Congenital combined vitamin K–dependent coagulation factors deficiency (VKCFD) is a rare autosomal recessive bleeding disorder. Patients with VKCFD have decreased activity in multiple vitamin K–dependent coagulation factors due to genetic mutations that limit the ability of the proteins to be carboxylated in a vitamin K–dependent manner. Since the discovery of the 2 vitamin K cycle enzymes GGCX (γ-glutamyl carboxylase) and VKOR (vitamin K epoxide reductase), > 30 GGCX mutations and 1 VKOR mutation have been identified from VKCFD patients.

The first clinical case of VKCFD was reported in 1966. The activity of the patient’s vitamin K–dependent coagulation factors (factors II, VII, IX, and X) was undetectable. High doses of vitamin K administration (15 mg/d) partially corrected the patient’s clotting activity. Thirteen years later, this patient’s clotting factors were reevaluated with immunologic assays, which suggested that abnormal forms of the clotting factors were present. As these studies were carried out before the identification of any of the enzymes in the vitamin K cycle, the molecular mechanism of the patient’s clinical phenotypes remained unclear.

To the editor:

Molecular basis of the first reported clinical case of congenital combined deficiency of coagulation factors

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The GGCX intronic mutation c.1889-6G>A is 4 bases upstream of exon 14 (Figure 1A). This mutation introduces an extra splicing-acceptor site (AG), which might affect the splicing of GGCX’s pre-mRNA splicing. To test the effect of this presumptive pre-mRNA splicing mutation, wild-type and mutant alleles of the genomic DNA fragments containing exon 14 along with its upstream and downstream intronic sequences were cloned into the exon 14 flanked by intronic sequences were cloned into a pSPL3 minigene splicing assay vector. These constructs were transfected into HEK293 cells, and the splicing products were amplified by reverse transcription polymerase chain reaction (RT-PCR) for sequencing analysis. (C) DNA electrophoresis of the splicing products from the empty vector, the wild-type GGCX, and the c.1889-6G>A mutant. Intronic insertion sequences are indicated in red. (E) Amino acid sequences of the C-terminus of the 2 identified GGCX truncation mutants.

To gain insights on how these GGCX mutations impacted patient clinical phenotypes, we characterized these mutations using our recently established CRISPR-Cas9–mediated GGCX knockout cell-based assay.6 We transiently expressed each of the mutants in the GGCX-deficient reporter cells, cultured the cells with 5 μg/mL vitamin K, and measured how efficiently the reporter protein was carboxylated. Figure 2A shows that the I553* mutant abolished reporter-protein carboxylation; however, the E630* mutant retained ~80% activity. The E630* mutant’s substantial residual activity cannot explain the patient’s clinical data, in which clotting activity of the vitamin K–dependent coagulation factors was decreased to undetectable levels.6 To better understand the details of how these mutations affect vitamin K–dependent carboxylation, we cultured the GGCX-deficient reporter cells expressing the GGCX mutant proteins with increasing concentrations of vitamin K. Figure 2B shows that the I553* mutant was unable to carboxylate the reporter protein at any of the vitamin K concentrations tested. At the vitamin K concentration where the wild-type GGCX–mediated carboxylation; however, the E630* mutant retained ~80% activity. The E630* mutant’s substantial residual activity cannot explain the patient’s clinical data, in which clotting activity of the vitamin K–dependent coagulation factors was decreased to undetectable levels.6 To better understand the details of how these mutations affect vitamin K–dependent carboxylation, we cultured the GGCX-deficient reporter cells expressing the GGCX mutant proteins with increasing concentrations of vitamin K. Figure 2B shows that the I553* mutant was unable to carboxylate the reporter protein at any of the vitamin K concentrations tested. At the vitamin K concentration where the wild-type GGCX–mediated carboxylation; however, the E630* mutant retained ~80% activity. 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reporter protein carboxylation up to 80%. This result is consistent with the patient’s clinical phenotype, as the initial coagulation activity deficiency was partially corrected by the administration of high doses of vitamin K. These results together suggest that with a null allele of *GGCX* (ie, the I553* mutation), the E630* mutation on the other allele is the causative mutation for the patient’s clinical phenotype.

In addition to the GGCX truncation mutations identified in this study, 4 other truncation mutations in the *GGCX* gene have been identified in patients/animals with bleeding disorders. These mutations result in terminations of GGCX at residues W315 (W315X), Q374 (Q374X), R687 (R687X), and R704 (R704X) (Figure 2C).

Because truncation of GGCX at residue I553 results in an inactive enzyme, we reasoned that termination of GGCX translation further toward the N-terminus (W315X and Q374X) would abolish GGCX activity. On the other hand, given that the GGCX molecule truncated at residue E630 retained ~80% activity, we suspected that termination of GGCX translation further toward the C-terminus (R687X and R704X) would have only minor effects on GGCX activity. This hypothesis was confirmed by the cell-based GGCX activity assay, which shows that the W315X and Q374X mutants lacked GGCX activity, whereas the R687X and R704X mutants retained ~95% activity (Figure 2D).

Together, these results suggest that in order for GGCX to be active, it must have at least 630 residues and that truncation of GGCX from its C-terminus can impact vitamin K binding, which is consistent with previous observations. GGCX is a glycoprotein with 5 functional glycosylation sites located at the C-terminus (Figure 2C). Truncation of GGCX at...
residue E630 not only removes the C-terminal sequences but also leaves the last 2 functional glycosylation sites (N605 and N627) at a position unfavorable for glycosylation.\textsuperscript{16,17} Consistent with our previous observation,\textsuperscript{15} a GGCX molecule with these 2 sites mutated (N605/627Q) migrates faster than the wild-type enzyme in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (supplemental Figure 1A). Our cell-based activity assay results showed that this mutant protein exhibits ~80% activity (supplemental Figure 1B) and required approximately twofold higher vitamin K concentration to reach half-maximal activity (supplemental Figure 1C). These results suggest that the last 2 glycosylation sites can have effects (albeit minor) on GGCX function.

GGCX has 5 transmembrane domains located at the N-terminus (residues 1-381), whereas its large C-terminus (residues 382-758) resides in the endoplasmic reticulum (ER) lumen (Figure 2C).\textsuperscript{18} Partial truncation of the C-terminal sequences of GGCX was expected thus to have only minor effects on ER targeting. Fluorescence confocal microscopy results showed that, as expected, GGCX and the E630* mutant clearly localized to the ER membrane (supplemental Figure 2). Under the same conditions, however, the I553* mutant showed significantly less fluorescent signal (indicating less intact protein expression). In addition, more fluorescent signal was observed outside the ER, suggesting mislocalization and probable misfolding of the mutant protein. The possible misfolding of the I553* mutant was further confirmed by the immunoblotting results (supplemental Figure 3), showing more protein bands for the mutant proteins (especially the I553* mutant) than the wild-type enzyme.

In summary, we identified 2 causative GGCX mutations from the first reported clinical case of a VKCFD patient. Functional characterization of these 2 mutations suggests that truncation of GGCX from its C-terminus affects not only substrate binding but also protein stability.

The online version of this article contains a data supplement.

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Contribution: D.-Y.J. created all the constructs and performed all the cell-based assays and GGCX mutation characterization; B.O.I. performed genomic DNA extraction, exome sequencing sample preparation, and data analysis; D.W.S. oversaw the whole project; and J.-K.T. designed the study, analyzed the data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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