td>
</tr>
<tr>
<td>10</td>
<td>EKLF-D-SgfI</td>
<td>CTA TCC GCC GCC GCC CGA GAC TCT G</td>
</tr>
<tr>
<td>11</td>
<td>EKLF-U-PmeI</td>
<td>CGA CCG CAT CGC CAT GGC CAC AGC</td>
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<tr>
<td>12</td>
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<td>CAA GGT TTA AAC TCA AAG GTG GCG</td>
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<td>14</td>
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<td>CTT CAT CTG</td>
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Materials and methods

Case report

A 30-year-old Japanese male patient was diagnosed with non iron deficiency anemia, and asked to present for further examination to determine the cause of his anemia. He had very mild microcytic anemia [Hb 13.2 g/dL, mean corpuscular volume (MCV) 73.0 fL, mean corpuscular hemoglobin (Hb) (MCH) 23.9 pg] with mild erythrocytosis (5.53 /C14, 1012/L). There were a number of target cells in his blood smear. The serum iron, unsaturated iron binding capacity (UIBC) and ferritin were all within normal range. The levels of Hb A2 and Hb F were slightly increased by 4.1 and 1.3%, respectively, suggesting a very mild -thal major.

DNA and RNA analysis

DNA was extracted from peripheral blood by the conventional phenol-chloroform method. The whole KLF1 gene was amplified by polymerase chain reaction (PCR) (Table 1, Figure 1), and subjected to sequencing analysis on an ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). The PCR was conducted by initial denaturation (94°C, 3 min.) and 35 cycles of denaturation (94°C, 40 seconds), annealing (60°C, 1 min.) and extension (72°C, 2 min.) followed by a final extension (72°C, 5 min.). The KLF1 RNA was extracted using TRIZOL Reagent (Invitrogen, Tokyo, Japan), and subjected to reverse transcriptase PCR (RT-PCR) in which the purified cDNA was amplified by EKLF-D-SgfI and EKLF-U-PmeI as primers (Table 1, Figure 1) under the condition of initial denaturation (94°C, 3 min.), 30 cycles of denaturation (94°C, 30 seconds), annealing (60°C, 30 seconds) and extension (72°C, 2 min.), and a final extension (72°C, 4 min.). The base sequence of the obtained cDNA obtained was analyzed.

Promoter-luciferase reporter assay (Figure 2)

The coding sequences of the wild and the mutated KLF1’s were incorporated into the downstream of the CMV-neo Flexi® vector (PromegaKK, Tokyo, Japan) of pF5A that strongly expresses EKLF (Figure 2A). The 413 bp-promoter of the normal β-globin gene (~1 to ~413) was incorporated into the upstream of the firefly luciferase gene in the reporter vector, or pGL4.10[luc2] vector (PromegaKK) (Figure 2B). The pGL4.74[hRluc/TK] vector (PromegaKK) that has the renilla luciferase gene was used as an internal standard (Figure 2C). The expression vector incorporated with wild or mutant KLF1 cDNA and the reporter carrying β-globin promoter were cotransfected to K562 cells using MicroPorator-mini MP-100 (Digital Bio Technology, AR Brown Co., Ltd., Tokyo, Japan), a transient transfection assay. The condition of the transfection and culturing were set according to established methods in the literature (10). Three kinds of in vitro expression models were designed: (1) only wild KLF1 expression vectors were used for normal models, (2) only mutant-incorporated expression vectors were used for mutation models, and (3) a mixture of the two in a 1:1 molar ratio were used for the patient model that corresponds to the heterozygote for the normal and mutated alleles. The Dual-luciferase Reporter Assay System® (Promega KK) was used for the dual-luciferase assay under the conditions recommended by the manufacturer. The fluorescent signal by firefly luciferase activity was measured after 24 hours incubation, and corrected to adjust for the renilla luciferase activity to get a relative luciferase activity. Thus, the relative luciferase activities of normal, mutant and patient models were compared.
Whole exome analysis by next-generation sequencing

Patient DNA, extracted from the blood sample by the phenol-chloroform method, was purified using PureLink Genomic DNA Mini Kit (Life Technologies, Carlsbad, CA, USA). We made the fragment library for NGS analysis using the 5500 SOLiD Fragment Library Core kit (Life Technologies). Exon regions were concentrated with TargetSeq/C212 Exome Enrichment Kit (Life Technologies), and were analyzed by a next-generation sequencer (SOLiD5500; Life Technologies). The enormous sequencing data obtained were mapped by LifeScope (Life Technologies). The hg19 of UCSC (University of California Santa Cruz) was used for the reference sequence. All mutation/variant and deletion data were analyzed by variant analysis (www.ingenuity.com/variants). We targeted our search for the mutation on the hemoglobinopathy.

Results

Krüppel-like factor 1 gene and cDNA analyses

The gene analysis of the KLF1 uncovered the KLF1 mutation of c.947G>A coding for p.C316Y as a heterozygote (Figure 3A). This mutation was explored for 100 alleles from normal individuals, and none of them carried this mutation. Thus, c.947G>A was not a polymorphism. The analysis of the cDNA revealed that mRNA derived from the mutant allele (c.947G>A) seemed to be present in almost the same amount as that from the normal allele (Figure 3B). Thus, it was suspected that the mutant EKLF protein (KLF1: p.C316Y) could be produced.

Promoter-luciferase reporter assay

The results of relative luciferase activity of the normal, mutant and patient models are presented in Figure 4. When the relative luciferase activity of the wild model was considered as 100.0%, the p.C316Y construct contributed only 7.0%, and the patient model that had equimolar construct of wild and mutant, 70.0%. Therefore, the promoter activity of the β-globin gene was reduced by the mutant KLF1, or p.C316Y.

Whole exome analysis

After mapping by LifeScope (Life Technologies), we could identify the sequencing data of 3.7 Gb. About 93.0% data was
mapped to the chromosome, indicating the accuracy of the data. Our patient had a mutation/variant of 44,070 and small deletion of 537 in the whole exon. All data were read more than five times and the coverage was more than 30, which indicates that these NGS data of our patient had sufficient analytic accuracy. All data were uploaded to the Ingenuity Company website (www.ingenuity.com) for variant analysis. The analyzed data disclosed five variants in the three genes ($KLF1$, $GRIN3B$, $CACNA1F$) that were related to the patient's thalassemia.

**Discussion**

Our study proved that the $KLF1$ mutation (c.947G $\rightarrow$ A) in a heterozygous state brings about very mild $\beta$-thal. This is a novel mutation showing a substitution of tyrosine for cysteine at codon 316, which is one of the cysteines that constitute the second ZF domain of KLF1 and essential for zinc (Zn) coordination. Thus, it is very likely that the p.C316Y affects the ZF motif and alters the DNA binding ability or affinity to the $\beta$-globin promoter (Figure 5), which results in the reduced $\beta$-globin gene expression in our patient. The phenotype of our patient may be milder than other $\beta$-thal patients with promoter mutations that reveal $\beta^+\text{-thal}$ (9). It has previously been reported that nonsense and frameshift mutations of $KLF1$ may make no KLF1 protein reveal haplosufficiency (11). Furthermore, missense mutations p.R328L, p.R328H, p.R331G located near our p.C316Y in the second ZF domain are expected to produce abnormal KLF1 protein that have no binding ability and are phenotypically almost normal (Figure 5) (12). In these mutants the KLF1 works in the expression of $\beta$-globin genes in a way of no ‘haplosufficiency.’ The reduced relative luciferase activity (70.0%) in our experiment suggests that p.C316Y may still retain some
However, our patient has only a mild type of abnormal p.E325K with binding ability to the promoter (12). Amounts of mutant mRNA and are expected to produce diverse genes. The patient with p.E325K has substantial second ZF domain, causes a CDA type associated with iron deficiency or uncertain if p.C316Y played a major role. Our patient had no of our p.C316Y patient is only slight (4.1%), and it was unpublished data, supports the link between KLF1 and hereditary persistence of fetal Hb (HPFH), in which some EKLF variants are variably related to elevated Hb F levels. They highlight the importance of the second DNA-binding ZF for normal KLF1 function where our p.C316Y resides. Our patient had a slightly elevated Hb F level (1.3%). If p.C316Y is associated with the increase of \( \gamma \)-globin gene expression, it would be in a very minor degree.

The slightly increased levels of Hb F and Hb A2 in our patient conform to those found in \( \beta \)-thal cases. Carriers of \( \beta^0 \)- or \( \beta^+ \)thal exhibit Hb A2 levels of 4.5–5.5% and 3.6–4.2%, respectively (9). Hb A2 levels tend to be higher in association with more severe \( \beta \)-thal phenotypes (9). However, the promoter that gives the \( \beta^+ \)-thal phenotype displays relatively higher Hb A2 levels (4.5–6.5%) in our laboratory (n = 29) than other \( \beta \)-thal mutants except for deletion types. Although our p.C316Y was associated with the \( \beta \)-globin promoter, its \( \beta \)-thal phenotype appears to be milder, and our Hb A2 level of 4.1% was a little lower than those of promoter mutants. Perseu et al. (5) reported that 52 of 145 normal individuals whose Hb A2 were borderline (3.3–3.8%) carried one of six different KLF1 mutations. Their MCV and MCH were normal or slightly reduced. Some of their mutants (p.L326R, p.T327S and p.K332Q) were on the same second ZF domain as our p.C316Y. However, the elevation of Hb A2 of our p.C316Y patient is only slight (4.1%), and it was uncertain if p.C316Y played a major role. Our patient had no iron deficiency or \( \delta \)-thal, which reduce Hb A2 levels, or hyperthyroidism and \( \alpha \)-globin gene triplication that may elevate it (9).

Some KLF1 mutations, such as compound heterozygotes for p.S270X and p.K332Q as well as 10 kinds of other KLF1 mutations are reported to be related to high levels of Hb F (4,6). The review by Giardine et al. (15) which includes unpublished data, supports the link between KLF1 and hereditary persistence of fetal Hb (HPFH), in which some EKLF variants are variably related to elevated Hb F levels. The GLT50 (reference range 22–55 seconds) is affected by the oxidation state of \( \beta \)-thal (16). The oxidation state seems to reflect the severity of the phenotype. The average of GLT50 of promoter mutants of –31 (A>G), other \( \beta^+ \)-thal, \( \beta^0 \)-thal and homozygous –31 in our laboratory are 97, 96, 112 and 154 seconds, respectively. The GLT50 was not prolonged in our p.C316Y, while half of the –31 in our laboratory did not demonstrate the prolongation of GLT50. This may be the reason why GLT50 was not prolonged in our patient.

The NGS analysis suggested that the mutations of the proband on three genes (KLF1, CACNA1F and GRIN3B) were related to hemoglobinopathies, in which the variant annotation and interpretation analyses were generated through the use of Qiagen’s Ingenuity® Variant Analysis™ software from Qiagen, Redwood City, CA, USA. However, GRIN3B has been reported to be associated with the treatment of pain in sickle cell anemia. The CACNA1F is reported as a gene associated with treatment for iron overload in \( \beta \)-thal. These mutations are not directly associated with hemoglobinopathies. Only the KLF1 mutation is related to \( \beta \)-thal.

We were unable to examine the InLu [Lu(\( \alpha \)-b-)] in our patient’s phenotype and aberrant expression of membrane proteins (reduced ICAM4, loss of CD44 and AQP1) seen in the p.E325K causes a CDA type associated with diverse
genes (13). Our patient revealed no hemolytic involvement, no remarkable increase in the levels of Hb A2 and Hb F, only mild β-thal, which was brought about by the KLF1: p.C316Y mutant.

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Authors F.K., Y.Y. and Y.H. collected the samples and clinical data; T.N., F.T., T.M. and T.T. performed research and analyzed data; T.N., F.T., M.F. and C.A. performed molecular diagnosis of thalassemia; T.N., Y.Y. and Y.H. designed the study, analyzed and interpreted the data and wrote the article.

Declaration of interest

This study was supported by JSPS KAKENHI Grant Number 25460686. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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