Regular Article

Nonsense-mediated mRNA decay was demonstrated in two hypofibrinogenemias caused by heterozygous nonsense mutations of *FGG*, Shizuoka III and Kanazawa II

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quantitative RT-PCR

Summary

We report two novel hypofibrinogenemias, Shizuoka III and Kanazawa II, which are caused by heterozygous mutations in *FGG*. Shizuoka III showed c.147delT and 147_149insACA in *FGG* exon 3 and a subsequent frameshift mutation, resulting in γ23X (stop codon), and Kanazawa II showed c.1205G>A in *FGG* exon 9, resulting in γ376X.

To determine whether the truncated γ-chains, γ23X and γ376X, were synthesized and participated in the assembly of fibrinogen, mutant-type cDNA vectors were transfected into Chinese hamster ovary (CHO) cells. Significant levels of mutant fibrinogen were not detected by ELISA in the culture media and cell lysates. Immunoblot analysis of cell lysates revealed that the mutant γ-chain of γ376X was observed but intact fibrinogen was not. On the other hand, mutant γ-chain was not observed in γ23X-expressing cells.

To demonstrate the involvement of the mechanisms of nonsense-mediated mRNA decay (NMD), we cloned wild- and mutant-type mini-genes containing γ23- or γ376-codon and transfected these into CHO cell lines in the absence or presence of cycloheximide (CHX) as an NMD inhibitor. mRNA levels were determined using real-time quantitative RT-PCR in CHO cells. In the absence of CHX, levels of mRNAs transcribed from the mutant gene were lower than from the wild-type gene whereas, in the presence of CHX, levels of mRNAs transcribed from the mutant gene increased dose-dependently.

Finally, these results demonstrated that aberrant mRNAs containing γ23X or γ376X are degraded by the NMD system and translation decrease in hepatocytes, resulting in hypofibrinogenemias.

1. Introduction

Fibrinogen is a 340 kDa plasma glycoprotein consisting of a dimeric molecule of 3 polypeptide chains, Aα, Bβ, and γ, synthesized and assembled into a disulfide-linked hexameric molecule in hepatocytes and secreted into the bloodstream at a concentration of 1.8-3.5 g/L [1]. Aα-, Bβ- and γ-chains are composed of 610, 461 and 411 residues, which are encoded by *FGA*, *FGB* and *FGG*, respectively. The three genes contain five exons for *FGA*, eight for *FGB*, and ten for *FGG*, respectively.

Genetic mutations in fibrinogen chain genes have been associated with either afibrinogenemia, hypofibrinogenemia, or dysfibrinogenemia, as listed in the fibrinogen variant database (http://site.geht.org/site/Pratiques-Professionnelles/Base-de-donnees-Fibrinogene/Base-de-donnees/Base-de-donnees-des-variants-du-Fibrinogene_40_.htm), and the molecular bases for the genetic and/or post-translational changes causing dysfibrinogenemia, hypofibrinogenemia and afibrinogenemia have been described [2].

Recently, we identified two novel hypofibrinogenemias, both associated with heterozygous nonsense mutations of *FGG*. One showed a nucleotide deletion and three insertions in *FGG* exon 3 (c.147delT, 147_149insACA) and a subsequent frameshift mutation, resulting in a premature stop codon (γ23X), and another showed a single point mutation of G>A in *FGG* exon 9 (at c.1205G>A), resulting in a nonsense mutation γ376X, and designated as fibrinogen Shizuoka III and Kanazawa II, respectively.

We have already reported that C-terminal residues of the γ-chain have an important role in fibrinogen assembly and secretion; in particular, the required length of the γ-chain is 387 residues, and 387Ile is essential [3]. Thus, it was supposed that truncated mutations of γ23X and γ376X do not assemble into an intact fibrinogen molecule and are not secreted into the bloodstream. In the last decade, the presence of an RNA surveillance system has been reported, namely nonsense codon-containing mRNAs originating from genetic mutations, abnormally spliced mRNAs or some edited mRNAs are degraded by the mechanism of so-called nonsense-mediated mRNA decay (NMD) before translation [4-6].

To determine whether the truncated γ-chains, γ23X or γ376X, are translated in the cells and participate in the assembly of fibrinogen, we constructed expression vectors containing mutant-type cDNA and transfected them into Chinese hamster ovary (CHO) cells. Furthermore, in order to analyze the involvement of the NMD system in the degradation of aberrant mRNAs, we constructed expression vectors containing γ23X or γ376X mini-genes, introduced them into CHO cells, and measured the levels of transcribed mRNAs in the absence or presence of cycloheximide (CHX) as an NMD inhibitor.

2. Materials and methods

This study was approved by the Ethics Review Board of Shinshu University School

of Medicine. After informed consent had been obtained from the patients, blood samples were collected for biochemical and genetic analyses.

2.1 Patients

The proposita of Shizuoka III was a 33-year-old woman who had unexplained infertility, and had no history of bleeding or thrombosis. The propositus of Kanazawa II was an 18-year-old man with Henoch-Schonleinpeliosis. In both cases, routine coagulation screening tests showed a markedly low concentration of plasma fibrinogen.

2.2 Coagulation screening tests

Prothrombin time (PT), activated partial thromboplastin time (APTT), and the fibrinogen concentration, which was determined by the thrombin time method, were measured with an automated analyzer, Coagrex-800 (Sekisui Medical Co., Tokyo, Japan). The immunological fibrinogen concentration was determined by a latex photometric immunoassay using anti-fibrinogen antibody-coated latex particles (Mitsubishi Chemical Medience Co., Tokyo, Japan) [7].

2.3 Polymerase chain reaction (PCR) and DNA sequence analysis

Genomic DNA was extracted from white blood cells using a DNA Extraction WB Kit (Wako Pure Chemical Ltd, Osaka, Japan), according to the manufacturer's instructions.

To analyze all exons and exon–intron boundaries of the fibrinogen genes, long-range PCR for *FGA*, *FGB* and *FGG* was performed using the TaKaRa LA Taq (Takara Bio Inc., Otsu, Japan) and the pair of primers for *FGA*: sense primer; 5'-CAGCCTAGCTTACCTAAGCACC-3' and antisense primer; 5'-GTTAAGGAAG AAATGCAAGGG-3', *FGB*: sense primer; 5'-CTCTTTGAGGAGTGCCCTAAC-3' and antisense primer; 5'-ACGTCTGCTTGAGAGTTTTAG-3', and *FGG*: sense primer; 5'-GAACTGGGACATGGGGGAAGT-3' and antisense primer; 5'-GCTTTGCAAGT CCATTGTC-3'. Amplified nucleotides of the Aα-, Bβ- and γ-chains were 5,618 bp, 8,000 bp and 8,934 bp, respectively. Twenty-eight primers were designed for sequence analysis of all exons and exon-intron boundaries. The PCR products were purified from agarose gels and directly sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism 3100 Genetic Analyzer (both from Applied Biosystems, Foster City, CA).

2.4 Construction of mutant expression vectors

The fibrinogen γ-chain expression vector pMLP-γ was altered by oligonucleotide-directed mutagenesis using the QuikChange II Site-Directed Mutagenic Kit (Stratagene, La Jolla, CA) and the following primer pairs (the altered bases are underlined): 5'-GT CCAACTACCTG<u>ACA</u>GGCATTGGAG-3' and 5'-CTCCAATGCC<u>TGT</u>CAGGTAGTT GGAC-3' for γ23X, and 5'-GGAAAACCCGGT<u>A</u>GTATTCCATGAAG -3' and 5'-CTT

CATGGAATACTACCGGGTTTCC-3' for γ376X, according to the instruction manuals.

2.5 Recombinant protein expression

Each of the expression vectors, pMLP-γ23X, pMLP-γ376X and pMLP-γN (wild-type, wt) was co-transfected with the histidinol selection plasmid (pMSV-his) into CHO cells that expressed normal human fibrinogen Aα- and Bβ-chains (AαBβ-CHO cells), using the standard calcium-phosphate coprecipitation method. The stable transfectants were selected as previously described [8].

2.6 Culture medium and cell lysate for immunological analysis

The fibrinogen concentrations of the culture media and cell lysates were determined by an enzyme-linked immunosorbent assay (ELISA) as described elsewhere [7]. SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions (10 % gel) or non-reducing conditions (8 % gel), and immunoblot analysis of fibrinogen or individual polypeptides were performed using enhanced chemiluminescence (ECL) detection reagents, and Hyperfilm-ECL (both from Amersham Pharmacia Biotech, Buckinghamshire, UK) as described previously [3].

2.7 Construction of mRNA expression vectors

To determine whether transcribed mRNAs from the γ23X or γ376X mutant genes were degraded by NMD, DNA fragments spanning from the 5' untranslated region (UTR) to exon 6 of *FGG* were amplified from the Shizuoka III proposita's genomic DNA using the primer pairs: *FGG*-5'UTR-F; 5'-CACCTATCCCAGGAGCTTAC-3' and *FGG*-Ex6 -R; 5'-GATGGAGTGTGTTTGAGAAG-3', and those from exon 8 to 3' UTR of *FGG* were amplified from the Kanazawa II propositus' genomic DNA using the primer pairs: *FGG*-Ex8-F; 5'-CACCATGTTCAAGGTGGGAC-3' and *FGG*-3' UTR-R; 5'-GGACAATGGACTTGCAAAGC-3'. As a positive control for NMD [9], the human beta globin gene (*HBB*) was amplified from normal genomic DNA using the primer pairs: *HBB*-5'UTR-F; 5′-CACCCTAGGGTTGGCCAATC-3' and *HBB*-3'UTR-R; 5'-CCCC AGTTTAGTAGTTGGAC-3'. PCR-amplified fragments (mini-genes) carrying the wild-type of Shizuoka III, Kanazawa II and *HBB* were 3,045, 2,623 and 1,636 bp, respectively. Subsequently, the DNA fragments with 3'- and 5'-protruding ends were converted into blunt-end DNA using a T4 DNA polymerase (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Purified blunt-end PCR products of Shizuoka III, Kanazawa II and *HBB* mini-genes were inserted into pcDNA3.1 vector (Invitrogen) and transfected into TOP10 high efficiency competent cells (Invitrogen) according to the manufacturer's instructions.

The nucleotide sequences of the plasmids were confirmed and named γ23wt, γ23X, γ376wt, γ376X and *HBB*39wt vectors. As a positive control for NMD [9], the *HBB*39wt

plasmid was altered by oligonucleotide-directed mutagenesis using the QuikChange II Site-Directed Mutagenic Kit and the following primer pairs (the altered base is underlined): 5'-GGTCTACCCTT GGTGACCCAGAGGTTC-3' and 5'-GAACC TCTGGGTCACCAAGGGTAGACC-3' for 39X, and named *HBB*39X.

2.8 Production of γ23, γ376 and *HBB* mRNAs

The expression vectors γ23wt, γ23X, γ376wt, γ376X, *HBB*39wt and *HBB*39X were introduced into CHO cells in a 6-well culture dish using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. Briefly, 250 μl antibiotic-free medium containing 40 ng vector DNA, 40ng fibrinogen Aα-chain expression vector pMLP-Aα (to correct transfection efficiency) and 4 μg pCR 2.1 plasmid vector (Invitrogen) containing the *HBB*39wt mini-gene (to adjust DNA concentration, but not transcribed to mRNA) were mixed with an equal volume of Lipofectamine 2000 reagent and transfected into CHO cells. To determine whether mutant transcripts are reduced by NMD, cells were treated with cycloheximide (CHX) (Sigma, St. Louis, MO) as an NMD inhibitor, at concentrations of 0, 20, 40, or 80 μg/mL for γ23 and γ376 or 0, 5, 10, or 20 μg/mL for *HBB*, respectively.

2.9 RNA extraction and RT-PCR

The CHO cells were harvested 18 h after transfection. Total cellular RNA was extracted from cells using a QIAamp RNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany), and contaminated DNA was deleted using a QIA shredder column and digested by DNase, according to the manufacturer's instructions. Reverse-transcriptase (RT) reactions were carried out at 42 °C for 1 h in 20 μL reaction mixture containing 0.5 μg extracted total RNA, 1X RT buffer, 0.5 mmol/L dNTP mixture, 0.5 μg oligo dT, 1 mmol/L dithiothreitol and 100 U reverse transcriptase from Moloney murine leukemia virus.

2.10 RNA isolation and real-time quantitative RT-PCR

Real-time quantitative RT-PCR (qRT-PCR) was carried out with gene-specific, double fluorescently labeled probes in an ABI PRISM 7700 Sequence detector (Applied Biosystems) [10], using FAM at the 5′-end nucleotide as a fluorescent reporter, and black hole quencher 1 (BHQ1) at the 3′-end nucleotide as a quencher. The primer and probe sequences were: γ23 forward primer: 5'-CAGGATGGATCTGGTTGGTGG-3', γ23 reverse primer: 5'-GGAGTAGATGCTTTTGAGTAAGTGC-3', γ23 probe: 5'-FAM-TGAACAAGTGTCACGCTGGCCATCTCAA-BHQ1-3', γ376 forward primer: 5'-TG GAGCCTGAGAGGTGACA-3', γ376 reverse primer: 5'-CAGTTGTCTCTGGTAG CA ACATAT-3'), γ376 probe: 5'-FAM-TGCTGACACTACAAGGCTCGGAGCTC-BHQ1 -3', *HBB* forward primer: 5'-GAGGAGAAGTCTGCCGTTACTG-3' and *HBB* reverse

primer: 5'-GACAGATCCCCAAAGGACTCAAA-3', *HBB* probe: 5'-FAM-CCAAGG GTAGACCACCAGCAGCCTG- BHQ1-3', Aα-chain forward primer: 5'-GGATCG TCTGCCTGGTCCTA-3', Aα-chain reverse primer: 5'-CCTTCAGCTAGAAAGTCA CCTTCA-3', Aα- chain probe: 5'-FAM-TGCAGTCCATGCTGTGCCCACCAC-BHQ1-3'. The reaction mixture contained 2 μL RT-PCR products, 1× Taq Man Universal PCR Master Mix (Applied Biosystems), 500 μmol/L each primer, and 250 μmol/L each probe in a total of 20 μL. Reaction mixtures were incubated at 50°C for 2 minutes and 95°C for 10 minutes, followed by 50 cycles of amplification with denaturation at 95°C for 15 seconds, and annealing and extension at 60°C for 1 minute. Levels of each mRNA were determined as copy numbers using standard curves (10^1-10^5 copies) constructed from an accurately determined plasmid vector, γ23wt/X, γ376wt/X, *HBB*39wt/X or pMLP-Aα. Moreover, to compensate for differences in cell numbers and the recovery rate of RNA, copy numbers of each target mRNA were corrected with the copy number of mRNA transcribed from pMLP-Aα.

3. Results

3.1 Coagulation screening tests

The PT of Shizuoka III was 13.2 and of Kanazawa II was 14.2 seconds (normal range: 10.8 to 13.2 seconds), and the APTT was 33.5 and 40.9 seconds, respectively (normal range: 23.0 to 38.0 seconds). The plasma fibrinogen concentration determined

by the thrombin time method of Shizuoka III was 0.79 and of Kanazawa II was 0.87 g/L, and those determined by the immunological method were 0.80 and 0.90 g/L, respectively (normal range: 1.80 to 3.50 g/L).

3.2 DNA sequence analysis

In Shizuoka III, we found a novel heterozygous mutation in *FGG* exon 3 comprising a deletion (T) plus 3 bp (ACA) insertion (c.147delT, 147_149insACA) (Fig. 1-A). This mutation is predicated to result in a frameshift and a premature termination codon (γ23 TGT:Cys>TGA:X). In Kanazawa II, we found a novel heterozygous mutation of G>A in *FGG* exon 9 (at c.1205G>A). This mutation results in a nonsense mutation at γ376 residue, TGG:Trp>TAG:X (Fig. 1-B).

3.3 Synthesis and secretion of recombinant variant fibrinogens in CHO cells

Two variant and normal fibrinogens were expressed in CHO cells as described in Materials and Methods. The fibrinogen concentrations in the culture media and cell lysates were measured by ELISA, and the results are shown in Fig. 2. In normal fibrinogen-expressing cell lines, fibrinogen concentrations (mean ± SD, n = 8) were 2,734 ± 817 ng/mL for the culture media and 1,710 ± 660 ng/mL for the cell lysates. Fibrinogen concentrations in the variant fibrinogen-expressing cell lines (mean ± SD, n = 8) were significantly lower than in the normal fibrinogen-expressing cell lines as follows: γ23X:

33.8 ± 5.7 (culture media), 7.0 ± 3.2 (cell lysates), γ376X: 31.5 ± 4.8 (culture media), 12.1 ± 2.7 (cell lysates) ng/mL, respectively (Fig. 2).

To ascertain the γ-chain synthesis and/or fibrinogen assembly, we performed immunoblot analyses under reducing or non-reducing conditions. Under reducing conditions, although normal fibrinogen-expressing cell lines showed Aα- Bβ-, γ-chain bands and several smaller species, γ23X-fibrinogen-expressing cell lines showed Aα-, Bβ-chain bands and no γ-chain band, and γ376X-fibrinogen-expressing cell lines showed Aα-, Bβ-chain bands and a lower molecular weight band than normal γ-chain band (Fig. 3A). Under non-reducing conditions, normal fibrinogen-expressing cell lines showed several cross-reacting bands, including intact fibrinogen, individual chains and their assembled bands, however, γ23X-fibrinogen-expressing cell lines showed no γ-chain and assembled bands, whereas γ376X-fibrinogen-expressing cell lines showed a lower molecular weight band than the normal γ-chain band and no assembled band (Fig. 3B).

3.4 Analysis of NMD

We quantified fibrinogen γ-chain and *HBB* mRNA concentrations in triplicate as described in Materials and Methods. mRNA levels of the fibrinogen γ-chain and *HBB* were normalized by exogenous mRNA levels of Aα-chain (transfection efficiency control) and shown in Fig. 4. The mRNA expression levels of wild-type cells were set to

100% in the absence or presence of CHX. In the absence of CHX, mutant cells showed lower mRNA levels than wild-type cells (100%), namely, γ23X; 71% (Fig. 4-A), γ376X; 57% (Fig. 4-B), and *HBB*39X; 36% (Fig. 4-C). In the presence of CHX, γ-chain mRNA expression levels of the γ23X cell lines increased dose-dependently and were more than two-fold (146%) at 40 μg/mL CHX (Fig. 4A). Those of the γ376X cell lines also increased dose-dependently and were more than 2.3-fold (134%) at 40 μg/mL CHX (Fig. 4B). On the other hand, in the NMD positive control experiment using *HBB*39X cell lines, the *HBB* mRNA expression levels increased more than 2.3-fold (85%) at 20 μg/mL CHX (Fig. 4C).

3. Discussion

We found two novel hypofibrinogenemias, both of which were caused by a heterozygous nonsense mutation in *FGG*. One was observed at c.147delT and c147_149insACA, resulting in γC23X, and the other was c.1206G>A, resulting in γW376X, and we designated these fibrinogens Shizuoka III and Kanazawa II, respectively.

We have already reported that the length of the γ-chain should be at least 387 residues for fibrinogen assembly and secretion[3]. To confirm that lower plasma fibrinogen levels of Shizuoka III and Kanazawa II than normal had been caused by shorter γ-chains, we prepared vectors containing γ23X- and γ376X-cDNAs and

transfected them into CHO cells, which synthesize normal human fibrinogen Aα- and Bβ-chains. Fibrinogen composed with γ23X or γ376X was not detected in the media or cell lysates. Immunoblot analysis indicated that intact fibrinogen was not observed in the cell lysates of γ23X or γ376X cells, but a mutant γ-chain of γ376X existed in the cells, whereas a mutant γ-chain of γ23X was not detected. We speculated that if the mutant γ-chain was synthesized in γ23X cells, the γ-chain molecule composed of only 22 amino acids was too small and flowed out of the gel on electrophoresis or could not react with the anti-fibrinogen polyclonal antibody, as it had none of the epitopes. Fibrinogen-expressing experiments using cDNA indicated that the mutant γ-chain synthesized, but was not assembled into fibrinogen in CHO cells.

Since nucleotide mutations of both Shizuoka III and Kanazawa II are located on exon 2 or exon 9 of *FGG*, respectively, we expected that NMD might cause lower levels of mRNA in a patient's hepatocytes. The biological role of NMD is thought to be to eject aberrant mRNA that encodes potentially deleterious truncated protein. Namely, naturally occurring nonsense codon-containing mRNAs derived from genetic mutations or abnormally spliced mRNAs are degraded by the mechanisms of NMD. At splicing, the exon junction complex (EJC) is deposited at the exon-exon junction, and the EJC interacts with many NMD regulation factors. If a premature termination codon is located in the 50-55 or more nucleotides upstream of the final exon-exon junction site of the gene, mRNA will be selectively degraded by the

mechanisms of NMD [4-6]. Recent research suggested that NMD has a more important role to abolish deleterious mRNAs arising from mutations and processing errors, and regulates the overall gene expression [4-6].

To confirm whether the lower levels of plasma fibrinogens of Shizuoka III and Kanazawa II were caused by the mechanisms of NMD, we quantified the amount of mRNA transcribed from the cloned wild-type and mutant-type mini-genes, including γ23X or γ376X. The mutant-type cell lines showed lower levels of mRNA than the wild-type in the absence of CHX, a compound acting as an NMD inhibitor. On the other hand, when CHX was added to the culture media, mRNA in mutant-type cell lines increased dose-dependently with CHX. We presumed that aberrant mRNAs bearing premature termination codon, γ23X and γ376X, were degraded by the mechanisms of NMD before translation.

In normal human hepatocytes, two kinds of γ-chain are translated from *FGG*, one constructed with 411 amino acids (85-92%), and the other with 427 amino acids, named γ'-chain (8-15%) [11]. The γ'-chain is an alternative splicing product and is translated by extension of exon 9 and terminating at codon 428. Since the premature termination codon, γ376X, is located on the last exon for the γ'-chain gene [12], there is a possibility that mRNA transcribed from the γ'-chain gene containing γ376X might avoid degradation by NMD. Therefore, we speculated that, in γ376X cells, CHX inhibition of mRNA degradation is not observed clearly; namely, mRNA does

not tend to increase as in γ23X cell lines. The presented data showed that, under our experimental conditions, transcription of the γ'-chain gene did not influence that of the γ-chain gene.

In conclusion, we reported two hypofibrinogenemias, Shizuoka III and Kanazawa II, both of which were caused by heterozygous nonsense mutation at the fibrinogen γ-chain, γ23X and γ376X, respectively. Molecular analyses of the genetic variants demonstrated that the transcribed aberrant mRNAs from both the γ23X and γ376X genes are degraded by NMD and not translated to the truncated polypeptide, therefore resulting in lower levels of plasma fibrinogen, hypofibrinogenemias.

Authorship

K. Soya performed research, analyzed data, and wrote the paper.

Y. Takezawa, N. Okumura and F. Terasawa designed the research and reviewed the paper.

Conflicts of Interest Statement

The authors state that they have no conflicts of interest.

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Figure legends

Figure 1. Nucleotide sequence of the patients' *FGG*.

A: Exon 3 of Shizuoka III *FGG*, B: Exon 9 of Kanazawa II *FGG*.

Figure 2. Synthesis and secretion of variant fibrinogens in cDNA-transfected CHO cell lines. Fibrinogen concentrations in the culture media and cell lysates were measured by ELISA, as described in Materials and methods. The mean values and standard deviations of each of the 8 cloned CHO cell lines. A: culture media, B: cell lysates.

Figure 3. Immunoblot analyses of CHO cell lysates. Samples of cell lysate were subjected to 10% SDS-PAGE under reducing conditions (A) or 8% SDS-PAGE under non-reducing conditions (B). The blots were developed with a polyclonal antibody to fibrinogen and reactive bands were detected by chemiluminescence, as described in Materials and methods. Lane 1: normal plasma fibrinogen, lane 2: Normal γ-expressing CHO cell line, lane 3-5: γ23X-expressing CHO cell lines, lane 6-8: γ376X-expressing CHO cell lines.

Figure 4. mRNA levels in mini-gene-transfected CHO cell lines. For mini-gene-transfected CHO cells in the absence or presence of CHX, γ-chain or *HBB*

mRNAs were measured by real-time quantitative RT-PCR and compensated for by the levels of Aα-chain mRNA, which was transcribed from a simultaneously transfected vector, pMLP-Aα. At each CHX concentration, mRNA levels in wild-type mini-gene-transfected cells were set to 100%. Gray bars: wild type, black bars: mutant type. Mini-gene-transfected CHO cells were as follows: A: γ23wt or γ23X, B: γ376wt or γ376X, and C: *HBB*39wt or *HBB*39X as an NMD positive control.

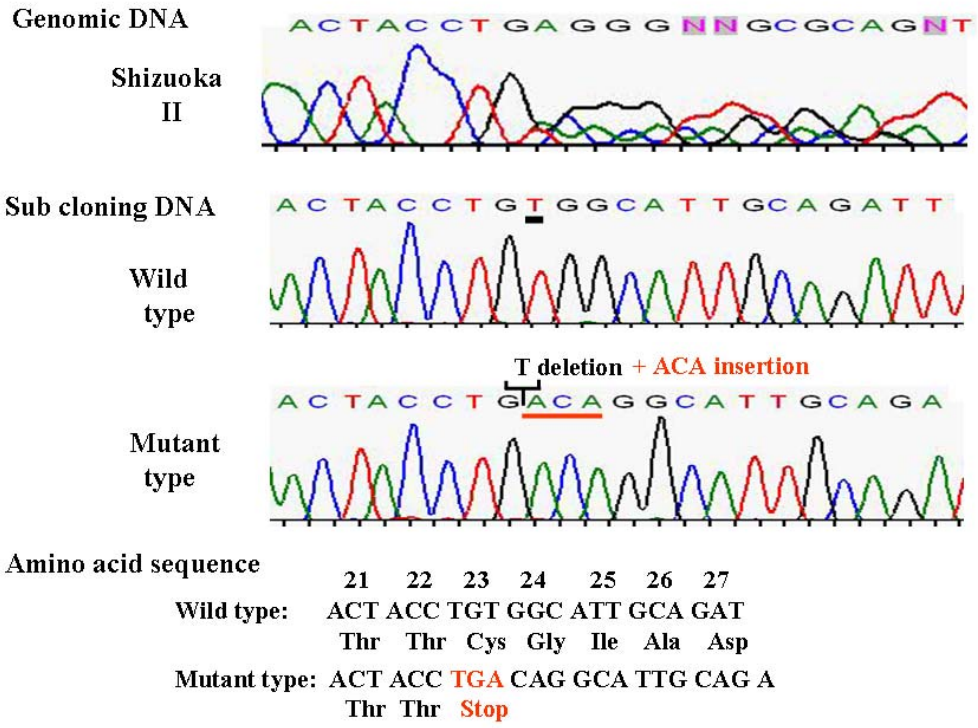
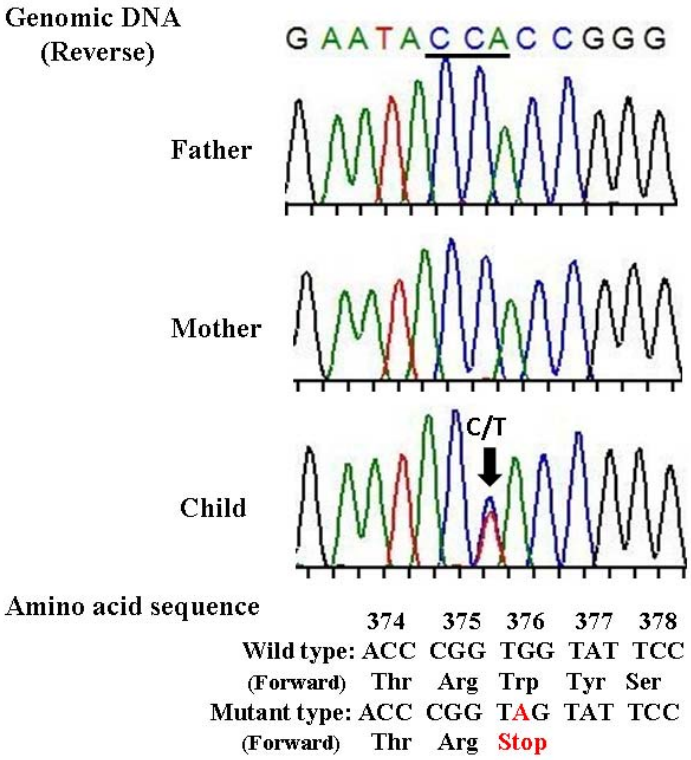

Genomic DNA
Shizuoka II
A C T A C C T G A G G G N N G C G C A G N T
Sub cloning DNA
Wild type
A C T A C C T G T G G C A T T G C A G A T T
T deletion + ACA insertion
Mutant type
A C T A C C T G A C A G G C A T T G C A G A
Amino acid sequence
21 22 23 24 25 26 27
Wild type: ACT ACC TGT GGC ATT GCA GAT
Thr Thr Cys Gly Ile Ala Asp
Mutant type: ACT ACC TGA CAG GCA TTG CAG A
Thr Thr Stop

Figure 1-A.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(Reverse)
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Father
Mother
C/T
Child
Amino acid sequence
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Mutant type: ACC CGG TAG TAT TCC
(Forward) Thr Arg Stop

Figure 1-B.

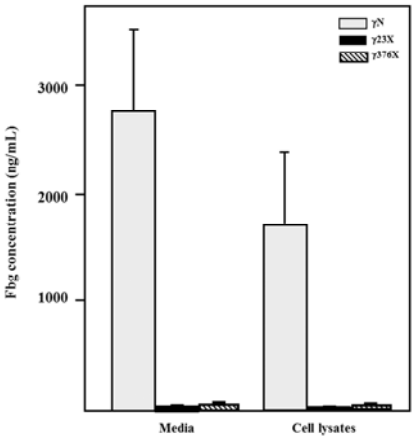
γN
γ23X
γ376X
3000
2000
1000
Fbg concentration (ng/mL)
Media
Cell lysates

Figure 2.

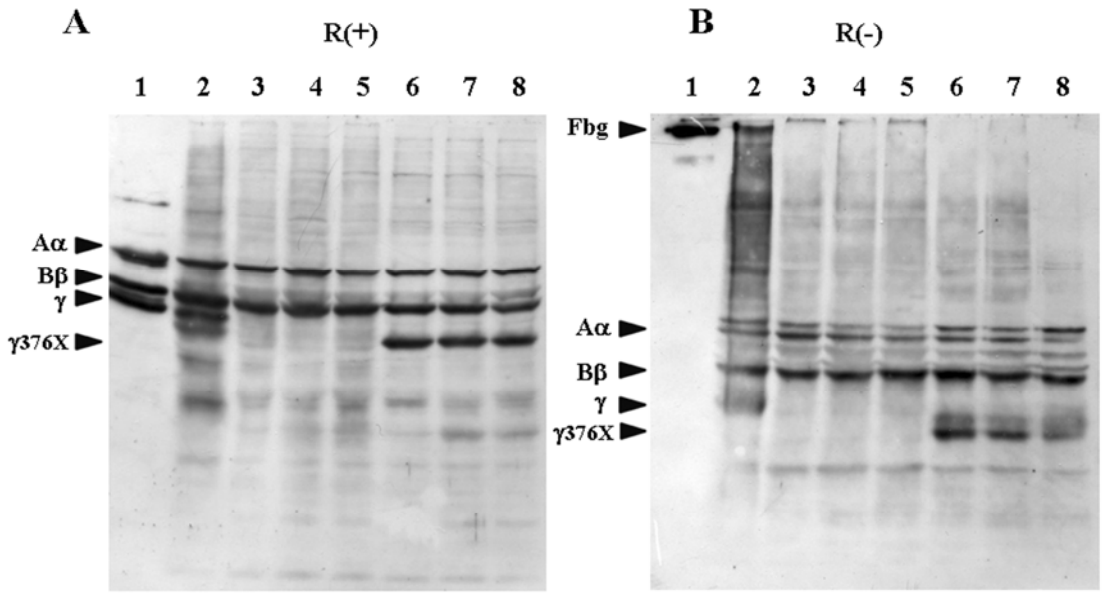

Figure 3.

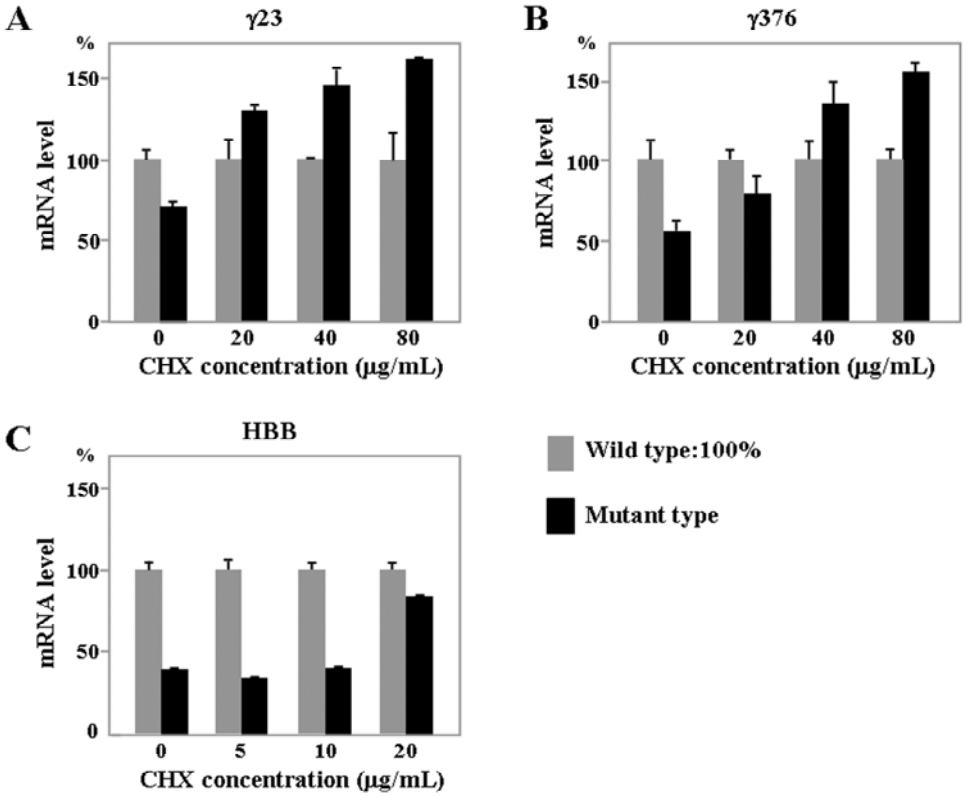
A
γ23
%
150
100
50
0
mRNA level
0
20
40
80
CHX concentration (μg/mL)
B
γ376
%
150
100
50
0
mRNA level
0
20
40
80
CHX concentration (μg/mL)
C
HBB
%
150
100
50
0
mRNA level
0
5
10
20
CHX concentration (μg/mL)
Wild type:100%
Mutant type

Figure 4.